Determination of antimicrobial resistance of Enterococcus strains isolated from pigs and their genotypic characterization by method of amplification of DNA fragments surrounding rare restriction sites (ADSRRS fingerprinting)

Aneta Nowakiewicz,*, Grażyna Ziółkowska, Aleksandra Trościąńczyk, Przemysław Zięba and Sebastian Gnat

Abstract

Purpose. In this study, we analysed phenotypic resistance profiles and their reflection in the genomic profiles of Enterococcus spp. strains isolated from pigs raised on different farms.

Methodology. Samples were collected from five pig farms (n=90 animals) and tested for Enterococcus. MICs of 12 antimicrobials were determined using the broth microdilution method, and epidemiological molecular analysis of strains belonging to selected species (faecalis, faecium and hirae) was performed using the ADSRRS-fingerprinting (amplification of DNA fragments surrounding rare restriction sites) method with a few modifications.

Results. The highest percentage of strains was resistant to tetracycline (73.4 %), erythromycin and tylosin (42.5 %) and rifampin (25.2 %), and a large number of strains exhibited high-level resistance to both kanamycin (25.2 %) and streptomycin (27.6 %). The strains of E. faecalis, E. faecium and E. hirae (n=184) revealed varied phenotypic resistance profiles, among which as many as seven met the criteria for multidrug resistance (30.4 % of strains tested). ADSRRS-fingerprinting analysis produced 17 genotypic profiles of individual strains which were correlated with their phenotypic resistance profiles. Only E. hirae strains susceptible to all of the chemotherapeutics tested had two different ADSRRS profiles. Moreover, eight animals were carriers of more than one genotype belonging to the same Enterococcus spp., mainly E. faecalis.

Conclusion. Given the possibility of transmission to humans of the high-resistance/multidrug resistance enterococci and the significant role of pigs as food animals in this process, it is necessary to introduce a multilevel control strategy by carrying out research on the resistance and molecular characteristics of indicator bacterial strains isolated from animals on individual farms.

INTRODUCTION

Enterococci are part of the natural intestinal biota of most mammals and birds. Because of their ubiquitous occurrence and ease of spread by both direct (contact with animals) and indirect (faeces, manure, wind or contaminated food) routes, bacteria of the genus Enterococcus are included among indicator microbes [1]. In addition to their natural drug resistance and the ease of transmission and acquisition of mobile genetic elements, not only within the same species but also in other species and genera, these indicator bacteria can reflect the degree of resistance carried by commensal biota, especially in food animals [2]. The occurrence of resistant Enterococcus strains in food animals is highly varied, depending on the animal species, the geographical region and the scope of antibacterial agents used in treatment and prevention [3]. The resistance profiles of these strains are generally similar to profiles of Enterococcus spp. isolated from nosocomial infections in humans and include resistance to aminoglycosides, macrolides, quinolones, streptogramins and tetracyclines [4]. The similarity of phenotypic profiles suggests that drug-resistant and multidrug-resistant strains of Enterococcus spp. occurring in the digestive tract of farm
animals and in food of animal origin play a key role in the spread of resistance in humans [1].

Epidemiological molecular testing enabling evaluation of the occurrence, origin and spread of different strains of bacteria in different environments [1, 5] is made more difficult by differences in the methods used and in how the results are interpreted.

For detection of resistance, classical microbiological methods are preferred over molecular techniques [6]. Methods based on direct detection of resistance genes do not always correspond to the phenotypic profiles of resistance [7]. Moreover, interpretation of phenotypic resistance data is varied (i.e. clinical breakpoints and epidemiological cut-off values) and depends on whether the goal is to evaluate therapeutic success or to detect evolutionary changes in resistance. These criteria are not always in agreement, which can lead to difficulties in comparative analysis of data obtained from different countries and different research groups. At the same time, the use of different criteria for sample selection and molecular analysis techniques with differing discriminatory potential impedes optimal and precise analysis of data from different research centres. The choice of an appropriate molecular technique is usually justified by several basic criteria: discriminatory power, total time and cost of analysis, the need to use specialized equipment, potential technical problems, ease of interpretation of results and repeatability [8]. Although a number of molecular techniques for epidemiological typing have been used, not all of them are characterized by repeatability and sufficiently high discriminatory potential desirable for analysis of local diversity or outbreak strains [8, 9]. These criteria are met by both PFGE and ADSRRS-fingerprinting (amplification of DNA fragments surrounding rare restriction sites) methods. PFGE method is still considered as the ‘gold standard’; however, it has some limitations; this method involves highly time-consuming procedures and requires qualified and experienced personnel for the laboratory work and data evaluation [8–11]. On the other hand, the ADSRRS-fingerprinting method gives comparable results and, in the case of analysis of vancomycin-resistant Enterococcus faecium, showed even a higher discriminatory power [11]. Moreover, this method is characterized by reliability and repeatability, and the interpretation of results is analogous to that in PFGE. Additionally, the use of the technique does not require prior knowledge of the sequences of analysed strains. The procedure involves the use of uncomplicated apparatus and basic techniques of molecular biology, which should not create technical problems. These features suggest that the ADSRRS-fingerprinting method can be useful for differentiation of micro-organisms within a species [12]. The ADSRRS-fingerprinting method has also been successfully used not only for molecular typing of bacteria of the genus Enterococcus [11] but also other bacterial species [13–15].

There are no data from Poland concerning the analysis of resistance of Enterococcus spp. isolated from farm animals, especially pigs. As a source of food, these animals can be an important vector of the spread of resistance in humans.

Molecular analysis of resistant strains and analysis of the spread of multidrug resistance (MDR) are essential processes for monitoring the growing worldwide development of resistance. The aim of the present study was to analyse phenotypic resistance profiles of Enterococcus spp. strains isolated from pigs from different sources (farms) and their genotypic characterization using the ADSRRS-fingerprinting method.

**METHODS**

**Sampling**

The study was conducted on five closed-cycle intensive pig farms (I–V) in southern and eastern Poland, spaced at least 30 km apart (the number of animals ranges from approximately 120 to 260 per farm). On all farms, the pigs were housed indoors on deep litter (straw). The farms had average health status, and the following information concerning the use of antibiotics for treatment and prevention (metaphylaxis) was available: penicillin, oxytetracycline, enrofloxacin, florfenicol and streptomycin were used on farms I, II, IV and V, in addition to lincomycin, spectinomycin, tiamulin and tylosin on farms I and V, while only oxytetracycline was used on farm III. The housing was disinfected at the end of the production cycle (with Virkon containing potassium peroxymonosulfate, sodium chloride and an inorganic buffer as active substances) on all farms.

The material for the study consisted of rectal swabs (n=90) from clinically healthy animals (sows n=26, piglets aged 2–4 weeks n=26 from farms I, IV and V, and fattening pigs n=38 from farms II, III and V) collected from March to June 2013. Faecal samples were collected from the rectum using a sterile cotton swab and stored at 5 to 8 °C for a maximum of 6 to 8 h before culture. There were 20 swabs collected from farms I and II, 12 each from farms III and IV and 26 from farm V (samples were taken proportionally to the number of animals on the farm).

**Isolation and identification**

The swabs were incubated in buffered peptone water for 12 h at 37 °C. The material was plated in the amount of 100 µl on Slanetz–Bartley agar (Biocorp) and incubated at 41 °C for 48 h. To demonstrate the possible existence of different Enterococcus spp. and/or different resistance profiles of isolates within individual animals, five most macroscopically different colonies from each sample were chosen for further analysis.

Identification to the genus Enterococcus was based on micromorphology (Gram staining), catalase tests, ability to hydrolyse bile aesculin and ability to grow in the presence of 6.5% NaCl. Species identification was performed using 16S–23S rRNA intergenic region restriction endonuclease analysis according to previously described protocols [16].

**Antimicrobial susceptibility testing**

Drug susceptibility of the isolates obtained was evaluated using the microdilution method (house kits prepared by ourselves were used) in accordance with the standards of the Clinical and Laboratory Standards Institute [17]. A
panel of 12 antimicrobials and the following breakpoints for classification as resistant was used: ampicillin \( \geq 16 \mu l \text{ ml}^{-1} \), chloramphenicol \( \geq 32 \mu l \text{ ml}^{-1} \), fluoroquinolones (ciprofloxacin and enrofloxacin) \( \geq 4 \mu l \text{ ml}^{-1} \) (for enrofloxacin, the breakpoint was defined according to CLSI [18]), erythromycin \( \geq 8 \mu l \text{ ml}^{-1} \), gentamicin \( \geq 512 \mu l \text{ ml}^{-1} \), rifampin \( \geq 4 \mu l \text{ ml}^{-1} \), streptomycin \( \geq 1024 \mu l \text{ ml}^{-1} \), tetracycline \( \geq 16 \mu l \text{ ml}^{-1} \) and vancomycin \( \geq 32 \mu l \text{ ml}^{-1} \). Since CLSI does not define criteria for kanamycin and tylosin, the breakpoints defined by the National Antimicrobial Resistance Monitoring System Animal Isolates (http://www.ars.usda.gov/News/docs.htm?docid=6750&page=3) were used: kanamycin \( \geq 64 \mu l \text{ ml}^{-1} \), streptomycin \( \geq 1024 \mu l \text{ ml}^{-1} \) and tylosin \( \geq 32 \mu l \text{ ml}^{-1} \). The range of concentrations used for each of the chemotherapeutics is presented in Table 1. Enterococcus faecalis ATCC 29212 and E. faecalis ATCC 51299 were used as quality controls [17, 19].

MDR was defined as a profile including acquired resistance to at least one agent in three or more antimicrobial classes [20].

**Statistical analysis**

Statistical analysis was performed in the R programme (version 3.1.1). Fisher’s exact test was used for inference of the relationships between Enterococcus spp. and resistance to the antimicrobials tested. In the analysed system, the occurrence of resistance was the tested variable, and the bacterial species was the grouping variable. The level of statistical significance for adoption of an alternative hypothesis about the dependence of variables was determined at a \( P<0.05 \) level.

**ADSRRS fingerprinting**

**DNA isolation**

DNA was isolated from 24 h bacterial cultures on Columbia blood lab agar (Biocorp) using ready-made kits – Bacterial and Yeast Genomic DNA purification kit (Eurx) – according to the manufacturer’s procedure and using mutanolysin (50 U per sample) (A&A Biotechnology) to achieve bacterial lysis.

The ADSRRS-fingerprinting procedure was performed as described by Krawczyk et al. [11] with a few modifications. The reaction of restriction endonuclease digestion of genomic DNA (150–250 ng) was carried out with 25 µl of a reaction mixture composed of 10 U XbaI, 5 U BglII and 5 µl Tango buffer (Thermo Scientific) at 37 °C, and digestion time was shortened to 60 min. For ligation, appropriate adapters [11] corresponding to the cohesive ends of restriction fragments and T4 ligase (0.5 µl) were used. The ligation reactions were carried out at room temperature for 60 min. The standard procedure for purification and precipitation of DNA following the enzymatic reactions (endonuclease digestion and ligation reaction of adapters) was replaced by thermal inactivation at 80 °C for 2 min and at 70 °C for 5 min. The PCR was carried out using 25 µl of a reaction mixture composed of 2 µl of the solution after the ligation reaction, 2.5 µl of 10× Shark reaction buffer [200 mM Tris/HCl pH 8.8, 100 mM KCl, 100 mM (NH₄)₂ SO₄, 1% TritonX-100], Hypernova DNA Polymerase Pwo 1 U, 2.5 µl of a dNTP mixture (the concentration of each nucleotide was 2.5 mM), 2 mM MgCl₂ (Blirt) and 50 pmol of each primer (GenoMed).

The reaction conditions for the thermal cycler (T Personal thermal cycler; Biometra) were as follows: initial cycle of 94 °C for 5 min, 72 °C for 5 min to fill in the ends of the DNA fragments and initial denaturation at 94 °C for 5 min (pre-PCR), followed by 22 cycles of 94 °C for 30 s, 60 °C for 30 s (for E. hirae and E. faecium) or 56 °C for 30 s (for E. faecalis) and 72 °C for 1.5 min, after which an extension cycle of 72 °C for 5 min was added (different annealing

<table>
<thead>
<tr>
<th>Agent</th>
<th>Range (µg ml⁻¹)</th>
<th>E. faecalis (n=87)</th>
<th>E. faecium (n=13)</th>
<th>Enterococcus hirae (n=84)</th>
<th>Other Enterococcus spp. (n=30)</th>
<th>Total (n=214)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.25–32</td>
<td>0.25–32</td>
<td>0.25–32</td>
<td>0.25–32</td>
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<td>0.25–32</td>
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</tbody>
</table>

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; RIF, rifampin; STR, streptomycin; TET, tetracycline; TYP, tylosin; VAN, vancomycin.

*Number/percentage of resistant strains.
temperatures were used for the particular species of Enterococcus spp. than in the PCR conditions described by Krawczyk et al. [11]). Electrophoretic separation of PCR products was carried out in 6% polyacrylamide gel (Sigma-Aldrich). Electrophoretic profiles were fixed using GelDoc2000 (BioRad).

The procedure was repeated three times for all strains.

BioNumerics 7.5 software (Applied Maths) was used for cluster analysis of strains by the unweighted pair group method with arithmetic mean. The similarity index of the isolates was calculated using the Jaccard correlation coefficient option of the software with position tolerance and optimization of 1%.

RESULTS

Occurrence and identification of Enterococcus spp.

A total of 450 isolates initially classified as representatives of the genus Enterococcus were obtained from 90 animals. Molecular verification limited further analyses to 430 Enterococcus isolates belonging to the species E. faecalis (n=177), E. hirae (n=172), E. faecium (n=27), Enterococcus casseliflavus (n=24), Enterococcus durans (n=13), Enterococcus gallinarum (n=10), Enterococcus thailandicus (n=6) and Enterococcus avium (n=1). The remaining isolates (n=20) (obtained from four animals) did not represent Enterococcus spp. (Table S1, available in the online Supplementary Material) and were excluded from further analysis.

The species profile of Enterococcus isolated from particular individuals was homogeneous for 67.4% (58/86) of the animals, which means that we isolated only one species of Enterococcus from this percentage of pigs. E. faecalis was the only species isolated from 48.3% of the animals (28/58), while E. hirae and E. casseliflavus were the only species present in 44.8% (26/58) and 6.9% (4/58) of the pigs, respectively. Two or three different species of Enterococcus were isolated simultaneously from the other animals (n=28) (Table S1).

Phenotypic susceptibility analysis

Phenotypic susceptibility analysis was performed with using the microdilution method. The results of the quality control strains (E. faecalis ATCC 29212 and E. faecalis ATCC 51299) were in CLSI acceptable quality control ranges. Preliminary testing of the drug resistance of Enterococcus spp. was used to eliminate analysis of duplicate isolates obtained from the same individual. Different MIC values were used as a selection criterion. Isolates derived from the same host with different MIC values for at least three antimicrobial agents were considered as distinct. Finally, we chose 214 isolates for further analysis.

Analysis of the results revealed a relationship between the distribution of Enterococcus spp. and the source of isolation (the farm); E. faecalis was the dominant species among isolates from farms IV and V (80 and 67.2%, respectively), and E. hirae mainly dominated in farm II (96%). In farm I, E. faecalis and E. hirae both occurred in the same percentage (46%) (Table S2).

Analysis of the resistance profiles of the selected isolates revealed a high percentage of isolates resistant to most of the antimicrobials tested. The highest percentage of Enterococcus spp. was resistant to tetracycline (73.4%), erythromycin and tylosin (42.5%) and rifampin (25.2%), and a large number of strains exhibited high-level resistance to both kanamycin (25.2%) and streptomycin (27.6%). Significantly lower resistance rates to chloramphenicol (18.2%), fluoroquinolones (10.7%) and high-level resistance to gentamicin (11.7%) were reported (Tables 1 and S3).

Comparative analysis and determination of the phenotypic resistance profiles of E. faecalis, E. faecium and E. hirae

Among the 214 Enterococcus isolates recovered, only the predominant and/or clinically relevant species, E. faecalis (n=87), E. faecium (n=13) and E. hirae (n=84), were considered in the further detailed comparative analysis.

Comparison of the resistance to the particular antimicrobials of E. faecalis and E. faecium showed statistically significant differences (P<0.05) in the case of chloramphenicol, ciprofloxacin, enrofloxacin, kanamycin, rifampin and streptomycin, while the difference between the resistance to specific antimicrobials of E. hirae and E. faecium was statistically significant for erythromycin, tylosin, streptomycin, kanamycin, gentamicin and rifampin. In the case of comparison of E. faecalis and E. hirae, only differences of resistance to vancomycin, ampicillin and tetracycline were not statistically significant (Table 1).

Based on the differences in the resistance to the particular antimicrobial agents within particular Enterococcus spp., it was shown that E. faecalis strains generated six separate resistance profiles; E. faecium, four profiles; and E. hirae, six profiles (Table 2). The qualitative and quantitative composition of antimicrobial agents was noted to be varied for the individual phenotypic resistance profiles: the presence of tetracycline (13/16 profiles), erythromycin and tylosin (9/16), high-level streptomycin resistance (6/16) and high-level kanamycin resistance (7/16) was noted most frequently. Profiles characterized by resistance to gentamicin (3/16) and rifampin (5/16) were observed only in the strains of E. faecalis and E. faecium, while resistance to chloramphenicol and/or fluoroquinolones was noted only in the E. faecalis strains (3/16). Susceptibility to all the antibiotics tested was found only in E. hirae isolates (Table 2).

MDR was noted in the case of 30.4% (n=56) of the total pool (n=184) of the isolated strains of E. faecalis, E. faecium and E. hirae. The highest percentage of MDR was observed in the E. faecium species (84.6%, 11/13). For E. faecalis, MDR was at a level of 42.5% (37/87), while for the E. hirae strains, it was considerably lower, i.e. approximately 9.5% (8/84). The level of MDR was also correlated with the source (Table 3) of the Enterococcus strains. The highest percentage of MDR was noted in farms V (56.7%, 34/60) and IV.
Genotypic analysis (ADSSRS fingerprinting)

Genotypic analysis of the strains tested \((n=184)\) revealed, depending on the strain, the presence of 10 to 25 bands ranging in size from 150 to 1000 bp (Figs 1 and S1 show selected representatives from different farms and/or individuals).

The ADSRRS-fingerprinting procedure was repeated three times for each strain. Particular strains produced identical electrophoretic profiles in each run of electrophoresis.

The profiles of the individual strains were grouped in species-specific clusters between which relatively low similarity was noted; the similarity coefficient was about 0.4 for all strains of the species \(E. \text{faecium}\) and 0.52 for \(E. \text{faecalis}\) and \(E. \text{hirae}\) (Figs 1 and S1).

Different genotypic profiles were noted within the individual species (\(E. \text{faecalis}, Ef\text{sA–EfsF};\) \(E. \text{faecium}, Ef\text{mA–Ef\text{mD}; and } E. \text{hirae}, EhA–EhG\)) (Table 2, Figs 1 and S1). Comparative analysis of the phenotypic resistance profiles and confrontation thereof with the genotypes of the strains tested (ADSSRS-fingerprinting profiles) showed that strains assigned to a particular genotype had an identical phenotypic resistance profile (Table 2).

Two different ADSRSS profiles, designated as \(EhF\) and \(EhG\), were shown only in the case of \(E. \text{hirae}\) strains susceptible to all of the chemotherapeutics tested, but the similarity between these profiles was relatively low, i.e. only 67.3\% (Figs 1 and S1).

The occurrence of more than one (two or three) genotypic profile of \(Enterococcus\) isolates belonging to the same species was also observed within the same individual. In the analysed pool of animals, there were eight individuals from which strains belonging to the same species exhibited two or three different profiles. A majority of the animals had strains belonging to \(E. \text{faecalis}: Ef\text{sC}+Ef\text{sE}, Ef\text{sA}+Ef\text{sD}, Ef\text{sA}+Ef\text{sD}+Ef\text{sF}\) and \(Ef\text{sA}+Ef\text{sB}\) and were present in four animals; concomitant presence of the \(Ef\text{sB}+Ef\text{sD}\) profiles was shown in two animals. In single individuals, profiles of \(E. \text{faecium} (Ef\text{mA}+Ef\text{mB})\) and \(E. \text{hirae} (EhA+EhB)\) were also found. Six of eight animals originated from farm V.

**DISCUSSION**

The study was carried out on five pig farms located in southern and eastern Poland. To obtain data that reflect the variation in \(Enterococcus\) strains in the farms as accurately as possible, five different colonies from the same animal

### Table 2. Resistance profiles of \(Enterococcus\) strains

<table>
<thead>
<tr>
<th>Phenotypic resistance profile*</th>
<th>CHL</th>
<th>CIP</th>
<th>ENR</th>
<th>ERY</th>
<th>GEN</th>
<th>KAN</th>
<th>RIF</th>
<th>STR</th>
<th>TET</th>
<th>TYL</th>
<th>ADSRSS genotypic profile</th>
<th>No. of strains</th>
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</thead>
<tbody>
<tr>
<td>(E. \text{faecalis} (n=87))</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>EfsA</td>
<td>14</td>
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<tr>
<td>CMGKRST</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>CFMKST</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
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<td>EhE</td>
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</table>

**Phenotypic resistance profile***

- CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; RIF, rifampin; STR, streptomycin; TET, tetracycline; TYL, tylosin.

*Profiles were created from the first letters of the names of antimicrobials (C, chloramphenicol; G, gentamicin; K, kanamycin; R, rifampin; S, streptomycin; T, tetracycline) or names of groups of antimicrobials: fluoroquinolones (F; ciprofloxacin and enrofloxacin) and macrolides (M; erythromycin and tylosin) to which given strains are resistant.
were selected and evaluated with regard to species identification and MIC. In most screening tests evaluating the resistance of indicator bacteria, the sample collection strategy is rather geared toward analysis of bacteria in the broadest possible pool of animals [21], and samples are collected from randomly selected individuals from the greatest possible number of herds or production groups. For this reason, there are no data fully reflecting both species distribution and the occurrence of particular resistance profiles in herds and in individuals.

Our study confirmed that, as in other countries, mainly E. faecalis, E. faecium and E. hirae were the dominant Enterococcus spp. colonizing the digestive tract of pigs [22, 23]. Because of the clinical significance of E. faecium and E. faecalis for humans (third and fourth most prevalent nosocomial pathogen worldwide) [24] and the high percentage of isolation of E. hirae, the essential analysis of the results focused on these species.

The phenotypic resistance profiles of strains varied within both the genus and the Enterococcus spp. Based on the clinical breakpoint, a total of 16 separate profiles were identified, 6 of which belonged to E. faecalis, 4 to E. faecium and 6 to E. hirae. Their distribution on the farms was also varied but very closely associated with the dominant Enterococcus spp. on the farms. On the farms dominated by E. faecalis and E. faecium (farms I, IV and V), the percentage of resistant strains, including multidrug-resistant strains, was high (from 30% to 56.7%). One of the factors affecting the profile of Enterococcus spp. in particular farms could be the age of the animals (in farms II and III, the samples were isolated only from fattening pigs, while the other farms were varied in terms of age groups), but it is also possible that other factors, such as diet, may also affect the composition of the species biota of the gastrointestinal tract [25].

The distribution of resistance profiles in the individual farms may also have been affected by the type and scope of antibiotic therapy [26]. On farm III, where only oxytetracycline had been used, a substantial percentage of the strains tested were susceptible to most of the antimicrobial agents; only resistance to tetracycline and/or rifampin was noted. However, the percentage of resistant strains on farm II (dominated by E. hirae) was very low despite the use of a panel of antimicrobials similar to that used on farm IV.

Molecular analysis showed that genotypic profiles obtained using the ADSRRS-fingerprinting method in the pool of the analysed Enterococcus strains are consistent with the patterns of phenotypic resistance based on clinical breakpoints. The phenotypic resistance profiles were found to be a reflection of their genotypic profiles, which was also confirmed by previous analysis conducted for E. faecalis and E. faecium strains isolated from poultry [27]. Only within the susceptible E. hirae strains were two separate genotypic profiles ( EhF and EhG) found for strains from two different farms. There may be several causes of this observation (but we did not analyse any of them in this study), for example, resistance to an antimicrobial agent that was not included in our study, the presence of silent genes or an inactive gene with a mutation which does not demonstrate expression of phenotypic resistance [28] or presence of other differences in the genome not linked to resistance.

Previous research in this area was generally based on the PFGE method as the gold standard in differentiating closely related strains [5, 24, 29-31]. Researchers have analysed the potential correlation between genotypic profiles (pulsotypes) of Enterococcus and the source of the strain (environmental, clinical or commensal) or resistance profiles, although their studies are generally limited to strains with the highest epidemiological impact, mainly vancomycin-resistant strains [29] or high-level aminoglycoside-resistant (HLAR) strains [5, 29]. Jackson et al. [30], Katsunuma et al. [31] and Dicuonzo et al. [24] have shown that strains with the same or very similar pulsotypes (and similar antibiograms) can be isolated from different sources (different individuals or farms), which suggests that bacteria with a specific resistance profile can move from one animal to another [30] and probably may also spread among pig farms. These observations are clearly confirmed in the present study. Varied resistance profiles (including MDR) with their corresponding genotypes within and between the farms and the occurrence of the same profiles in different farms (e.g. EfsA in farms IV and V; EfmA in

### Table 3. Distribution of genotypic profiles and MDR profiles in farms

<table>
<thead>
<tr>
<th>Farm no. (n=184)*</th>
<th>Enterococcus/no. of strains</th>
<th>MDR profiles/no. of strains</th>
<th>ADSRRS profiles/no. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (50)</td>
<td>E. faecalis/23 CFMGKST/9</td>
<td>EjfC/9</td>
<td>EhF/3</td>
</tr>
<tr>
<td></td>
<td>E. faecium/4 MKST/3</td>
<td>EjfD/13</td>
<td>EhB/3</td>
</tr>
<tr>
<td></td>
<td>E. hirae/23 MST/3</td>
<td>EfmA/3</td>
<td>EAD/1</td>
</tr>
<tr>
<td>II (49)</td>
<td>E. faecalis/0</td>
<td>EfmC/1</td>
<td>EME/25</td>
</tr>
<tr>
<td></td>
<td>E. faecium/1</td>
<td>EhF/22</td>
<td>EMe/23</td>
</tr>
<tr>
<td>III (4)</td>
<td>E. faecalis/1</td>
<td>EjhF/1</td>
<td>EhG/3</td>
</tr>
<tr>
<td></td>
<td>E. faecium/0</td>
<td>EjhF/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. hirae/3</td>
<td>EjhF/1</td>
<td></td>
</tr>
<tr>
<td>IV (21)</td>
<td>E. faecalis/20 CMGKRST/6</td>
<td>EjhA/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. faecium/1</td>
<td>EjfA/14</td>
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<tr>
<td></td>
<td>E. hirae/0</td>
<td>EjfA/14</td>
<td></td>
</tr>
<tr>
<td>V (60)</td>
<td>E. faecalis/43 CMGKRST/8</td>
<td>EjhA/8</td>
<td></td>
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<tr>
<td></td>
<td>CEMKST/14</td>
<td>EjfB/19</td>
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<tr>
<td></td>
<td>E. faecium/7</td>
<td>EjfD/19</td>
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<td>EfmA/5</td>
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<tr>
<td></td>
<td>E. hirae/10</td>
<td>EhmB/2</td>
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</table>

*Total number of strains belonging to E. faecalis, E. faecium and E. hirae.
farms I and IV) may be an indication of the ease of spread and exchange of Enterococcus strains. Moreover, in isolated cases, the presence of strains belonging to the same species, but with two or three different genotypic profiles, was noted in individuals, which confirms that more than one type of strains belonging to the same Enterococcus spp. can colonize a single host. The causes of the spread of Enterococcus strains, particularly MDR strains, often to distant locations are varied and include introduction of new animals to the farm, transport [1], the role of free-living animals as indirect vectors [32] or personnel involved in animal care [1]. Isolation of strains from different farms having the same resistance profiles can also be linked to animals located on different farms having come from a common source.

Fig. 1. Dendrogram of genetic relatedness among E. faecium, E. faecalis and E. hirae strains (dendrogram shows selected representatives from different farms and/or individuals). *Letters correspond to genotypic/phenotypic resistance profiles; roman numerals indicate the farm number; arabic numerals designate the strain number (from different farms and/or different animals).
Analysis of the overall level of resistance to particular antimicrobials showed that resistance of Enterococcus isolates tested in this study is comparable with other countries, including European countries [1, 22, 26, 33, 34].

The highest occurrence of resistance was noted mainly for tetracycline and macrolides, which due to the widespread use of these antimicrobials in pigs is a phenomenon observed in many countries [1, 22, 33, 34, 35]. Despite the ban on the use of antibiotics (including tylosin) as growth promoters introduced by the European Union in 2006, the drugs are still used for treatment. Resistance among enterococci to macrolides and cross-resistance to erythromycin are thought to be due to tylosin use in this group of animals [25]. Our results seem to confirm this phenomenon through the occurrence of simultaneous resistance to tylosin and erythromycin in all strains resistant to macrolides. The relatively high level of resistance of Enterococcus spp. to rifampin in the pigs in the present study, as in other animals, is noted fairly often [32], although this drug is used only sporadically in animals [36]. Such high resistance may be a consequence of point mutations in the β-subunit of RNA polymerase induced by environmental stress, e.g. low glucose levels or temperature shock [37, 38].

Although the percentage of HLAR strains isolated from humans has been decreasing in many countries, the number of these strains isolated from production animals (including pigs) is still high. The high-level aminoglycoside resistance to streptomycin and kanamycin was noted in 43 and 85% of the strains of E. faecalis and E. faecium, respectively, which is comparable to the distribution of HLAR strains in other countries of Europe [1, 33], North America [35] and Asia [22]. The presence of high-level streptomycin resistance and high-level kanamycin resistance strains in the E. hirae population in our study may indicate the growing importance of this microbe as a potential resistance indicator.

The level of resistance to gentamicin, despite the fact that this antimicrobial agent is relatively seldom used in pigs, is high, i.e. nearly twice as high as the percentage of high-level gentamicin-resistant strains of E. faecalis and E. faecium noted in other countries. For example, in Canada and the USA, it does not exceed 15% [26, 35]. In some European countries (e.g. Portugal and Lithuania), these values are similar [33, 34].

Elevated resistance indices in comparison with most other countries were also noted in the case of resistance of E. faecalis to chloramphenicol (about 50% of the strains in the present study) [33, 34, 39].

Resistance to fluoroquinolones in Enterococcus strains isolated from pigs in other countries is quite diverse. Our results are significantly higher (23%) in comparison to those reported from most countries, where the proportion of resistant strains of E. faecalis does not exceed 10% [26, 33–35]; however, there are data demonstrating that 85% to 100% of Enterococcus isolates are resistant to ciprofloxin [1].

Most studies of Enterococcus resistance to fluoroquinolones are based on analysis of breakpoints for ciprofloxacin. In our study, we tested enrofloxacin, used exclusively in animals, and ciprofloxacin, generally used in humans. All strains resistant to enrofloxacin were simultaneously resistant to ciprofloxacin, which is consistent with the results obtained by Hershberger et al. [26] and Kuo et al. [40], who showed that a wide use of enrofloxacin in animals can select Enterococcus strains that are resistant to ciprofloxacin and probably to other fluoroquinolones.

In conclusion, because of the prevalence and the high resistance/MDR of strains of E. faecalis, E. faecium and E. hirae in herds, the possibility of their direct transmission to humans, particularly in the case of MDR strains, and the significant role of pigs as food animals in this process, it is necessary to introduce a multilevel control strategy by carrying out research on the resistance and molecular characteristics of indicator bacterial strains isolated from animals on individual farms. Apparently, because of the presence of strains with varied resistance and genotypic profiles in individual animals (we found two different MDR strains in three individuals), analysis of more than one strain derived from a single individual should be considered.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


