# Susceptibility of bacteria of the *Enterococcus* genus isolated on Lithuanian poultry farms

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**ABSTRACT**: The aim of this study was to test and analyse the antimicrobial susceptibility of *Enterococcus* isolates from Lithuanian poultry farms. Investigations were carried out during the years 2008–2009. The sampling sites, located all over the country, included eight poultry farms of large capacity. All samples were collected from broilers. *Enterococcus* spp. were isolated from intestines immediately after slaughtering. A total of 160 samples were collected, 20 samples from each farm. The MICs (Minimum Inhibitory Concentrations) of eleven antimicrobial agents were determined for each of the isolates using the broth microdilution method with specific microtitre plate panels (Trek Diagnostic Systems, Inc.). Susceptibility according to clinical breakpoints of chloramphenicol, linezolid, erythromycin, penicillin, quinupristin/dalfopristin, tetracycline, vancomycin, ciprofloxacin and nitrofurantoin was evaluated. One hundred and forty seven samples (92%) from a total of 160 tested samples were positive for *Enterococcus* spp., however, only 74 strains were selected as non-duplicate isolates. The most predominant species were identified as *E. faecium* (38%), *E. faecalis* (17.5%), *E. gallinarum* (12%) and *E. casseliflavus* (12%). The most frequent resistance properties were resistances to tetracycline (75.6%), erythromycin (56.8%) and ciprofloxacin (41.9%). No strains resistant to vancomycin and linezolid were found. High percentages of susceptibility to chloramphenicol (82.4%) and penicillin (71.6%) were also observed. A high MIC of tigecycline (≥ 1 mg/l) to 12.2% of enterococci was determined during this study. 44.6% of tested strains had a high MIC (≥ 64 mg/l) to tylosin. There was no significant correlation found between resistances of different species to different antimicrobial agents *in vitro.*

**Keywords**: antibiotics; antimicrobial agents; clinical breakpoints; resistance; food safety

*Enterococcus* species are ubiquitous, commensal inhabitants of the gastrointestinal tract of mammals, birds, insects, and reptiles. These organisms are particularly challenging to eliminate because of their ability to adapt to environmental stresses (Hayes et al., 2003). They are frequently isolated from environmental sources such as soil, surface waters, and raw plant and animal products, where their intrinsic ruggedness allows them to persist and spread in the environment (Johnston and Jaykus, 2004).

During the last decades, enterococci have emerged as important nosocomial pathogens. Their role in such infections has increased due to their ability to acquire resistance to various antimicrobial agents, which renders them difficult to treat (Linden and Miller, 1999; Iversen et al., 2002; Kolar et al., 2008). This has resulted in an increased interest in identifying reservoirs of both antimicrobial-resistant strains of enterococci and genes coding for resistance properties (Rice et al., 1995; Kolar et al., 2000; Manson et al., 2004; Olson et al., 2006).

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Enterococci are commonly resistant to macrolides, cephalosporins and tetracycline and often exhibit high-level resistance to gentamicin, precluding synergy with β-lactams or glycopeptides which are important in human medicine (Lukasova and Sustackova, 2003; Brown et al., 2008). Some studies provide evidence for dissemination of resistant enterococci from animals to man and, probably more importantly, the exchange of resistance genes between poultry and human enterococci (van den Bogaard et al., 2002). Multidrug resistance is common among enterococci and presents a formidable treatment problem (Donabedian et al., 2003). Food animals are implicated as reservoirs for antibiotic-resistant enterococci, following the use of antimicrobial growth promoters and prophylactics (Bates et al., 1993; Waites et al., 2006).

Poultry production has an intensive manufacturing cycle and thus can hardly dispense with antimicrobial agents. This leads to increased antimicrobial resistance of pathogenic and commensal microbiota. Resistant strains of enterococci could also originate from humans and some studies have shown that isolates in poultry originated from humans with a history of poultry exposure (Borgen et al., 2002). Carriage of resistant enterococci in healthy poultry slaughterers and farmers has been described (Borgen et al., 2000), and transmission of resistant strains is likely to occur between poultry and humans in these settings (Borgen et al., 2002). The overall resistance in broilers was correlated with the resistance in broiler farmers and in poultry slaughterers (van den Bogaard et al., 2002).

The antimicrobial resistance of *Enterococcus* varies depending on the geographical site, national and local antimicrobial usage politics, and usage intensity. Uncontrolled usage of antimicrobial agents is recognized as the most important factor that favours the development and spread of resistant microorganisms (Moreno et al., 2000; Acar and Rostel, 2001; Burch, 2005).

The aim of this study was to analyse the level of antimicrobial susceptibility of *Enterococcus* spp. from Lithuanian poultry farms.

## MATERIAL AND METHODS

### Clinical material and isolates

Investigations were carried out during the years 2008–2009. The sampling sites, located all over the country, included eight poultry farms of large capacity. Samples taken with cotton swabs (Transwab, UK) were collected from the intestines immediately after slaughtering. A total of 160 samples were collected – 20 samples from each farm. All samples were collected from broilers. Slanetz-Bartley Agar+TTC, Aesculine Bile Agar and Pfizer Selective Enterococcus Agar (Liofilchem, Italy) were used for inoculation of clinical material. Media were incubated for 48 hours at +35°C. Isolates were characterized as enterococci based on growth and morphological characteristics, Gram staining, catalase production, tolerance to 6.5% NaCl and growth at 45°C, the production of pyrrolidonyl arylamidase, and hydrolysis of esculin in the presence of bile. Control strains included American Type Culture Collection strains *E. faecalis* ATCC 29212, *E. durans* ATCC 49479, *E. avium* ATCC 14025 and *E. hirae* ATCC 10541. Species identification was performed using the RapID STR identification system (Remel, USA). Results were interpreted using a PC and ERIC software (Remel).

### Susceptibility testing

The MICs (Minimum Inhibitory Concentrations) of eleven antimicrobial agents were determined for each of the isolates using the broth microdilution method based on the recommendations in the document M07-A8, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically of the Clinical and Laboratory Standards Institute (2009-1), with custom-made microtitre plates (Trek Diagnostic Systems, Inc., Westlake, Ohio). The antimicrobial agents and test ranges (2-fold dilution series) were as follows: tigecycline, 0.015 to 0.5 μg/ml; chloramphenicol, 2 to 32 μg/ml; erythromycin, 0.5 to 8 μg/ml; penicillin, 0.5 to 16 μg/ml; quinupristin/dalfopristin, 1 to 32 μg/ml; tetracycline, 4 to 32 μg/ml; vancomycin, 0.5 to 32 μg/ml; tylosin tartrate 0.5 to 32 μg/ml; ciprofloxacin, 0.12 to 4 μg/ml; linezolid, 0.5 to 8 μg/ml; and nitrofurantoin, 2 to 32 μg/ml. Fifty microlitres of a culture suspension in Mueller-Hinton broth containing approximately $5 \times 10^8$ CFU of each isolate/L was inoculated into microtitre plates containing the test antimicrobial agents and incubated at 37°C for 18 h ± 1 h in ambient air. *E. faecalis* strain ATCC 29212 was used as a quality control. The plates were removed and read manually for growth to score the MIC determinations ac-

cording to the breakpoints described in the CLSI document M100-S19, Performance Standards for Antimicrobial Susceptibility Testing (2009-2), except for tigecycline and tylosin tartrate, as there are no CLSI *Enterococcus* interpretive criteria available for these antibiotics. Strains of the same species from the same farm with similar antibiograms, were considered to be duplicate isolates and only a single isolate was subjected to further evaluation.

Statistical analysis was performed using the statistical package Instat (GraphPad Sofware).

## RESULTS

One hundred and forty seven samples (92%) from a total of 160 tested were positive for *Enterococcus* spp. In most cases various types of colonies grew on Slanetz-Barley Agar. However, only a single colony from each sample was taken for further investigations. Finally, 74 strains of *Enterococcus* were selected as non-repeatable isolates from the poultry farms. A wide variety of *Enterococcus* species were identified among the selected isolates. The most predominant species were *E. faecium* (38%), *E. faecalis* (17.5%), *E. gallinarum* (12%) and *E. casseliflavus* (12%). Nine of the strains remained unidentified because of weak or ambiguous biochemical reactions.

The *in-vitro* susceptibility of the isolated strains varied according to the antimicrobial agents tested (Table 1). The most frequent resistances were to tetracycline (75.6%), erythromycin (56.8%) and ciprofloxacin (41.9%). For certain antimicrobial agents a law percentage of susceptible, but a considerable percentage of strains classified as intermediate was detected. For example, only 16.2% of all enterococci were susceptible to ciprofloxacin, because of the high numbers of intermediate isolates. No strains resistant to vancomycin and linezolid were found. A single isolate were classified as intermediate to vancomycin and linezolid, whereas the remaining 73 isolates were susceptible to these antibiotics. High percentages of susceptible strains were recorded for chloramphenicol (82.4%) and penicillin (71.6%).

## DISCUSSION

The past few years have witnessed increasing interest in enterococci. These bacteria are found in the intestine of nearly all animals, from cockroaches to humans. The predominant species inhabiting the intestine varies. In humans and some animals *E. faecalis* predominates in some instances and *E. faecium* in others (Rice et al., 1995). However, there is a wider range of *Enterococcus* species commonly found in poultry. *E. avium*, *E. casseliflavus*, *E. cecorum*, *E. durans*, *E. gallinarum*, *E. hirae*, *E. malodoratus* and other species have been isolated from poultry (Debnam et al.,

Table 1. Minimum inhibitory concentrations distribution (%) among *Enterococcus* spp. strains, isolated from poultry ($n$ = 74)

| Antimicrobial agents | MIC distribution (%) (mg/l) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.03 | 0.06 | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |
| Tigecycline | 0 | 9.5 | 25.7 | 40.5 | 12.2 | 12.2 | | | | | | | |
| Chloramphenicol | | | | | | | 0 | 16.2 | 66.2 | 12.2 | 2.7 | 2.7 | |
| Erythromycin | | | | | 23.0 | 8.1 | 10.8 | 1.4 | 9.5 | 47.3 | | | |
| Penicillin | | | | | 9.5 | 5.4 | 16.2 | 28.4 | 12.2 | 16.2 | 12.2 | | |
| Quinu/dalfo* | | | | | | 31.1 | 29.5 | 16.4 | 23.0 | 0 | 0 | 0 | |
| Tetracycline | | | | | | | | 24.3 | 0 | 0 | 6.8 | 68.9 | |
| Vancomycin | | | | | 33.8 | 32.4 | 31.1 | 1.4 | 1.4 | 0 | 0 | 0 | |
| Tylosin | | | | 2.7 | 0 | 9.5 | 24.3 | 9.5 | 5.4 | 1.4 | 2.7 | 44.6 | |
| Ciprofloxacin | | | 0 | 0 | 1.4 | 14.9 | 41.9 | 28.4 | 13.5 | | | | |
| Linezolid | | | | | 0 | 18.9 | 79.7 | 1.4 | 0 | 0 | | | |
| Nitrofurantoin | | | | | | | 0 | 0 | 6.8 | 9.5 | 13.5 | 54.1 | 16.2 |

yellow cells = susceptible; orange cells = intermediate susceptible; purple cells – resistant
*$n$ = 61: *E. faecalis* was exclued from testing as intrinsically resistant to quinupristin/dalfopristin

2005). Despite the wide species variety in poultry, *E. faecalis* and *E. faecium* still predominate (Hayes et al., 2003; Devriese et al., 2008). Bacteria of this genus, which represent the leading causes of nosocomial bacteremia, surgical wound infection, and urinary tract infection, are becoming resistant to many and sometimes all standard therapies (Jarvis et al., 1996; Huycke et al., 1998). Enterococci often acquire antibiotic resistance through exchange of resistance-encoding genes carried on conjugative transposons, pheromone-responsive plasmids, and other broad-host-range plasmids (Rice et al., 1995). Antimicrobial resistance in enterococci is of two types: inherent/intrinsic resistance and acquired resistance. Intrinsic resistance is species characteristics and thus present in all members of a species and is chromosomally mediated. Enterococci exhibit intrinsic resistance to penicillinase susceptible penicillin (low level), penicillinase resistant penicillins, cephalosporins, lincosamides, nalidixic acid, low levels of aminoglycoside and low levels of clindamycin (Marothi et al., 2005). New antimicrobial agents such as linezolid and tigecycline were developed are generally clinically effective against enterococci. However, instances of susceptibility to these antimicrobial agents have been described in the past few years (Kainer et al., 2007; Werner et al., 2008). Our data on avian isolates showed decreased susceptibility of enterococci to almost all tested antimicrobial agents. Only linezolid and vancomycin were highly effective against enterococci *in vitro*. We found that the most frequent or high-level resistance was associated with the most intensively used antimicrobial agents in the last decade in Lithuania. Such antimicrobial agents include tetracycline, tylosin and fluoroquonolones. After the patent protection of Baytril® was terminated, enrofloxacin was introduced into poultry farms on a large scale. The lack of a strategy to control antimicrobial usage in the country led to uncontrolled usage of such important antimicrobial agents such as fluoroquinolones or cephalosporins. This could be one of the reasons for such frequent antimicrobial resistance to fluoroquinolones in Lithuania. Only 16.2% of tested enterococci were susceptible to ciprofloxacin. Our previous studies on susceptibility of *E. coli* and *Salmonella enterica* to fluoroquinolones proved the relationship between high resistance and intensive usage of certain antimicrobial agents (Ruzauskas et al., 2007). Unexpectedly high MIC's to the novel antimicrobial agent tigecycline has been determined since the publication of this report. Tigecycline is a member of the new group of glycylcyclines and is a promising new antibiotic of last resort, active against many bacteria including *Enterococcus* spp. (Olson et al., 2006; Waites et al., 2006; Werner et al., 2008). However, 12.2% of enterococci demonstrated high MIC values (≥ 1 mg/l). The disc diffusion method was used for additional testing of susceptibility to this antibiotic and the obtained results correlated with results obtained using the microdilution method. Resistance to tigecycline is uncommon among enterococci and only a few described cases of enterococcal resistance to tigecycline have been described in recent years. For example, Werner et al. (2008) reported resistance of enterococci to tigecycline in German hospitals in 2007 for the first time.

Analysis of MIC ranges demonstrated high resistance to those antimicrobial agents that had been used most in the country. For example, 68.9% of enterococci were resistant to tetracycline and 44.6% to tylosin at a high concentration – 64 mg/l or more.

Analysis on susceptibility of different species of enterococci to separate antimicrobial agents during this study was also performed. No significant correlation between resistance of different species and different antimicrobial agents was determined *in vitro*. The only significant frequency of resistance was determined with *E. faecalis* to a standard breakpoint of quinupristin/dalfopristin. All isolates of *E. faecalis* demonstrated resistance to this combination of antimicrobial agents. However, this species of enterococci have natural species resistance to quinupristin-dalfopristin (Singh et al., 2002; Phillips et al., 2004) and that property was confirmed in our study. There is no clinical breakpoint to quinupristin/dalfopristin established for *E. faecalis*. By this reason this species (with regard to susceptibility to quinupristin/dalfopristin) was excluded from the results that are presented in Table 1. In any case, the results showed that 31.1% of enterococci other than *E. faecalis* were resistant to quinupristin/dalfopristin – the combination that is widely used for clinical treatment of humans (Lamb et al., 1999). This combination is not used in veterinary practice, but other streptogramins such as virginiamycin had been used for animal treatment or prophylaxis for a long time (McDonald et al., 2001). Resistance of different *Enterococcus* species isolated from animal products to quinupristin/dalfopristin is widely described by other authors (Soltani et al., 2000; Peters et al., 2003).

The obtained results on the susceptibility of *Enterococcus* spp. of animal origin were interpreted

using CLSI-approved clinical breakpoints intended for human treatment. Thus, the results should not be used to predict therapeutic outcome when used to treat infections in animals. However, the results indicate a potential risk both to humans and animals: a high number of enterococci had decreased susceptibility to different antimicrobial agents. This could be potentially hazardous to humans and animals, e.g., in the case of meat consumption after inappropriate food processing (Moreno et al., 2000; Hayes et al., 2004; Koluman et al., 2009).

Special attention must be paid to resistance to antibiotics such as tigecycline and quinupristin/dalfopistin that are used exclusively in human medicine and appropriate measures must be taken to control the spread of resistant enterococci to humans.

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