

Antimicrobial resistance and genetic diversity of *Enterococcus faecalis* from yolk sac infections in broiler chicks

Dagmara Stępień-Pyśniak ^{*,1} Tomasz Hauschild,[†] Marta Dec,^{*} Agnieszka Marek,^{*} Michał Brzeski,[‡] and Urszula Kosikowska[§]

^{*} Department of Veterinary Prevention and Avian Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Lublin, 20-612 Poland; [†] Department of Microbiology and Biotechnology, Faculty of Biology, University of Białystok, Białystok, 15-245 Poland; [‡] Veterinary Cabinet - Brzeski and partners, Giżycko, 11-500 Poland; and [§] Department of Pharmaceutical Microbiology, Medical University in Lublin, Lublin, 20-093 Poland

ABSTRACT Despite restrictions on the use of antibiotics in poultry, the percentage of multidrug resistant bacteria, isolated from both adult birds and chicks, remains high. These bacteria can spread between countries via hatching eggs or chicks. Antibiotic resistant bacteria can also pose a threat to hatchery and farm workers or to consumers of poultry. The aim of the study was to perform a phenotypic and genotypic analysis of the drug resistance of *E. faecalis* isolates from yolk sac infections in broiler chicks from Poland and the Netherlands and to determine their genetic diversity. The tests revealed resistance to antibiotics from category D, that is, tetracycline (69.7%); category C – lincomycin (98.7%), erythromycin (51.3%), aminoglycosides (high-level streptomycin and kanamycin resistance – 10.5% and 3.95%, respectively), and chloramphenicol (7.9%); and category B – ciprofloxacin (25% with resistance or intermediate resistance). No resistance to penicillin,

ampicillin, high-level gentamicin, tigecycline, or linezolid was noted. Various combinations of the *erm*(B), *tet*(M), *tet*(L), *tet*(O), *ant*(6)-Ia, *aph*(3)-IIIa, *ant*(4')-Ia, *cat*, and *msr*(A/B) genes were detected in all isolates (irrespective of the drug-resistance phenotype). Among isolates that carried the *tet*(M) and/or the *tet*(L) gene, 28% also had the *Int-Tn* gene, in contrast with isolates possessing *tet*(O). There were 28 sequence types and 43 PFGE restriction patterns. About 60% of isolates were of sequences types ST59, ST16, ST116, ST282, ST36, and ST82. Nine new sequence types were shown (ST836-ST844). In conclusion, broiler chicks can be a source of drug-resistant sequence types of *E. faecalis* that are potentially hazardous for people and animals. Restrictive programs for antibiotic use in broiler breeding flocks should be developed to decrease drug resistance in day-old chicks and reduce economic losses during rearing.

Key words: yolk sac infections, *Enterococcus faecalis*, antibiotic resistance, broiler chicks

2021 Poultry Science 100:101491
<https://doi.org/10.1016/j.psj.2021.101491>

INTRODUCTION

Good breeding practices in raising breeding flocks and in hatching of broilers are essential for obtaining chicks of good biological quality (Tona et al., 2005). Inappropriate practices, both in breeding flocks and in hatchery management, can lead to embryo death or bacterial infections after hatching, particularly yolk sac infections (Yassin et al., 2009). Bacteria can cause infections both during the development of the egg in the hen's

reproductive system (vertical transmission) and immediately after the egg is laid (contact with material in the nest), as well as during storage or transport of the eggs before they are set (horizontal transmission) (Higeyni, 2014). Unhygienic conditions before and during hatching can contribute to bacterial infections in newly hatched chicks and cause infections of the navel and yolk sac (Khan et al., 2004). The main, natural route of infection for bacteria causing omphalitis is the unhealed navel. Bacteria may also enter the hatching egg, for example, the yolk sac, as a result of in ovo vaccination (Landman et al., 2000). Yolk sac infections are the cause of significant economic losses in poultry farming due to increased chick mortality, increased culling of weak birds in the flock, and chronic infections during rearing (Amare et al., 2013).

Among Gram-positive bacteria, the most commonly identified species isolated from hatching eggs, dead

© 2021 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received June 28, 2021.

Accepted September 9, 2021.

¹Corresponding author: dagmara.stepien@up.lublin.pl

embryos, and chicks up to 1 wk of age is *Enterococcus faecalis* (Olsen et al., 2012b; Stępień-Pyśniak et al., 2016; Dolka et al., 2017; Karumarathna et al., 2017). Some studies have found that a high frequency of *E. faecalis* in chicks in the first 24 h of life is associated with horizontal transmission of these bacteria between chicks in the hatchery (Fertner et al., 2011; Olsen et al., 2012a). Apart from omphalitis or yolk sac infection, during rearing of poultry *E. faecalis* has been associated with amyloid arthropathy, amyloidosis, arthritis, femoral head necrosis, joint lesions, pododermatitis, endocarditis, septicemia, pulmonary hypertension syndrome (ascites), oophoritis, and salpingitis/peritonitis (Christensen and Bisgaard, 2016).

Despite adherence to biosecurity principles and implementation of alternative methods to control infections in poultry flocks, the use of antibiotics remains a widespread strategy to maintain the health of animals, including poultry. This is reflected in sales of antibiotics, which remain high in many European countries (EMA, 2019). According to the European Medicines Agency (EMA) report (2019), the most commonly sold antibiotics for food animals in European countries are tetracyclines and penicillins (about 30% each). Other classes of antibiotics included in the report are (<10% for each class) macrolides, lincosamides, sulphonamides, trimethoprim, polymyxins, aminoglycosides, pleuromutins, and fluoroquinolones. This is especially dangerous because many antibiotics used in food animals, including poultry, belong to the same classes or groups that are often used in human medicine (EFSA, 2019; EMA, 2019). Data published by the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) indicate that antimicrobials used to treat diseases and transmitted from animals to people become less and less effective. For this reason, the EMA has published a classification of antibiotics based primarily on the demand for a given class or subclass of antimicrobial agents in human medicine and on the risk of spread of resistance to these substances from animals to people (EMA, 2019). The classification includes antimicrobials from the World Health Organisation's (WHO) list of critically important antimicrobials and divides them into the following four categories: A – Avoid, B – Restrict, C – Caution, and D – Prudence.

The use of antibacterial agents creates selective pressure for the emergence of resistant strains, both pathogenic and commensal, for example, *Enterococcus*. Enterococci exhibit a variety of mechanisms of intrinsic and acquired resistance to the major classes of antibiotics of clinical use, as well as efficient genetic exchange mechanisms that facilitate the dissemination of antibiotic resistance genes (Rehman et al., 2018). Moreover, a bacterial cell often possesses more than one mechanism to resist an antibiotic, and co-operation between different resistance mechanisms often generates high-level resistance. Enterococci have become resistant to antimicrobials through a number of mechanisms: efflux pumps – *tet(K)*, *tet(L)*, *mef(A/E)*, *msr(A/B)*; modification of target molecule: modification of the ribosomal target – *erm(A)*, *erm(B)*, *erm(T)*, alteration in penicillin-binding protein (PBP) –

pbp5, altered cell wall precursors – *vanA* and *vanB*, ribosomal protection – *tet(M)* and *tet(O)*; antibiotic inactivation: aminoglycoside modifying enzymes – *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3')-IIIa*, *ant(4')-Ia*, *ant(6)-Ia*, chloramphenicol-acetyl-transferases – *cat*, β -lactamase – *blaZ* and nucleotidyltransferase – *lnuB* (van Hoek et al., 2011).

Although previous studies have focused on transmission and genetic diversity of *E. faecalis* during hatch (Fertner et al., 2011), very little is known about this species having clinical implications in chicks in the post-hatching period (Olsen et al., 2012a,b). It should be pointed out here that many resistant pathogens, including opportunistic ones, such as bacteria of the genus *Enterococcus*, can spread between countries due to sales of hatching eggs or chicks. Furthermore, many studies have presented an analysis of the drug resistance of *Enterococcus* bacteria from poultry of slaughter age. The authors of these studies often do not report what therapeutic procedures, if any, have been used in these birds, including antimicrobial agents. Therefore, we conducted an analysis of the drug resistance of *E. faecalis* isolates from broiler chicks from 2 countries with different rearing procedures for these birds, and which before death had exhibited untreated symptoms of yolk sac inflammation.

Due to the scarcity of information on the problem presented above, the aim of the study was to carry out a phenotypic and genotypic analysis of the drug resistance of *E. faecalis* isolates from yolk sac infections in broiler chicks from Poland and the Netherlands and determining their genetic diversity in terms of risk to broiler chick rearing and human health.

MATERIALS AND METHODS

Bacterial Isolates

The material for the study was collected during post-mortem examination from broiler chicks showing lesions characteristic of yolk sac infection. The surface of the affected organ was first decontaminated by searing and then the contents of the yolk sac were taken using a sterile cotton swab. The bacteria was isolated on Bile Esculin Azide Lab-Agar (BIOMAXIMA, Poland) at 37°C for 24 to 48 h under microaerophilic conditions (Stępień-Pyśniak et al., 2016). Cases in which abundant colony growth was observed in pure culture were selected for further analysis. The bacteria were initially identified with standard procedures and a commercial biochemical test – STREPTOtest 24 (Erba Lachema, Czech Republic). Then all isolates were grown in Brain Heart Infusion (BHI) Broth (OXOID, Hampshire, UK) and 20% glycerol was added as a cryoprotector to store at –80°C until further analysis. After revival, the identification was verified by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) using an Ultraflex extreme spectrometer and MALDI-Biotyper 3.0 software (Daltonik Bruker, Germany) and by *rpoA* gene sequencing, as previously described (Stępień-Pyśniak et al., 2021). Finally, a collection of 76 *E. faecalis* isolates (35 Polish

isolates and 41 Dutch isolates) from yolk sac infections in broiler chicks were included in the study.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of the 76 *E. faecalis* isolates was tested by broth microdilution using 13 antimicrobials. Susceptibility to vancomycin (0.25–128 mg l⁻¹), linezolid (0.25–128 mg l⁻¹), penicillin (0.25–128 mg l⁻¹), ampicillin (0.125–64 mg l⁻¹), gentamicin (2–1024 mg l⁻¹), streptomycin (4–2,048 mg l⁻¹), kanamycin (4–2,048 mg l⁻¹), tetracycline (0.25–128 mg l⁻¹), tigecycline (0.03–2 mg l⁻¹), erythromycin (0.125–64 mg l⁻¹), lincomycin (0.5–256 mg l⁻¹), ciprofloxacin (0.125–64 mg l⁻¹), and chloramphenicol (0.25–128 mg l⁻¹) (Oxoid, UK) was determined. *E. faecalis* ATCC 29212 was used as a quality control strain.

Susceptibility tests were performed according to recommendations by the Clinical and Laboratory Standards Institute (CLSI, 2015a). MICs (minimal inhibitory concentrations) were evaluated based on the interpretative criteria of CLSI (CLSI, 2015c) supplement VET01S for vancomycin, penicillin, ampicillin, erythromycin, tetracycline, doxycycline, and chloramphenicol for *Enterococcus* spp.; CLSI (CLSI, 2015b) document M100-S25 for ciprofloxacin for *Enterococcus* spp.; and Comité de l'antibiogramme de la Société Française de Microbiologie (CA-SFM) (CA-SFM, 2015) for lincomycin and all aminoglycosides for *Streptococcus* spp.

Antibiotic Resistance Genes

Genomic DNA was extracted using a commercial Genomic Mini kit (A&A Biotechnology, Gdańsk, Poland). To improve the nucleic acid extraction efficiency, lysozyme was used in the enzymatic lysis step.

PCR was used to demonstrate the presence of resistance genes and Tn916/Tn1545-like transposons (integrase gene *Int-Tn*). The primers for resistance genes and annealing temperatures used are listed in Table S1.

Screening of 7 tetracycline and macrolide resistance genes (*tet(M)*, *tet(L)*, *tet(K)*, *tet(O)*, *erm(A)*, *erm(B)*, and *mef(A/E)*) and 6 aminoglycoside resistance genes (*aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3')-IIIa* and *ant(4)-Ia*) was carried out using multiplex PCR in a final volume of 25 μL of reaction mixture according to Vakulenko et al. (2003) and Malhotra-Kumar et al. (2005). The reagents used in the multiplex PCR mixtures were purchased from AmpliKIT Allegro Taq (Novazym, Poznań, Poland).

The remaining resistance genes and the gene encoding integrase were identified by uniplex PCR, in which the reaction mixture was composed of 1 μL (~20 ng) of DNA as a template, 12.5 μL of 2× Taq PCR Master Mix (Qiagen, Wrocław, Poland), 2.5 μL of 10× primer mix (2 μM of each primer), and RNase-free water (Qiagen) to a final volume of 20 μL.

PCR was conducted using a DNA Mastercycler (Eppendorf, Hamburg, Germany). The following

conditions were used: pre-denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at temperatures specified in Table S1 for 60 s, extension at 72°C for 1 min, and a final extension at 72°C for 8 min. Then the PCR products were resolved by electrophoresis on a 1.5% agarose-Tris-borate-EDTA gel containing 1 μL of GelView, a safe DNA gel stain (Novazym, Poland), per 50 mL and then visualized using a gel imaging analysis system with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Molecular Typing by Pulsed-Field Gel Electrophoresis

Whole-cell DNA for PFGE was prepared as described previously (Hauschild and Schwarz, 2003) with some modifications. The cells were digested with 5 mg of lysozyme (Sigma-Aldrich, Poznan, Poland) for 6 h and then with 1 mg of pronase E (Sigma-Aldrich, Poznan, Poland) for 6 h. Slices of DNA-containing agarose plugs were subjected to restriction endonuclease digestion in a total volume of 150 μL containing 30 U of *SmaI* (Thermo Scientific, Waltham, MA). DNA was digested at 30°C for 4 h. The *SmaI* fragments were electrophoretically separated in a 1% (w/v) agarose gel (Sigma-Aldrich, Poland) using a CHEF Mapper System (BIO-RAD Laboratories). The gel was exposed to a pulse time of 5–30 s at 6 V/cm for 24 h. A 50–1,000 kb pulsed-field DNA marker (Sigma-Aldrich) served as a size standard. The relatedness of the strains was determined based on generally accepted criteria (Tenover et al., 1995).

Multilocus Sequence Typing

Multilocus sequence typing of the *E. faecalis* isolates was performed using primers and procedures established by Ruiz-Garbajosa et al. (2006). The PCR amplicons of 7 housekeeping genes were sequenced by automated sequencing with an ABI PRISM 3130 genetic analyser (Applied Biosystems, Forest City, CA), edited and aligned using DNAMAN ver. 4.13 software (Lynnon BioSoft, Canada), and compared with a reference set of alleles and the *Enterococcus faecalis* MLST web-based database sited at the University of Oxford (Jolley and Maiden, 2010). Alleles without a match in the database were submitted for assignment. Isolates were defined by their alleles at the seven loci (allelic profile), and each allelic profile was assigned to a sequence type, either as already defined in the database or as designated by the website curator for new registrations.

RESULTS

Antimicrobial Susceptibility Testing and Detection of Resistance Genes

Table 1 shows the distribution of phenotypic resistance in *E. faecalis* isolates, taking into account the categories of antibiotics. Antibiotic resistance and/or

Table 1. Distribution of phenotypic resistance and intermediate resistance in *E. faecalis* strains, broken down by category of antibiotic.

AMEG [†] category	Antibiotic class, subclasses	Antibiotic*	Poland		Netherlands		Totaln = 76 (%)
			<i>E. faecalis</i> = 35 (%)	<i>E. faecalis</i> = 41 (%)	R	I	
Category A 'Avoid'	Glycopeptides	Vancomycin				2 (4.9)	2 (2.6)
		Oxazolidinones	Linezolid				0 (0)
		Glycylcyclines	Tigecycline				0 (0)
Category B 'Restrict'	Quinolones: fluoroquinolones and other quinolones	Ciprofloxacin	7 (20)		1 (2.4)	11 (26.8)	19 (25)
Category C 'Caution'	Aminoglycosides (except spectinomycin)	Gentamicin					0 (0)
		Streptomycin	4 (11.4)		4 (9.8)		8 (10.5)
		Kanamycin**	1 (2.9)		2 (4.9)		3 (3.95)
		Lincomycin	30 (85.7)	4 (11.8)	39 (95.1)	2 (4.9)	75 (98.7)
		Erythromycin**	9 (25.7)	4 (11.8)	17 (41.5)	9 (22)	39 (51.3)
Category D 'Prudence'	Lincosamides Macrolides (not including ketolides) Amphenicols Aminopenicillin, without beta-lactamase inhibitors Tetracyclines Penicillins: Natural, narrow spectrum penicillins (beta-lactamase-sensitive penicillins)	Chloramphenicol**	2 (5.7)		2 (4.9)	2 (4.9)	6 (7.9)
		Ampicillin					0 (0)
		Tetracycline	23 (65.7)		30 (73.1)		53 (69.7)
		Benzympenicillin					0 (0)

[†]AMEG, Antimicrobial Advice ad hoc Expert Group; R, resistant isolates; I, intermediate resistant isolates.

*Example of antibiotic(s) included in European Medicines Agency category: in macrolides – tylosin, tulathromycin; in aphenicols – florfenicol, thiamphenicol.

**The analysis of antibiotic resistance included erythromycin, chloramphenicol and kanamycin according to Clinical and Laboratory Standards Institute (CLSI) / Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) recommendations, but for the purposes of interpretation we assigned these substances to the appropriate class/subclass. The numbers in front of the brackets indicate the number of resistant or intermediate resistant isolates, respectively; while the values in the brackets represent the percentage of positive isolates (R or I) within isolates from a given country. The last column shows the sum of R and I from both countries and the percentage in relation to the total isolates tested.

intermediate resistance were detected in 100% of Polish and Dutch *E. faecalis* isolates. Multiple-drug resistance was observed for 82.9% (34/41) of Dutch and 74.3% (26/35) of Polish enterococci.

According to the established criteria, the highest resistance in *E. faecalis* isolates was noted for antibiotics belonging to category C – lincomycin (98.7%; 75/76) and erythromycin (51.3%; 39/76) – and category D – tetracycline (69.7%; 53/76). In addition, medium to low resistance was also observed for category B – ciprofloxacin (25%; 19/76, resistance and intermediate resistance together), category C – aminoglycosides (high level of streptomycin and kanamycin resistance – 10.5% (8/76) and 3.95% (3/76), respectively) and chloramphenicol (7.9%; 6/76). None of *E. faecalis* isolates showed resistance to linezolid, penicillin, ampicillin, tigecycline, or high-level gentamicin. However, intermediate resistance to vancomycin was noted in 2.6% (2/76) of isolates.

Table 2 shows the phenotypic and genotypic resistance patterns of the *E. faecalis* isolates, as well as the presence of the integrase gene (*Int-Tn*) of the Tn916-1545 family of transposons. Ten of the 22 investigated resistance genes were detected in the enterococci. Only 1 Dutch (H34) and 3 Polish (P3, P24, and P35) isolates did not possess any of the 22 resistance genes. The *vanA*, *vanB*, *blaZ*, *pbp5*, *tet(K)*, *mef(A/E)*, *erm(A)*, *erm(T)*, *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, and *aph(2'')-Id* genes were not found in any of the *E. faecalis* isolates.

None of the high-level streptomycin-resistant isolates harboured the *ant(6)-Ia* gene, but it was found in 5.7% (2/35) of streptomycin-susceptible Polish isolates. Among the one Polish and 2 Dutch isolates resistant to

high-level kanamycin, only the Dutch strains carried the *aph(3')-IIIa* gene. In addition, the *ant(4')-Ia* gene was detected in 59.2% of isolates; 51.2% (21/41) of Dutch and 68.6% (24/35) of Polish isolates.

The *erm(B)* gene was harboured by 70.6% (12/17) and 55.6% (5/9) of erythromycin-resistant Dutch and Polish isolates, respectively. This gene was also found in one erythromycin-susceptible Polish isolate (P38). Besides the *erm(B)* gene, one Dutch isolate additionally harboured the *msr(A/B)* gene.

The *tet(M)* gene, with or without the *tet(L)* gene, was found in 61% (25/41) of Dutch and 25.7% (9/35) of Polish isolates. Other Polish isolates (37.1%; 13/35) carried the *tet(O)* gene with the *tet(M)* or *tet(L)* gene in 2 cases. The *tet(O)* gene was also harboured by 5 Dutch strains, of which one also carried the *tet(M)* gene and another both *tet(M)* and *tet(L)*.

The *Int-Tn* gene was demonstrated in 39% (16/41) of Dutch isolates and 14.3% (5/35) of Polish isolates that contained the *tet(M)* gene alone or both the *tet(M)* and *tet(L)* genes. In addition, the *Int-Tn* gene was carried by one tetracycline-susceptible Polish strain that was negative for *tet* genes. It is worth noting that none of the strains harboring the *tet(O)* gene was positive for the *Int-Tn* gene.

The *cat* gene was detected in 2 Dutch *E. faecalis* strains resistant to chloramphenicol and one with intermediate resistance. In addition, the *cat* gene was found in 8.6% (3/35 strains) of Polish chloramphenicol-susceptible strains.

The *lnu(B)* gene was detected only in one (2.4%) lincomycin-resistant Dutch strain.

Table 2. Phenotypic and genotypic resistance patterns and genetic diversity of *E. faecalis* from broiler chicks with yolk sac infections.

Origin of strains [†]	AMEG [‡] categories	ST* [‡]	Pulsotype	Phenotypic resistance profile [‡]	Genotypic resistance profile
H5	A B C C C C D	838	16d	<i>van</i> , <i>cip</i> , STR, TET, <i>chl</i> , ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>lnu(B)</i> , <i>erm(B)</i> , <i>Int-Tn</i>
H30	A B C C	387	20	<i>van</i> , <i>cip</i> , <i>ery</i> , LIN	<i>ant(4')-Ia</i>
P27	B C C C D	59	10	CIP, KAN, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>ant(4')-Ia</i>
H41	B C C C D	116	16a	<i>cip</i> , STR, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>cat</i> , <i>Int-Tn</i>
P32	B C C D	93	21	CIP, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>ant(6')-Ia</i> , <i>cat</i>
P29	B C C D	836	28	CIP, TET, ERY, LIN	<i>tet(M)</i> , <i>erm(B)</i> , <i>ant(4')-Ia</i> , <i>Int-Tn</i>
P31	B C C D	836	28	CIP, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>ant(4')-Ia</i> , <i>Int-Tn</i>
P34	B C C D	843	25	CIP, TET, ERY, LIN	<i>tet(L)</i> , <i>erm(B)</i> , <i>cat</i>
H17	B C C D	16	13b	<i>cip</i> , TET, ERY, LIN	<i>tet(M)</i> , <i>ant(4')-Ia</i>
H31	B C C D	36	1	<i>cip</i> , TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>tet(O)</i> , <i>erm(B)</i>
H43	B C C D	82	26a	<i>cip</i> , TET, <i>ery</i> , LIN	<i>tet(M)</i> , <i>Int-Tn</i>
H25	B C C D	116	16e	<i>cip</i> , TET, <i>ery</i> , LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>Int-Tn</i>
H25	B C C D	116	16e	<i>cip</i> , TET, <i>ery</i> , LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>Int-Tn</i>
H29	B C C D	116	17	CIP, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>Int-Tn</i>
H42	B C C D	363	14	<i>cip</i> , TET, ERY, LIN	<i>tet(M)</i> , <i>ant(4')-Ia</i> , <i>Int-Tn</i>
H27	B C C D	387	20	<i>cip</i> , TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>ant(4')-Ia</i>
H28	B C C D	529	4	CIP, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>msr(A/B)</i> , <i>ant(4')-Ia</i>
H32	B C C	59	9b	<i>cip</i> , <i>ery</i> , LIN	<i>ant(4')-Ia</i>
P33	B C D	16	15	CIP, TET, LIN	<i>tet(M)</i> , <i>aph(3')-IIIa</i> , <i>ant(4')-Ia</i> , <i>ant(6')-Ia</i> , <i>Int-Tn</i>
H26	B C D	16	13e	<i>cip</i> , TET, LIN	<i>tet(M)</i> , <i>ant(4')-Ia</i> , <i>Int-Tn</i>
P38	B D	529	4	CIP, TET	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
H3	C C C C D	116	16a	STR, TET, CHL, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>cat</i> , <i>Int-Tn</i>
H16	C C C C D	116	16b	STR, TET, CHL, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>cat</i> , <i>Int-Tn</i>
P26	C C C D	837	31	TET, CHL, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>ant(4')-Ia</i>
P39	C C C D	840	3	TET, CHL, ERY, LIN	<i>tet(L)</i> , <i>tet(O)</i>
H11	C C C D	245	23	KAN, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aph(3')-IIIa</i> , <i>ant(4')-Ia</i>
H24	C C C D	245	23	KAN, TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aph(3')-IIIa</i> , <i>ant(4')-Ia</i>
P35	C C C	165	6	STR, <i>ery</i> , LIN	0
P37	C C C	302	13f	STR, <i>ery</i> , LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
H20	C C C	842	18	<i>chl</i> , <i>ery</i> , LIN	<i>ant(4')-Ia</i> , <i>cat</i>
P3	C C D	36	2	TET, ERY, LIN	0
P24	C C D	36	2	TET, <i>ery</i> , LIN	0
P6	C C D	65	11	TET, ERY, LIN	<i>tet(O)</i>
P36	C C D	82	26a	STR, TET, LIN	<i>tet(M)</i> , <i>Int-Tn</i>
P28	C C D	82	26a	STR, TET, LIN	<i>tet(M)</i> , <i>Int-Tn</i>
H33	C C D	32	30	TET, <i>ery</i> , <i>lin</i>	<i>tet(O)</i> , <i>ant(4')-Ia</i>
H2	C C D	65	11	TET, ERY, LIN	<i>tet(L)</i> , <i>tet(O)</i>
H4	C C D	100	12a	TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
H1	C C D	100	12a	TET, ERY, LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
H18	C C D	116	16c	TET, <i>ery</i> , LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>Int-Tn</i>
H35	C C D	202	7	TET, <i>ery</i> , LIN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>ant(4')-Ia</i>
H6	C C D	282	24	TET, ERY, LIN	<i>erm(B)</i> , <i>ant(4')-Ia</i>
H34	C C D	839	12b	TET, ERY, <i>lin</i>	0
P4	C C	116	22	<i>ery</i> , LIN	<i>Int-Tn</i>
H36	C C	82	26a	<i>ery</i> , LIN	<i>tet(M)</i> , <i>Int-Tn</i>
P19	C D	59	9a	TET, LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P25	C D	59	9a	TET, LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P20	C D	59	9a	TET, LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P18	C D	59	9a	TET, <i>lin</i>	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P15	C D	59	9a	TET, LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P23	C D	59	9a	TET, LIN	<i>tet(M)</i> , <i>tet(O)</i> , <i>ant(4')-Ia</i>
P13	C D	59	9a	TET, LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P17	C D	59	9a	TET, LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P16	C D	59	9a	TET, LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
H19	C D	4	5	TET, LIN	<i>tet(M)</i> , <i>ant(4')-Ia</i> , <i>Int-Tn</i>
H38	C D	16	13c	TET, LIN	<i>tet(M)</i> , <i>ant(4')-Ia</i> , <i>Int-Tn</i>
H22	C D	36	1	TET, LIN	<i>tet(O)</i>
H39	C D	36	1	TET, LIN	<i>tet(O)</i>
H7	C D	49	8	TET, LIN	<i>ant(4')-Ia</i>
H13	C D	82	26a	TET, LIN	<i>tet(M)</i> , <i>Int-Tn</i>
H15	C D	844	27	TET, LIN	<i>tet(M)</i> , <i>Int-Tn</i>
P7	C	16	13b	LIN	<i>ant(4')-Ia</i>
P8	C	16	13b	LIN	<i>ant(4')-Ia</i>
P14	C	59	9a	LIN	<i>tet(O)</i> , <i>ant(4')-Ia</i>
P12	C	282	24	LIN	<i>ant(4')-Ia</i>
P11	C	282	24	<i>lin</i>	<i>ant(4')-Ia</i>
P10	C	282	24	<i>lin</i>	<i>ant(4')-Ia</i>
P22	C	282	24	LIN	<i>ant(4')-Ia</i>
P9	C	282	24	<i>lin</i>	<i>ant(4')-Ia</i>
P21	C	282	24	LIN	<i>ant(4')-Ia</i>
H10	C	4	29	LIN	<i>tet(M)</i> , <i>ant(4')-Ia</i>
H23	C	16	13b	LIN	<i>ant(4')-Ia</i>
H40	C	16	13d	LIN	<i>ant(4')-Ia</i>
H37	C	16	13f	LIN	<i>ant(4')-Ia</i>

(continued)

Table 2 (*Continued*)

Origin of strains [‡]	AMEG [†] categories	ST [*]	Pulsotype	Phenotypic resistance profile [‡]	Genotypic resistance profile
H14	C	245	19	LIN	<i>ant(4′)-Ia</i>
H8	C	302	13a	LIN	<i>ant(4′)-Ia</i>
H12	C	841	26b	LIN	<i>tet(M)</i> , <i>Int-Tn</i>

[‡]Origin of strains: H – Dutch, P – Polish.

[†]AMEG, Antimicrobial Advice ad hoc Expert Group (Category A ‘Avoid’, Category B, ‘Restrict’, Category C ‘Caution’ and Category D ‘Prudence’) – the individual letters indicate the AMEG category profile in relation to the phenotypic resistance profile of the isolate.

^{*}ST, sequence type.

[‡]CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; KAN, kanamycin; LIN, lincomycin; STR, streptomycin; TE, tetracycline; VAN, vancomycin; uppercase – resistance; lower case - intermediate resistance.

MLST and PFGE Typing

Among the *E. faecalis* strains, 28 sequence types and 43 PFGE restriction patterns were noted (Table 2). About 60% of isolates belonged to 6 sequence types: ST59, ST16, ST116, ST282, ST36 and ST82. The other 22 sequence types (ST4, ST32, ST49, ST65, ST93, ST100, ST165, ST202, ST245, ST302, ST363, ST387, ST529, ST836, ST837, ST838, ST839, ST840, ST841, ST842, ST843, and ST844) were represented by 1–3 isolates. MLST analysis confirmed the presence of as many as 9 new sequence types (ST836–ST844). Even greater genetic diversity in the population was determined based on PFGE restriction patterns (43 pulsotypes).

DISCUSSION

Despite restrictions on the use of antibiotics in poultry, the percentage of bacteria resistant to multiple antibiotics is still high. According to Enne et al. (2001), drug resistance in microorganisms may persist even when antibiotics are not used. Bacteria of the genus *Enterococcus* are characterized by relatively rapid acquisition (through horizontally mobile elements including conjugative plasmids, integrons, and transposons) and spread of resistance to antibiotics, which provides selective benefits for their survival and spread in the environment, including adaption to new hosts (Hegstad et al., 2010; Yang et al., 2019). This may be indicated by the troublesome fact that young birds are already a source of multidrug-resistant bacteria in the post-hatching period (Jiménez-Belenguer et al., 2016; Stępień-Pyśniak et al., 2016; Moreno et al., 2019). Nearly 80% of *E. faecalis* isolates in the present study were multidrug-resistant. This means that the vast majority of isolates from day-old broiler chicks with yolk sac infections were resistant to 2 to 7 antibiotics (including isolates with intermediate susceptibility to a given antibiotic). Our study showed that *E. faecalis* isolates were most often resistant to lincomycin, tetracycline, erythromycin, and ciprofloxacin. A lower level of resistance to other antimicrobial agents was found, which included resistance to chloramphenicol and to high-level kanamycin and streptomycin. As observed previously, the percentage of phenotypic resistance to these drugs among enterococci isolated from poultry of different ages (Maasjost et al., 2015; Stępień-

Pyśniak et al., 2016; Woźniak-Biel et al., 2019), as well as poultry meat (Kim et al., 2019) was at similar levels, which may indicate the persistence of resistant bacteria in broilers. This limits or even eliminates the possibilities of treating bacterial diseases during rearing. It may also pose a threat to public health due to the likelihood of multidrug-resistant microbes colonizing hatchery and farm workers, and even consumers, if infectious diseases in humans are treated with antibiotics from the same groups/classes (categories B, C, and D) that are also used in poultry. It is also worth noting that resistant and even multidrug resistant enterococci also contaminate poultry manure (Hayes et al., 2004), which is often used for organic field fertilization. These resistant bacteria in the manure would eventually enter various ecosystems and pose a risk to wildlife (Stępień-Pyśniak et al., 2018; Dec et al., 2020; Nowakiewicz et al., 2020).

However, it is satisfactory that no resistance has been found to penicillin, ampicillin, high-level gentamicin, tigecycline, or linezolid, which are used in specific therapeutic regimens for severe enterococcal infections in hospitalized patients (Arias et al., 2010).

On the other hand, an intermediate resistance to vancomycin (2.6% – category A) was found in Dutch isolates, as this antibiotic is the drug of choice for treating human infections with multidrug-resistant *Enterococcus* spp. However, in comparison with our results, Woźniak-Biel et al. (2019) and Dolka et al. (2016) reported a higher percentage of intermediate susceptibility to vancomycin or even resistance in enterococci isolated from poultry from large-scale farms in Poland. The in vitro resistance to vancomycin noted by the authors may have been due to the method used to determine drug susceptibility, that is, the Kirby–Bauer disc diffusion method. Similarly, Bertelloni et al. (2015) observed a certain percentage of vancomycin-resistant enterococci from healthy laying hens of different hobby poultry flocks using the disc diffusion method, while the microdilution technique used for the same isolates did not confirm resistance to this antibiotic. Therefore, the authors suggest that the results of the Kirby–Bauer method for vancomycin should be confirmed by other tests (Bertelloni et al., 2015). Although each of these 2 phenotypic methods is recommended by CLSI (CLSI, 2015a) for the assessment of bacterial susceptibility in routine laboratory work, both have advantages and

disadvantages (Khan et al., 2019). Therefore, it would seem prudent to use more accurate methods, such as genotyping or even genomic sequencing, in the questionable cases of susceptibility assessment, the molecular mechanisms of resistance, and the importance of mobile genetic elements in the spreading of resistance, especially for antibiotics of last resort (Turner et al., 2021).

In our study, at the molecular level, various combinations of the antimicrobial resistance genes *erm*(B), *tet*(M), *tet*(L), *tet*(O), *ant*(6)-*Ia*, *aph*(3′)-*IIIa*, *ant*(4′)-*Ia*, *cat* and *msr*(A/B) (among 22 genes tested) were detected in the genomes of isolates that were susceptible, intermediate-resistant and resistant to antibiotics. In addition, the *Int-Tn* gene encoding the integrase responsible for transposition was found.

Phenotypic resistance to tetracycline in the present study was associated with the presence of the genes *tet*(M), *tet*(L), and *tet*(O). The *tet*(M) gene, with or without *tet*(L), was noted in nearly half of the strains tested. The *tet*(O) gene was also detected in many of the isolates, mainly from Poland. In addition, 28% isolates containing the *tet*(M) gene alone or with *tet*(L) also carried the *Int-Tn* gene. It should be noted that none of the isolates possessing the *tet*(O) gene contained the *Int-Tn* gene. Tremblay et al. (2011) reported that the *tet*(O) resistance gene was present on a small and transferable plasmid of about 11 kb in *E. faecalis* isolates from the caecal contents of broiler chickens and meat turkeys. The authors also demonstrated co-localization of the *erm*(B) and *tet*(O) genes on this plasmid. In the present study, the *tet*(M) and *tet*(L) genes were usually found to occur together and in the same isolate as the *erm*(B) gene, whereas *erm*(B) was observed together with *tet*(O) in only one isolate. On the other hand, the gene *erm*(B) was previously shown to be frequently associated with *tet*(M) in the highly mobile conjugative transposon *Tn916/Tn1545* (*Int-Tn* gene), dominant in clinical strains of Gram-positive bacteria (De Leener et al., 2004). Other authors have also noted that resistance to macrolides (erythromycin) in *E. faecalis* strains of various origins was mainly associated with the gene *erm*(B), the erythromycin ribosomal methylase gene (Cauwerts et al., 2007), which confers cross-resistance to antibiotics of the MLS_B group (macrolides, lincosamides and streptogramin B). In our study, the most frequently detected gene in the *E. faecalis* isolates from yolk sac infections in broiler chickens showing phenotypic resistance to erythromycin was *erm*(B). What is more, this gene was also found in a small percentage of isolates susceptible to this antibiotic. Only in one erythromycin-resistant isolate from the Netherlands was *erm*(B) found together with *msr*(A/B), which encodes the protein of the ABC transporter family responsible for active pumping of the drug from the cell (an ATP-dependent efflux pump), often detected in *Staphylococcus* isolates (Pyzik et al., 2019). In addition, only one of the lincomycin-resistant isolates carrying the *erm*(B) was also found to contain the *lnu*(B) gene encoding nucleotidyltransferase, which modifies the antibiotic and determines resistance only to lincosamides. Similarly, Nowakiewicz et al.

(2017) detected the gene *lnu*(B) in lincomycin-resistant isolates of *E. faecalis*. In addition, the authors showed that *erm*(B) usually occurred together with *lnu*(B), which was in agreement with our results.

Resistance to high-level aminoglycosides of therapeutic importance in humans is mediated by the acquisition of genes encoding aminoglycoside-modifying enzymes (Kobayashi et al., 2001; Vakulenko et al., 2003). In the present study, none of the *E. faecalis* isolates from yolk sac infections in broilers carried the bifunctional gene *aac*(6′)-*Ie-aph*(2′′)-*Ia* determining resistance to high-level gentamicin, which was also confirmed by the phenotypic drug resistance tests. Despite that, the presence of genes determining resistance to high-level streptomycin and kanamycin was detected. None of the streptomycin-resistant isolates possessed the *ant*(6)-*Ia* gene, but it was found in 2 streptomycin-susceptible strains from Poland. In line with our results, Sharma et al. (2020) did not determine a mechanism of resistance in streptomycin-resistant clinical *E. cecorum* isolates from poultry. However, among the three isolates resistant to high-level kanamycin, the *aph*(3′)-*IIIa* gene was found in the two isolates from the Netherlands, but not in the isolate from Poland. The failure to detect the *ant*(6)-*Ia* or *aph*(3′)-*IIIa* gene may have been due to the presence of variants of these genes that cannot be detected with the primers that were used, or it may indicate the presence of other genes determining resistance to aminoglycosides (European Medicines Agency, 2018) that may circulate in the *E. faecalis* population in poultry. An earlier study conducted in Poland (Stępień-Pyśniak et al., 2019) showed that resistance to high-level gentamicin in *E. faecium* isolated from the digestive tract of a woodpecker was determined by the presence of the gene *aph*(2′′)-*Id*, and not *aac*(6′)-*Ie-aph*(2′′)-*Ia*, which is the most widespread among Gram-positive bacteria (Hauschild et al., 2008; Dec et al., 2017; Nowakiewicz et al., 2017; Woźniak-Biel et al., 2019). Similarly, Dec et al. (2017) showed the presence of the *aph*(2′′)-*Ic* gene among gentamicin-resistant and susceptible *Lactobacillus* spp. isolates from the digestive tract of broiler chickens. It should also be noted that both the *ant*(6)-*Ia* gene and the *aph*(3′)-*IIIa* gene are located on transposons, and thus can easily be transferred to bacteria of the same species or even a different species (Hegstad et al., 2010), resulting in the spread of resistance. In addition, among analysed isolates we found the widespread presence (about 60% of isolates) of the *ant*(4′)-*Ia* gene (a plasmid-encoded aminoglycoside nucleotidyltransferase), mediating resistance to low concentrations of kanamycin, neomycin, tobramycin and amikacin, in contrast to Dec et al. (2020), which noted its sporadic occurrence (8%) in enterococci from wild mammals living in the Apuan Alps Regional Park (Tuscany, Italy). Higher incidence of *ant*(4′)-*Ia* was also reported among clinical *Staphylococcus aureus* isolates in Poland (Hauschild et al., 2008).

Resistance to chloramphenicol is usually caused by synthesis of chloramphenicol acetyltransferase, determined by the presence of the *cat* gene, which leads to

inactivation of the drug. In our study, the *cat* gene was present in 2.7% of strains resistant to chloramphenicol, 1.3% of strains with intermediate susceptibility, and 4% of susceptible strains. Similarly, Hummel et al. (2007) noted that the *cat* gene may be present in bacteria susceptible to chloramphenicol. Moreover, as they did not observe expression of the *cat* gene in such strains in either induced or non-induced conditions, they speculated that a mutation in the regulatory region may be responsible for inhibition of expression of the *cat* gene in phenotypically susceptible isolates.

Among *E. faecalis* isolates from yolk sac infections in broiler chickens, sequence types were noted that had previously been found in various pathological states in the parents of the broilers (e.g., ST16, ST32 and ST82) (Gregersen et al., 2010), as well as in the digestive tract of newly hatched broiler chicks (ST4, ST16, ST59, ST82, ST116, and ST245) (Olsen et al., 2012a) and healthy chicks that were to become layers (ST4, ST32, ST82, and ST100) (Fertner et al., 2011). According to the database on the MLST website (PubMLST, 2019), ST36, ST59, ST82, ST170, ST171, ST172, and ST174 of *E. faecalis* have been also associated with amyloid arthropathy, and ST34, ST82, ST174, and ST177 have been associated with first-week mortality in layers (Olsen et al., 2012b).

Types ST36, ST59 and ST82 identified in avian *E. faecalis* strains have also been linked to human infections, which may represent a hazard to human health through transmission of these isolates (PubMLST, 2019).

It should be emphasized that sequence type ST16 isolated from yolk sac infections was previously characterized as an epidemic clone in hospitals in Poland (Kawalec et al., 2007) and in some other European countries (Kuch et al., 2012). Its presence has also been demonstrated in animals, including poultry, pigs and cattle (PubMLST, 2019). According to the PubMLST database, *E. faecalis* sequence types ST4, ST16, ST59, ST82, ST116, and ST245 have also been noted in hospitalized patients. Furthermore, some data suggest that poultry meat or poultry can be a source of *E. faecalis* in humans (Del Grosso et al., 2000; Templer et al., 2008; Olsen et al., 2012c; Poulsen et al., 2012).

CONCLUSIONS

The data obtained in the study indicate that day-old broiler chicks that have not received antimicrobial treatment can be a source of infection with resistant and even multidrug-resistant strains of *E. faecalis*, posing a potential threat to humans and animals.

The *E. faecalis* isolates derived from yolk sac infections in broiler chicks were most often resistant to category D, C, and B antibiotics, which are also used to treat humans. The bacteria were susceptible to vancomycin and linezolid, considered antibiotics of last resort, as well as to tigecycline, which belongs to category A (Avoid).

The high resistance to tetracycline noted in the isolates, as well as the high percentage of isolates with genetic determinants of resistance to this antibiotic (*tet*(M) and *tet*(O) – ribosomal protection and *tet*(L) – efflux of the tetracycline from the bacterial cell), may be linked to the common use of tetracycline in poultry. The data presented in the paper may provide the basis for considering restrictions on the use of tetracyclines in poultry.

Like the *tet*(M) and *tet*(L) genes, *erm*(B) was detected in many isolates and associated with conjugative transposons, which may also explain the wide dissemination of these genes in the environment. Among the *E. faecalis* isolates susceptible to the antibiotics tested, including high-level streptomycin and chloramphenicol, there were strains with genes determining resistance to these antibiotics.

In addition, these results show that 1-day-old broiler chicks can be infected with diverse sequence types of *E. faecalis* associated with different lesions in poultry, including first week mortality, regardless of country of origin.

More accurate characterisation of *E. faecalis* isolates, as indicator bacteria, in hatcheries and taking appropriate countermeasures (e.g., biosecurity, restrictive antibiotic use programs in broiler breeding flocks, improving prevention programs in broiler parent flocks) could perhaps contribute to decreasing losses during chick rearing and would reduce the risk of spreading drug-resistant strains in the poultry producing environment.

ACKNOWLEDGMENTS

This work was supported by the National Science Centre, Poland under Grant no. 2017/01/X/NZ6/00430.

DISCLOSURES

There are no known conflicts of interest associated with this publication.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2021.101491](https://doi.org/10.1016/j.psj.2021.101491).

REFERENCES

- Amare, A., A. M. Amin, A. Shiferaw, S. Nazir, and H. Negussie. 2013. Yolk sac infection (omphalitis) in Kombolcha Poultry Farm, Ethiopia. *Am. Eur. J. Sci. Res.* 8:10–14.
- Arias, C. A., G. A. Contreras, and B. E. Murray. 2010. Management of multidrug-resistant enterococcal infections. *Clin. Microbiol. Infect.* 16:555–562.
- Bertelloni, F., C. Salvadori, A. Moni, D. Cerri, P. Mani, and V. V. Ebani. 2015. Antimicrobial resistance in *Enterococcus* spp. isolated from laying hens of backyard poultry flocks. *Ann. Agric. Environ. Med.* 22:665–669.

- CA-SFM. 2015. Comité de l'antibiogramme de la Société Française de Microbiologie. Partie Vét. 11-12. Accessed Oct. 2021. http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM_VET2015.pdf.
- Cauwerts, K., A. Decostere, E. M. De Graef, F. Haesebrouck, and F. Pasmans. 2007. High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the erm(B) gene. *Avian Pathol.* 36:395–399.
- Christensen, H., and M. Bisgaard. 2016. Editor in Chief. William. 2016. *Streptococcus and Enterococcus species*. Pages 127-138 in *Laboratory Manual for the Isolation, Identification, and Characterization of Avian Pathogens*. M. Susan, ed. 6th ed. American Association of Avian Pathologists, Inc., Athens, GA.
- CLSI. 2015a. Document M07-A10. Methods for Dilution Antimicrobial Susceptibility Test for Bacteria that Grow Aerobically; Approved Standard – tenth edition. Clinical and Laboratory Standards Institute, Wayne, PA.
- CLSI. 2015b. Document M100-S25. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
- CLSI. 2015c. Performance Standards for Antimicrobial Disc and Dilution Test for Bacteria Isolated From Animals. 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA CLSI supplement VET01S.
- De Leener, E., A. Martel, A. Decostere, and F. Haesebrouck. 2004. Distribution of the erm(B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb. Drug Resist.* 10:341–345.
- Dec, M., D. Stępień-Pyśniak, S. Gnat, F. Fratini, R. Urban-Chmiel, D. Cerri, S. Winiarczyk, and B. Turchi. 2020. Antibiotic susceptibility and virulence genes in *Enterococcus* isolates from wild mammals living in Tuscany. *Italy. Microb. Drug Resist.* 26:505–519.
- Dec, M., R. Urban-Chmiel, D. Stępień-Pyśniak, and A. Wernicki. 2017. Assessment of antibiotic susceptibility in *Lactobacillus* isolates from chickens. *Gut Pathogens.* 9:54.
- Del Grosso, M., A. Caprioli, P. Chinzari, M. C. Fontana, G. Pezzotti, A. Manfrin, E. D Giannatale, E. Goffredo, and A. Pantosti. 2000. Detection and characterization of vancomycin-resistant enterococci in farm animals and raw meat products in Italy. *Microb. Drug Resist.* 6:313–318.
- Dolka, B., D. Chrobak-Chmiel, L. Makrai, and P. Szeleszczuk. 2016. Phenotypic and genotypic characterization of *Enterococcus cecorum* strains associated with infections in poultry. *BMC Vet. Res.* 12:129.
- Dolka, B., M. Gołębowska–Kosakowska, K. Krajewski, P. Kwieciński, T. Nowak, J. Szubstarski, J. Wilczyński, and P. Szeleszczuk. 2017. Occurrence of *Enterococcus* spp. in poultry in Poland based on 2014-2015 data. *Med. Weter.* 73:220–224.
- EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). 2019. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. *EFSA J.* 17:5598 278 pp.
- EMA (European Medicines Agency), 2018. Reflection paper on use of aminoglycosides in animals in the European Union: development of resistance and impact on human and animal health, (EMA/CVMP/AWP/721118/2014). Accessed Oct. 2021. https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-use-aminoglycosides-animals-european-union-development-resistance-impact-human_en.pdf.
- EMA (European Medicines Agency), 2019. European surveillance of veterinary antimicrobial consumption, 2019. Sales of veterinary antimicrobial agents in 31 European countries in 2017, (EMA/294674/2019).
- Enne, V. I., D. M. Livermore, P. Stephens, and L. M. Hall. 2001. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *Lancet.* 357:1325–1328.
- Fertner, M. E., R. H. Olsen, M. Bisgaard, and H. Christensen. 2011. Transmission and genetic diversity of *Enterococcus faecalis* among layer chickens during hatch. *Acta Vet. Scand.* 53:56.
- Gregersen, R. H., A. Petersen, H. Christensen, and M. Bisgaard. 2010. Multilocus sequence typing of *Enterococcus faecalis* isolates demonstrating different lesion types in broiler breeders. *Avian Pathol.* 39:435–440.
- Hauschild, T., P. Sacha, P. Wiczorek, M. Zalewska, K. Kaczyńska, and E. Tryniszewska. 2008. Aminoglycosides resistance in clinical isolates of *Staphylococcus aureus* from a University Hospital in Białystok, Poland. *Folia Histochem. Cytobiol.* 46:225–228.
- Hauschild, T., and S. Schwarz. 2003. Differentiation of *Staphylococcus sciuri* strains isolated from free-living rodents and insectivores. *J. Vet. Med. B.* 50:241–246.
- Hayes, J. R., L. L. English, L. E. Carr, D. D. Wagner, and S. W. Joseph. 2004. Multiple-antibiotic resistance of *Enterococcus* spp. isolated from commercial poultry production environments. *Appl. Environ. Microbiol.* 70:6005–6011.
- Hegstad, K., T. Mikalsen, T. M. Coque, G. Werner, and A. Sundsfjord. 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. *Clin. Microbiol. Infect.* 16:541–554.
- Higenyi, J. 2014. Microbial contamination load of hatching eggs in Butaleja, eastern Uganda. *Anim. Vet. Sci.* 2:22.
- Hummel, A. S., C. Hertel, W. H. Holzapfel, and C. M. Franz. 2007. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Appl. Environ. Microbiol.* 73:730–739.
- Jiménez-Belenguer, A., E. Doménech, A. Villagrà, A. Fenollar, and M. A. Ferrús. 2016. Antimicrobial resistance of *Escherichia coli* isolated in newly-hatched chickens and effect of amoxicillin treatment during their growth. *Avian Pathol.* 45:501–507.
- Jolley, K. A., and M. C. J. Maiden. 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinform.* 11:595.
- Karumarathna, R., S. Popowich, M. Wawryk, B. Chow-Lockerbie, K. A. Ahmed, C. Yu, M. Liu, K. Goonewardene, T. Gunawardana, S. Kurukulasuriya, A. Gupta, P. Willson, N. Ambrose, M. Ngeleka, and S. Gomis. 2017. Increased incidence of enterococcal infection in nonviable broiler chicken embryos in western canadian hatcheries as detected by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *Avian Dis.* 61:472–480.
- Kawalec, M., Z. Pietras, E. Daniłowicz, A. Jakubczak, M. Gniadkowski, W. Hryniewicz, and R. J. L. Willems. 2007. Clonal structure of *Enterococcus faecalis* isolated from Polish hospitals: characterization of epidemic clones. *J. Clin. Microbiol.* 45:147–153.
- Khan, K. A., S. A. Khan, A. Aslam, M. Rabbani, and M. Y. Tipu. 2004. Factors contributing to yolk retention in poultry: a review. *Pak. Vet. J.* 24:46–51.
- Khan, Z. A., M. F. Siddiqui, and S. Park. 2019. Current and emerging methods of antibiotic susceptibility testing. *Diagnostics (Basel).* 9:49.
- Kim, Y. B., K. W. Seo, H. Y. Jeon, S. K. Lim, H. W. Sung, and Y. J. Lee. 2019. Molecular characterization of erythromycin and tetracycline-resistant *Enterococcus faecalis* isolated from retail chicken meats. *Poult. Sci.* 98:977–983.
- Kobayashi, N., M. Alam, Y. Nishimoto, S. Urasawa, N. Uehara, and N. Watanabe. 2001. Distribution of aminoglycoside resistance genes in recent clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus avium*. *Epidemiol. Infect.* 126:197–204.
- Kuch, A., R. J. Willems, G. Werner, T. M. Coque, A. M. Hammerum, A. Sundsfjord, I. Klare, P. Ruiz-Garbajosa, G. S. Simonsen, M. van Luit-Asbroek, W. Hryniewicz, and E. Sadowy. 2012. Insight into antimicrobial susceptibility and population structure of contemporary human *Enterococcus faecalis* isolates from Europe. *J. Antimicrob. Chemother.* 67:551–558.
- Landman, W., K. Veldman, G. Mevius, and P. Doornenbal. 2000. Contamination of Marek's disease vaccine suspensions with *Enterococcus faecalis* and its possible role in amyloid arthropathy. *Avian Pathol.* 29:21–25.
- Maasjost, J., K. Mühlendorfer, S. Cortez de Jäckel, and H. M. Hafez. 2015. Antimicrobial susceptibility patterns of *Enterococcus faecalis* and *Enterococcus faecium* isolated from poultry flocks in Germany. *Avian Dis.* 59:143–148.
- Malhotra-Kumar, S., C. Lammens, J. Piessens, and H. Goossens. 2005. Multiplex PCR for simultaneous detection of

- macrolide and tetracycline resistance determinants in streptococci. *Antimicrob. Agents Chemother.* 49:4798–4800.
- Moreno, M. A., S. García-Soto, M. Hernández, C. Bárcena, D. Rodríguez-Lázaro, M. Ugarte-Ruiz, and L. Domínguez. 2019. Day-old chicks are a source of antimicrobial resistant bacteria for laying hen farms. *Vet. Microbiol.* 230:221–227.
- Nowakiewicz, A., P. Zięba, S. Gnat, A. Trościańczyk, M. Osińska, D. Łagowski, U. Kosior-Korzecka, and I. Puzio. 2020. A significant number of multi-drug resistant *Enterococcus faecalis* in wildlife animals; long-term consequences and new or known reservoirs of resistance? *Sci. Total Environ.* 705:135830.
- Nowakiewicz, A., G. Ziółkowska, P. Zięba, S. Gnat, A. Trościańczyk, and Ł. Adaszek. 2017. Characterization of multidrug resistant *E. faecalis* strains from pigs of local origin by ADSRRS-fingerprinting and MALDI-TOF MS; evaluation of the compatibility of methods employed for multidrug resistance analysis. *PLoS One.* 12: e0171160.
- Olsen, R. H., H. Christensen, and M. Bisgaard. 2012a. Transmission and genetic diversity of *Enterococcus faecalis* during hatch of broiler chicks. *Vet. Microbiol.* 160:214–221.
- Olsen, R. H., C. Frantzen, H. Christensen, and M. Bisgaard. 2012b. An investigation on first-week mortality in layers. *Avian Dis.* 56:51–57.
- Olsen, R. H., H. C. Schönheyde, H. Christensen, and M. Bisgaard. 2012c. *Enterococcus faecalis* of human and poultry origin share virulence genes supporting the zoonotic potential of *E. faecalis*. *Zoonoses Public Health.* 59:256–263.
- Poulsen, L. L., M. Bisgaard, N. T. Son, N. V. Trung, H. M. An, and A. Dalsgaard. 2012. *Enterococcus faecalis* clones in poultry and in humans with urinary tract infections. *Vietnam Emerg. Infect. Dis.* 18:1096–1100.
- PubMLST, 2019. Public databases for molecular typing and microbial genome diversity. *Enterococcus faecalis* MLST databases. Accessed Feb. 2019. <https://pubmlst.org/efaecalis/>.
- Pyzik, E., A. Marek, D. Stępień-Pyśniak, R. Urban-Chmiel, Ł. Jarosz, and I. Jagiełło-Podebska. 2019. Detection of antibiotic resistance and classical enterotoxin genes in coagulase-negative staphylococci isolated from poultry in Poland. *J. Vet. Res.* 63:183–190.
- Rehman, M. A., X. Yin, R. Zaheer, N. Goji, K. K. Amoako, T. McAllister, J. Pritchard, E. Topp, and M. S. Diarra. 2018. Genotypes and phenotypes of enterococci isolated from broiler chickens. *Front. Sustain. Food Syst.* 2:83.
- Ruiz-Garbajosa, P., M. J. Bonten, D. A. Robinson, J. Top, S. R. Nallapareddy, C. Torres, T. M. Coque, R. Cantón, F. Baquero, B. E. Murray, R. del Campo, and R. J. L. Willems. 2006. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. *J. Clin. Microbiol.* 44:2220–2228.
- Sharma, P., S. K. Gupta, J. B. Barrett, L. M. Hiott, T. A. Woodley, S. Kariyawasam, J. G. Frye, and C. R. Jackson. 2020. Comparison of antimicrobial resistance and pan-genome of clinical and non-clinical *Enterococcus cecorum* from poultry using whole-genome sequencing. *Foods.* 9:686.
- Stępień-Pyśniak, D., T. Hauschild, M. Dec, A. Marek, and R. Urban-Chmiel. 2019. Clonal structure and antibiotic resistance of *Enterococcus* spp. from wild birds in Poland. *Microb. Drug Resist.* 25:1227–1237.
- Stępień-Pyśniak, D., T. Hauschild, M. Dec, A. Marek, R. Urban-Chmiel, and U. Kosikowska. 2021. Phenotypic and genotypic characterization of *Enterococcus* spp. from yolk sac infections in broiler chicks with a focus on virulence factors. *Poult. Sci.* 100:100985.
- Stępień-Pyśniak, D., T. Hauschild, A. Nowaczek, A. Marek, and M. Dec. 2018. Wild birds as a potential source of known and novel multilocus sequence types of antibiotic-resistant *Enterococcus faecalis*. *J. Wildl. Dis.* 54:219–228.
- Stępień-Pyśniak, D., A. Marek, T. Banach, Ł. Adaszek, E. Pyzik, J. Wilczyński, and S. Winiarczyk. 2016. Prevalence and antibiotic resistance of *Enterococcus* strains isolated from poultry. *Acta Vet. Hung.* 64:148–163.
- Templer, S. P., P. Rohner, and A. Baumgartner. 2008. Relation of *Enterococcus faecalis* and *Enterococcus faecium* isolates from foods and clinical specimens. *J. Food Protect.* 71:2100–2104.
- Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33:2233–2239.
- Tona, K., V. Bruggeman, O. Onagbesan, F. Bamelis, M. Gbeassor, K. Mertens, and E. Decuyper. 2005. Day-old chick quality: relationship to hatching egg quality, adequate incubation practice and prediction of broiler performance. *Avian Poult. Biol. Rev.* 16:109–119.
- Tremblay, C. L., A. Letellier, S. Quessy, M. Boulianne, D. Daignault, and M. Archambault. 2011. Multiple-antibiotic resistance of *Enterococcus faecalis* and *Enterococcus faecium* from cecal contents in broiler chicken and turkey flocks slaughtered in Canada and plasmid colocalization of *tetO* and *ermB* genes. *J. Food Protect.* 74:1639–1648.
- Turner, A. M., J. Y. H. Lee, C. L. Gorrie, B. P. Howden, and G. P. Carter. 2021. Genomic insights into last-line antimicrobial resistance in multidrug-resistant *Staphylococcus* and vancomycin-resistant *Enterococcus*. *Front. Microbiol.* 12:637656.
- Vakulenko, S. B., S. M. Donabedian, A. M. Voskresenskiy, M. J. Zervos, S. A. Lerner, and J. W. Chow. 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrob. Agents Chemother.* 47:1423–1426.
- van Hoek, A. H., D. Mevius, B. Guerra, P. Mullany, A. P. Roberts, and H. J. Aarts. 2011. Acquired antibiotic resistance genes: an overview. *Front. Microbiol.* 2:203.
- Woźniak-Biel, A., G. Bugła-Płoskońska, J. Burdzy, K. Korzekwa, S. Ploch, and A. Wieliczko. 2019. Antimicrobial resistance and biofilm formation in *Enterococcus* spp. isolated from humans and turkeys in Poland. *Microb. Drug Resist.* 25:277–286.
- Yang, Y., A. J. Ashworth, C. Willett, K. Cook, A. Upadhyay, P. R. Owens, S. C. Ricke, J. M. DeBruyn, and P. A. Moore Jr.. 2019. Review of antibiotic resistance, ecology, dissemination, and mitigation in U.S. broiler poultry systems. *Front. Microbiol.* 10:2639.
- Yassin, H., A. G. Velthuis, M. Boerjan, and J. van Riel. 2009. Field study on broilers' first-week mortality. *Poult. Sci.* 88:798–804.