*Polish Journal of Veterinary Sciences Vol. 15, No. 3 (2012), 431-438*

DOI 10.2478/v10181-012-0067-6

*Original article*

# Antibiotic resistance genes and virulence factors in *Enterococcus faecium* and *Enterococcus faecalis* from diseased farm animals: pigs, cattle and poultry

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## Abstract

Eighty enterococcal isolates (*E. faecium*, n=38, *E. faecalis*, n=42) from diseased farm animals (swine, cattle, poultry) in Lithuania have been studied for the prevalence of antibiotic resistance and for resistance and virulence genetic determinants. 86% of *E. faecium* and 71% of *E. faecalis* isolates were multidrug resistant (resistant to three or more unrelated antibiotics). Resistance to aminoglycosides, tetracycline and erythromycin was found most frequently in both species (61%, 69%) and was linked to *aph*(3')-IIIa, *aac*(6')-Ie-*aph*(2")-Ia, *ant*(6)-Ia (aminoglycoside resistance), *tetM*, *tetL* (tetracycline resistance), *ermA*, *ermB* (erythromycin resistance) gene combinations, which were supplemented with chloramphenicol resistance genes *catA7*, *catA8* (*E. faecalis*) and *catA9* (*E. faecium*). All *E. faecalis* isolates harboured genes coding for virulence factors *agg*, *esp*, *fsr gelE* alone or in combinations with the high prevalence of *esp* gene in isolates from cattle (63%) and pigs (79%). The origin-dependent incidence of *agg* gene variants *prgB* and *asp1* was observed. The results indicate the existence of a large pool of potentially virulent and multidrug resistant *E. faecalis* in diseased farm animals posing risk to humans.

**Key words**: *Enterococcus faecium, Enterococcus faecalis,* antibiotic resistance, virulence genes

## Introduction

The importance of multidrug-resistant enterococci as infection agents in animals has not reached the level of human infection and it is still a matter of debate whether enterococci are part of the natural intestinal flora of most mammals and birds. Nevertheless, enterococci have been implicated in a number of infections in animals, mainly mastitis in cattle, diarrhea in swine and cattle, as well as endocarditis and septicaemia in poultry (Ok et al. 2009, Bisgaard et al. 2010, Nam et al. 2010). Animal (mouse, rat, rabbit) models have been used for enterococci urinary tract infection and endocarditis studies (Singh et al. 2009, Thurlow et al. 2010). Several such studies demonstrated a synergistic virulent interaction between *Enterococcus faecalis* and other bacteria (Montravers et al. 1997, Lavigne et al. 2008).

The mechanisms of antibiotic resistance in enterococci is widely studied, however, pathogenesis is

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poorly understood even in human infection (Berti et al. 1998, Roberts et al. 2004). Several factors important for virulence have been characterized. Among them, pheromone-inducible multifunctional aggregation substance AS (*asa1*, *prgB*, *asp1* gene variants and *asc10* on pathogenicity island), extracellular surface protein Esp (encoded by *esp* gene on pathogenicity island), a group of hydrolitic enzymes including hyaluronidase and gelatinase (encoded by the plasmidic $hyl_{Efm}$ gene and chromosomal *gelE* gene), secreted virulence factor such as cytolisin (encoded by $cylL_L$ $cylL_s$ genes on pathogenicity island or on the plasmid) and *fsr* locus, which regulates other genes important for virulence (Kreft et al. 1992, Rice et al. 2003, Thurlow et al. 2010). The antibiotic resistance and virulence genetic determinants of enterococci isolated from food-producing and wild animals have been studied, however, those isolated from diseased animals are little understood (Poeta et al. 2005, Diarra et al. 2010, Han et al. 2011). The aim of this work was to characterize the *Enterococcus* isolates obtained from diseased farm animals (cattle, swine and poultry) in Lithuania. Antibiotic susceptibility testing and PCR methodology was applied to study the prevalence of genes coding for resistance and virulence determinants.

## Materials and Methods

### Bacterial isolates

Specimens from 16 animal farms (ten of swine, four of cattle and two of poultry) located in 7 counties (Kaunas, Vilnius, Klaipėda, Tauragė, Alytus, Panevėžys and Utena) of Lithuania were collected during period 2005-2008 and analyzed in this study. Animals of different species were geographically separated with no animal transfer between farms. Diarrhoea was the main manifestation of the disease in all animal species. Rectal and cloacal swabs were taken using cotton swabs with transport media (Transwab, UK). No antimicrobial drugs were used for animal treatment at least for 2 weeks before taking the samples. Slanetz-Bartley Agar+TTC, Aesculine Bile Agar and Pfizer Selective *Enterococcus* Agar (Liofilchem, Italy) were used for the isolation and presumptive identification of enterococci. The media were incubated for 48 hours at 35°C. Final identification was performed using RapID STR identification system (Remel, USA). Results were interpreted using ERIC software (Remel, USA). Only one randomly selected isolate per diseased animal from one herd or flock was used for further testing.

### Susceptibility testing

Antibiotic susceptibility testing was performed by the disk diffusion (Kirby-Bauer) test according to the CLSI guidelines (CLSI 2006). Disks were obtained from Oxoid (UK). The isolates were interpreted as susceptible or resistant according to the clinical breakpoints using CLSI recommendations. High-level resistance was considered for aminoglycosides (see antibiotic concentrations). Intermediate susceptible strains were considered as resistant. The antimicrobials used for the susceptibility testing of *Enterococcus* spp. isolates were as follows (μg/disk): kanamycin (30 μg), gentamicin (120 μg), streptomycin (300 μg), penicillin (10 U), ciprofloxacin (5 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), vancomycin (30 μg), nitrofurantoin (300 μg). *E. faecalis* ATCC29212 was used as the reference strain.

### Detection of antibiotic resistance and virulence genes

Bacterial colonies were suspended in 100 μl of sterile water, boiled, centrifuged briefly and the supernatants were used as DNA templates in PCR. The following genes were tested: *tetL*, *tetM*, *tetK*, *tetO*, *tetS* (tetracycline resistance, Ng et al. 2001), *aph*(3')IIIa, *ant*(6)Ia, *aac*(6')-*ap*h(2")-Ia, *aph*(2')Ib, *aph*(2')Ic, *aph*(2')Id, *ant*(4')Ia (aminoglycoside resistance Vakulenko et al. 2003), *catA7*, *catA8*, *catA9* (chloramphenicol resistance), *ermA*, *ermB*, *ermC*, *ermTR*, *mefAB*, *msrAB* (macrolide resistance) and *cylL*, *gelE*, *fsr, esp* (*E. faecium* and *E. faecalis*), $hyl_{Efm}$ (virulence factors). The primers used for this study are listed in Table 1. Multiplex PCR was carried out in a Thermocycler (Eppendorf) for 30 cycles with denaturation at 94°C 30 s, annealing at 58°C 1 min and polymerization at 72°C 1 min. The PCR reaction mix (25 μl) and the primers concentrations were as recommended by the *Taq* polymerase supplier (AB Fermentas). The amplification products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining to assess the sizes of amplicons. To amplify *agg* gene variants *asa1*, *prgB, asp1* and *asc10,* primers were designed according to the published sequences. The gene variant of *agg* was determined by DNA sequencing.

### Statistical methods

Differences in the prevalence of antibiotic resistance between different groups were assessed using Chi-square or Fisher exact test. A P-value of $< 0.05$

Table 1. Primers used in this study.

| Gene | Multiplex group | Primer sequence 5' – 3' (forward/reverse) | Amplicon length (bp) |
|---|---|---|---|
| *catA7* | I | CCAGCAAACTACGTATAGCAT/CGGTATGGTGTTTTCAGGTAT | 355 |
| *catA8* | I | GGATATGAACTGTATCCTGCT/AATGAAACATGGTAACCATCAC | 461 |
| *catA9* | I | ATGGTTCGGGGAAATTGTTTC/AAGCCAGTCATTAGGCCTATC | 278 |
| *ermA* | II | GAAGCGGTAAACCCCTCTG/ACCCAAAGCTCGTTGCAGAT | 216 |
| *ermB* | II | ATTGGAACAGGTAAAGGGCAT/ATCTGGAACATCTGTGGTATG | 447 |
| *ermC* | II | GAAATCGGCTCAGGAAAAGG/AGCAAACCCGTATTCCACGA | 293 |
| *ermTR* | III | AATTGGGTCAGGAAAAGGACA/TCAGTAACATTCGCATGCTTC | 561 |
| *mefAB* | III | AGTATCATTAATCACTAGTGCC/GTTCTTCTGGTACTAAAAGTGG | 347 |
| *msrAB* | III | CAAATGGTGTAGGTAAGACAAC/TGATGTAAACAAAATGATTCCAG | 395 |
| *agg* | IV | CACGTAATTCTTGCCCACCA/CAAGCATTATTGGCAGCGTT | 520 |
| *hyl* | IV | CAGAAGAGCTGCAGGAAATG/GACTGACGTCCAAGTTTCCA | 276 |
| *cylL* | IV | GTGTTGAGGAAATGGAAGCGAT/GGCTAGTTTCACTAGCCCTCT | 424 |
| *gelE* | V | AGGGATGCCCATCTTGTCAGT/CGTTCCTTTATACCGTGCTCGA | 391 |
| *fsr* | V | CGCCAGAGATTTCACCTGACT/ATGACGAAACATCGCTAGCTCT | 218 |
| *esp* | V | AGCGGGAACAGGTCACAAAGC/ CCACGTCGAAAGTTCGATTTCC | 419 |

Table 2. Antibiotic resistance of *E. faecium* and *E. faecalis* isolates from different origin.

| Antibiotic | No of resistant isolates (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Poultry[a] | Pigs | | Cattle | | Total | |
| | *E. faecium* (N=17) | *E. faecium* (N=9) | *E. faecalis* (N=14) | *E. faecium* (N=12) | *E. faecalis* (N=27) | *E. faecium* (N=38) | *E. faecalis* (N=42) |
| KAN | 8 (47) | 6 (67) | 13 (93) | 12 (100) | 21 (78) | 26 (68) | 34 (81) |
| GEN | 4 (24) | 2 (22) | 9 (64) | 1 (8) | 8 (30) | 7 (18) | 17 (40) |
| STR | 4 (24) | 7 (78) | 12 (86) | 12 (100) | 23 (85) | 23 (60) | 35 (83) |
| ERY | 11 (65) | 8 (89) | 12 (86) | 12 (100) | 19 (70) | 31 (82) | 31 (77) |
| TET | 12 (71) | 7 (78) | 12 (86) | 12 (100) | 24 (89) | 31 (82) | 36 (86) |
| PEN | 10 (59) | 4 (44) | – | 10 (83) | 1 (4) | 24 (63) | 1 (2) |
| NIT | 11 (65) | 6 (67) | – | 1(8) | – | 18 (47) | – |
| CHL | 2 (12) | – | 2 (14) | 2 (17) | 12 (44) | 4 (11) | 14 (33) |
| CIP | 7 (41) | – | 1 (7) | 8 (67) | 11 (41) | 15 (39) | 12 (29) |
| VAN | – | – | – | – | – | – | – |

[a] – single *E. faecalis* isolate recovered from poultry was susceptible to all antibiotics tested.

was considered to be statistically significant. Statistical analyses were performed using GraphPad Prism (GraphPad software, San Diego, USA).

## Results

### Antibiotics resistance

A total of 80 *Enterococcus* isolates were recovered from diseased animals. Twelve *E. faecium* and 27 *E. faecalis* isolates were recovered from cattle, 9 *E. faecium* and 14 *E. faecalis* isolates from swine and 17 *E. faecium* and one *E. faecalis* isolate from poultry. Other species of enterococci (*E. hirae*/*durans*) were found in low numbers and were not included in this study. Five isolates (6%, of them 3% of *E. faecalis* and 10% of *E. faecium*) were susceptible to all antibiotics tested. Table 2 shows the frequency of antibiotic-resistant enterococcal isolates regarding their species and origin. *E. faecium* and *E. faecalis* isolates from diseased animals were more frequently resistant to erythromycin (82% and 77%, respectively), tetracycline (82% and 86%), kanamycin (68% and 81%), streptomycin (60% and 83%) and less frequently resistant to ciprofloxacin (39% and 29%). Chloramphenicol and gentamicin-resistance was more frequent among isolates of *E. faecalis*, as compared to *E. faecium* (33% versus 11% and 40% versus 18%, $P<0.05$), whereas incidence of penicillin-resistant *E. faecium* was as higher compared to *E. faecalis* (63% versus 2%, $P<0.05$). Significant part of *E. faecalis* and *E. faecium*

Table 3. Prevalence of antibiotic resistance genes in *E. faecalis* and *E. faecium* isolates.

| No of isolates | Resistance phenotype[a] | No of isolates carrying resistance genes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *aph*(3')-IIIa | *aac-aph* | *ant*(6)-Ia | *erm* | | *tet* | | *cat* | | | |
| | | | | | A | B | M | L | A7 | A8 | A9 | |
| | | *E. faecalis*[b] (42) | | | | | | | | | | |
| 1 | KT | | | | | | 1 | | | | | |
| 4 | KTS | 4 | | 4 | | | 3 | 3 | | | | |
| 1 | KSE | 1 | | 1 | | 1 | | | | | | |
| 1 | KSET | 1 | | 1 | | 1 | | 1 | | | | |
| 1 | KSETCpP | 1 | | 1 | | 1 | 1 | 1 | | | | |
| 3 | KSETC | 3 | | 3 | | 3 | 2 | 3 | 3 | | | |
| 5 | KSETCCp | 5 | | 5 | | 5 | 5 | 4 | 5 | | | |
| 1 | KSTCCp | 1 | | 1 | | | 1 | 1 | | 1 | | |
| 1 | KGSETCCp | 1 | 1 | 1 | | 1 | 1 | | | 1 | | |
| 2 | KGSETC | 2 | 2 | 2 | | 2 | 2 | | 1 | 1 | | |
| 11 | KGSET | 11 | 11 | 11 | | 11 | 11 | 1 | | | | |
| 2 | KGSETCp | 2 | 2 | 2 | | 2 | 2 | | | | | |
| 1 | GSET | | | 1 | | 1 | 1 | 1 | | | | |
| 2 | SETCCp | | | 2 | | 2 | 1 | 2 | | 2 | | |
| 1 | ET | | | | | 1 | 1 | 1 | | | | |
| | | *E. faecium*[c] (38) | | | | | | | | | | |
| 1 | KET | 1 | | | | 1 | 1 | 1 | | | | |
| 1 | KECpPN | 1 | | | 1 | 1 | | | | | | |
| 1 | KETCpPN | 1 | | | | 1 | | 1 | | | | |
| 1 | KSE | 1 | | 1 | | 1 | | | | | | |
| 1 | KSEP | 1 | | 1 | | | | | | | | |
| 1 | KSET | 1 | | 1 | | 1 | 1 | 1 | | | | |
| 3 | KSETPN | 3 | | 3 | | 3 | 3 | 3 | | | | |
| 3 | KSETP | 3 | | 3 | | 3 | 3 | 3 | | | | |
| 6 | KSETCpP | 6 | | 6 | | 4 | 5 | 2 | | | | |
| 1 | KSETCp | 1 | | 1 | | 1 | | 1 | | | | |
| 1 | KSETC | 1 | | 1 | | 1 | 1 | | | | 1 | |
| 1 | KGSETCCp | 1 | 1 | 1 | | 1 | 1 | 1 | | | 1 | |
| 1 | KGSETCCpN | | 1 | 1 | | 1 | 1 | 1 | | 1 | | |
| 1 | KGSETCpPN | 1 | | 1 | | 1 | | 1 | | | | |
| 1 | KGSETPN | 1 | 1 | 1 | | 1 | 1 | 1 | | | | |
| 1 | KGECpPN | | 1 | | 1 | 1 | | | | | | |
| 1 | KGN | 1 | 1 | | | | | | | | | |
| 1 | GSETP | | | 1 | | 1 | 1 | 1 | | | | |
| 1 | ST | | | | | | 1 | | | | | |
| 1 | ETC | | | | | 1 | 1 | 1 | | | 1 | |
| 2 | ETPN | | | | 1 | 1 | 1 | 2 | | | | |
| 1 | ETP | | | | | 1 | | | | | | |
| 1 | ETN | | | | | 1 | 1 | | | | | |
| 1 | TCpN | | | | | | | 1 | | | | |
| 1 | TP | | | | | | | 1 | | | | |
| 1 | TPN | | | | | | | 1 | | | | |

[a] – K, kanamycin, G, gentamicin, S, streptomycin, P, penicillin, C, chloramphenicol, Cp, ciprofloxacin, E, erythromycin, T, tetracycline, N, nitrofurantoin.
[b,c] – resistance genes were not determined in five and two isolates.

isolates were resistant to kanamycin, streptomycin or to all aminoglycosides tested (38% and 42%).

Multidrug-resistant (resistant to three or more unrelated antibiotics) were 86% of *E. faecium* and 71% of *E. faecalis* isolates. Forty seven percent of *E. faecium* and 21% of *E. faecalis* isolates were resistant to five or more antibiotics. Resistance to aminoglycosides, erythromycin and tetracycline, was most frequent in both *E. faecium* (61%) and *E. faecalis* (69%). Vancomycin-resistant enterococci were not detected in this study.

In general, the prevalence of enterococci resistant to different classes of antibiotics was similar within three animal groups tested, except for poultry isolates, which were more susceptible to streptomycin than isolates from pigs and cattle (78% versus 17% and 10%,

Table 4. Prevalence of virulence genes in *E. faecalis* from different origin.

| Virulence gene | No of isolates | No of resistant isolates[a] | | | | | | | | Origin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | K | G | S | E | T | C | Cp | P | Poultry | Pigs | Cattle |
| *agg*[b] | 1 | 1 | | 1 | | 1 | | | | | | 1 |
| *esp* | 8 | 8 | 8 | 8 | 8 | 8 | 1 | 3 | | | 4 | 4 |
| *agg(asp1) esp* | 3 | 3 | 3 | 3 | 3 | 3 | 1 | | | | 3 | |
| *agg(prgB) esp* | 1 | 1 | 1 | 1 | 1 | 1 | | | | | 1 | |
| *fsr gelE* | 10 | 3 | 1 | 5 | 5 | 7 | 2 | 3 | 1 | | 2 | 8 |
| *esp fsr gelE* | 3 | 3 | 3 | 3 | 3 | 3 | 1 | | | | 1 | 2 |
| *agg*[b] *fsr gelE* | 1 | | | | | | | | | 1 | | |
| *agg(asp1) fsr gelE* | 1 | 1 | | 1 | 1 | 1 | | | | | 1 | |
| *agg(prgB) fsr gelE* | 1 | 1 | | | | | | | | | | 1 |
| *agg*b *esp fsr gelE* | 2 | 2 | | 2 | 1 | 2 | 2 | 2 | | | | 2 |
| *agg(asp1) esp fsr gelE* | 3 | 3 | 1 | 3 | 3 | 2 | 1 | | | | 2 | 1 |
| *agg(prgB) esp fsr gelE* | 8 | 8 | | 8 | 6 | 8 | 6 | 4 | | | | 8 |
| Total | 42 | 34 | 17 | 35 | 31 | 36 | 14 | 12 | 1 | 1 | 14 | 27 |

[a] – K, kanamycin, G, gentamicin, S, streptomycin, E, erythromycin, T, tetracycline, C, chloramphenicol, Cp, ciprofloxacin, P, penicillin;
[b] – DNA sequence not determined.

P<0.05). Also, isolates from pigs were more susceptible to ciprofloxacin (96% versus 61% and 50%, P<0.05) and *E. faecium* isolates from cattle were more frequently susceptible to nitrofurantoin (92% versus 35% and 33%, P<0.05). All isolates of *E. faecalis* were susceptible to nitrofurantoin irrespectively of animal group.

## Antibiotic resistance genes

Antibiotic resistance genes were studied by PCR and results are summarized in Table 3. Aminoglycoside-resistant enterococcal isolates carried the following genes: *aph*(3')-IIIa gene confering resistance to kanamycin (94% (32/34) of *E. faecalis* and 92% (24/26) of *E. faecium*)), *aac*(6')-Ie-*aph*(2')-Ia gene confering resistance to kanamycin and gentamicin (94% (16/17) of *E. faecalis* and 71% (5/7) of *E. faecium*); and *ant*(6)-Ia gene, confering resistance to streptomycin (100% (35/35) of *E. faecalis* and 96% (22/23) of *E. faecium*). All erythromycin-resistant *E. faecalis* and 87% (27/31) of *E. faecium* isolates had *ermB* gene. Two *E. faecium* isolates harboured *ermB* with *ermA* gene.

Tetracycline-resistant isolates carried either *tetM* (47% (17/36) of *E. faecalis*, 19% (6/31) of *E. faecium*), *tetL* (8% (3/36) of *E. faecalis*, 23% (7/31) of *E. faecium*) or a combination of *tetM tetL* genes (42% (15/36) of *E. faecalis*, 52% (16/31) of *E. faecium*). Other tetracycline resistance genes (*tetO*, *tetK* and *tetS*) were not detected. Chloramphenicol-resistant isolates carried either *catA7* (64% (9/14) of *E. faecalis*), *catA8* (36% (5/14) of *E. faecalis*, 25% (1/4) *E. faecium*) or *catA9* determinants (75% (3/4) *E. faecium*). The *catA9* gene was significantly associated with *E. faecium* (P<0.05). No significant differences in gene distribution were observed between groups of isolates of different origin.

In total, 24 and 18 different resistance gene combinations were detected in 36 and 37 isolates of *E. faecium* and *E. faecalis*, respectively.

## Virulence genes

The present study revealed various combinations of genes coding for virulence factors in *E. faecalis* isolates (Table 4). No such genes were found in *E. faecium* isolates. Especifically, *agg*, *esp* and *fsr gelE* genes were identified in 50% (21/42), 67% (28/42) and 69% (29/42) of *E. faecalis* isolates, respectively. Sequencing of *agg*-specific amplicons showed that ten and seven isolates carried *prgB*-like (99% identity) and *asp1*-like (99% identity) gene, respectively. The variant of *agg* gene was not defined for four isolates. Most likely, these isolates carried two different *agg* variants. Thirty one percent (13/42) of *E. faecalis* isolates carried four virulence genes (*agg, esp, fsr* and *gelE*) and most of them (N=11) have been recovered from diseased cattle. Interestingly, most of these isolates were gentamicin-susceptible, while gentamicin-resistant isolates had mainly *esp* (16/17) gene and genes coding for *agg* (5/17) or *fsr gelE* locus (5/17). None of the enterococci isolates had *cylL* and $hyl_{Efm}$.

## Discussion

Eighty isolates of *E. faecium* and *E. faecalis* were obtained from the cattle and swine with the symptoms of diarrhoea in this study. We did not determine all aetiological agents, whereas diarrhoea in calves is commonly caused by ETEC or Rota-virus, Corona-virus (Ok et al. 2009). However, several studies showed that commensal enterococci play an important role in animal infection. Single *Enterococcus* spp. or mixed infections were detected in faeces taken from the diarrheic neonatal calves (Ok et al. 2009). Post-mortem findings revealed valvular endocarditis and septicemia caused by *E. faecalis* or *E. faecium* in hens and broiler (Hedegaard et al. 2009, Bisgaard et al. 2010). Enterococci have been shown to increase virulence potential during a polymicrobial infection in animal models suggesting a synergistic efect between enterococci and virulent bacteria (Montravers et al. 1997, Lavigne et al. 2008). Altogether, the data show that the role of enterococci in animal infection could be underestimated and not investigated.

This study demonstrates that enterococci isolated from diseased farm animals in Lithuania were most frequently resistant to aminoglycosides (streptomycin, neomicin and kanamicin), tetracyclines (including doxycycline), and macrolides (tylosin). These antibiotics were the most intensively used during the last decade in animal treatment. The lack of antimicrobial policy in previous years in the country led to uncontrolled usage of critically important antimicrobial agents such as fluorochinolones (enrofloxacin) which were introduced on a large scale leading to a high prevalence of resistant enterococcal isolates in certain animal groups (41% and 67% of isolates from diseased poultry and cattle, respectively). We observed the high percentage of isolates resistant to gentamicin, an antibiotic of human importance, among *E. faecalis* recovered from diseased cattle and pigs (30% and 64%, respectively). Gentamicin is used parenterally for mammals only, however, gentamicin resistant *E. faecium* poultry isolates were identified in this study. Chloramphenicol was banned for treatment of production animals in the country since 1997, however, 44% of *E. faecalis* cattle isolates were chloramphenicol-resistant. The antimicrobial resistance of commensal enterococci isolated from livestock and poultry products in Lithuania was investigated recently (Ružauskas et al. 2009, Ružauskas et al. 2010). Importantly, resistance rates observed in this study are higher, indicating that the emergence of resistant strains in diseased animals could be promoted by the pressure of antibiotics used for animal treatment and metaphylaxis (aminoglycosides, tetracyclines, tylosin and fluoroquinolones). Improper use of animicrobial agents can lead to the failure of treatment in the presence of multidrug-resistant bacteria.

Various antibiotic resistance mechanisms leading to enzymatic modification of the target, antibiotic itself or drug efflux, have been described and well documented in enterococci. Resistance genes identified in this study are similar to those reported earlier in enterococcal isolates of different origin: animals, food and clinical isolates (Aarestrup et al. 2000, Poeta et al. 2006, Abbassi et al. 2007, Diarra et al. 2010). This is not surprising, since resistance determinants are shown to reside on highly transferable plasmids or transposons and are believed to be effectively exchanged between enterococci of different origin as demonstrated in animal model and in human intestine (Lester et al. 2006, Dahl et al. 2007).

It is of interest to note that most of the *E. faecalis* isolates harboured at least one gene coding for virulence factor. Thirty one percent of *E. faecalis* isolates had four of such genes. The *esp* gene coding for enterococcal surface protein was frequently detected, in contrast to other studies where high prevalence of *gelE* gene, coding for zinc-metalloprotease, has been reported (Poeta et al. 2005, Diarra et al. 2010, Han et al. 2011). The virulence gene *esp* has been shown to reside on a large pathogenicity islands (PAI) of *E. faecium* and *E. faecalis* (McBride et al. 2009). The Esp protein is expressed on the surface of the bacteria and is important for initial adherence during biofilm formation and urinary tract colonization (Heikens et al. 2007). In our study high prevalence of *esp* was determined in *E. faecalis* isolates from diseased pigs (79%) and cattle (63%), similar to that reported in clinical isolates, but not in isolates of animal origin (Hallgren et al. 2009). *esp*-positive *E. faecium* isolates were believed to be limited to hospital-acquired clones, however such isolates from swine and environmental samples were described recently, but not obtained in this study (Layton et al. 2009).

The virulence gene *esp* has been proposed as a marker of the presence of the PAI (Shankar et al. 2006). However, we did not observe other PAI-borne genes contributing to virulence, *asc10* (aggregation substance) and *cytL* (cytolisin), in *esp*-positive isolates. This is in agreement with other studies showing that enterococcal PAI appears to be highly variable in gene content and might even be truncated (Shankar et al. 2006, McBride et al. 2009).

In this study 50% of *E. faecalis* isolates were identified to possess *agg* gene coding for another important multifunctional virulence factor, aggregation substance (AS) of *E. faecalis*. The AS protein mediates adherence to different intestinal epithelial cells and cultured renal tubular cells, survival in polymorphonuclear leukocytes and macrophages (Sartingen et al. 2000). Several gene variants of *agg* are known: *asp1*, *prgB*, *asa1,* located on pheromone-inducible conjugative plasmids (pPD1, pCF10, pAD1, respectively) and *asc10,* located on PAI. All gene variants

encode enterococcal AS protein which directs tight contact between donor and recipient cells enabling the plasmid transfer at high frequency during conjugation (Kozlowicz et al. 2006). The incidence of *agg* virulence gene was reported to range between 26.5% and 79% in *E. faecalis* isolates from chicken, wild animal feaces and clinical isolates, (Poeta et al. 2005, Hallgren et al. 2009, Han et al. 2011). Plasmid-borne *prgB*-like and *asp1*-like genes were found in *E. faecalis* in this study. Interestingly, *prgB*-like gene prevailed among isolates from cattle, whereas *asp1*-like gene was most frequent among isolates from swine. AS proteins, encoded by *asp1*, *prgB*, *asa1,* share over 90% amino acid sequence homology, although their variable region displays only 30-40% similarity (Wirth 1994). There are no published data regarding specificity of the interaction of AS with eukaryotic host proteins. Some indirect evidences for this come from the observations that different AS proteins, encoded by *asa1* and *prgB,* bind differently to eukaryotic extracellular matrix proteins (Rozdzinski et al. 2001, Chuang et al. 2009). The AS protein, encoded by *asa1*, was clearly shown to contribute to endocarditis in rabbit but not in the rat (Berti et al. 1998, Chuang et al. 2009). These data suggest that the interaction specificity between bacterium and eukaryotic cell, aided by AS proteins, could result in the prevalence of *E. faecalis* with particullar AS-carrying plasmid in the host of certain species.

In this study 69% of *E. faecalis* isolates were found to carry both *gelE* and *fsr* loci, in agreement with other studies on *E. faecalis* isolated from broiler chickens, swine, cattle and wild animals (Poeta et al. 2005, Macovei et al. 2009, Diarra et al. 2010, Han et al. 2011). Zinc-metalloprotease, encoded by *gelE*, cleaves a broad range of substrates and appears to contribute to biofilm formation and pathogenesis of endocarditis caused by *E. faecalis* (Thurlow et al. 2010). Expression of *gelE* requires regulatory system encoded by the closely located *fsrABDC* operon (Hancock and Perego 2004). *fsr* locus has been shown to activate expression of a variety of other genes likely involved in virulence. The role of *gelE* and *fsr* loci in *E. faecalis* virulence has been demonstrated in mammalian infection model (Mohamed and Murray 2006). The presence of *gelE fsr* virulence genes appears to be common in isolates from both healthy and diseased animals as shown in this and other studies (Poeta et al. 2005, Macovei et al. 2009, Diarra et al. 2010, Han et al. 2011).

Present phenotypic and genotypic study shows high prevalence of antibiotic resistance and the PAI- and plasmid-encoded virulence genes in enterococci isolates from diseased farm animals in Lithuania. Interestingly, *E. faecium* from production animals is considered not a human hazard because most of *E. faecium* isolates causing outbreaks in hospitals belong to clonal complex CC17, whereas isolates recovered from animals belong to other clonal complexes. For *E. faecalis* the situation appears different as the isolates of the same sequence type have been detected both in human and animal origin (reviewed in (Hammerum et al. 2010).

In conclusion, the existence of a reservoir of the *Enterococcus* spp., which can be implicated in causing infections in animals, calls for careful monitoring due to possible transmission of genetic elements conferring resistance and virulence from animal to human pathogenic strains or *vice versa*.

## Acknowledgements

The Lithuanian State and Sciences Foundation (Grant No. C-07061) supported this work.

## References

Aarestrup FM, Agerso Y, Gerner-Smidt P, Madsen M, Jensen LB (**2000**) Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn Microbiol Infect Dis 37: 127-137.

Abbassi MS, Achour W, Hassen AB (**2007**) High-level gentamicin-resistant *Enterococcus faecium* strains isolated from bone marrow transplant patients: accumulation of antibiotic resistance genes, large plasmids and clonal strain dissemination. Int J Antimicrob Agents 29: 658-664.

Berti M, Candiani G, Kaufhold A, Muscholl A, Wirth R (**1998**) Does aggregation substance of *Enterococcus faecalis* contribute to development of endocarditis? Infection 26: 48-53.

Bisgaard M, Bojesen AM, Christensen JP, Christensen H (**2010**) Observations on the incidence and aetiology of valvular endocarditis in broiler breeders and detection of a newly described taxon of *Pasteurellaceae*, *Avibacterium endocarditidis*. Avian Pathol 39: 177-181.

Chuang ON, Schlievert PM, Wells CL, Manias DA, Tripp TJ, Dunny GM (**2009**) Multiple functional domains of *Enterococcus faecalis* aggregation substance Asc10 contribute to endocarditis virulence. Infect Immun 77: 539-548.

CLSI (**2006**) Performance standards for antimicrobial susceptibility testing: Sixteenth information supplement m100-s16. Clinical and Laboratory Standarts Institute, Wayne, PA.

Dahl KH, Mater DD, Flores MJ, Johnsen PJ, Midtvedt T, Corthier G, Sundsfjord A (**2007**) Transfer of plasmid and chromosomal glycopeptide resistance determinants occurs more readily in the digestive tract of mice than *in vitro* and exconjugants can persist stably *in vivo* in the absence of glycopeptide selection. J Antimicrob Chemother 59: 478-486.

Diarra MS, Rempel H, Champagne J, Masson L, Pritchard J, Topp E (**2010**) Distribution of antimicrobial resistance and virulence genes in *Enterococcus* spp. and characterization of isolates from broiler chickens. Appl Environ Microbiol 76: 8033-8043.

Hallgren A, Claesson C, Saeedi B, Monstein HJ, Hanberger H, Nilsson LE (**2009**) Molecular detection of aggregation substance, enterococcal surface protein, and cytolysin

genes and *in vitro* adhesion to urinary catheters of *Enterococcus faecalis* and *E. faecium* of clinical origin. Int J Med Microbiol 299: 323-332.

Hammerum AM, Lester CH, Heuer OE (**2010**) Antimicrobial-resistant enterococci in animals and meat: A human health hazard? Foodborne Pathog Dis 7: 1137-1146.

Han D, Unno T, Jang J, Lim K, Lee SN, Ko G, Sadowsky MJ, Hur HG (**2011**) The occurrence of virulence traits among high-level aminoglycosides resistant Enterococcus isolates obtained from feces of humans, animals, and birds in South Korea. Int J Food Microbiol 144: 387-392.

Hancock LE, Perego M (**2004**) The *Enterococcus faecalis fsr* two-component system controls biofilm development through production of gelatinase. J Bacteriol 186: 5629--5639.

Hedegaard L, Christensen H, Chadfield MS, Christensen JP, Bisgaard M (**2009**). Association of *Streptococcus pluranimalium* with valvular endocarditis and septicaemia in adult broiler parents. *Avian Pathol* 38: 155-160.

Heikens E, Bonten MJ, Willems RJ (**2007**) Enterococcal surface protein Esp is important for biofilm formation of *Enterococcus faecium* E1162. J Bacteriol 189: 8233-8240.

Kozlowicz BK, Dworkin M, Dunny GM (**2006**) Pheromone-inducible conjugation in *Enterococcus faecalis*: a model for the evolution of biological complexity? Int J Med Microbiol 296: 141-147.

Kreft B, Marre R, Schramm U, Wirth R (**1992**) Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. Infect Immun 60: 25-30.

Lavigne JP, Nicolas-Chanoine MH, Bourg G, Moreau J, Sotto A (**2008**) Virulent synergistic effect between *Enterococcus faecalis* and *Escherichia coli* assayed by using the *Caenorhabditis elegans* model. PLoS One 3: e3370.

Layton BA, Walters SP, Boehm AB (**2009**) Distribution and diversity of the enterococcal surface protein (*esp*) gene in animal hosts and the Pacific coast environment. J Appl Microbiol 106: 1521-1531.

Lester CH, Frimodt-Moller N, Sorensen TL, Monnet DL, Hammerum AM (**2006**) *In vivo* transfer of the *vanA* resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. Antimicrob Agents Chemother 50: 596-599.

Macovei L, Ghosh A, Thomas VC, Hancock LE, Mahmood S, Zurek L (**2009**) *Enterococcus faecalis* with the gelatinase phenotype regulated by the *fsr* operon and with biofilm-forming capacity are common in the agricultural environment. Environ Microbiol 11: 1540-1547.

McBride SM, Coburn PS, Baghdayan AS, Willems RJ, Grande MJ, Shankar N, Gilmore MS (**2009**) Genetic variation and evolution of the pathogenicity island of *Enterococcus faecalis*. J Bacteriol 191: 3392-3402.

Mohamed JA, Murray BE (**2006**) Influence of the *fsr* locus on biofilm formation by *Enterococcus faecalis* lacking *gelE*. J Med Microbiol 55: 1747-1750.

Montravers P, Mohler J, Saint Julien L, Carbon C (**1997**) Evidence of the proinflammatory role of *Enterococcus faecalis* in polymicrobial peritonitis in rats. Infect Immun 65: 144-149.

Nam HM, Lim SK, Moon JS, Kang HM, Kim JM, Jang KC, Kim JC, Kang MI, Joo YS, Jung SC (**2010**) Antimicrobial resistance of enterococci isolated from mastitic bovine milk samples in Korea. Zoonoses Public Health 57: 59--64.

Ng LK, Martin I, Alfa M, Mulvey M (**2001**) Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes 15: 209-215.

Ok M, Guler L, Turgut K, Ok U, Sen I, Gunduz IK, Birdane MF, Guzelbektes H (**2009**) The studies on the aetiology of diarrhoea in neonatal calves and determination of virulence gene markers of *Escherichia coli* strains by multiplex PCR. Zoonoses Public Health 56: 94-101.

Poeta P, Costa D, Rodrigues J, Torres C (**2006**) Antimicrobial resistance and the mechanisms implicated in faecal enterococci from healthy humans, poultry and pets in Portugal. Int J Antimicrob Agents 27: 131-137.

Poeta P, Costa D, Saenz Y, Klibi N, Ruiz-Larrea F, Rodrigues J, Torres C (**2005**) Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. J Vet Med B Infect Dis Vet Public Health 52: 396-402.

Rice LB, Carias L, Rudin S, Vael C, Goossens H, Konstabel C, Klare I, Nallapareddy SR, Huang W, Murray BE (**2003**) A potential virulence gene, *hylEfm*, predominates in *Enterococcus faecium* of clinical origin. J Infect Dis 187: 508-512.

Roberts JC, Singh KV, Okhuysen PC, Murray BE (**2004**) Molecular epidemiology of the *fsr* locus and of gelatinase production among different subsets of *Enterococcus faecalis* isolates. J Clin Microbiol 42: 2317-2320.

Rozdzinski E, Marre R, Susa M, Wirth R, Muscholl-Silberhorn A (**2001**) Aggregation substance-mediated adherence of *Enterococcus faecalis* to immobilized extracellular matrix proteins. Microb Pathog 30: 211-220.

Ružauskas M, Sužiedėlienė E, Šiugždinienė R, Šeputienė V, Povilonis J (**2010**) Antimicrobial resistance of *Enterococcus* spp. spread in poultry products in Lithuania. J Food Safety 30: 902-915.

Ružauskas M, Virgailis M, Šugždinienė R, Sužiedėlienė E, Šeputienė V, Daugelavičius R, Zienius D, Šengaut J, Pavilonis A (**2009**) Antimicrobial resistance of *Enterococcus* spp. isolated from livestock in Lithuania. Vet Archiv 79: 439-449.

Sartingen S, Rozdzinski E, Muscholl-Silberhorn A, Marre R (**2000**) Aggregation substance increases adherence and internalization, but not translocation, of *Enterococcus faecalis* through different intestinal epithelial cells *in vitro*. Infect Immun 68: 6044-6047.

Shankar N, Baghdayan AS, Willems R, Hammerum AM, Jensen LB (**2006**) Presence of pathogenicity island genes in *Enterococcus faecalis* isolates from pigs in Denmark. J Clin Microbiol 44: 4200-4203.

Singh KV, Lewis RJ, Murray BE (**2009**) Importance of the *epa* locus of *Enterococcus faecalis* OG1RF in a mouse model of ascending urinary tract infection. J Infect Dis 200: 417-420.

Thurlow LR, Thomas VC, Narayanan S, Olson S, Fleming SD, Hancock LE (**2010**) Gelatinase contributes to the pathogenesis of endocarditis caused by *Enterococcus faecalis*. Infect Immun 78: 4936-4943.

Vakulenko SB, Donabedian SM, Voskresenskiy AM, Zervos MJ, Lerner SA, Chow JW (**2003**) Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. Antimicrob Agents Chemother 47: 1423-1426.

Wirth R (**1994**) The sex pheromone system of *Enterococcus faecalis*. More than just a plasmid-collection mechanism? Eur J Biochem 222: 235-246.