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Doctoral Study of Molecular Biosciences

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GENOMIC ANALYSIS AND ANTIMICROBIAL RESISTANCE OF *CAMPYLOBACTER JEJUNI* STRAINS ISOLATED FROM HUMANS AND BIRDS IN CROATIA

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GENOMIC ANALYSIS AND ANTIMICROBIAL RESISTANCE OF *Campylobacter jejuni* STRAINS ISOLATED FROM HUMANS AND BIRDS IN CROATIA

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Thesis performed at: University Hospital for Infectious Diseases "Dr. Fran Mihaljević", Zagreb

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Abstract:

This study focuses on *Campylobacter jejuni*, a leading cause of foodborne illness, with significant impacts on human and veterinary health, as well as the agricultural sector due to increasing antibiotic resistance. Using a One Health approach, the research explores the pathogen's epidemiology, ecology, and resistance mechanisms across three key reservoirs: wild birds, broilers, and humans. A total of 62 isolates were analyzed, including strains from humans with diarrhea, broilers, and wild birds, focusing on genetic profiles, clonal distribution, and resistance patterns. Key findings indicate that *C. jejuni* exhibit notable genetic diversity while maintaining some reservoir associations. Phylogenetic analysis revealed a close genetic relationship between strains from humans and broilers, suggesting shared lineages and resistance mechanisms. A phylogenetic connection between human and wild bird strains was identified within a cluster of *C. jejuni* strains belonging to ST822, highlighting genetic links between these reservoirs. Additionally, the study detected sequence type ST1949 in wild birds for the first time, previously reported only in humans and broilers. This finding not only suggests a potential epidemiological link between these reservoirs but also further underscores the significant influence of this niche on the epidemiology of *C. jejuni* and its transmission dynamics. The study emphasizes the importance of whole-genome sequencing in identifying resistance genes, predicting resistance profiles, tracking clonal complexes, and providing early outbreak warnings. These findings highlight the need for continuous surveillance and a One Health approach to mitigate antimicrobial resistance and better and better understand *C. jejuni* epidemiology in human, veterinary, and environmental contexts.

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GENOMSKA ANALIZA I REZISTENCIJE NA ANTIBIOTIKE U *Campylobacter jejuni* SOJEVA IZOLIRANIH IZ LJUDI I PTICA U HRVATSKOJ

Silvija Šoprek Strugar

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Kratki sažetak doktorskog rada:
Ova studija istražuje *Campylobacter jejuni*, vodećeg uzročnika bolesti prenosivih hranom, koji ima značajan utjecaj na ljudsko i veterinarsko zdravlje te pogađa poljoprivredni sektor zbog sve veće rezistencije na antibiotike. Koristeći pristup Jednog zdravlja, istraživanje analizira epidemiologiju, ekologiju i mehanizme rezistencije na antibiotike ovog patogena u tri ključna rezervoara: divlje ptice, brojleri i ljudi. Ukupno je analizirano 62 izolata, uključujući sojeve izolirane iz ljudi s dijarejom, brojlere i divlje ptice, s naglaskom na genetske profile, klonalnu distribuciju i obrasce rezistencije na antibiotikeKljučni podatci ukazuju da *C. jejuni* pokazuje značajnu genetsku raznolikost uz određenu povezanost s pojedinim rezervoarima. Filogenetska analiza otkrila je blisku genetsku povezanost između sojeva iz ljudi i brojlera, što ukazuje na zajedničke loze i mehanizme otpornosti. Filogenetska analiza detektirala je povezanost između sojeva iz ljudi i divljih ptica unutar klastera ST822. U ovoj je studiji prvi put detektiran *C. jejuni* ST1949 kod divljih ptica, tip koji je ranije bio zabilježen samo kod ljudi i brojlera. Ovi nalazi dodatno ističu ulogu divljih ptica kao važne niše u prijenosnom lancu *C. jejuni* i širenju rezistencije na antibiotike Studija potvrđuje vrijednost sekvenciranja cijeloga genoma u identificiranju gena rezistencije na antibiotike, predviđanju fenotipskih profila rezistencije na iste, praćenju pojavnosti klonalnih kompleksa te posjedično ranom otkrivanju i upozoravanju na potencijalne epidemije. Ovi rezultati naglašavaju potrebu za kontinuiranim nadzorom i primjenom pristupa Jednog zdravlja kako bi se ublažilo širenje antimikrobne otpornosti i bolje razumjela epidemiologija *C. jejuni* u ljudskom i okolišnom kontekstu

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1. INTRODUCTION

1.1. *Campylobacter jejuni* – Taxonomy, Microbiology, Species Identification and Genotyping

1.1.1. Taxonomy

Originally known as *Vibrio jejuni* due to its resemblance to *Vibrio* spp., *Campylobacter jejuni* underwent a name change in 1963 when Seabald and Vernon proposed the transition to the Campylobacter genus as they detected significant biological differences with *Vibrio* spp. (1). These unique biological distinctions originated from its low guanine and cytosine content, non-fermentative metabolism, and its preference for microaerophilic growth conditions. As early as 1886, pediatrician Theodor Escherich identified *Campylobacter* spp. in diarrhea samples from children. The first isolation of *C. jejuni* took place in Brussels, Belgium, from stool samples obtained from a patient experiencing diarrhea (2–4).

In modern taxonomy, this bacterium is categorized within the phylum Proteobacteria, the class Epsilonproteobacteria, and the family *Campylobacteraceae* (5).

1.1.2. Microbiology

Campylobacter jejuni, a member of the *Campylobacter* genus, is a Gram-negative, highly motile, fastidious bacterium with characteristically curved, spiral, or S-shaped cells. *C. jejuni* requires microaerophilic conditions for growth, which involve incubating with 5-10% carbon dioxide and reduced oxygen levels, generally around 5-10%. The optimal temperature for the growth of this thermophilic species ranges between 37 and 42°C. High motility is achieved through a single flagellum located at one or both poles. Based on its specific metabolic processes, it is defined as oxidase positive, non-fermentative bacterium (4,6,7).

Its natural habitat is very diverse. *C. jejuni* naturally inhabits the intestinal tracts of poultry, cattle, swine, rodents, domestic animals such as cats and dogs, as well as wild birds and various other wildlife (8–10).

The genome of *C. jejuni* strain NCTC11168 was published in 2000, revealing a size of 1,641,481 base pairs (30.6% G+C content), which was projected to encode 1,654 proteins and 54 stable RNA species (11). *C. jejuni* has a built-in capacity for natural genetic transformation, allowing it to incorporate donor sequences into its genome through homologous recombination, so it freely uptakes foreign DNA containing genetic information responsible for antibiotic resistance (12). *C. jejuni* exhibits remarkable genetic heterogeneity and strain variability, largely attributed to frequent genetic recombination and the noted phenomenon of natural genetic transformation (13). This pathogen is acknowledged for its extensive diversity, encompassing approximately 11,884 unique sequence types (STs) and over 45 clonal complexes (11).

1.1.3. Species Identification

The preliminary identification of *Campylobacter* spp. from primary culture typically involves assessing their characteristic colonial appearance, performing a Gram stain, evaluating growth in the presence of oxygen, and conducting an oxidase test. Colonies typically display a unique appearance, often resembling spilled milk, as they tend to spread or swarm, although significant variations are common. On blood agar, *Campylobacter* spp. colonies typically appear as non-hemolytic, greyish, smooth, and slightly raised colonies. When cultured on selective agar like Charcoal cefoperazone deoxycholate agar, colonies typically appear grey/white or creamy grey, with a moist appearance. In Gram stain, *Campylobacter* spp. appear as curved or spiral-shaped, Gram-negative rods. They often exhibit a "seagull wing" or "comma" shape due to their spiral form. Pleomorphism is common, meaning they can vary in size and shape within a single culture often increasing with ageing of cultures. In preliminary diagnostics, laboratories employ additional tests like the indole test, and the Voges-Proskauer test. Since all *Campylobacter* spp. are oxidase positive and indole negative, they are also expected to produce negative results in the Voges-Proskauer test, due to their inability to ferment or oxidize carbohydrates. For species identification, laboratories perform several conventional phenotypic tests, such as the hippurate test, which detects the presence of hippuricase, an enzyme possessed by most *C. jejuni* strains. Additionally, laboratories assess sensitivity to cefazolin, where *C. jejuni* typically exhibits resistance and the indoxyl acetate test to assess hydrolase activity. Species differentiation using conventional methods can be challenging due to the absence of discriminating tests (4,14,15). However, the introduction of novel methods such as MALDI-ToF and molecular tests enables rapid and accurate differentiation (16,17).

1.1.4. Genotyping

Genotyping in *C. jejuni* refers to the process of characterizing and analyzing the genetic variation within the bacterial species. This genetic variation can provide valuable insights into the epidemiology, evolution, and pathogenesis of *C. jejuni* infections. There are several methods used for genotyping *C. jejuni* strains (18,19), each with its own advantages and applications:

- PCR-Based Typing Methods: Various PCR-based techniques, such as PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), Random Amplification of Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP), can be used to genotype *C. jejuni* strains based on specific genetic markers or genomic regions. These methods are often faster and less resource-intensive than sequencing-based approaches but may have lower discriminatory power.
- Pulsed-Field Gel Electrophoresis (PFGE): PFGE is a molecular typing technique that separates DNA fragments based on their size using an electric field. By digesting genomic DNA with restriction enzymes and analyzing the resulting banding patterns, researchers can differentiate between *C. jejuni* strains. PFGE is particularly useful for outbreak investigations and source attribution studies due to its high discriminatory power. However, challenges remain with data interpretation and reproducibility.
- Multilocus Sequence Typing (MLST): MLST is a widely used genotyping method that involves sequencing seven housekeeping genes from bacterial isolates. By comparing the sequences of these genes, researchers can assign unique sequence types (STs) to individual strains. MLST data are stored in publicly accessible databases, allowing for the comparison of strains globally and the tracking of strain dissemination over time.
- Whole Genome Sequencing (WGS): WGS provides the most comprehensive approach to genotyping *C. jejuni* strains by sequencing the entire bacterial genome. This allows for the identification of single nucleotide polymorphisms (SNPs), insertions, deletions, and other genetic variations at high resolution. WGS data can be used for phylogenetic analysis, population structure studies, and the identification of virulence factors and antibiotic resistance genes.

Genotyping of *C. jejuni* strains is essential for understanding the transmission patterns of campylobacteriosis and guiding public health measures. By identifying related strains,

tracking outbreak clusters, and identifying sources of infection, genotyping can help prevent and control the spread of *C. jejuni* infections, ultimately reducing the burden of campylobacteriosis on human health.

1.1.5. *C. jejuni* Virulence Factors and Pathogenesis of Infections Caused by *C. jejuni*

1.1.5.1. Virulence Factors of *C. jejuni*

C. jejuni possesses numerous distinct virulence factors that enhance its ability to cause disease. Among these are cytolethal distending toxin (CDT), flagella, adhesins, capsular polysaccharides, lipooligosaccharide (LOS), and the type III and type VI secretion system (T3SS, T6SS) (20–22).

Cytolethal distending toxin is a potent cytotoxin produced by *C. jejuni*, which induces DNA damage and cell cycle arrest in host cells, leading to cell distention and death. This toxin is implicated in tissue damage and inflammation during infection.

Flagella enable *C. jejuni* to be highly motile, which facilitates its movement through the mucous layer and adherence to host epithelial cells. Flagellar motility is essential for *C. jejuni* colonization and invasion of the intestinal mucosa.

C. jejuni expresses various adhesins, including Campylobacter adhesion to fibronectin (CadF) and *Campylobacter jejuni* adherence and invasion protein A (FlpA), which promote the binding of bacteria to host cells, facilitating colonization and invasion. It also produces capsular polysaccharides that aid in immune evasion by inhibiting phagocytosis and complement activation. These polysaccharides also play a role in biofilm formation and persistence in the host environment. With LOS molecules being present on the outer membrane of *C. jejuni,* cells can induce host immune responses and contribute to inflammation during infection. LOS variation among different strains influences the severity of disease and host immune responses.

It can secrete various proteins, including proteases and toxins, which contribute to tissue damage, inflammation, and disease progression. These secreted proteins facilitate host cell invasion and manipulation of host signaling pathways.

Some *C. jejuni* strains harbor T3SS or T6SS, complex molecular systems capable of delivering virulence factors directly into host cells or amplifying virulence through processes such as pore formation, contact-dependent cytotoxicity targeting red blood cells, and actin cross-linking. These complexes are transmembrane proteins that enable the translocation of bacterial effectors across the host cell membrane, modulating host cell signaling pathways and promoting bacterial survival and pathogenesis. These effector proteins play roles in cellular invasion, intracellular survival, and modulation of host immune responses.

1.1.5.2. Pathogenesis of Infections Caused by *C. jejuni*

The primary sources of Campylobacter infection involve the consumption of contaminated food, raw or undercooked poultry, unpasteurized milk, and dairy products (23,24). Another transmission route identified is the consumption of water contaminated by feces from infected humans, animals, and unpasteurized milk, which has been linked to numerous local infection outbreaks (25). Direct contact with infected animals or their environments can also transmit *Campylobacter* spp. Once *C. jejuni* colonizes the intestinal tract, particularly the jejunum, it adheres to the intestinal epithelial cells. The bacterium employs various virulence factors to facilitate its pathogenesis, including adhesion proteins, invasion mechanisms, and toxin production. Inflammatory responses triggered by the infection contribute to the clinical symptoms experienced during illness. The pathophysiology of *C. jejuni* infections can be summarized into several key processes:

- Adherence and Invasion: *C. jejuni* attaches to the mucosal surface of the intestine through its adhesins, such as CadF and FlpA, and flagella. It then invades the intestinal epithelial cells through a process involving cytoskeletal rearrangements and membrane ruffling.
- Tissue Damage: *C. jejuni* produces various virulence factors, including CDT and secreted proteins, which induce cellular damage and inflammation in the intestinal mucosa. The toxins can cause DNA damage, induce a block in the cell cycle, and promote apoptosis in host cells, leading to tissue destruction and disruption of the intestinal barrier function.
- Inflammatory Response: The interaction between *C. jejuni* and the host immune system triggers an inflammatory response characterized by the recruitment of immune cells, such as neutrophils and macrophages, to the site of infection. This inflammatory cascade contributes to the clinical manifestations of infection, including diarrhea, abdominal pain, and fever.
- Toxin-Mediated Effects: *C. jejuni* toxins, such as CDT and LOS, can directly stimulate pro-inflammatory cytokine production by host cells, exacerbating the inflammatory response and tissue damage. These toxins can also disrupt intestinal epithelial barrier function, leading to increased intestinal permeability and fluid loss.

- Systemic Spread: In severe cases or in immunocompromised individuals, *C. jejuni* may disseminate beyond the gastrointestinal tract, leading to bacteremia and the potential for extraintestinal manifestations, such as septicemia, meningitis, or reactive arthritis.

1.2. Clinical Presentations of Infections Caused by *C. jejuni*

The illness exhibits a broad spectrum of symptoms, primarily affecting the gastrointestinal tract. Typical symptoms within this tract include watery or bloody diarrhea, abdominal cramps, nausea, frequently associated with headaches, and elevated fever. Although it is usually presented as a mild, self-limiting disease, resolving spontaneously within 7 to 10 days, Campylobacter infection can lead to life-threatening conditions such as bacteremia, endocarditis, meningitis, and other extraintestinal infections. In rare instances, *C. jejuni* infection can trigger a severe autoimmune disorder such as Guillain-Barré syndrome (GBS), Miller-Fischer syndrome, or reactive arthritis which can lead to long-term and chronic complications, significantly impacting the quality of life (26–31).

Although *C. jejuni* infections usually resolve spontaneously, they often require symptomatic therapy, electrolyte and volume replacement. In severe cases, such as bacteremia, meningitis, and other extraintestinal infections where antibiotic therapy is necessary, the available choices for effective treatment are becoming increasingly limited due to the rising resistance to available antibiotics (27,32).

1.3. Antibiotic Resistance Mechanisms in *C. jejuni*

Recognized mechanisms of resistance in *C. jejuni* isolates include target modification, drug modification or inactivation by enzyme production, active efflux and reduced permeability (33).

Fluoroquinolone resistance primarily arises from mutations in the target genes within the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV, though it can also be mediated by efflux mechanisms (34,35). Macrolide resistance in Campylobacter is primarily driven by mutations in the 23S rRNA gene, which alter the ribosomal target binding site, reducing the antibiotic's ability to bind and inhibit protein synthesis. In some cases, resistance can also occur through target methylation or enzymatic modification of the drug, although these mechanisms are less common in *Campylobacter* spp. (12,34–36).

The mechanisms underlying Campylobacter resistance to certain β-lactams, such as ampicillin and some extended-spectrum cephalosporins, are diverse and not fully understood. Generally, the majority of *Campylobacter* spp. strains are considered to be resistant to β-lactam antimicrobial agents, especially the penicillins and narrow-spectrum cephalosporins, except for some carbapenems. The first β-lactamase encoding gene in *Campylobacter* spp. was found to be located on a chromosome and was linked to a class D β-lactamase production (37,38). The corresponding gene, from a human clinical isolate, was cloned and characterised to code for a class D β-lactamase, OXA-61, conferring resistance to ampicillin, penicillin, and carbenicillin (39,40). Meanwhile, several enzymes were identified and characterized based on their varying activity against eight different beta-lactam antibiotics. In addition to these enzymatic modifications, β-lactam resistance in *Campylobacter* spp. can also occur through active efflux, particularly via efflux pumps like CmeABC. This mechanism allows the bacteria to actively expel the antibiotic, reducing its intracellular concentration and effectiveness, thereby further diminishing susceptibility to beta-lactams and contributing to resistance (41,42).

Multiple aminoglycosides-modifying enzymes, including aminoglycoside phosphotransferase types I, III, IV, and VII, aminoglycoside adenyltransferase, and 6-aminoglycoside adenyltransferase, are described in *Campylobacter* spp. Aminoglycoside resistance is primarily mediated by enzymes such as aminoglycoside acetyltransferases, phosphotransferases, and nucleotidyltransferases, which modify the antibiotic by altering specific hydroxyl or amino groups. These changes reduce the affinity of aminoglycosides for the A-site of 16S rRNA, diminishing the drug's ability to interfere with bacterial protein

synthesis (43,44). Resistance to tetracyclines in *Campylobacter* spp. is conferred by the *tet*(O) gene, which is widely present in both *C. jejuni* and *C. coli* (45–47). Chloramphenicol resistance is conferred by a plasmid-carried *cat* gene that encodes acetyltransferase, which modifies chloramphenicol in a way that prevents it from binding to ribosomes (48).

1.4. Epidemiology of *C. jejuni* and Burden of Diseases

C. jejuni is one of the most common causes of bacterial gastroenteritis worldwide, and its epidemiology is closely monitored by organizations such as the European Food Safety Authority (EFSA), the European Centre for Disease Prevention and Control (ECDC), the Centre for Disease Control and Prevention (CDC), and the World Health Organization (WHO). Surveillance data collected by EFSA and ECDC, together with CDC and WHO, highlight the significant public health impact of *C. jejuni* infections (49,50).

As outlined in EFSA and ECDC reports, campylobacteriosis is estimated to affect around 246,000 people annually in the European Union alone, while in the United States, the estimate stands at 1.5 million illnesses each year. Globally, incidences have been steadily increasing in both industrialized and developing countries in recent years. The World Health Organization (WHO) estimates the global incidence of human infections at 200 cases per 100,000 people, while the overall EU/EEA notification rate in 2022 was 46.9 cases per 100 000 population with regional differences (e.g., 147 cases per 100,000 in the Luxemburg and 27 cases per 100,000 in the Netherlands). A clear seasonal pattern is observed, with cases reaching their peak during the summer months, especially in Europe, where the highest numbers are typically seen in July. Climate change is expected to drive a rise in incidences in Northern Europe in the coming decades (51).

While it is generally observed that campylobacteriosis is associated with low mortality rates, ranging from 0.3 to 2.9% in developed countries regardless of age, diarrhea presents a significant concern in less developed nations. In these regions, diarrhea is among the leading causes of mortality, particularly affecting children. According to the WHO, diarrhea ranks as the second highest cause of death in children under the age of 5 worldwide, resulting in the loss of 525,000 young lives annually. These factors are reflected in the hospitalization rate of around 20% in Europe (52,53). It is associated with a financial burden estimated by EFSA at approximately 2.4 billion euros annually just for the EU.

Although *C. jejuni* infections typically resolve on their own, they often require symptomatic treatment, including electrolyte and fluid replacement. Despite antibiotic therapy not being generally indicated, the issue of overprescription arises, with antibiotics frequently administered even in less severe cases (53). The incidence of human Campylobacter infections is increasing worldwide, as well as the proportion of isolates resistant to fluoroquinolones and/or macrolides. Consequently, treatment options for this infection are becoming increasingly

limited, highlighting an urgent need for alternative therapeutic approaches and more effective strategies to manage resistance. Furthermore, the situation becomes more intricate since in cases of severe systemic infections caused by species of this genus (bacteremia, meningitis, and other extraintestinal infections), the choice of effective antibiotics for treatment is becoming increasingly limited (26,27,32,54). To conclude, Campylobacter infections pose a significant global health challenge due to the persistent rise in antimicrobial resistance trends, impacting both human and veterinary medicine, as well as agriculture sector.

C. jejuni has demonstrated a concerning level of antibiotic resistance, which, as mentioned, can make treatment strategies more challenging. The growing rates of antimicrobial resistance, especially to fluoroquinolones and macrolides, are largely driven by the extensive use of these drugs in animal production and overuse or the indiscriminate use of antibiotics in both human healthcare and agriculture. These practices contribute to the selection and spread of resistant bacteria in various environments, exacerbating the global resistance problem (55). Common antibiotics used to treat *C. jejuni* infections, such as fluoroquinolones and tetracyclines are already encountering high rates of resistance, while for macrolides the increasing trend in resistance rates is observed.

Data from human and animal *C. jejuni* isolates in Croatia align closely with the average resistance rates observed across the European Union. The first national report on the susceptibility of *C. jejuni* isolates from humans in Croatia was published in 2013, revealing a 50% resistance rate to fluoroquinolones and a 26% resistance rate to tetracycline (56). Since then, resistance to both ciprofloxacin and tetracycline has steadily increased, with fluoroquinolone resistance rates now stabilizing around 70% and tetracycline resistance reaching approximately 40% in recent years. In contrast, erythromycin resistance has remained consistently low, at or below 3% throughout this period (57) (49).

Antibiotic resistance surveillance in the veterinary sector in Croatia began in 2015, initially focusing on two key classes of antibiotics commonly used in both veterinary and human medicine: quinolones and tetracyclines. Surveillance for macrolide resistance was introduced in 2017, followed by carbapenems in 2021. Resistance rates have varied depending on the animal species from which the isolates were recovered. An analysis of *C. jejuni* isolates from broilers in 2015 revealed ciprofloxacin resistance rates of 76% and tetracycline resistance rates of 29% (58)(59). Over the years, there has been a consistent increase in resistance across both antibiotic classes, with current fluoroquinolone resistance levels stabilizing around 85% and tetracycline resistance reaching approximately 40% (57,60,61).

Notably, macrolide resistance in broilers is significantly higher than in humans, reaching 13% in 2022. While resistance rates to quinolones and tetracyclines have shown a marked increase since 2015, additional years of monitoring will be required to fully assess trends in macrolide and carbapenem resistance.

Based on the available data, it can be inferred that campylobacteriosis is a widespread disease which significantly contributes to the burden of foodborne illnesses. Given all these factors, *C. jejuni* has emerged as a particularly compelling species for research. Its widespread presence across various animal and human populations complicates efforts to control and prevent its dissemination. Beyond the typical routes of transmission, *C. jejuni* extends its reach to a diverse range of wild animal species, including marine environments and wastewater systems. The survival and spread of *C. jejuni* in the environment are influenced by factors such as water quality, temperature fluctuations, and interactions with wildlife. It can persist in water, soil, and biofilms, serving as reservoirs for transmission to susceptible hosts, including humans. By applying the One Health approach, an interdisciplinary strategy that examines the interconnectedness of human health, animal well-being, and the environment, we can better understand the fundamental links between *C. jejuni* infections in humans and its presence in wild birds. This approach also sheds light on the potential transfer of antimicrobial resistance (AMR) across different ecosystems. The presence of AMR in wild animals is likely tied to human activities and the resulting pressure on ecosystems, leading some animals to act as "sentinels" for the spread of AMR in the environment. Among birds, gulls are considered a good example for AMR studies due to their specific movement patterns, as they readily acquire resistance genes from the environment or resistant bacteria and quickly transmit them to ecosystems with low resistance rates. With the changing biology of birds caused by anthropization of ecosystems, animal species coexist today as "urban wildlife," increasingly influenced by human presence. One Health approach also enables the study of genetic variability and mutual relationships between them. Defining the genetic determinants of antibiotic resistance and linkage to their clonal relatedness of these bacteria can enable a better understanding of both the acquisition of resistance genes and their spread in the ecosystem. Everything said can lead to new insights into their pathogenesis and epidemiology which is of great importance for public health and food safety.

2. GOAL AND PURPOSE OF THE RESEARCH

2.1. The Goal of the Research

The goal of the study is to define the genomic and phenotypic characteristics of *C. jejuni* strains and to compare differences and similarities across various reservoirs from which they are isolated.

Hypothesis of the research:

1. The specificity of *C. jejuni* clone distribution to individual reservoirs

2. The co-occurrence of strains of *C. jejuni* in both humans and broiler chickens within identical clonal complexes/phylogenetically connected

3. *C. jejuni* strains originating from humans and broiler chickens share identical multiple genetic determinants of antibiotic resistance.

4. Observation of decreased rates of antibiotic resistance in *C. jejuni* strains isolated from wild birds.

2.2. The Purpose of Research

The purpose of this research is to shed light on previously unexplored aspects of the pathogenesis, ecology, and epidemiology of *C. jejuni*, with a specific emphasis on interactions between various reservoirs and the role of antimicrobial resistance in these processes. Through the integration of genetic, phenotypic, and ecological data, the research aims to provide a holistic insight into the intricate mechanisms of spread and adaptation of these bacteria across diverse habitats. This research highlights the role of WGS as a method that can increase the understanding of the underlying mechanisms of resistance and may have a crucial impact in situations where the pathogen cannot be tested for susceptibility to antibiotics with standard procedures. Whole Genome Sequencing can play a crucial role as an alert system for epidemic occurrences, specifically through the identification of clusters of related strains. By enabling early detection of potentially concerning clustering patterns, WGS enhances routine laboratory work with a proactive approach to epidemic monitoring and control. This makes it an invaluable tool in recognizing and responding to infectious threats, thereby supporting timely interventions to curb the spread of infectious agents.

The findings of this research could significantly contribute to the development of strategies for managing campylobacteriosis, enhancing the understanding of connections between human health, wild animals, the environment, and ultimately advancing public health and food safety.

This study represents the first genomic investigation of strains of the species *C. jejuni* collected from different reservoirs, including strains of human origin, strains from broiler chickens, and wild birds in Croatia. Through this research, knowledge about the epidemiology, resistance patterns of *C. jejuni* strains in Croatia, and the mutual influence of different and new reservoirs on the development of resistance in *C. jejuni*, as well as the spread of resistance genes within the entire ecosystem, will be expanded, contributing to the containment of antimicrobial resistance in general.

3. MATERIALS AND METHODS

3.1.Strain collection

3.1.1. Origin of Isolates

Collection consisted of 62 *C. jejuni* isolates, including 46 from human patients with clinical symptoms of diarrhea, 5 from broilers (Gallus gallus), and 10 from wild birds, specifically 2 from Yellow-legged Gulls (Larus michahellis) and 8 from White Storks (Ciconia ciconia).

Human isolates were collected during 2019 and 2020 from patients presenting with clinical symptoms of diarrhea, originating from four independent laboratory centers across Croatia. Additionally, all *C. jejuni* isolates from primary sterile samples available in the collection were included in the study. A total of 46 strains were analyzed, including 10 isolates from primary sterile samples and 35 from stool samples, collected during the same period. The isolates were geographically distributed as follows: 24 from Zagreb, 10 from Split, 11 from Pula, and 1 from Osijek.

Broiler isolates were obtained as part of the Croatian national monitoring program for Campylobacter in broilers, All procedures were carried out in accordance with the amendment to Regulation (EC) 2073/2005, incorporating the Campylobacter process hygiene criterion, as well as Regulation (EC) No 2160/2003.

Wild bird isolates were collected during ecological monitoring studies conducted between November 2016 and March 2020 from cloacal swabs. Gull isolates were obtained from Yellow-legged Gulls (Larus michahellis) breeding in the Croatian Adriatic region. One strain was isolated from an adult bird nesting in a rooftop colony in Zadar, while another was collected from a chick on the islet of Mrkan near Dubrovnik. Stork isolates were primarily derived from White Stork (Ciconia ciconia) chicks in Lonjsko Polje Nature Park, specifically from the villages of Jasenovac and Puska. An additional strain was obtained from a chick near a rubbish tip in Jakuševec, Zagreb.

3.1.2. Bacterial Isolation and Cultivation

Following collection at the Croatian Veterinary Institute laboratory, *C. jejuni* strains were streaked onto Columbia blood agar supplemented with 10% defibrinated sheep blood (Biomerieux, Marcy-l'Étoile, France) and incubated under microaerobic conditions (CampyGen, Thermo Fisher Scientific, Oxoid Limited, Hants, UK) at 42°C for 48 hours. Revived strains were sub-cultured on the same medium and subsequently stored at −80°C for additional analyses as needed. All strains were analyzed through collaborative efforts between the Croatian Veterinary Institute and the University Hospital for Infectious Diseases laboratories.

3.1.3. Species Confirmation

Species confirmation was performed using a multiplex PCR assay as described by Wang et al. (17). This method enables simultaneous detection of genes from five major clinically relevant *Campylobacter* species. DNA extraction was conducted on fresh overnight cultures grown on blood agar under microaerobic conditions. A full loop of pure culture was resuspended in 100 µL of PCR-grade water, and DNA was extracted using the NucleoSpin Microbial DNA Mini Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol.

3.2. Antimicrobial Susceptibility Testing

Strains were revived and sub-cultured on blood agar supplemented with 10% defibrinated sheep blood. After overnight incubation, the *C. jejuni* cultures were utilized for antimicrobial susceptibility testing (AST) using the broth microdilution method. AST was performed for six clinically and epidemiologically relevant antimicrobials in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. EUCAMP3 microplates (Sensititer, Trek Diagnostic Systems Ltd., East Grinstead, West Sussex, UK) were used for the analysis.

Epidemiological cut-off values (ECOFFs) were applied as interpretative thresholds to identify non-wild-type strains potentially harboring resistance mechanisms. EUCAST ECOFFs were used to assess susceptibility to erythromycin (ERY; 1–512 mg/L), ciprofloxacin (CIP; 0.12–32 mg/L), and tetracycline (TET; 0.5–64 mg/L). For ertapenem (ERTA; 0.12–4 mg/L), gentamicin (GEN; 0.25–16 mg/L), and chloramphenicol (CHL; 2–64 mg/L), EFSA ECOFFs were employed due to the absence of EUCAST data for these antibiotics. The *C. jejuni* ATCC 33,560 reference strain was used for quality control to ensure the reliability of the results.

3.3. Whole Genome Seqencing (WGS)

Genomic DNA from 62 C. jejuni isolates was sequenced at MicrobesNG (Birmingham, UK) using the Illumina platform with 2 × 250 bp paired-end reads. The raw trimmed reads and assembled FASTA files were provided alongside a basic bioinformatics analysis pipeline. Kraken version 1.1 software identified the closest available reference genome, and the BWA mem tool version 0.7.11 mapped reads to the reference genome to assess data quality. De novo assembly was performed using SPAdes version 3.15.5, with reads mapped back to the resulting contigs to generate additional quality metrics.

3.3.1. Genetic Distance Analysis

Multi-locus sequence typing (MLST) was applied to de novo assemblies to determine sequence types (ST) and clonal complexes (CC) using the Campylobacter PubMLST database (http://pubmlst.org/campylobacter, accessed on 15 December 2022). To expand the analysis, core genome MLST (cgMLST) was conducted on ST 21 *C.jejuni* strains using cgMLSTfinder (https://cge.food.dtu.dk/services/cgMLSTFinder/, accessed on 15 December 2022) with a loci scheme of 1343 genes. Raw reads in FASTQ format were input for cgMLST analysis, and relatedness among isolates was visualized using a minimum spanning tree generated with Grapetree version 1.5.0.

3.3.2. Antimicrobial Resistance Gene Identification

In silico antimicrobial susceptibility testing (AST) was performed on de novo assemblies using the Resistance Gene Identifier (RGI) from CARD (https://card.mcmaster.ca/home, accessed on 16 December 2022) and ResFinder 4.1 (https://cge.food.dtu.dk/services/ResFinder, accessed on 16 December 2022). Default parameters were applied to identify resistance determinants, with a threshold of 98% for gene identification and 100% for minimum length. Acquired antimicrobial resistance genes and chromosomal point mutations were analyzed on all available *C. jejuni* genomes.

3.3.3. Statistical analysis

Categorical variables were represented as counts and percentages, while numerical variables were given as medians and ranges. The association between categorical variables was tested with the chi-square test or Fisher's exact test, as appropriate. Numerical variables were non-parametrically distributed and compared with the Mann–Whitney U test. All tests were two-tailed with the significance level set to 95%, and a p-value of <0.05 was considered statistically significant. Descriptive statistical analysis was used to further characterize the isolates and assess the frequency of sequence types (ST) depending on the reservoir. Statistical analysis and data visualization were performed in R (version 4.4.1.).

4. RESULTS

A total of 62 *C. jejuni* strains were collected from three distinct reservoirs: humans, broilers, and wild birds. However, due to unreadable sequences identified in three human-derived strains during WGS analysis, these were excluded from the final statistical evaluation to ensure the accuracy and reliability of the results. In the final statistical analysis, 59 isolates of *C. jejuni* were included.

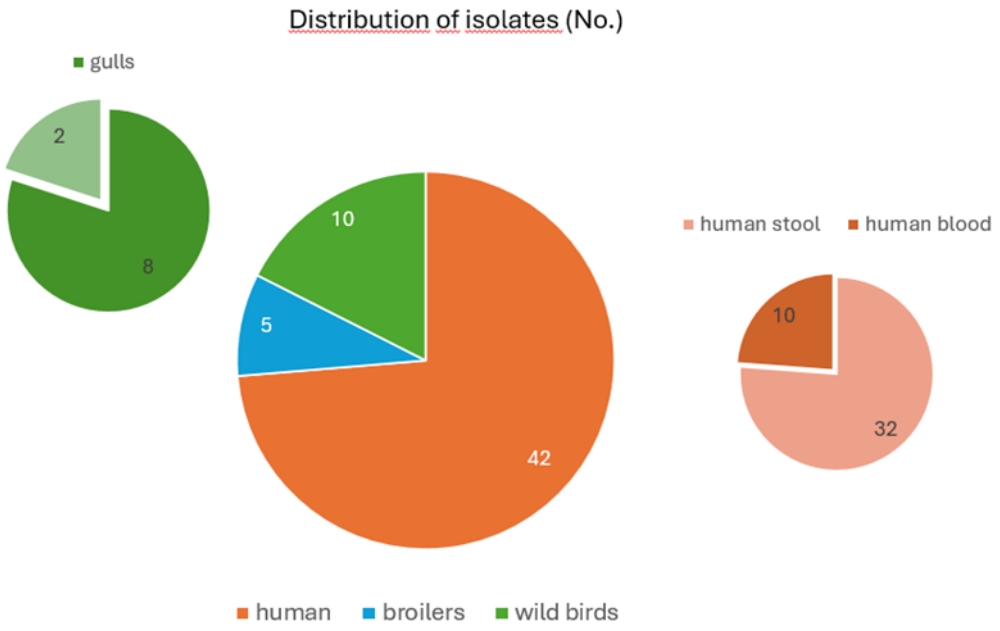
The study analyzed 42 human-derived strains, which included 10 isolates from primary sterile samples (9 from blood cultures and 1 from cerebrospinal fluid, samples ZGI01-ZGI10) and 35 from stool samples. All strains from primary sterile samples, along with 10 stool isolates, were collected in Zagreb at the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" (samples ZG01-ZG10). Additionally, 10 stool isolates were obtained from Split (Public Health Institute of Split-Dalmatia County, samples ST2-ST12), 1 from Osijek (University Hospital Center Osijek, sample OS01), and 9 from Pula (Teaching Institute for Public Health of Istria County, samples PU01-PU11).

The strains originating from gulls were isolated from breeding Yellow-legged Gulls, Larus michahellis, in the Croatian part of the Adriatic Sea. Namely, 1-C-258 was isolated from an adult bird breeding on a rooftop colony in the city of Zadar and 1-C-381 was obtained from pullus sampled on the islet of Mrkan, near Dubrovnik. Most of the stork isolates are from chicks of White Storks, Ciconia ciconia, hatched in the Lonjsko Polje Nature Park (1-C-402, 1-C-405 and 1-C-406 from village Jasenovac; 1-C-410, 1-C-411 and 1-C-412 from village Puška), while 1-C-423 was isolated from a storklet from Jakuševec, Zagreb, breeding just near the rubbish tip.

The broiler isolates were taken from the Croatian Veterinary Institute collection of *C. jejuni* strains found in Gallus gallus chicks bred for meat production while implementing national monitoring programs for *Campylobacter* spp. in broilers. The broiler isolates consisted of two strains from Međimurje County, (CP01, CP14), two from Brod-Posavina County (CP03, CP10), and one from Varaždin County (CP11). Everything was performed according to the amendment of Regulation (EC) 2073/2005 to include Campylobacter process hygiene criterion; regulation (EC) No 2160/2003.

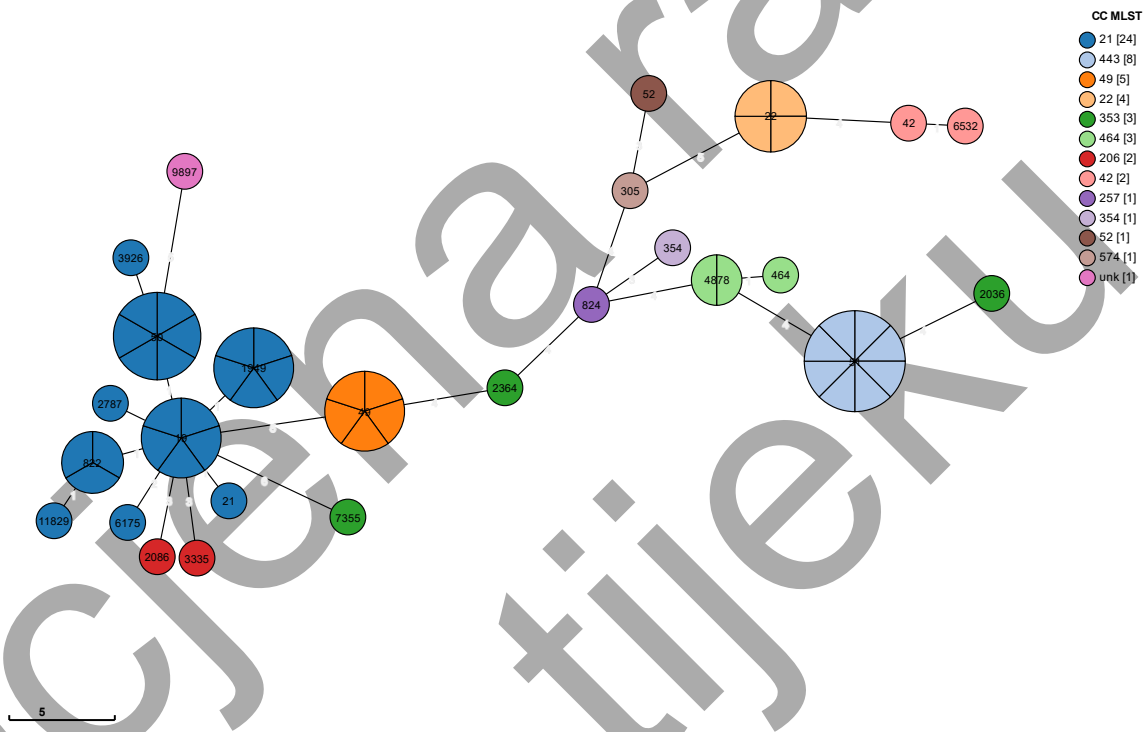
Isolate distribution across different reservoirs is presented in Figure 1, illustrating the data on *C. jejuni* isolates and their respective sources.

Figure 1. Isolate Distribution Across Different Reservoirs



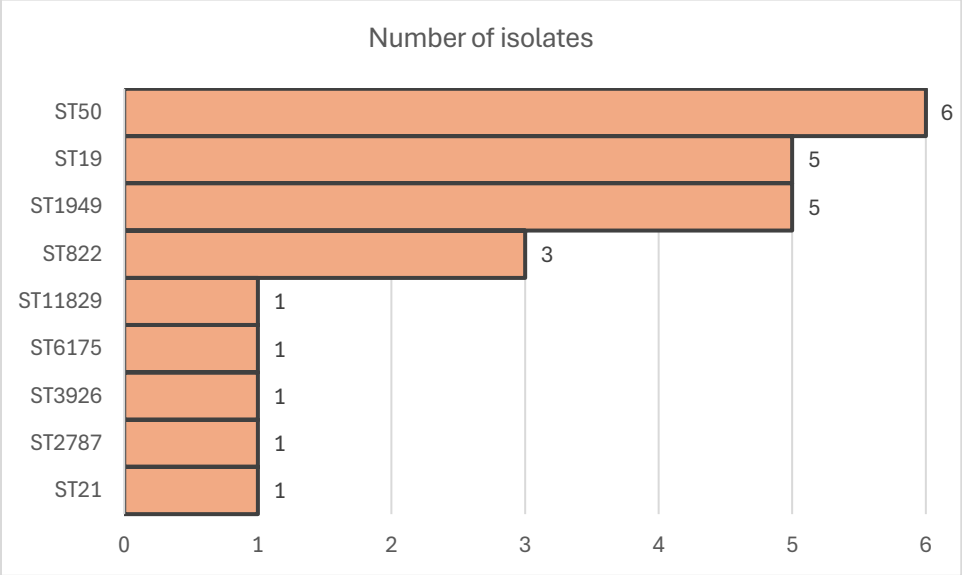
A total of 12 clonal complexes (CCs) were identified. Consequently, 26 different sequence types (STs) were detected, with one remaining undetermined (Figure 2).

Figure 2 Minimum spanning tree (MST) showing relationships between tested strains according to MLST data. Distinct colors are used to distinguish various Clonal Complexes (CCs) identified in *C. jejuni* isolates. The numbers inside the circles represent the identified STs. The number of isolates within each ST is shown by the division of the circle into segments, with each segment representing a portion of the total isolates. The size of each segment visually reflects the proportion of isolates in that ST.



Within the analyzed CC21 *C. jejuni* strains, a total of nine distinct STs were identified, highlighting the genetic diversity within this clonal complex. The distribution of these STs among the isolates is presented in Figure 3, providing an overview of their prevalence.

Figure 3. Distribution of sequence types (ST) within clonal complex 21 (N = 24).



Additionally, a statistical analysis was conducted specifically within the CC21 of *C. jejuni* isolates to further explore potential associations and variations in sequence type distribution between host sources.

The statistical analysis included a comparison of STs within CC21 and the reservoir in which *C. jejuni* isolates were found, specifically assessing differences in ST distribution between human, broiler, and wild bird isolates. Fisher's exact test was applied to determine the statistical significance of these associations, ensuring a robust evaluation of potential reservoir-specific patterns. The results of this analysis are presented in Table 1.

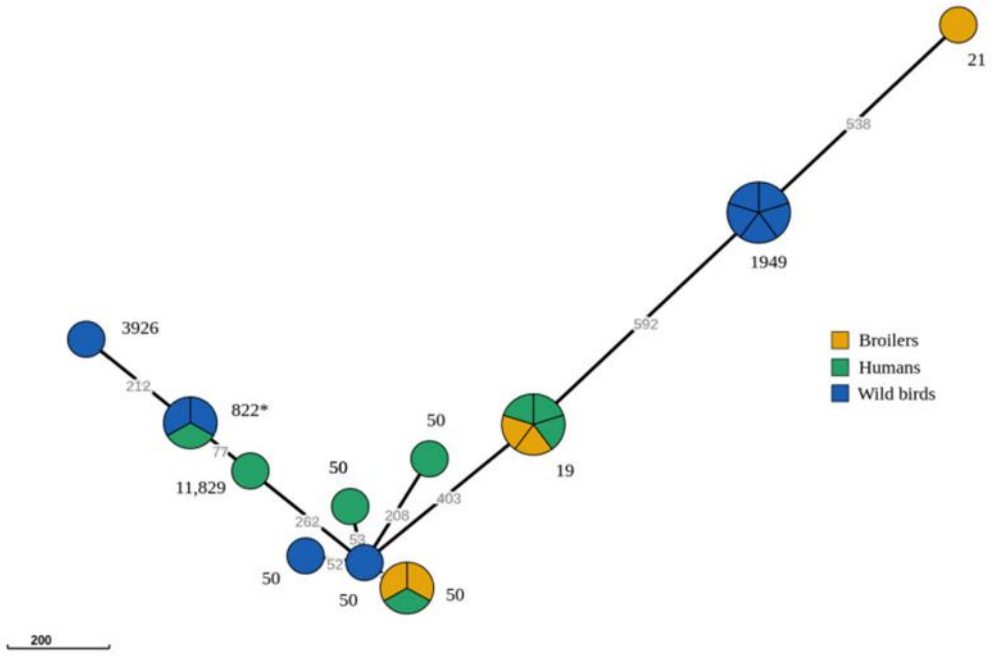
Table 1. Association between clonal complex 21 STs and organism type.

ST	N (%) wild birds	N (%) human+broilers	p*
50	2 (20%)	4 (28.57%)	0.999
1949	**5 (50%)**	**0 (0.0%)**	**0.005**
19	0 (0%)	5 (35.71%)	0.053
822	2 (20%)	1 (7.14%)	0.565
3926	1 (10%)	0 (0.0%)	0.417
21	0 (6.7%)	1 (7.14%)	0.999
6175	0 (0.0%)	1 (7.14%)	0.375
2787	0 (0.0%)	1 (7.14%)	0.375
11829	0 (0.0%)	1 (7.14%)	0.375

ST1949 was found to be significantly more prevalent in wild birds compared to other analyzed reservoirs (50.0% vs. 0.0%, p = 0.005), indicating a strong association with this host

group. No statistically significant differences were observed for other STs, with p-values exceeding the conventional significance threshold ($p > 0.05$). However, ST19 showed a marginally non-significant trend towards being more common in the human and broiler category (0.0% vs. 35.71%, $p = 0.053$), suggesting a possible association that may warrant further investigation with a larger dataset.

Figure 4. Minimum spanning tree (MST) revealing genomic relationship amongst *C. jejuni* ST-21 CC based on cgMLST profiles. Different colors represent different reservoirs of strains. The numbers next to the samples correlate with the identified STs. The asterisk mark indicates clusters that are phenotypically defined as fluoroquinolones wild-type strains.



Additionally, the distribution of STs among human samples was examined to identify patterns or clustering . A comparison of STs isolated from blood samples versus those obtained from other human sources was also conducted to explore potential associations with sample origin (Figure 5, Table 2). These analyses were conducted using Fisher's exact test, to evaluate statistical significance.

Figure 5. Distribution of sequence types (ST) in human isolates (N = 42).

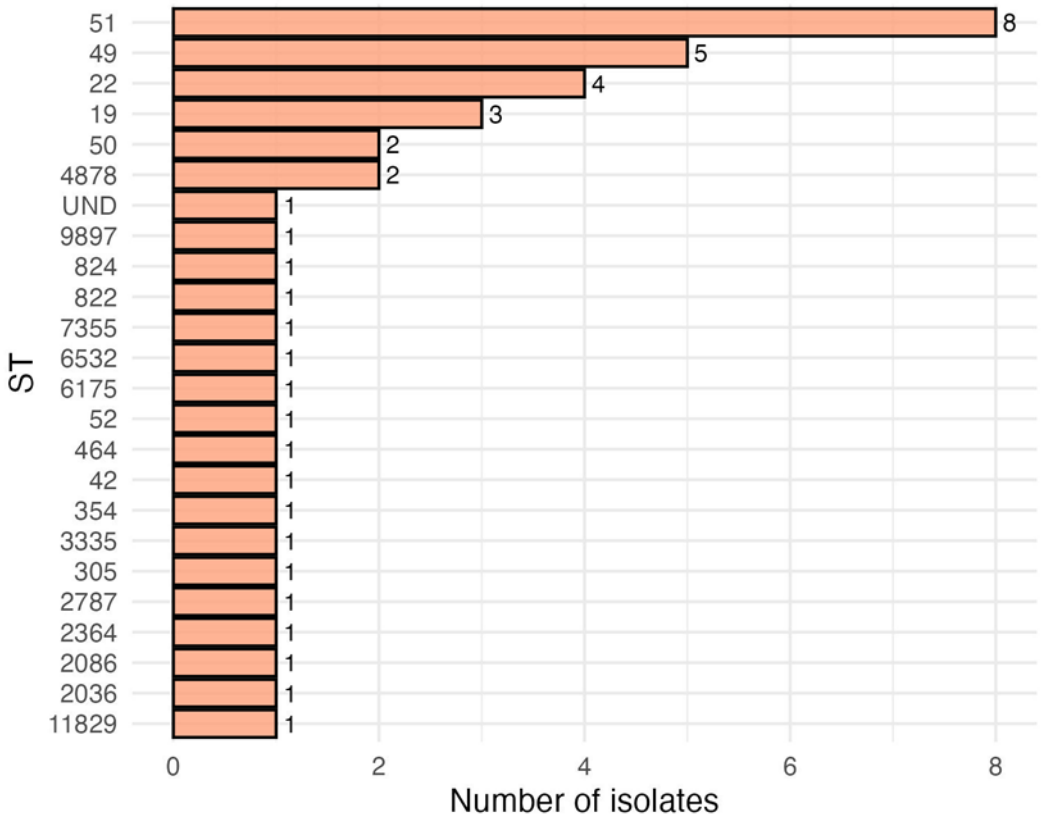


Table 2. Association between STs and human sample type.

ST	N (%) blood	N (%) other samples	OR (95% CI)	p*
51	1 (11.1%)	7 (21.9%)	0.45 (0.01-4.54)	0.659
49	5 (55.5%)	0 (0.0%)	/	**<0.001**
22	2 (22.2%)	2 (6.3%)	4.09 (0.25-65.91)	0.204
19	0 (0.0%)	3 (9.4%)	/	0.999
50	0 (0.0%)	2 (6.3%)	/	0.999
4878	0 (0.0%)	2 (6.3%)	/	0.999
11829	1 (11.1%)	0 (0.0%)	/	0.238
822	0 (0.0%)	1 (3.1%)	/	0.999
2787	0 (0.0%)	1 (3.1%)	/	0.999
6175	0 (0.0%)	1 (3.1%)	/	0.999
52	0 (0.0%)	1 (3.1%)	/	0.999
2364	0 (0.0%)	1 (3.1%)	/	0.999
9897	0 (0.0%)	1 (3.1%)	/	0.999
3335	0 (0.0%)	1 (3.1%)	/	0.999
42	0 (0.0%)	1 (3.1%)	/	0.999
2006	0 (0.0%)	1 (3.1%)	/	0.999
354	0 (0.0%)	1 (3.1%)	/	0.999
464	0 (0.0%)	1 (3.1%)	/	0.999
6532	0 (0.0%)	1 (3.1%)	/	0.999
305	0 (0.0%)	1 (3.1%)	/	0.999
824	0 (0.0%)	1 (3.1%)	/	0.999
2036	0 (0.0%)	1 (3.1%)	/	0.999
7355	0 (0.0%)	1 (3.1%)	/	0.999

ST49 was significantly more common in blood samples compared to other sample types (55.5% vs. 0.0%, **$p < 0.001$**), indicating a strong association with bloodstream infections. ST22 showed a higher occurrence in blood samples than other sources (22.2% vs. 6.3%); however, this association did not reach statistical significance (OR = 4.09, 95% CI: 0.25–65.91, p = 0.204). No statistically significant differences were observed for other STs, with p-values exceeding the conventional threshold (**$p > 0.05$**), suggesting no strong associations between these STs and sample type

A total of five *C. jejuni* isolates belonging to sequence type (ST) 1949 were recovered from samples collected from White Storks (*Ciconia ciconia*) in the Lonjsko Polje Nature Park, Croatia. The isolates were distributed across two sampling locations within the park: Jasenovac and Puška. Specifically, two isolates (1c404 and 1c406) were obtained from samples collected in Jasenovac, while three isolates (1c410, 1c411, and 1c412) were recovered from samples collected in Puška. Genetic characterization revealed that all five isolates belonged to the multilocus sequence typing (MLST) clonal complex (CC) 21. Core genome MLST (cgMLST) analysis further confirmed that all isolates exhibited an identical cgMLST profile, designated as 7821. These findings are summarized in Table 3.

Table 3. Selected details on ST 1949 *C.jejuni* isolates

Isolate No	ORIGIN	Location of the bird	MLST CC	ST	cgMLST
1c404	White Stork, Ciconia ciconia	Lonjsko polje, Jasenovac	21	1949	7821
1c406	White Stork, Ciconia ciconia	Lonjsko polje, Jasenovac	21	1949	7821
1c410	White Stork, Ciconia ciconia	Lonjsko polje, Puška	21	1949	7821
1c411	White Stork, Ciconia ciconia	Lonjsko polje, Puška	21	1949	7821
1c412	White Stork, Ciconia ciconia	Lonjsko polje, Puška	21	1949	7821

Table 4. Distributions of MICs values among tested strains

Isolate ID	Antimicrobial agent (Class) - MIC					
	Erythromycin (Macrolide)	**Ciprofloxacin** (Fluoroquinolones)	**Tetracycline** (Tetracycline)	**Gentamicin** (Aminoglycoside)	**Chloramphenicol** (Amphenicol)	**Ertapenem** (Carbapenem)
ZG01	≤1	16	≤0,5	≤0,25	4	≤0,12
ZG02	≤1	8	≤0,5	0,5	≤2	0,25
ZG03	≤1	8	≤0,5	0,5	≤2	0,5
ZG04	≤1	16	≤0,5	0,5	≤2	≤0,12
ZG06	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ZG07	≤1	8	≤0,5	≤0,25	≤2	≤0,12
ZG08	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ZG10	≤1	≤0,12	≤0,5	0,5	≤2	0,25
ZG11	≤1	8	≤0,5	≤0,25	≤2	0,25
ZG12	≤1	8	≤0,5	0,5	≤2	0,5
ZG13	≤1	16	≤0,5	≤0,25	≤2	≤0,12
ZG14	≤1	8	≤0,5	≤0,25	≤2	0,25
ZGI01	≤1	8	≤0,5	0,5	≤2	≤0,12
ZGI02	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ZGI03	≤1	16	≤0,5	0,5	≤2	0,25
ZGI04	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ZGI05	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ZGI06	≤1	16	≤0,5	0,5	≤2	≤0,12
ZGI07	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ZGI08	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ZGI09	≤1	≤0,12	≤0,5	≤0,25	≤2	≤0,12
ZGI10	≤1	8	≤0,5	0,5	≤2	0,25
OS1	≤1	8	≤0,5	≤0,25	≤2	≤0,12
PU01	≤1	16	≤0,5	0,5	≤2	0,25
PU03	≤1	16	64	0,5	≤2	0,25
PU04	≤1	8	64	0,5	≤2	0,25
PU05	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
PU06	≤1	8	32	0,5	≤2	0,25
PU07	≤1	8	≤0,5	≤0,25	≤2	≤0,12
PU09	≤1	16	64	≤0,25	≤2	0,25
PU10	≤1	8	64	0,5	≤2	≤0,12
PU11	≤1	16	≤0,5	≤0,25	≤2	0,25
ST10	≤1	16	64	0,5	≤2	0,25
ST11	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ST12	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
ST2	≤1	≤0,12	≤0,5	≤0,25	≤2	≤0,12
ST3	≤1	8	≤0,5	0,5	≤2	0,25
ST4	≤1	16	≤0,5	≤0,25	≤2	0,25
ST6	≤1	8	≤0,5	≤0,25	≤2	≤0,12
ST7	≤1	16	64	≤0,25	4	0,5
ST8	≤1	16	≤0,5	≤0,25	≤2	0,25
ST9	≤1	8	64	≤0,25	≤2	0,5
1c258	≤1	8	≤0,5	0,5	≤2	0,25
1c381	≤1	8	≤0,5	0,5	≤2	≤0,12
1c402	4	≤0,12	≤0,5	0,5	≤2	≤0,12
1c404	≤1	8	≤0,5	≤0,25	≤2	≤0,12

Continuated						
1c405	≤1	8	≤0,5	0,5	≤2	≤0,12
1c406	≤1	8	≤0,5	≤0,25	≤2	≤0,12
1c410	≤1	8	≤0,5	0,5	≤2	≤0,12
1c411	≤1	8	≤0,5	≤0,25	≤2	0,25
1c412	≤1	8	≤0,5	≤0,25	≤2	0,25
1c423	≤1	≤0,12	≤0,5	0,5	≤2	≤0,12
CP01	≤1	16	≤0,5	0,5	4	0,25
CP03	4	8	≤0,5	≤0,25	≤2	≤0,12
CP10	≤1	16	≤0,5	0,5	4	0,25
CP11	≤1	8	≤0,5	≤0,25	≤2	0,25
CP14	≤1	8	≤0,5	≤0,25	≤2	0,25

While initial approach was to use the t-test to compare antibiotic MIC values between avian and human isolates, the specific characteristics of the data required the adoption of an alternative method, the Mann-Whitney u-test. The Mann-Whitney u-test, unlike the t-test, is a non-parametric alternative that does not assume normal distribution or equal variances. It is particularly suited for unevenly distributed or small, heterogeneous samples, allowing for a robust comparison of differences in resistance profiles between the two groups while effectively accommodating the non-normality of the data.

Table 5. Distribution of antibiotic resistance scores in humans and broilers vs. wild birds.

	Mean (median, IQR)		
Antibiotic	**Wild birds**	**Humans + broilers**	**U, p***
Ciprofloxacin	6.42 (8, 8-8)	8.37 (8, 0.12-16)	277, 0.347
Gentamicin	0.38 (0.25, 0.25-0.5)	0.49 (0.5, 0.25-0.5)	267.5, 0.426
Erythromycin	1.30 (1, 1-1)	1.06 (1, 1-1)	216.5, 0.236
Ertapenem	0.16 (0.12, 0.12-0.22)	0.21 (0.12, 0.12-0.25)	285.5, 0.235
Tetracycline	0.5 (0.5, 0.5-0.5)	10.62 (0.5, 0.5-0.5)	195, 0.828

*Mann-Whitney U test

No statistically significant differences ($p > 0.05$) were observed between the two groups for any of the tested antibiotics. While median values remained similar across groups, slight variations were noted in the mean resistance scores. Ciprofloxacin showed a higher mean resistance score in humans + broilers (8.37) compared to wild birds (6.42), but the difference was not statistically significant ($U = 277$, $p = 0.347$). Similarly, gentamicin resistance was slightly higher in humans + broilers (mean = 0.49) than in wild birds (mean = 0.38), though without statistical significance ($U = 267.5$, $p = 0.426$).

For erythromycin and ertapenem, wild birds exhibited slightly higher mean resistance scores (1.30 and 0.16, respectively) compared to humans + broilers (1.06 and 0.21), but again, no significant association was observed ($p = 0.236$ and $p = 0.235$, respectively). Tetracycline resistance showed the largest discrepancy in mean values (wild birds: 0.5 vs. humans + broilers: 10.62), yet this difference was not statistically significant ($U = 195$, $p = 0.828$).

These findings indicate that, while variations in resistance profiles exist, no statistically significant differences were detected between the two groups for the tested antibiotics, suggesting similar resistance patterns across reservoirs.

Table 6. Distribution of antibiotic resistance scores in the analysed organisms.

	Mean (median, IQR)		
Antibiotic	**Birds**	**Humans**	**U, p***
Ciprofloxacin	8.02 (8, 8-8)	8.04 (8, 0.12-16)	315, 0.999
Gentamicin	0.38 (0.5, 0.25-0.5)	0.41 (0.5, 0.25-0.5)	350, 0.465
Erythromycin	1.40 (1, 1-1)	1.00 (1, 1-1)	273, **0.018**
Ertapenem	0.19 (0.25, 0.12-0.25)	0.20 (0.12, 0.12-0.25)	298, 0.735
Tetracycline	0.5 (0.5, 0.5-0.5)	11.57(0.5, 0.5-64)	262, 0.077

*Mann-Whitney U test

The comparison of antibiotic resistance scores between birds and humans was performed using the Mann-Whitney U test, a non-parametric method suitable for non-normally distributed data.

Isolates from birds exhibited a significantly higher reisistance to erythromycin than isolates from humans ($p = 0.018$).

For ciprofloxacin, gentamicin, ertapenem, and tetracycline, no statistically significant differences were found between birds and humans ($p > 0.05$). Ciprofloxacin resistance was nearly identical between both groups ($U = 315$, $p = 0.999$). Gentamicin resistance was slightly higher in humans (mean = 0.41) compared to birds (mean = 0.38), but this difference was not statistically significant ($U = 350$, $p = 0.465$). Ertapenem resistance showed minor variations, with birds exhibiting a slightly higher median resistance score, though the difference was also non-significant ($U = 298$, $p = 0.735$).

For tetracycline, isolates from humans showed a substantially higher mean resistance score (11.57) compared to birds (0.5), but this difference did not reach statistical significance ($U = 262$, $p = 0.077$), possibly due to data variability or sample size constraints.

Table 7. Phenotypic and genotypic results of tested strains (RD—resistance determinants, W—wild-type, NW—non-wild-type).

Isolate Nr.	Chloramphenicol (Amphenicol)		Erythromycin (Macrolide)		Gentamicin (Aminoglycoside)		Ciprofloxacin (Fluoroquinolone)		Tetracycline (Tetracycline)		Ertapenem (Beta-Lactam)	
	16 mg/L	RD	8 mg/L	RD	2 mg/L	RD	0.5 mg/L	RD	1 mg/L	RD	1 mg/L	
OS1	W	-	W	-	W	-	NW	-	W	-	W	
PU01	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
PU03	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	NW	*tet* (O/32/O)	W	
PU04	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	NW	*tet* (O/32/O)	W	
PU05	W	-	W	-	W	-	W	-	W	-	W	
PU06	W	-	W	-	W	*ant* (6)-Ia	NW	*gyrA T86I aca-ata*	NW	-	W	
PU07	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
PU09	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	NW	*tet* (O/32/O)	W	
PU10	W	-	W	-	W	*ant* (6)-Ia	NW	*gyrA T86I aca-ata*	NW	*tet* (O/32/O)	W	
PU11	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ST10	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	NW	-	W	
ST11	W	-	W	-	W	-	W	*gyrA T86I aca-ata*	W	-	W	
ST12	W	-	W	-	W	-	W	-	W	-	W	
ST2	W	-	W	-	W	-	W	-	W	-	W	
ST3	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ST4	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ST6	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ST7	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	NW	*tet* (O/32/O)	W	
ST8	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ST9	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	NW	*tet*(L)	W	
ZG01	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZG02	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZG03	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZG04	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	

ZG06	W	-	W	-	W	-	W	-	W	-	W	
ZG07	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZG08	W	-	W	-	W	*aac* (3)-XI	W	*gyrA T86I aca-ata*	W	-	W	
ZG10	W	-	W	-	W	-	W	-	W	-	W	
ZG11	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZG12	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZG13	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZG14	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZGI01	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZGI02	W	-	W	-	W	-	W	*gyrA T86I aca-ata*	W	-	W	
ZGI03	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
ZGI04	W	-	W	-	W	-	W	-	W	-	W	
ZGI05	W	-	W	-	W	-	W	-	W	-	W	
ZGI06	W	-	W	-	W	-	NW	*gyr*A T86I aca-ata	W	-	W	
ZGI07	W	-	W	-	W	-	W	-	W	-	W	
ZGI08	W	-	W	-	W	-	W	-	W	-	W	
ZGI09	W	-	W	-	W	-	W	-	W	-	W	
ZGI10	W	-	W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
CP01	W		W	-	W	-	NW	*gyr*A T86I aca-ata	W	-	W	
CP03	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
CP10	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
CP11	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
CP14	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
1c258	W		W	-	W	-	NW	-	W	-	W	
1c381	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
1c402	W		W	-	W	-	W	-	W	-	W	
1c404	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
1c405	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
1c406	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
1c410	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	

1c411	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
1c412	W		W	-	W	-	NW	*gyrA T86I aca-ata*	W	-	W	
1c423	W		W	-	W	-	W	-	W	-	W	

Resistance to ciprofloxacin (fluoroquinolones) was genotypically the most represented and extremely high with mutation in *gyr*A detected in 43 out of the 49 tested strains 86%). All the tested strains have the same T86I mutation. It is a point mutation resulting in a substitution of ACA with ATA in the quinolone resistance-determining region (QRDR) of the *gyr*A gene. Four strains without a detected *gyr*A mutation clustered specifically to the cgST-115 clone, found in two stork strains and two human strains.

A total of six distinct genetic determinants of antimicrobial resistance (AMR) were identified among the analyzed *C. jejuni* isolates. The *gyrA* mutation, associated with quinolone resistance, was the most frequently detected determinant, conferring reduced susceptibility to fluoroquinolones through alterations in the DNA gyrase enzyme. Resistance to tetracyclines was linked to the presence of the *tet*(L) gene and *tet*(O/32/O) hybrid gene, both of which encode ribosomal protection proteins that mitigate the inhibitory effects of tetracyclines. Additionally, resistance to aminoglycosides was associated with the presence of *ant(6)-Ia* and *aac(3)-XI* genes, which encode aminoglycoside-modifying enzymes that inactivate the antibiotic, thereby conferring resistance. Furthermore, a genetic determinant belonging to the OXA-61-like group was identified, which confers resistance to narrow-spectrum beta-lactams. However, this determinant was not considered significant in the context of this study, as it is not associated with resistance to carbapenems.

Table 8. Comparison between phenotypic and genotypic antimicrobial resistance

ANTIMICROBIAL AGENT (CLASS)	PHENOTYPE SUSCEPTIBLE		PHENOTYPE RESISTANT	
	Genotype RESISTANT	Genotype SUSCEPTIBLE	Genotype RESISTANT	Genotype SUSCEPTIBLE
CHLORAMPHENICOL (AMPHENICOL)	0	59	0	0
ERYTHROMYCIN (MACROLIDE)	0	59	0	0
GENTAMICIN (AMINOGLYCOSIDE)	3	56	0	0
CIPROFLOXACIN (FLUOROQUINOLONE)	3	12	42	2
TETRACYCLINE (TETRACYCLINE)	0	51	5	3
ERTAPENEM (BETA-LACTAME)	0	59	0	0

Among the six tested antimicrobial agents, four exhibited complete concordance between genotypic and phenotypic susceptibility, with all isolates being classified as susceptible (100%). Genotypic antimicrobial resistance (AMR) was detected in only two antimicrobial classes: fluoroquinolones (ciprofloxacin) and tetracyclines (tetracycline).

Genotypic resistance to ciprofloxacin (fluoroquinolone) was the most prevalent, observed in 77.8% of phenotypically resistant isolates (42 out of 54 resistant samples, $p < 0.001$). Resistance determinants to tetracycline was detected in 5 out of 8 phenotypically resistant samples (62.5%), showing a significant but lower concordance ($p = 1.1 \times 10^{-5}$). No genotypic determinants were identified for phenotypic resistance to other tested antimicrobial agents, including chloramphenicol, erythromycin, gentamicin, and ertapenem, all of which showed complete genotypic susceptibility.

These findings indicate that, while fluoroquinolone and tetracycline resistance are well-supported by genetic markers, other important resistance mechanisms may also play a role, including efflux pump overexpression, porin loss, and additional regulatory adaptations that influence antimicrobial susceptibility.

The analysis of phenotypic and genotypic antimicrobial resistance revealed several discrepancies between the two methods, highlighting potential limitations in genetic-based resistance prediction and the presence of alternative resistance mechanisms. One of the key

findings was the absence of known genetic resistance determinants in certain phenotypically resistant isolates, particularly for ciprofloxacin and tetracycline. Two ciprofloxacin-resistant isolates lacked corresponding genotypic markers, suggesting the involvement of alternative resistance mechanisms such as efflux pumps or mutations outside commonly screened resistance genes. Similarly, three tetracycline-resistant isolates did not carry identifiable genetic resistance determinants, indicating the possibility of novel resistance genes or epigenetic modifications contributing to resistance.

Conversely, some isolates exhibited genotypic resistance without phenotypic confirmation, particularly for gentamicin, where three isolates harbored known resistance genes but remained phenotypically susceptible. This discrepancy could be attributed to low expression levels of the resistance genes or compensatory mutations that mitigate resistance at the functional level. In contrast, complete concordance was observed for chloramphenicol, erythromycin, and ertapenem, where all isolates were classified as susceptible by both genotypic and phenotypic analyses.

The degree of genotype-phenotype concordance varied across antimicrobial agents. The highest agreement was found for ciprofloxacin resistance, where 77.8% of phenotypically resistant isolates carried corresponding genotypic markers ($p < 0.001$). For tetracycline resistance, the concordance was lower (62.5%, $p = 1.1 \times 10^{-5}$), suggesting the involvement of additional resistance factors. In contrast, gentamicin resistance genes were detected in only a small proportion of isolates (5.1%) and did not always correlate with phenotypic resistance.

These findings underscore the complexity of AMR expression and suggest that genotypic testing alone may not always be sufficient to predict phenotypic resistance accurately. The observed discrepancies emphasize the need for complementary phenotypic testing and further investigation into regulatory, mutational, efflux-mediated, and other resistance mechanisms that influence antimicrobial susceptibility. Nevertheless, WGS has proven to be a valuable tool for predicting resistance, providing important insights into the genetic determinants of AMR and serving as a reliable method for surveillance and risk assessment.

5. DISCUSSION

This thesis is primarily focused on performing genetic analysis of *C. jejuni* isolates obtained from various specific niches, encompassing human, broiler, and wild bird isolates. The objectives were to investigate clonal distribution, detect potential overlaps, and characterize antibiotic susceptibility patterns. The rationale for selecting this bacterium for the study was derived from several factors: its innate genetic transformability, its widespread prevalence in infections, and most importantly, its role as a predominant species causing campylobacteriosis—a zoonotic disease of major global public health concern. Additionally, with the growing global concern regarding antimicrobial resistance, this bacterium has become even more relevant for further investigation.

Several authors in Croatia have conducted genetic studies on *C. jejuni*, including both surveillance of antibiotic resistance and epidemiological analysis. Carev and colleagues conducted a study on human *Campylobacter* spp. strains, focusing specifically on the Split-Dalmatia County region. The research emphasized the microbiological characteristics and antibiotic resistance profiles of the strains, while genetic analysis was used to further investigate the epidemiological features of campylobacteriosis (62,63). Strains of *C. jejuni* were collected as part of a population-based laboratory surveillance program for campylobacteriosis in the Split-Dalmatia County from May 2012 to May 2013. The study included 153 strains from stool samples of hospitalized gastroenteritis patients, as well as non-hospitalized patients. The results showed that 60% of the strains were resistant to ciprofloxacin, while 24% showed resistance to tetracycline. Among tetracycline-resistant strains, 89% were co-resistant to ciprofloxacin, with a significant increase in such isolates after 2010. Pulsed Field Gel Electrophoresis genotyping revealed that these *C. jejuni* strains were associated with several clonal PFGE genotypes (*Sma*I S12, 17, 18, and 24).

Mikulić and colleagues conducted a similar study on *C. jejuni* strains, but focused exclusively on strains isolated from chicken meat (64). They examined strains from several regions in Croatia, collected from chicken meat samples from various retail outlets to determine incidence and identify genotypes. The analysis of 241 samples revealed *Campylobacter* spp. in 74% of the samples, indicating a notably high prevalence rate. Most of the isolates were linked to *C. jejuni*, found in 53% of the samples, while *C. coli* was detected in 15%. Through MLST analysis, several STs were identified among eight *C. jejuni* strains and four *C. coli* isolates. The analysis demonstrated significant polyclonality, identifying the following STs in *C. jejuni*: ST25, ST51, ST918, ST2036, ST4878, and the novel ST6182. They were associated with the following clonal complexes: CC460, CC443, CC45, CC48, CC353, and CC464.

Cobo-Díaz et al. published an extensive analysis of *C. jejuni* and *C. coli* strains based on data from WGS in public repositories (65). The aim of their research was to define the association between the most common CCs and STs, compare the prevalence of antimicrobial resistance genes, and correlate resistance patterns with countries/continents, sources of isolation, and clonal relationships, while assessing changes in these patterns over the past 20 years. They analyzed a total of 39,798 *C. jejuni* genomes and 11,920 *C. coli* genomes. The analysis of *C. jejuni* genomes revealed 41 different CCs, with the most prevalent being ST21 (23.8% of analyzed genomes), followed by ST353 and ST45. The most common sources of *C. jejuni* isolates were humans, followed by chickens, cows, and sheep. Resistance to aminoglycosides and macrolide antibiotics was low (6.7%; 0.5%), while resistance to quinolones was 28.7%. On a global scale, the distribution of *C. jejuni* CCs varied across continents. ST21 was dominant in North America, Europe, and Asia; ST353 in South America and Africa, while ST354 prevailed in Oceania.

Although campylobacteriosis is primarily associated with domestic and breeding animals, recent findings suggest that wild birds, particularly gulls and storks, as well as different water bodies and the broader environment, also play an increasingly recognized role as reservoirs and dissemination sites of *Campylobacter s*pp., as well as serving as specific niches for acquiring and spreading resistance genes.

While existing knowledge suggests that the clonal distribution and sensitivity of *C. jejuni* strains in humans should closely align with those found in broilers and other domestic animals, this research aimed not only to confirm these established facts but also to investigate relationship between human and less-explored ecological niches. Specifically, focus was placed on the roles of wild animals, specifically wild birds, to better understand their significant impact on the epidemiology of *C. jejuni.* The aim was to demonstrate overlaps between wild birds, humans, and broilers. Over time, the wild birds' ecological niche is increasingly exposed to selective antibiotic pressure from human food remnants and water sources, which are significant reservoirs for antibiotics, resistant bacteria, and resistance genes, contributing to resistance development and horizontal gene transfer. The identification of *C. jejuni* strains clustering within the CC21 circulating among humans, broilers, and wild birds underscores the significant role these avian species play in the transmission chain. With the ongoing urbanization of ecosystems and climate change, wild animal species are compelled to adapt to their ecology, coexisting today as urban wildlife within human settlements. This adaptation often leads to the loss of their natural habitats, bringing them into closer proximity with humans,

domestic animals, and wastewater. Consequently, they are increasingly exposed to antibiotic pressure from environmental sources, wastewater, and human food, heightening the risk not only of acquiring but also developing new resistance genes. Given their capacity for long-distance travel within short timeframes, wild birds are presumed to facilitate the promotion and acquisition of resistance genes, thereby disseminating pathogens and resistant bacterial strains across diverse geographical areas. Their mobility enables them to act as sentinels for the spread of both resistant and particularly virulent strains.

The collection of *C. jejuni* human strains presented in this research reveals a polyclonal genetic background which aligns with current data. The study identified 15 clonal complexes, and 23 sequence types of *C. jejuni* strains, with ST51, ST50, and ST49 being the most predominant. This high genetic diversity aligns with other studies on MLST sequence types of *C. jejuni* isolated from humans. Notably, ST51, one of the predominant sequence types in our findings, is among the top ten STs isolated in Europe (42, 43). European studies have identified several predominant sequence types circulating among patients with campylobacteriosis, including ST21, ST22, ST45, ST48, ST53, ST257, and ST267 (66–70). Out of the STs predominant in Europe, in this research, we identified only the ST22. Despite the polyclonal diversity, *C. jejuni* strains from primary sterile human specimens appear to be genetically more uniform. Given that the population of collected *C. jejuni* strains of human origin included those isolated from blood, one of the study's objectives was to compare these blood-derived strains with those originating from the gastrointestinal system. It is very rare to find virulent invasive strains in humans, however, invasive strains can still appear. The aim of this research was to identify differences in antibiotic susceptibility between these two populations of strains, ascertain whether the blood-derived strains are more susceptible or resistant, and determine if certain sequence types were more frequently represented among blood isolates, which might indicate specific virulence factors enabling them to penetrate the bloodstream successfully. One of the hypotheses was that invasive *C. jejuni* strains would be more susceptible to antibiotics than those isolated from the gastrointestinal system. Hypothesis was based on the widely accepted concept that, while resistance genes are essential for bacterial survival in the presence of antibiotics, they simultaneously impose a fitness burden, often leading to reduced virulence and pathogenicity. Also, *C. jejuni* strains causing more severe clinical manifestations do not typically remain on mucosal surfaces for extended periods, thereby reducing the opportunity for genetic exchange with surrounding microbiota and limiting exposure to antibiotic pressure leading to resistance selection. This study’s analysis shows that *C. jejuni* strains obtained from

blood cultures are genetically more uniform, displaying an epidemiologically more homogeneous pattern, exhibiting lower rates of quinolone resistance compared to the overall average. The majority of strains clustered within the same clone, ST49 (CC49), and were phenotypically and genotypically characterized as wild type. It is evident that the *C. jejuni* strain ST49 has emerged as the predominant blood derived strain suggesting that this might be a strain with more virulence determinants. Furthermore, the analysis supported the hypothesis that invasive *C. jejuni* strains display a more susceptible phenotype compared to those originating from the gastrointestinal tract. This can be explained by the fact that *C. jejuni* strains that persist on mucosal surfaces, leading to subclinical or prolonged infections, or colonize the mucosa and cause relapses of infections due to immune deficiencies, have a higher potential to develop resistance. This is either due to antibiotic pressure or genetic material exchange with surrounding bacteria, as they tend to live longer on gastrointestinal mucosa. The only STs most commonly reported in patients with confirmed campylobacteriosis in Europe identified in this study were ST22 and ST51. The frequency of particular genotypes generally varies between countries and is influenced by multiple factors. Possible factors influencing this genotypic diversity and epidemiology of different STs include variations in food sources, animal reservoirs, seasons, and different levels of zoonotic transmissions and rates of horizontal gene transfer (71,72).

Besides the identification of the most common ST types collected in the study, a significant prevalence of *C. jejuni* isolates belonging to the CC21 was observed, spanning across multiple niches, including strains from humans, broilers, and wild birds. After identifying the clustering of isolates within CC21, and their presence in overlapping niches, including humans, broilers, and wild birds, the subsequent study concentrated on this clonal complex. This observation led to the hypothesis that a common *C. jejuni* clone might be present across these niches. The analysis of strains within the CC21 confirmed the persistence of typical polyclonality, even within a single clonal complex, with the distribution of *C. jejuni* ST types being highly specific to particular reservoirs. Within CC21 among *C. jejuni* strains originating from wild birds, several sequence types were identified. ST1949 emerged as the most prevalent, observed exclusively in White Storks, and showed a strong association with fluoroquinolone resistance. This represents the first documented occurrence of ST1949 in a wild bird population (11), as it has previously been found only in *C. jejuni* isolates from humans and broilers. These findings suggest that ST1949 may provide further evidence of a potential link between wild birds and other niches explored in this study. The phenomenon of clonal clustering observed in

this study can likely be attributed to the fact that all these storks shared the same natural habitat, Lonjsko Polje. Considering the information provided, along with the fact that ST1949 has so far been observed only in *C. jejuni* isolates from humans and broilers, this shared environment suggests a possible epidemiological link. It indicates that storks may have acquired this strain through exposure to contaminated human waste and environmental waters. Among the other sequence types of *C. jejuni* strains from wild birds, ST50 and ST822 emerged as particularly interesting, as these STs were found across all the observed epidemiological niches. The cgMLST allele profile analysis of ST50 strains demonstrated genetic relatedness exclusively among strains within the wild bird niche, with no genetic connection to ST50 strains from humans and broilers. In contrast, a clear genetic relatedness was observed between ST50 strains originating from humans and broilers, highlighting a distinct separation between the wild bird niche and the other sources, which aligns with previous research. Finally, the phylogenetic analysis of strains within the ST822 cluster, which were found in both humans and storks, confirmed a potential overlap between strains from humans and wild birds. According to the cgMLST allele profile analysis, this cluster was identified to be structured from identical isolate types (cgST115) indicating the confirmed genetic relatedness, thereby supporting one of the primary hypotheses of the study. These findings suggest potential transmission pathways and shared ecological niches between these hosts. The ST822 isolates were also indistinguishable by their results in phenotypic antibiotic susceptibility tests and analysis of potential resistance gene carriage, defined as wild-type strains with no acquired resistance.

The results of this research suggest that the *C. jejuni* strains within CC21 exhibit high resistance to fluoroquinolones which is consistent with findings from an earlier investigation (23). Notably, this high resistance was not observed in strains belonging to ST822 and the singleton ST6175, which were classified as wild-type strains. All detected mutations in the *gyrA* gene were characterized by the T86I point mutation, specifically the nucleotide change from ACA to ATA. This mutation is the most prevalent worldwide and is especially common in strains found in humans and poultry (23,24). While prior research has linked CC21 to significant tetracycline resistance, this research did not observe this association (25). Based on *in vitro* antibiotic susceptibility tests for other classes of tested antibiotics (macrolides, tetracyclines, aminoglycosides, chloramphenicol) and genetic analysis of resistance determinants, we can conclude that the *C. jejuni* strains from CC21 have not acquired additional resistance genes.

Considering the clinically important antibiotics for campylobacteriosis, resistance to quinolones, tetracycline and macrolides was analyzed in all study strains. Other classes of antibiotics have also emerged as significant areas of study. In this context, it was important to investigate resistance to carbapenems and aminoglycosides as these antibiotics are used to treat severe cases. Evaluating resistance to macrolides among isolates was essential, as macrolides are the primary choice of antibiotics for treating *C. jejuni*. In Croatia, *C. jejuni* consistently shows low resistance rates to macrolides, despite the high use of these antibiotics in human medicine and the import of poultry from EU/EEA countries, where macrolide resistance rates are significantly higher (up to 60%). Special attention was given to monitoring *C. jejuni* and its susceptibility to carbapenems, given that carbapenemase-producing strains currently pose one of the most urgent global concerns within *Enterobacterales*, *Pseudomonas*, and *Acinetobacter* species.

Due to the extensive historical use of fluoroquinolones in both veterinary practices and meat production to enhance growth, it was anticipated that resistance in *C. jejuni* to this antibiotic group would arise and propagate under selective pressure. Once it emerged in broilers, it was reasonable to anticipate that *C. jejuni* isolates with fluoroquinolone resistance would eventually be transmitted to humans, either through the consumption of the meat or close contact with the birds. Moreover, wastewater could disperse antibiotics and resistant bacteria into various aquatic reservoirs accessible to wild animals, thereby facilitating the spread of resistance within these ecological niches.

Given the long-term selective pressure from antibiotics on both animals and humans mentioned before, it was expected that the genetic determinants of resistance to both fluoroquinolones and tetracyclines would be present in strains originating from both human and broiler reservoirs. Considering their strong epidemiological connection, a similar occurrence of these resistance genes was anticipated in both sources, and at the same time higher compared to those originating from wild birds. However, a markedly high resistance to fluoroquinolones was found in *C. jejuni* isolates originating from all three observed niches, including those from wild birds. While the majority of STs in the study exhibited resistance to fluoroquinolones, several STs were found to be highly associated with quinolone resistance, namely, ST51 (CC443), ST50 (CC21), and ST22 (CC22). Unlike in our study, the Fiedoruk group detected genetic determinants associated with fluoroquinolones resistance in just 32% of the ST51 strains (54). Conversely, the strains defined as ST822 (CC21) and ST-49 (CC49) were classified

as wild-type, exhibiting MICs below the ECOFF for all antibiotics tested. Diverse findings were described throughout the studies.

While the high fluoroquinolone resistance rates among *C. jejuni* isolates from humans (69%) and broilers (100%) was anticipated and consistent with national data over the years, the high fluoroquinolone resistance found in wild birds (80%) was surprising. This unexpected finding can be explained by the fact that nearly all isolates from the storks belonged to the same sequence type, ST1949 (CC21), which was found to be strongly associated with fluoroquinolone resistance. This suggests that ST1949 may provide additional evidence of a potential link between wild birds and other niches examined in this study. Unfortunately, we were unable to confirm this connection, as ST1949 was observed exclusively in wild birds, preventing us from conducting a comprehensive phylogenetic analysis. This phenomenon of clonal clustering is likely due to the fact that all these storks shared the same natural habitat, Lonjsko Polje. This shared environment suggests an epidemiological link, indicating that storks may have acquired the strain from exposure to contaminated human waste and water sources.

Wastewater management in Kutina is known as a significant environmental concern, especially due to its impact on Lonjsko Polje, a protected wetland. The city faces challenges in managing industrial and municipal wastewater, which frequently enters watercourses that flow into the wetland, resulting in contamination. Additionally, a significant source of pollution is the untreated sewage from Repušnica, which accumulates in the wetland and degrades water quality, posing risks to local wildlife and compromising traditional uses of the area, such as livestock watering and fishing (73). Such contact can result in gene mutations developing under antibiotic pressure or direct acquisition of resistance genes from human waste or wastewater, thereby spreading resistance within their ecological niches and beyond. According to these data, it could be inferred that wild birds are under more significant selective antibiotic pressure due to changes in their ecology than previously thought.

Given that national surveillance data show a high level of tetracycline resistance in *C. jejuni* strains from both humans and broilers, the analysis was expected to confirm this high resistance. However, this was not observed in this study. While prior research has linked CC21 with significant tetracycline resistance, this study did not observe such association. The analysis of strains in this study revealed that tetracycline resistance was detected exclusively in human strains, with a resistance rate of 19%, which is considerably lower than the national average of 40% for human-derived isolates. This resistance was predominantly linked to the presence of the mosaic gene *tet*(O/32/O). Alongside this gene, the *tet*(L) gene was also identified, indicating

multiple genetic mechanisms contributing to resistance. The analysis also revealed that several strains with MIC higher than the ECOFF lacked identifiable genetic determinants that could account for their resistance. This suggests the potential for an unidentified resistance mechanism, possibly involving novel mutations, efflux pump activity, or membrane porin impermeability. These findings highlight the complexity of antibiotic resistance and suggest that some strains may harbor yet undiscovered genetic factors contributing to their resistance profiles.

Phenotypic analysis indicated that all tested strains in the aminoglycoside antibiotic group were classified as wild types, displaying MICs below the defined ECOFF values. However, resistance determinants were detected in 3 isolates. The mutations identified in the genetic analysis were *ant(6)-Ia* and *aac(3)-XI*. For aminoglycoside resistance, genetic markers such as *ant(6)Ia* and *aac(3)-XI* mutations were detected, with *ant(6)-Ia* being the most prevalent. This finding is not unexpected. The mere presence of a gene for enzyme production does not necessarily imply that the strain actively produces the enzyme. Additionally, it is well-known that these genes encode enzymes with varying affinities for different antibiotics within the same class (74,75). Consequently, phenotypic resistance may be observed against certain members of this antibiotic group, while others remain unaffected. Given that gentamicin was used as the class representative while testing susceptibility to aminoglycosides, it can be inferred that the detected resistance determinants do not exhibit a high affinity for the tested antibiotic.

All strains in the study were phenotypically tested for susceptibility to carbapenems, with none showing MICs above the ECOFF, classifying them all as wild type. However, genes associated with potential beta-lactam resistance, particularly *bla*OXA, were detected in most isolates, with several variants identified. The majority of these genes belong to the *bla*OXA-61-like group, with the OXA-193 variant being the most prevalent. These genes encode enzymes classified as class D β-lactamases, OXA-61. Although enzymes in Ambler class D β-lactamases often demonstrate ability to hydrolyze carbapenems, no phenotypic resistance to carbapenems was observed, as noted in previous studies. This result was not surprising, as this group of enzymes are primarily known as oxacillinases, targeting β-lactams with a narrower spectrum of activity compared to carbapenems (76). This particular gene group originates from Pseudomonas and is known to be responsible for resistance to narrow-spectrum β-lactams. In conclusion, although genetic determinants were identified in the strains, all tested strains in the

study still exhibited low MICs for carbapenems. This suggests that the presence of the *bla*OXA-61 gene group alone is not sufficient to confer phenotypic resistance to carbapenems.

One of the main objectives of this study was to evaluate the effectiveness of WGS in providing reliable diagnostic information. The data analysis demonstrated a strong correlation between genotypic markers and the phenotypic expression of antibiotic resistance, emphasizing the potential of WGS as a valuable tool in routine laboratory work, particularly for predicting resistance. This is especially important in cases where standard antibiotic susceptibility testing methods are insufficient. Furthermore, even when traditional procedures provide the necessary information, WGS offers a significant advantage during epidemic situations by rapidly identifying clusters, enabling quicker detection and timely alerts of clustering events.

The whole genome sequencing proved to be a valuable tool in investigating the underlying mechanisms of antibiotic resistance. Through the integration of genetic, phenotypic, and ecological data, this research provided a holistic insight into the intricate mechanisms of spread and adaptation of these bacteria across diverse habitats.

6. CONCLUSIONS

The collection of *C. jejuni* strains presented in this study reveals a polyclonal genetic background. However, *C. jejuni* strains from primary sterile human specimens appear to be genetically more uniform, showing an epidemiologically more homogenous pattern.

The *C. jejuni* strains isolated from blood cultures exhibited lower rates of resistance to quinolones compared to the overall average.

Distribution of *C. jejuni* clones is highly specific to certain reservoirs.

Despite the clonal diversity and reservoir specificity, the phylogenetic analysis expectedly reveals a close genetic relationship between *C. jejuni* strains from humans and broilers. This is also true for CC21, one of the most ubiquitous clonal complexes among *C. jejuni* strains. Within CC21 the genetic relatedness of *C. jejuni* strains from humans to those from wild birds was documented to a lesser degree and was confined to a cluster of *C. jejuni* strains belonging to ST822 (CC21).

In this research, the majority of *C. jejuni* strains from clonal complex CC21 originating from wild birds clustered within ST1949 (CC1949) which was found to be strongly associated with fluoroquinolone resistance. This sequence type was identified exclusively in *C. jejuni* strains isolated from White Storks. This represents the first documented occurrence of ST1949 in a wild bird population.

The analysis revealed that the strains included in this study generally exhibited a high level of resistance to quinolones. The only identified genetic determinant associated with quinolone resistance was the *gyrA* T86I (ACA→ATA) point mutation, resulting in threonine to isoleucine substitution, the most prevalent mutation worldwide.

Several STs strongly associated with resistance to quinolones were identified, and these included ST51 (CC443), ST50 (CC21), ST22 (CC22) and ST1949 (CC1949).

Genetic determinants for tetracycline resistance, namely *tet*(O/32/O) and *tet*(L), were identified in the strains analyzed. Notably, tetracycline resistance was exclusively observed in strains derived from human sources.

For aminoglycoside resistance, genetic markers such as *ant(6)Ia* and *aac(3)XI* were detected in the analyzed strains. Despite the presence of aminoglycoside resistance genes in some strains, their MICs were below ECOFFs, classifying them as wild-type strains.

In the collection of isolates studied, genes associated with potential beta-lactam resistance, *bla*OXA, were found in most isolates, with *bla*OXA-61 being the most common. Several variants were identified, with *bla*OXA-193 being the most prevalent. However, no phenotypic carbapenem resistance was detected as the presence of the *bla*OXA-61-like or *bla*OXA-184-like subfamily of genes found in the study strains is insufficient by itself to confer phenotypic resistance to carbapenems.

A strong correlation was observed between genetic markers identified through WGS and phenotypic expression of antibiotic resistance with the exception of aminoglycosides, highlighting the reliability of whole genome sequencing in predicting resistance profiles.

The study hypothesized that *C. jejuni* strains from humans and broilers would exhibit higher antibiotic resistance rates or carry more resistance genes compared to strains originating from wild birds. However, the data from our study did not support the proposed hypothesis.

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8. SUMMARY

This research focuses on *C. jejuni*, a prominent foodborne pathogen that stands out as the most commonly reported zoonotic infection. According to EFSA and ECDC reports, campylobacteriosis impacts approximately 246,000 people annually in the European Union and an estimated 1.5 million people annually in the United States. Although generally self-limiting with low mortality rates, campylobacteriosis poses a serious health risk in developing nations, where diarrhea remains a leading cause of death, especially among children.

While the incidence of human *Campylobacter* spp. infections is rising globally, the situation with antibiotic resistance is worsening, with an increasing proportion of strains showing resistance to fluoroquinolones, macrolides, and other antibiotics, thereby further limiting available treatment options. This growing antimicrobial resistance presents a substantial challenge to human and veterinary health and affects the agricultural sector. Current data indicate that campylobacteriosis is a prevalent disease, contributing significantly to the overall burden of foodborne illness worldwide.

Consequently, this research was designed to shed light on previously unexplored aspects of the pathogenesis, ecology, and epidemiology of *C. jejuni*, with a specific emphasis on interactions between various reservoirs and the role of antimicrobial resistance in these processes. It also details the genetic profiles, antibiotic resistance, and clonal distribution of *C. jejuni* strains in Croatia.

The study employs the One Health approach, which is essential for understanding the complex interactions among human, animal, and environmental health. This approach demonstrates the impact of various ecosystems on the epidemiology of *C. jejuni.* The research places particular focus on broilers and wildlife, especially wild birds—niches that have not been thoroughly explored until now. It shows how these environments support the life cycle of *C. jejuni* and contribute to the spread of infection, as well as to the development and transmission of antimicrobial resistance.

The samples for this randomised studies included *C. jejuni* strains collected from three primary reservoirs: wild birds (specifically gulls and storks), broilers, and humans showing symptoms of diarrhea. All available strains from gulls, storks, and human blood culture isolates were included in the study, along with randomly collected strains from the gastrointestinal tracts of broilers and from patients with symptomatic diarrhea from 2021–2022. A total of 62 isolates were included in the study: 10 from wild birds (2 from gulls, 8 from storks), 5 from broilers,

and 47 human isolates, of which 10 were *C. jejuni* strains isolated from blood cultures. Human strains were obtained from four independent microbiological laboratories across different regions in Croatia - Zagreb, Osijek, Pula, and Split, while bird strains (from both wild birds and broilers) were collected from the *C. jejuni* strain collection at the Croatian Veterinary Institute.

All *C. jejuni* strains from this study were confirmed using multiplex PCR testing, and antibiotic susceptibility testing was performed by microdilution method. The raw data quality will be assessed using FastQC, followed by Kraken, which will identify the closest available reference genome. Sequenced fragments will then be mapped to this reference genome using BWA-MEM to ensure data accuracy, with Trimmomatic used to remove nucleotide identification adapters. Spades will then assemble the sequenced fragments into a complete genome. Subsequently, ResFinder will be used to identify resistance determinants, focusing on resistance genes and mutations linked to antimicrobial resistance. Sequence alignment algorithms will help match known alleles for each gene locus, allowing for the determination of each strain's ST (Sequence Type) and CC (Clonal Complex), providing insights into the genetic diversity and relationships of *C. jejuni* strains. Finally, MLST (Multilocus Sequence Typing) data will support the construction of a phylogenetic tree using software like Grapetree. The assembled strain sequences were then compared to reference databases, such as PubMLST, to identify the closest matches using sequence alignment algorithms. This comparison enabled the determination of each strain's association with a clonal complex and identification of its sequence type, uncovering genetic diversity and relationships among *C. jejuni* strains. Furthermore, whole-genome sequencing data were utilized to detect specific antibiotic resistance mechanisms and resistance genes by referencing publicly available genetic databases.The primary research hypotheses proposed that the distribution of *C. jejuni* sequence types would be specific to particular reservoirs. However, it was anticipated that *C. jejuni* strains identified in humans and broiler chickens would share the same clonal complexes, reflecting a close genetic relationship, whereas strains isolated in wild birds will belong to distinct lineages. The strains of human and broiler but not wild bird origin were expected to harbor identical genetic determinants of antibiotic resistance, coding for common resistance mechanisms. The study's findings confirmed that the distribution of *C. jejuni* clones is indeed highly specific to certain reservoirs. Phylogenetic analysis further revealed a close genetic relationship between *C. jejuni* strains from humans and broilers, supporting the hypothesis of shared lineage. This genetic similarity was also underscored by high levels of quinolone resistance across strains, all of which shared a consistent mutation in the *gyrA* T86I (ACA→ATA), reinforcing the

existence of common resistance mechanisms within this clonal grouping. Additionally, certain sequence types (STs) were found to be common between humans and wild birds, further highlighting potential overlaps in their evolutionary pathways. The concept of wild birds serving as an important epidemiological niche closely linked to humans and other typical reservoirs of *Campylobacter jejuni* is further supported by the findings of this study. Specifically, for the first time, ST-1949 was identified in wild birds, a sequence type previously described exclusively in humans and broilers.

In contrast, it was hypothesized that *C. jejuni* strains isolated from wild birds would exhibit lower rates of antibiotic resistance, reflecting a unique resistance profile within wildlife reservoirs. However, this study did not meet expectations. Resistance analysis revealed an unexpectedly high proportion of fluoroquinolone-resistant strains, even among those isolated from wild birds. This resistance is likely due to the fact that most of these strains belong to sequence type ST1949, which was found to be strongly associated with fluoroquinolone resistance.

Although the study demonstrated that the distribution of *C. jejuni* clones is highly specific to certain reservoirs, phylogenetic analysis expectedly revealed a close genetic relationship between *C. jejuni* strains from humans and broilers. Although the genetic connection between human and wild bird strains was less pronounced, it was clearly demonstrated in this study within ST822 strains, highlighting their role as a significant niche in the transmission chain of *C. jejuni* infections and the spread of antibiotic resistance. This study also presents the first report of *C. jejuni* ST1949 (CC1949) in wild birds, a sequence type previously detected only in human and broiler isolates, suggesting that ST1949 may offer further evidence of a potential link between wild birds and other niches explored in this research. These findings underscore the important role of wild birds in the transmission of resistant *C. jejuni* strains, acting as sentinels for AMR spread. These results reinforce the importance of continuous surveillance and a One Health approach to understanding and controlling the epidemiology of *C. jejuni*, especially in light of growing antibiotic resistance concerns.

The study also highlighted a strong correlation between genotypic markers and the phenotypic expression of antibiotic resistance, accurately predicting resistance profiles. Whole genome sequencing proved to be an effective tool for diagnosing and monitoring *C. jejuni* resistance patterns, as well as for identifying and providing early warnings of potential outbreaks.

9. SAŽETAK

Ovo istraživanje fokusira se na *C. jejuni*, vodećeg baterijskog uzročnika bolesti prenosivih hranom, koji ujedno predstavlja i najčešće prijavljivanu zoonozu. Prema izvještajima EFSA-e i ECDC-a, kampilobakterioza godišnje pogađa oko 246.000 ljudi u Europskoj uniji i oko 1,5 milijuna ljudi u Sjedinjenim Američkim Državama. Iako je bolest uglavnom samoograničavajuća i s niskim mortalitetom, kampilobakterioza predstavlja ozbiljan zdravstveni rizik u zemljama u razvoju, gdje je dijareja jedan od vodećih uzroka smrti, osobito kod djece.

Dok incidencija infekcija *Campylobacter* spp. kod ljudi globalno raste, sve je ozbiljnija i situacija s antibiotskom rezistencijom, budući da sve veći udio sojeva pokazuje otpornost na fluorokinolone, makrolide i druge antibiotike, dodatno ograničavajući dostupne opcije liječenja. Ova rastuća otpornost na antibiotike predstavlja značajan izazov za ljudsko i veterinarsko zdravlje te utječe na poljoprivredni sektor. Trenutni podaci pokazuju da je kampilobakterioza učestala bolest koja znatno doprinosi ukupnom opterećenju bolestima prenosivim hranom diljem svijeta.

Sukladno tome, ovo je istraživanje osmišljeno kako bi rasvijetlilo ranije neistražene aspekte patogeneze, ekologije i epidemiologije *C. jejuni*, s posebnim naglaskom na interakcije između različitih rezervoara i ulogu antimikrobne rezistencije u tim procesima. Također donosi podatke o genetičkim profilima, antibiotskoj rezistenciji i klonalnoj distribuciji sojeva *C. jejuni* u Hrvatskoj.

Istraživanje koristi pristup Jednog zdravlja, ključnog za razumijevanje složenih interakcija između ljudskog, životinjskog i okolišnog zdravlja, čime se pokazuje utjecaj različitih ekosustava na epidemiologiju *C. jejuni*. Istraživanje se posebno fokusira na brojlere i divlje ptice – niše koje do sada nisu bile temeljito istražene. Proučava kako ti okoliši podupiru životni ciklus *C. jejuni* te doprinose širenju infekcija i razvoju te prijenosu rezistencije na antibiotike.

Uzorke za ovu randomiziranu studiju činili su sojevi *C. jejuni* prikupljeni iz tri glavna rezervoara: divlje ptice (posebno galebovi i rode), brojleri i ljudi s simptomima dijareje. U istraživanju su obuhvaćeni svi dostupni sojevi iz galebova, roda i uzoraka ljudske krvi, kao i nasumično odabrani sojevi iz gastrointestinalnih trakta brojlera i pacijenata s simptomatskom dijarejom prikupljeni tijekom 2021. i 2022. godine. U istraživanju je uključeno ukupno 62 izolata: 10 iz divljih ptica (2 iz galebova, 8 iz roda), 5 iz brojlera i 47 iz ljudskih izolata, od kojih je 10 bilo iz *C. jejuni* sojeva izoliranih iz uzoraka krvi. Ljudski sojevi dobiveni su iz četiri

nezavisna mikrobiološka laboratorija iz različitih regija Hrvatske - Zagreb, Osijek, Pula i Split, dok su ptičji sojevi (iz divljih ptica i brojlera) prikupljeni iz zbirke sojeva *C. jejuni* Hrvatskog veterinarskog instituta.

Svi *C. jejuni* sojevi iz ovog istraživanja potvrđeni su primjenom multiplex PCR testiranja, a testiranje osjetljivosti na antibiotike provedeno je metodom mikrodilucije. Sirovi podaci će biti procijenjeni putem FastQC-a, nakon čega će Kraken identificirati najbliži dostupni referentni genom. Sekvencirani fragmenti bit će mapirani na ovaj referentni genom pomoću BWA-MEM kako bi se osigurala točnost podataka, dok će Trimmomatic ukloniti adaptere za identifikaciju nukleotida. Spades će zatim sastaviti sekvencirane fragmente u potpuni genom. Nakon toga, ResFinder će identificirati gene otpornosti, fokusirajući se na gene otpornosti i mutacije povezane s antimikrobnom rezistencijom. Algoritmi za poravnanje sekvenci pomoći će u podudaranju poznatih alela za svaki genski lokus, omogućujući određivanje ST (tip sekvence) i CC (klonski kompleks) svakog soja, pružajući uvid u genetičku raznolikost i odnose među sojevima *C. jejuni*. Na kraju, podaci iz MLST-a (tipizacija višestrukih lokusa) podržat će izradu filogenetskog stabla pomoću softvera poput Grapetree. Sastavljene sekvence sojeva zatim će biti uspoređene s referentnim bazama podataka, poput PubMLST-a, kako bi se identificirali najbliži podudari pomoću algoritama za poravnanje sekvenci. Ova usporedba omogućila je određivanje povezanosti svakog soja s klonskim kompleksom i identifikaciju njegovog tipa sekvence, otkrivajući genetičku raznolikost i odnose među sojevima *C. jejuni*. Nadalje, podaci o cjelokupnom genomu korišteni su za otkrivanje specifičnih mehanizama rezistencije na antibiotike i gena otpornosti korištenjem javno dostupnih genetičkih baza podataka.

Primarna hipoteza istraživanja predložila je da će distribucija sekvenčnih tipova *C. jejuni* biti specifična za određene rezervoare. Međutim, očekivalo se da će sojevi *C. jejuni* identificirani kod ljudi i brojlera dijeliti iste klonske komplekse, odražavajući blisku genetičku povezanost, dok će sojevi izolirani kod divljih ptica pripadati različitim linijama. Očekivalo se da će sojevi ljudskog i brojlerskog podrijetla, ali ne i divljih ptica, sadržavati identične genetičke determinante rezistencije na antibiotike, kodirajući uobičajene mehanizme rezistencije. Rezultati istraživanja potvrdili su da je distribucija klonova *C. jejuni* visoko specifična za određene rezervoare. Filogenetska analiza dodatno je otkrila blisku genetičku povezanost između sojeva *C. jejuni* iz ljudi i brojlera, podržavajući hipotezu o zajedničkoj liniji. Ova genetička sličnost također je naglašena visokim razinama rezistencije na kinolone među

sojevima, koji su svi dijelili konzistentnu mutaciju u genu *gyrA* T86I (ACA→ATA), potvrđujući postojanje zajedničkih mehanizama rezistencije unutar ove klonske skupine.

S druge strane, pretpostavilo se da će sojevi *C. jejuni* izolirani iz divljih ptica pokazivati niže stope rezistencije na antibiotike, što bi odražavalo jedinstven profil rezistencije u divljim rezervoarima. Međutim, ovo istraživanje nije ispunilo očekivanja. Analiza rezistencije otkrila je neočekivano visok udio sojeva otpornih na fluorokinolone, čak i među onima izoliranim iz divljih ptica. Ova rezistencija vjerojatno je posljedica činjenice da većina ovih sojeva pripada sekvenčnom tipu ST1949, koji je pokazao snažnu povezanost s rezistencijom na fluorokinolone.

Iako je istraživanje pokazalo da je distribucija klonova *C. jejuni* visoko specifična za određene rezervoare, filogenetska analiza očekivano je otkrila blisku genetičku povezanost između sojeva *C. jejuni* iz ljudi i brojlera. Iako je genetička povezanost između sojeva iz ljudi i divljih ptica bila manje izražena, jasno je dokazana u ovoj studiji unutar ST822 sojeva, naglašavajući njihovu ulogu kao značajne niše u prijenosu infekcija *C. jejuni* i širenju rezistencije na antibiotike. Ova studija također predstavlja prvo izvješće o pojavi *C. jejuni* ST1949 (CC1949) u divljim pticama, sekvenčnog tipa koji je prethodno otkriven samo u ljudskim i brojlerskim izolatima, što sugerira da ST1949 može ponuditi daljnje dokaze o potencijalnoj poveznici između divljih ptica i drugih niša istraženih u ovom istraživanju. Ovi nalazi naglašavaju važnu ulogu divljih ptica u prijenosu rezistentnih sojeva *C. jejuni*, djelujući kao čuvari širenja AMR-a. Rezultati dodatno podcrtavaju važnost kontinuiranog nadzora i pristupa Jednog zdravlja za razumijevanje i kontrolu epidemiologije *C. jejuni*, posebno s obzirom na sve veću zabrinutost zbog rezistencije na antibiotike.

Studija je također istaknula snažnu povezanost između genotipskih markera i fenotipske ekspresije rezistencije na antibiotike, što omogućuje preciznu predikciju profila rezistencije. Sekvenciranje cijelog genoma pokazalo se kao učinkovit alat za dijagnosticiranje i praćenje obrazaca rezistencije kod *C. jejuni*, kao i za identifikaciju i rano upozoravanje na moguće epidemije.

10.CURRICULUM VITAE AND PUBLICATION LIST

a. Curriculum Vitae

Silvija Šoprek Strugar was born on April 2, 1982, in Zagreb, Croatia. She completed her medical studies at the University of Zagreb Medical School, graduating in 2008. Upon completing her medical degree, in 2008 Silvija started her trainee program in medical microbiology and parasitology at the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" in Zagreb.

Since completing her trainee program in 2013, she has been a member of the clinical microbiology team at the University Hospital for Infectious Diseases "Dr. Fran Mihaljević" in Zagreb. Over the past 11 years, she has played a crucial role in the hospital's diagnostic and research activities. Her responsibilities involve diagnosing infectious diseases, antibiotic and diagnostic stewardship, and prevention of hospital-acquired infections.

In 2021, she enrolled in an interdisciplinary postgraduate doctoral program in Molecular Biosciences, conducted in collaboration between the University of J. J. Strossmayer, the Ruđer Bošković Institute in Zagreb, and the University of Dubrovnik. In 2023, she successfully defended her doctoral thesis titled “Genomic analysis and antimicrobial resistance of *Campylobacter jejuni* strains in Croatia“ under the mentorship of Associate Professor Arjana Tambić Andrašević and Luka Jurinović, PhD, senior scientific associate.

Her research endeavors are diverse, reflecting her broad interests in resistance mechanisms in bacteria and in epidemiological studies of gastrointestinal pathogens. She has collaborated on several significant projects related to these areas.

Beyond her clinical and research achievements, Silvija has embraced a vital role in education. Since 2024, she has been appointed as an associate assistant at the Faculty of Medicine, Croatian Catholic University in Zagreb. She is actively involved in teaching within the graduate program of the Medical School, specifically contributing to the collegium on Etiological Factors in Disease, within the microbiology field.

Since 2014, she has shared her expertise on a global scale as a WHO expert consultant, offering her insights and knowledge to international audiences. She has been an influential figure in the Croatian Society of Clinical Microbiology (HKDM), where she served as the president and a founding member of the HDKM Trainee Association from 2014 to 2017. Her commitment to addressing antimicrobial resistance is reflected in her roles as an executive committee member of the HDKM Study Group for Antibiotic Resistance and as a Croatian

National focal point for EARS-data management for antimicrobial resistance (AMR) data since 2011. She contributes to international efforts in this field as a member of the ESCMID Study Group for Antimicrobial Resistance Surveillance (ESGARS) and the ESCMID Emerging and Environmental Foodborne Waterborne Infections Study Group (EEFWISG), which share overlapping goals in combating antimicrobial resistance and monitoring foodborne and waterborne infections.

Silvija's commitment to continuous professional development is evident through her participation in numerous advanced workshops and courses organized by leading institutions like ESCMID, WHO and Study group for Antibiotic Resistance of the Croatian Society of Clinical Microbiology. These training programs have focused on essential topics such as antimicrobial resistance, diagnostic stewardship, and laboratory quality system implementation, ensuring that she remains at the forefront of clinical microbiology and infectious disease control.

Silvija Šoprek Strugar's career is a testament to her dedication to medical microbiology, her significant contributions to scientific research, and her passion for educating the next generation of healthcare professionals. Her journey reflects an unwavering commitment to excellence in all aspects of her work.

b. Publication List

a) List of published chapters in the book (serial publication)

1. Šoprek S, Tambić Andrašević A. Praćenje rezistencije na antibiotike u invazivnih izolata. U: Tambić Andrašević A, Tambić T, ur. Osjetljivost i rezistencija bakterija na antibiotike u Republici Hrvatskoj u 2021.g. Zagreb: AMZH; 2021., str. 90-102.

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7. Tambić Andrašević A, Šoprek S. Praćenje rezistencije na antibiotike u invazivnih izolata. U: Tambić Andrašević A, Tambić T, ur. Osjetljivost i rezistencija bakterija na antibiotike u Republici Hrvatskoj u 2015.g. Zagreb: AMZH; 2016. str. 75-87.

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9. Tambić Andrašević A, Šoprek S. Praćenje rezistencije na antibiotike u invazivnih izolata. U: Tambić Andrašević A, Tambić T, ur. Osjetljivost i rezistencija bakterija na antibiotike u Republici Hrvatskoj u 2013.g. Zagreb: AMZH; 2014. str. 72-84.

10. Tambić Andrašević A., Šoprek S Praćenje rezistencije na antibiotike u invazivnih izolata. U: Tambić Andrašević A, Tambić T, ur. Osjetljivost i rezistencija bakterija na antibiotike u Republici Hrvatskoj u 2012.g. Zagreb: AMZH; 2013. str. 68-78.

11. Tambić Andrašević A, Šoprek S. Praćenje rezistencije na antibiotike u invazivnih izolata. U: Tambić Andrašević A, Tambić T, ur. Osjetljivost i rezistencija bakterija na antibiotike u Republici Hrvatskoj u 2011.g. Zagreb: AMZH; 2012. str. 69-79.

12. Tambić Andrašević A, Šoprek S. Praćenje rezistencije na antibiotike u invazivnih izolata. U: Tambić Andrašević A, Tambić T, ur. Osjetljivost i rezistencija bakterija na antibiotike u Republici Hrvatskoj u 2010.g. Zagreb: AMZH; 2011. str. 65-74.

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b) List of papers published in journals:

1. Šoprek S, Ujević J, Kompes G, Jurinović L, Tambić Andrašević A. First Report of Campylobacter jejuni Strains Belonging to ST-21 Clonal Complex Isolated from Human, Poultry and Wild Birds in Croatia: Antimicrobial Resistance and Genetic Distance. Microorganisms [Internet] 2023;11(8):1884. Available from: http://dx.doi.org/10.3390/microorganisms11081884

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3. Rubić Ž, Jelić M, Šoprek S, Tarabene M, Ujević J, Goić-Barišić I, i sur. Molecular characterization of colistin resistance genes in a high-risk ST101/KPC-2 clone of Klebsiella pneumoniae in a University Hospital of Split, Croatia. Int Microbiol 2023:1-7.

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