Enterococcus saigonensis sp. nov., isolated from retail chicken meat and liver

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Two Gram-stain-positive strains, VE80ᵀ and VE116, which were resistant to vancomycin, were isolated from retail chicken meat and liver in Ho Chi Minh, Vietnam, respectively. These strains were characterized by sequence analyses of 16S rRNA, RNA polymerase α-subunit (rpoA), ATP synthase α-subunit (atpA), and phenylalanyl-tRNA synthase α-subunit (pheS) genes, determination of DNA G+C content, cellular fatty acid methyl ester analysis, DNA–DNA hybridization, and conventional morphological and biochemical tests. Strains VE80ᵀ and VE116 had 99.6 % 16S rRNA gene sequence similarity with Enterococcus canintestini LMG 13590ᵀ, and 99.1 % 16S rRNA gene sequence similarity with Enterococcus dispar ATCC 51266ᵀ. However, the two isolates could be clearly differentiated from these reference strains by the low sequence similarities (86.1–86.8 %) of the atpA gene, low DNA–DNA relatedness (<22.8 %), and differences in the production of acid from melezitose and methyl α-D-glucoside. Based on the results obtained in the present study, these two isolates are considered to represent a novel species of the genus Enterococcus, for which the name Enterococcus saigonensis sp. nov., is proposed. The type strain is VE80ᵀ (=JCM 31193ᵀ =CCUG 68827ᵀ).

The genus Enterococcus consists of 52 species (List of Prokaryotic names with Standing in Nomenclature; http://www.bacterio.net/index.html) (Parte, 2014) at the time of writing that are part of the normal intestinal microbiota of humans and animals, and are generally considered to have low pathogenic potential. However, due to the recent emergence of multi-antimicrobial-resistant strains of enterococci, particularly vancomycin-resistant enterococci (VRE), these organisms are capable of causing serious clinical and nosocomial infections (Nilsson, 2012).

In the European Union and Asia, the use of the vancomycin analogue avoparcin, a glycopeptide antibiotic, as a growth promoter and prophylactic agent in animal feed has been associated with the increased detection of VRE in farm animals (Borgen et al., 2000; Chan et al., 2008; Jung et al., 2007). Although VRE strains originating from food products have not been conclusively linked to clinical infections, several studies have shown the possible spread of VRE from animals to the human population via the food chain (Borgen et al., 2000; Seong et al., 2004). Therefore, it is important to routinely monitor food-producing animals and retail food products for the presence of VRE.

In our routine monitoring for antibiotic-resistant Enterococcus strains in Ho Chi Minh, Vietnam, two enterococcal...
strains were isolated from retail chicken meat and liver. Sequencing analyses of several housekeeping genes demonstrated that these two strains might belong to a novel enterococcal species. The purpose of the present study was to determine the taxonomic position of these two isolates.

In December 2012, 25-gram samples of chicken meat (n=13), chicken liver (n=4) and duck meat (n=3), which were purchased at four supermarkets in Ho Chi Minh, Vietnam, were aseptically removed from the packaging and placed in sterile plastic bags (30×19 cm). After adding 225 ml buffer peptone water (BPW; Oxoid), the samples were homogenized for 1 min and were then incubated for 18±2 h at 37±1 °C for the enrichment of enterococci. Ten microlitres of the cultures were spread on Enterococcus agar plates (BD) supplemented with and without 128 µg ml−1 vancomycin (Sigma), and the plates were then incubated at 37±1 °C for 48±3 h for the isolation of individual colonies. Suspected enterococcal colonies were confirmed as enterococci by positive Gram staining, absence of catalase, and a positive result for aesculin hydrolysis. Additionally, the isolates were also identified as members of the genus *Enterococcus* by PCR using *Enterococcus* genus-specific primers (Ke et al., 1999). Two of the identified strains, designated VE80T and VE116, which were isolated from chicken meat and liver, respectively, were deposited in the Japan Collection of Microorganisms (JCM)(RIKEN BioResource Center) and the Culture Collection, University of Gothenburg (CCUG).

To determine the minimum inhibitory concentrations (MICs) of vancomycin and teicoplanin, the E-test method (bioMérieux) with 5 % sheep blood Mueller-Hinton agar (BD) was performed under aerobic conditions, according to the manufacturer’s instructions. Results were interpreted by following the criteria outlined by the Clinical and Laboratory Standards Institute (2015). Additionally, the presence of vancomycin-resistance genes among strains VE80T and VE116 was also determined by PCR as previously reported (Bell et al., 1998; Harada et al., 2010). The E-tests showed that the strains were resistant to vancomycin (MIC of >256 µg ml−1) and susceptible to teicoplanin (VE80T, MIC of 8 µg ml−1; VE116, MIC of 4 µg ml−1). Moreover, strains VE80T and VE116 were positive for the vanA gene.

It is well known that 16S rRNA gene sequences have limited discriminating power for several closely related enterococcal species, and RNA polymerase α-subunit (rpoA), ATP synthase α-subunit (atpA) and phenylalanyl-tRNA synthase α-subunit (pheS) gene sequences are much more discriminatory than 16S rRNA gene sequences for enterococcal species differentiation (Naser et al., 2005a, b, c). Therefore, in this study, to determine the phylogenetic relationships of strains VE80T and VE116, analyses of the 16S rRNA gene, rpoA, atpA and pheS sequences of these strains were performed using direct sequencing, or cloning and sequencing methods. Briefly, strains were grown in BBL trypticase soy broth (TSB; BD) for 20 h at 36 °C. To prepare DNA template for PCR, 100 µl of the culture was centrifuged at 5000 g for 5 min to collect the cells, which were resuspended in 85 µl of 50 mM NaOH, incubated at 95 °C for 5 min, and then neutralised by the addition of 15 µl of 1 M Tris-HCl (pH 7.5). After centrifugation of the suspension at 20 000 g for 5 min, the supernatants were collected for use as DNA template. PCR amplification and direct DNA sequencing of the 16S rRNA and pheS genes, which constitutes a discriminative target sequence for differentiating enterococcal species, were performed using primers described previously (Naser et al., 2005b; Woo et al., 2001, 2004). DNA sequencing of the amplified fragments was performed using the dideoxy chain termination method with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). For the PCR amplification of rpoA and atpA, the primer pairs rpoA-21F and rpoA-23R, and atpA-20F and atpA-27R, respectively, were used. The sequences of these primers have been described elsewhere (Naser et al., 2005a, b). All PCR amplifications were carried out in a 25-µl reaction mixture containing 2.0 µl DNA template, 2.5 µl 10× PCR buffer (Takara), 0.2 mM dNTPs (Takara), 0.125 µl TaKaRa Ex Taq HS (Takara), 0.4 µM of each PCR primer, and sterile distilled water. The amplification conditions consisted of an initial denaturation step at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, followed by a final elongation step at 72 °C for 5 min. The amplified products were cloned using the TOPO TA cloning Kit (Invitrogen) according to the manufacturer’s protocol and were confirmed by sequencing. Additionally, to compare with reference sequences obtained from the GenBank database (National Centre for Biotechnology Information; NCBI) (Bilofsky & Burks, 1988), the target and reference sequences were aligned, and phylogenetic trees were reconstructed using the neighbour-joining method with GENETYX software (v. 11; GENETYX).

The partial sequences of the 16S rRNA (1395 bp), rpoA (672 bp), atpA (1074 bp) and pheS (460 bp) genes of strains VE80T and VE116 were analysed using the EzTaxon program (http://www.ezbiocloud.net/eztaxon) (Chun et al., 2007; Kim et al., 2012) or BLAST (Basic Local Alignment Search Tool) on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997). Strain VE80T shared 100, 100, 99.9 and 100 % similarity of the 16S rRNA gene, rpoA, atpA, and pheS sequences, respectively, with strain VE116.

Phylogenetic analysis of the 16S rRNA gene sequence with the neighbour-joining method suggested that strains VE80T and VE116 belonged to the genus *Enterococcus* and formed a distinct branch with *Enterococcus canintestini* LMG 13590T and *Enterococcus dispar* ATCC 51266T as the nearest neighbours (Fig. 1). The 16S rRNA gene sequence of *E. canintestini* LMG 13590T had the highest similarity (99.6 %) with strains VE80T and VE116, followed by that of *E. dispar* ATCC 51266T with 99.1 % similarity. Moreover, EzTaxon results showed that strains VE80T and VE116 had >97 % similarities with 24 other enterococcal species.

3780 International Journal of Systematic and Evolutionary Microbiology 66
The *rpoA* gene sequence of *E. canintestini* LMG 13590\(^T\) shared the highest similarity (98.1\%) with strains VE80\(^T\) and VE116, followed by that of *E. dispar* LMG 13521\(^T\) with 97.3\% similarity. The *atpA* gene sequence of *E. dispar* LMG 13521\(^T\) had the highest degree of similarity (86.6–86.8\%) with strains VE80\(^T\) and VE116, followed by *E. canintestini* LMG 13590\(^T\) with 86.1–86.3\% similarity. Moreover, the *pheS* gene sequence of *E. canintestini* LMG 13590\(^T\) had the highest similarity (86.7\%) with strains VE80\(^T\) and VE116, followed by that of *E. dispar* LMG 13521\(^T\) with 85.8\% similarity. Naser et al. (2005c) demonstrate that all enterococcal species are clearly differentiated on the basis of *rpoA*, *atpA* and *pheS* gene sequences, with a maximum of 97, 92 and 86\% similarity, respectively. Consequently, it is found that strains VE80\(^T\) and VE116 represent one novel species in the genus *Enterococcus* based on the *rpoA* gene sequences. However, since the *rpoA* and *pheS* gene sequences of strains VE80\(^T\) and VE116 did not meet the criteria established by Naser et al. (2005c), additional experiments such as DNA–DNA hybridization were required to determine relatedness among strains VE80\(^T\) and VE116, and the type strains of *E. canintestini* and *E. dispar*. The phylogenetic trees of *rpoA*, *atpA* and *pheS* gene sequences are available as Fig. S1 (available in the online Supplementary Material).

Determination of DNA G+C content was performed by high-performance liquid chromatography (HPLC) analysis. Strains were inoculated into 45 ml brain heart infusion (BHI) broth (BBL) and incubated overnight at 37 °C with shaking at 160 r.p.m. The culture was then centrifuged at 5000 g for 15 min and the obtained pellet was used for total genomic DNA isolation using a FastDNA SPIN Kit for Soil (MP Biomedicals), according to the manufacturer’s instructions. The DNA samples were concentrated by isopropanol precipitation and used for the determination of DNA G+C content using a DNA–GC kit (Yamaso Shyou) with slight modifications to the manufacturer’s protocol. Briefly, the DNA samples were hydrolysed with nuclease P1 provided in the kit for 30 min at 60 °C, and were then dephosphorylated with alkaline phosphatase (*Escherichia coli*; Toyobo) for 60 min at 36 °C. The treated DNA samples were separated by HPLC under the following conditions: column, COSMOSIL 5C18-MS-II (150×4.6 mm ID); solvent, 20 mM NH\(_4\)H\(_2\)PO\(_4\) with 5\% acetonitrile; flow rate, 1.0 ml min\(^{-1}\); column temperature, 40 °C; and wavelength, 270 nm. The standard mixture provided in the kit was used as the calibration reference and was dephosphorylated with alkaline phosphatase (*Escherichia coli*; Toyobo) for 60 min at 36 °C prior to use. The DNA G+C

![Phylogenetic tree based on 16S rRNA gene sequences showing the taxonomic positions of strains VE80\(^T\) and VE116 among members of the genus *Enterococcus*. The tree was generated using the neighbour-joining method and Kimura’s two-parameter substitution model. Bootstrap values >50\%, based on 1000 replications, are shown at branch nodes. *Tetragenococcus solitarius* NCIMB 12902\(^T\) was used as an outgroup. GenBank accession numbers are shown in parentheses. Bar, 5\% sequence divergence.](http://ijs.microbiologyresearch.org)
content of strains VE80<sup>T</sup> and VE116 was determined to be 36.8 and 37.5 mol%, respectively.

Cells of strains VE80<sup>T</sup>, VE116, *Enterococcus canintestini* JCM 19616<sup>T</sup> and *E. dispar* NBRC 100678<sup>T</sup> were harvested from cultures grown in BBL trypticase soy agar (TSA) supplemented with 5% sheep blood (BD) after incubation at 37 °C for 24 h under microaerophilic conditions and used for chemotaxonomic analysis. Cellular fatty acid methyl esters (FAMEs) were obtained from approximately 40 mg wet cells by saponification, methylation and extraction using the method of Miller (1982) with minor modifications (Kuykendall et al., 1988). FAMEs were analysed by gas chromatography (HP 6890 Series gas chromatograph system; Agilent Technologies) and identified using the Microbial Identification System (Sherlock version 6.2; MIDI) based on the TSBA6 library. The major fatty acids (>10% of total fatty acids) of strains VE80<sup>T</sup> and VE116 were summed feature 8, C<sub>16:0</sub> and summed feature 3 (Table 1). The cellular fatty acid profiles of strains VE80<sup>T</sup> and VE116 were generally similar to those of *Enterococcus canintestini* JCM 19616<sup>T</sup> and *E. dispar* NBRC 100678<sup>T</sup> (Table 1).

DNA–DNA hybridization was performed among strains VE80<sup>T</sup>, VE116, *E. canintestini* JCM 19616<sup>T</sup> and *E. dispers* NBRC 100678<sup>T</sup> by the microplate method described by Ezaki et al. (1989) with slight modification (Goris et al., 1998). Briefly, strains were inoculated in 100 ml BHI broth (BD) and incubated overnight at 37 °C with shaking at 160 r.p.m. The culture was then centrifuged at 2500 g for 15 min and the obtained pellet was used for total genomic DNA isolation using a Power Microbial Maxi DNA Isolation Kit (MO Bio) according to the manufacturer’s instructions. All reciprocal hybridizations were also carried out. Strains VE80<sup>T</sup> and VE116 showed low DNA–DNA relatedness to both *E. canintestini* JCM 19616<sup>T</sup> and *E. dispers* NBRC 100678<sup>T</sup> (<22.8%), however, the DNA–DNA relatedness between strains VE80<sup>T</sup> and VE116 was >75.1%. These data suggested that strains VE80<sup>T</sup> and VE116 represent a single novel species of the genus *Enterococcus*, when the results were interpreted by a recommended standard for delineating bacterial species (Goris et al., 2007; Wayne et al., 1987).

Phenotypic analysis of strains VE80<sup>T</sup> and VE116 was performed using conventional tests and commercial identification kits. Catalase and oxidase activities were tested using a 3% (v/v) H<sub>2</sub>O<sub>2</sub> solution and cytochrome oxidase test strip (Nissui), respectively, after cultivating the strains on a BHI agar plate for 24 h at 36 °C. Motility was determined in modified Difco motility medium (Facklam & Elliott, 1995). Growth at different temperatures and in the presence of 6.5% NaCl was tested in BHI broth (BD) for 14 days. The haemolytic reaction was determined on TSA supplemented with 5% sheep blood (Facklam & Elliott, 1995). Lancefield D antigen was determined using a Streptococcal Grouping Kit (Oxoid). Substrate utilization, fermentation/oxidation profile, acid production and other phenotypic characteristics were determined using the API 20 Strep, API rapidID 32 Strep system and API 50 CH strips with API 50 CHL medium (bioMérieux). The API 50 CH strips were incubated for 48 h at 36 °C according to the manufacturer’s instructions. There was no difference between the phenotypic and biochemical profiles of strain VE80<sup>T</sup> and those of strain VE116. Additionally, these profiles were distinctive from those of 26 other related enterococcal strains having >97% 16S rRNA gene sequence similarity with strains VE80<sup>T</sup> and VE116 (Table 2). Additional features of strains VE80<sup>T</sup> and VE116 are given in the species description.

### Description of *Enterococcus saigonensis* sp. nov.

*Enterococcus saigonensis* (sa.i.gon.en’sis. N.L. masc. adj. saigonensis referring to Saigon, the geographic area where the type strain was isolated).
Table 2. Phenotypic characteristics that differentiate *Enterococcus saigonensis* sp. nov. from other related enterococci

| Strains | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Growth at/in: | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 °C | + | ND | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| 45 °C | + | ND [+*] | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| 6.5% (w/v) NaCl | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Yellow pigment | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Production of: | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Alkaline phosphatase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Arginine dihydrolase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| α-Galactosidase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Pyrrolidonyl arylamidase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Acid from: | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Arabinose (l-arabinose) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| 2-Ketogluconate (l-mannitol) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Mannitol | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Melezitose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Methyl α-D-glucoside (methyl α-D-glucopyranoside) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Raffinose (l-ribose) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Ribose (l-sorbose) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| Sucrose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |

*Data from Kim et al. (2013).
†Growth at 42 °C.
The species description is based on two strains. Cells are Gram-stain-positive, facultatively anaerobic, non-motile, non-spore-forming, spherical or ovoid cocci, occurring as single cells, pairs of cells, or chains of three cells. Colonies formed on TSA supplemented with 5% sheep blood after incubation for 48 h at 36 °C are non-pigmented, circular, smooth, shiny, milky, 2–3 mm in diameter, and are unaffected by the absence or presence of 5% CO₂. The colonies have weak α-haemolytic activity. After incubation for 48 h at 36 °C on enterococcosel agar plates (BD), colonies with positive aesculin reaction are formed. Although growth at 10 °C in BHI broth is slower than at 36 °C, the growth rate is similar at 36 and 45 °C. Cells grow in BHI broth supplemented with 6.5% NaCl and do not react with Lancefield group D antigen antisera. The strains are negative for oxidase and catalase activities. The strains are positive for Voges–Proskauer test, aesculin, pyrrolidonyl arylamidase, arginine dihydrolase, α-galactosidase, leucine aminopeptidase and β-glucosidase. Acid is produced from ribose (D-ribose), lactose, trehalose, starch, raffinose, maltose, melibiose, methyl β-D-glucopyranoside, D-tagatose, glycerol, D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, sucrose, melezitose, gentiobiose and 2-ketogluconate by the strains. However, the strains are negative for hippurate hydrolysis, β-galactosidase, β-glucuronidase, alkaline phosphatase, alanine-phenylalanine-proline arylamidase, N-acetyl-β-glucosaminidase, glycine-tryptophan arylamidase, β-mannosidase and urease. Additionally, acid is not produced from erythritol, arabinose (L-arabinose), D-arabinose, D-xylitol, L-xylitol, D-adenitol, methyl β-D-xylopyranoside, sorbose (L-sorbose), L-rhamnose, dulcitol, inositol, mannitol (D-mannitol), D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucoside (methyl α-D-glucopyranoside), inulin, glycosyl, xylitol, turanose, D-lyxose, L-fucose, D-fucose, D-arabitol, L-arabitol, glucuronate, 5-ketogluconate, cyclodextrin and pullulan. Resistant to vancomycin and susceptible to teicoplanin, and possess the vanA gene. The fatty acid profile contains C₁₂:0, C₁₄:0, C₁₆:0, C₁₈:1ω9c, C₁₈:0, summed feature 3 (C₁₆:1ω7c and/or C₁₆:1ω6c), summed feature 5 (C₁₈:2ω6c and/or anteiso-C₁₈:0) and summed feature 8 (C₁₈:1ω6c and/or C₁₈:1ω7c). The DNA G+C content ranges from 36.8 to 37.5 mol%. The species is differentiated from other enterococcal species by atpA gene sequences.

The type strain is VE80T (=JCM 31193T=CCUG 68827T) isolated from retail chicken meat in Ho Chi Minh, Vietnam. The DNA G+C content of the type strain is 36.8 mol%. An additional strain of the species is VE116 (=JCM 31194=CCUG 68828) and is included in the species description.

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References


