University of Zagreb

Faculty of Veterinary Medicine

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INSIGHT INTO THE RE-EMERGENCE OF *LEPTOSPIRA* spp. SEROGROUP POMONA

DOCTORAL DISSERTATION

Zagreb, 2025

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Supervisor:

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I Z J A V A

Ja, Iva Benvin, 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.

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(potpis studenta)

Zagreb, 2025.

This dissertation was prepared in the Department of Microbiology and Infectious Diseases with Clinic of the Faculty of Veterinary Medicine, Croatia, under the supervision of Professor Nenad Turk, PhD. One part of the research was conducted at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, within the framework of the Zoonotic Disease Integrated Action (ZODIAC) project of the International Atomic Energy Agency (IAEA), whose funding and coordination of training greatly contributed to the development of this scientific work.

INFORMATION ON SUPERVISOR

Professor Nenad Turk works at the Department of Microbiology and Infectious Diseases with Clinic, Veterinary Faculty, University of Zagreb. He finished PhD at the Veterinary Faculty, University of Zagreb, in the field of molecular epizootiology of pathogenic bacteria. The topic of his dissertation was the molecular characterisation of different strains of pathogenic bacteria Leptospira spp. Scientific activity is focused on the investigation of infectious diseases in animals, particularly zoonoses as well as the investigation of the role of stem cells in therapy. His role as a mentor was in the development of the idea of the proposed investigation as well as an adviser in all aspects necessary for the preparation of this dissertation. Up to now, according to Scopus, he published 73 scientific papers with 957 citations and an h-index of 16.

Najveću zahvalnost upućujem svojem mentoru, prof. dr. sc. Nenadu Turku, na uloženom trudu, nesebičnoj podršci i vodstvu tijekom čitavog razdoblja doktorata. Njegovi savjeti, ispravci i poticaji nisu bili samo profesionalna smjernica, već i dragocjen izvor motivacije, ohrabrenja i stalne inspiracije za moj daljnji rad.

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ABSTRACT

Leptospirosis is a re-emerging zoonosis of global importance caused by pathogenic spirochetes of the genus *Leptospira*, affecting humans, domestic and wild animals. Recent evidence indicates an increasing dominance of *Leptospira* spp. serogroup Pomona in various hosts, which is associated with severe clinical manifestations. In this dissertation, it was hypothesised that serogroup Pomona is re-emerging as the most virulent within the pathogenic *Leptospira* complex and is becoming a dominant infectious entity in different animal species. The research was based on three studies that included horses, cats and small rodents as representative hosts. Horses and cats represent two extremes in susceptibility to *Leptospira* spp. infection and clinical manifestation, while rodents serve as the main reservoir and play a crucial role in environmental persistence. In horses, a ten-year serological study of 61,724 samples revealed a seroprevalence of 10.8 %, with the serogroup Pomona emerging as the most prevalent and showing a clear increasing trend over time. In contrast to previous reports, most horses appeared clinically healthy, suggesting a possible evolutionary adaptation of Pomona to the equine host. In cats, a one-year survey of 54 pets revealed a remarkable seroprevalence of 18.5 %, with Pomona again identified as the most common serogroup. Importantly, the results show that infected cats can develop clinical signs, particularly in immunocompromised individuals or with comorbidities, and the results suggest a possible link between Pomona seropositivity and respiratory manifestations. Finally, whole genome sequencing of 48 isolates collected from small rodents over a 14-year period revealed that the Croatian isolates form a distinct, geographically restricted lineage of the *L. kirschneri* serogroup Pomona, most likely corresponding to the serovar Mozdok, suggesting long-term adaptation to the host. Overall, the convergence of results from horses, cats and small rodents shows that Pomona is not a sporadic occurrence but a re-emerging serogroup with established ecological stability, high pathogenic potential and significant impact in the context of One Health. These findings form the basis for the development of improved diagnostics, the design of targeted preventive measures and the prioritisation of future vaccine development to contain the growing threat from this serogroup.

Keywords: leptospirosis; *Leptospira* spp.; serogroup Pomona; horses; cats; small rodents; seroprevalence; whole genome sequencing; re-emergence; Croatia; One Health

PROŠIRENI SAŽETAK

UVOD: Leptospiroza je reemergentna zarazna bolest brojnih domaćih i divljih životinja te čovjeka, uzrokovana patogenim bakterijama iz roda *Leptospira*. Globalno je rasprostranjena zoonoza, često klinički neprepoznata, a time i zapostavljena bolest. Leptospire su genetski i imunološki heterogeni mikroorganizmi, trenutačno podijeljeni na 69 vrsta i više od 300 patogenih serovarova organiziranih u 30 seroloških skupina. Varijacije u virulenciji serovarova ili sojeva leptospira, prijemljivost i imunosni status domaćina te infektivna doza, dovode do različitih kliničkih očitovanja koja mogu varirati od blažih ili subkliničkih do izrazito teških oblika sa smrtnim ishodom. Sitni glodavci poput štakora, miševa i voluharica glavni su rezervoari leptospira ključni za njihovo održavanje u prirodi. Nakon infekcije kontinuirano ili povremeno izlučuju leptospire urinom tijekom cijeloga života. Ljudi i životinje mogu se zaraziti izravnim kontaktom s inficiranim urinom ili neizravno putem kontaminirane vode ili tla. Kod nekih životinjskih vrsta dolazi do prilagodbe određenim serovarovima, što rezultira asimptomatskim infekcijama te takve životinje predstavljaju održavajuće domaćine. Kod drugih vrsta životinja infekcija istim serovarovima može izazvati teške kliničke posljedice te one predstavljaju slučajne domaćine. Konji i mačke predstavljaju dvije krajnosti u smislu osjetljivosti na infekciju leptospirama i kliničkog očitovanja. Konji se smatraju izrazito imunogenom vrstom ili hiperreaktivnim domaćinima jer razvijaju snažan imunološki odgovor na *Leptospira* spp., što rezultira značajnom proizvodnjom protutijela i izraženim kliničkim očitovanjima. Nasuprot tome, smatralo se da mačke ne obolijevaju od leptospiroze i da su rezistentne budući da su često u kontaktu s glodavcima i infekcije su obično asimptomatske ili blage. Osim toga, konji proizvode značajno veći volumen alkalnog urina, što pogoduje preživljavanju leptospira radi čega se smatraju značajnim izlučivačima, dok mačke proizvode manju količinu kiselog urina, nepovoljnog pH za leptospire radi čega se mačke ne smatraju značajnim izlučivačima. Zanimljivo je da obje vrste mogu predstavljati održavajuće i slučajne domaćine. Trenutno nije poznato koji serovarovi uzrokuju slučajne infekcije, a koji su razvili prilagodbe određenim vrstama. Obzirom na to da sitni glodavci predstavljaju glavne rezervoare leptospira, njihova uloga u epizootio/epidemiološkom ciklusu leptospiroze u Republici Hrvatskoj istraživana je dugi niz godina. Oni ne samo da omogućuju dugotrajno preživljavanje i održavanje leptospira u prirodi, već pružaju uvid u trenutačno cirkulirajuće serovarove patogenih leptospira. Serološka skupina Icterohaemorrhagiae oduvijek se smatrala najpatogenijom unutar širokog spektra patogenih leptospira i povezivala se s teškim kliničkim oboljenjima. Međutim, posljednjih dvadesetak godina posebno se ističe serološka skupina

Pomona, koja se povezuje s pojavom novog kliničkog oblika leptospiroze - plućnog hemoragijskog sindroma kod pasa, konja i ljudi. Pretpostavlja se da je serološka skupina Pomona trenutno najpropulzivnija od svih leptospira u smislu patogenosti. Dosadašnja istraživanja u Hrvatskoj ukazuju na povezanost teških slučajeva leptospiroze i serološke skupine Pomona, osobito kod pasa i ljudi. Dijagnostika bolesti otežana je radi složene taksonomije roda *Leptospira*. Zbog nespecifičnih kliničkih znakova i poteškoća u izdvajanju leptospira na hranidbenim podlogama, dijagnostika i epizootio/epidemiološka istraživanja uglavnom se temelje na serološkim i molekularnim metodama. Standardni serološki test je mikroskopski test aglutinacije (engl. *microscopic agglutination test*, MAT), a od molekularnih metoda u svrhu dijagnostike najviše su u upotrebi lančana reakcija polimerazom (engl. *polymerase chain reaction*, PCR) i lančana reakcija polimerazom u stvarnom vremenu (engl. *real-time polymerase chain reaction*, qPCR). PCR proizvodi iz pozitivnih kliničkih uzoraka mogu se sekvencirati kako bi se identificirala vrsta leptospira koja uzrokuje infekciju. Međutim, koncentracija DNA *Leptospira* spp. u kliničkim uzorcima često je vrlo niska, što utječe na dobivanje visokokvalitetnih podataka putem sekvenciranja. S druge strane, uspješno izdvajanje *Leptospira* spp. na hranidbenim podlogama omogućuje primjenu naprednih molekularnih tehnika, poput sekvenciranja cijeloga genoma (engl. *whole genome sequencing*, WGS), za identifikaciju i detaljnu analizu specifičnih sojeva. Sekvenciranje cijeloga genoma omogućava proučavanje razlika u strukturi genoma, što je posebno korisno kada se analiziraju sojevi *Leptospira* spp. izdvojeni iz specifičnih geografski ili ekološki različitih područja. Genomska obilježja lokalnih sojeva omogućuju usporednu analizu s drugim sekvenciranim genomima patogenih *Leptospira* spp., što doprinosi razvoju dijagnostičkih testova i vakcina, razumijevanju raznolike patogenosti *Leptospira* spp. te pruža genetske i epizootio/epidemiološke informacije koje mogu unaprijediti postojeće spoznaje o infekcijama izazvanim patogenim leptospirama. U Republici Hrvatskoj leptospiroza je endemska bolest i predstavlja značajni javnozdravstveni problem. Iako je jedna od najčešćih zoonoza globalnog značaja, leptospiroza ostaje nedovoljno dijagnosticirana i zapostavljena bolest, što zahtijeva sustavno praćenje i daljnja istraživanja.

HIPOTEZA I CILJEVI: Ovo istraživanje temelji se na hipotezi da serološka skupina Pomona postepeno preuzima vodeću ulogu najpatogenije leptospire unutar kompleksa patogenih leptospira te da njezin spektar djelovanja postaje dominantan u različitim vrstama životinja. Da bi se ova hipoteza potvrdila ili opovrgnula, postavljeni su opći i specifični ciljevi. Opći cilj istraživanja je dokazati reemergentnost *Leptospira* spp. serološke skupine Pomona. Specifični

ciljevi uključuju istraživanje seroprevalencije u konja i mačaka, dvije životinjske vrste koje predstavljaju krajnosti u prijemljivosti leptospirama, zatim utvrđivanje zastupljenosti serološke skupine Pomona kao vjerojatno infektivne u konja i mačaka te analizu cijeloga genoma *Leptospira* spp. serološke skupine Pomona u sojeva izdvojenih iz sitnih glodavaca kao glavnih rezervoara leptospira tijekom dužeg vremenskog perioda i utvrđivanje njihova genomska obilježja te raznolikost.

MATERIJAL I METODE: Ovaj doktorski rad temelji se na tri znanstvena rada koja obuhvaćaju istraživanja provedena na konjima, mačkama i sitnim glodavcima u Republici Hrvatskoj. U prvom znanstvenom radu analizirano je ukupno 61 724 ostatnih uzoraka seruma konja iz arhive Laboratorija za leptospire, prikupljenih u razdoblju od deset godina (2012.–2022.). Reprezentativni uzorci potjecali su od klinički naizgled zdravih konja različitih pasmina, dobi i spola iz različitih geografskih područja Hrvatske. U drugom znanstvenom radu obrađeni su ostatni uzorci pune krvi, seruma i urina od ukupno 54 mačke koje su tijekom jednogodišnjeg razdoblja (2022.–2023.) zaprimljene u Sveučilišnu veterinarsku bolnicu radi različitih kliničkih indikacija. Za sve mačke bili su dostupni uzorci seruma i urina, dok su uzorci pune krvi prikupljeni od ukupno 27 mačaka koje su zadovoljile barem jedan od sljedećih kriterija: izostanak antibiotske terapije, prisutnost imunosupresivnog stanja (retrovirusne infekcije, tumori, dijabetes melitus, imunosupresivna terapija) ili hematološki poremećaji koji ukazuju na anemiju, trombocitopeniju i/ili leukocitozu. Uzorci su prikupljeni u svrhu rutinske obrade pacijenta, a pohranjeni u Laboratoriju Klinike za unutarnje bolesti i Bakteriološkom laboratoriju. Ostatni uzorci seruma pohranjeni su na -20 °C do serološke analize, dok su ostatni uzorci pune krvi i urini pohranjeni na 4 °C i unutar 24 sata preliminarno obrađeni centrifugiranjem i ispiranjem u svrhu pripreme za daljnji protokol izdvajanja DNA. Dio ostatnih uzoraka urina odmah je pohranjen na na -20 °C do izdvajanja DNA. Podaci o epizootiološkim čimbenicima, kliničkim znakovima i laboratorijskim nalazima zabilježeni su za svaku mačku pretraživanjem zapisa iz ambulantnog protokola. U trećem znanstvenom radu analizirano je 48 izolata *Leptospira* spp. serološke skupine Pomona izdvojenih iz bubrega sitnih glodavaca. Uzorci su prikupljeni tijekom četrnaestogodišnjeg razdoblja (2005.–2018.) na različitim lokacijama u Hrvatskoj i dio su zbirke patogenih leptospira Laboratorija za leptospire gdje su se održavali u Korthof i Fletcher hranidbenim podlogama. Vrste sitnih glodavaca *Apodemus agrarius* i *Microtus lavernedii* bilo je moguće morfološki determinirati, dok su *Apodemus flavicollis* i *Apodemus sylvaticus* morfološki nerazlučivi te je determinacija vrste učinjena PCR metodom za mitohondrijski gen *citokrom b* uz daljnje sekvenciranje. Uzorci seruma iz prvog i

drugog znanstvenog rada pretraženi su referentnom serološkom metodom mikroskopske aglutinacije na prisutnost protutijela za leptospire. Za uzorke seruma konja koristio panel antigena od osam serovarova patogenih *Leptospira* spp., a za uzorke mačaka panel od 12 patogenih serovarova. Pripremljena su dvostruka serijska razrjeđenja s fosfatnim puferom te nakon inkubacije, rezultat je očitan mikroskopom s tamnim vidnim poljem. Konačan titar predstavljalo je najveće razrjeđenje seruma koje pokazuje najmanje 50 % aglutiniranih leptospira. Granična vrijednost za mačke je iznosio titar 1:50, dok za konje 1:200 za serovar Bratislava i 1:400 za ostale serovarove. Vjerojatno infektivna serološka skupina je određena najvišim titrom za jedan ili više serovarova koji pripadaju određenoj serološkoj skupini. Molekularne metode provedene u drugom radu uključivale su izdvajanje DNA, *real-time* PCR i konvencionalni PCR s naknadnim Sanger sekvenciranjem. Statistička analiza podataka iz prvog i drugog znanstvenog rada provedena je uporabom programa R, Statistica i MedCalc. Izolati *Leptospira* spp. iz trećeg rada, serološki su tipizirani panelom od 14 referentnih hiperimunih seruma proizvedenih na kunićima (*OIE Reference Laboratory for Leptospirosis*, AMC, Nizozemska). Istraživani izolat pripadao je onoj serološkoj skupini s čijim je hiperimunim serumom aglutinacija vidljiva u najvećem titru. Izolati koji su pripadali serološkoj skupini Pomona analizirani su sekvenciranjem cijeloga genoma na Illumina MiSeq platformi, a rezultati su obrađeni bioinformatičkim alatima korištenjem Nextflow radnog tijeka bioinformatičkog tima laboratorija *Zoonoses and Select Agent Laboratory* (ZSAL), Centra za kontrolu i prevenciju bolesti (CDC), Atlanta, Georgia, SAD. Provedene genomske analize obuhvatile su prosječnu sličnost nukleotida (engl. *average nucleotide identity*, ANI), pangenomsku analizu, tipiziranje na osnovi multilokusnih sekvenci (engl. *multilocus sequence typing*, MLST), sekvenciranje ključnih regija genoma (engl. *core genome multilocus sequence typing*, cgMLST), analizu polimorfizama pojedinačnih nukleotida cijeloga genoma (engl. *whole genome single nucleotide polymorphism*, whole genome SNP) te filogenetske analize.

REZULTATI I RASPRAVA: Provedenim istraživanjima ovaj doktorski rad daje detaljan uvid u epizootiološku situaciju leptospiroze u Republici Hrvatskoj gdje je ova bolest endemski prisutna, s posebnim naglaskom na serološku skupinu Pomona. U desetogodišnjem serološkom istraživanju provedenom na 61 724 seruma konja zabilježena je ukupna seroprevalencija od 10,8 %. Najčešće utvrđena vjerojatno infektivna serološka skupina bila je Pomona koja je tijekom promatranog razdoblja pokazala jasan trend porasta i postupno preuzela dominaciju nad ostalim serološkim skupinama. Većina serološki pretraženih konja bila je naizgled klinički zdrava, što otvara mogućnost da se Pomona djelomično prilagodila na konje kao domaćina,

održavajući se u populaciji uz minimalna klinička očitovanja, za razliku od ostalih studija koje su zabilježile povezanost infekcije serološkom skupinom Pomona i izraženih kliničkih očitovanja poput periodične oftalmije (engl. *equine recurrent uveitis*, ERU) i akutnih pobačaja. U jednogodišnjem istraživanju provedenom na 54 mačke utvrđena je seroprevalencija od 18,5 %, pri čemu je Pomona ponovno bila najzastupljenija serološka skupina. Rezultati su pokazali da mačke, iako se tradicionalno smatraju rezistentnima na leptospirozu, mogu razviti kliničke znakove bolesti, osobito u slučajevima imunosupresije i prisutnih komorbiditeta. Najčešće su zabilježeni simptomi poput anoreksije, letargije, povraćanja te respiratornih poremećaja, pri čemu je u seropozitivnih mačaka uočena potencijalna povezanost respiratornih očitovanja sa serološkom skupinom Pomona kao vjerojatno infektivnom. Učestalost urinarnog izlučivanja leptospira bila je niska (1,85 %), no i ovakav nalaz potvrđuje da mačke mogu doprinijeti širenju uzročnika u okolišu, iako vjerojatno u ograničenom opsegu radi općenito kiselog pH urina i visokog osmolaliteta koji otežavaju preživljavanje leptospira u urinu mačaka. Analizom 48 izolata *Leptospira* spp. serološke skupine Pomona prikupljenih iz bubrega sitnih glodavaca tijekom četrnaestogodišnjeg razdoblja potvrđena je njihova pripadnost vrsti *L. kirschneri*, najvjerojatnije serovaru Mozdok. Genomskim analizama (ANI, pangenomska analiza, cgMLST, SNP i filogenetske analize) utvrđeno je da hrvatski izolati čine zasebnu, genetski homogenu i geografski ograničenu liniju. MLST-om je utvrđena pripadnost sekvencijskom tipu ST-98, a cgMLST-om je ustanovljena podjela u sedam genotipskih klastera (označenih kao A-G) s jasnim geografskim obrascima u većine uzoraka. Filogenetskom analizom temeljenom na polimorfizmima pojedinačnih nukleotida (SNP) među hrvatskim uzorcima uočena su grupiranja povezana s geografskom lokacijom, dok je u usporedbi s drugim *L. kirschneri* dostupnim genomima iz Nacionalnog centra za biotehnološke informacije (NCBI) ustanovljeno grupiranje s nekoliko uzoraka iz Brazila i nepoznate lokacije koji su također pripadali ST-98 te su determinirani kao *L. kirschneri* serovar Mozdok. Unatoč tome, svi hrvatski izolati čine filogenetski dobro podržanu monofiletičku skupinu, što upućuje na njihovu blisku genetsku srodnost i sugerira postojanje regionalno dominantne linije koja cirkulira u Republici Hrvatskoj. Ovi nalazi potvrđuju važnu ulogu glodavaca kao primarnih domaćina serološke skupine Pomona te čine molekularni dokaz njezine dugotrajne evolucijske prisutnosti u Republici Hrvatskoj. Kombinirani rezultati iz konja, mačaka i glodavaca jasno pokazuju da Pomona nije sporadična pojava, već reemergentna serološka skupina sa stabilnim ekološkim ciklusom, visokim patogenim potencijalom i kliničkom važnošću. Ovi nalazi imaju šire implikacije u okviru pristupa ‘Jedno zdravlje’ jer ukazuju na stalnu izloženost različitih

životinjskih vrsta, ali i ljudi, ovoj serološkoj skupini. Stoga rezultati naglašavaju potrebu za integriranim dijagnostičkim i preventivnim strategijama, kao i razvojem učinkovitih cjepiva protiv serološke skupine Pomona.

ZAKLJUČCI: Zaključci ovih triju znanstvenih radova objavljenih u okviru doktorskog rada potvrđuju da je Pomona reemergentna serološka skupina koja je dominantna u različitim vrstama životinja te preuzima vodeću ulogu najpatogenije leptospire unutar kompleksa patogenih leptospira u Hrvatskoj. Prvi rad prikazuje da je Pomona tijekom posljednjeg desetljeća postala najčešće zastupljena serološka skupina u konja, uz jasan trend porasta koji ukazuje na dinamičnu promjenu u epizootiologiji leptospiroze u Hrvatskoj. Nadalje, drugi rad potvrđuje da se Pomona pojavila i u mačaka kao vodeća serološka skupina, uz dokaze da infekcija može rezultirati kliničkim očitovanjima, osobito u imunosuprimiranih životinja, što upućuje na podcijenjenu ulogu mačaka u epizootiologiji leptospiroze. Na kraju, treći rad donosi rezultate sekvenciranja cijeloga genoma izolata *Leptospira* spp. iz glodavaca koji su potvrdili da hrvatski izolati čine zasebnu, geografski ograničenu liniju *L. kirschneri* serološke skupine Pomona, najvjerojatnije serovara Mozdok, što ukazuje na dugotrajnu prilagodbu rezervoaru i potvrđuje njihovu ulogu kao značajnog izvora infekcija. Kombinirani nalazi u konja, mačaka i glodavaca pružaju jasan dokaz da Pomona nije sporadična pojava, već reemergentna serološka skupina s utvrđenom ekološkom stabilnošću, visokim patogenim potencijalom i značajem u okviru koncepta ‘Jedno zdravlje’.

KLJUČNE RIJEČI: leptospiroza; *Leptospira* spp.; serološka skupina Pomona; konji; mačke; sitni glodavci; seroprevalencija; molekularne metode; sekvenciranje cijeloga genoma; reemergentnost; Republika Hrvatska; Jedno zdravlje

ABBREVIATIONS

A.	*Apodemus*
ANI	Average Nucleotide Identity
BIGSdb	Bacterial Isolate Genome Sequence Database
CAAT	Cross-Agglutination Absorption Tests
CBC	Complete Blood Count
CDC	Centers for Disease Control and Prevention
cgMLST	Core Genome Multilocus Sequence Typing
cgST	Core Genome Sequence Type
CI	Confidence Interval
DIC	Disseminated Intravascular Coagulation
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERU	Equine Recurrent Uveitis
GFF3	General Feature Format version 3
IS	Insertion Sequence
L.	*Leptospira*
LPHS	Leptospiral Pulmonary Haemorrhagic Syndrome
LPS	Lipopolysaccharide
M.	*Microtus*
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MAT	Microscopic Agglutination Test
MLST	Multilocus Sequence Typing
MLVA	Multiple-Locus Variable-Number Tandem Repeat analysis

NAAT	Nucleic Acid Amplification Test
NCBI	National Center for Biotechnology Information
ND	Nondetermined
NGS	Next-Generation Sequencing
OR	Odds Ratio
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
qPCR	Real-time Polymerase Chain Reaction
RefSeq	Reference Sequence Database
RFLP	Restriction Fragment Length Polymorphism
SBS	Sequencing by Synthesis
SNP	Single Nucleotide Polymorphism
spp.	Species pluralis
ST	Sequence Type
VNTR	Variable Number of Tandem Repeats
WGS	Whole Genome Sequencing
ZSAL	Zoonoses and Select Agent Laboratory

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1. INTRODUCTION

1.1. General overview of leptospirosis

Leptospirosis is a re-emerging infectious disease with worldwide distribution caused by pathogenic bacteria of the genus *Leptospira* (LEVETT, 2001). This ubiquitous disease affects humans, domestic and wild animals and has even been found in birds, amphibians, reptiles and fish (PICARDEAU, 2017). Although leptospirosis is a widespread zoonotic infection that occurs on all continents except Antarctica, it is often underdiagnosed and neglected (PAPPAS et al., 2008). Global factors such as climate change and urbanisation, together with environmental and socio-economic conditions, have a significant impact on the increasing trend of leptospirosis, with warm, humid climates, heavy rainfall, flooding and inadequate housing infrastructure increasing the risk of infection (HARTSKEERL et al., 2011; BAHAROM et al., 2024; MUÑOZ-ZANZI et al., 2025).

Leptospirosis is a systemic disease with variable clinical signs, usually characterised by fever, renal and hepatic insufficiency, pulmonary manifestations and reproductive failure (ADLER and DE LA PEÑA MOCTEZUMA, 2010). Worldwide, leptospirosis is estimated to cause more than 1 million severe human cases per year, resulting in approximately 58,900 deaths and 2.9 million disability-adjusted life years (DALYs) lost per year (COSTA et al., 2015). A comparable incidence of infection and the role it plays in causing serious illness and death has been estimated in companion animals, livestock and wildlife (SYKES et al., 2022). In addition to the impact on public health, leptospirosis also causes considerable financial and economic losses. In particular, the infection of livestock leads to a significant global economic burden due to reduced productivity, reproductive losses and increased veterinary costs (CARVALHO et al., 2024).

1.2. History of leptospirosis

The significance of leptospirosis has been known for more than a century, with important milestones marking the history of the disease. Although many ancient texts are thought to describe diseases consistent with leptospirosis, the modern history of the disease began in 1886 when Adolph Weil provided the first detailed clinical description, characterizing it by jaundice, splenomegaly, renal dysfunction, conjunctivitis and skin rashes (WEIL, 1886). In recognition of his contribution, the icteric form of the disease in humans is still referred to as Weil's disease. The causative agent was first observed in 1907 by Stimson, who used Levaditi silver staining to detect spirochetes in the kidneys of patients who had died of yellow fever and named the

organism *Spirochaeta interrogans* (STIMSON, 1907). The first successful isolation of the pathogen took place in Japan in 1915, when blood from infected patients was injected intraperitoneally into guinea pigs (INADA and IDO, 1915). Almost simultaneously and independently of this, the agent was isolated in the same way in Germany by two groups of doctors who examined infected German soldiers (HÜBENER and REITER, 1915; UHLENHUTH and FROMME, 1915).

In Croatia, leptospirosis was first confirmed in a dog in 1926 (BABIĆ, 1927), while the first human case was reported in 1935 (ANTUNOVIĆ-MIKAČIĆ, 1935). Croatia occupies a notable place in the history of leptospirosis research, as it was the second country in the world to detect an infection in horses, with *Leptospira* Pomona being isolated in 1951 (ZAHARIJA, 1953). Remarkably, Croatia was also the first country in Europe and the third in the world to document leptospirosis in cats. Carrier status in clinically healthy cats was first reported in 1974 with Australis (MODRIĆ, 1978), followed by evidence of Pomona, Icterohaemorrhagiae and Bataviae in 1977 (MODRIĆ, 1979).

Since then, advances in microbiology and molecular biology have increased knowledge of the complex taxonomy, epidemiology and clinical manifestations and highlighted leptospirosis as a zoonosis of significant importance to veterinary medicine and public health worldwide.

1.3. Etiology of leptospirosis

1.3.1. Taxonomy and systematics of leptospires

Leptospires are spiral-shaped bacteria belonging to the genus *Leptospira*, the family *Leptospiraceae* and the order *Spirochaetales* (FAINE et al., 1999).

Due to their remarkable ability to adapt to different hosts and environmental conditions, leptospires are genetically and immunologically heterogeneous microorganisms. In the past, the genus *Leptospira* was divided into two species: pathogenic (*L. interrogans*) and saprophytic (*L. biflexa*), based on phenotypic characteristics such as growth at 13 °C and in the presence of 8-azaguanine, and the ability to form spherical cells in 1 M NaCl (JOHNSON and FAINE, 1984). However, the phenotypic approach proved to be unreliable and insufficient (ALEXANDER et al., 2015).

The heterogeneity among leptospires has led to the development of two complementary classification systems: a serological system based on differences in the carbohydrate component of the outer lipopolysaccharide membrane and a genomic system based on genetic relatedness. Traditionally, leptospires have been divided into two main groups by serological typing:

pathogenic (*L. interrogans*) and saprophytic (*L. biflexa*), with serovars defined using cross-agglutination absorption tests (CAAT) with homologous antigen (DIKKEN and KMETY, 1978; KMETY and DIKKEN, 1993). The serovars are categorised into serogroups on the basis of their antigenic relatedness. Accordingly, serological typing has shown that the genus comprises more than 60 serovars of *L. biflexa* grouped into three serogroups, and more than 300 serovars of *L. interrogans* grouped into 30 serogroups (FAINE and STALLMAN, 1982; KMETY and DIKKEN, 1993).

With the advances in molecular methods and sequence analysis, the genus has been subdivided into 69 genomic species that are further classified into four subclades: pathogenic (P1), intermediate pathogenic (P2) and two saprophytic groups (S1 and S2) (Figure 1) (VINCENT et al., 2019; KORBA et al., 2021; FERNANDES et al., 2022).

It is important to emphasise that the serological and genomic classification systems do not always correlate directly with each other, which is why both systems are still in use. Indeed, it is now recognised that serogroup and serovar do not reliably correspond to *Leptospira* species, as pathogenic and non-pathogenic serovars can occur within the same species (ALEXANDER et al., 2015).

Differences in the pathogenicity of the species and serovars, the susceptibility and immune response of the hosts and the infectious dose lead to different clinical manifestations, ranging from mild or subclinical infections to severe, life-threatening outcomes (LEVETT, 2001; ADLER and DE LA PEÑA MOCTEZUMA, 2010).

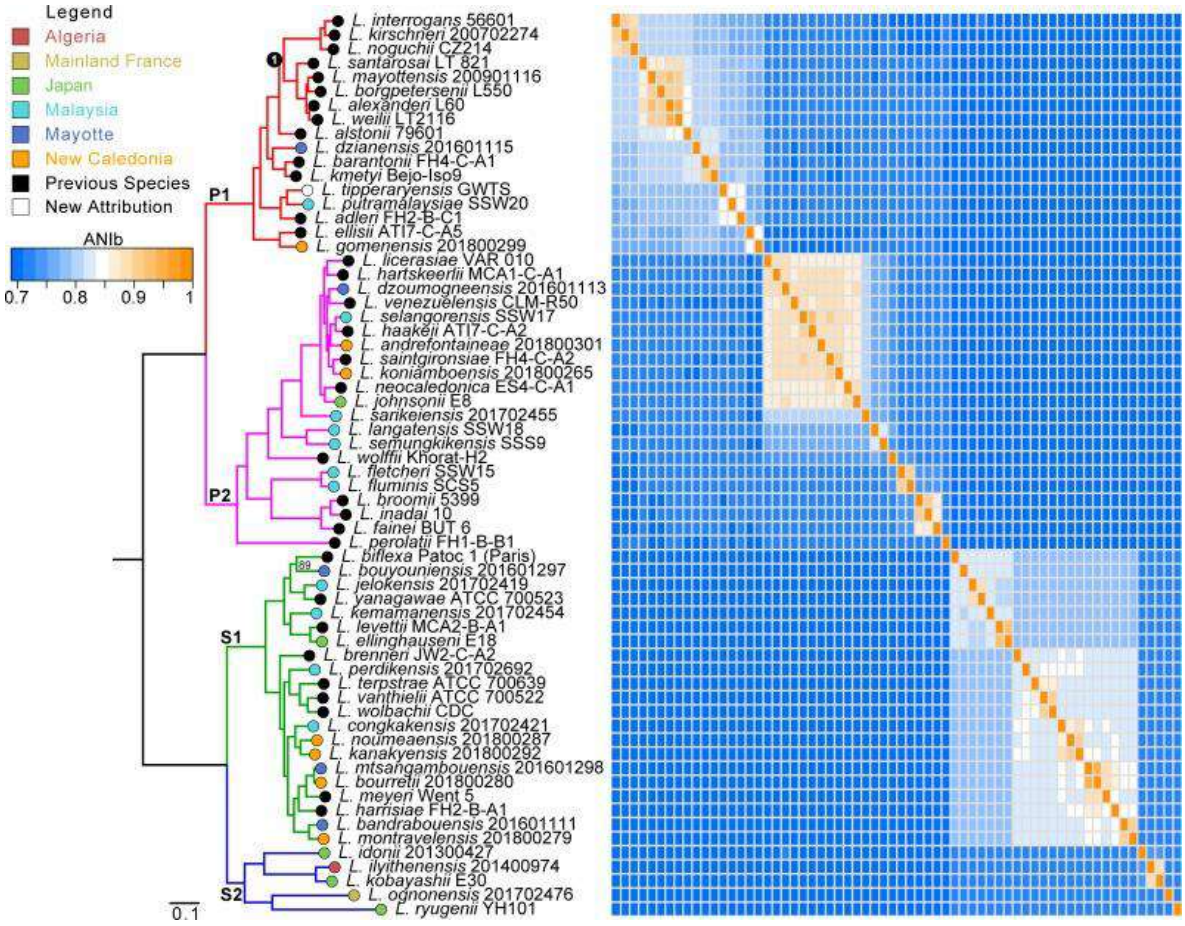

Figure 1. Phylogenetic tree based on the sequences of 1371 genes inferred as orthologues. The matrix represents the calculated ANIb values for all genomic sequences. The branches are coloured according to their affiliation to the four main subclades: P1 (red), P2 (purple), S1 (green) and S2 (blue). According to the legend, a coloured circle represents the geographical origin of each of the newly described species. Node 1 indicates the node from which the pathogenic species most frequently involved in human diseases originate (VINCENT et al., 2019).

1.3.2. Morphological and phenotypic characteristics of leptospires

Leptospires are thin, filamentous, highly motile bacteria with a length of about 6–20 µm and a width of about 0.1 µm, with characteristic hook-shaped ends (ADLER and DE LA PEÑA MOCTEZUMA, 2010). In native preparations, leptospires can be visualised most effectively using dark-field microscopy (CAMERON, 2015). Alternatively, they can also be observed under a light microscope using special silver-based staining techniques such as Levaditi, Warthin-Starry or Dieterle (FAINE et al., 1999). Leptospires have a double membrane structure with a cytoplasmic membrane and a peptidoglycan cell wall overlaid by an outer membrane, in which lipopolysaccharide (LPS) is the main antigen, structurally and immunologically similar to that of Gram-negative bacteria (Figure 2B) (CULLEN et al., 2004). Motility is enabled by

two periplasmic flagella with polar insertions located in the periplasmic space that allow active movement by anti-clockwise rotation, resulting in a spiral-shaped end, and by clockwise rotation, resulting in a hook-shaped end (Figure 2A) (WOLGEMUTH et al., 2006). Leptospires are obligate aerobes that grow optimally at 28–30 °C and a pH value of 7.2–7.6. Pathogenic leptospires can grow at 37 °C, but not at low temperatures compared to saprophytic strains that can grow at 11–13 °C (CAMERON, 2015).

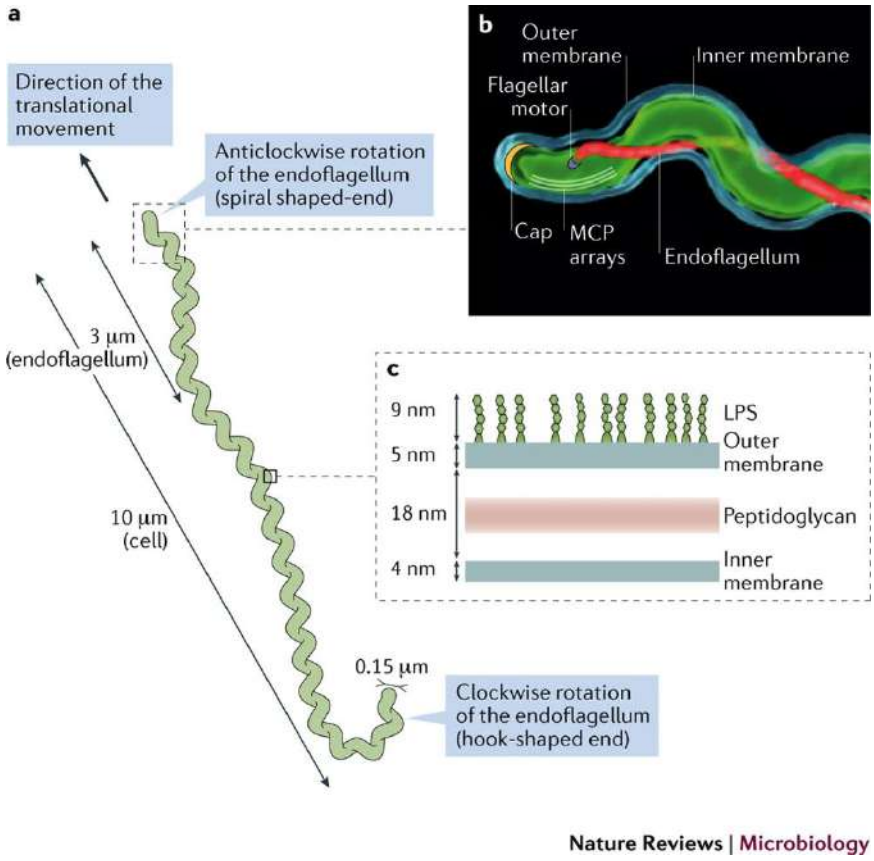

Figure 2. Cell wall morphology and envelope architecture of *Leptospira interrogans*.
(A) Schematic representation of the spiral cell shape and the endoflagella responsible for motility. (B) Schematic representation of the envelope structure with inner and outer membranes, peptidoglycan layer and surface-exposed LPS (PICARDEAU, 2017).

For *in vitro* cultivation, they require media supplemented with long-chain fatty acids as the main carbon source, ammonium salts as the main nitrogen source and additional nutrients such as thiamine (vitamin B1), biotin (vitamin B7), phosphorus, calcium, magnesium, iron, copper, manganese and sulphate. As essential fatty acids contain toxic substances, detoxification is achieved by the addition of serum albumin (rabbit serum or bovine serum) or

polysorbates (Tween) (FAINE et al., 1999). Vitamin B12 was previously thought to be essential for the growth of pathogenic leptospires at 37 °C, but the discovery of the complete vitamin B12 biosynthetic operon has shown that it is not required (NASCIMENTO et al., 2004).

Leptospires can be stored long-term in semi-solid medium, but storage in liquid nitrogen is the preferred method for maintaining virulence (CAMERON, 2015).

1.3.3. General features of the Leptospira genome

The genome of *Leptospira* usually consist of at least two circular replicons, a large circular chromosome (cI) and a smaller replicon (cII). It is characterised by a guanine-cytosine content (G+C) of 35–42 % and a relatively large genome size of 3.9 to 4.6 Mbp, which varies depending on the species and strain (PICARDEAU, 2015). This genomic variability gives *Leptospira* an improved adaptability to different hosts and environmental conditions (PICARDEAU et al., 2008).

The larger chromosome (cI) with about 4,277 kb and a G+C content of 35 % has a gene density of 75-92 % and contains housekeeping genes. The smaller replicon (cII) with a size of about 350 kb and a G+C content of 35 % carries essential genes such as *metF* and *asd*, which code for the enzyme's methylenetetrahydrofolate reductase and aspartate semialdehyde dehydrogenase (ZUERNER et al., 1993; BOURHY and SAINT GIRONS, 2000). In addition, a third circular replicon (p74) with a size of 74 kb and a G+C content of 36 % was identified only in *L. biflexa* by whole genome sequencing (PICARDEAU et al., 2008). These additional replicons, cII and p74, can be considered as "chromids" (HARRISON et al., 2010) as they carry core genes that are normally located on larger chromosomes in other *Leptospira* species and share similar nucleotide composition and codon usage with large chromosomes (PICARDEAU et al., 2008). Pangenome analyses have shown the presence of a conserved "core genome" (essential genes) and a large "accessory genome" that contributes to genetic diversity and virulence (XU et al., 2016; VINCENT et al., 2019).

Comparative genome analyses have provided important insights into the diversity of saprophytic and pathogenic *Leptospira* species. These analyses have shown that the genomes of *L. biflexa* and *L. borgpetersenii* are similar in size, but the gene density is much higher in *L. biflexa*, which is due to insertion sequence (IS)-mediated genome erosion in *L. borgpetersenii*. This gene loss has led to a reduction in the size of the *L. borgpetersenii* genome by approximately 700 kb, which is associated with greater host dependence and diminished environmental survival (BULACH et al., 2006). In contrast, the larger genome of *L. interrogans* provides additional genetic information required for survival in both mammalian hosts and aquatic environments, whereas *L. biflexa* is more restricted to aquatic niches, and *L.*

borgpetersenii to mammalian hosts (PICARDEAU et al., 2008). Moreover, comparative genomics of pathogenic (*L. interrogans* and *L. borgpetersenii*) and saprophytic (*L. biflexa*) species has identified pathogen-specific genes (Figure 3), with genes coding for proteins of unknown function being significantly overrepresented. Among the proteins unique to *L. interrogans*, 78 % have no known function, indicating the possible presence of as yet undiscovered pathogenic mechanisms unique to *Leptospira* (PICARDEAU et al., 2008; ADLER et al., 2011). The genome of *L. biflexa* contains only five IS elements, in contrast to *L. interrogans* (36–69 IS elements, depending on strain) and *L. borgpetersenii* (167 IS elements) (PICARDEAU et al., 2008). A higher number of IS elements indicates genomic plasticity in *Leptospira* species, reflecting a less stable genome that is more prone to rearrangements and emphasises the evolutionary adaptability of pathogenic leptospires.

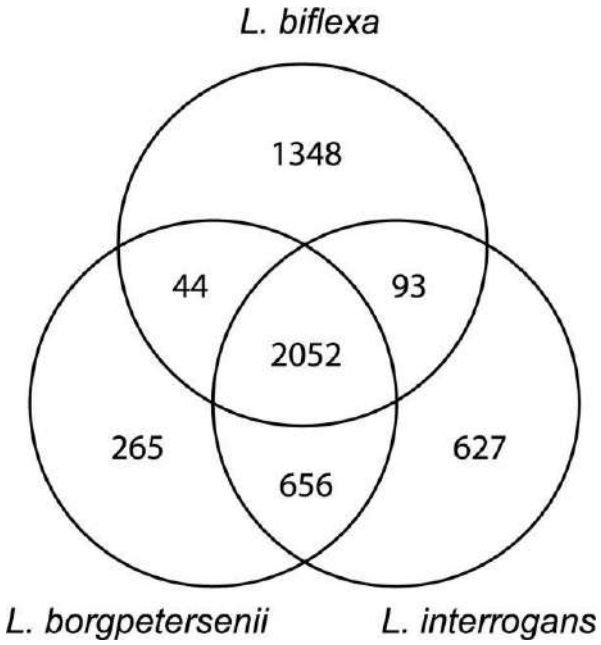

Figure 3. Comparative genomics of *Leptospira* spp. Venn diagram representing numbers of shared and species-specific genes of *L. interrogans*, *L. borgpetersenii*, and *L. biflexa* (PICARDEAU et al., 2008).

1.4. Epizootiology of leptospirosis

Small rodents such as rats, mice and voles serve as the main reservoirs for *Leptospira* spp. and play a crucial role in the persistence of the disease in the environment. Once infected, they are asymptomatic carriers, which excrete leptospires in their urine continuously or intermittently throughout their lifespan (LEVETT, 2001; ADLER and DE LA PEÑA

MOCTEZUMA, 2010). In humans and animals, infection occurs through mucous membranes and microlesions of the skin directly via infected urine or indirectly via contaminated water or soil (BARANTON and OLD, 1995; KO et al., 2009; MWACHUI et al., 2015). Direct transmission can also occur through the ingestion of infected animals or tissues, as well as via placental or venereal routes. Leptospires colonise and persist in the epithelial cells of the proximal renal tubules and are subsequently excreted in the urine (KO et al., 2009; ADLER and DE LA PEÑA MOCTEZUMA, 2010) and contaminate the environment, where they may remain pathogenic for as long as six to twenty months (ANDRE-FONTAINE et al., 2015; BIERQUE et al., 2020). Excretion in the urine of incidental hosts is usually of limited duration but may persist for up to six months after infection. Under favourable environmental conditions such as a neutral pH, humidity, and moderate temperatures, they can remain infectious for several months (LEVETT, 2001).

The ability of *Leptospira* spp. to form biofilms plays an important role in their survival in the environment and their persistence in the host (SILVA DIAS and PINNA, 2025). A biofilm is a structured community of microorganisms embedded in a self-produced extracellular polymeric matrix that adheres to surfaces and provides physical and chemical protection. Once attached, leptospires develop microcolonies that secrete a protective matrix, leading to the formation of a mature biofilm (TOYOFUKU et al., 2016). This ability favours the survival and dissemination, enabling leptospires to persist for prolonged periods in water, soil or on surfaces by protecting them from ultraviolet radiation, pH fluctuations, desiccation and other environmental stressors (THIBEAUX et al., 2020; SILVA DIAS and PINNA, 2025). Biofilm formation is also thought to contribute to the colonisation of renal tubules in animal hosts, supporting bacterial persistence and chronic urinary excretion. Furthermore, biofilm-associated leptospires show increased resistance to antimicrobial agents and the host immune response, which further enhances their epidemiological importance (CARVALHO et al., 2023; REZENDE MIRES DE CARVALHO et al., 2023; SILVA DIAS and PINNA, 2025).

Some animals had coadaptation with some serovars causing minimal pathological damage and asymptomatic infections, and these animals represent maintenance hosts. For example, the brown rat (*Rattus norvegicus*) is considered the maintenance host for the serovar Icterohaemorrhagiae, cattle and sheep for the serovar Hardjo, horses for the serovar Bratislava, pigs for the serovars Pomona, Tarassovi, and possibly Bratislava, and dogs for the serovar Canicola (ELLIS, 2015). Such hosts are an important source of infection as they may excrete leptospires in their urine for years, and leptospires often show tropism for other tissues, including the genital tract (ELLIS et al., 1986a, 1986b). However, under certain conditions,

such as immunocompromised states or the presence of comorbidities, maintenance hosts can develop a pronounced clinical disease (ELLIS, 2015).

In contrast, the same serovars may cause severe clinical consequences in other species that are considered incidental hosts. Incidental hosts usually develop acute clinical disease, and the shedding of leptospires is limited to a certain period after recovery, during which they may act as convalescent carriers (ELLIS, 2015). Humans are incidental hosts and are not a significant source of infection, although leptospires may be excreted in urine for several weeks (CHOW et al., 2012; HAAKE and LEVETT, 2015).

1.5. Susceptibility of animal hosts: Horses and cats as opposite animal models

The development and progression of leptospirosis are determined by the virulence of the infecting strain, the susceptibility of the host and the size of the inoculum (KO et al., 2009).

Horses and cats represent two extremes in terms of susceptibility to infection with *Leptospira* spp. and clinical manifestations. Horses are considered highly immunogenic or hyperreactive hosts that develop strong immune responses with significant antibody production and occasionally prominent clinical signs (ELLIS, 2015). Leptospirosis in horses manifests as lethargy, anorexia, jaundice, anaemia and renal dysfunction as well as reproductive disorders such as abortion, stillbirth or birth of infected foals (VERMA et al., 2013). Severe pulmonary forms such as acute respiratory distress syndrome and severe pulmonary haemorrhagic syndrome have also been reported (HAMOND et al., 2011; BROUX et al., 2012). A significant manifestation is equine recurrent uveitis (ERU), which is characterised by periodic ophthalmia and possible blindness (VERMA et al., 2010).

Cats, on the other hand, were considered resistant to leptospirosis because they frequently come into contact with rodents and are usually asymptomatic or show only mild signs (ELLIS, 2015). However, under certain conditions, such as a weakened immune system or the presence of comorbidities, cats may develop more pronounced clinical signs (MOREIRA DA SILVA et al., 2020; MAZZOTTA et al., 2023). When clinical signs occur in cats, these may include polyuria, polydipsia, lethargy, anorexia, gastrointestinal disturbances, haematuria, uveitis, lameness, weight loss, ascites and inflammatory skin lesions (MURILLO et al., 2020; MIOTTO et al., 2024). Kidney and liver damage have also been reported (RODRIGUEZ et al., 2014). Moreover, pulmonary haemorrhages have been described in both naturally and experimentally infected cats (BRYSON and ELLIS, 1976; MODRIĆ, 1978).

Although rats excrete the highest concentration of *Leptospira* per millilitre of urine (median = 5.7×10^6 cells), the total daily excretion in large animals is much greater (5.1×10^8

to 1.3 × 10^9 cells) due to the higher volume of urine they produce (BARRAGAN et al., 2017). This underlines that the volume, together with the physicochemical properties of the urine, determines the epizootiological/epidemiological relevance of the different animal hosts. In this context, horses produce a large amount of alkaline urine, which favours the survival of leptospires and makes them one of the most significant shedders, while cats produce a smaller amount of acidic urine in which pathogenic leptospires do not survive well (LEVETT, 2001). Although cats are not considered significant shedders, it has been reported that naturally infected cats can shed leptospires in urine under certain conditions, even without clinical signs (RODRIGUEZ et al., 2014; WEIS et al., 2017; ZAIDI et al., 2018). The estimated overall prevalence of *Leptospira* spp. in cat urine is 3.7 % (RICARDO et al., 2023) and 8 % (MIOTTO et al., 2024). Recent studies on seroprevalence in cats show varying exposure, with reported rates ranging from 0.26 % (GRIPPI et al., 2023) to 18.18 % (ALASHRAF et al., 2019) depending on geographical location. On the other hand, no meta-analyses or systematic reviews of seroprevalence or urinary excretion in horses have been conducted to date. However, some recent studies report urinary excretion in 8 % of asymptomatic horses (HAMOND et al., 2024) and up to 55 % of horses with acute disease onset (RAMSAY et al., 2024). In addition, recent studies have reported high seropositivity in horses, ranging from 28.57 % (DEWES et al., 2020) to 97.2 % (RIZZO et al., 2022).

Interestingly, both species can be both maintenance and incidental hosts. Currently, which serovars are responsible for incidental infections and which have developed adaptations to each species remain unknown.

1.6. Pathogenesis of leptospirosis

After entering the host, leptospires multiply rapidly in the bloodstream within a day of infection and may circulate for up to a week. They disseminate to several organs, especially the kidneys and liver, where replication and inflammation cause tissue damage (ADLER and DE LA PEÑA MOCTEZUMA, 2010). Bacteraemia usually begins 1–2 days after infection, with leptospires detectable in the blood, most organs and cerebrospinal fluid, and typically persists until circulating antibodies appear, which is usually within the first week (ELLIS, 2015). At this stage, the bacteria are generally cleared from the bloodstream and most tissues by opsonophagocytosis. However, leptospires colonise the proximal renal tubules, where they presumably survive by forming an amorphous biofilm-like structure (KO et al., 2009). They multiply in this niche and are excreted either intermittently or continuously in the urine, with a bacterial load of up to 10^8/ml (ADLER and DE LA PEÑA MOCTEZUMA, 2010). Leptospires

can also colonise the oviduct and uterus of females, the male genital tract (ELLIS et al., 1986a, 1986b; OLIVEIRA et al., 2007) and the mammary glands (THIERMANN, 1982).

The pathogenesis of leptospirosis is multifactorial and remains incompletely understood. The most important virulence factors include motility, adhesion and toxin production. Among the most extensively studied proteins are LipL32, OmpL1, Lig and Len, which are thought to be virulence factors and play a central role in pathogenesis. These surface proteins facilitate adhesion to components of the host extracellular matrix (fibronectin, elastin, collagen) and contribute to immune defence by conferring resistance to pathways of complement activation (KO et al., 2009; MURRAY, 2015). In addition, the OmpA-like outer membrane protein Loa22 is a key virulence factor that is essential for disease initiation (RISTOW et al., 2007). Motility mediated by endoflagella enables leptospires to rapidly penetrate mucous membranes and connective tissue and spread rapidly in the bloodstream (JOHNSON, 2018). The primary pathogenic mechanisms include damage to the endothelium of small blood vessels, leading to local ischaemia and necrosis in target organs such as the kidneys, liver and lungs (ADLER and DE LA PEÑA MOCTEZUMA, 2010; MURRAY, 2015). These processes may also lead to meningitis, myositis and placentitis. The secretion of numerous enzymes that degrade the membranes of the host cells further exacerbates the clinical manifestations (NASCIMENTO et al., 2004). Leptospirosis activates the coagulation cascade and causes thrombocytopenia and disseminated intravascular coagulation (DIC) (CHIERAKUL et al., 2008). It is hypothesised that severe disease progression is often associated with an exaggerated host immune response, including excessive release of cytokines, leading to tissue destruction (CAGLIERO et al., 2018). Furthermore, haemolysins have been detected in several leptospiral serovars, with genes encoding sphingomyelinases identified in pathogenic species. At least seven sphA-like genes have been described, including a putative pore-forming haemolysin, SphH, which is thought to play a role in tissue injury and haemolysis (ADLER and DE LA PEÑA MOCTEZUMA, 2010).

Immune-mediated reactions also contribute to chronic ocular complications, such as equine recurrent uveitis (ERU), in which cross-reactive antibodies against the leptospiral proteins LruA and LruB, which share epitopes with ocular tissues, are thought to play a role in immunopathogenesis (VERMA et al., 2010). In addition, one study suggests that ERU is associated with biofilm-associated intraocular leptospiral infection (ACKERMANN et al., 2021). Another severe manifestation is leptospiral pulmonary haemorrhagic syndrome (LPHS), which is increasingly recognised as a major cause of mortality in dogs and humans (KOHN et al., 2010; NICODEMO and DUARTE-NETO, 2021). LPHS is thought to be caused by toxins that target endothelial cells and bind to vascular cadherins, resulting in endothelial damage,

increased permeability and disruption of intercellular junctions (MEDEIROS et al., 2010; NICODEMO and DUARTE-NETO, 2021). It is characterised by acute and progressive intra-alveolar bleeding associated with coagulation abnormalities such as thrombocytopenia and DIC, vascular injury and immune-mediated mechanisms (MEDEIROS et al., 2010; SCHULLER et al., 2015).

1.7. Diagnosis of leptospirosis

The diagnosis of leptospirosis is a challenge due to the complex taxonomy of the genus *Leptospira*. Diagnostic methods for direct detection of the causative agent include culture, histopathology, immunostaining of tissues and clinical specimens, and nucleic acid amplification tests (NAATs). Indirect methods comprise serological approaches such as the microscopic agglutination test (MAT), the enzyme-linked immunosorbent assay (ELISA) and lateral flow assays (SYKES et al., 2022). Direct methods are particularly valuable in the early phase of the disease, while indirect methods have a higher sensitivity in the later course of the infection (Figure 4). Accordingly, these methods are interdependent and diagnosis should not rely on a single test but rather on a combination of factors, including potential exposure, clinical presentation, laboratory findings and the results of multiple diagnostic methods (NALLY et al., 2020; PHILIP et al., 2020).

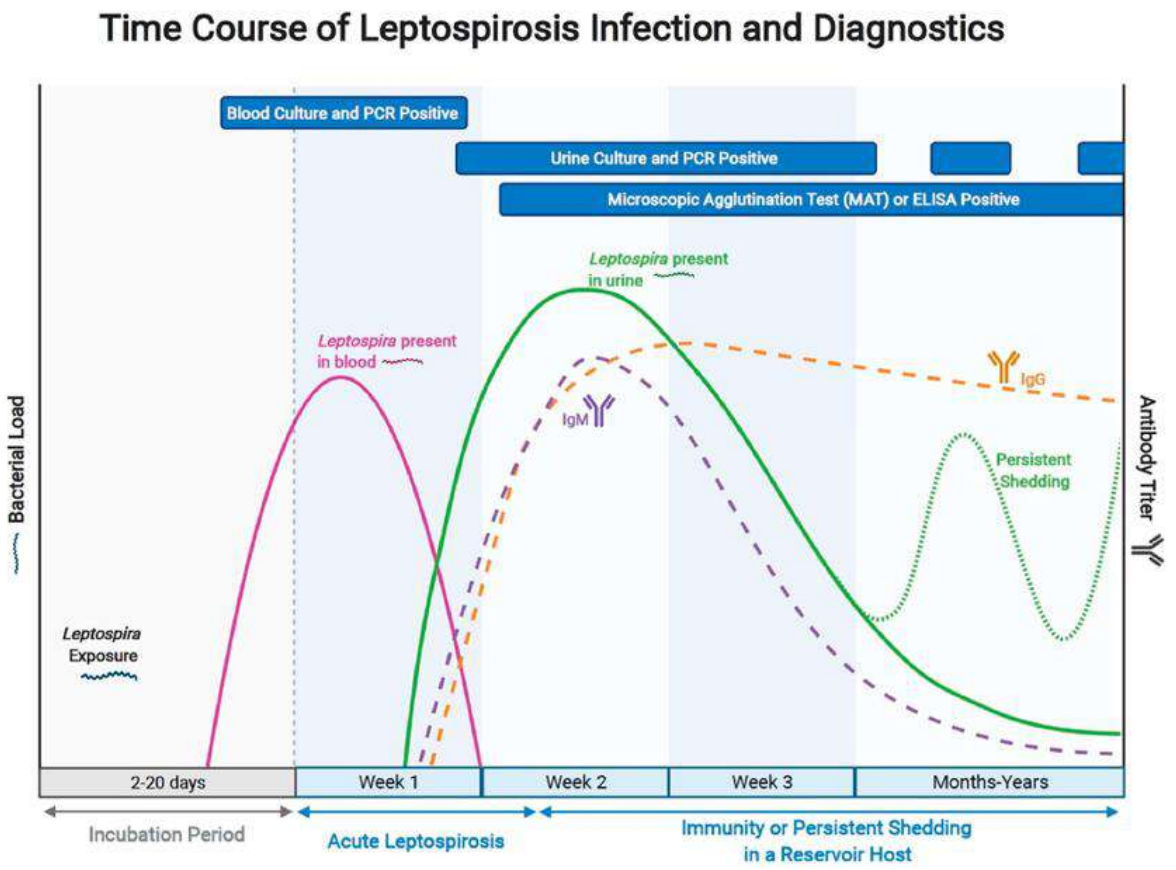

Figure 4. Schematic representation of the kinetics of leptospiral infection and the corresponding diagnostic tools, illustrating the temporal dynamics of leptospiremia, leptospiruria, antibody production, and test applicability (SYKES et al., 2022).

1.7.1. Culture and direct examination

The cultural isolation of *Leptospira* spp. is a definitive test for the diagnosis of the disease, but the method is technically demanding. Its limitations include the slow growth of leptospires, frequent contamination and the requirement for specialised growth conditions (SYKES et al., 2022). In addition, samples must be collected before antibiotics are administered, and storage conditions prior to inoculation also strongly influence the success of isolation. For example, the viability of leptospires in urine decreases rapidly, making culture difficult if the sample is left for more than two hours (ZARANTONELLI et al., 2018), while tissue samples are prone to autolysis if they are not inoculated on the same day (MILLER et al., 1990). To improve the chances of successful isolation, it is recommended to inoculate samples at different dilutions and use antimicrobials such as 5-fluorouracil or a combination of sulfamethoxazole, trimethoprim, amphotericin B, fosfomycin and 5-fluorouracil to reduce contamination (FAINE et al., 1999; LEVETT, 2001; CHAKRABORTY et al., 2011). Inoculated cultures are incubated

at 28–30 °C and examined under a dark-field microscope weekly for up to 13 weeks before being discarded (GALLOWAY and GULVIK, 2023), making this approach time-consuming for routine diagnostics. On the other hand, improvements in culture media and inoculation methods (CHAKRABORTY et al., 2011; HORNSBY et al., 2020; NARKKUL et al., 2020) have increased the successful isolation of *Leptospira* spp. which is a great advantage as it enables subsequent analyses that allow accurate identification of the infecting species and serogroup.

Although leptospires can be directly observed in clinical specimens under the darkfield microscope as thin, coiled, motile organisms, this method is unsuitable for routine diagnostics due to its low sensitivity (requires ~10^4 organisms/L) and specificity and is prone to misinterpretation of fibrin or protein filaments in blood and urine (GALLOWAY and GULVIK, 2023). To improve detection in direct examination, alternative approaches such as silver staining, Warthin-Starry staining, immunofluorescence, immunoperoxidase, immunohistochemistry or *in situ* hybridisation may be used, although these methods also carry the risk of false-positive and false-negative results (MUSSO and LA SCOLA, 2013).

1.7.2. Serological methods

Due to the non-specific clinical signs and the difficulties in culture isolation and direct examination, the laboratory diagnosis and epizootiological/epidemiological investigation of leptospirosis are mainly based on serological and molecular methods (MUSSO and LA SCOLA, 2013; PICARDEAU, 2013; ANONYMOUS, 2021).

The microscopic agglutination test (MAT) is the standard serological test and the most commonly used method for the diagnosis of leptospirosis. It has a high sensitivity and specificity and detects both IgM and IgG antibodies. An initial antibody titre ≥1:400 or ≥1:800 in endemic areas in simptomatic patients or a fourfold increase in paired sera is considered indicative of acute infection (FAINE et al., 1999). In some rare cases, seroconversion may take longer, which is why repeated sampling is recommended (GALLOWAY and GULVIK, 2023). A major limitation of MAT is that it cannot distinguish between antibodies resulting from infection or vaccination and frequent cross-reactions between different serogroups, especially in the acute phase. Paradoxical reactions may also occur, where the highest titres are directed against a serogroup unrelated to the infecting one. Another challenge is the requirement for a panel of live leptospiral cultures that are prevalent in certain geographical regions (ADLER and DE LA PEÑA MOCTEZUMA, 2010). Due to its technical complexity and demanding interpretation, MAT is often limited to national and regional reference laboratories (GALLOWAY and GULVIK, 2023). Nevertheless, the MAT is the most suitable test for

epizootic/epidemiological studies and can best provide information on the serogroups prevalent in the population and specific geographical areas (GALLOWAY and GULVIK, 2023).

The enzyme-linked immunosorbent assay (ELISA) offers a rapid and automatable alternative to MAT, but with lower sensitivity and specificity, so that it should not be used as the only diagnostic method (ADLER and DE LA PEÑA MOCTEZUMA, 2010). Lateral flow assays and IgM dipsticks are also available and show comparable sensitivity to ELISA. However, positive results must be confirmed by MAT, especially in endemic areas where antibodies may be present long after exposure (GALLOWAY and GULVIK, 2023).

1.7.3. Molecular methods

Nucleic acid amplification tests (NAATs) such as polymerase chain reaction (PCR) are commonly used to diagnose leptospirosis from blood, plasma, serum, urine, aqueous humour, cerebrospinal fluid and tissue samples (LEVETT, 2015). The main advantage of NAATs is their ability to confirm infection during the acute phase when treatment is most effective (RIEDIGER et al., 2017). The use of real-time PCR is recommended due to its superior sensitivity, specificity and low risk (BOURHY et al., 2011; MUSSO and LA SCOLA, 2013). In addition, it enables quantification (LOURDAULT et al., 2009). Targeting genes that are specific for pathogenic *Leptospira* spp. such as *lipL32* further increases the specificity of the test (STODDARD et al., 2009). PCR-based diagnosis detects leptospiral DNA in clinical material and allows identification to species level, although it cannot determine the infecting serogroup or serovar (BOURHY et al., 2011). However, molecular characterisation is sometimes possible directly from clinical samples (WEISS et al., 2016; MENDOZA and RIVERA, 2021;).

1.7.4. Identification and typing methods

The identification of *Leptospira* spp. is performed using serological methods or sequence-based techniques. Among the serological methods, the cross-agglutinin absorption test (CAAT) is traditionally used to identify serovars (DIKKEN and KMETY, 1978). However, this method is technically demanding and is only carried out in a few laboratories. The MAT, on the other hand, reliably identifies the presumptive infectious serogroup, but not the specific serovar (LEVETT, 2003). Due to the challenges associated with the serological identification of leptospiral isolates, many molecular methods for identification and subtyping have been developed, such as restriction endonuclease digestion of chromosomal DNA, restriction fragment length polymorphism (RFLP) analysis, pulsed-field gel electrophoresis (PFGE), 16S ribosomal RNA sequencing, matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS), multiple-locus variable-number tandem repeat analysis

(MLVA), detection of variable number of tandem repeats (VNTR), multilocus sequence typing (MLST), core genome multilocus sequence typing (cgMLST), and average nucleotide identity (ANI) based on whole genome sequencing (GALLOWAY and GULVIK, 2023). Among these molecular methods, PFGE can enable identification at the serovar level (GALLOWAY and LEVETT, 2010). However, all these molecular methods require high quality DNA in sufficient concentration, which is often a limitation in clinical samples due to the typically low bacterial load. This affects the quality of the sequencing data and prevents successful strain characterisation.

1.7.5. Advanced approaches: whole genome sequencing

The history of DNA sequencing has made remarkable progress over the last five decades, from the first laborious methods that enabled the reading of short DNA fragments to today's technologies that can sequence entire genomes. It all began with the development of DNA sequencing with chain-terminating inhibitors introduced by Sanger and colleagues in 1977, which remained the gold standard for DNA sequencing for more than two decades (SANGER et al., 1977). Sanger sequencing was the key to creating the first complete bacterial genomes and laid the foundation for comparative bacterial genomics.

The advent of next-generation sequencing (NGS) technologies in the early 2000s represented a breakthrough in genomics, enabling the rapid and cost-effective sequencing of multiple bacterial genomes (GOODWIN et al., 2016). More recently, third-generation long-read platforms such as PacBio SMRT and Oxford Nanopore have further advanced genome assembly by generating complete, closed bacterial genomes; however, they are limited by lower raw data accuracy compared to Illumina short-read sequencing (RHOADS and AU, 2015; TYLER et al., 2018). Today, the combination of short-read and long-read sequencing represents the gold standard for whole-genome sequencing (WGS), offering both accuracy and completeness.

The application of WGS to *Leptospira* spp. began with the sequencing of *L. interrogans* in 2003 (REN et al., 2003), followed by *L. borgpetersenii* in 2006 (BULACH et al., 2006). These studies have already demonstrated how comparative genomics can reveal major biological differences, such as the larger genome of *L. interrogans*, which supports environmental survival, versus the reduced genome of *L. borgpetersenii*, reflecting adaptation to a host-dependent lifestyle (PICARDEAU et al., 2008). Since then, an increasing number of *Leptospira* genomes have been sequenced, establishing that the genus possesses an open pangenome, with the capacity to acquire new genes through horizontal gene transfer (JORGE et al., 2018; VINCENT et al., 2019).

With the introduction of WGS, extended typing methods of *Leptospira* spp. such as cgMLST were developed. In contrast to classical MLST, where only a small number of housekeeping genes are analysed, cgMLST is based on 545 genes representing key regions of the genome. It offers a much higher resolution and enables the identification of serogroups and closely related serovars (GUGLIELMINI et al., 2019).

A common limitation of WGS is the inability to determine the infecting serovar. Recently, approaches based on the prediction of the serovar using the *rfb* cluster, which encodes genes for O-antigen biosynthesis, have gained attention (GIRAUD-GATINEAU et al., 2025). Several studies indicate that the gene composition of the *rfb* cluster correlates with the determination of the *Leptospira* serovar (NIEVES, 2022; CHINCHILLA et al., 2023; FERREIRA, 2024). While this method is promising, it requires further validation across different genomes and closely related serovars may still be difficult to distinguish (GIRAUD-GATINEAU et al., 2025).

Today, WGS is an important tool for analysing bacterial evolution, diversity and pathogenicity. Nevertheless, genomic sequence analyses have shown that *Leptospira* spp. possess an open pangenome, reflecting their ability to acquire new genetic material through horizontal gene transfer. This increases their genetic potential to adapt and infect a wide range of host species (SYKES et al., 2022). Furthermore, WGS enables the study of differences in genome structure, which is particularly useful for analysing strains isolated from geographically or ecologically diverse areas (JORGE et al., 2018). It not only provides deeper insights into the genetic characteristics of local strains, but also supports the development of improved diagnostics and vaccines, linking basic genomic research with applied clinical and epidemiological outcomes.

1.8. Leptospirosis in Croatia

Leptospirosis is an endemic disease in Croatia and has significant veterinary and public health concern. Between 1990 and 2007, the mean annual incidence was 1.83 cases per 100,000 inhabitants, with peaks exceeding 2.5 per 100,000 every three to four years (BALEN TOPIC et al., 2010), while in the subsequent period from 2009 to 2014, the mean incidence decreased slightly to 1.53 cases per 100,000 inhabitants (HABUS et al., 2017). Despite this decline, Croatia is still one of the European countries with the highest incidence of leptospirosis in humans. Although it is one of the most common zoonoses worldwide, leptospirosis in Croatia remains underestimated and often underdiagnosed, which emphasises the need for systematic surveillance and further research.

1.8.1. The role of small rodents and circulating Leptospira serogroups in Croatia

The role of small rodents as primary reservoirs in the epizootiological and epidemiological cycle of leptospirosis in Croatia has been studied over the years (BORČIĆ et al., 1982, 1983; MILAS et al., 2002; TURK et al., 2003; ŠTRITOF MAJETIĆ et al., 2014; HABUS et al., 2017). They not only enable the long-term survival and persistence of *Leptospira* spp. in the environment, but also provide insight into the currently circulating pathogenic serovars of *Leptospira* spp.

Epizootiologically, the risk of disease is strongly linked to the presence and abundance of rodent populations as one of the main reservoirs of leptospirosis (MILAS et al., 2002). Appropriate weather conditions can further increase forest vegetation and biomass, support rodent populations in forest ecosystems and directly influence their density and distribution (TURK et al., 2009).

While *Leptospira* spp. can infect a wide range of animal species, only a limited number of serovars tend to persist within a particular geographic area. Each serovar is typically maintained in certain reservoir hosts, whereas other species are infected incidentally upon exposure (ELLIS, 2015). For example, the black-striped field mouse (*Apodemus agrarius*) is considered a reservoir for the serovars Pomona and Mozdok, the common vole (*Microtus lavernedii*) for Grippotyphosa, the brown rat (*Rattus norvegicus*) for Icterohaemorrhagiae and the house mouse (*Mus musculus*) for Sejroe (MILAS et al., 2002; TURK et al., 2003; ŠTRITOF MAJETIĆ et al., 2014).

The serogroup Icterohaemorrhagiae is considered the most pathogenic of the broad spectrum of pathogenic leptospires (LEVETT, 2001; ELLIS, 2015). In addition, serogroups capable of producing haemolysins, such as Pomona or Icterohaemorrhagiae, are frequently associated with severe clinical manifestations in both animals and humans (ADLER and DE LA PEÑA MOCTEZUMA, 2010; ELLIS, 2015). In Croatia, the most frequently reported presumptive infectious serogroups in humans are Sejroe, Pomona, Australis and Icterohaemorrhagiae (PERIĆ et al., 2005; CVITKOVIĆ, 2007; BALEN TOPIC et al, 2010; HABUS et al, 2017), while the most common serogroups in various animal species are Australis, Pomona, Grippotyphosa, Sejroe, Icterohaemorrhagiae and Bataviae (TURK et al., 2003; ŠTRITOF MAJETIĆ et al., 2014; HABUS et al., 2017).

1.8.2. Increasing trend of the serogroup Pomona in different hosts

In the last two decades, the serogroup Pomona has gained importance, as it is considered one of the most pathogenic among *Leptospira* spp. It has been associated with a severe clinical form of leptospirosis - pulmonary haemorrhagic syndrome (LPHS). This is further supported

by studies in Croatia that have linked severe clinical outcomes of leptospirosis to infections with the serogroup Pomona, particularly in humans (unpublished data) and dogs (HABUŠ et al., 2017; HABUŠ et al., 2020), although the specific serovars responsible within this serogroup remain unclear. This serogroup consists of eight serovars, Altodouro, Kennewicki, Kunming, Mozdok, Pomona, Proechimys, Tropica and Tsaratsovo, which are distributed across five distinct species: *L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. noguchii* and *L. santarosai* (SEMENOVA, 1965; GALE et al., 1966; MANEV, 1976; SULZER et al., 1982; ZHANG et al., 1987; BOURHY et al., 2012; PAIVA-CARDOSO et al., 2013). Pigs have been considered the main carriers of the serogroup Pomona, especially for the serovars Pomona and Kennewicki, which in the past caused widespread clinical disease and became endemic in North and South America, Australia, New Zealand and several parts of Europe (ELLIS, 2012). On the other hand, small rodents, particularly the black-striped field mouse (*A. agrarius*), are recognised as important reservoirs for the serovar Mozdok (ŠTRITOF MAJETIĆ et al., 2014). Although vaccination programmes and the switch to indoor housing systems in certain regions have contributed to a decline in swine-associated Pomona outbreaks (BOLIN and CASSELLS, 1992; RIBOTTA et al., 1999), rodent-origin incidental infections and outbreaks with the serovars Mozdok and Pomona are still reported in Europe (ARENT et al., 2017a; BERTASIO et al., 2020; GAJDOV et al., 2024). Importantly, recent studies indicate an increasing prevalence of Pomona in various domestic and wild hosts worldwide (GUEDES et al., 2021; ALIBERTI et al., 2022; HELMAN et al., 2023; PETAKH and KAMYSHNYI., 2025), and during the last decade, Pomona has emerged as the most common serogroup detected in clinical cases of leptospirosis in Croatia (ŠTRITOF MAJETIĆ et al., 2012; HABUS et al., 2017; TADIĆ et al., 2024; ZEČEVIĆ et al., 2024).

2. HYPOTHESIS AND OBJECTIVES

The hypothesis of this doctoral dissertation was that the Pomona serogroup was emerging as the most virulent within the pathogenic *Leptospira* spp. complex and that its presence and pathogenicity were becoming dominant in different animal species.

In order to confirm or refute this hypothesis, the following objectives were defined:
The general objective of this study was to demonstrate the re-emergence of *Leptospira* spp. serogroup Pomona.
The specific objectives of this study were:
1. To investigate seroprevalence in horses and cats, two animal species representing extremes in susceptibility to leptospires.
2. To investigate the prevalence of the serogroup Pomona as a presumptive infectious serogroup in horses and cats.
3. To analyse the whole genome of *Leptospira* spp. serogroup Pomona in strains isolated over time from small rodents, which are the primary reservoirs for leptospires, and to determine their genomic characteristics and diversity.

3. MATERIALS AND METHODS

3.1. Materials

This doctoral dissertation was based on three scientific papers that encompassed material originating from different animal species in Croatia. Scientific Papers I, II and III analysed equine serum samples, feline blood and urine samples, as well as bacterial cultures of *Leptospira* spp. isolated from small rodents, respectively.

For Paper I, a total of 61,724 residual equine sera from the archive of the Laboratory for Leptospires, Faculty of Veterinary Medicine, University of Zagreb, were used. Representative sera were selected based on their availability in the archive over a ten-year period, between January 2012 and December 2022. The samples originated from apparently healthy horses of various breeds, ages, and sexes from different geographical regions of Croatia, where the endemicity of leptospirosis had previously been confirmed.

Paper II analysed blood and urine samples collected from a total of 54 cats presented for various reasons at the Veterinary Teaching Hospital of the University of Zagreb between December 2022 and December 2023. The samples were collected for diagnostic purposes, including haematology, serum biochemistry, urinalysis and/or bacteriological examination of urine, and were subsequently stored in the laboratory of the Clinic for Internal Diseases and in the Bacteriological Laboratory, Faculty of Veterinary Medicine, University of Zagreb, until processed for this study. From all 54 cats, serum and urine samples were available, while EDTA-anticoagulated blood samples were collected from 27 cats that met at least one of the following selection criteria: no antibiotic administration, immunocompromising conditions (retroviral infection, tumours, diabetes mellitus, immunosuppressive therapy), or blood count abnormalities such as anaemia, thrombocytopenia, and/or leukocytosis. Data on epizootiological factors and clinical signs were obtained by reviewing records from the patient protocol. Therefore, breed, age, sex, clinical signs, and retroviral status were recorded for each cat. Information on living conditions (indoor/outdoor, presence of other pets in the household), a possible immunocompromised state and laboratory results (complete blood count (CBC), biochemistry profile and urinalysis) were recorded for almost all cats, and cats were categorised into three groups according to the primary diagnosis established at the Veterinary Teaching Hospital: kidney and urinary tract diseases, diseases associated with immunosuppression, and other conditions such as trauma, hyperthyroidism, heart disease, and hypertensive retinopathy. The procedure for sample storage and pretreatment for DNA extraction is described in detail in Paper II. Briefly, serum samples were stored at −20 °C until serologically analysed, while

EDTA-anticoagulated blood was stored at 4 °C and processed with pretreatment extraction steps within 24 h. Urine samples were collected either by cystocentesis (n=36), catheterisation (n=7), or free-flow collection (n=11). Urine samples were stored either immediately at −20 °C (n=26) or at 4 °C for a maximum of 24 h (n=28) until subjected to pretreatment. The pretreatment extraction steps of urine included centrifugation and resuspension in Tris-EDTA solution, followed by the DNA extraction protocol.

Paper III analysed a total of 48 archived isolates of *Leptospira* spp. originating from the kidneys of small rodents. These isolates were collected over a 14-year period (2005-2018) from various regions of Croatia and were part of the collection of pathogenic leptospires in the Laboratory for Leptospires, Faculty of Veterinary Medicine, University of Zagreb. The isolates were maintained in Korthof and Fletcher media until analysis. Data on host species, collection location, and sampling dates were available for all isolates and is attached as Supplementary Material (Table S2) in Paper III. The small rodent species, including *Apodemus agrarius* and *Microtus lavernedii*, were identified based on morphological characteristics, while *Apodemus flavicollis* and *Apodemus sylvaticus*, which are morphologically indistinguishable, were differentiated using polymerase chain reaction (PCR) targeting the mitochondrial *cytochrome b* gene, followed by sequencing of the PCR products. Species differentiation was previously performed for 45 samples (unpublished data), while it was performed for three samples in this study using primers L14771 (5'-CAACATTCGTAAAACCCACC-3') and H15149 (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3') and the protocol described by TADIN et al. (2012).

3.2. Microscopic agglutination test (MAT)

The serological reference method of microscopic agglutination, described in Papers I and II, was used to test serum samples for the presence of antibodies against leptospires. The antigen panel consisted of reference strains of different serovars of live leptospires adapted to the species tested and to previous epizootiological and epidemiological analyses (DIKKEN and KMETY, 1978). Accordingly, a panel of eight pathogenic *Leptospira* spp. serovars was used for equine sera (Table 1), while a panel of twelve pathogenic serovars was used for feline samples (Table 2). For this method, *Leptospira* spp. cultures cultivated in Korthof medium for up to 10 days old, having a density of 2–4 × 10^8 leptospires/mL, were used as antigens. Serial dilutions of the tested sera were prepared in microtitre plates with phosphate-buffered saline (PBS), starting with a dilution of 1:50. After a two-hour incubation at 28–30 °C, the results were read under a darkfield microscope. A serologically positive reaction was determined by

the presence of live, non-agglutinated leptospires compared to the negative control. The endpoint titre was defined as the highest serum dilution that showed at least 50 % agglutination of leptospires. The endpoint titre was set at 1:50 in cats, at 1:200 for horses for serovar Bratislava, and at 1:400 for horses for the other serovars. The presumptive infectious serogroup was determined on the basis of the highest titre detected for one or more serovars belonging to a certain serogroup. Based on the results of this method, the seroprevalence was calculated and defined as the frequency of serologically positive samples in relation to the total number of samples analysed, expressed as a percentage.

Table 1. The panel of *Leptospira* spp. serovars used as antigens for serological screening of equine serum samples.

No.	Serogroup	Serovar	Strain	Genomic species
1	Grippotyphosa	Grippotyphosa	Moskva V	*L. kirschneri*
2	Sejroe	Sejroe	M84	*L. borgpetersenii*
3	Australis	Bratislava	Jež Bratislava	*L. interrogans*
4	Pomona	Pomona	Pomona	*L. interrogans*
5	Canicola	Canicola	Hond Utrecht IV	*L. interrogans*
6	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*L. interrogans*
7	Sejroe	Saxkoebing	Mus 24	*L. interrogans*
8	Bataviae	Bataviae	Swart	*L. interrogans*

Table 2. The panel of *Leptospira* spp. serovars used as antigens for serological screening of feline serum samples.

No.	Serogroup	Serovar	Strain	Genomic species
1	Grippotyphosa	Grippotyphosa	Moskva V	*L. kirschneri*
2	Sejroe	Sejroe	M84	*L. borgpetersenii*
3	Australis	Bratislava	Jež Bratislava	*L. interrogans*
4	Pomona	Pomona	Pomona	*L. interrogans*
5	Canicola	Canicola	Hond Utrecht IV	*L. interrogans*
6	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*L. interrogans*
7	Tarassovi	Tarassovi	Perepelitsin	*L. borgpetersenii*
8	Sejroe	Saxkoebing	Mus 24	*L. interrogans*
9	Ballum	Ballum	Mus 127	*L. borgpetersenii*
10	Bataviae	Bataviae	Swart	*L. interrogans*
11	Javanica	Poi	Poi	*L. borgpetersenii*
12	Sejroe	Hardjo	Hardjobovis	*L. interrogans*

3.3. Serological typing of *Leptospira* spp. isolates

The affiliation of the *Leptospira* spp. culture isolates to specific serogroups was tested using a panel of 14 reference hyperimmune sera (Table 3) prepared in rabbits (OIE Reference Laboratory for Leptospirosis, AMC, Amsterdam, The Netherlands) according to the standard procedure (DIKKEN and KMETY, 1978). Antigens, serial dilutions, incubation, and evaluation of the reactions were carried out in the same way as for the MAT method and was thoroughly described in Paper III. A positive reaction was determined as agglutination of at least 50 % of the leptospires. The infectious serogroup of each culture was determined on the basis of the hyperimmune serum showing the highest agglutination titre.

Table 3. Panel of 14 reference hyperimmune sera used for serological typing of *Leptospira* spp. isolates.

No.	Serogroup	Serovar	Strain
1	Grippotyphosa	Grippotyphosa	Moskva V
2	Sejroe	Sejroe	M 84
3	Australis	Bratislava	Jež Bratislava
4	Pomona	Pomona	Pomona
		Mozdok	5621
5	Canicola	Canicola	Hond Utrecht IV
6	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
7	Tarassovi	Tarassovi	Perepelitsin
8	Sejroe	Saxkoebing	Mus 24
9	Ballum	Ballum	Mus 127
10	Bataviae	Bataviae	Swart
11	Javanica	Poi	Poi
12	Sejroe	Hardjo	Sponselee
13	Autumnalis	Rachmati	Rachmat
14	Hebdomadis	Hebdomadis	Hebdomadis

3.4. Detection of pathogenic *Leptospira* spp. using molecular techniques

3.4.1 DNA extraction

After the pretreatment extraction steps of feline EDTA-anticoagulated blood and urine samples, described in detail in Paper II, the pellets were resuspended in 180 µL of Lysis Buffer T1 and 25 µL of proteinase K. Total DNA was then extracted using the NucleoSpin Tissue Mini Kit for DNA from Cells and Tissues (MACHEREY-NAGEL GmbH & Co. KG, Germany), following the manufacturer's protocols, with a final elution volume of 100 µL to increase the

DNA concentration. The extracted DNA was stored at −20 °C until testing. The purity and concentration of DNA were determined for all extracted samples using the BioDrop µLITE spectrophotometer.

Leptospira spp. cultures up to 10 days old, having a density of 2–4 × 10^8 bacteria/mL in Korthof medium, were sent to the Zoonoses and Select Agent Laboratory, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, where further analyses and processing were conducted to identify, characterise, and study the isolates. Pretreatment of the cultures for DNA extraction, including centrifugation and resuspension in PBS, is described in detail in Paper III. DNA extraction was subsequently performed using the Maxwell CSC 48 automated extraction system (Promega Corporation, Madison, WI, USA) with the Maxwell® RSC Cultured Cells DNA Kit (Promega Corporation, Madison, WI, USA). The extracted DNA was stored at −20 °C until testing.

3.4.2. Real-time polymerase chain reaction (qPCR)

Real-time PCR protocol is thoroughly described in Paper II. The extracted DNA was analysed using the Rotor-Gene Q system (Qiagen, Hilden, Germany) with a TaqMan probe targeting the *lipL32* gene. The QuantiFast Pathogen PCR + IC Kit (Qiagen, Hilden, Germany), which includes an internal control assay, was used. Primers LipL32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and LipL32-286R (5′-GAA CTC CCA TTT CAG CGA TT-3′), with the probe LipL32-189P (FAM-5′-AA AGC CAG GAC AAG CGC CG-3′-BHQ1), and amplification protocol were applied as described by STODDARD et al. (2009). Samples were tested in duplicate, and a Ct value below 35 in both replicates was considered positive, indicating urinary excretion of *Leptospira* spp.

3.4.3. Conventional polymerase chain reaction (PCR)

Conventional PCR was performed on the extracted DNA from feline EDTA-anticoagulated blood and urine using the primers LipL32 F (5′-ATC TCC GTT GCA CTC TTT GC-3′) and LipL32 R (5′-ACC ATC ATC ATC ATC GTC CA-3′), as described by AHMED et al. (2006), which amplify a 474-bp fragment of the *lipL32* gene. Each amplification reaction was carried out in the T100™ thermal cycler (Bio-Rad, USA) under the conditions described in detail in Paper II. The amplified DNA was electrophoresed through a 1 % agarose gel and compared with a molecular size marker.

3.4.4. Sanger sequencing

Positive results of conventional PCR were sequenced to confirm the presence of pathogenic *Leptospira* species as detailed in Paper II. Sanger sequencing was performed at

Macrogen Europe Inc. (Amsterdam, The Netherlands). The obtained sequences were aligned using Clustal X (version 2.0), analysed with BioEdit software (version 7.7), and compared with the GenBank nucleotide database using the BLAST programme of the National Center for Biotechnology Information (NCBI).

3.4.5. Whole genome sequencing (WGS)

Strains isolated from the kidneys of small rodents, identified by serological typing as belonging to the serogroup Pomona, were analysed by whole genome sequencing, as described in detail in Paper III. The workflow included DNA extraction, with a minimum concentration of 3.3 ng/µL and purity of A260/A280 between 1.8 and 2.0, followed by library preparation in which the genomic DNA samples were converted into fragment libraries compatible with sequencing on the MiSeq instrument. Library preparation was carried out using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), while sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq v3 600-cycle kit (2 × 300 bp reads). The MiSeq system applies Sequencing by Synthesis (SBS) technology, which detects individual bases as they are incorporated into growing DNA strands.

3.4.6. Genome assembly

For the whole genome sequencing samples analysed in Paper III, genome assembly was performed using the Nextflow v24.04.2 assembly pipeline with default parameters developed by the bioinformatics team at the Zoonoses and Select Agent Laboratory (ZSAL), Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA (Figure 5) This pathogen-agnostic, general-purpose workflow was specifically designed for the genome assembly of Illumina paired-end sequencing data and was applied to ensure accurate and efficient data assembly. To improve assembly quality, contigs shorter than 500 bp were removed and only genomes with a minimum sequencing coverage of 39.5× (median 53.3×, range 39.5×–74.4×) were retained. These quality control steps ensured that only high-quality assemblies were used for genomic analyses.

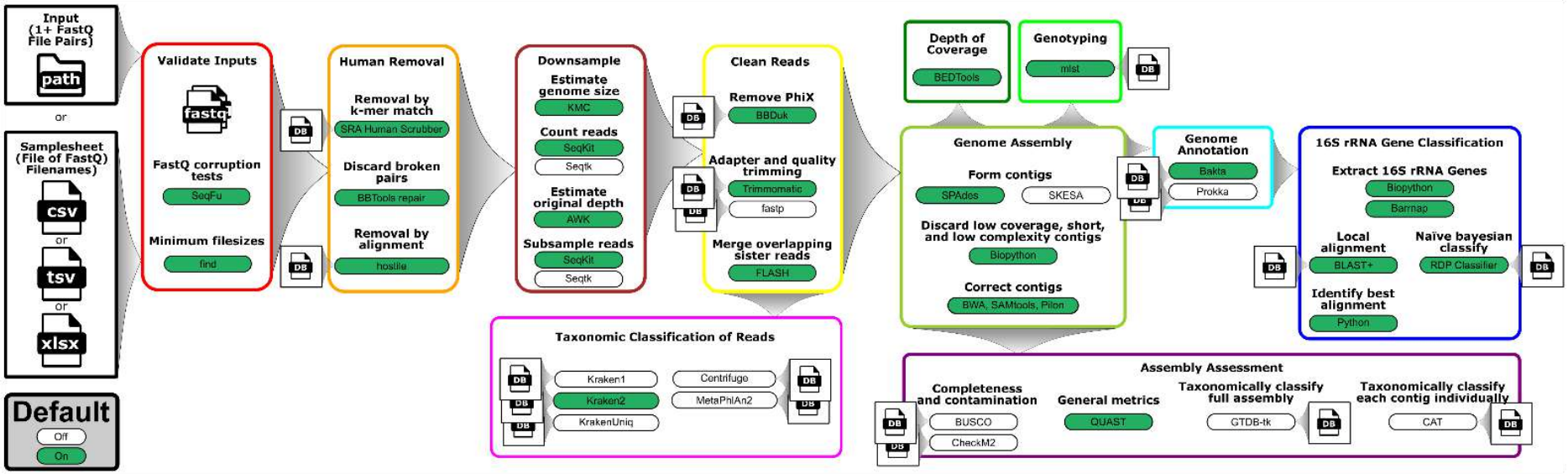

Figure 5. General schematic of the steps in the Nextflow-based genome assembly workflow used for *Leptospira* spp. whole-genome sequencing (Paper III). The diagram outlines the key computational steps — from raw paired-end Illumina FastQ inputs through quality filtering, assembly to contig correction and post-assembly quality assessment (https://github.com/bacterial-genomics/wf-paired-end-illumina-assembly, v3.0.0).

3.4.7. Genomic analyses

The genomic analyses included four complementary approaches: average nucleotide identity (ANI), pangenome analysis, core genome multilocus sequence typing (cgMLST), and whole genome single nucleotide polymorphism (SNP) analysis. Computational resources for genomic analyses are described in detail in Section 2.3.6 of Paper III.

3.4.7.1. Average nucleotide identity (ANI)

To assess genomic relatedness among isolates, ANI was computed using the automated workflow available at the bacterial-genomics GitHub repository (https://github.com/bacterial-genomics/wf-ani, v1.0.0), which implements three complementary ANI methods: Biopython v1.6.8, FastANI v1.33, and SKANI v0.1.3 (COCK et al., 2009; SHAW and YU, 2023). Two datasets were analyzed: 118 genomes comprising 70 reference *Leptospira* spp. strains from NCBI RefSeq and 48 Croatian isolates, and 99 genomes including the 48 Croatian isolates and 51 *L. kirschneri* reference genomes. Analyses were performed with Nextflow workflow manager to ensure reproducibility and scalability (DI TOMMASO et al., 2017). Default parameters were employed and ANI values >95 % were considered indicative of the same genomic species, in line with accepted prokaryotic species thresholds.

3.4.7.2. Pangenome analysis

Pangenome analysis of the 48 Croatian *Leptospira* genomes was performed to characterize core and accessory genome components. Assemblies were filtered to remove contigs <500 bp and annotated with Bakta v1.9.4 (SCHWENGERS et al., 2021). The annotated GFF3 files were processed with Panaroo v1.3.4 in strict mode (95 % identity threshold) to account for gene presence–absence variation and assembly errors (TONKIN-HILL et al., 2020). Outputs included a gene presence–absence matrix and definitions of core and accessory genomes. Gene presence–absence patterns were visualized using R v4.3.1 to explore genomic diversity and to identify genes associated with specific clusters or traits. The pangenome was further analyzed to estimate core genome size and overall gene repertoire.

3.4.7.3. Core genome multilocus sequence typing (cgMLST)

To establish a cgMLST scheme, a gene-by-gene approach was applied using chewBBACA v3.3.10 (SILVA et al., 2018). Coding sequences were predicted with Prodigal v2.6.3, and loci were filtered with BLAST Score Ratio (threshold 0.6) to to retain non-paralogous, high-quality loci. Only loci present in ≥95 % of genomes were included in the final schema. A preliminary *Leptospira* cgMLST schema from BIGSdb v1.51.4 (JOLLEY et al., 2010) served as the basis for locus selection. Allele calling was then performed on the 48

Croatian isolates, generating allelic profiles and identifying novel alleles. The final schema and profiles were integrated into a local BIGSdb instance, enabling comparative analysis and core genome sequence type (cgST) assignment across isolates.

3.4.7.4. Whole genome single nucleotide polymorphism (SNP) analysis

Genomic variation at the single nucleotide level was further assessed through whole-genome SNP analysis, using the wf-assembly-snps pipeline (https://github.com/bacterial-genomics/wf-assembly-snps, v1.0.3) implemented in Nextflow v24.04.2 (DI TOMMASO et al., 2017). This reference-free workflow applies Parsnp v1.5.6 (TREANGEN et al., 2014) for core genome alignment and SNP identification and was run both on the 48 Croatian *Leptospira* assemblies and on an extended dataset including 23 additional genomes from NCBI RefSeq. Parsnp identified high-confidence core genome SNPs and generated multiple sequence alignments, which were used to build phylogenetic trees with IQ-TREE v2.4.0 (MINH et al., 2020). SNP alignments and phylogenetic trees were further analyzed and visualized in R packages (ape, ggtree) to explore evolutionary relationships and clustering among isolates.

3.4.8. Phylogenetic analysis

A maximum likelihood phylogeny was inferred with IQ-TREE v2.4.0 (MINH et al., 2020) using the core SNP alignment generated from the 48 Croatian isolates and 23 additional *L. kirschneri* genomes obtained from NCBI RefSeq. The best-fitting substitution model was determined automatically using ModelFinder based on the Bayesian Information Criterion. Branch support was assessed using 1000 ultrafast bootstrap replicates (MINH et al., 2020). The phylogenetic tree was then visualized and annotated in R with ggtree package v3.10.0 (YU et al., 2017), which enabled the exploration of clustering patterns, evolutionary relationships, and potential geographic or host-associated structure among the isolates.

3.5. Statistical Analysis

The data from Papers I and II were statistically analysed and detailed descriptions of each statistical software, method, and criterion applied can be found in the respective individual papers. Briefly, the data were processed using the statistical software R 4.4.0 (R Core Team, 2024), Statistica v14 (TIBCO Software Inc., 2020), and the MedCalc Odds Ratio Calculator (https://www.medcalc.org/calc/odds_ratio.php). Descriptive statistics were presented as numbers and percentages. The odds ratio (OR) test was applied to calculate the effects of individual risk factors (frequencies), with results expressed as OR with a 95 % confidence interval (CI). For multinomial risk factors (more than two categories), logistic regression was used to calculate the OR. For quantitative variables, the normal distribution was assessed using

the Kolmogorov-Smirnov test. Depending on the number of groups, either a t-test or a one-way ANOVA was applied, or their non-parametric variants, with results presented as arithmetic means and standard deviations or medians and interquartile ranges. Statistical significance was determined at a p-value of <0.05.

3.6. Ethics approval and consent to participate

The scientific research included in the doctoral dissertation was evaluated and approved by the Faculty Council of the Faculty of Veterinary Medicine, University of Zagreb, at its 6th regular session in the academic year 2024/2025, based on the proposal of the Committee for Ethics in Veterinary Medicine, held on January 22, 2025 (Editorial number: 251-61-01/139-25-20; Class: 640-01/25-02/01). The owners provided written informed consent before sampling.

4. RESULTS

4.1. Paper I

BENVIN, I.; MOJČEC PERKO, V.; MALJKOVIĆ, M.M.; HABUŠ, J.; ŠTRITOF, Z.; HAĐINA, S.; PERHARIĆ, M.; ZEČEVIĆ, I.; CVETNIĆ, M.; TURK, N. (2023): Serological Surveillance of Equine Leptospirosis in Croatia in the Period From 2012 to 2022: A Key Insight into the Changing Epizootiology. J. Equine Vet. Sci. 127, 104844. https://doi.org/10.1016/j.jevs.2023.104844

The results presented in Paper I (BENVIN et al., 2023) meet specific objectives 1 and 2 by providing a comprehensive serological overview of equine leptospirosis in Croatia over a ten-year period. Out of 61,724 serum samples tested, 6,665 (10.80 %) were seropositive, with annual prevalence ranging from 5.00 % to 15.94 %. Titres ranged from 1:400 (1:200 for serovar Bratislava) to 1:51,200. The distribution of infectious serogroups showed that Pomona was the most common presumptive infectious serogroup (41.98 %), followed by Grippotyphosa (31.34 %), Sejroe (8.03 %), Icterohaemorrhagiae (7.05 %) and Bratislava (6.47 %). This study is the first in Europe in which such high seropositivity for Pomona was found in apparently healthy horses, indicating the increasing epizootiological importance of the disease. These results show that horses in Croatia are particularly exposed to *Leptospira* spp. and confirm the changing epizootiology and re-emergence of the serogroup Pomona as the dominant infectious serogroup in this species (Figure 6).

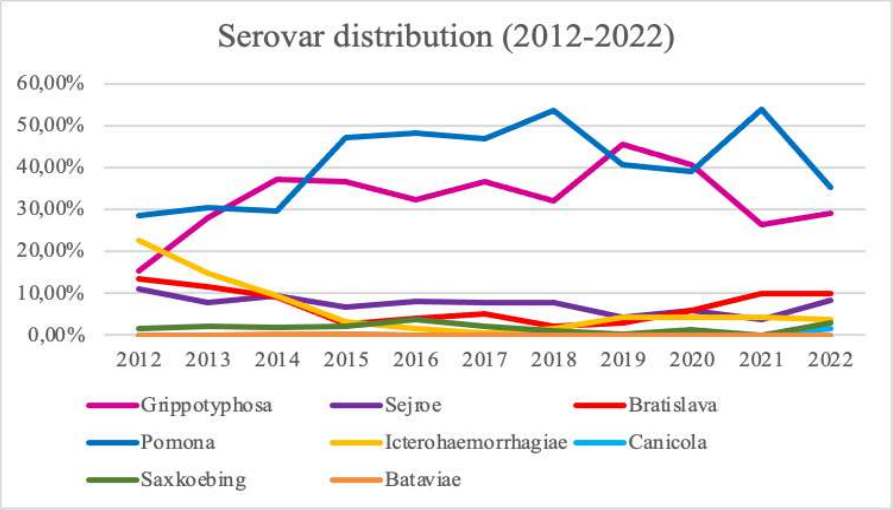

Figure 6. Distribution of presumptive infective *Leptospira* spp. serovar within each year of the investigated period, showing changing epizootiology (adapted from Figure 3 in BENVIN et al., 2023).

4.2. Paper II

BENVIN, I.; FITZ, D.; MOJČEC PERKO, V.; MAURIĆ MALJKOVIĆ, M.; ĐURIĆ, V.; HABUŠ, J.; ŠTRITOF, Z.; PERHARIĆ, M.; HAĐINA, S.; ZEČEVIĆ, I.; TURK, N. (2024): Insights into *Leptospira* spp. infection in pet cats in Croatia: Clinical, serological and molecular findings with emphasis on the potential important role of serogroup Pomona. Acta Trop. 260, 107465. https://doi.org/10.1016/j.actatropica.2024.107465

The results presented in Paper II (BENVIN et al., 2024) also address specific objectives 1 and 2 by analysing seroprevalence and presumptive infectious serogroups in cats in Croatia. Out of 54 serum samples tested, 18.52 % were seropositive, with titres ranging from 1:50 to 1:12,800. Pomona was the most common presumptive infectious serogroup (40 %), followed by Sejroe, Icterohaemorrhagiae, Australis and Javanica (Table 4). In addition, one cat (1.85 %) was confirmed by PCR and sequencing to shed pathogenic *Leptospira* spp. in its urine, while all tested EDTA-anticoagulated blood samples were negative for the *lipl32* gene. Importantly, several risk factors for seropositivity were identified in the study, including immunocompromised conditions, multi-cat households and renal disease. Clinical associations were also observed, as seropositive cats, particularly those that were seropositive for Pomona, were more likely to have respiratory signs and radiographic lung changes. These results provide new evidence that cats may play a role in the epizootiology of leptospirosis in Croatia and confirm the emergence of the serogroup Pomona as the dominant infectious serogroup in various animal species. Furthermore, the results indicate that cats can also develop a pronounced clinical disease and show high antibody titres, especially those that were Pomona seropositive. Importantly, these cats often had an established alternative diagnosis, suggesting that feline leptospirosis is underdiagnosed and neglected in Croatia.

Table 4. Individual MAT titre results and presumptive infectious serogroup from seropositive cat (adapted from Table 1 in BENVIN et al., 2024).

	Serovars								Presumptive infectious serogroup
Cat	Pomona	Icterohaemorrhagie	Tarassovi	Bratislava	Saxkoebing	Sejroe	Gryppotyphosa	Poi	
1	-	50	-	-	-	-	-	-	Icterohaemorrhagie
2	-	-	100	-	-	-	-	100	ND
3	100	-	-	-	-	-	-	-	Pomona
4	-	-	-	100	-	-	-	-	Australis
5	-	-	-	-	200	-	-	200	ND
6	-	-	-	100	200	50	-	-	Sejroe
7	12800	-	-	-	-	-	6400	-	Pomona
8	1600	-	-	-	-	-	200	-	Pomona
9	100	-	-	-	-	-	-	-	Pomona
10	-	-	-	-	-	-	-	100	Javanica

ND = nondetermined

4.3. Paper III

BENVIN, I.; PAISIE, T. K.; CAETANO VARANDA, I.; WEINER, Z. P.; STODDARD, R. A.; GEE, J. E.; GULVIK, C. A.; MARSTON, C. K.; MOJČEC PERKO, V.; ŠTRITOF, Z.; HABUŠ J.; MARGALETIĆ, J.; VUCELJA, M.; BJEDOV, L.; TURK, N. (2025): Whole Genome Characterization of *Leptospira kirschneri* Serogroup Pomona in Croatia: Insights into Its Diversity and Evolutionary Emergence. Pathogens, 14, 860. https://doi.org/10.3390/pathogens14090860

The results presented in Paper III (BENVIN et al., 2025) contribute to specific objective 3 by providing the characterisation of the whole genome of *Leptospira* spp. serogroup Pomona in strains isolated from the primary reservoirs, small rodents, and by determining their genomic characteristics and diversity. A total of 48 strains were obtained from small rodents over a period of 14 years and all were serologically confirmed as belonging to the serogroup Pomona. ANI and MLST analyses assigned all isolates to *L. kirschneri* ST-98, which corresponds to the serovar Mozdok or Tsaratsovo. Core genome MLST (cgMLST) further resolved the population into seven genotype clusters (A-G), many of which showed clear geographic structuring, while SNP-based phylogenetic analyses revealed monophyletic clades associated with specific regions, suggesting localised clonal expansions. Comparative analyses with global *L. kirschneri* genomes showed that the Croatian isolates formed a distinct, geographically restricted lineage that was genetically cohesive but distinct from other international strains (Figure 7). Pangenome analysis confirmed a stable core genome with limited variation in accessory genes, further

supporting the clonality of the isolates. Overall, these results indicate the existence of a regionally dominant and potentially host-adapted lineage of *L. kirschneri* serogroup Pomona in Croatia, underlining its evolutionary emergence and epizootiological significance.

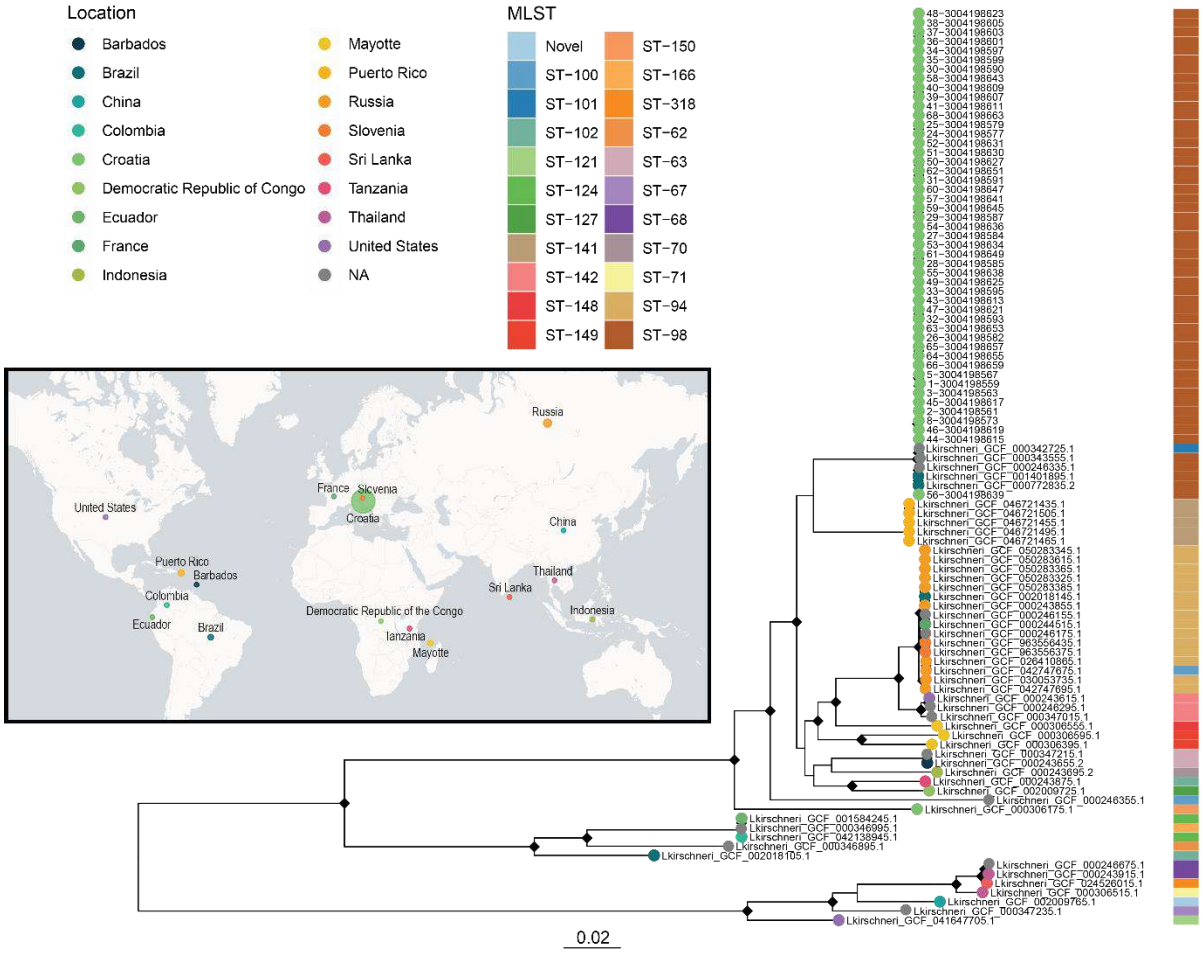

Figure 7. Maximum-likelihood phylogeny of *Leptospira kirschneri* isolates from Croatia and global references, showing the distinct monophyletic clade formed by Croatian Pomona isolates. The phylogenetic tree was reconstructed from a core SNP alignment of 99 genomes, including 48 Croatian isolates from rodents and 51 international references. The Croatian isolates are closely clustered, indicating the presence of a geographically restricted and genetically cohesive lineage distinct from other global strains (according to Figure 5 in BENVIN et al., 2025).

5. DISCUSSION

Leptospirosis is widely distributed among both animals and humans in Croatia, where it occurs both in natural and increasingly in synanthropic foci. Croatia ranks first among European countries and 13th in the world in terms of endemic leptospirosis (PAPPAS et al., 2008). The climatic conditions and geomorphological diversity of the country contribute to an exceptionally high biodiversity, which in turn harbours large rodent populations that serve as the main reservoir for leptospires. These ecological factors characterise the endemic nature of leptospirosis in Croatia and ensure active transmission cycles in different geographical regions (MILAS et al., 2002; TURK et al., 2003).

The outcome of a leptospiral infection is determined by complex interactions between host and pathogen, including host age, immune response and infectious dose as well as intrinsic differences in virulence between serovars (LEVETT, 2001). Previous genomic studies have shown that not all pathogenic *Leptospira* spp. have the same pathogenic potential, with genetic variations in virulence factors contributing to different clinical outcomes (PICARDEAU, 2017; JORGE et al., 2018). Although serogroup Icterohaemorrhagiae is traditionally considered the most pathogenic within the genus *Leptospira* and is associated with severe clinical manifestations (LEVETT, 2001), the importance of serogroup Pomona has recently been increasingly emphasised. Pigs are considered to be the most important maintenance hosts, particularly for the serovar Pomona, which has caused widespread disease and became endemic in North and South America, Australia, New Zealand and parts of Europe in the past (ELLIS, 2012). On the other hand, small rodents, especially *A. agrarius*, are considered a reservoir for the serovar Mozdok (ŠTRITOF MAJETIĆ et al., 2014). In addition, sporadic cases and outbreaks with Pomona have been reported in several European countries, including Spain and the United Kingdom, underlining the epizootiological importance of the disease beyond endemic regions (ARENT et al., 2017a; ARENT et al., 2017b). In addition, a Spanish study showed the genetic heterogeneity of serovar Pomona and identified three different types (ARENT et al., 2017a). Importantly, serious clinical consequences such as pulmonary haemorrhagic syndrome in dogs (HABUŠ et al., 2020), uveitis in horses (TIROSH-LEVY et al., 2021) and even acute reproductive disorders, so-called "abortion storms" in cattle (GROOMS, 2006), have been associated with Pomona infection, underlining the pathogenic potential and the need for continuous surveillance.

Horses, which are considered highly immunogenic animals and were systematically monitored as part of the national surveillance programme for leptospirosis, provided the

opportunity to test samples collected throughout the entire country, including both healthy and clinically affected animals. This broad sampling enabled the most objective possible insight into the epidemiological and epizootiological situation of leptospirosis in Croatia. By analysing 61,724 samples collected over a ten-year period (2012–2022), it was possible to obtain a representative picture of the situation. The results showed that infection with *Leptospira* spp. is still widespread in horses in Croatia, with 6,665 seropositive samples and a seroprevalence of 10.8 %. Most importantly, Pomona emerged as the most prevalent serogroup with 41.98 % of seropositive animals, followed by Grippotyphosa, Sejroe, Icterohaemorrhagiae, Bratislava, and Saxkoebing. Furthermore, Pomona showed a clear increasing trend over time, indicating a dynamic shift in the epizootiology of leptospirosis. While recent studies in Europe report that other serogroups such as Grippotyphosa (WASIŃSKI et al., 2021), Bratislava (VERA et al., 2019) or Sejroe (ŻMUDZKI et al., 2025) are most prevalent in horses, the Croatian situation seems to be unique with its strong dominance of Pomona. This difference is probably due to ecological and reservoir-specific factors, including the abundance of certain rodent hosts and environmental conditions that favour the persistence of Pomona.

The clinical relevance of Pomona infection in horses has been emphasised in recent literature. TIROSH-LEVY et al. (2021) reported that most Pomona-seropositive horses had clinical signs of equine recurrent uveitis (ERU), while FAGRE et al. (2020) found that almost all horses with high Pomona titres (≥800) had uveitis, ERU or another ophthalmic ailment. Furthermore, HAMOND et al. (2024) reported that infection with serovar Pomona was associated with acute spontaneous abortion. In contrast, the majority of horses examined in this study were apparently healthy, raising the possibility that Pomona may have undergone an evolutionary adaptation to the equine host. This adaptation could allow the persistence of the infection with limited clinical signs while posing a risk of urinary excretion. Given that horses are long-lived animals that are often kept in close contact with other domestic animals and humans, their role as potential carriers and contributors to environmental contamination by leptospires should not be underestimated. Further studies are needed to clarify whether Pomona is indeed adapting to a subclinical course in horses or whether clinical outcomes are underdiagnosed in this population.

In contrast, cats have traditionally been neglected due to the perception of natural resistance and the assumption that infections remain asymptomatic. However, due to their instinctive predatory behaviour and frequent contact with rodents, cats are inevitably exposed to leptospires in the environment. Considering that *Leptospira* spp. infections in cats have not been the focus of research in Croatia for many years, we wanted to determine the current status

of *Leptospira* spp. infection in this species, especially in pet cats that have the closest contact with humans, and to clarify the question of whether infection in cats is associated with clinical manifestations and under what circumstances. To address this, pet cats were examined immediately after the comprehensive study in horses in the period 2022–2023 to provide a contemporary status. A seroprevalence of 18.5 % was found, which is significantly higher than in most comparable recent European studies that included pet cats (LEHTLA et al., 2020; ŽÁKOVSKÁ et al., 2020; DONATO et al., 2022), and once again Pomona was identified as the most common serogroup, followed by Sejroe, Icterohaemorrhagie, Australis and Javanica. Apart from our study, only one study in Europe reported Pomona as the most common serogroup in cats (LEHTLA et al., 2020). Importantly, the results also showed that infected cats can develop clinical signs, and the most common clinical findings were anorexia, lethargy, vomiting, respiratory symptoms, anaemia and leukocytosis. Furthermore, the highest titres and suggestive symptoms of clinical leptospirosis were observed in immunocompromised cats with severe systemic comorbidities, which is also described by MAZZOTTA et al. (2023). In addition, all cats with respiratory symptoms were seropositive for Pomona and showed anaemia at the same time. This observation is consistent with reports of leptospirosis causing pulmonary involvement in other species, including pulmonary haemorrhage and acute respiratory distress syndrome in horses (BROUX et al., 2012), dogs (SCHULLER et al., 2015) and humans (NICODEMO and DUARTE-NETO, 2021). The association between Pomona and such manifestations suggests that cats may not be as resistant to clinical disease as previously thought.

The role of cats as potential shedders of leptospires also warrants attention. In this study, urinary shedding of leptospires was found in only one cat (1.85 %) that was seronegative but presented with acute kidney injury, elevated renal biochemical parameters and ultrasonographic signs of glomerulonephritis. The possibility of typical rapid drop in titres in cats (SHOPHET, 1979; MARKOVICH et al., 2012), coupled with the prolonged excretion of leptospiral DNA after confirmed infection (WEIS et al., 2017), could explain the seronegative result in this cat. This case shows that cats may excrete leptospires while showing clinical disease, even when no antibodies are detectable in serum. Factors such as the acidic pH and high osmolality of cat urine may reduce the survival of leptospires and the detectability of DNA (RUBINI and WOLF, 1957; LEVETT, 2001), which could contribute to the low prevalence of shedding observed. These factors also influence the concentration and quality of DNA in urine samples. Consequently, identification of *Leptospira* species by sequencing was not possible due to the low DNA concentration and poor DNA quality, which is a common limitation in clinical

samples with low bacterial load. Nevertheless, the possibility that cats may act as sources of infection cannot be ruled out. Overall, these results challenge the long-held view of resistance in cats and suggest that cats should be considered more carefully in the epizootiology of leptospirosis, especially given their close and frequent contact with humans.

To further investigate the reasons for the apparent re-emergence and increased pathogenicity of serogroup Pomona, it was necessary to examine its characteristics in natural reservoirs. Small rodents are the most important reservoirs of leptospires, acting as asymptomatic carriers that maintain the bacteria in the environment through lifelong urinary excretion. Therefore, they represent an ideal model for studying long-term evolutionary dynamics. For this purpose, archival cultures of *Leptospira* spp. serogroup Pomona isolated from rodent kidneys over a period of 14 years were analysed. This time frame included the years prior to the surveillance of horses (2005–2012) as well as the overlapping surveillance period (2012–2018), allowing sufficient time to investigate whether genomic changes in Pomona might have contributed to the trends observed in horses and cats. This study specifically selected isolates from rodents identified by serotyping as belonging to the serogroup Pomona.

Genomic analyses further clarified the taxonomic position of these isolates and classified them as *L. kirschneri* based on high similarity values obtained by ANI. The assignment of the isolates to ST-98 using classical MLST suggests that the isolates belong to a single species *L. kirschneri,* most likely serovar Mozdok, and a single sequence type (ST-98). However, the subdivision into seven distinct genotype clusters (labelled A-G) identified by cgMLST, based on allelic similarity and minimum spanning tree topology, suggests remarkable genetic diversity within the population, with many clusters associated with geographic locations. This geographic structuring suggests that local ecological conditions and the distribution of rodent hosts play an important role in shaping genetic variation within Pomona populations. However, cgMLST provided only a broad resolution, and further analysis with SNP-based phylogeny revealed additional fine-scale differences and identified distinct sub-clusters within cgMLST groups.

Phylogenetic analyses based on SNP data provided further evidence for this adaptation and revealed a monophyletic clade of Croatian isolates that is clearly distinct from other *L. kirschneri* strains reported worldwide. This indicates that the Pomona lineage circulating in Croatia is geographically restricted and likely host-adapted. The classification of isolates as *L. kirschneri*, serogroup Pomona and most likely serovar Mozdok, although Tsaratsovo cannot be completely ruled out, is of particular significance, since serovar Mozdok has been associated

with rodent reservoirs in Europe, especially *A. agrarius*, which were prevalent in this study. Such reservoir-pathogen associations support the idea of long-term host adaptation, which could explain the persistence of Pomona in Croatia and its spread to other species.

The discovery of a distinct and geographically confined Pomona lineage in rodents adds weight to the findings in horses and cats. In horses, Pomona was the most prevalent serogroup with an increasing trend, while in cats Pomona was the leading serogroup associated with clinical disease in some individuals. The fact that the same serogroup dominates in both incidental hosts and natural reservoirs suggest a stable ecological cycle that maintains Pomona in the environment and continuously exposes other species to infection. This convergence between host species is strong evidence that Pomona is not a sporadic or transient occurrence, but rather a re-emerging serogroup that is ecologically and evolutionarily entrenched in Croatia, thereby reinforcing the central hypothesis of this dissertation.

Genomic comparisons with global *L. kirschneri* isolates further highlight the significance of these findings. Only a few strains outside Croatia, such as those from Brazil and one of unknown origin, clustered with the Croatian lineage as ST-98, indicating a very limited global distribution and supporting the idea of geographic confinement and long-term *in situ* evolution. Since all 48 Croatian isolates belong to MLST ST-98 and are phylogenetically clustered with the *L. kirschneri* serovar Mozdok strains, it is plausible that they all represent the *L. kirschneri* serogroup Pomona serovar Mozdok and less likely the serovar Tsaratsovo. Interestingly, a closely related isolate classified as ST-101 and associated with serovar Mozdok was previously shown to cause severe pulmonary hemorrhagic lesions in an experimental hamster model, suggesting a high virulence potential within this lineage (MORENO et al., 2016). These observations are consistent with our previous findings in which the serogroup Pomona was frequently associated with severe disease, particularly leptospiral pulmonary haemorrhagic syndrome (LPHS) in dogs (HABUŠ et al., 2017; HABUŠ et al., 2020), although the role of individual serovars remains unclear. Given the high incidence of leptospirosis and the predominance of Pomona in clinical cases in Croatia, these results underscore its high pathogenic potential and the need for further investigation within One Health concept to clarify its pathogenicity, host range and environmental persistence. This observation, together with reports linking Pomona and Mozdok to severe clinical manifestations in animals and humans, emphasises the pathogenic capacity of this serogroup and reinforces the relevance of our findings.

Taken together, the results of this dissertation provide a coherent picture of Pomona as a re-emerging serogroup with broad epidemiological and clinical significance. In horses, Pomona

has established itself as the most prevalent serogroup, with an increasing trend observed even in apparently healthy individuals. In cats, traditionally overlooked in the context of leptospirosis, Pomona has emerged as the leading serogroup, with infections associated with clinical manifestations in certain circumstances, particularly in immunocompromised animals. In rodents, the primary reservoirs, Pomona has been confirmed as a distinct, geographically confined lineage of *L. kirschneri*, most likely corresponding to the serovar Mozdok, which is a stable ecological source of spillover infections. This convergence between reservoir and incidental hosts emphasises the strength of the central hypothesis that Pomona has re-emerged as a dominant and potentially highly virulent serogroup in Croatia.

The broader implications of these findings become clear when viewed through the lens of the One Health framework. The simultaneous presence of Pomona in wildlife reservoirs, livestock species and companion animals create multiple transmission pathways that ultimately pose a threat to human health. The presence of Pomona in pet cats, which live in close contact with humans, raises particular concern regarding household exposure. Likewise, the role of horses as long-lived carriers that frequently shed leptospires into the environment increases the risk of environmental contamination and incidental infections in other species. In combination, these factors highlight the need for integrated surveillance and control strategies that link veterinary and human health.

Ecological factors have a further influence on the persistence and transmission of Pomona. Changes in rodent population dynamics, often related to climate change, land use change and urbanisation, are likely to affect the prevalence of certain serogroups in natural reservoirs. Mixed farming systems, where horses, cats and other companion and domestic animals live together and share environments contaminated with rodent urine, increase the potential for cross-species transmission. In such contexts, Pomona, as a serogroup with proven adaptability and pathogenic potential, is particularly well positioned to persist and spread. These ecological and anthropogenic influences must therefore be considered when developing preventive measures and diagnostic approaches.

At the same time, this research opens several important avenues for further investigation. In cats, the relatively small sample size does not allow definite conclusions, but the observed findings emphasise the need to better determine the clinical relevance of Pomona infections in this species, especially in relation to respiratory manifestations. Future work should extend comparative analyses across different species, including humans, dogs, pigs, and cattle, and include more comprehensive genomic studies to clarify the associations between virulence and clinical outcomes across species. Given that there are currently no licensed vaccines against the

serogroup Pomona available in Croatia, these findings also emphasise the urgent need for the development and implementation as part of preventive strategies. Future priorities should also include defining the role of individual serovars in pathogenesis, implementation of whole genome sequencing directly on clinical samples, monitoring the impact of environmental changes on reservoir dynamics and developing integrated prevention and control strategies.

In conclusion, this dissertation shows that Pomona has re-emerged as the most virulent and dominant serogroup within the pathogenic *Leptospira* spp. complex in Croatia. By including data from horses, cats and rodents, Pomona is shown to be an ecologically stable and clinically significant serogroup with important implications for human and animal health. The recognition of a geographically restricted lineage of *L. kirschneri* serogroup Pomona emphasises the need for country-specific surveillance, while the documented clinical impact in cats and high prevalence in horses calls for increased vigilance in veterinary practise. These findings underline the importance of a One Health concept approach that fully recognises the interconnectedness of reservoir hosts, domestic and companion animals and human health. Ultimately, the re-emergence of Pomona in Croatia demonstrates the evolutionary resilience and pathogenic capacity of this serogroup and emphasises its importance as a priority veterinary and public health target.

6. CONCLUSIONS

1. The serogroup Pomona has re-emerged as the most virulent and dominant lineage within the pathogenic *Leptospira* spp. in Croatia.
2. Pomona was identified as the most prevalent serogroup in horses, with a clear increasing trend in the last decade, indicating a dynamic shift in the epizootiology of leptospirosis.
3. Pomona also emerged in cats as the leading serogroup, with evidence that infection can lead to the clinical disease, particularly in immunocompromised individuals, indicating that leptospirosis is underdiagnosed in cats and that cats may play a more important role in the epidemiology than previously thought.
4. In rodents as primary reservoirs, whole genome sequencing revealed that Croatian isolates form a distinct, geographically restricted lineage of the *L. kirschneri* serogroup Pomona, most likely serovar Mozdok, suggesting long-term host adaptation and supporting its role as a primary reservoir and stable ecological source of spillover infections.
5. The convergence of findings in horses, cats and rodents is clear evidence that Pomona is not a sporadic occurrence, but a re-emerging serogroup with established ecological stability, high pathogenic potential and importance in the context of One Health concept.

7. BIBLIOGRAPHY

ACKERMANN, K., R. KENNGOTT, M. SETTLES, H. GERHARDS, J. MAIERL, B. WOLLANKE (2021): *In vivo* biofilm formation of pathogenic *Leptospira* spp. in the vitreous humor of horses with recurrent uveitis. Microorganisms 9, 1915. https://doi.org/10.3390/microorganisms9091915

ADLER, B., A. DE LA PEÑA MOCTEZUMA (2010): *Leptospira* and leptospirosis. Vet. Microbiol. 140, 287-296. https://doi.org/10.1016/j.vetmic.2009.03.012

ADLER, B., M. LO, T. SEEMANN, G. L. MURRAY (2011): Pathogenesis of leptospirosis: the influence of genomics. Vet. Microbiol. 153, 73-81. https://doi.org/10.1016/j.vetmic.2011.02.055

AHMED, N., S. M. DEVI, M. DE LOS Á. VALVERDE, P. VIJAYACHARI, R. S. MACHANG'U, W. A. ELLIS, R. A. HARTSKEERL (2006): Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. Ann. Clin. Microbiol. Antimicrob. 5, 28. https://doi.org/10.1186/1476-0711-5-28

ALASHRAF, A. R., S. F. LAU, K. H. KHOR, S. KHAIRANI-BEJO, A. R. BAHAMAN, M. A. ROSLAN, M. S. A. RAHMAN, S. H. GOH, R. RADZI (2019): Serological detection of anti-*Leptospira* antibodies in shelter cats in Malaysia. Top. Companion Anim. Med. 34, 10-13. https://doi.org/10.1053/j.tcam.2018.12.002

ALEXANDER, D. C., P. N. LEVETT, C. Y. TURENNE (2015): Molecular taxonomy. In: Molecular Medical Microbiology (Dongyou Liu, Ed.), 2nd ed., Elsevier, London, 369-379. https://doi.org/10.1016/B978-0-12-397169-2.00021-4

ALIBERTI, A., V. BLANDA, V. DI MARCO LO PRESTI, G. MACALUSO, P. GALLUZZO, C. BERTASIO, C. SCIACCA, F. ARCURI, R. D'AGOSTINO, D. IPPOLITO, F. PRUITI CIARELLO, A. TORINA, F. GRIPPI (2022): *Leptospira interrogans* serogroup Pomona in a dairy cattle farm in a multi-host zoo-technical system. Vet. Sci. 9, 83. doi:10.3390/vetsci9020083. https://doi.org/10.3390/vetsci9020083

ANDRE-FONTAINE, G., F. AVIAT, C. THORIN (2015): Waterborne leptospirosis: survival and preservation of the virulence of pathogenic *Leptospira* spp. in fresh water. Curr. Microbiol. 71, 136-142. https://doi.org/10.1007/s00284-015-0836-4

ANONYMOUS (2021): 3.01.12 LEPTOSPIROSIS. In: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022.

ANTUNOVIĆ-MIKAČIĆ, S. (1935): O prvom slučaju Weilove bolesti na našem Primorju. (The first case of Weil's disease on our coastal region). Lij. Vjes. 57, 377. (in Croatian).

ARENT, Z. J., C. GILMORE, J. M. SAN-MIGUEL AYANZ, L. QUEVEDO NEYRA, F. J. GARCÍA-PEÑA (2017a): Molecular epidemiology of *Leptospira* serogroup Pomona infections among wild and domestic animals in Spain. EcoHealth 14, 48-57. https://doi.org/10.1007/s10393-017-1210-8

ARENT, Z., C. GILMORE, A. M. BARLOW, L. SMITH, W. A. ELLIS (2017b): *Leptospira interrogans* serogroup Pomona infections in the UK: Is there a real threat for farm animals? Vet. Rec. 180, 424. https://doi.org/10.1136/vr.103891

BABIĆ, I. (1927): Typhus canum u Zagrebu. (Typhus canum in Zagreb). Jug. Vet. Glas. 7, 21. (in Croatian).

BAHAROM, M., N. AHMAD, R. HOD, M. H. JA'AFAR, F. S. ARSAD, F. TANGANG, R. ISMAIL, N. MOHAMED, M. F. M. RADI, Y. OSMAN (2024): Environmental and occupational factors associated with leptospirosis: a systematic review. Heliyon 10, e23473. https://doi.org/10.1016/j.heliyon.2023.e23473

BALEN TOPIC, M., J. HABUS, Z. MILAS, E. CELJUSKA TOSEV, Z. STRITOF, N. TURK (2010): Human leptospirosis in Croatia: current status of epidemiology and clinical characteristics. Trans. R. Soc. Trop. Med. Hyg. 104, 202-206. https://doi.org/10.1016/j.trstmh.2009.05.018

BARANTON, G., I. G. OLD (1995): The spirochaetes: a different way of life. Bull. l'Institut Pasteur 93, 63-95. https://doi.org/10.1016/0020-2452(96)81485-0

BARRAGAN, V., N. NIETO, P. KEIM, T. PEARSON (2017): Meta-analysis to estimate the load of *Leptospira* excreted in urine: beyond rats as important sources of transmission in low-income rural communities. BMC Res. Notes 10, 71. https://doi.org/10.1186/s13104-017-2384-4

BENVIN, I.; MOJČEC PERKO, V.; MALJKOVIĆ, M.M.; HABUŠ, J.; ŠTRITOF, Z.; HAĐINA, S.; PERHARIĆ, M.; ZEČEVIĆ, I.; CVETNIĆ, M.; TURK, N. (2023): Serological Surveillance of Equine Leptospirosis in Croatia in the Period From 2012 to 2022: A Key Insight into the Changing Epizootiology. J. Equine Vet. Sci. 127, 104844. https://doi.org/10.1016/j.jevs.2023.104844

BENVIN, I.; FITZ, D.; MOJČEC PERKO, V.; MAURIĆ MALJKOVIĆ, M.; ĐURIĆ, V.; HABUŠ, J.; ŠTRITOF, Z.; PERHARIĆ, M.; HAĐINA, S.; ZEČEVIĆ, I.; TURK, N. (2024): Insights into *Leptospira* spp. infection in pet cats in Croatia: Clinical, serological and molecular findings with emphasis on the potential important role of serogroup Pomona. Acta Trop. 260, 107465. https://doi.org/10.1016/j.actatropica.2024.107465

BENVIN, I.; PAISIE, T. K.; CAETANO VARANDA, I.; WEINER, Z. P.; STODDARD, R. A.; GEE, J. E.; GULVIK, C. A.; MARSTON, C. K.; MOJČEC PERKO, V.; ŠTRITOF, Z.; HABUŠ J.; MARGALETIĆ, J.; VUCELJA, M.; BJEDOV, L.; TURK, N. (2025): Whole Genome Characterization of *Leptospira kirschneri* Serogroup Pomona in Croatia: Insights into Its Diversity and Evolutionary Emergence. Pathogens, 14, 860. https://doi.org/10.3390/pathogens14090860

BERTASIO, C., A. PAPETTI, E. SCALTRITI, S. TAGLIABUE, M. D'INCAU, M. B. BONIOTTI (2020): Serological survey and molecular typing reveal new *Leptospira* serogroup Pomona strains among pigs of Northern Italy. Pathogens 9, 332. https://doi.org/10.3390/pathogens9050332

BIERQUE, E., M. E. SOUPÉ-GILBERT, R. THIBEAUX, D. GIRAULT, L. GUENTAS, C. GOARANT (2020): *Leptospira interrogans* retains direct virulence after long starvation in water. Curr. Microbiol. 77, 3035-3043. https://doi.org/10.1007/s00284-020-02128-7

BOLIN, C. A., J. A. CASSELLS (1992): Isolation of *Leptospira interrogans* serovars Bratislava and Hardjo from swine at slaughter. J. Vet. Diagn. Invest. 4, 87-89. https://doi.org/10.1177/104063879200400121

BORČIĆ, B., H. KOVAČIĆ, Z. ŠEBEK, B. ALERAJ, N. TVRTKOVIĆ (1982): Small terrestrial mammals as reservoir of leptospires in Sava Valley (Croatia). Folia Parasitol. 29, 177-182.

BORČIĆ, B., H. KOVAČIĆ, Z. ŠEBEK, B. ALERAJ, N. TVRTKOVIĆ (1983): Small terrestrial mammals as reservoir of leptospires in Drava Valley. Vet. Arhiv 53, 41-49.

BOURHY, P., I. SAINT GIRONS (2000): Localization of the *Leptospira interrogans metF* gene on the CII secondary chromosome. (brief report). https://doi.org/10.1111/j.1574-6968.2000.tb09349.x

BOURHY, P., S. BREMONT, F. ZININI, C. GIRY, M. PICARDEAU (2011): Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. J. Clin. Microbiol. 49, 2154-2160. https://doi.org/10.1128/JCM.02452-10

BOURHY, P., L. COLLET, T. LERNOUT, F. ZININI, R. A. HARTSKEERL, H. VAN DER LINDEN, J. M. THIBERGE, L. DIANCOURT, S. BRISSE, C. GIRY, F. PETTINELLI, M. PICARDEAU (2012): Human *Leptospira* isolates circulating in Mayotte (Indian Ocean) have unique serological and molecular features. J. Clin. Microbiol. 50, 307-311. https://doi.org/10.1128/JCM.05931-11

BROUX, B., S. TORFS, B. WEGGE, P. DEPREZ, G. VAN LOON (2012): Acute respiratory failure caused by *Leptospira* spp. in five foals. J. Vet. Intern. Med. 26, 684-687. https://doi.org/10.1111/j.1939-1676.2012.00902.x

BRYSON, D. G., W. A. ELLIS (1976): Leptospirosis in a British domestic cat. J. Small Anim. Pract. 17, 459-465. https://doi.org/10.1111/j.1748-5827.1976.tb06986.x

BULACH, D. M., R. L. ZUERNER, P. WILSON, T. SEEMANN, A. MCGRATH, P. A. CULLEN, J. DAVIS, M. JOHNSON, E. KUCZEK, D. P. ALT, B. PETERSON-BURCH, R. L. COPPEL, J. I. ROOD, J. K. DAVIES, B. ADLER (2006): Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. Proc. Natl. Acad. Sci. USA 103, 14560-14565.https://doi.org/10.1073/pnas.0603979103

CAGLIERO, J., S. Y. A. M. VILLANUEVA, M. MATSUI (2018): Leptospirosis pathophysiology: into the storm of cytokines. Front. Cell. Infect. Microbiol. 8, 204. https://doi.org/10.3389/fcimb.2018.00204

CAMERON, C. E. (2015): Leptospiral structure, physiology, and metabolism. In: *Leptospira* and leptospirosis (Adler, B., Ed.), Curr. Top. Microbiol. Immunol. 387, Springer, Berlin, Heidelberg, 21-41. https://doi.org/10.1007/978-3-662-45059-8_3

CARVALHO, R. R. M., C. SILVA DIAS, L. N. PAZ, T. M. L. FIRES, C. P. FIGUEIRA, K. A. DAMASCENO, M. H. PINNA (2023): Biofilm formation in vitro by *Leptospira interrogans* strains isolated from naturally infected dogs and their role in antimicrobial resistance. Heliyon 9, e13802. https://doi.org/10.1016/j.heliyon.2023.e13802

CARVALHO, H. G. A. C., D. M. SILVA, G. R. D. RODRIGUES, A. H. GAMEIRO, R. F. DOS SANTOS, C. RAINERI, A. M. C. LIMA (2024): Estimation of economic losses due to leptospirosis in dairy cattle. Prev. Vet. Med. 229, 106255. https://doi.org/10.1016/j.prevetmed.2024.106255

CHAKRABORTY, A., S. MIYAHARA, S. Y. A. M. VILLANUEVA, M. SAITO, N. G. GLORIANI, S. I. YOSHIDA (2011): A novel combination of selective agents for isolation of *Leptospira* species. Microbiol. Immunol. 55, 494-501. https://doi.org/10.1111/j.1348-0421.2011.00347.x

CHIERAKUL, W., P. TIENTADAKUL, Y. SUPUTTAMONGKOL, V. WUTHIEKANUN, K. PHIMDA, R. LIMPAIBOON, N. OPARTKIATTIKUL, N. J. WHITE, S. J. PEACOCK, N. P. DAY (2008): Activation of the coagulation cascade in patients with leptospirosis. Clin. Infect. Dis. 46, 254-260. https://doi.org/10.1086/524664

CHINCHILLA, D., C. NIEVES, R. GUTIÉRREZ, V. SORDOILLET, F. J. VEYRIER, M. PICARDEAU (2023): Phylogenomics of *Leptospira santarosai*, a prevalent pathogenic species in the Americas. PLoS Negl. Trop. Dis. 17(11): e0011733. https://doi.org/10.1371/journal.pntd.0011733

CHOW, E., J. DEVILLE, J. NALLY, M. LOVETT, K. NIELSEN-SAINES (2012): Prolonged *Leptospira* urinary shedding in a 10-year-old girl. Case Rep. Pediatr. 2012, 169013. https://doi.org/10.1155/2012/169013

COCK, P. J., T. ANTAO, J. T. CHANG, B. A. CHAPMAN, C. J. COX, A. DALKE, I. FRIEDBERG, T. HAMELRYCK, F. KAUFF, B. WILCZYNSKI, M. J. DE HOON (2009): Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics 25, 1422-1423. https://doi.org/10.1093/bioinformatics/btp163

COSTA, F., J. E. HAGAN, J. CALCAGNO, M. KANE, P. TORGERSON, M. S. MARTINEZ-SILVEIRA, C. STEIN, B. ABELA-RIDDER, A. I. KO (2015): Global morbidity and mortality of leptospirosis: a systematic review. PLoS Negl. Trop. Dis. 9, e0003898. https://doi.org/10.1371/journal.pntd.0003898

CULLEN, P. A., D. A. HAAKE, B. ADLER (2004): Outer membrane proteins of pathogenic spirochetes. FEMS Microbiol. Rev. 28, 291-318. https://doi.org/10.1016/j.femsre.2003.10.004

CVITKOVIĆ, A. (2007): Human leptospirosis in Slavonski Brod, 1995-2005. Acta Med. Croatica 61, 349-353.

DEWES, C., T. P. FORTES, G. B. MACHADO, P. S. PACHECO, J. P. M. SILVA, A. C. P. SEIXAS NETO, S. R. FÉLIX, É. F. DA SILVA (2020): Prevalence and risk factors associated with equine leptospirosis in an endemic urban area in southern Brazil. Braz. J. Dev. 6, 58380-58390. https://doi.org/10.34117/bjdv6n8-302

DI TOMMASO, P., M. CHATZOU, E. W. FLODEN, P. PRIETO BARJA, E. PALUMBO, C. NOTREDAME (2017): Nextflow enables reproducible computational workflows. Nat. Biotechnol. 35, 316-319. https://doi.org/10.1038/nbt.3820

DIKKEN, H., E. KMETY (1978): Serological typing methods of leptospires. In: Methods in Microbiology (T. Bergan, J. R. Norris, Eds.), Academic Press, New York, 259-307. https://doi.org/10.1016/S0580-9517(08)70493-8

DONATO, G., M. MASUCCI, K. HARTMANN, M. G. A. GORIS, A. A. AHMED, J. ARCHER, A. ALIBRANDI, M. G. PENNISI (2022): *Leptospira* spp. prevalence in cats from Southern Italy with evaluation of risk factors for exposure and clinical findings in infected cats. Pathogens 11, 1129. https://doi.org/10.3390/pathogens11101129

ELLIS, W. A., J. G. SONGER, J. MONTGOMERY, J. A. CASSELLS (1986a): Prevalence of *leptospira interrogans* serovar Hardjo in the genital and urinary tracts of non-pregnant cattle. Vet. Rec. 118, 11-13. https://doi.org/10.1136/vr.118.1.11

ELLIS, W. A., P. J. MCPARLAND, D. G. BRYSON, A. B. THIERMANN, J. MONTGOMERY (1986b): Isolation of leptospires from the genital tract and kidneys of aborted sows. Vet. Rec. 118, 294-295. https://doi.org/10.1136/vr.118.11.294

ELLIS, W. A. (2012): Leptospirosis. In: Diseases of Swine (Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J., Stevenson, G. W., Eds.), 10th ed., Wiley, New York, 770-788.

ELLIS, W. A. (2015): Animal leptospirosis. In: *Leptospira* and leptospirosis (Adler, B., Ed.), Curr. Top. Microbiol. Immunol. 387, Springer, Berlin, Heidelberg, 99-137. https://doi.org/10.1007/978-3-662-45059-8_6

FAGRE, A. C., C. E. MAYO, K. L. PABILONIA, G. A. LANDOLT (2020): Seroprevalence of *Leptospira* spp. in Colorado equids and association with clinical disease. J. Vet. Diagn. Invest. 32, 718-721. https://doi.org/10.1177/1040638720943155

FAINE, S., N. D. STALLMAN (1982): Amended descriptions of the genus *Leptospira*. Int. J. Syst. Bacteriol. 32, 461-463. https://doi.org/10.1099/00207713-32-4-461

FAINE, S., B. ADLER, C. BOLIN, P. PÉROLAT (1999): *Leptospira* and Leptospirosis, 2nd ed., MediSci, Melbourne.

FERNANDES, L. G. V., N. E. STONE, C. C. ROE, M. G. A. GORIS, H. VAN DER LINDEN, J. W. SAHL, D. M. WAGNER, J. E. NALLY (2022): *Leptospira sanjuanensis* sp. nov., a pathogenic species isolated from soil in Puerto Rico. Int. J. Syst. Evol. Microbiol. 72, 005560. https://doi.org/10.1099/ijsem.0.005560

FERREIRA, L.C.A. (2024): Genetic structure and diversity of the rfb locus of pathogenic species of the genus *Leptospira*. Life Sci. Alliance 7, e202302478. https://doi.org/10.26508/lsa.202302478

GAJDOV, V., G. JOKIĆ, S. SAVIĆ, M. ZEKIĆ, T. BLAŽIĆ, M. RAJKOVIĆ, T. PETROVIĆ (2024): Genotyping of *Leptospira* spp. in wild rats leads to first time detection of *L. kirschneri* serovar Mozdok in Serbia. Front. Microbiol. 15, 1379021. https://doi.org/10.3389/fmicb.2024.1379021

GALE, N. B., A. D. ALEXANDER, L. B. EVANS, R. H. YAGER, R. A. METHENEY (1966): An outbreak of leptospirosis among U.S. Army troops in the Canal Zone. II. Isolation and characterization of the isolates. Am. J. Trop. Med. Hyg. 15, 64-70. https://doi.org/10.4269/ajtmh.1966.15.64

GALLOWAY, R. L., P. N. LEVETT (2010): Application and validation of PFGE for serovar identification of *Leptospira* clinical isolates. PLoS Negl. Trop. Dis. 4, e824. https://doi.org/10.1371/journal.pntd.0000824

GALLOWAY, R. L., C. A. GULVIK (2023): *Leptospira*. In: ClinMicroNow, Wiley, 1-9. https://doi.org/10.1002/9781683674849.mcm0061

GIRAUD-GATINEAU, A., K. DAGBO, H. PĚTROŠOVÁ, C. WERTS, F. J. VEYRIER, M. PICARDEAU (2025): Shaping the future of *Leptospira* serotyping. J. Med. Microbiol. 74, 002059. https://doi.org/10.1099/jmm.0.002059

GOODWIN, S., J. D. MC PHERSON, W. R. MC COMBIE (2016): Coming of age: ten years of next-generation sequencing technologies. Nat. Rev. Genet. 17, 333-351. https://doi.org/10.1038/nrg.2016.49

GRIPPI, F., V. CANNELLA, G. MACALUSO, V. BLANDA, G. EMMOLO, F. SANTANGELO, D. VICARI, P. GALLUZZO, C. SCIACCA, R. D'AGOSTINO, I. GIACCHINO, C. BERTASIO, M. D'INCAU, A. GUERCIO, A. TORINA (2023): Serological and molecular evidence of pathogenic *Leptospira* spp. in stray dogs and cats of Sicily (South Italy), 2017-2021. Microorganisms 11, 385. https://doi.org/10.3390/microorganisms11020385

GROOMS, D. L. (2006): Reproductive losses caused by bovine viral diarrhea virus and leptospirosis. Theriogenology 66(3), 624-628. https://doi.org/10.1016/j.theriogenology.2006.04.016

GUEDES, I. B., G. O. DE SOUZA, J. F. DE PAULA CASTRO, M. B. BURILLI CAVALINI, A. F. DE SOUZA FILHO, A. L. P. MAIA, E. A. DOS REIS, A. CORTEZ, M. B. HEINEMANN (2021): *Leptospira interrogans* serogroup Pomona strains isolated from river buffaloes. Trop. Anim. Health Prod. 53, 194. https://doi.org/10.1007/s11250-021-02623-4

GUGLIELMINI, J., P. BOURHY, O. SCHIETTEKATTE, F. ZININI, S. BRISSE, M. PICARDEAU (2019): Genus-wide *Leptospira* core genome multilocus sequence typing for strain taxonomy and global surveillance. PLoS Negl Trop Dis 13(4): e0007374. https://doi.org/10.1371/journal.pntd.0007374

HAAKE, D. A., P. N. LEVETT (2015): Leptospirosis in humans. In: *Leptospira* and leptospirosis (Adler, B., Ed.), Curr. Top. Microbiol. Immunol. 387, Springer, Berlin, Heidelberg, 65-97. https://doi.org/10.1007/978-3-662-45059-8_5

HABUS, J., Z. PERSIC, S. SPICIC, S. VINCE, Z. STRITOF, Z. MILAS, Z. CVETNIC, M. PERHARIC, N. TURK (2017): New trends in human and animal leptospirosis in Croatia, 2009-2014. Acta Trop. 168, 1-8. https://doi.org/10.1016/j.actatropica.2017.01.002

HABUŠ, J., A. GUDAN, B. ŠKRLIN, Z. ŠTRITOF, V. MOJČEC PERKO, S. HAĐINA, V. STEVANOVIĆ, M. PERHARIĆ, K. MARTINKOVIĆ, D. HUBER, Z. MILAS, N. TURK (2017): Radiological and necropsy findings in dogs with leptospirosis pulmonary haemorrhagic syndrome. Proc. 10th Int. Leptospirosis Soc. Conf., Palmerston North, 27.11.-01.12.2017, 236.

HABUŠ, J., Z. POLJAK, Z. ŠTRITOF, V. MOJČEC PERKO, Z. MILAS, M. PERHARIĆ, K. MARTINKOVIĆ, S. HAĐINA, V. STEVANOVIĆ, V. STAREŠINA, N. TURK (2020): Prognostic factors for survival of canine patients infected with *Leptospira* spp. Vet. Arhiv 90, 111-128.

HAMOND, C., G. MARTINS, J. REIS, E. KRAUS, A. PINNA, W. LILENBAUM (2011): Pulmonary hemorrhage in horses seropositive to leptospirosis. Pesqui. Vet. Bras. 31, 413-415. https://doi.org/10.1590/S0100-736X2011000500008

HAMOND, C., E. N. ADAM, N. E. STONE, K. LECOUNT, T. ANDERSON, E. J. PUTZ, P. CAMP, J. HICKS, T. STUBER, H. VAN DER LINDEN, D. O. BAYLES, J. W. SAHL, L. K. SCHLATER, D. M. WAGNER, J. E. NALLY (2024): Identification of equine mares as reservoir hosts for pathogenic species of *Leptospira*. Front. Vet. Sci. 11, 1346713. https://doi.org/10.3389/fvets.2024.1346713

HARRISON, P. W., R. P. LOWER, N. K. KIM, J. P. YOUNG (2010): Introducing the bacterial chromid: not a chromosome, not a plasmid. Trends Microbiol. 18, 141-148. https://doi.org/10.1016/j.tim.2009.12.010

HARTSKEERL, R. A., M. COLLARES-PEREIRA, W. A. ELLIS (2011): Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. Clin. Microbiol. Infect. 17, 494-501. https://doi.org/10.1111/j.1469-0691.2011.03474.x

HELMAN, S. K., A. F. N. TOKUYAMA, R. O. MUMMAH, N. E. STONE, M. W. GAMBLE, C. E. SNEDDEN, B. BORREMANS, A. C. R. GOMEZ, C. COX, J. NUSSBAUM, I. TWEEDT, D. A. HAAKE, R. L. GALLOWAY, J. MONZÓN, S. P. D. RILEY, J. A. SIKICH, J. BROWN, A. FRISCIA, J. W. SAHL, D. M. WAGNER, J. W. LYNCH, K. C. PRAGER, J. O. LLOYD-SMITH (2023): Pathogenic *Leptospira* are widespread in the urban wildlife of southern California. Sci. Rep. 13, 14368. https://doi.org/10.1038/s41598-023-40322-2

HORNSBY, R. L., D. P. ALT, J. E. NALLY (2020): Isolation and propagation of leptospires at 37 °C directly from the mammalian host. Sci. Rep. 10, 9620. https://doi.org/10.1038/s41598-020-66526-4

HÜBENER, E. A., H. REITER (1915): Beiträge zur Ätiologie der Weil'schen Krankheit. Dtsch. Med. Wochenschr. 41, 1275-1277. https://doi.org/10.1055/s-0029-1192227

INADA, R., Y. IDO (1915): Eine zusammenfassende Mitteilung über die Entdeckung des Erregers der Weil'schen Krankheit. Tokyo. Ijishinski 1808.

JOHNSON, R. C., S. FAINE (1984): Order I. *Spirochaetales*: Family II. *Leptospiraceae* Hovind-Hougen 1979. In: Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams & Wilkins, Baltimore, 62-67.

JOHNSON, D. I. (2018): *Leptospira* spp. In: Bacterial Pathogens and Their Virulence Factors (Johnson, D. I., Ed.), Springer, Cham, 289-294. https://doi.org/10.1007/978-3-319-67651-7_21

JOLLEY, K. A., M. C. J. MAIDEN (2010): BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinform. 11, 595. https://doi.org/10.1186/1471-2105-11-595

JORGE, S., F. S. KREMER, N. R. OLIVEIRA, G. O. S. V. NAVARRO, A. M. GUIMARÃES, C. D. SÁNCHEZ, R. D. D. S. WOLOSKI, K. F. RIDIERI, V. F. CAMPOS, L. S. PINTO, O. A. DELLAGOSTIN (2018): Whole-genome sequencing of *Leptospira interrogans* from southern Brazil: genetic features of a highly virulent strain. Mem. Inst. Oswaldo Cruz 113, 80-86. https://doi.org/10.1590/0074-02760170130

KMETY, E., H. DIKKEN (1993): Classification of the species *Leptospira interrogans* and history of its serovars. University Press, Groningen.

KO, A. I., C. GOARANT, M. PICARDEAU (2009): *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat. Rev. Microbiol. 7, 736-747. https://doi.org/10.1038/nrmicro2208

KOHN, B., K. STEINICKE, G. ARNDT, A. D. GRUBER, B. GUERRA, A. JANSEN, B. KASER-HOTZ, R. KLOPFLEISCH, F. LOTZ, E. LUGE, K. NÖCKLER (2010): Pulmonary abnormalities in dogs with leptospirosis. J. Vet. Intern. Med. 24, 1277-1282. https://doi.org/10.1111/j.1939-1676.2010.0585.x

KORBA, A. A., H. LOUNICI, M. KAINIU, A. T. VINCENT, J. F. MARIET, F. J. VEYRIER, C. GOARANT, M. PICARDEAU (2021): *Leptospira ainlahdjerensis* sp. nov., *L. ainazelensis* sp. nov., *L. abararensis* sp. nov. and *L. chreensis* sp. nov. from water sources in Algeria. Int. J. Syst. Evol. Microbiol. 71, e005056. https://doi.org/10.1099/ijsem.0.005148

LEHTLA, A., K. MUST, B. LASSEN, T. ORRO, P. JOKELAINEN, A. VILTROP (2020): *Leptospira* spp. in cats in Estonia: Seroprevalence and risk factors for seropositivity. Vector Borne Zoonotic Dis. 20(7), 524-528. https://doi.org/10.1089/vbz.2019.2555

LEVETT, P. N. (2001): Leptospirosis. Clin. Microbiol. Rev. 14, 296-326. https://doi.org/10.1128/CMR.14.2.296-326.2001

LEVETT, P. N. (2003): Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. Clin. Infect. Dis. 36, 447-452. https://doi.org/10.1086/346208

LEVETT, P. N. (2015): *Leptospira*. In: Manual of Clinical Microbiology (Jorgensen, J. H., Pfaller, M. A., Carroll, K. C. et al., Eds.), 11th ed., ASM Press, Washington, DC, 1028-1036. https://doi.org/10.1128/9781555817381.ch58

LOURDAULT, K., F. AVIAT, M. PICARDEAU (2009): Use of quantitative real-time PCR for studying the dissemination of *Leptospira interrogans* in the guinea pig infection model. J. Med. Microbiol. 58, 648-655. https://doi.org/10.1099/jmm.0.008169-0

MANEV, C. (1976): Serological characteristics of the *Leptospira* serogroup Pomona. I. Factor analysis of the reference strains. Zentralbl. Bakteriol. Orig. A 236, 316-322.

MARKOVICH, J. E., L. ROSS, E. MCCOBB (2012): The prevalence of leptospiral antibodies in free roaming cats in Worcester County, Massachusetts. J. Vet. Intern. Med. 26(3), 688-689. https://doi.org/10.1111/j.1939-1676.2012.00900.x

MAZZOTTA, E., G. DE ZAN, M. COCCHI, M. B. BONIOTTI, C. BERTASIO, T. FURLANELLO, L. LUCHESE, L. CEGLIE, L. BELLINATI, A. NATALE (2023): Feline susceptibility to leptospirosis and presence of immunosuppressive co-morbidities: first European report of *L. interrogans* serogroup Australis sequence type 24 in a cat and survey of exposure in outdoor cats. Trop. Med. Infect. Dis. 8, 54. https://doi.org/10.3390/tropicalmed8010054

MEDEIROS, F. R., A. SPICHLER, D. A. ATHANAZIO (2010): Leptospirosis-associated disturbances of blood vessels, lungs and hemostasis. Acta Trop. 115, 155-162. https://doi.org/10.1016/j.actatropica.2010.02.016

MENDOZA, M. V., W. L. RIVERA (2021): Application of simplified MLST scheme for direct typing of clinical samples from human leptospirosis cases. PLoS ONE 16, e0258891. https://doi.org/10.1371/journal.pone.0258891

MILAS, Z., N. TURK, V. STAREŠINA, J. MARGALETIĆ, A. SLAVICA, D. ŽIVKOVIĆ, Z. MODRIĆ (2002): The role of myomorphous mammals as reservoirs of leptospira in the pedunculate oak forests of Croatia. Vet. Arhiv 72, 119-129.

MILLER, D. A., M. A. WILSON, G. W. BERAN (1990): The effect of storage time on isolation of *Leptospira interrogans* from bovine kidneys. J. Vet. Diagn. Invest. 2, 11-14. https://doi.org/10.1177/104063879000200112

MINH, B. Q., H. A. SCHMIDT, O. CHERNOMOR, D. SCHREMPF, M. D. WOODHAMS, A. VON HAESELER, R. LANFEAR, E. TEELING (2020): IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. 37, 1530-1534. https://doi.org/10.1093/molbev/msaa015

MIOTTO, B. A., Q. C. CAMELO, A. C. M. M. GROLLA, A. B. M. DE OLIVEIRA, M. M. B. SILVA, M. K. HAGIWARA, S. B. ESTEVES (2024): Current knowledge on leptospirosis in cats: a systematic review with meta-analysis. Res. Vet. Sci. 174, 105292. https://doi.org/10.1016/j.rvsc.2024.105292

MODRIĆ, Z. (1978): Natural and experimental leptospirosis in the cat. Vet. Arhiv 48, 147-156. (in Croatian).

MODRIĆ, Z. (1979): Leptospirosis in the cat (*Felis domestica*) in Baranja. Summaries XXI World Veterinary Congress, Moscow, 4, 50.

MOREIRA DA SILVA, J., S. PRATA, T. D. DOMINGUES, R. O. LEAL, T. NUNES, L. TAVARES, V. ALMEIDA, N. SEPÚLVEDA, S. GIL (2020): Detection and modelling of anti-*Leptospira* IgG prevalence in cats from Lisbon and correlation to retroviral infections and clinical changes. Vet. Anim. Sci. 10, 100144. https://doi.org/10.1016/j.vas.2020.100144

MORENO, L. Z., F. S. KREMER, F. MIRAGLIA, A. P. LOUREIRO, M. R. ESLABAO, O. A. DELLAGOSTIN, W. LILENBAUM, A. M. MORENO (2016): Comparative genomic analysis of Brazilian *Leptospira kirschneri* serogroup Pomona serovar Mozdok. Mem. Inst. Oswaldo. Cruz. 111, 539-541. https://doi.org/10.1590/0074-02760160174

MURILLO, A., M. G. A. GORIS, A. AHMED, R. CUENCA, J. PASTOR (2020): Leptospirosis in cats: current literature review to guide diagnosis and management. J. Feline Med. Surg. 22, 216-228. https://doi.org/10.1177/1098612X20903601

MURRAY, G. L. (2015): The molecular basis of leptospiral pathogenesis. In: *Leptospira* and leptospirosis (Adler, B., Ed.), Curr. Top. Microbiol. Immunol. 387, Springer, Berlin, Heidelberg, 139-185. https://doi.org/10.1007/978-3-662-45059-8_7

MUSSO, D., B. LA SCOLA (2013): Laboratory diagnosis of leptospirosis: a challenge. J. Microbiol. Immunol. Infect. 46, 245-252. https://doi.org/10.1016/j.jmii.2013.03.001

MUÑOZ-ZANZI, C., A. DREYFUS, U. LIMOTHAI, W. FOLEY, N. SRISAWAT, M. PICARDEAU, D. A. HAAKE (2025): Leptospirosis - improving healthcare outcomes for a neglected tropical disease. Open Forum Infect. Dis. 12, ofaf035. https://doi.org/10.1093/ofid/ofaf035

MWACHUI, M. A., L. CRUMP, R. HARTSKEERL, J. ZINSSTAG, J. HATTENDORF (2015): Environmental and behavioural determinants of leptospirosis transmission: a systematic review. PLoS Negl. Trop. Dis. 9, e0003843. https://doi.org/10.1371/journal.pntd.0003843

NALLY, J. E., A. A. A. AHMED, E. J. PUTZ, D. E. PALMQUIST, M. G. A. GORIS (2020): Comparison of real-time PCR, culture and FAT for detection of *Leptospira borgpetersenii* in urine of cattle. Vet. Sci. 7, 66. https://doi.org/10.3390/vetsci7020066

NARKKUL, U., J. THAIPADUNGPANIT, P. SRILOHASIN, P. SINGKHAIMUK, M. THONGDEE, S. CHAIWATTANARUNGRUENGPAISAN, P. KRAIROJANANAN, W. PAN-NGUM (2020): Optimization of culture protocols to isolate *Leptospira* spp. from environmental water and factors associated with their presence. Trop. Med. Infect. Dis. 5, 94. https://doi.org/10.3390/tropicalmed5020094

NASCIMENTO, A. L., S. VERJOVSKI-ALMEIDA, M. A. VAN SLUYS, C. B. MONTEIRO-VITORELLO, L. E. CAMARGO, L. A. DIGIAMPIETRI, R. A. HARTSKEERL, P. L. HO, M. V. MARQUES, M. C. OLIVEIRA, J. C. SETUBAL, D. A. HAAKE, E. A. L. MARTINS (2004): Genome features of *Leptospira interrogans* serovar Copenhageni. Braz. J. Med. Biol. Res. 37, 459-478. https://doi.org/10.1590/S0100-879X2004000400003

NICODEMO, A. C., A. N. DUARTE-NETO (2021): Pathogenesis of pulmonary hemorrhagic syndrome in human leptospirosis. Am. J. Trop. Med. Hyg. 104, 1970-1972. https://doi.org/10.4269/ajtmh.20-1000

NIEVES, C. (2022): Horizontal transfer of the *rfb* cluster in *Leptospira* is a genetic determinant of serovar identity. Life Sci. Alliance 6, e202201480. https://doi.org/10.26508/lsa.202201480

OLIVEIRA, S. J. D., F. BORTOLANZA, D. T. PASSOS, J. A. S. PIRES NETO, L. C. B. FALLAVENA, W. T. DE A. (2007): Molecular diagnosis of *Leptospira* spp. in culled sows. Braz. J. Vet. Res. Anim. Sci. 44, 18-23. https://doi.org/10.11606/issn.1678-4456.bjvras.2007.26655

PAIVA-CARDOSO, M. D., Z. ARENT, C. GILMORE, R. HARTSKEERL, W. A. ELLIS (2013): Altodouro, a new *Leptospira* serovar of the Pomona serogroup isolated from rodents in northern Portugal. Infect. Genet. Evol. 13, 211-217. https://doi.org/10.1016/j.meegid.2012.09.013

PAPPAS, G., P. PAPADIMITRIOU, V. SIOZOPOULOU, L. CHRISTOU, N. AKRITIDIS (2008): The globalization of leptospirosis: worldwide incidence trends. Clin. Microbiol. Infect. 12, 351-357. https://doi.org/10.1016/j.ijid.2007.09.011

PERIĆ, L., D. ŠIMAŠEK, J. BARBIĆ, N. PERIĆ, V. PRUS, V. ŠIŠLJAGIĆ, L. ZIBAR (2005): Human leptospirosis in eastern Croatia, 1969-2003: epidemiological, clinical, and serological features. Scand. J. Infect. Dis. 37, 738-741. https://doi.org/10.1080/00365540510012170

PETAKH, P., O. KAMYSHNYI (2025): Serogroup distribution of *Leptospira* among humans and rodents in Zakarpattia Oblast, Ukraine (2018-2023). Microorganisms 13, 614. https://doi.org/10.3390/microorganisms13030614

PHILIP, N., N. B. AFFENDY, S. N. MASRI, Y. Y. MUHAMAD, L. T. L. THAN, Z. SEKAWI, V. K. NEELA (2020): Combined PCR and MAT improves the early diagnosis of leptospirosis. PLoS ONE 15, e0239069. https://doi.org/10.1371/journal.pone.0239069

PICARDEAU, M., D. M. BULACH, C. BOUCHIER, R. L. ZUERNER, N. ZIDANE, P. J. WILSON, S. CRENO, E. S. KUCZEK, S. BOMMEZZADRI, J. C. DAVIS, A. MCGRATH, M. J. JOHNSON, C. BOURSAUX-EUDE, T. SEEMANN, Z. ROUY, R. L. COPPEL, J. I. ROOD, A. LAJUS, J. K. DAVIES, C. MÉDIGE, B. ADLER (2008): Genome sequence of the saprophyte *Leptospira biflexa* provides insights into evolution and pathogenesis. PLoS ONE 3, e1607. https://doi.org/10.1371/journal.pone.0001607

PICARDEAU, M. (2013): Diagnosis and epidemiology of leptospirosis. Med. Mal. Infect. 43, 1-9. https://doi.org/10.1016/j.medmal.2012.11.005

PICARDEAU, M. (2015): Genomics, proteomics, and genetics of *Leptospira*. In: *Leptospira* and leptospirosis (Adler, B., Ed.), Curr. Top. Microbiol. Immunol. 387, Springer, Berlin, Heidelberg, 43-63. https://doi.org/10.1007/978-3-662-45059-8_4

PICARDEAU, M. (2017): Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nat. Rev. Microbiol. 15, 297-307. https://doi.org/10.1038/nrmicro.2017.5

RAMSAY, L., C. EBERHARDT, A. SCHOSTER (2024): Acute leptospirosis in horses: a retrospective study of 11 cases (2015-2023). J. Vet. Intern. Med. 38, 2729-2738. https://doi.org/10.1111/jvim.17184

REN, S.-X., G. FU, X.-G. JIANG, R. ZENG, Y.-G. MIAO, H. XU, Y.-X. ZHANG, H. XIONG, G. LU, L.-F. LU, H.-Q. JIANG, J. JIA, Y.-F. TU, J.-X. JIANG, W.-Y. GU, Y.-Q. ZHANG, Z. CAI, H.-H. SHENG, H.-F. YIN, Y. ZHANG, G.-F. ZHU, M. WAN, H.-L. HUANG, Z. QIAN, S.-Y. WANG, W. MA, Z.-J. YAO, Y. SHEN, B.-Q. QIANG, Q.-C. XIA, X.-K. GUO, A. DANCHIN, I. SAINT GIRONS, R. L. SOMERVILLE, Y.-M. WEN, M.-H. SHI, Z. CHEN, J.-G. XU, G.-P. ZHAO (2003): Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature 422, 888-893. https://doi.org/10.1038/nature01597

REZENDE MIRES DE CARVALHO, R., C. SILVA DIAS, L. N. NOGUEIRA PAZ, T. M. DE L. FIRES, C. P. FIGUEIRA, K. A. DAMASCENO, M. HANZEN PINNA (2023): Biofilm formation in vitro by *Leptospira interrogans* strains isolated from naturally infected dogs and their role in antimicrobial resistance. Heliyon 9, e13802. https://doi.org/10.1016/j.heliyon.2023.e13802

RIBOTTA, M., R. HIGGINS, D. PERRON (1999): Swine leptospirosis: low risk of exposure for humans. Can. Vet. J. 40, 809-810. https://doi.org/10.1111/j.1528-1157.1999.tb00785.x

RICARDO, T., L. AZÓCAR-AEDO, M. SIGNORINI, M. A. PREVITALI (2023): Leptospiral infection in domestic cats: Systematic review with meta-analysis. Prev. Vet. Med. 212, 105851. https://doi.org/10.1016/j.prevetmed.2023.105851

RIEDIGER, I. N., R. A. STODDARD, G. S. RIBEIRO, S. M. NAKATANI, S. D. R. MOREIRA, I. SKRABA, A. W. BIONDO, M. G. REIS, A. R. HOFFMASTER, J. M. VINETZ, A. I. KO, E. A. WUNDER JR. (2017): Rapid diagnosis of urban epidemic leptospirosis using a lipL32 real-time PCR assay. PLoS Negl. Trop. Dis. 11, e0005940. https://doi.org/10.1371/journal.pntd.0005940

RISTOW, P., P. BOURHY, F. W. MC BRIDE, C. P. FIGUEIRA, M. HUERRE, P. AVE, I. SAINT GIRONS, A. I. KO, M. PICARDEAU (2007): The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog. 3, e97. https://doi.org/10.1371/journal.ppat.0030097

RIZZO, H., L. L. L. ROCHA, D. D. M. DINIZ, G. S. LIMA, T. K. S. JESUS, J. W. PINHEIRO JÚNIOR, V. CASTRO (2022): Seroprevalence of *Leptospira* spp. in horses from Rio Grande do Norte, Brazil. Pesqui. Vet. Bras. 42, e07035. https://doi.org/10.1590/1678-5150-pvb-6784

RHOADS, A., K. F. AU (2015): PacBio sequencing and its applications. Genomics Proteomics Bioinformatics 13(5), 278-289. https://doi.org/10.1016/j.gpb.2015.08.002

RODRIGUEZ, J., M. C. BLAIS, C. LAPOINTE, J. ARSENAULT, L. CARIOTO, J. HAREL (2014): Serologic and urinary PCR survey of leptospirosis in healthy cats and in cats with kidney disease. J. Vet. Intern. Med. 28, 284-293. https://doi.org/10.1111/jvim.12287

RUBINI, M. E., A. V. WOLF (1957): Refractometric determination of total solids and water of serum and urine. J. Biol. Chem. 225(2), 869-876. https://doi.org/10.1016/S0021-9258(18)64885-9

SANGER, F., S. NICKLEN, A.R. COULSON (1977): DNA sequencing with chain-terminating inhibitors. PNAS 74(12), 5463-5467. https://doi.org/10.1073/pnas.74.12.5463

SCHULLER, S., J. J. CALLANAN, S. WORRALL, T. FRANCEY, A. SCHWEIGHAUSER, B. KOHN, R. KLOPFLEISCH, H. POSTHAUS, J. E. NALLY (2015): Immunohistochemical detection of IgM and IgG in lung tissue of dogs with leptospiral pulmonary haemorrhage syndrome. Comp. Immunol. Microbiol. Infect. Dis. 40, 47-53. https://doi.org/10.1016/j.cimid.2015.04.002

SCHWENGERS, O., L. JELONEK, M. A. DIECKMANN, S. BEYVERS, J. BLOM, A. GOESMANN (2021): Bakta: Rapid and standardized annotation of bacterial genomes via alignment-free sequence identification. Microb. Genom. 7, 685. https://doi.org/10.1099/mgen.0.000685

SEMENOVA, L. P. (1965): New serological subtype of the Pomona *Leptospira* group: L. pomona Mozdok. J. Hyg. Epidemiol. Microbiol. Immunol. 9, 233-239.

SHAW, J., Y. W. YU (2023): Fast and robust metagenomic sequence comparison through sparse chaining with skani. Nat. Methods 20, 1661-1665. https://doi.org/10.1038/s41592-023-02018-3

SHOPHET, R. (1979): A serological survey of leptospirosis in cats. N. Z. Vet. J. 27(11), 236-246. https://doi.org/10.1080/00480169.1979.34662

SILVA, M., M. P. MACHADO, D. N. SILVA, M. ROSSI, J. MORAN-GILAD, S. SANTOS, M. RAMIREZ, J. A. CARRIÇO (2018): chewBBACA: A complete suite for gene-by-gene schema creation and strain identification. Microb. Genom. 4, e000166. https://doi.org/10.1099/mgen.0.000166

SILVA DIAS, C. S., M. H. PINNA (2025): *Leptospira* biofilms: implications for survival, transmission, and disease management. Appl. Environ. Microbiol. 91, e01914-24. https://doi.org/10.1128/aem.01914-24

STIMSON, A. M. (1907): Note on an organism found in yellow-fever tissue. Public Health Rep. 22, 541. https://doi.org/10.2307/4559008

STODDARD, R. A., J. E. GEE, P. P. WILKINS, K. MCCAUSTLAND, A. R. HOFFMASTER (2009): Detection of pathogenic *Leptospira* spp. through TaqMan PCR targeting lipL32. Diagn. Microbiol. Infect. Dis. 64, 247-255. https://doi.org/10.1016/j.diagmicrobio.2009.03.014

SULZER, K., V. POPE, F. ROGERS (1982): New leptospiral serotypes isolated during 1964-1970 from the Western Hemisphere. Rev. Latinoam. Microbiol. 24, 15-17.

SYKES, J. E., K. L. REAGAN, J. E. NALLY, R. L. GALLOWAY, D. A. HAAKE (2022): Role of diagnostics in epidemiology, management, surveillance, and control of leptospirosis. Pathogens 11, 495. https://doi.org/10.3390/pathogens11040395

ŠTRITOF MAJETIĆ, Z., J. HABUŠ, Z. MILAS, V. MOJČEC PERKO, V. STAREŠINA, N. TURK (2012): Serological survey of canine leptospirosis in Croatia - the changing epizootiology of the disease. Vet. Arhiv 82, 183-191.

ŠTRITOF MAJETIĆ, Z., R. GALLOWAY, E. RUŽIĆ SABLJIĆ, Z. MILAS, V. MOJČEC PERKO, J. HABUŠ, J. MARGALETIĆ, R. PERNAR, N. TURK (2014): Epizootiological survey of small mammals as *Leptospira* reservoirs in Eastern Croatia. Acta Trop. 131, 111-116. https://doi.org/10.1016/j.actatropica.2013.12.009

TADIĆ, M., D. KONJEVIĆ, V. MOJČEC PERKO, Z. ŠTRITOF, I. ZEČEVIĆ, I. BENVIN, Z. MILAS, N. TURK, M. BUJANIĆ, S. HAĐINA, J. HABUŠ (2024): The occurrence of *Leptospira* spp. serogroup Pomona infections in wild boars. Vet. Stanica 56, 225-233. https://doi.org/10.46419/vs.56.2.8

TADIN, A., N. TURK, M. KORVA, J. MARGALETIĆ, R. BECK, M. VUCELJA, J. HABUŠ, P. SVOBODA, T. AVŠIČ ŽUPANC, H. HENTTONEN, A. MARKOTIĆ (2012): Multiple co-infections of rodents with hantaviruses, *Leptospira*, and *Babesia* in Croatia. Vector-Borne Zoonotic Dis. 12, 388-392. https://doi.org/10.1089/vbz.2011.0632

THIBEAUX, R., M.-E. SOUPÉ-GILBERT, M. KAINIU, D. GIRAULT, E. BIERQUE, J. FERNANDES, H. BÄHRE, A. DOUYÈRE, N. ESKENAZI, J. VINH, M. PICARDEAU, C. GOARANT (2020): The zoonotic pathogen *Leptospira interrogans* mitigates environmental stress through c-di-GMP-controlled biofilm production. npj Biofilms Microbiomes 6, 39. https://doi.org/10.1038/s41522-020-0134-1

THIERMANN, A. B. (1982): Experimental leptospiral infections in pregnant cattle with organisms of the Hebdomadis serogroup. Am. J. Vet. Res. 43, 780-784. https://doi.org/10.2460/ajvr.1982.43.05.780

TIROSH-LEVY, S., M. BAUM, G. SCHVARTZ, B. KALIR, O. PE'ER, A. SHNAIDERMAN-TORBAN, M. BERNSTEIN, S. E. BLUM, A. STEINMAN (2021): Seroprevalence of *Leptospira* spp. in horses in Israel. Pathogens 10(4), 408. https://doi.org/10.3390/pathogens10040408

TONKIN-HILL, G., N. MACALASDAIR, C. RUIS, A. WEIMANN, G. HORESH, J. A. LEES, R. A. GLADSTONE, S. LO, C. BEAUDOIN, R. A. FLOTO, S. D. W. FROST, J. CORANDER, S. D. BENTLEY, J. PARKHILL (2020): Producing polished prokaryotic pangenomes with the Panaroo pipeline. Genome Biol. 21, 180. https://doi.org/10.1186/s13059-020-02090-4

TOYOFUKU, M., T. INABA, T. KIYOKAWA, N. OBANA, Y. YAWATA, N. NOMURA (2016): Environmental factors that shape biofilm formation. Biosci. Biotechnol. Biochem. 80, 7-12. https://doi.org/10.1080/09168451.2015.1058701

TREANGEN, T. J., B. D. ONDOV, S. KOREN, A. M. PHILLIPPY (2014): The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 15, 524. https://doi.org/10.1186/s13059-014-0524-x

TURK, N., Z. MILAS, J. MARGALETIĆ, V. STAREŠINA, A. SLAVICA, N. RIQUELME SERTOUR, E. BELLENGER, G. BARANTON, D. POSTIC (2003): Molecular characterisation of *Leptospira* strains isolated from small rodents in Croatia. Epidemiol. Infect. 130, 159-166. https://doi.org/10.1017/S0950268802008026

TURK, N., J. MARGALETIĆ, A. MARKOTIĆ (2009): Forest ecosystem and zoonoses. In: Wildlife: Destruction, Conservation and Biodiversity (Harris, J. D., Brown, P. L., Eds.), Nova Science, New York, 3-47.

TYLER, A.D., L. MATASEJE, C.J. URFANO, L. SCHMIDT, K.S. ANTONATION, M.R. MULVEY, C.R. CORBETT (2018): Evaluation of Oxford Nanopore's MinION sequencing device for microbial whole genome sequencing applications. Sci. Rep. 8(1): 10931. https://doi.org/10.1038/s41598-018-29334-5

UHLENHUTH, P., W. FROMME (1915): Weitere experimentelle Untersuchungen über die sog. Weil'sche Krankheit. Med. Klin. 11, 1264.

VERA, E., S. TADDEI, S. CAVIRANI, J. SCHIAVI, M. ANGELONE, C. S. CABASSI, E. SCHIANO, F. QUINTAVALLA (2019): *Leptospira* seroprevalence in Bardigiano horses in Northern Italy. Animals 10(1), 23. https://doi.org/10.3390/ani10010023

VERMA, A., P. KUMAR, K. BABB, J. F. TIMONEY, B. STEVENSON (2010): Cross-reactivity of antibodies against leptospiral recurrent uveitis-associated proteins A and B (LruA and LruB) with eye proteins. PLoS Negl. Trop. Dis. 4, e771. https://doi.org/10.1371/journal.pntd.0000778

VERMA, A., B. STEVENSON, B. ADLER (2013): Leptospirosis in horses. Vet. Microbiol. 167, 61-66. https://doi.org/10.1016/j.vetmic.2013.04.012

VINCENT, A. T., O. SCHIETTEKATTE, C. GOARANT, V. K. NEELA, E. BERNET, R. THIBEAUX, N. ISMAIL, M. K. N. M. KHALID, F. AMRAN, T. MASUZAWA, R. NAKAO, A. A. KORBA, P. BOURHY, F. J. VEYRIER, M. PICARDEAU (2019): Revisiting the taxonomy and evolution of pathogenicity of the genus *Leptospira* through the prism of genomics. PLoS Negl. Trop. Dis. 13, e0007270. https://doi.org/10.1371/journal.pntd.0007270

WASIŃSKI, B., K. PASCHALIS-TRELA, J. TRELA, M. CZOPOWICZ, J. KITA, M. ŻYCHSKA, A. CYWIŃSKA, I. MARKOWSKA-DANIEL, C. CARTER, L. WITKOWSKI (2021): Serological survey of *Leptospira* infection in Arabian horses in Poland. Pathogens 10(6), 688. https://doi.org/10.3390/pathogens10060688

WEIL, A. (1886): Ueber eine eigenthümliche, mit Milztumor, Icterus und Nephritis einhergehende, acute Infectionskrankheit. Dtsch. Arch. Klin. Med. 39, 209.

WEIS, S., A. RETTINGER, M. BERGMANN, J. R. LLEWELLYN, N. PANTCHEV, R. K. STRAUBINGER, K. HARTMANN (2017): Detection of *Leptospira* DNA in urine and presence of specific antibodies in outdoor cats in Germany. J. Feline Med. Surg. 19, 470-476. https://doi.org/10.1177/1098612X16634389

WEISS, S., A. MENEZES, K. WOODS, A. CHANTHONGTHIP, S. DITTRICH, A. OPOKUBOATENG, M. KIMULI, V. CHALKER (2016): An Extended Multilocus Sequence Typing (MLST) Scheme for Rapid Direct Typing of *Leptospira* from Clinical Samples. 1-11. https://doi.org/10.1371/journal.pntd.0004996

WOLGEMUTH, C. W., N. W. CHARON, S. F. GOLDSTEIN, R. E. GOLDSTEIN (2006): The flagellar cytoskeleton of the spirochetes. J. Mol. Microbiol. Biotechnol. 11, 221-227. https://doi.org/10.1159/000094056

XU, Y., Y. ZHU, Y. WANG, Y. F. CHANG, Y. ZHANG, X. JIANG, X. ZHUANG, Y. ZHU, J. ZHANG, L. ZENG, M. YANG, S. LI, S. WANG, Q. YE, X. XIN, G. ZHAO, H. ZHENG, X. GUO, J. WANG (2016): Whole genome sequencing revealed host adaptation-focused genomic plasticity of pathogenic *Leptospira*. Sci. Rep. 6, 20020. https://doi.org/10.1038/srep20020

YU, G., D. K. SMITH, H. ZHU, Y. GUAN, T. T. Y. LAM (2017): ggtree: An R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol. Evol. 8, 28-36. https://doi.org/10.1111/2041-210X.12628

ZAIDI, S., A. BOUAM, A. BESSAS, D. HEZIL, H. GHAOUI, K. AIT-OUDHIA, M. DRANCOURT, I. BITAM (2018): Urinary shedding of pathogenic *Leptospira* in stray dogs and cats, Algiers: a prospective study. PLoS ONE 13, e0197068. https://doi.org/10.1371/journal.pone.0197068

ZAHARIJA, I. (1953): Leptospirosis Pomona in horses found in 1951 in Croatia. Vet. Arhiv 23, 297-302. (in Croatian).

ZARANTONELLI, L., A. SUANES, P. MENY, F. BURONI, C. NIEVES, X. SALABERRY, C. BRIANO, N. ASHFIELD, C. D. S. SILVEIRA, F. DUTRA, C. EASTON, M. FRAGA, F. GIANNITTI, C. HAMOND, M. MACÍAS-RIOSECO, C. MENÉNDEZ, A. MORTOLA, M. PICARDEAU, J. QUINTERO, C. RÍOS, V. RODRÍGUEZ, A.

ROMERO, G. VARELA, R. RIVERO, F. SCHELOTTO, F. RIET-COREA, A. BUSCHIAZZO; GRUPO DE TRABAJO INTERINSTITUCIONAL DE LEPTOSPIROSIS CONSORTIUM (2018): Isolation of pathogenic *Leptospira* strains from naturally infected cattle in Uruguay reveals high serovar diversity and risk for humans. PLoS Negl. Trop. Dis. 12, e0006694. https://doi.org/10.1371/journal.pntd.0006694

ZEČEVIĆ, I., M. PICARDEAU, S. VINCE, S. HAĐINA, M. PERHARIĆ, Z. ŠTRITOF, V. STEVANOVIĆ, I. LOHMAN JANKOVIĆ, I. BENVIN, J. HABUŠ (2024): Association between exposure to *Leptospira* spp. and abortion in mares in Croatia. Microorganisms 12, 1039. https://doi.org/10.3390/microorganisms12061039

ZHANG, F. Z., P. R. LONG, P. Y. MENG, J. J. WANG (1987): Identification of *Leptospira kunming* of the Pomona serogroup. Wei Sheng Wu Xue Bao 27, 88-91.

ZUERNNER, R. L., J. L. HERRMANN, I. SAINT GIRONS (1993): Comparison of genetic maps for two *Leptospira interrogans* serovars provides evidence for two chromosomes and intraspecies heterogeneity. J. Bacteriol. 175, 5445-5451. https://doi.org/10.1128/jb.175.17.5445-5451.1993

ŽÁKOVSKÁ, A., P. SCHÁNILEC, F. TREML, M. DUŠKOVÁ, C. F. AGUDELO (2020): Seroprevalence of antibodies against *Borrelia burgdorferi* s.l. and *Leptospira interrogans* s.l. in cats in the district of Brno and its environs, the Czech Republic. Ann. Agric. Environ. Med. 27(3), 356-360. https://doi.org/10.26444/aaem/122804

ŻMUDZKI, J., M. OSTROWSKA, Z. ARENT, M. FRANT, M. KOCHANOWSKI, A. NOWAK, S. ZĘBEK, D. KALINOWSKI, K. PODGÓRSKA (2025): Seroprevalence of equine leptospirosis in Poland (2019-2023). Equine Vet. J. https://doi.org/10.1111/evj.70069

8. PUBLISHED SCIENTIFIC PAPERS

8.1. Paper I: "Serological Surveillance of Equine Leptospirosis in Croatia in the Period From 2012 to 2022: A Key Insight Into the Changing Epizootiology"

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Original Research

Serological Surveillance of Equine Leptospirosis in Croatia in the Period From 2012 to 2022: A Key Insight Into the Changing Epizootiology

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ABSTRACT

Leptospirosis is re-emerging zoonotic bacterial disease of global importance that affects domestic and wild animals and humans. Due to the public health importance, control of disease in Croatia is being implemented by monitoring the seroprevalence of equine leptospirosis and it is regulated by the law. In the period from 2012 to 2022, a total of 61,724 serum samples from apparently healthy horses were admitted to the Laboratory for leptospires, Faculty of Veterinary Medicine University of Zagreb. Serum samples were tested for *Leptospira* spp. antibodies using the microscopic agglutination test (MAT). Samples were considered seropositive with a cut-off titre 1:200 for Bratislava and 1:400 for other *Leptospira* spp. serovars. Out of 61,724 serum samples tested, 6,665 (10.80%) were found seropositive for at least one *Leptospira* serovar. In the studied period, seroprevalence varied between 5.00% and 15.94%. The highest seroprevalence was found for serovar Pomona (41.98%) and serovar Grippotyphosa (31.34%), followed by Sejroe (8.03%), Icterohaemorrhagiae (7.05%) and Bratislava (6.47%). Results indicated that horses in Croatia are particularly exposed to *Leptospira* spp. infections. The most prevalent presumed infective serovar was Pomona increasing each year. Investigated horses were randomly selected and healthy and most seropositive horses have anamnestic titre due to previous infection. This is the first study in Europe reporting such high seropositivity for the serovar Pomona in apparently healthy horses. According to the results of the present study, the question arises of the possible evolutionary adaptation of the pathogenic serovar Pomona as dominant for horses.

1. Introduction

Leptospirosis is a re-emerging infectious disease caused by various pathogenic *Leptospira* spp. with a worldwide distribution [1,2]. This ubiquitous disease affects humans, domestic and wild animals and has even been found in birds, amphibians, reptiles, and fish [3]. The most important reservoirs are rodents, which excrete leptospires in the urine continuously or intermittently throughout their lifetime and represent a main source of infection [1,4]. Based on serological classification, *Leptospira* spp. is divided into numerous serovars [1]. Incidental infection with serovars transmitted from other infected animal hosts that are carriers results in certain clinical signs depending on the species and infective serovars [5]. Leptospires enter the host via mucous membranes and microlesions of the skin directly by contact with infected urine or indirectly through a contaminated environment (soil and water) [6–8]. After the bacteremic stage, leptospires colonise the proximal tubule epithelial cells of the kidneys and are excreted in the urine [4,7], contaminating the environment where it may retain pathogenicity for as long as six to 20 months [9,10]. Excretion in the urine of incidental hosts is usually of limited duration but may persist for up to six months after infection. Some animals had coadaptation with some serovars which tend to be maintained in specific hosts causing minimal pathological damage and this infection is asymptomatic [4,6].

Animal welfare/ethical statement: Considering the importance of leptospirosis for public health in Croatia, disease control has been carried out for years by monitoring the seroprevalence of equine leptospirosis, and the results of the last decade are presented in this study. Therefore, this study doesn't have an ethical statement because blood samples were taken by local veterinary practitioners from different parts of Croatia within the framework of routine mandatory measures for the protection of domestic animals from infectious and parasitic diseases ordered by the Veterinary Directory of the Ministry of Agriculture of the Republic of Croatia.

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Leptospirosis in horses is usually subclinical, but where clinical signs do occur, they range from lethargy, anorexia, jaundice, anaemia, and impaired renal function to abortion, stillbirth, and the birth of infected foals [5,11]. Compared with adult horses, icteric leptospirosis mainly occurs in foals [11]. The increasingly described acute respiratory distress syndrome and severe pulmonary haemorrhagic syndrome associated with leptospirosis in humans are also reported in horses but are still insufficiently researched [12,13]. An important clinical outcome of leptospirosis in horses is equine recurrent uveitis (ERU) with signs of periodic ophthalmia and blindness [14,15].

Considering that the clinical signs are unspecific and definitive diagnosis based on isolation of *Leptospira* spp. is challenging to obtain and time-consuming, epidemiological studies and diagnostics are mostly based on serology [16–18]. The standard serological test is the microscopic agglutination test (MAT), in which antibodies in serum react with live antigens of different *Leptospira* spp. serovars [16–18].

Studies on seroprevalence and isolation of *Leptospira* spp. indicate that horses are susceptible to different serovars that cause incidental infections. Serovar Bratislava is the most prevalent serovar in horses worldwide and it has been postulated that both incidental and maintained infections can occur [5]. Except for Bratislava [19–22], the most common serovars identified in recent studies as causing incidental infections worldwide are Icterohaemorrhagiae [23], Canicola [24], Grippotyphosa [14], Ballum [25,26] and Pomona [27,28].

An increase or decrease in the prevalence of leptospirosis in animals follows the trend of leptospirosis in humans, as well as the trend of the most frequent serovar. The most prevalent infective serovars in humans are Sejroe [29], Australis [29,30], Icterohaemorrhagiae [29,31], Grippotyphosa [30,31], and Saxkoebing [30].

Although leptospirosis is one of the most common zoonotic infections and a worldwide problem for veterinary and public health, this disease is globally underestimated and underdiagnosed [2,32]. In Croatia, leptospirosis is an endemic disease and has significant importance for public health. That's why disease control has been carried out for years by state monitoring the seroprevalence of equine leptospirosis, and the results of the last decade are presented in this study.

2. Materials and Methods

2.1. Study Design

In the period from January 2012 to December 2022, blood samples were taken once by local veterinary practitioners from different areas of Croatia within the framework of routine mandatory measures for the protection of domestic animals from infectious and parasitic diseases ordered by the Veterinary Directorate of the Ministry of Agriculture of the Republic of Croatia.

2.2. Animals

A total of 61,724 equine serum samples were randomly collected from apparently healthy animals based on the previously established prevalence of leptospirosis in certain regions and submitted to the Laboratory for leptospires, Faculty of Veterinary Medicine the University of Zagreb. The animals belonged to different breeds, age groups, and sexes.

2.3. Microscopic Agglutination Test (MAT)

2.3.1. Reference Method

Serum samples were tested for *Leptospira* spp. antibodies using the microscopic agglutination test (MAT). The serological assay was performed following the standard procedure in accordance with World Health Organization (WHO) [17] and the International Leptospirosis Society (ILS) instructions.

2.3.2. Panel of Antigens

MAT was performed with a panel of antigens for screening of healthy horses consisting of eight pathogenic *Leptospira* spp. serovars: Grippotyphosa, Sejroe, Bratislava, Pomona, Canicola, Icterohaemorrhagiae, Saxkoebing, and Bataviae. The antigen panel was adapted to the tested animal species and was composed of reference strains of serovar obtained from The Leptospirosis Reference Centre (KIT Biomedical Research, Amsterdam, Netherlands), whose presence in Croatia had been confirmed by previous epidemiological and epizootiological analyses (Table 1) [33–38].

2.3.3. Preparation of Samples

Before performing MAT, the antigen's density, mobility, and purity were checked. Cultures of *Leptospira* spp. up to 10 days old, with a density of 2×10^8 bacteria/mL and without contamination were used for this research. All serum samples were serially diluted in phosphate buffer solution (PBS) in a microtiter plate, starting with a dilution of 1:50.

2.3.4. Interpretation of Results

The endpoint titre was the highest serum dilution that showed agglutination of at least 50% of the leptospires, compared to the negative antigen control. Samples were considered positive according to Croatian regulations at a cut-off value of 1:200 for Bratislava and 1:400 for other *Leptospira* spp. serovars. Presumptive infective serovars were determined by identifying the highest titres to one or more serovars belonging to a certain serogroup. Seropositive samples indicate the suspicion of leptospirosis or possible convalescent shedding, and it is notifiable, regardless of clinical status.

2.4. Data Analysis

Statistical analyses were performed using Statistica v.13 (TIBCO Software Inc., 2017), MedCalc for Windows, version 20.218 (MedCalc Software, Ostend, Belgium) and R 4.2.2 (R Core Team, Vienna, Austria, 2022). Descriptive statistics are presented as numbers and percentages. Odds ratio (OR) with 95% confidence intervals (95% CI) was used for the risk of a single year compared to the entire period. Differences were considered statistically significant for $P < .05$.

3. Results

Out of 61,724 serum samples tested, 6,665 (10.80%; 95%CI = 10.55–11.05) had agglutinating antibodies against at least one *Leptospira* spp. serovar and were considered seropositive. In the investigated period, the most frequent serovar was serovar Pomona in 2,798 (41.98%; 95% CI = 40.79–43.18) samples and serovar Grippotyphosa in 2,089 (31.34%; 95% CI = 30.23–32.47) samples, followed by Sejroe in 535 (8.03%; 95% CI = 7.39–8.71), Icterohaemorrhagiae in 470 (7.05%; 95% CI = 6.45–7.69), Bratislava 431 (6.47%; 95% CI = 5.89–7.08) and Saxkoebing in 128 (1.92%; 95% CI = 1.6–2.28) samples (Fig. 1). There were 188 (2.82%; 95% CI = 2.44–3.25) serum samples with agglutination antibodies in the same titre to two or more serovars belonging to different serogroups. In this case, identifying the presumptive infective serovar was not possible, so these serum samples were classified as nondetermined. Titres ranged from 1:400 (1:200 for serovar Bratislava) to 1:51200.

From 2012 to 2022, seroprevalence varied between 5.00% (95% CI = 4.19–5.91; 2022) and 15.94% (95% CI = 15.06–16.85; 2012) (Fig. 2). A significant decrease in seroprevalence was observed in

Table 1
The panel of antigens for serological screening of healthy horses

No.	Serogroup	Serovar	Strain	Genomic Species
1	Grippotyphosa	Grippotyphosa	Moskva V	*L. kirschneri*
2	Sejroe	Sejroe	M84	*L. borgpetersenii*
3	Australis	Bratislava	Jež Bratislava	*L. interrogans*
4	Pomona	Pomona	Pomona	*L. interrogans*
5	Canicola	Canicola	Hond Utrecht IV	*L. interrogans*
6	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*L. interrogans*
7	Sejroe	Saxkoebing	Mus 24	*L. interrogans*
8	Bataviae	Bataviae	Swart	*L. interrogans*

Table 2
Seroprevalence of equine leptospirosis in Croatia (2012–2022): odds ratio, 95% confidence interval and *P*-values of a particular year compared to the investigated period

Year	N Positive Samples/N	OR	95% CI	*P-Value*
2012	1,042/6,536	1.57	1.46–1.68	<.0001
2013	484/6,497	0.73	0.66–0.80	<.0001
2014	622/5,000	1.17	1.08–1.28	.0003
2015	762/6,452	1.11	1.02–1.20	.01
2016	1,060/10,735	0.91	0.85–0.97	.004
2017	422/3,885	1.01	0.91–1.12	.9
2018	1,220/12,123	0.92	0.87–0.99	.017
2019	277/2,293	1.14	0.9985–1.29	.053
2020	414/3,075	1.29	1.16–1.43	<.0001
2021	232/2,527	0.84	0.73–0.96	.01
2022	130/2,601	0.43	0.36–0.52	<.0001

CI, confidence interval; N, total number, OR, odds ratio.

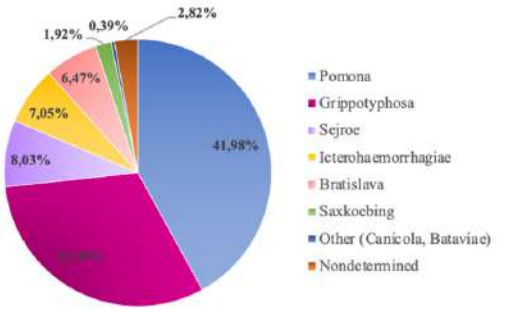

Fig. 1. The distribution of presumptive infective *Leptospira* spp. serovar among a total number of seropositive samples in the period from 2012 to 2022 (N = 6,665).

2013 (7.45%; OR = 0.73, 95% CI = 0.66–0.80; $P < .0001$; Table 2), followed by a renewed increase in 2014 (12.44%; OR = 1.17, 95% CI = 1.08–1.28, $P = .0003$; Table 2). In subsequent years (2015–2021), seroprevalence ranged from 9.18% (95% CI = 8.08–10.37) to 13.46% (95% CI = 12.28–14.72), with the odds ratio shown in Table 2. In 2022, 130 horses were seropositive, with a seroprevalence of 5.00% (95% CI = 4.19–5.91; OR = 0.43; $P < .0001$; Table 2). The total number of investigated horses varied, moderately decreasing each year (Fig. 2).

During the studied period, Grippotyphosa was found as the most presumptive infective serovar in 2014 (37.30%; 95% CI = 33.49–41.23), 2019 (45.49%; 95% CI = 39.52–51.55), and 2020 (40.58%; 95% CI = 35.81–45.48), while the second most prevalent serovar in those years was Pomona (Fig. 3). In the other years, the most prevalent presumptive infective serovar was Pomona, and the second most prevalent was Grippotyphosa with the exception in 2012 when Icterohaemorrhagiae (22.55%; 95% CI = 20.05–25.21) followed Pomona (28.60%; 95% CI = 25.87–31.45) (Fig. 3). In 2020, the difference in the distribution of serovars as the most presumptive infective between Pomona (39.13%; 95% CI = 34.4–44.02) and Grippotyphosa (40.58%; 95% CI = 35.81–45.48) was minimal.

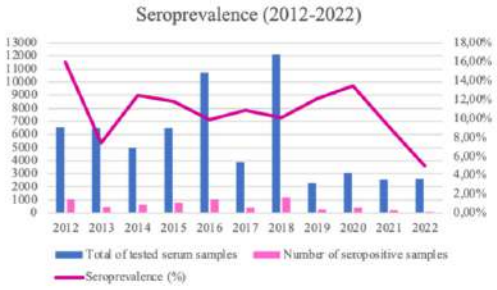

Fig. 2. Number of positive samples in total tested samples from 2012 to 2022.

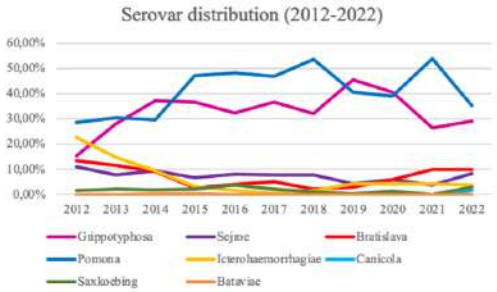

Fig. 3. Distribution of presumptive infective *Leptospira* spp. serovar within each year of the investigated period.

4. Discussion

The results of this study indicate that *Leptospira* spp. infections in horses are still highly present in Croatia. Leptospirosis persists in natural foci and Croatia is considered as the first endemic country in Europe and 13th in the World [2]. Unfortunately, equine leptospirosis is still underdiagnosed and quite underestimated due to its subclinical manifestation, and just a few studies in Europe report seroprevalence in horses [14,22,39,40]. Nevertheless, the aforementioned studies in Europe and most recent studies from the rest of the world [21,23–25,27] indicate a high seropositivity of equine leptospirosis, ranging from 28.57% to 97.2%. All studies on the serological prevalence of equine leptospirosis indicate frequent infections, although the disease is usually asymptomatic in horses. In addition, horses are long-living animals and are at risk of being convalescents that excrete various pathogenic *Leptospira* spp. into the environment. Moreover, there are numer-

ous forms of cohabitation with other animals and humans and thus serve as potentially important sources of infection.

In our study, 6,665 (10.80%) of 61,724 serum samples tested were seropositive. Seroprevalence ranged from 5.00% established in 2022 and 15.94% in 2012. A slight decrease in seroprevalence was observed compared to earlier studies [19,29,41,42]. On the other hand, it is important to emphasise that despite the continuous occurrence of leptospirosis in horses, the total number of horses tested in the state surveillance program decreased yearly due to a lack of state funding or different priorities in mandatory measures, mostly because of COVID-19 pandemia.

A significant decrease was recorded in 2013, but due to extreme weather conditions and severe flooding in mainland Croatia, the prevalence increased to 12.44% in 2014. Throughout the study period, we recorded the highest seroprevalence for serovar Pomona (41.98%) and Grippotyphosa (31.34%), followed by Sejroe, Icterohaemorrhagiae, Bratislava, and Saxkoebing. In contrast to this, other studies in Europe reported Grippotyphosa [14,39], Bratislava [22] and Pyrogenes [40] as the most prevalent serovars in horses. Furthermore, compared with previous studies in Croatia [19,29,41,43], we found significant variation in presumptive infective serovars. Namely, a significant increase in the occurrence of serovar Grippotyphosa and Pomona was observed, with a remarkable decrease in the frequency of serovars Bratislava and Australis, which were the most presumptive infective serovars in previous studies. During the investigated period, Sejroe and Icterohaemorrhagiae are also continuously present serovars, but a slightly reduced frequency of Icterohaemorrhagiae was noticed. That was probably connected with variation in different small rodent species abundance serving as reservoirs of particular *Leptospira* spp. serovars and it is probably due to climate changes influencing forests and field biotopes.

Epizootiological, the disease risk is mainly related to the presence of the rodent population as one of the main reservoirs of leptospirosis [33]. Appropriate weather conditions also positively affect the increase in forest vegetation and biomass that the rodents of forest ecosystems feed on, which additionally directly influences their presence and number [44]. Considering the presence of certain rodent reservoirs in a particular geographical area only a limited number of serovars are endemic in a specific region or country [5]. Black-striped field mouse (*Apodemus agrarius*) is considered a reservoir host for serovar Pomona, common vole (*Microtus arvalis*) for Grippotyphosa, yellow-necked field mouse (*Apodemus flavicollis*) for Bratislava and Saxkoebing, rat (*Rattus norvegicus*) for Icterohaemorrhagie, and the house mouse (*Mus musculus*) for Sejroe [34,37,45]. Black-striped field mouses and yellow-necked field mouses are reported to be dominant reservoirs for leptospires in the natural foci of leptospirosis in Croatia [33,34,37]. However, there is still a questionable difference in rodent species population numbers through the years. It is important to know potential reservoirs and their belonging serovars to select antigens for performing in MAT, because antigens should be representative strains of the serogroups present in particular geographical areas and known to be maintained to the host species under test [18].

Except for the rodent population's presence, leptospirosis risk is also related to climate change and urbanisation [46]. Horses in Croatia usually live in pasture or stable, and farms are often mixed with other domestic animals. A mixed livestock management system can cause environmental contamination with different *Leptospira* spp. strains which can lead to maximum survival of those strains in the environment and incidental infection, respectively. Therefore, a risk factor for *Leptospira* spp. infection in horses is contact with domestic and wild animals, in addition to the presence of rodents and water sources.

The most prevalent presumptive infective serovar in 2014 was Grippotyphosa (37.30%). Habus et al. also reported increased serological reactivity against serovar Grippotyphosa in 2014 in all pasture animals. This increase was associated with extremely wet conditions, resulting in leptospires, excreted by main reservoirs rodents, being spread and maintained in the environment for a long time [38,47,48]. In 2019 and 2020, Grippotyphosa was also found as the most prevalent presumptive infective serovar. It is interesting to note that according to publicly available data from Croatian Meteorological and Hydrological Service, 2019 was also recorded as an extremely warm and very wet year with high precipitation. In addition, 2020 was recorded as very warm, but mostly with average amounts of precipitation, and in this year difference in distribution as presumptive infective serovar between Grippotyphosa (40.58%) and Pomona (39.13%) was very small. Contrary to that, all other years in the investigated period were recorded as very to extremely warm with an average amount of precipitation in addition to some small parts of dry or rainy geographical areas. During those years, the most prevalent presumptive infective serovar was Pomona increasing each year.

This report is essential and indicative because Pomona is the most common serovar associated with the clinical manifestation of leptospirosis in horses. The first report of leptospirosis in Croatia in 1953 was in a clinically ill horse caused by serovar Pomona [49]. Tirosh-Levy et al. reported an outbreak of *Leptospira* spp. serogroup Pomona in humans and cattle in 2017 and 2018, which is also confirmed in horses and most seropositive horses had clinical signs of equine recurrent uveitis (ERU). Likewise, Fagre et al. found the highest seropositivity with serovar Pomona in horses with uveitis, ERU or another ophthalmic ailment, but serovar Bratislava in horses without clinical illness. Serovar Pomona is mostly associated with an agent of leptospirosis in swine and cattle. It is also possible that some wild animals, such as wild boars, are the source of infection.

For proving the acute status of the infection in horses and detecting them as leptospiral carriers, it would be desirable to detect leptospires in urine using polymerase chain reaction (PCR) [50,51]. Unfortunately, due to the large number of horses, we were unable to test all seropositive animals with PCR. However, for scientific purposes, we tested a certain number of animals every year and found the presence of the *LipL32* gene of pathogenic *Leptospira* spp. in many urines of horses. Moreover, we isolated one isolate by urine culture, which was serologically determined as Pomona (data still not published).

In our study, horses were randomly selected and apparently healthy, and most seropositive horses have anamnestic titre due to previous infection. This is the first study in Europe reporting such high seropositivity for the serovar Pomona in apparently healthy horses. It is known that some serovars cause latent infections or a mild form of the disease, most likely due to mutual adaptation of the host and a certain serovar. According to the results of the present study, the question arises of the possible evolutionary adaptation of the pathogenic serovar Pomona as dominant for horses.

Finally, the testing of horses is part of a multidisciplinary approach being conducted with the purpose of surveillance and control of leptospirosis in Croatia. Considering all these factors, the One Health approach is required to identify pathogens in humans, animals, and the environment as well as their mutual interactions in a particular area. The results of an interdisciplinary approach may be useful in understanding the epidemiology of leptospirosis and investigating the potential changes in this disease's epizootiology and its challenges.

Declaration of Competing Interest

None.

References

[1] Leptospirosis Levett PN. Clin Microbiol Rev 2001;14(2):296–326.
[2] Pappas G, Papadimitriou P, Siozopoulou V, Christou L, Akritidis N. The globalization of leptospirosis: worldwide incidence trends. Int J Infect Dis 2008;12(4):351–7.
[3] Picardeau M. Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nat Rev Microbiol 2017;15(5):297–307.
[4] Adler B, de la Peña Moctezuma A. Leptospira and leptospirosis. Vet Microbiol 2010;140(3-4):287–96.
[5] Ellis WA. Animal leptospirosis. In: Adler B, editor. Leptospira and leptospirosis, current topics in microbiology and immunology. Berlin Heidelberg: Springer-Verlag; 2015. p. 99–137.
[6] Baranton G, Old IG. The spirochaetes: a different way of life. Bull Inst Pasteur 1995;93(2):63–95.
[7] Ko AI, Goarant C, Leptospira Picardeau M. The dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol 2009;7(10):736–47.
[8] Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. Environmental and behavioural determinants of leptospirosis transmission: a systematic review. PLoS Negl Trop Dis 2015;9(9):1–15.
[9] Andre-Fontaine G, Aviat F, Thorin C. Waterborne leptospirosis: survival and preservation of the virulence of pathogenic *Leptospira* spp. in fresh water. Curr Microbiol 2015;71(1):136–42.
[10] Bierque E, Soupé-Gilbert ME, Thibeaux R, Girault D, Guentas L, Goarant C. *Leptospira interrogans* retains direct virulence after long starvation in water. Curr Microbiol 2020;77(10):3035–43.
[11] Verma A, Stevenson B, Adler B. Leptospirosis in horses. Vet Microbiol 2013;167(1- 2):61–6.
[12] Broux B, Torfs S, Wegge B, Deprez P, van Loon G. Acute respiratory failure caused by *Leptospira* spp. in 5 foals. J Vet Intern Med 2012;26(3):684–7.
[13] Hamond C, Martins G, Reis J, Kraus E, Pinna A, Lilenbaum W. Pulmonary hemorrhage in horses seropositive to leptospirosis. Pesqui Vet Bras 2011;31(5):413–15.
[14] Wasiński B, Paschalis-Trela K, Trela J, Czopowicz M, Kita J, Żychska M, et al. Serological survey of *Leptospira* infection in Arabian horses in Poland. Pathogens 2021;10(688):1–6.
[15] Verma A, Kumar P, Babb K, Timoney JF, Stevenson B. Cross-reactivity of antibodies against leptospiral recurrent uveitis-associated proteins A and B (LruA and LruB) with eye proteins. PLoS Negl Trop Dis 2010;4(8):1–9.
[16] Picardeau M. Diagnosis and epidemiology of leptospirosis. Med Mal Infect 2013;43(1):1–9.
[17] . Chapter 3.1.12. leptospirosis. In: Manual of diagnostic tests and vaccines for terrestrial animals; 2022. p. 1–13.
[18] Musso D, La Scola B. Laboratory diagnosis of leptospirosis: a challenge. J Microbiol Immunol Infect 2013;46(4):245–52.
[19] Turk N, Milas Z, Habuš J, Štritof Majetić Z, Mojčec Perko V, Barbić L, et al. Equine leptospirosis in Croatia-occurrence of subclinical infections. Vet Arh 2013;83(3):253–62.
[20] Baverud V, Gunnarsson A, Engvall EO, Franzén P, Egenvall A. Leptospira seroprevalence and associations between seropositivity, clinical disease and host factors in horses. Acta Vet Scand 2009;51(15):1–10.
[21] Trimble AC, Blevins CA, Beard LA, Deforno AR, Davis EG. Seroprevalence, frequency of leptospiuria, and associated risk factors in horses in Kansas, Missouri, and Nebraska from 2016-2017. PLoS One 2018;13(10):1–12.
[22] Vera E, Taddei S, Cavirani S, Schiavi J, Angelone M, Cabassi CS, et al. Leptospira seroprevalence in bardigiano horses in northern Italy. Animals 2020;10(1):23.
[23] Rizzo H, Rocha LLL, Diniz DDM, Lima GS, Jesus TKS, Pinheiro Júnior JW, et al. Seroprevalence of *Leptospira* spp. in horses from Rio Grande do Norte. Brazil Pesqui Vet Bras 2022;42:1–6.
[24] Dewes C, Fortes TP, Machado GB, Pacheco PS, Silva JPM, Seixas Neto ACP, et al. Prevalence and risk factors associated with equine leptospirosis in an endemic urban area in southern Brazil. Brazilian J Dev 2020;6(8):58380–90.
[25] Bastiani MP, Lovato LT, von Laer AE, Pötter L, Rodrigues RO, de Souza BC, et al. Occurrence of *Leptospira* spp. and factors associated with the infection in horses from a military contingent in the Rio Grande do Sul State. Brazil Brazilian J Vet Res Anim Sci 2021;58:e180884.
[26] Tadich TA, Tapia C, González D. Seroprevalence of *Leptospira* spp. in working horses located in the central region of Chile. J Equine Vet Sci 2016;38:14–18.
[27] Fagre AC, Mayo CE, Pabilonia KL, Landolt GA. Seroprevalence of *Leptospira* spp. in Colorado equids and association with clinical disease. J Vet Diagnostic Investig 2020;32(5):718–21.
[28] Tirosh-Levy S, Baum M, Schvartz G, Kalir B, Pe'er O, Shnaiderman-Torban A, et al. Seroprevalence of *Leptospira* spp. in horses in Israel. Pathogens 2021;10(4):1–9.
[29] Habus J, Persic Z, Spicic S, Vince S, Stritof Z, Milas Z, et al. New trends in human and animal leptospirosis in Croatia, 2009–2014. Acta Trop 2017;168:1–8.
[30] Balen Topic M, Habus J, Milas Z, Celjuska Tosev E, Stritof Z, Turk N. Human leptospirosis in Croatia: current status of epidemiology and clinical characteristics. Trans R Soc Trop Med Hyg 2010;104:202–6.
[31] Mišić-Majerus L, Kišek TC, Ružić-Sabljić E. Leptospirosis and characterization of Leptospira isolates from patients in Koprivnica-Križevci County, Croatia from 2000-2004. Access Microbiol 2023;5(4):acmi000431.
[32] Hartskeerl RA, Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. Clin Microbiol Infect 2011;17(4):494–501.
[33] Milas Z, Turk N, Staresina V, Margaletić J, Slavica A, Živković D, et al. The role of myomorphous mammals as reservoirs of leptospira in the pedunculate oak forests of Croatia. Vet Arh 2002;72(3):119–29.
[34] Turk N, Milas Z, Margaletic J, Staresina V, Slavica A, Riquelme-Sertour N, et al. Molecular characterization of *Leptospira* spp. strains isolated from small rodents in Croatia. Epidemiol Infect 2003;130(1):159–66.
[35] Modrić Z, Adorić D, Ropac D, Brkić N. Antibodies to leptospires in horse sera in Sinj and surroundings. Vet stanica 2004;35:145–50 Croatian.
[36] Cvetnić Ž, Jukić B, Špičić S. Distribution of equine leptospirosis in the Republic of Croatia from 1994 to 2003. Vet stanica 2004;35:67–75 Croatian.
[37] Štritof Z. [Molecular epizootiology of leptospirosis in mouse-like rodents] [dissertation]. Zagreb: Faculty of Veterinary Medicine University of Zagreb; 2010. Croatian.
[38] Stritof Majetic Z, Galloway Z, Ruzic-Sabljic E, Milas Z, Mojcec V, Habus J, Margaletic J, Pernar R, Turk N. Epizootiological survey of small mammals as *Leptospira* spp. reservoirs in Eastern Croatia. Acta Trop 2014;131:111–16.
[39] Arent ZJ, Kedzierska-Mieszkowska S. Seroprevalence study of leptospirosis in horses in northern Poland. Vet Rec 2013;172(10):269.
[40] Blatti S, Overesch G, Gerber V, Frey J, Hüssy D. Seroprevalence of *Leptospira* spp. in clinically healthy horses in Switzerland. Schweiz Arch Tierheilkd 2011;153(10):449–56.
[41] Habuš J, Cvetnić Z, Milas Z, Štritof Z, Balen-Toplč M, Margaletić J, et al. Seroepidemiological and seroepizootiological investigation of leptospirosis in Croatia in 2007. Infektoloski Glas 2008;28(4):183–8 Croatian.
[42] Turk N, Milas Z, Mojcec V, Ruzic-Sabljic E, Staresina V, Stritof Z, et al. Molecular analysis of *Leptospira* spp. isolated from humans by restriction fragment length polymorphism, real-time PCR and pulsed-field gel electrophoresis. FEMS Microbiol Lett 2009;300(2):174–9.
[43] Milas Z, Štritof Majetić Z, Habuš J, Mojčec Perko V, Staršina V, Barbić L, et al. The occurrence and maintenance of *Leptospira* serovars Australis and Bratislava in domestic and wild animals in Croatia. Vet Arh 2013;83(4):357–69.
[44] Turk N, Margaletic J, Markotic A. Forest ecosystem and zoonoses. In: Harris JD, Brown PL, editors. Wildlife: destruction, conservation and biodiversity. New York: Nova Science Publishers; 2009. p. 3–47.
[45] Milas Z, Turk N, Štritof Majetić Z, Habuš J, Mojčec Perko V, Barbić L, et al. Diagnosis and control of equine leptospirosis in Croatia. Hrvat Vet Vjesn 2013;21(5-6):31–7 Croatian.
[46] Lau CL, Smythe LD, Craig SB, Weinstein P. Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? Trans R Soc Trop Med Hyg 2010;104(10):631–8.
[47] Habuš J, Peršić Z, Vince S, Vucelja M, Štritof Z, Mojčec V, Milas Z, Margaletić J, Turk N. Leptospirosis and climate change. In: Korn H, Stadler J, Marselle M, Bonn A, editors. Proceedings of European conference "biodiversity and health in the face of climate change: challenges, opportunities and evidence gaps"; 2018. p. 126–7.
[48] Štritof Z, Habuš J, Mojčec V, Milas Z, Hađina S, Perharić M, Staršina V, Turk N. Fluctuation of seroprevalence in equine population in Croatia. In: 3rd European leptospirosis society scientific meeting on leptospirosis and other rodent borne haemorrhagic fevers; 2018. p. 45.
[49] Zaharija I. Leptospirosis Pomona in horses found in 1951 in Croatia. Vet arh 1953;23:297–302 Croatian.
[50] Hamond C, Martins G, Loureiro AP, Pestana C, Lawson-Ferreira R, Medeiros MA, Lilenbaum W. Urinary PCR as an increasingly useful tool for an accurate diagnosis of leptospirosis in livestock. Vet Res Commun 2014;38(1):81–5.
[51] Di Azevedo MIN, Lilenbaum W. An overview on the molecular diagnosis of animal leptospirosis. Lett Appl Microbiol 2021;72(5):496–508.

8.2. Paper II: "Insights into *Leptospira* spp. infection in pet cats in Croatia: Clinical, serological and molecular findings with emphasis on the potential important role of serogroup Pomona"

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Insights into *Leptospira* spp. infection in pet cats in Croatia: Clinical, serological and molecular findings with emphasis on the potential important role of serogroup Pomona

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ABSTRACT

Leptospirosis, a globally re-emerging zoonosis caused by pathogenic *Leptospira* spp., poses a significant threat to public health. Leptospirosis in cats is often neglected due to its high underdiagnosis. Therefore, the role of cats in disease transmission and bacterial maintenance in the environment remains unclear. For this study, 54 serum samples, 54 urine samples and 27 EDTA-anticoagulated blood samples from pet cats presenting to the Veterinary Teaching Hospital due to health problems were used. The serum samples were tested for antibodies against 12 pathogenic serovars of *Leptospira* spp. using the microscopic agglutination test (MAT). EDTA-anticoagulated blood and urine samples were tested for the *lipL32* gene of pathogenic *Leptospira* spp. by conventional (PCR) and real-time (qPCR) polymerase chain reaction. Agglutinating antibodies were detected in 18.52% (10/54) of the sera with a titre range of 1:50 to 1:12800. The most common serogroup was Pomona, followed by Sejroe, Icterohaemorrhagiae, Australis and Javanica. *Leptospira* spp. DNA was found in 1.85% (1/54) of the urine samples, while all EDTA-anticoagulated blood samples were negative. A statistically significant difference in seropositivity regarding lifestyle was found between outdoor/indoor and indoor-only cats, while the presence of another cat in the household significantly increased the likelihood of seropositivity. Cats with immunocompromising conditions showed a significantly increased risk of seropositivity, especially those undergoing immunosuppressive treatment. In addition, respiratory signs and changes in lung structure associated with the presence of leptospiral antibodies, and these cats were more likely to be infected with the Pomona serogroup. Moreover, cats with anaemia, leucocytosis, and thrombocytopenia were significantly more likely to have antibodies against *Leptospira* spp., while seropositive cats had significantly lower urine-specific gravity compared to seronegative cats. The results underline the importance of raising awareness of feline leptospirosis in veterinary care and recognising pet cats as potential carriers of leptospires. Further research is needed to clarify the specific role of the Pomona serogroup as a potentially highly evolutionary drifting serogroup in terms of pathogenicity and to clarify the zoonotic potential of infected cats, which is crucial for the implementation of effective public health measures and veterinary interventions.

1. Introduction

Leptospirosis is a worldwide zoonosis caused by pathogenic spirochetes of the genus *Leptospira* (Levett, 2001). This re-emerging infectious disease has been identified in various species, highlighting its ubiquity and potential public health impact (Bharti et al., 2003; Picardeau, 2017). Rodents play a crucial role as they shed leptospires in their urine continuously or intermittently throughout their lifespan and thus represent the most important reservoirs (Levett, 2001; Adler and de la Peña Moctezuma, 2010). Infection occurs through mucous membranes

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and microlesions of the skin directly with infected urine, the ingestion of infected animals or tissues as well as via placental and venereal transmission or indirectly via a contaminated environment (soil, food and water) (Baranton and Old, 1995; Adler and de la Peña Moctezuma, 2010). Following leptospiraemia, leptospires colonise the proximal renal tubular epithelial cells and are subsequently excreted in the urine (Ko et al., 2009; Adler and de la Peña Moctezuma, 2010), leading to environmental contamination (Bharti et al., 2003). Naturally infected cats can excrete leptospires in their urine, with prevalence rates of up to 67.8% reported (Markovich et al., 2012; Chan et al., 2014; Rodriguez et al., 2014; Weis et al., 2017; Zaidi et al., 2018; Gomard et al., 2019; Sprißler et al., 2019; Alashraf et al., 2020; Dorsch et al., 2020; Moreira da Silva et al., 2020; Murillo et al., 2020a). According to recent systematic reviews and meta-analyses by Ricardo et al. (2023) and Miotto et al. (2024), the overall prevalence rates of *Leptospira* spp. in cat urine are 3.7% and 8%, respectively.

Seroprevalence in cats worldwide varies between 0.26% (Grippi et al., 2023) and 66.6% (Natarajaseenivasan and Raja, 2002), depending on the geographical location of the study. The overall estimates of seroprevalence are 11% (Miotto et al., 2024) and 11.7% (Ricardo et al., 2023), while in asymptomatic cats it is 9.7% (Ricardo et al., 2024). Exposure to several serogroups has been identified, including Icterohaemorrhagiae, Canicola, Grippotyphosa, Pomona, Hardjo, Autumnalis, Ballum and Bratislava (Schuller et al., 2015; Ricardo et al., 2024). Which serovars are responsible for incidental infections and which have developed adaptations to feline species remain unknown.

Although serological evidence suggests exposure of cats to leptospires, cases of clinical leptospirosis in cats are rarely documented (Agunloye and Nash, 1996; Arbour et al., 2012). Infected cats are usually asymptomatic or show only mild signs. However, when clinical manifestation occurs, the following signs are reported: polyuria, polydipsia, lethargy, anorexia, vomiting, diarrhoea, haematuria, uveitis, lameness, weight loss, ascites, pain on handling, and inflammatory lesions on the skin and digits (Murillo et al., 2020b; Miotto et al., 2024). Kidney and liver disease have also been reported in cats with leptospirosis (Millán et al., 2009; Rodriguez et al., 2014), and interstitial nephritis appears to be the most common clinical manifestation after both natural and experimental infection (Fessler and Morter, 1964; Rees, 1964; Modrić, 1974; Bryson and Ellis, 1976; Modrić and Bambir, 1991; Arbour et al., 2012). The potential role of *Leptospira* spp. infection in the development of chronic kidney disease in cats has been discussed (Hartmann et al., 2020). In addition, subpleural and intra-alveolar haemorrhages in cats with leptospirosis were described in one study (Bryson and Ellis, 1976) and disseminated parenchymal haemorrhages in the lungs of experimentally infected cats in another study (Modrić, 1978). Clinicopathological findings during leptospiraemia may initially include leucopenia, but over time this develops into leucocytosis characterised by neutrophilia with a left shift (Sykes et al., 2011; Schuller et al., 2015). Infected cats often exhibit moderate to severe azotemia, slight increases in serum liver enzymes, and possible electrolyte imbalances (Sykes et al., 2011; Rodriguez et al., 2014; Murillo et al., 2020b). Reported findings in urinalysis include hyposthenuria, haematuria and proteinuria (Arbour et al., 2012; Beaudu-Lange and Lange, 2014).

Given the non-specific clinical signs and the difficulty of isolating *Leptospira* spp., epidemiological studies and laboratory diagnosis of leptospirosis rely mainly on serological and molecular methods (Musso and La Scola, 2013; Picardeau, 2013; Anonymus, 2021). The microscopic agglutination test (MAT) is a widely used serological method for the diagnosis of leptospirosis (Musso and La Scola, 2013; Picardeau, 2013; Anonymus, 2021). The polymerase chain reaction (PCR) identifies leptospiral DNA in a sample and can determine the *Leptospira* species, but not the infecting serogroup or serovar (Bourhy et al., 2011). Due to the higher sensitivity, specificity and low risk of contamination, real-time PCR techniques are recommended (Bourhy et al., 2011; Musso and La Scola, 2013; Waggoner and Pinsky, 2016). The inclusion of genes that are only specific for pathogenic *Leptospira* spp. such as *lipL32* can increase the specificity of the test (Stoddard et al., 2009).

Although leptospirosis is a prevalent zoonotic infection worldwide, it is often underestimated and underdiagnosed (Pappas et al., 2008; Hartskeerl and Ellis, 2011). The extent to which cats contribute to environmental contamination with leptospires remains unknown and overlooked. The main objective of this study is to investigate the seroprevalence and urinary excretion of *Leptospira* spp. in pet cats in Croatia. Additionally, risk factors, clinical signs, laboratory results in infected cats, and the potentially involved serogroups were evaluated.

2. Materials and methods

2.1. Study design

In the period from December 2022 to December 2023, blood and urine samples were collected from cats that were patients of the Veterinary Teaching Hospital of the University of Zagreb for various reasons. The samples were subjected to the general tests of haematology, serum biochemistry and urinalysis and/or bacteriological examination of urine. The samples were stored in the laboratory of the Clinic for Internal Diseases and in the Bacteriological Laboratory, Faculty of Veterinary Medicine, University of Zagreb, until they were processed for this study.

2.2. Cats

Serum and urine samples were collected from a total of 54 cats presenting to the Veterinary Teaching Hospital due to health problems. EDTA-anticoagulated blood was collected and tested only from cats that met at least one of the following selection criteria: no antibiotic administration, immunocompromising conditions (retrovirus infection, tumours, diabetes mellitus, under immunosuppressive therapy) and if the blood count indicated anaemia, thrombocytopenia and/or leucocytosis. Based on these criteria, 27 EDTA-anticoagulated blood samples were collected. Most of the samples were from cats with kidney and urinary tract disease. Breed, age, sex, and clinical signs were recorded for each cat. All cats were tested for feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) using a commercial test (SNAP Combo FeLV/FIV test®, IDEXX). For most cats, the living conditions (outdoor/indoor, other pets in the household) and a possible immunocompromised state were recorded. In addition, the laboratory results of the complete blood count (CBC), biochemical profile and urinalysis were recorded for almost all cats, with the exception of one CBC and three urinalyses. Cats were categorised into three groups based on the primary diagnosis under which they were treated at the Veterinary Teaching Hospital: kidney and urinary tract diseases, diseases related to immunosuppression and other diseases such as trauma, hyperthyroidism, heart disease and hypertensive retinopathy, while this was not determined in one cat.

2.3. Urine and blood collection and DNA extraction

2.3.1. Blood

The blood samples were collected in EDTA tubes for DNA extraction and in serum tubes for serological testing. The serum samples were stored at −20 °C until analysed. The blood specimens in EDTA were stored at 4 °C and centrifuged at 100 rcf for 15 min within 24 h. The supernatant was pipetted into a sterile Eppendorf tube, centrifuged at 14,100 rcf for an additional 45 min and the pellet was frozen at −20 °C until DNA extraction.

2.3.2. Urine

Urine samples were collected using three different methods: cystocentesis ($n = 36$), catheterisation ($n = 7$) or free-flow collection ($n = 11$). The minimum volume of urine collected was 1 ml, which was stored either immediately frozen at −20 °C ($n = 26$) or at 4 °C for a maximum

of 24 h ($n = 28$) until further processing. The samples were centrifuged at 10,000 rcf at 4 °C for 10 min. To enhance the yield of bacterial DNA (Munch et al., 2019), the urine pellet was then resuspended in 1 ml Tris-EDTA solution (pH 8.0, 10 mM Tris, 1 mM EDTA) and centrifuged again at 10,000 rcf for 10 min. The urine pellet was either frozen at −20 °C or immediately subjected to a DNA extraction protocol.

2.3.3. DNA extraction

After the pretreatment extraction steps of EDTA-anticoagulated blood and urine samples, the pellets were resuspended in 180 µL Lysis Buffer T1 and 25 µL proteinase K. Total DNA was then extracted using the NucleoSpin Tissue, Mini Kit for DNA from Cells and Tissues (MACHEREY-NAGEL GmbH & Co. KG, Germany). All further extraction procedures were performed according to the manufacturer's protocols with a final elution volume of 100 µL to increase the DNA concentration. The extracted DNA was stored at −20 °C until testing. The purity and concentration of DNA was determined for all extracted samples (BioDrop µLITE spectrophotometer).

2.3. Detection of Leptospira spp. antibodies

2.3.1. Reference method

The serum samples were tested for antibodies against *Leptospira* spp. using the microscopic agglutination test (MAT). The serological test was performed in the Laboratory for Leptospirosis of the Faculty of Veterinary Medicine, University of Zagreb, according to the standard procedure in accordance with the instructions of the World Health Organisation (WHO) (Anonymus, 2021) and the International Leptospirosis Society (ILS).

2.3.2. Panel of antigens

The MAT was performed with an antigen panel consisting of 12 pathogenic serovars of *Leptospira* spp.: Grippotyphosa, Sejroe, Bratislava, Pomona, Canicola, Icterohaemorrhagiae, Tarassovi, Saxkoebing, Ballum, Bataviae, Poi and Hardjo. The antigen panel consisted of reference strains of serovars from the Leptospirosis Reference Centre (KIT Biomedical Research, Amsterdam, The Netherlands). Previous epidemiological and epizootiological analyses have proven the presence of these serovars in Croatia (Milas et al., 2002; Turk et al., 2003; Stritof Majetić et al., 2012; Stritof Majetic et al., 2014; Habus et al., 2017; Benvin et al., 2023).

2.3.3. Test procedure and result interpretation

For this test, up to 10-day-old cultures of *Leptospira* spp. with a density of 2×10^8 bacteria/ml and without contamination were used. In the test procedure, all serum samples were serially diluted in a phosphate buffer solution (PBS), starting with an initial dilution of 1:50. The endpoint titre was the highest serum dilution showing 50% agglutination compared to the negative antigen control. At a cut-off value of 1:50, the samples were categorised as positive. The presumptive infectious serogroup was determined by identifying the highest titres for one or more serovars belonging to a particular serogroup. Seropositive samples indicate suspected leptospirosis or possible convalescence.

2.4. Detection of pathogenic Leptospira spp. by molecular techniques

2.4.1. Real-time lipL32 polymerase chain reaction (qPCR)

For the real-time PCR reaction, the extracted DNA was analysed in Rotor-Gene Q (Qiagen, Hilden, Germany) using a TaqMan probe for the *lipL32* gene. In this study, the QuantiFast Pathogen PCR + IC kit (Qiagen, Hilden, Germany) was used, which includes an internal control assay. The primer set used in this study consists of LipL32–45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and LipL32–286R (5′-GAA CTC CCA TTT CAG CGA TT-3′) to amplify a fragment of 242 bp, which was detected with the probe LipL32–189P (FAM-5′-AA AGC CAG GAC AAG CGC CG-3′-BHQ1) (Stoddard et al., 2009). The amplification protocol consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of amplification (95 °C for 5 s and 60 °C for 30 s). All samples were tested in duplicate and each run included a negative control with PCR water and a positive control with leptospiral DNA. The sample was considered positive if the cycle threshold (Ct) was below 35 and was recorded in duplicate. A positive result was interpreted as an indication of urinary excretion.

2.4.2. Conventional polymerase chain reaction (PCR) and DNA sequencing

The polymerase chain reaction was performed with the entire extracted DNA using primers: LipL32 F (5′-ATC TCC GTT GCA CTC TTT GC-3′) and LipL32 (5′- ACC ATC ATC ATC ATC GTC CA-3′) as described by Ahmed et al. (2006), which can amplify a 474-bp DNA fragment of the *lipL32* gene. Each amplification reaction was performed in the T100TM thermal cycler (Bio-Rad, USA) with the following steps: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 1 min, followed by extension at 72 °C for 7 min. The amplified DNA was electrophoresed through a 1% agarose gel and compared with a molecular size marker. A positive PCR result was sequenced to confirm the presence of pathogenic *Leptospira* spp. Sanger sequencing was performed by Macrogen Europe Inc (Amsterdam, The Netherlands). The resulting sequences were aligned with Clustal X (version 2.0), analysed using BioEdit software (version 7.7) and compared with the GenBank nucleotide databases using the BLAST programme of the National Centre for Biotechnology Information (NCBI).

2.5. Data analysis

Statistical analyses were performed with Statistica v.14 (TIBCO Software Inc., 2020), Medcalc Odds Ratio Calculator v.22 (MedCalc Software Ltd.) and R 4.2.2 (R Core Team, Vienna, Austria, 2022). Descriptive statistics are presented as numbers and percentages. Odds ratio (OR) with 95% confidence intervals (95% CI) were used for single bivariate risk factors. Logistic regression analysis was used to calculate ORs for multinomial risk factors. When complete separation occurred for multinomial variables (cells with 0), Firth correction was applied using the logistf package. The *t*-test was used for continuous variables after checking their normal distribution with the Kolmogorov-Smirnov test, and results were presented as mean ± standard deviation. Differences were considered significant at $P < 0.05$.

3. Results

Of the 54 serum samples tested, 10 (18.52%; 95% CI = 9.25–31.43) had agglutinating antibodies against at least one serovar of *Leptospira* spp. and were considered seropositive. The most common serogroup was Pomona in four (40.0%; 95% CI = 12.16–73.76) samples, while Sejroe, Icterohaemorrhagie, Australis and Javanica were considered presumptive infectious serogroups in one serum sample (Table 1). In two serum samples with agglutination antibodies in the same titre against two serovars belonging to different serogroups, it was not possible to identify the presumptive infectious serogroup, so these serum samples were classified as nondetermined. The titres ranged from 1:50 to 1:12800 (Table 1).

Among all cats included in the study, 68.52% (37/54) were male, of which 48.15% (26/54) were neutered, while 31.48% (17/54) were female and 20.37% (11/54) were neutered. The median age of all cats tested was 6.5 years with a range of six months to 17 years. The median age of the seropositive and seronegative cats was 8 and 6 years, respectively. Most cats were mixed breeds ($n = 44$), while some were purebred ($n = 10$). There were no significant sex-, age- and breed-related differences between the groups (Table 2).

Regarding lifestyle, there was no difference in seropositivity between outdoor and indoor cats ($P = 0.26$), whereas a statistically significant difference in seropositivity was found between outdoor/indoor cats and indoor-only cats ($P = 0.03$). In addition, the presence of another cat in

Table 1
MAT titre results from seropositive cats.

Cat	Serovars								Presumptive infectious serogroup
	Pomona	Icterohaemorrhagie	Tarassovi	Bratislava	Saxkoebing	Sejroe	Gryppotyphosa	Poi	
1	–	1:50	–	–	–	–	–	–	Icterohaemorrhagie
2	–	–	1:100	–	–	–	–	1:100	ND
3	1:100	–	–	–	–	–	–	–	Pomona
4	–	–	–	1:100	–	–	–	–	Australis
5	–	–	–	–	1:200	–	–	1:200	ND
6	–	–	–	1:100	1:200	1:50	–	–	Sejroe
7	1:12800	–	–	–	–	–	1:6400	–	Pomona
8	1:1600	–	–	–	–	–	1:200	–	Pomona
9	1:100	–	–	–	–	–	–	–	Pomona
10	–	–	–	–	–	–	–	1:100	Javanica

ND = nondetermined.

Table 2
Association of the serological status and potential risk factors.

Variable	Category	n	n positive (%)	n negative (%)	Odds ratio	95% CI	P value
Sex	Female	17	4 (23.53)	13 (76.47)	Ref.		
	Male	37	6 (16.22)	31 (83.78)	0.63	0.15–2.61	0.52
Reproductive status	Neutered male	26	5 (19.23)	21 (80.77)	Ref.		
	Intact male	11	1 (9.09)	10 (90.91)	0.56	0.05–3.34	0.54
	Intact female	6	0 (0.0)	6 (100.0)	0.3	0.002–3.29	0.37
	Neutered female	11	4 (36.36)	7 (63.64)	2.35	0.51–10.75	0.27
Age (years)	Young adult (1–6)	26	4 (15.38)	22 (84.62)	Ref.		
	Kitten (<1)	1	0 (0.0)	1 (100.0)	0.78	0.005–17.84	0.88
	Mature (7–10)	14	4 (28.57)	10 (71.43)	0.51	0.07–2.83	0.44
	Senior (>10)	13	2 (15.38)	11 (84.62)	0.47	0.1–2.14	0.32
Breed	Purebred	10	3 (30.0)	7 (70.0)	Ref.		
	Mixed breed	44	7 (15.91)	37 (84.09)	0.44	0.09–2.13	0.31
Lifestyle	Indoor-only	18	1 (5.56)	17 (94.44)	Ref.		
	Outdoor	10	2 (20.0)	8 (80.0)	4.25	0.36–99.78	0.26
	Outdoor/indoor	17	7 (41.18)	10 (58.82)	11.9	1.76–240.02	0.03
	N/A	9					
Outdoor access	Indoor-only	18	1 (5.56)	17 (94.44)	Ref.		
	Outdoor access	27	9 (33.33)	18 (66.67)	8.5	0.97–74.43	0.053
	N/A	9					
Other pets in the household	No	10	0 (0.0)	10 (100.0)	Ref.		
	Cat	20	8 (40.0)	12 (60.0)	14.28	1.46–1928.18	0.02
	Cat and dog	1	0 (0.0)	1 (100.0)	7.0	0.03–1591.53	0.39
	Dog	2	0 (0.0)	2 (100.0)	4.2	0.02–881.2	0.51
	N/A	21					
Immunocompromising condition	No	40	4 (10.0)	36 (90.0)	Ref.		
	Yes	14	6 (42.86)	8 (57.14)	6.75	1.54–29.62	0.01
FIV	Negative	50	8 (16.0)	42 (84.0)	Ref.		
	Positive	4	2 (50.0)	2 (50.0)	5.25	0.64–42.91	0.12
FeLV	Negative	52	9 (17.31)	43 (82.69)	Ref.		
	Positive	2	1 (50.0)	1 (50.0)	4.78	0.27–83.72	0.28
Immunosuppressive treatment	No	51	8 (15.69)	43 (84.31)	Ref.		
	Yes	3	2 (66.67)	1 (33.33)	10.75	0.87–133.12	0.06
Diabetes mellitus	No	51	9 (17.65)	42 (82.35)	Ref.		
	Yes	3	1 (33.33)	2 (66.67)	2.33	0.19–28.6	0.51
Tumours	No	49	8 (16.33)	41 (83.67)	Ref.		
	Yes	5	2 (40.0)	3 (60.0)	3.42	0.49–23.85	0.22
Contact with rodents	No	12	2 (16.67)	10 (83.33)	Ref.		
	Yes	2	1 (50.0)	1 (50.0)	5	0.21–117.9	0.32
	N/A	40					
Primary diagnosis	Others	8	0 (0.0)	8 (100.0)	Ref.		
	Kidney and urinary tract diseases	36	8 (22.22)	28 (77.78)	5.07	0.53–680.52	0.19
	Diseases related with immunosuppression	9	2 (22.22)	7 (77.78)	5.67	0.38–831.87	0.23
	N/A	1					
Kidney and urinary tract diseases	None	15	0 (0.0)	15 (100.0)	Ref.		
	Urinary tract disease	20	1 (5.0)	19 (95.0)	2.38	0.12–358.07	0.58
	Kidney disease	19	9 (47.37)	10 (52.63)	28.05	2.98–3762.67	0.001
Antibiotic administration	No	40	9 (22.5)	31 (77.5)	Ref.		
	Yes	13	1 (7.69)	12 (92.31)	0.29	0.03–2.52	0.26
	N/A	1					

n = number, CI = confidence interval, Ref. = Reference category.
N/A = not available (excluded from statistical analysis).
P value for the overall significance of the variable on the seropositivity. P value <0.05 was considered statistically significant.

the household significantly increased the probability of seropositivity ($P = 0.02$) (Table 2).

Circumstances associated with immunocompromising conditions that were considered in this study include immunosuppressive treatments, FIV and FeLV infections, tumours and diabetes mellitus. It was found that cats affected by immunocompromising conditions had a significantly increased risk of seropositivity ($P = 0.01$).

There was no difference in seropositivity in relation to the primary diagnosis. On the other hand, cats with kidney or urinary tract disease, which were not necessarily treated under this diagnosis as the primary diagnosis, were 28.05 (95% CI = 2.98–3762.67; $P = 0.001$) times more likely to be infected with leptospires (Table 2).

The recorded clinical signs that could be associated with *Leptospira* spp. infection in cats are summarised in Table 3. An association was found between respiratory signs and recognisable changes in lung structure, as determined by radiographs, and the presence of antibodies to leptospires.

The analysis results for the laboratory findings of the CBC, biochemical profile and urinalysis are shown in Table 4. Cats with anaemia, leucocytosis and thrombocytopenia were 5.14-, 6.5- and 5.71-fold more likely to have antibodies against *Leptospira* spp. respectively. On the other hand, no significant difference was found between the seropositive and seronegative cats in the selected serum biochemistry and urinalysis variables. A significant difference was only found between the mean urine specific gravity (USG) of seropositive and seronegative cats ($P = 0.04$). Thus, seropositive cats (1.021 ± 0.01) have a significantly lower USG value than seronegative cats (1.033 ± 0.02).

Given that Pomona was the most common serogroup, an additional analysis was performed between two groups based on seropositivity for Pomona ($n = 4$) or other serogroups ($n = 6$) according to risk factors, clinical signs and laboratory findings. The risk factors previously found to be different compared to seronegative cats and the recorded clinical signs in each of the seropositive cats are shown in Table 5, while the changes in laboratory findings are shown in Table 6. No significant difference was observed in the selected risk factors, clinical signs and laboratory findings of urinalysis and serum biochemical variables between cats infected with Pomona or other serogroups. However, none of the cats infected with the Pomona serogroup had elevated creatinine levels and a statistically significant difference was found compared to cats infected with other serogroups ($P = 0.02$). In addition, it was found that seropositive cats with respiratory signs and changes in lung structure were 30.33 and 15 times more likely to be infected with the Pomona serogroup, respectively.

All tested EDTA-anticoagulated blood samples ($n = 27$) were negative for the *lipL32* gene by conventional and real-time PCR. On the other hand, of the 54 urine samples, only one urine sample (1.85%; 95% CI = 0.05–9.89) was positive for leptospiral DNA by conventional PCR. Sequencing of the PCR product and BLAST analysis of the extracted DNA confirmed pathogenic *Leptospira* spp. in the urine (GenBank accession number PP915794). The cat with urinary shedding was a nine-year-old neutered mixed-breed cat presented with inappetence, lethargy and vomiting. Clinical and laboratory findings showed hyperthermia, elevated urea, creatinine and ALT levels. The cat lived in a multi-cat household and was FIV and FeLV negative. Urine was collected by cystocentesis and bacteriological culture was negative. Urinalysis revealed that the urine was moderately concentrated, which may be considered inappropriately dilute urine as the cat was dehydrated, with a normal pH (6.0), while the UPC ratio indicated proteinuria.

Table 3
Association of the serological status and clinical signs.

Variable	Category	n	n positive (%)	n negative (%)	Odds ratio	95% CI	P value
Anorexia	No	25	5 (20.0)	20 (80.0)	Ref.		
	Yes	28	5 (17.86)	23 (82.14)	0.87	0.22–3.45	0.84
	N/A	1					
Lethargy	No	16	1 (6.25)	15 (93.75)	Ref.		
	Yes	38	9 (23.68)	29 (76.32)	4.66	0.54–40.29	0.16
PU/PD	No	41	6 (14.63)	35 (85.37)	Ref.		
	Yes	8	2 (25.0)	6 (75.0)	1.94	0.32–12	0.47
	N/A	5					
Vomiting	No	31	6 (19.35)	25 (80.65)	Ref.		
	Yes	23	4 (17.39)	19 (82.61)	0.88	0.22–3.55	0.85
Diarrhoea	No	52	9 (17.31)	43 (82.69)	Ref.		
	Yes	2	1 (50.0)	1 (50.0)	4.78	0.27–83.72	0.28
Fever	No	43	8 (18.6)	35 (81.4)	Ref.		
	Yes	8	1 (12.5)	7 (87.5)	0.63	0.07–5.82	0.68
	N/A	3					
Hypothermia	No	41	8 (19.51)	33 (80.49)	Ref.		
	Yes	10	1 (10.0)	9 (90.0)	0.46	0.05–4.16	0.49
	N/A	3					
Respiratory signs	No	49	7 (14.29)	42 (85.71)	Ref.		
	Yes	5	3 (60.0)	2 (40.0)	9.0	1.27–63.89	0.03
Changes in lungs	No	49	6 (12.24)	43 (87.76)	Ref.		
	Yes	4	4 (100.0)	0 (0.0)	60.23	2.89–1253.89	0.008
	N/A	1					
Thoracic or/and abdominal effusion	No	45	8 (17.78)	37 (82.22)	Ref.		
	Yes	6	2 (33.33)	4 (66.67)	2.31	0.36–14.88	0.38
	N/A	1					
Jaundice	No	49	9 (18.37)	40 (81.63)	Ref.		
	Yes	4	0 (0.0)	4 (100.0)	0.47	0.02–9.57	0.63
	N/A	1					
Inflammatory lesions on the skin and digits	No	51	9 (17.31)	43 (82.69)	Ref.		
	Yes	2	1 (50.0)	1 (50.0)	4.78	0.27–83.72	0.28
Lameness	No	52	10 (19.23)	42 (80.77)	Ref.		
	Yes	2	0 (0.0)	2 (100.0)	0.81	0.04–18.16	0.89
Neurological signs	No	51	10 (19.61)	41 (80.39)	Ref.		
	Yes	3	0 (0.0)	3 (100.0)	0.56	0.03–11.8	0.71

n = number, CI = confidence interval, Ref. = Reference category.
N/A = not available (excluded from statistical analysis).
P value for the overall significance of the variable on the seropositivity. P value <0.05 was considered statistically significant.

Table 4
Association of the serological status and the laboratory results (complete blood count, biochemical profile and urinalysis).

Variable	Category	n	n positive (%)	n negative (%)	Odds ratio	95% CI	P value
Anaemia	No	41	5 (12.2)	36 (87.8)	Ref.		
	Yes	12	5 (41.67)	7 (58.33)	5.14	1.17–22.61	0.03
	N/A	1					
Leucocytosis	No	45	6 (13.33)	39 (86.67)	Ref.		
	Yes	8	4 (50.0)	4 (50.0)	6.5	1.27–33.2	0.02
	N/A	1					
Leucopenia	No	37	7 (18.92)	30 (81.08)	Ref.		
	Yes	16	3 (18.75)	13 (81.25)	0.99	0.22–4.44	0.99
	N/A	1					
Thrombocytopenia	No	47	7 (14.89)	40 (85.11)	Ref.		
	Yes	6	3 (50.0)	3 (50.0)	5.71	0.95–34.24	0.056
	N/A	1					
Elevated urea	No	23	3 (13.04)	20 (86.96)	Ref.		
	Yes	31	7 (22.58)	24 (77.42)	1.94	0.44–8.52	0.38
Elevated creatinine	No	33	4 (12.12)	29 (87.88)	Ref.		
	Yes	21	6 (28.57)	15 (71.43)	2.9	0.71–11.88	0.14
Electrolyte imbalance	No	38	5 (13.16)	33 (86.84)	Ref.		
	Yes	16	5 (31.25)	11 (68.75)	3.0	0.73–12.35	0.13
Elevated liver enzymes	No	38	5 (13.16)	33 (86.84)	Ref.		
	Yes	13	3 (23.08)	10 (76.92)	1.98	0.4–9.77	0.4
Elevated total bilirubin	No	48	9 (18.75)	39 (81.25)	Ref.		
	Yes	4	0 (0.0)	4 (100.0)	0.46	0.02–9.34	0.61
Haematuria	No	40	8 (20.0)	32 (80.0)	Ref.		
	Yes	12	2 (16.67)	10 (83.33)	0.8	0.15–4.4	0.8
	N/A	2					
UPCR	Nonproteinuric	19	7 (36.84)	12 (63.16)	Ref.		
	Borderline proteinuria	13	1 (7.69)	12 (92.31)	0.14	0.007–0.98	0.09
	Proteinuria	19	2 (10.53)	17 (89.47)	0.2	0.03–1.01	0.07
	N/A	3					
USG	Concentrated	21	2 (9.52)	19 (90.48)	Ref.		
	Moderately concentrated	23	6 (26.09)	17 (73.91)	3.35	0.67–25.05	0.17
	Isosthenuria	5	1 (20.0)	4 (80.0)	2.37	0.1–31.76	0.52
	Dilute	2	1 (50.0)	1 (50.0)	9.5	0.3–322.98	0.16
	N/A	3					

n = number, CI = confidence interval, Ref. = Reference category, UPCR = urine protein creatinine ratio, USG = urine specific gravity.
N/A = not available (excluded from statistical analysis).
P value for the overall significance of the variable on the seropositivity. P value <0.05 was considered statistically significant.

Ultrasonography revealed suspected glomerulonephritis and the patient was treated with a diagnosis of acute kidney injury.

4. Discussion

In general, leptospirosis is widespread among animals and humans in Croatia and occurs both in natural and increasingly in synanthropic foci. Due to the climatic conditions and geomorphology, Croatia is one of the European countries with an exceptionally high biodiversity. This biodiversity supports a large population of rodents, the main reservoirs of leptospires, and contributes to the endemic character of leptospirosis in the country (Milas et al., 2002; Turk et al., 2003; Stritof, 2010). Considering the predatory behaviour of cats and rodents as the first link in their food chain, it is to be expected that they frequently encounter leptospires. However, in the absence of clinical signs in most cases, leptospirosis in cats is often neglected and underestimated, and it is still uncertain whether their health is at risk and what role they play in the transmission of leptospirosis.

The results of this study show a considerable seroprevalence of *Leptospira* spp. infection among pet cats presented to the Veterinary Teaching Hospital of the University of Zagreb. Of the 54 sera tested, 10 (18.52%) were seropositive. This seroprevalence is higher than the overall seroprevalence of 11.7% and 11% reported by Ricardo et al. (2023) and Miotto et al. (2024), respectively. In addition, more recent studies worldwide indicate varying seropositivity rates of leptospirosis in cats, with rates of 0.26% (Grippi et al., 2023), 4.1% (Murillo et al., 2020a), 5.4% (Sprißler et al., 2019), 8.6% (Palerme et al., 2019), 9.2% (Žákovská et al., 2020), 10.53% (Mazzotta et al., 2023), 12.8% (Lehtla et al., 2020), 15.3% (Donato et al., 2022), and 18.18% (Alashraf et al., 2019).

Furthermore, most studies in Europe report *Leptospira* spp. infections in stray cats (Agunloye and Nash, 1996; Millán et al., 2009; Obrenović et al., 2014; Weis et al., 2017; Murillo et al., 2020a; Grippi et al., 2023; Mazzotta et al., 2023), and only a small number mention it in privately owned cats (Mylonakis et al., 2005; Lehtla et al., 2020; Žákovská et al., 2020; Donato et al., 2022). The seroprevalence determined in our study is higher than in the above-mentioned recent studies in Europe involving pet cats, but lower than the seroprevalence of 33.3% reported by Mylonakis et al. (2005).

The cut-off dilution used in this study was 1:50, which is lower than in some other studies where 1:100 was considered the cut-off dilution (Murillo et al., 2020b). It is noteworthy that only one cat had a titre of 1:50, while all others had a higher titre. Furthermore, cats appear to respond to infection with a lower antibody titre, not exceeding 1:100, and may show even lower antibody responses compared to dogs (Shophet and Marshall, 1980; Agunloye and Nash, 1996; Mylonakis et al., 2005; Markovich et al., 2012; Rodriguez et al., 2014; Shropshire et al., 2016; Sprißler et al., 2019). As there is no consensus on the appropriate cut-off value, we considered a dilution of 1:50 to be appropriate, in line with Croatian regulations for dogs. However, this could potentially lead to a higher prevalence in our study if a lower cut-off value had been used.

Overall, the prevalent presence of antibodies in cats was against serogroup Pomona, followed by Sejroe, Icterohaemorrhagie, Australis and Javanica. Earlier studies on leptospirosis in cats in Croatia have also found a high seroprevalence for serogroup Pomona, followed by serogroups Icterohaemorrhagiae, Sejroe, Grippotyphosa and Javanica (Modrić, 1978; Modrić and Bambir, 1991). In contrast, other studies in Europe report Australis (Obrenović et al., 2014; Weis et al., 2017), Icterohaemorrhagie (Millán et al., 2009; Grippi et al., 2023),

Table 5
Selected risk factors and clinical signs in each of the seropositive cats.

Cat	Risk factors				Clinical signs										
	Lifestyle	Other pets in the household	Immunocompromising condition	Kidney and urinary tract diseases	Anorexia	Lethargy	PU/PD	Vomiting	Diarrhoea	Fever	Hypothermia	Respiratory signs	Changes in lungs	Thoracic or abdominal effusion	Inflammatory lesions on the skin and digits
1	indoor	cats	Immunosuppressive treatment	Kidney disease	–	–	N/A	–	–	N/A	N/A	–	–	–	+
2	outdoor/ indoor	cats	FIV	Kidney disease	+	+	+	–	–	–	–	–	–	–	–
3	outdoor	cats	Tumour and Immunosuppressive treatment	Kidney disease	+	+	–	+	–	–	–	+	+	+	–
4	outdoor/ indoor	cats	–	Kidney disease	+	+	–	–	–	–	+	–	–	–	–
5	outdoor/ indoor	cats	–	Kidney disease	+	+	–	+	–	–	–	–	–	–	–
6	outdoor/ indoor	cats	–	Kidney disease	–	+	–	+	–	–	–	–	–	–	–
7[a]	outdoor/ indoor	N/A	Diabetes mellitus and FIV	Kidney disease	–	+	+	+	+	–	–	+	+	+	–
8	outdoor/ indoor	cats	Tumour	Urinary tract disease	–	+	–	–	–	–	–	–	–	–	–
9	outdoor	N/A	FeLV	Kidney disease	–	+	N/A	–	–	+	–	+	+	–	–
10	outdoor/ indoor	cats	–	Kidney disease	+	+	–	–	–	–	–	–	+	–	–

Numbering of cats corresponds to Table 1.
+ = present, - = absent, N/A = not available, PU/PD = polyuria and polydipsia.
[a] This cat had changes in the lungs, kidneys and liver established by X-ray and ultrasound.

Table 6
Laboratory results of complete blood count, serum biochemistry and urinalysis as well as correspondant presumptive infectious serogroup in each of the seropositive cats.

			Complete blood count				Biochemical profile				Urinalysis			
Cat	Presumptive infectious serogroup	Anaemia	Leucocytosis	Leucopenia	Thrombocytopenia	Elevated urea	Elevated creatinine	Electrolyte imbalance	Elevated liver enzymes	Haematuria	pH	UPCR	USG
1	Icterohaemorrhagiae	-	-	+	-	+	+	-	N/A	-	7	nonproteinuric	concentrated
2	ND	+	+	-	-	+	+	+	+	-	6	nonproteinuric	isosthenuria
3	Pomona	+	+	-	-	-	-	-	+	-	6.5	nonproteinuric	moderately concentrated
4	Australis	-	-	+	-	+	+	+	-	+	7	proteinuria	moderately concentrated
5	ND	-	-	-	-	+	+	+	N/A	-	5.5	nonproteinuric	moderately concentrated
6	Sejroe	+	-	-	-	+	+	+	-	-	5.5	borderline	moderately concentrated
7	Pomona	+	+	-	+	+	-	-	-	-	6	nonproteinuric	concentrated
8	Pomona	-	-	-	+	-	-	-	+	+	7.5	nonproteinuric	dilute
9	Pomona	+	+	-	+	-	-	+	+	-	6.5	proteinuria	moderately concentrated
10	Javanica	-	-	+	-	+	+	-	-	-	6	nonproteinuric	moderately concentrated

Numbering of cats corresponds to Table 1. None of the seropositive cats had jaundice, lameness, neurologic signs, and elevated total bilirubin, so these variables were excluded.
+ = present, - = absent, N/A = not available, UPCR = urine protein creatinine ratio, USG = urine specific gravity.

Grippotyphosa (Zákovská et al., 2020; Mazzotta et al., 2023), Javanica (Donato et al., 2022), Autumnalis (Mylonakis et al., 2005) and Cynopteri (Murillo et al., 2020a) as the most common serogroups in *Leptospira* spp. infections in cats. Apart from our study, only one study in Europe reported Pomona as the most common serogroup in cats (Lehtla et al., 2020). According to recent studies on equine leptospirosis in Croatia, Pomona has been identified as the most common serogroup in the last ten years, in addition to Grippotyphosa (Benvin et al., 2023). Furthermore, other studies conducted in Croatia have shown that Pomona is the most prevalent presumed infective serogroup in dogs (Štritof Majetić et al., 2012; Habus et al., 2017). In addition to Pomona, seropositivity was also observed for the serogroups Grippotyphosa, Sejroe, Australis and Icterohaemorrhagiae in dogs, horses, pigs and ruminants (Habus et al., 2017). Our study therefore suggests that the likelihood of leptospiral infection in cats is influenced by the presence of leptospires in other domestic animals. Moreover, habitat sharing by different animal species may lead to environmental contamination with different strains of *Leptospira*, which may maximise survival of these strains in the environment or lead to incidental infection (Ellis, 2015).

In our study, no breed- and sex-specific differences were found in relation to infection with *Leptospira* spp. Although older age was mentioned as a risk factor in several studies (Mylonakis et al., 2005; Rodriguez et al., 2014; Sprißler et al., 2019), this association did not reach statistical significance in our study. The presence of another cat in the household was identified as a risk factor for infection with *Leptospira* spp. as described in a study by Rodriguez et al. (2014). Although no other statistically significant difference in lifestyle was found in this study, except for outdoor/indoor cats compared to indoor-only cats, it was observed that cats with outdoor access were more likely to be infected with leptospires, which is consistent with the findings of Ricardo et al. (2023).

Typically, clinical signs appear to be rare in infected cats (Murillo et al., 2020b; Hartmann, 2022; Miotto et al., 2024). The significantly higher seropositivity in cats with kidney disease compared to cats with other diseases of the urinary system or without any disease of the kidneys or urinary system suggests that leptospirosis may be an underdiagnosed cause of kidney disease in cats, emphasising the need for increased awareness and consideration of this possibility. In our study, the most frequently observed clinical signs in seropositive cats were anorexia, lethargy, vomiting and respiratory signs, followed by PU/PD, while fever, hypothermia, diarrhoea and inflammatory lesions on the skin and digits were only observed in one cat. Nevertheless, for the first time, we found an association between leptospiral infection and respiratory signs in cats, as well as changes in the lungs, which were detected by X-ray examination. Of the cases observed, two cats were dyspnoeic, while one cat presented with a cough, and all radiographic findings were suggestive of a loss of lung translucency associated with pulmonary infiltrates. Interestingly, all cats with respiratory signs were anaemic and tested seropositive for the Pomona serogroup. The involvement of leptospirosis in pulmonary manifestations can range from subtle clinical features to severe conditions such as pulmonary haemorrhage and acute respiratory distress syndrome (Gulati and Gulati, 2012), as has been reported in dogs, horses and humans. This suggests that the potential impact of leptospirosis on the respiratory tract in cats requires further investigation.

Further on, our results suggest that cats in an immunocompromised state are more likely to have anti-leptospiral serum antibodies. While the specific immunocompromising condition with the greatest impact on seropositivity remains undetermined, it was observed that cats undergoing immunosuppressive treatment have an increased susceptibility to leptospiral infection and the presence of antibodies. In contrast to our study, in which retroviral infection was not identified as a significant risk factor for seropositivity, Moreira da Silva et al. (2020) found that FIV is significant in this regard. However, there were only a limited number of FIV- and FeLV-positive cats in our study, suggesting that further investigations with a larger sample size need to be conducted.

Moreover, the highest titres and suggestive symptoms of clinical leptospirosis were observed in immunocompromised cats with severe systemic comorbidities, which is also described by Mazzota et al. (2023). Therefore, further research is needed to determine which immunocompromising conditions have the greatest impact.

Laboratory changes in cats with leptospirosis may resemble those commonly seen in affected dogs (Hartmann, 2022). In the present study, we found significant associations between anaemia and leucocytosis in cats infected with leptospires. Furthermore, cats with thrombocytopenia were more likely to be infected with leptospires. Although we investigated possible associations between serum biochemistry, urinalysis parameters and leptospiral seropositivity, no significant correlations were observed, with the exception of a significantly lower USG value in seropositive cats compared to seronegative cats.

Based on the results obtained, we performed a comparison of risk factors, clinical manifestations and laboratory results between cats that tested positive for the Pomona serogroup and those that tested positive for other serogroups. Our analysis showed that seropositive cats with respiratory signs and lung changes were more likely to be infected with serogroup Pomona.

Antibodies against the Pomona serogroup were detected several times in high dilutions (1:12800 and 1:1600), indicating a possible active infection. Both cat #7 and cat #8 were immunocompromised, with cat #7 having diabetes mellitus and FIV, while cat #8 had a spleen tumour. Cat #7 showed a wide range of symptoms including lethargy, polyuria/polydipsia (PU/PD), vomiting, diarrhoea and dyspnoea. Blood work showed anaemia, leucocytosis and thrombocytopenia, while serum biochemistry showed only elevated urea levels and normal urine results. In particular, radiographic changes in the lungs (reduced transparency with loss of alveolar pattern) and ultrasound changes in the kidneys (suggestive of nephropathy, most consistent with glomerulonephritis), spleen (suggestive of splenomegaly) and liver (suggestive of hepatopathy, most consistent with vacuolar hepatopathy) were observed in this cat. Similar observations were made in an earlier study in which histological changes in the liver, kidneys and lungs were documented in cats experimentally infected with the Pomona serogroup (Modrić, 1978). In contrast, cat #8 showed symptoms consistent with lower urinary tract disease and lethargy, with laboratory findings limited to thrombocytopenia, haematuria and dilute urine.

The outcome of an acute infection is influenced by factors such as the age and immune response of the host as well as the virulence and size of the inoculum of the pathogen (Levett, 2001). Furthermore, it has been observed that not all pathogenic *Leptospira* spp. exhibit the same virulence, as shown by previous whole genome sequencing studies that have revealed variations in genetic characteristics and virulence factors between strains of pathogenic leptospires (Picardeau, 2017; Jorge et al., 2018). The significance of this study lies in the high seropositivity for the Pomona serogroup, which has various clinical manifestations, emphasising the complicated dynamics of leptospirosis infection. However, the small size of the Pomona seropositive sample in our study represents a limitation in confirming the observed associations between clinical signs and Pomona seropositivity. Therefore, further future studies are needed to draw more definitive conclusions about the association between clinical signs and Pomona seropositivity in cats.

The MAT is the most widely used diagnostic test for acute leptospirosis; however, it does not determine carrier status, as low antibody titres can occur in chronically infected animals (Schuller et al., 2015). Cats usually show a rapid immune response to the infection, often followed by a rapid drop in titres (Shophet, 1979; Markovich et al., 2012). Elevated titres may indicate either a recent or active infection or a potential re-infection. On the other hand, a positive PCR in blood in conjunction with consistent clinical signs strongly suggests acute leptospirosis, while a positive PCR in urine indicates renal excretion, which can occur in both acutely infected animals and chronic renal carriers (Schuller et al., 2015).

In our study, we used molecular methods specifically targeting the *lipL32* gene, which is unique to pathogenic *Leptospira* spp. (Stoddard et al., 2009). Overall, all EDTA-anticoagulated blood samples were negative by both conventional and real-time PCR, and only one cat was identified as shedding pathogenic *Leptospira* spp. Given that leptospiraemia is transient and typically only occurs in the early stages of the disease, while urinary excretion is delayed and intermittent following acute infection (Levett, 2001), a negative result in these samples does not rule out leptospirosis (Schuller et al., 2015). This could also explain the absence of leptospiral DNA shedding in the urine of all seropositive cats. Furthermore, the negative results could be due to recent antibiotic treatment. Moreover, the low number of PCR-positive urine samples could also be due to the low pH and higher osmolality of cat urine. In particular, the increased osmolality creates an unfavourable environment for bacterial growth, in contrast to that of dogs and humans (Rubini and Wolf, 1957). In addition, leptospires degrade relatively rapidly in acidic urine (Levett, 2001), which could reduce the quality and quantity of amplifiable DNA and thus make the detection of urine excretion more difficult in many cats. However, various urinary tract infections can increase the pH of urine, so *Leptospira* infection with urine from cats is likely possible (Hartmann et al., 2013). Nevertheless, freezing urine samples could also reduce the sensitivity of PCR compared to fresh urine (Branger et al., 2005). The discrepancy between the results of conventional and real-time PCR in the urine sample could be due to a low bacterial load, as real-time PCR may not detect very low DNA concentrations despite its high sensitivity and its efficiency could be further impaired by inhibitors present in the urine (Kubista et al., 2006; Sidstedt et al., 2015). On the other hand, conventional PCR detected leptospiral DNA, which was confirmed by Sanger sequencing of the *lipL32* gene, specific to pathogenic *Leptospira*. However, identification of the *Leptospira* species present in the urine proved challenging in our study and the species could not be determined. The limitations in detecting *Leptospira* species with advanced molecular methods are primarily due to the often low bacterial load in clinical samples, which leads to low DNA concentrations and poor DNA quality. These methods require good DNA quality, but clinical samples such as blood and urine often have degraded DNA or insufficient bacterial presence, highlighting the challenges for reliable detection.

In this study, a cat with leptospiruria tested negative for antibodies, and the PCR test performed with EDTA-anticoagulated blood also gave negative results. Similarly, previous studies in seronegative cats have observed urinary excretion confirmed by culture or molecular methods (Fessler and Morter, 1964; Shophet, 1979; Chan et al., 2014; Spriβler et al., 2019; Alashraf et al., 2020; Murillo et al., 2023). The possibility of prolonged shedding of leptospiral DNA after a confirmed infection (Weis et al., 2017), coupled with the typical rapid decline in titres observed in cats, could explain the seronegative result in this cat. Moreover, a cat that has been previously exposed to the bacteria may not only have low antibody titres, but also a complete absence of titre. This result depends on several factors, including the infecting serovar and the cat's possible adaptation to that serovar over time, resulting in either low or no antibody titres (Adler, 2014).

A limitation of the study was the lack of follow-up, which could potentially lead to false-negative results due to intermittent shedding. Although urine samples were repeatedly collected or bacteriologically analysed from some cats ($n = 15$) participating in this study and the archived samples were also tested for pathogenic leptospires as part of this study, all results were negative. Nevertheless, our results show that cats can be susceptible to infection and possibly act as carriers. In addition, MAT can sometimes present a challenge in accurately identifying the infectious serogroup of *Leptospira* spp. due to cross-reactions and paradoxical reactions. Sera from cats that were repeatedly tested for general serum biochemical tests ($n = 21$) were also repeatedly serologically analysed for the presence of antibodies against leptospires. Among the repeatedly tested seropositive cats ($n = 7$), the highest titre was found for the same serogroups listed in this article, suggesting that the mentioned serogroups can be considered as presumptive infectious

serogroups.

In conclusion, the findings underscore the critical need for heightened attention to feline leptospirosis in veterinary care. Furthermore, the realisation that domestic cats are potential carriers of leptospires is crucial for the development of effective public health strategies aimed at the control and prevention of leptospirosis. While naturally infected cats can shed pathogenic *Leptospira* spp. in their urine, the potential for zoonotic transmission remains unclear. The specific role of the serogroup Pomona as an evolutionary highly drifting serogroup in terms of pathogenicity should also be clarified. Therefore, further research is essential to deepen our understanding of the role these animals play in the environmental transmission cycle.

Ethics approval

This study received institutional approval from The Committee for Ethics in Veterinary Medicine of the Faculty of Veterinary Medicine, University of Zagreb (640–01/23–17/26; 251–61–41–23–01). The owners of the cats provided written informed consent before the samples were taken.

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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.

Data availability

Data will be made available on request.

References

Adler, B., 2014. Current Topics in Microbiology and Immunology, 1st ed. Springer, Berlin/Heidelberg, Germany.

Adler, B., de la Peña Moctezuma, A., 2010. *Leptospira* and leptospirosis. Vet. Microbiol. 140 (3–4), 287–296. https://doi.org/10.1016/j.vetmic.2009.03.012.

Agunloye, C.A., Nash, A.S., 1996. Investigation of possible leptospiral infection in cats in Scotland. JSAP 37, 126–129.

Ahmed, N., Devi, S.M., de los A Valverde, M., Vijayachari, P., Machang'u, R.S., Ellis, W. A., Hartskeerl, R.A., 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. Ann. Clin. Microbiol. Antimicrob. 5, 28. https://doi.org/10.1186/1476-0711-5-28.

Alashraf, A.R., Lau, S.F., Khor, K.H., Khairani-Bejo, S., Bahaman, A.R., Roslan, M.A., Rahman, M.S.A., Goh, S.H., Radzi, R., 2019. Serological Detection of Anti-Leptospira Antibodies in Shelter Cats in Malaysia. Top. Companion Anim. Med. 34, 10–13. https://doi.org/10.1053/j.tcam.2018.12.002.

Alashraf, A.R., Lau, S.F., Khairani-Bejo, S., Khor, K.H., Ajat, M., Radzi, R., Roslan, M.A., Rahman, M.S.A., 2020. First report of pathogenic *Leptospira* spp. Isolated from urine and kidneys of naturally infected cats. PLoS ONE 15 (3), e0230048. https://doi.org/10.1371/journal.pone.0230048.

Anonymus, 2021. 3.01.12_LEPTO OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022.

Arbour, J., Blais, M.C., Carioto, L., Sylvestre, D., 2012. Clinical leptospirosis in three cats (2001-2009). JAAHA 48 (4), 256–260. https://doi.org/10.5326/JAAHA-MS-5748.

Baranton, G., Old, I.G., 1995. The spirochaetes: a different way of life. Bulletin de l'Institut Pasteur 93 (2), 63–95. https://doi.org/10.1016/0020-2452(96)81485-0.

Beaudu-Lange, C., Lange, E., 2014. Unusual clinical presentation of leptospirosis in a cat. Rev. Vet. Clin. 49 (3), 115–122. https://doi.org/10.1016/j.anicom.2014.05.001.

Benvin, I., Perko, V.M., Maljković, M.M., Habuš, J., Štritof, Z., Hađina, S., Perharić, M., Zečević, I., Cvetnić, M., Turk, N., 2023. Serological Surveillance of Equine Leptospirosis in Croatia in the Period From 2012 to 2022: a Key Insight Into the Changing Epizootiology. J. Equine Vet. Sci. 127, e104844. https://doi.org/10.1016/j.jevs.2023.104844.

Bharti, A.R., Nally, J.E., Ricaldi, J.N., Matthias, M.A., Diaz, M.M., Lovett, M.A., Levett, P. N., Gilman, R.H., Willig, M.R., Gotuzzo, E., Vinetz, J.M., 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis. 3, 757–771. http://infection.thelancet.com.

Bourhy, P., Bremont, S., Zinini, F., Giry, C., Picardeau, M., 2011. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. J. Clin. Microbiol. 49 (6), 2154–2160. https://doi.org/10.1128/JCM.02452-10.

Branger, C., Blanchard, B., Fillonneau, C., Suard, I., Aviat, F., Chevallier, B., André-Fontaine, G., 2005. Polymerase chain reaction assay specific for pathogenic *Leptospira* based on the gene *hap1* encoding the hemolysis-associated protein-1. FEMS Microbiol. Lett. 243 (2), 437–445. https://doi.org/10.1016/j.femsle.2005.01.007.

Bryson, D.G., Ellis, W.A., 1976. Leptospirosis in a British domestic cat. JSAP 17 (7), 459–465. https://doi.org/10.1111/j.1748-5827.1976.tb06986.x.

Chan, K.W., Hsu, Y.H., Hu, W.L., Pan, M.J., Lai, J.M., Huang, K.C., Chou, S.J., 2014. Serological and PCR detection of feline *Leptospira* in Southern Taiwan. Vector Borne Zoonotic Dis 14 (2), 118–123. https://doi.org/10.1089/vbz.2013.1324.

Donato, G., Masucci, M., Hartmann, K., Goris, M.G.A., Ahmed, A.A., Archer, J., Alibrandi, A., Pennisi, M.G., 2022. *Leptospira* spp. prevalence in cats from Southern Italy with evaluation of risk factors for exposure and clinical findings in infected cats. Pathog 11, 1129. https://doi.org/10.3390/pathogens11101129.

Dorsch, R., Ojeda, J., Salgado, M., Monti, G., Collado, B., Tomckowiack, C., Tejeda, C., Muller, A., Eberhard, T., Klaasen, H.L.B.M., Hartmann, K., 2020. Cats shedding pathogenic *Leptospira* spp.-An underestimated zoonotic risk? PLoS ONE 15 (10), e0239991. https://doi.org/10.1371/journal.pone.0239991.

Ellis, W.A., 2015. Animal Leptospirosis. In: Adler, B. (Ed.), Current Topics in Microbiology and Immunology. Springer-Verlag, Berlin, Germany, pp. 99–137.

Fessler, J.F., Morter, R.L., 1964. Experimental feline leptospirosis. Cornell Vet. 54, 176–190.

Gomard, Y., Lagadec, E., Humeau, L., Pinet, P., Bureau, S., Da Silva, D., Turpin, M., Soulaimana Mattoir, Y., Georger, S., Mavingui, P., Tortosa, P., 2019. Feral cats do not play a major role in leptospirosis epidemiology on Reunion island. Epidemiol. Infect. 147, e97. https://doi.org/10.1017/S0950268819000190.

Grippi, F., Cannella, V., Macaluso, G., Blanda, V., Emmolo, G., Santangelo, F., Vicari, D., Galluzzo, P., Sciacca, C., D'Agostino, R., Giacchino, I., Bertasio, C., D'Incau, M., Guercio, A., Torina, A., 2023. Serological and Molecular Evidence of Pathogenic *Leptospira* spp. in Stray Dogs and Cats of Sicily (South Italy), 2017–2021. Microorganisms 11, 385. https://doi.org/10.3390/microorganisms11020385.

Gulati, S., Gulati, A., 2012. Pulmonary manifestations of leptospirosis. Lung India 29 (4), 347–353. https://doi.org/10.4103/0970-2113.102822.

Habus, J., Persic, Z., Spicic, S., Vince, S., Stritof, Z., Milas, Z., Cvetnic, Z., Perharic, M., Turk, N., 2017. New trends in human and animal leptospirosis in Croatia, 2009–2014. Acta Trop. 168, 1–8. https://doi.org/10.1016/j.actatropica.2017.01.002.

Hartmann, K., Egberink, H., Pennisi, M.G., Lloret, A., Addie, D., Belák, S., Boucraut-Baralon, C., Frymus, T., Gruffydd-Jones, T., Hosie, M.J., Lutz, H., Marsilio, F., Möstl, K., Radford, A.D., Thiry, E., Truyen, U., Horzinek, M.C., 2013. *Leptospira* Species Infection in Cats: ABCD guidelines on prevention and management. J. Feline Med. Surg. 15 (7), 576–581. https://doi.org/10.1177/1098612X13489217.

Hartmann, K., Pennisi, M.G., Dorsch, R., 2020. Infectious agents in feline chronic kidney disease. Adv. Small Anim. Med. Surg. 1, 189–206. https://doi.org/10.1016/j.yasa.2020.07.013.

Hartmann, K., 2022. Guideline for *Leptospira* spp. infection in cats. https://www.abcdcatsvets.org/guideline-for-leptospira-spp-infection-in-cats/?pdf=5069.

Hartskeerl, R.A., Ellis, W.A., 2011. Emergence, control and re-emerging leptospirosis : dynamics of infection in the changing world. Clin. Microbiol. Infect. 17 (4), 494–501. https://doi.org/10.1111/j.1469-0691.2011.03474.x.

Jorge, S., Kremer, F.S., De Oliveira, N.R., Navarro, G.D.O.S.V., Guimarães, A.M., Sanchez, C.D., Woloski, R.D.dos S., Ridieri, K.F., Campos, V.F., Pinto, L.da S., Dellagostin, O.A., 2018. Whole-genome sequencing of *Leptospira interrogans* from southern Brazil: genetic features of a highly virulent strain. Mem. Inst. Oswaldo Cruz. 113 (2), 80–86. https://doi.org/10.1590/0074-02760170130.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. Mol. Asp. Med. 27 (2–3), 95–125. https://doi.org/10.1016/j.mam.2005.12.007.

Ko, A.I., Goarant, C., Picardeau, M., 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat. Rev. Microbiol. 7 (10), 736–747. https://doi.org/10.1038/nrmicro2208.

Lehtla, A., Must, K., Lassen, B., Orro, T., Jokelainen, P., Viltrop, A., 2020. *Leptospira* spp. in Cats in Estonia: seroprevalence and Risk Factors for Seropositivity. Vector Borne Zoonotic Dis. 20 (7), 524–528. https://doi.org/10.1089/vbz.2019.2555.

Levett, P.N., 2001. Leptospirosis. Clin. Microbiol. Rep. 14 (2), 296–326. https://doi.org/10.1128/CMR.14.2.296.

Markovich, J.E., Ross, L., McCobb, E., 2012. The Prevalence of Leptospiral Antibodies in Free Roaming Cats in Worcester County, Massachusetts. J. Vet. Intern. Med. 26 (3), 688–689. https://doi.org/10.1111/j.1939-1676.2012.00900.x.

Mazzotta, E., De Zan, G., Cocchi, M., Boniotti, M.B., Bertasio, C., Furlanello, T., Lucchese, L., Ceglie, L., Bellinati, L., Natale, A., 2023. Feline susceptibility to leptospirosis and presence of immunosuppressive co-morbidities: first European Report of L. interrogans serogroup australis sequence type 24 in a cat and survey of *Leptospira* exposure in outdoor cats. Trop. Med. Infect. Dis. 8, 54. https://doi.org/10.3390/tropicalmed8010054.

Milas, Z., Turk, N., Staresina, V., Margaletić, J., Slavica, A., Živković, D., Modrić, Z., 2002. The role of myomorphous mammals as reservoirs of *leptospira* in the pedunculate oak forests of Croatia. Vet. Arh. 72 (3), 119–129.

Millán, J., Candela, M.G., López-Bao, J.V., Pereira, M., Jiménez, M.Á., León-Vizcaíno, L., 2009. Leptospirosis in wild and domestic carnivores in natural areas in Andalusia, Spain. Vector Borne Zoonotic Dis 9 (5), 549–554. https://doi.org/10.1089/vbz.2008.0081.

Miotto, B.A., Camelo, Q.C., Grolla, A.C.M.M., de Oliveira, A.B.M., Silva, M.M.B., Hagiwara, M.K., Esteves, S.B., 2024. Current knowledge on leptospirosis in cats: a systematic review with metanalysis on direct detection, serological response, and clinical data. Res. Vet. Sci. 174, 105292. https://doi.org/10.1016/j.rvsc.2024.105292.

Modric, Z., 1974. Experimental Leptospirosis in the Cat, 12. Folia Faculty of Medicine, University Comenianae, Bratislava, pp. 257–262. Supplementum.

Modrić, Z., 1978. Natural and experimental leptospirosis in the cat. Vet. Arh. 48 (3), 147–156. Croatian.

Modrić, Z., Bumbir, S., 1991. Leptospirosis in the domestic cat (*Felis domestica* Briss.) in Slavonia. Vet. Arh. 61 (5), 283–288. Croatian.

Moreira da Silva, J., Prata, S., Domingues, T.D., Leal, R.O., Nunes, T., Tavares, L., Almeida, V., Sepúlveda, N., Gil, S., 2020. Detection and modeling of anti-Leptospira IgG prevalence in cats from Lisbon area and its correlation to retroviral infections, lifestyle, clinical and hematologic changes. Vet. Sci. 10, e100144. https://doi.org/10.1016/j.vas.2020.100144.

Munch, M.M., Chambers, L.C., Manhart, L.E., Domogala, D., Lopez, A., Fredricks, D.N., Srinivasan, S., 2019. Optimizing bacterial DNA extraction in urine. PloS ONE 14 (9), e0222962. https://doi.org/10.1371/journal.pone.0222962.

Murillo, A., Cuenca, R., Serrano, E., Marga, G., Ahmed, A., Cervantes, S., Caparrós, C., Vieitez, V., Ladina, A., Pastor, J., 2020a. *Leptospira* detection in cats in Spain by serology and molecular techniques. Int. J. Environ. Res. Public Health 17 (5), 1600. https://doi.org/10.3390/ijerph17051600.

Murillo, A., Goris, M., Ahmed, A., Cuenca, R., Pastor, J., 2020b. Leptospirosis in cats: current literature review to guide diagnosis and management. J. Feline Med. Surg. 22 (3), 216–228. https://doi.org/10.1177/1098612X20903601.

Murillo, A., Pastor, J., Serrano, E., Tvarijonaviciute, A., Cerón, J., Goris, M., Ahmed, A., Cuenca, R., 2023. Acute phase proteins and total antioxidant capacity in free-roaming cats infected by pathogenic leptospires. BMC Vet. Res. 19, 148. https://doi.org/10.1186/s12917-023-03697-y.

Musso, D., La Scola, B., 2013. Laboratory diagnosis of leptospirosis: a challenge. J. Microbiol. Immunol. Infect. 46 (4), 245–252. https://doi.org/10.1016/j.jmii.2013.03.001.

Mylonakis, M.E., Bourtzi-Hatzopoulou, E., Koutinas, A.F., Petridou, E., Saridomichelakis, M.N., Leontides, L., Siochu, A., 2005. Leptospiral seroepidemiology in a feline hospital population in Greece. Vet. Rec. 156 (19), 615–616. https://doi.org/10.1136/vr.156.19.615.

Natarajaseenivasan, K., Raja, S., 2002. Leptospirosis among Rice Mill Workers of Salem, South India. Jpn. J. Infect. Dis. 55 (5), 170–173. https://www.researchgate.net/publication/10974680.

Obrenović, S., Radojičić, S., Stević, N., Bogunović, D., Vakanjac, S., Valčić, M., 2014. Seroprevalence of cat leptospirosis in Belgrade (Serbia). Acta Vet 64 (4), 510–518. https://doi.org/10.2478/acve-2014-0047.

Palerme, J.S., Lamperelli, E., Gagne, J., Cazlan, C., Zhang, M., Olds, J.E., 2019. Seroprevalence of *Leptospira* spp., Toxoplasma gondii, and Dirofilaria immitis in free-roaming cats in Iowa. Vector Borne Zoonotic Dis. 19 (3), 193–198. https://doi.org/10.1089/vbz.2017.2255.

Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., Akritidis, N., 2008. The globalization of leptospirosis : worldwide incidence trends. J. Glob. Infect. Dis. 12 (4), 351–357. https://doi.org/10.1016/j.ijid.2007.09.011.

Picardeau, M., 2013. Diagnosis and epidemiology of leptospirosis. Med. Mal. Infect. 43 (1), 1–9. https://doi.org/10.1016/j.medmal.2012.11.005.

Picardeau, M., 2017. Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nat. Rev. Microbiol. 15 (5), 297–307. https://doi.org/10.1038/nrmicro.2017.5.

Rees, H.G., 1964. Leptospirosis in a cat. N. Z. Vet. J. 12 (3), 64. https://doi.org/10.1080/00480169.1964.33553. –64.

Ricardo, T., Azócar-Aedo, L., Signorini, M., Previtali, M.A., 2023. Leptospiral infection in domestic cats: systematic review with meta-analysis. Prev. Vet. Med. 212, e105851. https://doi.org/10.1016/j.prevetmed.2023.105851.

Ricardo, T., Azócar-Aedo, L.I., Previtali, M.A., Monti, G., 2024. Seroprevalence of pathogenic *Leptospira* serogroups in asymptomatic domestic dogs and cats: systematic review and meta-analysis. Front. Vet. Sci. 11, 1301959. https://doi.org/10.3389/fvets.2024.1301959.

Rodriguez, J., Blais, M.C., Lapointe, C., Arsenault, J., Carioto, L., Harel, J., 2014. Serologic and urinary PCR survey of leptospirosis in healthy cats and in cats with kidney disease. J. Vet. Intern. Med. 28 (2), 284–293. https://doi.org/10.1111/jvim.12287.

Rubini, M.E., Wolf, A.V., 1957. Refractometric determination of total solids and water of serum and urine. J. Biol. Chem. 225 (2), 869–876.

Schuller, S., Francey, T., Hartmann, K., Hugonnard, M., Kohn, B., Nally, J.E., Sykes, J., 2015. European consensus statement on leptospirosis in dogs and cats. J. Small Anim. Pract. 56 (3), 159–179. https://doi.org/10.1111/jsap.12328.

Shophet, R., 1979. A serological survey of leptospirosis in cats. N. Z. Vet. J. 27 (11), 236–246. https://doi.org/10.1080/00480169.1979.34662.

Shophet, R., Marshall, R.B., 1980. An experimentally induced predator chain transmission of *Leptospira ballum* from mice to cats. Br. Vet. J. 136 (3), 265–270. https://doi.org/10.1016/S0007-1935(17)32291-1.

Shropshire, S.B., Veir, J.K., Morris, A.K., Lappin, M.R., 2016. Evaluation of the *Leptospira* species microscopic agglutination test in experimentally vaccinated cats and *Leptospira* species seropositivity in aged azotemic client-owned cats. J. Feline Med. Surg. 18 (10), 768–772. https://doi.org/10.1177/1098612X15593902.

Sidstedt, M., Jansson, L., Nilsson, E., Noppa, L., Forsman, M., Rådström, P., Hedman, J., 2015. Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. Anal. Biochem. 487, 30–37. https://doi.org/10.1016/j.ab.2015.07.002.

Sprißler, F., Jongwattanapisan, P., Luengyosluechakul, S., Pusoonthornthum, R., Prapasarakul, N., Kurilung, A., Goris, M., Ahmed, A., Reese, S., Bergmann, M., Dorsch, R., Klaasen, H.L.B.M., Hartmann, K., 2019. *Leptospira* infection and shedding in cats in Thailand. Transbound. Emerg. Dis. 66 (2), 948–956. https://doi.org/10.1111/tbed.13110.

Stoddard, R.A., Gee, J.E., Wilkins, P.P., McCaustland, K., Hoffmaster, A.R., 2009. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the *LipL32* gene. Diagn. Microbiol. Infect. Dis. 64 (3), 247–255. https://doi.org/10.1016/j.diagmicrobio.2009.03.014.

Stritof Majetic, Z., Galloway, R., Ruzic Sabljic, E., Milas, Z., Mojcec Perko, V., Habus, J., Margaletic, J., Pernar, R., Turk, N., 2014. Epizootiological survey of small mammals as *Leptospira* spp. reservoirs in Eastern Croatia. Acta Trop 131 (1), 111–116. https://doi.org/10.1016/j.actatropica.2013.12.009.

Sykes, J.E., Hartmann, K., Lunn, K.F., Moore, G.E., Stoddard, R.A., Goldstein, R.E., 2011. 2010 ACVIM Small Animal Consensus Statement on Leptospirosis: diagnosis, Epidemiology, Treatment, and Prevention. J. Vet. Intern. Med. 25 (1), 1–13. https://doi.org/10.1111/j.1939-1676.2010.0654.x.

Stritof, Z., 2010. Molecular Epizootiology of Leptospirosis in Mouse-Like Rodents. Faculty of Veterinary Medicine University of Zagreb, Zagreb.

Štritof Majetić, Z., Habuš, J., Milas, Z., Mojcec Perko, V., Staresina, V., Turk, N., 2012. Serological survey of canine leptospirosis in Croatia - the changing epizootiology of the disease. Vet. Arh. 82 (2), 183–191.

Turk, N., Milas, Z., Margaletic, J., Staresina, V., Slavica, A., Riquelme-Sertour, N., Bellenger, E., Baranton, G., Postic, D., 2003. Molecular characterization of *Leptospira* spp. strains isolated from small rodents in Croatia. Epidemiol. Infect. 130 (1), 159–166. https://doi.org/10.1017/S0950268802008026.

Waggoner, J.J., Pinsky, B.A., 2016. Molecular diagnostics for human leptospirosis. Curr. Infect. Dis. Rep. 29 (5), 440–445. https://doi.org/10.1097/QCO.0000000000000295.

Weis, S., Rettinger, A., Bergmann, M., Llewellyn, J.R., Pantchev, N., Straubinger, R.K., Hartmann, K., 2017. Detection of *Leptospira* DNA in urine and presence of specific antibodies in outdoor cats in Germany. J. Feline Med. Surg. 19 (4), 470–476. https://doi.org/10.1177/1098612X16634389.

Zaidi, S., Bouam, A., Bessas, A., Hezil, D., Ghaoui, H., Ait-Oudhia, K., Drancourt, M., Bitam, I., 2018. Urinary shedding of pathogenic *leptospira* in stray dogs and cats, Algiers: a prospective study. PLoS ONE 13 (5), e0197068. https://doi.org/10.1371/journal.pone.0197068.

Žákovská, A., Schánilec, P., Treml, F., Dušková, M., Agudelo, C.F., 2020. Seroprevalence of antibodies against borrelia burgdorferi S. L. and leptospira interrogans S. L. in cats in district of brno and its environs, the Czech Republic. Ann. Agric. Environ. Med. 27 (3), 356–360. https://doi.org/10.26444/aaem/122804.

8.3. Paper III: "Whole Genome Characterization of *Leptospira kirschneri* Serogroup Pomona in Croatia: Insights into Its Diversity and Evolutionary Emergence"

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Article

Whole Genome Characterization of *Leptospira kirschneri* Serogroup Pomona in Croatia: Insights into Its Diversity and Evolutionary Emergence

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Abstract

Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira* spp. with small rodents serving as the main reservoir. In Croatia, the serogroup Pomona has been detected most frequently, but its genomic diversity remains insufficiently characterized. This study presents the first whole genome sequencing analysis of 48 Croatian *Leptospira* spp. isolates collected from small rodents over a 14-year period. Serological typing confirmed that all the isolates belonged to the serogroup Pomona. Genomic analysis assigned them to *L. kirschneri* based on high genomic similarity using average nucleotide identity (ANI). The isolates were assigned to ST-98 using traditional multilocus sequence typing (MLST), while cgMLST identified seven genotype clusters, many of which showed geographic structuring. Phylogenetic analyses based on single nucleotide polymorphisms (SNPs) supported this structure and revealed a monophyletic clade of Croatian isolates distinct from other global *L. kirschneri* strains. Serological typing, MLST, and phylogenetic clustering support classification of the isolates as *L. kirschneri*, serogroup Pomona, most likely serovar Mozdok, although serovar Tsaratsovo cannot be excluded. These results indicate the existence of a geographically restricted and potentially host-adapted lineage of *L. kirschneri* in Croatia. The integration of ecological, serological, and genomic data in this study emphasizes the value of whole genome sequencing for understanding the population biology of *Leptospira* spp. serogroup Pomona. Moreover, it supports targeted, country-specific surveillance and control strategies for leptospirosis through the identification of circulating serovars and species in reservoir hosts, in line with a One Health approach.

Keywords: *Leptospira kirschneri*; serogroup Pomona; whole genome sequencing; phylogenetic analysis; small rodents; One Health

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1. Introduction

Leptospirosis is a globally distributed, re-emerging infectious disease caused by pathogenic bacteria of the genus *Leptospira* [1]. It affects a wide range of domestic and wild animals as well as humans, posing significant public health concerns [2]. Leptospires are immunologically and genetically heterogeneous microorganisms. The genus *Leptospira* is currently classified into 69 species grouped into four subclades, with more than 300 serovars of pathogenic *Leptospira* organized into 30 serogroups [2,3]. Differences in the pathogenicity of the species and serovars, the susceptibility and immune response of the hosts, and the infectious dose lead to different clinical manifestations ranging from mild or subclinical infections to severe, life-threatening outcomes [1,4].

Small rodents serve as the main reservoirs of *Leptospira* spp. and play a crucial role in the persistence of the disease in the environment. Once infected, they become asymptomatic carriers that shed leptospires in their urine continuously or intermittently throughout their lifetime, serving as an important source of infection [1,4]. In humans and animals, infection occurs through mucous membranes and microlesions of the skin via infected urine or contaminated water or soil [5,6]. Following the bacteriemic stage, the leptospires colonize the renal proximal tubular epithelial cells and are excreted in the urine [4,6], contaminating the environment, where pathogenicity may be retained for up to 20 months [7,8].

Hemolysin-producing serogroups such as Pomona or Icterohaemorrhagiae are commonly associated with pronounced clinical symptoms in both animals and humans [4,9]. Among them, the serogroup Pomona is of particular importance due to its increasing trend of occurrence in various domestic and wild animal species worldwide [10–16]. Moreover, over the last decade, research has identified the serogroup Pomona as the most prevalent serogroup in various hosts in Croatia [17–19]. The serogroup Pomona consists of eight serovars—Altodouro, Kennewicki, Kunming, Mozdok, Pomona, Proechimys, Tropica, and Tsaratsovo—which are distributed across five distinct species: *L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. noguchii*, and *L. santarosai* [20–26]. Pigs have been considered the main carriers of the serogroup Pomona, particularly for the serovar Pomona [27], whereas black-striped field mice (*Apodemus agrarius*) are the primary reservoirs for the serovar Mozdok [28].

The complex taxonomy of the genus *Leptospira* makes diagnosis challenging. Most laboratory diagnoses and epizootiologic/epidemiologic studies rely on serologic and molecular methods [29–31]. The microscopic agglutination test (MAT) identifies the presumptive infectious serogroup but not the specific serovar [32], while molecular techniques can determine the *Leptospira* spp. species, but not the infecting serogroup or serovar [33]. Sequencing PCR products from positive clinical samples is feasible but often hampered by the low abundance of leptospiral DNA, which can compromise sequencing quality. Furthermore, the successful isolation of *Leptospira* spp. in media enables the use of advanced molecular techniques such as whole genome sequencing (WGS) to identify and thoroughly characterize specific strains. WGS enables the analysis of genomic variation, which is particularly valuable for comparing strains from geographically or ecologically diverse regions [34].

In Croatia, leptospirosis is an endemic disease and a significant veterinary and public health concern. The role of small rodents in the epizootiologic and epidemiologic cycle of leptospirosis in Croatia has been studied over the years [17,28,35–37]. These rodents enable the long-term survival and persistence of *Leptospira* spp. in the environment, and characterization of the strains they harbor provides insight into the currently circulating pathogenic *Leptospira* species and serovars.

The aim of this study is to analyze the whole genomes of *Leptospira* spp. strains belonging to the serogroup Pomona, isolated over time from small rodents, to characterize their genomic features and diversity. A deeper understanding of the genomic characteristics of

the *Leptospira* spp. serogroup Pomona should provide valuable insights into its pathogenic potential, epidemiology, and evolutionary emergence.

2. Materials and Methods

2.1. Investigated Leptospira spp. Isolates

A total of 48 archived isolates of *Leptospira* spp. isolated from the kidneys of small rodents were used for this study. These isolates were collected over a 14-year period from various regions of Croatia, with available data on the small rodent species, collection location, and date of sampling. The small rodent species *Apodemus agrarius* and *Microtus lavernedii* were identified on the basis of morphological characteristics, while *Apodemus flavicollis* and *Apodemus sylvaticus*, which are morphologically indistinguishable, were differentiated using polymerase chain reaction (PCR) targeting the mitochondrial *cytochrome b* gene, followed by sequencing of the PCR products. The cultures were part of the collection of pathogenic leptospires in the Laboratory of Leptospires at the Faculty of Veterinary Medicine, University of Zagreb, where they were maintained in Korthof and Fletcher media.

2.2. Serological Typing of Leptospira spp. Isolates

The affiliation of the analyzed isolates of *Leptospira* spp. to specific serogroups was tested using a panel of 14 reference hyperimmune sera (Table S1) prepared in rabbits (OIE Reference Laboratory for Leptospirosis, AMC, Amsterdam, The Netherlands) according to a standard procedure [38]. *Leptospira* spp. cultures cultivated in Korthof medium for up to 10 days, having a density of 2–4 $\times$ 10^8 bacteria/mL, were used as antigens. Serial dilutions of the tested sera were prepared in microtiter plates with phosphate-buffered saline (PBS), starting with a dilution of 1:50. After a 2-hour incubation at 28–30 °C, the results were read under a darkfield microscope. A serologically positive reaction was determined by the presence of agglutinated leptospires compared to the negative control. The endpoint titer was the highest serum dilution showing at least 50% agglutination of the leptospires. The infectious serogroup of the culture was determined based on the hyperimmune serum showing the highest agglutination titer.

2.3. Genomic Characterization of Leptospira spp. Strains

2.3.1. DNA Extraction

Leptospira spp. cultures up to 10 days old having a density of 2–4 $\times$ 10^8 bacteria/mL in Korthof media were sent to the Zoonoses and Select Agent Laboratory, Centers for Disease Control and Prevention, Atlanta, GA, USA, where further analyses and processing were conducted to identify, characterize, and study the strains.

Pretreatment of the *Leptospira* spp. cultures for DNA extraction included centrifugation at 4000 rpm for 15 min, followed by removal of the supernatant. The resulting pellets were then resuspended in 400 µL PBS. DNA extraction was subsequently performed using the Maxwell CSC 48 automated extraction system (Promega Corporation, Madison, WI, USA) with the Maxwell® RSC Cultured Cells DNA Kit (Promega Corporation, Madison, WI, USA).

2.3.2. Whole Genome Sequencing (WGS)

Strains isolated from the kidneys of small rodents determined with serological typing and belonging to the *Leptospira* spp. serogroup Pomona were analyzed by whole genome sequencing. The workflow for whole genome sequencing included DNA extraction (minimum concentration of 3.3 ng/µL and purity of A260/A280 between 1.8 and 2.0) and library preparation using the Nextera XT DNA Library Preparation Kit (Illumina, San

Diego, CA, USA). Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq v3 600 cycle kit (2 × 300 bp reads) (Illumina, San Diego, CA, USA).

2.3.3. Genome Assembly

Genome assembly of the sequenced samples was conducted using a Nextflow v24.04.2 assembly pipeline with default parameters developed by the bioinformatics team at the Zoonoses and Select Agent Laboratory (ZSAL) of the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA (https://github.com/bacterial-genomics/wf-paired-end-illumina-assembly, v3.0.0). This pathogen-agnostic, general-purpose workflow is specifically designed for genome assembly of Illumina paired-end sequence data and was employed to ensure accurate and efficient data assembly. To improve assembly quality, the assemblies were filtered to remove contigs shorter than 500 bp, and all genomes retained had a minimum sequencing coverage of 39.5× (median 53.3×, range 39.5×–74.4×). These quality control steps ensured that only high-quality assemblies were used for the downstream analyses.

2.3.4. Genomic Analyses

Average Nucleotide Identity (ANI)

To assess genomic relatedness among isolates, average nucleotide identity (ANI) was computed using the automated workflow available at the bacterial-genomics GitHub repository (https://github.com/bacterial-genomics/wf-ani, v1.0.0). The workflow implements three different ANI calculation methods—Biopython v1.6.8 [39], FastANI v1.33 (ParBLiSS/FastANI: Fast Whole-Genome Similarity (ANI) Estimation), and SKANI v0.1.3 [40]—providing comprehensive and robust pairwise identity metrics for genome comparisons.

In total, 118 genomes are included in Figure 1, with 70 representing recognized *Leptospira* spp. downloaded from the NCBI RefSeq database (RefSeq: NCBI Reference Sequence Database) as species type strains and the 48 sequenced *Leptospira* spp. isolates obtained from rodent populations sampled across locations in Croatia. Another dataset having a total of 99 genomes was also constructed, using the 48 *Leptospira* spp. samples collected in Croatia and 51 *L. kirschneri* isolates downloaded from NCBI's RefSeq database (RefSeq: NCBI Reference Sequence Database) (Figure 2).

The analysis was performed using the Nextflow workflow manager [41], facilitating reproducibility and scalability. Briefly, the workflow involved the following key steps: preparation of genome input files in FASTA format; all pairwise ANI calculations among reference and Croatian isolate genomes, using Biopython, FastANI, and SKANI. Default parameters were employed, and ANI values above 95% were used as the threshold to define genomic species boundaries, consistent with accepted standards for prokaryotic species delineation.

Pangenome Analysis

A pangenome analysis was performed on the 48 Croatian *Leptospira* spp. genome assemblies to characterize core and accessory genome components. Prior to annotation, the assemblies were filtered to remove contigs shorter than 500 bp to minimize low-quality sequences. Genome annotation was then conducted using Bakta v1.9.4, a rapid prokaryotic genome annotation tool that provides standardized GFF3 output files [42]. The annotated GFF3 files were subsequently processed using Panaroo v1.3.4 [43], a robust pipeline for pangenome analysis that accounts for gene presence–absence variation and sequence fragmentation. Panaroo was run in strict mode with a 95% sequence identity threshold for gene clustering to minimize false positives and correct for assembly or annotation

errors, while all the other parameters were left at their default values. The resulting output included a gene presence–absence matrix and definitions of core and accessory genome components. Gene presence–absence patterns were visualized using R v4.3.1 with the packages ggplot2 v3.5.1 and dplyr v1.1.4 to explore genomic diversity and to identify genes associated with specific clusters or traits. The pangenome was further analyzed to estimate the size of the core genome and the total gene repertoire across all the isolates.

Core Genome Multilocus Sequence Typing (cgMLST)

A gene-by-gene approach was used to define a core genome multilocus sequence typing (cgMLST) scheme using chewBBACA v3.3.10 [44]. Coding sequences (CDSs) were predicted for the 48 Croatian *Leptospira* spp. genome assemblies using Prodigal v2.6.3, followed by clustering based on BLAST Score Ratio (BSR) analysis to retain non-paralogous, high-quality loci [45]. The default BSR threshold of 0.6 implemented in chewBBACA was applied to filter paralogous loci. Incomplete loci were further excluded by requiring presence in at least 95% of genomes, forming the final cgMLST schema. A preliminary cgMLST schema for *Leptospira* was downloaded from BIGSdb v1.51.4 [46] as the basis for locus selection.

Allele calling was performed on the same 48 assemblies using the curated schema, generating allelic profiles and identifying novel alleles. The resulting allele table was used to extract the core genome loci and calculate presence–absence matrices.

The final cgMLST schema and allele profiles were integrated into a local BIGSdb v1.51.4 instance [46]. The locus definitions and allele sequences were uploaded through the schema definition interface, and the allelic profiles were used to assign core genome sequence types (cgSTs). This framework facilitated querying, clustering, and comparative analysis of allelic diversity across the Croatian isolates.

Whole Genome Single Nucleotide Polymorphism (SNP) Analysis

To investigate the genomic variation among the isolates, the wf-assembly-snps pipeline (https://github.com/bacterial-genomics/wf-assembly-snps, v1.0.3) was applied, a reproducible workflow built using Nextflow v24.04.2 [41]. This pipeline performs reference-free SNP calling and phylogenetic reconstruction from whole genome assemblies using Parsnp v1.5.6 [47].

The analysis was conducted in two phases. First, the pipeline was run on the 48 Croatian *Leptospira* spp. assembled genomes. Second, the same pipeline was executed using an expanded dataset that included the 48 Croatian genomes along with 23 additional *Leptospira* spp. assemblies obtained from NCBI RefSeq. In both runs, draft assemblies in FASTA format were used as the input.

Parsnp was used to align the core genome regions of the assemblies, identify high-confidence SNPs, and generate a multiple sequence alignment. The SNP alignment was used for phylogenetic tree creation with the program IQ-TREE v2.4.0 [48].

Phylogenetic trees and SNP alignments were further analyzed and visualized using R packages such as ape v5.8.1 [49] and ggtree v3.10.0 [50] to assess evolutionary relationships and genetic clustering among the isolates.

2.3.5. Phylogenetic Analysis

A maximum likelihood phylogeny was inferred using IQ-TREE v2.4.0 [48] from the core SNP alignment generated from the 48 *Leptospira* spp. isolates collected in Croatia and 23 additional *L. kirschneri* genomes obtained from the NCBI RefSeq database. The best-fitting substitution model was determined automatically using ModelFinder based on the Bayesian Information Criterion. Branch support was assessed using 1000 ultrafast bootstrap replicates [48].

The resulting phylogenetic tree was visualized and annotated in R using the ggtree package v3.10.0 [50]. Tree topology and bootstrap values were used to assess clustering patterns, evolutionary relationships, and potential geographic or host-associated structure among the isolates.

2.3.6. Computational Resources

Analyses were conducted on a high-performance computing cluster. Workflow execution was containerized using Docker to maintain consistency and reproducibility across all the steps. All the scripts and data processing procedures are publicly accessible on GitHub, promoting transparency and reproducibility.

3. Results

3.1. Leptospira spp. Isolates and Serological Typing

Detailed information on the individual isolates, including rodent species, sampling locations and dates, is provided in the Supplementary Materials (Table S2). Rodent species identification revealed that 42 isolates were from *Apodemus agrarius*, 5 from *Apodemus flavicollis* and 1 from *Microtus lavernedii*. All 48 isolates of *Leptospira* spp. tested with a panel of 14 hyperimmune reference sera showed agglutination exclusively with the serogroup Pomona, with titers $\geq$ 3200, clearly indicating a significantly higher reactivity to Pomona and confirming that all the isolates belonged to this serogroup.

3.2. Genome Assembly Quality and Metrics

High-quality whole genome sequencing data were obtained for all 48 *Leptospira* spp. isolates collected in Croatia (Table S3). Depth of coverage ranged from 39.5× to 74.4× (median: 53.3×), providing strong support for accurate de novo assembly. The assemblies consisted of between 50 and 95 contigs (median: 58), with total genome lengths spanning 4,389,636 bp to 4,428,928 bp (median: 4,413,607 bp), consistent with the expected size range for *L. kirschneri*. N50 values, reflecting assembly contiguity, ranged from 82,966 bp to 218,205 bp (median: 175,746 bp). GC content was highly uniform across all the samples, having a mean of 35.87% $\pm$ 0.004%, aligning with published values for this species. Collectively, these metrics demonstrate the high quality and consistency of the assemblies, supporting their use in comparative genomics and phylogenetic analyses.

3.3. Average Nucleotide Identity Confirms Species Delineation and Intraspecific Diversity

The ANI analysis was used to assess the genomic relatedness of the 48 *Leptospira* spp. isolates from Croatia in the context of both inter- and intraspecies diversity (Figures 1 and 2). Figure 1 presents the ANI values for a dataset of 118 genomes, which includes the 48 Croatian *Leptospira* spp. isolates and 70 publicly available genomes representing all recognized *Leptospira* spp. type strains with available whole genome sequences from the NCBI RefSeq database (Table S4). The heatmap revealed a distinct genomic cluster containing all the Croatian isolates, with bidirectional ANI values exceeding 95% in comparisons with the *L. kirschneri* reference genome, confirming their species-level classification. These isolates also formed a discrete clade, clearly separated from other *Leptospira* species, which exhibited ANI values below the 95% threshold, reaffirming species-level divergence.

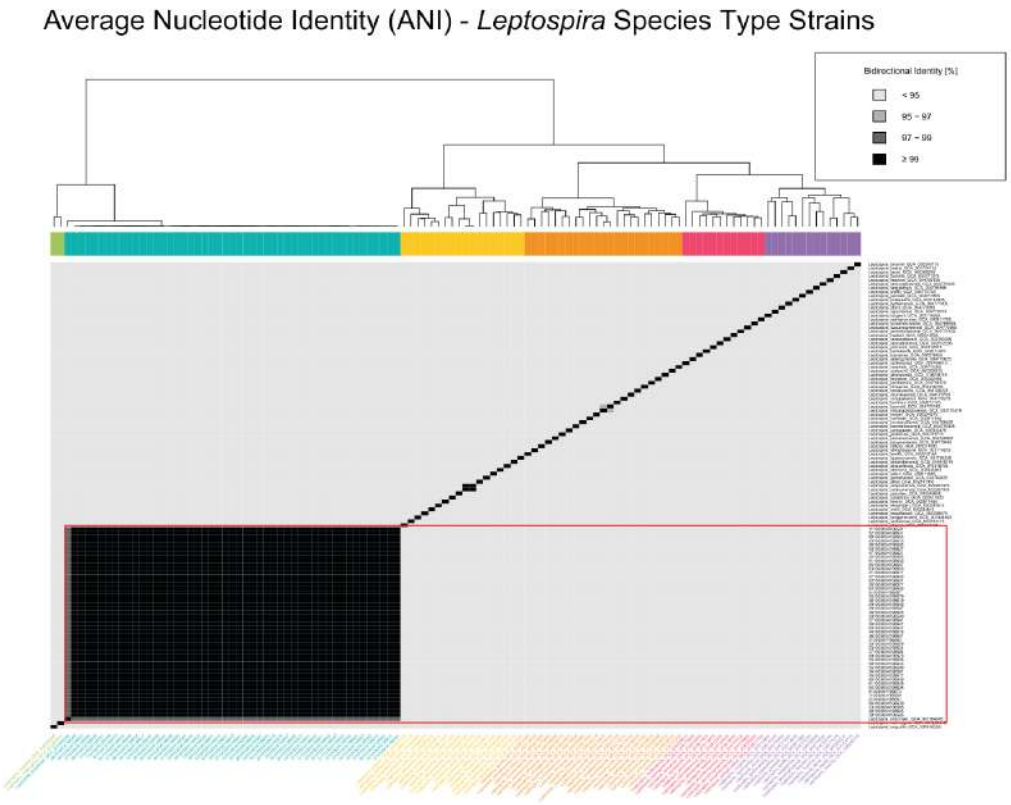

Figure 1. Average nucleotide identity (ANI) heatmap of *Leptospira* species type strains. Pairwise bidirectional ANI values are shown for representative genomes across diverse *Leptospira* species, including Croatian *L. kirschneri* isolates and reference type strains obtained from NCBI RefSeq (Table S4). The heatmap is color-coded by percent identity, with darker shades indicating higher similarity: <95% (lightest), 95–97%, 97–99%, and ≥99% (darkest). The Croatian isolates form a distinct cluster having ≥99% identity, consistent with high intra-lineage similarity. Dendrograms adjacent to the heatmap depict hierarchical clustering of genomes based on pairwise ANI values, and side bars represent clustering patterns across the dataset.

Figure 2 focuses on a higher-resolution comparison among 99 *L. kirschneri* genomes, including the 48 Croatian isolates and 51 additional *L. kirschneri* genomes from the NCBI RefSeq database (Table S5). Within this subset, the Croatian isolates formed a tightly clustered group with pairwise ANI values consistently exceeding 99%, indicative of a highly clonal population. Several additional subclusters were observed among non-Croatian isolates, with ANI values ranging from 97% to 99%, reflecting a broader spectrum of genomic diversity within *L. kirschneri*. Notably, despite their close identity, the Croatian isolates remained genetically distinct from all the other global reference strains, reinforcing their phylogeographic cohesion.

This pattern was further supported by pangenome analysis (Figure S1), which revealed that the Croatian isolates shared a large and stable core genome, consistent with clonal structure. While accessory gene variation was detected, it was relatively limited and

showed no clear association with sampling location or other metadata, underscoring the genetic uniformity of this regional lineage.

Overall, the ANI analysis supports the assignment of all the Croatian isolates to *L. kirschneri* and highlights their high genomic similarity, while also contextualizing them within the broader diversity of the genus and species. These results corroborate phylogenetic and MLST-based findings and further demonstrate the utility of ANI for high-resolution bacterial population genomics.

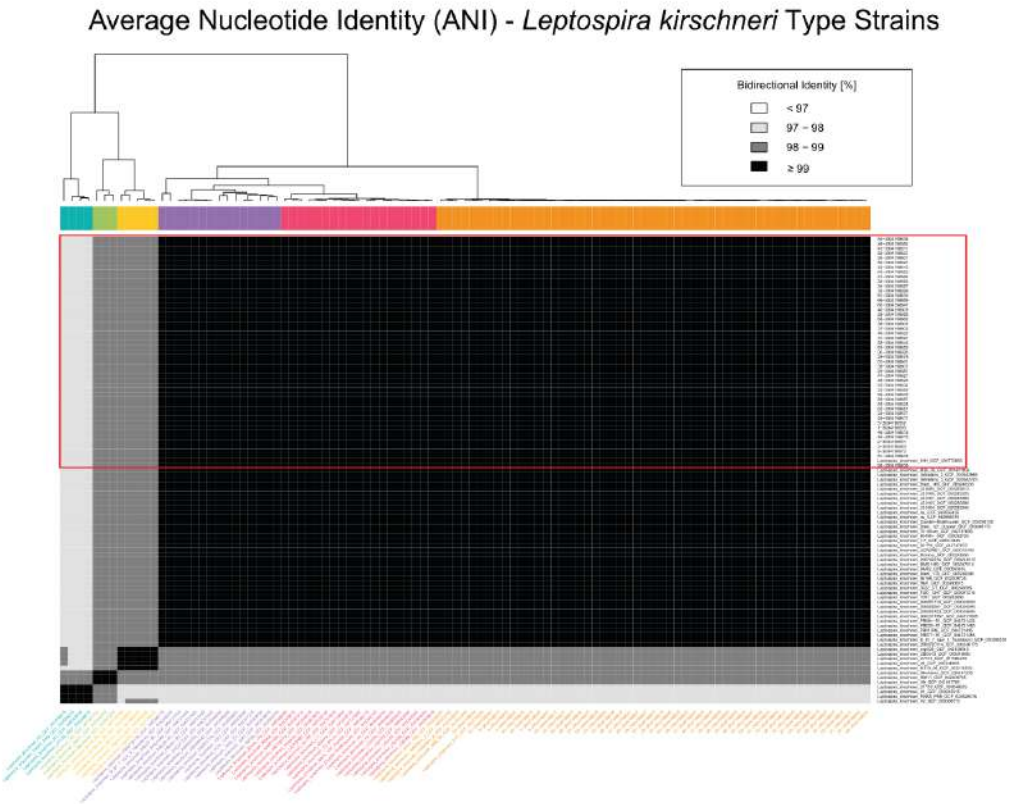

Figure 2. Average nucleotide identity (ANI) heatmap of *Leptospira kirschneri* genomes. Pairwise ANI values are shown for 99 *L. kirschneri* genomes, including 48 isolates from Croatia and 51 publicly available genomes from the NCBI RefSeq database (Table S5). The heatmap is color-coded by percent identity, with darker shades representing higher similarity: <95% (lightest), 95–97%, 97–99%, and ≥99% (darkest). The Croatian isolates form a tightly clustered group having ≥99% identity, consistent with high intra-lineage similarity, but exhibit lower identity values (95–98%) when compared to other *L. kirschneri* genomes, highlighting the genetic distinctiveness of the Croatian lineage. The dendrogram adjacent to the matrix represents hierarchical clustering based on pairwise ANI values, and colored side bars indicate the resulting genomic clusters.

3.4. Multilocus Sequence Typing (MLST) and Core Genome MLST (cgMLST)

All 48 *Leptospira* isolates from Croatia were assigned to sequence type ST-98 according to MLST scheme 3, indicating a highly clonal population structure at the seven-locus level (Table S6). This sequence type is affiliated with the serogroup Pomona and the genomic

species *L. kirschneri* and is consistent with the serovars Mozdok or Tsaratsovo. Core genome MLST (cgMLST) analysis resolved the isolates into seven genotype clusters (designated A–G) based on allelic similarity and minimum spanning tree topology (Figure 3). These clusters corresponded to the following cgSTs: A (cgST-1016), B (cgST-1012, 1016, 1017), C (cgST-1017), D (cgST-1016, 1017), E (cgST-1012), F (cgST-959, 1017), and G (cgST-1011, 1013, 1849) (Table S3).

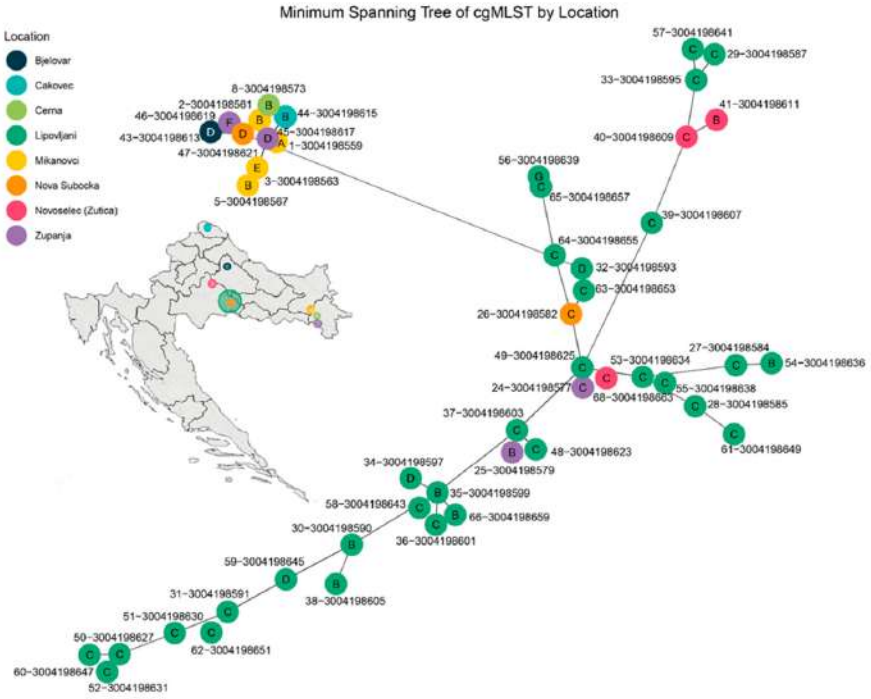

Figure 3. Minimum spanning tree (MST) of *Leptospira kirschneri* isolates based on core genome multilocus sequence typing (cgMLST). The MST was constructed from allele profiles of 48 isolates collected from rodents across eight locations in Croatia. Each node represents a single isolate and is color-coded by sampling location. Genotype clusters (A–G) are labeled within the nodes and were defined based on allelic similarity and MST topology. Edges represent the smallest number of allelic mismatches between profiles. Genotype C (cgST-1017) occupies the central portion of the network. Genotype B includes cgSTs 1012, 1016, and 1017; Genotype D includes cgSTs 1016 and 1017; Genotype E corresponds to cgST-1012; Genotype F includes cgSTs 959 and 1017; and Genotype G includes cgSTs 1011, 1013, and 1849.

Distinct geographic patterns were observed among the genotype clusters. Genotype C exhibited strong spatial clustering, predominating in Lipovljani and also present in Bjelovar and Županja, suggesting localized transmission or persistence. Genotype B had the broadest geographic distribution, detected in Čakovec, Cerna, Mikanovci, Lipovljani, Županja, and Novoselec (Žutica), indicating either a more widespread reservoir or greater mobility across regions. Genotype D showed intermediate clustering, primarily in Lipovljani, with additional isolates from Bjelovar and Županja. Genotypes A and E were each confined to Mikanovci, supporting localized occurrence. Genotype F was restricted to Nova Subocka, while Genotype G was found only in Čakovec. These findings highlight a significant correlation between genotype structure and geographic origin, pointing to

spatially distinct transmission patterns and region-specific reservoirs within the Croatian *Leptospira* population.

3.5. Phylogenetic Relationships of Croatian Isolates

A ML phylogeny was reconstructed from the core SNP alignment of the 48 *Leptospira* spp. isolates collected from various locations across Croatia. The tree revealed several well-supported clades, with key nodes marked by black diamonds indicating ultrafast bootstrap support values exceeding 90% (Figure 4). These high-confidence branches suggest robust evolutionary relationships among the isolates. Clustering patterns were strongly associated with geographic origin, as denoted by colored tip circles corresponding to the sampling sites. Notably, isolates from Lipovljani and Mikanovci formed distinct monophyletic groups, reflecting localized evolutionary divergence.

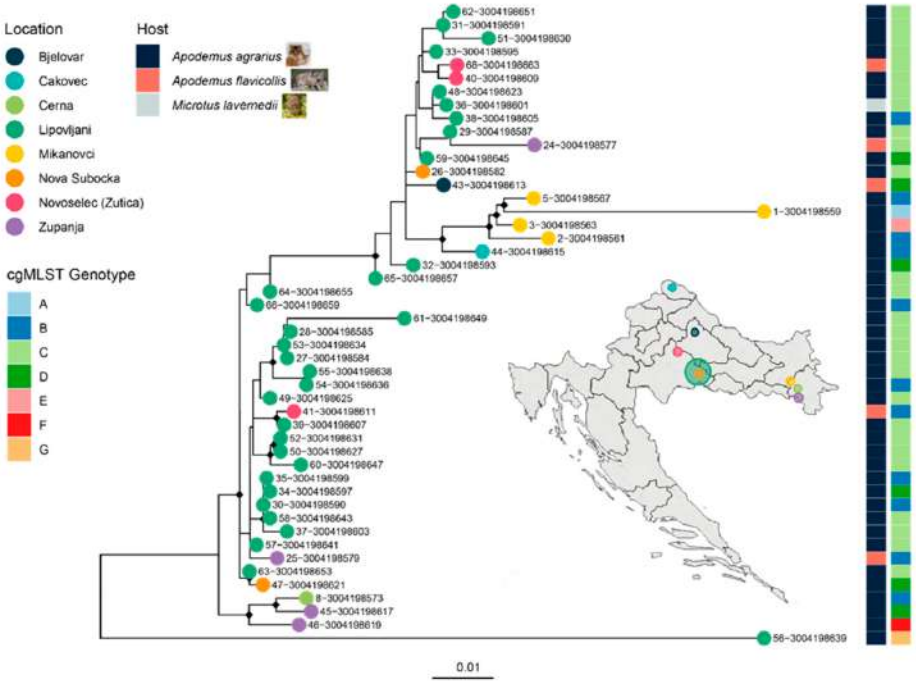

Figure 4. Maximum likelihood (ML) phylogeny of 48 *Leptospira kirschneri* isolates from Croatia, annotated by sampling location, host species, and cgMLST genotype. A maximum likelihood tree was constructed from a core SNP alignment of 48 *L. kirschneri* genomes collected from rodent hosts across multiple locations in Croatia. Tip colors correspond to geographic origin, as shown in the legend. The rightmost heatmaps indicate cgMLST genotype groups and host species (blue: *Apodemus agrarius*, red: *Apodemus flavicollis*, grey: *Microtus lavernedii*). Small rodent icons depict representative host species sampled. A map of Croatia (inset) highlights the sampling locations, with circle size proportional to the number of isolates from each site. Genotype groups defined by cgMLST are consistent with clade topology, supporting the presence of localized clonal expansions. The spatial clustering of strains and limited host diversity suggest restricted circulation within rodent reservoirs and geographic foci.

The heatmap adjacent to the phylogeny annotated each isolate by host species and cgMLST genotype group (A–G), revealing tight concordance between phylogenetic struc-

ture and genotype. For instance, Genotype C isolates, predominantly from Lipovljani, clustered into a single, well-supported clade. Similarly, Genotype B showed some spatial dispersion but maintained phylogenetic cohesion.

3.6. Phylogenetic Placement of Croatian *L. kirschneri* Isolates in a Global Context

A ML phylogeny was constructed from a core SNP alignment of 99 *L. kirschneri* genomes, including 48 isolates from Croatia and 51 publicly available reference genomes from the NCBI RefSeq database (Figure 5). The resulting tree revealed a strongly supported monophyletic clade comprising all the Croatian isolates, indicating their close genetic relatedness and suggesting the presence of a regionally dominant lineage circulating in the country.

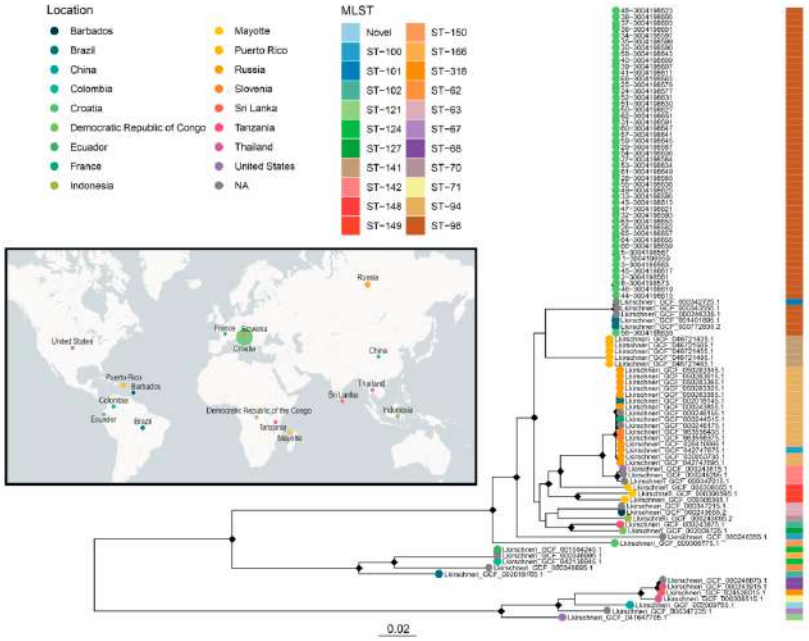

Figure 5. Maximum likelihood phylogeny of 99 *Leptospira kirschneri* genomes from Croatia and global sources, annotated by location, cgMLST genotype, host, and sequence type. A maximum likelihood tree was generated from a core SNP alignment of 99 *L. kirschneri* genomes, including 48 rodent-derived isolates from Croatia and 51 publicly available assemblies from the NCBI RefSeq database. Tips are colored by geographic origin (map and leftmost legend), with the accompanying sidebar denoting MLST sequence type. The NCBI RefSeq genomes originate from diverse global locations, spanning Africa, Asia, Europe, and the America, representing a wider range of MLST types, including ST-121, ST-124, ST-127, and others. The inset world map illustrates the geographic distribution of all the sampled isolates, highlighting the distinct separation between the Croatian and global *L. kirschneri* lineages, with circle size proportional to the number of isolates from each geographical location.

In contrast, the global reference genomes represented a diverse array of sequence types and geographic origins, spanning Brazil, China, Russia, the Democratic Republic of the Congo, Colombia, Puerto Rico, Ecuador, the United States, France, Slovenia, Thailand, Sri Lanka, Indonesia, Tanzania, Mayotte, and Barbados. These genomes encompassed multiple MLST sequence types, including ST-121, ST-124, ST-127, ST-141, and several

novel STs, highlighting the global diversity of *L. kirschneri*. It is noteworthy that all the Croatian isolates were assigned to MLST ST-98, a sequence type shared with only a few global strains from Brazil and an unknown location. The same strains were also closely clustered in the phylogenetic analysis with the Croatian isolates, all of which had previously been identified as the *L. kirschneri* serovar Mozdok. This double concordance in sequence type and phylogenetic position supports the hypothesis of a geographically confined but globally dispersed lineage representing the *L. kirschneri* serogroup Pomona, likely the serovar Mozdok or Tsaratsovo, that has undergone long-term in situ evolution in Croatia, with limited evidence of international dissemination based on a few closely related global strains.

4. Discussion

Leptospirosis is one of the most common zoonotic infections of global importance but is still frequently underdiagnosed and neglected. In Croatia, leptospirosis is an important endemic zoonosis, occurring both in natural and increasingly in synanthropic foci. The unique combination of favorable climate and complex geomorphology of the country favors high species diversity, including a dense and diverse rodent population, which is the main reservoir of pathogenic *Leptospira* spp. [28,37]. These ecological conditions contribute to the endemic nature of the disease and maintain active transmission cycles in different geographical regions.

In previous studies, the serogroup Pomona has been consistently identified as the most widespread in both domestic and wild animals in Croatia using methods such as MAT, PFGE, MLST, and cgMLST [17–19,28,51]. Small rodents are considered important reservoirs responsible for the maintenance and spread of leptospires in these natural foci. Among them, the black-striped field mouse (*Apodemus agrarius*) has been established as the dominant reservoir host for the serogroup Pomona, while the yellow-necked field mouse (*Apodemus flavicollis*) is mainly associated with the serogroup Australis [28,37]. The composition of rodent species in this study was consistent with previous results, with *A. agrarius* accounting for 42 of the 48 isolates, further supporting the dominant role of *A. agrarius* as a reservoir for the serogroup Pomona serovar Mozdok in Croatia. Similar observations have been observed in other European countries, where the serovar Mozdok is maintained by small rodents [52,53]. While *A. agrarius* dominated overall in our study, isolates of *A. flavicollis* and *M. lavernedii* also formed distinct clusters, raising the possibility of limited host specificity or niche adaptation within the *L. kirschneri* strains. Whether these patterns reflect ecological constraints, selective pressures, or recent host jumps warrants further investigation.

Although the serogroup Pomona includes eight serovars distributed among five *Leptospira* species, the most frequently detected serovars in Croatia were Mozdok, Tsaratsovo, and Pomona [28,37,54]. Notably, Mozdok and Tsaratsovo belong to *L. kirschneri*, whereas Pomona belongs to *L. interrogans*. In this study, serological typing confirmed that all 48 isolates belong to the serogroup Pomona, confirming the dominant role of this serogroup in Croatia.

This study represents the first detailed whole-genome investigation of *Leptospira* isolates from Croatia and provides unprecedented resolution for species confirmation, strain-level comparison, and population structure analysis. The ANI analyses confirmed the species-level assignment of all 48 isolates to *L. kirschneri*, with bidirectional ANI values of 97–99%, both among the Croatian isolates and relative to the reference *L. kirschneri* genomes, while maintaining clear separation from other species. This indicates a highly clonal and distinct population circulating in Croatia.

Despite this overall clonality, cgMLST revealed seven genotype clusters, many of which were associated with specific geographic locations. These patterns were mirrored by SNP-based ML phylogenetic analyses, which revealed strong geographic structuring, particularly in regions such as Lipovljani and Mikanovci. This spatial structuring of cgMLST genotypes suggests localized transmission patterns and potential environmental or ecological segregation among the *L. kirschneri* populations in Croatia. The observed congruence between the cgMLST genotypes and phylogenetic clades suggests localized microevolution or ecological separation within the Croatian rodent reservoir system. Overall, these results suggest that *L. kirschneri* in Croatia exhibits structured genetic diversity driven by both geographic and host-related factors, with a strong phylogenetic signal supported by high bootstrap confidence. However, interpretation of geographic specificity should be made cautiously, given the limited availability of *L. kirschneri* genomes from outside Croatia for broader comparison.

While the cgMLST was useful for identifying broader genotype clusters, the minimum spanning tree did not fully recapitulate the fine-scale relationships observed in the SNP-based phylogeny. This discrepancy likely reflects the inherent difference in resolution between the two methods: cgMLST compares allelic variation across a fixed set of loci, collapsing all within-allele sequence variation, whereas SNP-based analysis captures single-nucleotide changes across the core genome. As a result, SNP phylogenies provide greater discriminatory power and more precise evolutionary inference, particularly among closely related isolates. The higher resolution of the SNP-based tree revealed distinct clustering within the cgMLST genotypes, emphasizing its suitability for inferring recent transmission events and microevolutionary patterns.

Phylogenetic clustering was consistent with both MLST designations and geographic origin, supporting the hypothesis that the Croatian L. *kirschneri* isolates form a regionally endemic lineage. A global comparison with publicly available *L. kirschneri* genomes confirmed that the Croatian isolates form a strongly supported monophyletic clade that is distinct from other global isolates in both SNP-based and ANI-based analyses. Only the Croatian isolates and a few global strains (from Brazil and an unknown location) were assigned to sequence type ST-98, indicating a limited global distribution and suggesting geographic confinement and possible long-term in situ evolution.

Ten isolates in this study were previously identified as the serovar Mozdok using PFGE [28], MLST, and monoclonal antibody tests. Since all 48 Croatian isolates belong to MLST ST-98 and are phylogenetically clustered with the *L. kirschneri* serovar Mozdok strains, it is plausible that they all represent the *L. kirschneri* serogroup Pomona serovar Mozdok, and less likely that they represent the serovar Tsaratsovo.

Interestingly, one isolate from the database, assigned to ST-101, clustered closely with the Croatian and the few global strains in the ST-98 group in the phylogenetic analysis. This ST-101 strain was previously identified as the *L. kirschneri* serogroup Pomona serovar Mozdok and caused pulmonary hemorrhagic lesions in an experimental hamster model, indicating high virulence [55]. This finding suggests the potential virulence of genetically related lineages within the serogroup Pomona. Moreover, other European studies further highlight the heterogeneous nature of the serogroup Pomona, indicating that both the *L. kirschneri* serovar Mozdok and the *L. interrogans* serovar Pomona can be associated with severe clinical manifestations in animals, underlining their pathogenic potential [52,53].

Additionally, these observations are in line with our previous clinical findings, where the serogroup Pomona has been frequently associated with severe disease manifestations, particularly leptospiral pulmonary hemorrhagic syndrome (LPHS) in dogs [56], although the specific serovars responsible within this serogroup remain unclear. Similar outcomes have also been reported in humans [57] and horses [58]. It is particularly relevant in our

context, given the high incidence of leptospirosis in Croatia and the predominance of the serogroup Pomona in clinical cases in Croatia, but further studies are needed to clarify the role of individual serovars in the pathogenesis of LPHS. Altogether, these findings suggest a high pathogenic potential of the *Leptospira* spp. serogroup Pomona, highlighting the need for further investigation within a One Health framework to better understand its pathogenicity, host range, and environmental persistence.

Overall, these results emphasize the utility of whole genome sequencing to advance our understanding of *Leptospira* epidemiology. In addition to resolving species- and strain-level relationships, the pangenome analysis provided further insight into genomic cohesion and adaptive potential within this lineage, revealing a largely conserved core genome having limited accessory gene diversity. Traditional typing methods such as MLST and serology are unable to resolve this fine-scale population structure and genomic dynamics, underscoring the added value of comprehensive genomic approaches.

Our data suggest the presence of a stable, geographically structured, and potentially host-associated lineage of the *L. kirschneri* serogroup Pomona in Croatia, with limited gene flow from other global lineages, most likely corresponding to the serovar Mozdok, although the serovar Tsaratsovo cannot be ruled out.

The integration of ecological, serological, and genomic data in this study provides a comprehensive framework for understanding the population biology of the *Leptospira* spp. serogroup Pomona, probably one of the most pathogenic serogroups with high evolutionary propulsion in terms of pathogenicity, in the context of One Health. The genomic characteristics of the local strains allow comparative analysis with other sequenced pathogenic genomes of *Leptospira* spp. and thus help in the development of further diagnostic tests and vaccines. In addition, this work helps to gain genetic and epidemiological insights that improve knowledge about pathogenic infections with *Leptospira* spp.

Future studies should be extended to environmental and clinical isolates as well as additional hosts to elucidate transmission routes and the potential for cross-species infections. The identification of this cohesive local lineage underscores the importance of targeted surveillance and localized control strategies for leptospirosis in Croatia.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pathogens14090860/s1. Figure S1: Presence–absence heatmap of pangenome gene content for 48 *Leptospira kirschneri* isolates from Croatia, with corresponding sampling location metadata.; Table S1: Panel of 14 reference hyperimmune sera used for serological typing of *Leptospira* spp. isolates, including corresponding serogroups, serovars, and reference strains.; Table S2: Metadata of *Leptospira* spp. isolates from small rodents: rodent species, sampling locations, collection dates, and associated SRA Information.; Table S3: Genome assembly quality and metrics for 48 Croatian *Leptospira kirschneri* isolates.; Table S4: Dataset of 118 genomes comprising 48 Croatian *Leptospira* isolates and 70 publicly available genomes representing all the recognized *Leptospira* type strains with whole-genome sequences available in the NCBI RefSeq database, used for the ANI analysis presented in Figure 1; Table S5: Dataset of 99 *Leptospira kirschneri* genomes comprising 48 Croatian isolates and 51 publicly available genomes from the NCBI RefSeq database used for the pairwise ANI analysis presented in Figure 2; Table S6: Multilocus sequence typing (MLST) and core genome MLST (cgMLST) profiles of 48 Croatian *Leptospira* spp. isolates.

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C.A.G.; supervision, Z.P.W. and N.T.; project administration, Z.P.W. and N.T.; funding acquisition, Z.P.W. and N.T. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Raw sequencing data for the 48 *L. kirschneri* isolates from Croatia generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1279268. Assembled genomes and additional supporting data are available from the corresponding author upon reasonable request.

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Abbreviations

The following abbreviations are used in this manuscript:

ANI	average nucleotide identity
BIGSdb	Bacterial Isolate Genome Sequence Database
cgMLST	core genome multilocus sequence typing
cgST	core genome sequence type
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GFF3	General Feature Format version 3
LPHS	leptospiral pulmonary hemorrhagic syndrome
MAT	microscopic agglutination test
ML	maximum likelihood
MLST	multilocus sequence typing
NCBI	National Center for Biotechnology Information
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RefSeq	Reference Sequence Database (NCBI)
SRA	Sequence Read Archive (NCBI)
SNP	single nucleotide polymorphism
ST	sequence type
WGS	whole genome sequencing

References

1. Levett, P.N. Leptospirosis. *Clin. Microbiol. Rev.* **2001**, *14*, 296–326. [CrossRef] [PubMed]
2. Picardeau, M. Virulence of the zoonotic agent of leptospirosis: Still terra incognita? *Nat. Rev. Microbiol.* **2017**, *15*, 297–307. [CrossRef]
3. Vincent, A.T.; Schiettekatte, O.; Goarant, C.; Neela, V.K.; Bernet, E.; Thibeaux, R.; Ismail, N.; Khalid, M.K.N.M.; Amran, F.; Masuzawa, T.; et al. Revisiting the taxonomy and evolution of pathogenicity of the genus *Leptospira* through the prism of genomics. *PLoS Negl. Trop. Dis.* **2019**, *13*, e0007270. [CrossRef] [PubMed]

4. Adler, B.; de la Peña Moctezuma, A. *Leptospira* and leptospirosis. *Vet. Microbiol.* **2010**, *140*, 287–296. [CrossRef]
5. Baranton, G.; Old, I.G. The spirochaetes: A different way of life. *Bull. Inst. Pasteur.* **1995**, *93*, 63–95. [CrossRef]
6. Ko, A.I.; Goarant, C.; Picardeau, M. *Leptospira*: The dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* **2009**, *7*, 736–747. [CrossRef]
7. Andre-Fontaine, G.; Aviat, F.; Thorin, C. Waterborne Leptospirosis: Survival and Preservation of the Virulence of Pathogenic *Leptospira* spp. in Fresh Water. *Curr. Microbiol.* **2015**, *71*, 136–142. [CrossRef]
8. Bierque, E.; Soupé-Gilbert, M.E.; Thibeaux, R.; Girault, D.; Guentas, L.; Goarant, C. *Leptospira interrogans* Retains Direct Virulence After Long Starvation in Water. *Curr. Microbiol.* **2020**, *77*, 3035–3043. [CrossRef]
9. Ellis, W.A. Animal Leptospirosis. In *Leptospira and Leptospirosis*; Adler, B., Ed.; Springer: Berlin/Heidelberg, Germany, 2015; pp. 99–137. [CrossRef]
10. Hamond, C.; Silveira, C.S.; Buroni, F.; Suanes, A.; Nieves, C.; Salaberry, X.; Aráoz, V.; Costa, R.A.; Rivero, R.; Giannitti, F.; et al. *Leptospira interrogans* serogroup Pomona serovar Kennewicki infection in two sheep flocks with acute leptospirosis in Uruguay. *Transbound. Emerg. Dis.* **2019**, *66*, 1186–1194. [CrossRef]
11. Bertasio, C.; Papetti, A.; Scaltriti, E.; Tagliabue, S.; D'Incau, M.; Boniotti, M.B. Serological Survey and Molecular Typing Reveal New *Leptospira* Serogroup Pomona Strains among Pigs of Northern Italy. *Pathogens* **2020**, *9*, 332. [CrossRef] [PubMed]
12. Guedes, I.B.; de Souza, G.O.; de Paula Castro, J.F.; Burilli Cavalini, M.; de Souza Filho, A.F.; Pinheiro Maia, A.L.; dos Reis, E.A.; Cortez, A.; Heinemann, M.B. *Leptospira interrogans* serogroup Pomona strains isolated from river buffaloes. *Trop. Anim. Health. Prod.* **2021**, *53*, 194. [CrossRef] [PubMed]
13. Tirosh-Levy, S.; Baum, M.; Schvartz, G.; Kalir, B.; Pe'er, O.; Shnaiderman-Torban, A.; Bernstein, M.; Blum, S.E.; Steinman, A. Seroprevalence of *Leptospira* spp. in Horses in Israel. *Pathogens* **2021**, *10*, 408. [CrossRef]
14. Aliberti, A.; Blanda, V.; Di Marco Lo Presti, V.; Macaluso, G.; Galluzzo, P.; Bertasio, C.; Sciacca, C.; Arcuri, F.; D'Agostino, R.; Ippolito, D.; et al. *Leptospira interrogans* Serogroup Pomona in a Dairy Cattle Farm in a Multi-Host Zootechnical System. *Vet. Sci.* **2022**, *9*, 83. [CrossRef]
15. Helman, S.K.; Tokuyama, A.F.N.; Mummah, R.O.; Stone, N.E.; Gamble, M.W.; Snedden, C.E.; Borremans, B.; Gomez, A.C.R.; Cox, C.; Nussbaum, J.; et al. Pathogenic *Leptospira* are widespread in the urban wildlife of southern California. *Sci. Rep.* **2023**, *13*, 14368. [CrossRef]
16. Petakh, P.; Kamyshnyi, O. Serogroup Distribution of *Leptospira* Among Humans and Rodents in Zakarpattia Oblast, Ukraine (2018–2023). *Microorganisms* **2025**, *13*, 614. [CrossRef]
17. Habus, J.; Persic, Z.; Spicic, S.; Vince, S.; Stritof, Z.; Milas, Z.; Cvetnic, Z.; Perharic, M.; Turk, N. New trends in human and animal leptospirosis in Croatia, 2009–2014. *Acta Trop.* **2017**, *168*, 1–8. [CrossRef]
18. Benvin, I.; Mojčec Perko, V.; Maljković, M.M.; Habuš, J.; Štritof, Z.; Hađina, S.; Perharić, M.; Zečević, I.; Cvetnić, M.; Turk, N. Serological Surveillance of Equine Leptospirosis in Croatia in the Period From 2012 to 2022: A Key Insight into the Changing Epizootiology. *J. Equine Vet. Sci.* **2023**, *127*, 104844. [CrossRef]
19. Benvin, I.; Fitz, D.; Mojčec Perko, V.; Maurić Maljković, M.; Đurić, V.; Habuš, J.; Štritof, Z.; Perharić, M.; Hađina, S.; Zečević, I.; et al. Insights into *Leptospira* spp. infection in pet cats in Croatia: Clinical, serological and molecular findings with emphasis on the potential important role of serogroup Pomona. *Acta Trop.* **2024**, *260*, 107465. [CrossRef]
20. Semenova, L.P. New serological subtype of the Pomona *Leptospira* group: L. Pomona mozdok. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1965**, *9*, 233–239. [PubMed]
21. Gale, N.B.; Alexander, A.D.; Evans, L.B.; Yager, R.H.; Metheney, R.A. An outbreak of leptospirosis among U.S. army troops in the Canal Zone. II. Isolation and characterization of the isolates. *Amer. J. Trop. Med. Hyg.* **1966**, *15*, 64–70. [CrossRef] [PubMed]
22. Manev, C. Serological characteristics of the *Leptospira* serogroup Pomona. I. Factor analysis of the reference strains. *Zentralbl. Bakteriol. Orig. A.* **1976**, *236*, 316–322.
23. Sulzer, K.; Pope, V.; Rogers, F. New leptospiral serotypes (serovars) from the Western Hemisphere isolated during 1964 through 1970. *Rev. Latinoam. Microbiol.* **1982**, *24*, 15–17.
24. Zhang, F.Z.; Long, P.R.; Meng, P.Y.; Wang, J.J. Identication of *Leptospira kunming* of the Pomona serogroup. *Wei. Sheng. Wu. Xue. Bao.* **1987**, *27*, 88–91. [PubMed]
25. Bourhy, P.; Collet, L.; Lernout, T.; Zinini, F.; Hartskeerl, R.A.; van der Linden, H.; Thiberge, J.M.; Diancourt, L.; Brisse, S.; Giry, C.; et al. Human leptospira isolates circulating in Mayotte (Indian Ocean) have unique serological and molecular features. *J. Clin. Microbiol.* **2012**, *50*, 307–311. [CrossRef] [PubMed]
26. Paiva-Cardoso, M.D.; Arent, Z.; Gilmore, C.; Hartskeerl, R.; Ellis, W.A. Altodouro, a new *Leptospira* serovar of the Pomona serogroup isolated from rodents in northern Portugal. *Infect. Genet. Evol.* **2013**, *13*, 211–217. [CrossRef]
27. Hagedoorn, N.N.; Maze, M.J.; Carugati, M.; Cash-Goldwasser, S.; Allan, K.J.; Chen, K.; Cossic, B.; Demeter, E.; Gallagher, S.; German, R.; et al. Global distribution of *Leptospira* serovar isolations and detections from animal host species: A systematic review and online database. *Trop. Med. Int. Health.* **2024**, *29*, 161–172. [CrossRef]

28. Stritof Majetic, Z.; Galloway, R.; Ruzic Sabljic, E.; Milas, Z.; Mojcec Perko, V.; Habus, J.; Margaletic, J.; Pernar, R.; Turk, N. Epizootiological survey of small mammals as *Leptospira* spp. reservoirs in Eastern Croatia. *Acta Trop.* **2014**, *131*, 111–116. [CrossRef] [PubMed]
29. Musso, D.; La Scola, B. Laboratory diagnosis of leptospirosis: A challenge. *J. Microbiol. Immunol. Infect.* **2013**, *46*, 245–252. [CrossRef]
30. Picardeau, M. Diagnosis and epidemiology of leptospirosis. *Med. Mal. Infect.* **2013**, *43*, 1–9. [CrossRef]
31. Anonymous. *3.01.12_LEPTO OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022*; World Organisation for Animal Health: Paris, France, 2021; pp. 1–13.
32. Levett, P.N. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clin. Infect. Dis.* **2003**, *36*, 447–452. [CrossRef]
33. Bourhy, P.; Bremont, S.; Zinini, F.; Giry, C.; Picardeau, M. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. *J. Clin. Microbiol.* **2011**, *49*, 2154–2160. [CrossRef]
34. Jorge, S.; Kremer, F.S.; De Oliveira, N.R.; Navarro, G.D.O.S.V.; Guimarães, A.M.; Sanchez, C.D.; Woloski, R.D.d.S.; Ridieri, K.F.; Campos, V.F.; Pinto, L.d.S.; et al. Whole-genome sequencing of *Leptospira interrogans* from southern Brazil: Genetic features of a highly virulent strain. *Mem. Inst. Oswaldo. Cruz.* **2018**, *113*, 80–86. [CrossRef]
35. Borcic, B.; Kovacic, H.; Sebek, Z.; Aleraj, B.; Tvrtkovic, N. Small terrestrial mammals as reservoir of leptospires in the Sava Valley (Croatia). *Folia Parasitol.* **1982**, *29*, 177–182.
36. Borcic, B.; Kovacic, H.; Sebek, Z.; Aleraj, B.; Tvrtkovic, N. Small terrestrial mammals as reservoirs of leptospires in the Drava Valley. *Vet. Arh.* **1983**, *53*, 41–49.
37. Turk, N.; Milas, Z.; Margaletic, J.; Staresina, V.; Slavica, A.; Riquelme-Sertour, N.; Bellenger, E.; Baranton, G.; Postic, D. Molecular characterization of *Leptospira* spp. strains isolated from small rodents in Croatia. *Epidemiol. Infect.* **2003**, *130*, 159–166. [CrossRef]
38. Dikken, H.; Kmety, E. Serological typing methods of leptospires. *Methods Microbiol.* **1978**, *11*, 260–295. [CrossRef]
39. Cock, P.J.A.; Antao, T.; Chang, J.T.; Chapman, B.A.; Cox, C.J.; Dalke, A.; Friedberg, I.; Hamelryck, T.; Kauff, F.; Wilczynski, B.; et al. Biopython: Freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* **2009**, *25*, 1422–1423. [CrossRef]
40. Shaw, J.; Yu, Y.W. Fast and robust metagenomic sequence comparison through sparse chaining with skani. *Nat. Methods.* **2023**, *20*, 1661–1665. [CrossRef]
41. Di Tommaso, P.; Chatzou, M.; Floden, E.W.; Barja, P.P.; Palumbo, E.; Notredame, C. Nextflow enables reproducible computational workflows. *Nat. Biotechnol.* **2017**, *35*, 316–319. [CrossRef]
42. Schwengers, O.; Jelonek, L.; Dieckmann, M.A.; Beyvers, S.; Blom, J.; Goesmann, A. Bakta: Rapid and standardized annotation of bacterial genomes via alignment-free sequence identification. *Microb. Genom.* **2021**, *7*, 685. [CrossRef] [PubMed]
43. Tonkin-Hill, G.; MacAlasdair, N.; Ruis, C.; Weimann, A.; Horesh, G.; Lees, J.A.; Gladstone, R.A.; Lo, S.; Beaudoin, C.; Floto, R.A.; et al. Producing polished prokaryotic pangenomes with the Panaroo pipeline. *Genome Biol.* **2020**, *21*, 180. [CrossRef]
44. Silva, M.; Machado, M.P.; Silva, D.N.; Rossi, M.; Moran-Gilad, J.; Santos, S.; Ramirez, M.; Carriço, J.A. chewBBACA: A complete suite for gene-by-gene schema creation and strain identification. *Microb. Genom.* **2018**, *4*, e000166. [CrossRef]
45. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinform.* **2009**, *10*, 421. [CrossRef]
46. Jolley, K.A.; Maiden, M.C.J. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinform.* **2010**, *11*, 595. [CrossRef]
47. Treangen, T.J.; Ondov, B.D.; Koren, S.; Phillippy, A.M. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* **2014**, *15*, 524. [CrossRef] [PubMed]
48. Minh, B.Q.; Schmidt, H.A.; Chernomor, O.; Schrempf, D.; Woodhams, M.D.; Von Haeseler, A.; Lanfear, R.; Teeling, E. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol. Biol. Evol.* **2020**, *37*, 1530–1534. [CrossRef]
49. Paradis, E.; Schliep, K. ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* **2019**, *35*, 526–528. [CrossRef] [PubMed]
50. Yu, G.; Smith, D.K.; Zhu, H.; Guan, Y.; Lam, T.T.Y. ggtree: An r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol. Evol.* **2017**, *8*, 28–36. [CrossRef]
51. Zečević, I.; Picardeau, M.; Vince, S.; Hađina, S.; Perharić, M.; Štritof, Z.; Stevanović, V.; Benvin, I.; Turk, N.; Lohman Janković, I.; et al. Association between Exposure to *Leptospira* spp. and Abortion in Mares in Croatia. *Microorganisms* **2024**, *12*, 1039. [CrossRef]
52. Arent, Z.; Gilmore, C.; Barlow, A.M.; Smith, L.; Ellis, W.A. *Leptospira interrogans* serogroup Pomona infections in the UK: Is there a real threat for farm animals? *Vet Rec.* **2017**, *180*, 424. [CrossRef] [PubMed]
53. Arent, Z.J.; Gilmore, C.; San-Miguel Ayanz, J.M.; Quevedo Neyra, L.; García-Peña, F.J. Molecular Epidemiology of *Leptospira* Serogroup Pomona Infections Among Wild and Domestic Animals in Spain. *EcoHealth* **2017**, *14*, 48–57. [CrossRef] [PubMed]
54. Habuš, J. Genetic Affinities of Pathogenic *Leptospira* Within a Natural Focus of Leptospirosis. Ph.D. Thesis, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia, 2013.

55. Moreno, L.Z.; Kremer, F.S.; Miraglia, F.; Loureiro, A.P.; Eslabao, M.R.; Dellagostin, O.A.; Lilenbaum, W.; Moreno, A.M. Comparative genomic analysis of Brazilian *Leptospira kirschneri* serogroup Pomona serovar mozdok. *Mem. Inst. Oswaldo Cruz.* **2016**, *111*, 539–541. [CrossRef] [PubMed]
56. Habuš, J.; Poljak, Z.; Štritof, Z.; Mojčec Perko, V.; Milas, Z.; Perharić, M.; Martinković, K.; Hađina, S.; Stevanović, V.; Starešina, V.; et al. Prognostic factors for survival of canine patients infected with *Leptospira* spp. *Vet. Arh.* **2020**, *90*, 111–128.
57. Gulati, S.; Gulati, A. Pulmonary manifestations of leptospirosis. *Lung India* **2012**, *29*, 347–353. [CrossRef]
58. Broux, B.; Torfs, S.; Wegge, B.; Deprez, P.; van Loon, G. Acute Respiratory Failure Caused by *Leptospira* spp. in 5 Foals. *J. Vet. Intern. Med.* **2012**, *26*, 684–687. [CrossRef]

9. BIOGRAPHY OF THE AUTHOR WITH BIBLIOGRAPHY OF PUBLISHED WORK

Iva Benvin was born on 1 July 1994 in Rijeka, Croatia. She completed her primary and secondary education in Mali Lošinj and graduated from the Faculty of Veterinary Medicine at the University of Zagreb in 2020. After graduation, she was employed as an assistant at the Department of Microbiology and Infectious Diseases with Clinic at the Faculty of Veterinary Medicine, University of Zagreb. Since then, she has been actively involved in clinical practise, teaching, as well as scientific and laboratory work in the department. She participates in teaching three courses: “Infectious diseases of domestic animals", “Ambulatory clinic,” and “Diseases and treatment of dogs and cats", both in Croatian and English.

Her main scientific and professional interests focus on infectious diseases of dogs and cats, with a special focus on leptospirosis. In 2021, she enrolled in the postgraduate doctoral programme in Veterinary Sciences at the Faculty of Veterinary Medicine, University of Zagreb. She completed additional clinical training at the Clinic for Internal Medicine at the Faculty of Veterinary Medicine at the University of Bern in Switzerland, attended the Ultrasound Diagnostic Course (Ultrasound I) at the OKEAN Veterinary Training System in Pančevo, Serbia, and continued her education at the Clinic for Small Animals at the Faculty of Veterinary Medicine at the Ludwig Maximilian University of Munich in Germany. She also participated in the Erasmus+ Staff Mobility for Teaching Assignment (STA) at the Hans Hoheisen Wildlife Research Station, Faculty of Veterinary Science, University of Pretoria, South Africa, where she held a seminar for students entitled “Leptospirosis – clinical and laboratory diagnostic approach”. In 2024, she completed a three-month scientific training at the Zoonoses and Select Agent Laboratory (ZSAL), Centers for Disease Control and Prevention (CDC) in Atlanta, USA, focussing on leptospiral diagnosis, whole genome sequencing and bioinformatic analysis.

She is a member of the Croatian Veterinary Chamber, the International Veterinary Students’ Association (IVSA) Alumni, the Editorial Board of the student journal “Veterinar” and the Media Relations Committee at the Faculty of Veterinary Medicine, University of Zagreb. She also contributes to online publications and database registrations for the journal “Veterinarski arhiv”.

To date, she has published three scientific papers as first author and more than 20 scientific and professional papers as co-author. She has actively participated in numerous national and international congresses. For her research work, she received the award for successful scientific research work in the PhD student category in the 2023/2024 academic year.

Scientific articles in WoS journals

BENVIN, I., T. K. PAISIE, I. CAETANO VARANDA, Z. P. WEINER, R. A. STODDARD, J. E. GEE, C. A. GULVIK, C. K. MARSTON, V. MOJČEC PERKO, Z. ŠTRITOF, J. HABUŠ, J. MARGALETIĆ, M. VUCELJA, L. BJEDOV, N. TURK (2025): Whole genome characterization of *Leptospira kirschneri* serogroup Pomona in Croatia: insights into its diversity and evolutionary emergence. Pathogens 14, 860. https://doi.org/10.3390/pathogens14090860

BENVIN, I.; FITZ, D.; MOJČEC PERKO, V.; MAURIĆ MALJKOVIĆ, M.; ĐURIĆ, V.; HABUŠ, J.; ŠTRITOF, Z.; PERHARIĆ, M.; HAĐINA, S.; ZEČEVIĆ, I.; TURK, N. (2024): Insights into *Leptospira* spp. infection in pet cats in Croatia: clinical, serological and molecular findings with emphasis on the potential important role of serogroup Pomona. Acta Trop. 260, 107465. https://doi.org/10.1016/j.actatropica.2024.107465

ZEČEVIĆ, I., M. PICARDEAU, S. VINCE, S. HAĐINA, M. PERHARIĆ, Z. ŠTRITOF, V. STEVANOVIĆ, I. LOHMAN JANKOVIĆ, **I. BENVIN**, J. HABUŠ (2024): Association between exposure to *Leptospira* spp. and abortion in mares in Croatia. Microorganisms 12, 1039–1045. https://doi.org/10.3390/microorganisms12061039

BENVIN, I., V. MOJČEC PERKO, M. M. MAURIĆ MALJKOVIĆ, J. HABUŠ, Z. ŠTRITOF, S. HAĐINA, M. PERHARIĆ, I. ZEČEVIĆ, M. CVETNIĆ, N. TURK (2023): Serological surveillance of equine leptospirosis in Croatia in the period from 2012 to 2022: a key insight into the changing epizootiology. J. Equine Vet. Sci. 127, 104844. https://doi.org/10.1016/j.jevs.2023.104844

STEVANOVIĆ, V., M. M. MAURIĆ MALJKOVIĆ, K. GRACIN, **I. BENVIN**, V. STAREŠINA, S. KOVAČ, A. ŠKRINJARIĆ, S. HAĐINA, I. ZEČEVIĆ, K. MARTINKOVIĆ, J. HABUŠ, Z. ŠTRITOF, M. PERHARIĆ, M. CVETNIĆ, LJ. BARBIĆ (2023): Seroprevalence of canine respiratory coronavirus in breeding kennels in Croatia. Vet. arhiv 93, 239–246. https://doi.org/10.24099/vet.arhiv.1602

STEVANOVIĆ, V., M. MAURIĆ MALJKOVIĆ, **I. BENVIN**, I. TABAIN, T. VILIBIĆ-ČAVLEK, Ž. HRUŠKAR, S. KOVAČ, I. SMIT, G. MILETIĆ, S. HAĐINA, V. STAREŠINA, L. RADIN, V. PLICHTA, B. ŠKRLIN, Z. VRBANAC, M. BRKLJAČIĆ, M. CVETNIĆ, J. HABUŠ, K. MARTINKOVIĆ, I. ZEČEVIĆ, G. JURKIĆ, I. FERENCAK, Z. ŠTRITOF, M. PERHARIĆ, L. BUCIĆ, LJ. BARBIĆ (2021): The emergence of SARS-CoV-2 within the dog population in Croatia: host factors and clinical outcome. Viruses 13, 1430. https://doi.org/10.3390/v13081430

VILIBIĆ-ČAVLEK, T., V. SAVIĆ, A. KLOBUČAR, T. FERENC, M. ILIĆ, M. BOGDANIĆ, I. TABAIN, V. STEVANOVIĆ, M. SANTINI, M. CURMAN POSAVEC, S. PETRINIĆ, **I. BENVIN**, I. FERENCAK, V. ROZAC, LJ. BARBIĆ (2021): Emerging trends in the West Nile virus epidemiology in Croatia in the 'One Health' context, 2011–2020. Trop. Med. Infect. Dis. 6, 140. https://doi.org/10.3390/tropicalmed6030140

STEVANOVIĆ, V., T. VILIBIĆ-ČAVLEK, I. TABAIN, **I. BENVIN**, S. KOVAČ, Ž. HRUŠKAR, M. MAURIĆ, L. MILAŠINČIĆ, L. ANTOLAŠIĆ, A. ŠKRINJARIĆ, V. STAREŠINA, LJ. BARBIĆ (2020): Seroprevalence of SARS-CoV-2 infection among pet animals in Croatia and potential public health impact. Transbound. Emerg. Dis. 68, 1767–1773. https://doi.org/10.1111/tbed.13924

VILIBIĆ-ČAVLEK, T., T. PETROVIĆ, V. SAVIĆ, L. BARBIĆ, I. TABAIN, V. STEVANOVIĆ, A. KLOBUČAR, A. MRZLJAK, M. ILIĆ, M. BOGDANIĆ, **I. BENVIN**, M. SANTINI, K. CAPAK, F. MONACO, E. LISTES, G. SAVINI (2020): Epidemiology of Usutu virus: the European scenario. Pathogens 9, 699. https://doi.org/10.3390/pathogens9090699

Scientific articles in Scopus journals

TADIĆ, M., D. KONJEVIĆ, V. MOJČEC PERKO, Z. ŠTRITOF, I. ZEČEVIĆ, **I. BENVIN**, Z. MILAS, N. TURK, M. BUJANIĆ, S. HAĐINA, J. HABUŠ (2024): The occurrence of *Leptospira* spp. serogroup Pomona infections in wild boars. Vet. stn. 56, 225–233. https://doi.org/10.46419/vs.56.2.8

STAREŠINA, V., M. PERHARIĆ, I. ZEČEVIĆ, **I. BENVIN**, K. MARTINKOVIĆ, Z. MILAS (2022): Vezikularni stomatitis. Vet. stn. 53, 637–643. https://doi.org/10.46419/vs.53.5.12

Scientific articles in other journals

HAĐINA, S., Z. ŠTRITOF, V. MOJČEC PERKO, **I. BENVIN**, I. ZEČEVIĆ, K. MARTINKOVIĆ, M. PERHARIĆ, V. STEVANOVIĆ, G. MILETIĆ, I. ĆORIĆ, S. PINTARIĆ, M. CVETNIĆ, J. HABUŠ (2024): Gljivične bolesti u veterinarskoj kliničkoj praksi – izazovi dijagnostike i liječenja. HVV. 32, 48–62.

ŠTRITOF, Z., K. MARTINKOVIĆ, J. HABUŠ, M. PERHARIĆ, V. STEVANOVIĆ, I. ZEČEVIĆ, **I. BENVIN**, S. PINTARIĆ, M. CVETNIĆ, S. HAĐINA (2023): Dermatofitoze pasa i mačaka. HVV. 31, 36–46.

RITOŠA, A., **I. BENVIN**, L. BARBIĆ, V. STEVANOVIĆ (2022): Epizootiološki čimbenici infekcije respiratornim koronavirusom pasa u Republici Hrvatskoj. Veterinar 60, 5–10.

KAMBER, M., **I. BENVIN**, M. PERHARIĆ (2022): Infekcija virusom mačje imunodeficijencije. Veterinar 60, 31–40.

BARBIĆ, L., V. STEVANOVIĆ, V. SAVIĆ, **I. BENVIN**, T. VILIBIĆ-ČAVLEK, J. MADIĆ (2021): Molecular epidemiology of equine influenza virus subtype H3N8 in Croatia (1968–2021). RAD CASA - Medical Sciences. 548, 28–33.

ROGIĆ, L., V. STEVANOVIĆ, **I. BENVIN**, P. JELIČIĆ, L. BARBIĆ (2021): Infekcije virusom hepatitisa E u konja, pasa i mačaka na području Republike Hrvatske. Veterinar 59, 32–38.

Conference papers (in Proceedings)

CVETNIĆ, M., V. MOJČEC PERKO, S. PINTARIĆ, S. HAĐINA, J. HABUŠ, **I. BENVIN**, Z. ŠTRITOF (2022): Antimicrobial susceptibility and multilocus sequence typing of *Campylobacter jejuni* isolates from dogs with diarrhea. Proceedings of the 4th International Conference of the European College of Veterinary Microbiology, 15-17 September, Bari, Italy, pp. 49–50.

OSTER, E., H. PAVASOVIĆ, S. FARAGUNA, **I. BENVIN**, O. SMOLEC, F. BOŽIĆ (2022): One Health: the importance of prudent and responsible use of antimicrobial medicines. Proceedings of the Conference Antimicrobial resistance in veterinary medicine: current state and perspectives, 21-23 June, Novi Sad, Serbia, pp. 148–156.

ZEČEVIĆ, I., M. PERHARIĆ, Z. ŠTRITOF, S. HAĐINA, **I. BENVIN**, O. SMOLEC, J. HABUŠ (2022): Antimicrobial susceptibility of *Staphylococcus pseudintermedius* isolated from dogs with pyoderma and otitis externa. Proceedings of the Conference Antimicrobial resistance in veterinary medicine: current state and perspectives, 21-23 June, Novi Sad, Serbia, pp. 88–93.

BENVIN, I., J. BROZ, A. SHEK VUGROVEČKI, D. ŠPOLJARIĆ, L. PAJURIN, D. JELENČIĆ, J. MILJKOVIĆ, S. MERKAŠ, G. MRŠIĆ, K. VLAHOVIĆ, I. STOLIĆ, M. POPOVIĆ (2017): Određivanje humanog DNK profila osoba u bliskom srodstvu na osnovi otiska prsta izuzetog sa površine karapaksa obične čančare (*Testudo hermanni*). Proceedings of the Veterinarski dani 2017, 25-28 October, Opatija, Croatia, pp. 287–295.

Conference abstracts (Book of Abstracts)

BENVIN, I., F. KAJIN, K. KLAŠTERKA, B. PIRKIĆ, S. HAĐINA, M. PERHARIĆ, I. ZEČEVIĆ, I. ĆORIĆ, G. MILETIĆ, P. DMITROVIĆ (2025): Unrecognized bite wound leading to chronic intracranial empyema in a stray kitten. Book of Abstracts of the 22nd

European Veterinary Emergency and Critical Care (EVECC) Congress, 5-7 June, Dubrovnik, Croatia.

BENVIN, I., D. FITZ, V. MOJČEC PERKO, M. MAURIĆ MALJKOVIĆ, B. BEER LJUBIĆ, J. HABUŠ, Z. ŠTRITOF, S. HAĐINA, M. PERHARIĆ, I. ZEČEVIĆ, V. STEVANOVIĆ (2024): Insights into clinical manifestations of *Leptospira* spp. infection in pet cats and associated risk factors. Book of Abstracts of the 13th Conference of the International Leptospirosis Society (ILS) and the 4th Meeting of the European Leptospirosis Society (ELS), 2-4 September, Brussels, Belgium, p. 128.

TURK, R., A. TRSTENJAK, **I. BENVIN**, B. BEER LJUBIĆ, N. TURK (2024): Haptoglobin and paraoxonase-1 in serum of sows with reproductive disorders caused by pathogenic *Leptospira* spp. Book of Abstracts of the Congress of the European Society of Veterinary Clinical Pathology (ESVCP), 28-31 August, Budapest, Hungary, p. 182.

ZEČEVIĆ, I., V. PLICHTA, M. PERHARIĆ, S. HAĐINA, V. MOJČEC PERKO, **I. BENVIN**, N. TURK, Z. ŠTRITOF, J. HABUŠ (2024): Ophthalmic manifestation of acute canine leptospirosis. Book of Abstracts of the 13th Conference of the International Leptospirosis Society (ILS) and the 4th Meeting of the European Leptospirosis Society (ELS), 2-4 September, Brussels, Belgium, p. 119.

BENVIN, I., I. ZEČEVIĆ, K. MARTINKOVIĆ, S. HAĐINA, J. HABUŠ, Z. ŠTRITOF, I. ĆORIĆ, V. STEVANOVIĆ, N. TURK, M. PERHARIĆ (2023): FeLV associated non-regenerative anaemia with co-infection with haemotropic mycoplasma – case report. Book of Abstracts of the 10th International Congress Veterinary Science and Profession, 5-7 October, Zagreb, Croatia, p. 92.

KOSTANIĆ, V., V. MOJČEC PERKO, A. ČIČMAK, Z. ŠTRITOF, J. HABUŠ, M. PERHARIĆ, **I. BENVIN**, I. ZEČEVIĆ, S. HAĐINA (2023): An insight into the antifungal susceptibility of *Candida* species from animals to four antifungal agents. Book of Abstracts of the 11th Trends in Medical Mycology (TIMM 2023), 20-23 October, Athens, Greece.

KAMBER, M., V. MOJČEC PERKO, Z. ŠTRITOF, J. HABUŠ, V. STEVANOVIĆ, M. PERHARIĆ, K. MARTINKOVIĆ, L. BARBIĆ, I. ZEČEVIĆ, **I. BENVIN**, S. HAĐINA (2023): Detection of tpi, tcdA and tcdB genes of *Clostridioides difficile* in feces of healthy dogs and dogs with clinical signs of gastrointestinal disease. Book of Abstracts of the 10th International Congress Veterinary Science and Profession, 5-7 October, Zagreb, Croatia, p. 147.

ZEČEVIĆ, I., S. HAĐINA, J. HABUŠ, K. MARTINKOVIĆ, Z. ŠTRITOF, V. STEVANOVIĆ, **I. BENVIN**, H. CAPAK, M. PERHARIĆ (2023): Therapeutic effect of the nucleoside analog GS-441524 in a cat with feline infectious peritonitis. Book of Abstracts of the 10th International Congress Veterinary Science and Profession, 5-7 October, Zagreb, Croatia, p. 82.

PERHARIĆ, M., B. JAGEC, J. HABUŠ, S. HAĐINA, Z. ŠTRITOF, V. STEVANOVIĆ, **I. BENVIN**, I. ZEČEVIĆ (2023): Epizootiology of retroviral infections in stray cats. Book of Abstracts of the 10th International Congress Veterinary Science and Profession, 5-7 October, Zagreb, Croatia, p. 80.

FUŠ, K., S. PINTARIĆ, S. HAĐINA, J. HABUŠ, M. PERHARIĆ, M. CVETNIĆ, I. ZEČEVIĆ, **I. BENVIN**, Z. ŠTRITOF (2023): Beta-hemolytic streptococci isolated from horses in Croatia. Book of Abstracts of the 10th International Congress Veterinary Science and Profession, 5-7 October, Zagreb, Croatia, p. 61.

DOJČINOVIĆ, E., D. CAUŠEVIĆ, S. PINTARIĆ, S. HAĐINA, J. HABUŠ, M. PERHARIĆ, M. CVETNIĆ, I. ZEČEVIĆ, **I. BENVIN**, Z. ŠTRITOF (2023): Antimicrobial susceptibility of bacteria isolated from urine of dogs with urinary tract infections. Book of Abstracts of the 10th International Congress Veterinary Science and Profession, 5-7 October, Zagreb, Croatia, p. 87.

BENVIN, I., V. MOJČEC PERKO, J. HABUŠ, Z. ŠTRITOF, V. STAREŠINA, S. HAĐINA, M. PERHARIĆ, I. ZEČEVIĆ, LJ. BARBIĆ, V. STEVANOVIĆ, N. TURK (2022): Monitoring of equine leptospirosis in Croatia in the period from 2012 to 2022. Book of Abstracts of the 12th International Leptospirosis Society (ILS) Conference, 13-16 November, Bangkok, Thailand, p. 97.

BENVIN, I., LJ. BARBIĆ, S. KOVAČ, I. TABAIN, T. VILIBIĆ-ČAVLEK, M. MAURIĆ, Ž. HRUŠKAR, V. STAREŠINA, B. BEER LJUBIĆ, S. HAĐINA, J. HABUŠ, Z. ŠTRITOF, K. MARTINKOVIĆ, M. PERHARIĆ, I. ZEČEVIĆ, V. STEVANOVIĆ (2022): Seroprevalence of SARS-CoV-2 infection among cats in Croatia. Book of Abstracts of the 24th Symposium of Epizootiologists and Epidemiologists, 27-29 April, Subotica, Serbia, pp. 110–111.

HAĐINA, S., V. MOJČEC PERKO, A. ČIČMAK, Z. ŠTRITOF, J. HABUŠ, V. STAREŠINA, **I. BENVIN**, V. STEVANOVIĆ, M. PERHARIĆ, K. MARTINKOVIĆ, I. ZEČEVIĆ, LJ. PINTER (2021): Disk diffusion susceptibility testing of *Malassezia pachydermatis* and *Candida* spp. isolated from animals against the three most commonly used antifungal

drugs. Journal of Fungi 7 (11), Conference Report, 10th Trends in Medical Mycology (TIMM), 8-11 October, Aberdeen, Scotland, pp. 291–292.

BENVIN, I., V. STEVANOVIĆ, S. KOVAČ, A. ŠKRINJARIĆ, V. STAREŠINA, K. MARTINKOVIĆ, I. ZEČEVIĆ, S. HAĐINA, J. HABUŠ, Z. ŠTRITOF, M. PERHARIĆ, M. CVETNIĆ, N. TURK, Z. MILAS, LJ. BARBIĆ (2021): Aetiology of viral gastroenteritis in dogs – prevalence and risk factors. Book of Abstracts of the 9th International Congress Veterinary Science and Profession, 9th October, Zagreb, Croatia, p. 90.

BENVIN, I., V. STEVANOVIĆ, T. VILIBIĆ-ČAVLEK, S. KOVAČ, LJ. BARBIĆ (2021): Rezultati praćenja proširenosti West Nile virusa u konja u Republici Hrvatskoj u razdoblju od 2010. do 2020. godine. Book of Abstracts of the Symposium "(Re-)Emerging Arboviruses in the Shadow of COVID-19 pandemic", 10-11 June, Zagreb, Croatia, pp. 26–27.

ZEČEVIĆ, I., Z. ŠTRITOF, S. HAĐINA, V. STEVANOVIĆ, M. PERHARIĆ, **I. BENVIN**, M. CVETNIĆ, K. MARTINKOVIĆ, J. HABUŠ (2021): Therapeutic potential of faecal microbiota transplantation in dogs. Book of Abstracts of the 9th International Congress Veterinary Science and Profession, 9th October, Zagreb, Croatia, p. 95.

MOHOROVIĆ, N., V. MOJČEC PERKO, LJ. PINTER, Z. ŠTRITOF, **I. BENVIN**, J. HABUŠ, V. STEVANOVIĆ, M. PERHARIĆ, K. MARTINKOVIĆ, I. ZEČEVIĆ, M. CVETNIĆ, S. HAĐINA (2021): Molecular identification of animal-derived *Candida* species and their susceptibility to miconazole determined by the broth microdilution method. Book of Abstracts of the 9th International Congress Veterinary Science and Profession, 9th October, Zagreb, Croatia, p. 93.

KAJMIĆ, A., A. JUTRIŠA, V. MOJČEC PERKO, J. HABUŠ, S. HAĐINA, S. PINTARIĆ, V. STEVANOVIĆ, M. PERHARIĆ, K. MARTINKOVIĆ, I. ZEČEVIĆ, M. CVETNIĆ, **I. BENVIN**, Z. ŠTRITOF (2021): High carriage rate of methicillin-resistant staphylococci in small animal veterinary clinicians – indirect evidence of zoonotic transmission. Book of Abstracts of the 9th International Congress Veterinary Science and Profession, 9th October, Zagreb, Croatia, p. 46.

CVETNIĆ, M., V. MOJČEC PERKO, D. BROZIĆ, S. PINTARIĆ, S. HAĐINA, J. HABUŠ, V. STEVANOVIĆ, M. PERHARIĆ, K. MARTINKOVIĆ, I. ZEČEVIĆ, **I. BENVIN**, Z. ŠTRITOF (2021): Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter upsaliensis* isolated from dog faeces. Book of Abstracts of the 9th International Congress Veterinary Science and Profession, 9th October, Zagreb, Croatia, p. 45.

BENVIN, I., LJ. BARBIĆ, S. KOVAČ, G. MILETIĆ, I. TABAIN, T. VILIBIĆ-ČAVLEK, M. MAURIĆ MALJKOVIĆ, Ž. HRUŠKAR, V. STAREŠINA, J. HABUŠ, Z. ŠTRITOF, K. MARTINKOVIĆ, M. PERHARIĆ, I. ZEČEVIĆ, G. JURKIĆ, V. STEVANOVIĆ (2021): The prevalence of SARS-CoV-2 virus among dogs in households with confirmed human infections. Book of Abstracts of the XXII/XXIII Symposium of Epizootiologists and Epidemiologists, 26-28 April, Subotica, Serbia, p. 127.

STEVANOVIĆ, V., **I. BENVIN**, S. KOVAČ, G. MILETIĆ, I. TABAIN, T. VILIBIĆ-ČAVLEK, M. MAURIĆ MALJKOVIĆ, Ž. HRUŠKAR, I. ŠMIT, L. RADIN, M. BRKLJAČIĆ, V. STAREŠINA, S. HAĐINA, Z. VRBANAC, B. ŠKRLIN, V. PLICHTA, I. FERENČAK, M. CVETNIĆ, LJ. BARBIĆ (2021): Seroprevalence of SARS-CoV-2 in dogs in the city of Zagreb and Zagreb County. Book of Abstracts of the XXII/XXIII Symposium of Epizootiologists and Epidemiologists, 26-28 April, Subotica, Serbia, p. 125.

STEVANOVIĆ, V., M. MAURIĆ MALJKOVIĆ, **I. BENVIN**, I. TABAIN, T. VILIBIĆ-ČAVLEK, S. KOVAČ, Ž. HRUŠKAR, V. STAREŠINA, I. ŠMIT, L. RADIN, M. BRKLJAČIĆ, S. HAĐINA, Z. VRBANAC, B. ŠKRLIN, V. PLIHTA, M. CVETNIĆ, J. HABUŠ, Z. ŠTRITOF, K. MARTINKOVIĆ, M. PERHARIĆ, I. ZEČEVIĆ, G. JURKIĆ, L. BUCIĆ, LJ. BARBIĆ (2021): The role of pet animals in the epidemiology of COVID-19 – what do we know today? Book of Abstracts of the Symposium "(Re-)Emerging Arboviruses in the Shadow of COVID-19 pandemic", 10-11 June, Zagreb, Croatia, pp. 61–62.

BARBIĆ, LJ., V. STEVANOVIĆ, T. VILIBIĆ-ČAVLEK, **I. BENVIN**, V. SAVIĆ, J. MADIĆ (2021): Domestic animals as sentinel for arbovirus surveillance. Book of Abstracts of the Symposium "(Re-)Emerging Arboviruses in the Shadow of COVID-19 pandemic", 10-11 June, Zagreb, Croatia, pp. 40–41.

STEVANOVIĆ, V., I. TABAIN, T. VILIBIĆ-ČAVLEK, **I. BENVIN**, S. KOVAČ, Ž. HRUŠKAR, M. MAURIĆ, LJ. MILAŠINČIĆ, LJ. ANTOLAŠIĆ, A. ŠKRINJARIĆ, V. STAREŠINA, LJ. BARBIĆ (2020): SARS-CoV-2 in pet animals. Book of Abstracts of the Symposium with International Participation "Zoonotic and vector-borne diseases in the One Health Context", 22-23 October, Zagreb, Croatia, p. 46.

BARBIĆ, L., V. STEVANOVIĆ, V. SAVIĆ, **I. BENVIN**, T. VILIBIĆ ČAVLEK, J. MADIĆ (2020): Javnozdravstveni značaj praćenja flavivirusnih infekcija u životinja. Book of Abstracts of the Symposium with International Participation "Zoonotic and vector-borne diseases in the One Health Context", 22-23 October, Zagreb, Croatia, pp. 55–56.

STEVANOVIĆ, V., T. VILIBIĆ-ČAVLEK, I. TABAIN, **I. BENVIN**, S. KOVAČ, Ž. HRUŠKAR, M. MAURIĆ MALJKOVIĆ, LJ. MILAŠINIĆ, LJ. ANTOLAŠIĆ, A. ŠKRINJARIĆ, V. STAREŠINA, LJ. BARBIĆ (2020): Učestalost i epidemiološki značaj infekcija kućnih ljubimaca SARS-CoV-2 virusom. Book of Abstracts of the Online Symposium "SARS-CoV-2 within One Health concept", 26th November, Zagreb, Croatia, p. 23.

BENVIN, I., LJ. PINTER, Z. ŠTRITOF, J. HABUŠ, V. MOJČEC PERKO, V. STEVANOVIĆ, M. PERHARIĆ, LJ. BARBIĆ, V. STAREŠINA, N. TURK, Z. MILAS, S. HAĐINA (2019): The optimization of inoculum size of *Malassezia pachydermatis* and its use in disk diffusion method for antifungal susceptibility testing. Book of Abstracts of the 8th International Congress Veterinary Science and Profession, 10-12 October, Zagreb, Croatia, p. 91.

HAĐINA, S., **I. BENVIN**, Z. ŠTRITOF, J. HABUŠ, V. STEVANOVIĆ, M. PERHARIĆ, K. MARTINKOVIĆ, V. MOJČEC PERKO, LJ. BARBIĆ, Z. MILAS, V. STAREŠINA, N. TURK, LJ. PINTER (2019): Screening of antifungal susceptibility of Malassezia pachydermatis isolates from dogs using disk diffusion test. Journal of Fungi 5, Conference Report, 9th Trends in Medical Mycology (TIMM), 11-14 October, Aberdeen, Scotland, pp. 136–137.