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Impact of commercial and autogenous *Escherichia coli* vaccine combination on broiler breeder stock performance, gross pathology, and diversity of *Escherichia coli* isolates

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Abstract

Avian colibacillosis is one of the main causes of economic losses in the poultry industry worldwide. Vaccination could help to prevent infection during the laying period on broiler breeder farms. Effective vaccination against avian pathogenic *Escherichia coli* (APEC) may be an essential step for protection of poultry flocks depending on the region where they are raised. The aim of this study was to investigate the additive protective effect of an autogenous *E. coli* vaccine in broiler breeders pre-vaccinated with a licensed *E. coli* vaccine (Poulvac®). Our field study was partially blinded and parallel group designed. Group 1 included 24 000 laying hens vaccinated by Poulvac®. Group 2 comprised 12 000 laying hens vaccinated by Poulvac® and additionally, by an autogenous *E. coli* vaccine via intramuscular application before transfer. The effectiveness of vaccination in both groups was evaluated according to the results of gross pathology, bacteriology (isolation and characterization of *E. coli*) and utility indicators. Based on the pathology, the occurrence of *E. coli* polyserositis syndrome (EPS), salpingoperitonitis syndrom (SPS), and haemorrhagic septicaemia was decreased in Group 2 compared to Group 1. The difference in the occurrence of EPS ($P < 0.001$) and SPS ($P = 0.0342$) was significant. The proportion of serotype O78 among *E. coli* isolated from Group 1 and Group 2 was also significant ($P = 0.0178$). The effective and multi-serotype vaccination program in order to expand heterologous protection of laying hens and combination of commercial and autogenous vaccines seems to be a promising preventive management tool.

Poultry, colibacillosis, APEC, autogenous vaccine

Avian pathogenic *Escherichia coli* (APEC) can be responsible for extraintestinal infections in poultry and, depending on the strain's virulence and the local lesions, may become systemic and culminate in colisepticaemia (Horn et al. 2012). Infection pressure (quantity of bacteria in direct contact with the bird), virulence factors and the bird's defence mechanisms interplay to determine the duration and severity of the disease. Even though gross lesions are characteristic for colisepticaemia, other bacteria also can occasionally produce septicaemic lesions (Nolan et al. 2013). It is necessary to isolate them from appropriate tissues and identify *E. coli* to confirm a diagnosis of colisepticaemia. Currently, accumulating evidence has led to the acceptance that certain clones of *E. coli* may act as primary pathogens and cause severe disease and high mortality despite high standard management, low stress levels, and the absence of concurrent diseases (Dziva et al. 2013; Pires dos Santos et al. 2013).

Reports of increased antimicrobial multi-resistance have forced the global poultry industry to consider some alternative methods of *E. coli* management. The kind of effective step for the poultry flock protection may be vaccination against colisepticaemia. Inactivated vaccines specific to serogroups are effective and their use in breeders has provided progeny with passive protection against homologous strains (Nolan et al. 2015).

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The aim of the presented study was to evaluate whether the use of the autogenous vaccine, together with the commercial one, would have an additive protective effect against colisepticaemia in vaccinated flocks. The assessment criteria consisted in the screening of the pathological signs of the disease together with performance indicators of lying flocks. In addition, we observed the autogenous vaccine effect on the spectrum of *E. coli* colonizing vaccinated birds through bacteriological isolation and characterization of isolates obtained from dead birds.

Materials and Methods

Breeding conditions and management of field study

Our research was conducted on a broiler breeder farm in the Czech Republic. In August 2017, 39 960 hens and roosters of the Cobb 500 genetic line were transferred into 6 halls with free run (including chicken nesting boxes) type of housing and capacity of 6 000 hens per hall.

This field study was partially blinded and parallel group designed. Group 1 included 24 000 laying hens vaccinated by licensed *E. coli* vaccine (Poulvac®, Zoetis, Parsippany, USA), by coarse spray according to the manufacturer's instructions: 2 ×, at 12 and 20 weeks of the birds' age. Group 2 comprised 12 000 laying hens vaccinated by licensed *E. coli* vaccine (Poulvac®) in spray (at the same age as group 1) and additionally with autogenous *E. coli* vaccine via intramuscular (i.m.) application before transfer (at the age of 18 weeks). The animals were treated with regard to animal welfare and in accordance with applicable regulations. All activities took place within the veterinary service on the monitored farm.

Vaccine specification

Commercial vaccine Poulvac® (Zoetis) is distributed in the form of a lyophilizate which is intended for the preparation of a suspension for the drinking water treatment or for the administration as a spray. It contains live strain *E. coli* of O78 serotype characterised as *aroA* gene deleted.

The autogenous vaccine was custom-made by the company AniCon Labor GmbH (Emstek, Germany) as a polyvalent inactivated vaccine for i.m. application. The two vaccine strains were isolated from the liver and spleen of a laying hen with colisepticaemia that came from the broiler breeder farm where the experiment was conducted. Both strains were subjected to whole-genome sequencing (WGS).

WGS of *E. coli* strains for autogenous vaccine

Genomic DNA was extracted by NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA libraries were prepared using The Nextera XT Library preparation kit (Illumina, San Diego, USA) and sequenced on NovaSeq 6000 (Illumina, San Diego, USA) platform producing 2 × 250 bp paired-end reads.

Trimmomatic v0.36 (Bolger et al. 2014) was used for removal of low-quality ends ($Q \leq 20$) and adaptor residues from the reads. *De novo* assembly (Bankevich et al. 2012) was performed using Shovill v1.0.4 (https://github.com/tseemann/shovill). Genetic content of the strains was identified using Centre for Genomic Epidemiology (CGE) tools (http://www.genomicepidemiology.org/): ResFinder v4.1 (Bortolaia et al. 2020; Zankari et al. 2020), PlasmidFinder v2.0 (Carattoli et al. 2014) and VirulenceFinder v2.0, with coverage and identity threshold set to 95%. Furthermore, typization was performed by SeroTypeFinder v2.0 (Joensen et al. 2015) and MLST v2.0 (Larsen et al. 2012). The ClermonTyping tool was used to classify isolates into phylogenetic groups (Beghain et al. 2018) and CSI Phylogeny v1.4 (Kaas et al. 2014) to determine genetic relatedness of the strains using the IMT5155 (Zhu Ge et al. 2014) as a reference. The analysed sequences are available in the Sequence Read Archive (SRA), under BioProject ID PRJNA839610.

Evaluation of health status and performance indicators

In both groups, the number of dead laying hens, utility indicators, feed and water consumption were monitored twice a day by the farm staff. The average weight and uniformity of both flocks were also monitored. All data were recorded in MS Excel sheets. Veterinary supervision included checking the health of the flocks, necropsying a representative number of carcasses, and taking samples for bacteriological examination. Veterinary inspections on the farm were carried out at weekly intervals. The observed clinical signs included apathy, depression, fluffy feathers, discharge from the beak or conjunctiva, dyspnoea or cough. Cachectic hens without secondary sexual characteristics were also regularly selected.

Gross autopsy and evaluation of findings

Postmortem examination was performed daily of dead or euthanized animals or frozen cadavers with clinical signs of colisepticaemia. The autopsy was performed outside the environment of breeding halls in the postmortem diagnostic premises according to a well-known procedure so that the internal organs were fully visible and could be removed for detailed examination (Galton et al. 1960; Barnes and Gross 1997). During the complete necropsy, special attention was paid to sampling birds with polyserositis (EPS) - liver, pericardium, spleen, lungs

and air sacs, yolk peritonitis (SPS) - ovary, fallopian tube or colisepticaemia - bone marrow. Each sampling was performed as aseptically as possible, using sterile instruments.

Sampling and bacteriological methods

Three samples (bone marrow, ovary and one visceral organ with pathological findings) were collected from each carcass with signs of colisepticaemia and polyserositis (peritonitis, perihepatitis, pericarditis, airsaculitis or haemorrhagic septicaemia) for the purpose of bacteriology examination of experimental flocks once per week during egg production. Samples were taken using sterile tampons and sent to the laboratory by courier service in a transport medium according to Amies (Copan, Brescia, Italy) within 24 h. A total of 219 samples were taken during the experiment. The samples were cultured on McConkey agar (Oxoid, Basingstoke, UK) for 24 h at 37 °C. Colonies that fermented lactose were sub-cultivated on Columbia blood agar (Oxoid, Basingstoke, UK) and identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry method (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany).

Characterization of *E. coli* cultures

Antigenic structure of *E. coli* isolates was determined by slide agglutination using specific O1, O8, O18 and O78 commercial antisera (Denka Seiken, Tokyo, Japan). *Escherichia coli* isolates were examined by disk diffusion test (DDT) according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2015) for susceptibility to 12 antibacterial substances (µg/disk): ampicillin (A, 10), amoxicillin-clavulanic acid (Ac, 20/10), cephalothin (Cf, 30), ceftazidime (Caz, 30), chloramphenicol (C, 30), ciprofloxacin (Cip, 5), gentamicin (Gn, 10), florfenicol (Ffc, 30), nalidixic acid (Na, 30), sulphonamide compounds (Su, 300), sulphamethoxazole-trimethoprim (Sxt, 23.75/1.25), tetracycline (T, 30), (Oxoid, Basingstoke, UK). Isolates were further tested for the presence of the *ompT, cvaC, iss, iroN, iutA, tsh, felA, frz* APEC virulence genes using polymerase chain reaction (PCR) (Ewers et al. 2005; Moulin-Schouleur et al. 2006; Dissanayake et al. 2014; Papouskova and Cizek 2020).

Data analysis

All data were entered into spreadsheets (MS Excel, Microsoft, Prague, Czech Republic); chi-square test of homogeneity was used for comparison between groups and Fisher's exact probability test was used where the assumption of the chi-square test did not hold. Statistical significance was accepted at $P < 0.05$.

Table 1. Virulence-associated genes detected in vaccine strains and their presumed functions.

Group	Gene	Function
Adhesins	*yfcV*	Fimbrial adhesin
	fimH	Type 1 fimbiral adhesin
	iheA	Adherence protein
	tsh	Temperature-sensitive haemagglutinin
Protectins	*kpsMII_K1*	Polysialic acid transport protein; Group 2 capsule
	kpsE	Capsule polysaccharide export inner-membrane protein
	neuC	Polysialic acid capsule biosynthesis protein
	traT	Outer membrane protein complement resistance
	iss	Increased serum survival
Iron transport	*chuA*	Outer membrane haemin receptor
	fyuA	Yersiniabactin siderophore receptor
	ireA	Siderophore receptor
	irp2	Yersinibactin peptide synthetase
	iroN	Salmochelin siderophore receptor
	iutA	Aerobactin siderophore receptor
	iucC	Aerobactin synthetase
	sitA	Iron transport protein
Miscellaneous	*ompT*	Outer membrane protease
	hlyF	Haemolysin F
	cvaC	Colicin V synthesis
	usp	Uropathogenic specific protein
	ibeA	Brain endothelium invasion

Results

Characterization of *E. coli* strains for autogenous vaccine

Pre-selection of *E. coli* isolates was made on the basis of detection of virulence associated genes: *ompT, cvaC, iss, iroN, iutA, tsh, felA* and *frz* by PCR. The two *E. coli* vaccine strains that were closely related (with a difference of 35 SNPs) belonged to the ST140 and B2 phylogroup. IncX1, IncFIB, and IncFIC replicons were detected. The strains were resistant to ampicillin and nalidixic acid, carrying resistance genes $bla_{TEM\text{-}1b}$ and *qnrS1*, presumably associated with plasmid IncX1, as they were found on the same contig as this replicon in the strain 3436. Besides, the *gyrA* gene mutation was associated with quinolone resistance. The strains were positive for all PCR-detected virulence genes apart from *felA*, and their presence was confirmed *in silico*. Virulence-associated genes detected in vaccine strains and their presumed functions are shown in Table 1.

Health status and utility indicators

By evaluating the total number of deaths in the observed period from the 30th to the 58th laying week, significant differences between Groups 1 and 2 were found ($P < 0.001$). A total of 379 and 272 birds died in Group 1 and 2, respectively. Nevertheless, Group 2 (treated with the commercial vaccine and revaccinated with the autogenous vaccine) showed lower overall losses; the decline of mortality was 1.8% (105 laying hens). Differences in the number of dead laying hens in each week during the laying period varied (Fig. 1). Furthermore, a different average count of eggs per hen in Group 1 (139.0 eggs) and Group 2 (141.4 eggs) was recorded. The course of the laying period as the laying intensity percentage is documented in Fig. 2.

Gross pathology

Evaluation of pathoanatomical findings is documented in Table 2. The level of mortality within the laying period (researched 29 weeks) was 379 hens in total (on average 13 hens per week) in Group 1 and 272 hens in total (average 9.4 hens per week) in Group 2. Cumulative

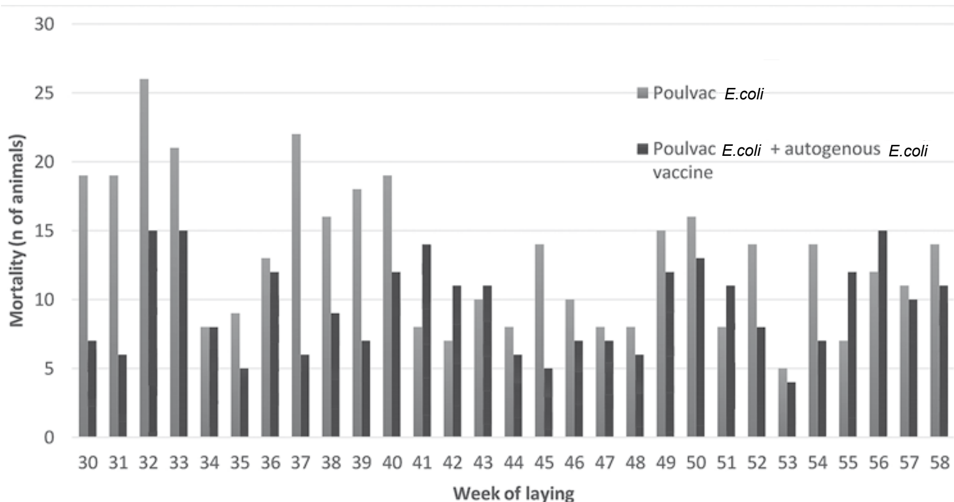

Fig. 1. Utility indicators – mortality values during the observed laying period

colibacillosis image (*E. coli* polyserositis syndrome - EPS, salpingoperitonitis syndrome - SPS, haemorrhagic septicaemia) in Group 2 (30.1%) in comparison with Group 1 (63.0%) was decreased. The difference shown was significant (P = 0.001). Also, the difference in the occurrence of the EPS ($P < 0.001$) and SPS (P = 0.0342) was significant. From carcasses with diagnosis of acute colibacillosis (53% in Group 1 vs. 26% in Group 2), 144 and 75 samples were obtained for bacteriology examination in Group 1 and Group 2, respectively.

Bacteriology

Bacterial isolation was considered positive if the bacterial swabs from the visceral organs, ovaries and bone marrow resulted in pure, fair, and florid growth of *E. coli* when cultured on Columbia blood agar and McConkey agar plates overnight. The results of bacteriological examination are documented in Table 3. A higher isolation rate of *E. coli* from the bone marrow (indicator of colisepticaemia) was found in Group 1 (74%) compared to Group 2 (54%). The proportional presence of *E. coli* serogroups from the bone marrow was examined. The difference between incidence of serogroup O78 was significant (P = 0.0178).

Table 3 also summarizes the results of testing for the presence of *ompT*, *cvaC*, *iss*, *iroN*, *iutA*, *tsh*, *felA*, *frz* genes encoding important virulence factors in APEC. *Escherichia coli* isolates positive for five and more genes occurred in 91% of Group 2 compared to 71% in Group 1.

Discussion

In future, production of high standard quality of hatch eggs on broiler breeder farms should be a prerequisite for antibiotic-free broiler production. Specific imunoprophylaxis can be considered an important tool in the global effort to reduce incidence of APEC infection and could prevent outbreaks of colibacillosis and chronic respiratory disease. Vaccination can support egg production and reduce the mortality rate in layers and breeder flocks.

For a vaccine aiming to protect broilers through vertical transfer of antibodies, high levels of circulating antibodies are crucial. In the present study, the transferability of the antibodies may also be questioned, as day-old broilers of vaccinated parents did not differ in antibody titres from day-old offspring of unvaccinated broiler breeders (Lili et al. 2016). The lack of correlation between IgY antibodies and the level of protection is not an uncommon observation in studies of bacterial infection.

Pressures surrounding the indiscriminate use of antibiotics are well established, with the World Health Organization naming resistance to antimicrobials a "major global threat" to human health, leading to a huge pressure on their use in poultry

Table 2. Gross pathology lesions in laying hens vaccinated with Poulvac® (Groups 1 and 2) and then revaccinated with autogenous *E. coli* vaccine (Group 2).

Group	Number of autopsies	Laying hens with gross lesions, % (no. positive)							
		E. coli polyserositis syndrome*	Salpingo-peritonitis syndrome*	Haemorrhagic septicaemia*	Traumatic injury	Cannibalism	Prolapse of cloaca rupture	Liver dystrophy, image	Cumulative colibacillosis
1	379	44.1 (167)+	10.0 (38)+	9.0 (34)	10.0 (38)	5.0 (19)	4.0 (15)	18.0 (68)	63.1 (239)+
2	272	19.8 (54)	5.1 (14)	5.9 (16)	18.0 (49)	21.0 (57)	4.0 (11)	26.0 (26)	30.8 (84)

*Colibacillosis image; + significant difference

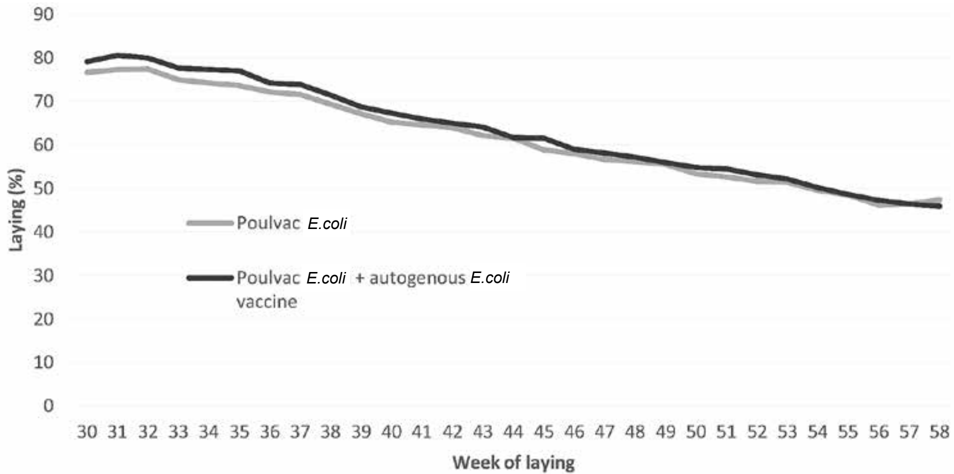

Fig. 2. Utility indicators – course of the laying period (laying intensity percentage)

Table 3. Results of culture examination and characterization of *E. coli* isolates from dissected laying hens vaccinated with Poulvac® (Groups 1 and 2) and then revaccinated with autogenous *E. coli* vaccine (Group 2).

Group	Sampled carcasses	*E. coli* positive bone marrow (%)	Proved serotypes					Virulence genes[1]	
			O1	O8	O18	O78⁺	Others	≥ 5	< 5
1	144	35 (73)	16	2	0	16	2	32	3
2	75	14 (54)	8	1	0	1	4	10	4

[1] tested genes (*ompT, cvaC, iss, iroN, iutA, tsh, felA, frz*); ⁺ significant difference

production. A number of developed countries have taken antibiotics considered critical to human health out of food animal production. One such antibiotic is colistin which was banned based on the evidence of a major increase in colistin resistance due to a plasmid-mediated *mcr-1* gene detected in *E. coli* isolates from poultry and swine (Quesada et al. 2016).

Our experience confirms the conclusions of previous fundamental studies (Ewers et al. 2005; Johnson et al. 2008; Schouler et al. 2012) that virulence genotyping is fairly reliable for virulence prediction which to some extent corresponds to the number of detected virulence-associated genes. On the other hand, when this approach is applied to the selection of potential vaccine strains, a more detailed characterization is strongly urged in order to ensure choosing truly relevant APEC strains.

Recent research shows the virulent APEC population to comprise several distinct genotypic lineages: predominantly strains belonging to the O78 serogroup and the C phylogenic group (sequence types ST23 and ST88), strains belonging to the O1 and O2 serogroups, mostly associated with the B2 phylogroup (ST95), and the ST117 (O-variable:H4) strains from the G phylogroup (Mehat et al. 2021). The vaccination scheme should cover at least these most predominant genotypes. The commercial vaccine contains the O78 antigen, protecting mostly against homologous challenge. The strains selected for the autogenous vaccine in this study belong to the ST140 from the clonal complex 95 and are closely related (difference in 95-105 SNPs) to a reference strain IMT5155 (O2:K1:H5-ST140-B2) which was highly virulent in chicken models and showed a certain zoonotic potential

(Zhu Ge et al. 2014). The ST95 strains are frequent in poultry, but also represent one of the dominant epidemic human ExPEC lineages causing urinary tract infections, bloodstream infections and neonatal meningitis (Poulsen et al. 2020; Denamur et al. 2021). However, the related ST140 seems to be more closely associated with poultry (Mehat et al. 2021).

Clinical studies have evaluated this live *E. coli* vaccine (Poulvac®) as effective after one dose for at least eight weeks against the O78 strain, and up to 20 weeks if is vaccinated twice (Zoetis). In our applied study, Groups 1 and 2 were vaccinated according to the recommended scheme twice by the live vaccine, and for a higher level of protection against heterologous *E. coli* strains, autogenous inactivated *E. coli* vaccine was added. An important viewpoint for the assessment of the level of protection of birds involves the half-life of disintegration antibodies during the laying period. Scheduled vaccination should be completed before the start of laying (24 weeks of age in broiler breeder flocks). Therefore, the expected protection time cannot exceed week 44 of age. Increased mortality after week 45 of age was found in both experimental groups.

Incorporation of appropriate autogenous APEC strain into the vaccination schedule can likely lead to reduction of incidence APEC strains with more than five genes of virulence (Group 1 – 92% vs. Group 2 – 71%) and the extension of protection against another serogroup.

Currently, the testing for virulence factors is done in multiplex PCR detecting eight gene encoding virulence factors. These eight virulence factors allow classification of *E. coli* isolated from poultry to be APEC or not (Ewers et al. 2005). Isolates that are positive for five or more out of the eight virulence genes are assumed to be APEC.

In conclusion, based on the pathology and bacteriology findings, the efficacy of combining the Poulvac® vaccine with the autogenous *E. coli* vaccine in field conditions was higher than when the commercial Poulvac® vaccine was applied alone. The use of the commercial vaccine in a flock, primarily protecting against the O78 serogroup, may reduce losses but also induce selection of other virulent genotypes, especially from the B2 phylogroup, as shown recently by Lozica et al. (2021). Autogenous vaccines offer a flexible solution as they are tailor-made for the particular flock and whole-genome sequencing is a perfect tool for monitoring the epidemiological situation and selection of relevant epidemic strains.

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