Modified 16S–23S rRNA intergenic region restriction endonuclease analysis for species identification of Enterococcus strains isolated from pigs, compared with identification using classical methods and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Aneta Nowakiewicz,1 Grażyna Ziółkowska,1 Przemysław Zięba,2 Aleksandra Trościąńczyk,1 Tomasz Banach3 and Cezary Kowalski4

1University of Life Sciences, Faculty of Veterinary Medicine, Institute of Biological Bases of Animal Diseases, Sub-Department of Veterinary Microbiology, Akademicka 12, 20-033 Lublin, Poland
2State Veterinary Laboratory, Droga Męczenników Majdanka 50, 20-325 Lublin, Poland
3University of Life Sciences, Faculty of Veterinary Medicine, Department of Epizootiology and Clinic of Infectious Diseases, Głęboka 30, 20-612 Lublin, Poland
4University of Life Sciences, Faculty of Veterinary Medicine, Department of Preclinical Veterinary Sciences, Sub-Department of Pharmacology, Akademicka 12, 20-033 Lublin, Poland

Fast and reliable identification of bacteria to at least the species level is currently the basis for correct diagnosis and appropriate treatment of infections. This is particularly important in the case of bacteria of the genus Enterococcus, whose resistance profile is often correlated with their species (e.g. resistance to vancomycin). In this study, we evaluated restriction endonuclease analysis of the 16S–23S rRNA gene intergenic transcribed spacer (ITS) region for species identification of Enterococcus. The utility of the method was compared with that of phenotypic methods [biochemical profile evaluation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)]. Identification was based on 21 Enterococcus reference strains, of the species E. faecalis, E. faecium, E. hirae, E. durans, E. casseliflavus, E. gallinarum, E. avium, E. cecorum and E. columbae, and 47 Enterococcus field strains isolated from pigs. Restriction endonuclease analysis of the ITS-PCR product using Hinfl, RsaI and MboI, restricted enzyme analysis of the ITS-PCR product using Hinfl, RsaI and MboI, in the order specified, enabled species differentiation of the Enterococcus reference and field strains, and in the case of the latter, the results of species identification were identical (47/47) to those obtained by MALDI-TOF MS. Moreover, as a result of digestion with MboI, a unique restriction profile was also obtained for the strains (3/3) identified by MALDI-TOF MS as E. thailandicus. In our opinion, restriction endonuclease analysis of the 16S–23S rRNA gene ITS region of Enterococcus may be a simple and relatively fast (less than 4 h) alternative method for identifying the species occurring most frequently in humans and animals.

INTRODUCTION

The genus Enterococcus comprises a ubiquitous group of over 30 bacterial species inhabiting extremely varied ecological niches. As commensal bacteria, they are part of the natural microbiota of the digestive tract in humans and animals. They are also isolated from soil, surface water, plants and foods of animal origin (Hayes et al., 2003). Enterococcus spp. are also included among indicator micro-organisms for determining water quality (faecal contamination) (Lleo et al., 2005; US Environmental Protection Agency, 1986).

The interest of clinical microbiologists, clinicians and epidemiologists is currently focused on bacteria of the genus Enterococcus as opportunistic microbes responsible for hospital infections in humans (the third most common causal agent after coagulase-negative Staphylococcus and...
Staphylococcus aureus) and sporadic infections in animals, including food animals (Kühn et al., 2000). In humans, they cause urinary tract infections, bacteraemia, endocarditis, peritonitis and wound infections (Sood et al., 2008), whilst in animals, they cause mainly diarrhoea in calves and mastitis in dairy cattle (Kühn et al., 2000). The species Enterococcus faecium and Enterococcus faecalis are responsible for 90% of infections (Sood et al., 2008). Other species, such as Enterococcus hirae, Enterococcus durans and Enterococcus avium, have been relatively rarely isolated from disease cases, but in the last decade a pronounced progressive increase in isolation of these species has been observed (Prakash et al., 2005).

Enterococcus spp. pose a serious threat to public health, due in part to their significant role in the dissemination and persistence of antimicrobial resistance (mainly to aminoglycosides, glycopeptides and β-lactam antibiotics) (Arias et al., 2010). Because of their varied virulence profile (Trivedi et al., 2011), as well as varied resistance among species to particular classes of chemotherapeutic agents, development of optimal therapy requires precise identification of the bacteria, to at least the species level. Commonly used classical identification methods, based mainly on morphology and biochemical characteristics, are extremely time consuming (Domig et al., 2003). The introduction of commercial tests and/or automatic identification methods (e.g. VITEK) has significantly reduced testing time, but their ability to differentiate to the species level is unsatisfactory (Moore et al., 2006). This is especially true for species with high phenotypic similarity (Devriese et al., 1993), newly discovered species and atypical strains.

The introduction of methods based on analysis of nucleic acids or proteins has made precise identification and classification of Enterococcus bacteria substantially faster and easier. Apart from the reference methods, i.e. multi-locus sequence analysis (MLSA) (Naser et al., 2005) and sequencing of 16S rRNA genes, for species identification of many bacterial species, including those of the genus Enterococcus (Domig et al., 2003), sequencing of selected genes has also been used (Poyart et al., 2000), as well as species-specific multiplex PCR (Jackson et al., 2004) and microarrays (Champagne et al., 2011). The methods used vary in terms of the number of differentiated species, difficulty of execution and cost.

Simple identification techniques, such as PCR-RFLP and species-specific PCR, are also available (Arias et al., 2006; Harwood et al., 2004; Velasco et al., 2004; Teng et al., 2001; Tyrrell et al., 1997), but these have either been used only for identification of a few Enterococcus spp. or their results are difficult to interpret.

In the present study, we adapted the intergenic transcribed spacer (ITS)-PCR technique used previously to identify Enterococcus spp. (Tyrrell et al., 1997), in combination with restriction endonuclease analysis, for species differentiation of Enterococcus strains isolated from domestic animals, and to assess identification agreement with selected classical diagnostics methods and with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

### METHODS

**Isolation of strains.** Reference strains were obtained from the following culture collections; American Type Culture Collection (ATCC), VA, USA; Netherlands Culture Collection of Bacteria (NCCB) Utrecht, the Netherlands; German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany; and Charité Berlin Culture Collection (CHB), Berlin, Germany. The study used 50 strains isolated from pig faeces and 21 reference strains of Enterococcus belonging to nine species (E. faecalis: ATCC 29212, ATCC 51299 vanB+®, NCCB 36031, DSM 20409; E. faecium: ATCC 35667, NCCB 46074, CHB 11037; E. avium: ATCC 49464, DSM 20063; E. gallinarum: ATCC 700425, DSM 20628; E. hirae: ATCC 10541, DSM 20160; E. casseliflavus: ATCC 700327, DSM 20382, DSM 7370; E. durans: ATCC 11576, DSM 20633; E. aerogenes: DSM 20683, DSM 20682; and E. columbae: DSM 7374). The field strains were isolated between June 2012 and May 2013 from the faeces of pigs (n=50) on breeding farms located in south-eastern and north-western Poland. The material in the form of smears was incubated in 2 ml brain–heart infusion (BHI) broth (Biocorp) for 3 h and then inoculated onto the selective medium (Sianetz and Bartley agar; Biocorp) and incubated at 37 °C for 48 h. One colony was selected from each culture for further identification. Preliminary identification to the genus Enterococcus was carried out on the basis of morphology (Gram staining) and basic biochemical characteristics [ability to grow in the presence of 6.5% NaCl, catalase test, ability to hydrolyse aesculin (bile aesculin agar; Biocorp) and pigment production]. Next, the species was determined using commercial tests: ENCOCCUTest (a short screening test based on eight characteristics) and STREPTOTest 24 (including 24 biochemical characteristics) (both tests provided by Erba Lachema). For both tests, the same ranges of values were used defining positive and negative identification: according to the manufacturer, the range of scores for positive identification is from 91 to 100% and is defined as GI (good identification); the range of values from 0 to 90% means only identification at the genus level or low probability of correct identification to the species level, and is defined as LD (low discrimination). For four Enterococcus spp. (E. faecium, E. durans, E. hirae and E. thailandicus) the ability to grow at pH 5.0 and growth at 10 °C were also determined.

**MALDI-TOF MS.** Bacteria were prepared for MS analysis according to a standard extraction protocol using formic acid, as recommended by the Bruker company (OP for Sample Preparation Using Formic Acid Extraction Method). A volume of 0.5 μl of the prepared material (full extraction of bacterial proteins) was applied to an MTP 384 ground steel target (BrukerDaltonics). An α-cyano-4-hydroxycinnamic acid matrix solution (suspended in a standard solution recommended by the manufacturer; BrukerDaltonics) was placed on each bacterial sample. The recommended BrukerDaltonics Bacterial Test Standard was applied in a configuration such that one location of the standard was positioned at the centre of four bacterial sample locations. Calibration was performed using a standard calibration mixture of an Escherichia coli extract (BrukerDaltonics) containing RNase A and myoglobin proteins. The spectrum of the bacteria was obtained in a positive linear manner, within a mass range of 2–20 kDa. The analysis was repeated three times for each sample. The bacterial spectra were acquired using flexControl 3.0 software, and then analysed using BrukerBiotyper 3.0.

For validation of the results, the following score values proposed by the manufacturer were used: a log(score) of ≥2 indicated good identification at the species level, a log(score) between 1.70 and 1.99
indicated a close relationship (at the genus level) and log(score) of <1.7 indicated unreliable identification.

**ITS-PCR.** ITS-PCR was carried out using the universal primers L1 and G1 (Jensen et al., 1993). DNA was isolated using 24 h bacterial cultures on BHI agar (Biocorp). Five bacterial colonies were suspended in 100 μl 1× TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.4) and the suspension was then subjected to a temperature of 95 °C for 15 min. The mixture was centrifuged for 3 min (10 500 g) (MiniSpin; Eppendorf). A 2 μl volume of the supernatant was used as a template for the reaction. The ITS-PCR was carried out in a T Personal thermal cycler (Biometra). The reaction was carried out using 25 μl reaction mixture composed of 12.5 μl Qiagen Taq PCR Master Mix (2.5 U Taq DNA polymerase, 200 μmol each nucleotide, 1.5 mM MgCl2) (Qiagen) and 15 pmol of each primer (Genomed S.A). The reaction conditions for the thermal cycler were as follows: initial cycle of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, after which an extension cycle of 72 °C for 7 min was added. Electrophoretic separation of PCR products was carried out simultaneously in 6 % polyacrylamide gel (Tyrrell et al., 1997) and 2 % agarose in 1× TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0) (Promega).

**RFLP.** Restriction endonucleases were selected on the basis of sequence analysis of the polymorphic ITS region between the genes encoding the 16S and 23S rRNA of the Enterococcus reference strains (http://www.ncbi.nlm.nih.gov/nuccore?term=16s-23s+enterococcus). The sequences underwent virtual restriction in Webcutter 2.0 software (http://rna.lundberg.gu.se/cutter2/). Five restriction endonucleases were initially selected: TaqI, HhaI, Rsal, MboI and Hinfl (Thermo Scientific). The PCR product was digested in 5 U enzyme per 8 μl product for 1 h at 37 °C, except for TaqI, whose activity required 65 °C for 2 h. Electrophoretic separation was carried out in 2 % agarose in 1× TBE buffer.

Due to the high phenotypic similarity between E. faecium and E. thailandicus, species-specific PCR for strains identified phenotypically (using biochemical tests) as E. faecium was conducted (amplification of the gene ddlE_faecium) (Dutka-Malen et al., 1995).

Agarose and polyacrylamide gels were documented using Gel Doc 2000 and analysed using Quantity One 4.2.0 software (Bio-Rad).

**RESULTS**

The classical phenotypic identification of the 50 isolates from pigs, classified preliminarily as Enterococcus (by growth on Slanetz and Bartley agar), was confirmed in 94 % (n=47) of cases. Three field strains were subsequently identified by MALDI-TOF MS as Streptococcus lutetiiensis. The commercial tests differentiated some of the Enterococcus isolates to the species level, with varying degrees of discrimination. The EN-COCCUStest determined only 19 % of strains (n=9) at a high level of identification (GI) with scores of 91.6–99.9 % (mean 95.6 %), which are acceptable results; these belonged to three Enterococcus spp.: E. faecium (n=5), E. durans (n=3) and E. avium (n=1) (Table S1 available in the online Supplementary Material). The STREPTOTest 24 had a greater ability to differentiate, with a high level of GI scores in the range 91.6–99.9 % (mean 97.9 %), obtained for 57 % of strains (n=27): E. faecium (n=5), E. durans (n=6), E. avium (n=1), E. faecalis (n=9) and E. hirae/dispar (n=6). The level of identification for the remaining strains with both tests was varied, ranging from 30.9 to 88.9 % (mean 67.4 %) and was characterized by the manufacturer as LD, an unacceptable result (Table S1).

**MALDI-TOF MS**

The isolates (n=50) were assigned in 94 % (n=47) of cases to eight species in the genus Enterococcus: E. faecalis (E. faecalis DSM 2570, n=6; E. faecalis DSM 6134, n=2; E. faecalis DSM 20409, n=1), E. faecium (E. faecium DSM 17050, n=5), E. durans (E. durans DSM 20633T, n=6), E. hirae (E. hirae DSM 20160T, n=6 and E. hirae DSM 3320, n=8), E. casseliflavus (E. casseliflavus DSM 7372, n=4), E. gallinarum (E. gallinarum DSM 20628T, n=4), E. avium (E. avium DSM 20063, n=1) and E. thailandicus (DSM 21767T, n=4). The three remaining strains (non-Enterococcus) were identified as Streptococcus lutetiiensis (Table S1). The level of identification for the vast majority of strains (41/50) was high: log(score) >2 (2.007–2.564, mean 2.281); in just nine cases the log(score) ranged from 1.813 to 1.972 (mean 1.897), for four strains of E. casseliflavus, two strains of E. gallinarum and three strains of E. thailandicus. The result of species-specific E. faecium PCR (ddlE_faecium) was positive for all (5/5) strains identified as E. faecium and negative for all (3/3) strains of E. thailandicus.

**ITS-PCR**

PCR was carried out for nine species of Enterococcus reference strains. Electrophoretic separation of the products in 6 % polyacrylamide gel (Fig. 1) and 2 % agarose gel (Fig. 2) yielded four clearly distinct profiles corresponding to Enterococcus spp. Irrespective of the type of gel visualization used, separate patterns were observed for E. durans, E. faecium and E. cocorum. In the case of 2 % agarose gels, the pattern for the remaining species was indistinguishable, whilst in the case of 6 % polyacrylamide gel, as described by Tyrrell et al. (1997), there was slight variation in the number of bands for E. gallinarum (the presence of additional bands) and in the size of bands for E. casseliflavus (smaller by about 40–50 bp) with respect to E. faecalis, E. avium, E. hirae and E. columbae. Electrophoresis in 6 % polyacrylamide gels showed additional high-molecular-size bands, as described by Tyrrell et al. (1997), running close to the upper edge of the gel (Fig. 1).

**RFLP**

Restriction analysis of ITS-PCR products enabled precise differentiation of the species of the Enterococcus reference strains. On the basis of preliminary research (unpublished data), three restriction endonucleases were established, Hinfl, Rsal and MboI, which, used in that order, were shown to be suitable for identifying nine Enterococcus species. Identification was carried out in three stages: digestion with Hinfl showed separate restriction patterns for E. faecalis, E. faecium, E. durans and E. cocorum (Fig. 3); digestion with Rsal distinguished another three species – E. casseliflavus, E. gallinarum and E. columbae (Fig. 4); and differentiation of
the two remaining species, *E. avium* and *E. hirae*, was obtained using *Mbo* I (Fig. 5). This procedure was used for species identification of 47 isolates of *Enterococcus* originating in pigs. Restriction endonuclease analysis made it possible to determine the species of the strains; the results were in agreement with those obtained using MALDI-TOF MS and corresponded to the restriction patterns of the species of the reference strains. The third stage of digestion, with *Mbo* I, additionally showed a restriction pattern distinct from that of *E. avium* and *E. hirae* for four strains identified by MALDI-TOF MS as *E. thailandicus* (Fig. 5).

**DISCUSSION**

The expansion in recent years of the number of *Enterococcus* spp. responsible for hospital infections (Reid et al., 2001; Chan et al., 2012) and their varied resistance profiles require the introduction of precise microbiological diagnostics. Current, simple procedures for species identification of bacteria focus mainly on distinguishing *E. faecium* and *E. faecalis*, which are responsible for over 90% of infections (Sood et al., 2008). Other species are generally distinguished by standard PCR using primers specific for each species (Jackson et al., 2004; Knijff et al., 2001), or by means of diagnostic methods that are significantly more expensive and not always available, such as MLSA, MALDI-TOF MS, sequencing of 16S rRNA genes and DNA–DNA hybridization (Domig et al., 2003).

The results of ITS-PCR obtained in the present study for reference strains of individual *Enterococcus* spp., which were in agreement with those obtained by Tyrrell et al. (1997), definitively identified only three species (*E. faecium, E. durans* and *E. cecorum*). In the remaining cases, there were no clearly visible differences or only slight ones – in the size or number of bands (e.g. *E. gallinarum*).

Separation of the ITS-PCR products in 6% polyacrylamide gel, as recommended by Tyrrell et al. (1997), provided maximum resolution but also exposed the presence of non-specific high-molecular-size bands and additional low-molecular-size bands, which appeared to be characteristic...
for each strain but not typical for a particular species of Enterococcus.

Visualization following PAGE using 6% gels of additional non-specific bands, together with the fact that the differences between the electrophoretic profiles of individual species were slight (40–50 bp), made the interpretation of results difficult in the case of a large and diversified pool of strains. The disadvantages of PAGE are toxicity and the time taken, especially for manual gel preparation. Although precast gels are available, their use can result in increased cost, as dedicated equipment is also required. We therefore sought to use agarose gels (2%) as an alternative. Separation in agarose gel (without restriction analysis of ITS-PCR products) showed a similar ability to differentiate, i.e. it definitively identified three Enterococcus spp., whilst the remaining species were not differentiated at all.

Increasing the sensitivity of ITS-PCR by introducing restriction endonuclease analysis in combination with visualization following agarose gel electrophoresis was the main purpose of our modification of the method described by Tyrrell et al. (1997). In the first stage, the endonuclease Hinfl made it possible to determine four characteristic patterns for four species: E. faecalis, E. faecium, E. durans and E. cecorum. Identification of the two most frequently isolated species, E. faecium and E. faecalis, from the pool of clinical isolates in the first stage of identification may significantly reduce the time required for diagnostics, including in testing for monitoring of drug resistance, which takes into account mainly these two species (SVARM Swedish Veterinary Antimicrobial Resistance Monitoring, NARMS National Antimicrobial Monitoring System).

The enzyme RsaI discriminated another three species (E. casseliflavus, E. gallinarum and E. columbae), and Mbol differentiated the remaining species of reference strains, E. hirae and E. avium.

The time required for the identification, despite the introduction of the additional step of digestion, is comparable to the time required to perform ITS-PCR according to Tyrrell et al. (1997). Genomic DNA isolation, ITS-PCR and restriction analysis carried out simultaneously for the three enzymes, and visualization on a 2% agarose gel together require less than 4 h. Interpretation of results is much easier and reproducible, because the differences in the size of the bands between profiles characteristic for different

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**Fig. 3.** RFLP patterns of ITS-PCR products with Hinfl digestion in a 2% agarose gel. Lanes: 1, DNA marker (Blirt); 2, E. faecalis ATCC 29212; 3, E. faecium ATCC 35667; 4, E. avium ATCC 49464; 5, E. durans ATCC 11576; 6, E. gallinarum ATCC 700425; 7, E. casseliflavus ATCC 700327; 8, E. columbae DSM 7374; 9, E. hirae ATCC 10541; 10, E. cecorum DSM 20683; 11, E. cecorum DSM 20682; 12, E. faecalis ATCC 51299 (vanB+); 13, E. faecium NCCB 46074; 14, E. thailandicus no. 36.

**Fig. 4.** RFLP patterns of ITS-PCR product with RsaI digestion in a 2% agarose gel. Lanes: 1, DNA marker (Gene Ruler ThermoScientific); 2, E. avium ATCC 49464; 3, E. hirae ATCC 10541; 4, E. thailandicus no. 36; 5, E. columbae DSM 7374; 6, E. casseliflavus ATCC 700327; 7, E. gallinarum ATCC 700425; 8, E. casseliflavus DSM 20382.
species are at least 100 bp (Figs 3–5). Furthermore, visualization using 2% agarose gels reduces variability within the species profiles for individual strains, as observed by Tyrrell et al. (1997).

The use of this method to identify clinical isolates from pigs was characterized by repeatability of species restriction patterns obtained for the reference strains and complete agreement with the species identification of strains using MALDI-TOF MS. In the present study, the results made it possible to evaluate the reproducibility and precision of both our own modification of ITS-PCR and methods based on morphology and biochemical characteristics.

It is also worth emphasizing that a unique restriction pattern was distinguished in the final stage, i.e. digestion with \( MboI \), which was characteristic for all four of the strains identified in MALDI-TOF as \( E. thailandicus \). Moreover, the phenotypic characteristics of these strains were also in agreement with the profile for this species determined by Tanasupawat et al. (2008) and Shewmaker et al. (2011). Due to the high phenotypic similarity of the species \( E. thailandicus \) and \( E. faecium \) and the lack of a reference strain of \( E. thailandicus \) in our collection, a negative result for the species-specific \( ddl \) \( E. faecium \) PCR served as additional confirmation of the identification.

Classical species identification based on morphology and biochemical characteristics enabled eight species to be distinguished in the 50 strains isolated from pigs; definitive identification, considered a positive result by the manufacturer, was obtained for 16 or 45% of strains, depending on the test. Difficulties in obtaining definitive identification were encountered mainly in the case of \( E. casseliflavus \), \( E. gallinarum \), \( E. thailandicus \) and \( E. hirae \); for instance, \( E. hirae \) was classified as \( E. faecalis ascaccharolyticus \), and \( E. thailandicus \) as \( E. faecium \). Similar results have been obtained by other authors (Jurković et al., 2006; Lavová et al., 2014), which can be explained by the high homogeneity of \( Enterococcus \) spp. and the high phenotypic variability within individual species.

In conclusion, the method developed enables rapid identification of the \( Enterococcus \) spp. isolated most frequently from people and animals and is relatively simple to execute. Its results were in full agreement with those obtained in parallel using MALDI-TOF MS. We believe that this method can be a significant alternative to current diagnostic ‘gold standards’, such as analysis of conserved sequences of DNA fragments or MALDI-TOF MS.

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