Characterization of enterococci of animal and environmental origin using phenotypic methods and comparison with PCR based methods

A. Brtkova[1], M. Filipova[1], H. Drahovska[2], H. Bujdakova[2]

[1]State Veterinary and Food Institute, Dolny Kubin, Slovak Republic
[2]Faculty of Natural Sciences, Comenius University, Bratislava, Slovak Republic

ABSTRACT: The purpose of this study was to evaluate the discriminatory power of *ddl*-PCR (D-alanin-D-alanin ligase PCR) and ITS-PCR (Internal Transcribed Spacer PCR) for accurate identification of enterococcal species in comparison with phenotypic assays. Results confirm previous published data that *ddl*-PCR simple approach allows rapid identification of two the most frequently isolated spp. *E. faecalis* and *E. faecium*. When identification points towards other enterococci then that mentioned above, the ITS-PCR seems to be suitable complementary assay. Correct identification of enterococci to species level is important because of different spp. susceptibility to some clinically important antibiotics. For example, it is necessary to distinquish acquired vancomycin resistance from inherent one that is less epidemiologically important. Additionally, both methods can be valuable in epidemiological studies following enterococcus resistance gene transfer within human population.

Keywords: *Enterococcus;* identification; D-alanin-D-alanin ligase PCR; Internal Transcribed Spacer PCR

The occurrence of environmental or animal enterococci resistant to different antimicrobial agents represents high risk for transmission of these bacteria to humans (Hayes et al., 2003; Moreno et al., 2006). Enormously increase of microorganisms resistant to different agents (Kolar et al., 2002; Sustackova et al., 2004; Appelbaum and Jacobs, 2005) has resulted in the decision of European Union to support the antimicrobial resistance research due to European surveillance plan (Directive 2003/99/EC). The rules for monitoring have been laid down in Directive 2003/99/EC and it has been transposed to Slovak Regulation No.626/2004. Scope of this regulation covers monitoring of zoonotic agents that could have a significant impact on the public health as well as (Slovak Regulation No.626/2004) can be a reservoir of antimicrobial resistance genes (Dzidic and Bedekovic, 2003). In the last two decades the genus *Enterococcus* has been believed to be one of the most common causative agens of nosocomial diseases in humans following staphylococci (DeLisle et al., 2003; Marothi et al., 2005; Hayden et al., 2006). Additionally, enterococci are employed as vectors in dissemination of resistance genes inside as well as outside of genus. They possess very effective mechanisms for exchange of genetic material and ability for resistance gene accumulation. This feature shifts them to the group of potential reservoirs of resistance genes with significant risk of resistance transfer to obligate pathogens, such a *Staphylococcus* spp. or *Listeria* spp. (Dzidic and Bedekovic, 2003; Belicova et al., 2007; Niederhausern et al., 2007; Paoletti et al., 2007) Additionally, some vancomycin-resistant enterococci belonging to the group of zoonoses (Slovak Regulation No.626/2004) can be transmitted to human via food chain. The members of enterococci are different in virulence and pathogenicity, meanwhile *E. faecalis* and *E. faecium* have dominant position in human diseases (Tacconelli and Cataldo, 2008; Hayden et al., 2008). Recent studies have revealed that the incidence of more unusual species such as *E. durans, E. hirae, E. gallinarum* and *E. casseliflavus/flavescens* has

Supported by the Slovak Ministry of Education (Grants No. VEGA 1/0320/09 and No. VEGA 1/0344/10).

significantly increased in clinical material (Blahova et al., 2007). For the most microbiological laboratories, the primary methods of identification of *Enterococcus* strains rely on phenotypic and biotyping characterization. It means cultivation of the isolates on specific cultivation media (Brain Infusion agar, Slanetz-Bartley agar, Bile-Aesculine agar) (Doming et al., 2003; Ramotar et al., 2006), characterization their morphology and identification by appropriate biotyping methods (En-coccus test, API20 Strep, Merlin system) (Delmans et al., 2008) based on determination of enzyme profile. Enterococci are intrinsically resistant to many antibiotics including β-lactams. Susceptibility to ampicillin may be of help as a species indicator. *E. faecium* isolates are significant less susceptible to ampicillin than *E. faecalis* isolates because of markedly lower affinity to penicillin-binding proteins for this antibiotic (Wiliamson, 1985; Agarwal et al., 2009).

Identification of enterococci using phenotypic means is a procedure taking for 1–2 days (Drahovska et al., 2002). In addition, time to time automated systems failly identify rare species (Tritz et al., 1990). For this reason, molecular techniques, randomly amplified polymorphic DNA analysis, ribotyping (Price et al., 1999), intergenic ribosomal PCR (Tyrell et al., 1997) have been developed to identify enterococci at the species level. Although these techniques are specific and sensitive, it is difficult to adapt them for common use in laboratories, because of consumables and requirement for highly skilled persons. The *ddl*-PCR (D-alanin-D-alanin ligase PCR) (Dutka-Malen et al., 1995) and *vanC* PCR (*vanC* operon coding for intrinsic resistant to a low level of glycopeptides) is an exception (Dutka-Malen et al., 1995) as they are useful in routine practice. The purpose of this study was to point at the discrimination power of the simple *ddl*-PCR and *vanC* PCR techniques and possibilities of the use of the highly sensitive ITS-PCR (Internal Transcribed Spacer PCR) in comparison with commercial biochemical tests in routine microbiological practice.

MATERIAL AND METHODS

Enterococcal isolates and reference strains

Isolates for microbiological examination (97 samples) were taken from the cloacal swabs of three-weeks old chickens and from the environment (chicken's waterfeed) originated from several farms in the North part of Slovakia.

Each cloacal swab was resuscitated in 9 ml Buffered Peptone Water (Oxoid, Basingstoke, Hants, United Kingdom) at 37 ± 1°C for 24 ± 2 h. One loop of the overnight suspension was inoculated on a selective medium containing bile, aesculin and aside (Slanetz Bartley agar, HiMedia, Mumbai, India) and incubated at 37 ± 1°C for 48 ± 2 h.

100 ml of waterfeed were filtrated through the membrane filter system (Milipore Ltd., Hertfordshire, UK). Used filter (0.47 μm pore size) was lying on a selective medium containing bile, aesculin and aside (Slanetz Bartley agar, HiMedia) and incubated at 37 ± 1°C for 48 ± 2 h. Suspected colonies of *Enterococcus* spp. were transferred to a non-selective medium containing sheep blood (Columbia agar, Oxoid). Samples were incubated at 37 ± 1°C for 24 ± 2 h. Presumptive isolates were Gram-positive and haemolytic negative cocci. They did not produce catalase and oxidase enzymes and produced enzyme pyrolidonylarylamidase (Mikrola-test, Pliva-LaChema, Brno, Czech Republic). Isolates were kept in cryotubes in accordance with instructions of manufacturer and stored at

–70°C until used. Standard reference strains used in this study are listed in the Table 1.

Biochemical methods of identification

Biochemical properties of *Enterococcus* spp. were determined by using En-coccus test (Pliva-LaChema) and API20 Strep (bioMerieux, Marcy l'Etoile, France). Before determination by En-coccus, the isolates were resuscitated on Columbia agar (Oxoid) for 24 h at 37 ± 1°C. After that, inoculum was prepared by suspending of overnight growth colonies and the suspension was adjusted to equal 2 McFarland standard (6×10^8 CFU/ml). Each isolate was inoculated into a set of En-coccus tests (Pliva-LaChema). All tests were performed as recommended by manufacturer. Code number profile obtained from the coloured change (after adding appropriate reagents) was evaluated by TNW Pr.6.5 Analytic Profile Index (Pliva-LaChema). Tests were conducted in a short period of time (max. 10 min) and by the same person. Accuracy and reproducibility were verified by the reference strains (Table 1).

Random selected isolates (20 isolates) were identified by using API20 Strep (bioMerieux). Before

Table 1. Reference strains used in this study

Reference strain	Origin	Specification
Enterococcus raffinosus CCM 4216	Czech Collection of Microorganisms, Brno, Czech Republic	En-coccus
Enterococcus durans CCM 5612	Czech Collection of Microorganisms, Brno, Czech Republic	En-coccus
Streptococcus uberis ATCC 700407	American Type Culture Collection, Manassas, Virginia, USA	API20 Strep
Enterococcus faecium CCM 6226	Czech Collection of Microorganisms, Brno, Czech Republic	*ddl*–PCR, ITS-PCR
Enterococcus faecalis CCM 4224	Czech Collection of Microorganisms, Brno, Czech Republic	*ddl*–PCR, ITS-PCR
Enterococcus gallinarum CCM 2397	Czech Collection of Microorganisms, Brno, Czech Republic	*vanC1,* ITS-PCR
Enterococcus casseliflavus CCM 2478	Czech Collection of Microorganisms, Brno, Czech Republic	*vanC2/C3,* ITS-PCR

determination by API20 Strep, the isolates were resuscitated on Blood agar (Oxoid) for 24 h at 37 ± 1°C. After that, inoculum was prepared by suspending of overnight growth colonies and the suspension was adjusted to equal four McFarland standard (1.2 × 10^9 CFU/ml). Each isolate was inoculated into a set of API20 Strep tests (bioMerieux). All tests were performed as recommended by manufacturer. Code number profile obtained from the coloured change (after adding appropriate reagents) was evaluated by API Analytic Profile Index (bioMerieux). For discrimination between *E. faecium and E. faecalis*, an additional test tube for assimilation of arabinose was performed according to Clark et al. (1993). Arabinose is utilized by *E. faecium*, but not by *E. faecalis* isolates. Acid production from ribose is the only biochemical test capable to discriminate *E. casseliflavus* from *E. flavescens* (Clark et al., 1993). Only *E. casseliflavus* is able to utilize ribose.

Molecular methods of identification

DNA preparation. Enterococci were cultivated on non-selective medium Columbia agar (Oxoid). The inoculum was prepared by suspending of overnight culture into the 100 µl STE (1M NaCl, 1M Tris, 0.5M EDTA, H_2O). Suspension was boiled 10 min at 95°C, and centrifuged 5 min at 13 000 × g. The supernatant containing DNA was used as a template.

The *ddl*-PCR and *vanC1* ligase genes, *vanC2/C3* ligase genes PCR were made according to Dutka-

Table 2. Oligodeoxynucleotide primers used in this study

Amplified gene	Size of PCR product (bp)	Sequence (5' to 3')	Reference
ddl $_{E. faecalis}$	941	5'-ATCAAGTACAGTTAGTCT-3' 5'-ACGATTCAAAGCTAACTG-3'	Dutka-Malen et al., 1995, Drahovska, 2002
ddl $_{E. faecium}$	550	5'-TAGAGACATTGAATATGCC-3' 5'-TCGAATGTGCTACAATC-3'	Dutka-Malen et al., 1995, Drahovska, 2002
vanC1	822	5'-GGTATCAAGGAAACCTC-3' 5'-CTTCCGCCATCATAGCT- 3'	Dutka-Malen et al., 1995, Janoskova et al., 2004
vanC2/C3	439	5'-CTCCTACGATTCTCTTG-3' 5'-CGAGCAAGACCTTTAAG-3'	Dutka-Malen et al., 1995, Janoskova et al., 2004
ITS	Various	5'-CAAGGCATCCACCGT-3' 5'-GAAGTCGTAACAAGG-3'	Jensen et al., 19993

Table 3. Results obtained from En-coccus and *ddl*-PCR + *vanC* PCR

	En-coccus	*ddl*-PCR + *van*C	Final identification
E. faecium	49	24	24
E. faecalis	0	34	34
E. gallinarum	0	4	4
E. casseliflavus/flavescens	0	35	35
Any of species mentioned above	48	0	0

Malen et al. (1995) and other autors (Drahovska, 2002; Janoskova and Kmet, 2004) as follows: reactions were performed by using DNA Personal Cycler 20Biometria (Whatman, Dassel, Germany) in 0.5 ml MicroAmp reaction tubes. *Taq* DNA polymerase was purchased from Hoffman-LaRoche, Basel, Switzerland. Amplifications were performed in mixtures containing 2.5 mmol/l $MgCl_2$ (Hoffman-LaRoche), 1 IU of *Taq* DNA polymerase (Hoffman-LaRoche), 200 µmol/l dNTP (Hoffman-LaRoche). The final reaction volume of 25 µl contained 50 pmol of each primer (Table 2, Applied Biosystems, Foster City, California, USA) and 2 µl enterococcal DNA. Reaction conditions for *ddl*-PCR and *vanC1*, *vanC2/C3*-PCR were as follows: an initial denaturation of 94°C for 1 min (amplicons $ddl_{E.\ faecalis}$, $ddl_{E.\ faecium}$) or 94°C for 2 min (amplicons *vanC1*, *vanC2/C3*) followed by 30 cycles: denaturation of 90°C for 30 s, anelation of 54°C for 30 s, polymerization of 72°C for 60 s, after which an extension cycle of 72°C for 8 min was added.

The ITS-PCR was done according to Drahovska et al. (2002) as follows: amplicons were generated by an initial denaturation of 94°C for 2 min followed by 30 cycles: denaturation of 90°C for 30 s, anelation of 54°C for 30 s, polymerization of 72°C for 60 s, after which an extension cycle of 72°C for 8 min was added.

For analysis, 10 µl aliquot of amplicon was electrophoresed on 1.5% agarose gel (Sigma-Aldrich, Brooklyn, New York, USA) using the horizontal electrophoresis apparature (Pharmacia, Stockholm, Sweden). The gels were stained with ethidium bromide (1 µg/ml) 20 min and photographed on a UV transilluminator – Gel-doc (Bio-Rad, Bratislava,

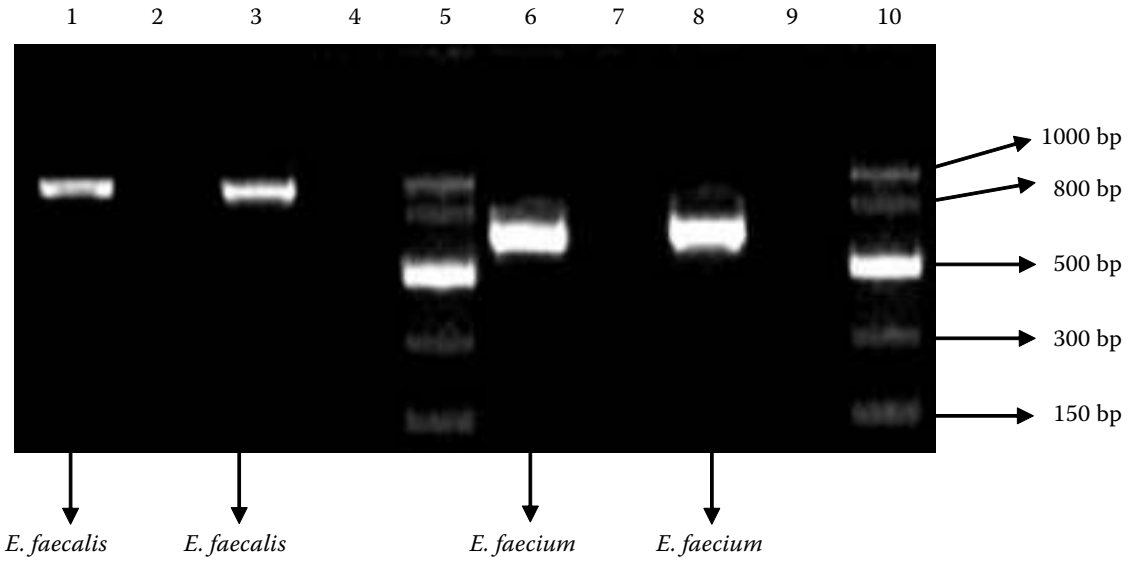

Figure 1. Detection of $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ by PCR. Line 1 = positive control CCM 4224 *E. faecalis*, line 2 = negative control – blank, line 3 = isolate *E. faecalis*, line 4 = negative control – blank, line 5 = DNA Marker C – 1000, 800, 500, 300, 150, 50 bp, line 6 = positive control CCM 6226 *E. faecium*, line 7 = negative control – blank, line 8 = isolate *E. faecium*, line 9 = negative control – blank, line 10 = DNA Marker C-1000, 800, 500, 300, 150, 50 bp

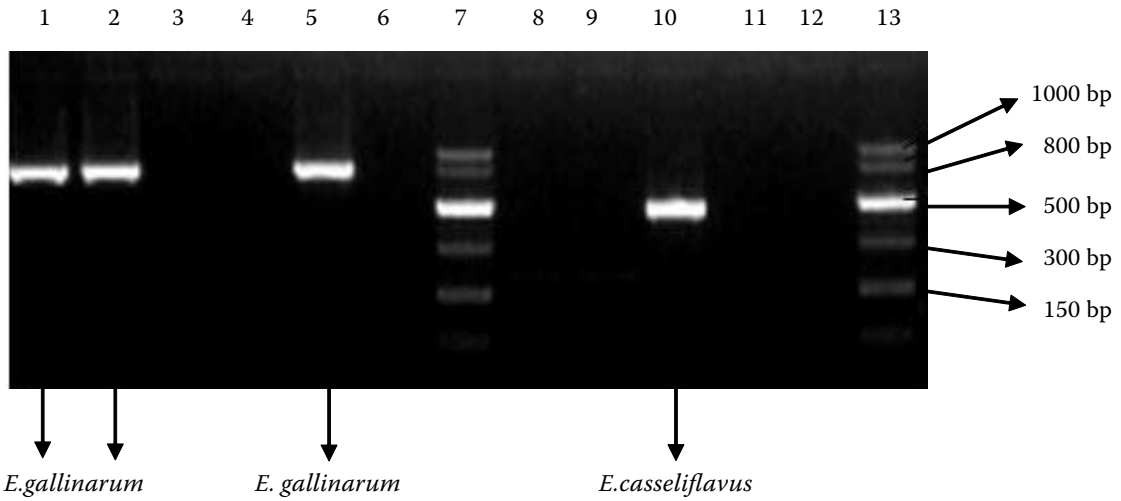

Figure 2. Detection of *vanC1* and *vanC2/C3* by PCR. Lines 1, 2 = *E. gallinarum* isolates, lines 3, 4 = negative control – blank, line 5 = positive control CCM 2397 *E. gallinarum*, line 6 = negative control – blank, line 7 = DNA Marker C-1000, 800, 500, 300, 150, 50 bp, lines 8, 9 = negative control – blank, line 10 = positive control CCM 2478 *E. casseliflavus*, lines 11, 12 = negative control – blank, line 13 = DNA Marker C – 1000, 800, 500, 300, 150, 50 bp

Slovakia). DNA Marker C – (SBS Genetech Co., Beijing, China) was used as a standard.

ITS-PCR products were purified by ethanol precipitation and resuspended in deionised water. Amplicons were separated on the automatic genetic analyzer ABI Prism 3100 Avant (Applied Biosystems) using 36 mm long cappilary, POP4 polymer and running time 1700 s. DNA fragments were sized based on Liz-500 DNA standard using GeneMapper 3.7 software (Applied Biosystems). The obtained results were exported and stored in Microsoft Excel (Redmond, Washington, USA). For species identification, ITS-PCR profiles of isolates were compared with the profiles of collection strains. Standard reference strains used in this study are listed in the Table 1.

RESULTS

There were total 97 isolates classified to the genus *Enterococcus.* All enterococci were initially assigned a species designation on the basis of commercially available biochemical En-coccus test. 49 out of 97 tested isolates were identified as *E. faecium* (Table 3). These isolates were also confirmed to be *E. faecium* by tube test for assimilation of arabinose. Randomly selected 20 isolates identified by En-coccus test as *E. faecium* were submitted for identification emploing API20 Strep. Using this test, eight isolates did not show enzymatic profile characteristic for *E. faecium* originally identified by En-coccus.

All 97 isolates were submitted for *ddl*-PCR. The 34 out of 97 isolates were identified as *E. faecalis* and 24 as *E. faecium* (Figure 1). The remaining 39 isolates were subjected for *vanC1*, *vanC2/C3* PCR to identify *E. gallinarum* and *E. casseliflavus/flavescens*. The PCR confirmed 35 isolates as *E. casseliflavus/flavescens* and four isolates as *E. gallinarum* (Figure 2). Acid production from ribose is the only biochemical test capable to discriminate *E. casseliflavus* from *E. flavescens* (Clark et al., 1993). Only *E. casseliflavus* is able to utilize ribose, 35 isolates were identified as *E. casseliflavus.*

Randomly selected 18 isolates were submitted for ITS-PCR. Using this method, we totally identified one *E. gallinarum* isolate, three isolates of *E. faecium* and 14 isolates of *E. faecalis*.

DISCUSSION

Enterococci are natural residents of the human and animal gastrointestinal tract, many species are found in soil, plants and food as well. These organisms also form an important part of the microflora of many cheeses, where they can reach level of 10^7 to 10^8 CFU/g. There is contradictory information on their role in flavour development in cheese with some studies showing that they have a positive effect, but others described negative ones (Becquet,

2003; Lund et al., 2006). *E. faecalis*, *E. faecium* and *E. durans* are still the most important species found in food, though recent results proved that *E. casseliflavus* may be also important. Many of these species withstand pasteurisation. Their presence in food has been questioned because they can easily transfered antibiotic resistance genes including that to vancomycin (Belicova et al., 2007; Niederhausern et al., 2007; Paoletti et al., 2007). In our collection of 97 enterococcal isolates, *E. faecalis* were confirmed as a dominant species. Results are in concern with study of Francz et al. (2003), found that *E. faecalis* belong to one of the predominant species in the Gram-positive cocci isolated from chicken samples collected at poultry abattoirs. On the other hand, the previous work from Slovakia published by Krocko et al. (2007) described *E. faecium* as the predominant species isolated from poultry abdomen. For identification, biochemical tests like En-coccus and API20 Strep were used. En-coccus test is commonly used in both clinical and veterinary microbiological laboratories. Results from this test showed big discrepancies in comparison with other biochemical tests (API20 Strep, Merlin System). It should be explained due to presence of low number (only eight) specific sugar substrates, while API20 Strep has a wide scale (20 substrates) with good discriminate value. Identification of animal and environmental enterococci is tricky because of large spectrum of species. In accordance with the newest data, there are 47 known enterococcal species of clinical, animal and environmental origin. Some authors declare that simple biochemical tests like En-coccus are not able to recognize some enterococci, especially unusual spp. En-coccus is able to identify only 19 enterococcal isolates. Lactose-negative strains of *E. faecalis* can be misidentified as *E. solitarius*. Distinguish supplementary biochemical test includes growth in 0.04% of tellurit, on which isolates of *E. faecalis* are able to growth (Ruoff et al., 1990). Utilization test of methyl-α-D-glucopyranosid is needed for separation between *E. gallinarum* and *E. faecium/E. faecalis* (Devriese et al., 1996). Conflicting outcomes have also been published regarding the utilization of sorbitol. Study of Day et al., (2001) showed that 16 out of 18 *E. faecium* isolates utilizated sorbitol, on the other hand, Facklam and Sahm (1995) indicated that over 97% isolates of *E. faecium* were not able to utilize sorbitol. Specific sugar substrates mentioned above are not in En-coccus test. Thus, problem with En-coccus test evocate application of additional tests, but identification is prolonged for next 1–2 days. Other problem with En-coccus test is associated with individual personal experiences and manual reading of the results. The interpretation is frequently fail because of insufficient colour change. In comparison with biochemical tests, single PCR seems to be more rapid, correct and not expensive method for identification of the most frequently occurred enterococcal isolates (Drahovska et al., 2004; Filipova, 2008).

Results obtained from En-coccus biochemical test and from *ddl*-PCR + *vanC* PCR are compared and summarized in Table 3.

Dutka-Malen et al. (1995) designed a PCR assay that allows simultaneous identification to the species level of clinically relevant enterococci (*Enterococcus faecium, E. faecalis, E. gallinarum, E. casseliflavus/flavescens*). This assay is based on specific amplification of internal fragments of *ddl* genes encoding D-alanine-D-alanine ligase of *E. faecalis* and *E. faecium*. Using this technique, we concluded that 34 isolates belonged to *E. faecalis* and 24 *E. faecium*. The remaining 39 isolates were not possible to involve into these species. These isolates were analysed for the presence of *vanC* operon coding for intrinsic resistance to a low level of glycopeptides. This resistance is characteristic for *E. gallinarum* – (*vanC1* ligase genes) and *E. casseliflavus/flavescens* – (*vanC2/C3* ligase genes). These species are very common in animal, soil, plants or food. In chicken's cloacal swabs, especially, *E. gallinarum* and *E. casselflavus/flavescens* is observed. Our results confirmed 35 isolates being identified as *E. casseliflavus/flavescens* and four isolates as *E. gallinarum*. Due to the close genomic pattern (*E. casseliflavus* and *E. flavescens* are phylogenetically closely related, as evidence by 16S rRNA sequence homologies of over 98.8%), it was necessary to distinguish these two species with an additional biochemical test – ribose utilization. Finally, 35 isolates were identified as *E. casseliflavus* and 0 isolates as *E. flavescens*. In spite of the facts that PCR for *vanC1, vanC2/C3* is simple molecular approach for rapid and precise identification of enterococci allowing to avoid the drawbacks of biochemical tests, it must be supplemented by ribose tube test, what takes more time. Additionally, this assay certainly fails to identify some enterococcal species outside the reach of the primers.

Barry et al. (1991) and Jensen et al. (1993) overcame the problem of minimal variability by examining the *16S-23S rDNA* (genes coding for *rRNA*)

intergenic region, referred to as the *I*nternally *T*ranscribed *S*pacer region (ITS-PCR). The point of this method is the amplification of the intergenic *23S* and *16S rRNA* gene regions, specific for the individual species of enterococci. It was suggested that this segment of DNA would be under minimal selective pressure compared to the selective pressure that *rRNA* genes would be under and therefore may allow for species identification due to enhanced variability between species within a genus. ITS-PCR is able to identify the majority of enterococci from the Facklam and Collins typing schemes (Facklam and Collins, 1989) – Group I (*E. avium*, *E. raffinosus*, *E. malodortatus*, *E. pseudoavium*), Group II (*E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. mundtii*, *E. gallinarum*) and Group III (*E. durans*, *E. hirae*). ITS-PCR is also used for the identification of phenotypic-aberant isolates (Tyrell et al., 1997). For this identification, we ramdomly selected 18 isolates. We identified one *E. gallinarum* isolate, three isolates of *E. faecium* and 14 isolates of *E. faecalis*. Identification of *E. faecalis* and *E. faecium* were in agreement with the result of *ddl*-PCR. In spite of the fact that we do not advocate the use of ITS-PCR in place of *ddl*-PCR in rapid identification of enterococci, it seems to be an important method for identification of the other species mentioned above. Moreover, this methodology could be used for epidemiological purpose like vancomycin-resistant enterococci outbreaks or in the reference laboratories for identification of problematic isolates. In conclusion, *ddl*-PCR is very precise, easy applicable and valid method useful for rapid routine laboratory identification of *E. faecalis* and *E. faecium* in comparison with commercial biochemical approaches.

REFERENCES

Agarwal J, Kalyan R, Singh M (2009): High-level aminoglycoside resistance and β-lactamase production in enterococci at a tertiary care hospital in India. Japan Journal of Infection Diseases, 62, 158–159.

Appelbaum PC, Jacobs MR (2005): Recently approved and investigational antibiotics for treatment of severe infections caused by Gram-positive bacteria. Current Opinion in Microbiology, 8, 510–517.

Barry T, Colleran G, Glennon M, Dunican LK, Gannon F (1991): The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Applied, 1, 51–56.

Becquet P (2003): EU assessment of enterococci as feed additives. International Journal of Food Microbiology, 88, 247–254.

Belicova E, Krizkova L, Krajcovic J, Jurkovic D, Sojka M, Ebringer L, Dubinsky R (2007): Antimicrobial susceptibility of *Enterococcus* species isolated from Slovak bryndza cheese. Folia Microbiologica, 52, 115–119.

Blahova J, Kralikova K, Krcmery Sr. V, Babalova M, Menkyna R, Glosova L, Knotkova H, Liskova A, Molokacova M, Vaculikova A, Bruckmayerova D, Rovny I (2007): Four years of monitoring antibiotic resistance in microorganisms from bacteremic patients. Journal of Chemotherapy, 19, 665–669.

Clark NC, Cooksey RC, Hill BC, Swenson JM, Tenover FC (1993): Characterization of glycopeptide-resistant enterococci from US hospitals. Antimicrobial Agents and Chemotherapy, 37, 2311–2317.

Day AM, Sandoe JET, Cove JH, Phillips-Jones MK (2001): Evaluation of biochemical test scheme for identifying clinical isolates of *Enterococus faecalis* and *Enterococcus faecium*. Letters in Applied Microbiology, 33, 392–396.

DeLisle S, Perl TM (2003): Vancomycin-resistant enterococci: a road map on how to prevent the emergence and transmission of antimicrobial resistance. Chest, 123, 504–518.

Delmans J, Chacornas JP, Robin F, Gianmarinaro P, Talm R, Bonnet R (2008): Evaluation of the Vitek 2 system with a variety of *Staphylococcus species*. Journal of Clinical Microbiology, 46, 311–313.

Devriese LA, Cruz Coloque JI, De Herdt P, Haesebrouck F (1996): Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats. Journal of Applied Microbiology, 73, 421–425.

Directive 2003/99/EC of European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. Official Journal of the European Union, 46, L 325, 31–40.

Doming KJ, Mayer HK, Kneifel W (2003): Methods used for the isolation, enumeration and identification of *Enterococcus* spp. 1. Media for isolation and enumeration. International Journal of Food Microbiology, 88, 165–188.

Drahovska H (2002): Molecular-biological methods of identification and typing of pathogens. [Academic dissertation.] Comenius University, Faculty of Natural Sciences, Bratislava, 172 pp.

Drahovska H, Kocinova D, Seman M, Turna J (2002): PCR – based methods for identification of *Enterococcus* species. Folia Microbiologica, 47, 649–653.

Drahovska H, Kocinova D, Seman M, Koncekova R, Trupl J, Turna J (2004): Antibiotic resistance and virulence factors among clinical and food enterococci isolated in Slovakia. Folia Microbiologica, 49, 763–768.

Dutka-Malen S, Evers S, Courvalin P (1995): Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. Journal of Clinical Microbiology, 33, 24–27.

Dzidic S, Bedekovic V (2003): Horizontal gene transfer – emerging multidrug resistance in hospital bacteria. Acta Pharmacologica, 24, 519–526.

Facklam RR, Collins MD (1989): Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. Journal of Clinical Microbiology, 27, 731–734.

Facklam R R, Sahm DF (1995): Enterococcus. Manual of Clinical Microbiology. 6th ed. American Society for Microbiology Press, Washington D.C. 308–314.

Filipova M. (2008): Mechanisms of aminoglycoside resistance and virulence factors in clinical isolates *Enterococcus faecalis* and *Enterococcus faecium*. [Academic dissertation.] Comenius University, Faculty of Natural Sciences, Bratislava, 164 pp.

Francz CM, Stiles ME, Schleifer KH, Holzapfel WH (2003): Enterococci in foods – a conundrum for food safety. International Journal of Food Microbiology, 88, 105–122.

Hayden MK, Bonten MJ, Blom DW, Lyle EA, van de Vijver DA, Weinstein RA (2006): Reduction in acquisition of vancomycin-resistant enterococci after enforcement of routine environmental cleaning measures. Journal of Clinical Infectious Diseases, 42, 1552–1560.

Hayden MK, Blom DW, Lyle EA, Moore CG, Weinstein RA (2008): Risk of hand or glove contamination after contact with patients colonized with vancomycin-resistant enterococci or the colonized patients environment. Infection Control and Hospital Epidemiology, 29, 149–154.

Hayes JR, English LL, Carter PJ (2003): Prevalence and antimicrobial resistance of *Enterococcus* species isolated from retail meats. Applied and Environmental Microbiology, 12, 7153–7160.

Janoskova A, Kmet V (2004): Vancomycin resistance in *Enterococcus* spp. Strains isolated from Alpine accentor and chamois. Acta Veterinaria, 73, 211–214.

Jensen MA, Webster JA, Strauss N (1993): Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Applied and Environmental Microbiology, 59, 945–952.

Kolar M, Pantucek R, Bardon J, Vagnerova I, Typovska H, Valka I, Doskar J. (2002): Occurrence of antibiotic-resistant bacterial strains isolated in poultry. Veterinarni Medicina, 47, 52–59.

Krocko M, Canigova M, Duckova V (2007): Occurence, isolation and antibiotic resistance of *Enterococcus* species isolated from raw pork, beef and poultry. Journal of Food and Nutrition Research, 46, 91–95.

Lund B, Billstrom H, Edlund C (2006): Increased conjugation frequencies in clinical *Enterococcus faecium* strains harbouring the enterococcal surface protein gene *esp*. Clinical Microbiology and Infection, 12, 588–591.

Marothi YA, Agnihotri H, Dubey D (2005): Enterococcal resistance – an overview. Indian Journal of Medicine Microbiology, 4, 214–219.

Moreno MRF, Sarantinopoulos P, Tsakalidou E, DeVuyst L (2006): The role and application of enterococci in food and health. International Journal of Food Microbiology, 106, 1–24.

Niederhausern S, Sabia C, Messi P, Guerrieri E, Manicardi G, Bondi M (2007): Van A-type vancomycin-resistant enterococi in equine and swine rectal swabs and in human clinical samples. Current Microbiology, 55, 240.

Paoletti C, Foglia G, Princivalli MS, Magi G, Guaglianone E, Donelli G, Pruzzo C, Biovasco F, Facinelli B (2007): Co-transfer of vanA and aggregation substance genes from *Enterococcus faecalis* isolates in intra and inter-species matings. Journal of Antimicrobial Chemotherapy, 59, 1005–1009.

Price TM, Wilson RD, Kulski JK (1999): Identification of enterococci by ribotyping with horseradish-peroxidase-labelled 16S rDNA probes. Journal of Microbiological Methods, 36, 147–155.

Ramotar K, Woods W, Larocque L, Toye B (2006): Comparison of phenotypic methods to identify enterococci intrinsically resistant to vancomycin (VanC VRE). Diagnostic Microbiology and Infectious Diseases, 36, 119–124.

Ruoff KL, De La Maza L, Murtagh MJ, Spargo JD, Ferraro M J (1990): Species identities of enterococci isolated from clinical specimens. Journal of Clinical Microbiology, 28, 435–437.

Slovak Regulation No. 626/2004 on the monitoring of zoonoses and zoonotic agents. Statue of SR, 268, 6242–6249.

Sustackova A, Napravnikova E, Schlegelova J (2004): Antimicrobial resistance of *Enterococcus* spp. isolates from raw beef and meat products. Folia Microbiologica, 49, 411–417.

Tacconelli E, Cataldo MA (2008): Vancomycin-resistant enterococci: transmission and control. International Journal of Antimicrobial Agents, 31, 99–106.

Tritz DM, Iwen PC, Woods GL (1990): Evaluation of MicroScan for identification of *Enterococcus* species. Journal of Clinical Microbiology, 28, 1477–1478.

Tyrell GJ, Bethune RN, Willey B, Low DE (1997): Species identification of enterococci via intergenic ribosomal PCR. Journal of Clinical Microbiology, 35, 1054–1060.

Williamson R, Le Bougu C, Gutmann L, Horaud T (1985): One or two low affinity penicillin-binding proteins may be responsibe for the range of susceptibility of *Enterococcus faecium* to benzylpenicillin. Journal of General Microbiology, 131, 1933–1940.

Received: 2009–10–20
Accepted after corrections: 2010–03–25

Corresponding Author:

Andrea Brtkova, State Veterinary and Food Institute, Janoskova 1611/58, 026 01 Dolny Kubin, Slovak Republic
Tel. +421 435 837 160, E-mail: brtkova@svpudk.sk