

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

Liča Lozica,^{*} Anamaria Ekert Kabalin,[†] Nada Dolenčić,[‡] Metka Vlahek,[‡] and Željko Gottstein^{*,1}

^{*}Department of Poultry Diseases With Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia;

[†]Department of Animal Breeding and Livestock Production, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia; and [‡]Galivet d.o.o, Prelog, Croatia

ABSTRACT *Escherichia coli* is the most common bacterial cause of infections in poultry farms. It is known for its genetic heterogeneity 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 multi-locus 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

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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

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¹Corresponding author: gottstei@vef.hr

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 heterogeneity 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	<i>chuA</i>	5'-ATGGTACCGGACGAACCAAC-3'	288
	chuA.2		5'-TGCCGCCAGTACCAAAGACA-3'	
	yjaA.1 b	<i>yjaA</i>	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'-AGTTTATCGCTCGGGTTCGC-3'	
AceK.f		5'-AACGCTATTCGCCAGCTTGC-3'	400	
ArpA1.r	<i>arpA</i>	5'-TCTCCCATACCGTACGCTA-3'		
Group E	ArpAgpE.f	<i>arpA</i>	5'-GATTCCATCTTGTCAAAAATATGCC-3'	301
	ArpAgpE.r		5'-GAAAAGAAAAAGAATTCCCAAGAG-3'	
Group C	trpAgpC.f	<i>trpA</i>	5'-AGTTTTATGCCAGTGCGAG-3'	219
	trpAgpC.r		5'-TCTGCGCCGGTACAGCCC-3'	

¹Reference (Clermont et al., 2013).

Table 3. Description of genotypes for phylogroup assignment.¹

Phylogroup	Quadruplex genotype			
	<i>arpA</i>	<i>chuA</i>	<i>yjaA</i>	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	+	+	+	+

¹Reference (Clermont et al., 2013).

²The 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).

³These 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 phylogenetic similarity of isolates from the same animal (Supplementary Table 1), the results agree with both previous reports, confirming homogeneity 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/C/E	B2/D/F
		A	B1	B2	C	D	E	F	Clades	Unknown		
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 ^{3**}	1 (7.1)	-	-	-	-	-	13 (92.9)	-	-	1 (7.1)	13 (92.9)
	4 ^{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)

¹Numbers in bold in each flock row represent phylogroups used in the autogenous vaccine for the next flock.

²Every 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 *** $P \leq 0.001$.

³Statistically 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 heterogeneity 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.

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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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