University of Zagreb

Faculty of Veterinary Medicine

Valentina Kunić

INTERSPECIES TRANSMISSION AND GENOMIC PROPERTIES OF PORCINE-ORIGINTED ROTAVIRUS A IN CROATIA

DOCTORAL THESIS

Zagreb, 2025

University of Zagreb

Faculty of Veterinary Medicine

Valentina Kunić

MEĐUVRSNI PRIJENOS I GENOMSKA SVOJSTVA ROTAVIRUSA A SVINJSKOG PODRIJETLA U HRVATSKOJ

DOKTORSKI RAD

Zagreb, 2025.

University of Zagreb

Faculty of Veterinary Medicine

Valentina Kunić

INTERSPECIES TRANSMISSION AND GENOMIC PROPERTIES OF PORCINE-ORIGINTED ROTAVIRUS A IN CROATIA

DOCTORAL THESIS

Supervisors:
Dragan Brnić, PhD, DVM
Prof Ljubo Barbić, PhD, DVM

Zagreb, 2025

University of Zagreb

Faculty of Veterinary Medicine

Valentina Kunić

MEĐUVRSNI PRIJENOS I GENOMSKA SVOJSTVA ROTAVIRUSA A SVINJSKOG PODRIJETLA U HRVATSKOJ

DOKTORSKI RAD

Mentori:
dr. sc. Dragan Brnić, dr. med. vet.
prof. dr. sc. Ljubo Barbić, dr. med. vet.

Zagreb, 2025.

VETERINARSKI FAKULTET

I Z J A V A

Ja, Valentina Kunić, potvrđujem da je moj doktorski rad izvorni rezultat mojega rada te da se u njegovoj izradi nisam koristila drugim izvorima do onih navedenih u radu.

_____

(potpis studenta)

Zagreb, 2025.

This dissertation was prepared in the Department of Virology of the Croatian Veterinary Institute, Croatia, within the project HRZZ-UIP-2017-05-8580: RECO – „*Rotaviruses in Croatian ecosystem: molecular epidemiology and zoonotic potential*“ funded by the Croatian Science Foundation. The work of Valentina Kunić was funded by the Croatian Science Foundation project DOK-2021-02-3623. The research for this thesis was conducted under the supervision of principal investigator Dragan Brnić, PhD, and Professor Ljubo Barbić, PhD.

INFORMATION ON SUPERVISORS

Dragan Brnić, Senior Research Associate, has been part of the Croatian Veterinary Institute since 2007 and has focused on virology research since 2010. He completed his PhD at the Faculty of Veterinary Medicine, University of Zagreb, with a thesis entitled "Detection and Phylogenetic Analysis of Astroviruses Isolated from Potential Animal Reservoirs in Croatia". As a principal investigator of the research project „Rotaviruses in Croatian ecosystem: molecular epidemiology and zoonotic potential", under which the present study was conducted, Dr. Brnić has taken on a mentorship role. He has authored 59 scientific publications and has presented his research at more than 60 scientific conferences worldwide. According to Scopus, his work has been cited over 570 times and he has an h-index of 13.

Ljubo Barbić is a Full Professor with tenure at the Department of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, University of Zagreb, where he has worked since 2001. He received his PhD at the same faculty in 2007 with a dissertation entitled "Phylogenetic and antigenic analysis of equine influenza viruses isolated in Croatia." His research is primarily focused on viral infectious diseases of animals, with particular emphasis on zoonoses. Within the framework of the research project "Rotaviruses in the Croatian ecosystem: molecular epidemiology and zoonotic potential", led by Dr Brnić as Principal Investigator, Professor Barbić served as the mentor for this dissertation. Throughout his career, he has supervised five doctoral candidates to successful completion. To date, he has published 97 papers in Scopus-indexed journals, which have collectively been cited 1,585 times, yielding an h-index of 22.

Izrada ovog doktorskog rada ne bi bila moguća bez kontinuirane podrške i angažmana mojih mentora, Dragana Brnića i Ljube Barbića, kojima ovim putem neizmjerno zahvaljujem. Zahvaljujem Draganu na svakodnevnom usmjeravanju, kolegijalnosti, savjetima, ali i ambiciji koja je u konačnici omogućila istraživanje ovdje predstavljene teme. Zahvaljujem Ljubi na pravovremenom angažmanu, stručnoj podršci i savjetima koji su cijeli proces učinili bržim i ugodnijim.

Veliko hvala mojim kolegicama na Hrvatskom veterinarskom institutu, posebno Marini, na ohrabrenjima i podršci tijekom posljednje četiri godine.

Zahvaljujem i svim mojim prijateljicama i prijateljima, roditeljima Željku i Vesni te ljubimici Sori, bez čije velike podrške ovaj pothvat ne bi bio moguć.

ABSTRACT

Rotavirus A (RVA) is a major cause of acute gastroenteritis in mammalian and avian species, especially offspring. Although sporadic interspecies transmission has been documented globally, research is largely human-focused, with far fewer genotyped strains from domestic pigs and, in particular, wildlife. Prior to this work, no animal-derived and only one human-derived RVA whole genome (G8P[8]) from Croatia had been published, leaving a major gap in understanding local RVA evolution. Therefore, this thesis aimed to investigate interspecies transmission and genomic characteristics of autochthonous porcine-originated RVAs (poRVAs) in domestic pigs, humans, and wildlife using a synchronized spatiotemporal One Health approach. Between 2018 and 2021, 2152 samples were collected in Croatia from five host populations: 445 domestic pigs, 441 wild boars, 533 red foxes, 131 golden jackals, and 602 hospitalized human patients. Samples were analyzed using VP2-based real-time RT-PCR and VP4/VP7 genotyping. RT-PCR products of untypable human strains and all animal strains underwent Sanger sequencing. The VP4/VP7 genotyping identified poRVAs in the aforementioned host species, indicating sporadic interspecies transmission between domestic pigs and humans or wildlife. Strains derived from humans, wildlife, and domestic pigs with matching genotypes were further subjected to NGS, followed by phylogenetic, reassortment and intragenic recombination analyses. The results determined the RVA prevalence of 49.9% in domestic pigs, 9.3% in wild boars, 15% in red foxes, and 36.6% in golden jackals. Human samples were RVA-positive by study design. The genotyping of VP7 and VP4 segments revealed 23 G/P combinations in domestic pigs (dominated by G5P[13] and G9P[23]) and four in wild boars (dominated by G3P[13]). Shared genotypes and close phylogenetic clustering indicated recurring interspecies transmission between domestic pigs and wild boars. Zoonotic transmission was investigated in humans through six G4P[6] strains, including three human- and three domestic pig-derived strains. All genome segments were of porcine origin, strongly suggesting porcine-to-human interspecies transmission. Further investigation in wildlife revealed poRVA strains sharing both G/P genotypes and porcine genogroup 1 constellation with domestic pig strains, providing additional evidence of interspecies transmission. This study presents the first complete RVA genome from golden jackals and the second from red foxes globally, as well as the first from wild boars outside Asia. In animals, infections with mixed RVA genotypes were found only in domestic pigs, perpetuating genotype diversity and suggesting their role as reservoirs. Additionally, one double-reassortant strain and intragenic recombination in multiple zoonotic and animal strains (in VP4, NSP1, NSP3 and NSP4 segments) further contributed to poRVA's genetic heterogeneity. Overall, these findings

confirm the hypothesis that interspecies transmission of RVAs, typical for domestic pigs, sporadically occurs in the Croatian ecosystem. This thesis provides the first comprehensive genomic characterization of autochthonous poRVAs in Croatia, addressing a knowledge gap in local RVA evolution, revealing the interspecies transmission and evolutionary mechanisms shaping their genetic properties.

KEYWORDS: *Rotavirus A*, molecular epidemiology, genetic diversity, interspecies transmission, zoonotic transmission, domestic pig, wildlife, recombination, reassortment, One Health

PROŠIRENI SAŽETAK

UVOD: Vrsta *Rotavirus alphagastroenteritidis/Rotavirus A* (RVA) glavni je uzročnik nebakterijskog akutnog gastroenteritisa (AGE) u sisavaca i ptica, osobito mladunčadi. Genom RVA čini dvolančana RNK (engl. double-stranded ribonucleic acid; dsRNA) sastavljena od 11 genskih segmenata koji kodiraju šest strukturnih (VP1–VP4, VP6 i VP7) i šest nestrukturnih proteina (NSP1–NSP6). Površinski proteini VP7 i VP4 definiraju binomnu nomenklaturu, označavajući genotipove G (glikozilirani) i P (protein osjetljiv na proteazu). Do danas je poznato 42 G i 58 P genotipova. Osim klasifikacije temeljene na VP4 i VP7 segmentima, klasifikacija cijelog genoma pruža osnovu za detaljnu genomsku analizu RVA, dodjeljujući genotip svakom genskom segmentu na temelju definiranih graničnih postotaka podudarnosti nukleotidnih slijedova. Tri genogrupe ljudskih RVA su *Wa-like, DS-1-like i AU-1-like* za koje se smatra da dijele zajedničko evolucijsko podrijetlo sa sojevima RVA podrijetla od svinja, goveda i mačaka. RVA se izlučuje u vrlo visokim koncentracijama putem izmeta te je izrazito kontagiozan. Prijenos se prvenstveno odvija fekalno-oralnim putem, iako je opisano i širenje putem sline te respiratornim putem. RVA je dokazan u širokom rasponu domaćina diljem svijeta. Iako je u pravilu specifičan za vrstu domaćina, ima sposobnost prelaska međuvrsnih barijera. Evolucijski mehanizmi RVA uključuju točkaste mutacije, genetsko preslagivanje i intragenske rekombinacije. Navedeni mehanizmi dovode do genetske raznolikosti RVA, što može dovesti do pojave novih sojeva. U ljudskoj populaciji, RVA je najistraženiji te može zahvatiti sve dobne skupine, a djeca mlađa od pet godina najranjivija su te se procjenjuje da su u toj dobnoj skupini ove infekcije odgovorne za približno 128.000 smrtnih slučajeva godišnje u svijetu. Za razliku od ljudi, RVA u domaćih životinja znatno je slabije istražen, dok je u divljih životinja nedostatak spoznaja, ovisno o vrsti, još izraženiji. U domaćih svinja (*Sus scrofa domesticus*) RVA je glavni uzročnik virusnog AGE-a, osobito u sisajuće i odbijene prasadi, što dovodi do značajnih ekonomskih gubitaka u svinjogojstvu. Iako je bolest najčešće samoograničavajuća, u prasadi može biti fatalna zbog dehidracije. Istraživanja u divljih svinja (*Sus scrofa*) pokazuju veliku genetsku raznolikost RVA te međuvrsni prijenos s domaćim svinjama, kao i filogenetsku srodnost pojedinih sojeva s onima dokazanima u ljudi. Divlji kanidi posebno su zanimljivi zbog prilagodbe urbanim i poluurbanim staništima, čime se povećava rizik prijenosa patogena na domaće životinje i ljude. Prethodno ustanovljena prevalencija RVA u crvenih lisica (*Vulpes vulpes*) u Hrvatskoj iznosila je 14,9%, a u zlatnih čagljeva (*Canis aureus*) 20,6%, što je uputilo na mogućnost da navedene vrste predstavljaju rezervoare RVA. Globalno je do danas opisan samo jedan cijeli genom RVA dokazan u crvene

lisice, dok cijeli RVA genomi podrijetlom od čagljeva dosad nisu opisani. Nadalje, dosada opisani cijeli genomi RVA dokazani u divljih svinja potječu isključivo iz Azije.

Patofiziološki, RV infekcija ponajprije zahvaća gastrointestinalni trakt, no radi se o sustavnoj infekciji te je RVA dokazan u mozgu, jetri, bubrezima, itd. Klinički znakovi nisu patognomonični te uključuju povišenu tjelesnu temperaturu i proljev bez krvi. Infekcija RVA oštećuje epitel tankog crijeva, uzrokujući malapsorpciju, osmotski proljev te elektrolitnu neravnotežu. Posljedično može nastati dehidracija sa šokom i smrtnim ishodom, osobito u mladih, pothranjenih i imunokompromitiranih jedinki. Odrasle jedinke najčešće su inficirane asimptomatski. Za kliničku dijagnostiku i genotipizaciju, zlatni standard predstavljaju molekularne tehnike, poput lančane reakcije polimerazom uz prethodnu reverznu transkripciju u stvarnom vremenu (engl. Reverse Transcription-quantitative Polymerase Chain Reaction; RT-qPCR) te konvencionalne lančane reakcije polimerazom uz prethodnu reverznu transkripciju (engl. Reverse Transcription-Polymerase Chain Reaction; RT-PCR). U istraživačkom kontekstu, najčešće korištena metoda jest sekvenciranje sljedeće generacije (engl. Next Generation Sequencing; NGS) koje omogućuje otkrivanje vrsno-specifičnih RVA genotipova u sekundarnim vrstama domaćina, infekcije miješanim genotipovima, kao i prepoznavanje genetskog preslagivanja i ranije neopisanih genotipova.

Liječenje se temelji na potpornoj i simptomatskoj terapiji, u odsutnosti etiološkog liječenja, uz primjenu antibiotika za liječenje sekundarnih bakterijskih infekcija. Profilaksa se zasniva na općim biosigurnosnim mjerama i cijepljenju. Cilj cijepljenja razlikuje se u ljudi i životinja. U ljudi podrazumijeva primarno poticanje aktivne imunosti nakon smanjenja razine majčinih protutijela, dok se u životinja zaštita temelji na poticanju pasivne imunizacije majčinim protutijelima preko kolostruma. U Republici Hrvatskoj istraživanja RVA dugo su bila usmjerena isključivo na ljudsku populaciju te od 2018. godine kreću istraživanja domaćih i divljih životinja te okoliša. Prethodno ovom istraživanju, cijeli genomi RVA životinjskog podrijetla iz Hrvatske nisu objavljeni, dok je od onih ljudskog podrijetla objavljen tek jedan (G8P[8]), što podrazumijeva manjak dostupnih informacija o lokalnoj evoluciji RVA. Iz navedenog proizlazi nužnost pristupa „Jednog zdravlja “, koji pruža nove spoznaje o evoluciji genoma RVA te potencijalnom utjecaju na učinkovitost postojećih cjepiva.

HIPOTEZA I CILJEVI: Ovo istraživanje temelji se na hipotezi da se međuvrsni prijenos RVA sojeva klasičnih za domaće svinje sporadično pojavljuje u ekosustavu Hrvatske. Opći cilj bio je istražiti međuvrsni prijenos i genomska svojstva autohtonih RVA sojeva svinjskog podrijetla (engl. porcine-originated *Rotavirus alphagastroenteritidis*; poRVA) u domaćih svinja, ljudi i divljih životinja kroz sinkroniziran prostorno-vremenski pristup “Jednog

zdravlja". Specifični ciljevi bili su odrediti prevalenciju i genetsku raznolikost RVA koji kruže u populaciji domaćih i divljih svinja; usporediti prevalenciju RVA u domaćih svinja između dvije skupine za svaki od čimbenika, načina uzgoja, dobi, spola te prisutnosti kliničkih znakova; istražiti zoonotski prijenos poRVA; istražiti međuvrsni prijenos poRVA između domaćih svinja i divljih životinja te procijeniti utjecaj genetskog preslagivanja i intragenskih rekombinacija na raznolikost cijelih genoma poRVA.

MATERIJAL I METODE: Na području Republike Hrvatske od 2018. do 2021. godine, primjenjujući pristup "Jednog zdravlja", ukupno je prikupljeno 2152 uzoraka fecesa i rektalnih briseva, podrijetlom od domaćih (n = 445) i divljih svinja (n = 441), crvenih lisica (n =533), zlatnih čagljeva (n =131) te hospitaliziranih ljudi (n = 602) s potvrđenom RVA infekcijom. Uzorkovanje svih domaćina provođeno je tijekom cijele godine, a uzorkovanje životinja geografski je obuhvatilo osam hrvatskih županija. Životinje su grupirane prema dobi, spolu i zdravstvenom statusu (prisustvo ili odsustvo proljeva). Domaće svinje uzorkovane su na 24 ekstenzivnih i osam intenzivnih gospodarstava, a većina uzoraka (98,2%) prikupljena je od listopada do ožujka. Divlje svinje uzorkovane su nakon redovnog odstrela u 15 lovišta smještenih u osam hrvatskih županija, a većina (78,9%) uzoraka prikupljena je od listopada do ožujka. Ljudski uzorci uglavnom su uzeti od djece mlađe od pet godina s prisutnim kliničkim znakovima akutnog gastroenteritisa, koja su posljedično primljena u Kliniku za infektivne bolesti „Dr. Fran Mihaljević" Zagreb, Klinički bolnički centar Osijek i Klinički bolnički centar Split. Uzorci fecesa divljih kanida prikupljeni su od crvenih lisica i zlatnih čagljeva odstrijeljenih u sklopu aktivnog nadzora kampanje oralnog cijepljenja protiv bjesnoće, u organizaciji Uprave za veterinarstvo i sigurnost hrane Ministarstva poljoprivrede, šumarstva i ribarstva Republike Hrvatske. Za razliku od domaćih svinja, u kojih je uzorkovanje bilo usmjereno uglavnom na mlađe dobne skupine, uzorci divljih životinja (divlje svinje, crvene lisice, zlatni čagljevi) prikupljeni su u skladu s lovnim propisima, odnosno većinom od odraslih životinja. Uzorci su prikupljani izravno iz rektuma lešina divljih kanida primljenih u Hrvatski veterinarski institut (HVI). Laboratorijska obrada slijedila je odmah nakon prijema uzoraka na HVI ili su oni bili pohranjeni na -20°C do daljnje obrade. Izdvajanje RNA odrađeno je iz supernatanta 20%-tne suspenzije fecesa/rektalnog brisa pomoću KingFisher™ Flex sustava s MagMAX™ CORE kompletom. Egzogena interna pozitivna kontrola (Xeno™ RNA Control) dodana je svakom uzorku kako bi se nadzirala moguća pojava PCR inhibitora. Prisutnost RVA dsRNA potvrđena je RT-PCR-om u stvarnom vremenu usmjerenim na dio VP2 gena konzerviranim među različitim RVA genotipovima koji inficiraju ljude i domaće životinje. Svi uzorci pozitivni na prisutnost RVA dsRNA bili su podvrgnuti genotipizaciji u svrhu

određivanja G (VP7) i P (VP4) genotipova. Za životinjske uzorke, zbog veće genetske raznolikosti, korišteni su višestruki setovi početnica i više protokola, dok je za ljudske uzorke korišten multipleks VP7/VP4 RT-PCR protokol Europske organizacije za istraživanje rotavirusa (Eurorotanet). Genotipizacija je provedena pomoću SuperScript™ III RT-PCR-a i GoTaq® G2 Master Mix kompleta, dok su rezultati (PCR produkti) vizualizirani kapilarnom elektroforezom QIAxcel Advanced. Svi uzorci životinjskog podrijetla pozitivni na prisutnost VP7 i VP4 segmenata RVA i netipizirani uzorci ljudskog podrijetla poslani su na Sanger sekvenciranje u Macrogen Europe (Amsterdam, Nizozemska). Nukleotidni slijedovi dobiveni Sanger sekvenciranjem analizirani su pomoću BLAST/ViPR alata, prateći granične vrijednosti genotipova. Tijekom procesa genotipizacije i analize podataka Sanger sekvenciranja, VP7 i VP4 RVA genotipovi klasično svinjskog podrijetla otkriveni su u ljudi i divljih životinja, što je potvrda pretpostavljenog sporadičnog međuvrsnog prijenosa poRVA u ekosustavu Hrvatske. Stoga su za NGS odabrani uzorci izdvojeni od ljudi (članak II) i više vrsta divljih životinja (divlje svinje, crvene lisice i zlatni čagljevi) (članak III) u kojima su poRVA dokazani, zajedno sa sojevima domaćih svinja s podudarnim genotipovima (članak I). Uzimajući u obzir dodatne praktične kriterije (npr. volumen prikupljenih uzoraka), za NGS je odabrano ukupno 25 uzoraka koji odgovaraju navedenim kriterijima (članak II, članak III). NGS je proveden korištenjem Illumina® NextSeq 500 platforme, nakon uklanjanja DNA te pretvorbom RNA u cDNA preko sinteze prvog i drugog lanca. Knjižnice su pripremljene Nextera XT DNA kompletom. Kvalitativna procjena knjižnica provedena je pomoću 2100 Bioanalyzer uređaja, a kvantitativna procjena pomoću QubitTM 4 fluorometra. NGS je proveden korištenjem NextSeq 500/550 High Output kompleta s postavkom 300 ciklusa, uz 150 očitanja uparenih krajeva. Bioinformatička analiza sirovih NGS očitanja provedena je uklanjanjem Illumina adaptera i mapiranjem očitanja na referentne nukleotidne slijedove, specifične za pojedini RVA genotip, pomoću CLC Genomics Workbench programa. Zadržani su konsenzusni nukleotidni slijedovi koji su zadovoljili kriterije pokrivenosti ≥90% i dubine ≥10×. Genotipovi su potvrđeni alatima BLASTn i ViPR. U slučajevima pojave praznina u konsenzus nukleotidnim slijedovima, korišten je *de novo* pristup sastavljanja nukleotidnih slijedova (članak II, članak III) i ciljani RT-PCR s novodizajniranim početnicama (članak III) sa svrhom popunjavanja praznina u onim nukleotidnim slijedovima u kojima je bilo moguće. U svrhu istraživanja evolucijskih odnosa između autohtonih sojeva poRVA predstavljenih u ovom doktorskom radu, izrađena su pojedinačna filogenetska stabla za VP7 i VP4 segmente (članak I) ili za svih 11 genskih segmenata RVA (članak II, članak III). U sva tri članka, korištenjem MEGA 11 programa, svi nukleotidni slijedovi višestruko su sravnjeni MUSCLE algoritmom (korištenjem zadanih

postavki). Za svako filogenetsko stablo primijenjeni su modeli supstitucije s najnižim rezultatom Bayesian informacijskog kriterija (engl. Bayesian Information Criterion; BIC) u kombinaciji s metodom maksimalne vjerojatnosti (engl. maximum likelihood; ML). Primijenjeno je 1000 pseudoreplikacija (engl. *bootstrap*) za procjenu pouzdanosti grananja svakog filogenetskog stabla koja su završno vizualizirana primjenom programa iTOL. U članku I, matrice postotaka podudarnosti nukleotidnih i aminokiselinskih slijedova te grafički prikaz vremenske raspodjele RVA genotipova koji cirkuliraju u domaćih svinja izračunati su u „R" programu korištenjem bio3d paketa, ggplot2 i Scatter Pie Plot alata. U člancima II i III, program CLC Genomics Workbench 22.0.2 korišten je za izračun matrica postotaka podudarnosti nukleotidnih i aminokiselinskih slijedova između prethodno poravnatih RVA nukleotidnih slijedova iz GenBank-a i autohtonih poRVA nukleotidnih slijedova. Određivanje genotipskih linija provedeno je u člancima I i II. U članku I, genotipske linije određene su prema prethodno objavljenim granicama za genotipove G1, G2, G3, G4, G6, G9, P[6] i P[8], zbog njihove visoke učestalosti u ljudi (G1-G4, G9 i P[8]) ili zbog bliske filogenetske povezanosti uočene između ljudskih i životinjskih sojeva RVA (G6 i P[6]). U članku II, različite G4 i P[6] linije također su određene na temelju prethodno objavljenih granica. Zbog nedosljednosti u nomenklaturi i nedostatka propisanih kriterija, genotipske linije nisu dodijeljene ostalim G i P genotipovima iz članka I, niti genima osim VP4 i VP7 u članku II. U članku III, G i P genotipske linije nisu određivane zbog nedosljednosti u nomenklaturi i nedostatka referentnih nukleotidnih slijedova RVA u divljih životinja što onemogućuje pouzdanu usporedbu. Analize intragenskih rekombinacija i genetskog preslagivanja opisane su u člancima II i III. Intragenska/homologna rekombinacijska analiza, uključujući i intragenotipske i intergenotipske slučajeve rekombinacija (za gene s miješanim genotipovima), provedena je korištenjem RDP programa. Primijenjeno je sedam metoda detekcije rekombinacija integriranih u navedeni program: RDP, GENECONV, MaxChi, Bootscan, Chimera, SiScan i 3Seq. Slučajevi rekombinacija utvrđeni s najmanje šest od sedam metoda smatrani su pozitivnim rekombinacijskim signalima. Slučajevi genetskog preslagivanja u članku II procijenjeni su tijekom filogenetske analize, uz izračune postotaka podudarnosti nukleotidnih i aminokiselinskih slijedova. Dodatno je u članku III korišten i Simplot++ program za „bootscan" analizu korištenjem algoritma susjednog sparivanja (engl. Neighbor-Joining). Deskriptivna statistika provedena je u članku I, uključujući analizu prevalencije RVA u domaćih i divljih svinja te usporedbu utjecaja različitih čimbenika na prevalenciju (tip uzgoja, dob, spol, prisutnost ili odsutnost kliničkih znakova). U tu svrhu korišten je SYSTAT program, a za kategoričku analizu podataka korišteni su $\chi 2$ test i log-linearni model (LLM). Za sve analize, $p < 0,05$ smatralo se statistički značajnim. U člancima II

i III, Bonferroni korekcija p-vrijednosti 0,05 primijenjena je u RDP softveru za utvrđivanje statistički značajnih intragenskih rekombinacija.

REZULTATI I RASPRAVA: Ovaj doktorski rad, u širokom rasponu potencijalnih RVA domaćina, usredotočen je na domaće svinje, ljude te divlje životinje prisutne u poluurbanim staništima u Hrvatskoj, uključujući divlje svinje, crvene lisice i zlatne čagljeve. U navedenih vrsta, prethodno su opisani RVA G i P genotipovi klasičnog svinjskog podrijetla, na temelju čega je pretpostavljen sporadični međuvrsni prijenos poRVA u Hrvatskoj. Za istraživanje značajki pojedinih gena, cijelih genoma i međuvrsnog prijenosa autohtonih poRVA u domaćih svinja, ljudi i divljih životinja korišten je prostorno-vremenski pristup te načela „Jednog zdravlja". Djelomični i cjeloviti nukleotidni slijedovi gena poRVA objavljeni u člancima I, II i III ovog doktorskog rada, validirani su prema unaprijed određenim kriterijima za klasifikaciju svih 11 dsRNA genskih segmenata RVA. Rezultati predstavljeni u članku I pružaju sveobuhvatne podatke o prevalenciji i genetskoj raznolikosti autohtonih RVA u domaćih i divljih svinja, kao i analizu prevalencije RVA u domaćih svinja u odnosu na različite epidemiološke čimbenike. Prevalencija RVA u domaćih svinja iznosi 49,9%, a u divljih svinja 9,3%. U domaćih svinja uočene su statistički značajne razlike u prevalenciji RVA prema načinu uzgoja i kliničkom statusu. Domaće svinje držane u intenzivnom uzgoju pokazale su značajno veću RVA prevalenciju (68,1%) u usporedbi s onima držanim u ekstenzivnom uzgoju (38,8%) ($p < 0,05$). Prema kliničkom statusu, u domaćih svinja s proljevom utvrđena je značajno veća prevalencija (71,5%) ($p < 0,05$) nego u domaćih svinja bez proljeva. Prema kliničkom statusu, skupina domaćih svinja s proljevom iskazala je značajno veću prevalenciju (71,5%) ($p < 0,05$) nego skupina domaćih svinja bez proljeva (37,1%). Navedeni rezultati podupiru postojeće spoznaje o RVA kao uzročniku proljeva u domaćih svinja te o olakšanom prijenosu virusa uslijed bliskog kontakta između svinja u intenzivnom uzgoju. Daljnja analiza podataka o prevalenciji pokazala je da spol nije utjecao na prevalenciju RVA. Relativno visoka ukupna prevalencija dokumentirana u članku I vjerojatno je rezultat veće zastupljenosti mlađih dobnih kategorija domaćih svinja uključenih u studiju te posljedično intenzivnije cirkulacije virusa. Međutim, unutar dvije mlađe dobne skupine (sisajuće i odbijene prasadi) nije uočena statistički značajna razlika u prevalenciji RVA. RVA sojevi u domaćih svinja pokazali su visok stupanj genetske raznolikosti, s osam utvrđenih G genotipova (G9, G5, G3, G1, G4, G2, G6, G11) i sedam P genotipova (P[13], P[23], P[8], P[6], P[32], P[7], P[11]). Navedeni G i P genotipovi formirali su 23 različite G/P kombinacije, najčešće G5P[13] i G9P[23] koje čine gotovo polovicu opisanih sojeva (49,6%). Veća genotipska raznolikost utvrđena je u intenzivnim uzgojima u usporedbi s ekstenzivno držanim svinjama, što je vjerojatno odraz intenzivne

proizvodnje i trgovine te posljedično bliskog kontakta među svinjama i cirkulacije raznih sojeva RVA. Osim toga, u domaćih svinja otkriveni su G4 i P[6], genotipovi koji se smatraju rijetkima i poznati su po zoonotskom potencijalu. Njihovo blisko filogenetsko svrstavanje sa zoonotskim RVA sojevima u ljudi pružilo je osnovu za istraživanje predstavljeno u članku II.

U divljih svinja, podaci o značaju RVA do sada su bili prilično oskudni, sa samo dva dostupna istraživanja iz Japana i Češke. Članak I do sada je najopsežnije istraživanje o RVA u divljih svinja, obuhvaćajući uzorak od 441 jedinke. Također, uzorkovanje divljih svinja provedeno je paralelno s uzorkovanjem domaćih svinja, pružajući prostorno-vremensku komponentu važnu za značaj filogenetskih usporedbi. Prevalencija RVA u divljih svinja utvrđena u članku I iznosila je 9,3%. Slično domaćim svinjama, dob i spol nisu bili značajni čimbenici za prevalenciju RVA. Genotipska raznolikost RVA u divljih svinja bila je niža u usporedbi s domaćim svinjama te je otkriveno pet G genotipova (G3, G5, G9, G6, G11) te jedan P genotip (P[13]). U članku I, genotip G3 po prvi puta je opisan u populaciji divljih svinja. Istovremeno, to je bio najzastupljeniji G genotip u hrvatskih divljih svinja te treći G genotip po zastupljenosti u domaćih svinja. Svi genotipovi otkriveni u divljih svinja također su otkriveni u domaćih, uz blisku filogenetsku povezanost, što podupire pretpostavljeni međuvrsni prijenos između domaćih i divljih svinja. Uzimajući u obzir sve navedeno, članak I pruža važne podatke o prevalenciji RVA, genetskoj raznolikosti i molekularnoj epidemiologiji, kao i značaj međuvrsnog prijenosa između domaćih i divljih svinja, pružajući osnovu za članak II i III.

Rezultati predstavljeni u članku II odnose se na istraživanje zoonotskog prijenosa autohtonih poRVA. Koliko je poznato, hrvatski sojevi RVA dokazani u ljudi do sada nisu podvrgnuti sekvenciranju cijelog genoma, osim jednog soja G8P[8] iz 2006. godine. Navedeno ukazuje na značajan nedostatak znanja o lokalnoj evoluciji RVA u Hrvatskoj. Rezultati otkrivaju kako je svih 11 genskih segmenata u svakom od šest G4P[6] RVA sojeva (tri izolirana iz domaćih svinja i tri iz čovjeka) imalo klasično svinjsko podrijetlo ili se radilo o sojevima RVA utvrđenim u drugoj vrsti domaćina (ljudima), a koji su slični svinjskim RVA sojevima. Navedeno upućuje na mogućnost da su G4P[6] sojevi otkriveni u djece rezultat međuvrsnog prijenosa s domaće svinje na čovjeka, a neizravan zoonotski prijenos preko okoliša smatra se najvjerojatnijim putem prijenosa, obzirom na izrazito mladu dob zaraženih ljudi. Šest G4P[6] sojeva, sadržavalo je RVA konstelaciju genogrupe 1, dok je filogenetska analiza svih genskih segmenata opisanih RVA sojeva otkrila njihovo svinjsko podrijetlo. Uz potvrdu zoonotskog prijenosa, dodatna analiza genoma otkrila je postojanje miješovitih RVA genotipova, genetsko preslagivanje te intragenske (homologne) inter- i intragenotipske rekombinacije. Ovim pristupom procijenjen je njihov utjecaj na cjelokupnu raznolikost genoma autohtonih poRVA. Svi hrvatski P[6] sojevi

bili su usko evolucijski povezani sa susjednim mađarskim zoonotskim P[6] sojevima, što naglašava utjecaj geolokacije na raznolikost sojeva RVA. Miješoviti RVA genotipovi potaknuli su pojavu genetskog preslagivanja i intragenskih rekombinacija utvrđenih u nekoliko sojeva. U dva ljudska soja slična svinjskim sojevima i jednom svinjskom soju utvrđena je pojava intragenskih rekombinacija barem u jednom od genskih segmenata VP4, NSP1 ili NSP3. Zanimljivo je da su u G4P[6] soju iz Dominikanske Republike, također genogrupe 1, utvrđene rekombinacije u istim genskim segmentima kao i tri spomenuta rekombinantna soja iz Hrvatske. Suprotno rezultatima ranije objavljenog istraživanja o prevalenciji intragenskih rekombinacija RVA, gdje analiza rekombinacija nije dala rezultate u segmentu NSP3, članak II izvještava o T1-T7 intergenotipskim rekombinacijama u sva tri rekombinantna NSP3 soja. To također govori o pojavi NSP3 rekombinacija u svakom soju u kojem su bili prisutni T1/T7 miješoviti genotipovi. Rezultati poput ovog dodatno podupiru spoznaju da miješoviti genotipovi doprinose evoluciji novih RVA sojeva i njihovoj genetskoj raznolikosti. Članak III usmjeren je na istraživanje međuvrsnog prijenosa poRVA sojeva između domaćih svinja i divljih životinja unutar ekosustava Hrvatske. Istražen je i utjecaj genetskog preslagivanja i intragenskih rekombinacija na genomsku raznolikost autohtonih poRVA sojeva temeljem cjelogenomske analize, kao i u članku II. Rezultati su otkrili konstelaciju svinjske genogrupe 1, s genotipovima površinskih proteina svojstvenim za domaće svinje u svim opisanim RVA sojevima. Nadalje, istraživanje pruža značajan uvid u raznolikost domaćina RVA, jer bilježi prvi cijeli genom RVA izdvojen iz zlatnog čaglja te drugi izdvojen iz crvenih lisica. Osim navedenog, sadrži i prve cijele genome RVA izdvojene iz divljih svinja izvan Azije. U članku III, utvrđena je prevalencija RVA u crvenih lisica (15%) i zlatnih čagljeva (36,6%), čime se nadopunjuju podaci o prevalenciji RVA u divljih i domaćih svinja prikazani u članku I. Rezultati potvrđuju postojanje međuvrsnog prijenosa, budući da je nekoliko poRVA sojeva otkrivenih u divljim životinjama bilo filogenetski blisko povezano s onima dokazanim u domaćih svinja što pretpostavlja divlje životinje kao prijemljive domaćine, ali i kao potencijalne rezervoare poRVA. Sveukupno, više čimbenika i kontaktnih točaka pridonosi međuvrsnom prijenosu između domaćih i divljih životinja, uključujući zajednička staništa, nedovoljnu biosigurnost ekstenzivnih uzgoja, bliski kontakt između domaćih svinja i divljih životinja, strvinarstvo, oportunističku prirodu divljih kanida i divljih svinja te preklapajuće trofičke niše zlatnih čagljeva i crvenih lisica. Svi navedeni čimbenici značajno olakšavaju te izravno ili neizravno omogućuju međuvrsni prijenos patogena koji inficiraju više vrsta domaćina. RVA pritom iskazuje sposobnost dugotrajnog preživljavanja u okolišu, zadržavajući infektivnost od nekoliko sati do nekoliko mjeseci izvan domaćina. Od 19 cijelih poRVA genoma opisanih u

članku III, najzastupljeniji VP7 genotip u divljih životinja bio je G3, u domaćih svinja G5, dok je zoonotski G4 izdvojen iz domaće svinje i crvene lisice. Najčešći VP4 genotip bio je P[13], dok je zoonotski P[6] potvrđen u domaće svinje i zlatnog čaglja. Nekoliko utvrđenih rekombinantnih sojeva upućuje na značajan doprinos intragenskih rekombinacija genetskoj raznolikosti poRVA, pri čemu su one zabilježene u segmentima gena VP4, NSP1 i NSP4, obuhvaćajući genotipove P[13], P[23], A8 i E9. Nedvosmisleni slučajevi genetskog preslagivanja nisu otkriveni. Istraživanje u okviru ovog doktorskog rada daje značajan doprinos razumijevanju lokalne evolucije RVA u Hrvatskoj, gdje nukleotidni slijedovi cijelih genoma RVA podrijetlom od životinja prethodno nisu objavljeni, dok je iz ljudskog uzorka objavljen tek jedan cijeli RVA genom (G8P[8]) 2006. godine. Istražene su nepoznanice vezane uz raznolikost RVA u populaciji svinja, prisutnost poRVA sojeva u populaciji ljudi i divljih životinja te filogenetske i genetske značajke poRVA, s ciljem donošenja zaključaka o pojavnosti i značaju međuvrsnog prijenosa poRVA unutar ekosustava Hrvatske. Nulta hipoteza glasila je da se međuvrsni prijenos RVA sojeva klasičnih za domaće svinje sporadično pojavljuje u ekosustavu Hrvatske. Rezultati predstavljeni u člancima I, II i III zajedno potvrđuju ovu hipotezu.

ZAKLJUČCI: Prevalencija RVA u Hrvatskoj u domaćih svinja u razdoblju od 2018. do 2021. godine bila je izrazito visoka (49,9 %), uz značajnu genotipsku raznolikost koja je obuhvaćala 23 različite G/P kombinacije. Populacija divljih svinja pokazala je nižu prevalenciju RVA (9,3 %) i manju genetsku raznolikost s četiri različite G/P kombinacije. Zastupljenost jednakih genotipova u domaćih i divljih svinja, uz njihovu blisku filogenetsku povezanost, pruža dokaze o opetovanom međuvrsnom prijenosu između ove dvije vrste. U domaćih svinja, tip uzgoja i klinički status utvrđeni su kao statistički značajni čimbenici koji utječu na prevalenciju RVA. Domaće svinje s velikih farmi te one koje su pokazivale kliničke znakove proljeva imale su znatno veću vjerojatnost pozitivnog testa na RVA. Nisu utvrđene značajne razlike u prevalenciji s obzirom na dob ili spol ni u jedne vrste. Zoonotski prijenos autohtonih poRVA u ekosustavu Hrvatske utvrđen je između domaćih svinja i ljudi, uz pretpostavku neizravnog zoonotskog prijenosa G4P[6] sojeva svinjskog podrijetla neizravno putem okoliša, obzirom na izrazito ranu dob zaraženih ljudi. Također, istraživanje međuvrsnog prijenosa autohtonih poRVA sojeva unutar ekosustava Hrvatske otkrilo je jasne dokaze prijenosa između domaćih svinja i divljih životinja. Ovaj doktorski rad ukazuje na potencijal divljih životinja i kao prijemljivih jedinki i kao rezervoara poRVA. Ovdje prikazani poRVA sojevi opisani u divljih životinja su prvi cjelogenomski podatci o RVA dokazanog u zlatnog čaglja te drugi podrijetlom iz crvenih lisica, ali i prvi cijeli genomi RVA iz divljih svinja izvan Azije. Svi oblici međuvrsnog prijenosa u

ovom doktorskom radu istraženi su sinkroniziranim prostorno-vremenskim pristupom prateći principe „Jednog zdravlja “. Nadalje, rezultati naglašavaju kontinuiranu prisutnost intragenskih rekombinacija i sporadičnog genetskog preslagivanja kao virusnih evolucijskih mehanizama koji doprinose genetskoj raznolikosti autohtonih poRVA. Sveukupno, ovaj doktorski rad daje značajne odgovore o nepoznanicama u lokalnoj evoluciji RVA te otkriva međuvrsni prijenos i evolucijski mehanizme koji utječu na genetska svojstva i raznolikost poRVA.

KLJUČNE RIJEČI: *Rotavirus A*, molekularna epidemiologija, genetska raznolikost, međuvrsni prijenos, zoonotski prijenos, domaća svinja, divlje životinje, rekombinacije, genetsko preslagivanje, Jedno zdravlje

ABBREVIATIONS

A	Interferon Antagonist
aa	Amino acid
Abs	Antibodies
AGE	Acute gastoenteritis
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
C	Core protein
Ca^{2+}	Calcium
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
DLP	Double-layered particle
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dsRNA	double-stranded RNA
E	Enterotoxin
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscope
FUT2	Fucosyltransferase 2
FUT3	Fucosyltransferase 3
G	Glycosylated
g	G-force/ times gravity
H	Phosphoprotein
HBGAs	Histo-blood group antigens
I	Inner capsid
ICA	Immunochromatographic assay
ICTV	International Committee on Taxonomy of Viruses
IFNs	Interferons
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgY	Immunoglobulin Y/ Yolk immunoglobulin
IL	Interleukin

IPC	Internal Positive Control
IRF	Interferon regulatory factor
kb	kilobase
LAT	Latex agglutination test
LLM	Log-linear model
M	Methyltransferase
MDA	Multiple displacement amplification
MDA5	Melanoma differentiation-associated protein 5
MHC	Major Histocompatibility Complex
ML	Maximum likelihood
mRNA	Messenger ribonucleic acid
N	NTPase / Nucleoside triphosphatase
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NGS	Next-generation sequencing
NIP	National immunization program
NK	Natural killer
nM	Nanomolars
NSPs	Non-structural proteins
nt	Nucleotide
NUTS	Nomenclature of territorial units for statistics
ORFs	Open reading frames
P	Protease sensitive
PAGE	Polyacrylamide gel electrophoresis
pi	Percentage identity
poRVA	Porcine-originated Rotavirus A
PRRs	Pattern recognition receptors
R	RNA-dependent RNA polymerase
RCWG	Rotavirus Classification Working Group
RECO	Rotaviruses in Croatian ecosystem: molecular epidemiology and zoonotic potential
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid

RNK	Ribonukleinska kiselina
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
RVA	Rotavirus A/*Rotavirus alphagastroenteritidis*
RVB	Rotavirus B/*Rotavirus betagastroenteritidis*
RVC	Rotavirus C/*Rotavirus tritogastroenteritidis*
RVD	Rotavirus D/*Rotavirus deltagastroenteritidis*
RVF	Rotavirus F/*Rotavirus phiagastroenteritidis*
RVG	Rotavirus G/*Rotavirus gammagastroenteritidis*
RVH	Rotavirus H/*Rotavirus aspergastroenteritidis*
RVI	Rotavirus I/*Rotavirus iotagastroenteritidis*
RVJ	Rotavirus J/*Rotavirus jotagastroenteritidis*
RVK	Rotavirus K/*Rotavirus kappagastroenteritidis*
RVL	Rotavirus L/*Rotavirus lambdagastroenteritidis*
RVs	Rotaviruses
SISPA	Sequence-independent single-primer amplification
T	Translation Enhancer
Ta	Annealing temperature
TLR3	Toll-like receptor 3
Tm	Melting temperature
ViPR	Virus Pathogen Resource
VPs	Viral proteins
w/v	Weight in volume
WGS	whole genome sequencing
WHO	World Health Organization

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Rotavirus History and Taxonomy

Rotaviruses (RVs) are enteropathogenic viruses that infect vertebrate hosts and are classified into the genus *Rotavirus* in the family *Sedoreoviridae, order Reovirales* (MATTHIJNSSENS et al., 2022). Based on serological reactivity of the VP6 protein and genetic variability of its coding VP6 gene, nine groups, also termed species of RVs, were distinguished (MATTHIJNSSENS et al., 2012). In 2024, the International Committee on Taxonomy of Viruses (ICTV) included two additional RV species in the genus *Rotavirus*, *Rotavirus kappagastroenteritidis* (RVK) and *Rotavirus lambdagastroenteritidis* (RVL). Also, new taxonomy names were given by the ICTV to all RV species and are summarized in Table 1 (ICTV, 2024).

Table 1. Taxonomy of the genus *Rotavirus* (ICTV, 2024).

Abbreviation	Previous species name	Current species name
RVA	Rotavirus A	*Rotavirus alphagastroenteritidis*
RVB	Rotavirus B	*Rotavirus betagastroenteritidis*
RVC	Rotavirus C	*Rotavirus tritogastroenteritidis*
RVD	Rotavirus D	*Rotavirus deltagastroenteritidis*
RVF	Rotavirus F	*Rotavirus phiagastroenteritidis*
RVG	Rotavirus G	*Rotavirus gammagastroenteritidis*
RVH	Rotavirus H	*Rotavirus aspergastroenteritidis*
RVI	Rotavirus I	*Rotavirus iotagastroenteritidis*
RVJ	Rotavirus J	*Rotavirus jotagastroenteritidis*
RVK	Rotavirus K	*Rotavirus kappagastroenteritidis*
RVL	Rotavirus L	*Rotavirus lambdagastroenteritidis*

Among the officially recognized species, *Rotavirus A* / *Rotavirus alphagastroenteritidis* (RVA) has the utmost importance in human and animal health, as the leading cause of non-bacterial acute gastroenteritis (AGE) in mammalian and avian species, especially offspring (ESTES and GREENBERG, 2013).

In 1973, *Rotavirus* was first discovered in human hosts in Australia (BISHOP et al., 1973). In 1974, Flewett et al. suggested the name *rotavirus* due to its characteristic wheel-like shape (Latin *rota* = “wheel”) observed under an electron microscope (EM) (FLEWETT et al.,

1974), and the name was officially adopted by the ICTV four years later. Following the discovery of RVs in mammalian hosts, they were also found in avian hosts, examining the intestinal contents of turkey poults using EM and finding particles morphologically identical to rotavirus (BERGELAND et al., 1977). In 2012, sequence-based species demarcation criteria, based on phylogenetic analyses and pairwise identity profiles of the VP6 encoding gene, were introduced, resulting in a 53% amino acid cut-off value to differentiate RVs per species (MATTHIJNSSENS et al., 2012). Among all RV species, RVA is the most significant in both human and veterinary medicine. This is attributed to its high prevalence and pathogenicity in humans, various mammals, and birds, as well as its remarkable genetic and antigenic diversity (HACKER et al., 2012; DORO et al., 2015). RVA is continuously reported as a leading cause of non-bacterial gastroenteritis in mammal and avian species, especially offspring. Nearly all morbidity and mortality caused by RVs are attributed to RVA (PATTON, 2012). Within five years of its identification, RVA was acknowledged as one of major causes of diarrhea in infants and young children globally, connected to around a third of required hospitalizations (ESTES and GREENBERG, 2013; OMATOLA and OLANIRAN, 2022). Today, RVA continues to be the leading cause of acute non-bacterial gastroenteritis in the said population, with decreasing but consistently high rates of hospitalizations and deaths globally (OMATOLA and OLANIRAN, 2022).

So far, species RVB and RVC have been found only in mammals, while species RVD, RVF, and RVG have been detected exclusively in birds (McNULTY, 2003; PINHEIRO et al., 2023). Rotavirus species A, B, C, E, and H have been confirmed to infect domestic pigs (VLASOVA et al., 2017; KUMAR et al., 2022). Although the RVE species was initially reported in domestic pigs (PEDLEY et al., 1986), it was subsequently excluded from the official ICTV species list due to the lack of original virus isolates and supporting sequence data (MATTHIJNSSENS et al., 2019; WALKER et al., 2020). RVH is notable for infecting mammals and was detected in humans, domestic pigs and bats (PUENTE et al., 2020), RVI was discovered in dogs in Hungary (MIHALOV-KOVÁCS et al., 2015), and RVJ was identified in bats in Serbia (BÁNYAI et al., 2017). Newly ICTV recognized species, RVK and RVL, were first detected in 2013 in the intestinal contents of common shrews (*Sorex araneus*) from Germany. Both reference strains originate from the same animal, which was co-infected with RVK and RVL (JOHNE et al., 2019; JOHNE et al., 2022; JOHNE et al., 2023; ICTV, 2024).

1.2. Structure

RVAs have a morphologically distinctive non-enveloped virion, 100 nm in diameter, with a three-layered protein capsid of icosahedral shape (DESSELBERGER, 2014). RVA's genome consists of 11 double-stranded RNA (dsRNA) gene segments with a total genome length of 18.5 kb. The 11 gene segments code for 12 viral proteins, six structural (VP1-VP4, VP6, and VP7) and six nonstructural proteins (NSP1-NSP6) (CRAWFORD et al., 2017). The structural viral proteins (VPs) constitute the viral particle, whereas the non-structural proteins (NSPs) are involved in the viral replication process or interact with host proteins, influencing viral pathogenesis and the host immune response (GELETU et al., 2021).

Table 2. RVA genes, names, and number of genotypes, cutoff values, protein functions and properties.

Coding Gene Segment/ Protein product	**Genotype Name/ Abbreviation** (MATTHIJNSSENS et al., 2008b)	**Number of genotypes** (RCWG, 2023)	**Genotype Cuttoff Value** (MATTHIJNSSENS et al., 2008b)	**Properties** (ESTES and GREENBERG, 2013; GÓMEZ-RIAL et al., 2020)
Segment 9/ VP7	**G**lycosylated / G	42	80%	G-type neutralizing antigen
Segment 4/ VP4	**P**rotease sensitive / P	58	80%	P-type neutralizing antigen, cell attachment, host range, virulence
Segment 6/ VP6	**I**nner capsid / I	32	85%	Serological grouping and subgrouping antigen
Segment 1/ VP1	**R**NA-dependent RNA polymerase / R	28	83%	RNA-dependent RNA polymerase RNA binding
Segment 2/ VP2	**C**ore protein / C	24	84%	RNA binding, required for replicase activity of VP1
Segment 3/ VP3	Methyltransferase / M	24	81%	Guanylyltransferase, methyltransferase, ssRNA binding, complex with VP1
Segment 5/ NSP1	Interferon **A**ntagonist / A	39	79%	Host interferon antagonist Anti-apoptosis
Segment 8/ NSP2	**N**TPase / N	28	85%	Forms viroplasms with VP1 and NSP5; NTPase; helix-destabilizing helicase
Segment 7/ NSP3	**T**ranslation Enhancer / T	28	85%	Viral translation enhancer, inhibition of host translation
Segment 10/ NSP4	**E**nterotoxin / E	32	85%	Enterotoxin, transmembrane protein, viroporin, virulence
Segment 11/ NSP5	p**H**osphoprotein / H	28	91%	Phosphoprotein, RNA binding

Note: NTPase = nucleoside triphosphatase

The VP7 and VP4 segments are the basis for the binomial nomenclature of RVs, providing the G (Glycosylated) and P (Protease-sensitive) genotypes, respectively (ESTES and GREENBERG, 2013). The Rotavirus Classification Working Group (RCWG) currently acknowledges 42 G and 58 P genotypes (RWCG, 2023). In addition to the binomial RV classification system, the complete genome-based classification was developed (MAUNULA and von BONSDORFF, 2002). It implies respective genotypes assigned to each genomic segment based on the predefined percentage identity cutoff values for nucleotide (nt) coding sequences of each VP and NSP (MATTHIJNSSENS et al., 2008a). Complete genome constellation nomenclature is described in scheme Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, with x presenting genotype number for VP7, VP4, VP6, VP1-3, NSP1-5, respectively (MATTHIJNSSENS et al., 2008a). The complete genome classification system was constructed as it allows direct determination of genetic relationships, providing an understanding of phylogenetic analyses to study the evolution of RVs (MATTHIJNSSENS et al., 2008b). Three main human RVA genotype constellations have been identified: Wa-like (genogroup 1), DS-1-like (genogroup 2), and AU-1-like (genogroup 3). Consequently, the complete genome classification system revealed genetic relationships among RVAs from different host species, including evidence that human RVAs belonging to the Wa-like genogroup have a common origin with porcine RVAs, while those belonging to the DS-1-like genogroup have a common origin with bovine RVAs. The less common AU-1 genogroup likely has a feline origin (MATTHIJNSSENS et al., 2008b). These genetic similarities between animal and human RVAs underscored the importance of a standardized naming system for use in medical, veterinary and public health contexts (MALIK et al., 2020).

In addition to the methods such as RNA-RNA hybridization assays, the development of next-generation sequencing (NGS) platforms provided the basis for the greater availability of the in-depth genomic analyses of viral genomes (MATTHIJNSSENS et al., 2008b; HOULDCROFT et al., 2017). In general, NGS in virology is considered to provide information on antigenic epitopes, virus evolution and evidence of recombination between different viral strains (HOULDCROFT et al., 2017). Through the utilization of the complete genome classification system of RVs (MATTHIJNSSENS et al., 2008b), the NGS enables the detection of the species specific RVA genotypes in a secondary host species, the detection of mixed genotype infections in the singular host, as well as allowing for the detection of reassortment events. Moreover, it helps in determination whether certain gene constellations play a role in RV host range restriction or virulence, and the identification of distinct or previously unknown genotypes (MATTHIJNSSENS et al., 2008b; DORO et al., 2015; HULL et al., 2020).

1.3. Evolution of Rotaviruses

RVAs are among the most genetically unstable and rapidly evolving viruses (MALIK et al., 2020). Their high evolutionary rate stems from several RNA-level mechanisms that contribute to genomic heterogeneity, driven by genomic drift and shift (LAGAN et al., 2023). Genetic drift occurs via point mutations, which are introduced during replication by the error-prone RNA-dependent RNA polymerase (VP1 protein), due to the lack of proofreading capability (ESTES and GREENBERG, 2013; DESSELBERGER, 2014). These mutations may accumulate over time or arise sporadically in key genomic regions, resulting in immune-evading variants that can lead to the emergence of novel strains (HAKIM et al., 2024). Genetic and antigenic shift primarily result from reassortment. This exchange of gene segments between RVA strains sometimes results in the assembly of chimeric human-animal RVA strains as a result of co-infection of the same host cell. Frequent co-infections with different genotypes are critical for reassortment to occur, a condition met by RVA as infectious units are often vesicles containing 5 to 15 viral particles rather than individual viruses (SANTIANA et al., 2018). This process facilitates significant RV evolutionary diversification, including interspecies transmission (MARTELLA et al., 2010). Although replication in non-natural hosts often results in evolutionary dead ends, reassortment increases the chances of generating variants capable of spreading in new populations (NGUYEN et al., 2024). In addition, reassortment is also relevant to vaccine efficacy when immune responses are G and P genotype-specific (GELETU et al., 2021).

Along with point mutations and genome reassortment, intragenic recombination events present another potential RVA's evolutionary diversifying mechanism. Although previously underestimated, recent studies indicate that recombination is a significant factor in RVA evolution (HOXIE and DENNEHY, 2020). The VP7 and VP4 proteins are under strong selective pressure for diversification, as their alteration helps evade the host immune response. Given the high co-infection rate with different RVA strains, recombination is likely to occur. Previously, intragenic recombination events have been detected in all gene segments except NSP3 (HOXIE and DENNEHY, 2020). While recombination can sometimes interfere with viral replication, its role in helping viruses evade immune detection can outweigh these drawbacks (HOXIE and DENNEHY, 2020). When recombination affects conserved epitopes, particularly those involved in host cell attachment, it may provide an evolutionary advantage by allowing the virus to temporarily escape antibody neutralization. Although these recombinant variants may initially display reduced fitness, further adaptation can restore or

even enhance their ability to compete with circulating RVA strains (HOXIE and DENNEHY, 2020).

In addition to genome diversification mechanisms listed above, RVA's broad host range, extensive genotype diversity across all gene segments, and frequent mixed infections (infections with more than one RVA strain) are additional co-factors for generating outstanding RVA genome heterogeneity. RVA co-infection rates exceed 20% in some developing countries, while in more developed regions, the rate is approximately 5% (PATTON, 2012).

1.4. Epidemiology

1.4.1.RVA Infection Dynamics

RVs are highly contagious pathogens, shedding up to 10^{10} – 10^{12} viral particles per milliliter of feces. Typically, the RVA viral shedding begins two days after the onset of the clinical signs. Symptomatic disease can last up to 7-8 days, continuously contaminating the environment, though some reports suggest the virus may be detected in feces for even longer (DHAMA et al., 2009; BERTONI et al., 2021). The virus can remain infectious for up to nine months at room temperature or for one hour at 60°C (GELETU et al., 2021). Transmission primarily occurs through the fecal-oral route, though salivary and possibly respiratory routes have also been suggested (DIAN et al., 2021; GHOSH et al., 2022). Infection spreads through direct contact with symptomatic or asymptomatic individuals or via contaminated objects, feed, or water (GELETU et al., 2021). Once inside the body, RVAs target mature enterocytes and enteroendocrine cells in the middle and tip regions of the small intestinal villi (CRAWFORD et al., 2017).

1.4.2. RVA in different hosts

Species of interest in this thesis include humans, domestic pigs, wild boars, red foxes and golden jackals. Within the human population, RVA can infect all age groups, with the most vulnerable group being children under five years of age. Nearly every child worldwide is expected to contract RVA at least once before the age of five (CRAWFORD et al., 2017). The global RVA mortality burden started decreasing after the early 2000s, counting more than 250,000 deaths, to an estimated 128,500 deaths in 2016 as more countries introduced vaccines into their National Immunization Programs (NIP) (TATE et al., 2016; TROEGER et al., 2018). Whole genome classification identified three main human RVA genogroups: Wa-like, DS-1-like, and AU-1-like. First, the most widespread human RVA strains belonging to the Wa-like genogroup (G1P[8], G3P[8], G4P[8], and G9P[8]) share the backbone genotypes with porcine

RVA strains of genogroup 1: R1-C1-M1-A1-N1-T1-E1-H1, and are considered to share a common origin (MATTHIJNSSENS et al., 2008b; PAPP et al., 2013a; THEUNS et al., 2015; SILVA et al., 2016). A close evolutionary relationship between human DS-1-like and bovine RVAs has been described, as these data suggest a common origin between the human DS-1-like and bovine RVAs (I2-R2-C2-M2-A2-N2-T2-E2-H2) (MATTHIJNSSENS et al., 2008b). The third, and the most rare, human AU-1-like genogroup 3 (I3-R3-C3- M3-A3-N3-T3-E3-H3), is believed to have a close evolutionary relationship with canine and feline RVA strains (MATTHIJNSSENS et al., 2008b).

In low-income countries, RV disease in humans is more frequently caused by uncommon RV strains and occurs at a younger age than in high-income countries (CRAWFORD et al., 2017). For example, the proportion of all RV hospitalizations that occur in infants by eight months of age is 43% in Africa but only 27% in Europe (CRAWFORD et al., 2017). In addition, seasonality correlates with the income level of a country, as more seasonal outbreaks are reported in high-income countries than in low-income countries (CRAWFORD et al., 2017). During the summer months, the efficiency of RVA transmission might be reduced, considering environmental conditions such as higher temperature and humidity, though RVA seasonality was proven only in human hosts and in the temperate climate (HUNGERFORD et al., 2016; KRAAY et al., 2018).

In domestic pigs, RVA is a major causative agent of viral AGE, particularly in suckling and weaned piglets, leading to substantial economic losses in the pork industry (CHANG et al., 2012). Regardless of the disease being mainly self-limiting, it can be fatal in young piglets due to dehydration, especially during outbreaks in intensive farm settings (PALMARINI, 2017). Intensive production environments often exhibit higher disease prevalence due to crowding, frequent animal introduction, and production-related stress, all of which increase the likelihood of pathogen spread (MANZOOR et al., 2023). In such conditions, RVA-induced mortality may be as high as 15% (DEWEY et al., 2003). The RVA prevalence in both clinically affected and asymptomatic pigs ranges from 3.3% to 67.3%, showing no consistent seasonal patterns, but displaying spatio-temporal variations and occasional re-emergence of certain genotypes (VLASOVA et al., 2017). The pig health management remains continuously challenged due to the RVA's ubiquity and environmental resilience (CHANG et al., 2012). Previously, RVA in domestic pigs showed remarkable genotype diversity, with more than 50 detected genotype combinations (DORO et al., 2015). Despite a variety of RVA genotypes discovered in pigs, G3, G4, G5, G9 and G11 in combination with P[5], P[6], P[7], P[13] and P[28] are most common (DORO et al., 2015; VLASOVA et al., 2017) and are considered as porcine genotypes. To

summarize RVA findings in domestic pigs in Europe, a study conducted between 2003 and 2007 in Denmark, Hungary, Slovenia, and Spain, revealed a broad diversity of porcine RVA genotypes, including 10 G types (G1–G6, G9–G12) and nine P types (P[6], P[7], P[8], P[9], P[10], P[13], P[23], P[27], P[32]) (MIDGLEY et al., 2012). G4 and P[6] were the most commonly detected genotypes across several countries, while in Slovenia, G3, G4, G5, and P[6] were particularly prevalent. Subsequent studies in Ireland, Poland, and the Netherlands confirmed ongoing diversity (e.g., G2, G5, G11, P[26]) and the absence of a consistently dominant genotype, highlighting the dynamic and regionally varied epidemiology of porcine RVA in Europe (WINIARCZYK et al., 2002; COLLINS et al., 2010; MIDGLEY et al., 2012). In the United Kingdom, analysis of samples collected from diarrheic pigs between 2010 and 2012 identified G4P[6] and G5P[7] as the most common combinations, suggesting some distinct differences in genotype distribution compared to the rest of Europe (CHANDLER-BOSTOCK et al., 2014). Domestic pigs have been suggested as reservoirs for RVAs and a source of newly adapted emerging strains for humans and other animals (DHAMA et al., 2009; WU et al., 2022).

In comparison with domestic animals, and especially humans, RVA in wildlife is far less studied. Nevertheless, previous data on RVA detection rates in wildlife suggests that they may serve as additional potential RVA reservoirs (MARTIN et al., 2011; ČOLIĆ et al., 2021; JOTA BAPTISTA et al., 2023). The research on wild boars (*Sus scrofa*) remains limited, albeit existing studies have demonstrated the genetic diversity of RVA strains circulating in wild boars, supporting evidence of interspecies transmission between them and domestic pigs. These findings also underscore the close phylogenetic relationship between certain wild boar RVA strains and those detected in humans (OKADERA et al., 2013; MOUTELÍKOVÁ et al., 2016). Although epidemiological data on RVAs in wild boars remains scarce, several VP7/VP4 genotypes had been identified. These include: G4P[25], G4P[6], G11P[13], G5P[13] detected in Czech Republic (MOUTELÍKOVÁ et al., 2016); G9P[23], G4P[23], G9P[13], G4P[6], G3P[23] in Japan (OKADERA et al., 2013; SHIZAWA et al., 2024); G3P[13], G9P[13], G5P[13] in China (LE et al., 2025).

Among wild canids, red foxes (*Vulpes vulpes*) hold particular interest due to their adaptation to urban and semi-urban habitats, increasing the risk of pathogen spread to other animals and humans (ZECCHIN et al., 2019). Thus far, research on RVAs in red foxes was limited to a single outcome garnered from negative-contrast EM (EVANS, 1984), along with the more recent revelation of RVA causing encephalitis (BUSI et al., 2017). In Croatia, red foxes were found to host 11 G and nine P genotypes, including those typically associated with

pigs (e.g., G5, G9, G11, P[13], P[23]), and had a prevalence of 14.9%, suggesting a reservoir possibility (ČOLIĆ et al., 2021). Data on golden jackals (*Canis aureus*) are even more scarce. The only known study, also from Croatia, reported a 20.6% prevalence and identified two G and three P genotypes (ČOLIĆ, 2021). To date, only one complete RVA genome has been obtained from a red fox (BUSI et al., 2017), and none from jackals. These findings highlight a significant knowledge gap regarding the role of wild canids in RVA transmission and the zoonotic potential of wild and domestic animal-derived RVA strains within the ecosystem.

1.4.3. Interspecies transmission

RVAs have been detected in a wide range of hosts worldwide. Some RVA genotypes are more common in certain species, and many of them are shared between different species (MARTELLA et al., 2010; McDONALD et al., 2016). Therefore, aside from the aforementioned genome variations in individual RVA genomes, another major RVA diversification factor is significant interspecies transmission potential. Although typically host-specific, RVA can cross species barriers as demonstrated experimentally in murine models infected with avian PO-13 strains (MORI et al., 2001). Field studies have also detected avian-like RVAs in calves with diarrhea (BRÜSSOW et al., 1992; ROHWEDDER et al., 1995) and in a red fox with encephalitis (BUSI et al., 2017). Interspecies transmission events involving porcine (e.g., G3, G4, G5, G11, P[6], P[7]) and avian genotypes (G17P[17], G18P[17]) being found in cattle have also been observed (DÍAZ ALARCÓN et al., 2022; GHOSH and KOBAYASHI, 2014). Furthermore, the multiple events of zoonotic transmission of porcine originated RVAs were detected globally (VLASOVA et al., 2017).

In parallel, domestic pigs can be infected with porcine and human RVA strains and develop clinical disease (SAIF et al., 1996). Additionally, similar polymorphic histo-blood group antigens (HBGAs) are observed in humans and animals, antigens A and H in pigs and humans specifically. That may provide an explanation why RVA strains of the P[6] genotype (that recognize H antigen) are commonly found in and transmitted between humans and pigs in different countries (MARTELLA et al., 2006; DORO et al., 2015; VLASOVA et al., 2017), and why P[6] displays accentuated zoonotic potential compared to other VP4 genotypes. Even though it is considered unusual in the human population, a G4P[6] genotype was discovered to reappear in humans globally (TACHAROENMUANG et al., 2021). Some RVA genotypes that adapted to human hosts, such as G9 or G12, are considered to be of porcine origin (MARTELLA et al., 2010).

Except for a standalone G or P genotypes, certain G/P genotype combinations are considered usual or unusual depending on the species in which they are detected. For example,

the G4P[6] genotype combination is regarded as an unusual combination in humans, but it is quite common in pigs (DORO et al., 2015). The detection of a rare genotype combination like this one in a secondary host species may indicate a recent interspecies transmission event. In such cases, whole-genome sequencing can be used as a method of choice for strain investigation (DORO et al., 2015).

Direct interspecies transmission, frequently involving reassortment, represents a key mechanism by which RVA crosses host barriers. However, the limited detection frequency of zoonotic strains suggests that such occurrences remain rare (MARTELLA et al., 2010). Furthermore, the majority of molecular epidemiology studies on RVA are conducted in human populations, most often in children hospitalized due to acute RVA infection. In such populations, only about 2% of strains have been identified as having a zoonotic origin (MIDGLEY et al., 2012), affirming that interspecies transmission of RVAs in humans occurs sporadically (DHAMA et al., 2009). However, even sporadic transmission can potentially influence the epidemiology and the protective efficacy of available vaccines (MARTELLA et al., 2010), especially since the successful viral adaptation to a human host has been described (NGUYEN et al., 2024). It is theorized that the currently detected rate of zoonotic transmission is significantly lower than the actual rate, as RVA strain surveillance is almost exclusively limited to individuals with symptomatic illness (DORO et al., 2015). Approximately 75% of emerging infectious diseases in humans originate from animals, with wildlife serving as primary reservoirs for some high-impact pathogens (WOAH, 2024). These diseases disproportionately affect socioeconomically disadvantaged populations, particularly in developing countries (MALIK et al., 2020). Therefore, a collaborative One Health approach to the ecosystem as a whole is needed to address the health of humans, animals, and the environment, especially considering multi-species pathogens like RVA (CUNNINGHAM et al., 2017; MALIK et al., 2020; WEGNER et al., 2022).

1.5. Pathogenesis

The triple-layered capsid structure of RV provides relative stability on the virion and delivery into the small intestine without inactivation (ESTES and GREENBERG, 2013). RVs primarily infect mature enterocytes located at the tips and middle of intestinal villi and enteroendocrine cells in the small intestine. After entering the host's organism, the RV attaches to cell surface receptors of targeted cells via its VP4 protein (CRAWFORD et al., 2017). Upon exposure to trypsin, the VP4 protein cleaves into VP5 and VP8 subunits. The VP8 interacts with cell membrane receptors (such as sialoglycans and HBGAs), allowing the virus to enter

the cell through endocytosis or direct fusion with enterocytes (DHAMA et al., 2009; LUNDGREN and SVENSSON, 2001). Considering differences in receptor usage for target cell entry, studies have distinguished human RVs from animal RVs, with most human RVs binding human HBGAs and animal RVs binding sialylated glycans (BÖHM et al., 2015; SAXENA et al., 2015). It has been found that RV binds different glycans in a genotype-dependent manner, and this interaction can even be strain-specific (ARIAS and LOPEZ, 2021). This interaction via polymorphic HBGA happens in red blood cells, mucosal secretions, and epithelia, biased by a particular rotavirus P genotype (OMATOLA and OLANIRAN, 2022). HBGAs, namely antigen A and Lewis antigen, have been suggested to be genetic factors that determine host susceptibility. In addition, both secretor status and Lewis status (regulated by the fucosyltransferase 2 (FUT2) and fucosyltransferase 3 (FUT3) enzymes, respectively) have been proposed to mediate susceptibility to infection and possibly response to vaccination in an RV genotype-dependent manner (NORDGREN et al., 2014; SAXENA et al., 2015). Additionally, the binding pattern of three human RVs (P[9], P[14], and P[25]) to the type A antigen was observed. Their VP8 proteins were proven to bind the A antigens of the porcine and bovine mucins, suggesting the A antigen as a possible factor for cross-species transmission of RVs (LIU et al., 2012).

As described previously, once internalized by the receptor-mediated endocytosis, the low concentration of Ca^{2+} ions in the endosome causes the outer capsid layer to detach, releasing a transcriptionally active double-layered particle (DLP) in the cytoplasm (DESSELBERGER, 2014). The next step is the messenger RNA (mRNA) transcription and translation of viral proteins. The RNA genome is packaged into newly made DLPs in specialized structures called viroplasms, formed from lipid droplets. The newly made DLPs bind to NSP4, which serves as an endoplasmic reticulum receptor. The NSP4 also acts as a viroporin to release Ca^{2+} from intracellular stores. The triple-layered particle maturation happens as transient membranes are removed and the outer capsid proteins VP4 and VP7 assemble. Progeny virions are released through cell lysis or the Golgi-independent non-classical vesicular transport mechanism (ESTES and GREENBERG, 2013; DESSELBERGER, 2014; CRAWFORD et al., 2017; GELETU et al., 2021).

After the cellular release, RV from the intestinal lumen can enter the bloodstream and lymphatic system, circulating to various organs, including the liver, heart, lungs, kidneys, and central nervous system (DIAN et al., 2021). Although its presence outside the gastrointestinal tract is confirmed in both animals and humans, its full impact on these organs remains unclear (DIAN et al., 2021). The presence of avian RVA in the tissue outside the gastrointestinal tract

was discovered in the pancreas and spleen of broilers; however, the ability of RVs to cause viremia was hypothesized as a reason (NUNEZ et al., 2016).

Nonetheless, the most common clinical manifestation of RV infection is gastrointestinal distress, which results from several mechanisms. These include malabsorption resulting from enterocyte destruction, ischemia of the intestinal villi, and the neuro-regulatory release of vasoactive substances from infected epithelial cells. In addition, NSP4 protein functions as an enterotoxin, triggering age- and dose-dependent diarrhea. It acts as a secretory agonist that increases Ca^{2+}-dependent cellular permeability and disrupts epithelial barrier integrity (VLASOVA et al., 2017). Through cell damage and death of the mature enterocytes, immature enterocytes migrate more rapidly from the intestinal crypts to the surface of the villi, while still not being able to absorb, causing the shortening of the intestinal villi (CRAWFORD et al., 2017). The destruction of enterocytes following viral replication reduces the absorptive surface area, resulting in unabsorbed glucose and loss of electrolytes, which leads to osmotic imbalance and fluid accumulation in the lumen. Additionally, increased fluid secretion from intestinal crypts leads to diarrhea and acidosis (CRAWFORD et al., 2017). Consequently, chloride, sodium, potassium, and water malabsorption occur, leading to rapid osmotic watery diarrhea with a loss of electrolytes and dehydration (CRAWFORD et al., 2017). Another diarrhea-inducing mechanism of RVA is through the NSP4 enterotoxin protein, which has similar activity in mammals and birds, despite significant amino acid (aa) differences observed between these strains (DHAMA et al. 2015). Recent studies suggest RVs exploit the host's paracrine purinergic signaling to generate intercellular calcium waves that amplify the dysregulation of host cells and alter gastrointestinal physiology, resulting in diarrhea (CHANG-GRAHAM et al., 2020). Finally, with nutrient malabsorption reducing the food conversion ratio and dehydration possibly leading to death, animal husbandry faces severe economic impacts (DHAMA et al., 2015).

1.6. Clinical signs, gross and histopathology lesions

RV infection presents with a broad clinical spectrum, ranging from asymptomatic or mild watery diarrhea to severe gastroenteritis with vomiting and high fever. This can lead to dehydration with shock, electrolyte imbalances, and potentially death, particularly in young children and undernourished individuals (DIAN et al., 2021). Adults are also frequently infected, but mostly asymptomatic. Main clinical signs include fever and diarrheal stools without blood, mucus, or leukocytes (ESTES and GREENBERG, 2013). RVA-induced AGE is generally more severe than many other diarrheal etiologies, necessitating hospitalization

more frequently. Illness typically lasts three to five days in immunocompetent individuals, sometimes with hospital stays ranging from two to 14 days (ESTES and GREENBERG, 2013). In case of death, it was mainly attributed to dehydration and severe electrolyte imbalance, with vomit aspiration being a rarer cause. Over the years, mortality rates have decreased significantly due to early and aggressive rehydration therapy (ESTES and GREENBERG, 2013).

RV infection mainly affects the gastrointestinal tract, but is not limited to it. Experimentally, during the acute phase of RV infection, both antigenemia and viremia were detected in animals and children, indicating that RV can reach a multitude of host compartments (RAMIG, 2007; GOMEZ-RIAL et al., 2018). Likewise, naturally occurring infection with wild-type RV in both humans and other animals, viremia, and systemic spread were reported. RV was detected in multiple organs, including the brain, liver, spleen, lungs, heart, kidneys, pancreas, thymus, adrenal gland, bladder, testis, and immune cells (DIAN et al., 2021). The systemic spread has been associated with neurological symptoms, hepatobiliary diseases, pancreatitis, thrombocytopenia, respiratory illness, myocarditis, renal failure, and autoimmune diseases such as type 1 diabetes and celiac disease (DIAN et al., 2021; XU et al., 2023). Neurological symptoms may include seizures, meningitis, encephalopathy, and encephalitis (DIAN et al., 2021). Viral RNA is frequently detected in the cerebrospinal fluid, although this may reflect systemic viremia rather than direct RV replication in the central nervous system (ESTES and GREENBERG, 2013). Moreover, benign seizures in young children perhaps occur due to the elevated temperature, as seen regularly in RV infections (ESTES and GREENBERG, 2013). The RV findings in upper and lower respiratory tract samples indicated respiratory involvement, which may precede or accompany gastrointestinal symptoms (DIAN et al., 2021). Nevertheless, extraintestinal RV pathogenesis has been largely overlooked in research, leaving its key aspects poorly understood.

Reports of intussusception after oral administration of various (particularly first-generation) live attenuated RV vaccines were an unexpected outcome of the effective RV vaccination program (ESTES and GREENBERG, 2013). Previously, ultrasound examinations of infants showed that RV infection may cause lymphoid hyperplasia and intestinal wall damage, potentially predisposing to intussusception (ROBINSON et al. 2004; ESTES and GREENBERG, 2013). However, later evidence suggests that neither natural RV infection nor modern vaccines significantly increase the intussusception risk (BURNETT et al., 2020).

Experimental infection of piglets with G9P[23] and G9P[7] strains demonstrated clinical signs such as diarrhea and virus shedding beginning on day 1 post-inoculation and continuing for eight to 10 days (KIM et al., 2013). Clinical signs in affected pigs include white-

yellow diarrhea and possible dehydration (DEWEY et al., 2003; CHANG et al., 2012). The RVA-induced AGE typically lasts up to three days and can lead to lower weaning weights and poor average daily weight gain in both colostrum-deprived and colostrum-fed piglets. In some cases, body weight can drop by as much as 59% by the ninth day post-infection. Recovery time varies, as three-day-old piglets usually recover from intestinal lesions within six to 10 days, while 21-day-old piglets recover more quickly, in about two to four days. Litters that have had preweaning RV diarrhea are more likely to suffer from postweaning diarrhea, as well as additional issues such as skin and respiratory problems and reduced growth rates (DEWEY et al., 2003). RVA infection in domestic pigs results in pathoanatomical lesions primarily affecting the small intestine. Macroscopically, the intestinal wall becomes thin and dilated, particularly in the jejunum and ileum, with luminal contents appearing watery or containing undigested feed or milk (LUNDGREN and SVENSSON, 2001; BURROUGH, 2024).

Histological studies, in both human and animal models (piglets, calves, lambs and mice), illustrated RV-induced intestinal damage, including villous atrophy, loss of epithelial microvilli, and intraepithelial lymphocytosis, immune cells infiltration in the lamina propria, and intestinal wall hypotrophy, leading to functional impairment of the intestinal barrier in the small intestine (CRAWFORD et al., 2017). RV infection commonly affects the caudal two-thirds of the small intestine, where segmental villous atrophy is observed. Villi become shortened, blunted, and are covered by immature cuboidal epithelial cells that replace destroyed enterocytes (LUNDGREN and SVENSSON, 2001; BRNIĆ et al., 2023). This structural damage significantly reduces the villus-height to crypt-depth ratio to around 5:1, impairing nutrient absorption and resulting in osmotic diarrhea. RV-induced damage is further characterized by irregular and sparse microvilli and infiltration of mononuclear cells into the lamina propria (VLASOVA et al., 2017). RVA antigens were also detected in the colon of RVA-infected pigs. Immunohistochemistry confirmed the presence of RVA antigens in the enterocytes and the crypts of Lieberkühn (Figure 1) (BRNIĆ et al., 2023). Findings included a moderate increase in lymphocytes within the lamina propria, necrosis of individual surface epithelial cells, and the mesocolon edema (BRNIĆ et al., 2023).

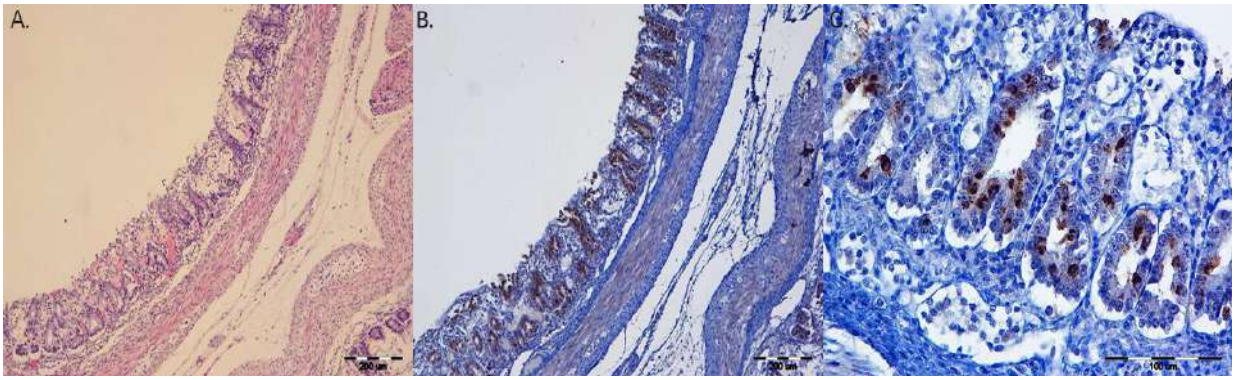


Figure 1. Histopathological and immunohistochemical findings in RVA infected piglets. **A.** Lesions in the colon were characterised by a moderate increase in the number of lymphocytes in the lamina propria, necrosis of individual surface epithelial cells, and oedema of the mesocolon (hematoxylin and eosin staining, 10x magnification). **B.** Positive immunohistochemistry reaction (brown colour) to RVA antigens was detected in the intestinal mucosa in colon (immunohistochemistry, 10x magnification). **C.** Intracytoplasmic brown granular staining was seen in the crypts of Lieberkűhn (immunohistochemistry, 40x magnification) (Source: BRNIĆ et al., 2023).

In addition to villus atrophy and crypt hyperplasia, substantial quantities of viral RNA were found in mesenteric lymph nodes, and viremia was confirmed by the detection of viral RNA in serum on days three and five days post-infection (KIM et al., 2013). Viral antigens can even be detected in the liver, lungs and choroid plexus, indicating a systemic spread of RVA in pigs (KIM et al., 2013). In general, villus shortening due to RV infection in pigs is less pronounced than when induced by coronaviruses (LUNDGREN and SVENSSON, 2001).

1.7. Diagnosis

Differential diagnosis in pigs includes the common causes of infectious diarrhea. This includes viruses (transmissible gastroenteritis virus, porcine epidemic diarrhea virus and other coronaviruses, other RV species, norovirus, other enteric viruses), bacteria (*Escherichia coli, Clostridium perfringens, Clostridium difficile, Enterococcus spp., Lawsonia intracellularis, Salmonella spp., Brachyspira spp.*), and parasites (*Cystoisospora suis, Cryptosporidium spp.* and nematodes). These agents can cause diarrhea ranging from mild to severe, potentially leading to high morbidity and mortality. Moreover, the underlying cause and clinical signs in

pigs or other hosts cannot be distinguished based on clinical presentation alone (KUMAR et al., 2022; LUPPI et al., 2023; BRNIĆ et al., 2023).

Molecular techniques such as real-time reverse transcription PCR (RT-qPCR) and conventional reverse transcription PCR (RT-PCR) are considered standard methods for the detection and genotyping of RVA in fecal or gastric samples. These assays offer high sensitivity and specificity, with multiplex RT-PCR enabling simultaneous detection of multiple RV species or genotypes (LUPPI et al., 2023). Real-time RT-qPCR assays usually target the VP6, VP2, and NSP3 gene segments (LOGAN et al., 2006; GUTIERREZ-AGUIRRE et al., 2008; MIJATOVIĆ RUSTEMPAŠIĆ et al., 2013). However, accurate surveillance depends on continuous updates of primers to compensate for RV genetic variability.

While virus isolation in cell culture is possible for RVA, it is laborious and time-consuming, prone to contamination, and as such, not requested for clinical diagnosis (OMATOLA and OLANIRAN, 2022). Commercially available ELISA kits serve as rapid screening tools for RVA antigen detection in feces, though equivalent assays for RVB and RVC are still lacking. In addition to antigen-based tests, histopathology can reveal intestinal lesions, and immunohistochemistry or in situ RNA hybridization techniques allow localization and differentiation of the virus within tissue samples. These complementary approaches support comprehensive diagnostic strategies, particularly in settings where subclinical infections or multiple enteric pathogens coexist (LUPPI et al., 2023).

Other methods can also be used, mainly in addition to the aforementioned methods. Firstly, the latex agglutination test (LAT) is a quick, simple test used to detect viral antigens in feces by observing the agglutination of latex particles coated with antibodies. Immunochromatographic assay (ICA), also known as a rapid test kit, is a rapid method suitable for field use, although it is generally less sensitive than ELISA or RT-PCR. However, ICAs present the method of choice for rapid clinical testing (EL-AGEERY et al, 2020). Polyacrylamide gel electrophoresis (PAGE) is used to identify RV by separating the viral RNA genome segments but is used less often (EL-AGEERY et al, 2020). Furthermore, EM allows for the direct visualization of the virus particles in feces but requires expensive equipment and skilled personnel (OMATOLA and OLANIRAN, 2022).

Sequencing technologies in virology have advanced through three main generations, each defined by its methodology and technological platform (HEATHER and CHAIN, 2016). The first generation, represented by Sanger sequencing, uses primer-based targeted sequencing and is known for its accuracy, but has limited throughput and shorter read lengths (HEATHER and CHAIN, 2016). Targeted sequencing of RVA can be used for singular gene segments or

for all 11 gene segments, in which case it can provide a complete genome. Gene-specific primers are designed to amplify each of the 11 genomic segments, allowing precise genotyping and detection (WHO, 2009). However, genetic variability often necessitates the redesign of primers or the use of degenerate primers to detect divergent strains, as seen in studies where variant-specific primers have improved genotyping accuracy (WHO, 2009). The second generation, also known as NGS, utilises Illumina platforms, which introduced massively parallel sequencing, enabling high-throughput and cost-effective analysis of large quantities of short DNA fragments (HEATHER and CHAIN, 2016). It enables various techniques for whole genome sequencing (WGS), such as target enrichment, PCR amplification and metagenomics shotgun sequencing (HOULDCROFT et al., 2017). The third generation, with technologies such as Oxford Nanopore and PacBio, allows for long-read sequencing (HEATHER and CHAIN, 2016). The development of NGS platforms, especially second and third generation, allows for abundant research and diagnostics opportunities by enabling comprehensive genomic characterization and overcoming limitations of primer-dependent methods.

Metagenomic sequencing bypasses specific pathogen amplification biases entirely in order to recover complete viral genomes directly from clinical specimens (WYLIE et al., 2018). It employs methods such as sequence-independent random amplification or sequence-independent adaptor-ligated dsRNA enrichment. Often used random amplification methods are multiple displacement amplification (MDA) or sequence-independent single-primer amplification (SISPA) (SMITS et al., 2014; VIBIN et al., 2018). The SISPA technique was used to successfully amplify RVA, RVC, and RVH genomes from metagenomic porcine samples, revealing evolutionary patterns undetectable by conventional methods (HULL et al., 2020). Metagenomics also enables broad surveillance of enteric viruses, as demonstrated in Dutch public health studies, where it detected RVA alongside norovirus, sapovirus, and enteroviruses in 39% of pediatric samples (SCHMITZ et al., 2023). For enhanced sensitivity, targeted capture panels like ViroCap enrich viral nucleic acids prior to sequencing, resulting in a consistent increase in viral read counts and enabling detection of antiviral resistance mutations (WYLIE et al., 2018). These high-throughput approaches provide critical data for updating molecular assays and tracking emerging variants across human and animal reservoirs (WHO, 2009; VIBIN et al., 2018).

1.8. Immunity

RVA infection impacts both the innate and adaptive immune responses. Upon RVA infection, mammalian cells, including intestinal epithelial cells, recognize viral dsRNA through

pattern recognition receptors (PRRs) (SPARRER and GACK, 2015). One key PRR involved is Toll-like receptor 3 (TLR3), which detects dsRNA and plays a critical role in the innate immune system. TLR3 is primarily located in the endosomes of immune cells such as dendritic cells and macrophages (SPARRER and GACK, 2015). Upon binding to dsRNA, TLR3 triggers a signalling cascade that leads to the production of type I interferons and other pro-inflammatory cytokines, which are crucial for fighting viral infections. Cytoplasmic, intracellular, PRRs RIG-I-like receptors (RLRs), including retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), mediate intracellular dsRNA detection. Engagement of these PRRs triggers downstream signaling leading to activation of IRF3 and NF-κB, resulting in the induction of type I and III interferons (IFNs) and pro-inflammatory cytokines like IL-6, IL-8 and IL-12 (ANGEL et al., 2012; HOLLOWAY and COULSON, 2013; DESSELBERGER, 2014; CLEMENTE et al., 2015). These responses inhibit early viral replication and recruit immune cells, notably macrophages and natural killer (NK) cells, to the site of infection. However, RVA has evolved potent immune evasion mechanisms. Its nonstructural protein NSP1 degrades key interferon regulatory factors (IRF3, IRF5, IRF7), suppressing IFN production and facilitating viral persistence (LIU et al., 2009). Meanwhile, the enterotoxic effects of NSP4 disrupt Ca^{2+} homeostasis and epithelial barrier integrity, resulting in diarrhea (DESSELBERGER, 2014).

Adaptive immunity is essential for viral clearance and long-term protection. Upon RV infection, acquired immune responses are triggered, including both, B cells producing virus-specific antibodies (Abs) and T cells recognizing RV epitopes on the surface of infected cells in MHC I and II antigen complexes (DESSELBERGER, 2014). During RV infection, Abs are produced against VP7, VP4, VP6, NSP3, and NSP4. However, the immune response to different proteins varies, and only VP7 and VP4 stimulate neutralizing Ab responses, many of which are neutralizing in vitro and protective in vivo (DESSELBERGER, 2014; KUMAR et al., 2022). Humoral responses, particularly the production of mucosal IgA targeting the outer capsid proteins VP4 and VP7, are the strongest correlates of protection. High intestinal and serum IgA titers correlate with immunity across species, including pigs and humans, rather than neutralizing antibody titers (DESSELBERGER and HUPPERTZ, 2011).

Humoral Abs boosted after repeated infection are directed against both serotype-specific and cross-reactive epitopes on VP4 and VP7 proteins, providing heterotypic protection (FRANCO et al., 2006). Cellular immunity also plays a role: CD8+ T cells secrete antiviral cytokines such as IFN-γ and TNF-α, aiding viral clearance (OMATOLA and OLANIRAN, 2022). Cross-reactive T cell epitopes present on VP4, VP6, and VP7 may contribute to

heterotypic protection. Nevertheless, RVA is a relatively poor inducer of robust cytotoxic T cell responses, especially in young human infants, where immune immaturity limits T cell memory development (DESSELBERGER and HUPPERTZ, 2011).

Piglets can be infected with porcine and human RV strains, resulting in disease either way (DESSELBERGER and HUPPERTZ, 2011). The absence of intrauterine immunoglobulin transfer in pigs makes neonatal piglets highly vulnerable, with protection relying heavily on passive lactogenic immunity (KUMAR et al., 2022). Passive immunity is primarily conferred to piglets transmammary, through high concentrations of IgG in colostrum and secretory IgA in both colostrum and milk. Among these, secretory IgA acts locally at the intestinal mucosal surface to neutralize RV (KUMAR et al., 2022). Strategies such as sow vaccination and natural planned exposure are employed to enhance maternal Ab levels and confer early protection. Additionally, the interaction between RVA infection, intestinal damage, and the gut microbiome in domestic pigs remains an area requiring further research. Understanding the complex interplay between RV and the host immune system is crucial for optimizing vaccine design, particularly to enhance mucosal IgA responses and cross-reactive immunity, and to overcome barriers observed in different host species and geographic settings (VLASOVA et al., 2017; KUMAR et al., 2022).

1.9. Vaccination

The goal of RV vaccination differs in humans and animals (MARTELLA et al., 2010). In humans, the primary objective is to induce active immunity once maternal Ab levels decline, ensuring protection during the early years of life. Conversely, in animals the primary method of protection is passive immunization through colostrum maternal antibodies. Commercial vaccines, particularly modified live vaccines, have demonstrated efficacy in reducing viral shedding and clinical disease during homologous challenges, though their effectiveness may vary against different RV strains (OMATOLA and OLANIRAN, 2022).

1.9.1. Vaccination in humans

The primary strategy for controlling RV infection currently relies on the use of live attenuated oral vaccines, particularly in countries with high child mortality rates. Several factors associated with the human host (e.g., malnutrition, HBGAs, concurrent administration with oral polio vaccine), pathogen (e.g., strain diversity, co-infections with other pathogens, and the viral load at exposure), and environment (enteropathy or dysbiosis of gut microbiome) have been suggested to possibly influence the differences in the efficacy of RV vaccines (OMATOLA and OLANIRAN, 2022). In addition, transplacentally acquired RV-specific IgG Abs in humans

protect newborns from infection and can interfere with immune responses to RV vaccination (APPAIAHGARI et al., 2014).

An advantage that RVA vaccination provides is the immune responses not only against the specific serotype included in the vaccine but also against heterologous serotypes (SCHWARTZ-CORNIL et al., 2002; DESSELBERGER, 2014). Since their World Health Organization (WHO) prequalification in 2008 and 2009, RotaTeq (RV5) and Rotarix (RV1) have become the most widely used vaccines for preventing rotavirus infections worldwide (BURKE et al., 2019). As of the end of 2018, Rotarix and RotaTeq have been enrolled in NIPs of 92 countries globally (BURKE et al., 2019), excluding Croatia (VRDOLJAK et al., 2019). Developed by GlaxoSmithKline Biologicals, Rotarix is an oral, monovalent vaccine introduced to the market in 2005 (DESSELBERGER, 2014). It contains a live-attenuated human G1P[8] RVA strain. In low-mortality countries, Rotarix prevented 90% of severe RVA diarrhea cases, and 51% of all-cause severe diarrhoea episodes. However, in high-mortality countries, Rotarix prevented 58% of severe RVA diarrhoea cases and 27% of severe all-cause diarrhoea cases (BERGMAN et al., 2021). In the same year, another oral vaccine launched by Merck and Co. Inc. as the pentavalent RotaTeq vaccine, which contains reassorted bovine-human RVA strains representing four common human G (G1, G2, G3, and G4) and one P genotype (P[8]) (DESSELBERGER, 2014). In low-mortality countries, RotaTeq prevented 97% of severe rotavirus diarrhoea cases. In medium-mortality countries, RotaTeq prevented 79% of severe rotavirus diarrhoea cases, while in high-mortality countries, RotaTeq prevented 57% of severe rotavirus diarrhoea cases with little to no difference in severe all cause diarrhoea cases (BERGMAN et al., 2021).

In 2018, the WHO prequalified two additional RV vaccines from India: Rotavac and Rotasiil. Rotavac is a monovalent vaccine containing a live-attenuated wild-type reassortant G9P[11] RV strain, that was developed by Bharat Biotech Ltd. Rotavac has not been assessed in any randomized controlled trials in countries with low or medium child mortality. In high-mortality countries, Rotavac prevented 57% of severe rotavirus diarrhoea cases and 16% of severe all-cause diarrhoea cases (BERGMAN et al., 2021). Rotasiil, developed by the Serum Institute of India Ltd., is a pentavalent vaccine containing human-bovine reassortant strains covering genotypes G1-G4 and G9. Both Rotavac and Rotasiil have been licensed internationally and have been introduced in India's NIP. Besides in India, Rotavac is currently used in Palestine and several African countries (OMATOLA and OLANIRAN, 2022).

As of early 2025, more than 131 countries had adopted either Rotarix or RotaTeq as part of their NIPs, including 127 countries that administer them routinely (IVAC VIEW-hub, 2025).

Only 13 countries in Europe provide a fully-funded program, and another five countries provide a partially-funded program. Partially funded implies either full funding for certain risk-groups and requires a parent co-payment for healthy children (Croatia) or is fully funded in specific regions (Sweden), or requires a parent co-payment (Belgium, Greece and Slovakia) (POELAERT et al., 2018).

Overall, Rotarix, RotaTeq, Rotasiil, and Rotavac are considered effective in preventing RV diarrhea. Relative effectiveness appears lower in high mortality rate countries in comparison with low mortality rate countries. Nevertheless, the absolute number of prevented cases is larger in high-mortality settings due to the higher baseline risk (BERGMAN et al., 2021). Importantly, no increased risk of serious adverse events, including intussusception, has been associated with any of the WHO-prequalified RV vaccines (BERGMAN et al., 2021). In addition to WHO-prequalified vaccines, two regionally licensed vaccines, Rotavin-M1 developed in Vietnam, and Lanzhou Lamb developed in China, are currently used nationally in Vietnam and India, respectively. However, WHO prequalification for these vaccines has not been received, and comprehensive large-scale efficacy trials and post-introduction impact evaluation are currently lacking for broad application (OMATOLA and OLANIRAN, 2022).

1.9.2. Animal Vaccination Strategies

In addition to minimizing RVA transmission through stringent hygiene practices, enhancing lactogenic immunity via vaccination remains the most effective strategy to prevent severe outcomes associated with RVA infection (PALMARINI, 2017). This strategy relies on maternal Abs, which are transferred either through the placenta (depending on the permeability of the placenta to maternal Abs) or via colostrum, offering short-term immunity against symptomatic RV infection. Since maternal Abs cannot transfer through the epitheliochorial (horses, pigs, etc.) or synepitheliochorial (ruminants, etc.) placentas, foals, piglets, and ruminant neonates are born without circulating maternal Abs and rely entirely on colostrum intake after birth to acquire passive immunity (CHUCRI et al., 2010). Therefore, pregnant animals are vaccinated in the later stages of pregnancy using either live attenuated or inactivated vaccines to boost lactogenic passive immunity in offspring (PAPP et al., 2013b, DORO et al., 2015).

In pigs, the variable efficacy of maternal RV vaccines observed in the field is influenced by several factors, including vaccine dose, viral strain, type of inactivating agent, choice of adjuvant, route of administration, and the level of RV exposure (VLASOVA et al., 2017). Interestingly, a live modified vaccine for active immunization of young piglets is available in the United States, following a strategy similar to that used in children. However, no such

vaccines are currently approved for use in pigs within the European Union, though importation from the USA remains an option (MONTEAGUDO et al., 2022).

Notably, aa variations of VP4 and VP7 antigenic epitopes may ultimately have an impact on vaccine efficacy, particularly if protection is based chiefly on G and P type specific responses (GELETU et al., 2021; PACKER and LITCHFIELD, 2025). The effective Ab titer of the G-specific neutralizing antiserum is affected by the aa composition of VP7 antigenic epitopes, even of the same G genotype (GELETU et al., 2021). In general, previous assumption was that vaccine efficacy was mainly influenced by protection based on specific G and P genotype responses, however new study has shown that backbone gene differences between RV strains influence vaccine effectiveness, highlighting the need for a broader approach to vaccine design (PACKER and LITCHFIELD, 2025).

1.10. Treatment and Non-vaccine Prevention Approaches

RV infection in pigs is managed primarily through supportive care, as there is no specific antiviral treatment available. Regardless, several potential anti-rotavirus drugs were described (OMATOLA and OLANIRAN, 2022). The main course of treatment for an RV infection is oral, subcutaneous, and intravenous rehydration (DESSELBERGER, 1999). To reduce losses, supportive care should involve administering fluids with glucose and electrolytes, using antibiotics to treat or prevent secondary bacterial infections, and providing warm, clean housing to lessen stress and the risk of additional infections (BURROUGH, 2024). Nutritional interventions, such as supplementing diets with spray-dried plasma have shown promise in alleviating intestinal damage and improving growth performance during and after infection (YAN et al., 2024). Probiotics, especially *Lactobacillus* and *Bifidobacterium* species, may help mitigate the severity of RVA infections by enhancing gut health and modulating immune responses, mechanisms still being under investigation (OMATOLA and OLANIRAN, 2022). In piglets, the orally administered L-glutamine has shown to improve fluid absorption (DESSELBERGER, 1999). In addition, oral administration of specific IgY appears to have considerable potential as a means of controlling diarrheal diseases and exerting growth-promoting activity in swine. The IgY technology is emerging as a promising alternative to antibiotics, with its key advantage being the ability to effectively control a wide range of pathogens (LI et al., 2015).

Since the development of RV vaccines proved to be difficult, largely due to the high antigenic variation of RVs, non-vaccine preventive strategies are crucial in management of RV outbreaks and include practices of good husbandry, and strict biosecurity. Biosecurity measures

do not make it possible to eradicate RVs from farming environments due to their ubiquity and resilience in the environment (CHANG et al., 2012). While enhanced hygiene practices can help reduce the incidence of RV infections, they are generally considered insufficient on their own. As a result, greater emphasis was placed on developing a strong local immune response, particularly because systemic immunity plays a relatively limited role compared to the critical importance of antibodies present in the intestinal lumen (MACLACHLAN and DUBOVI, 2016). Therefore, the ingestion of colostrum and milk rich in RV-specific Abs provides effective protection for piglets (MACLACHLAN and DUBOVI, 2016).

In conclusion, thorough and timely investigation of diarrhea outbreaks is essential for implementing effective pig health and biosecurity measures. Evaluating the impact of past outbreaks on specific production metrics can provide valuable insights, helping to inform and guide future management improvements (BRNIĆ et al., 2023).

1.11. Rotavirus Research in Croatia

In the past, research of the RVAs in Croatia had primarily focused on human-originated RVAs. The first insights into the prevalence of specific genotype combinations date back to the 2005 and 2006 RV seasons. During this period, the most frequently detected genotype combinations in humans were G1P[8] (21.8%), G2P[4] (19.2%), G4P[8] (12.6%), G8P[8] (6.8%), and G3P[8] (5%) (TCHEREMENSKAIA et al., 2007). This distribution pattern was considered uncommon, with a notably high prevalence of the G8P[8] genotype combination, which in 2006 appeared at an unusually high rate, predominantly among children under one year of age (TCHEREMENSKAIA et al., 2007; DELOGU et al., 2013). Additionally, a retrospective study was conducted at the Clinical Hospital Center Split, analyzing hospitalized preschool children with RVA-caused AGE from 2006 to 2008, during which a 35.12% contracted nosocomial RVA infections, with a median Vesikari score of 12 (VLASTELICA et al., 2010). In 2008, a notification of RV infection in Croatia became mandatory (MESZNER et al., 2013), while the active immunization against RV infection was introduced in 2011 just for risk populations. This recommendation addresses that infants should be vaccinated within the first six months of age with two (for RV1) or three (for RV5) doses of vaccine (TEŠOVIĆ et al., 2012). The next comprehensive study on RVA genotype diversity in the pediatric population was conducted between July 2012 and July 2014 (VRDOLJAK et al., 2019). During this period, G1P[8] emerged as the most prevalent genotype again (60.5%), commonly observed in countries where RV vaccination is not included in the NIP. It was followed by G2P[4] (21.2%), with various other genotype combinations each accounting for less than 4%. Genotype

prevalence was provided without the aspect of phylogenetic analysis (VRDOLJAK et al., 2019).

Until recently, no studies had been conducted in Croatia on RVs in animals or the environment. However, in April 2018, a comprehensive One Health RVA surveillance program of RVA in domestic and wild animals, humans, and environmental samples (RECO) was launched (BRNIĆ et al., 2018; ŠIMIĆ et al., 2019). This project marked the first study of its kind in Croatia to examine RVA in both animals and the environment, while also continuing the surveillance of genotype diversity in the human population. The RECO project lasted five years and yielded new insights, particularly regarding the molecular epidemiology and zoonotic potential of autochthonous RVA strains (BRNIĆ et al., 2018).

Prior to this doctoral thesis, in the scope of the RECO project, several studies about human RVAs were conducted from 2018 to 2022 (VILIBIĆ ČAVLEK et al., 2021), and indicated a dominance of genotype G3 (54%) with rising season-to-season prevalence of G3 equine-like (G3e) lineage. Furthermore, the P[8] genotype was detected in 79% of samples. On the contrary, strains with a zoonotic background were infrequent, with only 1.6% (BRNIĆ et al., 2022a). Furthermore, the circulation of human–animal reassortant strains in Croatia has been hypothesized by the sporadic detection of typical bovine genotypes G6, G8, G10, and P[14] in the human population (BRNIĆ et al., 2020; VILIBIĆ ČAVLEK et al., 2021). The zoonotic background of autochthonous RVA strains was especially evident for genotype G10 (BRNIĆ et al., 2019).

Master theses on animal and environmental RVAs, in the scope of the RECO project, discovered the high genetic heterogeneity of circulating strains in different domestic (DŽAKULA, 2019) and wild animal species (ČOLIĆ, 2021). In addition, the reported RVA prevalence in bivalve molluscan shellfish from December 2019 to January 2021 was 23% (17/74) (BRNIĆ et al., 2022b). The same study detected the presence of RVA genetic material in 22.2% (2/9) of surface water samples and 100% (21/21) of wastewater samples, suggesting possible environmental contamination (BRNIĆ et al., 2022b).

The aim of the Master's thesis from 2021, about genotyping of RVAs detected in wildlife in the Croatian territory, was to determine the RVA genotypes circulating in populations of red foxes (*Vulpes vulpes*), golden jackals (*Canis aureus*), wild boars (*Sus scrofa*), yellow-legged gulls (*Larus michahellis*), and black-headed gulls (*Larus ridibundus*). Using real-time RT-PCR, the presence of the RVA genes was confirmed in 11% of all analyzed samples. The genotyping results indicate a remarkable diversity and heterogeneity of RVA among wild animals in Croatia, while phylogenetic analysis suggests the potential for

interspecies transmission and underscores the importance of further RVA research in wild animal populations (ČOLIĆ, 2021). To our knowledge, the RVs of golden jackals (*Canis aureus*) have not been researched globally. The only available data are from Croatia, where a prevalence of 20.6% was reported, along with two G and three P genotypes (ČOLIĆ, 2021).
In the following study about the prevalence, molecular epidemiology, and genetic diversity of RVA strains circulating in the red fox (*Vulpes vulpes*) population in Croatia, 370 fecal samples were collected from 2018 to 2019. The results revealed the RVA prevalence of 14.9%, while the circulating RVA strains showed a remarkable genetic diversity in terms of 11 G and nine P genotypes, including G5, G9, G11, P[13] and P[23], considered to have a porcine origin. These were discovered along with a 14.9% prevalence (ČOLIĆ et al., 2021). These findings indicate a complexity behind the previous interspecies transmission events in the Croatian ecosystem, offering new insights into the possible role of foxes in the RVA epidemiology and the theory that they may serve as reservoirs for various RVA strains.

To elaborate further on enteric viruses in domestic pigs, including RVA and RVB, two diarrhea outbreaks on a large farrow-to-finish holding and subsequent circulation of outbreak-related enteric viruses were investigated (BRNIĆ et al., 2023).

Currently, significant knowledge gaps exist regarding autochthonous RVA in animals or the environment. Only limited data are available on the presence of domestic animal-derived RVA strains in humans and wild animal populations. Furthermore, information on their phylogenetic and whole-genome characteristics, as well as insights into interspecies transmission within the Croatian ecosystem, remains scarce. Considering this, it is crucial to downsize the current knowledge gaps about RVA prevalence and genomic diversity across human, domestic and wildlife populations. Conclusively, addressing the knowledge gaps may help to assess the occurrence of interspecies transmission and the putative influence on the protectiveness of currently available vaccines.

2. HYPOTHESIS AND OBJECTIVES

The hypothesis: The interspecies transmission of RVAs, typical for domestic pigs, sporadically occurs in the Croatian ecosystem.

General objective: To investigate interspecies transmission and genomic properties of autochthonous porcine-originated RVAs (poRVAs) in domestic pigs, humans and wild animals through a synchronized spatiotemporal One Health approach.

Specific objectives:

1. To determine the prevalence and genetic diversity of RVAs circulating in domestic pigs and wild boars.
2. To compare the prevalence of RVA in domestic pigs between two groups for each of the factors of farm type, age, sex and the presence of clinical signs.
3. To investigate the zoonotic transmission of poRVAs.
4. To explore the interspecies transmission of poRVAs among wild animals and domestic pigs.
5. To evaluate the influence of gene reassortment and intragenic recombination on poRVAs complete genome diversity.

3. MATERIAL AND METHODS

3.1. Sampling

The poRVA genomes analyzed in this doctoral thesis were obtained from samples collected in Croatia over three consecutive years (2018–2021), as part of the broader One Health RVA surveillance project *Reco*- "Rotaviruses in Croatian Ecosystem: molecular epidemiology and zoonotic potential". Sampling was carried out continuously throughout this period, encompassing, but not limited to, the RV seasons of 2018/2019, 2019/2020, and 2020/2021. Moreover, sampling comprised both in-season and out-of-season periods, ensuring year-round RVA surveillance. Each individual was sampled only once. During this surveillance, 445 fecal samples or rectal swabs were collected from domestic pigs (*Sus scrofa domesticus*), 441 from wild boars (*Sus scrofa*), 533 from red foxes (*Vulpes vulpes*), 131 from golden jackals (*Canis aureus*), and 602 from humans (*Homo sapiens*). In total, 2152 fecal material or rectal swab samples were processed. The sampling plan targeted a minimum of 420 samples per group to enable detection of an estimated RVA prevalence of approximately 30% in domestic animals and 9% in wildlife, with 95% confidence, a margin of error between 5% and 8%, and assumed test sensitivity and specificity of 95% and 99%, respectively (SERGEANT, 2018). For sample size calculations, red foxes and golden jackals were grouped under the mutual category of wild canids. Humans were excluded from the sample size calculation, since these samples came exclusively from symptomatic individuals hospitalized due to RVA infection. Nevertheless, the sample size of human samples matched that of the other species.

Domestic pigs were sampled at large industrial and small backyard holdings in Croatia. Most of their samples (98.2%) were collected during October to March. According to the NUTS-2 classification, sampled domestic pigs originated from seven counties located in Continental Croatia (Pannonian Croatia, Northern Croatia, and the City of Zagreb) and one county (Split-Dalmatia County) located in Adriatic Croatia. Domestic pigs included in the present study were locally bred on 24 small backyard holdings (n = 276) and eight large holdings (n = 169). Holdings that breed imported weanling and fattening pigs were excluded from the study. The age, gender and status of diarrhea were registered at the time of sampling. Domestic pigs were divided into four age groups: suckling piglets (<28 days; n = 231), weanling pigs (29–84 days; n = 177), fattening pigs (>85 days, n = 28) and sows (n = 9). The sex was reported for 385 domestic pigs, comprising 178 females and 207 males. Diarrhea was observed in 165 domestic

pigs (37.1%), while the remaining 280 domestic pigs (62.9%) showed no gastrointestinal clinical signs and were considered clinically healthy. Wild boars were sampled after regular hunting in 15 hunting areas in Croatia. The hunting areas were located in eight counties in three regions of Continental Croatia (Pannonian Croatia, Northern Croatia, and the City of Zagreb). On the other hand, three age groups were defined for wild boars: <1 year (n = 151), 1–2 years (n = 135) and >2 years (n = 155), based on farrowing date. The sex was reported for 440 wild boars (223 females and 217 males). Diarrhea was registered in only eight wild boars (1.8%), while 433 wild boars (98.2%) were free of clinical signs regarding the gastrointestinal tract. Like in domestic pigs, the majority (78.9%) of wild boar samples were collected during the autumn/winter months (October to March). Samples were collected from individual animals using rectal swabs for domestic pigs, and with a plastic scoop attached to the container lid for fecal or intestinal content from wild boars.

Human samples mostly included children under 5 years of age with present clinical signs of acute gastroenteritis, consequently admitted to the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" Zagreb, Clinical Hospital Center Osijek, and Clinical Hospital Center Split. The collected human stool samples were initially tested for the presence of rotaviral and adenoviral antigens in their respective hospital centers, using a commercial immunochromatographic assay, the Rota-AdenoGnost (BioGnost, Zagreb, Croatia).

Fecal samples from wild canids were collected from red foxes and golden jackals hunted as a part of active surveillance conducted during the anti-rabies oral vaccination campaign, organized by the Veterinary and Food Safety Directorate of the Croatian Ministry of Agriculture, Forestry and Fisheries. In contrast to domestic pigs, where sampling targeted mostly younger age groups, wildlife samples (wild boars, red foxes, and golden jackals) were collected upholding hunting regulations, resulting in the majority being adult animals. Samples were collected directly from the rectum of wild canid carcasses received at the Croatian Veterinary Institute. Upon collection, all samples were transferred to the Croatian Veterinary Institute for subsequent laboratory testing, maintaining a cold chain while in transportation. The samples were further processed immediately after reception or stored at −20°C.

3.2. *Rotavirus A* detection and genotyping

3.2.1. RNA Extraction

RNA was extracted from the supernatant of 20% w/v fecal/rectal swab suspension, which was prepared using Medium 199 (Sigma Aldrich, St. Louis, USA), vortexed and centrifuged at 14,000g. The RNA extraction procedure was performed on the KingFisher™

Flex purification system (ThermoFisher Scientific, Waltham, USA) using the MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, Waltham, USA) following the manufacturer's instructions for fecal samples known as the complex workflow. The exogenous Internal Positive Control (IPC) RNA, Xeno™ RNA Control (ThermoFisher Scientific, Waltham, USA), was added to each sample (2µL) to supervise the appearance of potential PCR inhibitors. The extracted RNA was stored at -80°C if not processed immediately.

3.2.2. Real-time RT-PCR

Detection of RVA dsRNA was performed using real-time RT-PCR targeting a fragment of the VP2 gene, which is conserved among various RVA genotypes infecting humans and domestic animals (GUTIÉRREZ-AGUIRRE et al., 2008). Nevertheless, this protocol was previously successfully applied for RVA detection in wildlife-related research (JAMNIKAR-CIGLENECKI et al., 2016; ČOLIĆ et al., 2021). Before performing one-step real-time RT-PCR, the RVA dsRNA was denatured at 95°C for 2 minutes in the presence of the primer mix (600 nM) and PCR-grade water. The final reaction mixture included the denatured RNA solution from the previous step, reagents of the VetMAX™-Plus One-Step RT-PCR Kit (ThermoFisher Scientific, Waltham, USA), the VP2-specific probe (200nM), and the VetMAX™ Xeno™ Internal Positive Control (IPC)—VIC™ Assay (ThermoFisher Scientific, Waltham, USA). The reaction setup and thermal cycling conditions were carried out according to the manufacturer's instructions. The runs were performed on a Rotor-Gene Q or QIAquant 96 5plex (Qiagen, Hilden, Germany). If inhibition was observed, the samples were diluted to 1:5 and retested.

3.2.3. VP7 and VP4 Genotyping

All VP2-positive samples underwent genotyping to determine G (VP7) and P (VP4) genotypes. For animal samples, due to higher genetic diversity, multiple primer sets and protocols were utilized (Table 3). In human samples, a multiplex VP7/VP4 RT-PCR (EUROROTANET, 2009; FUJII et al., 2019) was used, with Sanger sequencing for untypable strains.

In PAPER I, the VP7 genotyping was performed utilizing a combination of VP7 Beg9 and VP7 End9 primers (GOUVEA et al., 1990) in the first round of RT-PCR followed by the nested PCR using VP7-up2 and VP7-down3 primers (ABE et al., 2009). The next approach was the RT-PCR using VP7-F and VP7-R primers, followed by the seminested PCR using VP7-F and VP7-RINT primers (EUROROTANET, 2009) if the result of the first RT-PCR reaction was negative. In some cases, we applied primers N-VP7F1 and N-VP7R1 in the first round of RT-PCR, and primers N-VP7F2 and N-VP7R2 in the nested PCR. These primer sets were designed for samples containing low RVA load (MIJATOVIC-RUSTEMPASIC et al., 2016).

The VP4 genotyping was a combination of three different approaches as well. One approach was the application of VP4-HeadF and VP4-1094R2 primers in the RT-PCR followed by the seminested PCR using VP4-HeadF and VP4-887R primers (ABE et al., 2009). The other one was a combination of VP4_1-17F and VP4R_DEG primers in the RT-PCR reaction (THEUNS et al., 2014). The last approach consisted of N-VP4F1 and N-VP4R1 in the RT-PCR, and N-VP4F2 and N-VP4R2 in the nested PCR (MIJATOVIC-RUSTEMPASIC et al., 2016).

In PAPERS I, II, III, all RT-PCR reactions were conducted with the utilization of SuperScript™III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (ThermoFisher Scientific, Waltham, USA). For the nested or seminested PCR, GoTaq® G2 Hot Start Colorless Master Mix (Promega, Madison, USA) was utilized. Primer concentrations and annealing temperatures used in each RT-PCR and nested or seminested PCR reaction were as recommended by the article, citing respective primer sequences, listed in the previous paragraph. Other conditions related to reaction mixture preparation and thermal cycling were applied according to the manufacturer's recommendations. Each reaction started with the initial dsRNA denaturation step at 95°C for 5 min, in which the extracted RNA was combined with the respective forward primer and PCR-grade water. Hereafter, the remaining reagents were added to the reaction mixture, which was run on the ABI 9700 GeneAmp thermal cycler (Applied Biosystems, Foster City, USA) or Biometra TRIO (Analytic Jena, Jena, Germany). PCR products were visualized on the QIAxcel Advanced System for capillary electrophoresis using the QIAxcel DNA Screening kit (Qiagen, Hilden, Germany). All animal-derived VP7 and VP4 PCR products were sent for Sanger sequencing, regardless of typeability.

Table 3. Primers for RVA VP7 and VP4 genotyping in animal-derived samples.

Primer ID*	Primer orientation	Primer sequence (5' – 3')	Location in genome (nt)	Tm (°C)	Product length (bp)
VP7-F	forward	ATGTATGGTATTGAATATACCAC	51-71	50.0	881
VP7-R	reverse	AACTTGCCACCATTTTTTCC	914-932	56.3	
VP7-RINT	reverse	ANAYNGANCCWGTYGGCCA	331-334	63.9	293
VP7 Beg9	forward	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	62.1	1062
VP7 End9	reverse	GGTCACATCATACAATTCTAATCTAAG	1036-1062	54.4	
VP7-up2	forward	GCTCCTTTTAATGTATGGTA	39-58	50.4	956
VP7-down3	reverse	GATCTYGATCTYTTGGACAT	976-995	54.1	
N-VP7F1	forward	TAGCTCCTTTTRATGTATGGTA	37-58	53.0	333
N-VP7R1	reverse	GTNGGCCATCCTTTNGT	354-370	58.1	
N-VP7F2	forward	ATGTATGGTATTGAATATACCAC	49-71	50.0	193
N-VP7R2	reverse	GTRTCCATDGATCCAGTNATTGG	220-242	59.1	
VP4_1-17F	forward	GGCTATAAAATGGCTTCGC	1-19	55.0	700
VP4R_DEG	reverse	TCYCTRTTRTATTGCATYTCYTTCC	?	57.9	
VP4 HeadF	forward	GGCTATAAAATGGCTTCGCTCATTTA	1-27	58.2	1100
VP4-1094R2	reverse	AATGCTTGTGARTCRTCCCARTAATC	1076-1101	60.4	
VP4-F	forward	TATGCTCCAGTNAATTGG	132-149	52.1	663
VP4-R	reverse	ATTGCATTTCTTTCCATAATG	775-795	47.7	
Rota-Seg4-s	forward	TCTAARACATCATTNTGGAARGA	766-788	54.7	312
Rota-Seg4-as	reverse	GCTTGTGAATCRTCCCARTTC	1057-1078	58.4	
N-VP4F1	forward	GGCTATAAAATGGYTTCNYT	1-20	52.9	257
N-VP4R1	reverse	ARYADCCARTAATCRNYDRTG	236-257	56.9	
N-VP4F1	forward	ATGGYTTCNYTMATTTATAGACA	10-32	52.6	214
N-VP4R2	reverse	GNTGGYTGATAWGGACCRCKA	203-224	62.0	

*citations for each primer pair are provided in subsection 3.2.3.

3.2.4. Sanger Sequencing and Genotype Assignment

RT-PCR and nested PCR products were purified with ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific, Waltham, USA) or Monarch DNA Gel Extraction Kit (New England Biolabs, Ipswich, USA) following the manufacturer's instructions. Subsequently, the samples were subjected to Sanger sequencing in forward and reverse directions using the services of Macrogen Europe (Amsterdam, the Netherlands). The RVA genotypes of VP7 and VP4 segments were assigned by following previously defined genotype cutoff values (MATTHIJNSSENS et al., 2008a), in addition to using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in combination with the ViPR tool (PICKETT et al., 2012), available at https://www.viprbrc.org/. During the genotyping process and Sanger sequencing data analysis, VP7 and VP4 RVA genotypes of typical porcine origin were detected in multiple species, leading to the presumed sporadic interspecies transmission of poRVAs in Croatia. These strains were investigated to expand the One Health perspective of poRVA interspecies transmission in the Croatian ecosystem. Therefore, samples from humans (PAPER II) and multiple wildlife species (wild boars, red foxes, and golden jackals) (PAPER III) in which poRVAs were detected, along with strains from domestic pigs with matching genotypes (PAPER I), were selected for NGS. Considering additional excluding practical criteria (e.g. quantity of collected samples), a total of 25 samples matching these criteria were selected for NGS (PAPER II, PAPER III).

3.3. NGS

Following administrative processing, all samples underwent initial laboratory procedures, including nucleic acid extraction, RVA VP2 real-time RT-PCR, VP7 and VP4 genotyping, Sanger sequencing and sequence analysis, as detailed in PAPERS I, II and III. During the genotyping process and Sanger sequencing data analysis, VP7/VP4 RVA genotypes of typical porcine origin were detected in multiple species. These strains were investigated to expand the One Health perspective of poRVA interspecies transmission in the Croatian ecosystem.

3.3.1. Library preparation and NGS

For PAPER II and PAPER III, rectal swab suspensions and fecal suspensions (20% w/v) prepared with Medium 199 (Sigma-Aldrich, St. Louis, USA) were used as a starting material for the NGS sample preparation. Suspensions were vortexed and centrifuged at 14,000g. The supernatant was used for nucleic acid extraction, which was performed on a Maelstrom 9600 device (TANBead Inc., Taoyuan City, Taiwan) using an OptiPure Viral Auto Plate (TANBead

Inc., Taoyuan City, Taiwan) extraction kit. In addition to the initial RVA VP2 detection by real-time RT-PCR (described in subsection 3.2.2.), a real-time RT-PCR assay using the LightMix Modular Rotavirus A assay (TIB Molbiol, Berlin, Germany) on a LightCycler 480 instrument (Roche, Basel, Switzerland) was also employed. Since viral RNA genome loads in metagenomic samples tend to be exceptionally low in concentration, DNA depletion was performed using the TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, USA). After the DNA removal, the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific™, Waltham, USA) was used for the first- and second-strand complementary DNA (cDNA) synthesis. Prepared cDNA was then purified utilizing the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, USA) to remove excess dNTPs and other reagents such as competing enzymes or buffer components. All procedures referenced above were performed following the respective manufacturer's instructions. The cDNA was finally quantified before proceeding with library preparation, using a Qubit™ 4 Fluorometer with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA). NGS libraries were constructed using a Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, USA) with barcoding respective samples with the IDT® for Illumina® Nextera DNA/RNA Unique Dual Indexes Set B and C (Illumina Inc., San Diego, USA) according to the manufacturer's instructions. After tagmentation and amplification, NGS libraries were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, USA). The quality and quantity of the purified libraries were assessed with a 2100 Bioanalyzer instrument (Agilent, Santa Clara, USA) using a High Sensitivity DNA Kit (Agilent, Santa Clara, USA), and a Qubit™ 4 Fluorometer using Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, USA), respectively. NGS was performed on lllumina® NextSeq 500 sequencer (Illumina Inc., San Diego, USA) utilizing the NextSeq 500/550 High Output Kit v 2.5 on 300 cycles (Illumina Inc., San Diego, USA) to produce 150 paired-end reads.

3.3.2. NGS data analysis

NGS data analysis, in the scope of PAPER II, and III, was performed using CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany). Representative reference sequences for each of the 11 RVA genomic segments, covering various genotypes, were selected from NCBI's Virus Variation Rotavirus Database (HATCHER et al., 2017) to build reference lists for each gene segment. Coding sequences (CDS) were assembled using a reference-based mapping process for each segment, reflecting the segmented nature of the RV genome. The workflow consisted of trimming raw reads of Illumina adapters, mapping trimmed reads to the segments reference lists, and extracting consensus sequences and mapping reports. Consensus

sequences were not considered for further investigation if they did not meet the previously defined minimum sequence length and identity criteria (MATTHIJNSSENS et al., 2008a) or distribution coverage of 90% and coverage depth of 10×. Final consensus sequences for every gene segment prior to the genotyping process were selected based on the mapping quality and the consequent full-length consensus sequence completeness. Genotypes were confirmed using final consensus sequences as queries, in the BLAST search tool3 in addition to the ViPR tool version 3.28.224 (PICKETT et al., 2012), and characterized following previously described guidelines defining genotype cutoff values (MATTHIJNSSENS et al., 2008a). During these searches, any consensus sequence that did not hold up to the respective genotype it was initially mapped to was herein discarded as a result of the mapping error. Strain names were assigned according to the RVA nomenclature uniformity guidelines administered by the RCWG. The CDSs that shared the highest percentage identity with each query or representatives of a certain group of sequences were used to assemble multiple sequence alignments and conduct evolutionary analyses in MEGA 11 software (TAMURA et al., 2021).

3.3.3. Addressing gaps in reference-based consensus assemblies

In PAPERS II and III, consensus sequence gaps were addressed when possible. In PAPER II, the approach included performing *de novo* assembly for the NGS samples and correlating contigs with the gapped reference-based consensus assemblies. The *de novo* assembly was performed in CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany), using default program settings. In addition to the first approach, in PAPER III the second approach was to design segment-specific primers (Table 4) if the first approach produced no results. The primers were designed using partial reference-based consensus assemblies, ensuring high specificity to enhance the completeness of the targeted consensus sequences. In accordance with the codon degeneracy, each primer pair was designed to cover multiple partial sequences. RVA gene segment-specific primers were designed to address gaps in reference-based consensus assemblies. Primer pairs that successfully amplified partial RVA CDSs for their respective segments as PCR products are listed in Table 4. In total, these primers improved the completeness of six reference-based assemblies.

Table 4. List of RVA gene segment-specific primers designed for addressing gaps in reference-based consensus assemblies. The Primer ID column quotes the targeted RVA gene segment for each primer. The table details the primer orientation, primer sequences, location in respective RVA gene segments, melting temperature (Tm), and product length. The location in the genome was determined considering the location in the Open Reading Frame (ORF) of the respective gene segments.

Primer ID	Primer orientation	Primer sequence (5' – 3')	Location in genome (nt)	Tm (°C)	Product length (bp)
VP1-F_12-31	forward	CTRTACWATGGGGAAGTAC	13–31	53.0	932
VP1-R_925-944	reverse	TCTTGAATCATYCTYGGTAT	925–944	50.2	
VP2-F_11-27	forward	GGYTCAATGGCGTACAG	11–27	52.4	493
VP2-R_485-503	reverse	TCAAYTTCCAATACCATCT	485–503	48.7	
VP4_(P6)-F_1774-1790	forward	GTATGGACGGAYGTYTC	1774–1790	50.0	586
VP4_(P6)-R_2343-2359	reverse	GGTCACATCCRCTATAG	2343–2359	50.0	
NSP1-F_13-31	forward	TTTATGAAAAGTCTTGTGG	13–31	48.7	481
NSP1-R_475-493	reverse	CACCATCSAATTCTAYYGA	475–493	50.9	
NSP3-F_22-39	forward	GTTGATGCTCAAGATGGA	22–39	51.6	932
NSP3-R_936-953	reverse	ATTCRTARTTGCATTGCC	936–953	47.0	

RT-PCR was applied using a SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Applied Biosystems, Waltham, USA) on a Biometra Trio thermocycler (Analytik Jena, Jena, Germany). The dsRNA denaturation step was done at 95°C for 5 minutes in which extracted RNA was combined with the respective forward primer and

PCR grade water. Hereafter, the remaining reagents were added to the reaction mixture and the thermal cycling conditions were as follows: reverse transcription at 45 °C, 30 min and denaturation at 94 °C, 2 min, followed by 40 cycles that included denaturation at 94 °C for 15 s. Annealing temperatures (Ta) cycles were applied for 30 s. The Ta was adjusted for each primer pair, corresponding to primer melting temperatures (Tm) according to MIQE guidelines (NOUR AND PFAFFL, 2020); continuing with 1 min elongation step at 68 °C. The final step was elongation at 68 °C for 5 min. Forward and reverse primer concentrations in the reaction mixtures were adjusted to 600 nmol/L. RT-PCR products were visualized on the QIAxcel Advanced System for capillary electrophoresis using the QIAxcel DNA Screening kit (Qiagen, Hilden, Germany), and sent to Sanger sequencing to Macrogen (Amsterdam, Netherlands). Finally, RT-PCR product sequences were matched with reference-based consensus assemblies to fill consensus gaps, where applicable.

3.4. Phylogenetic analysis and pairwise identity matrices

To investigate the evolutionary relationship between autochthonous poRVA strains presented in this thesis, individual phylogenetic trees for VP7/VP4 (PAPER I), or for the 11 RVA genomic segments (PAPER II, PAPER III) were constructed. Therefore, the representative strains from GenBank were selected based on their high percentage identity with the herein presented query sequences and comparability based on geolocation, origin, host, or lineage for comparison purposes. In each PAPER, the evolutionary history was inferred using the maximum-likelihood (ML) method for each multiple sequence alignment obtained by the MUSCLE algorithm, both acquired utilizing MEGA 11 software (TAMURA et al., 2021). In PAPER I, two substitution models with the lowest Bayesian Information Criterion (BIC) score were applied: T92+G+I (all VP7 and VP4 sequences of P[13], P[23] and P[32] genotypes) and T92+G (VP4 sequences of P[6], P[7], P[8] and P[11] genotypes). The substitution models yielding the lowest BIC scores in the PAPER II dataset were as follows: T92+G (VP6, NSP2, NSP4, NSP5), T92+G+I (VP7, NSP1, NSP3), TN93+G+I (VP2), GTR+G+I (VP1, VP3), and HYK+G+I (VP4). In the PAPER III dataset, substitution models demonstrating the lowest BIC value were T92 + G + I (VP7, VP6, NSP2, NSP3, NSP5), GTR + G + I (VP4, VP2, VP3, NSP1), TN93 + G + I (VP1), and T92 + G (NSP4).

In all three PAPERS, the bootstrap analysis with 1,000 replicates was used to assess the branching support for each ML tree. At the same time, the evolutionary history was inferred using the maximum-likelihood (ML) method for each multiple sequence alignment obtained by the MUSCLE algorithm (using default settings), both performed in MEGA 11 software

(TAMURA et al., 2021). The phylogenetic trees were visualized and annotated using the iTOL version 6.5.8. in PAPER I and II, and iTOL version 7 in PAPER III (LETUNIC and BORK, 2021).

In the PAPER I, the nt and aa pairwise identity matrices and graphical overview of the temporal distribution of RVA genotypes circulating in domestic pigs were calculated in R using the bio3d package, ggplot2 and Scatter Pie Plot (GRANT et al., 2006; WICKHAM, 2016; YU, 2021; R CORE TEAM, 2022). In PAPERS II and III, CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany) was used to calculate pairwise identity matrices among the previously aligned RVA sequences from the GenBank and the autochthonous poRVAs.

3.5. Lineage demarcation

Lineage demarcation was conducted in the PAPERS I and II. In PAPER I, lineage demarcation for a particular genotype was set by the previously recommended classification for G1, G2, G3, G4, G6, G9, P[6] and P[8] genotypes (PHAN et al., 2007a; PHAN et al., 2007b; STEYER et al., 2008; AFRAD et al., 2014; JAMNIKAR-CIGLENECKI et al.; 2016; KATZ et al., 2019; WANDERA et al., 2021; BONURA et al., 2022). It was done so due to their high frequency in humans (G1-G4, G9 and P[8]) or due to the close phylogenetic relatedness observed between human and animal RVA strains (G6 and P[6]). Due to the overall inconsistency in nomenclature and the lack of consensus on lineage demarcation, lineages were not assigned for other G and P genotypes reported in PAPER I. In PAPER II, different G4 lineages were determined based on lineage attribution from WANDERA et al. (2021). Lineages of the P[6] genotype were assigned according to the attributions described by MARINGA et al. (2020) and WANDERA et al. (2021). Lineage determination for backbone RVA gene segments was not performed due to the general inconsistency in the nomenclature and/or the absence of consensus in the lineage demarcation.

In PAPER III, G and P genotype lineages were not defined due to inconsistencies in nomenclature and the lack of consensus on lineage demarcation criteria. Additionally, the RVA genomes derived from wildlife reported in PAPER III were either the first or among the earliest published, resulting in a lack of sufficient reference sequences to support reliable lineage demarcation for these strains.

3.6. Intragenic recombination and reassortment analysis

Intragenic recombination and reassortment analyses were conducted in PAPER II and III, since these analyses are whole-genome based. In PAPER II, utilizing the BLAST tool, we identified and downloaded complete BLAST search results for each of the 11 gene segments of six G4P[6] Croatian strains, including their respective mixed genotypes where applicable. Multiple sequence alignment sets were constructed as described earlier (subsection 3.4.). In PAPER III, intragenic recombination analysis was also performed on each of the 11 RVA gene segments, on the same taxa used in the phylogenetic analysis for each RVA gene segment. Intragenic/homologous recombination analysis, including both intragenotype and intergenotype (for genes with apparent mixed genotypes), was conducted using RDP software (v.4.101 in PAPER II and v.5.64 in PAPER III). Seven integrated recombination detection methods were applied: RDP, GENECONV, MaxChi, Bootscan, Chimera, SiScan, and 3Seq (MARTIN et al., 2015). For every detected recombination event, the UPGMA method integrated in RDP constructed breakpoint-defined major and minor parent phylogenetic trees (data not shown). The term "parent" does not identify the exact evolutionary progenitors of recombinant strains, but rather represents groups of RVA strains from which the actual progenitors may have originated. Only recombination events predicted by at least six of the seven methods were considered as positive homologous recombination signals (HOXIE and DENNEHY, 2020). Since ancestral state reconstruction was not conducted, sequences with detected recombination were retained in the phylogenetic analysis without removing recombinant regions. This was done to illustrate the phylogenetic effects of recombination-induced genotype divergence.

Reassortment events in PAPER II were evaluated during the phylogenetic analysis, alongside nt and aa percentage identity calculations. To explore reassortment more thoroughly, in PAPER III, complete genome concatenation was conducted in CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany) for 10 RVA genomes with fully acquired ORFs for all 11 gene segments. Multiple sequence alignment of concatenated genomes was acquired as previously described in subsection 3.4. The concatenated ORFs were uploaded to Simplot++ software (SAMSON et al., 2022) for bootscan analysis using the following parameters: a window size of 200 bp, a step size of 200 bp, 500 repetitions, the Kimura 2-Parameter distance model, and a percentage of permuted trees calculated using the Neighbor-Joining algorithm.

3.7. Statistical Analysis

In PAPER I descriptive statistics (prevalence) and comparison of the type of holding (farm/backyard), age and gender in affected (diarrheic) and non-affected animals (non-diarrheic) were performed in SYSTAT Software v.13.2 (Systat Software, Inc., Chicago, USA).

For the categorical data analysis , the χ^2 test and log-linear model (LLM) were used. For all analyses, $p < 0.05$ was considered statistically significant. In PAPERS II and III, a Bonferroni-corrected p-value threshold of 0.05 was applied in the RDP software to identify statistically significant intragenic intra- and intergenotype recombination events.

3.8. Data availability

3.8.1. Deposited RVA sequences

RVA nt and aa sequences characterized in the PAPER I are deposited in the GenBank under accession numbers OL440064-OL440111, ON017591-ON017611, ON647404-ON647430, ON721080-ON721102, and OP136969.

RVA nt and aa sequences characterized in PAPER II were submitted to the GenBank with adjacent accession numbers: D230: OQ440159-OQ440170; D329: OQ440171-OQ440184; D572: OQ440185-OQ440195; S243: OQ440196-OQ440210; S338: OQ440211-OQ440223; and S344: OQ440224-OQ440236 (listed in PAPER II-Supplementary Table 4, link available in subsection 3.8.).

RVA nt and aa sequences characterized in PAPER III sequences were submitted to the GenBank with adjacent accession numbers: PQ299823- PQ300023 and PQ273712- PQ273720 (listed in PAPER III-Supplementary table 1, link available in subsection 3.8.2.).

3.8.2. Supplementary material

Additional data related to PAPERS II and III are provided in their respective supplementary materials. Supplementary material adjacent to PAPER II is available online at https://www.frontiersin.org/articles/10.3389/fmicb.2023.1194764/full#supplementarymaterial . It comprises the following content: Supplementary table 1. Reference-mapping data; Supplementary table 2. Nucleotide and amino acid percentage identity data; Supplementary table 3. Nucleotide and amino acid percentage identity data for VP7 and VP4 mixed genotypes; Supplementary table 4. GenBank accession numbers of deposited sequences, and Supplementary figure 1. The phylogenetic tree of the detected mixed genotypes G1, G4, G5, G11 in the VP7 (A) and P[6], P[8], P[13] in the VP4 (B) gene segments.

The supplementary material characterized in PAPER III is available online at https://www.sciencedirect.com/science/article/pii/S004896972501650X?dgcid=author. It comprises the following content: Supplementary table 1. GenBank accession numbers; Supplementary table 2. Pairwise comparison; Supplementary table 3. Number and distribution of RVA-positive and sampled individuals by host, county and year of sampling in Croatia;

Supplementary Figure 1. Recombination analysis with wild-canid major parent NSP3 strains, and Supplementary methods 1. Addressing gaps in reference-based consensus assemblies.

3.9. Ethics approval and consent to participate

The scientific research on animal samples included in this doctoral thesis was evaluated and approved by the Board of Ethics of the Croatian Veterinary Institute, reference number Z-VI-4-5206/17, and the Committee for Ethics in Veterinary Medicine of the Faculty of Veterinary Medicine, University of Zagreb, class number: 640-01/23-17/45, and editorial number: 251-61-41-23-01. Human samples were collected from children under 5 years of age with present clinical signs of acute gastroenteritis, consequently admitted to the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" Zagreb, Clinical Hospital Center Osijek, Clinical Hospital Center Split, and the Institute of Public Health of Osijek-Baranja County. Each establishment issued its Ethics Committee approval for participation in the research conducted for this doctoral thesis, under the reference numbers 01-157-2-2018, R2-640/2018, 2181-147-01/06/M.S.-17-2, and 381-17-152, respectively.

4. RESULTS

4.1. PAPER I

BRNIĆ, D., D. ČOLIĆ, V. KUNIĆ, N. MALTAR-STRMEČKI, N. KREŠIĆ, D. KONJEVIĆ, M. BUJANIĆ, I. BAČANI, D. HIŽMAN, L. JEMERŠIĆ (2022): *Rotavirus A* in Domestic Pigs and Wild Boars: High Genetic Diversity and Interspecies Transmission. Viruses 14, 9, 2028, doi: 10.3390/v14092028

The results presented in PAPER I address Specific Objectives 1 and 2 by providing comprehensive data on the prevalence and genetic diversity of autochthonous RVA in domestic pigs and wild boars, as well as analyzing RVA prevalence across various epidemiological factors in domestic pigs. RVA was detected in 49.9% of domestic pigs and 9.3% of wild boars, with prevalence by county depicted in Figure 1. For domestic pigs, all eight large industrial holdings and 20 out of 24 small backyard holdings were positive for RVA in at least one sampled animal. Statistically significant differences in RVA prevalence were observed by farm type and clinical status: large commercial farms exhibited a significantly higher prevalence (68.1%) compared to backyard holdings (38.8%), and diarrheic pigs were significantly more likely to test positive (71.5%) than non-diarrheic pigs (37.1%). RVA strains in domestic pigs displayed high genetic diversity, with eight G genotypes (G9, G5, G3, G1, G4, G2, G6, G11) and seven P genotypes (P[13], P[23], P[8], P[6], P[32], P[7], P[11]) identified. They formed 23 different G/P combinations, most commonly G5P[13] and G9P[23], together comprising nearly half of the characterized strains (49.6%), with higher genotype diversity being found on large holdings. Detected genotypes differed between RV seasons, as depicted in the temporal distribution of genotypes (Figure 2). Furthermore, PAPER I revealed notable intragenotype diversity among poRVAs and suggested the presence of potentially novel VP7 and VP4 lineages (Figures 3 and 4). In addition, the G4 and P[6] genotypes, considered rare and noted for zoonotic potential, were detected in domestic pigs. The most prominent result was their close phylogenetic clustering with human strains previously reported as zoonotic (Figure 3B and 4A), providing a foundation for the investigation presented in PAPER II.

In wild boars, the RVA genetic diversity was lower compared to domestic pigs, as five G genotypes (G3, G5, G9, G6, G11) and only one P genotype (P[13]) were detected. Notably, in PAPER I, the G3 genotype was described for the first time in wild boars. At the same time, it was the most prevalent G genotype in Croatian wild boars, and the third G genotype in Croatian domestic pigs. All genotypes detected in wild boars were also detected in domestic

pigs, sharing a high nt pi and close phylogenetic relatedness (Figure 3A, 3B and 4B), marking the most prominent putative interspecies transmission between domestic pigs and wild boars. Possible interspecies transmission between domestic pigs and other species was also detected. A small number of samples presented with bovine-like G6 and P[11] genotypes, implying interspecies transmission between bovines and domestic pigs, which is also evident from their phylogenetic clustering (Figures 3B and 4A). One of the most interesting findings was the emergence of G1P[8] strains (n = 7), considered a typical human genotype combination, and as such marking the possible reverse zoonotic transmission events. Both G1 and P[8] genotypes found in domestic pigs clustered within typical human lineages (Figures 3A and 4A) and were detected during the same sampling season (2020/2021) in several holdings in three different counties.

4.2. PAPER II

KUNIĆ, V., T. MIKULETIČ, R. KOGOJ, T. KORITNIK, A. STEYER, S. ŠOPREK, G. TEŠOVIĆ, V. KONJIK, I. ROKSANDIĆ KRIŽAN, M. PRIŠLIN, D. BRNIĆ (2023): Interspecies transmission of porcine-originated G4P[6] *Rotavirus A* between pigs and humans: a synchronized spatiotemporal approach. Front. Microbiol. 14 , 1194764, doi: 10.3389/fmicb.2023.1194764

The results presented in PAPER II address Specific Objective 3 by investigating the zoonotic transmission of autochthonous poRVA. The study employed a synchronized spatiotemporal approach, analyzing whole-genome sequences of G4P[6] RVA strains collected from symptomatic children under the two years of age and weanling piglets with diarrhea in Croatia between 2018 and 2021. Initial screening identified three human-derived and three domestic pig-derived G4P[6] strains, which were subjected to NGS and comprehensive complete genome analysis. The findings revealed that all 11 gene segments in each of the six strains were of porcine or porcine-like origin, strongly indicating that the G4P[6] strains detected in children resulted from porcine-to-human interspecies transmission. Six porcine-originated G4P[6] strains displayed a genogroup 1 constellation, while phylogenetic analysis revealed that in every genomic segment, these strains were genetically closely related to porcine-like human RVAs or porcine-originated strains (Figure 1-4). Notably, further genetic analysis revealed that the diversity of Croatian G4P[6] strains was shaped by both reassortment and recombination events. Therefore, the results presented in PAPER II also address Specific Objective 5 by evaluating the roles of gene reassortment and intragenic recombination in

shaping the complete genome diversity of poRVA strains. Human-derived strain D572 did not show similarity to any available human-derived R1 or A8 sequences. This was evident from its complete phylogenetic separation from human-derived strains and clustering exclusively with porcine-originated R1 and A8 strains, making it a putative porcine/porcine-like human reassortant strain in VP1 (Figure 3A) and NSP1 (Figure 4A) gene segments. In addition to reassortment, this study detected evidence of intragenic/homologous recombination within the VP4, NSP1, and NSP3 gene segments across several strains (Table 3). The final result included VP2 sequence insertions in the 38-41 aa region, where five out of six C1 strains presented with different insertions.

4.3. PAPER III

KUNIĆ, V., LJ. BARBIĆ, J. ŠIMIĆ, T. MIKULETIČ, R. KOGOJ, T. KORITNIK, A. STEYER, D. KONJEVIĆ, M. BUJANIĆ, M. PRIŠLIN ŠIMAC, D. BRNIĆ (2025): Interspecies transmission and genome heterogeneity of porcine-originated *Rotavirus A* between domestic pigs and wildlife in the Croatian ecosystem. Sci. Total Environ. 994, 180010, doi: 10.1016/j.scitotenv.2025.180010

The results presented in PAPER III address Specific Objectives 4 and 5. It focuses on the interspecies transmission of poRVA strains between domestic pigs and wild animals within the Croatian ecosystem, while evaluating the impact of gene reassortment and intragenic recombination on genome diversity based on a complete genome analysis of autochthonous poRVA strains. Results revealed porcine genogroup 1 constellation, with surface protein genotypes characteristic of porcine hosts in all presented RVA strains (Table 2). Furthermore, the study provides valuable insights into RVA host diversity, presenting the first complete RVA genome data from golden jackals and the second from red foxes globally. In addition, it presents the first complete RVA genomes from wild boars outside of Asia to date. PAPER III reports the RVA prevalence in red foxes (15%) and golden jackals (36.6%), complementing the wild boar and domestic pig RVA prevalence data reported in PAPER I. The findings revealed clear evidence of interspecies transmission, as several poRVA strains detected in wildlife were phylogenetically closely related to those found in domestic pigs, confirming that wildlife serves as both recipients and potential reservoirs of poRVAs (Figures 3, 5, and 7). Notably, in the 19 complete genomes characterized in the PAPER III, G3 was the dominant VP7 genotype in wildlife, G5 in domestic pigs, while the zoonotic G4 genotype was identified in domestic pig and a red fox (Table 2). The most prevalent VP4 genotype was P[13], and the zoonotic P[6]

genotype was identified in a domestic pig and a golden jackal (Table 2). Mixed-genotype infections, involving VP7, VP4, and NSP4 segments, were found exclusively in domestic pigs, while no mixed genotypes occurred in wildlife-derived RVAs (Table 2). Comprehensive genome analysis revealed that intragenic recombination contributed significantly to poRVA genetic diversity, with several recombinant strains. Recombination events were detected in VP4, NSP1, and NSP4 gene segments, encompassing genotypes P[13], P[23], A8 and E9 (Figure 4). Moreover, the wild canid-derived RVAs influenced recombination events in human-derived zoonotic strains (Supplementary Figure 1). As for reassortment analysis, no unequivocal reassortment events were detected since each discovered segment was conclusive with RVA genogroup 1 constellation and porcine origin. Lastly, VP2 sequence insertions in occurred at 37–41 aa positions (Figure 6) in 10 out of 19 C1 strains.

5. DISCUSSION

RVA is a significant cause of viral acute gastroenteritis in mammals and birds, with sporadic zoonotic events (MARTELLA et al., 2010; ESTES and GREENBERG, 2013). It poses a persistent public health challenge due to its capacity for genome reassortment and intragenic recombination (McDONALD et al., 2016; HOXIE and DENNEHY, 2020; HAKIM et al., 2024). Mixed genotype infections propel these evolutionary mechanisms as they drive the emergence of novel strains, sporadically resulting in interspecies transmission. Despite demonstrated sporadic interspecies transmission potential of poRVAs, the specific role of wildlife in these dynamics remains poorly understood.

RVA genomes analyzed in this doctoral thesis were obtained from samples collected over three consecutive years (2018–2021) in Croatia, as part of a broader One Health RVA research project (BRNIĆ et al., 2018). Among the wide range of potential RVA hosts, this thesis focused on domestic pigs, humans, and naturally occurring wildlife species in Croatia, namely wild boars, red foxes, and golden jackals, in which VP7/VP4 genotypes of typical porcine origin had previously been detected. That discovery led to the presumed sporadic interspecies transmission of poRVAs in Croatia. Therefore, the One Health spatiotemporal approach was employed to investigate genome characteristics and interspecies transmission of autochthonous poRVAs in domestic pigs, humans, and wildlife in the Croatian ecosystem. Initially, partial and complete genomes of the poRVA strains in PAPER I, II and III of this doctoral thesis had to comply with the criteria stipulated by Matthijnssens et al. for the classification of RVAs using all 11 genomic RNA segments (MATTHIJNSSENS et al., 2008a). After defining partial and whole genome sequences of autochthonous poRVA strains among domestic pigs, humans and wild animals, further investigation of their genetic properties was warranted to establish their interspecies transmission and evolutionary relationship.

In preexisting research, domestic pigs have shown remarkable genotype diversity as RVA hosts, with more than 50 detected genotype combinations (DORO et al., 2015). Furthermore, a shared ancestral link between human Wa-like RVAs and porcine genogroup 1 RVAs was recognized (MATTHIJNSSENS et al., 2008b). In comparison with domestic pigs, far fewer studies have focused on RVA in wild boars. Nevertheless, existing research supports the occurrence of interspecies transmission of RVAs between domestic pigs and wild boars, and highlights the close phylogenetic relationship of some poRVA strains detected in humans with those detected in wild boars (OKADERA et al., 2013; MOUTELÍKOVÁ et al., 2016).

Considering all aforementioned, PAPER I aimed to reveal the concurrent prevalence, molecular epidemiology, genetic diversity and possible interspecies transmission between domestic pigs and wild boars in Croatia during three consecutive years (2018-2021). It focused on the first and the second specific objectives of this thesis. In the scope of the first specific objective, PAPER I determined the prevalence and genetic diversity of RVA strains circulating in domestic pigs and wild boars in Croatia. This was achieved by investigating comprehensive data on the prevalence and genetic diversity of autochthonous RVA in domestic pigs and wild boars from 2018-2021, indicating recurring interspecies transmission of poRVA strains between domestic pigs and wild boars. The second specific objective was achieved by comparing the prevalence of RVA in domestic pigs between two groups for each factor: farm type, age, sex and the presence of clinical signs. The observed RVA prevalence in domestic pigs was 49.9%, aligning with previous reports from the USA (MARTHALER et al., 2014), Spain (MONTEAGUDO et al., 2022), and Italy (FERRARI et al., 2022). These studies were also consistent with PAPER I in the detection method used for determining RVA prevalence, all employing real-time RT-PCR. Since the detection method can significantly influence the results, the Taiwanese study, which used an Enzyme Immunoassay for initial screening, followed by end-point RT-PCR for confirmation, reported a much lower and non-comparable prevalence rate (WU et al., 2022). The relatively high overall RVA prevalence reported in PAPER I is likely influenced by the predominance of younger age categories (suckling and weanling pigs), which favour more frequent RVA circulation. Further analysis of the prevalence data showed that sex and age group (suckling versus weanling) are not significant risk factors for RVA infection. In contrast, significantly higher RVA prevalence was detected in domestic pigs from large commercial holdings compared to those from small backyard farms. Moreover, diarrheic animals showed a significantly higher RVA prevalence than healthy ones., supporting the existing evidence that RVA is a causative agent of diarrhea in domestic pigs (PALMARINI, 2017) and that close contact between pigs in intensive farming facilitates viral transmission (MAES et al., 2020).

Data on the RVA significance in wild boars have been rather scarce so far with only two available reports from Japan (OKADERA et al., 2013) and the Czech Republic (MOUTELÍKOVÁ et al., 2016). PAPER I is the most comprehensive study to date, encompassing a sample set of 441 animals. It is also noteworthy that the sampling was performed in parallel with domestic pigs, which provides a temporal component important for relevant phylogenetic comparisons. The RVA prevalence in the present study (9.3%) was higher compared to those of the two previous studies, primarily due to a different approach to

RVA detection. We applied the real-time RT-PCR compared to the conventional end-point RT-PCRs applied by others (OKADERA et al., 2013; MOUTELÍKOVÁ et al., 2016), which are usually less sensitive. The method we implemented has been previously successfully applied in RVA-related research on domestic animals and wildlife (GUTIÉRREZ-AGUIRRE et al., 2008; JAMNIKAR-CIGLENECKI et al., 2016; ČOLIĆ et al., 2021). Nevertheless, the unknown range of VP2 genotypes that this method detects, and the fact that the assay design was limited to only human strains of C1 and C2 genotypes, might have underestimated the prevalence in both species. Moreover, the prevalence in wild boars might be even higher, as we did not have access to the youngest age categories, where higher RVA circulation is expected, due to hunting regulations. Similar to domestic pigs, age and gender were not significant factors for RVA prevalence.

Genetic diversity of RVA in domestic pigs was high, with eight identified G (G1-G6, G9, G11) and seven P genotypes (P[6]–P[8], P[11], P[13], P[23], P[32]). In wild boars, genotype diversity was somewhat lower, with five detected G (G3, G5, G6, G9, G11) and one P genotype (P[13]). The genotyping procedures were more challenging for wild boar samples, since 63.4% of RVA-positive wild boars had Cq values greater than 32, indicating low RVA genome concentrations in these samples. Such low viral loads may be indicative of the latent infection and a possible reservoir trait (MANDL et al., 2015), but they also pose challenges for sequencing (HOULDCROFT et al., 2017). On the other hand, the higher genetic diversity of RVA strains in domestic pigs bred on large holdings in comparison with small backyard holdings is likely a reflection of intensive production and trade practices, diverse RVA strain circulation, and close contact among pigs (CHANG et al., 2012; PALMARINI, 2017). The actual genetic diversity may be underestimated, considering that the diverse RVA strains could potentially affect primer specificity due to possible primer mismatch and genotyping incongruities. Apart from the remarkable genetic diversity of each segment (VP7/VP4), we observed a striking 23 different genotype combinations, which is higher than previously reported in Denmark, Hungary, Slovenia, and Spain combined (n = 21) (MIDGLEY et al., 2012). However, this number was lower than the 33 genotype combinations previously reported in Poland (KOZYRA et al., 2019).

In the PAPER I dataset, interspecies transmission was detected between domestic pigs and each of the following species: wild boars, humans, and cattle. It was the most prominent between domestic pigs and wild boars. All genotypes detected in wild boars (G3, G5, G6, G9, G11, and P[13]) were also detected in domestic pigs, and some of those genotypes were already proven relevant to wild boars (OKADERA et al., 2013; MOUTELÍKOVÁ et al., 2016). To the

best of our knowledge, as reported in PAPER I, the G3 genotype was identified for the first time in the wild boar population, where it emerged as the most dominant genotype. Simultaneously, the G3 was the third most prevalent in domestic pigs within the same study. Furthermore, possible sporadic interspecies transmission between domestic pigs and other species was detected. Sporadic occurrence of typical bovine G6 and P[11] genotypes and the emergence of typical human G1 and P[8] strains marked the possible bovine-porcine and reverse zoonotic human-porcine transmission events. Finally, G4 and P[6] genotypes, considered as rare and noted for their accentuated zoonotic potential (PAPP et al., 2013a), were detected in Croatian domestic pigs. Although less prevalent, these genotypes underscored their zoonotic potential by exhibiting close phylogenetic relationships with porcine-like RVA strains identified in humans (PAPER I, Figures 3B and 4A), thereby providing a foundation for the investigation presented in PAPER II.

PAPER I provided important baseline data on RVA prevalence, genetic diversity, and molecular epidemiology, as well as the extent of interspecies transmission between domestic pigs and wild boars. These findings are critical for understanding RVA epidemiology in swine populations and underscore the need for targeted control measures, including vaccine development, particularly given the lack of an approved porcine RVA vaccine in the EU.

The PAPER II investigates whole genomes of RVA of porcine origin in humans and domestic pigs that were initially found to share the same G4P[6] genotype. Consequently, it expanded on the One Health perspective by investigating potential zoonotic transmission of poRVA between domestic pigs and humans, thereby addressing the third specific objective of this thesis. Three samples from children under two years of age containing typical porcine-originated G and P genotypes, along with three samples from domestic pigs with matching genotypes (PAPER II, Table 1), were selected for NGS to acquire complete RVA genomes. To our knowledge, Croatian RVA strains in humans have not been subjected to complete genome sequencing thus far, except for one G8P[8] strain from 2006 (DELOGU et al., 2013), which indicates a significant knowledge gap in RVA evolution in Croatia. By characterizing whole genomes and multiple mixed genotypes in VP7, VP4 and NSP3 gene segments, PAPER II further corroborated the host and genotype diversity of the poRVA in Croatia (PAPER II, Table 2). The findings revealed that all 11 gene segments in each of the six RVA strains were of porcine or porcine-like origin, strongly indicating that the G4P[6] strains detected in children likely resulted from porcine-to-human interspecies transmission. In addition to phylogenetic analysis confirming zoonotic transmission of G4P[6] RVA strains, in-depth genome analysis revealed the mixed RVA infections, gene reassortment, and intragenic (homologous) inter- and

intragenotype recombination events. This approach addressed the fifth specific objective by evaluating the influence of gene reassortment and intragenic recombination on the complete genome diversity of autochthonous poRVAs. PAPER II highlighted how genetically intertwined an unusual zoonotic G4P[6] RVA genotype can be in porcine and human populations concurrently, accentuating the influence of animal RVAs on the evolution and recurrence of heterotypic RVAs in humans. Notably, porcine RVA strains exhibited a porcine genogroup 1 origin in all gene segments, with typical porcine genotypes, such as I5, A8, T7, and E9, standing out. Three porcine-like human G4P[6] strains displayed a Wa-like genogroup 1 constellation. At the same time, phylogenetic analysis revealed that in every genomic segment, these strains were genetically closely related to porcine-like human RVAs or porcine-originated strains. Human RVA Wa-like genogroup constellation is known to share its origin with porcine RVA genogroup 1 strains (MATTHIJNSSENS et al., 2008b; STEYER et al., 2008;MARTELLA et al., 2010; PAPP et al., 2013b). Considering surface protein coding gene segments, the G4 genotype has been proven to infect humans sporadically, and for pigs, it is the third most prevalent VP7 genotype in pigs (DORO et al., 2015). The same is accurate for P[6], which is also a major porcine genotype. Nevertheless, human porcine-like RVA P[6] strains have been identified in a very sporadic pattern in Europe, but recurrence was continuous (BÁNYAI et al., 2004; MARTELLA et al., 2006; STEYER et al., 2008; PAPP et al., 2013a; VRDOLJAK et al., 2019). All these P[6] strains were closely evolutionary connected to neighboring Hungarian zoonotic P[6] strains, underlining the influence of regional geolocation on RVA strain diversity.

The timing of detection of human-derived G4[6] strains was uncommon as all three G4P[6] strains were detected in symptomatic children in the summer months, an RVA out-of-season period in Croatia. This is consistent with reports from Europe showing increased rates of mixed and rare genotypes out-of-season (HUNGERFORD et al., 2016). Similar findings were also reported in Southern Italy; a 6-month-old child infected with the zoonotic G4P[6] RVA strain paired with the Wa-like backbone constellation, was also hospitalized in August. The foreign origin of this strain was further hypothesized (IANIRO et al., 2019). Similar to neighboring Italy, Croatia is a Mediterranean country with an immense amount of tourism in July and August, thus, the import of an unusual zoonotic strain at that time was hypothesized. However, based on the pairwise nt identities and phylogenetic relatedness of Croatian porcine and human-derived G4P[6] strains in the majority of gene segments, these cases are likely the result of independent events of indirect zoonotic interspecies transmission within Croatia. Moreover, the recombination analysis on multiple RVA segments provided additional evidence

in favor of this conclusion. Environmental transmission may have played a role in the epidemiology of these infections, as direct piglet-child transmission was deemed highly unlikely due to the very young age of infected children. RVA mixed genotypes, detected in G4P[6] complete genomes, propelled an incidence of reassortment events and intragenic homologous recombination that occurred in a few strains (PAPER II, Table 3). Due to the divergence of the D572 strain in VP1 and NSP1 segments from the rest of the human and porcine-like human strains, as well as clustering with exclusively porcine-derived strains in these segments, it most likely signifies the occurrence of reassortment between typical porcine and porcine-like human RVA strains (PAPER II, Figures 3A, 4A). No human-derived VP1 and NSP1 sequences similar to the D572 strain were available in GenBank for comparison, pointing out a lack of known human-derived evolutionary relatives of the D572 R1 and A8 segments, reaffirming its classification as a putative porcine/porcine-like human reassortant. It is accepted that heterologous RVAs of the porcine origin or porcine–human RVA reassortants had sporadically occurred and successfully spread among humans (MARTELLA et al., 2010). This kind of human-to-human transmission is generally short-lived since the heterologous RVA strains do not spread horizontally as efficiently among their non-specific hosts (MATTHIJNSSENS et al., 2006). Consequently, the significance of zoonotic transmission is potentially overlooked because clinically hospitalized symptomatic individuals are the focal point of RVA strain surveillance (VILIBIC-CAVLEK et al., 2021). However, successful virus adaptation to a human host has been documented (NGUYEN et al., 2024), underscoring the potential public health risks posed by unresearched animal RVAs. Moreover, two human porcine-like strains and one porcine strain have shown recombination events in at least one of the gene segments (VP4, NSP1, or NSP3). Interestingly, a G4P[6] RVA strain with a Wa-like constellation detected in the Dominican Republic was reported with the recombination events in the same genome segments as these three Croatian recombinants (ESONA et al., 2017). Conversely to the comprehensive study on RVA intragenic recombination prevalence, where recombination analysis of the NSP3 gene segment yielded no evidence of recombination (HOXIE and DENNEHY, 2020), PAPER II reported T1–T7 intergenotype recombination events in all three NSP3 recombinant strains. This also means that the NSP3 recombination was detected in every strain presented with a T1/T7 mixed genotype (PAPER II - Table 2, Table 3). Findings like this further endorse the cognition that mixed genotypes predispose the evolution of novel RVA strains (ESTES and GREENBERG, 2013). Zoonotic transmission events like these highlight the importance of continuous surveillance of animal RVAs and raise awareness on the role of animal RVAs in the evolution of strains affecting the human population.

In PAPER III, the focus was on exploring the interspecies transmission of poRVAs between domestic pigs and wild animals. During the genotyping process, VP7/VP4 RVA genotypes of typical porcine origin were detected in species other than domestic pigs, including wild boars, red foxes, and golden jackals, leading to the presumption of sporadic interspecies transmission of autochthonous poRVAs between domestic pigs and wildlife. In PAPER II, zoonotic spillover in Croatia was corroborated, underscoring the need for further investigation of poRVAs to enhance understanding of interspecies transmission and support the One Health perspective within the Croatian ecosystem. Therefore, samples from wildlife species in which poRVAs were detected, along with strains from domestic pigs with matching genotypes, were selected for NGS to acquire complete RVA genomes. Considering additional excluding practical criteria (e.g. quantity of collected samples), a total of 19 samples matching these criteria were selected (PAPER III, Table 1 in Figure 1). In addition, PAPER III evaluated the influence of gene reassortment and intragenic inter- and intragenotype recombination on the complete genome diversity of autochthonous poRVAs. Hence, PAPER III addressed the fourth and the fifth specific objectives of this thesis. Notably, the analysis of complete poRVA genomes confirmed the interspecies transmission events inferred from VP7 and VP4 gene data, previously hypothesized by ČOLIĆ et al., 2021, and PAPER I. Domestic pig-derived RVAs showed significant genetic heterogeneity, as mixed genotypes in VP7, VP4, and NSP4 genes were found only in domestic pigs, likely a consequence of intensive production and trade, diverse RVA strain circulation, and close contact among pigs (CHANG et al., 2012; PALMARINI, 2017). This finding is in accordance with the remarkable G and P genotype diversity in domestic pigs described in PAPER I. Most Croatian poRVAs phylogenetically clustered with each other or with other European RVA strains of porcine origin across all gene segments, underlining the influence of regional geolocation on RVA strain diversity as mentioned in PAPER II. To date, research efforts have been predominantly focused on human RVAs, with domestic pig-derived RVA strains being genotyped roughly 100 times less frequently (PAPP et al., 2013a). This information gap is even more pronounced when considering the limited data on genotyped RVA strains circulating in wildlife (GHOSH and KOBAYASHI, 2014). The importance of considering host species when evaluating disease model systems for multi-species pathogens is well-supported by One Health research, as understanding this dynamic is crucial for accurately predicting disease emergence and informing effective prevention strategies (SINGH et al., 2023; RUI et al., 2024). The potential significance of animal-derived RVA strains may be underestimated, especially considering the frequent wildlife origin of emerging infectious diseases (CUNNINGHAM et al., 2017;

VILIBIC-CAVLEK et al., 2021). Approximately 75% of emerging infectious diseases in humans originate from animals, with wildlife serving as primary reservoirs for some high-impact pathogens (WOAH, 2024). Therefore, a One Health-based spatiotemporal approach is crucial for understanding the genetic interconnectedness of multi-species pathogens, like RVA, in various human and animal populations.

Despite their potential role in RVA transmission, research on wild canid-derived RVA is scarce, with only two studies focused on red foxes (EVANS, 1984; BUSI et al., 2017). The previous research about the genetic diversity of RVA strains circulating in red foxes in Croatia discovered a remarkable 11 G and nine P RVA genotypes, including G5, G9, G11, P[13] and P[23] considered to have a porcine origin**.** These were discovered along with a 14.9% prevalence, suggesting a reservoir possibility (ČOLIĆ et al., 2021). To our knowledge, the RVs of golden jackals have not been researched. The only available data are from Croatia, where a prevalence of 20.6% was reported, along with two G and three P genotypes (ČOLIĆ et al., 2021). To our understanding, only one available RVA complete genome was acquired from red fox (BUSI et al., 2017), and none from jackals on a global scale. This highlights a significant knowledge gap regarding the role of wild canids in RVA circulation. Furthermore, the zoonotic potential of porcine-like RVAs (poRVAs) originating from wild animals within the Croatian ecosystem remains largely unexplored. To the best of our knowledge, PAPER III presents the first reported complete RVA genome in golden jackal and the second in red fox worldwide. Additionally, the complete RVA genomes reported in wild boars are the first documented outside of Asia (SHIZAWA et al., 2024; LE et al., 2025). The concern regarding infectious disease transmission from domestic animals to wildlife has been well recognized (AGUIRRE, 2009; MARTIN et al., 2011). Domestic pigs have already been suggested as reservoirs for RVAs and a source of newly adapted emerging strains for humans and other animals (DHAMA et al., 2009; WU et al., 2022; PAPER I; PAPER II). Nevertheless, previous data on RVA detection rates in wildlife suggest that they may serve as additional potential RVA reservoirs (MARTIN et al., 2011; ČOLIĆ et al., 2021; JOTA BAPTISTA et al., 2023). The current study shows the close evolutionary relationship between wild canid- and wild boar-derived RVAs (PAPER III - Figure 1), which aligns with the fact that these animals share the same habitat and, at times, even prey-predator dynamic (BASSI et al., 2012). The trophic niche ranges of the golden jackal and red fox in the Pannonian ecoregion proved to be very narrow, with a mean food overlap index of 73% (LANSZKI et al., 2006). Based on prey remains found in scat, the golden jackals and red foxes are known for predation upon wild boar piglets (LANSZKI et al., 2006). The wild canid-derived RVAs from this study consistently exhibit porcine RVA origin

across all gene segments, clustering closely with RVAs derived from either domestic pigs or wild boars. Pig populations may also act as intermediate hosts, amplifying infectious agents transmitted from other wild or domestic animal species, and then transmitting them to humans, as described for the Nipah virus (FOURNIÉ et al., 2015). Nevertheless, in each gene segment, at least one fox-derived RVA strain clustered closely with zoonotic poRVAs from human hosts (PAPER III - Figure 3, 5, and 7). Furthermore, wild canid-derived RVAs were identified as either major or minor parents in five out of seven recombinant strains detected in the present dataset, including two zoonotic NSP3 recombinants reported in PAPER II (PAPER II - Supplementary Figure 1). Considering all of the above, current results may imply that the evolutionary relationship may exist between Croatian wildlife-derived RVAs and zoonotic human-derived RVAs of porcine origin without the domestic pig as the intermediate host. A more conclusive portrayal of RVA geoevolutionary patterns and reservoir determination remains limited due to the current lack of domestic pig- and wildlife-derived complete RVA genomes, both from the affected area and globally. In contrast to the intensive pork industry, in Croatia, the pig farming sector is largely composed of small, traditional rural farms, with fewer than ten sows and less than three hectares of land, accounting for up to 75% of all pig holdings (WELLBROCK, 2008). Due to their size and resources, these farms fall under biosafety category 1 and generally lack the capacity to implement effective biosecurity measures, increasing the risk of pathogen transmission among multiple susceptible species. Rural farming, especially with outdoor or free-range systems, is more vulnerable to predation by foxes and jackals due to insufficient protective barriers (FLEMING et al., 2016). Furthermore, in rural outdoor farms, wild boars and domestic pigs can interact and even interbreed (ANDERSON et al., 2019). Overall, there are multiple factors and contact points between these animals, such as shared habitat, insufficient barriers for outdoor farms, interactions between domestic pigs and wildlife, scavenging and opportunistic nature of wild canids and wild boars, overlapping trophic niches of golden jackals and red foxes, etc. All mentioned factors significantly influence and enable interspecies transmission of multi-species pathogens. Therefore, direct or indirect interspecies transmission through environmental exposure may serve as a potential RVA infection source for domestic animals and wildlife alike. RVA can survive for prolonged periods in the environment, preserving infectivity for several hours to several months outside the host (D'SOUZA et al., 2008). Although RVA is primarily transmitted via the fecal-oral route, it is also recognized as a foodborne and waterborne virus (SVENSSON, 2000; DHAMA et al., 2009; KRAAY et al., 2018). Lately, increasing attention has been given to the waterborne transmission of RVA, taking into account environmental conditions such as temperature and

humidity (KRAAY et al., 2018). In Croatia, a study from December 2019 to January 2021 detected RVA in 22.2% (2/9) of surface water samples and 100% (21/21) of wastewater samples (BRNIĆ et al., 2022b), suggesting possible environmental contamination. Similar results were reported in neighbouring Slovenia, where 60.3% of surface water samples tested positive for at least one enteric virus, including rotaviruses, noroviruses, and astroviruses, indicating widespread environmental contamination (STEYER et al., 2011). These contaminated environments may serve as hotspots for the transmission of enteric viruses to wildlife, while also posing a potential risk to public health. These aforementioned wildlife-urban interface (WUI) sites, dispersed throughout Croatia and Europe (SCHUG et al., 2023), combined with the rising wild canid and wild boar density in Europe (STATHAM et al., 2018; COLOMER et al., 2024), emphasize the importance of wildlife surveillance for multispecies pathogens like RVA (SCHUG et al., 2023; JIMÉNEZ-RUIZ et al., 2024). The recurrent zoonotic transmission and recombination potential of poRVAs in Croatia, reported in PAPERS II and III, further emphasize this concern.

The PAPER III highlights the spatiotemporal recurrence of poRVAs in Croatian wildlife over several years. A comprehensive complete RVA genome analysis provided evidence on interspecies transmission of poRVAs. However, it remains unclear whether these RVAs successfully adapt to non-dominant hosts long-term or if such interspecies transmission events are transient. Integrating wildlife into RVA studies is crucial from both conservation medicine and One Health perspectives, emphasizing the interconnectedness of ecological and human health. Studies like this are essential to address the knowledge gap about the role that wildlife holds in RVA epidemiology, particularly their role as reservoirs of emerging and potentially zoonotic RVA strains. Applying One Health principles and a spatiotemporal approach can advance our understanding of the evolutionary dynamics of poRVA, facilitating the assessment of interspecies transmission impacts on vaccine efficacy.

This doctoral thesis addressed the aforementioned knowledge gaps by investigating the diversity of RVA in the porcine population, the presence of poRVA strains in human and wild animal populations, and the phylogenetic and whole-genome characteristics of poRVAs, with the aim of drawing conclusions about their interspecies transmission within the Croatian ecosystem. The hypothesis of this thesis stated that interspecies transmission of RVAs, typical of domestic pigs, sporadically occurs within the Croatian ecosystem. The results presented in PAPERS I, II, and III collectively confirm this hypothesis.

6. CONCLUSIONS

- The results reveal that *Rotavirus A* (RVA) is present in both species, the domestic pig and wild boar, with a higher prevalence found in the domestic pig population. The observed genotype overlap, together with the close phylogenetic relationship, provides strong evidence for recurrent interspecies transmission between the two species, despite the higher genotype diversity observed in domestic pigs. In domestic pigs, farm type and clinical status were statistically significant factors affecting RVA prevalence. Animals from large holdings and those exhibiting clinical signs of diarrhea were considerably more likely to test RVA-positive. On the contrary, age and sex in either species did not influence the prevalence.
- Zoonotic transmission of autochthonous porcine-originated RVA (poRVA) in the Croatian ecosystem was corroborated through a synchronized spatiotemporal approach. Three human-derived porcine-like G4P[6] strains exhibited close phylogenetic clustering with domestic pig-derived strains in all gene segments, accentuating their porcine origin. Indirect zoonotic transmission via environmental route was considered the most plausible, given the young age of infected humans.
- The investigation of interspecies transmission of autochthonous poRVA strains within the Croatian ecosystem revealed clear evidence of transmission between domestic pigs and wild animals. RVA strains detected in wildlife were phylogenetically closely related to those found in domestic pigs, highlighting the ecological interconnectedness between domestic and wild animals in the transmission dynamics of RVA. The present study indicates the potential of wildlife to act as both reservoirs and recipients of poRVAs.
- PoRVA strains characterized in wildlife present the first complete RVA genome data from golden jackals and the second from red foxes worldwide. Additionally, this study reports the first complete RVA genomes from wild boars outside of Asia.
- Intragenic recombination events proved to be significant drivers of genome diversity in autochthonous poRVAs. This is based on several exhibited intragenic recombination found in poRVA strains derived from humans, domestic pigs and wildlife. One human-derived porcine-like strain exhibited a unique plausible double reassortant profile in VP1 and NSP1, lacking close human-derived phylogenetic relatives, thus highlighting its exclusive porcine origin. These results highlight the perpetual role of intragenic recombination and sporadic reassortment as viral evolutionary mechanisms shaping the genetic diversity of autochthonous poRVAs.

7. BIBLIOGRAPHY

ABE, M., N. ITO, S. MORIKAWA, M. TAKASU, T. MURASE, T. KAWASHIMA, Y. KAWAI, J. KOHARA, M. SUGIYAMA (2009): Molecular epidemiology of rotaviruses among healthy calves in Japan: Isolation of a novel bovine rotavirus bearing new P and G genotypes. Virus Res 144, 250–257, doi: https://doi.org/10.1016/j.virusres.2009.05.0055

AFRAD, M. H., J. MATTHIJNSSENS, S. F. AFROZ, P. RUDRA, L. NAHAR, R. RAHMAN, M. E. HOSSAIN, S. R. RAHMAN, T. AZIM, M. RAHMAN (2014): Differences in lineage replacement dynamics of G1 and G2 rotavirus strains versus G9 strain over a period of 22 years in Bangladesh. Infect Genet Evol. 28, 214–222, doi: https://doi.org/10.1016/j.meegid.2014.10.002

AGUIRRE, A. A. (2009): Wild canids as sentinels of ecological health: A conservation medicine perspective. Parasit Vectors 2. doi: https://doi.org/10.1186/1756-3305-2-S1-S7

ANDERSON, D., R. TOMA, Y. NEGISHI, K. OKUDA, H. ISHINIWA, T.G. HINTON, K. NANBA, H.B. TAMATE and S. KANEKO (2019): Mating of escaped domestic pigs with wild boar and possibility of their offspring migration after the Fukushima Daiichi Nuclear Power Plant accident. Sci Rep 9. doi: https://doi.org/10.1038/s41598-019-47982-z

ANGEL, J., M. A. FRANCO and H. B. GREENBERG (2012): Rotavirus immune responses and correlates of protection. Curr Opin Virol. 2, 419–425. doi:10.1016/j.coviro.2012.05.003

APPAIAHGARI, M. B., R. GLASS, S. SINGH, S. TANEJA, T. RONGSEN-CHANDOLA, N. BHANDARI, S. MISHRA and S. VRATI (2014): Transplacental rotavirus IgG interferes with immune response to live oral rotavirus vaccine ORV-116E in Indian infants. Vaccine 32, 651–656. doi:10.1016/j.vaccine.2013.12.017

ARIAS, C. F. and S. LÓPEZ (2021): Rotavirus cell entry: not so simple after all. Curr Opin Virol. 49, 42–48, doi: https://doi.org/10.1016/j.coviro.2021.03.0111

BÁNYAI, K., V. MARTELLA, F. JAKAB, B. MELEGH, G. SZÜCS (2004): Sequencing and phylogenetic analysis of human genotype P[6] rotavirus strains detected in Hungary provides evidence for genetic heterogeneity within the P[6] VP4 gene. J Clin Microbiol 42, 4338–4343. doi: https://doi.org/10.1128/JCM.42.9.4338-4343.2004

BÁNYAI, K., G. KEMENESI, I. BUDINSKI, F. FÖLDES, B. ZANA, S. MARTON, R. VARGA-KUGLER, M. OLDAL, K. KURUCZ and F. JAKAB (2017): Candidate new

rotavirus species in Schreiber's bats, Serbia. Infect Genet Evol. 48, 19–26. doi:10.1016/j.meegid.2016.12.002

BASSI, E., E. DONAGGIO, A. MARCON, M. SCANDURA and M. APOLLONIO (2012): Trophic niche overlap and wild ungulate consumption by red fox and wolf in a mountain area in Italy. Mamm. Biol. 77, 369–376. doi:10.1016/j.mambio.2011.12.002

BERGELAND, M. E., J. P. MCADARAGH and I. STOTZ (1977): Rotaviral enteritis in turkey poults. In: Proceedings of the 26th Western Poultry Diseases Conference. Davis (CA): University of California, pp. 129-130.

BERGMAN, H., N. HENSCHKE, D. HUNGERFORD, F. PITAN, D. NDWANDWE, N. CUNLIFFE and K. SOARES-WEISER (2021): Vaccines for preventing rotavirus diarrhoea: vaccines in use. Cochrane Database of Systematic Reviews 2021. doi: https://doi.org/10.1002/14651858.CD008521.pub6

BERTONI, E. A., M. BOK, C. VEGA, G. M. MARTINEZ, R. CIMINO and V. PARREÑO (2021): Influence of individual or group housing of newborn calves on rotavirus and coronavirus infection during the first 2 months of life. Trop Anim Health Prod 53. doi: https://doi.org/10.1007/s11250-020-02540-y

BISHOP, R. F., G. P. DAVIDSON, I. H. HOLMES and B. J. RUCK (1973): Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet 302, 1281-1283. doi: 10.1016/S0140-6736(73)92867-5

BÖHM, R., F. E. FLEMING, A. MAGGIONI, V. T. DANG, G. HOLLOWAY, B. S. COULSON, M. VON ITZSTEIN and T. HASELHORST (2015): Revisiting the role of histo-blood group antigens in rotavirus host-cell invasion. Nat Commun 6. doi: https://doi.org/10.1038/ncomms6907

BONURA, F., K. BÁNYAI, L. MANGIARACINA, C. BONURA, V. MARTELLA, G. M. GIAMMANCO, S. DE GRAZIA (2022): Emergence in 2017–2019 of novel reassortant equine-like G3 rotavirus strains in Palermo, Sicily. Transbound Emerg Dis. 69, 813–835, doi: https://doi.org/10.1111/tbed.14054

BRNIĆ D., I. ŠIMIĆ, N. KREŠIĆ, I. ŠKOKO, I. LOJKIĆ (2018): Rotaviruses in Croatian ecosystem: molecular epidemiology and zoonotic potential - Reco. Power of Viruses Book of Abstracts, Poreč, Croatia, pp. 47-47.

BRNIĆ, D., A. KOVAČEVIĆ, N. KREŠIĆ, I. ŠIMIĆ, I. ŠKOKO, V. KONJIK, M. SIPL, J. JUKIĆ GUĆ, V. KRŽELJ, S. ŠOPREK, G. TEŠOVIĆ (2019): Rotavirus A infection in hospitalized children in Croatia during season 2018/2019: the emergence of equine-like

G3P[8] and G1P[8] intergenogroup reassortant strains. 9th Croatian Infectious diseases congress (CROCMID), Šibenik, Croatia, pp. 156-157.

BRNIĆ, D., A. KOVAČEVIĆ, D. ČOLIĆ, I. ŠIMIĆ, I. ŠKOKO, N. KREŠIĆ (2020): Molekularna epidemiologija i zoonotski potencijal rotavirusa A dokazanih u ekosustavu Republike Hrvatske. Symposium with international participation Zoonoze i bolesti koje se prenose vektorima u kontekstu "Jednog zdravlja", Zagreb, Croatia, pp. 42-43

BRNIĆ, D., D. ČOLIĆ, V. KUNIĆ, N. MALTAR-STRMEČKI, N. KREŠIĆ, D. KONJEVIĆ, M. BUJANIĆ, I. BAČANI, D. HIŽMAN, L. JEMERŠIĆ (2022): *Rotavirus A* in Domestic Pigs and Wild Boars: High Genetic Diversity and Interspecies Transmission. Viruses 14, 9, 2028, doi: 10.3390/v14092028

BRNIĆ, D., V. KUNIĆ, T. MIKULETIČ, A. STEYER, R. KOGOJ, A. KOVAČEVIĆ, N. KREŠIĆ, V. KONJIK, SIPL, I. ROKSANDIĆ KRIŽAN, J. JUKIĆ GUĆ, V. KRŽELJ, S. ŠOPREK, G. TEŠOVIĆ (2022a): Rotavirus A infection in hospitalized children in Croatia during three seasons: the predominance of G3 genotype and the emergence of intergenogroup reassortant strains. 14th International dsRNA Virus Symposium, Banff, Canada. Available online at: https://www.croris.hr/crosbi/publikacija/prilog-skup/725370

BRNIĆ, D., I. LOJKIĆ, I. ŠKOKO, N. KREŠIĆ, I. ŠIMIĆ, T. KEROS, M. GANJTO, D. ŠTEFANAC, B. VIDUKA, D. KARŠAJ, D. ŠTILER, B. HABRUN and L. JEMERŠIĆ (2022b): SARS-CoV-2 circulation in Croatian wastewaters and the absence of SARS-CoV-2 in bivalve molluscan shellfish. Environ Res 207. doi: https://doi.org/10.1016/j.envres.2021.112638

BRNIĆ, D., D. VLAHOVIĆ, A. GUDAN KURILJ, N. MALTAR-STRMEČKI, I. LOJKIĆ, V. KUNIĆ, L. JEMERŠIĆ, I. BAČANI, G. KOMPES, R. BECK, T. MIKULETIČ and A. STEYER (2023): The impact and complete genome characterisation of viruses involved in outbreaks of gastroenteritis in a farrow-to-finish holding. Sci Rep. 13. doi: https://doi.org/10.1038/s41598-023-45994-4

BRÜSSOW, H., O. NAKAGOMI, G. GERNA and W. EICHHORN (1992): Isolation of an avian-like group A rotavirus from a calf with diarrhea. J Clin Microbiol. 30, 67-73. 10.1128/jcm.30.1.67-73.1992

BURKE, R. M., J. E. TATE, C. D. KIRKWOOD, A. D. STEELE and U. D. PARASHAR (2019): Current and new rotavirus vaccines. Curr Opin Infect Dis. 32, 435–444. doi:10.1097/qco.0000000000000572

BURNETT, E., U. D. PARASHAR and J. E. TATE (2020): Associations of Intussusception With Adenovirus, Rotavirus, and Other Pathogens: A Review of the Literature. Pediatr Infect Dis J. 39, 1127–1130. doi:10.1097/inf.0000000000002860

BURROUGH, E. R. (2024): Rotaviral Enteritis in Pigs. In: MSD Veterinary Manual, Merck and Co., Inc., Rahway, N. J., USA. Available online at: https://www.msdvetmanual.com/digestive-system/intestinal-diseases-in-pigs/rotaviral-enteritis-in-pigs , accessed on May 4th 2025

BUSI, C., V. MARTELLA, A. PAPETTI, C. SABELLI, D. LELLI, G. L. ALBORALI, L. GIBELLI, D. GELMETTI, A. LAVAZZA, P. CORDIOLI and M. B. BONIOTTI (2017): Group a rotavirus associated with encephalitis in red fox. Emerg Infect Dis 23, 1535–1538. doi:10.3201/eid2309.170158

CHANDLER-BOSTOCK, R., L. R. HANCOX, S. NAWAZ, O. WATTS, M. ITURRIZA-GOMARA and K. M. MELLITS (2014): Genetic diversity of porcine group A rotavirus strains in the UK. Vet Microbiol. 173, 27–37. doi:10.1016/j.vetmic.2014.06.030

CHANG, K. S., Y. KIM, L. J. SAIF (2012): "Rotavirus and reovirus," in Diseases of Swine, Eds. J. J., Zimmerman, L. A., Karriker, A., Ramirez, K. J., Schwartz, G.W., Stevenson. Wiley-Blackwell: Ames, USA, pp. 621–634.

CHANG-GRAHAM, A. L., J. L. PERRY, M. A. ENGEVIK, K. A. ENGEVIK, F. J. SCRIBANO, J. T. GEBERT, H. A. DANHOF, J. C. NELSON, J. S. KELLEN, A. C. STRTAK, N. P. SASTRI, M. K. ESTES, R. A. BRITTON, J. VERSALOVIC and J. M. HYSER (2020): Rotavirus induces intercellular calcium waves through ADP signaling. Science, 1979, 370, doi: https://doi.org/10.1126/science.abc3621

CHUCRI, T. M., J. M. MONTEIRO, A. R. LIMA, M. L. B. SALVADORI, J. R. K. JUNIOR and M. A. MIGLINO (2010): A review of immune transfer by the placenta. J Reprod Immunol 87, 14–20. doi:10.1016/j.jri.2010.08.062

CLEMENTE, M. G., J. T. PATTON, R, A. ANDERS, R. H. YOLKEN, K. B. SCHWARZ (2015): Rotavirus infects human biliary epithelial cells and stimulates secretion of cytokines IL-6 and IL-8 via MAPK pathway. Biomed Res Int. 2015, 697238, doi: https://doi.org/10.1155/2015/697238

ČOLIĆ, D., 2021. Genotipizacija rotavirusa A iz divljih životinja s područja Republike Hrvatske. Master's thesis. Faculty of Veterinary Medicine, University of Zagreb. Available online at: https://urn.nsk.hr/urn:nbn:hr:217:900539

ČOLIĆ, D., N. KREŠIĆ, Ž. MIHALJEVIĆ, T. ANDREANSZKY, D. BALIĆ, M. LOLIĆ and D. BRNIĆ (2021): A remarkable genetic diversity of rotavirus a circulating in red fox

population in Croatia. Pathogens 10, 4, 485, doi: https://doi.org/10.3390/pathogens10040485

COLLINS, P. J., V. MARTELLA, R. D. SLEATOR, S. FANNING and H. O'SHEA (2010): Detection and characterisation of group A rotavirus in asymptomatic piglets in southern Ireland. Arch Virol 155, 1247–1259. doi:10.1007/s00705-010-0713-1

COLOMER, J., G. MASSEI, D. ROOS, C. ROSELL and J. D. RODRÍGUEZ-TEIJEIRO (2024): What drives wild boar density and population growth in Mediterranean environments? Sci Total Environ, 931, 2024, 172739, doi: https://doi.org/10.1016/j.scitotenv.2024.172739

CRAWFORD, S. E., S. RAMANI, J. E. TATE, U. D. PARASHAR, L. SVENSSON, M. HAGBOM, M. A. FRANCO, H. B. GREENBERG, M. O'RYAN, G. KANG, U. DESSELBERGER and M. K. ESTES (2017): Rotavirus infection. Nat Rev Dis Primers 3, 2017, 17083, doi: https://doi.org/10.1038/nrdp.2017.83

CUNNINGHAM, A. A., P. DASZAK and J. L. N. WOOD (2017): One health, emerging infectious diseases and wildlife: Two decades of progress? Phil Trans R Soc. B 372: 20160167, doi: http://dx.doi.org/10.1098/rstb.2016.0167

D'SOUZA, R. M., G. HALL and N. G. BECKER (2008): Climatic factors associated with hospitalizations for rotavirus diarrhoea in children under 5 years of age. Epidemiol Infect 136, 56–64. doi:10.1017/s0950268807008229

DELOGU, R., A. LO PRESTI, F. M. RUGGERI, E. CELLA, M. GIOVANETTI, M. CICCOZZI, S. LJUBIN-STERNAK, S. BUKOVSKI-SIMONOSKI, A. LUKIC-GRLIC, G. IANIRO and L. FIORE (2013): Full-genome characterization of a G8P[8] rotavirus that emerged among children with diarrhea in Croatia in 2006. J Clin Microbiol, 51, 5, doi: https://doi.org/10.1128/jcm.00396-13https://doi.org/10.1128/JCM.00396-13

DESSELBERGER, U. (1999): Rotavirus Infections Guidelines for Treatment and Prevention. Drugs, 58 (3): 447-452.

DESSELBERGER, U. (2014): Rotaviruses. Virus Res 190, 75–96. doi:10.1016/j.virusres.2014.06.016

DESSELBERGER, U. and H. I. HUPPERTZ (2011): Immune responses to rotavirus infection and vaccination and associated correlates of protection. Journal of Infectious Diseases 203, 188–195. doi:10.1093/infdis/jiq031

DEWEY C., S. CARMAN, T. PASMA, G. JOSEPHSON, B. McEWEN (2003): Relationship between group A porcine rotavirus and management practices in swine herds in Ontario. Can Vet J. 44, 8, 649–653.

DHAMA, K., M. SAMINATHAN, K. KARTHIK, R. TIWARI, M. Z. SHABBIR, N. KUMAR, Y. S. MALIK and R. K. SINGH (2015): Avian rotavirus enteritis – an updated review. Vet Q, 35, 142–158. doi:10.1080/01652176.2015.1046014

DHAMA, K., R. S. CHAUHAN, M. MAHENDRAN and S. V. S. MALIK (2009): Rotavirus diarrhea in bovines and other domestic animals. Vet Res Commun, 33, 1, 1–23, doi: 10.1007/s11259-008-9070-x

DIAN, Z., Y. SUN, G. ZHANG, Y. XU, X. FAN, X. YANG, Q. PAN, M. PEPPELENBOSCH and Z. MIAO (2021): Rotavirus-related systemic diseases: clinical manifestation, evidence and pathogenesis. Crit Rev Microbiol, 47, 5, 580–595. doi:10.1080/1040841x.2021.1907738

DÍAZ ALARCÓN, R.G., D.J. LIOTTA and S. MIÑO (2022): Zoonotic RVA: State of the Art and Distribution in the Animal World. Viruses 14, 11, 2554. doi: 10.3390/v14112554

DORO, R., S.L. FARKAS, V. MARTELLA and K. BANYAI (2015): Zoonotic transmission of rotavirus: Surveillance and control. Expert Rev Anti Infect Ther 13, 11, 1337–1350. doi:10.1586/14787210.2015.1089171

DŽAKULA, S., 2019. Utvrđivanje prisutnosti i proširenosti rotavirusa A u ljudi i različitih vrsta domaćih životinja na jednom obiteljskom gospodarstvu. Master's thesis. Faculty of Veterinary Medicine, University of Zagreb. Available online at: https://urn.nsk.hr/urn:nbn:hr:178:328845

EL-AGEERY, S. M., R. ALI, N. T. ABOU EL-KHIER, S. A. RAKHA and M. S. ZEID (2020): Comparison of enzyme immunoassay, latex agglutination and polyacrylamide gel electrophoresis for diagnosis of rotavirus in children. Egypt. J. Basic Appl. Sci. 7, 47–52. doi:10.1080/2314808x.2020.1716185

ESONA, M. D., S. ROY, K. RUNGSRISURIYACHAI, J. SANCHEZ, L. VASQUEZ, V. GOMEZ, L. A. RIOS, M. D. BOWEN and M. VAZQUEZ (2017): Characterization of a triple-recombinant, reassortant rotavirus strain from the Dominican republic. J Gen Virol. 98, 134–142. doi:10.1099/jgv.0.000688

ESTES, M. K. and H. B. GREENBERG (2013). "Rotaviruses," in Fields Virology. 6th Edn. eds. D. M. Knipe and P. M. Howley (Philadelphia, PA: Lippincott Williams and Wilkins), pp. 1347–1401.

EUROROTANET (2009). Rotavirus detection and typing methods. Available online at: https://www.eurorotanet.com/project-information/documents-and-methods/ (accessed April 20, 2022).

EVANS, R. H. (1984): Rotavirus-associated diarrhea in young raccoons (Procyon lotor), striped skunks (Mephitis mephitis) and red foxes (Vulpes vulpes). J Wildl Dis. 20, 79–85. doi:10.7589/0090-3558-20.2.79

FERRARI, E., C. SALOGNI, V. MARTELLA, G. L. ALBORALI, A. SCABURRI and M. B. BONIOTTI (2022): Assessing the Epidemiology of Rotavirus A, B, C and H in Diarrheic Pigs of Different Ages in Northern Italy. Pathogens 11, 467, doi: https://doi.org/10.3390/pathogens11040467

FLEMING, P. A., S. J. DUNDAS, Y. Y. W. LAU and J. R. PLUSKE (2016): Predation by red foxes (Vulpes vulpes) at an outdoor piggery. Animals 6, 60, doi: https://doi.org/10.3390/ani6100060

FLEWETT, T. H., A. S. BRYDEN, H. DAVIES, G. N. WOODE, J. C. BRIDGER, J. M. DERRICKS (1974): Relation between viruses from acute gastroenteritis of children and newborn calves. Lancet, 2 (7872):61-3. doi: 10.1016/s0140-6736(74)91631-6

FOURNIÉ, G., L. KEARSLEY-FLEET, J. OTTE and D. U. PFEIFFER (2015): Spatiotemporal trends in the discovery of new swine infectious agents. Vet Res. 46, 114, doi: https://doi.org/10.1186/s13567-015-0226-8

FRANCO, M. A., J. ANGEL and H. B. GREENBERG (2006): Immunity and correlates of protection for rotavirus vaccines. Vaccine 24, 2718–2731. doi:10.1016/j.vaccine.2005.12.048

FRIEDRICH, R., E. RAPPOLD, C. BOGDAN, J. HELD (2018): Comparative analysis of the wako B-glucan test and the fungitell assay for diagnosis of candidemia and Pneumocystis jirovecii pneumonia. J Clin Microbiol. 56, 9, e00464-18, doi: 10.1128/JCM.00464-18

FUJII, Y., Y. HAI DOAN, R. M. WAHYUNI, M. I. LUSIDA, T. UTSUMI, I. SHOJI, K. KATAYAMA (2019): Improvement of rotavirus genotyping method by using the semi-nested multiplex-PCR with new primer set. Front Microbiol. 29, 10, 2019, doi: https://doi.org/10.3389/fmicb.2019.00647

GELETU, U. S., M. A. USMAEL and F. D. BARI (2021): Rotavirus in Calves and Its Zoonotic Importance. Vet Med Int. 2021, 639701, 18, doi: https://doi.org/10.1155/2021/6639701

GHOSH, S. and N. KOBAYASHI (2014): Exotic rotaviruses in animals and rotaviruses in exotic animals. Virus disease 25, 158–172. doi:10.1007/s13337-014-0194-z

GHOSH, S., M. KUMAR, M. SANTIANA, A. MISHRA, M. ZHANG, H. LABAYO, A. M. CHIBLY, H. NAKAMURA, T. TANAKA, W. HENDERSON, E. LEWIS, O. VOSS, Y. SU, Y. BELKAID, J. A. CHIORINI, M. P. HOFFMAN and N. ALTAN-BONNET (2022): Enteric viruses replicate in salivary glands and infect through saliva. Nature 607, 345–350. doi:10.1038/s41586-022-04895-8

GÓMEZ-RIAL, J., I. RIVERO-CALLE, A. SALAS and F. MARTINÓN-TORRES (2020): Rotavirus and autoimmunity. J Infect. 81, 183–189, doi:10.1016/j.jinf.2020.04.041

GOUVEA, V., R. I. GLASS, P. WOODS, K. TANIGUCHI, H. F. CLARK, B. FORRESTER, Z. Y. FANG (1990): Polymerase Chain Reaction Amplification and Typing of Rotavirus Nucleic Acid from Stool Specimens. J Clin Microbiol. 28, 2, 276-82, doi: 10.1128/jcm.28.2.276-282.1990

GRANT, B. J., A. P. C. RODRIGUES, K. M. EL SAWY, J. A. MCCAMMON, L. S. D. CAVES (2006): Bio3d: An R package for the comparative analysis of protein structures. Bioinformatics 22, 2695–2696, doi: https://doi.org/10.1093/bioinformatics/btl461

GUTIÉRREZ-AGUIRRE, I., A. STEYER, J. BOBEN, K. GRUDEN, M. POLJŠAK-PRIJATELJ and M. RAVNIKAR (2008): Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. J Clin Microbiol. 46, 2547–2554. doi:10.1128/jcm.02428-07

HACKER J., U. DOBRINDT, R. KURTH (Eds.) (2012): Genome Diversity and Evolution of Rotaviruses. Genome Plast. Infect. Dis. Asm Press. 214–241, ISBN 978-1-55581-708-4

HAKIM, M. S., F. M. GAZALI, S. A. WIDYANINGSIH and M. K. PARVEZ (2024): Driving forces of continuing evolution of rotaviruses. World J Virol. 13, 2, 93774, doi: https://doi.org/10.5501/wjv.v13.i2.93774

HATCHER, E. L., S. A. ZHDANOV, Y. BAO, O. BLINKOVA, E. P. NAWROCKI, Y. OSTAPCHUCK, A. A. SCHAFFER, J. RODNEY BRISTER (2017): Virus Variation Resource-improved response to emergent viral outbreaks. Nucleic Acids Res 45, D482–D490, doi: https://doi.org/10.1093/nar/gkw1065

HEATHER, J. M. and B. CHAIN (2016): The sequence of sequencers: The history of sequencing DNA. Genomics 107, 1–8. doi:10.1016/j.ygeno.2015.11.003

HOLLOWAY, G. and B. S. COULSON (2013): Innate cellular responses to rotavirus infection. J Gen Virol. 94, 1151–1160. doi:10.1099/vir.0.051276-0

HOULDCROFT, C. J., M. A. BEALE and J. BREUER (2017): Clinical and biological insights from viral genome sequencing. Nat Rev Microbiol. 15, 183–192. doi:10.1038/nrmicro.2016.182

HOXIE, I. and J. J. DENNEHY (2020): Intragenic recombination influences rotavirus diversity and evolution. Virus Evol. 6, 1, vez059, doi: https://doi.org/10.1093/ve/vez059

HULL, J. J. A., M. QI, A. M. MONTMAYEUR, D. KUMAR, D. E. VELASQUEZ, S. S. MOON, L. C. MAGAÑA, N. BETRAPALLY, T. F. F. NG, B. JIANG and D. MARTHALER (2020): Metagenomic sequencing generates the whole genomes of porcine rotavirus A, C, and H from the United States. PLoS One 15, 12, e0244498, doi: https://doi.org/10.1371/journal.pone.0244498

HUNGERFORD, D., R. VIVANCOS, J.M. READ, V. E. PITZER, N. CUNLIFFE, N. FRENCH, M. ITURRIZA GÓMARA, S. ABERLE, M. VAN RANST, J. MATTHIJNSSENS, M. ZELLER, Z. MLADENOVA, S. MIDGLEY, T. K. FISCHER, V. P. SYRIOPOULOU, D. M. KOUKOU, K. BÁNYAI, R. DÓRÓ, J. KAPLON, P. POTHIER, L. MAUNULA, F. M. RUGGERI, L. FIORE, V. USONIS, I. IVASKEVICIENE, H. VENNEMA, A. KRONEMAN, M. POLJSAK-PRIJATELJ, A. STEYER, J. BUESA, G. CILLA, M. MONTES, M. BRYTTING, L. SCHLOSS, A. M. MARQUES, D. J. ALLEN and S. NAWAZ (2016): In-season and out-of-season variation of rotavirus genotype distribution and age of infection across 12 European countries before the introduction of routine vaccination, 2007/08 to 2012/13. Euro Surveill. 21, 30106, doi: 10.2807/1560-7917.ES.2016.21.2.30106.

IANIRO, G., R. MICOLANO, M. CONTE, M. LABIANCA, G. VACCARI and M. MONINI (2019): Detection of an animal-derived G4P[6] group A rotavirus strain in a symptomatic child, in Italy. Virus Res. 260, 7–11, doi:10.1016/j.virusres.2018.11.003

INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES (ICTV) (2024): Rotavirus Taxonomy. Available online: https://ictv.global/taxonomy, accessed on 25th May 2024

IVAC VIEW-hub (2025): Global Vaccine Introduction and Implementation Report. Available online at: https://view-hub.org/resources, accessed on June 25th 2025

JAMNIKAR-CIGLENECKI, U., U. KUHAR, S. STURM, A. KIRBIS, N. RACKI and A. STEYER (2016): The first detection and whole genome characterization of the G6P[15] group A rotavirus strain from roe deer. Vet Microbiol. 191, 52–59. doi:10.1016/j.vetmic.2016.05.019

JIMÉNEZ-RUIZ, S., N. SANTOS, J. A. BARASONA, A. E. FINE and F. JORI (2024): Editorial: Pathogen transmission at the domestic-wildlife interface: a growing challenge that requires integrated solutions. Front Vet Sci. 11, 1415335, doi: 10.3389/fvets.2024.14153355

JOHNE, R., K. SCHILLING-LOEFFLER, R. G. ULRICH, S. H. TAUSCH (2022): Whole Genome Sequence Analysis of a Prototype Strain of the Novel Putative Rotavirus Species L. Viruses 14, 3, 462, doi: 10.3390/v14030462.

JOHNE, R., S. H. TAUSCH, J. GRÜTZKE, A. FALKENHAGEN, C. PATZINA-MEHLING, M. BEER, D. HÖPER, R. G. ULRICH (2019): Distantly Related Rotaviruses in Common Shrews, Germany, 2004-2014. Emerg Inf Dis. 25, 12, 2310–2314, doi: 10.3201/eid2512.191225

JOHNE, R., S. H. TAUSCH, R. G. ULRICH and K. SCHILLING-LOEFFLER (2023): Genome analysis of the novel putative rotavirus species K. Virus Res 334, 199171, doi: 10.1016/j.virusres.2023.199171

JOTA BAPTISTA, C., F. SEIXAS, J. M. GONZALO-ORDEN and P. A. OLIVEIRA (2023): Wild boar (Sus scrofa) as a potential reservoir of infectious agents in Portugal: a review of two decades (2001–2021). Eur J Wildl Res 69, 101, 2023, doi: https://doi.org/10.1007/s10344-023-01732-9

KATZ, E. M., M. D. ESONA, N. S. BETRAPALLY, L. A. DE LA CRUZ DE LEON, Y. R. NEIRA, G. J. REY, M. D. BOWEN (2019): Whole-gene analysis of inter-genogroup reassortant rotaviruses from the Dominican Republic: Emergence of equine-like G3 strains and evidence of their reassortment with locally-circulating strains. Virology 534, 114–131, doi: https://doi.org/10.1016/j.virol.2019.06.007

KIM, H. H., J. G. PARK, J. MATTHIJNSSENS, H. J. KIM, H. J. KWON, K. Y. SON, E. H. RYU, D. S. KIM, W. S. LEE, M. KANG, D. K. YANG, J. H. LEE, S.J. PARK and K. O. CHO (2013): Pathogenicity of porcine G9P[23] and G9P[7] rotaviruses in piglets. Vet Microbiol 166, 123–137. doi:10.1016/j.vetmic.2013.05.024

KOZYRA, I., J. KOZYRA, A. DORS and A. RZEŻUTKA (2019): Molecular chracterisation of porcine group A rotaviruses: Studies on the age-related occurrence and spatial distribution of circulating virus genotypes in Poland. Vet Microbiol. 232, 105–113. doi:10.1016/j.vetmic.2019.03.026

KRAAY, A. N. M., A. F. BROUWER, N. LIN, P. A. COLLENDER, J. V. REMAIS and J. N. S. EISENBERG (2018): Modeling environmentally mediated rotavirus transmission:

The role of temperature and hydrologic factors. Environ Sci. 115, 12, E2782-E2790, doi: https://doi.org/10.1073/pnas.1719579115

KUNIĆ, V., T. MIKULETIČ, R. KOGOJ, T. KORITNIK, A. STEYER, S. ŠOPREK, G. TEŠOVIĆ, V. KONJIK, I. ROKSANDIĆ KRIŽAN, M. PRIŠLIN, D. BRNIĆ (2023): Interspecies transmission of porcine-originated G4P[6] *Rotavirus A* between pigs and humans: a synchronized spatiotemporal approach. Front Microbiol. 14, 1194764, doi: 10.3389/fmicb.2023.1194764

KUNIĆ, V., LJ. BARBIĆ, J. ŠIMIĆ, T. MIKULETIČ, R. KOGOJ, T. KORITNIK, A. STEYER, D. KONJEVIĆ, M. BUJANIĆ, M. PRIŠLIN ŠIMAC, D. BRNIĆ (2025): Interspecies transmission and genome heterogeneity of porcine-originated *Rotavirus A* between domestic pigs and wildlife in the Croatian ecosystem. Sci. Total Environ. 994, 180010, 15, doi: 10.1016/j.scitotenv.2025.180010

KUMAR, D., F. K. SHEPHERD, N. L. SPRINGER, W. MWANGI and D. G. MARTHALER (2022): Rotavirus Infection in Swine: Genotypic Diversity, Immune Responses, and Role of Gut Microbiome in Rotavirus Immunity. Pathogens 11, 10, 1078. doi: 10.3390/pathogens11101078

LAGAN, P., M. H. MOONEY and K. LEMON (2023): Genome analyses of species A rotavirus isolated from various mammalian hosts in Northern Ireland during 2013−2016. Virus Evol. 9, 2, vead039, doi: https://doi.org/10.1093/ve/vead0399

LANSZKI, J., M. HELTAI and L. SZABÓ (2006): Feeding habits and trophic niche overlap between sympatric golden jackal (Canis aureus) and red fox (Vulpes vulpes) in the Pannonian ecoregion (Hungary). Can J Zool 84, 1647–1656, doi:10.1139/z06-147

LE, X., Y. TAO, B. WANG, Y. HOU, Y. NING, J. HOU, R. WANG, Q. LI and X. XIA (2025): Diversity and Potential Cross-Species Transmission of Rotavirus A in Wild Animals in Yunnan, China. Microorganisms 13, 1, 145, doi: https://doi.org/10.3390/microorganisms13010145

LETUNIC, I. and P. BORK (2021): Interactive Tree of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49, 1, W293–W296, doi: https://doi.org/10.1093/nar/gkab301

LI, X., L. WANG, Y. ZHEN, S. LI and Y. XU (2015): Chicken egg yolk antibodies (IgY) as non-antibiotic production enhancers for use in swine production: A review. J Anim Sci Biotechnol. 6, 40, 2015, doi: https://doi.org/10.1186/s40104-015-0038-8

LIU, K., X. YANG, Y. WU and J. LI (2009): Rotavirus strategies to evade host antiviral innate immunity. Immunol Lett. 127, 13–18, doi:10.1016/j.imlet.2009.08.007

LIU, Y., P. HUANG, M. TAN, Y. LIU, J. BIESIADA, J. MELLER, A. A. CASTELLO, B. JIANG and X. JIANG (2012): Rotavirus VP8*: Phylogeny, Host Range, and Interaction with Histo-Blood Group Antigens. J Virol. 86, 9899–9910, doi:10.1128/jvi.00979-12

LOGAN, C., J. J. O'LEARY and N. O'SULLIVAN (2006): Real-time reverse transcription-PCR for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. J Clin Microbiol. 44, 3189–3195, doi:10.1128/jcm.00915-06

LUNDGREN, O. and L. SVENSSON (2001): Pathogenesis of Rotavirus diarrhea. Microbes Infect. 3, 13, 1145-56, doi: 10.1016/s1286-4579(01)01475-7

LUPPI, A., G. D'ANNUNZIO, C. TORREGGIANI and P. MARTELLI (2023): Diagnostic Approach to Enteric Disorders in Pigs. Animals 13, 3, 338, doi: 10.3390/ani13030338.

MacLACHLAN N. J., E. J. DUBOVI (Eds.) (2016): Epidemiology and Control of Viral Diseases. In Fenner's Veterinary Virology. 5th Ed. Boston: Academic Press, 131–153.

MAES, D. G. D., J. DEWULF, C. PIÑEIRO, S. EDWARDS and I. KYRIAZAKIS (2020): A critical reflection on intensive pork production with an emphasis on animal health and welfare. J Anim Sci. 98, S15–S26, doi:10.1093/jas/skz362

MALIK, Y. S., S. BHAT, P. S. DAR, S. SIRCAR, K. DHAMA and R. K. SINGH (2020): Evolving Rotaviruses, Interspecies Transmission and Zoonoses. Open Virol J. 14, 1–6, doi:10.2174/1874357902014010001

MANDL, J. N., R. AHMED, L. B. BARREIRO, P. DASZAK, J. H. EPSTEIN, H. W. VIRGIN and M. B. FEINBERG (2015): Reservoir host immune responses to emerging zoonotic viruses. Cell 160, 20–35, doi:10.1016/j.cell.2014.12.003

MANZOOR, S., Z. SYED and M. ABUBABAKAR (2023): Global Perspectives of Intensive Animal Farming and Its Applications. Intensive Animal Farming - A Cost-Effective Tactic. IntechOpen, doi: http://dx.doi.org/10.5772/intechopen.112271

MARINGA, W. M., P. N. MWANGI, J. SIMWAKA, E. M. MPABALWANI, J. M. MWENDA, I. PEENZE, M. D. ESONA, M. JEFFREY MPHAHLELE, M. L. SEHERI, M. M. NYAGA (2020): Molecular characterisation of a rare reassortant porcine-like g5p[6] rotavirus strain detected in an unvaccinated child in Kasama, Zambia. Pathogens 9, 8, 663, doi: 10.3390/pathogens9080663

MARTELLA, V., K. BÁNYAI, J. MATTHIJNSSENS, C. BUONAVOGLIA and M. CIARLET (2010): Zoonotic aspects of rotaviruses. Vet Microbiol 140, 246–255, doi:10.1016/j.vetmic.2009.08.028

MARTELLA, V., K. BÁNYAI, M. CIARLET, M. ITURRIZA-GÓMARA, E. LORUSSO, S. DE GRAZIA, S. ARISTA, N. DECARO, G. ELIA, A. CAVALLI, M. CORRENTE, A.

LAVAZZA, R. BASELGA and C. BUONAVOGLIA (2006): Relationships among porcine and human P[6] rotaviruses: Evidence that the different human P[6] lineages have originated from multiple interspecies transmission events. Virology 344, 509–519, doi:10.1016/j.virol.2005.08.029

MARTHALER, D., N. HOMWONG, K. ROSSOW, M. CULHANE, S. GOYAL, J. COLLINS, J. MATTHIJNSSENS and M. CIARLET (2014): Rapid detection and high occurrence of porcine rotavirus A, B, and C by RT-qPCR in diagnostic samples. J Virol Methods 209, 30–34, doi:10.1016/j.jviromet.2014.08.018

MARTIN, C., P. P. PASTORET, B. BROCHIER, M. F. HUMBLET and C. SAEGERMAN (2011): A survey of the transmission of infectious diseases/infections between wild and domestic ungulates in Europe. Vet Res. 42, 70, doi: https://doi.org/10.1186/1297-9716-42-70

MARTIN, D. P., B. MURRELL, M. GOLDEN, A. KHOOSAL, B. MUHIRE (2015): RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol. 1, 1, vev003, doi: https://doi.org/10.1093/ve/vev0033

MATTHIJNSSENS, J., M. RAHMAN, V. MARTELLA, Y. XUELEI, S. DE VOS, K. DE LEENER, M. CIARLET, C. BUONAVOGLIA and M. VAN RANST (2006): Full Genomic Analysis of Human Rotavirus Strain B4106 and Lapine Rotavirus Strain 30/96 Provides Evidence for Interspecies Transmission. J Virol. 80, 3801–3810. doi:10.1128/jvi.80.8.3801-3810.2006

MATTHIJNSSENS, J., M. CIARLET, M. RAHMAN, H. ATTOUI, K. BÁNYAI, M. K. ESTES, J. R. GENTSCH, M. ITURRIZA-GÓMARA, C. D. KIRKWOOD, V. MARTELLA, P. P. C. MERTENS, O. NAKAGOMI, J. T. PATTON, F. M. RUGGERI, L. J. SAIF, N. SANTOS, A. STEYER, K. TANIGUCHI, U. DESSELBERGER and M. VAN RANST (2008a): Recommendations for the classification of group a rotaviruses using all 11 genomic RNA segments. Arch Virol. 153, 1621–1629, doi:10.1007/s00705-008-0155-1

MATTHIJNSSENS, J., M. CIARLET, E. HEIMAN, I. ARIJS, T. DELBEKE, S. M. MCDONALD, E. A. PALOMBO, M. ITURRIZA-GÓMARA, P. MAES, J. T. PATTON, M. RAHMAN and M. VAN RANST (2008b): Full Genome-Based Classification of Rotaviruses Reveals a Common Origin between Human Wa-Like and Porcine Rotavirus Strains and Human DS-1-Like and Bovine Rotavirus Strains. J Virol 82, 3204–3219, doi:10.1128/jvi.02257-07

MATTHIJNSSENS, J., P. H. OTTO, M. CIARLET, U. DESSELBERGER, M. VAN RANST, R. JOHNE (2012): VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. Arch Virol. 157, 1177–1182, doi: https://doi.org/10.1007/s00705-012-1273-3

MATTHIJNSSENS J., R. JOHNE, J. T. PATTON, K. BÁNYAI (2019): Remove one species (Rotavirus E) from the genus Rotavirus. 2019.023M, available at: https ://ictv.globa l/ICTV/proposals/2019.023M.zip (accessed on 17th April 2025)

MATTHIJNSSENS, J., H. ATTOUI, K. BÁNYAI, C. P. D. BRUSSAARD, P. DANTHI, M. DEL VAS, T. S. DERMODY, R. DUNCAN, Q. FĀNG, R. JOHNE, P. P. C. MERTENS, F. M. JAAFAR, J. T. PATTON, T. SASAYA, N. SUZUKI, T. WEI, S. G. SIDDELL, E. J. LEFKOWITZ, S. SABANADZOVIC, P. SIMMONDS, F. M. ZERBINI, D. B. SMITH and J. H. KUHN (2022): ICTV Virus Taxonomy Profile: Sedoreoviridae 2022. J Gen Virol. 103, 10, doi: 10.1099/jgv.0.001782

MAUNULA, L. and C. H. VON BONSDORFF (2002): Frequent Reassortments May Explain the Genetic Heterogeneity of Rotaviruses: Analysis of Finnish Rotavirus Strains. J Virol. 76, 11793–11800, doi:10.1128/jvi.76.23.11793-11800.2002

McDONALD, S. M., M. I. NELSON, P. E. TURNER and J. T. PATTON (2016): Reassortment in segmented RNA viruses: Mechanisms and outcomes. Nat Rev Microbiol. 14, 448–460, doi:10.1038/nrmicro.2016.46

McNULTY, M. S. (2003): Rotavirus infections. In: Diseases of poultry. Eds. Saif, Y. M., Barnes, H. J., Glisson, J. R., Fadly, A. M., McDougald, L .R., Swayne, D. E. Ames (IA): Iowa State Press (308-317).

MÉSZNER, Z., I. ANCA, F. ANDRÉ, R. CHLIBEK, M. ČIŽMAN, P. GRZESIOWSKI, A. MANGAROV, M. POKORN, R. PRYMULA, D. RICHTER, N. SALMAN, P. ŠIMURKA, E. TAMM, G. TEŠOVIĆ, I. URBANCIKOVA, V. USONIS and D. ZAVADSKA (2013): Rotavirus vaccination in central Europe. J Pediatr Gastroenterol Nutr. 56, 586–596, doi:10.1097/mpg.0b013e31828dc35d

MIDGLEY, S. E., K. BÁNYAI, J. BUESA, N. HALAIHEL, C. K. HJULSAGER, F. JAKAB, J. KAPLON, L. E. LARSEN, M. MONINI, M. POLJŠAK-PRIJATELJ, P. POTHIER, F. M. RUGGERI, A. STEYER, M. KOOPMANS and B. BÖTTIGER (2012): Diversity and zoonotic potential of rotaviruses in swine and cattle across Europe. Vet Microbiol. 156, 238–245, doi:10.1016/j.vetmic.2011.10.027

MIHALOV-KOVÁCS E., Á. GELLÉRT, S. MARTON, S. L. FARKAS, E. FEHÉR, M. OLDAL, F. JAKAB, V. MARTELLA, K. BÁNYAI (2015): Candidate New Rotavirus

Species in Sheltered Dogs, Hungary. Emerg. Infect. Dis. 21, 660–663, doi: 10.3201/eid2104.141370

MIJATOVIC-RUSTEMPASIC, S., K. I. TAM, T. K. KERIN, J. M. LEWIS, R. GAUTAM, O. QUAYE, J. R. GENTSCH and M. D. BOWEN (2013): Sensitive and specific quantitative detection of rotavirus a by one-step real-time reverse transcription-PCR assay without antecedent double-stranded-RNA denaturation. J Clin Microbiol 51, 3047–3054. doi:10.1128/jcm.01192-13

MIJATOVIC-RUSTEMPASIC, S., M. D. ESONA, A. L. WILLIAMS, M. D. BOWEN (2016): Sensitive and specific nested PCR assay for detection of rotavirus A in samples with a low viral load. J Virol Methods 236, 41–46 doi: https://doi.org/10.1016/j.jviromet.2016.07.007

MONTEAGUDO, L. V., A. A. BENITO, S. LÁZARO-GASPAR, J. L. ARNAL, D. MARTIN-JURADO, R. MENJON and J. QUÍLEZ (2022): Occurrence of Rotavirus A Genotypes and Other Enteric Pathogens in Diarrheic Suckling Piglets from Spanish Swine Farms. Animals 12, 3, 251, doi: 10.3390/ani12030251.

MORI, Y., M. A. BORGAN, N. ITO, M. SUGIYAMA and N. MINAMOTO (2002): Sequential analysis of non-structural protein NSP4s derived from Group A avian rotaviruses. Virus Res. 89, 145-151, doi: 10.1016/S0168-1702(02)00112-0

MOUTELÍKOVÁ, R., L. DUFKOVÁ, J. KAMLER, J. DRIMAJ, R. PLHAL and J. PRODĚLALOVÁ (2016): Epidemiological survey of enteric viruses in wild boars in the Czech Republic: First evidence of close relationship between wild boar and human rotavirus A strains. Vet Microbiol. 193, 28–35, doi:10.1016/j.vetmic.2016.08.003

NGUYEN H. T., T., T. NAKAGOMI, H. MANH VU, T. THU THI NGUYEN, A. THI HAI DAO, A. THE NGUYEN, J.E. BINES, S. THOMAS, V. GRABOVAC, C. KATAOKA-NAKAMURA, T. TAICHIRO, F. HASEBE, T. KODAMA, M. KANEKO, H. THI THANH DANG, H. THI DUONG, D. DUCANH and O. NAKAGOMI (2024): Evolution of DS-1-like G8P[8] rotavirus A strains from Vietnamese children with acute gastroenteritis (2014-21): Adaptation and loss of animal rotavirus-derived genes during human-to-human spread. Virus Evol. 10, 1, veae045, doi: 10.1093/ve/veae045

NORDGREN, J., S. SHARMA, F. BUCARDO, W. NASIR, G. GÜNAYDIN, D. OUERMI, L. W. NITIEMA, S. BECKER-DREPS, J. SIMPORE, L. HAMMARSTRÖM, G. LARSON and L. SVENSSON (2014): Both lewis and secretor status mediate susceptibility to rotavirus infections in a rotavirus genotype-dependent manner. Clin. Infect. Dis. 59, 1567–1573, doi:10.1093/cid/ciu633

NOUR A. M. A. and M. W. PFAFFL (2020): MIQE and qPCR: How to apply MIQE Guidelines – a Visual, Interactive and Practical qPCR guide. 4th ed. bioMCC. ISBN: 9783000488061

NUÑEZ, L. F. N., S. H. SANTANDER PARRA, C. S. ASTOLFI-FERREIRA, C. CARRANZA, D. I. D. DE LA TORRE, A. C. PEDROSO and A. J. P. FERREIRA (2016): Detection of enteric viruses in pancreas and spleen of broilers with runting-stunting syndrome (RSS). Pesq Vet Bras. 36, 595–599. doi:10.1590/s0100-736x2016000700006

OKADERA, K., M. ABE, N. ITO, S. MORIKAWA, A. YAMASAKI, T. MASATANI, K. NAKAGAWA, S. YAMAOKA and M. SUGIYAMA (2013): Evidence of natural transmission of group A rotavirus between domestic pigs and wild boars (Sus scrofa) in Japan. Infect Genet Evol. 20, 54–60, doi:10.1016/j.meegid.2013.07.029

OMATOLA, C. A. and A. O. OLANIRAN (2022): Rotaviruses: From Pathogenesis to Disease Control— A Critical Review. Viruses 14, 5, 875, doi: 10.3390/v14050875

PACKER, E. and G. LITCHFIELD (2025): Viral 'backbone' underlies variation in rotavirus vaccine effectiveness. Available online at: https://elifesciences.org/subjects/microbiology-infectious-disease, accessed on 15th of May 2025

PALMARINI, M. Reoviridae. In Fenner's Veterinary Virology, 5th ed.; MacLachlan, N. J., Dubovi, E. J., Eds.; Academic Press: Boston, MA, USA, 2017, 299–317.

PAPP, H., B. LÁSZLÓ, F. JAKAB, B. GANESH, S. DE GRAZIA, J. MATTHIJNSSENS, M. CIARLET, V. MARTELLA and K. BÁNYAI (2013a): Review of group A rotavirus strains reported in swine and cattle. Vet Microbiol 165, 190–199. doi:10.1016/j.vetmic.2013.03.020

PAPP, H., R. BORZÁK, S. FARKAS, P. KISFALI, G. LENGYEL, P. MOLNÁR, B. MELEGH, J. MATTHIJNSSENS, F. JAKAB, V. MARTELLA and K. BÁNYAI (2013b): Zoonotic transmission of reassortant porcine G4P[6] rotaviruses in Hungarian pediatric patients identified sporadically over a 15year period. Infect Genet Evol. 19, 71–80, doi:10.1016/j.meegid.2013.06.013

PATTON, J. T. (2012): Rotavirus Diversity and Evolution in the Post-Vaccine World. Discov Med. 13, 68, 85–97.

PEDLEY S., J. C. BRIDGER, D. CHASEY, M. A. MCCRAE (1986): Definition of two new groups of atypical rotaviruses. J Gen Virol. 67, 1, 131-7.

PHAN, T. G., P. KHAMRIN T. D. QUANG, S. K. DEY, S. TAKANASHI, S. OKITSU, N. MANEEKARN, H. USHIJIMA (2007a): Detection and Genetic Characterization of

Group A Rotavirus Strains Circulating among Children with Acute Gastroenteritis in Japan. J Virol 81, 4645–4653. https://doi.org/10.1128/jvi.02342-06

PHAN, T. G., S. OKITSU, N. MANEEKARN, H. USHIJIMA (2007b): Genetic heterogeneity, evolution and recombination in emerging G9 rotaviruses. Infect Genet Evol. 7, 656–663, doi: https://doi.org/10.1016/j.meegid.2007.05.001

PICKETT, B. E., E. L. SADAT, Y. ZHANG, J. M. NORONHA, R. B. SQUIRES, V. HUNT, M. LIU, S. KUMAR, S. ZAREMBA, Z. GU, L. ZHOU, L., C. N. LARSON, J. DIETRICH, E. B. KLEM, R. H. SCHEUERMANN (2012): ViPR: An open bioinformatics database and analysis resource for virology research. Nucleic Acids Res 40, D593–D598, doi: https://doi.org/10.1093/nar/gkr859

PINHEIRO, M. S., J. B. L. DIAS, M. P. PETRUCCI, C. E. P. F. TRAVASSOS, G. S. MENDES and N. SANTOS (2023): Molecular Characterization of Avian Rotaviruses F and G Detected in Brazilian Poultry Flocks. Viruses 15, 5, 1089, doi: https://doi.org/10.3390/v15051089

POELAERT, D., P. PEREIRA, R. GARDNER, B. STANDAERT and B. BENNINGHOFF (2018): A review of recommendations for rotavirus vaccination in Europe: Arguments for change. Vaccine 36, 2243–2253, doi:10.1016/j.vaccine.2018.02.080

PUENTE, H., M. CORTEY, P. J. G. DE NOVA, Ó. MENCÍA-ARES, M. GÓMEZ-GARCÍA, I. DÍAZ, H. ARGUELLO, M. MARTÍN, P. RUBIO and A. CARVAJAL (2021): First identification and characterization of rotavirus H in swine in Spain. Transbound Emerg Dis. 68, 3055–3069, doi:10.1111/tbed.13992

R CORE TEAM R: A language and Environment for Statistical Computing. Available online: https://www.R-project.org/, accessed on June 8th 2022.

RAMIG, R. F. (2007): Systemic rotavirus infection. Expert Rev Anti Infect Ther 5, 591–612, doi:10.1586/14787210.5.4.591

RCWG (2023): List of Accepted Genotypes by Rotavirus Classification Working Group. Available online: https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg (accessed on 28 June 2025).

ROBINSON C. G., M. HERNANZ SCHULMAN, Y. ZHU, M. R. GRIFFIN, W. GRUBER, K. M. EDWARDS (2004): Evaluation of Anatomic Changes in Young Children with Natural Rotavirus Infection: Is Intussusception Biologically Plausible? J Infect Dis. 189, 8, 1382–1387, doi: https://doi.org/10.1086/3826555

ROHWEDDER, A., K. I. SCHÜTZ, N. MINAMOTO and H. BRÜSSOW (1995): Sequence analysis of pigeon, turkey, and chicken rotavirus VP8* identifies rotavirus 993/83,

isolated from calf feces, as a pigeon rotavirus. Virology 210, 231-235, doi: 10.1006/viro.1995.1338

RUI, J., K. LI, H. WEI, X. GUO, Z. ZHAO, Y. WANG, W. SONG, B. ABUDUNAIBI and T. CHEN (2024): MODELS: a six-step framework for developing an infectious disease model. Infect Dis Poverty 13, 30, doi: https://doi.org/10.1186/s40249-024-01195-3

SAIF, L. J., L. A. WARD, L. YUAN, B. I. ROSEN and T. L. TO (1996): The gnotobiotic piglet as a model for studies of disease pathogenesis and immunity to human rotaviruses. Arch Virol Suppl. 12, 153-61, doi: 10.1007/978-3-7091-6553-9_17

SAMSON, S., É. LORD, V. MAKARENKOV (2022): SimPlot++: a Python application for representing sequence similarity and detecting recombination. Bioinformatics 38, 3118–3120, doi: https://doi.org/10.1093/bioinformatics/btac287

SANTIANA, M., S. GHOSH , B. A. HO, V. RAJASEKARAN , W. L. DU , Y. MUTSAFI, D. A. DE JÉSUS-DIAZ, S. V. SOSNOVTSEV, E. A. LEVENSON, G. I. PARRA, P. M. TAKVORIAN, A. CALI, C. BLECK, A. N. VLASOVA, L. J. SAIF, J. T. PATTON, P. LOPALCO, A. CORCELLI, K. Y. GREEN, N. ALTAN-BONNET (2018): Vesicle-Cloaked Virus Clusters Are Optimal Units for Interorganismal Viral Transmission. Cell Host Microbe 24, 208-220, e8, doi: 10.1016/j.chom.2018.07.006

SAXENA, K., S. E. BLUTT, K. ETTAYEBI, X. L. ZENG, J. R. BROUGHMAN, S. E. CRAWFORD, U. C. KARANDIKAR, N. P. SASTRI, M. E. CONNER, A.R. OPEKUN, D. Y. GRAHAM, W. QURESHI, V. SHERMAN, J. FOULKE-ABEL, J. IN, O. KOVBASNJUK, N. C. ZACHOS, M. DONOWITZ and M. K. ESTES (2016): Human Intestinal Enteroids: a New Model To Study Human Rotavirus Infection, Host Restriction, and Pathophysiology. J Virol. 90, 43–56, doi:10.1128/jvi.01930-15

SCHMITZ, D., F. ZWAGEMAKER, B. VAN DER VEER, H. VENNEMA, J. F. J. LAROS, M. P. G. KOOPMANS, M. DE GRAAF and A. KRONEMAN (2023): Metagenomic Surveillance of Viral Gastroenteritis in a Public Health Setting. Microbiol Spectr. 11, e0502222, doi: 10.1128/spectrum.05022-22

SCHUG, F., A. BAR-MASSADA, A. R. CARLSON, H. COX, T. J. HAWBAKER, D. HELMERS, P. HOSTERT, D. KAIM, N. K. KASRAEE, S. MARTINUZZI, M. H. MOCKRIN, K. A. PFOCH and V. C. RADELOFF (2023): The global wildland–urban interface. Nature 621, 94–99, doi:10.1038/s41586-023-06320-0

SCHWARTZ-CORNIL, I., Y. BENUREAU, H. GREENBERG, B. A. HENDRICKSON and J. COHEN (2002): Heterologous Protection Induced by the Inner Capsid Proteins of

Rotavirus Requires Transcytosis of Mucosal Immunoglobulins. J Virol. 76, 8110–8117. doi:10.1128/jvi.76.16.8110-8117.2002

SERGEANT, E. S. G. (2018): Epitools Epidemiological Calculators. Ausvet. Available at: http://epitools.ausvet.com.au (accessed on 18th January 2022)

SHIZAWA, S., F. FUKUDA, Y. KIKKAWA, T. OI, H. TAKEMAE, T. MASUDA, H. ISHIDA, H. MURAKAMI, S. SAKAGUCHI, T. MIZUTANI, M. NAGAI and M. OBA (2024): Genomic diversity of group A rotaviruses from wild boars and domestic pigs in Japan: wide prevalence of NSP5 carrying the H2 genotype. Arch Virol 169, 3, 63, doi: 10.1007/s00705-023-05954-9

SILVA, F. D. F., F. GREGORI and S. M. McDONALD (2016): Distinguishing the genotype 1 genes and proteins of human Wa-like rotaviruses vs. porcine rotaviruses. Infect Genet Evol. 43, 6–14, doi:10.1016/j.meegid.2016.05.014

ŠIMIĆ, I., A. KOVAČEVIĆ, N. KREŠIĆ, I. ŠKOKO, V. KONJIK, J. J. GUĆ, V. KRŽELJ, G. TEŠOVIĆ and D. BRNIĆ (2019): Molecular characteristics and zoonotic potential of rotaviruses. Paediatr Croat. 63, 63–70. doi:10.13112/pc.2019.10

SINGH, B. B., M. P. WARD and N. K. DHAND (2023): Host characteristics and their influence on zoonosis, disease emergence and multi-host pathogenicity. One Health 17, 100596, doi: https://doi.org/10.1016/j.onehlt.2023.100596

SMITS, S. L., R. BODEWES, A. RUIZ-GONZALEZ, W. BAUMGÄRTNER, M. P. KOOPMANS, A. D. M. E. OSTERHAUS and A. C. SCHÜRCH (2014): Assembly of viral genomes from metagenomes. Front Microbiol. 18, 5, 2014, doi: https://doi.org/10.3389/fmicb.2014.00714

SPARRER, K. M. J. and M. U. GACK (2015): Intracellular detection of viral nucleic acids. Curr Opin Microbiol. 26, 1–9. doi:10.1016/j.mib.2015.03.001

STATHAM, M. J., C. J. EDWARDS, K. NORÉN, C. D. SOULSBURY and B. N. SACKS (2018): Genetic analysis of European red foxes reveals multiple distinct peripheral populations and central continental admixture. Quat Sci Rev. 197, 257–266, doi:10.1016/j.quascirev.2018.08.019

STEYER, A., K. G. TORKAR, I. GUTIÉRREZ-AGUIRRE and M. POLJŠAK-PRIJATELJ (2011): High prevalence of enteric viruses in untreated individual drinking water sources and surface water in Slovenia. Int J Hyg Environ Health 214, 392–398, doi:10.1016/j.ijheh.2011.05.006

STEYER, A., M. POLJŠAK-PRIJATELJ, D. BARLIČ-MAGANJA and J. MARIN (2008): Human, porcine and bovine rotaviruses in Slovenia: Evidence of interspecies transmission and genome reassortment. J Gen Virol. 89, 1690–1698, doi:10.1099/vir.0.2008/001206-0

SVENSSON, L. (2000): Diagnosis of foodborne viral infections in patients, Int J Food Microbiol. 59, 117-26, doi: 10.1016/s0168-1605(00)00281-6

TACHAROENMUANG, R., R. GUNTAPONG, S. UPACHAI, P. SINGCHAI, S. FUKUDA, T. IDE, R. HATAZAWA, K. SUTTHIWARAKOM, S. KONGJORN, N. ONVIMALA, T. LUECHAKHAM, K. RUCHUSATSAWAST, Y. KAWAMURA, B. SRIWANTHANA, K. MOTOMURA, M. TATSUMI, N. TAKEDA, T. YOSHIKAWA, T. MURATA, B. UPPAPONG, K. TANIGUCHI and S. KOMOTO (2021): Full genome-based characterization of G4P[6] rotavirus strains from diarrheic patients in Thailand: Evidence for independent porcine-to-human interspecies transmission events. Virus Genes 57, 338–357, doi:10.1007/s11262-021-01851-y

TAMURA, K., G. STECHER, S. KUMAR (2021): MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 38, 3022–3027, doi: https://doi.org/10.1093/molbev/msab120

TATE, J. E., A. H. BURTON, C. BOSCHI-PINTO, U. D. PARASHAR, M. AGOCS, F. SERHAN, L. DE OLIVEIRA, J. M. MWENDA, R. MIHIGO, P. RANJAN WIJESINGHE, N. ABEYSINGHE, K. FOX and F. PALADIN (2016): Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013. Clin Infect Dis. 62, S96–S105, doi:10.1093/cid/civ1013

TCHEREMENSKAIA, O., G. MARUCCI, S. DE PETRIS, F. M. RUGGERI, D. DOVECAR, S. L. STERNAK, I. MATYASOVA, M. K. DHIMOLEA, Z. MLADENOVA, L. FIORE, A. KRAIGHER, M. BUJKO, M. TRKOV, S. BUKOVSKI-SIMONOSKI, A. LUKIC-GRLIC, N. SOJKOVA, P. PAZDIORA, E. KAKARRIQI, S. BINO, D. BEBECI, N. KORSUN and S. GYUROVA (2007): Molecular epidemiology of rotavirus in Central and Southeastern Europe. J Clin Microbiol. 45, 2197–2204. doi:10.1128/jcm.00484-07

TEŠOVIĆ, G. (2012): Childhood vaccinations in Croatia. Period. Biol. 114, 2, 149–166.

THEUNS, S., E. HEYLEN, M. ZELLER, I. D. M. ROUKAERTS, L. M. B. DESMARETS, M. VAN RANST, H. J. NAUWYNCK and J. MATTHIJNSSENS (2015): Complete Genome Characterization of Recent and Ancient Belgian Pig Group A Rotaviruses and

Assessment of Their Evolutionary Relationship with Human Rotaviruses. J Virol. 89, 1043–1057, doi:10.1128/jvi.02513-14

THEUNS, S., L. M. B. DESMARETS, E. HEYLEN, M. ZELLER, A. DEDEURWAERDER, I. D. M. ROUKAERTS, M. VAN RANST, J. MATTHIJNSSENS, H. J. NAUWYNCK (2014): Porcine group A rotaviruses with heterogeneous VP7 and VP4 genotype combinations can be found together with enteric bacteria on Belgian swine farms. Vet Microbiol. 172, 23–34, doi: https://doi.org/10.1016/j.vetmic.2014.04.002

TROEGER, C., I. A. KHALIL, P. C. RAO, S. CAO, B. F. BLACKER, T. AHMED, G. ARMAH, J. E. BINES, T. G. BREWER, D. V. COLOMBARA, G. KANG, B. D. KIRKPATRICK, C. D. KIRKWOOD, J. M. MWENDA, U. D. PARASHAR, W. A. PETRI, M. S. RIDDLE, A. D. STEELE, R. L. THOMPSON, J. L. WALSON, J.W. SANDERS, A. H. MOKDAD, C. J. L. MURRAY, S. I. HAY and R. C. REINER (2018): Rotavirus Vaccination and the Global Burden of Rotavirus Diarrhea among Children Younger Than 5 Years. JAMA Pediatr. 172, 958–965. doi:10.1001/jamapediatrics.2018.1960

VIBIN, J., A. CHAMINGS, F. COLLIER, M. KLAASSEN, T. M. NELSON and S. ALEXANDERSEN (2018): Metagenomics detection and characterisation of viruses in faecal samples from Australian wild birds. Sci Rep 8, 8686, doi: https://doi.org/10.1038/s41598-018-26851-1

VILIBIC-CAVLEK, T., L. BARBIC, A. MRZLJAK, D. BRNIC, A. KLOBUCAR, M. ILIC, N. JANEV-HOLCER, M. BOGDANIC, L. JEMERSIC, V. STEVANOVIC, I. TABAIN, S. KRCMAR, M. VUCELJA, J. PRPIC, M. BOLJFETIC, P. JELICIC, J. MADIC, I. FERENCAK and V. SAVIC (2021): Emerging and neglected viruses of zoonotic importance in Croatia. Pathogens 10, 1–24, doi:10.3390/pathogens10010073

VLASOVA, A. N., J. O. AMIMO and L. J. SAIF (2017): Porcine rotaviruses: Epidemiology, immune responses and control strategies. Viruses 9, 3, 48, doi: 10.3390/v9030048

VLASTELICA Ž., M. ROGULJ, V. KRŽELJ, I. IVIĆ, L. STEMBERGER, J. PETRIĆ, T. KOVAČEVIĆ, B. RUNTIĆ , A. NOVAK, G. TEŠOVIĆ (2010): Rotavirusne infekcije djece liječene u kliničkom bolničkom centru split tijekom trogodišnjeg razdoblja. Paediatr Croat. 54, Supl 1, 177-181.

VRDOLJAK, M., M. GUŽVINEC, V. TRKULJA, I. BUTIĆ, I. IVIĆ, V. KRŽELJ, M. TONKIĆ, M. HEGEDUŠ JUNGVIRTH, M. PAYERL PAL and G. TEŠOVIĆ (2019): Distribution of rotavirus genotypes in three Croatian regions among children ≤5 years of age (2012–2014). Int J Infect Dis. 89, 3–9, doi:10.1016/j.ijid.2019.09.008

WALKER, P. J., S. G. SIDDELL, E. J. LEFKOWITZ, A. R. MUSHEGIAN, E. M. ADRIAENSSENS, D. M. DEMPSEY, B. E. DUTILH, B. HARRACH, R. L. HARRISON, R. C. HENDRICKSON, S. JUNGLEN, N. J. KNOWLES, A. M. KROPINSKI, M. KRUPOVIC, J. H. KUHN, M. NIBERT, R. J. ORTON, L. RUBINO, S. SABANADZOVIC, P. SIMMONDS, D. B. SMITH, A. VARSANI, F. M. ZERBINI and A. J. DAVISON (2020): Changes to virus taxonomy and the Statutes ratified by the International Committee on Taxonomy of Viruses (2020). Arch Virol. 165, 2737–2748, doi:10.1007/s00705-020-04752-x

WANDERA, E. A., R. HATAZAWA, N. TSUTSUI, N. KUROKAWA, C. KATHIIKO, M. MUMO, E. WAITHIRA, M. WACHIRA, B. MWAURA, J. NYANGAO, S. A. KHAMADI, J. NJAU, S. FUKUDA, T. MURATA, K. TANIGUCHI, Y. ICHINOSE, S. KANEKO, S. KOMOTO (2021): Genomic characterization of an African G4P[6] human rotavirus strain identified in a diarrheic child in Kenya: Evidence for porcine-to-human interspecies transmission and reassortment. Infect Genet Evol. 96, 105133, doi: https://doi.org/10.1016/j.meegid.2021.105133

WEGNER, G. I., K. A. MURRAY, M. SPRINGMANN, A. MULLER, S. H. SOKOLOW, K. SAYLORS and D. M. MORENS (2022): Averting wildlife-borne infectious disease epidemics requires a focus on socio-ecological drivers and a redesign of the global food system. EClinicalMedicine 47, 101386, doi: 10.1016/j.eclinm.2022.101386

WELLBROCK, W. (2008): Animal Production Systems Pig welfare in Croatia: A critical reflection on the EU pig welfare directives. Master thesis, Animal Sciences, Wageningen University. Available at: https://www.researchgate.net/publication/40095486_Pig_welfare_in_Croatia_a_critical_reflection_on_the_EU_welfare_directives

WICKHAM, H. (2016): Ggplot2: Elegant Graphics for Data Analysis; Springer: New York, NY, USA. ISBN 978-3-319-24277-4.

WINIARCZYK, S., P. S. PAUL, S. MUMMIDI, R. PANEK and Z. GRADZKI (2002): Survey of porcine rotavirus G and P genotype in Poland and the United States using RT-PCR. J Vet Med. B 49, 373–378, doi:10.1046/j.1439-0450.2002.00572.x

WORLD HEALTH ORGANIZATION (2009): Manual of rotavirus detection and characterization methods. WHO/IVB/08.17. Geneva, Switzerland.

WORLD ORGANISATION FOR ANIMAL HEALTH (2024): The Importance of the One Health Approach in Tackling Emerging and Re-emerging Zoonotic Epidemics and Pandemics. Licence: CC BY-SA 3.0 IGO, doi: https://doi.org/10.20506/woah.3480.

WU, F. T., L. T. C. LIU, B. JIANG, T. Y. KUO, C. Y. WU and M. H. LIAO (2022): Prevalence and diversity of rotavirus A in pigs: Evidence for a possible reservoir in human infection. Infect Genet Evol. 98, 105198, doi: 10.1016/j.meegid.2021.105198

WYLIE K. M., T. N. WYLIE, R. BULLER, B. HERTER, M. T. CANNELLA, G. A. STORCH (2018): Detection of Viruses in Clinical Samples by Use of Metagenomic Sequencing and Targeted Sequence Capture. J Clin Microbiol. 56, 12, e01123-18, doi: 10.1128/JCM.01123-18

XU, X., Y. LUO, C. HE, Z. DIAN, H. MI, J. YANG, Y. FENG, Z. MIAO and X. XIA (2023): Increased Risk of Neurological Disease Following Pediatric Rotavirus Infection: A Two-Center Case-Control Study. Journal of Infectious Diseases 227, 1313–1321. doi:10.1093/infdis/jiac486

YAN, H., B. DONG, X. LI, J. HE, B. YU, X. MAO, J. YU, Y. LUO, J. LUO, A. WU, J. PU, Q. WANG, H. WANG, J. CRENSHAW, Y. SHEN and D. CHEN (2024): Spray-dried plasma protects against rotavirus-induced gastroenteritis via regulating macrophage and T cells divergence in weanling pigs. Front Vet Sci. 16, 11, 2024, doi: https://doi.org/10.3389/fvets.2024.1467108

YU, G. Scatterpie: Scatter Pie Plot, R Package Version 0.1.7, 2021. Available online: https://CRAN.R-project.org/package=scatterpie (accessed on 15 July 2022).

ZECCHIN, B., M. DE NARDI, P. NOUVELLET, C. VERNESI, M. BABBUCCI, B. CRESTANELLO, Z. BAGÓ, T. BEDEKOVIĆ, P. HOSTNIK, A. MILANI, C. A. DONNELLY, L. BARGELLONI, M. LORENZETTO, C. CITTERIO, F. OBBER, P. DE BENEDICTIS and G. CATTOLI (2019): Genetic and spatial characterization of the red fox (Vulpes vulpes) population in the area stretching between the Eastern and Dinaric Alps and its relationship with rabies and canine distemper dynamics. PLoS One 14, 3, e0213515, doi: 10.1371/journal.pone.0213515

8. PUBLISHED SCIENTIFIC PAPERS

8.1. PAPER I

BRNIĆ, D., D. ČOLIĆ, V. KUNIĆ, N. MALTAR-STRMEČKI, N. KREŠIĆ, D. KONJEVIĆ, M. BUJANIĆ, I. BAČANI, D. HIŽMAN, L. JEMERŠIĆ (2022): *Rotavirus A* in Domestic Pigs and Wild Boars: High Genetic Diversity and Interspecies Transmission. Viruses, 14 (2022), 9, 2028, 20. doi: 10.3390/v14092028

viruses

MDPI

Article

Rotavirus A in Domestic Pigs and Wild Boars: High Genetic Diversity and Interspecies Transmission

Dragan Brnić [1,*,†], Daniel Čolić [1,2,†], Valentina Kunić [1], Nadica Maltar-Strmečki [3], Nina Krešić [1], Dean Konjević [4], Miljenko Bujanić [4], Ivica Bačani [5], Dražen Hižman [6,7] and Lorena Jemeršić [1]

1 Virology Department, Croatian Veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia
2 Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Baldiri Reixac 10, 08028 Barcelona, Spain
3 Laboratory for Electron Spin Spectroscopy, Division of Physical Chemistry, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia
4 Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia
5 Animal Feed Factory Ltd., Dr. Ivana Novaka 11, 40000 Čakovec, Croatia
6 Belje Agro-Vet plus Ltd., Kokingrad 4, Mece, 31326 Darda, Croatia
7 Rusagro, LLC "Tambovsky bacon", Bazarnaya 104, 392036 Tambov, Russia
* Correspondence: brnic@veinst.hr; Tel.: +385-1-6123-674
† These authors contributed equally to this work.

Abstract: Rotavirus A (RVA) is an important pathogen for porcine health. In comparison to humans, RVA in domestic animals and especially in wildlife is under researched. Therefore, the aim of the present study was to investigate the prevalence, genetic diversity, molecular epidemiology and interspecies transmission of RVA in domestic pigs and wild boars. During the three consecutive RVA seasons (2018–2021) we collected 445 and 441 samples from domestic pigs and wild boars, respectively. Samples were tested by real-time RT-PCR, and RVA-positive samples were genotyped in VP7 and VP4 segments. Our results report an RVA prevalence of 49.9% in domestic pigs and 9.3% in wild boars. Outstanding RVA genetic diversity was observed in VP7 and VP4 segments, especially in domestic pigs exhibiting a striking 23 different RVA combinations (G5P[13] and G9P[23] prevailed). Interspecies transmission events were numerous between domestic pigs and wild boars, sharing G3, G5, G6, G9, G11 and P[13] genotypes. Furthermore, our data indicate that such transmission events involved even bovines (G6, P[11]) and, intriguingly, humans (G1P[8]). This study contributes to the basic knowledge that may be considered important for vaccine development and introduction, as a valuable and currently missing tool for efficient pig health management in the EU.

Keywords: rotavirus A; domestic pig; wild boar; genotype; genetic diversity; molecular epidemiology; phylogenetic analysis; interspecies transmission; Croatia

check for updates

Citation: Brnić, D.; Čolić, D.; Kunić, V.; Maltar-Strmečki, N.; Krešić, N.; Konjević, D.; Bujanić, M.; Bačani, I.; Hižman, D.; Jemeršić, L. Rotavirus A in Domestic Pigs and Wild Boars: High Genetic Diversity and Interspecies Transmission. *Viruses* **2022**, *14*, 2028. https://doi.org/10.3390/v14092028

Academic Editor: Grzegorz Woźniakowski

Received: 18 August 2022
Accepted: 11 September 2022
Published: 13 September 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Rotaviruses (RV), the species *Rotavirus A* (RVA) in particular, represent a major healthcare burden worldwide [1]. They are a significant enteric pathogen in intensive animal farming as well, especially among younger animals [2]. The focus of the research community has been primarily oriented towards human rotaviruses, with 30 and 100 times fewer genotyped RVA strains of bovine and swine origin, respectively [3]. The knowledge gap is even wider on rotaviruses circulating in wildlife [4].

The family *Reoviridae* and the genus *Rotavirus* encompass a diverse group of rotavirus species; *Rotavirus A–D* and *Rotavirus F–J* [5]. The RVA is by far the species with the most significant impact on human and animal health [6]. These dsRNA viruses possess a genome consisting of 11 segments, which are enclosed in a triple-layered capsid [7]. The rotavirus nomenclature is binomial and based on the two genomic segments encoding two outer capsid structural proteins, VP7 and VP4, which define the G and P genotypes, respectively.

However, the nomenclature based on the genotype assignment for all 11 segments has been developed [8]. Currently, there are at least 41 G and 57 P genotypes recognized by the Rotavirus Classification Working Group (RCWG) [9].

Direct interspecies transmission and transmission coupled with reassortment are considered to be two major routes for rotaviruses to cross the host barrier [10]. The segmented form of the rotavirus genome is a prerequisite for the occurrence of new chimeric reassortant strains, very often of human and animal origin with many examples involving domestic animals [11]. However, the importance of wildlife in such interspecies transmission events might be underestimated since the research on that topic has largely been neglected by scientists worldwide.

Rotaviruses provide an everlasting challenge for pig health management due to their ubiquitous nature and high resistance in the environment [12]. The disease they cause is usually self-limiting gastroenteritis, which can be fatal in young piglets due to dehydration, with outbreaks being especially severe in intensive farming systems [2]. The prevalence of RVA in clinically affected and asymptomatic pigs ranges between 3.3 and 67.3%, without evidence of seasonality, but with spatio-temporal variations and the re-emergence of certain genotypes [13]. Despite being under-researched, RVAs circulating in domestic pigs exhibit high diversity with at least 50 known genotype combinations [6]. Domestic pigs are considered to be the origin of some RVA genotypes circulating in humans, such as G9 and G12 genotypes [11]. Moreover, it is considered that human Wa-like and porcine RVAs share a common ancestor [14]. Apart from reducing RVA build-up through strict hygiene measures, the importance of boosting lactogenic immunity with vaccination remains the most important tool for confronting RVA infection adverse outcomes [2]. Nevertheless, currently, there is no authorized vaccine against porcine RVA in the European Union, yet some pig producers rely on vaccine importation from the USA [15]. In light of the high genetic diversity of porcine RVAs, there are concerns regarding vaccine efficacy on heterologous strains [13,15].

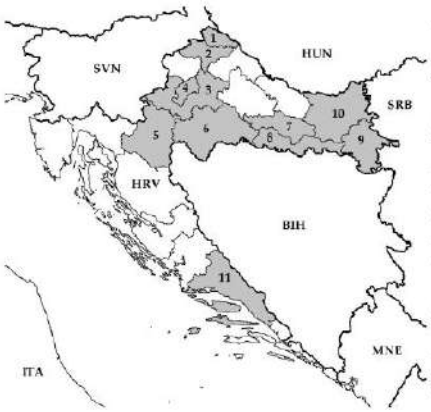
RVA in wild boars, to the best of our knowledge, has been scarcely researched so far with only two papers dealing with molecular epidemiology and genetic diversity of circulating strains. Both advocate interspecies transmission of RVAs between domestic pigs and wild boars [16,17]. In addition, the close phylogenetic relatedness to certain RVA strains detected in humans was described [16,17].

The aim of the present study was to reveal the concurrent prevalence, molecular epidemiology, genetic diversity and possible interspecies transmission events of RVA strains circulating in domestic pig and wild boar populations in Croatia during three consecutive RVA seasons.

2. Materials and Methods

2.1. Sampling

From 2018 to 2021 (covering rotavirus seasons 2018/2019, 2019/2020 and 2020/2021), we sampled 445 domestic pigs and 441 free-living wild boars (*Sus scrofa*). All animals were sampled only once. Most of the samples (98.2% of domestic pigs and 78.9% of wild boars) were collected during the autumn/winter months (October to March). Sampled animals originated from 10 counties (seven for domestic pigs and eight for wild boars) of the Continental Croatia (Pannonian Croatia, Northern Croatia and City of Zagreb according to the NUTS-2 classification) and in one county (Split-Dalmatia County: domestic pigs) of the Adriatic Croatia (Figure 1). Domestic pigs included in the present study were locally bred on 24 small backyard holdings ($N = 276$) and eight large holdings ($N = 169$). Holdings breeding imported weanling and fattening pigs were not included in the present study. Some holdings were visited for sampling more than once, but different animals were sampled each time. Wild boars were sampled after regular hunting in 15 hunting areas located in eight counties (Figure 1).



Map area	County	No. of domestic pigs (No./% RVA positive)	RVA genotypes, domestic pigs	No. of wild boars (No./% RVA positive)	RVA genotypes, wild boars
1	Medimurje	98 (49/50)	G1, G2, G3, G5, G6, G9; P[7], P[8], P[11], P[13], P[23], P[32]	11 (0)	N/A
2	Varaždin	17 (6/35.3)	G2, G4; P[23], P[32]	0	N/A
3	Zagreb County	20 (15/75)	G4, G3; P[13]	115 (16/13.9)	G3, G9, G11; P[13]
4	City of Zagreb	0	N/A	115 (7/6.1)	G3, G6, G9
5	Karlovac	0	N/A	21 (2/9.5)	G3
6	Sisak-Moslavina	43 (17/39.5)	G2, G3; P[13]	31 (0)	N/A
7	Požega-Slavonia	13 (0)	N/A	0	N/A
8	Brod-Posavina	0	N/A	36 (1,2.8)	G6
9	Vukovar-Srijem	152 (91/59.9)	G1, G2, G3, G4, G5, G9, G11; P[6], P[8], P[13], P[23]	49 (4/8.2)	G5, G11; P[13]
10	Osijek-Baranja	65 (34/52.3)	G1, G2, G3, G5, G9; P[6], P[7], P[8], P[13], P[23]	63 (11/17.5)	G3, G5; P[13]
11	Split-Dalmatia	37 (10/27)	G2, G3; P[13], P[23]	0	N/A
Total		445 (222/49.9)		441 (41/9.3)	

Figure 1. Geographical distribution of sampling sites and the results of RVA detection and genotyping (VP7 and VP4) in domestic pigs and wild boars. Counties included in the present study are marked in grey. N/A stands for Not Applicable in the case there were no samples taken or all collected samples were negative on RVA. The map source is available at: https://commons.wikimedia.org/wiki/File:Croatia_location_map.svg (NordNordWest; CC BY-SA 3.0; accessed on 11 January 2019).

All samples were taken from individual animals by rectal swabs (young age categories of domestic pigs) or by a plastic scoop attached to a container's lid when fecal samples or intestinal content were sampled (wild boars). The age, gender and status of diarrhea were registered at the time of sampling. Domestic pigs were divided into four age groups: suckling piglets (<28 days; $N = 231$), weanling pigs (29–84 days; $N = 177$), fattening pigs (>85 days, $N = 28$) and sows ($N = 9$). On the other hand, three age groups were defined for wild boars: <1 year ($N = 151$), 1–2 years ($N = 135$) and >2 years ($N = 155$). The gender was reported for 385 domestic pigs (178 females and 207 males) and 440 wild boars (223 females and 217 males). Diarrhea was registered in 165 domestic pigs (37.1%) and in only eight wild boars (1.8%). Other animals were observed as healthy regarding their gastrointestinal tract: 280 domestic pigs (62.9%) and 433 wild boars (98.2%). All samples were transferred to the laboratory maintaining the cold chain and stored at −20 °C until further use.

2.2. RNA Extraction and Real-Time RT-PCR

RNA was extracted from the supernatant of 20% *w/v* fecal suspension, which was prepared using Medium 199 (Sigma Aldrich, St. Louis, MO, USA), vortexed and centrifuged at 14,000× *g*. The RNA extraction procedure was performed on the KingFisher™ Flex purification system (ThermoFisher Scientific, Waltham, MA, USA) using the MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) by following the manufacturer's instructions (complex workflow). The exogenous Internal Positive Control (IPC) RNA, Xeno™ RNA Control (ThermoFisher Scientific, Waltham, MA, USA), was added to each sample (2 µL) to supervise the appearance of potential PCR inhibitors. The extracted RNA was stored at −80 °C if not processed immediately.

Detection of the RVAs dsRNA was performed by real-time RT-PCR, which amplifies the fragment of VP2 segment of different RVA genotypes infecting humans and domestic animals [18]. Nevertheless, this protocol was previously successfully applied for RVA detection in wildlife-related research [19,20]. The reaction mixture setup and thermal cycling conditions were described previously [20]. The runs were performed on a Rotor-Gene Q or QIAquant 96 5plex (Qiagen, Hilden, Germany). If inhibition was observed, the samples were diluted at 1:5 and retested.

2.3. VP7 and VP4 Genotyping

All VP2 positive samples were subjected to genotyping in order to define the G genotype (VP7) and P genotype (VP4). Several approaches were applied to overcome possible bottlenecks due to the RVA strain genetic diversity.

The VP7 genotyping was performed with a combination of VP7 Beg9 and VP7 End9 primers [21] in the first round of RT-PCR followed by the nested PCR using VP7-up2 and VP7-down3 primers [22]. The next approach was the RT-PCR using VP7-F and VP7-R primers, followed by the seminested PCR using VP7-F and VP7-RINT primers [23] if the result of the first RT-PCR reaction was negative. In some cases, we applied primers N-VP7F1 and N-VP7R1 in the first round of RT-PCR, and primers N-VP7F2 and N-VP7R2 in the nested PCR. These primer sets were designed for samples containing low RVA load [24].

The VP4 genotyping was a combination of three different approaches as well. One approach was the application of VP4-HeadF and VP4-1094R2 primers in the RT-PCR followed by the seminested PCR using VP4-HeadF and VP4-887R primers [22]. The other one was a combination of VP4_1-17F and VP4R_DEG primers in the RT-PCR reaction [25]. The last approach consisted of N-VP4F1 and N-VP4R1 in the RT-PCR, and N-VP4F2 and N-VP4R2 in the nested PCR [24].

All RT-PCR reactions were conducted with the utilization of SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (ThermoFisher Scientific, Waltham, MA, USA). For the nested or seminested PCR, GoTaq® G2 Hot Start Colorless Master Mix (Promega, Madison, WI, USA) was utilized. Primer concentrations and annealing temperatures used in each RT-PCR and nested or seminested PCR reaction were as recommended by the corresponding article. Other conditions, related to the reaction mixture setup and thermal cycling, were as recommended by the reagent's manufacturer. Each reaction started with the initial dsRNA denaturation step at 95 °C for 5 min in which extracted RNA was combined with the respective forward primer and PCR grade water. Hereafter, the remaining reagents were added to the reaction mixture, which was run on the ABI 9700 GeneAmp thermal cycler (Applied Biosystems, Foster City, CA, USA) or Biometra TRIO (Analytic Jena, Jena, Germany). Reactions were visualized on the QIAxcel Advanced System for capillary electrophoresis using the QIAxcel DNA Screening kit (Qiagen, Hilden, Germany).

2.4. Genotype Assignment and Phylogenetic Analysis

The purification of RT-PCR and PCR products was performed by ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific, Waltham, MA, USA) or Monarch DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA, USA) as described previously [20]. Subsequently, the samples were subjected to direct Sanger sequencing in both directions using the services of Macrogen Europe (Amsterdam, the Netherlands). The RVA genotypes of VP7 and VP4 segments were assigned by following previously defined cutoffs [8] and using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 24 February 2022) in combination with the ViPR tool [26] available at https://www.viprbrc.org/ (accessed on 24 February 2022).

The phylogenetic analysis was performed with the selected sequences of adequate quality and length, which represented a certain genotype, lineage and origin. In the analysis, we included the selected number of referent sequences obtained from the GenBank. The MUSCLE algorithm was utilized for the purpose of building a multiple sequence alignment, preceding a phylogenetic analysis conducted with the maximum-likelihood (ML) method. Two substitution models with the lowest BIC score were applied: T92+G+I (all VP7 and VP4 sequences of P[13], P[23] and P[32] genotypes) and T92+G (VP4 sequences of P[6]–P[8] and P[11] genotypes). The branching support of the ML tree was assessed by the bootstrap analysis with 1000 repetitions. These analyses were performed in MEGA11 software [27]. The phylogenetic trees were visualized and annotated using iTOL (version 6.5.8) [28]. The nucleotide pairwise identity matrix and graphical overview of the temporal distribution of RVA genotypes circulating in domestic pigs were calculated in R using the bio3d package, ggplot2 and Scatter Pie Plot [29–32]. The lineage designation of a certain genotype was set

by the previously recommended classification for G1, G2, G3, G4, G6, G9, P[6] and P[8] genotypes [19,33–39] due to their high frequency in humans (G1-G4, G9 and P[8]) or due to the close phylogenetic relatedness observed between human and animal RVA strains (G6 and P[6]). As a result of the general inconsistency in the nomenclature and/or the absence of consensus in the lineage demarcation, we opted not to define lineages for other G and P genotypes reported within the present study.

RVA sequences characterized in the present study are deposited to the GenBank under accession numbers OL440064-OL440111, ON017591-ON017611, ON647404-ON647430, ON721080-ON721102 and OP136969.

2.5. Statistical Analysis

Descriptive statistics (prevalence) and comparison of the type of holding (farm/backyard), age and gender in affected (diarrheic) and non-affected animals (non-diarrheic) were performed in SYSTAT 13 For Windows© Version No.13.2, Systat Software, Inc. 2017. For the categorical data analysis, χ^2 test and log-linear model (LLM) were used. For all analyses, $p < 0.05$ was considered statistically significant.

3. Results

3.1. RVA Prevalence in Domestic Pigs and Wild Boars

Our results demonstrate that RVA is a highly prevalent pathogen in domestic pigs with 49.9% (222/445; 95% CI, 45.1–54.6%) positive samples. It was significantly more prevalent ($p < 0.00001$) in pigs bred on large holdings (115/169; 68.1%) compared to small backyard holdings (107/276, 38.8%). Moreover, the prevalence was significantly ($p < 0.00001$) higher in diarrheic animals (118/165, 71.5%) compared to those that were healthy, i.e., without gastrointestinal symptoms (104/280, 37.1%). On the other hand, statistical significance was not found in the RVA prevalence between suckling (113/231, 48.8%) and weanling pigs (93/177, 52.5%) ($p < 0.468$; older age categories were excluded from the analysis due to the small sample number) and between females (83/178, 46.6%) and males (99/207, 47.8%) ($p < 0.895$). All large holdings ($N = 8$) and 20 out of 24 small backyard holdings were positive for RVA in at least one sampled animal.

Wild boars were mostly RVA negative since only 41 of 441 tested animals were positive, which gives an RVA prevalence of 9.3% (95% CI, 6.8–12.4%). All positive wild boars were within the healthy group. The age group was not a significant factor ($p < 0.341$) for the RVA prevalence in wild boars since RVA was detected in 11.9% (18/151) of animals under one year of age, in 8.9% (12/135) of those between one and two years of age, and in 7.1% (11/155) of animals older than two years of age. Similarly, gender was not a significant factor ($p < 0.291$) either, since RVA was detected in 10.7% (24/223) of female compared to 7.8% (17/217) of male wild boars.

RVA prevalence on the county level for domestic pigs and wild boars is shown in Figure 1.

The observed Cq values for the RVAs detected in domestic pigs were in 55% (122/222) and 45% (100/222) under and over 32, respectively. In wild boars, a Cq lower than 32 was observed in 36.6% (15/41) of samples compared to 63.4% (26/41) of samples in which it was higher than 32. The result of IPC amplification reveals the general absence of PCR inhibitors in the majority of samples of domestic pigs (99.3%, 442/445) and wild boars (95.7%, 422/441). Those samples that were retested in 1:5 dilutions (22 in total: 3 and 19 in domestic pig and wild boar sample sets, respectively) were mostly RVA negative (20/22). Only two RVA positives were detected in diluted samples that originated from domestic pigs.

3.2. VP7 and VP4 Genotype Diversity in RVA Strains Circulating in Domestic Pigs and Wild Boars

The genotyping procedure, described in Materials and Methods, was at least partially successful (G or P genotype was defined) in 176 out of 222 (79.3%) RVA-positive samples of domestic pigs and in 24 out of 41 (58.5%) RVA-positive samples of wild boars.

In domestic pigs, we determined the circulation of eight and seven different G and P genotypes, respectively. The G genotype was detected in 163 samples. Namely, G9 (N = 42, 25.8%), G5 (N = 40, 24.5%), G3 (N = 30, 18.4%), G1 (N = 19, 11.7%), G4 (N = 16, 9.8%), G2 (N = 14, 8.6%), G6 (N = 1, 0.6%) and G11 (N = 1, 0.6%). The G3, G5 and G9 genotypes combined were the most dominant genotypes (68.7%). The P genotype was determined in 140 samples as follows: P[13] (N = 60, 42.9%), P[23] (N = 55, 39.3%), P[8] (N = 8, 5.7%), P[6] (N = 6, 4.3%), P[32] (N = 5, 3.6%), P[7] (N = 3, 2.1%) and P[11] (N = 3, 2.1%). The most dominant genotypes were P[13] and P[23] with a combined share of 82.1%. The geographical distribution of RVA G and P genotypes circulating in domestic pigs was shown in Figure 1 and the temporal distribution in Figure 2. Not all genotypes were present in all three RVA seasons covered by this study; for instance, the G1 and P[8] genotypes were detected only in the season 2020/2021, G6 in the season 2018/2019 and G11 in the season 2019/2020. The G/P genotype combination was defined for 127 RVA strains in domestic pigs. In total, there were 23 different genotype combinations with G5P[13] and G9P[23] being the most prevalent (49.6%). Genetic diversity was the highest on large holdings where we detected up to six different G and six different P genotypes in one holding. On the contrary, the genetic diversity was lower on backyard holdings with up to three and two different G and P genotypes per holding, respectively. If we consider only one sampling time point per holding, the observed genetic diversity of circulating RVA strains was at four different G and six different P genotypes on large holdings and up to three different G and two different P genotypes on small backyard holdings.

In wild boars, the genetic heterogeneity of circulating RVA strains was lower with five different G genotypes and only one P genotype detected during three consecutive RVA seasons. The G genotype was detected in 23 and the P genotype in 13 wild boar samples. The most frequent G genotype was G3 (N = 12), followed by G5 (N = 4), G9 (N = 3), G6 (N = 2) and G11 (N = 2). All 13 samples with the successful detection of the P genotype, belonged to the P[13] genotype. The G/P genotype combination was defined for 12 RVA strains, namely, G3P[13] (N = 8), G5P[13] (N = 2), G9P[13] (N = 1) and G11P[13] (N = 1). The geographical distribution of RVA G and P genotypes detected in wild boars was shown in Figure 1, and the temporal distribution was described in Section 3.3.

Even though successful and reliable genotype definitions can be achieved for shorter sequences [23,24], a small portion of G genotypes and all P genotypes did not meet the previously defined requirements [8]; hence, these genotypes are to be considered as candidate genotypes of already established ones.

3.3. The Results of Phylogenetic Analysis of RVA Strains in Domestic Pigs and Wild Boars

3.3.1. VP7 Genotyping

G1

The RVA strains of this common human genotype were detected in season 2020/2021 (Figure 2) on four domestic pig holdings in three counties (Figure 1). All these porcine G1 strains were closely phylogenetically related since they share 99.9–100% nucleotide (nt) sequence identity and 99.6–100% amino acid (aa) sequence identity. They branched within lineage I (Figure 3A), which is a common lineage for the majority of human RVA strains with which they share 96.8–98.5% on the nt and 97–98.1% on the aa level. The G1 genotype was detected in combination with P[7], P[8], P[11] and P[23].

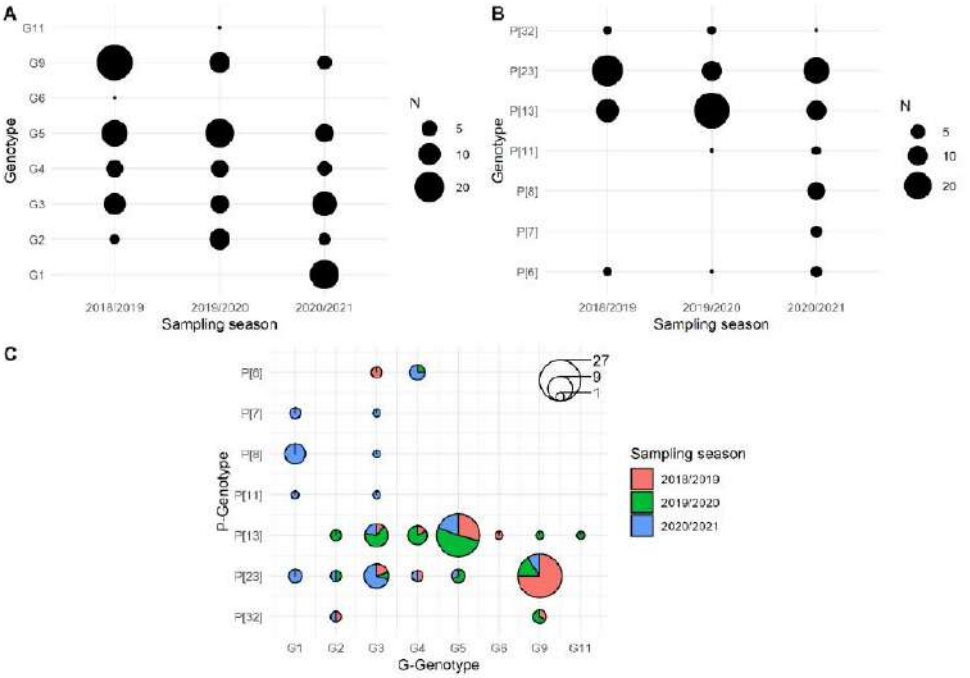


Figure 2. Temporal distribution of RVA's G genotypes (**A**), P genotypes (**B**) and G/P genotype combinations (**C**) detected in domestic pigs. The circle sizes are proportional to the number of detected RVA strains.

G2

The G2 RVA strains detected in domestic pigs within the present study were placed in the potentially novel lineage (Figure 3B), since they share less than 90% nt identity and 91.8–93.5% aa identity with the closest porcine RVA strains. Nevertheless, they are more closely related to porcine than to human strains (Figure 3B). If we observe only G2 strains from the present study, they share a high resemblance with 98.7–99.9% and 98.9–100% on the nt and aa levels, respectively. These G2 strains were detected on seven holdings in six counties (Figure 1), covering all three sampling seasons (Figure 2). They come in combination with P[13], P[23] and P[32] genotypes.

G3

Genotype G3 was the third most abundant genotype circulating in domestic pigs, and the most prevalent genotype in wild boars. These RVA strains form a diverse group of sequences, sharing 85.9–100% sequence identity on the nt level and 93.1–100% on the aa level. Two clusters of RVA strains detected in the present study can be recognized; the first one with mixed strains originating from domestic pigs and wild boars and the second one observed only in domestic pigs (Figure 3A). Within the first cluster, RVA strains from domestic pigs and wild boars share sequence identity in the range between 90.3 and 97.5% on the nt and 94.7–98.5% on aa level. The second cluster might represent a distinct lineage since sequence identities were lower than 89% (Figure 3A). All RVA strains from our study were quite clearly separated from the two lineages circulating in humans, lineage I representing classical human RVA strains, and lineage IX representing equine-like RVA strains (Figure 3A). The phylogenetically closest RVA strains to Croatian autochthonous strains were those detected in domestic pigs, sharing the highest 91.7% nt and 96.9% aa identity with the Slovakian RVA strain TOPC23 (MN203555). The strains of G3 genotype

in domestic pigs were detected in eight holdings and three counties (Figure 1) during all three sampling seasons (Figure 2). They circulated in combination with six out of seven P genotypes detected in our study (P[6]–P[8], P[11], P[13] and P[23]). On the other hand, wild boar RVA strains were detected in six hunting grounds and four counties (Figure 1) during all sampling seasons, as well. More precisely, three, eight and one G3 strain in the 2018/2019, 2019/2020 and 2020/2021 seasons, respectively. Since the circulation of only one P genotype in wild boars was determined, the G3 strains combined solely with the P[13] genotype.

G4

RVA strains of this genotype were detected only in domestic pigs in all three sampling seasons (Figure 2). They were circulating in seven holdings and three counties (Figure 1). These strains were quite diverse, clustering in three separate groups (Figure 3B), which may represent three different lineages due to the low nucleotide sequence identity, ranging between 82.6 and 85.8%. Nevertheless, if the lineage designation described in Wandera et al. [39] was applied, these strains would cluster within lineage VI, which is in contrast with the demarcation threshold of previously established lineages (I–V). The G4 strains detected in the current study, share a high phylogenetic relationship with the different RVA strains detected in domestic pigs, wild boars and humans (Figure 3B). The latter are considered to be a zoonotic spillover [40]. The G4 genotype was found in combination with P[6], P[13] and P[23] RVA strains.

G5

This typically porcine RVA genotype was confirmed to circulate in both species: domestic pigs and wild boars. It was present during all three sampling seasons in domestic pigs (Figure 2) and during the last two sampling seasons in wild boars (three strains in the season 2019/2020 and one in the season 2020/2021) The RVA strains of this genotype clustered in three groups (Figure 3A) potentially representing three distinctive lineages since the nucleotide identity is in the range between 83 and 88.4%. The first group includes strains from domestic pigs and wild boars, while the other two were detected only in domestic pigs. The RVA strains of domestic pigs and wild boars described in the present study are highly phylogenetically related (Figure 3), sharing 95.5–97.4% and 96.7–98.9% on the nt and aa levels, respectively. Overall, this genotype was detected in the largest number of holdings (N = 14) from six counties (Figure 1), when domestic pigs are considered. Within the wild boar population, the G5 genotype was detected in two hunting grounds from two counties (Figure 1). When we look at combinations with the P genotype, they were less diverse compared to the other prevalent G genotypes. Accordingly, in domestic pigs, we observed only combinations with P[13] and P[23] genotypes, whereas G5P[13] was the sole combination observed in wild boars.

G6

The G6 genotype, a common RVA genotype in cattle, was detected in one domestic pig and two wild boars during the season 2018/2019 (Figure 2), each from a different county (Figure 1). The strain detected in the domestic pig was found in combination with the P[13] genotype. The length of sequences derived from wild boars was not informative enough for the inclusion of these strains into the phylogenetic analysis. Nevertheless, the genotype assignment was reliable [24]. The sequence detected in a domestic pig was closely related to a bovine RVA strain from Hungary and a bovine-like RVA strain detected in a child from Slovenia (97.7%/99.2% on the nt/aa level) and clustered within the lineage V (Figure 3B).

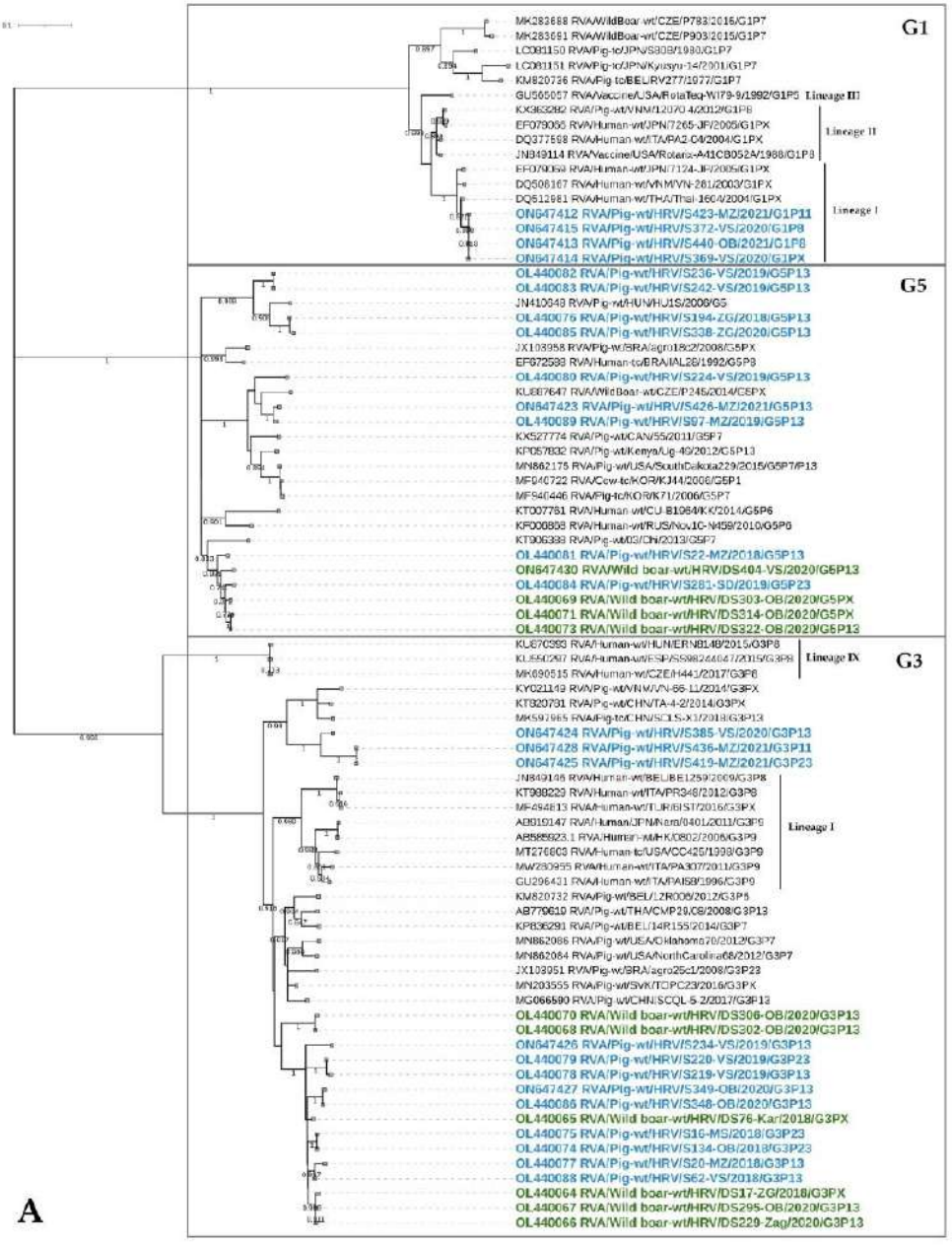


Figure 3. *Cont.*

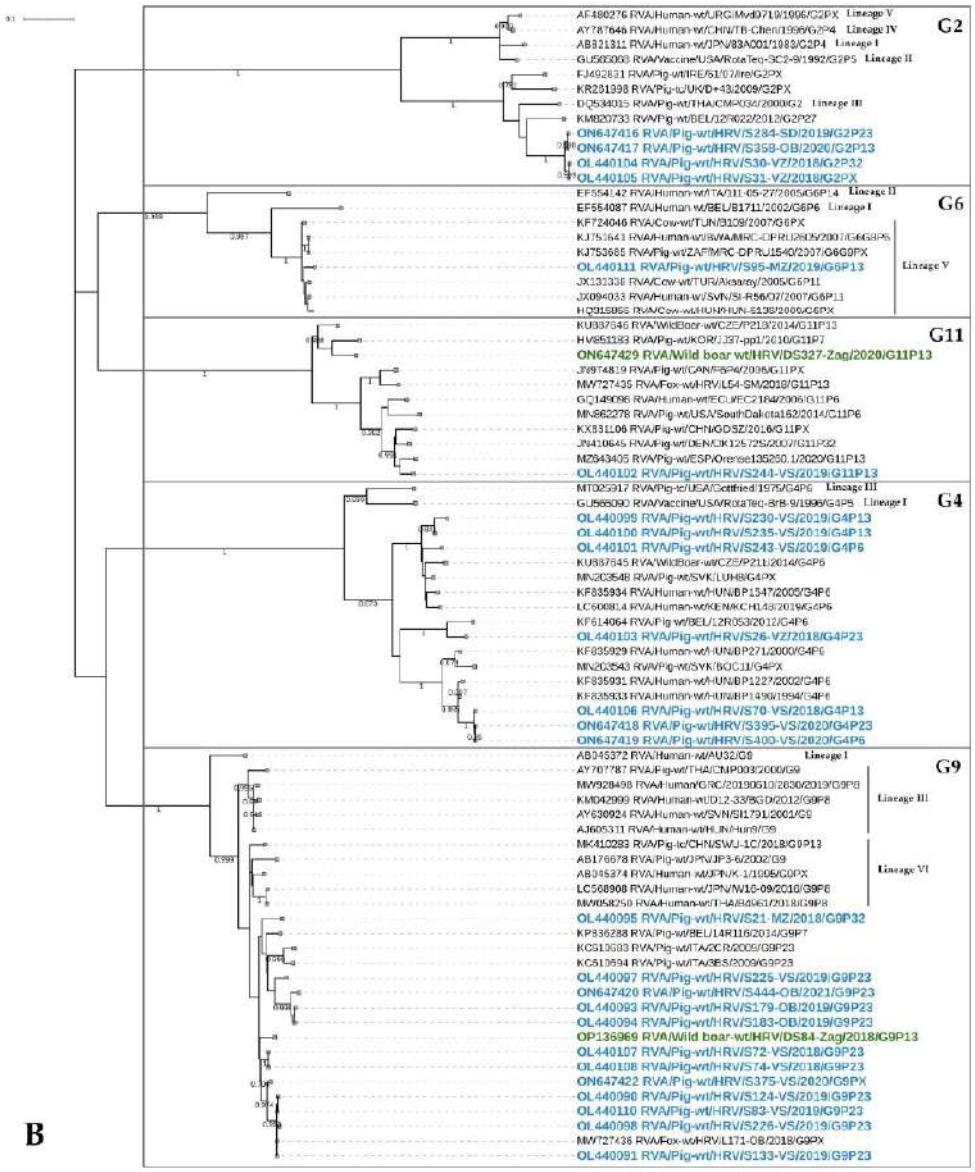


Figure 3. Phylogenetic relationship between RVA strains of G1, G3, G5 (**A**) and G2, G4, G6, G9, G11 (**B**) genotypes. The strains from the present study that were derived from domestic pigs and wild boars are marked in blue and green, respectively. The accession numbers of all strains, including referent strains from the GenBank, are designated within taxa. Based on the partial VP7 sequences (~800 nt), both trees were generated by the ML method and T92+G+I model in MEGA 11 software. The branching stability of each phylogenetic tree was assessed by 1000 bootstrap replicates (values indicated adjacent to the nodes if >0.7). The scale bar represents the number of substitutions per site. In displaying RVA strain nomenclature within taxa, the brackets for the P genotype were omitted for the sake of simplicity.

G9

This genotype was the most prevalent genotype in domestic pigs and the third most prevalent genotype in wild boars. It was detected in 10 domestic pig holdings located in three counties, whilst wild boar G9 strains were detected in three hunting grounds from three counties (Figure 1). It circulated during each sampling season of domestic pigs (Figure 2), and in the first (2018/219, N = 2) and third (2020/2021, N = 1) sampling seasons of wild boars. Despite being the most prevalent, this genotype was found in combination with only P[13], P[23] and P[32] genotypes. In wild boars, it combined with P[13], the sole P genotype detected in that species. All G9 RVA sequences from the present study were closely related (92.2–99.5%/94.7–100% on the nt/aa level) and showed high identities (95.3%/95% on the nt/aa level) among wild boar and domestic pig strains (Figure 3B). Together with the Italian and Belgian domestic pig strains, they form a potentially novel lineage as already proposed [41]. As a matter of interest, the strain derived from the red fox, described in our concurrent study [20], phylogenetically clustered together with porcine strains from this study sharing high sequence identities with several strains (99.7/99.5% on the nt/aa level) (Figure 3B). Human and porcine G9 strains from the current study were clearly phylogenetically distinct (Figure 3B).

G11

The low prevalent, porcine-related genotype was confirmed in only one domestic pig and two wild boars during seasons 2019/2020 and 2020/2021, respectively, and geographically originated from two counties. Indeed, the majority of related referent RVA sequences were of porcine origin with an evident close phylogenetic relationship (Figure 3B). Our G11 RVA strains detected in a domestic pig and wild boars were clustered in clearly distinguished lineages (Figure 3B) sharing only 86% nucleotide sequence identity. Likewise, for the G9 genotype, it is interesting to note that this genotype was detected in a red fox during our concurrent study [20]. However, the red fox G11 strain clusters in a separate lineage (Figure 3B) considering the low 86.4% and 87.7% nucleotide identities it shares with wild boar and domestic pig strains, respectively.

3.3.2. VP4 Genotyping

P[6]

The P[6] genotype was not among the most prevalent VP4 RVA genotypes in domestic pigs during the present study. However, the importance of these strains lies in the close phylogenetic connection to human P[6] strains from Hungary and Slovenia within lineages IV and V, respectively (Figure 4A). With these human strains, they share nt/aa identities of 94.1–97.8%/90.3–97.7%. These human RVA strains are reported to represent the event of porcine-to-human zoonotic transmission [33,40]. The six P[6] strains from the present study originated in two counties (Figure 1) and four holdings and were detected in all three sampling seasons (Figure 2). These strains were detected in combination with G3 and G4 genotypes.

P[7]

This primarily porcine genotype was detected in three domestic pigs in the last sampling season (2020/2021) in two holdings from two counties (Figure 1). Our strains shared the highest similarity with the wild boar strain from the Czech Republic (95.9%/92.6% on the nt/aa level) (Figure 4A). However, none of the wild boar samples in our study presented this genotype. These three P[7] strains were detected in combination with human-like G1 and G3 genotypes.

P[8]

Likewise the P[7] genotype, this genotype emerged in the 2020/2021 season (Figure 2). It is considered to be the most common genotype in humans; hence, the expected high sequence identities (up to 99.5/98.6 on the nt/aa level) were observed with human strains

of lineage III (Figure 4A). Nevertheless, the close phylogenetic relatedness was identified with porcine strains detected in the United Kingdom and Taiwan (Figure 4A). When we look at the mutual relatedness of our P[8] strains, they share 96.2–100% and 93–100% on the nt and aa levels, respectively. These RVA strains emerged in three holdings and three counties (Figure 1) and were combined with human-like G1 and G3 genotypes.

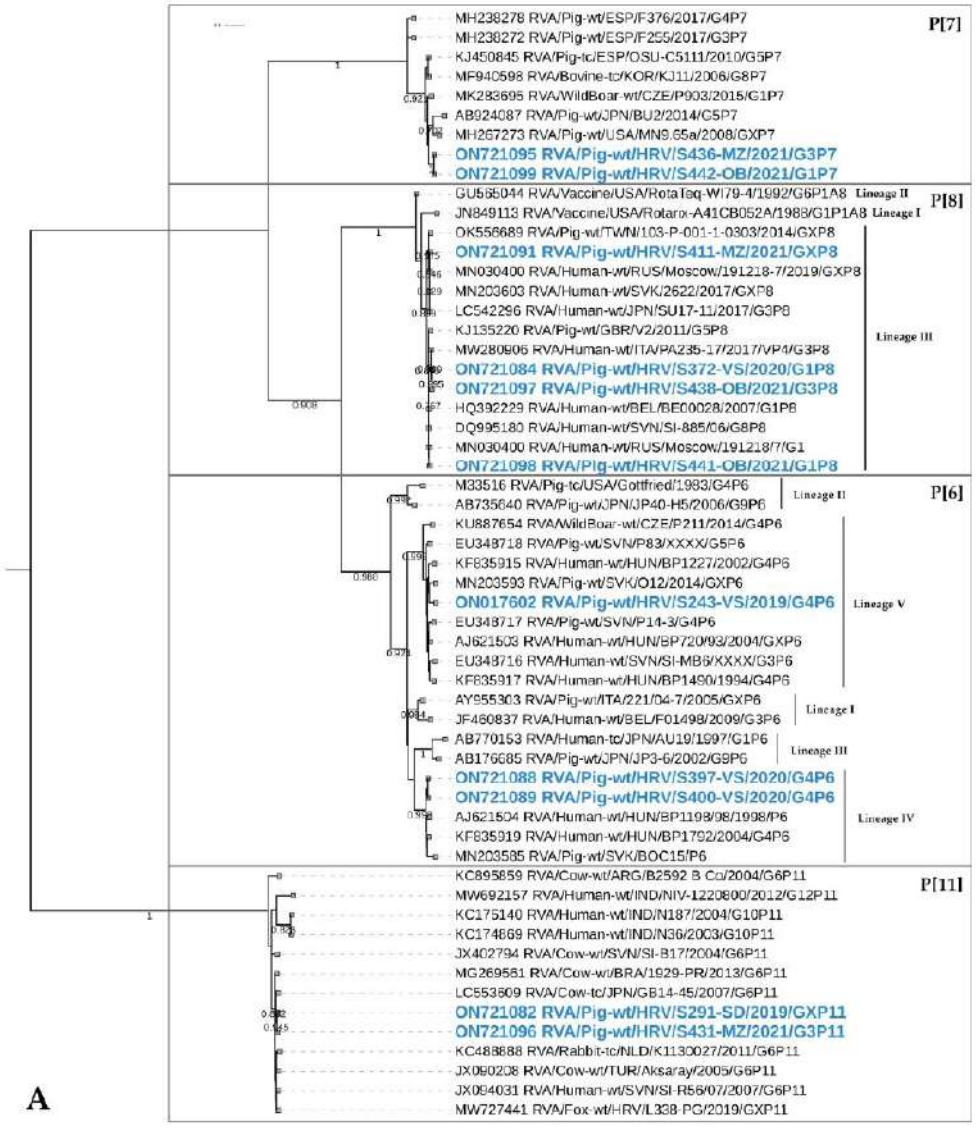


Figure 4. *Cont.*

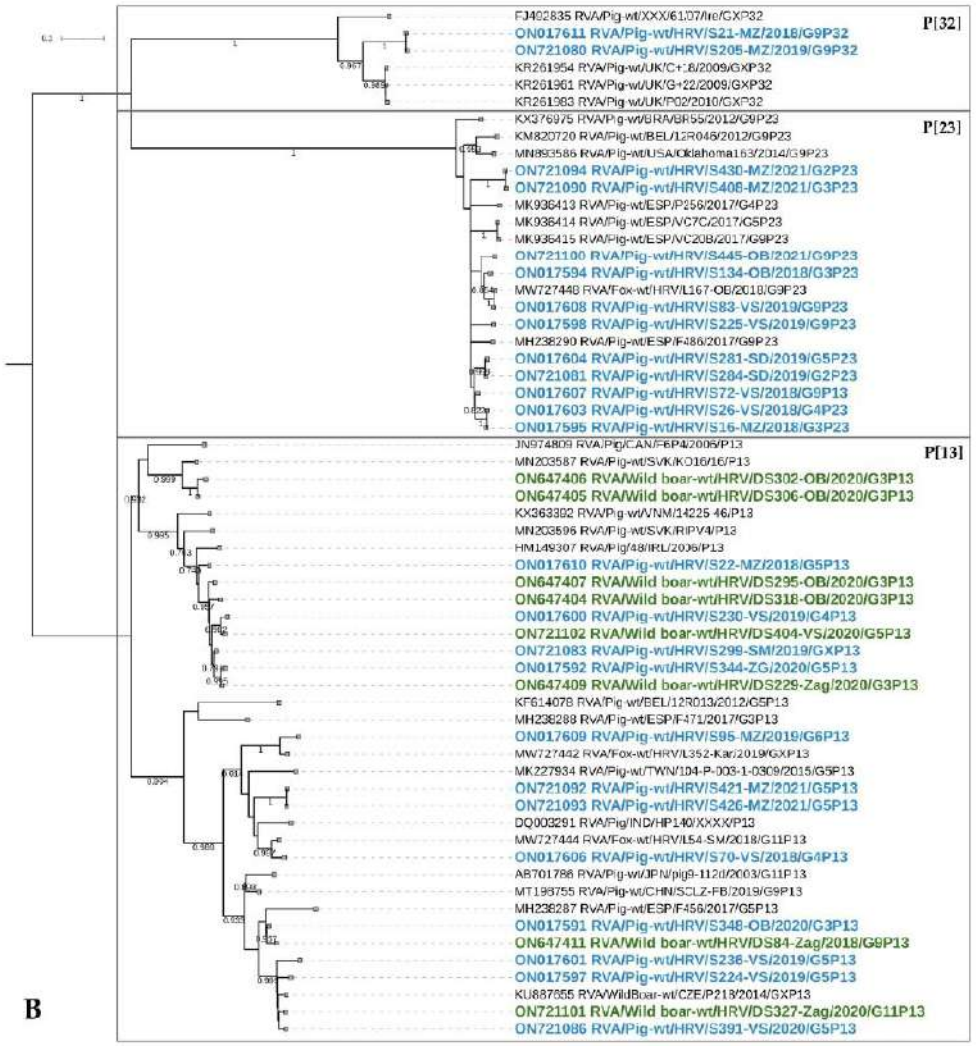


Figure 4. Phylogenetic relationship between RVA strains of P[6], P[7], P[8], P[11] (**A**) and P[13], P[23], P[32] (**B**) genotypes. The strains from the present study that were derived from domestic pigs and wild boars are marked in blue and green, respectively. The accession numbers of all strains, including referent strains from the GenBank, are designated within taxa. Based on the partial VP4 sequences (~650 nt), both trees were generated by the ML method and T92+G (**A**) or T92+G+I (**B**) model in MEGA 11 software. The branching stability of each phylogenetic tree was assessed by 1000 bootstrap replicates (values indicated adjacent to the nodes if >0.7). The scale bar represents the number of substitutions per site. In displaying RVA strain nomenclature within taxa, the brackets for the P genotype were omitted for the sake of simplicity.

P[11]

The P[11] genotype is considered to be one of the most frequent bovine genotypes. However, in the present study, we detected it in three domestic pigs from two counties (Figure 1). These strains elicited a high sequence identity with different RVA strains circulating in cattle (95.3–98% on the nt and 93.9–99.5% on the aa level) (Figure 4A). Our

previous investigation reports the presence of this genotype in red foxes, which is closely phylogenetically related to domestic pig strains from the present study (97.1%/98.1% on the nt/aa level) (Figure 4A). The P[11] genotype was determined in combination with human-like G1 and G3 genotypes.

P[13]

One of the two most numerous P genotypes characterized in the present study was detected in 16 domestic pig holdings located in five counties (Figure 1) in all three sampling seasons (Figure 2). Moreover, this genotype was detected in wild boars in five hunting grounds from three counties during all sampling seasons (one, nine and three strains in the 2018/2019, 2019/2020 and 2020/2021 seasons, respectively). It was detected in combination with seven different G genotypes (all except human-like G1) in domestic pigs and four different G genotypes (all except G6) in wild boars. The phylogenetic analysis revealed that P[13]-circulating RVA strains have the highest intragenotype diversity of all genotypes detected in the present study. In total, six clusters (Figure 4B) were defined: one cluster with wild boar strains (DS302-OB as representative strain), three clusters with strains from domestic pigs (S95-MZ, S421-MZ and S70-VS) and two clusters of combined RVA strains originating in both species (S22-MZ/DS229-Zag and S348-OB/DS327-Zag). The highest sequence identity between these clusters was 88.9% and 89.3% on the nt and aa levels, respectively. It is evident that the closest relatives to our P[13] are RVA referent strains originating from domestic pigs and wild boars (Figure 4B). The interesting observation is the close resemblance of two red fox RVA strains described in our previous study [20] and strains/clusters from domestic pigs S70-VS and S95-MZ (nt sequence identity approx. 95%) (Figure 4B). The phylogenetic grouping of domestic pig and wild boar strains within two clusters was confirmed with high sequence identities of up to 98.6%/98.1% on the nt/aa level in cluster S22-MZ/DS229-Zag and 97.4%/96.8% on the nt/aa level in cluster S348-OB/DS327-Zag.

P[23]

The second most numerous P genotype, detected only in domestic pigs, showed a significantly lower genetic heterogeneity since all sequences are grouped within a single cluster (Figure 4B) with identities ranging between 90.1–99.8% on the nt and 92.9–99.5% on the aa level. This genotype was detected in 11 holdings in five counties (Figure 1) during all three seasons (Figure 2). Furthermore, it was found circulating in combination with six G genotypes (G1-G5, G9). Interestingly, one fox strain from our previous study [20] was phylogenetically closely related to domestic pig RVA strains (Figure 4B) sharing up to 99.7/100% identity on the nt/aa level.

P[32]

This rare genotype was confirmed circulating in Croatia, but with restricted regional importance since it was detected only in five strains originating in three holdings from two neighboring northernmost counties (Figure 1). It circulated in all three seasons (Figure 2) and came in combination with G2 and G9 RVA strains. The closest P[32] strains from the GenBank were those from the UK (Figure 4B) and Switzerland (not shown) sharing less than 89% identity on the nt level and 90% on the aa level. The evident separate clustering indicates a possible circulation of a distinct lineage.

4. Discussion

The present study brings a comprehensive concurrent investigation of the prevalence, molecular epidemiology and genetic diversity of circulating RVA strains in domestic pigs and wild boars during three consecutive RVA seasons. The concurrent spatio-temporal study of RVA strains circulating in certain reservoir species and the environment has previously been recognized as important in order to draw relevant conclusions on their prevalence and health impact [3,42]. Furthermore, this is one of the rare studies on RVAs in

domestic pigs in this part of the world (South East Europe), and only the third in general, to the best of our knowledge, where RVAs in wild boars have been considered.

The prevalence in domestic pigs presented in the current study is relatively high (49.9%), similar to previously described studies in Spain [15], Italy [43] and the USA [44]. Most certainly, the predominantly represented young age categories of suckling and weanling pigs, favor the more frequent circulation of RVAs. Moreover, the used method of detection has a substantial influence on the final result. Similar to our study, Spanish, Italian and US researchers [15,43,44] used the real-time RT-PCR based method, while in contrast, Taiwanese researchers used the Enzyme Immunoassay as screening and end-point RT-PCR as the confirmatory method [45], which finally resulted in a significantly lower prevalence that is incomparable. Our results indicate that there is no significant difference in the prevalence between the suckling and weanling age categories and between females and males, but the difference in RVA prevalence was significant in diarrheic compared to healthy animals, and in those bred on large holdings compared to the small backyard holdings. RVAs are known causative agents of diarrhea in mammals [2], and our research brings more to that knowledge.

Data on the RVA significance in wild boars have been rather scarce so far with only two available reports from Japan [16] and the Czech Republic [17]. Our study is the most comprehensive to date, encompassing a sample set of 441 animals. It is also noteworthy that the sampling was performed in parallel with domestic pigs, which provides a temporal component important for relevant phylogenetic comparisons. RVA prevalence in the present study (9.3%) was higher compared to those two previous studies, primarily due to having a different approach to RVA detection. We applied the real-time RT-PCR compared to the conventional end-point RT-PCRs applied by others [16,17], which are usually less sensitive. The method we implemented has been previously successfully applied in RVA-related research on domestic animals and wildlife [18–20]. Nevertheless, the unknown range of VP2 genotypes that this method detects, and the fact that the assay design was limited to only human strains of C1 and C2 genotypes, might have underestimated the prevalence in both species. Moreover, the prevalence in wild boars might be even higher, since, due to the hunting regulations, we did not have access to the youngest age categories where the higher RVA circulation is expected. Similar to domestic pigs, age and gender were not significant factors for RVA prevalence.

Genetic diversity in domestic pigs was high in both genomic segments, with eight different G (G1-G6, G9 and G11) and seven different P genotypes (P[6]–P[8], P[11], P[13], P[23] and P[32]). In wild boars, the RVA strains were less genetically diverse with five detected G (G3, G5, G6, G9 and G11) and one detected P genotype (P[13]). The genotyping protocols were more challenging for wild boar samples, similar to what we previously reported in another wildlife species, i.e., red foxes [20]. The impact of the low RVA genomic concentrations (63.4% of RVA positive wild boars with Cq > 32), and the presence of diverse RVA strains influencing primer specificity, should not be excluded. Therefore, the underlying RVA genetic diversity might be even higher. On the other hand, the higher genetic diversity of RVA strains that was discovered in domestic pigs bred on large holdings, compared to the small backyard holdings, was predictable due to the more frequent animal movements in that type of holding.

The most dominant G genotypes found to be circulating in domestic pigs during the course of the study were G9, G5 and G3 and they account for 68.7% of all sequenced RVA strains. Considering the P genotypes, only two genotypes, more precisely P[13] and P[23], equaled 82.1% in total. All these genotypes are common in domestic pigs with varying spatio-temporal prevalence [3]. Previous reports indicate the substantial dominance of the G5P[7] genotype combination in domestic pigs worldwide [3]. Our results report a changing pattern, with G5P[13] and G9P[23] being the most abundant with 49.6% of all detected genotype combinations (Figure 2C). The combination G9P[23] was recently reported to be among the most frequent in Germany [46] and Spain [15]. Apart from the remarkable genetic diversity of each segment (VP7/VP4), we observed a striking number

($N = 23$) of different genotype combinations, higher than previously reported data for four countries combined ($N = 21$) [10], but lower than what was previously reported in Poland ($N = 33$) [47]. Moreover, a distinguished intragenotype diversity, i.e., the circulation of several lineages (genotypes G3-G5, G11, P[6] and P[13]) or the existence of possibly novel lineages (genotypes G2, G3 and P[32]), was further observed. The orientation of our study to locally bred domestic pigs, excluding the holdings with imported weanling and fattening pigs, might have contributed to these results. Nevertheless, the import of domestic pigs, which is common in Croatia, primarily from Western European countries, still has an important impact on animal health and the introduction of novel viral pathogens or certain genomic variants [48].

Among other less prevalent genotypes, the most interesting finding is the emergence of G1P[8] strains ($N = 7$), which are commonly observed in humans [49]. Both genotypes which were found in domestic pigs clustered within typical human lineages (Figures 3A and 4A) and were detected during the last sampling season (2020/2021) in several holdings in three counties. This is not the first time such possible reverse zoonotic events, including G1 and/or P[8] strains, have been reported in domestic pigs [45,47,50–52]. Notably, our investigation of other samples collected on these holdings revealed that G1 and P[8] strains were involved in reassortment events with other circulating RVA strains of different genotypes. However, it seems that the VP7 is more readily involved in such reassortment events compared to the VP4, since G1 was found in combination with three additional different P genotypes (P[7], P[11] and P[23]) in a total of six reassortant strains, compared to only one G3P[8] reassortant strain. A possible reason lies in the VP7 segment having the lowest host-species barrier of all RVA segments, despite the proposition that VP4 may be the segment that reassorts more frequently [53]. Therefore, it is not surprising that in the present study, G1 strains were detected more often ($N = 19$) in domestic pigs compared to the P[8] strains ($N = 8$), indicating possible enhanced host adaptation. Most certainly, the host adaptation was further driven by reassortment with the already present genotypes. Such assumptions need to be further investigated on a larger sample set by implementing multiplex RT-PCR and whole genome sequencing. Sanger sequencing applied in the present study may underestimate the presence of certain genotypes in mixed infections. Therefore, the combination of different approaches may result in more certain conclusions.

The porcine-to-human spillover was often reported for the G4 and P[6] strains [40], which were not among the highly prevalent RVA genotypes within the present study. Nevertheless, their close phylogenetic relatedness with different referent porcine-like RVA strains in humans (Figures 3B and 4A) speaks in favor of their zoonotic potential.

The interesting finding of the current study is the circulation of the P[32] genotype, which was first detected in Ireland [54] and Denmark [10] and further in the UK [50], Germany [46], Poland [47] and Switzerland [55]. Even though it was confirmed circulating in all three sampling seasons (Figure 2), it was restricted to only the two northernmost counties (Figure 1) and to only five samples, indicating its regional potential. These strains were rather distantly related to other available P[32] strains from the GenBank (<89% nucleotide sequence identity), which may indicate the circulation of this certain lineage for some extended period of time within the area. Continuous monitoring of RVA strains in domestic pigs, which is generally not present, would provide more information on the importance of such underrepresented genotypes.

Apart from possible interspecies transmission with humans (G1P[8]), we identified a small number of samples with G6 and P[11] genotypes, implying interspecies transmission events with bovines, which is also evident from their phylogenetic clustering (Figures 3B and 4A). Both genotypes were detected as reassortants with typical porcine G3 and P[13] genotypes. Similar findings were reported elsewhere [3], confirming the existence of similar, but sporadic porcine–bovine reassortant strains.

Nevertheless, the most prominent number of interspecies transmission events in the current study was observed between domestic pigs and wild boars. More precisely, all genotypes detected in wild boars (G3, G5, G6, G9, G11 and P[13]) were detected in domestic

pigs as well. The evidence that supports the statement that the natural transmission of RVAs between these two species actually occurs is the high sequence identities and phylogenetic relatedness depicted in Figures 3A,B and 4B. Most of these RVA genotypes (G5, G9, G11 and P[13]), which we discovered circulating in the wild boar population, were already confirmed relevant for that species [16,17]. The exception was primarily the G3 genotype, which was detected for the first time in wild boars and it was in fact the most dominant genotype within the present study. Considering that the G3 genotype was the third most prevalent genotype in domestic pigs, it is not a surprise. Moreover, the RVA strains of the G3 genotype are believed to have the broadest host range [56]. The extent of inclusion of these two species in the natural transmission of certain RVA genotypes was observed by the previous detection of G4, P[6] and P[23] genotypes in wild boars in the Czech Republic and Japan [16,17] and, additionally, the G1 and the P[7] in the Czech Republic (unpublished genotypes available in the GenBank) (Figures 3A and 4A).

An interesting observation on how porcine RVA strains impact the genetic heterogeneity of RVAs retrieved from another, yet distant, wildlife species was described in our previous investigation conducted on red foxes [20]. When fox RVA strains were compared to strains derived from the current study, it was evident they share porcine-related G9, G11, P[13] and P[23] genotypes with the prominent phylogenetic relatedness for the three most common genotypes (G9, P[13] and P[23]) (Figures 3B and 4B). Since both species, wild boars and red foxes, tend to enter rural and urban areas, their contact with different animal species and their pathogens is expected. The contact of wildlife with domestic pigs and their manure is foreseen, especially in the matter of small backyard holdings, which usually have low biosecurity conditions.

In conclusion, our results contribute to the basic knowledge of RVA prevalence, genetic diversity and molecular epidemiology and to the extent of interspecies transmission events in domestic pigs and wild boars. Such baseline data may be considered important for the development and introduction of RVA vaccines in domestic pigs, an essential tool for pig health management. This is especially valuable for countries in the European Union where currently no authorized RVA vaccine for domestic pigs is commercially available. Lastly, the continuous monitoring of RVA in different species allows the prompt detection of new emerging variant strains that could become important for human health and the future effectiveness of vaccines currently in use.

Author Contributions: Conceptualization, D.B.; methodology, D.Č., V.K., N.K. and N.M.-S.; software, D.Č., V.K., D.B. and N.M.-S.; validation, D.B. and D.Č.; formal analysis, D.Č and N.M.-S.; investigation, N.K., D.K., M.B., I.B. and D.H.; resources, D.B. and L.J.; data curation, D.B. and D.Č.; writing—original draft preparation, D.B.; writing—review and editing, D.Č., V.K. and L.J.; visualization, D.Č. and D.B.; supervision, D.B.; project administration, D.B.; funding acquisition, D.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Croatian Science Foundation installation research project Reco "Rotaviruses in Croatian Ecosystem: molecular epidemiology and zoonotic potential", grant number HRZZ-UIP-2017-05-8580.

Institutional Review Board Statement: This study was approved by the Board of Ethics of the Croatian Veterinary Institute (protocol code Z-VI-4-5206/17, approved on 11 December 2017).

Data Availability Statement: The datasets used and/or analyzed within the frame of the present study can be provided by the corresponding author upon a justified request.

Acknowledgments: The authors would like to thank Ivana Pišćak, Vladimir Margeta, Davor Balić, Vlatko Rukavina, Marin Franjić, Tomislav Latin, Tomislav Mitrović, Ivana Ferčić and Krešimir Kušenić for their technical assistance.

Conflicts of Interest: The authors declare no conflict of interest. The funding body had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Troeger, C.; Khalil, I.A.; Rao, P.C.; Cao, S.; Blacker, B.F.; Ahmed, T.; Armah, G.; Bines, J.E.; Brewer, T.G.; Colombara, D.V.; et al. Rotavirus Vaccination and the Global Burden of Rotavirus Diarrhea Among Children Younger Than 5 Years. *JAMA Pediatr.* **2018**, *172*, 958–965. [CrossRef] [PubMed]
2. Palmarini, M. Reoviridae. In *Fenner's Veterinary Virology*, 5th ed.; MacLachlan, N.J., Dubovi, E.J., Eds.; Academic Press: Boston, MA, USA, 2017; pp. 299–317.
3. Papp, H.; Laszlo, B.; Jakab, F.; Ganesh, B.; De Grazia, S.; Matthijnssens, J.; Ciarlet, M.; Martella, V.; Banyai, K. Review of group A rotavirus strains reported in swine and cattle. *Vet. Microbiol.* **2013**, *165*, 190–199. [CrossRef] [PubMed]
4. Ghosh, S.; Kobayashi, N. Exotic rotaviruses in animals and rotaviruses in exotic animals. *VirusDisease* **2014**, *25*, 158–172. [CrossRef] [PubMed]
5. ICTV. Rotavirus Taxonomy. 2021. Available online: https://talk.ictvonline.org/taxonomy/ (accessed on 25 May 2022).
6. Doro, R.; Farkas, S.L.; Martella, V.; Banyai, K. Zoonotic transmission of rotavirus: Surveillance and control. *Expert Rev. Anti Infect. Therap.* **2015**, *13*, 1337–1350. [CrossRef] [PubMed]
7. Crawford, S.E.; Ramani, S.; Tate, J.E.; Parashar, U.D.; Svensson, L.; Hagbom, M.; Franco, M.A.; Greenberg, H.B.; O'Ryan, M.; Kang, G.; et al. Rotavirus infection. *Nat. Rev. Dis. Prim.* **2017**, *3*, 17083. [CrossRef]
8. Matthijnssens, J.; Ciarlet, M.; Rahman, M.; Attoui, H.; Banyai, K.; Estes, M.K.; Gentsch, J.R.; Iturriza-Gomara, M.; Kirkwood, C.D.; Martella, V.; et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Arch. Virol.* **2008**, *153*, 1621–1629. [CrossRef]
9. RCWG. List of Accepted Genotypes by Rotavirus Classification Working Group. 2021. Available online: https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg (accessed on 28 May 2022).
10. Midgley, S.E.; Banyai, K.; Buesa, J.; Halaihel, N.; Hjulsager, C.K.; Jakab, F.; Kaplon, J.; Larsen, L.E.; Monini, M.; Poljsak-Prijatelj, M.; et al. Diversity and zoonotic potential of rotaviruses in swine and cattle across Europe. *Vet. Microbiol.* **2012**, *156*, 238–245. [CrossRef]
11. Martella, V.; Banyai, K.; Matthijnssens, J.; Buonavoglia, C.; Ciarlet, M. Zoonotic aspects of rotaviruses. *Vet. Microbiol.* **2010**, *140*, 246–255. [CrossRef]
12. Chang, K.-O.; Saif, J.L.; Kim, Y. Reoviruses (Rotaviruses and Reoviruses). In *Diseases of Swine*, 10th ed.; Zimmerman, J., Karriker, L., Ramirez, A., Schwartz, K., Stevenson, G., Eds.; Wiley-Blackwell: Ames, IA, USA, 2012; pp. 621–634.
13. Vlasova, N.A.; Amimo, O.J.; Saif, J.L. Porcine Rotaviruses: Epidemiology, Immune Responses and Control Strategies. *Viruses* **2017**, *9*, 48. [CrossRef]
14. Matthijnssens, J.; Ciarlet, M.; Heiman, E.; Arijs, I.; Delbeke, T.; McDonald, S.M.; Palombo, E.A.; Iturriza-Gomara, M.; Maes, P.; Patton, J.T.; et al. Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. *J. Virol.* **2008**, *82*, 3204–3219. [CrossRef]
15. Monteagudo, L.V.; Benito, A.A.; Lázaro-Gaspar, S.; Arnal, J.L.; Martin-Jurado, D.; Menjon, R.; Quílez, J. Occurrence of Rotavirus A Genotypes and Other Enteric Pathogens in Diarrheic Suckling Piglets from Spanish Swine Farms. *Animals* **2022**, *12*, 251. [CrossRef] [PubMed]
16. Okadera, K.; Abe, M.; Ito, N.; Morikawa, S.; Yamasaki, A.; Masatani, T.; Nakagawa, K.; Yamaoka, S.; Sugiyama, M. Evidence of natural transmission of group A rotavirus between domestic pigs and wild boars (Sus scrofa) in Japan. *Infect. Genet. Evol.* **2013**, *20*, 54–60. [CrossRef] [PubMed]
17. Moutelikova, R.; Dufkova, L.; Kamler, J.; Drimaj, J.; Plhal, R.; Prodelalova, J. Epidemiological survey of enteric viruses in wild boars in the Czech Republic: First evidence of close relationship between wild boar and human rotavirus A strains. *Vet. Microbiol.* **2016**, *193*, 28–35. [CrossRef] [PubMed]
18. Gutiérrez-Aguirre, I.; Steyer, A.; Boben, J.; Gruden, K.; Poljšak-Prijatelj, M.; Ravnikar, M. Sensitive Detection of Multiple Rotavirus Genotypes with a Single Reverse Transcription-Real-Time Quantitative PCR Assay. *J. Clin. Microbiol.* **2008**, *46*, 2547–2554. [CrossRef]
19. Jamnikar-Ciglenecki, U.; Kuhar, U.; Sturm, S.; Kirbis, A.; Racki, N.; Steyer, A. The first detection and whole genome characterization of the G6P[15] group A rotavirus strain from roe deer. *Vet. Microbiol.* **2016**, *191*, 52–59. [CrossRef] [PubMed]
20. Čolić, D.; Krešić, N.; Mihaljević, Ž.; Andreanszky, T.; Balić, D.; Lolić, M.; Brnić, D. A Remarkable Genetic Diversity of Rotavirus A Circulating in Red Fox Population in Croatia. *Pathogens* **2021**, *10*, 485. [CrossRef]
21. Gouvea, V.; Glass, R.I.; Woods, P.; Taniguchi, K.; Clark, H.F.; Forrester, B.; Fang, Z.Y. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* **1990**, *28*, 276–282. [CrossRef]
22. Abe, M.; Ito, N.; Morikawa, S.; Takasu, M.; Murase, T.; Kawashima, T.; Kawai, Y.; Kohara, J.; Sugiyama, M. Molecular epidemiology of rotaviruses among healthy calves in Japan: Isolation of a novel bovine rotavirus bearing new P and G genotypes. *Virus Res.* **2009**, *144*, 250–257. [CrossRef]
23. Eurorotanet. Rotavirus Detection and Typing 2009; p. 25. Available online: https://www.eurorotanet.com/project-information/documents-and-methods/ (accessed on 2 April 2018).
24. Mijatovic-Rustempasic, S.; Esona, M.D.; Williams, A.L.; Bowen, M.D. Sensitive and specific nested PCR assay for detection of rotavirus A in samples with a low viral load. *J. Virol. Methods* **2016**, *236*, 41–46. [CrossRef]

25. Theuns, S.; Desmarets, L.M.B.; Heylen, E.; Zeller, M.; Dedeurwaerder, A.; Roukaerts, I.D.M.; Van Ranst, M.; Matthijnssens, J.; Nauwynck, H.J. Porcine group a rotaviruses with heterogeneous VP7 and VP4 genotype combinations can be found together with enteric bacteria on Belgian swine farms. *Vet. Microbiol.* **2014**, *172*, 23–34. [CrossRef]
26. Pickett, B.E.; Sadat, E.L.; Zhang, Y.; Noronha, J.M.; Squires, R.B.; Hunt, V.; Liu, M.; Kumar, S.; Zaremba, S.; Gu, Z.; et al. ViPR: An open bioinformatics database and analysis resource for virology research. *Nucleic Acids Res.* **2011**, *40*, D593–D598. [CrossRef]
27. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef]
28. Letunic, I.; Bork, P. Interactive Tree Of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* **2021**, *49*, W293–W296. [CrossRef] [PubMed]
29. Grant, B.J.; Rodrigues, A.P.; ElSawy, K.M.; McCammon, J.A.; Caves, L.S. Bio3d: An R package for the comparative analysis of protein structures. *Bioinformatics* **2006**, *22*, 2695–2696. [CrossRef] [PubMed]
30. R Core Team. R: A language and Environment for Statistical Computing. Available online: https://www.R-project.org/ (accessed on 8 June 2022).
31. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016; ISBN 978-3-319-24277-4.
32. Yu, G. Scatterpie: Scatter Pie Plot, R Package Version 0.1.7, 2021. Available online: https://CRAN.R-project.org/package=scatterpie (accessed on 15 July 2022).
33. Steyer, A.; Poljsak-Prijatelj, M.; Barlic-Maganja, D.; Marin, J. Human, porcine and bovine rotaviruses in Slovenia: Evidence of interspecies transmission and genome reassortment. *J. Gen. Virol.* **2008**, *89*, 1690–1698. [CrossRef] [PubMed]
34. Katz, E.M.; Esona, M.D.; Betrapally, N.S.; De La Cruz De Leon, L.A.; Neira, Y.R.; Rey, G.J.; Bowen, M.D. Whole-gene analysis of inter-genogroup reassortant rotaviruses from the Dominican Republic: Emergence of equine-like G3 strains and evidence of their reassortment with locally-circulating strains. *Virology* **2019**, *534*, 114–131. [CrossRef]
35. Bonura, F.; Bányai, K.; Mangiaracina, L.; Bonura, C.; Martella, V.; Giammanco, G.M.; De Grazia, S. Emergence in 2017–2019 of novel reassortant equine-like G3 rotavirus strains in Palermo, Sicily. *Transbound. Emerg. Dis.* **2022**, *69*, 813–835. [CrossRef]
36. Afrad, M.H.; Matthijnssens, J.; Afroz, S.F.; Rudra, P.; Nahar, L.; Rahman, R.; Hossain, M.E.; Rahman, S.R.; Azim, T.; Rahman, M. Differences in lineage replacement dynamics of G1 and G2 rotavirus strains versus G9 strain over a period of 22 years in Bangladesh. *Infect. Genet. Evol.* **2014**, *28*, 214–222. [CrossRef]
37. Phan, T.G.; Khamrin, P.; Quang, T.D.; Dey, S.K.; Takanashi, S.; Okitsu, S.; Maneekarn, N.; Ushijima, H. Detection and Genetic Characterization of Group A Rotavirus Strains Circulating among Children with Acute Gastroenteritis in Japan. *J. Virol.* **2007**, *81*, 4645–4653. [CrossRef]
38. Phan, T.G.; Okitsu, S.; Maneekarn, N.; Ushijima, H. Genetic heterogeneity, evolution and recombination in emerging G9 rotaviruses. *Infect. Genet. Evol.* **2007**, *7*, 656–663. [CrossRef]
39. Wandera, E.A.; Hatazawa, R.; Tsutsui, N.; Kurokawa, N.; Kathiiko, C.; Mumo, M.; Waithira, E.; Wachira, M.; Mwaura, B.; Nyangao, J.; et al. Genomic characterization of an African G4P[6] human rotavirus strain identified in a diarrheic child in Kenya: Evidence for porcine-to-human interspecies transmission and reassortment. *Infect. Genet. Evol.* **2021**, *96*, 105133. [CrossRef] [PubMed]
40. Papp, H.; Borzák, R.; Farkas, S.; Kisfali, P.; Lengyel, G.; Molnár, P.; Melegh, B.; Matthijnssens, J.; Jakab, F.; Martella, V.; et al. Zoonotic transmission of reassortant porcine G4P[6] rotaviruses in Hungarian pediatric patients identified sporadically over a 15 year period. *Infect. Genet. Evol.* **2013**, *19*, 71–80. [CrossRef] [PubMed]
41. Monini, M.; Zaccaria, G.; Ianiro, G.; Lavazza, A.; Vaccari, G.; Ruggeri, F.M. Full-length genomic analysis of porcine rotavirus strains isolated from pigs with diarrhea in Northern Italy. *Infect. Genet. Evol.* **2014**, *25*, 4–13. [CrossRef] [PubMed]
42. Hungerford, D.; Vivancos, R.; Read, J.M.; Pitzer, V.E.; Cunliffe, N.; French, N.; Iturriza-Gomara, M. In-season and out-of-season variation of rotavirus genotype distribution and age of infection across 12 European countries before the introduction of routine vaccination, 2007/08 to 2012/13. *Euro Surveill.* **2016**, *21*, 30106. [CrossRef]
43. Ferrari, E.; Salogni, C.; Martella, V.; Alborali, G.L.; Scaburri, A.; Boniotti, M.B. Assessing the Epidemiology of Rotavirus A, B, C and H in Diarrheic Pigs of Different Ages in Northern Italy. *Pathogens* **2022**, *11*, 467. [CrossRef]
44. Marthaler, D.; Homwong, N.; Rossow, K.; Culhane, M.; Goyal, S.; Collins, J.; Matthijnssens, J.; Ciarlet, M. Rapid detection and high occurrence of porcine rotavirus A, B, and C by RT-qPCR in diagnostic samples. *J. Virol. Method.* **2014**, *209*, 30–34. [CrossRef]
45. Wu, F.-T.; Liu, L.T.-C.; Jiang, B.; Kuo, T.-Y.; Wu, C.-Y.; Liao, M.-H. Prevalence and diversity of rotavirus A in pigs: Evidence for a possible reservoir in human infection. *Infect. Genet. Evol.* **2022**, *98*, 105198. [CrossRef]
46. Wenske, O.; Rückner, A.; Piehler, D.; Schwarz, B.-A.; Vahlenkamp, T.W. Epidemiological analysis of porcine rotavirus A genotypes in Germany. *Vet. Microbiol.* **2018**, *214*, 93–98. [CrossRef]
47. Kozyra, I.; Kozyra, J.; Dors, A.; Rzeżutka, A. Molecular chracterisation of porcine group A rotaviruses: Studies on the age-related occurrence and spatial distribution of circulating virus genotypes in Poland. *Vet. Microbiol.* **2019**, *232*, 105–113. [CrossRef]
48. Brnić, D.; Šimić, I.; Lojkić, I.; Krešić, N.; Jungić, A.; Balić, D.; Lolić, M.; Knežević, D.; Hengl, B. The emergence of porcine epidemic diarrhoea in Croatia: Molecular characterization and serology. *BMC Vet. Res.* **2019**, *15*, 249. [CrossRef]
49. Banyai, K.; Laszlo, B.; Duque, J.; Steele, A.D.; Nelson, E.A.; Gentsch, J.R.; Parashar, U.D. Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: Insights for understanding the impact of rotavirus vaccination programs. *Vaccine* **2012**, *30* (Suppl. 1), A122–A130. [CrossRef] [PubMed]
50. Chandler-Bostock, R.; Hancox, L.R.; Nawaz, S.; Watts, O.; Iturriza-Gomara, M.; Mellits, K.H. Genetic diversity of porcine group A rotavirus strains in the UK. *Vet. Microbiol.* **2014**, *173*, 27–37. [CrossRef] [PubMed]

51. Flores, P.S.; Costa, F.B.; Amorim, A.R.; Mendes, G.S.; Rojas, M.; Santos, N. Rotavirus A, C, and H in Brazilian pigs: Potential for zoonotic transmission of RVA. *J. Vet. Diagn. Investig.* **2021**, *33*, 129–135. [CrossRef] [PubMed]
52. Amimo, J.O.; Junga, J.O.; Ogara, W.O.; Vlasova, A.N.; Njahira, M.N.; Maina, S.; Okoth, E.A.; Bishop, R.P.; Saif, L.J.; Djikeng, A. Detection and genetic characterization of porcine group A rotaviruses in asymptomatic pigs in smallholder farms in East Africa: Predominance of P[8] genotype resembling human strains. *Vet. Microbiol.* **2015**, *175*, 195–210. [CrossRef]
53. Hoxie, I.; Dennehy, J.J. Rotavirus A Genome Segments Show Distinct Segregation and Codon Usage Patterns. *Viruses* **2021**, *13*, 1460. [CrossRef]
54. Collins, P.J.; Martella, V.; Buonavoglia, C.; O'Shea, H. Identification of a G2-like porcine rotavirus bearing a novel VP4 type, P[32]. *Vet. Res.* **2010**, *41*, 73. [CrossRef]
55. Baumann, S.; Sydler, T.; Rosato, G.; Hilbe, M.; Kümmerlen, D.; Sidler, X.; Bachofen, C. Frequent Occurrence of Simultaneous Infection with Multiple Rotaviruses in Swiss Pigs. *Viruses* **2022**, *14*, 1117. [CrossRef]
56. Arana, A.; Montes, M.; Jere, K.C.; Alkorta, M.; Iturriza-Gómara, M.; Cilla, G. Emergence and spread of G3P[8] rotaviruses possessing an equine-like VP7 and a DS-1-like genetic backbone in the Basque Country (North of Spain), 2015. *Infect. Genet. Evol.* **2016**, *44*, 137–144. [CrossRef]

8.2. PAPER II

KUNIĆ, V., T. MIKULETIČ, R. KOGOJ, T. KORITNIK, A. STEYER, S. ŠOPREK, G. TEŠOVIĆ, V. KONJIK, I. ROKSANDIĆ KRIŽAN, M. PRIŠLIN, D. BRNIĆ (2023): Interspecies transmission of porcine-originated G4P[6] *Rotavirus A* between pigs and humans: a synchronized spatiotemporal approach. Front. Microbiol. 14 (2023), 1194764, 14. doi: 10.3389/fmicb.2023.1194764

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 22 May 2023
DOI 10.3389/fmicb.2023.1194764

OPEN ACCESS

EDITED BY
Jue Liu,
Yangzhou University, China

REVIEWED BY
Saumya Anang,
Dana–Farber Cancer Institute, United States
Joshua Oluoch Amimo,
The Ohio State University, United States

*CORRESPONDENCE
Dragan Brnić
brnic@veinst.hr

RECEIVED 27 March 2023
ACCEPTED 25 April 2023
PUBLISHED 22 May 2023

CITATION
Kunić V, Mikuletič T, Kogoj R, Koritnik T, Steyer A, Šoprek S, Tešović G, Konjik V, Roksandić Križan I, Prišlin M, Jemeršić L and Brnić D (2023) Interspecies transmission of porcine-originated G4P[6] rotavirus A between pigs and humans: a synchronized spatiotemporal approach.
Front. Microbiol. 14:1194764.
doi: 10.3389/fmicb.2023.1194764

Interspecies transmission of porcine-originated G4P[6] rotavirus A between pigs and humans: a synchronized spatiotemporal approach

Valentina Kunić[1], Tina Mikuletič[2], Rok Kogoj[2], Tom Koritnik[3], Andrej Steyer[3], Silvija Šoprek[4], Goran Tešović[4,5], Vlatka Konjik[6], Ivana Roksandić Križan[6], Marina Prišlin[1], Lorena Jemeršić[1] and Dragan Brnić[1]*

[1]Virology Department, Croatian Veterinary Institute, Zagreb, Croatia, [2]School of Medicine, Institute for Microbiology and Immunology, University of Ljubljana, Ljubljana, Slovenia, [3]Public Health Microbiology Department, National Laboratory of Health, Environment, and Food, Ljubljana, Slovenia, [4]Department for Pediatric Infectious Diseases, University Hospital for Infectious Diseases "Dr. Fran Mihaljević", Zagreb, Croatia, [5]School of Medicine, University of Zagreb, Zagreb, Croatia, [6]Clinical Hospital Center Osijek, Osijek, Croatia

As a leading viral cause of acute gastroenteritis in both humans and pigs, rotavirus A (RVA) poses a potential public health concern. Although zoonotic spillover of porcine RVA strains to humans is sporadic, it has been detected worldwide. The origin of chimeric human–animal strains of RVA is closely linked to the crucial role of mixed genotypes in driving reassortment and homologous recombination, which play a major role in shaping the genetic diversity of RVA. To better understand how genetically intertwined porcine and zoonotic human-derived G4P[6] RVA strains are, the present study employed a spatiotemporal approach to whole-genome characterization of RVA strains collected during three consecutive RVA seasons in Croatia (2018–2021). Notably, sampled children under 2 years of age and weanling piglets with diarrhea were included in the study. In addition to samples tested by real-time RT-PCR, genotyping of VP7 and VP4 gene segments was conducted. The unusual genotype combinations detected in the initial screening, including three human and three porcine G4P[6] strains, were subjected to next-generation sequencing, followed by phylogenetic analysis of all gene segments, and intragenic recombination analysis. Results showed a porcine or porcine-like origin for each of the eleven gene segments in all six RVA strains. The G4P[6] RVA strains detected in children most likely resulted from porcine-to-human interspecies transmission. Furthermore, the genetic diversity of Croatian porcine and porcine-like human G4P[6] strains was propelled by reassortment events between porcine and porcine-like human G4P[6] RVA strains, along with homologous intragenotype and intergenotype recombinations in VP4, NSP1, and NSP3 segments. Described concurrent spatiotemporal approach in investigating autochthonous human and animal RVA strains is essential in drawing relevant conclusions about their phylogeographical relationship. Therefore, continuous surveillance of RVA, following the One Health principles, may provide relevant data for assessing the impact on the protectiveness of currently available vaccines.

KEYWORDS

rotavirus A, human, zoonosis, domestic pig, G4P[6], reassortment, recombination, Croatia

1. Introduction

Rotavirus A (RVA) group is continuously reported as a leading cause of non-bacterial gastroenteritis in mammal and avian species, especially offspring. In humans, it can infect neonates, older children, and sometimes adults, with children younger than 5 years being the most affected (Trojnar et al., 2009; Estes and Greenberg, 2013; Desselberger, 2014). Global RVA mortality burden started decreasing after the early 2000s, counting more than 250,000 deaths, to estimated 128,500 deaths in 2016 as more countries introduced vaccines into their National Immunization Programs (NIP) (Tate et al., 2016; Troeger et al., 2018). The most common symptoms associated with RVA-induced acute gastroenteritis (AGE) typically include profuse diarrhea, vomiting, and fever. The need for hospital care often stems from dehydration and reduced ability for oral intake (Dennehy, 2008; Crawford et al., 2017). RVAs are also a major causative agent of viral AGE in pigs, mainly in suckling and weaned pig age groups, causing substantial financial costs to the pork industry (Chang et al., 2012). *Rotavirus A* species belongs to the *Rotavirus* genus within the *Reoviridae* family, whose genome consists of double-stranded RNA arranged in 11 genome segments. The VP7 and VP4 segments are the basis for the binomial nomenclature of rotaviruses, providing the G and P genotypes, respectively (Estes and Greenberg, 2013). However, whole-genome-based classification has been developed and increasingly used in recent years (Maunula and von Bonsdorff, 2002). The respective genotypes are assigned to each genomic segment based on the percentage identity cutoff values for nucleotide (nt) coding sequences of each viral (VP) and non-structural protein (NSP) (Matthijnssens et al., 2008a). This whole-genome classification aims to detect the genetic relationships between RVAs derived from different host species, reassortment events, and previously undetected genotypes (Matthijnssens et al., 2008b). Reassortment and recombination events are driving rotavirus diversification, which sometimes results in the emergence of chimeric human-animal strains. It is well-known that some RVA genotypes are more common in certain species, and many of them are shared between different species (Martella et al., 2010; McDonald et al., 2016). The human Wa-like and porcine RVAs are considered to have a common origin source since genogroup 1 genes found in the human RVA strains with the Wa-like constellation (i.e., I1-R1-C1-M1-A1-N1-T1-E1-H1) are also frequently found in porcine RVA strains (Matthijnssens et al., 2008b; Papp et al., 2013a; Theuns et al., 2015; Silva et al., 2016). Moreover, certain G/P genotype combinations can be considered usual or unusual for the given species. Therefore, the G4P[6] genotype combination is considered an unusual combination in humans, but it is quite common in pigs (Doro et al., 2015). Detection of a rare genotype combination like this one in a secondary host species may indicate a recent interspecies transmission event. In such cases, whole-genome sequencing can be used as a method of choice for strain investigation (Doro et al., 2015). Even though it is considered unusual in the human population, a G4P[6] genotype was discovered to reappear globally (Tacharoenmuang et al., 2021). A previous epidemiological study about the occurrence of RVA genotypes in children in Croatia reports a single case of the G4P[6] genotype (Vrdoljak et al., 2019). In our recent study on RVAs circulating in domestic pigs and wild boars, G4P[6] combination showed its modest appearance in domestic pigs, with the overall prevalence of G4 and P[6] strains among genotyped samples of only 9.8 and 4.3%, respectively (Brnić et al., 2022).

The present study aimed to comparatively analyze whole genomes of G4P[6] RVA strains detected in symptomatic children and pigs in Croatia with the synchronized spatiotemporal approach. It offers an insight into G4P[6] RVAs circulating in both populations during the same timeframe and relatively small geographical region, giving an opportunity for drawing adequate conclusions on the possible interspecies transmission, reassortment, and intragenic recombination events, which individually and collectively boost RVA genetic diversity in Croatian ecosystem.

2. Material and methods

2.1. Sampling

Stool samples and rectal swabs were collected from RVA-infected children and domestic pigs, respectively, which were sampled from 2018 to 2021, accounting for RVA seasons 2018/2019, 2019/2020, and 2020/2021. Sampling was conducted continuously, comprising rotavirus in-season and out-of-season samples. Mostly, children under 5 years of age with present clinical signs of acute gastroenteritis, consequently admitted to the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" Zagreb and Clinical Hospital Center Osijek, were included in this study. The collected stool samples were initially tested for the presence of rotaviral and adenoviral antigens using a single commercial immunochromatographic assay, the Rota-AdenoGnost (BioGnost, Zagreb, Croatia). During the same timeframe, the piglets with or without diarrhea were sampled, each by individual rectal swabbing, at large industrial and small backyard holdings in multiple counties as described in our recent work (Brnić et al., 2022). The piglets whose samples are reported in this research showed clinical signs of acute gastroenteritis at the time of sampling. Collected stool and swab samples were transferred to the Croatian Veterinary Institute for subsequent laboratory testing, maintaining a cold chain while in transportation. The samples were further processed immediately after reception or stored at −80°C. Detailed information about the sampled individuals is shown in Table 1.

2.2. Molecular diagnostics

Molecular diagnostics including RNA extraction, VP2 real-time RT-PCR, VP4, and VP7 genotyping were conducted within the scope of the initial screening of samples and are described in our study on RVAs in domestic pigs and wild boars (Brnić et al., 2022). For the human samples, the exception was the VP4/VP7 genotyping which was performed with the application of a multiplex VP7 and VP4 RT-PCR (EuroRotaNet, 2009[1]; Fujii et al.,

1 https://www.eurorotanet.com/project-information/documents-and-methods/ (accessed April 20, 2022)

TABLE 1 Data about human and domestic pig samples included in the present study.

RVA strain ID	Gender	Age	Sampling time	Diarrhea	Vesikari score	RVA Vaccine	County	Country
RVA/Human-wt/HRV/D230-ZG/2019/G4P[6]	f	1 y and 10 m	August/2019	yes	11	No	City of Zagreb	Croatia
RVA/Human-wt/HRV/D329-OB/2019/G4P[6]	f	1 y and 9 m	July/2019	yes	*ND**	No	Osijek-Baranja	Croatia
RVA/Human-wt/HRV/D672-ZG/2021/G4P[6]	f	1 y and 4 m	July/2021	yes	9	No	City of Zagreb	Croatia
RVA/Pig-wt/HRV/S243-VS/2019/G4P[6]	m	30–40 days	November/2019	yes	*N/A**	No	Vukovar-Srijem	Croatia
RVA/Pig-wt/HRV/S338-Z/2020/G4P[6]	f	37 days	March/2020	yes	*N/A**	No	Zagreb	Croatia
RVA/Pig-wt/HRV/S344-Z/2020/G4P[6]	f	37 days	March/2020	yes	*N/A**	No	Zagreb	Croatia

*ND–not determined since a child was not hospitalized. She was admitted to the pediatric ambulatory care unit, with symptoms resolved after 2 days.
*N/A–not applicable.

2019) complemented with the Sanger sequencing of untypable strains. Based on genotyping results, six G4P[6] strains, three of human and three of porcine origin, were selected for next-generation sequencing (NGS).

2.3. Library preparation and NGS

Three individual sequencing runs were performed chronologically as samples were collected. Firstly, 20% *w/v* fecal and swab suspensions prepared with Medium 199 (Sigma-Aldrich, St. Louis, USA) were used as a starting material. Suspensions were sent to the Institute of Microbiology and Immunology, Slovenia, where sample preparation and NGS were conducted. Nucleic acid extraction from the supernatant of 20% *w/v* fecal and swab suspension was performed on a Maelstrom 9600 device (TANBead Inc., Taoyuan City, Taiwan) using an OptiPure Viral Auto Plate (TANBead Inc., Taoyuan City, Taiwan) extraction kit, followed by the real-time RT-PCR detection of RVA by LightMix Modular assay (TIB Molbiol, Berlin, Germany) on a LightCycler 480 instrument (Roche, Basel, Switzerland). Since viral genome loads in metagenomic samples tend to be exceptionally low in concentration, DNA depletion was performed using TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, USA). After DNA removal, the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific™, Waltham, USA) was used for the first- and second-strand cDNA synthesis. Prepared dsDNA was then purified utilizing GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, USA) to remove excess dNTPs and other reagents such as competing enzymes or buffer components. All procedures referenced above were performed following the respective manufacturer's instructions. Complementary DNA (cDNA) was finally quantified before proceeding with library preparation, using a Qubit™ 4 Fluorometer with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA).

NGS libraries were constructed using a Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, USA) with barcoding respective samples with the IDT® for Illumina® Nextera DNA/RNA Unique Dual Indexes Set B and C (Illumina Inc., San Diego, USA) according to the manufacturer's instructions.

After tagmentation and amplification, NGS libraries were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, USA). The quality and quantity of the purified libraries were assessed with a 2100 Bioanalyzer instrument (Agilent, Santa Clara, USA) using a High Sensitivity DNA Kit (Agilent, Santa Clara, USA), and a Qubit™ 4 Fluorometer using Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, USA), respectively. NGS was performed on Illumina® NextSeq 500 sequencer (Illumina Inc., San Diego, USA) utilizing the NextSeq 500/550 High Output Kit v 2.5 on 300 cycles (Illumina Inc., San Diego, USA) to produce 150 paired-end reads. The herein-described procedure was applied for all three individual sequencing runs.

2.4. NGS data analysis

Data analysis for the NGS was performed using a CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany). For each of the 11 RVAs genomic segments, representative sequences of various genotypes were selected using NCBI's Virus Variation Rotavirus Database[2] (Hatcher et al., 2017). Those were used for building reference lists for each gene segment, regardless of the genotype. Genomes were assembled utilizing the reference-based mapping process for each gene segment individually due to the segmented nature of the rotavirus genome. The workflow consisted of trimming raw reads of Illumina adapters, mapping trimmed reads to all the reference lists, and finally extracting consensus sequences and mapping reports. Consensus sequences were not considered for further investigation if they did not meet the previously defined minimum sequence length and identity criteria (Matthijnssens et al., 2008a) or distribution coverage of 90% and coverage depth of 10×. Any occurring sequence gaps were managed by performing a *de novo* assembly on the same samples and correlating relevant contigs with the relevant reference-based consensus assemblies. Additional mapping data containing accession numbers of each genotype reference sequence used for the mapping process can be found in Supplementary Table 1 for

2 https://www.ncbi.nlm.nih.gov/genomes/VirusVariation/Database/nph-select.cgi?taxid=28875 (accessed January 9, 2023).

each RVA strain characterized in the present study. Final consensus sequences for every gene segment prior to the genotyping process were selected based on the mapping quality and the consequent full-length consensus sequence completeness. Genotypes were confirmed using final consensus sequences as queries, in the BLASTn search tool[3] in addition to the ViPR tool version 3.28.22[4] (Pickett et al., 2011), and characterized following previously described guidelines defining genotype cutoff values (Matthijnssens et al., 2008a). During these searches, any consensus sequence that did not hold up to the respective genotype it was initially mapped to was herein discarded as a result of the mapping error. Strain names were assigned according to the RVA nomenclature uniformity guidelines administered by the Rotavirus Classification Working Group[5] (RCWG). The CDSs that shared the highest percentage identity with each query or representatives of a certain group of sequences were used to assemble multiple sequence alignments and conduct evolutionary analyses in MEGA 11 software (Tamura et al., 2021).

2.5. Phylogenetic analysis and pairwise comparison

To investigate the evolutionary relationship between human and porcine RVA G4P[6] genotype strains, we constructed individual phylogenetic trees for each of the 11 RVA genomic segments, alongside the calculation of pairwise identity matrices. Therefore, we chose the representative strains from GenBank based on their high percentage identity with our query sequences and comparability based on geolocation, origin, host, or lineage for comparison purposes. The evolutionary history was inferred using the maximum-likelihood (ML) method for each multiple sequence alignment obtained by the MUSCLE algorithm, both acquired utilizing MEGA 11 software (Tamura et al., 2021). Substitution models that demonstrated the lowest BIC score values were as follows: T92+G (VP6, NSP2, NSP4, NSP5), T92+G+I (VP7, NSP1, NSP3), TN93+G+I (VP2), GTR+G+I (VP1, VP3), and HYK+G+I (VP4). The bootstrap analysis with 1,000 replicates was used to assess the branching support for each ML tree. Different G4 lineages were determined based on lineage attribution from Wandera et al. (2021). Different P[6] genotype lineages were determined based on the lineage attribution from Maringa et al. (2020) and Wandera et al. (2021). For graphical editing and annotation of phylogenetic trees, we used iTOL version 6[6] (Letunic and Bork, 2021). Furthermore, CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany) was used for calculating pairwise identity matrices among the previously aligned RVA sequences from the GenBank and the strains from the present study (Supplementary Tables 2, 3). Obtained nt and amino acid (aa) sequences of complete CDS for each RVA gene segment, including additional genotypes in mixed infections, were submitted to the GenBank with adjacent accession numbers: D230: OQ440159-OQ440170; D329: OQ440171-OQ440184; D572: OQ440185-OQ440195; S243: OQ440196-OQ440210; S338: OQ440211-OQ440223; and S344: OQ440224-OQ440236 (Supplementary Table 4).

2.6. Intragenic recombination analysis

Utilizing the BLASTn tool, we identified and downloaded complete BLAST search results for each of the 11 genome segments of six G4P[6] Croatian strains, including their respective mixed genotypes where applicable. Multiple sequence alignment sets were constructed as described earlier, with the number of sequences analyzed per gene alignment ranging from 110 to 383. The RDP4 v.4.101 software was used to perform intragenotype (for each gene) and intergenotype (for genes with apparent mixed genotypes) recombination analysis by applying seven integrated recombination detection methods: RDP, GENECONV, MaxChi, Bootscan, Chimera, SiScan, and 3Seq (Martin et al., 2015). For every detected recombination event, the UPGMA method constructed the breakpoint-defined major and minor parent phylogenetic trees (data not shown). The term parent in this context does not point out the exact evolutionary progenitors of the recombinant strains, it rather signifies a group of RVA strains from which the actual progenitors might have originated. Only putative homologous recombination predicted by at least six program methods was considered positive recombination signals (Hoxie and Dennehy, 2020).

3. Results

3.1. NGS results and the whole-genome constellation of RVA strains

Illumina NextSeq 500 platform yielded 23.5×10^6 reads (~122 bp average length), 18.1×10^6 reads (~110 bp av. Length), 28.5×10^6 reads (~110 bp av. length), 29.6×10^6 reads (~115 bp av. length), 24.7×10^6 reads (~147 bp av. length), and 16.8×10^6 reads (~111 bp av. length) for strains D230, D329, S243, S338, S344, and D572, respectively. Complete coding sequences were successfully determined for all 11 gene segments of all sequenced strains, and their respective mixed genotypes, except for the P[13] genotype of the S243 strain, which was a partial CDS (97%). A number of mapped reads, average coverage, and other reference mapping-related data are summarized in Supplementary Table 1 for each reported RVA strain. Concatenated 11-gene segmented genome constellations are presented in Table 2. Croatian G4P[6] porcine-like human RVA strains displayed a Wa-like genogroup constellation, and porcine G4P[6] strains displayed an RVA genogroup 1 constellation. Each gene segment of both human and domestic pig RVA strains uncovered the evolutionary connection with porcine and porcine-like human strains from neighboring countries but also with some distant global RVA strains. Pairwise identity matrices for each gene segment can be found in Supplementary Table 2.

3 https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed December 14, 2022).

4 https://www.bv-brc.org/ (accessed December 20, 2022).

5 https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg (accessed December 16, 2022).

6 https://itol.embl.de/ (accessed February 24, 2023).

TABLE 2 Whole-genome constellations of six Croatian RVA G4P[6] strains.

RVA strain ID	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-wt/HRV/D230-ZG/2019/G4P[6]	G4	P[6]	I1	R1	C1	M1	A1	N1	T1/T7	E1	H1
RVA/Human-wt/HRV/D329-OB/2019/G4P[6]	G4/G1	P[6]/P[8]	I1	R1	C1	M1	A1	N1	T1/T7	E1	H1
RVA/Human-wt/HRV/D572-ZG/2021/G4P[6]	G4	P[6]	I1	R1	C1	M1	A8	N1	T7	E1	H1
RVA/Pig-wt/HRV/S243-VS/2019/G4P[6]	G4/G5/G11	P[6]/P[13]	I5	R1	C1	M1	A8	N1	T1/T7	E9	H1
RVA/Pig-wt/HRV/S338-Z/2020/G4P[6]	G4/G5	P[6]/P[13]	I5	R1	C1	M1	A1	N1	T7	E1	H1
RVA/Pig-wt/HRV/S344-Z/2020/G4P[6]	G4/G5	P[6]/P[13]	I5	R1	C1	M1	A1	N1	T7	E1	H1

Mixed genotypes are designated for the respective genomic segment.

3.2. Phylogenetic and recombination analysis

3.2.1. VP7

Croatian G4 strains presented in this research belonged to lineage VI (Figure 1), which shared an nt percentage identity of approximately 83–86% with other G4 lineages. Intralineage nt percentage identity was larger than 86%. Our G4 strains (both porcine and human detected) formed a separate cluster within lineage VI with two porcine-derived strains from the Czech Republic and Slovakia and three G4 zoonotic strains detected in humans in Hungary and Kenya (Figure 1). Sequences in this cluster shared high nt similarities (94–100%), revealing that the three human-derived G4 Croatian strains have a porcine origin. Phylogenetic analysis for mixed genotypes that occurred in the VP7 segment, listed in Table 2, can be found in Supplementary Figure 1A. In addition, respective nt and aa % identities are located in Supplementary Table 3. The RDP4 recombination analysis detected no intragenotype or intergenotype recombination events in this gene segment.

3.2.2. VP4

Phylogenetic analysis of the VP4 segment grouped Croatian P[6] strains within the lineage V, among the zoonotic P[6] strains from Hungary, multiple zoonotic P[6] African strains, and P[6] strains detected in European pigs (Figure 2). Three Croatian pig RVA strains formed a separate clade, human-detected strains also, all of which shared the greatest nt similarity within their respective clades, while the D329 P[6] strain displayed porcine origin closest to Hungarian zoonotic P[6] (Figure 2). Genotype P[6] displayed intragenotype differences much like the G4; hence, nt percentage identities between different lineages ranged from 82 to 88%, and within the lineage V from 90 to 99.6%. Recombination analysis of the VP4 segment resulted in identifying the porcine S338 strain as an intragenotype P[6] recombinant of two human P6 strains of zoonotic porcine origin, from Hungary and Russia as major and minor parents (Table 3). The recombination event has been detected with six of seven RDP4 detection methods, therefore, strongly supported. Phylogenetic analysis for mixed genotypes that occurred in the VP4 segment, listed in Table 2, and can be found in Supplementary Figure 1B. In addition, respective nt and aa % identities are located in Supplementary Table 3.

3.2.3. VP6

Two different genotypes were established in this segment, I5 for domestic pig-derived, and I1 for human-derived strains (Figure 3D). Intragenotype I5 nt similarity fell in the range of 92.8–99.8%. Among the typical porcine I5 genotype, two clusters of different sources of origin can be recognized, one that includes strain S243 mixed with other European strains (from Italy, Belgium, and Spain) and one of remote origin that includes strains S338 and S344 mixed with North American strain (Figure 3D). Genotype I1 human-derived strains presented as porcine-originated, since these strains branch together with Hungarian and Kenyan porcine-like strains previously reported as zoonotic (Papp et al., 2013a; Wandera et al., 2021) with nt similarity surpassing 95%. The RDP4 recombination analysis detected no intragenotype or intergenotype recombination events in this gene segment.

3.2.4. VP1

Six Croatian G4P[6] strains were identified as genotype R1, although they proved to be a diverse group of sequences with nt similarity ranging from 86 to 100%. Human-derived strain D572 clustered with various European porcine R1 strains (94–96% nt identity) including an autochthonous S243 domestic pig-derived strain, and with the USA Gottfried, a representative strain for the porcine-originated Wa-like G4P[6] constellation (Figure 3A). Strain D572 R1 did not prove similar to any human-derived R1 available sequences (<87%). This complete phylogenetic separation from human-derived R1 strains suggests a VP1 porcine/porcine-like human reassortment event. The second human-derived R1 strain, D230, clustered with Hungarian zoonotic porcine-like strains (Figure 3A). Interestingly, the human-detected D329 strain also displayed a porcine-like origin but was similar to porcine and porcine-like R1 strains from South Korea and Nepal (Figure 3A). The remaining two porcine-detected strains (S344, S348) were mixed with other European porcine strains in a different R1 lineage (Figure 3A). The RDP4 recombination analysis detected

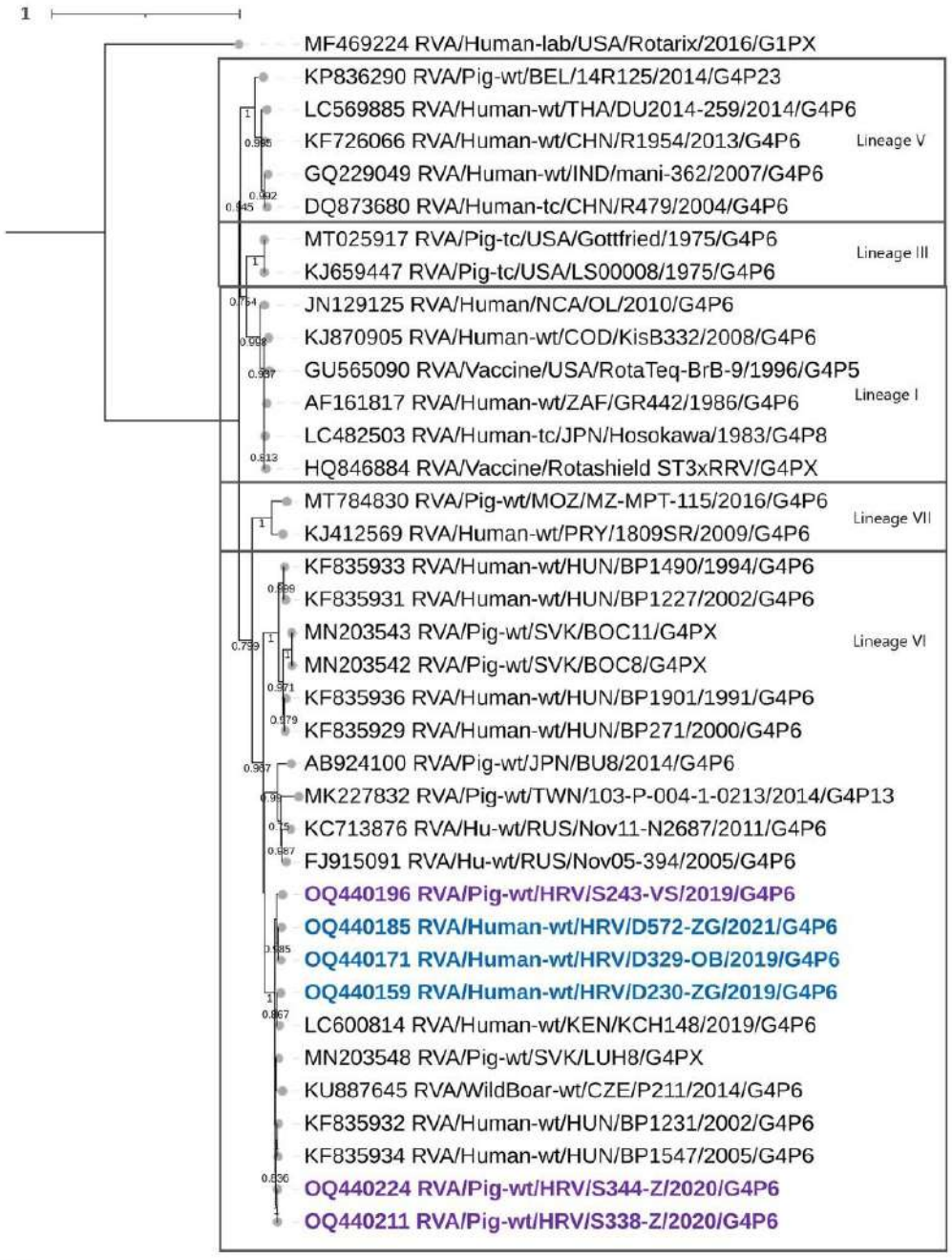


FIGURE 1
The phylogenetic tree of the full-length VP7 segment G4 genotype CDS sequences. The strains from the present study were bolded and marked in purple (for pig-derived strains) and blue (for human-derived strains). Accession numbers of all strains are included in the taxa labels. The tree was generated by the ML method and T92+G+I model in MEGA 11 software. The bootstrap analysis with 1,000 replicates was used to assess the branching support (showed values > 0.7). The scale bar represents the number of substitutions per site. Rotarix G1 strain as an outgroup.

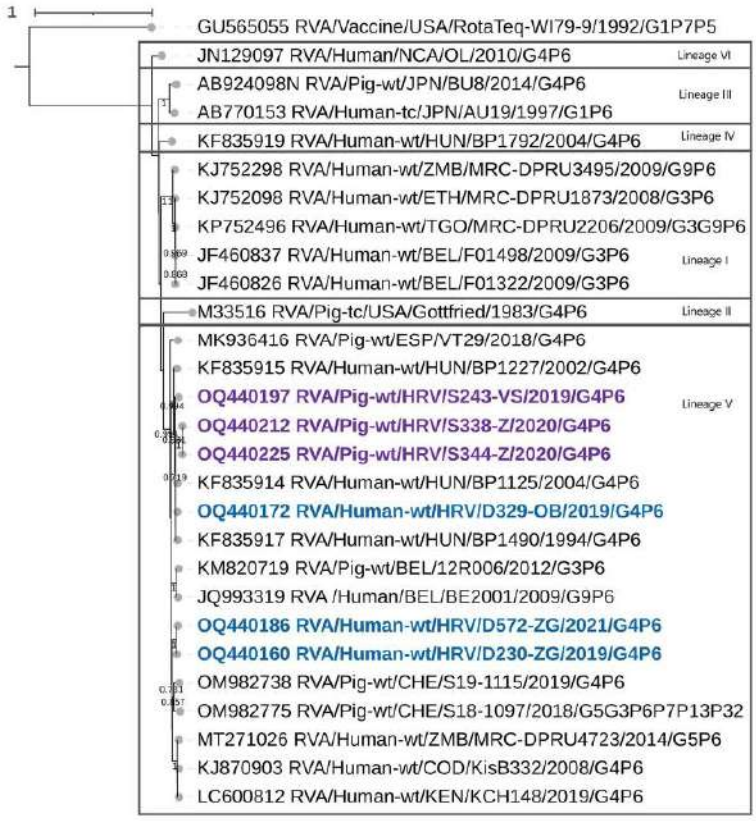


FIGURE 2
The phylogenetic tree of the full-length VP4 segment P[6] genotype CDS sequences. The strains from the present study were bolded and marked in purple (pig-derived strains) and in blue (human-derived strains). Accession numbers of all strains are included in the taxa labels. The tree was generated by the ML method and the HYK+G+I model in MEGA 11 software. The bootstrap analysis with 1,000 replicates was used to assess the branching support (showed values > 0.7). The scale bar represents the number of substitutions per site. RotaTeq P[5] strain stands as an outgroup.

TABLE 3 RVA intragenotype and intergenotype recombination data.

Recombinant strain	S338 P6	D329 A1	D329 T7	S243 T7	D230 T1
Recombination type	Intragenotype	Intragenotype	Intergenotype	Intergenotype	Intergenotype
Major parent	KF835917 RVA/Human-wt/HUN/1490/1994/G4P[6]	KF835940 RVA/Human-wt/HUN/BP1231/2002/G4P[6]A1	KF723308 RVA/Pig-wt/ITA/519RE/2010/G5P23T7	D572 T7	OM982754 RVA/Pig-wt/CHE/S18-1463/2018/G9P[32]T1
Minor parent	JX156399 RVA/Human-wt/RUS/N2687/2011/G4P[6]	ON992465 RVA/Human-wt/CHN/JL18221043/2018/G9P[8]A1	D329 T1	S243 T1	D230 T7
Starting breakpoint*	1,858	885	246	1/838	310
Ending breakpoint*	2,156	971	575	174/943	692
No. of detection methods confirming recombination event	6/7	7/7	7/7	7/7	7/7

*Breakpoint confidence interval 99%.

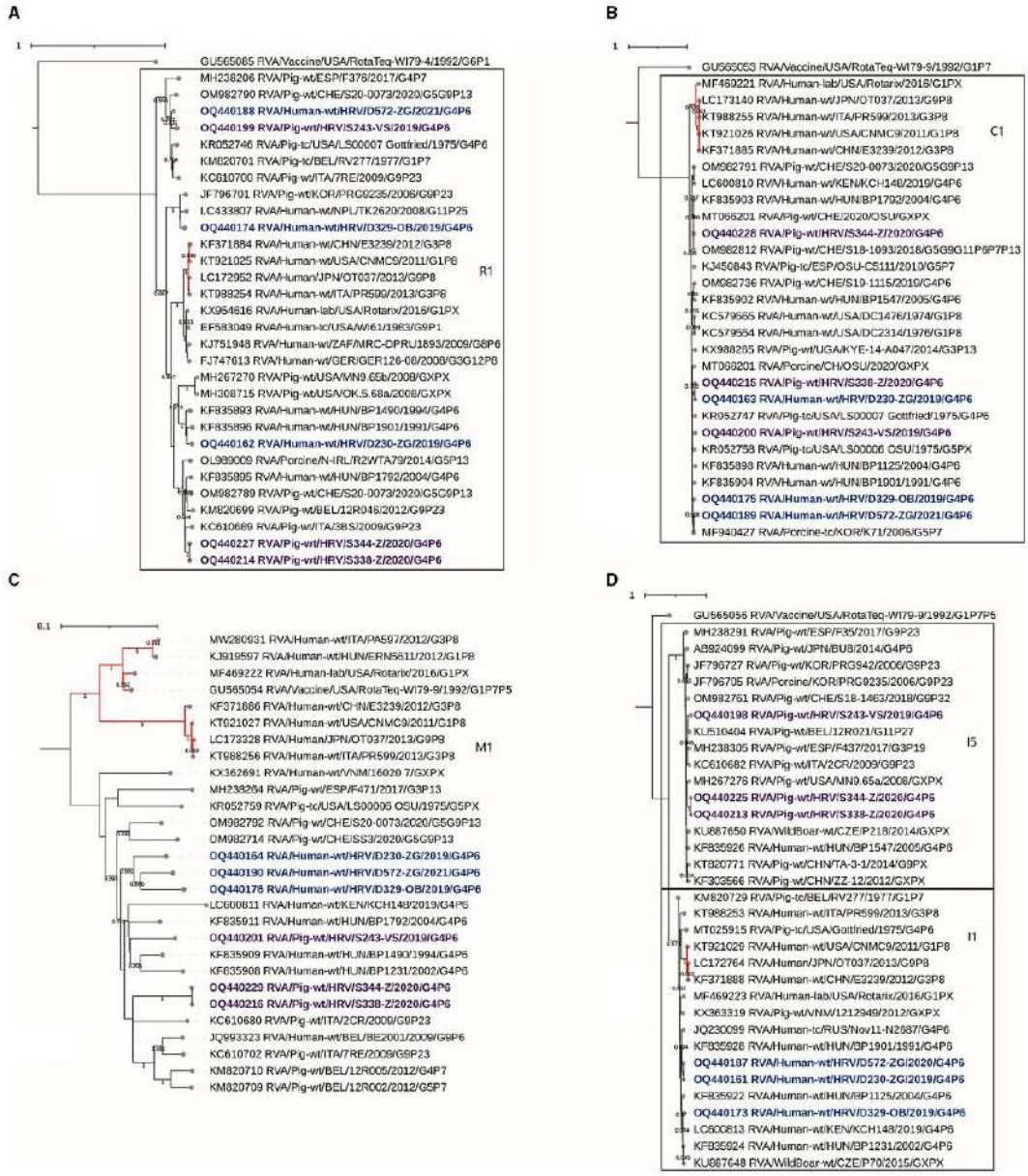


FIGURE 3
The phylogenetic tree of the full-length backbone viral proteins CDS sequences: VP1 **(A)**, VP2 **(B)**, VP3 **(C)**, and VP6 **(D)**. The strains from the present study were bolded and marked in purple (pig-derived strains) and in blue (human-derived strains). Accession numbers of all strains are included in the taxa labels. The branches colored in red are designated to typically human RVA strains to accentuate the separation between porcine and human-originated RVAs of the same genotype. The tree was generated in MEGA 11 software by the ML method, models TN93+G+I, and T92+G, respectively. The bootstrap analysis with 1,000 replicates was used to assess the branching support (showed values > 0.7). The scale bar represents the number of substitutions per site. RotaTeq genogroup 2 strains stand as an outgroup, besides VP3 **(C)**, where it shares the M1 genotype.

no intragenotype or intergenotype recombination events in this gene segment.

3.2.5. VP2

All presented Croatian strains genotyped as C1 shared a porcine or porcine-like origin. A few typical human strains with Wa-like backbone constellation, including the Rotarix vaccine strain, were added to phylogenetic analysis for comparison purposes, and have formed a separate cluster, thus outlining the phylogenetic distance between human and porcine-originated genotype C1 (Figure 3B). Two porcine-derived Croatian strains, S338 and S344, sampled at the same time and on the same holding, separated into different clusters in this gene segment (Figure 3B). What probably influenced this separation is the insertion of S amino acid at position 41 in the amino acid sequence for strain S338. Moreover, additional insertions were observed in other Croatian C1 sequences, such as NNKN amino acids at positions 38–41 for strains D329 and S243, and KAS amino acids at positions 39–40 for strain D230. Listed insertions were sequenced with the high coverage for each nt position. The typical genotype C1 strains of human origin shared an insertion similar to the strains D329 and S243, differing in two amino acids (KNRN). In contrast, the insertion described for the D230 strain has not been, to the best of our knowledge, described yet. Regardless of the mentioned differences, nt similarity among porcine- and human-derived C1 strains ranged from 93 to 99%. The RDP4 recombination analysis detected no intragenotype or intergenotype recombination events in this gene segment.

3.2.6. VP3

Croatian strains shared an M1 genotype consistent with porcine or porcine-like origin. Croatian human-originated M1 sequences clustered separately as shown in Figure 3C but phylogenetically connected to porcine RVA strains. Domestic pig-derived strains S344 and S338 clustered separately from strain S243, but all were phylogenetically related to porcine or human RVA strains of porcine origin. All six strains described in the present study displayed the highest nt identity with Hungarian zoonotic porcine-like strains, but interestingly, except for the porcine-derived S338 and S344 strains mutually, none of the strains shared more than 94.3% similarity with another autochthonous or database-accessed strain. The said divergence of herein included M1 genotype strains was also suggested by the phylogram branching pattern and branch lengths (Figure 3C). The RDP4 recombination analysis detected no intragenotype or intergenotype recombination events in this gene segment.

3.2.7. NSP1

Within the NSP1 gene segment, the Croatian strains clustered into two genotypes, A1 and A8 (Figure 4A). A typical porcine A8 genotype was found in one porcine and one human-derived strain. The latter, the human-derived D572 A8 strain, was presented as a putative porcine/porcine-like human reassortment event, possessing a typical porcine genotype. Consequently, it separated phylogenetically with porcine strains, forming a clade with the porcine A8 strain detected in Switzerland (89.4% nt identity) supported by a high bootstrap value, while it barely reached the genotype cutoff value with other A8 strains. We accentuate the proximity of the Swiss porcine strain in this case since the reassortment suggests a putative evolutionary connection between D572 and porcine strains from Switzerland, which already occurred in the VP1 segment (Figure 3A). This finding marks a second reassortment event for the D572 strain, making it a porcine-human RVA reassortant in VP1 (Figure 3A) and NSP1 (Figure 4A). No available human-derived A8 sequences that would be similar to this strain were available in GenBank for comparison, pointing out a lack of known human-derived evolutionary relatives of the D572 A8 strain. In genotype A1, taxons branched in two separate directions, one of typical human-originated A1, and the other of porcine-like-originated A1. One human and two porcine Croatian sequences formed a clade in a porcine-like A1 cluster, while the human D329 strain branched individually within the same A1 cluster, with evident divergence, showing the highest nt identity of only 87.7% with a zoonotic porcine-like strain from Kenya (Figure 4A). The explanation for D329 A1 phylogenetic divergence was found in the NSP1 gene segment recombination analysis. It was identified as an intragenotype A1 recombinant between two human A1 strains, one of which was a zoonotic porcine-like origin strain from Hungary serving as a major parent, and in the role of the minor parent, there was a Chinese A1 strain of typical human origin (Table 3). This outcome is completely cohesive with the fact that in the D329 sample, along with the porcine-like G4P[6], G1P[8] genotype combination was also present, which is a typical human RVA genotype combination (Supplementary Figure 1). This recombination event was strongly supported by seven of seven RDP4 detection methods.

3.2.8. NSP2

In the NSP2 phylogeny, Croatian strains were genotyped as N1 genotype, while clustering in three different branches (Figure 4B). One formed a cluster consisting of Croatian strains, two human and two porcine-derived, paired with a Hungarian zoonotic porcine-like strain. These four Croatian strains shared the highest percentage nt identity of approximately 97%, underlying an obvious connection between autochthonous porcine and porcine-like human strains. Furthermore, this cluster shared high identities at the nt level (>95%) with East Asian strains. Another strain, D572, also demonstrated phylogenetic proximity to far-eastern strains in a form of a clade with Chinese and South Korean pig strains (Figure 4B). The third putative source of origin was presented by two North American porcine N1 genotype strains, including Gottfried, in the same clade as the Croatian porcine S243 strain, as supported by pairwise nt identity comparison, and high bootstrap support (Figure 4B). The RDP4 recombination analysis detected no intragenotype or intergenotype recombination events in this gene segment.

3.2.9. NSP3

The translation enhancer gene segment is presented in two genotypes, T7 and T1. In addition to VP7 and VP4 segments, mixed genotypes were also detected in NSP3 (Table 2, Figure 4C).

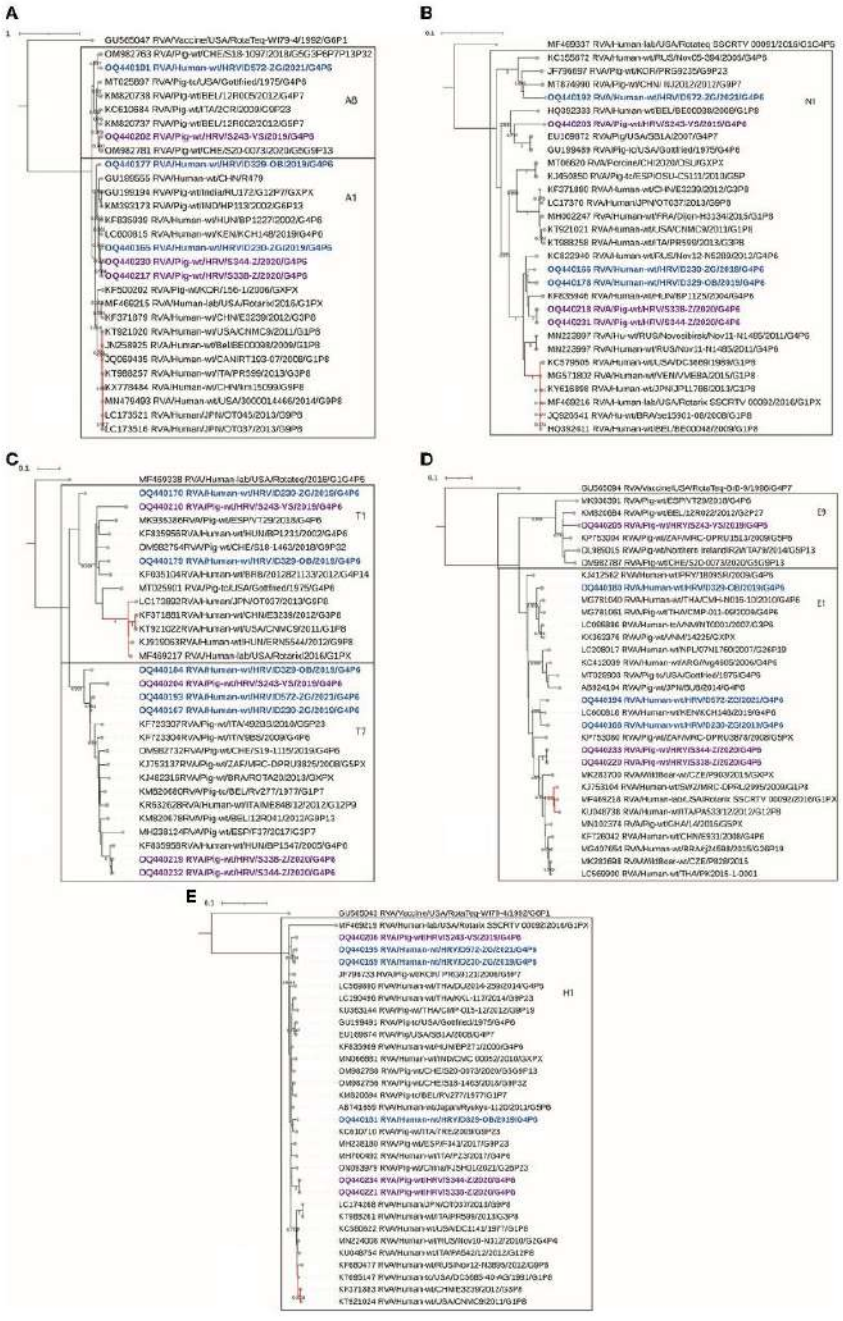


FIGURE 4
The phylogenetic tree of the full-length backbone non-structural proteins CDS sequences; NSP1 **(A)**, NSP2 **(B)**, NSP3 **(C)**, NSP4 **(D)**, and NSP5 **(E)**. The strains from the present study were bolded and marked in purple (for pig-derived strains) and blue (for human-derived strains). Accession numbers of all strains are included in the taxa labels. The branches colored in red are designated to typically human RVA strains to accentuate the separation between porcine and human-originated RVAs of the same genotype. The tree was generated in MEGA 11 software by the ML method, models T92+G+I **(A, C)** and T92+G **(B, D, E)**. The bootstrap analysis with 1,000 replicates was used to assess the branching support (showed values > 0.7). The scale bar represents the number of substitutions per site. RotaTeq genogroup 2 strains stand as an outgroup.

All Croatian G4P[6] strains presented with typical porcine T7 genotype, grouping with other European and global porcine T7 strains (89–98%). Two human-derived strains (D230, D329) and one porcine strain (S243) were presented as a mix of T1/T7 genotypes. These strains shared the highest identity (92%) with other porcine and porcine-like human T1 strains (Figure 4C). In the T1 phylogram, the branches forming a clade with T1 RVA strains of human origin are highlighted in red to accentuate a divergence of porcine and porcine-like human strains detected in the present study. Moreover, the NSP3 segment headlined in recombination analysis, since every mixed genotype strain was also presented as an intergenotype T1–T7 recombinant (Table 3). This finding was also obvious in the phylogenetic tree as these strains occupied sequestered branches falling on the edges of their respective genotypes (Figure 4C). Human strain D329 T7 was profiled as a recombinant of the Italian porcine T7 strain (major parent) and D329 T1 (minor parent). Furthermore, human strain D230 T1 was detected to be a recombinant between a Swiss porcine T1 strain (major parent) and D230 T7 (minor parent). Finally, one more recombination event took place in the NSP3 segment, with a porcine strain S243 T7 as a recombinant, having S243 T1 as a minor, and another Croatian human porcine-like strain D572 T7 as a major parent (Table 3). Every listed recombination event was strongly supported because detection was achieved with six or seven RDP4 integrated detection methods.

3.2.10. NSP4

Genome analysis of the RVA enterotoxin segment demonstrated two genotypes, E9 and E1. The porcine strain S243 presented with a typical porcine E9 genotype and is evidently related to a variety of European porcine strains (Figure 4D). The other five Croatian RVA strains described in the present study are positioned in the E1 genotype. Red-branching clade including the Rotarix vaccine strain shows the E1 genotype of human-origin RVAs (Figure 4D). Croatian E1 strains are positioned among porcine or porcine-like human strains in three different subclades, expressing high nt identity (92–100%) with autochthonous, European, African, Asian, and even Latin American strains, making it difficult to presume exact origin. The RDP4 recombination analysis detected no intragenotype or intergenotype recombination events in this gene segment.

3.2.11. NSP5

All Croatian strains of human and porcine origin were genotyped as H1 genotypes showing over 97% nt identity among intragenotype strains (Figure 4E). Nevertheless, three clusters of Croatian H1 sequences can be determined phylogenetically. Two porcine sequences (S344, S388) branched with Chinese porcine H1 strain, one porcine (S243) and two human H1 porcine-like strains (D230, D572) sequestered in a separate clade, and finally, a human-derived porcine-like D329 strain branched with porcine H1 strain detected in Italy (Figure 4E). For origin reference, H1 strains of human RVA origin were marked in a red branching pattern and hence illustrated a separation from porcine-like H1 strains. The RDP4 recombination analysis detected no intragenotype or intergenotype recombination events in this gene segment.

4. Discussion

In the present study, we sequenced and analyzed the whole genomes of six Croatian RVA G4P[6] strains detected in children under 2 years of age with AGE symptoms and in weanling piglets with diarrhea, during a synchronized spatiotemporal 3-year study (2018–2021). The aim was to illustrate how genetically intertwined an unusual zoonotic G4P[6] RVA genotype can be in both populations concurrently, accentuating the influence that the animal rotaviruses have on the evolution and recurrence of heterotypic RVAs in humans. Expectedly, porcine RVA strains displayed to have a porcine genogroup 1 origin in all gene segments, with typical porcine genotypes such as I5, A8, T7, and E9 standing out. Three porcine-like human G4P[6] strains displayed a Wa-like genogroup 1 constellation, while phylogenetic analysis revealed that in every genomic segment, these strains were genetically closely related to porcine-like human RVAs or porcine-originated strains. Human RVA Wa-like genogroup constellation is known to share its origin with porcine RVA genogroup 1 strains (Matthijnssens et al., 2008b; Steyer et al., 2008; Martella et al., 2010; Papp et al., 2013b). Considering surface protein coding gene segments, the G4 genotype has also been proven to infect humans and pigs, predominantly as a part of the G4P[8] genotype combination in humans, and as a third most prevalent VP7 genotype in pigs (Doro et al., 2015). The same is accurate for P[6], which is also a major porcine genotype. Nevertheless, human porcine-like RVA P[6] strains have been identified in a very sporadic pattern in Europe, but recurrence was continuous (Bányai et al., 2004; Martella et al., 2006; Steyer et al., 2008; Papp et al., 2013a; Vrdoljak et al., 2019). In the present study, G4 genotype strains clustered within lineage VI as defined by Wandera et al. (2021). However, as we have already hypothesized, G4 lineage VI could actually be formed of three distinct lineages if the lineages I–V demarcation threshold was applied (Brnić et al., 2022), but the consensus threshold criterion for lineage definition is currently unknown. In actuality, all G4 lineage VI strains presented in that study branched into three groups, possibly marking different lineages (Brnić et al., 2022). Nevertheless, despite the linage notation, all human-derived G4 strains from the present study are of porcine origin (Figure 1). This could also be said for strains of the P[6] genotype which clustered within the lineage V (Figure 2). In our previous study on porcine RVAs in Croatia, two porcine P[6] strains clustered within lineage IV in addition to lineage V strains (Brnić et al., 2022). All these P[6] strains were closely evolutionary connected to neighboring Hungarian zoonotic P[6] strains, underlining the influence of regional geolocation on RVA strain diversity.

The timing of detection of human-derived G4[6] strains was uncommon as all three G4P[6] strains were detected in symptomatic children in the summer months, an RVA out-of-season period in Croatia. This comes in agreement with earlier reports that emphasize an increase in mixed and rare genotype rates in multiple European countries in out-of-season months (Hungerford et al., 2016). Similar findings were also reported in Southern Italy; a 6-month-old child infected with the zoonotic G4P[6] RVA strain paired with the Wa-like backbone constellation, was also hospitalized in August. The foreign origin of this strain was further hypothesized (Ianiro et al., 2019). Similar to neighboring Italy, Croatia is a Mediterranean country with an immense amount

of tourism in July and August, thus, the import of an unusual zoonotic strain at that time could be hypothesized. However, based on the pairwise nt identities and phylogenetic relatedness of Croatian porcine and human-derived G4P[6] strains in the majority of gene segments, we believe that these cases are the result of independent events of indirect zoonotic interspecies transmission within Croatia. Moreover, the recombination analysis on multiple RVA segments provided additional evidence in favor of this conclusion. In all three human RVA cases, most probably an indirect RVA transmission occurred because of the very young age of infected children, where a direct piglet-child transmission is deemed highly unlikely. Environmental transmission might have played a role in the epidemiology of these infections. Since our human samples were collected during the summer months, the efficiency of RVA transmission might be reduced in higher temperature conditions (Kraay et al., 2018).

RVA mixed genotypes detected in Croatian porcine and porcine-like human G4P[6] strains propelled an incidence of reassortment events and intragenic homologous recombinations occurred in a few strains (Table 3). Due to the divergence of the D572 strain in VP1 and NSP1 segments from the rest of the human and porcine-like human strains, as well as clustering with exclusively porcine-derived strains in these segments, it most likely signifies the occurrence of reassortment between typical porcine and porcine-like human RVA strains (Figures 3A, 4A). No human-derived VP1 and NSP1 sequences that would be similar to the D572 strain were available in GenBank for comparison, pointing out a lack of known human-derived evolutionary relatives of D572 R1 and A8 strains, reaffirming D572 as a putative porcine/porcine-like human reassortant. It is accepted that heterologous RVAs of the porcine origin or porcine–human RVA reassortants had sporadically occurred and successfully spread among humans (Martella et al., 2010). Nevertheless, this kind of human-to-human transmission is generally short-lived since the heterologous RVA strains do not spread horizontally as efficiently among their non-specific hosts (Matthijnssens et al., 2006). Consequently, the significance of zoonotic transmission is potentially overlooked because clinically hospitalized symptomatic individuals are the focal point of RVA strain surveillance (Vilibic-Cavlek et al., 2021).

Moreover, two human porcine-like strains and one porcine strain have shown recombination events in at least one of the gene segments (VP4, NSP1 or NSP3). Interestingly, a G4P[6] RVA strain with a Wa-like constellation detected in the Dominican Republic was reported with the recombination events in the same genome segments as these three Croatian recombinants (Esona et al., 2017). Conversely to the comprehensive research of rotavirus A intragenic recombination prevalence, where recombination analysis of the NSP3 gene segment gave no results (Hoxie and Dennehy, 2020), herein we report T1–T7 intergenotype recombination among all three NSP3 recombinant strains, which also means that the NSP3 recombination was present in every strain presented with a T1/T7 mixed genotype. Findings like this further endorse the cognition that mixed genotypes predispose the evolution of novel RVA strains (Estes and Greenberg, 2013).

Finally, the VP2 nt sequence insertions in the 1–134 region are quite common (Matthijnssens et al., 2008a), and insertions detected in Croatian C1 sequences were found in the same region.

In conclusion, zoonotic interspecies transmission like these highlights the importance of continuous surveillance of animal RVAs and raises awareness on the role of animal RVAs in the evolution of strains affecting the human population. Such events of zoonotic transmission may have a short-term and long-term impact on the protectiveness of currently available vaccines. Thus, it is important to monitor the possible emergent liabilities which stem from the interconnection of human–animal RVAs. In that process, a One Health approach in RVA research brings an immense contribution.

Data availability statement

The data presented in the study are deposited in the NCBI GenBank repository. Accession numbers are listed in Material and Methods and in the Supplementary Table 4.

Ethics statement

The studies involving human participants were reviewed and approved by Board of Ethics of the University Hospital for Infectious Diseases Dr. Fran Mihaljević Zagreb; Board of Ethics of the Clinical Hospital Center Osijek; and Board of Ethics of the Institute for Public Health of Osijek-Baranja County. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by Board of Ethics of the Croatian Veterinary Institute.

Author contributions

VKu and DB contributed to the conception and design of the study. SŠ, GT, VKo, and IRK provided human samples, assessed the Vesikari score, and collected the guardian's or next of kin's informed consent. TM and RK performed NGS runs. VKu and TK organized the database and conducted NGS data analysis. VKu wrote the first draft of the manuscript. RK, DB, AS, MP, and LJ wrote sections of the manuscript. DB was responsible for funding acquisition and project administration. All authors contributed to the manuscript revision, read, and approved the submitted version.

Funding

This research was funded by the Croatian Science Foundation installation research project Reco "Rotaviruses in Croatian Ecosystem: molecular epidemiology and zoonotic potential," Grant No. HRZZ-UIP-2017-05-8580. The work of VK was funded by the Croatian Science Foundation project DOK-2021-02-3623.

Acknowledgments

We would like to thank the technical laboratory staff of the Croatian Veterinary Institute, especially Ivana Pišćak for their

contribution. Also we would like to thank Croatian Veterinary Institute for financing VK's NGS and NGS data analysis education, through funds from the Ministry of Science and Education, Republic of Croatia.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1194764/full#supplementary-material

SUPPLEMENTARY TABLE 1
Reference-mapping data.

SUPPLEMENTARY TABLE 2
Nucleotide and amino acid percentage identity data.

SUPPLEMENTARY TABLE 3
Nucleotide and amino acid percentage identity data for VP7 and VP4 mixed genotypes.

SUPPLEMENTARY TABLE 4
GenBank accession numbers of deposited sequences.

SUPPLEMENTARY FIGURE 1
The phylogenetic tree of the detected mixed genotypes G1, G4, G5, G11 in the VP7 (**A**) and P[6], P[8], P[13] in the VP4 (**B**) gene segments. The strains from the present study were bolded and marked in purple (pig-derived strains) and in blue (human-derived strains). Accession numbers of all strains are included in the taxa labels. The tree was generated by the ML method, and T92+G+I model in MEGA 11 software. The bootstrap analysis with 1000 replicates was used to assess the branching support (showed values > 0.7). The scale bar represents the number of substitutions per site.

References

Bányai, K., Martella, V., Jakab, F., Melegh, B., and Szücs, G. (2004). Sequencing and phylogenetic analysis of human genotype P[6] rotavirus strains detected in Hungary provides evidence for genetic heterogeneity within the P[6] VP4 gene. *J. Clin. Microbiol.* 42, 4338–4343. doi: 10.1128/JCM.42.9.4338-4343.2004

Brnić, D., Colić, D., Kunić, V., Maltar-Strmečki, N., Krešić, N., Konjević, D., et al. (2022). Rotavirus A in domestic pigs and wild boars: high genetic diversity and interspecies transmission. *Viruses* 14, 2028. doi: 10.3390/v14092028

Chang, K. S., Kim, Y., and Saif, L. J. (2012). "Rotavirus and reovirus," in *Diseases of Swine*, eds. J. J., Zimmerman, L. A., Karriker, A., Ramirez, K. J., Schwartz, G.W., Stevenson (United Kingdom: Wiley-Blackwell) 621–634.

Crawford, S. E., Ramani, S., Tate, J. E., Parashar, U. D., Svensson, L., Hagbom, M., et al. (2017). Rotavirus infection. *Nat. Rev. Dis. Primers* 3, 83. doi: 10.1038/nrdp.2017.83

Dennehy, P. H. (2008). Rotavirus vaccines: An overview. *Clin. Microbiol. Rev.* 21, 198–208. doi: 10.1128/CMR.00029-07

Desselberger, U. (2014). Rotaviruses. *Virus Res.* 190, 75–96. doi: 10.1016/j.virusres.2014.06.016

Doro, R., Farkas, S. L., Martella, V., and Banyai, K. (2015). Zoonotic transmission of rotavirus: Surveillance and control. *Expert Rev. Anti. Infect. Ther.* 13, 1337–1350. doi: 10.1586/14787210.2015.1089171

Esona, M. D., Roy, S., Rungsrisuriyachai, K., Sanchez, J., Vasquez, L., Gomez, V., et al. (2017). Characterization of a triple-recombinant, reassortant rotavirus strain from the Dominican Republic. *J. Gen. Virol.* 98, 134–142. doi: 10.1099/jgv.0.000688

Estes, M. K., and Greenberg, H. B. (2013). "Rotaviruses," in *Fields Virology*. 6th Edn. eds. D. M. Knipe and P. M. Howley (Philadelphia, PA: Lippincott Williams and Wilkins) 1347–1401.

EuroRotaNet (2009). *Rotavirus detection and typing methods*. Available online at: https://www.eurorotanet.com/project-information/documents-and-methods/ (accessed April 20, 2022).

Fujii, Y., Hai Doan, Y., Wahyuni, R. M., Lusida, M. I., Utsumi, T., Shoji, I., et al. (2019). Improvement of rotavirus genotyping method by using the semi-nested multiplex-PCR with new primer set. *Front. Microbiol.* 10, 647. doi: 10.3389/fmicb.2019.00647

Hatcher, E. L., Zhdanov, S. A., Bao, Y., Blinkova, O., Nawrocki, E. P., Ostapchuck, Y., et al. (2017). Virus Variation Resource–improved response to emergent viral outbreaks. *Nucleic Acids Res.* 45, D482–D490.

Hoxie, I., and Dennehy, J. J. (2020). Intragenic recombination influences rotavirus diversity and evolution. *Virus Evol.* 6, 59. doi: 10.1093/ve/vez059

Hungerford, D., Vivancos, R., Read, J. M., Pitzer, V. E., Cunliffe, N., French, N., et al. (2016). In-season and out-of-season variation of rotavirus genotype distribution and age of infection across 12 European countries before the introduction of routine vaccination, 2007/08 to 2012/13. *Euro Surveill.* 21, 310106. doi: 10.2807/1560-7917.ES.2016.21.2.30106

Ianiro, G., Micolano, R., Conte, M., Labianca, M., Vaccari, G., and Monini, M. (2019). Detection of an animal-derived G4P[6] group A rotavirus strain in a symptomatic child, in Italy. *Virus Res.* 260, 7–11. doi: 10.1016/j.virusres.2018.11.003

Kraay, A. N. M., Brouwer, A. F., Lin, N., Collender, P. A., Remais, J. V., and Eisenberg, J. N. S. (2018). Modeling environmentally mediated rotavirus transmission: The role of temperature and hydrologic factors. *PNAS* 115. doi: 10.1073/pnas.1719579115

Letunic, I., and Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. doi: 10.1093/nar/gkab301

Maringa, W. M., Mwangi, P. N., Simwaka, J., Mpabalwani, E. M., Mwenda, J. M., Peenze, I., et al. (2020). Molecular characterisation of a rare reassortant porcine-like G5P[6] rotavirus strain detected in an unvaccinated child in Kasama, Zambia. *Pathogens* 9, 1–22. doi: 10.3390/pathogens9080663

Martella, V., Bányai, K., Ciarlet, M., Iturriza-Gómara, M., Lorusso, E., De Grazia, S., et al. (2006). Relationships among porcine and human P[6] rotaviruses: Evidence that the different human P[6] lineages have originated from multiple interspecies transmission events. *Virol. J.* 344, 509–519. doi: 10.1016/j.virol.2005.08.029

Martella, V., Bányai, K., Matthijnssens, J., Buonavoglia, C., and Ciarlet, M. (2010). Zoonotic aspects of rotaviruses. *Vet. Microbiol.* 140, 246–255. doi: 10.1016/j.vetmic.2009.08.028

Martin, D. P., Murrell, B., Golden, M., Khoosal, A., and Muhire, B. (2015). RDP4: Detection and analysis of recombination patterns in virus genomes. *Virus Evol.* 1. doi: 10.1093/ve/vev003

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S. M., et al. (2008b). Full genome-based classification of rotaviruses reveals a common origin between human wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. *J. Virol.* 82, 3204–3219. doi: 10.1128/JVI.02257-07

Matthijnssens, J., Ciarlet, M., Rahman, M., Attoui, H., Bányai, K., Estes, M. K., et al. (2008a). Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Arch. Virol.* 153, 1621–1629. doi: 10.1007/s00705-008-0155-1

Matthijnssens, J., Rahman, M., Martella, V., Xuelei, Y., De Vos, S., De Leener, K., et al. (2006). Full genomic analysis of human rotavirus strain B4106 and lapine rotavirus strain 30/96 provides evidence for interspecies transmission. *J. Virol.* 80, 3801–3810. doi: 10.1128/JVI.80.8.3801-3810.2006

Maunula, L., and von Bonsdorff, C.-H. (2002). Frequent reassortments may explain the genetic heterogeneity of rotaviruses: analysis of finnish rotavirus strains. *J. Virol.* 76, 11793–11800. doi: 10.1128/JVI.76.23.11793-11800.2002

McDonald, S. M., Nelson, M. I., Turner, P. E., and Patton, J. T. (2016). Reassortment in segmented RNA viruses: Mechanisms and outcomes. *Nat. Rev. Microbiol.* 14, 448–460. doi: 10.1038/nrmicro.2016.46

Papp, H., Borzák, R., Farkas, S., Kisfali, P., Lengyel, G., Molnár, P., et al. (2013a). Zoonotic transmission of reassortant porcine G4P[6] rotaviruses in Hungarian pediatric patients identified sporadically over a 15year period. *Infect. Genet. Evol.* 19, 71–80. doi: 10.1016/j.meegid.2013.06.013

Papp, H., László, B., Jakab, F., Ganesh, B., De Grazia, S., Matthijnssens, J., et al. (2013b). Review of group A rotavirus strains reported in swine and cattle. *Vet. Microbiol.* 165, 190–199. doi: 10.1016/j.vetmic.2013.03.020

Pickett, B. E., Sadat, E. L., Zhang, Y., Noronha, J. M., Squires, R. B., Hunt, V., et al. (2011). ViPR: An open bioinformatics database and analysis resource for virology research. *Nucleic Acid Res.* 40, D593–D598. doi: 10.1093/nar/gkr859

Silva, F. D. F., Gregori, F., and McDonald, S. M. (2016). Distinguishing the genotype 1 genes and proteins of human Wa-like rotaviruses vs. porcine rotaviruses. *Infect. Genet. Evol.* 43, 6–14. doi: 10.1016/j.meegid.2016.05.014

Steyer, A., Poljšak-Prijatelj, M., Barlič-Maganja, D., and Marin, J. (2008). Human, porcine and bovine rotaviruses in Slovenia: Evidence of interspecies transmission and genome reassortment. *J. Gen. Virol.* 89, 1690–1698. doi: 10.1099/vir.0.2008/001206-0

Tacharoenmuang, R., Guntapong, R., Upachai, S., Singchai, P., Fukuda, S., Ide, T., et al. (2021). Full genome-based characterization of G4P[6] rotavirus strains from diarrheic patients in Thailand: Evidence for independent porcine-to-human interspecies transmission events. *Vir. Gen.* 57, 338–357. doi: 10.1007/s11262-021-01851-y

Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 38, 3022–3027. doi: 10.1093/molbev/msab120

Tate, J. E., Burton, A. H., Boschi-Pinto, C., Parashar, U. D., Agocs, M., Serhan, F., et al. (2016). Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013. *Clin Infect. Dis.* 62, S96–S105. doi: 10.1093/cid/civ1013

Theuns, S., Heylen, E., Zeller, M., Roukaerts, I. D. M., Desmarets, L. M. B., van Ranst, M., et al. (2015). Complete Genome Characterization of Recent and Ancient Belgian Pig Group A Rotaviruses and Assessment of Their Evolutionary Relationship with Human Rotaviruses. *J. Virol.* 89, 1043–1057. doi: 10.1128/JVI.02513-14

Troeger, C., Khalil, I. A., Rao, P. C., Cao, S., Blacker, B. F., Ahmed, T., et al. (2018). Rotavirus Vaccination and the Global Burden of Rotavirus Diarrhea among Children Younger Than 5 Years. *JAMA Pediatr.* 172, 958–965. doi: 10.1001/jamapediatrics.2018.1960

Trojnar, E., Otto, P., and Johne, R. (2009). The first complete genome sequence of a chicken group A rotavirus indicates independent evolution of mammalian and avian strains. *Virol.* 386, 325–333. doi: 10.1016/j.virol.2009.01.034

Vilibic-Cavlek, T., Barbic, L., Mrzljak, A., Brnic, D., Klobucar, A., Ilic, M., et al. (2021). Emerging and neglected viruses of zoonotic importance in Croatia. *Pathog.* 10, 1–24. doi: 10.3390/pathogens10010073

Vrdoljak, M., GuŽvinec, M., Trkulja, V., Buti,ć, I., Ivi,ć, I., KrŽelj, V., et al. (2019). Distribution of rotavirus genotypes in three Croatian regions among children ≤5 years of age (2012–2014). *Int. J. Infect. Dis.* 89, 3–9. doi: 10.1016/j.ijid.2019.09.008

Wandera, E. A., Hatazawa, R., Tsutsui, N., Kurokawa, N., Kathiiko, C., Mumo, M., et al. (2021). Genomic characterization of an African G4P[6] human rotavirus strain identified in a diarrheic child in Kenya: Evidence for porcine-to-human interspecies transmission and reassortment. *Infect. Genet. Evol.* 96, 105133. doi: 10.1016/j.meegid.2021.105133

8.3. PAPER III

KUNIĆ, V., LJ. BARBIĆ, J. ŠIMIĆ, T. MIKULETIČ, R. KOGOJ, T. KORITNIK, A. STEYER, D. KONJEVIĆ, M. BUJANIĆ, M. PRIŠLIN ŠIMAC, D. BRNIĆ (2025): Interspecies transmission and genome heterogeneity of porcine-originated Rotavirus A between domestic pigs and wildlife in the Croatian ecosystem. Sci. Total Environ. 994 (2025), 180010, 15. doi: 10.1016/j.scitotenv.2025.180010

Science of the Total Environment 994 (2025) 180010

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Interspecies transmission and genome heterogeneity of porcine-originated *Rotavirus A* between domestic pigs and wildlife in the Croatian ecosystem

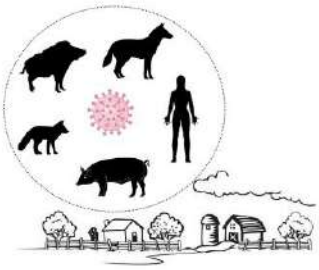
Valentina Kunić [a], Ljubo Barbić [e], Jakob Šimić [a,b], Tina Mikuletič [c], Rok Kogoj [c], Tom Koritnik [d], Andrej Steyer [d], Dean Konjević [e], Miljenko Bujanić [e], Marina Prišlin Šimac [a], Dragan Brnić [a,*]

[a] Virology Department, Croatian Veterinary Institute, Zagreb, Croatia
[b] Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia
[c] Institute for Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
[d] Public Health Microbiology Department, National Laboratory of Health, Environment, and Food, Ljubljana, Slovenia
[e] Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

HIGHLIGHTS

- Wildlife poRVAs matched domestic pig strains, confirming interspecies transmission.
- PoRVAs showed genetic heterogeneity and involvement in several recombination events.
- The first complete RVA genomes found in wild boars outside of Asia.
- The first complete RVA genome found in golden jackal, and the second in red fox.
- Findings emphasize wildlife's role in poRVA epidemiology and the need for further surveillance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Philip Hopke

Keywords:
Rotavirus A
Interspecies transmission
Molecular epidemiology
Wildlife
Domestic pig
Recombination
One Health

ABSTRACT

Rotavirus A (RVA) is a major cause of non-bacterial gastroenteritis in mammals and birds, with sporadic zoonotic events. Despite well-documented interspecies transmission of porcine-originated RVA strains (poRVAs), the role of wildlife in transmission dynamics remains underexplored. Using a One Health spatiotemporal approach, this study investigated the genetic interconnectedness of poRVAs in domestic pigs and wildlife in Croatia (2018–2021). Fecal samples or rectal swabs from 445 domestic pigs, 441 wild boars, 533 red foxes, and 131 golden jackals underwent RNA extraction, RT-PCR, VP7/VP4 genotyping, and Sanger sequencing. From these, nineteen samples carrying poRVA genotypes in wildlife and matching genotypes in domestic pigs were selected for the NGS and complete RVA genome analysis. Results revealed porcine genogroup 1 constellation, with surface protein genotypes characteristic of porcine hosts in all detected RVA strains. Wildlife-derived poRVAs proved evolutionary related to domestic pig-derived strains, verifying previously hypothesized interspecies transmission. Among VP7 genotypes, G3 dominated in wildlife, G5 in domestic pigs, and G4 showed zoonotic potential. In VP4, P[13] was the most prevalent, while P[23] and P[6] exhibited recombination events. Mixed-genotype RVAs appeared only in domestic pigs encompassing VP7, VP4, and NSP4 gene segments. This study

* Corresponding author at: Croatian Veterinary Institute, Virology Department, Savska cesta 143, Zagreb 10000, Croatia.
E-mail address: brnic@veinst.hr (D. Brnić).

https://doi.org/10.1016/j.scitotenv.2025.180010
Received 20 March 2025; Received in revised form 21 June 2025; Accepted 27 June 2025
Available online 10 July 2025

provides insights into RVA host diversity, presenting the first complete RVA genome data from golden jackals and the second from red foxes globally. In addition, it presents the first complete RVA genomes from wild boars outside of Asia to date. Wildlife-derived RVAs showed evolutionary links to domestic pig strains, including zoonotic strains, highlighting the role of wildlife in RVA dissemination. These findings emphasize the need for expanded animal RVA surveillance to better understand environmental transmission, zoonotic risks, and control strategies within a One Health framework.

1. Introduction

Rotavirus A (RVA) is the leading cause of non-bacterial gastroenteritis in mammalian and avian species (Estes and Greenberg, 2013), with an estimated 128,000 deaths annually in children under the age of five (Troeger et al., 2018). It is a multispecies virus infecting a wide range of hosts, including humans, domestic animals, and wildlife. The RVA's double-stranded RNA (dsRNA) genome consists of 11 gene segments encoding six structural (VP1–VP4, VP6, and VP7) and six non-structural (NSP1–NSP6) proteins (Crawford et al., 2017). The VP7 and VP4 surface proteins define its binomial nomenclature, designating the G (Glycosylated) and P (Protease-sensitive) genotypes, respectively (Estes and Greenberg, 2013), with 42 G and 58 P genotypes currently recognized (Rotavirus Classification Working Group, 2023). Additionally, the whole genome-based classification provides the basis for in-depth genomic analysis of the RVA genome, assigning genotypes to each gene segment based on predefined nucleotide (nt) percentage identity (pi) cutoff values (Matthijnssens et al., 2008a). Transmission primarily occurs through the fecal-oral route, though salivary and possibly respiratory routes have also been suggested (Dian et al., 2021; Ghosh et al., 2022). Infection spreads through direct contact with symptomatic or asymptomatic individuals or via contaminated objects, feed, or water. Degradation of natural habitats exacerbates multi-species pathogen transmission risks by forcing wild animals into closer proximity with humans and domestic animals, increasing opportunities for cross-contamination via shared water sources, discarded food or excrements (de Barros et al., 2018; Senica et al., 2024). RVs are highly contagious, with infected individuals shedding large quantities of virus in feces and contaminating the environment, where RVA can preserve infectivity for several hours to several months outside the host (D'Souza et al., 2008; Geletu et al., 2021). This underscores the ecological connectivity of RVA indirect transmission, where contaminated environment, possible reservoir hosts, and overlapping trophic niches perpetuate interspecies spillover risks. Environmental transmission route is especially relevant in wildland-urban interface (WUI) regions where wildlife, domestic animals and humans share habitats, facilitating interspecies and zoonotic transmission of multi-species pathogens, like RVA (Malik et al., 2020). The global incidence of zoonotic diseases is steadily increasing, with several potential risk factors identified, such as urbanization, deforestation, changes in population dynamics and human encroachment in wildlife habitats (Cunningham et al., 2017; Desvars-Larrive et al., 2024). Therefore, a collaborative One Health approach to the ecosystem as a whole is needed to address the health of humans, animals, and the environment, especially considering multi-species pathogens in shared habitats (Cunningham et al., 2017; Wegner et al., 2022). Combined with a spatiotemporal approach, it provides context for sampling all investigated species conducted within the same timeframe and geographic area (Mathian and Sanders, 2014; Gálvez et al., 2024).

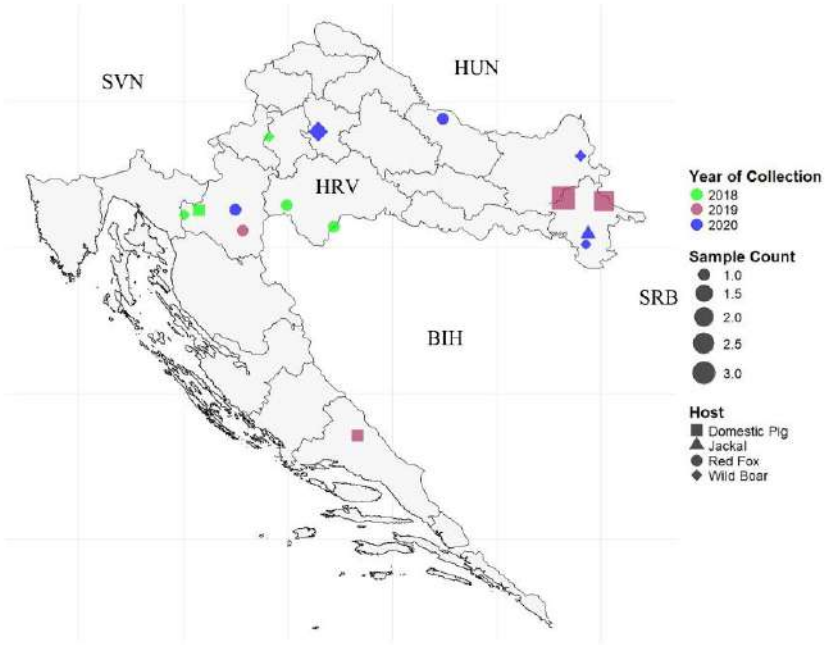
To date, research efforts have been predominantly focused on human RVAs, with domestic pig-derived RVA strains being genotyped roughly 100 times less frequently (Papp et al., 2013a). The information gap is even more pronounced when considering the limited data on genotyped RVA strains circulating in wildlife (Ghosh and Kobayashi, 2014). The importance of considering host species when evaluating disease model systems for multi-species pathogens is well-supported by One Health research, as understanding this dynamic is crucial for accurately predicting disease emergence and informing effective prevention strategies (Singh et al., 2023; Rui et al., 2024). The potential importance of wildlife-derived RVA strains may be underestimated, especially considering the frequent wildlife origin of emerging infectious diseases (Cunningham et al., 2017; Vilibic-Cavlek et al., 2021). Approximately 75 % of emerging infectious diseases in humans originate from animals, with wildlife serving as primary reservoirs for some high-impact pathogens (World Organisation for Animal Health, 2024). Therefore, One Health-based spatiotemporal approach is crucial for understanding the genetic interconnectedness of RVAs in various human and animal populations. In the swine industry, particularly in suckling and weanling pigs, RVA outbreaks can cause significant losses due to dehydration, especially in intensive farm settings (Chang et al., 2012; Palmarini, 2017). Domestic pigs exhibit remarkable genotype diversity as RVA hosts, with over 50 genotype combinations identified (Doro et al., 2015). Globally, the most common porcine RVA genotypes include G5 (46 %), G3 (11 %), and G4 (10 %) for VP7, while VP4 genotypes are dominated by P[7] (47 %), P[6] (16 %) and P[13] (3 %) (Doro et al., 2015). Conversely, far less research was conducted on RVAs in wild boars. Nonetheless, existing research endorsed the occurrence of RVA interspecies transmission between domestic pigs and wild boars, along with highlighting the close phylogenetic relationship of some RVA strains detected in humans (Okadera et al., 2013; Moutelíková et al., 2016; Brnić et al., 2022a). Wild canids hold particular interest due to their presence in semi-urban habitats, potentially posing a risk of spreading disease to human and animal populations (Zecchin et al., 2019). Despite their potential role in RVA transmission, research on wild canid-derived RVA is scarce, with only two studies focused on red foxes (Evans, 1984; Busi et al., 2017). The golden jackal has notably expanded across Europe, including Croatia and neighboring countries (Spassov and Acosta-Pankov, 2019; Krofel et al., 2023; Bijl et al., 2024), while red foxes remain widespread across the continent (Statham et al., 2018). Previous Croatian studies reported an RVA prevalence of 14.9 % in red foxes and 20.6 % in golden jackals with a remarkable RVA genetic diversity in terms of 11 G and nine P circulating genotypes, including those of typical porcine origin (Colić et al., 2021; Colić, 2021). To the best of our knowledge, studies on RVA in golden jackals have not been conducted so far, leaving a significant knowledge gap regarding the wild canids' role in RVA epidemiology. This study aimed to analyze porcine-originated complete RVA genomes from Croatian domestic pigs and wildlife utilizing a spatiotemporal One-Health approach to assess the genetic interconnectedness of porcine-originated RVAs (poRVAs) and their potential interspecies transmission within the Croatian ecosystem. Additionally, we aimed to use comprehensive complete RVA genome analysis to verify previously hypothesized interspecies transmission based on surface protein coding VP7/VP4 genes (Colić et al., 2021; Brnić et al., 2022a). The decribed complete RVA genomes derived from wildlife may contribute to narrowing the knowledge gap about wildlife influence on RVA epidemiology in the context of the One Health framework.

2. Methods

2.1. Sampling and molecular diagnostics

Nineteen complete poRVA genomes analyzed in this study were obtained from samples collected over three consecutive years (2018–2021) in Croatia, as part of a broader One Health RVA

surveillance program. In total, 445 fecal samples or rectal swabs were collected from domestic pigs (*Sus scrofa domesticus*), 441 from wild boars (*Sus scrofa*), 533 from red foxes (*Vulpes vulpes*), and 131 from golden jackals (*Canis aureus*). Each animal was sampled only once. In domestic pigs, suckling and weaning categories were sampled, each by individual rectal swabbing, while fecal samples from wild boars were sampled after regular hunting practices. Both domestic pigs and wild boars were sampled in multiple counties in Croatia, on various industrial and small backyard holdings and different hunting areas, as described by Binić et al., 2022a. Upon collection, all samples were transferred to the



	ID	Host	Month	Year	Country	Sex	Age	Diarrhea
	S55	Domestic Pig	12	2018	Croatia	F	20 days	No
	S219	Domestic Pig	11	2019	Croatia	Unknown	23 days	Yes
	S224	Domestic Pig	11	2019	Croatia	M	40 days	Yes
	S225	Domestic Pig	11	2019	Croatia	M	40 days	Yes
	S236	Domestic Pig	11	2019	Croatia	Unknown	14 days	Yes
	S244	Domestic Pig	11	2019	Croatia	M	40 days	Yes
	S280	Domestic Pig	12	2019	Croatia	M	22 days	No
	L54	Red Fox	7	2018	Croatia	Unknown	3-4 years	No
	L62	Red Fox	7	2018	Croatia	Unknown	6 months	No
	L352	Red Fox	10	2019	Croatia	M	4-5 years	No
	L465	Red Fox	10	2020	Croatia	F	Unknown	No
	L533	Red Fox	11	2020	Croatia	F	1-2 years	No
	C48	Jackal	2	2020	Croatia	F	Unknown	No
	DS76	Wild Boar	11	2018	Croatia	M	Under 1 year	No
	DS84	Wild Boar	11	2018	Croatia	F	Under 1 year	No
	DS229	Wild Boar	1	2020	Croatia	M	1-2 years	No
	DS306	Wild Boar	1	2020	Croatia	F	Under 1 year	No
	DS327	Wild Boar	6	2020	Croatia	M	1-2 years	No
	DS404	Wild Boar	12	2020	Croatia	F	Under 1 year	No

Fig. 1. Map of Croatia illustrating the spatiotemporal distribution of sampled animals. Different shapes represent animal species: squares for domestic pigs, diamond shape for wild boars, circle for red foxes, and a triangle for a jackal. The color coding indicates the sampling year, with green representing 2018, pink representing 2019, and blue representing 2020 (legend shown). Sample count per location spot is indicated in a shown legend. Below the map, Table 1 with sample information is provided. The image was generated with RStudio (2024.12.1). The map was acquired from the GADM repository (https://gadm.org/, accessed on February 18th, 2025).

Croatian Veterinary Institute for further molecular diagnostics. Fecal samples from wild canids were collected from red foxes and golden jackals hunted in the scope of the active surveillance of the indirect anti-rabies oral vaccination campaign, organized by the Veterinary and Food Safety Directorate of the Croatian Ministry of Agriculture. Fecal samples were collected directly from the rectum of wild canid carcasses received at the Croatian Veterinary Institute. In contrast to domestic pigs in which younger categories were sampled, all wildlife (wild boars, red foxes, and golden jackals) fecal samples were collected upholding hunting regulations, i.e., mostly adults were sampled.

All samples underwent initial sample processing, including nucleic acid extraction, RVA VP2 real-time RT-PCR, VP7 and VP4 genotyping, Sanger sequencing and sequence analysis, performed as previously described (Colić, 2021; Colić et al., 2021; Brnić et al., 2022a). The RVA prevalence rates in investigated species were based on results obtained by the VP2 real-time RT-PCR protocol (Colić et al., 2021; Brnić et al., 2022a). The RVA prevalence was 49.9 % in domestic pigs, 9.3 % in wild boars (Brnić et al., 2022a), 15 % in red foxes (previously published prevalence was 14.9 % on 370 samples (Colić et al., 2021)) and 36.6 % in golden jackals (previously published prevalence was 20.6 % on 34 samples (Colic, 2021)). During the genotyping process, VP7/VP4 RVA genotypes of typical porcine origin were detected in multiple species, leading to the presumed sporadic interspecies transmission of poRVAs in Croatia. Since in previous research a zoonotic spillover of poRVAs in Croatia was already detected (Kunić et al., 2023), we further investigated these strains to expand on the One Health perspective of poRVA interspecies transmission in the Croatian ecosystem. Therefore, samples from multiple wildlife species in which poRVAs were detected (wild boars, red foxes, and golden jackals), along with strains from domestic pigs with matching genotypes were selected for next-generation sequencing (NGS). Considering additional excluding practical criteria (e.g. quantity of collected samples), a total of 19 samples matching these criteria were selected for NGS (Fig. 1).

2.2. Library preparation and NGS

For NGS sample preparation, fecal and swab suspensions (20 % *w/v*) prepared as described earlier served as the starting material. The nucleic acid extraction, DNA depletion, cDNA synthesis, purification, and quantification steps, as well as NGS library preparation, were conducted using previously described protocols (Kunić et al., 2023). In brief, key steps included nucleic acid extraction using the Maelstrom 9600 device with OptiPure Viral Auto Plates (TANBead Inc., Taoyuan City, Taiwan), while cDNA synthesis was conducted with the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific, Waltham, USA). Libraries were constructed using the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, USA), barcoded with IDT for Illumina Nextera DNA/RNA Unique Dual Indexes (Illumina Inc., San Diego, USA), and purified utilizing Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, USA). Library quality and quantity were assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, USA) and a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, USA). Sequencing was performed on the Illumina NextSeq 500 system with a NextSeq 500/550 High Output Kit v2.5 (Illumina Inc., San Diego, USA) on 300 cycles, to generate 150 paired-end reads.

2.3. NGS data analysis

NGS data analysis was performed using CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany). Representative sequences for each of the 11 RVA genomic segments, covering various genotypes, were selected from NCBI's Virus Variation Rotavirus Database (Hatcher et al., 2017) to build reference lists for each gene segment. Coding sequences (CDS) were assembled using a reference-based mapping process for each segment, reflecting the segmented nature of the rotavirus genome. The workflow consisted of trimming raw reads of Illumina adapters, mapping trimmed reads to the segments reference lists, and extracting consensus sequences and mapping reports. Consensus sequences were not considered for further investigation if they did not meet the previously defined minimum sequence length and identity criteria (Matthijnssens et al., 2008a) or distribution coverage of 90 % and coverage depth of 10×. All RVA genotypes were confirmed using final consensus sequences as queries in both the BLASTn search tool and the ViPR tool version 3.28.224 (Pickett et al., 2011), following previously defined genotype cutoff values (Matthijnssens et al., 2008a). During genotyping, any consensus sequence that mismatched the initially mapped genotype was discarded as a mapping error. Obtained nt and amino acid (aa) sequences of acquired CDS for each RVA gene, were submitted to the GenBank with accession numbers listed in Supplementary Table 1.

2.4. Phylogenetic analysis, pairwise comparison, and Simplot analysis

To explore the evolutionary relationship between domestic pig- and wildlife-derived porcine-like RVA strains, we constructed individual phylogenetic trees for each of the 11 RVA genomic segments. Representative strains from the GenBank were selected for comparison with herein presented RVAs based on their high pi with our query sequences, their geolocation, or host origin. The evolutionary history was inferred using the maximum-likelihood (ML) method for each multiple sequence alignment generated with the MUSCLE algorithm (using default settings), both performed in MEGA 11 software (Tamura et al., 2021). The substitution models yielding the lowest Bayesian Information Criterion (BIC) scores were T92 + G + I (VP7, VP6, NSP2, NSP3, NSP5), GTR + G + I (VP4, VP2, VP3, NSP1), TN93 + G + I (VP1), and T92 + G (NSP4), respectively. Bootstrap analysis with 1000 replicates was used to assess branching support for each ML tree. Phylogenetic trees were graphically edited and annotated using iTOL version 7 (Letunic and Bork, 2021). Pairwise nt and aa identity matrices between GenBank and study RVA sequences, using the same datasets as for phylogenetic analysis, were calculated with CLC Genomics Workbench 22.0.2 and are presented in Supplementary Table 2. Additionally, for the samples in which whole RVA genome completeness was acquired (S219, S224, S225, S236, L465, C48, DS76, DS84, DS229, DS327), we did a whole genome concatenation of each gene segment open reading frames (ORFs) in CLC Genomics Workbench 22.0.2. Among those, where a mixed genotype was present (Table 2), a dominant genotype with the most mapped reads per respective gene segment was selected for concatenation. Finally, concatenated ORFs were aligned as described earlier and uploaded to Simplot++ software for a Simplot analysis (Samson et al., 2022).

2.5. Intragenic recombination and reassortment analysis

Intragenic recombination analysis was performed on all taxa used in the phylogenetic analysis (Fig. 3, Fig. 5, Fig. 7) for each RVA gene segment. Multiple sequence alignment sets were constructed as described earlier. Recombination analysis, including both intragenotype and intergenotype (for genes with apparent mixed genotypes, Table 2), was conducted using RDP v.5.64 software. Seven integrated recombination detection methods were applied: RDP, GENECONV, MaxChi, Bootscan, Chimera, SiScan, and 3Seq (Martin et al., 2015). For each detected recombination event, the RDP-integrated UPGMA method constructed breakpoint-defined phylogenetic trees for major and minor parent strains (data not shown). Herein, "parent" does not indicate the exact evolutionary progenitors of recombinant strains but rather represents groups of RVAs from which the progenitors may have originated. Only recombination events predicted by at least six methods were considered positive signals of homologous recombination (Hoxie and Dennehy, 2020). Since ancestral state reconstruction was not conducted, sequences with detected recombination were retained in the phylogenetic analysis without removing the recombinant parts to illustrate the phylogenetic effects of recombination-induced genotype divergence.

Table 2
Whole genome constellations of Croatian typical porcine and porcine-like RVA strains.[1]

Host	Sample ID	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
	**S55**	G5	P[13]	I5	R1	C1	M1	A8	N1	T1	E1	H1
	S219	G3/G5/G9	P[13]/P[23]	I5	R1	C1	M1	A8	N1	T1	E1/E9	H1
	S224	G5/G9	P[13]/P[23]	I5	R1	C1	M1	A8	N1	T1	E9	H1
	S225	G4/G9	P[6]/P[23]	I5	R1	C1	M1	A8	N1	T1	E9	H1
	S236	G5	P[13]	I5	R1	C1	M1	A8	N1	T1	E1	H1
	S244	G11	P[13]	I5	R1	C1	M1	A8	N1	T7	E1	H1
	S280	G5	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1
	**L54**	G11	P[13]	I5	R1	C1	M1	A1	N1	T7	E1	H1
	L62	G3	P[13]	I5	R1	C1	M1	A8	N1	T1	EX	HX
	L352	G4	P[13]	I5	R1	C1	M1	A1	N1	T1	E1	H1
	L465	G3	P[13]	I5	R1	C1	M1	A8	N1	T7	E1	H1
	L533	G5	P[13]	I5	R1	C1	M1	AX	NX	T7	EX	H1
	**C48**	G3	P[6]	I5	R1	C1	M1	A8	N1	T1	E1	H1
	**DS76**	G3	P[13]	I5	R1	C1	M1	A8	N1	T1	E1	H1
	DS84	G9	P[13]	I5	R1	C1	M1	A8	N1	T1	E1	H1
	DS229	G3	P[13]	I5	R1	C1	M1	A8	N1	T7	E1	H1
	DS306	G3	P[13]	I5	R1	C1	M1	A8	N1	T7	EX	H1
	DS327	G11	P[13]	I5	R1	C1	M1	A8	N1	T1	E1	H1
	DS404	G5	P[13]	I5	R1	C1	M1	A8	N1	T7	EX	H1

[1] Gene segments that did not meet the minimum criteria for genotype assignment outlined by Matthijnssens et al., 2008a, remained undetermined and are shown as "X".

Detection of reassortment events was considered during the phylogenetic analysis approach, along with the nt and aa pi calculation. In addition, in the samples in which whole RVA genome completeness was acquired (S219, S224, S225, S236, L465, C48, DS76, DS84, DS229, DS327) a complete genome concatenation and multiple sequence alignment were conducted as described earlier. Therein, concatenated ORFs were uploaded to Simplot++ software (Samson et al., 2022) for a bootscan analysis. Selected bootscan parameters included window size of 200 bp, step size of 200 bp, 500 repetitions, Kimura 2 Parameter distance model, and % of permuted trees calculated utilizing the Neighbor-Joining algorithm.

3. Results

3.1. NGS results and RVA whole-genome constellation

The number and the average length of reads yielded by Illumina NextSeq 500 platform for presented RVA strains (Table 2) ranged from 3.5×10^6–4.0×10^7 and ~90–118 bp, respectively. Complete CDS for all 11 gene segments were successfully determined in 10 sequenced porcine-originated RVA strains (S219, S224, S225, S236, L465, C48, DS76, DS84, DS229, DS327), and a Simplot analysis of their concatenated ORFs is shown in Fig. 2. For the rest, partial CDS was acquired in at least one gene segment. Gene segments that did not meet the minimum criteria for genotype assignment outlined by Matthijnssens et al., 2008a, remained undetermined and are shown as "X" (Table 2) (Matthijnssens et al., 2008a). Data of all the obtained nt and aa RVA CDS sequences of determined genotypes (Table 2) are submitted in the GenBank with their accession numbers listed in Supplementary Table 1. Moreover, mixed genotypes were detected in three domestic pig-derived RVA strains (S219, S224, S225), including VP7, VP4, and NSP4 gene segments, while no mixed genotypes occurred in wildlife-derived RVAs (Table 2). As for reassortment analysis, no unequivocal reassortment events were detected since each discovered segment was conclusive with RVA genogroup 1 constellation and porcine origin.

All RVA strains exhibited an RVA genogroup 1 constellation, paired with typically porcine I5, A8, T7, and E9 genotypes in the backbone VP6, NSP1, NSP3, and NSP4 segments, respectively. Additionally, typically porcine VP7 and VP4 genotypes were detected across all investigated species. Phylogenetic analysis for each gene segment is comprised in Figs. 3, 5, and 7, while nt and aa pairwise identity matrices are comprised in Supplementary Table 2, both containing complete strain name and accession numbers for each strain used in the analysis. Additional information about the sample pool investigated to acquire RVA genomes described in Table 2 were summarized in Supplementary Table 3. Importantly, the complete poRVA genome analysis provided conclusive evidence supporting the previously proposed interspecies transmission events based on VP7 and VP4 gene data (Colić et al., 2021; Brnić et al., 2022a).

3.2. Phylogeny and pairwise identity analysis

3.2.1. Phylogenetic and pairwise identity analysis of surface protein coding RVA genes

In the VP7 gene segment, RVA strains from this study were detected in domestic pigs, wild boars, red foxes, and a golden jackal presented with G5, G11, G3, G9, and G4 genotypes (Fig. 3A). In the given dataset, Croatian G5 RVAs branched out in two separate branches, one where two strains formed a clade with Czechian wild boar G5 strain, also the closest in nt similarity with S219 and S224, 90.8 % and 91.3 %, respectively. In addition to domestic pig-derived strains published in our previous study, another G5 branch included a newly identified fox-derived G5 L533 strain which shared the highest nt similarity of 98.78 % with the domestic pig-derived S338 strain and 93.48 % with the Swiss SS3 strain, further supporting a porcine origin.

In contrast to the G5 genotype, herein presented wildlife-derived G11 RVAs displayed more heterogeneous and divergent from domestic pig-derived G11 strains. For example, the Canadian G11 derived from a domestic pig was the most similar strain to the fox-derived L54 G11, sharing a 93.58 % nucleotide similarity (Fig. 3A). On the other hand, the closest Croatian RVA to fox-derived L54 was domestic pig-derived S244 with considerably less nt similarity of 88.69 %. Furthermore, the wild boar-derived DS327 strain sequestered in a separate branch with European pig and wild boar-derived RVAs, distanced from the other Croatian RVAs and displayed the highest similarity to Slovakian pig-derived G11 strain (91.03 %). On the other hand, domestic pig-derived G11 S244 strain claded with previously reported domestic pig-derived S243 strain, as they branched out separately from wildlife-derived G11 strains.

Within the given dataset, the G3 genotype (Fig. 3A) stood out as the most frequently detected VP7 genotype in wildlife hosts. Croatian pig and wildlife-derived G3 strains branched out together, except for the wild boar-derived DS306 strain, which was the closest to the Japanese wild boar-derived G3 strain with 92.86 % nt similarity. The mentioned cluster of Croatian G3 RVAs included two red fox-derived strains (L62,

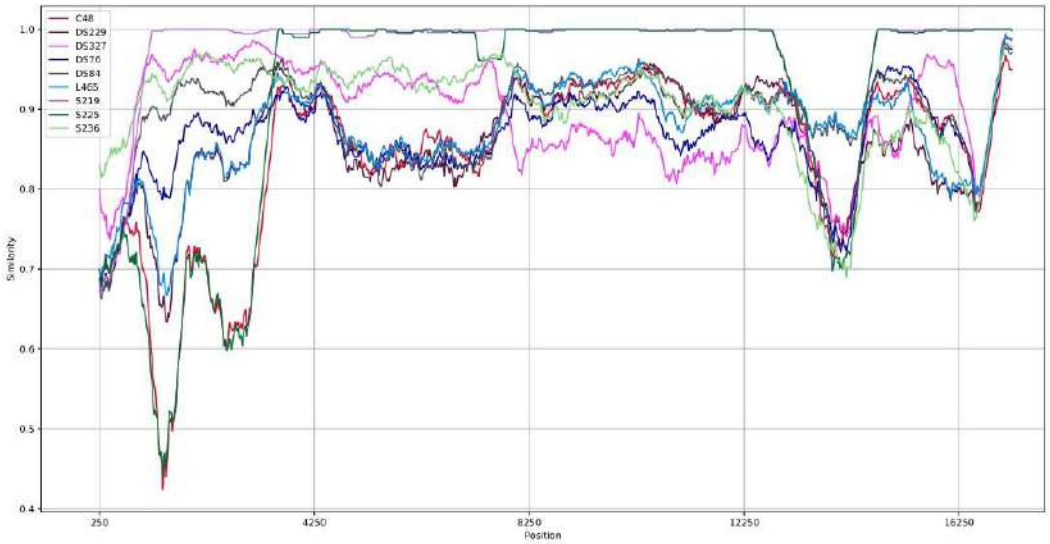


Fig. 2. SimPlot analysis comparing the concatenated ORFs of the domestic pig-derived S224 RVA genome with other complete RVA genomes in the present study (S219, S225, S236, L465, C48, DS76, DS84, DS229 and DS327). For RVA strains with mixed genotypes (Table 2), the dominant genotype is presented, defined as the one with the highest number of mapped reads per respective gene segment. Analysis was carried out using the Kimura 2 parameter, with a window size of 200 bp and a step size of 50 bp.

L465), one golden jackal (C48), two wild boar (DS76, DS229), and one domestic pig-derived G3 strain (S219) (Fig. 3A). The nt similarity for these RVA strains was high and ranged between 95.62 % and 97.86 %, with the closest match being the Swiss domestic pig-derived G3 strain (S18) (94.6 %). In the VP7 gene segment, this is the third example of Swiss porcine RVAs being the closest phylogenetic relatives to Croatian poRVAs.

The G9 genotype occurred in three domestic pig-derived RVAs that branched out together (Fig. 3A). Interestingly, G9 was not present as a single VP7 genotype in Croatian domestic pig-derived RVAs, only in mixed genotype constellations (Table 2). Furthermore, G9 occurred in a wild boar-derived DS84 strain while branching very distantly from mentioned strains, and most similar (95.11 %) to mixed genotype Swiss domestic pig-derived SS3 strain, already mentioned for G5 genotype in connection to Croatian fox-derived L533 RVA strain. The G9 also showed less geographic divergence since European porcine RVAs proved mutually similar, but Chinese and North American RVAs branched out separately, hence showing a different dynamic than G11 (Fig. 3A).

The G4 genotype proved heterogeneous and putatively with the highest zoonotic potential of all typical porcine genotypes. Three human-derived G4 strains, reported as zoonotic porcine-originated strains in our previous study (Kunić et al., 2023), have herein clustered with fox and domestic pig-derived G4 strains from the present study (Fig. 3A). The fox-derived L352 G4 strain exhibited the highest nt similarity to the zoonotic, human-derived G4 strains D329 (95.82 %) and D572 (95.3 %) from our previous study, suggesting a closer relationship to human than to domestic pig-derived G4 strains. Although both the Croatian fox- and human-derived G4 RVAs are clearly of porcine origin, this raises questions about the primary source of infection. The remaining domestic pig-derived G4 strain S225 clustered with other Croatian domestic pig-derived, and Hungarian zoonotic human-derived G4 strains (Fig. 3A).

In the VP4 gene segment, RVA strains from this study were characterized with P[13], P[23] and P[6] genotypes. Porcine-originated P[13] genotype proved the most frequent among animal hosts in the Croatian ecosystem with five strains identified in domestic pigs, five in foxes, and six in wild boars (Table 2). The VP4 phylogram (Fig. 3B) formed two main branches, each with two sub-branches, with RVA strains from the present study exhibiting nt pairwise identities ranging from 82.01 % to 99.44 %. All five fox-derived P[13] strains (L62, L54, L352, L533, and L465) clustered in multiple clades either with domestic pig- or wild boar-derived P[13] RVAs, underlining potential interspecies transmission between porcine and wild canid hosts. Wild boar-derived DS306 strain branched out separately from other Croatian RVAs displaying significant divergence, sharing only 86.48 % nt pairwise identity with fox-derived L533 strain, as the closest reported evolutionary relative. Furthermore, wild boar-derived DS229 P[13] strain presented as an intragenotype recombinant (Fig. 3B, Fig. 4A).

Conversely to P[13], the P[23] genotype was not detected in wildlife hosts, only in domestic pigs, and displayed less intragenotype heterogeneity than P[13], attributing the nt similarity range from 88.05 % to 98.46 % between RVAs from the present study. However, two intergenotype recombination events were detected in P[23], the first intergenotype recombinant being domestic pig-derived P[23] S224 strain (Fig. 3B, Fig. 4B), which consequently resulted in its recombination-induced phylogenetic separation from the other P[23] RVAs, sharing only 91.22 nt similarity with S225 P23 as the closest evolutionary relative (Fig. 3B). Secondly, the S225 P[23] strain (Fig. 4C) presented as an intergenotype recombinant.

The P[6] genotype, much like the G4 in the VP7 segment, displayed the greatest zoonotic potential in the given dataset. Domestic pig-derived S225 strain branched out individually, sharing the closest nt similarity of 91.71 % with the human-derived zoonotic D230 P[6] strain characterized in our previous study (Kunić et al., 2023) (Fig. 3B). Moreover, another zoonotic P[6] strain (D329) from the said study, clustered with the jackal-derived C48 P[6] strain, sharing a high nt similarity of 96.82 %.

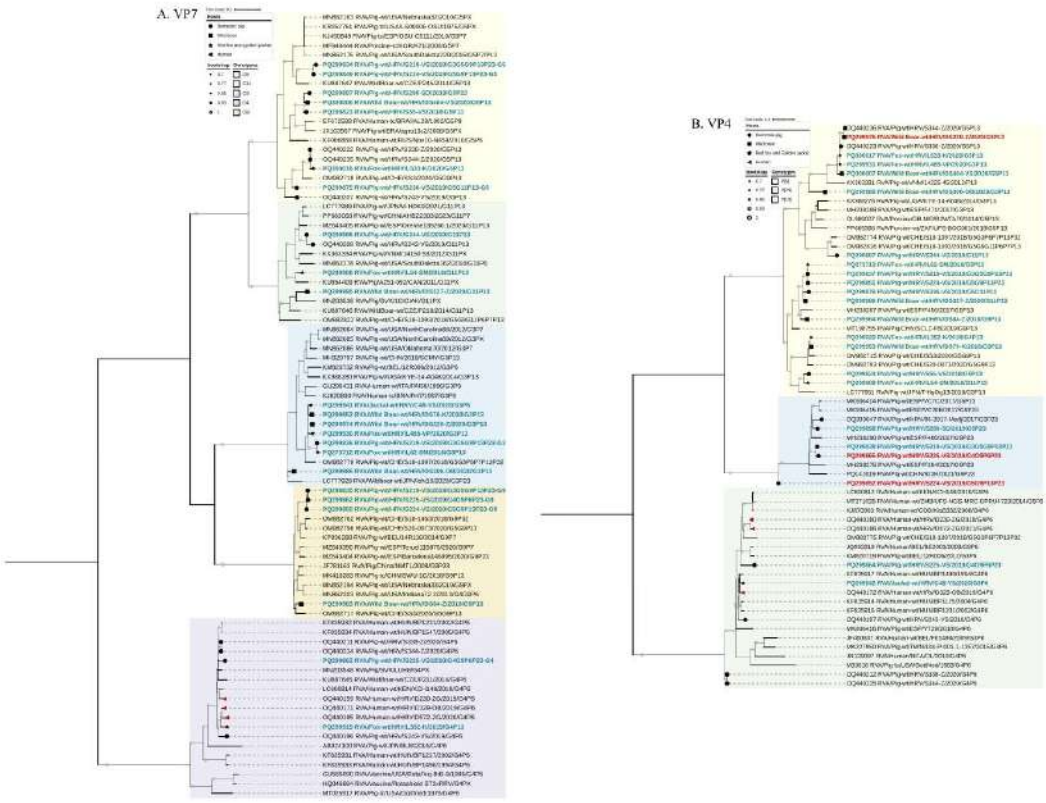


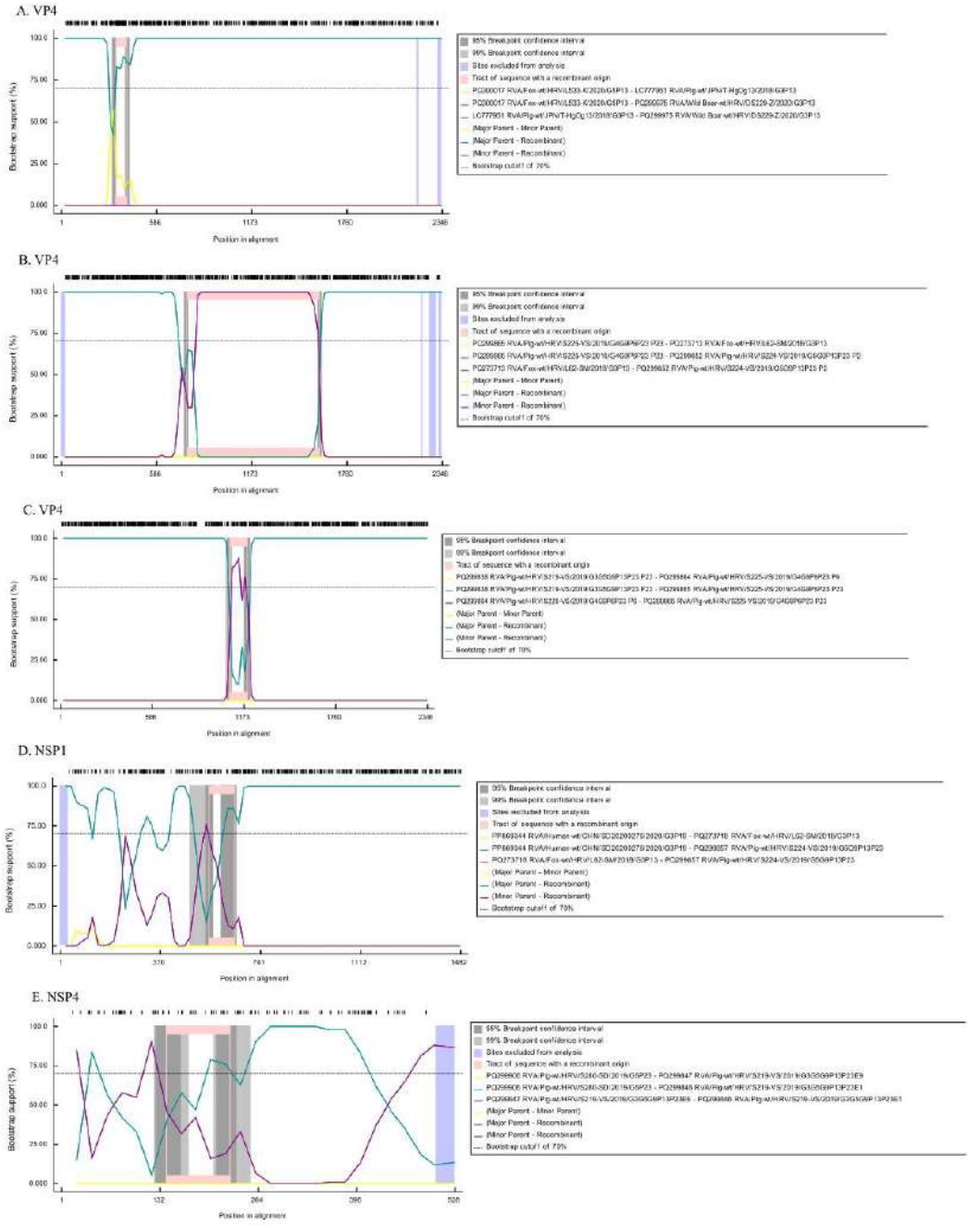
Fig. 3. Phylogenetic trees of the surface protein-coding VP7 (A) and VP4 (B) nucleotide sequences. Hosts, genotypes, and bootstrap values are indicated in the legend. Host symbols are displayed only adjacent to the RVA strains reported in this study and previously reported Croatian RVA strains. Strains from the present study (Table 2) are highlighted in bold and labeled in blue, while recombinant strains are highlighted in bold and labeled in red. Accession numbers for all RVA strains are included in the taxa labels. The phylogenetic trees were constructed using the Maximum Likelihood (ML) method in MEGA 11 software. The nucleotide substitution models with the lowest BIC scores used for VP7 and VP4 were T92 + G + I and GTR + G + I, respectively. Branching support was assessed through bootstrap analysis with 1000 replicates, and only bootstrap values >0.7 are displayed. The scale bar represents the number of substitutions per site.

3.2.2. Phylogenetic and pairwise identity analysis of backbone VP RVA genes

The RVA strains from the present study exhibited a wide nt similarity range across the backbone VP segments, demonstrating both conservation and divergence. The nt similarity across VP1, VP2, VP3, and VP6 segments ranged as follows: VP1 (85.5 % to 99.9 %), VP2 (86.26 % to 99.93 %), VP3 (86.28 % to 99.96 %), and VP6 (91.12 % to 99.5 %). Despite the variations in genetic similarity, all segments consistently affirmed a predominant porcine origin, with strains clustering alongside domestic pig-derived and zoonotic human-derived RVA strains of porcine origin, further emphasizing interspecies transmission and zoonotic potential of poRVAs.

In the VP1 segment, RVA strains from the present study were classified as the R1 genotype with notable genetic heterogeneity highlighted by the phylogenetic clustering of RVA strains into four main branches (Fig. 5A). However, all strains were confirmed to have a porcine origin, as they grouped with domestic pig strains or zoonotic human-derived RVA strains of porcine origin. A certain level of divergence was observed in three clades, where wildlife-derived RVA strains branched out separately from domestic pig-derived strains.

In the VP2 segment, RVA strains from the present study presented as the C1 genotype. The VP2 phylogram displayed two main branches, with the majority of herein presented RVAs showing little phylogenetic divergence from one another, as domestic pig-derived and wildlife-derived strains generally clustered together. Some wildlife-derived strains (L54, DS84, DS327) clustered in proximity to zoonotic human-derived RVA strains of porcine origin (Fig. 5B). In a second main branch, two wildlife-derived strains (L352, DS306) displayed more divergence, as they separated from the rest of the Croatian strains while clustering with porcine strains from Russia and China making their evolutionary ancestral origin uncertain. Despite this phylogenetic closeness among the majority of Croatian C1 strains, certain genetic diversification was evident through insertions observed in 10 sequences from the present study. Insertions were observed in C1 sequences derived from three domestic pigs (S225, S236, S244), one red fox (L352), and six wild boars (DS76, DS84, DS229, DS306, DS327, DS404). These insertions occurred at amino acid positions 37–41 of the VP2 segment and the graphical representation of their respective nt and aa sequences can be found in Fig. 6. Listed insertions were sequenced with the high coverage for each nt position. No connections were observed



(caption on next page)

Fig. 4. Recombination analysis. Recombination analysis was performed on taxa identified in the phylogenetic analysis (Fig. 3, Fig. 7) for each RVA gene segment using RDP v.5.64 software. Seven integrated recombination detection methods were applied, including RDP, GENECONV, MaxChi, Bootscan, Chimera, SiScan, and 3Seq. Bootscan analysis results for each recombinant strain are shown in Figs. A–E: A. Wild boar-derived DS229 P[13] strain: Intragenotype recombinant detected by six out of seven RDP5 detection methods. B. Domestic pig-derived S224 P[23] strain: Intergenotype recombinant detected by all seven RDP5 detection methods. C. Domestic pig-derived S225 P[23] strain: Intergenotype recombinant detected by all seven RDP5 detection methods. D. Domestic pig-derived S224 A8 strain: Intragenotype recombinant detected by all seven RDP5 detection methods. E. Domestic pig-derived S219 E1 strain: Intergenotype recombinant detected by five out of seven RDP5 detection methods.

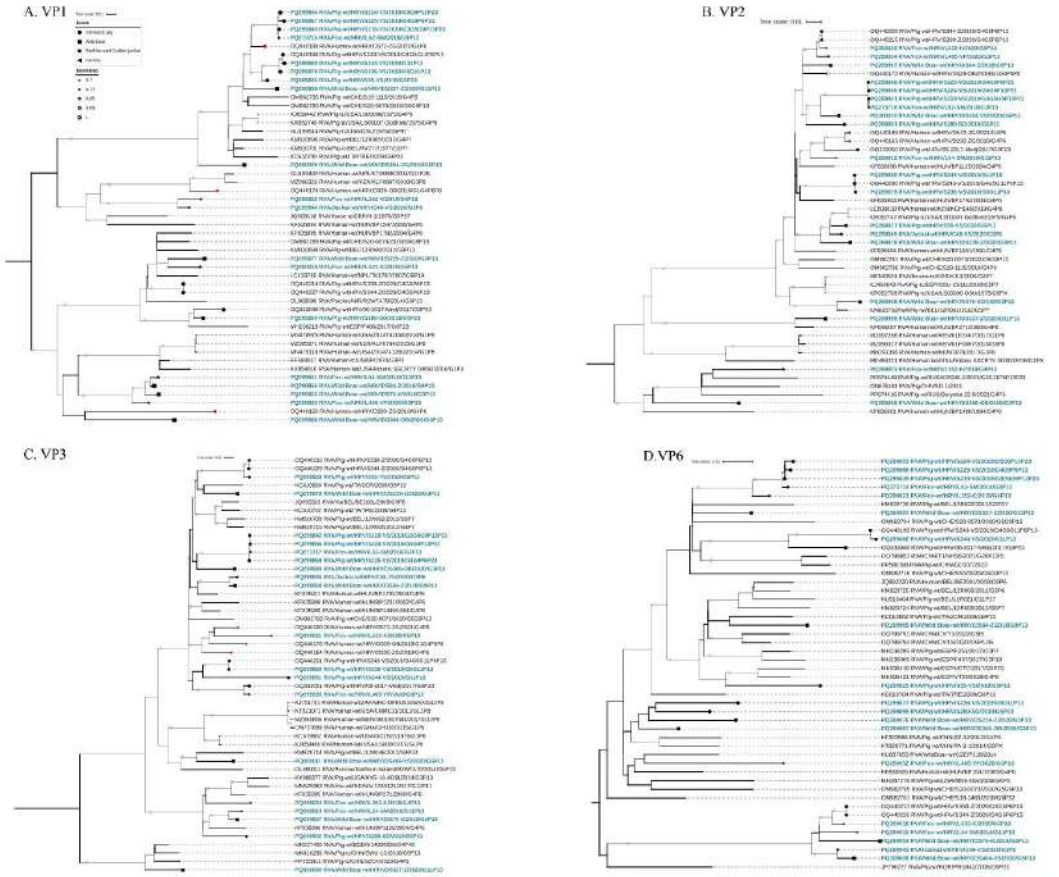


Fig. 5. Phylogenetic trees of the VP1 (A), VP2 (B), VP3 (C) and VP6 (D) nucleotide sequences. All taxons presented in forementioned gene segments share one genotype per gene, as follows: R1, C1, M1 and I5. Hosts and bootstrap values are indicated in the legend. Host symbols are displayed adjacent to only the RVA strains from the present study and previously reported Croatian RVAs. RVAs from the present study (Table 2) are highlighted in bold and labeled in blue. Accession numbers for all RVA strains are included in the taxa labels. The phylogenetic trees were constructed using the Maximum Likelihood (ML) method in MEGA 11 software. The nucleotide substitution models with the lowest BIC scores were TN93 + G + I (VP1), T92 + G + I (VP2, VP6) and GTR + G + I (VP3). Branching support was assessed through bootstrap analysis with 1000 replicates, and only bootstrap values >0.7 are displayed. The scale bar represents the number of substitutions per site.

considering the spatiotemporal distribution of the strains containing these insertions within the VP2 sequences, as these samples corresponded with different sampling locations, years, and host species (Fig. 1).

In the VP3 segment, RVA strains from the present study shared a porcine or porcine-like originated M1 genotype. Initially, M1 strains formed two main branches (Fig. 5C). Firstly, the heterogeneous branch with diversified clustering pattern, and secondly, the branch where Croatian wild boar-derived DS327 clustered with three domestic pig-derived strains from East Asia, sharing >93 % nt similarity. The DS327 evolutionary ancestral origin remains uncertain considering the current lack of known phylogenetically and geographically close evolutionary relatives. Two wild canid-derived strains (L352, L533) exhibited a close phylogenetic relationship with zoonotic human-derived strains of porcine origin from Hungary and Croatia (Fig. 5C).

In the VP6 gene segment, RVA strains from the present study

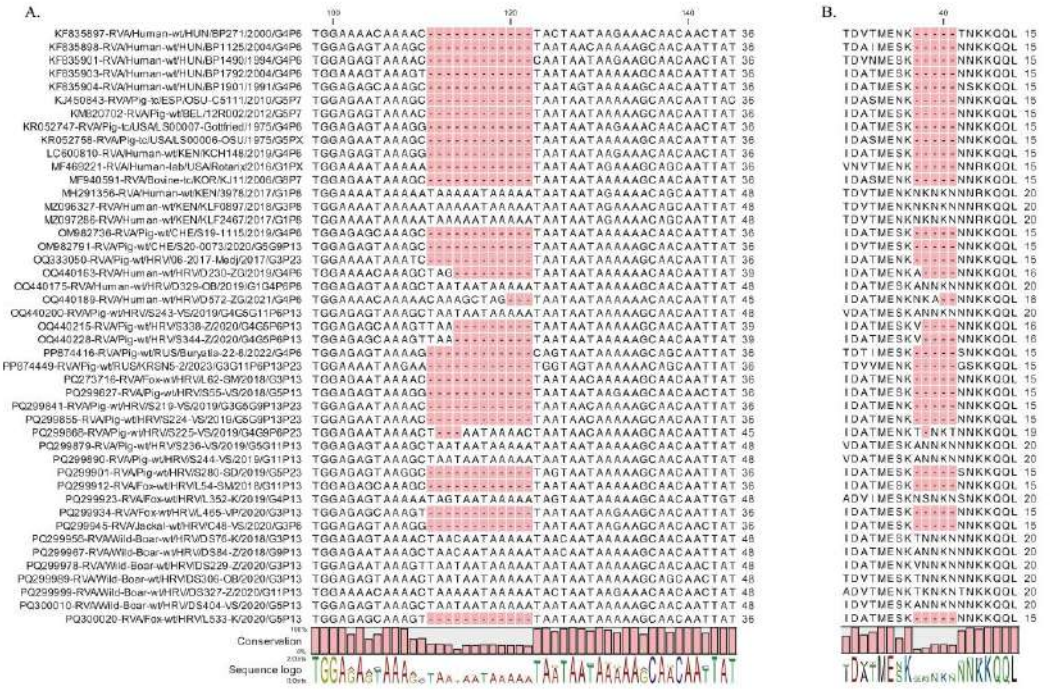


Fig. 6. Graphical representation of insertions in nucleotide (A) and protein (B) multiple sequence alignments of the VP2 segment. Insertions occurred at amino acid positions 37–41. The multiple sequence alignment was generated using the MUSCLE algorithm with default settings in MEGA 11 software, and the graphic representation was produced using CLC Genomics Workbench 22.0.2.

exhibited a typical porcine I5 genotype (Fig. 5D). With an intragenotype nt similarity in the range of 91.12 % to 99.5 %, it stood out as the most homogenous backbone VP genotype. Croatian I5 domestic pig- and wild boar-derived RVAs clustered among themselves, or with Italian, Spanish, and Swiss I5 domestic pig-derived RVAs. One cluster comprised solely of Croatian I5 sequences phylogenetically sequestered in a separate branch with no foreign RVAs, suggesting a more local evolutionary pathway.

3.2.3. *Phylogenetic and pairwise identity analysis of backbone NSP RVA genes*

Backbone NSP segments exhibited a wide nt similarity range, comprising of both heterogeneous and homogenous genotypes. Despite variations in genetic similarity, all segments indicated a porcine origin of wildlife RVA strains, predominantly clustering with domestic pig-derived and zoonotic human-derived RVA strains of porcine origin.

In the NSP1 segment, RVAs from the present study were presented as A1 and A8 genotypes (Fig. 7A). The typical porcine A8 genotype was the most common, exempting only two fox-derived A1 strains. The A8 RVAs from the present study exhibited a broad nt similarity range of 81.04–99.53 %, dispersing in ramified branching pattern with strong bootstrap support (Fig. 7A), making the A8 the most diverse genotype of this study. Furthermore, A8 sequestered in two main branches, possibly standing for two genotype lineages, considering the highest nt similarity between the respective branches is 85.09 %, between domestic pig-derived strains S55 and S224. The respective lowest value is 78.88 % between human-derived A8 reassortant D572 and South African domestic pig-derived MRC-DPRU1557 A8 strain and between D572 and Swiss domestic pig-derived S20 strain. The said lowest value falls slightly beneath the genotype cutoff value of 79 % (Matthijnssens et al., 2008a), accentuating the significant genetic heterogeneity of this genotype. In both branches (Fig. 7A), wildlife-derived A8 RVAs from this study clustered with domestic pig-derived strains, further underscoring their porcine origin in all investigated species. The domestic pig-derived S224 strain phylogenetically separated from Croatian strains and clustered with two Chinese strains, making its evolutionary ancestral origin uncertain, likely due to its identification as an intragenotype recombinant (Fig. 4D). In the A1 genotype, two fox-derived strains (L54, L352) clustered with previously reported Croatian domestic pig-derived strains S338, and S344 with the strong branching support (Fig. 7A). The nt similarity in the said cluster ranged from 95.14 % to 98.79 %, affirming their porcine origin.

In the NSP2 segment, RVA strains from the present study were genotyped as N1 genotype with an intragenotype nt similarity range of 86.58 % to 99.9 %, instructing a similar heterogeneity pattern as the majority of the backbone gene segments. The N1 clustered into three main branches. In each, most RVA strains from the present study, domestic pig- and wildlife-derived alike, clustered together or with previously reported European poRVAs, underscoring interspecies transmission (Fig. 7B). However, zoonotic potential was also evident, since wildlife-derived strains (L54, L465, DS327, DS306, DS404) clustered with zoonotic human-derived RVAs of porcine origin from Russia, Kenya, and Croatia.

In the NSP3 segment, RVA strains from the present study divided into the T1 and T7 genotypes. For the T1, the intragenotype nt similarity varied 88–100 %, and 91.4–96.71 % for the T7 genotype. Most of the T1

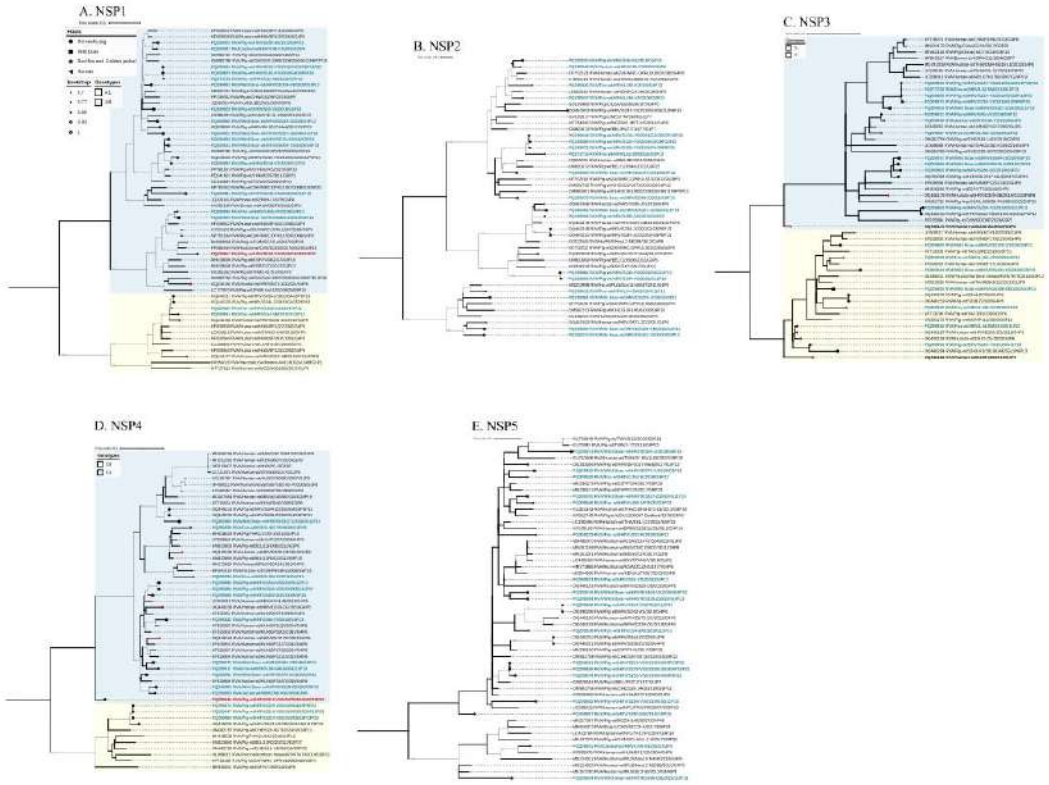


Fig. 7. Phylogenetic trees of the NSP1 (A), NSP2 (B), NSP3 (C), NSP4 (D) and NSP5 (E) RVA genomic segments based on nucleotide sequences. Hosts and bootstrap values are indicated in the legend. Host symbols are displayed adjacent to both the RVA strains from the present study and previously reported Croatian RVAs. RVAs from the present study (Table 2) are highlighted in bold and labeled in blue, while recombinant strains from the present study are highlighted in bold and labeled in red. In NSP3, recombinant strains from the previous study with major parent strains from the present study are highlighted in bold and labeled in black. Accession numbers for all RVA strains are included in the taxa labels. The phylogenetic trees were constructed using the Maximum Likelihood (ML) method in MEGA 11 software. The nucleotide substitution models with the lowest BIC scores were as follows: T92 + G + I (NSP2, NSP3, NSP5), GTR + G + I (NSP1) and T92 + G (NSP4). Branching support was assessed through bootstrap analysis with 1000 replicates, and only bootstrap values >0.7 are displayed. The scale bar represents the number of substitutions per site.

RVAs from the present study clustered together, reinforcing the porcine origin of wildlife-derived Croatian strains. Conversely, in the T7 genotype, herein reported RVAs followed a more phylogenetically dispersed pattern (Fig. 7C). Nevertheless, both T1 and T7 wild canid-derived strains demonstrated putatively increased zoonotic potential, evident in phylogenetic (Fig. 7C) and recombination analysis (Supplementary Fig. 1) alike.

Within the NSP4 segment, the majority of the RVA strains from the present study were characterized as the E1 genotype, including all wildlife-derived strains, while only three strains derived from domestic pigs were genotyped as E9. The E1 exhibited an intragenotype nt similarity of 87.12–99.24 %, with Croatian domestic pig- and wildlife-derived strains clustering together, indicating a porcine origin. Interestingly, domestic pig-derived S219 E1 phylogenetically branched out between the E1 and E9 genotypes indicating a recombination event (Fig. 7D, Fig. 4E). The typically porcine E9 genotype was present in three herein-reported domestic pig-derived strains, in S224 and S225 as the only NSP4 genotype, and in S219 as a mixed E1/E9 genotype (Table 2, Fig. 7D). These three E9 strains shared a 100 % nt similarity, making the E9 the most homogenous genotype in this dataset.

In the NSP5 segment, RVAs from the present study belonged to the H1 genotype, with the nt similarity range of 94.78 % to 100 %, indicating a relatively homogenous nature and porcine origin. In the H1 phylogram, two main branches with onward ramified branching patterns were observed (Fig. 7E). Across the H1 phylogram two domestic pig- (S55, S236), three wild canid- (C48, L54, L533), and two wild boar-derived (DS84, DS306) strains phylogenetically positioned in proximity, or even formed a clade with human-derived zoonotic RVAs of porcine origin, geographically close and distant alike (Fig. 7E).

3.3. Recombination analysis

In the present study, recombination events were identified in the VP4, NSP1, and NSP4 segments. Interestingly, the recombination potential of poRVAs was also confirmed in the role of major parents for the NSP3 recombinants from the previous study (Kunić et al., 2023). The jackal-derived C48 (T1) and fox-derived L54 (T7) strains were identified as major parental sources for these human-derived T1/T7 intergenotype

recombinant D230 T1 (Supplementary Fig. 1A) and D329 T7 strains (Supplementary Fig. 1B). The following sections detail the recombination events detected in the VP4, NSP1, and NSP4 segments.

In the VP4 segment, putative recombination events occurred in genotypes P[13] and P[23]. To begin with, in the P13 genotype wild boar-derived DS229 P[13] strain presented as an intragenotype recombinant (Fig. 3B, Fig. 4A) between fox-derived L533 P[13] strain and Japanese domestic pig-derived P[13] strain (T-HgOg13) as major and minor parents, respectively (Fig. 3B; Fig. 4A). The recombination event was strongly supported as it was identified by six out of seven RDP5 detection methods. Furthermore, two intergenotype recombination events were detected in the P[23] genotype. Firstly, the domestic pig-derived P[23] S224 strain presented as an intergenotype recombinant between the domestic pig-derived S225 P[23] strain and fox-derived L62 P[13] strain as major and minor parents, respectively (Fig. 3B; Fig. 4B). Consequently, the recombination-induced divergence of the S224 P[23] strain led to its phylogenetic separation from the rest of the P[23] RVAs, sharing only 91.22 nt similarity with its major parent as the closest evolutionary relative (Fig. 3B). Interestingly, the S225 P[23] strain posing as a major parent in the above-mentioned recombinant, presented as an intergenotype recombinant itself (Fig. 4C). Domestic pig-derived S219 P[23] strain was detected as a major parent while the S225 P[6] strain, mixed with P[23] genotype (Table 2), posed as the minor parent (Fig. 3B, Fig. 4C). Both P[23] recombination events were detected with all seven recombination detecting methods, hence strongly supported. Conversely, no recombination events were detected in the P[6] genotype.

In the NSP1 segment, The RDP5 analysis produced a positive recombination signal within the A8 genotype. The domestic pig-derived S224 strain was detected as an intragenotype A8 recombinant, by all seven RDP5 integrated methods. The Chinese human-derived SD20200276 strain and fox-derived L62 presented as major and minor parents, respectively (Fig. 4D). This resulted in S224 being divergently sequestered from the rest of the RVAs from this study while clustering with two Chinese strains, one dog- and one human-derived (Fig. 7A). On the other hand, no recombination events were detected in the A1 genotype.

In the NSP3 gene, no recombinant strains were detected among RVAs from the current study. Nevertheless, two previously reported T1/T7 intergenotype recombinant human-derived D230 and D329 strains (Kunić et al., 2023), were also detected as T1/T7 intergenotype recombinants within the herein presented dataset (Fig. 7C, Supplementary Fig. 1A, Supplementary Fig. 1B). Notably, the jackal-derived C48 T1 and the fox-derived L54 T7 strains were detected as major parents for mentioned zoonotic recombinants, further highlighting the complex genetic interconnectedness and zoonotic potential of poRVA strains.

In the NSP4 gene, the domestic pig-derived S219 E1 strain was detected as an intergenotype recombinant by five out of seven RDP5 methods. On the NSP4 phylogram (Fig. 7D), S219 phylogenetically branched out between the E1 and E9 genotypes, whereas its mixed genotype S219 E9 (Table 2) was detected as a minor, while domestic pig-derived S280 E1 presented as a major parent (Fig. 4E). Although this recombination event was detected by five/seven RDP5 integrated methods, it was clearly depicted in the E1 phylogram (Fig. 7D), supporting its validity as a positive recombination signal.

3.4. Discussion

During this study, we sequenced and analyzed the whole genomes of 19 RVA strains detected in domestic pigs, wild boars, red foxes, and a golden jackal over a three-year period (2018–2021). Animals were sampled utilizing the spatiotemporal approach, and the acquired RVA genomes were compared and characterized following One Health principles, aiming to explore genetic interconnectedness and interspecies transmission of poRVAs within the Croatian ecosystem. Notably, the analysis of complete poRVA genomes confirmed the previously hypothesized interspecies transmission events inferred from VP7 and VP4 genes data (Ćolić et al., 2021; Bmić et al., 2022a). To the best of our knowledge, this study presents the first complete RVA genome in a golden jackal and the second in a red fox (Busi et al., 2017) on a global scale. Additionally, the complete RVA genomes reported in wild boars are the first documented outside of Asia (Shizawa et al., 2024; Le et al., 2025). Each described RVA strain (Table 2) exhibited a typical porcine RVA constellation, i.e. porcine genogroup 1 constellation paired with backbone genotypes such as I5, A8, T7, and E9, which are characteristic of porcine hosts (Matthijnssens et al., 2008b; Doro et al., 2015). Regarding the surface protein-coding gene segments VP7 was identified with G3, G4, G5, G9, and G11 genotypes, whileVP4 was identified with P[6], P[13], and P[23] genotypes, all of which are typically found in porcine hosts (Matthijnssens et al., 2008b; Doro et al., 2015). Domestic pig-derived RVAs showed significant genetic heterogeneity, as mixed genotypes in VP7, VP4, and NSP4 genes were found only in domestic pigs, likely a consequence of intensive production and trade, diverse RVA strain circulation, and close contact among pigs (Chang et al., 2012; Palmarini, 2017). Most Croatian poRVAs phylogenetically clustered with each other or with other European strains across all gene segments.

The intensification of the pork industry and trade accelerates the spread of porcine-originated infectious agents (Fournie et al., 2015). As live pig transport and pork products may impact the WUI environment, the risk of future pathogen spillover should be considered (Fournié et al., 2015). The wildlife movement in the shared WUI environment is presumed to account for pathogen transmission between countries, as wildlife was described to carry pathogens across national borders (Yon et al., 2019). Interestingly, in a few segments (VP3, NSP1, NSP4, and NSP5), some Croatian RVAs clustered with strains from Asian countries, including Japan, China, Taiwan, and Russia (Fig. 5C, Fig. 7A, Fig. 7D, Fig. 7E). This evolutionary link may result from a significant underreporting of animal-derived RVA sequences globally, making it difficult to presume the exact strain origin. NSP5 was the only segment where human and animal-derived strains were more phylogenetically intertwined (Fig. 7E), as it lacks distinct host-specific clustering between human and porcine hosts (Silva et al., 2016).

The concern regarding infectious disease transmission from domestic animals to wildlife has been well recognized (Aguirre, 2009; Martin et al., 2011). Domestic pigs have already been suggested as reservoirs for RVAs and a source of newly adapted emerging strains for humans and other animals (Dhama et al., 2009; Wu et al., 2022). Nevertheless, previous data on RVA detection rates in wildlife suggests that they may serve as additional potential RVA reservoirs (Martin et al., 2011; Ćolić et al., 2021; Jota Baptista et al., 2023). The current study shows the close evolutionary relationship between wild canid- and wild boar-derived RVAs (Fig. 1) which aligns with the fact these animals share the same habitat and, at times, even prey-predator dynamic (Bassi et al., 2012). The trophic niche range of golden jackal and red fox in the Pannonian ecoregion proved to be very narrow with a mean food overlap index of 73 % (Lanszki et al., 2006). Based on prey remains found in scat, the golden jackals and red foxes are known for predation upon wild boar piglets (Lanszki et al., 2006). The wild canid-derived RVAs from this study consistently exhibit porcine RVA origin across all gene segments, clustering closely with RVAs derived from either domestic pigs or wild boars. Pig populations may also act as intermediate hosts, amplifying infectious agents transmitted from other wild or domestic animal species, and then transmitting them to humans, as described for the Nipah virus (Fournié et al., 2015). Nevertheless, in each gene segment, at least one fox-derived RVA strain clustered closely with zoonotic poRVAs from human hosts (Fig. 3, Fig. 5, Fig. 7). Furthermore, wild canid-derived RVAs were identified as either major or minor parents in five out of seven recombinant strains detected in the present dataset, including two zoonotic NSP3 recombinants from our previous study (Kunić et al., 2023) (Supplementary Fig. 1). Considering all of the above, current results may imply that the evolutionary relationship may exist between

Croatian wildlife-derived RVAs and zoonotic human-derived RVAs of porcine origin without the domestic pig as the intermediate host. A more conclusive portrayal of RVA geoevolutionary patterns and reservoir determination remains limited due to the current lack of domestic pig- and wildlife-derived complete RVA genomes, both from the affected area and globally. In contrast to the intensive pork industry, in Croatia, the pig farming sector is largely composed of small, traditional rural farms, with fewer than 10 sows and less than three hectares of land, accounting for up to 75 % of all pig holdings (Wellbrock, 2008). Due to their size and resources, these farms fall under biosafety category 1 and generally lack the capacity to implement effective biosecurity measures important for spread of various pathogens among multiple susceptible species. Rural farming, especially with outdoor or free-range systems, is more vulnerable to predation by foxes and jackals due to unsufficient protective barriers (Fleming et al., 2016). Furthermore, in rural outdoor farms, wild boars and domestic pigs can interact and even interbreed (Anderson et al., 2019).

Overall, there are multiple factors and contact points between these animals such as shared habitat, insufficient barriers for outdoor farms, interactions between domestic pigs and wildlife, scavenging and opportunistic nature of wild canids and wild boars, overlapping trophic niches of golden jackals and red foxes, etc. All mentioned factors significantly influence and enable interspecies transmission of multispecies pathogens. Therefore, direct or indirect interspecies transmission through environmental exposure may serve as a potential RVA infection source for domestic animals and wildlife alike. RVA can survive for prolonged periods in the environment, preserving infectivity for several hours to several months outside the host (D'Souza et al., 2008). Although RVA is primarily transmitted via the fecal-oral route, it is also recognized as a foodborne and waterborne virus (Svensson, 2000; Dhama et al., 2009; Kraay et al., 2018). Lately, increasing attention has been given to the waterborne transmission of RVA, taking into account environmental conditions such as temperature and humidity (Kraay et al., 2018). In Croatia, a study from December 2019 to January 2021 detected RVA in 22.2 % (2/9) of surface water samples and 100 % (21/21) of wastewater samples (Brnić et al., 2022b), suggesting possible environmental contamination. Similar results were reported in neighboring Slovenia, where 60.3 % of surface water samples tested positive for at least one enteric virus, including rotaviruses, noroviruses, and astroviruses, indicating widespread environmental contamination (Steyer et al., 2011). These contaminated environments may serve as hotspots for the transmission of enteric viruses to wildlife, while also posing a potential risk to public health. The aforementioned WUI sites, dispersed throughout Croatia and Europe (Schug et al., 2023), combined with the rising wild canid and wild boar density in Europe (Statham et al., 2018; Colomer et al., 2024), emphasize the importance of wildlife surveillance for multispecies pathogens like RVA (Schug et al., 2023; Jiménez-Ruiz et al., 2024). RVA infections stemming from interspecies transmission, including zoonotic transmission, are generally considered limited in non-dominant host species (Martella et al., 2010). Certain genotypes are known to exhibit host tropism (Matthijnssens et al., 2008a; Papp et al., 2013b; Doro et al., 2015; Lagan et al., 2023). However, successful virus adaptation to a human host has been documented (Hoa-Tran et al., 2024), underscoring the potential public health risks posed by underresearched animal RVAs. The recurrent zoonotic transmission and recombination potential of poRVAs in Croatia further emphasize this concern (Kunić et al., 2023).

To conclude, this study provides the first detection of RVA in a previously unreported host, the golden jackal, and highlights the spatiotemporal recurrence of poRVAs in Croatian wildlife over several years. A comprehensive complete RVA genome analysis provided evidence on interspecies transmission of poRVAs. However, it remains unclear whether these RVAs successfully adapt to non-dominant hosts long-term or if such interspecies transmission events are transient. Integrating wildlife into RVA studies is crucial from both conservation medicine and One Health perspectives, emphasizing the interconnectedness of ecological and human health. Studies like this are essential to address the knowledge gap of the role that wildlife holds in RVA epidemiology, particularly their role as reservoirs of emerging and potentially zoonotic RVA strains. Applying One Health principles and spatiotemporal approach can advance our understanding of the evolutionary dynamics of poRVA, facilitating the assessment of interspecies transmission impacts on vaccine efficacy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.180010.

CRediT authorship contribution statement

Valentina Kunić: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ljubo Barbić:** Supervision, Conceptualization. **Jakob Simić:** Visualization, Formal analysis. **Tina Mikuletic:** Resources, Methodology. **Rok Kogoj:** Resources, Methodology. **Tom Koritnik:** Visualization, Validation, Methodology, Formal analysis. **Andrej Steyer:** Writing – review & editing, Resources. **Dean Konjević:** Writing – review & editing, Resources. **Miljenko Bujanić:** Resources. **Marina Prislin Simac:** Writing – review & editing. **Dragan Brnić:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Funding

This research was funded by the Croatian Science Foundation installation research project Reco "Rotaviruses in Croatian Ecosystem: molecular epidemiology and zoonotic potential" Grant No. HRZZ-UIP-2017-05-8580. The work of VK was funded by the Croatian Science Foundation project DOK-2021-02-3623.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Croatian Veterinary Institute for financing VK's NGS and data analysis education, through funds from the Ministry of Science and Education, Republic of Croatia.

Data availability

Data will be made available on request.

References

Aguirre, A.A., 2009. Wild canids as sentinels of ecological health: a conservation medicine perspective. Parasit. Vectors 2 (Suppl 1), S7, 1–8. https://doi.org/10.1186/1756-3305-2-S1-S7.

Anderson, D., Toma, R., Negishi, Y., Okuda, K., Ishiniwa, H., Hinton, T.G., Nanba, K., Tamate, H.B., Kaneko, S., 2019. Mating of escaped domestic pigs with wild boar and possibility of their offspring migration after the Fukushima Daiichi nuclear power plant accident. Sci. Rep. 9, 11537. https://doi.org/10.1038/s41598-019-47982-z.

Bassi, E., Donaggio, E., Marcon, A., Scandura, M., Apollonio, M., 2012. Trophic niche overlap and wild ungulate consumption by red fox and wolf in a mountain area in Italy. Mamm. Biol. 77 (5), 369–376. https://doi.org/10.1016/j.mambio.2011.12.002.

Bijl, H., Schally, G., Márton, M., Heltai, M., Csányi, S., 2024. From invaders to residents: the golden jackal (Canis aureus) expansion in Hungary since the mid-1990s. PLoS One 19 (7), e0306489. https://doi.org/10.1371/journal.pone.0306489.

Brnić, D., Čolić, D., Kunić, V., Maltar-Strmecki, N., Kresić, N., Konjević, D., Bujanić, M., Bacani, I., Hizman, D., Jemersić, L., 2022a. Rotavirus a in domestic pigs and wild boars: high genetic diversity and interspecies transmission. Viruses 14 (9), 2028. https://doi.org/10.3390/v14092028.

Brnić, D., Lojkić, I., Škoko, I., Kresić, N., Simić, I., Keros, T., Ganjto, M., Stefanac, D., Viduka, B., Karsaj, D., Štiler, D., Habrun, B., Jemeršić, L., 2022b. SARS-CoV-2 circulation in Croatian wastewaters and the absence of SARS-CoV-2 in bivalve molluscan shellfish. Environ. Res. 207, 112638. https://doi.org/10.1016/j.envres.2021.112638.

Busi, C., Martella, V., Papetti, A., Sabelli, C., Lelli, D., Alborali, G.L., Gibelli, L., Gelmetti, D., Lavazza, A., Cordioli, P., Boniotti, M.B., 2017. Group a rotavirus associated with encephalitis in red fox. Emerg. Infect. Dis. 23 (9), 1535–1538. https://doi.org/10.3201/eid2309.170158.

Chang, K.S., Kim, Y., Saif, L.J., 2012. Reoviruses (Rotaviruses and Reoviruses). In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, 11th ed. Wiley-Blackwell, United Kingdom, pp. 621–634.

Čolić, D., 2021. Genotyping of Rotavirus A from wild animals in Croatia. Master's thesis. Faculty of Veterinary Medicine, University of Zagreb. https://urn.nsk.hr/urn:nbn:hr:217:900539.

Čolić, D., Krešić, N., Mihaljević, Ž., Andreanszky, T., Balić, D., Lolić, M., Brnić, D., 2021. A remarkable genetic diversity of rotavirus a circulating in red fox population in Croatia. Pathogens 10 (4), 485. https://doi.org/10.3390/pathogens10040485.

Colomer, J., Massei, G., Roos, D., Rosell, C., Rodríguez-Teijeiro, J.D., 2024. What drives wild boar density and population growth in Mediterranean environments? Sci. Total Environ. 931, 172739. https://doi.org/10.1016/j.scitotenv.2024.172739.

Crawford, S.E., Ramani, S., Tate, J.E., Parashar, U.D., Svensson, L., Hagbom, M., Franco, M.A., Greenberg, H.B., O'Ryan, M., Kang, G., Desselberger, U., Estes, M.K., 2017. Rotavirus infection. Nat. Rev. Dis. Primers 3, 17083. https://doi.org/10.1038/nrdp.2017.83.

Cunningham, A.A., Daszak, P., Wood, J.L.N., 2017. One health, emerging infectious diseases and wildlife: two decades of progress? Philos. Trans. R. Soc. B. https://doi.org/10.1098/rstb.2016.0167.

de Barros, B., Chagas, E.N., Bezerra, L.W., Ribeiro, L.G., Barboza Duarte, J.W., Pereira, D., da Penha, E.T., Silva, J.R., Melo Bezerra, D.A., Bandeira, R.S., Costa Pinheiro, H.H., dos Santos Guerra, S.def., J. R., de Paula Souza e Guimarães, Mascarenhas, J.D.A.P., 2018. Rotavirus a in wild and domestic animals from areas with environmental degradation in the Brazilian Amazon. PLoS One 13. https://doi.org/10.1371/journal.pone.0209005.

Desvars-Larrive, A., Vogl, A.E., Puspitarani, G.A., Yang, L., Joachim, A., Käsbohrer, A., 2024. A one health framework for exploring zoonotic interactions demonstrated through a case study. Nat. Commun. 15. https://doi.org/10.1038/s41467-024-49967-7.

Dhama, K., Chauhan, R.S., Mahendran, M., Malik, S.V.S., 2009. Rotavirus diarrhea in bovines and other domestic animals. Vet. Res. Commun. 33, 1–23. https://doi.org/10.1007/s11259-008-9070-x.

Dian, Z., Sun, Y., Zhang, G., Xu, Y., Fan, X., Yang, X., Pan, Q., Peppelenbosch, M., Miao, Z., 2021. Rotavirus-related systemic diseases: clinical manifestation, evidence and pathogenesis. Crit. Rev. Microbiol. https://doi.org/10.1080/1040841X.2021.1907738.

Doro, R., Farkas, S.L., Martella, V., Banyai, K., 2015. Zoonotic transmission of rotavirus: surveillance and control. Expert Rev. Anti-Infect. Ther. 13 (11), 1337–1350. https://doi.org/10.1586/14787210.2015.1089171.

D'Souza, R.M., Hall, G., Becker, N.G., 2008. Climatic factors associated with hospitalizations for rotavirus diarrhoea in children under 5 years of age. Epidemiol. Infect. 136 (1), 56–64. https://doi.org/10.1017/S0950268807008229.

Estes, M.K., Greenberg, H.B., 2013. Rotaviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 6th edn. Lippincott Williams & Wilkins, Philadelphia, USA, pp. 1347–1401.

Evans, R.H., 1984. Rotavirus-associated diarrhea in young raccoons (Procyon lotor), striped skunks (Mephitis mephitis) and red foxes (Vulpes vulpes). J. Wildl. Dis. 20, 79–85. https://doi.org/10.7589/0090-3558-20.2.79.

Fleming, P.A., Dundas, S.J., Lau, Y.Y.W., Pluske, J.R., 2016. Predation by red foxes (Vulpes vulpes) at an outdoor piggery. Animals 6. https://doi.org/10.3390/ani6100060.

Fournié, G., Kearsley-Fleet, L., Otte, J., Pfeiffer, D.U., 2015. Spatiotemporal trends in the discovery of new swine infectious agents. Vet. Res. 46, 114. https://doi.org/10.1186/s13567-015-0226-8.

Gálvez, À., Peres-Neto, P.R., Castillo-Escrivà, A., Bonilla, F., Camacho, A., García-Roger, E.M., Iepure, S., Miralles, J., Monros, J.S., Olmo, C., Picazo, A., Rojo, C., Rueda, J., Sasa, M., Segura, M., Armengol, X., Mesquita-Joanes, F., 2024. Spatial versus spatio-temporal approaches for studying metacommunities: a multi-taxon analysis in Mediterranean and tropical temporary ponds. Proc. R. Soc. B Biol. Sci. 291. https://doi.org/10.1098/rspb.2023.2768.

Geletu, U.S., Usmael, M.A., Bari, F.D., 2021. Rotavirus in calves and its zoonotic importance. Vet Med Int. https://doi.org/10.1155/2021/6639701.

Ghosh, S., Kobayashi, N., 2014. Exotic rotaviruses in animals and rotaviruses in exotic animals. VirusDis 25, 158–172. https://doi.org/10.1007/s13337-014-0194-z.

Ghosh, S., Kumar, M., Santiana, M., Mishra, A., Zhang, M., Labayo, H., Chibly, A.M., Nakamura, H., Tanaka, T., Henderson, W., Lewis, E., Voss, O., Su, Y., Belkaid, Y., Chiorini, J.A., Hoffman, M.P., Altan-Bonnet, N., 2022. Enteric viruses replicate in salivary glands and infect through saliva. Nature 607, 345–350. https://doi.org/10.1038/s41586-022-04895-8.

Hatcher, E.L., Zhdanov, S.A., Bao, Y., Blinkova, O., Nawrocki, E.P., Ostapchuck, Y., Schäffer, A.A., Brister, J.R., 2017. Virus variation resource–improved response to emergent viral outbreaks. Nucleic Acids Res. 45, 482–490. https://doi.org/10.1093/nar/gkw1065.

Hoa-Tran, T.N., Nakagomi, T., Minh Vu, H., Thu Thi Nguyen, T., Thi Hai Doo, A., The Nguyen, A., Bines, J.E., Thomas, S., Grabovac, V., Kataoka-Nakamura, C., Taichiro, T., Hasebe, F., Kodama, T., Kaneko, M., Thi Thanh Dang, H., Thi Duong, H., Duc Anh, D., Nakagomi, O., 2024. Evolution of DS-1-like G8P[8] rotavirus a strains from Vietnamese children with acute gastroenteritis (2014-21): adaptation and loss of animal rotavirus-derived genes during human-to-human spread. Virus Evol. 10 (1), vead045. https://doi.org/10.1093/ve/vead045.

Hoxie, I., Dennehy, J.J., 2020. Intragenic recombination influences rotavirus diversity and evolution. Virus Evol 6 (1), vez059. https://doi.org/10.1093/ve/vez059.

Jiménez-Ruiz, S., Santos, N., Barasona, J.A., Fine, A.E., Jori, F., 2024. Editorial: pathogen transmission at the domestic-wildlife interface: a growing challenge that requires integrated solutions. Front. Vet. Sci. 11, 1415335. https://doi.org/10.3389/fvets.2024.1415335.

Jota Baptista, C., Seixas, F., Gonzalo-Orden, J.M., Oliveira, P.A., 2023. Wild boar (Sus scrofa) as a potential reservoir of infectious agents in Portugal: a review of two decades (2001–2021). Eur. J. Wildl. Res. 69, 101. https://doi.org/10.1007/s10344-023-01732-9.

Kraay, A.N.M., Brouwer, A.F., Lin, N., Collender, P.A., Remais, J.V., Eisenberg, J.N.S., 2018. Modeling environmentally mediated rotavirus transmission: the role of temperature and hydrologic factors. Proc. Natl. Acad. Sci. 115 (12), E2782–E2790. https://doi.org/10.1073/pnas.1719579115.

Krofel, M., Berce, M., Berce, T., Kryštufek, B., Lamut, S., Tarman, J., Fležar, U., 2023. New mesocarnivore at the doorstep of Central Europe: historic development of golden jackal (Canis aureus) population in Slovenia. Mamm. Res. 68, 329–339. https://doi.org/10.1007/s13364-023-00686-2.

Kunić, V., Mikuletič, T., Kogoj, R., Koritnik, T., Steyer, A., Šoprek, S., Tesović, G., Konjik, V., Roksandić Križan, I., Prislin, M., Jemeršić, L., Brnić, D., 2023. Interspecies transmission of porcine-originated G4P[6] rotavirus a between pigs and humans: a synchronized spatiotemporal approach. Front. Microbiol. 14, 1194764. https://doi.org/10.3389/fmicb.2023.1194764.

Lagan, P., Mooney, M.H., Lemon, K., 2023. Genome analyses of species a rotavirus isolated from various mammalian hosts in Northern Ireland during 2013–2016. Virus. Evolution 9 (2), vead039. https://doi.org/10.1093/ve/vead039.

Lanszki, J., Heltai, M., Szabó, L., 2006. Feeding habits and trophic niche overlap between sympatric golden jackal (Canis aureus) and red fox (Vulpes vulpes) in the Pannonian ecoregion (Hungary). Can. J. Zool. 84, 1647–1656. https://doi.org/10.1139/Z06-147.

Le, X., Tao, Y., Wang, B., Hou, Y., Ning, Y., Hou, J., Wang, R., Li, Q., Xia, X., 2025. Diversity and potential cross-species transmission of rotavirus a in wild animals in Yunnan, China. Microorganisms 13 (1), 145. https://doi.org/10.3390/microorganisms13010145.

Letunic, I., Bork, P., 2021. Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49 (1), W293–W296. https://doi.org/10.1093/nar/gkab301.

Malik, Y.S., Bhat, S., Dar, P.S., Sircar, S., Dhama, K., Singh, R.K., 2020. Evolving rotaviruses, interspecies transmission and Zoonoses. Open Virol J 14, 1–6. https://doi.org/10.2174/1874357902014010001.

Martella, V., Bányai, K., Matthijnssens, J., Buonavoglia, C., Ciarlet, M., 2010. Zoonotic aspects of rotaviruses. Vet. Microbiol. 140 (3–4), 246–255. https://doi.org/10.1016/j.vetmic.2009.08.028.

Martin, C., Pastoret, P.P., Brochier, B., Humblet, M.F., Saegerman, C., 2011. A survey of the transmission of infectious diseases/infections between wild and domestic ungulates in Europe. Vet. Res. 42, 70. https://doi.org/10.1186/1297-9716-42-70.

Martin, D.P., Murrell, B., Golden, M., Khoosal, A., Muhire, B., 2015. RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol. 1 (1), vev003. https://doi.org/10.1093/ve/vev003.

Mathon, H., Sanders, L., 2014. Spatio-Temporal Approaches: Geographic Objects and Change Process. ISTE Ltd and John Wiley and Sons, Inc (ISBN: 978-1-848-21552-8).

Matthijnssens, J., Ciarlet, M., Rahman, M., Attoui, H., Bányai, K., Estes, M.K., Gentsch, J. R., Iturriza-Gómara, M., Kirkwood, C.D., Martella, V., Mertens, P.P.C., Nakagomi, O., Patton, J.T., Ruggeri, F.M., Saif, L.J., Santos, N., Steyer, A., Taniguchi, K., Desselberger, U., Van Ranst, M., 2008a. Recommendations for the classification of group a rotaviruses using all 11 genomic RNA segments. Arch. Virol. 153, 1621–1629. https://doi.org/10.1007/s00705-008-0155-1.

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S.M., Palombo, E.A., Iturriza-Gómara, M., Maes, P., Patton, J.T., Rahman, M., Van Ranst, M., 2008b. Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J. Virol. 82 (7), 3204–3219. https://doi.org/10.1128/jvi.02257-07.

Moutelíková, R., Dufková, L., Kamler, J., Drimaj, J., Plhal, R., Prodělalová, J., 2016. Epidemiological survey of enteric viruses in wild boars in the Czech Republic: first evidence of close relationship between wild boar and human rotavirus a strains. Vet. Microbiol. 193, 28–35. https://doi.org/10.1016/j.vetmic.2016.08.003.

Okadera, K., Abe, M., Ito, N., Morikawa, S., Yamasaki, A., Masatani, T., Nakagawa, K., Yamaoka, S., Sugiyama, M., 2013. Evidence of natural transmission of group a rotavirus between domestic pigs and wild boars (Sus scrofa) in Japan. Infect. Genet. Evol. 20, 54–60. https://doi.org/10.1016/j.meegid.2013.07.029.

Palmarini, M., 2017. In: MacLachlan, N.J., Dubovi, E.J. (Eds.), Reoviridae. In Fenner's veterinary virology. academic press, Boston, MA, USA, pp. 299–317, 5th ed.

Papp, H., László, B., Jakab, F., Ganesh, B., De Grazia, S., Matthijnssens, J., Ciarlet, M., Martella, V., Bányai, K., 2013a. Review of group a rotavirus strains reported in swine and cattle. Vet. Microbiol. 165 (3–4), 190–199. https://doi.org/10.1016/j.vetmic.2013.03.020.

Papp, H., Borzák, R., Farkas, S., Kisfali, P., Lengyel, G., Molnár, P., Melegh, B., Matthijnssens, J., Jakab, F., Martella, V., Bányai, K., 2013b. Zoonotic transmission of reassortant porcine G4P[6] rotaviruses in Hungarian pediatric patients identified sporadically over a 15year period. Infect. Genet. Evol. 19, 71–80. https://doi.org/10.1016/j.meegid.2013.06.013.

Pickett, B.E., Sadat, E.L., Zhang, Y., Noronha, J.M., Squires, R.B., Hunt, V., et al., 2011. ViPR: an open bioinformatics database and analysis resource for virology research. Nucleic Acid Res. 40 (1), D593–D598. https://doi.org/10.1093/nar/gkr859.

Rotavirus Classification Working Group, 2023. List of accepted genotypes. Rega Instituut KU Leuven. Available online: https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg, accessed on January 7th, 2025.

Rui, J., Li, K., Wei, H., Guo, X., Zhao, Z., Wang, Y., Song, W., Abudunaibi, B., Chen, T., 2024. MODELS: a six-step framework for developing an infectious disease model. Infect. Dis. Poverty 13. https://doi.org/10.1186/s40249-024-01195-3.

Samson, S., Lord, E., Makarenkov, M., 2022. SimPlot++: a Python application for representing sequence similarity and detecting recombination. Bioinformatics 38 (11), 3118–3120. https://doi.org/10.1093/bioinformatics/btac287.

Schug, F., Bar-Massada, A., Carlson, A.R., Cox, H., Hawbaker, T.J., Helmers, D., Hostert, P., Kaim, D., Kasraee, N.K., Martinuzzi, S., Mockrin, M.H., Pfoch, K.A., Radeloff, V.C., 2023. The global wildland–urban interface. Nature 621, 94–99. https://doi.org/10.1038/s41586-023-06320-0.

Senica, P., Zele Vengust, D., Vengust, G., Kuhar, U., 2024. Genomic revelations: investigating rotavirus a presence in wild ruminants and its zoonotic potential. Front Vet Sci 11. https://doi.org/10.3389/fvets.2024.1429654.

Shizawa, S., Fukuda, F., Kikkawa, Y., Oi, T., Takemae, H., Masuda, T., Ishida, H., Murakami, H., Sakaguchi, S., Mizutani, T., Nagai, M., Oba, M., 2024. Genomic diversity of group a rotaviruses from wild boars and domestic pigs in Japan: wide prevalence of NSP5 carrying the H2 genotype. Arch. Virol. 169, 63. https://doi.org/10.1007/s00705-023-05954-9.

Silva, F.D.F., Gregori, F., McDonald, S.M., 2016. Distinguishing the genotype 1 genes and proteins of human Wa-like rotaviruses vs. porcine rotaviruses. Infect. Genet. Evol. 43, 6–14. https://doi.org/10.1016/j.meegid.2016.05.014.

Singh, B.B., Ward, M.P., Dhand, N.K., 2023. Host characteristics and their influence on zoonosis, disease emergence and multi-host pathogenicity. One Health 17. https://doi.org/10.1016/j.onehlt.2023.100596.

Spassov, N., Acosta-Pankov, I., 2019. Dispersal history of the golden jackal (Canis aureus moreoticus Geoffroy, 1835) in Europe and possible causes of its recent population explosion. Biodivers. J. 7. https://doi.org/10.3897/BDJ.7.e34825. E34825.

Statham, M.J., Edwards, C.J., Norén, K., Soulsbury, C.D., Sacks, B.N., 2018. Genetic analysis of European red foxes reveals multiple distinct peripheral populations and central continental admixture. Quat. Sci. Rev. 197, 257–266. https://doi.org/10.1016/j.quascirev.2018.08.019.

Steyer, A., Godič Torkar, K., Gutiérrez-Aguirre, I., Poljšak-Prijatelj, M., 2011. High prevalence of enteric viruses in untreated individual drinking water sources and surface water in Slovenia. Int. J. Hyg. Environ. Health 214 (5), 392–398. https://doi.org/10.1016/j.ijheh.2011.05.006.

Svensson, L., 2000. Diagnosis of foodborne viral infections in patients. Int. J. Food Microbiol. 59 (1–2), 117–126. https://doi.org/10.1016/S0168-1605(00)00281-6.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38 (7), 3022–3027. https://doi.org/10.1093/molbev/msab120.

Troeger, C., Khalil, I.A., Rao, P.C., Cao, S., Blacker, B.F., Ahmed, T., Armah, G., Bines, J. E., Brewer, T.G., Colombara, D.V., Kang, G., Kirkpatrick, B.D., Kirkwood, C.D., Mwenda, J.M., Parashar, U.D., Petri, W.A., Riddle, M.S., Steele, A.D., Thompson, R. L., Walson, J.L., Sanders, J.W., Mokdad, A.H., Murray, C.J.L., Hay, S.I., Reiner, R.C., 2018. Rotavirus vaccination and the global burden of rotavirus diarrhea among children younger than 5 years. JAMA Pediatr. 172 (10), 958–965. https://doi.org/10.1001/jamapediatrics.2018.1960.

Vilibic-Cavlek, T., Barbic, L., Mrzljak, A., Bmic, D., Klobucar, A., Ilic, M., Janev-Holcer, N., Bogdanic, M., Jemersic, L., Stevanovic, V., Tabain, I., Kremar, S., Vucelja, M., Pepic, J., Boljfetic, M., Jelicic, P., Madic, J., Ferencak, I., Savic, V., 2021. Emerging and neglected viruses of zoonotic importance in Croatia. Pathogens. https://doi.org/10.3390/pathogens10010073.

Wegner, G.I., Murray, K.A., Springmann, M., Muller, A., Sokolow, S.H., Saylors, K., Morens, D.M., 2022. Averting wildlife-borne infectious disease epidemics requires a focus on socio-ecological drivers and a redesign of the global food system-NC-ND license. https://doi.org/10.1016/j.

Wellbrock, W., 2008. Pig Welfare in Croatia: A Critical Reflection on the EU Pig Welfare Directives. Master's thesis.. Wageningen University of Animal Sciences.

World Organisation for Animal Health, 2024. The Importance of the One Health Approach in Tackling Emerging and Re-emerging Zoonotic Epidemics and Pandemics. Licence: CC BY-SA 3.0 IGO. https://doi.org/10.20506/woah.3480.

Wu, F.T., Liu, L.T.C., Jiang, B., Kuo, T.Y., Wu, C.Y., Liao, M.H., 2022. Prevalence and diversity of rotavirus a in pigs: evidence for a possible reservoir in human infection. Infect. Genet. Evol. 98, 105198. https://doi.org/10.1016/j.meegid.2021.105198.

Yon, L., Duff, J.P., Ågren, E.O., Erdélyi, K., Ferroglio, E., Godfroid, J., Hars, J., Hestvik, G., Horton, D., Kuiken, T., Lavazza, A., Markowska-Daniel, I., Martel, A., Neimanis, A., Pasmans, F., Price, S.J., Ruiz-Fons, F., Ryser-Degiorgis, M.P., Widén, F., Gavier-Widén, D., 2019. Recent changes in infectious diseases in european wildlife. J. Wildl. Dis. https://doi.org/10.7589/2017-07-172.

Zecchin, B., De Nardi, M., Nouvellet, P., Vernesi, C., Babbucci, M., Crestanello, B., Bagó, Z., Bedeković, T., Hostnik, P., Milani, A., Donnelly, C.A., Bargelloni, L., Lorenzetto, M., Citterio, C., Obber, F., De Benedictis, P., Cattoli, G., 2019. Genetic and spatial characterization of the red fox (Vulpes vulpes) population in the area stretching between the eastern and Dinaric Alps and its relationship with rabies and canine distemper dynamics. PLoS One 14, e0213515. https://doi.org/10.1371/journal.pone.0213515.

9. BIOGRAPHY OF THE AUTHOR WITH BIBLIOGRAPHY OF PUBLISHED WORK

Valentina Kunić was born on April 30th, 1993, in Zagreb, Croatia. After graduating from the III. Gymnasium Ivan Kušlan, Zagreb, she enrolled in the Faculty of Veterinary Medicine at the University of Zagreb. Valentina graduated in 2018 and started to work in B. Braun Adria d. o. o. as a Sales representative for the Hospital care program until September 2021, when she joined the Department of Virology at Croatian Veterinary Institute. She was employed as a research assistant on the project "Reco: *Rotaviruses in Croatian ecosystem: molecular epidemiology and zoonotic potential*", funded by the Croatian Science Foundation. The work of Valentina was funded by the Croatian Science Foundation project DOK-2021-02-3623. In 2022, she enrolled in the postgraduate doctoral program in Veterinary Sciences at the Faculty of Veterinary Medicine, University of Zagreb. In 2024, Valentina participated in 28th International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology (VEME 2024), held at Fiocruz, Brasilia. In addition, she completed several training programs, including next generation sequencing, bioinformatics analyses, cell culture and flow cytometry training. Valentina has published 9 scientific papers, with an h-idex of 4, and has participated in 14 international conferences.

Scientific articles in WoS journals

KUNIĆ, V., LJ. BARBIĆ, J. ŠIMIĆ, T. MIKULETIČ, R. KOGOJ, T. KORITNIK, A. STEYER, D. KONJEVIĆ, M. BUJANIĆ, M. PRIŠLIN ŠIMAC, D. BRNIĆ (2025): Interspecies transmission and genome heterogeneity of porcine-originated Rotavirus A between domestic pigs and wildlife in the Croatian ecosystem. Sci. Total Environ. 994, 2025, 180010. doi:10.1016/j.scitotenv.2025.180010

PRIŠLIN ŠIMAC, M., Š. NALETILIĆ, V. KOSTANIĆ, **V. KUNIĆ**, T.M. ZOREC, M. POLJAK, D. VLAJ, R. KOGOJ, N. TURK, D. BRNIĆ (2024): Canid alphaherpesvirus 1 infection alters the gene expression and secretome profile of canine adipose-derived mesenchymal stem cells in vitro. Virol. J. 21, 336, 18. doi:10.1186/s12985-024-02603-8

PRIŠLIN, M., A. BUTORAC, R. BERTOŠA, **V. KUNIĆ**, I. LJOLJE, P. KOSTEŠIĆ, D. VLAHOVIĆ, Š. NALETILIĆ, N. TURK, D. BRNIĆ (2024): In vitro aging alters the gene expression and secretome composition of canine adipose-derived mesenchymal stem cells. Front. Vet. Sci. 11, 1387174, 12. doi:10.3389/fvets.2024.1387174

KUNIĆ, V., T. MIKULETIĆ, R. KOGOJ, T. KORITNIK, A. STEYER, S. ŠOPREK, G. TEŠOVIĆ, V. KONJIK, I. ROKSANDIĆ KRIŽAN, M. PRIŠLIN, D. BRNIĆ (2023):

Interspecies transmission of porcine-originated G4P[6] rotavirus A between pigs and humans: a synchronized spatiotemporal approach. Front. Microbiol. 14, 1194764, 14. doi:10.3389/fmicb.2023.1194764

BRNIĆ, D., D. VLAHOVIĆ, A. GUDAN KURILJ, N. MALTAR-STRMEČKI, I. LOJKIĆ, **V. KUNIĆ**, L. JEMERŠIĆ, I. BAČANI, G. KOMPES, R. BECK, T. MIKULETIČ, A. STEYER (2023): The impact and complete genome characterisation of viruses involved in outbreaks of gastroenteritis in a farrow-to-finish holding. Sci. Rep. 13, 18780, 14. doi:10.1038/s41598-023-45994-4

BRNIĆ, D., D. ČOLIĆ, V. KUNIĆ, N. MALTAR-STRMEČKI, N. KREŠIĆ, D. KONJEVIĆ, M. BUJANIĆ, I. BAČANI, D. HIŽMAN, L. JEMERŠIĆ (2022): Rotavirus A in domestic pigs and wild boars: high genetic diversity and interspecies transmission. Viruses 14, 9, 2028, 20. doi:10.3390/v14092028

PRIŠLIN, M., D. VLAHOVIĆ, P. KOSTEŠIĆ, I. LJOLJE, D. BRNIĆ, N. TURK, I. LOJKIĆ, **V. KUNIĆ**, T. KARADJOLE, N. KREŠIĆ (2022): An outstanding role of adipose tissue in canine stem cell therapy. Animals 12, 9, 1088, 19. doi:10.3390/ani12091088

Scientific articles in Scopus journals

PRIŠLIN, M., Š. NALETILIĆ, M. HOHŠTETER, **V. KUNIĆ**, N. KREŠIĆ, N. TURK, I. LOJKIĆ, L. JEMERŠIĆ, D. BRNIĆ (2024): Patologija pseće herpesviroze. Vet. Stanica 55, 1, 99–110. doi:10.46419/vs.55.1.7

KUNIĆ, V., Ž. GOTTSTEIN, M. PRIŠLIN, V. SAVIĆ, D. BRNIĆ (2024): Health repercussions of avian rotaviruses on poultry and fancy pigeons. Vet. Stanica 55, 6, 677–689. doi:10.46419/vs.55.6.3

Conference papers (in Proceedings)

KOSTANIĆ, V., **V. KUNIĆ**, M. PRIŠLIN ŠIMAC, M. LOLIĆ, T. SUKALIĆ, D. BRNIĆ (2024): Klinički i epidemiološki uvid u akutni gastroenteritis uzrokovan rotavirusom A i koronavirusom kod goveda. Zbornik radova, 7. Hrvatski veterinarski kongres, Dubrovnik, Croatia, pp. 145–145.

PRIŠLIN ŠIMAC, M., **V. KUNIĆ**, V. KOSTANIĆ, R. BECK, D. JURKOVIĆ ŽILIĆ, S. DUVNJAK, I. REIL, I. LOJKIĆ, D. BRNIĆ (2024): Procjena kontaminacije patogenima u pripravcima mezenhimskih matičnih stanica porijeklom iz masnog tkiva pasa. 7. Hrvatski veterinarski kongres, Dubrovnik, Croatia, Abstract book, pp. 123–124.

KUNIĆ, V., T. MIKULETIĆ, T. KORITNIK, A. STEYER, D. KONJEVIĆ, M. BUJANIĆ, M. PRIŠLIN ŠIMAC, D. BRNIĆ (2024): Porcine-originated rotavirus A shows significant genetic heterogeneity and interspecies transmission potential. 9th European Rotavirus Biology Meeting, Abstract Book, pp. 49–49.

PRIŠLIN ŠIMAC, M., Š. NALETILIĆ, V. KOSTANIĆ, **V. KUNIĆ**, T.M. ZOREC, M. POLJAK, D. VLAJ, R. KOGOJ, N. TURK, D. BRNIĆ (2024): In vitro interaction between canid alphaherpesvirus 1 and canine adipose-derived mesenchymal stem cells: impact on cell gene expression and secretome profile. Power of Viruses 2024, Zadar, Croatia. Book of Abstracts, pp. 48–48.

PRIŠLIN, M., A. BUTORAC, R. BERTOŠA, **V. KUNIĆ**, I. LJOLJE, P. KOSTEŠIĆ, D. VLAHOVIĆ, Š. NALETILIĆ, N. TURK, D. BRNIĆ (2024): In vitro ageing changes the gene expression and secretome proteomic profile of canine adipose-derived mesenchymal stem cells. International Society for Stem Cell Research, Hamburg, Germany. Poster Abstract Guide 2024, 659–659.

PRIŠLIN, M., N. KREŠIĆ, D. VLAHOVIĆ, I. LOLJE, P. KOSTEŠIĆ, N. TURK, **V. KUNIĆ**, I. LOJKIĆ, D. BRNIĆ (2023): Primjena mezenhimskih matičnih stanica masnog tkiva u liječenju bolesti pasa. Veterinarski dani 2023, Osijek, Croatia, Abstract book, pp. 53.

BRNIĆ, D., I. ŠKOKO, **V. KUNIĆ**, J. PRPIĆ, L. JEMERŠIĆ (2023): Viral contaminants of bivalve molluscan shellfish harvested from production areas in Croatia: the results of a three-year study. 58th Croatian and 18th International Symposium of Agronomists, Abstract book, pp. 195–195.

KUNIĆ, V., T. MIKULETIĆ, R. KOGOJ, T. KORITNIK, A. STEYER, S. ŠOPREK, G. TEŠOVIĆ, V. KONJIK, I. ROKSANDIĆ KRIŽAN, M. PRIŠLIN, L. JEMERŠIĆ, D. BRNIĆ (2023): Interspecies transmission of G4P[6] rotavirus A between pigs and humans revealed by synchronized spatiotemporal approach in Croatia. MEEGID XVI, Dresden, Germany, Abstract Book O5.2:145.

BRNIĆ, D., D. VLAHOVIĆ, A. GUDAN KURILJ, I. LOJKIĆ, **V. KUNIĆ**, I. BAČANI, N. MALTAR-STRMEČKI, A. STEYER, L. JEMERŠIĆ (2023): Outbreaks of viral gastroenteritis in domestic pig farming: the impact on production results and complete genome characterisation of implicated pathogens. Thai J. Vet. Med. 53 Suppl. 2, pp. O21–O22.

PRIŠLIN, M., T. M. ZOREC, D. VLAHOVIĆ, Š. NALETILIĆ, I. LJOLJE, P. KOSTEŠIĆ, **V. KUNIĆ**, N. TURK, D. BRNIĆ (2023): In vitro interaction of canine adipose-derived mesenchymal stem cells with canid alphaherpesvirus: influence on cell differentiation

genes and variations of viral genome. ISSCR Int. Symp. “From Concepts to Clinic,” Sao Paulo, pp. 76–77.

BRNIĆ, D., **V. KUNIĆ**, N. KREŠIĆ, J. JUKIĆ GUĆ, V. KRŽELJ, V. KONJIK, I. ROKSANDIĆ KRIŽAN, S. ŠOPREK, G. TEŠOVIĆ (2022): Pristup “Jednog zdravlja” u istraživanju rotavirusa A: značaj životinjskih sojeva u epidemiologiji rotavirusnih infekcija u djece. O-90.

KUNIĆ, V., T. MIKULETIĆ, A. STEYER, R. KOGOJ, S. ŠOPREK, G. TEŠOVIĆ, V. KONJIK, J. JUKIĆ GUĆ, V. KRŽELJ, N. KREŠIĆ, D. BRNIĆ (2022): Whole genome characterization of unusual Rotavirus A strains with zoonotic background detected in hospitalized children in Croatia. 14th International dsRNA Virus Symposium, Banff, Canada

BRNIĆ, D., **V. KUNIĆ**, T. MIKULETIĆ, A. STEYER, R. KOGOJ, A. KOVAČEVIĆ, N. KREŠIĆ, V. KONJIK, M. SIPL, I. ROKSANDIĆ KRIŽAN, J. JUKIĆ GUĆ, V. KRŽELJ, S. ŠOPREK, G. TEŠOVIĆ (2022): Rotavirus A infection in hospitalized children in Croatia during three seasons: the predominance of G3 genotype and the emergence of intergenogroup reassortant strains. 14th International dsRNA Virus Symposium, Banff, Canada.

KUNIĆ, V., S. ŠOPREK, G. TEŠOVIĆ, J. JUKIĆ GUĆ, V. KRŽELJ, N. KREŠIĆ, L. JEMERŠIĆ, D. BRNIĆ (2022): Overview of the Rotavirus A genotypes circulating in hospitalized children in wider Zagreb and Split areas during 2020 and 2021. 7th Croatian Congress of Microbiology with International Participation, Sveti Martin na Muri, Croatia. Book of Abstracts, pp. O 51.