LONGITUDINAL STUDY ON ESCHERICHIA COLI GENE VARIABILITY AFTER APPLICATION OF AUTOGENOUS VACCINE IN BROILER BREEDER FLOCKS

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Supervisor:
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I Z J A V A

Ja, Liča Lozica, potvrđujem da je moj doktorski rad izvorni rezultat mojega rada te da se u njegovoj izradi nisam koristio/-la drugim izvorima do onih navedenih u radu.

_____

(potpis studenta)

Zagreb, 2022.

This doctoral dissertation was developed at the Department of Poultry Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Croatia, under the supervision of associate professor Željko Gottstein, PhD. Part of the research was funded by the University of Zagreb, Croatia, under grant „Longitudinal epizootiological study of the virulence-associated genes by whole-genome sequencing of the pathogens with aim to improve biosafety measures and immunoprophylaxis programs on poultry farms“.

INFORMATION ON SUPERVISOR

Associate professor Željko Gottstein, PhD, has worked at the Department of Poultry Diseases with Clinic, University of Zagreb, since March 2002. His field of interest are viral poultry diseases, avian immunology, molecular diagnostics and vaccine development. He has published 38 scientific papers and 113 conference presentations. According to Google Scholar his scientific work counts 286 citations, while on Scopus the number of citations is 166.

Zahvaljujem mentoru na svim profesionalnim i prijateljskim savjetima, na dugim terenskim razgovorima i stalnom izvoru novih ideja za postojeća i buduća istraživanja. Hvala najviše na strpljenju i smirenosti.

Hvala svim koautorima bez kojih ovih radova ne bi bilo. Hvala i svim prijateljima i kolegama koji su pomogli razgovorima i razmjenom dobrih i loših iskustava.

Najveće hvala obitelji na stalnom ohrabrivanju i vjerovanju u mene. Bez vas ne bi bila tu gdje jesam. Hvala na svakodnevnim razgovorima, razvedravanju i motivaciji.
Hvala što ste uvijek tu.

ABSTRACT

Escherichia coli (E. coli) is the most common bacterial cause of decreased production and high mortality rates in the poultry industry. Avian pathogenic *E. coli* (APEC) is one of the extraintestinal *E. coli* (ExPEC) subpathotypes, characterized by high genetic diversity. Regardless of their diversity, ExPEC strains share many common virulence factors that allow them to colonize tissues outside of the intestine. APEC genetic diversity complicates the efficacy of the immunoprophylaxis programs, which are the foundation of poultry health protection. In this research, 115 *E. coli* strains were longitudinally isolated from the chicken carcasses diagnosed with colibacillosis. The strains originated from four and five flocks, on Farm A and Farm B, respectively, which are part of the same broiler breeder company. The selected strains originated from the flocks vaccinated with commercial, combination of commercial and autogenous, or solely autogenous vaccines. The strains were sequenced, individually analysed and mutually compared based on the phylogenetic groups, multilocus sequence types (MLST), virulence-associated genes (VAGs), antimicrobial resistance (AMR) genes, and core-genome single-nucleotide polymorphisms (cgSNPs). The aim of this dissertation was to investigate the effect of autogenous vaccines on the genetic heterogeneity of *E. coli* strains on poultry farms. The results showed that autogenous vaccine application gradually induced lower genetic heterogeneity of the isolates on both farms, based on the prevalence of phylogenetic groups, STs and cgSNP phylogeny. In total, 23 sequence types (STs) were detected, with 52.58% of the isolates belonging to two clonal complexes. Nevertheless, the average number of virulence genes per isolate increased on both farms, while the prevalence of the antimicrobial resistance genes decreased. Out of the highly prevalent STs, ST95, ST390 and ST131 had the highest average number of VAGs per isolate. The overall results of the research confirmed that application of autogenous vaccines affects the genetic heterogeneity and virulence profiles of *E. coli.*

Key words: *Escherichia coli,* APEC, poultry, virulence, autogenous vaccine, WGS, MLST, SNP, AMR

PROŠIRENI SAŽETAK

UVOD: Kolibaciloza predstavlja značajan problem u peradarskoj industriji, uzrokujući velike ekonomske gubitke i smanjenje dobrobiti životinja na farmama. Uzrokovana je bakterijom *Escherichia coli (E. coli),* koja može biti primarni ili sekundarni patogen, često u koinfekciji s drugim mikroorganizmima kao što su *Mycoplasma* ili virus zaraznog bronhitisa, ili uslijed različitih oblika imunosupresije. Težina infekcije ovisi o različitim čimbenicima kao što su soj, mjesto ulaska patogena i opće zdravstveno stanje životinje. U pilića zaraza najčešće nastaje putem nezacijeljenog pupčanog tračka u valionici ili vertikalnim prijenosom od zaraženog roditeljskog jata. Kod kokoši u proizvodnji, najčešće lezije uključuju poliserozitis, peritonitis, salpingitis i salpingitis-peritonitis sindrom (SPS), pri čemu infekcija nastaje ascendentno putem jajovoda. Iako infekcija može biti lokalizirana, češće se radi o sistemskoj infekciji, odnosno septikemiji. Izbijanje bolesti je vrlo često povezano s početkom proizvodnje jaja, pa se septikemija kod kokoši nesilica smatra bolešću koja je potaknuta stresom.

Sojevi *E. coli* mogu se podijeliti u crijevne i izvancrijevne (engl. extraintestinal pathogenic *E. coli*, ExPEC), pri čemu ptičji patogeni sojevi (engl. avian pathogenic *E. coli*, APEC) pripadaju skupini izvancrijevnih sojeva. Svi ExPEC sojevi sadržavaju čimbenike virulencije koji im omogućavaju preživljavanje i kolonizaciju tkiva izvan crijeva, ali su genetski vrlo raznoliki, što komplicira detaljnu klasifikaciju i dijagnostiku. Geni virulencije u APEC sojevima vrlo često su grupirani na otocima patogenosti (engl. pathogenicity islands, PAIs) koji mogu biti smješteni na kromosomima ili plazmidima. S obzirom da izrazito virulentni APEC sojevi vrlo često sadržavaju takve plazmide, oni se smatraju njihovom karakteristikom, iako dosadašnjim istraživanjima nije uspješno utvrđeno koji pojedini geni virulencije su svojstveni za APEC sojeve. Rezultati istraživanja različitih ExPEC podskupina pokazali su da su svi takvi sojevi genetski vrlo slični, što je stvorilo temelj za istraživanje zoonotskog potencijala APEC sojeva.

U svrhu tipizacije sojeva *E. coli* korištene su mnoge metode, od kojih se u današnje vrijeme vrlo često primjenjuje filogenetska tipizacija temeljena na dokazu gena koji se smatraju reprezentativnima za pojedine filogenetske skupine. Prema trenutnoj podjeli, postoji osam skupina, te tzv. „cryptic clade“ I-V, pri čemu se skupine B2, D, F i G smatraju patogenima, dok ostale čine komenzalni sojevi. Primjena takve

metode omogućava brzu dijagnostiku i monitoring sojeva *E. coli* na farmama. U istraživanjima i dijagnostici, sve češće se primjenjuje tehnologija sekvenciranja novih generacija (engl. next-generation sequencing, NGS), koja ima veliki potencijal u populacijskoj genomici i molekularnoj epidemiologiji. Primjenom metoda sekvenciranja moguće je pratiti evoluciju bakterijskih populacija, te njihovu prilagodbu različitim domaćinima.

Zaštita peradi na farmama temelji se na provedbi strogih biosigurnosnih mjera i primjeni cjepiva. S obzirom da su sojevi *E. coli* genetski vrlo raznoliki, komercijalne vrste cjepiva su često nedovoljno učinkovite jer pružaju zaštitu od nekolicine sojeva. Iz toga razloga, sve češće se koriste autogena cjepiva koja su proizvedena od sojeva izdvojenih na farmama na kojima će se i primjenjivati, pa su zato vrlo specifična i učinkovita. Dosadašnja istraživanja pokazala su da primjena autogenoga cjepiva ima pozitivan utjecaj na zdravlje životinja i njihove proizvodne rezultate. Longitudinalnim praćenjem genetske strukture populacije *E. coli* na farmama na kojima se primjenjuje autogeno cjepivo, može se dobiti uvid o utjecaju cjepiva na raznolikost i virulenciju sojeva.

HIPOTEZA: Osnovni cilj bio je istražiti utjecaj primjene autogenoga cjepiva na genski sastav i selekciju sojeva *E. coli.* Analiza longitudinalno izdvojenih sojeva omogućila bi detekciju genskih varijacija kroz nekoliko jata, uzimajući u obzir utjecaj primjene autogenoga cjepiva. Hipoteza istraživanja bila je da kontinuirano cijepljenje jata autogenim cjepivom utječe na gensku heterogenost i profile virulencije *E. coli*.

MATERIJALI I METODE: Istraživanje je provedeno na ukupno 115 sojeva *E. coli* izdvojenih iz lešina kokoši s dvije odvojene farme roditeljskih jata teške linije genetike Ross 308. Sojevi su izdvojeni iz četiri i pet jata, na Farmi A, odnosno Farmi B, koje su dio iste tvrtke. Obje farme imale su kontinuirane probleme uzrokovane kolibacilozom, zbog čega je započeta primjena autogenoga cjepiva. Prvo jato na obje farme cijepljeno je komercijalnim cjepivom, dok je primjena autogenoga cjepiva posebno dizajniranog za svako jato započela u drugom jatu. Uzorci su prikupljeni tijekom patomorfoloških pretraga uginulih kokoši u sklopu kontinuiranog nadzora na farmi i u slučaju izbijanja bolesti. Uzorci su uključivali obriske makroskopski promijenjenih organa, te su nasađeni na tri vrste čvrstih hranjivih podloga. Identifikacija bakterija provedena je na

temelju morfološke pretrage i biokemijskih karakteristika, a potvrđena MALDI-TOF metodom masene spektrometrije (engl. matrix-assisted laser desorption/ionization- time of flight mass spectrometry). Svi uzorci su čuvani u Brain Heart Infusion bujonu s dodatkom 50%-tnog glicerola pri temperaturi od -20 ºC. Odabir sojeva za daljnje analize temeljen je na organu podrijetla i dobi životinje. Ciljani organi bili su peritoneum, jetra, jajovod i koštana srž, s obzirom da su najčešće zahvaćeni u slučajevima kolibaciloze. Kada sojevi iz odabranih organa nisu bili dostupni, analizirani su sojevi iz drugih često zahvaćenih tkiva kao što su pluća i perikard. Svi uzorci izdvojeni su iz životinja starijih od 21 tjedna, kada najčešće dolazi do izbijanja bolesti. Sojevi su uspoređivani na temelju filogenetskih skupina, održavateljskih gena (engl. housekeeping genes), skupine odabranih gena virulencije koji su prethodno istraživani kao moguće odrednice virulencije ExPEC sojeva, detektiranih stečenih gena rezistencije, te jednonukleotidnih polimorfizama (engl. single-nucleotide polymorphism, SNP) čitavog genoma istraživanih sojeva.

REZULTATI I DISKUSIJA: Rezultati istraživanja pokazali su postepeno smanjivanje genske heterogenosti sojeva *E. coli* nakon kontinuirane primjene autogenoga cjepiva. Tipizacija sojeva u filogenetske skupine pokazala je nešto veću prevalenciju komenzalnih sojeva na Farmi A (49.02%) – uzimajući u obzir i udio netipiziranih sojeva (5.88%). Na Farmi B bili su značajno učestaliji patogeni sojevi (82.3%), te je posljednje jato bilo izrazito homogeno sa 100%-tnom prevalencijom skupine B2. Na Farmi A došlo je i do porasta prevalencije komenzalnih sojeva, to jest skupine „cryptic clade“ u posljednjem jatu (46.2%), koju karakterizira fiziološki visoka prevalencija u probavnom sustavu ptica.

Filogenetske analize temeljene na MLST (engl. multilocus sequence typing) i cgSNP (engl. core-genome single-nucleotide polymorphism) metodama potvrdile su da primjena autogenoga cjepiva uzrokuje smanjenje heterogenosti sojeva, dok je analiza gena virulencije pokazala suprotno. Kontinuirana primjena cjepiva na Farmi A dovela je do značajnog porasta prosječnog broja gena virulencije po izolatu kroz vrijeme, dok se na Farmi B, usprkos početnom porastu, taj broj do kraja istraživanja blago smanjio. Uspoređujući prosječan broj gena virulencije svih jata, na Farmi B je od početka bio značajno veći nego na Farmi A. Pojedini čimbenici virulencije prethodno vezani isključivo uz uropatogene sojeve (engl. uropathogenic *E. coli*, UPEC), dokazani su u

velikom broju istraživanih izolata, što je također jedan od mogućih pokazatelja da infekcija može nastati ascendentno putem jajovoda. Detektiran je porast prevalencije čimbenika virulencije s funkcijom vezanja i transporta željeza na obje farme. U usporedbi s drugim skupinama gena virulencije, udio čimbenika transporta željeza bio je značajno viši. Također je detektirana visoka prevalencija toksin-kodirajućih gena (>85%), pa tako i bakteriocina, što ukazuje na kompetitivni fenotip istraživanih bakterija, te moguću dominaciju jednog ili nekoliko sojeva prilikom izbijanja kolibaciloze na farmama. Osobitosti virulencije sojeva zavisne su o dokazanim ST profilima (engl. sequence type), potvrđujući mogućnost primjene MLST metode u istraživanju raznolikosti virulencije sojeva *E. coli*. Suprotno tome, pojavnost gena antimikrobne rezistencije (AMR) nije bila specifična za određeni ST.

Analiza gena AMR pokazala je najvišu prevalenciju gena *mdf(A)* (100%) i sit operona (71.3%). Ekspresija gena *mfd(A)* omogućava multirezistentnost sojeva na različite skupine antimikrobnih pripravaka, što ukazuje na to da su se sojevi prilagodili uvjetima okoliša posjedovanjem gena AMR širokog spektra. S druge strane, *sit* operon geni bitni su čimbenici virulencije zbog njihove funkcije u vezanju i transportu metalnih iona i rezistenciji na vodikov peroksid, što predstavlja važnu ulogu u patogenezi kolibaciloze. Prevalencija većine ostalih detektiranih AMR gena bila je značajno niža, te je iznosila do 4.35%, osim za gen *tet(A)* koji je detektiran u ukupno 17 izolata (14.78%). Praćenjem frekvencije AMR kroz vrijeme može se uočiti pad prevalencije, što je zasigurno i rezultat primjene autogenoga cjepiva koje je smanjilo potrebu za primjenom antimikrobnih pripravaka u svrhu terapije kolibaciloze na istraživanim farmama.

ZAKLJUČAK: Primjena autogenoga cjepiva utjecala je na prevalenciju filogenetskih skupina istraživanih izolata *E. coli*, pri čemu je došlo do eliminacije slabije virulentnih sojeva homolognih cijepnim sojevima i perzistencije patogenih, ali uz uspješnu kontrolu kliničke manifestacije na farmama. Kontinuirana primjena uzrokovala je smanjenje genetske raznolikosti sojeva, što je dokazano Clermont metodom filotipizacije, te filogenetskim analizama temeljenima na MLST i cgSNP metodama. Suprotno tome, nije došlo do smanjenja genske raznolikosti čimbenika virulencije. Prosječan broj navedenih gena na Farmi A se značajno povećao, dok se na Farmi B blago smanjio. Profili gena virulencije odgovarali su specifičnim ST-ovima i filogenetskim skupinama

E. coli. Detektirana su 23 ST profila, od čega su ST95, ST117, ST390 i ST23 činili 62.61% od ukupnog broja izolata. Od navedenih, ST95, ST390 i ST131 imali su najveći prosječan broj gena virulencije po izolatu. Dok je većina ST profila bila prisutna samo na jednoj farmi, ST117 je izdvojen na objema farmama, što ukazuje na mogućnost vertikalnog prijenosa iz djedovskih jata ili uzgojnih peradnjaka.

Analiza gena antimikrobne rezistencije pokazala je najveću zastupljenost gena *mdf(A)* i sit operona, koji pružaju zaštitu protiv širokog spektra antimikrobnih pripravaka i omogućavaju patogenezu same infekcije. Ostali geni AMR detektirani su u bitno manjem broju i njihova pojavnost nije bila vezana uz specifičan ST. Rezultati ukazuju i na mogući sinergistički učinak terapije i autogenoga cjepiva čime se slabije virulentni ali rezistentniji sojevi uspješno kontroliraju imunosnim sustavom potaknutim cjepivom, dok se patogeni ali manje rezistentni sojevi u slučaju izbijanja bolesti mogu kontrolirati terapijom.

Ključne riječi: *Escherichia coli,* APEC, perad, kokoš, virulencija, autogeno cjepivo, WGS, MLST, SNP, AMR

ABBREVIATIONS

µL	microlitre, a metric unit of volume equal to one-millionth (10^{-6}) of a litre
AEEC	attaching and effacing *Escherichia coli*
AMR	antimicrobial resistance
AFEC	avian fecal commensal *Escherichia coli*
APEC	avian pathogenic *Escherichia coli*
bp	base pair
CC	clonal complex
CGE	Center for Genomic Epidemiology
cgSNPs	core-genome single-nucleotide polymorphisms
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EnPEC	endometrial pathogenic *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
ExPEC	extraintestinal pathogenic *Escherichia coli*
MLEE	multilocus enzyme electrophoresis
min	minute
MLST	multilocus sequence typing
MPEC	mammary pathogenic *Escherichia coli*
NCBI	National Center for Biotechnology Information
NGS	next-generation sequencing
NMEC	neonatal meningitis *Escherichia coli*
PAI	pathogenicity island
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pmol	picomole, a metric unit of molar mass equal to one-

	billionth (10^{-9}) of amount of substance
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
s	second
SPS	salpingitis-peritonitis syndrome
SEPEC	sepsis-associated *Escherichia coli*
SNP	single-nucleotide polymorphism
STEC	Shiga toxin (*stx)*-producing *Escherichia coli*
ST	sequence type
TraDIS	transposon-directed insertion site sequencing
UPEC	uropathogenic *Escherichia coli*
v	version
VAG	virulence-associated gene
WGS	whole-genome sequencing
wk	week

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1. INTRODUCTION

1.1. Colibacillosis

Escherichia coli (*E. coli*) presents a major problem in the poultry industry, causing great economic losses and affecting the welfare of birds on farms (NOLAN et al., 2020; POULSEN et al., 2020). It was considered a secondary pathogen, usually in coinfection with *Mycoplasma* or viral agents such as infectious bronchitis virus, but current opinion is that *E. coli* can also be a primary pathogen (NOLAN et al., 2020). The localization and severity of the infection varies depending on the strain, site of entry and general health of the bird (NOLAN et al., 2020; CHRISTENSEN et al., 2021). *E. coli* is most commonly acquired through the respiratory system, navel, skin, or by bacteria ascending through the cloaca and oviduct (LANDMAN et al., 2013; GUABIRABA and SCHOULER, 2015; POULSEN et al., 2020). In young chicks, the most common lesion is omphalitis, which occurs because of the contamination of the unhealed navel in the hatchery or by vertical transmission from the hens with oophoritis or salpingitis (POULSEN et al., 2017; NOLAN et al., 2020; CHRISTENSEN et al., 2021). However, in adult egg-laying hens the most common lesions are polyserositis, peritonitis, salpingitis and salpingitis-peritonitis syndrome (SPS) (Figures 1, 2 and 3) (JORDAN et al. 2005; LI et al., 2017). One of the localized forms of colibacillosis is coliform cellulitis, characterized by sheets of caseated exudate in the subcutaneous tissue. The lesions are usually located on the skin over the abdomen or between the thigh and midline, and can be accompanying other forms of localized infection (ELFADIL et al., 1996; GOMIS et al., 1997; ONDERKA et al., 1997). In case the bacteria enter the circulation, the infection results in colisepticaemia, which may be differentiated depending on the place of entry to the circulation (NOLAN et al., 2020). In the respiratory-origin form of colisepticaemia, *E. coli* is usually a secondary pathogen, spreading to circulation through the respiratory mucosa damaged by other microorganisms or ammonia, or because of the impaired immune response caused by other ongoing infections (NAGARAJA et al., 1983; MATTHIJS et al., 2009). Generally, the systemic form of colibacillosis occurs in stressed and immunocompromised birds (GUABIRABA and SCHOULER, 2015). As the majority of outbreaks are associated with the onset of egg production, layer colisepticaemia is

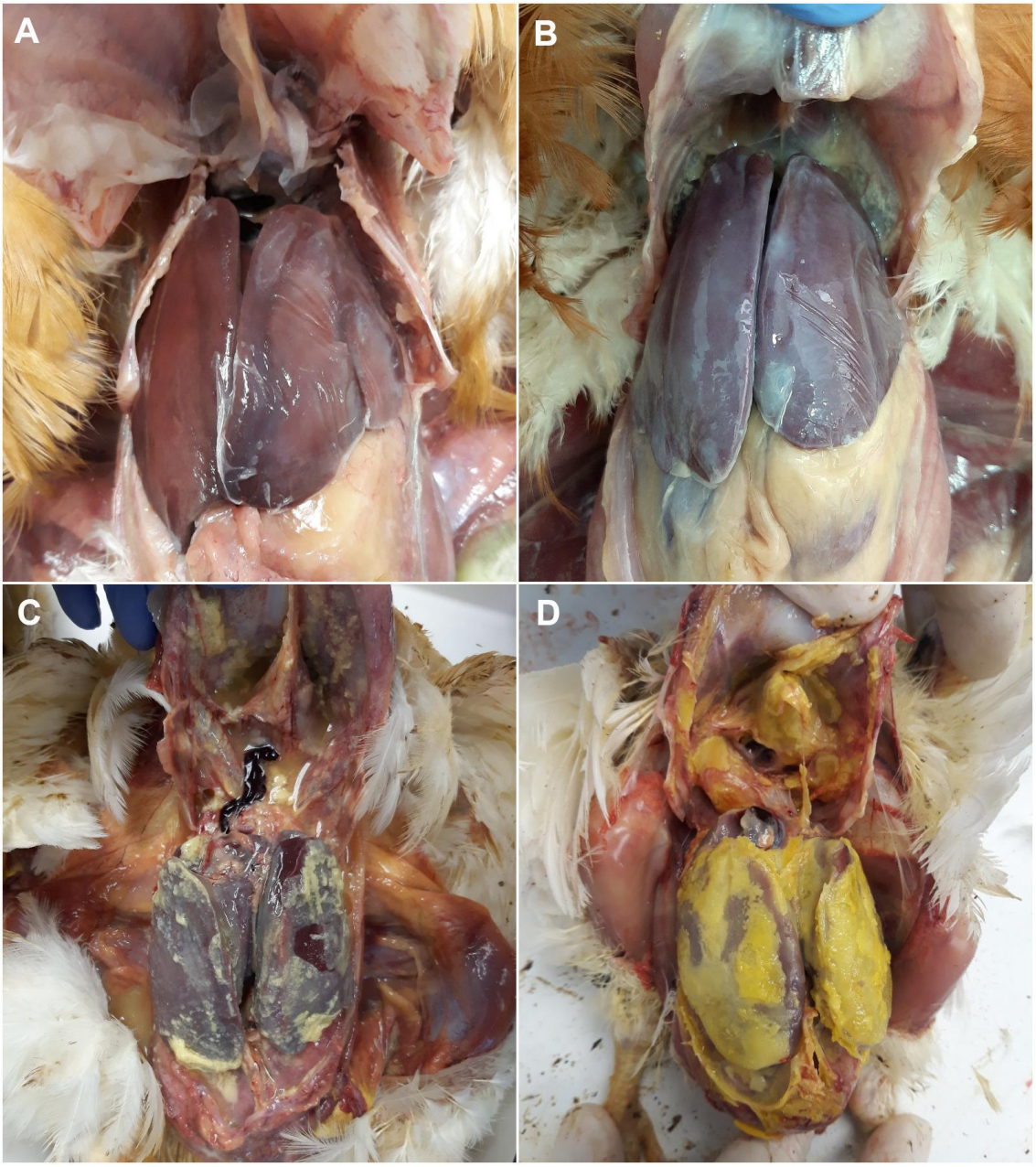

Figure 1. *In situ* view of the organs after opening the coelomic cavity showing different severity of colibacillosis. **A.** Serous perihepatitis. **B.** Serofibrinous polyserositis (perihepatitis, airsacculitis, pericarditis) **C.** Fibrinous polyserositis (perihepatitis, airsacculitis, pericarditis). **D.** Fibrinous perihepatitis, airsacculitis and pericarditis, characterized by thick yellow exudate on the affected organs. (Photo: L. Lozica, Faculty of Veterinary Medicine, University of Zagreb)

considered a disease of young birds triggered by stress (ZANELLA et al., 2000). It can lead to synovitis, osteomyelitis or osteoarthritis. In that case, the symptoms usually include lameness and poor growth due to inability to approach the feeders, so the affected birds are often victims of cannibalism (NOLAN et al., 2020). As opposed to *E. coli* infection in mammals, enteritis caused by *E. coli* is a rare manifestation in poultry. It can result from an infection with enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteropathogenic (EPEC) or enteroinvasive *E. coli* (EIEC), collectively known as attaching and effacing *E. coli* (AEEC), as opposed to other forms of colibacillosis which are caused by extraintestinal pathogenic *E. coli* (ExPEC) (NOLAN et al., 2020).

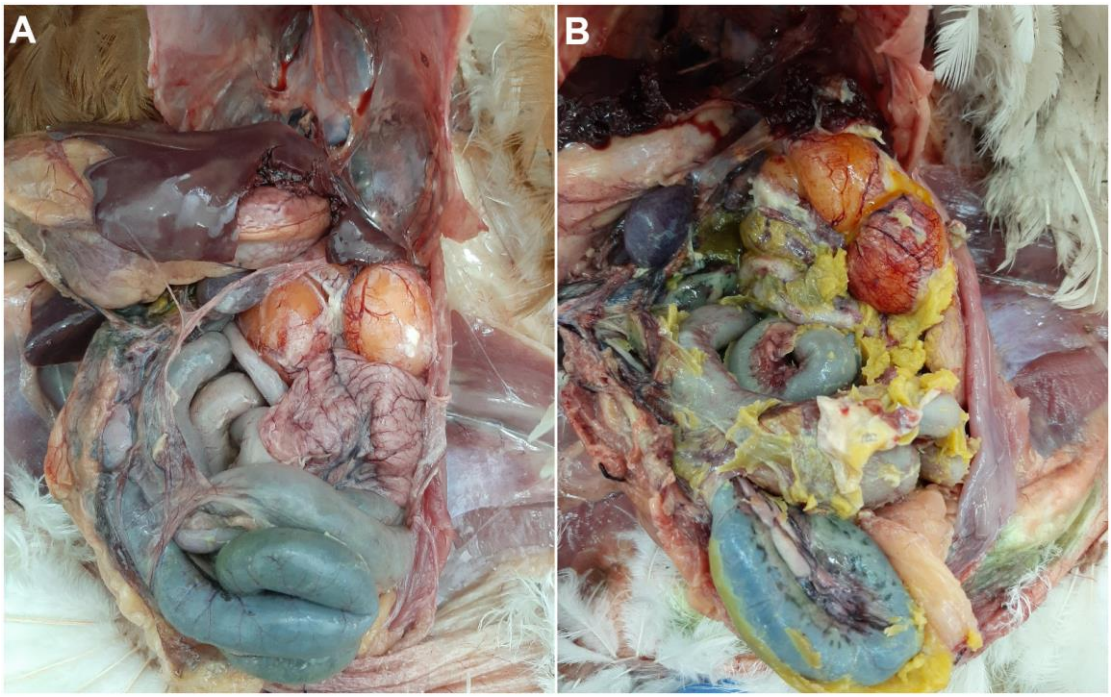

Figure 2. *E. coli* peritonitis and oophoritis. **A.** Serofibrinous exudate on the serous membranes in the peritoneal cavity. **B.** Thick, fibrinous exudate on the follicles and serous membranes in the peritoneal cavity. (Photo: L. Lozica, Faculty of Veterinary Medicine, University of Zagreb)

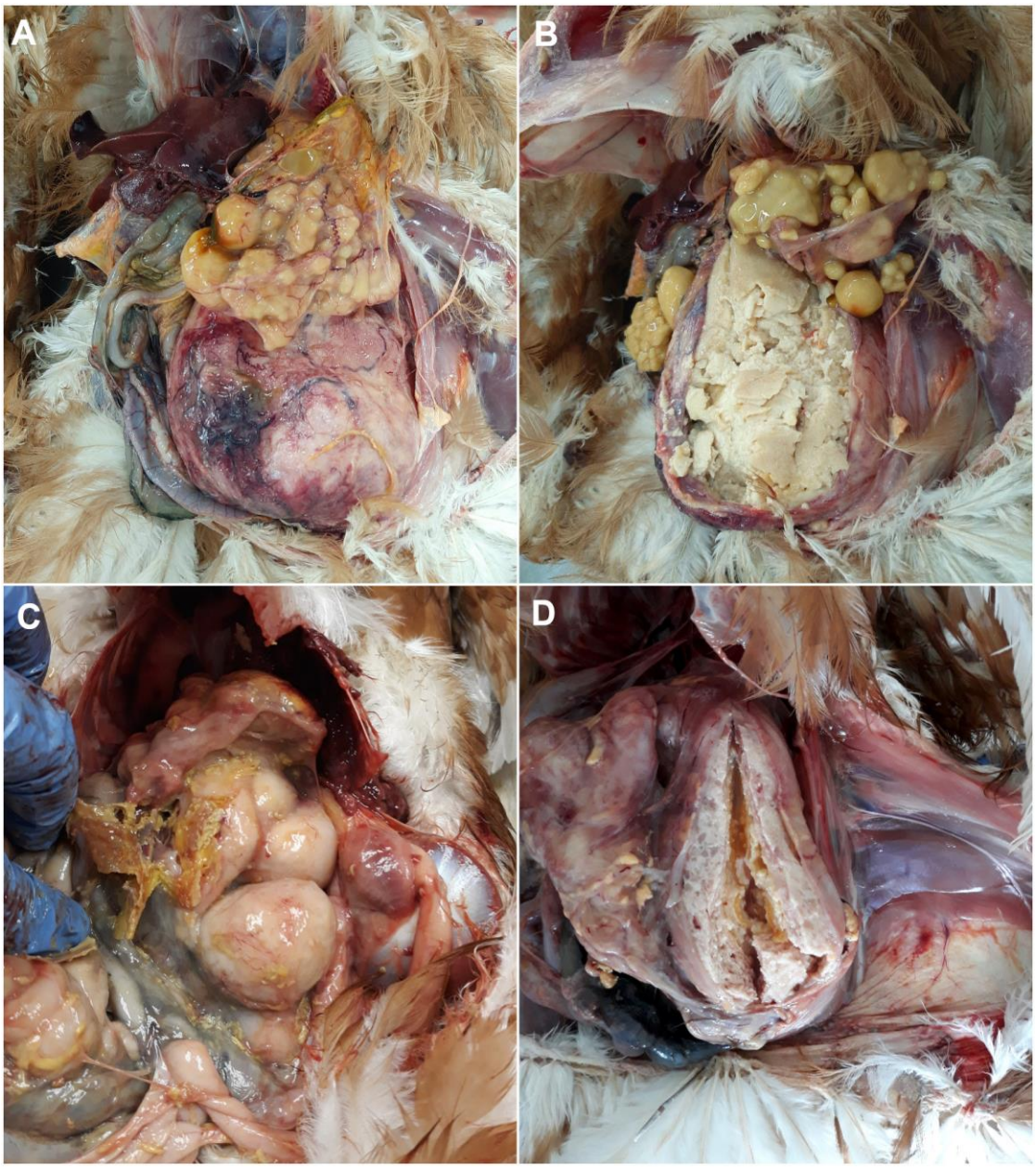

Figure 3. Severe oophoritis and salpingitis-peritonitis syndrome (SPS) in laying hens. **A.** Oophoritis and salpingitis characterized by caseation of the ovaries and follicles, and expansion of the oviduct, respectively. **B.** Caseous exudate in the ruptured follicles and lumen of the opened oviduct. **C.** Degenerated ovaries and SPS characterized by distended, thin-walled oviduct filled with caseous exudate and sheets of caseated exudate in the peritoneal cavity. **D.** Cross section of the large caseous mass in the oviduct. (Photo: L. Lozica, Faculty of Veterinary Medicine, University of Zagreb)

1.2. Strain classification and genetic diversity

E. coli is classified into approximately 180 serotypes based on somatic (O), capsular (K), fimbrial (F) and flagellar (H) antigens (NOLAN et al., 2020). Whole genome consists of 4.5-5.5 Mb, which includes 4000-5500 genes, of which over 1700 genes are involved in the virulence (ANTÃO, 2010). It can be divided to intestinal and extraintestinal pathogenic *E. coli* (ExPEC). To date, ExPEC group includes four confirmed subpathotypes - avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and sepsis-associated pathogenic *E. coli* (SEPEC) (SAROWSKA et al., 2019), and two emerging - mammary pathogenic *E. coli* (MPEC) and endometrial pathogenic *E. coli* (EnPEC) (SHPIGEL et al., 2008; SHELDON et al., 2010). Although all mentioned groups share some of the virulence factors that allow them to colonize different tissues outside of the intestine (RODRIGUEZ-SIEK et al., 2005b; EWERS et al., 2007; MOULIN-SCHOULEUR et al., 2007; NOLAN et al., 2019; POULSEN et al., 2020), they exhibit a high genome diversity (JØRGENSEN et al., 2019), which can complicate allocation to subpathotypes (Figure 4). Diverse phenotypes of *E. coli* are considered a result of the genome plasticity, which allows a large number of different gene combinations (TOUCHON et al., 2009; CLERMONT et al., 2011). The greatest overlap in virulence profiles has been detected between APEC and UPEC (RODRIGUEZ-SIEK et al., 2005b). UPEC is a very common cause of urinary tract infections in humans and animals. It has developed the ability to colonize the environment outside of the host's intestine, most frequently the urinary tract, but can easily spread to bloodstream and cause septicaemia (TERLIZZI et al., 2017).

Virulence-associated genes (VAGs) in APEC are often clustered into pathogenicity islands (PAIs), mostly plasmid-located (JOHNSON et al., 2006). Many research groups are focused on the importance of certain VAGs and their role in the pathogenesis of *E. coli* infection (RODRIGUEZ-SIEK et al., 2005a; DZIVA and STEVENS, 2008; JOHNSON et al., 2010; DE OLIVEIRA et al., 2015). To date, there are no defined virulence determinants for APEC, although recent research has suggested the virulence is dependent on the sequence variations of the plasmid genes, not their occurrence (MAGEIROS et al., 2021).

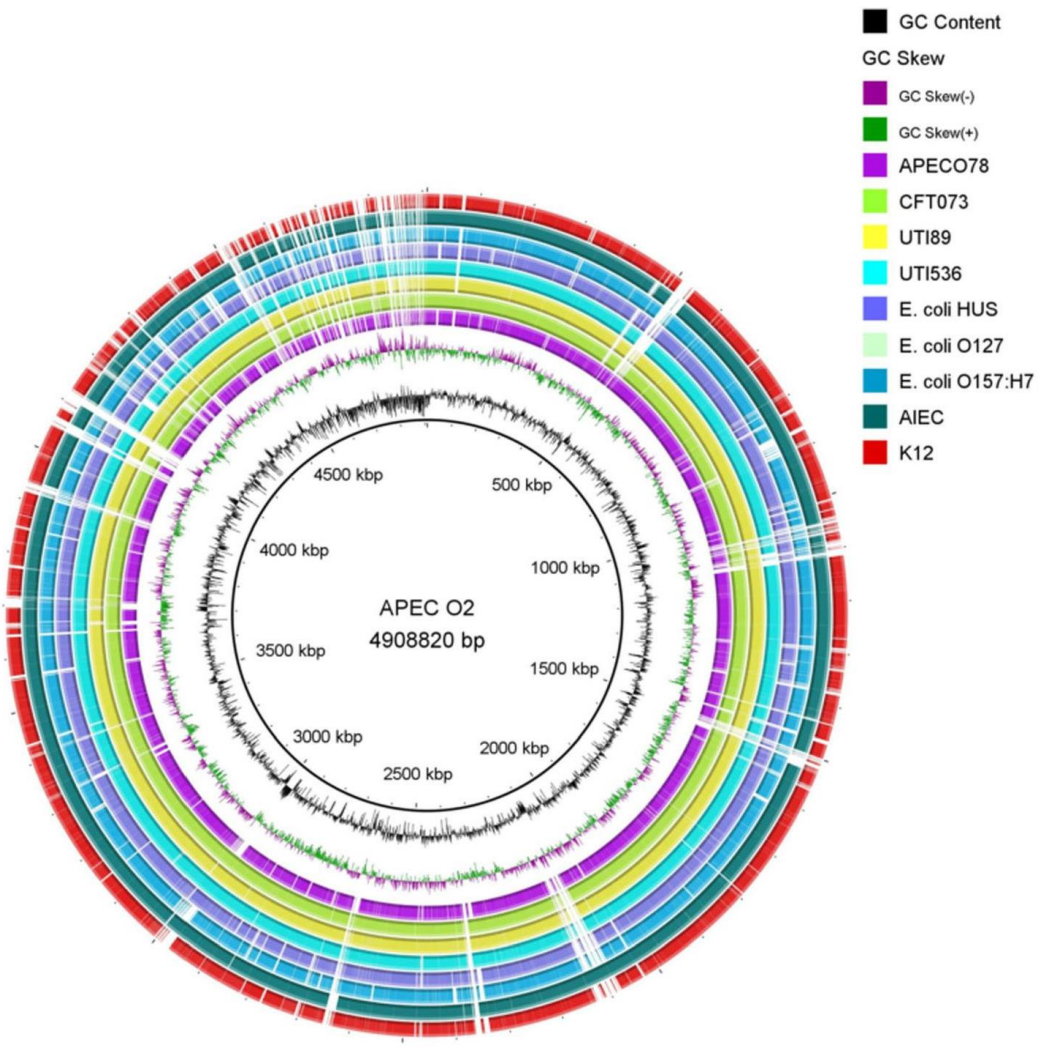

Figure 4. Genomic comparison of APEC O2 with other strains of *E. coli.* The analysis included genome wide comparison of APEC O2 (AY214164) with APECO78 (CP004009.1), three isolates of uropathogenic *E. coli* – UTI CFT073 (NC_004431.1), UTI89 (NC_007946.1) and UTI536 (NC_008253.1), three isolates of intestinal pathogenic *E. coli* – *E. coli* HUS (PRJNA68275), *E. coli* O127 (PRJNA204937) and *E. coli* O157:H7 (GCA_000008865.1), AIEC (GCA_000183345.1) and a non-pathogenic *E. coli* K12 (GCA_000005845.2). Solid colour of concentric rings indicates genomic areas also present in APEC O2 (inner black circle), whereas absence of colour in a ring indicates absence of the region. Source: Jørgensen et al. (2017).

1.3. Molecular typing methods

In the late 1980s, many researchers were investigating the efficacy of multilocus enzyme electrophoresis (MLEE) in the bacterial population genetics, systematics and epidemiology (SELANDER et al., 1987). This method was based on using 35 enzyme loci to differentiate six phylogroups – A, B1, B2, C, D and E of *E. coli*, in a panel of 72 strains representative of the species diversity. Afterwards, these phylogroups have been confirmed by various methods such as rDNA restriction fragment length polymorphism (RFLP), multilocus sequence typing (MLST) and whole-genome sequencing (WGS) (ESCOBAR-PÁRAMO et al., 2006, TOUCHON et al., 2009, CLERMONT et al., 2019). Additionally, pulsed-field gel electrophoresis (PFGE) has been used to produce DNA fingerprints of bacterial strains in order to compare the isolates.

Extensive phylotyping analyses of *E. coli* have shown there are eight phylogenetic groups including A, B1, B2, C, D, E, F and G with cryptic clades I-V (CLERMONT et al., 2019). Allocation to said phylogroups is based on the presence of certain genes, which are considered distinctive. Analyses based on phylotyping methods have been routinely used for research, as well as diagnostic purposes, and many studies have confirmed lineages B2 and D to be the most common among the highly virulent strains (PICARD et al., 1999; CARLOS et al., 2010; PIRES-DOS-SANTOS et al., 2013), with group G now being the most prevalent in poultry (CLERMONT et al., 2019; MEHAT et al., 2021). In the longitudinal epidemiological study on pullets and layers, which was based on serotypization and PFGE, no single clonality among the isolates was found in regard with the geographical region, age of the host or pathological lesions (PAUDEL et al., 2016). Moreover, research on the risk of *E. coli* transmission from broiler breeders with salpingitis to broilers revealed that only specific clones are transmitted to progeny where they cause first week mortality, whereas horizontal transmission in the hatchery occurs with both pathogenic and apparently non-pathogenic strains (POULSEN et al., 2017). There is still no clear evidence which factors are most responsible for the virulence of ExPEC, but former studies have reported they include adhesins, invasins, protectins, iron acquisition systems, toxins, two-component systems, a quorum-sensing (QS) system, transcriptional regulators, secretion systems and genes associated with metabolism (KATHAYAT et al., 2021). Current classification of *E. coli* pathogenic strains is not unified. Certain pathotypes are defined by the organ (e.g.

UPEC), host of the infection (e.g. APEC) or specific genes (e.g. Shiga toxin (*stx)*-producing *E. coli,* STEC) (DENAMUR et al., 2020; MEHAT et al., 2021). WGS has a great potential for investigating the population genomics and molecular epidemiology of *E. coli* from different niches, ultimately clarifying the classification (MEHAT et al., 2021). Next-generation sequencing (NGS) has provided new insights in the evolutionary processes affecting bacterial populations (SHEPPARD et al., 2018). NGS-based population studies have revealed signatures of host adaptation in the bacterial genomes, which included mutations and horizontal acquisition of genetic elements from the host-specific gene pools (SHEPPARD et al., 2018; MEHAT et al., 2021). Such studies investigating the evolutionary history can also be used for identification of outbreak sources, and defining the specific genetic factors determining the success of *E. coli* infection in different host species (MEHAT et al., 2021).

In 2019, transposon-directed insertion site sequencing (TraDIS) strategy was used to screen for virulence-essential genes in avian and mammalian models of *E. coli*-related septicaemia (ZHANG et al., 2019). Several genes and gene clusters were identified as essential for systemic infection and host adaptation. From 280 detected essential genes in avian models, *mprA* and *nhaA* were identified as vital genes for ExPEC virulence and considered as possible targets for the development of broadly conserved vaccine antigens (ZHANG et al., 2019).

Since the prices for whole-genome sequencing have decreased, it is now more available, cost-effective and gradually becoming more applicable in the clinical diagnostics and epidemiological investigations of pathogens (WU et al., 2016; RONCO et al., 2017; BLANC et al., 2020). As *E. coli* is the most important model in biochemical genetics, molecular biology and biotechnology, it has been selected as one of the first organisms for WGS (BLATTNER et al., 1997). Although extensive genome analyses have been performed in order to compare the APEC strains from various serogroups and phylogroups, still no single APEC strain can be considered as the representative of the subpathotype.

1.4. Treatment

Treatment of colibacillosis is based on the application of antimicrobials, usually through drinking water. Unfortunately, the effect is often only temporary, especially if

the infection is secondary (LANDMAN and VAN ECK, 2017). If the treatment with antimicrobials is unavoidable, it should be prescribed according to the results of the antimicrobial susceptibility testing. Apart from the obvious negative effect of the disease on health of the birds, excessive use of antimicrobials also leads to clonal dissemination and acts as selective pressure for the development of antibiotic resistance (IEVY et al., 2020; CHRISTENSEN et al., 2021). Besides the influence of the class of antimicrobials and the route of their administration, the colonization of birds with antibiotic resistant *E. coli* is also affected by the initial prevalence and fitness of the strains (CHANTZIARAS, et al., 2017; CHRISTENSEN et al., 2021). Therefore, the overall effect of antimicrobials on the microbiota and common pathogens in micro-locations such as poultry barns or farms should always be considered.

Nowadays, many farms lean toward antibiotic-free production, and use alternative products such as herbal supplements, organic acids, probiotics or bacteriophage therapy for the control of colibacillosis (CHRISTENSEN et al., 2021). Despite the decreased use of antimicrobials, multidrug resistant bacteria often persist on farms, which maintains a concern for the health of people, animals, and the environment. By relying less on the antimicrobial therapy and investing more in the basic prevention strategies including bio-exclusion, -management and -containment, and carefully planned vaccination programs, the long-term effect of the prudent use of antimicrobials should alleviate the selection pressure and positively influence the antimicrobial resistance.

1.5. Vaccination

Given the heterogeneity of APEC serogroups and quantity of genes involved in the pathogenesis of colibacillosis, there is little or no cross-protection between different strains (GUABIRABA and SCHOULER, 2015; KOUTSIANOS et al., 2020). As poultry health protection is based on good biosecurity practices, adequate immunoprophylaxis programs and controlled production, being aware of the current state in the field is essential. Improvement of the immunoprophylaxis programs on farms should reduce the need for antimicrobials, and consequently reduce the selection pressure for multidrug-resistant strains (BÉLANGER et al., 2011), but there are several challenges in developing the efficacious *E. coli* vaccine. It has to be able to induce cross-protection against various virulent APEC serotypes, it should be deliverable

through mass immunization and, ideally, it should be economically feasible. Commercially available vaccines are often ineffective, mostly because of the high diversity of APEC strains. For that reason, application of autogenous vaccines that are specifically designed for each flock on the farm has become frequent (LANDMAN et al., 2014). In that way, the isolates used for the vaccine are compatible with strains in the specific flock and consequently more effective in respect to the level of provided protection against *E. coli*. Live attenuated vaccines are more appealing because they are available for mass administration and therefore easier to apply, as opposed to inactivated vaccines, which have to be administered individually. Several factors have to be taken into consideration in determining the efficacy of an inactivated vaccine, such as serotypes included in the vaccine, type of adjuvant, method of inactivation, route and frequency of administration, and age of the flock at the time of vaccination (GHUNAIM et al., 2014). On the other hand, subunit vaccines prepared from one or few immunogenic epitopes have proved to be efficacious against both homologous and heterologous challenges, although there are not enough studies confirming the field success. Nowadays, whole-genome sequences enable us to discover new virulence factors suitable for development of new subunit vaccines that could be more effective and provide cross-protection between a wider range of serogroups.

1.6. Economic and public health significance

E. coli infections are one of the most frequently reported diseases in the poultry production worldwide (GUABIRABA and SCHOULER, 2015; NOLAN et al., 2020). The economic impact of the disease includes direct profit losses through mortalities and loss of meat and eggs, but also indirect losses through the cost of treatment, immunoprophylaxis, diagnostics, destruction of dead birds, downgrading and condemnation at processing (GUABIRABA and SCHOULER, 2015; LANDMAN and VAN ECK, 2015; KATHAYAT et al., 2021).

Apart from the economic significance, colibacillosis presents a public health problem. APEC strains can act as reservoirs of virulence and antimicrobial resistance genes, which are mostly horizontally transmitted on plasmids and other mobile genetic elements (EWERS et al., 2007; SINGER et al., 2007; BÉLANGER et al., 2011;

MANGES et al., 2012; MANGES et al., 2016; NJI et al., 2021). Extensive and irresponsible use of antibiotics in agriculture has greatly contributed to the transmission of resistance genes and global development of multidrug-resistant bacteria. Transmission of genes may occur between different ExPEC strains, pathogenic and commensal strains, or even different bacterial species (SKYBERG, 2006; BÉLANGER et al., 2011; MANGES et al., 2016; NJI et al., 2021). Careless use of antimicrobials in the poultry industry is a major problem, presenting an additional danger to human health as these highly resistant strains can be transmitted through contaminated natural environment or food contamination during meat processing (JOHNSON et al., 2009; BÉLANGER et al., 2011; MELLATA, 2013; MANGES et al., 2016; KATHAYAT et al., 2021; NJI et al., 2021).

2. AIMS AND HYPOTHESES

The aim of this research was to determine the effect of autogenous vaccine application on the genetic profile of *E. coli* strains residing on studied poultry farms. The research included strains longitudinally isolated from the daily mortalities in several consecutive flocks, which enabled detection of the genetic variations occurring over time. The strains were compared based on their phylogenetic groups, housekeeping genes, a set of virulence-associated genes that have previously been studied as possible virulence determinants for ExPEC, antimicrobial resistance genes and core-genome single-nucleotide polymorphisms (cgSNPs).

The hypothesis of the dissertation was that application of the autogenous vaccine affects the strain selection and virulence profiles of *E. coli* present on the farms, consequently influencing the overall diversity and pathogenicity of *E. coli* strains.

Besides the scientific value, the obtained research results will contribute to better understanding of how to improve the selection of strains for the production of autogenous vaccines, which would provide better protection of the flocks and maintain the quality of poultry production and food safety for public health.

3. MATERIAL AND METHODS

The research included 115 *E. coli* strains isolated from the poultry carcasses on two production farms located in Donji Pustakovec (Farm A) and Palinovec (Farm B), that are part of the same broiler breeder company. Four and five flocks, from Farm A and B, respectively, were selected for this study. Both farms repeatedly reported problems with colibacillosis, which were ultimately controlled through implementation of autogenous vaccine to the immunoprophylaxis program. Autogenous vaccines were manufactured using several *E. coli* strains isolated from the previous flock on the farm (Table 1 – Paper I. and III., Table II – Paper II.), and therefore every flock was vaccinated with a specifically designed vaccine. The samples were acquired during necropsies of the daily mortalities diagnosed with colibacillosis. The pathomorphological examinations were done as a part of regular monitoring of poultry health on farms or during outbreaks. Weekly mortality rates above 0.4% were considered as outbreaks and the description of strains was provided in the Supplementary Table S1 (Paper I.) and Supplementary Table S1 (Paper II.).

3.1. Sampling and bacteria identification

The swabs were taken from pathomorphologically changed organs during necropsies and streaked directly on basal, enriched and selective types of growth media. After overnight incubation at 37 ºC, identification of the bacteria was done based on the morphological and biochemical characteristics, and confirmed using Bruker Microflex LT MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Afterwards, all samples were stored in Brain Heart Infusion broth (Oxoid, Basingstoke, UK) with 50% glycerol (Fagron Hrvatska d.o.o., Donja Zelina, Croatia) at -20 ºC until further analyses.

3.2. Selection of bacterial strains

The strains for the analyses were selected based on the target organs and age of the birds. Target organs were peritoneum, liver, oviduct and bone marrow as they are most often affected with colibacillosis. In case the strains from the targeted organs were

not available, strains from other tissues such as lungs and pericardium were selected for the analyses (Supplementary Table S1 – Paper I., Supplementary Table S1 – Paper II.). All strains were isolated from the birds older than 21 weeks of age, after the relocation to the production poultry houses and revaccination procedure, when colibacillosis usually start emerging.

3.3. DNA isolation

Bacterial DNA for phylogenetic group determination was isolated using Chelex 100 (BioRad, Hercules, CA, USA). The isolation was performed according to the manufacturer's instructions and stored at -20 °C.

DNA for whole-genome sequencing was isolated using Maxwell RSC Cultured Cells DNA kit (Promega, Madison, WI, USA) and MagAttract HMW DNA kit (Qiagen, Hilden, Germany) for the first 40 and remaining 75 strains, respectively, according to the manufacturer's instructions.

3.4. Phylogenetic group determination

DNA samples were analysed following the slightly adapted PCR protocol developed by Clermont et al. (2013), as described in Paper I. The procedure included quadruplex reaction and additional PCR reaction with specific primers if needed, in order to allocate the investigated strains to phylogroups A, B1, B2, C, D, E, F or cryptic clades I-V.

3.5. Library preparation and whole-genome sequencing

Sequencing library was prepared based on the Illumina technologies and following the manufacturer’s recommendations. Forty isolates were sequenced using MiSeq (Illumina, San Diego, CA, USA) with paired-end 250 bp strategy at the Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, while the remaining 75 isolates were

sequenced using NovaSeq 6000 platform (Illumina, Beijing, China) with paired-end 150 bp strategy at the Novogene Co. Ltd., Cambridge, United Kingdom. The whole genome sequences analysed in this research were deposited to the National Center for Biotechnology Information (NCBI) as BioProject under the accession number PRJNA681385.

3.6. Genomic analyses

3.6.1. MLST and phylogenetic analysis

Assembling and initial editing of the raw sequencing reads was performed using the Assembler v1.2 (LARSEN et al., 2012) on the Center for Genomic Epidemiology (CGE) online platform. Sequence typing analysis was done using the MLST tool on the same platform (LARSEN et al. 2012), after which the obtained sequences of the housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) were concatenated, aligned and phylogenetically analysed using the Molecular Evolutionary Genetics Analysis software (MEGA X) v10.0.5. (KUMAR et al., 2018).

3.6.2. Analysis of the virulence-associated and antimicrobial resistance genes

Database of 84 virulence-associated genes was constructed using the MyDbFinder tool on the CGE platform. The created database included genes that were considered important virulence determinants for ExPEC, and was later used for the analysis of the researched isolates.

Antimicrobial resistance (AMR) genes were detected using ResFinder v4.1. (BORTOLAIA et al., 2020) on the CGE platform, after which the correlation of their prevalence with the application of antimicrobial treatment was statistically analysed.

3.6.3. Single-nucleotide polymorphism analysis

Single-nucleotide polymorphism (SNP) analysis was performed with raw sequence reads, which were previously assembled using SPAdes v3.13.1. After the

analysis, Parsnp v1.2 was used to construct the core-genome SNP tree, which included the investigated and reference genomes (Supplementary Table S2 – Paper III.).

3.7. Statistical analyses

Statistical analyses were done using Statistica v13.2.0.17. (TIBCO Software Inc., Tulsa, OK, USA) and R v4.0.5. (R Core Team, Vienna, Austria).

4. PUBLISHED PAPERS

4.1. PAPER I.: "Phylogenetic characterization of avian pathogenic *Escherichia coli* strains longitudinally isolated from broiler breeder flocks vaccinated with autogenous vaccine"

The article was published in the journal Poultry Science (Elsevier, Amsterdam, the Netherlands), 100(5), pp. 1-6, in May 2021. DOI number of the article is 10.1016/j.psj.2021.101079.

The full paper and its supplementary material can be accessed through the following link: https://www.sciencedirect.com/science/article/pii/S0032579121001139

Phylogenetic characterization of avian pathogenic *Escherichia coli* strains longitudinally isolated from broiler breeder flocks vaccinated with autogenous vaccine

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ABSTRACT *Escherichia coli* is the most common bacterial cause of infections in poultry farms. It is known for its genetic heterogenicity that complicates the protection of poultry health through immunoprophylaxis. In farms with continuous problems with colibacillosis, autogenous *E. coli* vaccine was implemented to the vaccination program instead of commercial vaccines. In this study, we investigated the effect of the autogenous vaccine on *E. coli* phylogroup diversity on 2 broiler breeder farms with 4 and 5 flocks, respectively. The first flocks on both farms were vaccinated with commercial vaccines, while application of autogenous vaccine was introduced in the second flock on both farms. In total, 113 strains were selected based on the target organs and age of chickens. Targeted organs were the peritoneum, liver, oviduct, and bone marrow, and analyzed strains were isolated from chickens older than 21 wk of age when problems with colibacillosis start emerging. The strains were phylotyped by PCR and allocated to phylogroups A, B1, B2, C, D, E, F or clades I–V. The results showed that autogenous vaccine could significantly affect the phylogroup shift of the strains. On farm A, application of the autogenous vaccine induced significantly lower prevalence ($P = 0.01$) of the phylogroups represented in the vaccine among the strains later isolated from the vaccinated flock, while on farm B, the results showed a decrease in the phylogenetic diversity with a dominant prevalence of group B2 despite the vaccine application. The results indicate that implementation of the autogenous vaccine can repress the majority of the strains, but also be unable to eliminate the presence of certain phylogroups, and thus lead to strain shift. Further detailed analyses of multilocus sequence typing and virulence genes will elucidate the pathogenic potential and selection of certain strains, with emphasis on B2 phylogroup.

Key words: *Escherichia coli*, PCR, phylotyping, poultry, autogenous vaccine

2021 Poultry Science 100:101079
https://doi.org/10.1016/j.psj.2021.101079

INTRODUCTION

Escherichia coli is the most common bacterial cause of infections in poultry farms, collectively known as colibacillosis (Jørgensen et al., 2019; Nolan et al., 2020). It has been considered as the secondary pathogen in coinfections with other microorganisms such as *Mycoplasma, Gallibacterium,* or infectious bronchitis virus or to some predisposing factors such as stress and inadequate housing, but recent studies agreed it is often a primary pathogen (Collingwood et al., 2014; Nolan et al., 2020). *E. coli* infection is most commonly acquired through the mucosal colonization of the respiratory or reproductive system or by faecal contamination of the eggs that leads to omphalitis and yolk sac infection (Landman et al., 2013; Guabiraba and Schouler, 2015). Such localized infections often develop into colisepticemia (Nolan et al., 2020), which results in high mortality rates and, consequently, significant economic losses.

E. coli can be divided into intestinal and extraintestinal (**ExPEC**) strains. The ExPEC group includes 4 pathotypes – avian pathogenic *E. coli* (**APEC**), uropathogenic *E. coli*, neonatal meningitis *E. coli*, and sepsis-associated *E. coli* (Sarowska et al., 2019). All pathotypes share some of the virulence factors, but they exhibit an extensive genetic diversity, which complicates early detection of the highly virulent strains. Virulence-associated genes in ExPEC are frequently encoded on pathogenicity islands, plasmids, and other

Received August 3, 2020.
Accepted February 22, 2021.

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mobile genetic elements (Sarowska et al., 2019), but studies have shown that most of the proposed genes in APEC are located on plasmids which enables easy dissemination of different traits among the strains (Johnson et al., 2006, 2008). As ExPEC strains share similar virulence profiles and clonal backgrounds, APEC is a potential zoonotic pathogen (Ewers et al., 2007; Mitchel et al., 2015; Sarowska et al., 2019) and should be researched using the One Health approach.

Clermont et al. (2019) have elaborated a phylotyping method for allocation of *E. coli* strains to different phylogroups. Because genetic heterogenicity often complicates planning of the adequate immunoprophylaxis program, phylotyping is a cost-effective method for determining the phylogenetic relationships between the strains, their diversity, and possible virulence of *E. coli* on farms. Many studies have reported a high prevalence of the ExPEC strains belonging to groups B2 and D (Picard et al., 1999; Clermont et al., 2000; Escobar-Páramo et al., 2006; Pires-dos-Santos et al., 2013; Cordoni et al., 2016). However, some studies have reported a higher prevalence of the strains in groups A and B1, which are considered commensal (Carlos et al., 2010; Solà-Ginés et al., 2015; Cordoni et al., 2016). Former research of APEC strains on Croatian poultry farms has shown that most of the strains were less common serotypes such as O8, O24, O73, O75, O83, and O172 (Gottstein et al., unpublished), and therefore, vaccination with available commercial vaccines is mostly unsuccessful. On farms with continuous problems with colibacillosis, autogenous *E. coli* vaccine was implemented to the vaccination program instead of commercial vaccines. Adaptation of the immunoprophylaxis program has resulted in enhanced production parameters and a significant decrease of morbidity and mortality rates (Gottstein et al., 2019). Because the application of autogenous vaccine has proved to be successful, we wanted to investigate its influence on the phylogenetic profiles of *E. coli* strains later isolated from the vaccinated flocks. In this study, we investigated the phylogenetic relationships of APEC strains longitudinally isolated from broiler breeder flocks before and after the implementation of autogenous *E. coli* vaccine to the immunoprophylaxis program on the farms. The objective was to investigate the effect of the autogenous vaccine on the phylogroup selection of *E. coli* strains in the studied flocks.

MATERIALS AND METHODS

Study Design

Two broiler breeder farms, Farm A and Farm B, with 4 and 5 flocks, respectively, were chosen for this study because of the previous history of severe colibacillosis, which was later controlled using autogenous vaccines. Flocks are later described with a number (flock number) and letter (farm) combination, for example flock 1A meaning flock 1 on farm A. Flock 1A was vaccinated with commercial *E. coli* vaccines and flocks 2A–4A with the autogenous *E. coli* vaccines, while flock 1B was vaccinated with commercial, flock 2B with a combination of commercial live and autogenous vaccines, and flocks 3B–5B only with autogenous vaccines (Table 1). Autogenous vaccines were made from 3 to 4 *E. coli* strains isolated from clinical colibacillosis cases from the previous flock, with the exception of flocks 1B and 2B where 8 and 6 strains were used, respectively. Hence, every flock, where the autogenous vaccine was used, was vaccinated with a specifically designed vaccine manufactured from the strains isolated in the previous flock.

Bacteria Isolation

Swabs were taken from pathomorphologically changed organs during necropsies of the daily mortalities as a part of regular monitoring of poultry health on farms or after high mortality outbreaks. Samples were then streaked directly on Columbia agar (Oxoid, Basingstoke, UK) enriched with 5% sheep blood (BioGnost, Zagreb, Croatia), Brilliant Green agar (Oxoid, Basingstoke, UK), and UTI Brilliance Clarity Chromogenic agar (Oxoid, Basingstoke, UK) and incubated aerobically at 37°C overnight. Identification was carried out based on morphologic characteristics and biochemical analyses, and afterward, it was confirmed using a Bruker Microflex LT MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). All the samples were stored in brain heart infusion broth (Oxoid, Basingstoke, UK) with 50% glycerol at −20°C until further analyses.

Selection of Strains

Altogether, 113 *E. coli* strains were selected for this study (Supplementary Table 1). One strain per bird was analyzed, with the exception of flocks 1B and 2B where 2 to 3 strains per bird were analyzed because of the lack of samples. The selection was based on the target organs the bacteria were isolated from and the age of birds. Targeted organs were the peritoneum, liver, oviduct, and bone marrow as they are most frequently affected. In case of insufficient amount of strains from selected organs, strains isolated from the lungs, pericardium, and subcutaneous caseous exudate were used. Because the vaccinations occurred no later than week 20, after the relocation process to production barns, we chose strains from hens older than 21 wk of age when problems with colibacillosis usually start emerging.

DNA Extraction and PCR Reactions

DNA extraction was performed using Chelex 100 (Hercules, CA) as per the manufacturer's instructions. After extraction, samples were stored at −20°C. Phylogroups were determined by slightly adapted PCR protocol developed by Clermont et al. (2013). Primer sequences for the PCR reactions are listed in Table 2. First, the quadruplex reaction was performed. Based on the results, an isolate was assigned to a certain phylogroup or additionally analyzed with C- or E-specific

Table 1. Description of *Escherichia coli* vaccination program for each flock in this study.

Farm	Flock	Number of analyzed strains per flock	Vaccination	Age at the time of vaccination
A	1	10	Commercial vaccines (live attenuated + inactivated 2x)	0 d 10 wk 18 wk
	2	14	Autogenous vaccine 2x	10 wk 18 wk
	3	14	Autogenous vaccine 2x	12 wk 19 wk
	4	13	Autogenous vaccine 2x	10 wk 19 wk
B	1	13	Commercial vaccines (live attenuated + inactivated 2x)	0 d 13 wk 19 wk
	2	8	Commercial vaccines (live attenuated 2x) Autogenous vaccine 2x	0 d 5 wk 11 wk 18 wk
	3	13	Autogenous vaccine 2x	9 wk 20 wk
	4	15	Autogenous vaccine 2x	10 wk 18 wk
	5	13	Autogenous vaccine 2x	9 wk 17 wk

primers as described in Table 3. The reaction mixture was composed of 6 µL GoTaq G2 Hot Start Green Master Mix (Promega, Madison, WI), 0.4 µL of each 10 pmol forward and reverse primers, 2 µL of DNA, and 3.8 µL or 6.2 µL of nuclease-free water (Promega, Madison, WI) for the quadruplex reaction or C/E-specific reaction, respectively (total volume = 15 µL). The cycling parameters for amplification included: 95°C for 5 min and then 30 cycles of denaturation at 95°C for 10 s, annealing at 59°C for 30 s (quadruplex and group C) or 57°C for 30 s (group E), elongation at 72°C for 30 s, and final extension step at 72°C for 5 min. PCR products were visualized on 1.5% agarose gel electrophoresis stained with Midori Green Advance (Nippon Genetics Europe GmbH, Düren, Germany).

Statistical Analyses

The statistical analyses were performed in Statistica 13.5.0.17. (TIBCO Software Inc.) software. The significance of differences in the frequency of strains between individual flocks (within farm) was analyzed using chi-square test with statistical significance set at level $P < 0.05$. In addition, Mann-Whitney U test was used to compare the results for B2 phylogroup on farm B with expected theoretical values, and *t*-test to compare total frequency of merged phylogroups on farm B.

RESULTS AND DISCUSSION

The objective of this study was to investigate the effect of the autogenous vaccine on the phylogroup selection of APEC strains in the studied flocks. Owing to high genetic diversity and poor cross-protection among *E. coli* strains, application of autogenous vaccines specifically produced for each flock has become frequent (Landman et al., 2014). Isolates used for the production of the vaccines were phylotyped as per the protocol designed by Clermont et al. (2013), together with the isolates chosen for this research. Our results indicate that autogenous vaccines have had an influence on the phylogroup selection of the strains on both farms, but the selection occurred in different directions. On farm A, application of the autogenous vaccine induced lower prevalence of the phylogenetic groups represented in that vaccine among the strains later isolated from the

Table 2. Primer sequences and sizes of PCR products used in PCR reactions.[1]

PCR reaction	Primer	Target	Primer sequence	PCR product (bp)
Quadruplex	chuA.a1	*chuA*	5′-ATGGTACCGGACGAACCAAC-3′	288
	chuA.2		5′-TGCCGCCAGTACCAAAGACA-3′	
	yjaA.1 b	*yjaA*	5′-CAAACGTGAAGTGTCAGGAG-3′	211
	yjaA.2 b		5′-AATGCGTTCCTCAACCTGTG-3′	
	TspE4.C2.1 b	TspE4.C2	5′-CACTATTCGTAAGGTCATCC-3′	152
	TspE4.C2.2 b		5′-AGTTTATCGCTGCGGGTCGC-3′	
	AceK.f		5′-AACGCTATTCGCCAGCTTGC-3′	400
	ArpA1.r	*arpA*	5′-TCTCCCCATACCGTACGCTA-3′	
Group E	ArpAgpE.f	*arpA*	5′-GATTCCATCTTGTCAAAATATGCC-3′	301
	ArpAgpE.r		5′-GAAAAGAAAAAGAATTCCCAAGAG-3′	
Group C	trpAgpC.f	*trpA*	5′-AGTTTTATGCCCAGTGCGAG-3′	219
	trpAgpC.r		5′-TCTGCGCCGGTCACGCCC-3′	

[1]Reference (Clermont et al., 2013).

Table 3. Description of genotypes for phylogroup assignment.[1]

Phylogroup	Quadruplex genotype			
	arpA	*chuA*	*yjaA*	TspE4.C2
A	+	−	−	−
B1	+	−	−	+
F	−	+	−	−
B2	−	+	+	−
B2	−	+	+	+
B2	−	+	−	+
A or C	+	−	+	−
D or E	+	+	−	−
D or E	+	+	−	+
E or clade I	+	+	+	−
Clade I or II	−	−	+	−
Clade III, IV or V	−	476[2]	−	−
Unknown[3]	−	−	−	+
Unknown	−	−	+	+
Unknown	+	−	+	+
Unknown	+	+	+	+

[1]Reference (Clermont et al., 2013).
[2]The quadruplex PCR reaction can result in strains belonging to cryptic clade III, IV or V yielding a 476 bp PCR product. If this is the case, such strains should be screened using the cryptic clade detection primers (Clermont et al., 2011b).
[3]These unassignable strains represent phylogroups that are very rare or are the results of large-scale recombinations and therefore untypable unless multilocus sequence typing (MLST) is performed.

next flock (Table 4). In flock 3A, there was a significant change in the prevalence of isolated phylogroups ($P = 0.00$). The prevalence of phylogroup A dropped because it was the only group present in the vaccine, while the prevalence of phylogroup F increased. The same occurred in Flock 4A that was vaccinated with isolates belonging to phylogroup F. Vaccination led to a highly significant change of prevalence ($P = 0.00$), which resulted in a decreased number of isolates in phylogroup F and considerable increase of clades I/II and phylogroup C, as well as distinct phylogenetic diversification of the strains. Because flock 2A was the first one in which autogenous vaccine was applied, phylogenetic groups were diverse with less obvious shift than in the next flocks, but the tendency of the vaccine effect could be seen. Prevalence of phylogroups A and B1 in flock 2A increased, phylogroups B2 and C were represented with only 1 isolate per group, while the prevalence of phylogroups D and F decreased. The applied autogenous vaccine contained isolates belonging to phylogroups B1, D, and F, which implies that strain shift was initiated from the beginning of autogenous vaccine application (Table 4). Interestingly, phylogroup B2 on farm B was the most prevalent with constantly increasing prevalence throughout the flocks, with the exception of flock 4B in which phylogroup F was dominant (Table 4). In flock 5B, the prevalence of B2 phylogroup was 100%, despite its application in the vaccine, which implies there were possibly several highly virulent and resistant strains in the initial flocks that subsisted. Although the manufactured vaccine for every flock contained strains belonging to phylogroup B2, strain shift went in the opposite direction contrary to the results on farm A. Statistical analyses showed a significant change of phylogroup prevalence in flocks 2–4A ($P = 0.00$), as opposed to farm B where vaccination with autogenous did not have the same effect, and there were no statistically significant changes in the prevalence ($P = 0.766$). However, significant resistance to reduction of the isolates belonging to B2 phylogroup was confirmed on farm B using Mann-Whitney U test. When compared to expected frequencies after autogenous vaccine application, based on the results achieved on farm A, phylogroup B2 strains on farm B showed significant resistance to reduction ($P = 0.0317$). Regarding phylotypic similarity of isolates from the same animal (Supplementary Table 1), the results agree with both previous reports, confirming homogenicity of the isolates (Poulsen et al., 2017) but also simultaneous infection with different strains (Paudel et al., 2016). Further analyses including the comparison of the isolates based on multilocus sequence typing and presence of virulence-associated genes will elucidate the reason for strain shift and reveal how related are the analyzed isolates. Extensive analysis of the virulence-associated genes could clarify which genes have the potential to be used in the production of commercial broad-spectrum subunit *E. coli* vaccines.

In the research by Horvatek Tomić et al. (2017), 32 strains from the same broiler breeder company that was studied in this research were phylogenetically characterized and the results showed a very high prevalence of B2 phylogroup (53.13%). The study was carried out as per the protocol reported by Clermont et al. (2000) which included phylogenetic analysis for identification of only 4 main groups – A, B1, B2 and D. Since then, phylotyping method has been improved and extended to identification of 7 phylogroups – A, B1, B2, C, D, E and F – with addition of cryptic clades I–V (Clermont et al., 2013), all of which could be determined using different sets of primers following given protocol for typing. A recent study by Clermont et al. (2019) has proposed novel phylogroup G, which is considered a sister group to B2, along with F group. Phylogenetic analyses have shown similarity among A, B1, C, and E groups and B2, D, F, and G groups, with highest relatedness of B2, F, and G groups (Clermont et al., 2013, 2019), which could explain the increased prevalence of phylogroup F in flock 4B. If groups on farms A and B are merged based on their phylogenetic relatedness and examined together, the results show the highest prevalence of group B2/D/F (45.1, 82.3%), following A/B1/C/E (37.3, 14.5%), clades I–V (11.8, 3.2%), and 3 unknown isolates (5.9%) on farm A (Table 4), which agrees with previous research (Horvatek Tomić et al., 2017). When analyzed statistically, results on farm B showed significantly higher prevalence of B2/D/F phylogroups ($P = 0.0003$), what is also in congruence with aforementioned resistance to reduction of B2 phylogroup (Table 4).

Various environmental factors additionally affect the genetic diversity of pathogens, as well as physiological microflora. Most of the former attempts to decrease the prevalence of colibacillosis in poultry farms were based on the excessive use of antibiotics, which led to

Table 4. Frequency of individual and merged phylogroups per flock and farm (number [%]).

Farm	Flock	Phylogroup										
		A	B1	B2	C	D	E	F	Clades	Unknown	A/B1/C/E	B2/D/F
A	1	4 (40)	**1**[1] (10)	-	-	**1** (10)	-	**4** (40)	-	-	5 (50)	5 (50)
	2[2]*	**6** (42.9)	2 (14.3)	1 (7.1)	1 (7.1)	-	-	1 (7.1)	-	3 (21.4)	9 (64.3)	2 (14.3)
	3***	1 (7.1)	-	-	-	-	-	**13** (92.9)	-	-	1 (7.1)	13 (92.9)
	4***	1 (7.7)	-	-	3 (23.1)	1 (7.7)	-	2 (15.4)	6 (46.2)	-	4 (30.8)	3 (23.1)
In total**		12 (23.5)	3 (5.9)	1 (2)	4 (7.8)	2 (3,9)	-	20 (39.2)	6 (11.8)	3 (5.9)	19 (37.3)	23 (45.1)
B	1	1 (7.7)	-	**7** (53.8)	-	-	1 (7.7)	**4** (30.8)	-	-	2 (15.4)	11 (84.6)
	2	**2** (25)	-	**5** (62.5)	1 (12.5)	-	-	-	-	-	3 (37.5)	5 (62.5)
	3	2 (15.4)	-	**9** (69.2)	-	-	-	2 (15.4)	-	-	2 (15.4)	11 (84.6)
	4	2 (13.3)	-	**4** (26.7)	-	-	-	**7** (46.7)	2 (13.3)	-	2 (13.3)	11 (73.3)
	5	-	-	13 (100)	-	-	-	-	-	-	-	13 (100)
In total		7 (11.3)	-	38 (61.3)	1 (1.6)	-	1 (1.6)	13 (21)	2 (3.2)	-	9[3B] (14.5)	51[A] (82.3)

[1]Numbers in bold in each flock row represent phylogroups used in the autogenous vaccine for the next flock.
[2]Every flock is compared with the previous flock on the same farm, with regard to the prevalence of individual phylogroups. Statistically significant values are indicated as follows: $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$.
[3]Statistically significant differences ($P = 0.0003$) between total merged phylogroups on farm B are indicated with different capital alphabet letters (A,B).

the development of multidrug-resistant strains that are hard to eradicate (Thomrongsuwannakij et al., 2020). On farm A, the number of therapies and the total number of hens which were under therapy decreased over time, what most likely affected the heterogenicity of the strains as well. Flock 1A was given antibiotics through drinking water 2 times during production period and flock 2A only once, while flocks 3A and 4A did not receive any therapy (Gottstein et al., 2019). On the contrary, on farm B, the number of therapies in the first 3 flocks was decreasing but in the last 2 flocks increased again because of the problems with necrotic enteritis that was most likely caused by low-quality feed. Overuse of antimicrobials can potentially induce a strain shift as well (Walk et al., 2007; Bibbal et al., 2009). Zakariazadeh et al. (2019) have reported in their study that overconsumption of antibiotics has resulted in alteration of commensal phylogenetic groups and has influenced the genetic structure of both commensal and pathogenic microflora. Owing to infection pressure on poultry farms, different stressful conditions can result in the emergence of infectious diseases that could possibly be prevented by applying basic management measures. *E. coli* strains that are classified into clades are phenotypically hard to differentiate from *E. coli sensu stricto* but genetically are highly divergent (Lescat et al., 2013). They have been considered commensal in the digestive tract of various species, with high prevalence in birds, as opposed to low prevalence in humans (Clermont et al., 2011a; Lescat et al., 2013). Our results on farm A show an increase of strains allocated to clades I/II in the last flock, which indicates a possible decrease of pathogenic strains' pressure over time after regular application of autogenous vaccine. In contrast, the results on farm B imply there is an increase of potentially highly virulent strains that subsisted on the farm, which resulted in decreased phylogenetic diversity, possibly as a result of the use of antimicrobials. Such highly virulent strains could be eliminated using alternative methods such as bacteriophage therapy and application of organic acids.

In conclusion, the results indicate that application of autogenous vaccine affects the phylogroup prevalence and phylogenetic relationships of APEC strains on poultry farms. This study investigated the distribution of different phylogroups in longitudinally sampled flocks, which showed that implementation of the autogenous vaccine could repress most phylogroups used in a vaccine but also enable selection of certain phylogroups and lead to strain shift.

ACKNOWLEDGMENTS

Part of this work was supported by the University of Zagreb, Croatia, under Grant "Longitudinal epizootiological study of the virulence-associated genes by whole genome sequencing of the pathogens with aim to improve biosafety measures and immunoprophylaxis programs on poultry farms." This article does not contain any studies with human participants or animals performed by any of the authors and all research was conducted in an ethical and responsible manner. The study was approved by the Committee on Research Ethics of the Faculty of Veterinary Medicine, University of Zagreb, and permission for sampling was granted by the farm.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2021.101079.

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4.2. PAPER II.: "Longitudinal study on the effect of autogenous vaccine application on the sequence type and virulence profiles of *Escherichia coli* in broiler breeder flocks"

The article was published in the journal Veterinary Microbiology (Elsevier, Amsterdam, the Netherlands), 259, pp. 1-12, in August 2021. DOI number of the article is 10.1016/j.vetmic.2021.109159.

The full paper and its supplementary material can be accessed through the following link: https://www.sciencedirect.com/science/article/pii/S0378113521001826

Veterinary Microbiology 259 (2021) 109159

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Longitudinal study on the effect of autogenous vaccine application on the sequence type and virulence profiles of *Escherichia coli* in broiler breeder flocks

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ARTICLE INFO

Keywords:
Escherichia coli
APEC
Poultry
Autogenous vaccine
Whole genome sequencing
Sequence type

ABSTRACT

Colibacillosis is one of the most common problems in the poultry industry. *Escherichia coli* strains on farms are often genetically diverse and therefore commercial vaccines provide little protection to the flocks. Here, we investigated the effect of the autogenous *E. coli* vaccines on the prevalence of 84 virulence-associated genes in *E. coli* isolated from four and five consecutive flocks on two broiler breeder farms, respectively. 115 *E. coli* isolates were sequenced using Illumina technologies, and compared based on both their set of housekeeping genes and their virulence profiles, defined through the composition of virulence genes. Predominantly, phylogenetic analysis showed obvious distinction between the isolates originating from different farms suggesting spatial-dependent transmission of pathogenic strains. We detected 23 sequence types, while 52.58 % of the isolates belonged to two clonal complexes. Analysis of the virulence genes showed highest prevalence (>85 %) of *feo*B, *usp*A, *usp*B, *usp*G, *usp*E, *fim*H, *omp*A, *ast*A, *foc*A, *hly*E, *usp*C, *crl*, *csg*A, *omp*T and *iss*, of which 50 % are toxin associated genes, demonstrating the importance of competition in the pathogenesis process. Interestingly, *usp* genes, which are primarily associated with uropathogenic *E. coli* strains, were detected in all investigated isolates. The heatmap analysis demonstrated that strains belonging to same phylogenetic groups often share similar virulence profiles, confirming the usefulness of quick tests for phylogenetic typing. However, our results suggest the need to update the list of the minimal predictors used for the identification of avian pathogenic strains. Overall results indicate that continuous application of autogenous vaccines led to lower genetic diversity of *E. coli* housekeeping genes, but not virulence genes.

1. Introduction

Colibacillosis is one of the most common problems in the poultry industry, causing decreased production and, consequently, major economic losses (Nolan et al., 2020). It is caused by *Escherichia coli (E. coli)*, which can be divided into intestinal and extraintestinal (ExPEC) strains. All ExPEC subpathotypes depend on the virulence factors to survive in the environment outside of the intestine. However, there are no conclusive criteria which virulence factors define different subpathotypes. To date, there are four confirmed subpathotypes including avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and sepsis-associated *E. coli* (SEPEC) (Sarowska et al., 2019), and two emerging- mammary pathogenic *E. coli* (MPEC) and endometrial pathogenic *E. coli* (EnPEC) (Shpigel et al., 2008; Sheldon et al., 2010). Many studies have reported specific sets of virulence factors that can be used as APEC predictors (Dziva and Stevens, 2008; Johnson et al., 2008a; Schouler et al., 2012; Dissanayake et al., 2014; De Oliveira et al., 2015). Therefore, various PCR multiplex assays have been developed in order to simplify the process of diagnostics (Ewers et al., 2005; Johnson et al., 2008a; Schouler et al., 2012; Cordoni et al., 2016; de Oliveira et al., 2020). Some research studies have concluded that all APEC-associated virulence factors are carried on plasmids, specifically ColV and ColBM (Johnson et al., 2006b; Skyberg et al., 2008; de Oliveira et al., 2015). As plasmid pathogenicity islands (PAIs) have been frequently detected in the highly virulent strains, they are considered a defining trait of the APEC subpathotype (Rodriguez-Siek et al., 2005).

Poultry health protection is based on the controlled production, good

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https://doi.org/10.1016/j.vetmic.2021.109159
Received 5 January 2021; Accepted 16 June 2021
Available online 19 June 2021

biosecurity practice and immunoprophylaxis programmes. However, many farms do not have the conditions or resources to implement strict biosecurity measures and maintain entirely closed production, so health protection often depends on the quality of the immunoprophylaxis programme, which has to be adjusted to the epidemiological area in which the farm is located. Farms usually use live or inactivated commercial *E. coli* vaccines that consist of one or a few strains such as O1, O2 and O78, which are considered most common (Ewers et al., 2004; Dziva and Stevens, 2008; Schouler et al., 2012; Nolan et al., 2020). However, *E. coli* strains causing disease on farms are often very diverse and commercial vaccines may not provide significant cross protection to be effective against this diversity. Currently, there is no specific vaccination procedure that is highly efficacious against multiple serotypes (Nolan et al., 2020). For that reason, many farms have started using autogenous vaccines that are designed specifically for each flock, and have proven to be efficient (Landman et al., 2014; Landman and van Eck, 2017).

Based on the significant improvement in the morbidity and mortality rates, as well as performance parameters, continuous application of the autogenous vaccines on Croatian farms has proven to be successful (Gottstein et al., 2019). Previous phylogenetic analyses have indicated that autogenous vaccines can induce strain shift on the farms, depending on the strain type (Lozica et al., 2021). In this longitudinal study, we investigated the prevalence of the selected virulence-associated genes in several consecutive flocks on two broiler breeder farms that have had continuous problems with colibacillosis despite the application of the commercial vaccines. The phylogenetic relationship of the isolates was assessed based on the set of *E. coli* housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA)* used for the multilocus sequence typing (MLST). The aim was to investigate the variability of virulence-associated genes over time after the implementation of the autogenous vaccine instead of the commercial *E. coli* vaccines to the immunoprophylaxis programme on the studied farms.

2. Material and methods

2.1. Study design

Two broiler breeder farms that are part of the same company- Farm A and Farm B, with four and five flocks, respectively, were chosen for this study. Each flock included 35,000 birds on average. Due to the repeated problems with colibacillosis, autogenous vaccine was implemented to the immunoprophylaxis programme instead of the commercial *E. coli* vaccines. Autogenous vaccines were produced using three to four *E. coli* strains isolated from the clinical cases from the previous flock, with the exception of Flocks 1 and 2 on Farm B where seven and six strains, respectively, were used in order to cover a wider range of the strains. Each flock was vaccinated with a specifically designed vaccine manufactured from the strains isolated in the previous flock. After the effect of autogenous vaccines on the health status (Table 1) and performance of the flocks proved to be successful (Gottstein et al., 2019), 115 *E. coli* strains originating from the flocks vaccinated with the commercial and/or autogenous vaccines were selected for whole-genome sequencing (Table 2).

2.2. Bacterial strain selection

Bacterial strains were isolated from the daily mortalities diagnosed with colibacillosis that were subjected to the pathomorphological examination as a part of the routine health surveillance or after unexpected rise of mortality rates (Supplementary Table S1). Weekly mortality rates above 0.4 % were considered as outbreaks. All isolates were recovered from the birds with lesions consistent with colibacillosis and the most frequently found were polyserositis, fibrinous peritonitis, pericarditis, pneumonia, salpingitis and septicaemia. As all the samples were recovered from the organs that were severely affected, the presumption was that the most virulent strains in each flock would be analysed. Identification of *E. coli* was confirmed by Bruker Microflex LT MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). All the samples were stored in Brain Heart Infusion broth (Oxoid, Basingstoke, UK) with 50 % glycerol at -20 °C until further analyses. Selection of the strains was based on the target organs they were isolated from and age of the birds. Target organs were peritoneum, liver, oviduct and bone marrow as they are most often affected with colibacillosis. In case the strains from targeted organs were not available, strains from other tissues such as lungs and pericardium were selected. All strains were isolated from birds older than 21 weeks of age, when problems with colibacillosis usually appear. Minimum three strains per flock used in the vaccine production were sequenced and included in the analyses (Supplementary Table S1). The selection procedure was expected to produce some bias toward more virulent strains of *E. coli,* but this was considered an advantage, as these are the strains whose virulence profiles are of the greatest interest.

Table 2
Description of *E. coli* vaccination program for each flock in this study[1].

Farm	Flock	Number of analysed strains per flock	Vaccination	Age at the time of vaccination
A	1	10	Commercial vaccines (live attenuated + inactivated 2x)	0 d 10 w 18 w
	2	14	Autogenous vaccine 2x	10 w 18 w
	3	13	Autogenous vaccine 2x	12 w 19 w
	4	14	Autogenous vaccine 2x	10 w 19 w
B	1	13	Commercial vaccines (live attenuated + inactivated 2x)	0 d 13 w 19 w
	2	7	Commercial vaccines (live attenuated 2x) Autogenous vaccine 2x	0 d 5 w 11 w 18 w
	3	15	Autogenous vaccine 2x	9 w 20 w
	4	16	Autogenous vaccine 2x	10 w 18 w
	5	13	Autogenous vaccine 2x	9 w 17 w

[1] Lozica et al. (2021).

2.3. DNA isolation

Bacterial DNA was isolated using Maxwell RSC Cultured Cells DNA Kit (Promega, Madison, WI, USA) and MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) for the first 40 and the remaining 75 strains,

Table 1
Average weekly mortality (%±SD) during the production period on both farms. Statistically significant differences are marked with lowercase superscripts.

Farm	A				B				
Flock	1	2	3	4	1	2	3	4	5
Average weekly mortality (%)	0.52 ± 0.23^{a}	0.38 ± 0.23^{bc} (p = 0.019)	0.31 ± 0.08^{c} (p = 0.002)	0.43 ± 0.18^{ab}	0.59 ± 0.2^{a}	0.47 ± 0.09^{a}	0.37 ± 0.34^{b} (p = 0.03)	0.28 ± 0.11^{bc} (p = 0.000003)	0.25 ± 0.09^{c} (p = 0.018)

respectively, according to the manufacturers' instructions.

2.4. Library preparation and whole-genome sequencing

Sequencing library was generated based on the Illumina technologies and following the manufacturer's recommendations. The library was analysed for size distribution and quantified using real-time PCR. Forty and 75 isolates were sequenced using MiSeq (Illumina, San Diego, CA, USA) with paired-end 250 bp strategy, and NovaSeq 6000 platform (Novogene Co. Ltd., Beijing, China) with paired-end 150 bp strategy, respectively.

2.5. MLST and phylogenetic analysis

Raw sequencing reads were assembled and trimmed using Assembler 1.2 on the Center for Genomic Epidemiology (CGE) online platform (Larsen et al., 2012). Next, sequence typing analyses were done using MLST tool on the same platform. Obtained sequences of the housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) were concatenated and aligned using ClustalW (Kumar et al., 2018). Afterwards, a phylogenetic tree was constructed by the Maximum Likelihood method and Tamura-Nei parameter model with gamma distributed rates with invariant sites using the Molecular Evolutionary Genetics Analysis software (MEGA X) version 10.0.5. (Tamura and Nei, 1993; Kumar et al., 2018). Four *E. coli* and one *E. fergusonii* genome sequences with the following accession numbers- CP000243, CP004009, CP007275, NC0026952 and CP055696, respectively, were used as outgroups for the construction of the phylogenetic tree. Whole-genome sequences analysed in this study were deposited to the National Center for Biotechnology Information (NCBI) as BioProject under the accession number PRJNA681385.

2.6. Analysis of the virulence-associated genes

We constructed a database of 84 virulence-associated genes, which was later used for the analysis of the researched isolates (see Table 4 for the list of virulence genes). The database was constructed based on the previous research done on the virulence profiles of APEC (Rodriguez-Siek et al., 2005; Johnson et al., 2006a; Johnson et al., 2008a; Jørgensen et al., 2019). Detection of the virulence-associated genes was done using MyDbFinder tool on the CGE online platform.

2.7. Heatmap construction

We have transformed gene presence/absence data into a colour scale *via* the heatmap() function in base R programming language (v4.0.3). The scaling option was switched off. By default, the heatmap() function performs hierarchical clustering on both sample and feature data and reorders it accordingly.

2.8. Principal coordinate analysis

To inspect patterns within the gene composition datasets, we used principal coordinate analysis (PCoA) as implemented in R language ade4 package (v1.7-15). The dissimilarity matrix for the PCoA was calculated using Jaccard distance between samples, as they consisted of the binary data representing the presence/absence of genes. The matrices were confirmed as euclidean using is.euclid() function of ade4. The ellipses were calculated and visualised using R car package (v3.0-10).

2.9. Statistical analyses

The statistical analyses were done in Statistica 13.5.0.17. (TIBCO Software Inc., Tulsa, OK, USA). The results were tested for the normality of data distribution using Smirnov-Kolmogorov test. Afterwards, the significance of differences in the frequency of STs between flocks within each farm, considering the STs used in the vaccine, was analysed using Wald-Wolfowitz Runs Test, while total virulence gene frequency per flocks and farms was tested with parametric ANOVA LSD test. Total grouped virulence gene frequency per flocks and average weekly mortality between flocks within each farm were tested using nonparametric Kruskal-Wallis test, with statistical significance set at level $p \leq 0.05$.

3. Results and discussion

3.1. MLST and phylogenetic analysis

Phylogenetic analysis found distinct differences between the isolates originating from different farms, in most cases (Fig. 1). This finding indicates a spatial-dependent transmission of the pathogenic *E. coli* strains. Eleven out of fourteen isolates (79 %) from Flock 4 on Farm A were grouped in one individual cluster, compared to the lower percentages in previous flocks, suggesting a decrease in strain diversity over time (Supplemetary Table S1). The phylogenetic analysis revealed high relatedness of our samples to APEC and other ExPEC subpathotypes used as outgroups, which was expected in light of the previous research (Ewers et al., 2007; Danzeisen et al., 2013; Pires-dos-Santos et al., 2013).

In total, 23 sequence types (ST) and one unknown ST were identified (Table 3). Four STs constituted more than 60 % of the total strains investigated, with ST95 and ST117 being the far most prevalent and constituting 42.24 %. Interestingly, ST95 was detected only on Farm B, while ST117 was the only diverse type that was present on both Farms in high quantity. ST95, ST69, ST23 and ST131 are very common causes of extraintestinal infections in human population and poultry (Bert et al., 2010; Danzeisen et al., 2013; Pires-dos-Santos et al., 2013; Stephens et al., 2017; Jørgensen et al., 2019), confirmed here by the highest prevalence of ST95 in our study. Previous research on *E. coli* genetic diversity in broiler breeder flocks in Denmark has showed that STs are not infection-type or host-specific, which suggests there may be a zoonotic potential (Bert et al., 2010; Pires-dos-Santos et al., 2013). Our data revealed high diversity of virulence genes in general, but within a small number of clonal complexes (CC), with 52.58 % of the isolates belonging to two clonal complexes. Virulence profiles are strongly ST-dependent (Fig. 2) which indicates a possible link between the phylogenetic groups and STs. ST117 has been reported as a causative agent of an increased incidence of colibacillosis cases on Nordic poultry farms from 2014 to 2016 (Ronco et al., 2017). In accordance with that, our data shows the highest prevalence of said ST in Flock 3 on Farm A, and Flock 4 on Farm B, after which the frequency abruptly decreases, presumably due to the application of autogenous vaccine, as previously reported (Lozica et al., 2021). Since its prevalence on both farms is high and the strains are genetically similar (Fig. 1), the origin of ST117 could be from the grandparent flocks (Ronco et al., 2017) or from the rearing houses they share. Over time, clonal complexes evolve and only highly virulent strains are able to subsist on farms. According to the prevalence data in the studied flocks, ST117 was probably not adapted as well as CC95, and consequently did not persist after adequate treatment. Global increase of the antimicrobial resistance to fluoroquinolones and extended-spectrum ß-lactamases has been linked to the emergence and worldwide spread of *E. coli* ST131, which is nowadays the predominant ExPEC lineage (Nicolas-Chanoine et al., 2014). As a carrier of a large number of resistance and virulence-associated genes, ST131 clonal complex has been frequently reported as a causative agent of human urinary infections and bacteraemia (Nicolas-Chanoine et al., 2014). In our study, it was detected only on Farm B, illustrating further the spatial dependence of *E. coli* transmission. Despite the presence of this highly virulent ST, the mortality rates in the Farm B significantly decreased over time (Table 1), possibly due to the use of vaccines. Lastly, the analyses showed a significant reduction of STs in the vaccine compared to the strains later isolated in the Flock 4 on Farm A ($p < 0.019$) and Flock 3 on Farm B ($p < 0.00002$), confirming that implementation of autogenous

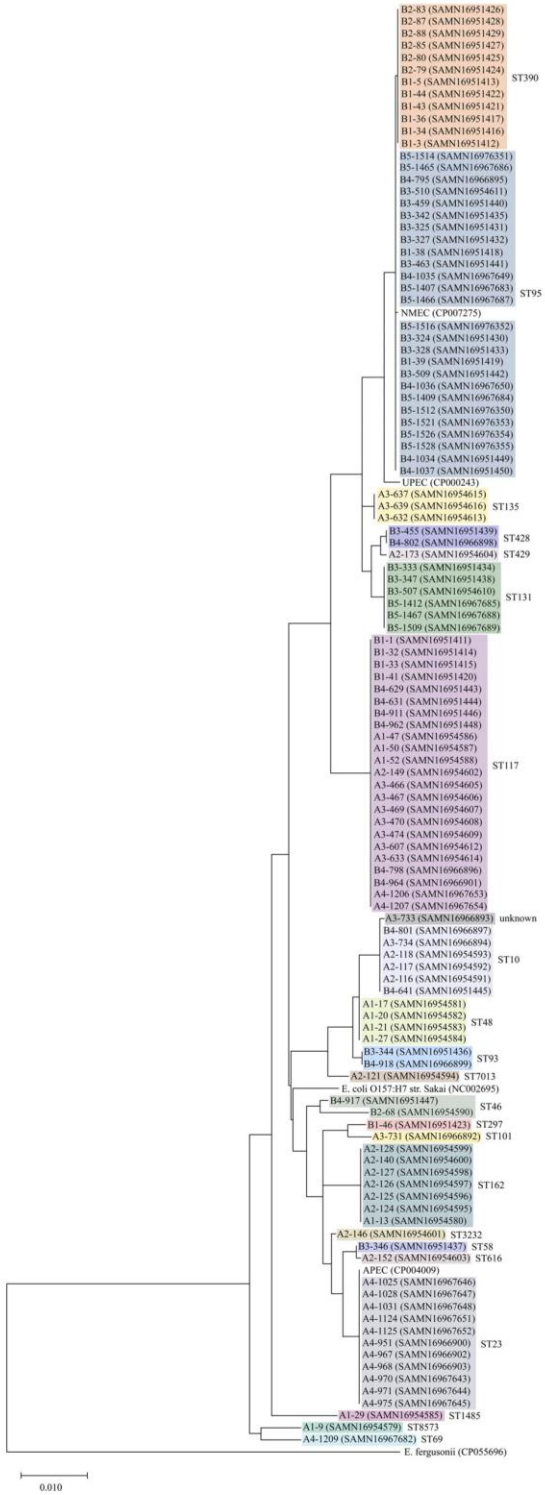

Fig. 1. Phylogenetic tree showing relatedness of the isolates from both farms. The letter represents the farm (A- Farm A; B- Farm B), while the number represents the flock. STs are marked with different colours. BioSample accession numbers are displayed in the brackets.

Table 3
Prevalence of sequence types (STs) according to the farm of origin and average number of virulence-associated genes (± SD) per ST.

ST	Farm A	Farm B	In total (%)	Average number of virulence genes (%)
10	4	2	6 (5.22)	22.33 ± 9.11 (26.27)
23	11	0	11 (9.57)	35.27 ± 11.04 (41.5)
46	0	1	1 (0.87)	18 (21.18)
48	4	0	4 (3.48)	14 ± 0.00 (16.47)
58	0	1	1 (0.87)	38 (44.71)
69	1	0	1 (0.87)	24 (28.24)
93	0	2	2 (1.74)	41 ± 5.66(48.24)
95	0	26	26 (22.61)	46 ± 7.60 (54.12)
101	1	0	1 (0.87)	48 (56.47)
117	13	10	23 (20)	41.65 ± 6.59 (49)
131	0	6	6 (5.22)	51.83 ± 8.77 (60.98)
135	3	0	3 (2.61)	41 ± 0.00 (48.24)
162	7	0	7 (6.09)	23.71 ± 7.63 (27.9)
297	0	1	1 (0.87)	21 (24.71)
390	0	12	12 (10.43)	45.25 ± 6.08 (53.24)
428	0	2	2 (1.74)	37 ± 11.31 (43.53)
429	1	0	1 (0.87)	54 (63.53)
616	1	0	1 (0.87)	35 (41.18)
746	0	1	1 (0.87)	34 (40)
1485	1	0	1 (0.87)	48 (56.47)
3232	1	0	1 (0.87)	37 (43.53)
7013	1	0	1 (0.87)	46 (54.12)
8573	1	0	1 (0.87)	38 (44.71)
unknown	1	0	1 (0.87)	35 (41.18)

vaccine repressed some of the STs that were used in the vaccine (Supplementary Table S1).

3.2. Analysis of the virulence-associated genes

The results showed highest prevalence (>85 %) of the virulence factors *feo*B, *usp*A, *usp*B, *usp*G, *usp*E, *fim*H, *omp*A, *ast*A, *foc*A, *hly*E, *usp*C, *crl*, *csg*A and *omp*T, which are located on the chromosome, with the exception of *iss* gene which is plasmid borne (Table 4). Half of the mentioned factors are toxins (see Table 4 for classification of gene functions), namely bacteriocin-related, which indicates that the competitive phenotype of the bacteria is very important in the pathogenesis process and for the survival of highly virulent strains. Association of the bacteriocin-encoding genes and virulence determinants in ExPEC strains has already been documented by Micenková et al. (2014)., who proved their positive correlation. The prevalence of toxin genes supports the idea of clonal selection and dominance of several strains per flock. Our data shows an increasing trend of the average number of virulence-associated genes per isolate on Farm A (Fig. 3A), while the trend on Farm B is minimally decreasing (Fig. 3B), probably due to the average frequency being initially very high on Farm B (Fig. 3C). A study investigating the diversity and genetic population overlap between the ST95 of human and avian origin (Jørgensen et al., 2019) has had results similar to ours. Although the prevalence of the investigated virulence-genes was variable, the results mainly corresponded to our findings.

According to the analysis based solely on the previously suggested minimal predictors for identification of APEC strains (Johnson et al., 2008a; de Oliveira et al., 2015), only 6/10 (60 %), 5/14 (37.71 %), 11/13 (84.62 %), 8/14 (57.14 %) of the isolates in the Flocks 1–4 on Farm A, and 11/13 (84.62 %), 6/7 (85.71 %), 15/15 (100 %), 12/16 (75 %) and 10/13 (76.92 %) of the isolates in the Flocks 1–5 on Farm B could be categorized as APEC. No correlation was detected between the organ of origin or the phylogroup and identification of the isolate as APEC. A considerably lower number of confirmed APEC strains was detected on Farm A, which is in agreement with the less severe clinical symptoms detected on that farm.

Iron is the essential micronutrient for bacteria as it enables their growth, colonization and survival in the host (Kronstad and Caza, 2013).

Table 4
Prevalence of the researched virulence-associated genes (%).

Group	Gene or operon	Farm A					Farm B						In total N = 115	Accession number
		Flock 1 n = 10	Flock 2 n = 14	Flock 3 n = 13	Flock 4 n = 14	In total A n = 51	Flock 1 n = 13	Flock 2 n = 7	Flock 3 n = 15	Flock 4 n = 16	Flock 5 n = 13	In total B n = 64		
Adhesin	*aat*A	3 (30)	2 (14.29)	7 (53.85)	5 (35.71)	**17 (33.33)**	6 (46.15)	0	0	8 (50)	0	**14 (21.88)**	**31 (26.96)**	DQ381420.1
	afa	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	HG941718
	bfp	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	KJ020710.1
	bmaE	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	M15677.1
	crl	10 (100)	13 (92.86)	13 (100)	14 (100)	**50 (98.03)**	11 (84.62)	7 (100)	8 (100)	16 (100)	13 (100)	**56 (98.21)**	**106 (92.17)**	LT903847
	*csg*A	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	11 (84.62)	7 (100)	13 (86.67)	16 (100)	13 (100)	**61 (95.31)**	**112 (97.39)**	NC_008563.1
	*fim*A	0	0	0	0	**0**	6 (46.15)	6 (85.71)	0	0	9 (69.23)	**21 (32.81)**	**21 (18.26)**	CP006830.1
	*fim*A1	6 (60)	11 (78.57)	11 (84.62)	14 (100)	**42 (82.35)**	3 (23.08)	4 (57.14)	5 (33.33)	10 (62.5)	13 (100)	**35 (54.67)**	**77 (66.96)**	CP006830.1
	*fim*H	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	13 (100)	7 (100)	14 (93.33)	16 (100)	13 (100)	**63 (98.44)**	**114 (99.13)**	NC_008563.1
	*foc*A	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	12 (92.31)	7 (100)	14 (93.33)	16 (100)	13 (100)	**62 (96.88)**	**113 (97.41)**	NC_000913.3
	foc cluster	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	S68237.1
	*gaf*D	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	L33969.1
	iha	1 (10)	6 (42.86)	3 (23.08)	0	**10 (19.61)**	0	0	4 (26.67)	1 (6.25)	1 (7.69)	**6 (9.38)**	**16 (13.91)**	NC_007365.1
	lpf	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	CP003034.1
	*pap*A	0	0	1 (7.69)	0	**1 (1.96)**	0	0	9 (60)	5 (31.25)	1 (7.69)	**15 (23.44)**	**16 (13.91)**	NZ_CP033092.2
	*pap*C	0	1 (7.14)	1 (7.69)	0	**2 (3.92)**	0	0	9 (60)	5 (31.25)	1 (7.69)	**15 (23.44)**	**17 (14.78)**	CP000468.1
	*sfa*S	0	0	0	0	**0**	4 (30.77)	6 (85.71)	0	0	9 (69.23)	**19 (29.69)**	**19 (16.52)**	CP006830.1
	tsh	1 (10)	1 (7.14)	3 (23.08)	0	**5 (9.8)**	0	0	4 (26.67)	1 (6.25)	6 (46.15)	**11 (17.19)**	**16 (13.91)**	DQ381420.1
Invasin	*aec*35	0	1 (7.14)	0	0	**1 (1.96)**	0	0	0	1 (6.25)	0	**1 (1.56)**	**2 (1.74)**	AY857617.1
	*aec*36	0	1 (7.14)	0	0	**1 (1.96)**	0	0	0	1 (6.25)	0	**1 (1.56)**	**2 (1.74)**	AY857617.1
	*aec*37	0	1 (7.14)	0	0	**1 (1.96)**	0	0	0	1 (6.25)	0	**1 (1.56)**	**2 (1.74)**	AY857617.1
	*ibe*A	0	1 (7.14)	3 (23.08)	0	**4 (7.84)**	5 (38.46)	6 (85.71)	4 (26.67)	1 (6.25)	12 (92.31)	**28 (43.08)**	**32 (27.83)**	AY248744.1
	tia	3 (30)	2 (14.29)	5 (38.46)	2 (14.29)	**12 (23.53)**	4 (30.77)	0	8 (53.33)	9 (56.25)	1 (7.69)	**22 (34.38)**	**34 (29.57)**	CP000468.1
Iron acquisition	*chu*A	5 (50)	2 (14.29)	10 (76.92)	3 (21.43)	**20 (39.22)**	11 (84.62)	6 (85.71)	13 (86.67)	12 (75)	13 (100)	**55 (85.94)**	**75 (65.22)**	NC_011750.1
	*eit*A	0	1 (7.14)	0	11 (78.57)	**12 (23.53)**	0	0	3 (20)	0	2 (15.38)	**5 (7.81)**	**17 (14.78)**	DQ381420.1
	*eit*B	0	1 (7.14)	0	11 (78.57)	**12 (23.53)**	0	0	3 (20)	0	2 (15.38)	**5 (7.81)**	**17 (14.78)**	DQ381420.1
	*eit*C	0	1 (7.14)	0	11 (78.57)	**12 (23.53)**	0	0	3 (20)	0	2 (15.38)	**5 (7.81)**	**17 (14.78)**	DQ381420.1
	*eit*D	0	1 (7.14)	0	11 (78.57)	**12 (23.53)**	0	0	3 (20)	0	2 (15.38)	**5 (7.81)**	**17 (14.78)**	DQ381420.1
	*feo*B	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	13 (100)	7 (100)	15 (100)	16 (100)	13 (100)	**64 (100)**	**115 (100)**	NC 008563.1
	*fyu*A	0	3 (21.43)	4 (30.77)			8 (61.54)				13 (100)		**71 (61.74)**	CP000468.1

(continued on next page)

Table 4 (*continued*)

Group	Gene or operon	Farm A					Farm B						In total N = 115	Accession number
		Flock 1 n = 10	Flock 2 n = 14	Flock 3 n = 13	Flock 4 n = 14	In total A n = 51	Flock 1 n = 13	Flock 2 n = 7	Flock 3 n = 15	Flock 4 n = 16	Flock 5 n = 13	In total B n = 64		
					12 (85.71)	**19 (37.25)**		6 (85.71)	14 (93.33)	11 (68.75)		**52 (81.25)**		
	*ire*A	3 (30)	1 (7.14)	8 (61.54)	2 (14.29)	**14 (27.45)**	3 (23.08)	0	7 (46.67)	12 (75)	1 (7.69)	**23 (35.94)**	**37 (32.17)**	CP000468.1
	*iro*B	6 (60)	5 (35.71)	11 (84.62)	7 (50)	**29 (56.86)**	12 (92.31)	6 (85.71)	15 (100)	8 (50)	12 (92.31)	**53 (82.81)**	**82 (71.3)**	DQ381420.1
	*iro*C	6 (60)	5 (35.71)	11 (84.62)	8 (57.14)	**30 (58.82)**	12 (92.31)	6 (85.71)	15 (100)	8 (50)	6 (46.15)	**47 (73.44)**	**77 (66.96)**	DQ381420.1
	*iro*D	6 (60)	5 (35.71)	11 (84.62)	8 (57.14)	**30 (58.82)**	9 (69.23)	5 (71.43)	15 (100)	8 (50)	10 (76.92)	**47 (73.44)**	**77 (66.96)**	DQ381420.1
	*iro*E	6 (60)	5 (35.71)	11 (84.62)	8 (57.14)	**30 (58.82)**	12 (92.31)	6 (85.71)	15 (100)	7 (43.75)	12 (92.31)	**52 (81.25)**	**82 (71.30)**	DQ381420.1
	*iro*N	6 (60)	5 (35.71)	11 (84.62)	8 (57.14)	**30 (58.82)**	9 (69.23)	5 (71.43)	14 (93.33)	8 (50)	8 (61.54)	**44 (68.75)**	**74 (64.35)**	DQ381420.1
	*irp*2	0	4 (28.57)	4 (30.77)	12 (85.71)	**20 (39.22)**	7 (53.85)	6 (85.71)	14 (93.33)	11 (68.75)	13 (100)	**51 (79.69)**	**71 (61.74)**	CP000468.1
	*iuc*A	5 (50)	3 (21.43)	6 (46.15)	8 (57.14)	**22 (43.14)**	12 (92.31)	6 (85.71)	15 (100)	10 (62.5)	5 (38.46)	**48 (75)**	**70 (60.87)**	AY545598.5
	*iuc*B	5 (50)	3 (21.43)	6 (46.15)	8 (57.14)	**22 (43.14)**	12 (92.31)	6 (85.71)	15 (100)	10 (62.5)	5 (38.46)	**48 (75)**	**70 (60.87)**	AY545598.5
	*iuc*C	5 (50)	3 (21.43)	6 (46.15)	8 (57.14)	**22 (43.14)**	12 (92.31)	6 (85.71)	15 (100)	10 (62.5)	5 (38.46)	**48 (75)**	**70 (60.87)**	AY545598.5
	*iuc*D	5 (50)	3 (21.43)	6 (46.15)	8 (57.14)	**22 (43.14)**	12 (92.31)	6 (85.71)	15 (100)	9 (56.25)	5 (38.46)	**47 (73.44)**	**69 (60)**	AY545598.5
	*iut*A	5 (50)	2 (14.29)	6 (46.15)	8 (57.14)	**21 (41.18)**	12 (92.31)	6 (85.71)	15 (100)	10 (62.5)	5 (38.46)	**48 (75)**	**69 (60)**	AY545598.5
	*sit*A	6 (60)	7 (50)	11 (84.62)	9 (64.29)	**33 (64.71)**	12 (92.31)	6 (85.71)	15 (100)	14 (87.5)	12 (92.31)	**59 (92.19)**	**92 (80)**	AY545598.5
	*sit*B	6 (60)	5 (35.71)	11 (84.62)	9 (64.29)	**31 (60.78)**	12 (92.31)	7 (100)	13 (86.67)	15 (93.75)	12 (92.31)	**59 (92.19)**	**90 (78.26)**	AY545598.5
	*sit*C	6 (60)	5 (35.71)	11 (84.62)	9 (64.29)	**31 (60.78)**	9 (69.23)	2 (28.57)	6 (40)	12 (75)	12 (92.31)	**41 (64.06)**	**72 (62.61)**	AY545598.5
	*sit*D	6 (60)	5 (35.71)	11 (84.62)	9 (64.29)	**31 (60.78)**	12 (92.31)	7 (100)	15 (100)	14 (87.5)	12 (92.31)	**60 (93.75)**	**91 (79.13)**	AY545598.5
	bor	0	0	0	11 (78.57)	**11 (21.57)**	1 (7.69)	0	0	1 (6.25)	0	**2 (3.13)**	**13 (11.30)**	KC253896.1
	iss	6 (60)	14 (100)	13 (100)	14 (100)	**47 (92.16)**	12 (92.31)	6 (85.71)	14 (93.33)	15 (93.75)	12 (92.31)	**59 (92.19)**	**106 (92.17)**	AY545598.5
	*kps*MT	1 (10)	1 (7.14)	3 (23.08)	1 (7.14)	**6 (11.76)**	2 (15.38)	2 (28.57)	6 (40)	4 (25)	13 (100)	**27 (42.19)**	**33 (28.7)**	X53819.1
Protectin/ serum resistance	*mch*C	1 (10)	7 (50)	7 (53.85)	0	**15 (29.41)**	0	0	4 (26.67)	1 (6.25)	2 (15.38)	**7 (10.94)**	**22 (19.13)**	CP053284
	*mch*F	1 (10)	9 (64.29)	7 (53.85)	4 (28.57)	**21 (41.17)**	6 (46.15)	5 (71.43)	13 (86.67)	5 (31.25)	3 (23.08)	**32 (50)**	**53 (46.09)**	CP054455.1
	*omp*A	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	12 (92.31)	7 (100)	15 (100)	16 (100)	13 (100)	**63 (98.44)**	**115 (99.13)**	NC_000913.3
	traT	6 (60)	5 (35.71)	10 (76.92)	2 (14.29)	**23 (45.1)**	12 (92.31)	6 (85.71)	15 (100)	13 (81.25)	6 (46.15)	**52 (81.25)**	**75 (65.22)**	NC_017659.1
	*yja*A	4 (40)	4 (28.57)	5 (38.46)	11 (78.57)	**24 (47.06)**	7 (53.85)	6 (85.71)	13 (86.67)	8 (50)	12 (92.31)	**46 (71.88)**	**70 (60.87)**	NC_000913.3
Toxin/ bacteriocin-related	*ast*A	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	12 (92.31)	7 (100)	14 (93.33)	16 (100)	13 (100)	**63 (96.88)**	**114 (99.13)**	NC_008563

(*continued on next page*)

Table 4 (*continued*)

Group	Gene or operon	Farm A					Farm B						In total N = 115	Accession number
		Flock 1 n = 10	Flock 2 n = 14	Flock 3 n = 13	Flock 4 n = 14	In total A n = 51	Flock 1 n = 13	Flock 2 n = 7	Flock 3 n = 15	Flock 4 n = 16	Flock 5 n = 13	In total B n = 64		
	cba	0	0	2 (15.38)	0	**2 (3.92)**	0	0	0	0	3 (23.08)	**3 (4.69)**	**5 (4.35)**	DQ381420.1
	cbi	0	1 (7.14)	7 (53.85)	0	**8 (15.69)**	0	0	0	1 (6.26)	3 (23.08)	**4 (6.25)**	**12 (10.43)**	DQ381420.1
	*cdt*B	0	1 (7.14)	4 (30.77)	0	**5 (9.8)**	0	0	1 (6.67)	0	9 (69.23)	**10 (15.63)**	**15 (13.04)**	NC_008563.1
	cib	2 (20)	3 (21.43)	2 (15.38)	0	**7 (13.73)**	8 (61.54)	6 (85.71)	12 (80)	4 (25)	2 (15.38)	**32 (50)**	**39 (33.91)**	HQ114281.1
	*cib*I	1 (10)	1 (7.14)	0	0	**2 (3.92)**	0	0	0	0	1 (7.69)	**1 (1.56)**	**3 (2.61)**	HQ114281.1
	cma	0	1 (7.14)	7 (53.85)	0	**8 (15.69)**	0	0	0	1 (6.25)	3 (23.08)	**4 (6.25)**	**12 (10.43)**	DQ381420.1
	cmi	0	1 (7.14)	7 (53.85)	0	**8 (15.69)**	0	0	0	1 (6.25)	3 (23.08)	**4 (6.25)**	**12 (10.43)**	DQ381420.1
	*cnf*1	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	U42629.1
	*cva*A	3 (30)	9 (64.29)	8 (1.54)	4 (28.57)	**24 (47.06)**	5 (38.46)	6 (85.71)	13 (86.67)	5 (31.25)	6 (46.15)	**35 (54.69)**	**59 (51.3)**	AY545598.5
	*cva*B	2 (20)	2 (14.29)	2 (15.38)	0	**6 (11.76)**	2 (15.38)	0	9 (60)	0	5 (38.46)	**16 (25)**	**22 (19.13)**	AY545598.5
	*cva*C	3 (30)	4 (28.57)	2 (15.38)	4 (28.57)	**13 (25.49)**	5 (38.46)	4 (57.14)	10 (66.67)	2 (12.5)	6 (46.15)	**27 (42.19)**	**40 (34.78)**	AY545598.5
	cvi	3 (30)	4 (28.57)	7 (53.85)	4 (28.57)	**18 (35.29)**	6 (46.15)	4 (57.14)	10 (66.67)	3 (18.75)	6 (46.15)	**29 (45.31)**	**47 (40.87)**	AY545598.5
	*hly*D	0	0	0	0	**0**	0	0	0	0	0	**0**	**0**	NC_012487.1
	*hly*E	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	12 (92.31)	7 (100)	15 (100)	15 (93.75)	13 (100)	**62 (96.88)**	**113 (98.26)**	CP006830.1
	hlyH	5 (50)	5 (35.71)	10 (76.92)	5 (35.71)	**25 (49.02)**	2 (15.38)	5 (71.43)	9 (60)	12 (75)	5 (38.46)	**33 (51.56)**	**58 (50.43)**	DQ381420.1
	*usp*A	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	13 (100)	7 (100)	15 (100)	16 (100)	13 (100)	**64 (100)**	**115 (100)**	NC_008563.1
	*usp*B	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	13 (100)	7 (100)	15 (100)	16 (100)	13 (100)	**64 (100)**	**115 (100)**	NC_008563.1
	*usp*C	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	11 (84.62)	7 (100)	15 (100)	16 (100)	13 (100)	**62 (96.88)**	**113 (98.26)**	NC_008563.1
	*usp*E	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	13 (100)	7 (100)	15 (100)	16 (100)	13 (100)	**64 (100)**	**115 (100)**	NC_008563.1
	*usp*G	10 (100)	14 (100)	13 (100)	14 (100)	**51 (100)**	13 (100)	7 (100)	15 (100)	16 (100)	13 (100)	**64 (100)**	**115 (100)**	NC 008563.1
	vat	3 (30)	1 (7.14)	8 (61.54)	2 (14.29)	**14 (27.45)**	6 (46.15)	5 (71.43)	7 (46.67)	11 (68.75)	6 (46.15)	**35 (54.69)**	**49 (42.61)**	CP023388.1
	*ets*A	6 (60)	5 (35.71)	7 (53.85)	6 (42.86)	**24 (47.06)**	12 (92.31)	6 (85.71)	15 (100)	7 (43.75)	5 (38.46)	**45 (70.31)**	**69 (60)**	DQ381420.1
	*ets*B	6 (60)	5 (35.71)	7 (53.85)	7 (50)	**25 (49.02)**	12 (92.31)	6 (85.71)	15 (100)	7 (43.75)	5 (38.46)	**45 (70.31)**	**70 (60.87)**	DQ381420.1
	*ets*C	6 (60)	5 (35.71)	7 (53.85)	7 (50)	**25 (49.02)**	11 (84.62)	6 (85.71)	13 (86.67)	7 (43.75)	5 (38.46)	**42 (65.63)**	**67 (58.26)**	DQ381420.1
Miscellaneous	*mal*X	4 (40)	3 (21.43)	10 (76.92)	2 (14.29)	**19 (37.25)**	7 (53.85)	6 (85.71)	9 (60)	11 (68.75)	13 (100)	**46 (71.88)**	**65 (56.52)**	AF003742.1
	*omp*T	3 (30)	11 (78.57)	13 (100)	13 (92.86)	**40 (78.43)**	11 (84.62)	7 (100)	14 (93.33)	15 (93.75)	13 (100)	**60 (93.75)**	**100 (86.96)**	NC_008563.1
	*omp*Tp	5 (50)	5 (35.71)	10 (76.92)	4 (28.57)	**24 (47.06)**	5 (38.46)	6 (85.71)	11 (73.33)	12 (75)	5 (38.46)	**39 (60.94)**	**63 (54.78)**	DQ381420.1
	pic	3 (30)	1 (7.14)	7 (53.85)	2 (14.29)	**13 (25.49)**	4 (30.77)	0	0	6 (37.5)	0	**10 (15.63)**	**23 (20)**	CP048920
	TspE4	5 (50)	10 (71.43)	11 (84.62)	2 (14.29)	**28 (54.9)**	12 (92.31)	6 (85.71)	14 (93.33)	12 (75)	13 (100)	**57 (89.06)**	**85 (73.91)**	AF222188.1

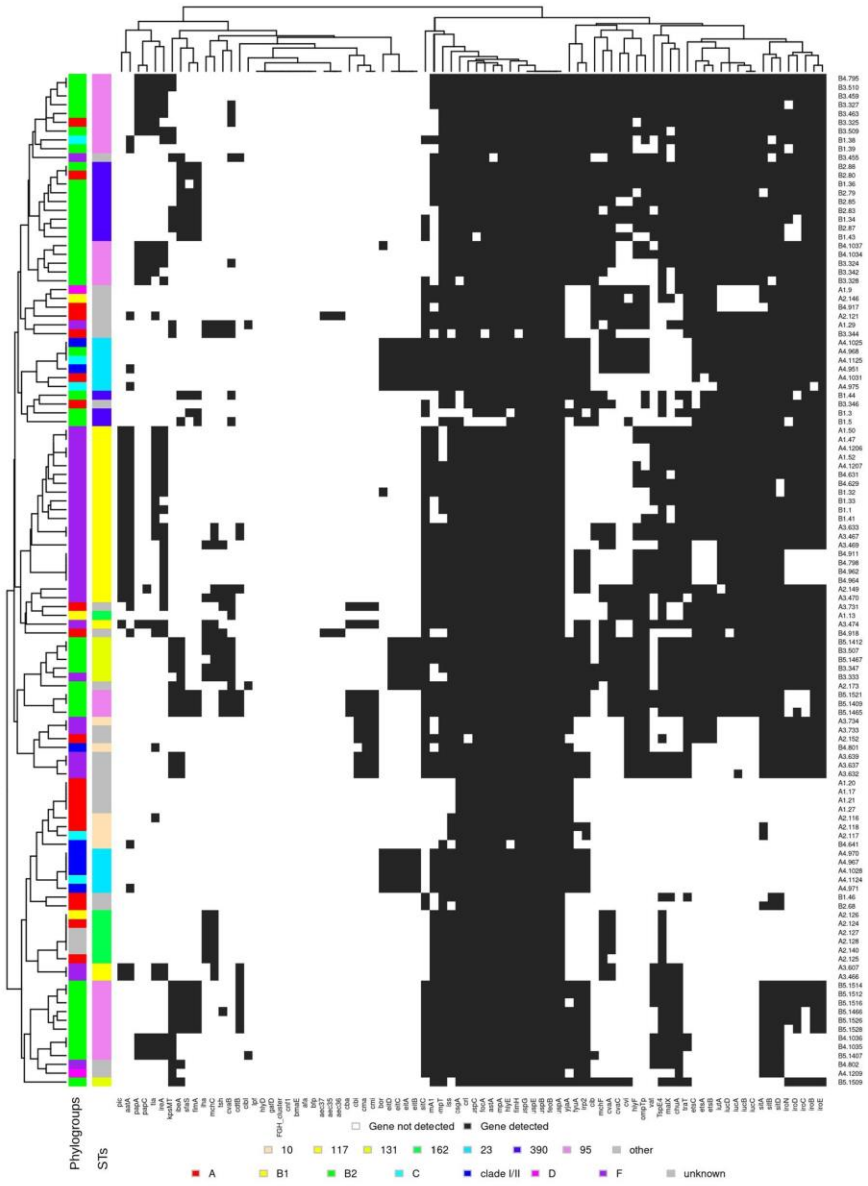

Fig. 2. Heatmap with dendrograms showing relatedness of the strains from both farms according to the presence/absence of the virulence genes. The rows of the legend represent heatmap, ST and phylogroup colours, respectively. Labels on the right represent farm, flock and isolate number.

The aerobactin system is one of the iron acquisition mechanisms that has been detected more frequently in the pathogenic than in the commensal *E. coli* strains (Gao et al., 2012). Our results showed a gradual increase in the prevalence of the iron acquisition factors on both farms (Fig. 4B, see Table 4 for the list of the factors). Their frequency in Flocks 1 and 3 on Farm B was significantly higher compared to other groups, and highest of all studied gene groups in the research (Fig. 4A).

Chouikha et al. (2006) have previously reported the importance of the *aec-35* to *aec-37* gene cluster in the virulence and carbohydrate metabolism. In their experiment, the mutant strain with a deletion of this gene cluster was less able to induce specific lesions of colibacillosis, bacteraemia and colonization of the liver. Interestingly, this gene cluster was detected in only two individual isolates (1.72 %) from different farms, although 56.9 % of the isolates originate from the liver and bone marrow, and have caused severe clinical symptoms characterized by fibrinous peritonitis, caseous exudate in the oviduct, splenomegaly and septicaemia. On the contrary, Pourbakhsh et al. (1997) have reported no difference in the chicken serum invasion between the mutant and wild-type *E. coli* strain in the *in vitro* experiments. This gene cluster is possibly involved in the early stages of infection through the respiratory system. Therefore, the low prevalence of this cluster in our collection may reflect the type of infection associated with the disease in the breeder layers, which is likely ascending through the oviduct.

Usp operon genes are associated primarily with UPEC causing pyelonephritis, prostatitis and bacteraemia. Previous research has showed enhanced infectivity in a mouse ascending urinary tract infection model, as they act as bacteriocins against other competitive strains (Črnigoj et al., 2014). In that way, they are responsible for the increased

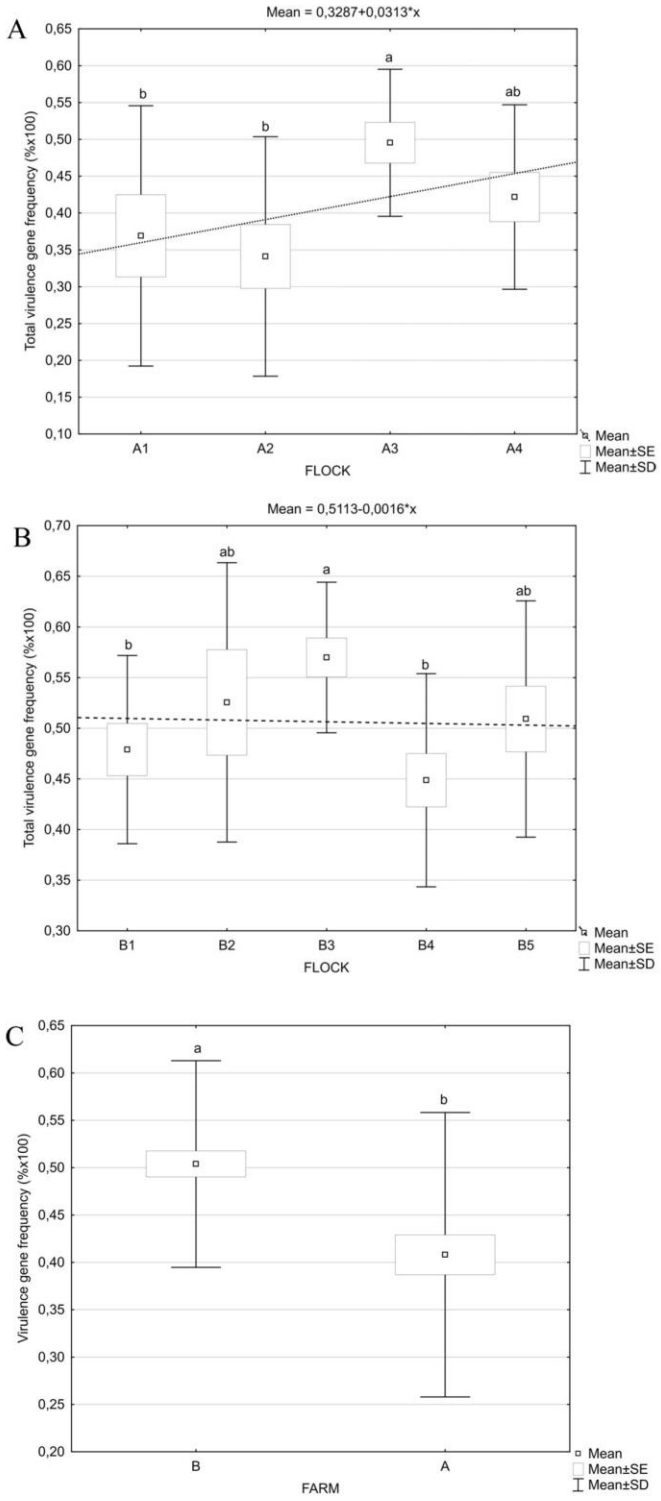

Fig. 3. Total virulence gene frequency in flocks on Farm A (A), Farm B (B), and average virulence gene frequency per farm (C). The dotted/dashed lines represent the frequency trend over time. The box plots marked with different lowercase letters (a, b, c) are significantly different ($p < 0.05$).

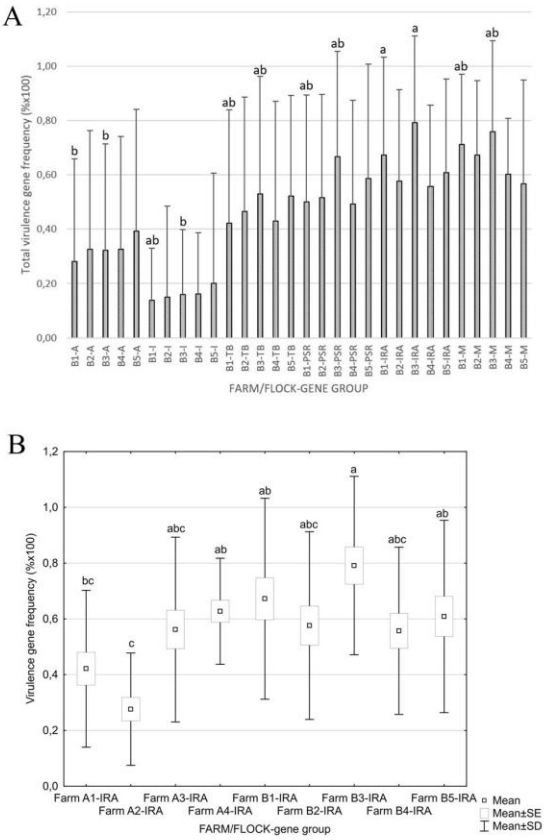

Fig. 4. Virulence gene frequency per gene group in each flock on Farm B (A), and virulence gene frequency for iron acquisition factors for each flock on Farm A and B (B). Abbreviations for gene groups are: A = adhesins, I = invasins, TB = toxins/bacteriocin-related factors, PSR = protectins/serum resistance factors, IRA = iron acquisition factors, M = miscellaneous. The bars marked with different lowercase letters (a, b) present statistically significant difference ($p < 0.05$) between gene groups within Flock 1 and 3 on Farm B (Fig. A), while the box plots marked with different lowercase letters (a, b, c) present statistically significant difference ($p < 0.05$) within IRA gene group between all flocks on both farms (Fig. B).

virulence and fitness of the pathogenic *E. coli* strains. Interestingly, in our case they were detected in 98.26–100 % of our isolates, depending on the gene (Table 4), which additionally indicates that infection possibly occurs more frequently ascendingly through the oviduct. Another gene frequently used as a PAI marker for UPEC strains is *malX* (Johnson et al., 2008b; Nowrouzian et al., 2009), which was detected in 56.03 % of our isolates (Table 4).

The average number of virulence-associated genes was significantly higher in phylogroups B2 and F (Supplementary Fig. S1), which was expected since said phylogroups are considered as common causes of ExPEC infections (Pires-dos-Santos et al., 2013; Stephens et al., 2017). The results also show a significant increase in average number of virulence genes in phylogroup A on both farms (Supplementary Fig. S2), as opposed to the persistently high amount in phylogroups B2 and F. That could be explained by the competitive exclusion principle formed by autogenous vaccine and/or more pathogenic strains, which allowed only more virulent strains to survive and persist in the environment. Additionally, horizontal gene transfer in the bacterial population with more pathogenic strains could result in higher gene frequency, which could be seen in the isolates belonging to phylogroup A on Farm B, with 10 % higher virulence gene frequencies' in the first two flocks compared to Farm A.

The analysis based on the heatmap with dendrograms shows high similarity of the strains belonging to same phylogroups and STs, in most cases, with regard to the presence or absence of the virulence genes (Fig. 2). Therefore, the results confirm the distinction of the phylogroups and STs based on the presence of certain sets of virulence-associated genes, and consequently, based on the virulence level of the isolates.

To additionally visualize and explore the gene presence/absence datasets, we used the PCoA method to project the samples onto two-dimensional plots (Fig. 5). The proximity of the sample points in the PCoA plots represents their similarity calculated on the basis of the virulence profiles - the more similar the samples with regard to the composition of virulence genes, the closer they are expected to group together. Otherwise, there is a great overlap between strain virulence profiles of Farm A and Farm B indicating similar virulence genes composition, when all the strains within a farm are taken into account (Fig. 5A). Similarities aside, the virulence genes composition is not the same between the two Farms, probably due to differences of locally transmitted *E. coli* strains. The farm-dependent differences of *E. coli* strains were also observable in the phylogenetic analysis as described above. Separate flocks show distinctive virulence profiles demonstrating that the virulence profiles change over time (Fig. 5B and C).

Our results are consistent with the previous report indicating continuous clonal selection of the virulent strains that successfully avoid the vaccine effect (Lozica et al., 2021). Further analyses will elucidate which of the analysed virulence-associated genes could be possible candidates for the subunit *E. coli* vaccines.

4. Conclusion

The results of this study show that many of the formerly used minimal predictors are possibly not sufficient for identification of the APEC strains, and should be updated with other virulence-associated genes linked to highly severe clinical symptoms. The results of the phylogenetic analysis indicate that continuous application of the autogenous vaccine gradually leads to lower genetic diversity of *E. coli* strains. As a result, the strains on investigated farms should now be more easily managed with regular vaccination using autogenous vaccine made with recent strains. There was no such effect on the diversity of virulence genes. Longitudinal data revealed an increase over time in average frequency of virulence-associated genes on Farm A, which had significantly lower average number of virulence genes per isolate than Farm B and less severe clinical manifestation. Virulence profiles corresponded to STs and phylogroups of the isolates, which confirms the accuracy of phylotyping and MLST analyses that are often used because they are more approachable and less expensive diagnostic tools. As less virulent strains can cause infections in the typically immunocompromised farm birds, *in vivo* assays should be performed in order to confirm the significance of certain virulence factors for the disease pathogenesis in healthy birds.

Funding

This study was partially supported by the University of Zagreb, Croatia, under Grant "Longitudinal epizootiological study of the virulence-associated genes by whole genome sequencing of the pathogens with aim to improve biosafety measures and immunoprophylaxis programs on poultry farms".

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors and all research was conducted in an ethical and responsible manner. The study was approved by the Committee on Research Ethics of the Faculty of Veterinary Medicine, University of Zagreb, Croatia, and permission for sampling was granted

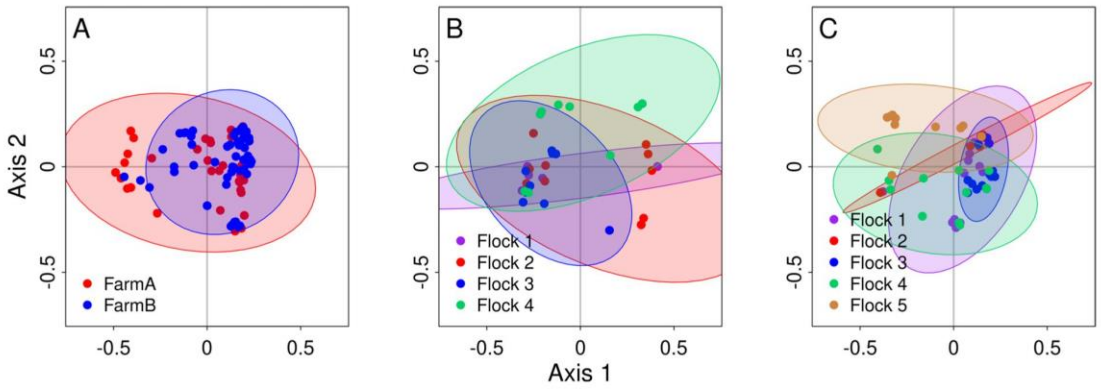

Fig. 5. Principal coordinate analysis (PCoA) of the *E. coli* isolates, based on the data on the presence/absence of 84 virulence genes. Drawn are the first two PCoA axes. Combinations of first/third, and second/third axes were also visualized and led to similar findings (Supplementary Fig. S1). Isolates are grouped per (A) farm (115 isolates), (B) flock in Farm A (51 isolates), (C) flock in Farm B (64 isolates). Ellipses used to visually describe data represent normal-probability contours (confidence level 0.95) of the sample groups.

by the farm.

Declaration of Competing Interest

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

Acknowledgments

The authors would like to thank Timothy Johnson for the insights on the selection of the virulence factors used for the analyses of the whole-genome sequences.

Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.vetmic.2021.109159.

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4.3. PAPER III.: "Genomic analysis of *Escherichia coli* longitudinally isolated from broiler breeder flocks after the application of an autogenous vaccine"

The article was published in the Special Issue „Avian Pathogens" of the journal Microorganisms (MDPI, Basel, Switzerland), 10(2), pp. 1-12, in February 2022. DOI number of the article is 10.3390/microorganisms10020377

The full paper and its supplementary material can be accessed through the following link: https://www.mdpi.com/2076-2607/10/2/377

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Genomic Analysis of *Escherichia coli* Longitudinally Isolated from Broiler Breeder Flocks after the Application of an Autogenous Vaccine

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Abstract: *Escherichia coli* is the main bacterial cause of major economic losses and animal welfare issues in poultry production. In this study, we investigate the effect of an autogenous vaccine on *E. coli* strains longitudinally isolated from broiler breeder flocks on two farms. In total, 115 *E. coli* isolates were sequenced using Illumina technologies, and compared based on a single-nucleotide polymorphism (SNP) analysis of the core-genome and antimicrobial resistance (AMR) genes they carried. The results showed that SNP-based phylogeny corresponds to a previous multilocus-sequence typing (MLST)-based phylogeny. Highly virulent sequence types (STs), including ST117-F, ST95-B2, ST131-B2 and ST390-B2, showed a higher level of homogeneity. On the other hand, less frequent STs, such as ST1485, ST3232, ST7013 and ST8573, were phylogenetically more distant and carried a higher number of antimicrobial resistance genes in most cases. In total, 25 antimicrobial genes were detected, of which the most prevalent were *mdf(A)* (100%), *sitABCD* (71.3%) and *tet(A)* (13.91%). The frequency of AMR genes showed a decreasing trend over time in both farms. The highest prevalence was detected in strains belonging to the B1 phylogenetic group, confirming the previous notion that commensal strains act as reservoirs and carry more resistance genes than pathogenic strains that are mostly associated with virulence genes.

Keywords: *Escherichia coli;* colibacillosis; poultry; autogenous vaccine; whole-genome sequencing; SNP; AMR

Citation: Lozica, L.; Villumsen, K.R.; Li, G.; Hu, X.; Maljković Maurić, M.; Gottstein, Ž. Genomic Analysis of *Escherichia coli* Longitudinally Isolated from Broiler Breeder Flocks after the Application of an Autogenous Vaccine. *Microorganisms* **2022**, *10*, 377. https://doi.org/10.3390/microorganisms10020377

Academic Editor: Kirill Sharshov

Received: 7 December 2021
Accepted: 3 February 2022
Published: 6 February 2022

1. Introduction

Escherichia coli (E. coli) is the main bacterial cause of major economic losses and animal welfare issues in poultry production [1,2]. Colibacillosis on poultry farms is controlled through vaccination programs, in addition to strict biosecurity standards and rigorous farm management [1]. There are several available commercial *E. coli* vaccines, but no vaccination procedure to date has proved to be highly efficacious due to the genetic diversity of the bacteria [1]. As the avian pathogenic *E. coli* (APEC) is closely related to other extraintestinal pathogenic *E. coli* (ExPEC) subpathotypes, which cause infections in the human population, colibacillosis in poultry also presents a danger to public health [2–8].

The localization and severity of the infection varies depending on the strain, site of entry and general health of the bird [1,9]. Usually, the most common lesions in adult birds

are airsacculitis, peritonitis, salpingitis and septicaemia, although in egg laying hens, peritonitis, salpingitis and salpingitis-peritonitis syndrome (SPS) have been considered the most prevalent, as the infection usually occurs by bacteria ascending through the cloaca [2,9–11].

Many studies have focused on the importance of certain virulence-associated genes (VAGs) and their role in the pathogenesis of *E. coli* infection [12–15]. The validation of specific sets of VAGs as definite predictors of *E. coli* virulence could help to improve the diagnostics process and enable quicker response during colibacillosis outbreaks. Currently, ColV plasmids are associated with the pathogenicity of *E. coli* and considered a defining trait of APEC [15–17]. However, Mageiros et al. (2021) have reported a high prevalence of the putative plasmid genes among both pathogenic and commensal *E. coli* strains in chickens, with a higher average number of plasmid genes per isolate in the commensal strains, suggesting that the virulence of *E. coli* is linked with the homologous sequence variations of the genes [18]. Several population genomics studies have detected signatures of adaptation to different hosts in the bacterial genomes, which are manifested as mutations or horizontal acquisition of genetic elements [8,19,20]. Additionally, the antimicrobial resistance (AMR) genes are often located on plasmids [21]. They are similarly easily acquired by horizontal transmission, which presents an additional growing concern for animal and human health [22].

Single-nucleotide polymorphisms (SNPs) are the most common form of genetic code variation in the genome [23]. They are considered the most useful biomarkers for disease diagnosis and prognosis because they can influence the rate of the disease progression and immune response of the host [23]. They can also be used to track transmission, predict important phenotypes of the bacteria and monitor disease outbreaks [24]. The aim of this study is to investigate the heterogeneity of the isolates using SNP analysis and to detect the prevalence and horizontal transmission of AMR genes between and within flocks on two broiler breeder farms after the application of the autogenous vaccine.

2. Material and Methods

2.1. Study Design

This study is a continuation of previous research on *E. coli* gene variability after the application of the autogenous vaccine [25]. While the previous study was focused on the frequency of virulence-associated genes and MLST, the focus of the present study was on the use of SNPs for the phylogenetic analysis and the effect of the autogenous vaccine on antimicrobial resistance gene prevalence.

Two broiler breeder farms that are part of the same company, Farm A and Farm B, with four and five flocks, respectively, were selected for the longitudinal research on *E. coli* gene variability after the application of the autogenous vaccine. The selected farms reported continuous problems with colibacillosis despite the regular use of commercial vaccines. From our suggestion, they started using an autogenous *E. coli* vaccine instead of the commercial vaccine, which proved to be successful [26]. Each flock was vaccinated with a specifically designed vaccine prepared from the strains isolated in the previous flock. In total, 115 *E. coli* strains originating from the flocks vaccinated with the commercial and/or autogenous vaccines were selected for whole-genome sequencing and further analyses (Table 1).

Table 1. Description of *E. coli* vaccination programs and antimicrobial treatment data (rearing and production period) for each flock in this study [1].

Farm	Flock	Number of Analysed Strains per Flock	Vaccination	Age at the Time of Vaccination	Treatment
A	1	10	Commercial vaccines (live attenuated + inactivated 2x)	0 d 10 w 18 w	Doxycycline
	2	14	Autogenous vaccine 2x	10 w 18 w	Doxycycline, tiamulin
	3	13	Autogenous vaccine 2x	12 w 19 w	-
	4	14	Autogenous vaccine 2x	10 w 19 w	Amoxicillin, doxycycline, enrofloxacin, oxytetracycline
B	1	13	Commercial vaccines (live attenuated + inactivated 2x)	0 d 13 w 19 w	Doxycycline, enrofloxacin, tylosin
	2	7	Commercial vaccines (live attenuated 2x) Autogenous vaccine 2x	0 d 5 w 11 w 18 w	Amoxicilllin, polymyxin E, tylosin
	3	15	Autogenous vaccine 2x	9 w 20 w	Doxycycline, polymyxin E
	4	16	Autogenous vaccine 2x	10 w 18 w	Doxycycline, enrofloxacin
	5	13	Autogenous vaccine 2x	9 w 17 w	Amoxicillin, enrofloxacin

[1] adapted from Lozica et al., 2021.

2.2. Bacterial Strain Selection and DNA Isolation

Bacterial strain selection, DNA isolation and sequencing were previously described in more detail [25]. Briefly, 115 *E. coli* strains were isolated from the daily mortalities diagnosed with colibacillosis. The carcasses were routinely subjected to the pathomorphological examination as a part of the health surveillance or during outbreaks. The strains were recovered from the birds with lesions consistent with colibacillosis and selected for whole-genome sequencing based on the tissue of origin and age of the birds. Target organs were the peritoneum, liver, oviduct, and bone marrow, as they are often severely affected by colibacillosis. In case the strains from targeted tissues were not available, strains from other organs, such as lungs and the pericardium, were selected. The strains originated from birds older than 21 weeks, when outbreaks caused by colibacillosis usually emerge. Three or more strains per flock used for the vaccine production were also sequenced and included in the analyses. DNA was isolated as previously described [25] and stored at −20 °C until further analyses. The isolates used in this study are described in Supplementary Table S1.

2.3. DNA Sequencing and Deposition

Sequencing libraries were created based on the Illumina technologies, following the manufacturer's recommendations. Afterwards, whole-genome sequencing was conducted using MiSeq (Illumina, San Diego, CA, U.S.A.) with paired-end 250 bp for 40 isolates, and NovaSeq 6000 platform (Novogene Co. Ltd., Beijing, China) with paired-end 150 bp strategy for the remaining 75 isolates. The whole-genome sequences analyzed in this study were deposited in the National Center for Biotechnology Information (NCBI) as a BioProject under the accession number PRJNA681385.

2.4. Phylogenetic Analysis

SPAdes (v3.13.1) was used to assemble the raw reads of the 115 *E. coli* isolates to generate 115 draft genomes. Parsnp (v1.2) was used to construct the SNP tree, including 115 draft genomes and reference genomes, which were chosen based on their genetic diversity. In total, 19 reference genomes belonging to different phylogenetic groups and STs were included in this analysis [27]. All other genomes were aligned to the genome of the isolate B4, one of the input 115 sequences, to detect core SNPs and generate the core-genome SNP tree [28] by using Parsnp (Supplementary Table S2).

2.5. Antimicrobial Resistance Gene Analysis

For the analysis of the AMR genes, raw reads were assembled and trimmed using Assembler v1.2. [29]. Acquired antimicrobial resistance genes were detected using ResFinder v4.1. [30], and the assembled genomes were analysed with 90% ID and 60% minimum length threshold.

2.6. Statistical Analysis

The statistical analyses were performed in Statistica 13.2.0.17. (TIBCO Software Inc., Tulsa, OK, U.S.A.) and R 4.0.5. (R Core Team, Vienna, Austria). The results were tested for normality of data distribution using the Kolmogorov–Smirnov test. The statistical significance of differences in the frequency of AMR genes between and within both farms was analysed using the Kruskal–Wallis test, with statistical significance set at level $p \leq 0.05$. Poisson regression analysis was used to calculate the incident rate, standard error (SE) and 95% confidence intervals (95% CI) between the antimicrobial treatment and AMR gene prevalence among different flocks.

3. Results and Discussion

The results of the phylogenetic analysis correspond to the MLST-based phylogeny from the previous research (Figure 1) [25]. Isolates belonging to identical STs and phylogenetic groups were distributed both within the same and adjacent phylogroups in the phylogenetic tree based on the core-genome SNP clustering. Highly pathogenic strains, including ST117-F, ST95-B2, ST131-B2, and ST390-B2, formed larger clusters and showed higher homogeneity between the isolates originating from the same farm. This indicates their possible resistance to vaccination, despite continuous inclusion in the vaccine, and longitudinal spread to the consecutive flocks, but with reduced general clinical implications and improved production traits on Farm B. Additionally, ST117-F clustered together regardless of the flock or farm of origin, which has already been observed and explained by the mutual origin of the strains from the grandparent flocks or from the shared rearing houses [25,31]. ST162 and ST23 formed separate clusters and were detected only on Farm A. Both STs included a variety of phylogenetic groups of which most are considered commensal, although they have previously been isolated from both diseased and healthy poultry [32–34]. Vaccination on Farm A significantly influenced the selection of strains in the consecutive flocks, but also showed to be non-protective against the heterologous strains. As the majority of the strains on Farm A were low pathogenic, they were probably more easily controlled by vaccination compared to Farm B, where the majority of the

strains were more pathogenic and the phylogroup B2 resisted vaccination. SNP genotyping generally reveals a more detailed clonal relationship between the investigated isolates [9]. In our study, it showed slightly more discriminatory results compared to the MLST-based phylogeny, showing distribution of several isolates with the same ST in adjacent clusters based on the core SNPs. The strains belonging to less frequent STs, such as ST1485, ST3232, ST7013 and ST8573, were phylogenetically more distant and usually clustered separately. However, their average number of detected AMR genes was variable, mostly depending on the phylogenetic group.

We identified 25 AMR genes, of which *mdf(A)* and *sitABCD* showed the highest prevalence on both farms (Table 2). Although *mdf(A)* has a broad-spectrum specificity, which includes six classes of antimicrobials, the phenotypes and the level of resistance it provides is unclear. Previous studies reported that *mdf(A)* encodes for a multidrug efflux pump and its expression confers multidrug resistance in *E. coli*, indicating that resistance could have occurred in isolates where we did not detect corresponding resistance genes [35,36]. Similar results were reported by Rafique et al. (2020), who detected the *mdf(A)* gene in all 92 investigated *E. coli* strains isolated from chickens in different Pakistani provinces [22].

The *SitABCD* system mediates the transport of iron and manganese. Its ability to obtain manganese contributes to the resistance to oxidative stress and protection against agents such as hydrogen peroxide [37]. Additionally, *sit* operon genes are often associated with clinical infections caused by ExPEC, and have a contributing role as virulence factors by mediating the metal ion transport [37,38]. ResFinder analyses of our isolates discovered multiple copies of *sitABCD* genes. Previous phylogenetic analyses of the *sit* operon originating from *E. coli* and *Shigella flexneri* revealed they are most likely acquired by several distinct genetic events involving horizontal gene transfer [38]. Said events probably contributed to the presence of multiple gene copies in a single isolate, from which some are plasmid borne, and some are carried on chromosomes, and led to their high occurrence in *E. coli* populations [16,38].

The gene *tet(A)* showed relatively high prevalence (13.91%), although it was detected only on Farm A and predominantly in Flock 4 (68.75%). The prevalence was probably a result of doxycycline application, which is commonly used as treatment for various bacterial poultry diseases. The dominance of *tet(A)* out of different tetracycline resistance genes was reported in *E. coli* isolated from chickens and several other animal species, as opposed to the strains isolated from humans where *tet(B)* was more prevalent [39–42].

Interestingly, mobilized colistin resistance (*mcr*) gene was not detected, although colistin (polymyxin E) has been used as antibiotic treatment on the studied farms (Table 1). It has been widely used to treat colibacillosis and as a growth promoter in the poultry industry for a long time [1,43,44]. Since polymyxins have been reintroduced as treatment for infections in humans and their application can influence the antimicrobial selective pressure [45], its use in veterinary medicine has been minimized [44].

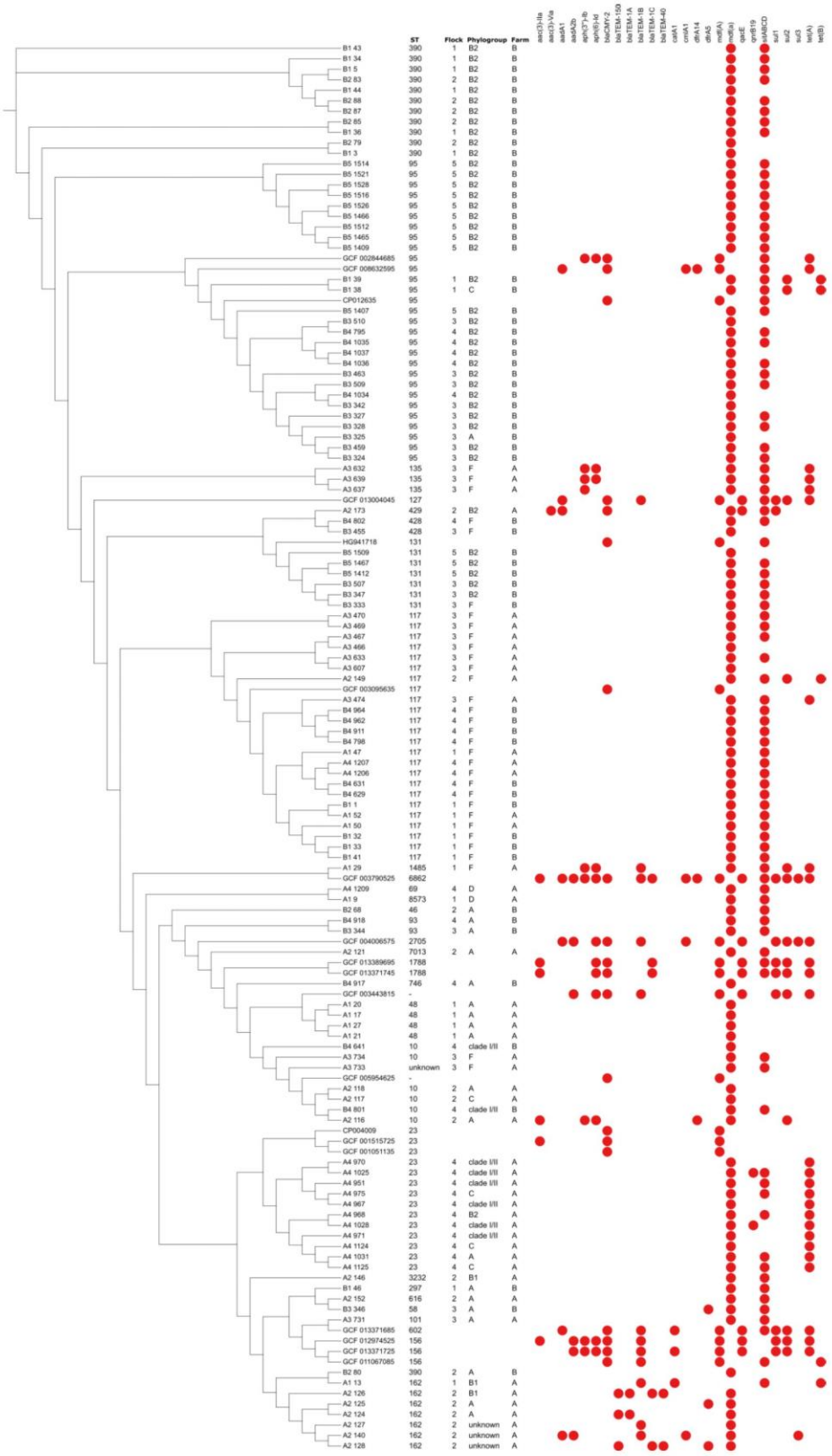
ST
Flock
Phylogroup
Farm
aac(3)-IIa
aac(3)-VIa
aadA1
aadA2b
aph(3'')-Ib
aph(6)-Id
blaCMY-2
blaTEM-150
blaTEM-1A
blaTEM-1B
blaTEM-1C
blaTEM-40
catA1
cmlA1
dfrA14
dfrA5
floR(A)
mdf(A)
qnrE
qnrB19
sulABCD
sul1
sul2
sul3
tet(A)
tet(B)

Figure 1. SNP-based phylogenetic tree showing distribution of AMR genes among 115 *E. coli* sequences. The analysis includes 19 reference *E. coli* genomes representing different STs and phylogenetic groups.

Table 2. Prevalence of the identified acquired AMR genes (*n* (%)).

AMR Gene	Phenotype	Number of AMR Genes on Farm A * *n* = 51	Number of AMR Genes on Farm B ** *n* = 64	Total Number of AMR Genes
aac(3)-IIa	apramycin, gentamicin, tobramycin, dibekacin, netilmicin, sisomicin	1 (1.96)	0	1 (0.87)
aac(3)-VIa	gentamicin	1 (1.96)	0	1 (0.87)
aadA1	spectinomycin	2 (3.92)	0	2 (1.74)
aadA2b	spectinomycin, streptomycin	1 (1.96)	0	1 (0.87)
aph(3″)-Ib	streptomycin	5 (9.8)	0	5 (4.35)
aph(6)-Id	streptomycin	5 (9.81)	0	5 (4.35)
$bla_{\text{CMY-2}}$	amoxicillin, amoxicillin + clavulanic acid, ampicillin, ampicillin + clavulanic acid, cefotaxime, cefotxitin, ceftazidime, piperacillin, piperacillin + tazobactam, ticarcillin, ticarcillin + clavulanic acid	1 (1.96)	0	1 (0.87)
$bla_{\text{TEM-1A}}$	amoxicillin, ampicillin, cephalothin, piperacillin, ticarcillin	3 (5.88)	0	3 (2.61)
$bla_{\text{TEM-1B}}$	amoxicillin, ampicillin, cephalothin, piperacillin, ticarcillin	4 (7.84)	0	4 (3.48)
$bla_{\text{TEM-1C}}$	amoxicillin, ampicillin, cephalothin, piperacillin, ticarcillin	2 (3.92)	0	2 (1.74)
$bla_{\text{TEM-40}}$	amoxicillin, amoxicillin + clavulanic acid, ampicillin, ampicillin + clavulanic acid, piperacillin, piperacillin + tazobactam, ticarcillin, ticarcillin + clavulanic acid	2 (3.92)	0	2 (1.74)
$bla_{\text{TEM-150}}$	unknown beta-lactam	3 (5.88)	0	3 (2.61)
catA1	chloramphenicol	1 (1.96)	0	1 (0.87)
cmlA1	chloramphenicol	1 (1.96)	0	1 (0.87)
dfrA5	trimethoprim	2 (3.92)	1 (1.56)	3 (2.61)
dfrA14	trimethoprim	1 (1.96)	0	1 (0.87)
mdf(A)	unknown macrolide, aminoglycoside,	51 (100)	64 (100)	115 (100)

qacE	tetracycline, fluoroquinolone, phenicol and rifamycin benzylkonium chloride, ethidium bromide, chlorhexidine, cetylpyridinium	1 (1.96)	0	1 (0.87)
qnrB19	ciprofloxacin	2 (3.92)	0	2 (1.74)
sitABCD	hydrogen peroxide	31 (60.78)	51 (79.69)	82 (71.3)
sul1	sulfamethoxazole	1 (1.96)	0	1 (0.87)
sul2	sulfamethoxazole	3 (5.88)	2 (3.13)	5 (4.35)
sul3	sulfamethoxazole	1 (1.96)	0	1 (0.87)
tet(A)	doxycycline, tetracycline	17 (33.33)	0	17 (14.78)
tet(B)	doxycycline, tetracycline, minocycline	2 (3.92)	2 (3.13)	4 (3.48)

*, ** Statistically significant difference in AMR gene frequency was detected between the farms ($p \leq 0.01$).

The frequency of AMR genes decreased over time on both farms, with a more apparent change on Farm A (Figure 2A). When considering the phylogenetic groups of the analysed isolates, the results showed the highest prevalence of AMR genes in the B1 phylogroup and strains belonging to untypeable phylogenetic groups (Figure 2B). The high prevalence of resistance genes in phylogroup B1 confirms the notion that commensal strains act as reservoirs of AMR genes. Previous studies indicated that commensal strains carry more AMR genes, as opposed to pathogenic strains that usually possess more virulence factors, but are more susceptible to antimicrobials [46,47]. This concept was confirmed by this study by the significantly higher frequency of AMR genes on the Farm A (Table 2), in addition to our previous study, which showed a significantly lower average frequency of VAGs on Farm A than on Farm B [25]. The strains of unknown phylogenetic groups were identified as ST162 (Supplementary Table S1). As other detected ST162 strains were identified as the A or B1 phylogenetic group, we assume that untypeable strains were variations of said commensal phylogroups, further confirming the highest prevalence of AMR genes in the commensal strains. Overall, the average number of AMR genes per isolate was 2.71 and 1.87 on Farm A and B, respectively, and there was no statistical significance in the prevalence of resistance genes between and within different studied flocks. The antimicrobial susceptibility testing of strains on the Farm A and B showed continuous decrease in resistance, especially on Farm A (unpublished data), which is consistent with the AMR gene results.

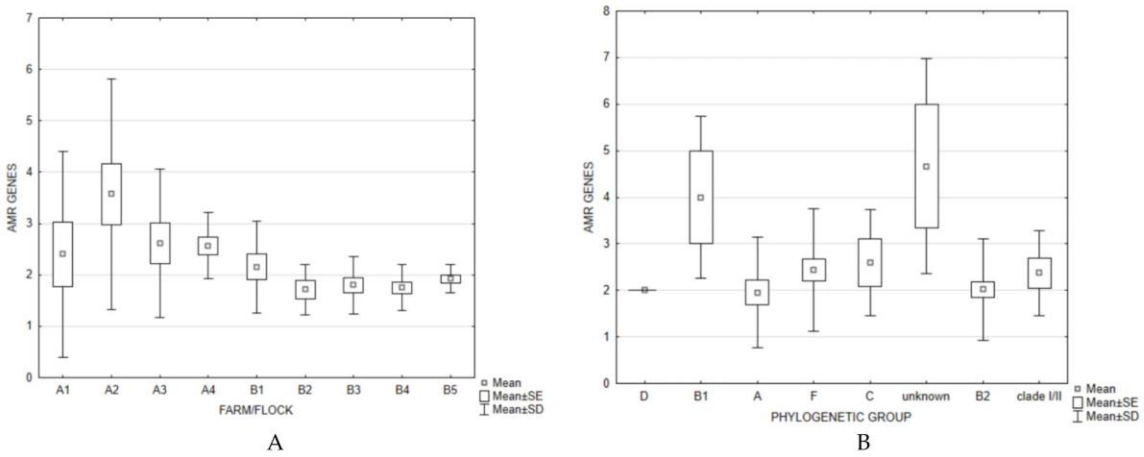

Figure 2. AMR gene frequency per flock on each farm (**A**) and per each detected phylogenetic group (**B**).

The results showed the treatment with antimicrobials possibly affected the prevalence of certain *E. coli* strains, in addition to the effect of autogenous vaccine application [48], although there was no correlation between the antimicrobials used in the flocks and the detected AMR genes (Supplementary Table S3). The implementation of the autogenous vaccine induced genetic homogenization of the isolates [26,48], which increased the selection pressure and alleviated the management of colibacillosis on the investigated farms. The present results correspond to previous studies, which reported that the application of autogenous vaccines alone or in combination with a commercial vaccine serves well as a preventive measure on poultry farms [49–51]. The results showed a probable positive interaction of the autogenous vaccine and antimicrobial treatment, which could have reduced the overall prevalence of AMR genes on the farms in the long term. The antimicrobials and the vaccine possibly interacted and formed a more powerful selection mechanism where strains with AMR genes, if not removed by treatment, could be controlled by the immune system. In addition, on the investigated farms, vaccination with autogenous vaccines reduced the need for treatment with antimicrobials, therefore the stimulation and emergence of resistant *E. coli* strains significantly decreased.

4. Conclusions

Genomic analysis showed that both MLST and SNP-based phylogeny can provide a detailed characterization of *E. coli* strains, taking into consideration their virulence and overall genetic relatedness. Although none of the STs or phylogenetic groups could be associated with specific AMR genes, strains belonging to more uncommon STs and commensal phylogenetic groups carried more AMR genes in most cases. The results showed the highest prevalence of *mdf(A)* and *sit* operon genes, which provide a wide-spectrum protection, indicating that the bacterial population was adapted over time by carrying less specialized genes. The autogenous vaccine induced the lower heterogeneity of the strains and possibly interacted with the antimicrobial treatment leading to the selection of strains with a lower amount of AMR genes over time, which is consistent with previous research.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/microorganisms10020377/s1,Table S1: Description of isolates used in this study. Isolates in bold were used for the production of autogenous vaccines for the next consecutive flock on the farm, Table S2: Description of reference genomes used for the SNP analysis, Table S3: The results of the Poisson regression analysis showing the incident rate, standard error

(SE) and 95% confidence intervals (95% CI) between the antimicrobial treatment and AMR gene prevalence among different flocks.

Author Contributions: Conceptualization, L.L. and Ž.G.; Methodology, L.L., Ž.G., and K.R.V.; Software, X.H. and M.M.M.; Validation, X.H. and G.L.; Formal Analysis, L.L., X.H., M.M.M., and Ž.G.; Investigation, L.L. and Ž.G.; Resources, L.L., Ž.G., K.R.V., X.H., and G.L.; Data Curation, X.H., G.L., and K.R.V.; Writing—Original Draft Preparation, L.L.; Writing—Review and Editing, Ž.G., K.R.V., and G.L.; Visualization, X.H., L.L., and Ž.G.; Supervision, Ž.G.; Project Administration, Ž.G.; Funding Acquisition, Ž.G. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partially supported by the University of Zagreb, Croatia, under Grant "Longitudinal epizootiological study of the virulence-associated genes by whole genome sequencing of the pathogens with the aim to improve biosafety measures and immunoprophylaxis programs on poultry farms".

Institutional Review Board Statement: This article does not contain any studies with human participants or animals performed by any of the authors and all research was conducted in an ethical and responsible manner. The study was approved by the institutional ethics committee according to the national Animal protection Act (OG 102/2017) (License: Class: 640-01/19-17/50, Reg no: 251-61-44-19-02).

Informed Consent Statement: The permission for sampling and further analysis of the samples were granted by the farm.

Data Availability Statement: The whole-genome sequences analysed in this study are publicly available as BioProject under the accession number PRJNA681385.

Conflicts of Interest: The authors declare no conflict of interest.

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5. DISCUSSION

E. coli is one of the most frequently reported causative agents of infection in the poultry industry (GUABIRABA and SCHOULER, 2015; NOLAN et al., 2020). In addition to its major impact on poultry production, it has a zoonotic potential and a role as a reservoir for extraintestinal infections in humans (JØRGENSEN et al., 2019). Therefore, comprehensive longitudinal analyses of the strains after implementation of autogenous vaccines to the vaccination program can provide information how to improve the selection of strains for the vaccine development in the future.

E. coli strains investigated in this study have shown high genomic diversity, as expected. The results of the phylogenetic analysis revealed that application of autogenous *E. coli* vaccine induced a strain shift and led to a decreased genetic diversity over time. Interestingly, the strain shift was not the same on both farms. On Farm A, the prevalence of specific phylogenetic groups was highly dependent on the strains used in the autogenous vaccine, as opposed to Farm B where phylogroup B2 showed resistance to reduction despite vaccination (Paper I.). In general, phylogenetic groups B2 and F are considered highly virulent, as opposed to A, B1 and clades I-V, which are considered commensal, especially the latter one, which is highly prevalent in the digestive tract of birds (LESCAT et al., 2013). The strain shift was probably additionally influenced by application of antimicrobials (Paper III.). Interaction of the autogenous vaccine and antimicrobials could have created a more powerful selection mechanism, where highly resistant strains were either removed by the treatment, or successfully controlled by the immune system. Although intensive poultry farming is based on strictly controlled production and good biosecurity practice, application of antimicrobials is inevitable in case of disease outbreaks. However, exposure to antimicrobials can lead to selection pressure and emergence of resistance (HEDMAN et al., 2020), while overuse of antimicrobials can induce a strain shift (WALK et al., 2007; BIBBAL et al., 2009; ZAKARIAZADEH et al., 2019). In this research, the overall frequency of AMR genes decreased on both farms, with a more obvious change on Farm A (Paper III.). The results also correspond to previous reports describing the higher prevalence of AMR genes in the commensal strains, as opposed to pathogenic strains that carry more VAGs, which was confirmed on Farm B (SMITH et al., 2007; CHAKRABORTY et al., 2015).

The results of the phylotyping and MLST analysis overlapped in most cases, confirming the efficacy of the phylogroup typing method (Paper II.). Said method is cost-effective and efficient in the everyday diagnostics, and should be used for continuous monitoring of the strain diversity and suspected virulence.

MLST and SNP-based phylogenetic analyses confirmed the spatial-dependant transmission of the strains, in most cases (Paper II., Paper III.). Strain diversity decreased over time, showing less genetic heterogeneity within the later flocks. Large clusters mostly contained *E. coli* lineages originating from multiple flocks on a single farm, additionally confirming the horizontal transmission of the strains. Out of 23 detected sequence types (STs), four constituted more than 60% of the total investigated strains, of which ST95 and ST117 were the most prevalent with 42.24%. ST23, ST95 and ST117 are considered the dominant lineages in poultry, although they are all adapted to colonize and cause an infection in poultry, livestock and humans, but to different extent (MEHAT et al., 2021). From the abovementioned, ST95 is more commonly associated with infections in humans. Here, ST95 showed the highest prevalence (22.61%), confirming the zoonotic potential in addition to the genetic similarity of the investigated strains to UPEC and NMEC isolates in the phylogenetic analysis (Paper II.). From the highly prevalent STs, ST23, ST95 and ST390 were identified only on one farm, while ST117 was the only lineage detected on both farms (Paper II., Paper III.). According to Ronco et al. (2016), the possible origin of ST117 are grandparent flocks, indicating the isolates were vertically transmitted to the progeny. In this case, both farms have the same rearing flocks, which could have been the source of this lineage that showed significantly high genetic homogeneity of the strains originating from both farms.

Core-genome SNP phylogeny showed high homogeneity between the highly virulent strains including ST117-F, ST95-B2, ST131-B2 and ST390-B2, which originate from the same farm (Paper III.). The results indicate these particular strains averted the effect of the autogenous vaccine, but the application led to reduced clinical implications and improved production performance of the birds. On the other hand, vaccination on Farm A significantly affected the selection of strains, but showed to be non-protective against the heterologous strains (Paper III.), as previously suggested by other researches (LANDMAN and VAN ECK, 2017; KOUTSIANOS et al., 2020).

Identification based on the previously suggested five minimal predictors (*iutA, hlyF, iss, iroN, ompT)* (JOHNSON et al., 2008; DE OLIVEIRA et al., 2015) showed that only 58.82% and 84.38% of the investigated isolates on Farm A and B, respectively, should be categorized as APEC. As these virulence factors were significantly more prevalent in the highly virulent *E. coli* strains, they are considered useful for distinguishing APEC from avian commensal fecal *E. coli* (RODRIGUEZ-SIEK et al., 2005a; JOHNSON et al., 2006; JOHNSON et al., 2008). However, other studies have reported that APEC contains various traits associated with intestinal *E. coli* pathotypes, further complicating the classification (NOLAN et al., 2020). The same was observed in this research. Out of 31 isolates that were not categorized as APEC, 58.06% of the strains belonged to the commensal phylogenetic groups. On the other hand, 32.26% of the strains belonged to the pathogenic phylogenetic groups, indicating the present set of predictors is insufficient and that phylotyping method according to the Clermont protocol (CLERMONT et al., 2013) is more effective for routine diagnostics.

Analysis of the VAGs showed the highest prevalence of the chromosome-borne genes, with the exception of *iss* gene, even though the plasmids containing PAIs are considered a defining trait of APEC (NOLAN et al., 2020). High prevalence of the bacteriocin-related toxins indicate a competitive phenotype of the bacteria, which enables selection of one or few dominant phenotypes in the host and development of a systemic infection (MAJEED et al., 2011). Colicins, i.e. bacteriocins produced by *E. coli* (CASCALES et al., 2007), often serve as a model for studying the mechanisms of bacteriocin action (MAJEED et al., 2011). In natural populations, 10-50% of *E. coli* produce colicins and their production is cross-induced (GORDON AND O'BRIEN, 2006; BARNES et al., 2007; MAJEED et al., 2011). In addition, strains belonging to phylogenetic group B2 often produce more than one type of bacteriocins (GORDON and O'BRIEN, 2006), which presumably contributes to their virulence. Although APEC are generally considered less toxigenic than mammalian pathogenic *E. coli* (NOLAN et al., 2020), in this research genes encoding toxins were highly prevalent, while colicins were present in up to 51.31% of the studied isolates, depending on the type of colicin (Paper II.).

The average number of VAGs per isolate showed an increasing trend on Farm A, in parallel to the increasing prevalence of clades I-V (Papers I., Paper II.), which are

considered commensal. On the contrary, average number of VAGs on Farm B was slightly decreasing, and the strains originating from the same farm showed high similarities based on the phylogeny (Paper II.). The overall virulence profiles of the investigated isolates were strongly ST dependant (Paper II.), confirming that MLST is an efficient method for detection of *E. coli* clones (CHRISTENSEN et al., 2021). The results obtained from the phylotyping method, MLST-phylogeny and VAG analysis were visualized in the heatmap (Paper II.), which additionally showed the relatedness of the strains and confirmed the overlap in the results from different investigation methods. Out of all gene groups studied in the research, iron acquisition factors have shown the highest prevalence (Paper II.). Various iron acquisition mechanisms, such as aerobactin, sit or iro systems, are more frequently detected in the pathogenic strains as they enable colonization of the host and are important in the pathogenesis of extraintestinal colibacillosis (GAO et al., 2012; CAZA and KRONSTAD, 2013; SAROWSKA et al., 2019; NOLAN et al., 2020). Therefore, their high prevalence was expected in the studied isolates.

The results of the AMR gene analysis showed the highest prevalence of *mdf(A)* and *sit* operon genes (Paper III.). Expression of *mdf(A)* confers the multidrug resistance (EDGAR et al., 1977; ONG et al., 2020), indicating the strains adapted to the environment by carrying broad-spectrum specificity AMR genes. On the other hand, *sit* operon genes mediate the transport of iron and manganese, and have a contributing role as virulence factors, ultimately affecting the development of clinical infections (SABRI et al., 2006; SABRI et al., 2008; NOLAN et al., 2020). Sit operon also increases resistance to the bactericidal effects of hydrogen peroxide and, consequently, resistance to oxidative stress (SABRI et al., 2006). Hydrogen peroxide is an effective mean of cleaning the drinking water systems on farms (HANCOCK et al., 2007; MAHARJAN, 2013), so the continuous exposure to the residual disinfectant possibly induced emergence of the corresponding resistance genes. As various classes of antimicrobials have been used on the investigated farms, application could have disrupted the prevalence of more specialized genes. Gene *tet(A)* also showed relatively high prevalence (14.78%), which was probably a result of the frequent use of doxycycline as antimicrobial treatment (Paper III.). Interestingly, it was detected only on Farm A, and predominantly in Flock 4 (64.71%), although it was used on both farms.

Several factors in the last decade could have contributed to the emergence of *E. coli* as a primary pathogen in poultry, including the reduced use of antimicrobials in livestock production and intensification of poultry husbandry (MEHAT et al., 2021). Use of antimicrobials on animal farms is difficult to control. Although the European Commission banned their use as growth promoters in feed in 2006 (Regulation (EC) No 1831/2003), they are still frequently applied in order to prevent common disease outbreaks. Unfortunately, their purchase and usage in animal production is not efficiently controlled, and the overuse is significantly contributing to the growing global problem of multidrug resistance. Thus, continuous monitoring of STs, VAGs and AMR gene transmission could provide a better insight in the evolution and adaptation of bacteria to current environment, and enable improvement of the disease control in the future.

6. CONCLUSIONS

1. Application of the autogenous vaccine affected the prevalence of phylogenetic groups and phylogenetic relationships of APEC strains. Implementation of the autogenous vaccine repressed most phylogenetic groups used in the vaccine, enabled selection of specific groups and led to a strain shift on Farm A.
2. Continuous application of autogenous vaccine gradually led to a lower genetic diversity of *E. coli* strains on the investigated farms. Higher genetic homogeneity of the isolates from the later flocks was confirmed based on the Clermont phylotyping method, MLST and cgSNP-based phylogenetic analyses.
3. Application of the autogenous vaccine did not lead to a lower genetic diversity of VAGs. The average frequency of VAGs on Farm A increased over time, while on Farm B it marginally decreased.
4. Virulence gene profiles corresponded to specific STs and phylogenetic groups of *E. coli* isolates. Out of the highly prevalent sequence types, ST95, ST390 and ST131 had the highest average number of VAGs per isolate.
5. In total, 23 sequence types were identified, of which ST95, ST117, ST390 and ST23 constituted 62.61% of the STs.
6. Most STs were present only on one farm, showing the transmission of *E. coli* is generally horizontal. ST117 was the only sequence type present on both farms, indicating the transmission possibly occurred vertically from the grandparent flocks or the rearing houses.
7. AMR gene analysis showed the highest prevalence of gene *mdf(A)*, which provides a wide-spectrum protection against six classes of antimicrobials, and *sit* operon genes that mediate the transport of iron and manganese and have an important role as virulence factors.
8. None of the STs and phylogenetic groups could be associated with specific AMR genes, but the overall prevalence of AMR genes was higher in the commensal strains.
9. Interaction between the autogenous vaccine and antimicrobial treatment possibly led to a gradual decrease of AMR genes on both farms.
10. Genomic analysis showed that both MLST and cgSNP-based phylogeny could provide a detailed clonal characterization of *E. coli* isolates.

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8. APPENDICES

Supplementary Table S1 from Paper I. Description of strains used in the study.

Farm	Flock	Phylogroup	Organ of origin	Age of the bird (wk)
A	1	**D**[a]	liver	33
		B1	liver	33
		A	liver	33
		A	liver	33
		A	liver	33
		A	liver	33
		F	lungs	33
		F	liver	35
		F	liver	35
		F	liver	35
	2	A	liver	34
		C	liver	34
		A	liver	34
		A	liver	34
		A	peritoneum	34
		A	peritoneum	34
		B1	peritoneum	34
		unknown	peritoneum	34
		unknown	peritoneum	34
		unknown	peritoneum	38
		B1	liver	43
		F	peritoneum	43
		A	lungs	47
		B2	oviduct	56
	3	F	peritoneum	25
		F	bone marrow	25
		F	oviduct	25
		F	peritoneum	25
		F	liver	25
		F	liver	25
		F	liver	44
		F	liver	49
		F	peritoneum	49
		F	liver	49
		F	bone marrow	49
		A	peritoneum	55
		F	peritoneum	55
		F	peritoneum	55
	4	clade I/II	peritoneum	25
		clade I/II	liver	25
		clade I/II	peritoneum	25

		clade I/II	liver	25
		C	oviduct	25
		clade I/II	liver	32
		clade I/II	bone marrow	32
		A	liver	32
		C	bone marrow	36
		C	bone marrow	36
		F	liver	41
		F	peritoneum	41
		D	peritoneum	41
B	1	◦**F**^{b}	liver	49
		◦**B2**	peritoneum	49
		B2	liver	49
		F	liver	62
		F	peritoneum	62
		•B2	peritoneum	62
		•B2	liver	62
		▫E	liver	62
		▫B2	peritoneum	62
		F	peritoneum	62
		▪B2	liver	62
		▪**B2**	peritoneum	62
		A	liver	62
	2	C	peritoneum	29
		A	peritoneum	39
		B2	peritoneum	41
		◊**A**	oviduct	41
		◊**B2**	subcutaneous caseous exudate	41
		∆**B2**	liver	41
		∆**B2**	oviduct	41
		∆**B2**	pericardium	41
	3	B2	liver	46
		B2	liver	46
		F	peritoneum	46
		B2	peritoneum	46
		A	peritoneum	46
		A	peritoneum	46
		B2	liver	46
		F	liver	53
		B2	peritoneum	53
		B2	liver	53
		B2	bone marrow	60
		B2	liver	60
		B2	bone marrow	60
	4	F	liver	22

	F	liver	22
	clade I/II	liver	22
	B2	bone marrow	35
	F	oviduct	35
	clade I/II	peritoneum	35
	F	bone marrow	35
	F	liver	41
	A	liver	41
	A	liver	41
	F	liver	51
	F	bone marrow	51
	B2	liver	58
	B2	peritoneum	58
	B2	bone marrow	58
5	B2	peritoneum	37
	B2	peritoneum	37
	B2	liver	37
	B2	bone marrow	39
	B2	bone marrow	39
	B2	bone marrow	39
	B2	peritoneum	43
	B2	liver	43
	B2	liver	43
	B2	liver	43
	B2	liver	43
	B2	peritoneum	43
	B2	liver	43

[a]Strains used for the production of autogenous vaccine are written in bold. Isolates from the present flock were always used for the production of the vaccine for the next flock.

[b]Strains marked with same symbols (◦/•/▫/▪/◊/∆) originate from the same bird.

Supplementary Table S1 from Paper II. Description of isolates used in this study. Isolates in bold were used for the production of autogenous vaccines for the next consecutive flock on the farm.

Farm-Flock	Isolate	Location of isolation	ST	Surveillance (S)/ Outbreak (O)	Accession number
A-1	**9**	liver	**8573**	O[a]	SAMN16954579
A-1	**13**	liver	**162**	O	SAMN16954580
A-1	17	liver	48	O	SAMN16954581
A-1	20	liver	48	O	SAMN16954582
A-1	21	liver	48	O	SAMN16954583
A-1	27	liver	48	O	SAMN16954584
A-1	29	lungs	1485	O	SAMN16954585
A-1	47	liver	117	S	SAMN16954586
A-1	**50**	liver	**117**	S	SAMN16954587
A-1	52	liver	117	S	SAMN16954588
A-2	116	liver	10	O	SAMN16954591
A-2	117	liver	10	O	SAMN16954592
A-2	**118**	liver	**10**	O	SAMN16954593
A-2	**121**	liver	**7013**	O	SAMN16954594
A-2	124	peritoneum	162	O	SAMN16954595
A-2	**125**	peritoneum	**162**	O	SAMN16954596
A-2	126	peritoneum	162	O	SAMN16954597
A-2	127	peritoneum	162	O	SAMN16954598
A-2	128	peritoneum	162	O	SAMN16954599
A-2	140	peritoneum	162	O	SAMN16954600
A-2	146	liver	3232	S	SAMN16954601

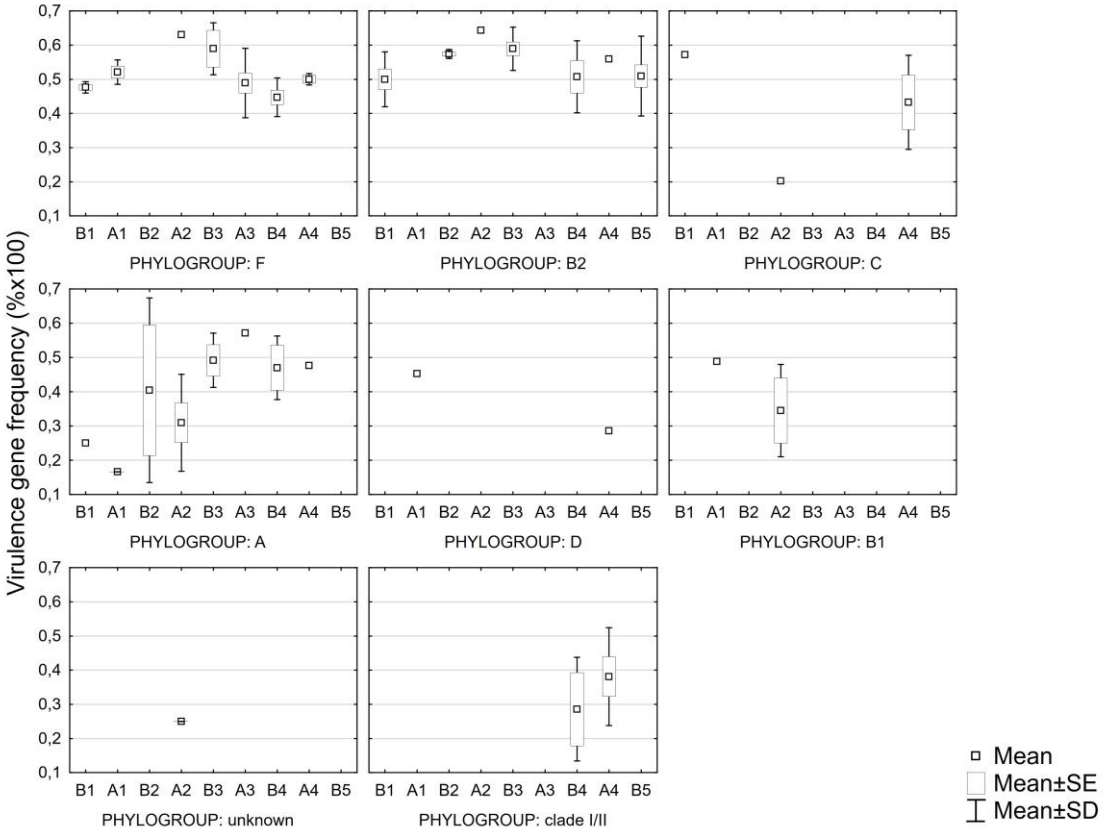
A-2	149	peritoneum	117	S	SAMN16954602
A-2	152	lungs	616	S	SAMN16954603
A-2	173	oviduct	429	S	SAMN16954604
A-3	466	peritoneum	117	O	SAMN16954605
A-3	**467**	bone marrow	**117**	O	SAMN16954606
A-3	469	oviduct	117	O	SAMN16954607
A-3	470	peritoneum	117	O	SAMN16954608
A-3	474	bone marrow	117	O	SAMN16954609
A-3	**607**	liver	**117**	S	SAMN16954612
A-3	**632**	liver	**135**	S	SAMN16954613
A-3	633	peritoneum	117	S	SAMN16954614
A-3	637	liver	135	S	SAMN16954615
A-3	**639**	bone marrow	**135**	S	SAMN16954616
A-3	731	peritoneum	101	S	SAMN16966892
A-3	733	peritoneum	unknown	S	SAMN16966893
A-3	734	peritoneum	10	S	SAMN16966894
A-4	951	peritoneum	23	O	SAMN16966900
A-4	967	liver	23	O	SAMN16966902
A-4	968	peritoneum	23	O	SAMN16966903
A-4	970	peritoneum	23	O	SAMN16967643
A-4	971	liver	23	O	SAMN16967644
A-4	975	oviduct	23	O	SAMN16967645
A-4	1025	liver	23	S	SAMN16967646
A-4	**1028**	bone marrow	**23**	S	SAMN16967647
A-4	1031	liver	23	S	SAMN16967648

A-4	**1124**	bone marrow	**23**	S	SAMN16967651
A-4	**1125**	bone marrow	**23**	S	SAMN16967652
A-4	1206	liver	117	O	SAMN16967653
A-4	**1207**	peritoneum	**117**	O	SAMN16967654
A-4	1209	peritoneum	69	O	SAMN16967682
B-1	**1**	liver	**117**	O	SAMN16951411
B-1	**3**	peritoneum	**390**	O	SAMN16951412
B-1	**5**	liver	**390**	O	SAMN16951413
B-1	**32**	liver	**117**	O	SAMN16951414
B-1	**33**	peritoneum	**117**	O	SAMN16951415
B-1	34	peritoneum	390	O	SAMN16951416
B-1	36	liver	390	O	SAMN16951417
B-1	38	liver	95	O	SAMN16951418
B-1	39	peritoneum	95	O	SAMN16951419
B-1	**41**	peritoneum	**117**	O	SAMN16951420
B-1	43	liver	390	O	SAMN16951421
B-1	**44**	peritoneum	**390**	O	SAMN16951422
B-1	**46**	liver	**297**	O	SAMN16951423
B-2	68	peritoneum	46	S	SAMN16954590
B-2	**79**	peritoneum	**390**	S	SAMN16951424
B-2	**80**	oviduct	**390**	S	SAMN16951425
B-2	**83**	subcutaneous caseous exudate	**390**	S	SAMN16951426
B-2	**85**	liver	**390**	S	SAMN16951427
B-2	**87**	oviduct	**390**	S	SAMN16951428
B-2	**88**	pericard	**390**	S	SAMN16951429

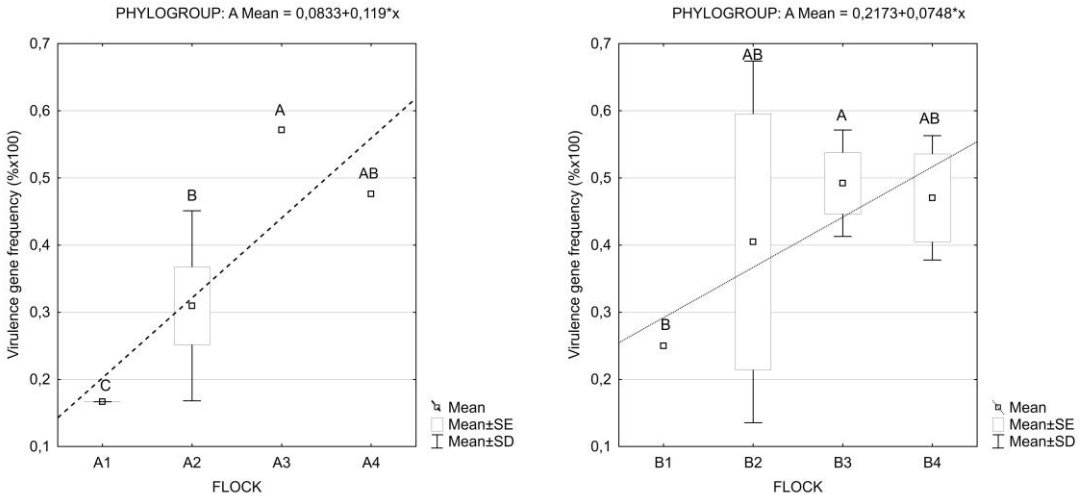
B-3	324	liver	95	O	SAMN16951430
B-3	325	peritoneum	95	O	SAMN16951431
B-3	327	peritoneum	95	O	SAMN16951432
B-3	328	liver	95	O	SAMN16951433
B-3	333	peritoneum	131	O	SAMN16951434
B-3	342	peritoneum	95	O	SAMN16951435
B-3	344	peritoneum	93	O	SAMN16951436
B-3	346	peritoneum	58	O	SAMN16951437
B-3	347	liver	131	O	SAMN16951438
B-3	455	liver	428	S	SAMN16951439
B-3	459	peritoneum	95	S	SAMN16951440
B-3	463	liver	95	S	SAMN16951441
B-3	**507**	bone marrow	**131**	S	SAMN16954610
B-3	**509**	liver	**95**	S	SAMN16951442
B-3	**510**	bone marrow	**95**	S	SAMN16954611
B-4	629	liver	117	S	SAMN16951443
B-4	631	liver	117	S	SAMN16951444
B-4	641	liver	10	S	SAMN16951445
B-4	795	bone marrow	95	S	SAMN16966895
B-4	798	oviduct	117	S	SAMN16966896
B-4	801	peritoneum	10	S	SAMN16966897
B-4	802	bone marrow	428	S	SAMN16966898
B-4	911	liver	117	O	SAMN16951446
B-4	917	liver	746	O	SAMN16951447
B-4	918	liver	93	O	SAMN16966899

B-4	962	liver	117	S	SAMN16951448
B-4	**964**	bone marrow	**117**	S	SAMN16966901
B-4	**1034**	liver	**95**	O	SAMN16951449
B-4	1035	peritoneum	95	O	SAMN16967649
B-4	**1036**	bone marrow	**95**	O	SAMN16967650
B-4	1037	liver	95	O	SAMN16951450
B-5	1407	peritoneum	95	O	SAMN16967683
B-5	1409	peritoneum	95	O	SAMN16967684
B-5	1412	liver	131	O	SAMN16967685
B-5	1465	bone marrow	95	O	SAMN16967686
B-5	1466	bone marrow	95	O	SAMN16967687
B-5	1467	bone marrow	131	O	SAMN16967688
B-5	1509	peritoneum	131	S	SAMN16967689
B-5	1512	liver	95	S	SAMN16976350
B-5	1514	liver	95	S	SAMN16976351
B-5	1516	liver	95	S	SAMN16976352
B-5	1521	liver	95	S	SAMN16976353
B-5	1526	peritoneum	95	S	SAMN16976354
B-5	1528	liver	95	S	SAMN16976355

[a]The samples were collected during an outbreak (O) or as a part of regular surveillance (S) of poultry health.



Supplementary Figure S1 from Paper II. Virulence gene frequency per flock for each phylogenetic group.



Supplementary Figure S2 from Paper II. Virulence gene frequency in phylogenetic group A for each flock on Farm A and B.

Supplementary Table S1 from Paper III. Description of isolates used in this study. Isolates in bold were used for the production of autogenous vaccines for the next consecutive flock on the farm.

Isolate	ST	Phylogroup	Accession number
A1_9[1]	8573	D	SAMN16954579
A1_13	162	B1	SAMN16954580
A1_17	48	A	SAMN16954581
A1_20	48	A	SAMN16954582
A1_21	48	A	SAMN16954583
A1_27	48	A	SAMN16954584
A1_29	1485	F	SAMN16954585
A1_47	117	F	SAMN16954586
A1_50	117	F	SAMN16954587
A1_52	117	F	SAMN16954588
A2_116	10	A	SAMN16954591
A2_117	10	C	SAMN16954592
A2_118	10	A	SAMN16954593
A2_121	7013	A	SAMN16954594
A2_124	162	A	SAMN16954595
A2_125	162	A	SAMN16954596
A2_126	162	B1	SAMN16954597
A2_127	162	unknown	SAMN16954598

A2_128	162	unknown	SAMN16954599
A2_140	162	unknown	SAMN16954600
A2_146	3232	B1	SAMN16954601
A2_149	117	F	SAMN16954602
A2_152	616	A	SAMN16954603
A2_173	429	B2	SAMN16954604
A3_466	117	F	SAMN16954605
A3_467	117	F	SAMN16954606
A3_469	117	F	SAMN16954607
A3_470	117	F	SAMN16954608
A3_474	117	F	SAMN16954609
A3_607	117	F	SAMN16954612
A3_632	135	F	SAMN16954613
A3_633	117	F	SAMN16954614
A3_637	135	F	SAMN16954615
A3_639	135	F	SAMN16954616
A3_731	101	A	SAMN16966892
A3_733	unknown	F	SAMN16966893
A3_734	10	F	SAMN16966894
A4_951	23	clade I/II	SAMN16966900
A4_967	23	clade I/II	SAMN16966902

A4_968	23	B2	SAMN16966903
A4_970	23	clade I/II	SAMN16967643
A4_971	23	clade I/II	SAMN16967644
A4_975	23	C	SAMN16967645
A4_1025	23	clade I/II	SAMN16967646
A4_1028	23	clade I/II	SAMN16967647
A4_1031	23	A	SAMN16967648
A4_1124	23	C	SAMN16967651
A4_1125	23	C	SAMN16967652
A4_1206	117	F	SAMN16967653
A4_1207	117	F	SAMN16967654
A4_1209	69	D	SAMN16967682
B1_1	117	F	SAMN16951411
B1_3	390	B2	SAMN16951412
B1_5	390	B2	SAMN16951413
B1_32	117	F	SAMN16951414
B1_33	117	F	SAMN16951415
B1_34	390	B2	SAMN16951416
B1_36	390	B2	SAMN16951417
B1_38	95	C	SAMN16951418
B1_39	95	B2	SAMN16951419

B1_41	117	F	SAMN16951420
B1_43	390	B2	SAMN16951421
B1_44	390	B2	SAMN16951422
B1_46	297	A	SAMN16951423
B2_68	46	A	SAMN16954590
B2_79	390	B2	SAMN16951424
B2_80	390	A	SAMN16951425
B2_83	390	B2	SAMN16951426
B2_85	390	B2	SAMN16951427
B2_87	390	B2	SAMN16951428
B2_88	390	B2	SAMN16951429
B3_324	95	B2	SAMN16951430
B3_325	95	A	SAMN16951431
B3_327	95	B2	SAMN16951432
B3_328	95	B2	SAMN16951433
B3_333	131	F	SAMN16951434
B3_342	95	B2	SAMN16951435
B3_344	93	A	SAMN16951436
B3_346	58	A	SAMN16951437
B3_347	131	B2	SAMN16951438
B3_455	428	F	SAMN16951439

B3_459	95	B2	SAMN16951440
B3_463	95	B2	SAMN16951441
B3_507	131	B2	SAMN16954610
B3_509	95	B2	SAMN16951442
B3_510	95	B2	SAMN16954611
B4_629	117	F	SAMN16951443
B4_631	117	F	SAMN16951444
B4_641	10	clade I/II	SAMN16951445
B4_795	95	B2	SAMN16966895
B4_798	117	F	SAMN16966896
B4_801	10	clade I/II	SAMN16966897
B4_802	428	F	SAMN16966898
B4_911	117	F	SAMN16951446
B4_917	746	A	SAMN16951447
B4_918	93	A	SAMN16966899
B4_962	117	F	SAMN16951448
B4_964	117	F	SAMN16966901
B4_1034	95	B2	SAMN16951449
B4_1035	95	B2	SAMN16967649
B4_1036	95	B2	SAMN16967650
B4_1037	95	B2	SAMN16951450

B5_1407	95	B2	SAMN16967683
B5_1409	95	B2	SAMN16967684
B5_1412	131	B2	SAMN16967685
B5_1465	95	B2	SAMN16967686
B5_1466	95	B2	SAMN16967687
B5_1467	131	B2	SAMN16967688
B5_1509	131	B2	SAMN16967689
B5_1512	95	B2	SAMN16976350
B5_1514	95	B2	SAMN16976351
B5_1516	95	B2	SAMN16976352
B5_1521	95	B2	SAMN16976353
B5_1526	95	B2	SAMN16976354
B5_1528	95	B2	SAMN16976355

[1]Labels of the isolates include farm, flock and isolate number (i.e. Farm A, Flock 1, isolate 9).

Supplementary Table S2 from Paper III. Description of reference genomes used for the SNP analysis.

Isolate	Phylogenetic group	ST	Accession number
ECCNB20-2	A	ST2705	GCA_004006575.1
EK2009	A	unknown	GCA_005954625.1
E308	A	unknown	GCA_003443815.1
AH62	A	ST1788	GCA_013371745.1
AH65	A	ST1788	GCA_013389695.1
AH25	B1	ST156	GCA_013371725.1
C21	B1	ST156	GCA_012974525.1
3R	B1	ST156	GCA_011067085.1
AH01	B1	ST602	GCA_013371685.1
HB37	B2	ST127	GCA_013004045.1
ExPECXM	B2	ST95	GCA_002844685.1
ST95-32	B2	ST95	GCA_008632595.1
ACN001	C	ST23	GCA_001051135.1
ACN002	C	ST23	GCA_001515725.1
ECCNB12-2	F	ST6862	GCA_003790525.1
104	F	ST117	GCA_003095635.1
APEC O78	C	ST23	CP004009
SF-088	B2	ST95	CP012635
EC958	B2	ST131	HG941718

Supplementary Table S3 from Paper III. The results of the Poisson regression analysis showing the incident rate, standard error (SE) and 95% confidence intervals (95% CI) between the antimicrobial treatment and AMR gene prevalence among different flocks. The first flock on both farms was used as a reference group for the analysis.

Farm	Flock	Incident rate	SE	2.5%	97.5%	p value
A	1	2.40	0.60	1.47	3.93	
	2	1.49	0.44	0.83	2.67	0.18
	3	1.09	0.32	0.62	1.93	0.77
	4	1.07	0.28	0.64	1.78	0.79
B	1	2.15	0.24	1.73	2.68	
	2	0.80	0.12	0.59	1.07	0.13
	3	0.84	0.11	0.64	1.09	0.19
	4	0.81	0.10	0.63	1.04	0.10
	5	0.89	0.11	0.71	1.12	0.34

9. BIOGRAPHY OF THE AUTHOR WITH BIBLIOGRAPHY OF PUBLISHED WORK

Liča Lozica was born on 5th of February 1993 in Korčula, Croatia. She attended elementary school, primary music school and general education high school in Korčula. She enrolled at the Faculty of Veterinary Medicine, University of Zagreb, in 2011. During her studies, she volunteered at several Departments of the Faculty of Veterinary Medicine, University of Zagreb, and a Small Animal Veterinary Clinic in Korčula. She was given multiple awards for excellent students of the city of Korčula, and a national scholarship for excellent students. In 2017, she was awarded the Rector's Prize for the research paper „Molecular characterization of APEC strains isolated from poultry farms in Croatia“. She graduated in June 2017 with the focus on companion, laboratory and exotic animal medicine. Since September 2017, she has been working as a research and teaching assistant at the Department of Poultry Diseases with Clinic, at the Faculty of Veterinary Medicine, University of Zagreb. In 2018, she enrolled in doctoral studies in Veterinary Sciences. Her work involves teaching students, routine diagnostic and field work on poultry farms, clinical work with pet birds and scientific research with a focus on bacterial poultry diseases. She is a member of the Croatian Veterinary Chamber, the American Association of Avian Pathologists and the World's Poultry Science Association. She has published 11 articles and 26 congress presentations. She participated in 20 conferences and symposia, and 12 workshops and courses for professional development.

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