Detection of *vanC$_1$* gene transcription in vancomycin-susceptible *Enterococcus faecalis*

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Here we report the presence and expression levels of the vanC$_1$ *and* vanC$_{2/3}$ *genes in vancomycin-susceptible strains of* Enterococcus faecalis. *The* vanC$_1$ *and* vanC$_{2/3}$ *genes were located in the plasmid DNA and on the chromosome, respectively. Specific mRNA of the* vanC$_1$ *gene was detected in one of these strains. Additionally, analysis of the* vanC *gene sequences showed that these genes are related to the* vanC *genes of* Enterococcus gallinarum *and* Enterococcus casseliflavus. *The presence of* vanC *genes is useful for the identification of* E. gallinarum *and* E. casseliflavus. *Moreover, this is the first report of* vanC *mRNA in* E. faecalis.

Key words: *Enterococcus faecalis* - vancomycin resistance - *vanC* gene - horizontal gene transfer

Enterococcus faecalis is part of the normal microbiota inhabiting the gastrointestinal tract of humans and animals and it is also present in soil, plants and food (Moreno et al. 2006, Riboldi et al. 2008, Cassenego et al. 2011). This pathogen is responsible for serious health problems and causes the majority of human enterococcal infections (Franz et al. 2003). An important feature of this species is its resistance to a wide range of antimicrobial agents. Animals may be an important reservoir of vancomycin resistant enterococci (VRE) because of the possibility of resistance genes being transferred to the human gut bacteria through the food chain and/or animal husbandry (Poeta et al. 2005).

Nine VRE genotypes have been described in enterococci (*vanA*, *-B*, *-C*, *-D*, *-E*, *-G*, *-L*, *-M* and *-N*). These genes encode intrinsic or acquired resistance determinants that result in changes in the peptidoglycan binding site and significantly reduce the strength of vancomycin binding (Courvalin 2006, Boyd et al. 2008, Xu et al. 2010, Lebreton et al. 2011). The VanC phenotype is chromosomally encoded by the *vanC$_1$* and *vanC$_{2/3}$* genes, which are intrinsic to *Enterococcus gallinarum* and *Enterococcus casseliflavus*, respectively, and therefore can be used for species identification (Park et al. 1997, French 1998, Ramotar et al. 2000, Courvalin 2006). These genes confer low-level resistance to vancomycin (2-32 µg/mL) and susceptibility to teicoplanin (0.5-1 µg/mL) (Dutka-Malen et al. 1992, 1995, Navarro & Courvalin 1994, Courvalin 2006).

The *vanC$_1$* gene was recently detected in a vancomycin-susceptible strain of *E. faecalis* (Schwaiger et al. 2012). Here we report the presence of the *vanC$_1$* and *vanC$_{2/3}$* genes and evaluate the expression of *vanC* genes by reverse transcription-polymerase chain reaction (RT-PCR) in *E. faecalis* isolates that were obtained from cloacal swabs of broilers; these strains were previously classified as vancomycin-intermediate resistant (Cassenego et al. 2011).

MATERIALS AND METHODS

E. faecalis strains - A total of 29 *E. faecalis* isolates from cloacal swabs of broilers that were classified as vancomycin-intermediate resistant by the disk diffusion method were screened for the presence of *van* genes by PCR (data not shown); three isolates (CB 114, CB 356 and CB 378) that were positive for the *vanC* gene were chosen for this study. The isolates were biochemically classified as *E. faecalis*, which was confirmed by PCR using species-specific primers for the D-alanine-D-alanine ligase (*ddl*)$_{E.faecalis}$ gene (Depardieu et al. 2004). The strains were also tested to exclude the species *E. casseliflavus* and *E. gallinarum* by PCR using the species-specific primer pairs *CA1*/*CA2* and *GA1*/*GA2*, respectively (Jackson et al. 2004).

Determination of the minimal inhibitory concentration (MIC) of vancomycin - The MIC of vancomycin was determined by the broth microdilution method (BMM) (0.125-32 µg/mL) according to the Clinical and Laboratory Standards Institute (CLSI 2010) and by the Epsilometer-test (E-test) (0-256 µg/mL) (bioMérieux®), fol-

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lowing the manufacturer's recommendations. All tests were performed in triplicate and the *E. faecalis* strains ATCC 29212 (vancomycin-susceptible) and *E. faecalis* ATCC 51299 (resistant to vancomycin) were used for quality control.

Extraction of DNA and PCR assays - Genomic DNA was extracted following the standard method of phenol (Invitrogen) extraction and ethanol (Pro Analysis) precipitation (Sambrook & Russell 2001) with minor modifications, as previously described (Moura et al. 2012). Plasmid DNA was extracted using standard miniprep methods (Sambrook & Russell 2001). The species identification of the *E. faecalis* isolates was confirmed by PCR using species-specific primers for the $ddl_{E.faecalis}$ *gene* (Depardieu et al. 2004). *E. faecalis* ATCC 51299 and *Enterococcus faecium* ATCC 53519 were used as positive and negative control strains, respectively. All strains were retested for the presence of *vanA*, *vanB* and $vanC_{1,2/3}$ by PCR. The oligonucleotides and PCR conditions used in this study for *vanA* and $vanC_1$ (Dutka-Malen et al. 1995), *vanB* (Depardieu et al. 2004) and $vanC_{2/3}$ (Satake et al. 1997) followed those reported by their respective authors. Reactions were performed in an Eppendorf Mastercycler thermal cycler under the following cycle conditions: 3 min at 94ºC, 30 cycles of 1 min at 94ºC, 1 min at 50ºC (*vanA*) or 54ºC (*vanB* and $vanC_{1,2/3}$) and 1 min at 72ºC and 5 min at 72ºC.

RNA extraction and analysis of vanC gene expression by RT-PCR - Briefly, 500 µL of overnight culture was inoculated into 50 mL 2xYT broth and incubated with agitation at 37ºC to an optical density at 600 nm of 0.3. A 3 mL aliquot was harvested by centrifugation for 10 min at 10,000 *g*, the supernatant was discarded and total RNA was extracted using TRIzol® (Invitrogen®), following the manufacturer's protocol. The total RNA was treated with RNase-free DNase I (Fermentas®) according to the manufacturer's recommendations.

Complementary DNA (cDNA) was synthesised from 1 µg of high-quality total RNA ($A_{260nm/280nm}$ of 1.80-2.0), following the manufacturer's instructions (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems®). Reverse transcriptase was omitted from the negative control. The cDNAs were used in the PCR amplification of the $vanC_1$ and $vanC_{2/3}$ genes in a final volume of 25 µL. The cDNAs were also used for PCR amplification of the 16S rRNA gene (Medeiros et al. 2010).

Sequencing of samples - To confirm the presence of $ddl_{E.faecalis}$, $vanC_1$ and $vanC_{2/3}$, the amplified products were submitted to nucleotide sequence analysis. The primers and PCR followed the protocols previously described (Dutka-Malen et al. 1995, Satake et al. 1997, Depardieu et al. 2004). The DNA fragments were purified using an Illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare-Buckinghamshire, United Kingdom). Sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in an ABI-PRISM 3100 Genetic Analyzer (ABI), according to the manufacturer's protocol. The nucleotide sequences obtained were compared with homologous nucleotide sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Three *E. faecalis* isolates (CB114, CB356 and CB378) were identified by a PCR strategy using species-specific primers to amplify the 475 bp $ddl_{E.faecalis}$ gene (data not shown). Moreover, the isolates were negative for the species *E. casseliflavus* and *E. gallinarum*.

The MIC determined by E-test exhibited an elliptical zone of inhibition within the range of 1.50-4.0 µg/mL and the strains were reclassified as vancomycin-susceptible (≤ 4.0 µg/mL). The BMM showed a MIC ranging from 4.0-1.0 µg/mL and all strains were also reclassified as vancomycin-susceptible by this method (Table).

All isolates were positive for the $vanC_1$ gene and the strains CB356 and CB378 harboured both the $vanC_1$ and $vanC_{2/3}$ genes. The $vanC_1$ gene was detected in plasmid DNA and the $vanC_{2/3}$ gene was present in the chromosomal DNA (data not shown).

DISCUSSION

The detection of more than one *van*-type gene in an *Enterococcus* strain has been reported in other studies, including the presence of $vanC_1$ + *vanA* or $vanC_1$ + *vanB* (Elsayed et al. 2001, Hassan et al. 2008). None of the strains tested positive by PCR for the *vanA* or *vanB*

TABLE

Phenotypic and genotypic characteristics of *Enterococcus faecalis* vanC-type isolates from cloacal swabs of broilers

	Phenotype[a]		Genotype				
Isolate	E-test	BMM	*ddl*	*vanA*	*vanB*	$vanC_1$	$vanC_{2/3}$
CB114	3.50 µg/mL	4.0 µg/mL	+	-	-	+	-
CB356	2.25 µg/mL	1.0 µg/mL	+	-	-	+	+
CB378	2.50 µg/mL	1.0 µg/mL	+	-	-	+	+
Parameters	≤ 4 µg/mL[b]	≤ 4 µg/mL[c]					

a: average values of triplicates; *b*: sensitive according to manufacturer; *c*: sensitive according to CLSI-M100-S20; BMM: broth microdilution method; *ddl:* D-alanine-D-alanine ligase; E-test: Epsilometer-test.

gene. The presence of the $vanC_1$ gene in vancomycin-susceptible *E. faecalis* strains isolated from pig manure samples was first described in Germany (Schwaiger et al. 2012). The detection of these *vanC* genes in *E. faecalis* is remarkable because they are thought to be intrinsic to *E. gallinarum* ($vanC_1$) and *E. casseliflavus* ($vanC_{2/3}$) and the *vanC* operon is chromosomally located in a transferable region, such as a transposon and/or integron (Dutka-Malen et al. 1992, Navarro & Courvalin 1994, Patel et al. 1997, 1998, Depardieu et al. 2004, Fisher & Phillips 2009). *E. faecalis* may have acquired *vanC* genes by horizontal transfer from *E. gallinarum* and *E. casseliflavus*, two natural inhabitants of the poultry gut. This flow of the *vanC* gene between species is important because the presence of this gene is often used to identify species and therefore, erroneous identification of species may be occurring. Furthermore, this flow also emphasises that the chromosomal location of a gene in intrinsically resistant strains does not necessarily protect against transfer to other species, thereby contributing to the diversification of species (Schwaiger et al. 2012).

RT-PCR experiments detected $vanC_1$-specific mRNA in only one strain (CB356) and did not detect $vanC_{2/3}$ mRNA (data not shown). Recently, a study using real-time RT-PCR assays also failed to detect a corresponding $vanC_1$ transcript in a $VanC_1$ genotype-positive strain (Schwaiger et al. 2012). A possible explanation for this result could be that a non-functional *vanC* gene cluster has been transferred from the bacterial community to CB114 and CB378 or it could reflect the action of a failed recombination event that inserted a non-functional gene and removed beneficial DNA (Lawrence et al. 2001).

The three partial $ddl_{E.faecalis}$, $vanC_1$ and $vanC_{2/3}$ gene sequences were deposited in GenBank (accessions JX220983, JX220984 and JX220985, respectively). The alignment of the sequences showed 99% identity to $ddl_{E.faecalis}$ of *E. faecalis* (GenBank ID U00457), 99% to $vanC_1$ of *E. gallinarum* (GenBank ID EU151770) and 99% to $vanC_{2/3}$ of *E. casseliflavus* (GenBank ID EU151764), respectively.

In conclusion, we have reported the first identification and mRNA expression of the *vanC* gene in a vancomycin-susceptible *E. faecalis* strain that was isolated from cloacal swabs of broilers in Brazil. Our results suggest that *E. faecalis* may have acquired the *vanC* genes by horizontal transfer from *E. gallinarum* and *E. casseliflavus*. These results are significant because the detection of the *vanC* gene is a useful tool for the detection of *E. gallinarum* and *E. casseliflavus*. We recommend that the $vanC_1$ and $vanC_{2/3}$ genes be used with caution as species-specific markers.

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