The first report of the vanC₁ gene in Enterococcus faecium isolated from a human clinical specimen

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The vanC₁ gene, which is chromosomally located, confers resistance to vancomycin and serves as a species marker for Enterococcus gallinarum. Enterococcus faecium TJ4031 was isolated from a blood culture and harbours the vanC₁ gene. Polymerase chain reaction (PCR) assays were performed to detect vanXYc and vanTc genes. Only the vanXYc gene was found in the E. faecium TJ4031 isolate. The minimum inhibitory concentrations of vancomycin and teicoplanin were 2 µg/mL and 1 µg/mL, respectively. Real-time reverse transcription-PCR results revealed that the vanC₁ and vanXYc genes were not expressed. Pulsed-field gel electrophoresis and southern hybridisation results showed that the vanC₁ gene was encoded in the chromosome. E. faecalis isolated from animals has been reported to harbour vanC₁ gene. However, this study is the first to report the presence of the vanC₁ gene in E. faecium of human origin. Additionally, our research showed the vanC₁ gene cannot serve as a species-specific gene of E. gallinarum and that it is able to be transferred between bacteria. Although the resistance marker is not expressed in the strain, our results showed that E. faecium could acquire the vanC₁ gene from different species.

Key words: Enterococcus gallinarum - Enterococcus faecium - vanC₁ gene

During the past two decades, glycopeptide-resistant enterococci, in particular Enterococcus faecium, have become increasingly widespread throughout the world and have been identified as multi-resistant opportunistic pathogens in hospitals and in the environment (e.g., in foods and animals) (Lebreton et al. 2011, Nomura et al. 2012). Since the first detection of vancomycin resistance in E. faecium in 1988, nine operons that confer resistance to glycopeptides have been distinguished based on the sequence of the structure for the resistance ligase (Leclercq et al. 1988, Uttley et al. 1988, Lebreton et al. 2011). These operons are classified according to the characteristics of the ligase gene, which can encode either a D-alanyl-D-lactate ligase or a D-alanyl-D-serine ligase. Genes that encode D-alanyl-D-lactate ligases include the vanA, vanB, vanD and vanM genes and those that encode D-alanyl-D-serine ligases include the vanC₁, vanC₃, vanC₅, vanE, vanG, vanL and vanN genes (Arthur et al. 1996, Courvalin 2006, Lebreton et al. 2011, Nomura et al. 2012). Resistance types can be acquired except for vanC-type resistance, which is thought to be intrinsic to Enterococcus gallinarum and Enterococcus casseliflavus. The vanC₁ cluster is composed of five genes: vanC₁, vanXYc, vanTc, vanRe and vanSc. Three of these genes are involved in inducing resistance according to the following mechanism: vanC₁ encodes a ligase that synthesises the dipeptide D-Ala-D-Ser, which is added to the UDP-MurNAc-tripeptide; vanXYc encodes a D,D-dipeptidase-carboxypeptidase that hydrolyses D-Ala-D-Ala and removes D-Ala from UDP-MurNAc-pentapeptide; vanT encodes a membrane-bound serine racemase that provides D-Ser to the synthetic pathway (Arias et al. 2000). The vanC₁ gene is thought to occur only in E. gallinarum and should therefore be useful for species identification (Ramotar et al. 2000). Furthermore, this gene is chromosomally located and has not been found in E. faecium until now. Since the vanC₁ gene was first identified in vancomycin-susceptible Enterococcus faecalis strains isolated from pig manure samples in Germany, vanC₁-type E. faecalis of animal origin has been reported in Spain and Brazil. This finding emphasises that the chromosomal location of a gene in intrinsically resistant strains does not necessarily prevent gene transfer to another species, which is in contrast to traditional views (Schwaiger et al. 2012, de Garínca et al. 2013, de Moura et al. 2013). So far, there has been one report of the genetic location of the vanC₁ gene isolated from cloacal swabs of broilers and this gene was detected on plasmid (de Moura et al. 2013). We also identified vanC₁-type E. faecium strain isolated from a blood culture.

In this study, E. faecium TJ4031 was susceptible to both vancomycin and teicoplanin, but harboured the vanC₁ resistance gene. We also presented evidence showing that the vanC₁ gene cluster was incomplete; the vanC₁ and vanXYc genes were not expressed. Additionally, the resistance gene in this clinical isolate was located on the chromosome.

MATERIALS AND METHODS

Strains - E. faecium TJ4031 was isolated in 2012 from a blood culture from an outpatient in our hospital. This isolate was initially identified by Gram staining and bio-
chemical reaction as previously described (Facklam & Collins 1989). Furthermore, the Vitek2-Compact system (bioMérieux, France) was used and Enterococcus-specific polymerase chain reaction (PCR) was performed (Jackson et al. 2004). This strain was also distinguished from E. gallinarum by determining the E. gallinarum sod gene using the species-specific Egaspe primer set. The primers used are listed as follows: EgaspeF, GAACCACAGGAGGCACTCA; EgaspeR, ACCAAGCCCGAGCAGA (Poyart et al. 2000). TJ430, which was confirmed as E. gallinarum isolated from a clinical specimen and E. faecalis ATCC29212, was used as positive and negative controls, respectively, for the detection and expression analysis of the vanC, vanXYc and vanTc genes. TJ430 was also used as control strain to analyse the location of the vanC gene. Salmonella H9812 was used as a marker for pulsed-field gel electrophoresis (PFGE).

Antibiotic susceptibility testing - Minimum inhibitory concentrations (MICs) were determined according to a standard agar dilution procedure, as recommended by the Clinical and Laboratory Standards Institute (CLSI 2012). E. faecalis ATCC29212 was used as a control strain.

Molecular typing and genetic techniques - TJ4031 was typed by subjecting genomic DNA to PFGE using the CHEF II Mapper system (Bio-Rad, France) (Lebretton et al. 2011). Total DNA was digested with Smal and then electrophoresed (6 V/cm, 17 h, pulse times of 1-17 s at 14°C) on an agarose gel. Total DNA from the H9812 marker was digested with XbaI. The plasmid size and content of the TJ4031 isolate were determined using the S1 nuclease method (Freitas et al. 2009a). The location of the vanC gene, vanC probe was constructed by labeling an internal amplification product of the vanC gene from TJ4031 with digoxigenin according to the manufacturer’s protocol (Roche, Switzerland). The primers used to amplify the product are listed as follows: vanC F, 5’-ATGGCTGATCCAAAGGACTG-3’; vanC R, 5’- AGGCAATGGTGCTGGGAGAC-3’.

The E. faecium TJ4031 isolate was subjected to multilocus sequence typing (MLST) analysis (Zheng et al. 2007, Freitas et al. 2009b). The alleles and sequence type (ST) were determined using the MLST database (faecium.mlst.net). The new ST identified in this study has been deposited in the database.

PCR - E. faecium isolates were species identified with PCR using Enterococcus-specific primers combined with species-specific primers for the E. gallinarum sod gene (Poyart et al. 2000, Jackson et al. 2004). E. faecium TJ4031 was also evaluated to determine the presence of vanA, vanB, vanC, vanC and vanXYc, vanTc (Patel et al. 1998, Schwaiger et al. 2012).

Sequence analysis - The band from the vanC-positive strain was excised from the gel and then sequenced. Sequence similarity was compared using the NCBI Basic Local Alignment Search Tool database (ncbi.nlm.nih.gov).

RNA extraction and reverse transcription-PCR (RT-PCR) - Total RNA from strain TJ4031 grown in brain-heart-yeast extract broth at 37°C to an absorbance at 600 nm of 0.8 was isolated using an RNeasy Mini Kit (Qiagen, Germany). Transcripts were obtained using a RevertAid First Strand cDNA system kit (Fermentas) according to the manufacturer’s instructions. Real-time RT-PCR was conducted in a final volume of 20 µL containing 10 µL SYBR Mix (NovoGene), 0.5 µM primers and 3 µL template cDNA. The reaction was performed using a Light Cycler 480 (Roche) under the following conditions: initial denaturation at 95°C for 7 min and 40 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 15 s, elongation at 72°C for 30 s and cooling at 40°C for 30 s. The primers used in this procedure are as follows: vanrtC1 F, ATTTGGTGTCTTGATGCG; vanrtC, R, CGGGAGTGCCCATGAAAA; vanXYc F, GCAAAA- CAGTGGGAACGAGCT; vanXYc R, ATCTCGAAATT-GAGGGCAGA. Non-reverse-transcribed PCR controls were used to indicate the absence of genomic DNA contamination. ATCC29212 and TJ430 were used as negative and positive control strains, respectively.

RESULTS

Characterisation of E. faecium TJ4031 - The clinical isolate TJ4031, which was isolated from a blood culture, was identified as E. faecium using conventional tests and the Vitek2-Compact system; species identity was confirmed by PCR. E. gallinarum was excluded as a possibility by a negative result in an E. gallinarum species-specific PCR. TJ4031 was susceptible to vancomycin (MIC = 2 µg/mL) and teicoplanin (MIC = 1 µg/mL). TJ4031 was also susceptible to chloramphenicol, linezolid, ciprofloxacin, levofloxacin and fosfomycin. By comparison, this strain was resistant to penicillin, rifampicin, erythromycin and nitrofurantoin. TJ4031 was positive for the vanC and vanXYc genes, but negative for the vanTc gene. The vanC gene sequence was deposited in GenBank (accessionKF849246).

Identification of gene expression by real-time RT-PCR - RT-PCR assays failed to detect the corresponding vanC and vanXYc gene transcripts in the vanC gene positive E. faecium TJ4031 strain. The positive and negative controls worked as expected.

Molecular typing - The E. faecium TJ4031 isolate was categorised as a new ST according to MLST analysis and designated as ST837 (AtpA, 52; Ddl, 5; Gdh, 1; PurK, 1; Gyd, 1; PstS, 1; Adk, 3). This new ST showed the greatest similarity to the reported MLST type ST547 (AtpA, 17, which is found in a hospital-adapted and epidemic E. faecium strain cluster.

Genetic location of vanC gene in E. faecium TJ4031 - Figure shows the restriction endonuclease pattern of the vanC genotype in the E. faecium TJ4031 strain after PFGE with Smal was performed. The location of the vanC gene was determined using southern blot hybridisation with a vanC probe. PFGE hybridisation analysis results showed that the vanC probe hybridised
t the chromosomal DNA band and that the size of the macrorestriction fragment was between 78.2-104.5 Kbp (Figure). However, SI-PFGE analysis results showed that a plasmid was absent in the TJ4031 isolate (data not shown). The results indicated that the vanC1 gene was located in the chromosome.

DISCUSSION

The vanC intrinsic resistance genotype is associated with several enterococcal species, including E. gallinarum (vanC1), E. casseliflavus (vanC1) and E. flavescens (vanC2). These vanC operons are chromosome associated and testing E. faecium or other Enterococcus species for the presence of vanC1 is considered unnecessary because this gene is thought to be species-specific for E. gallinarum (Leclercq et al. 1992, Schwaiger et al. 2012). However, the vanC1 gene has also been detected in E. faecalis strains isolated from pig manure samples (Germany), sheep bulk tank milk samples (Spain) and cloacal swabs of broilers (Brazil).

In our study, expected biochemical reactions were observed in the vanC1 genotype-positive E. faecium TJ4031. E. faecium was also analysed using the Vitek2-Compact system. Moreover, the Enterococcus and E. gallinarum-specific PCR results of this study are consistent with those of other bacteriological studies (Patel et al. 1998, Arias et al. 2000, Zheng et al. 2007, de Moura et al. 2013). Our study failed to detect the corresponding vanC1 and vanXYc genes in a vanC1 genotype-positive strain with real-time RT-PCR assays and similar results have been previously reported (Schwaiger et al. 2012, de Moura et al. 2013). This result could be attributed to a non-functional vanC1 gene cluster that has been transferred from a bacterial community to our strain or to a failed recombination event that inserted a non-functional gene and removed beneficial DNA (Lawrence et al. 2001, de Moura et al. 2013). In a previous study, the vanC1 gene was found on a plasmid (de Moura et al. 2013). However, our study showed that the vanC1 genotype of the E. faecium TJ4031 isolate contained no plasmid; this procedure was repeated in triplicate to verify our initial findings. The possible explanation is that megaplasmids, which cannot be detected with current techniques, or bacteria without plasmids may be present. Furthermore, the vanC1 gene was successfully hybridised to the chromosome band using southern blot, showing that this gene was located in this chromosome.

E. faecium TJ4031 may have acquired the vanC1 gene via a horizontal gene transfer from a natural carrier (E. gallinarum) or from a carrier of animal origin (E. faecalis) (Schwaiger et al. 2012, de Garnica et al. 2013, de Moura et al. 2013) because strains from human-adapted CCs that cause enterococcal infection may be recovered from farm and companion animals and strains from CCs commonly found among animals have also been isolated from humans. Furthermore, other studies have revealed several cases of animal-human transmission of vancomycin-resistant enterococci, resulting in frequent infections of healthy humans that closely interact with animals (Damborg et al. 2009, Freitas et al. 2009b, 2011, Willems & van Schaik 2009, Larsen et al. 2010).

This study was the first to identify the vanC1 gene in a vancomycin-susceptible E. faecium strain isolated from the blood culture of a patient in China. Our result is important because the vanC1 gene is often used to identify E. gallinarum; without this gene, species may be erroneously identified. This result also emphasises that the chromosomal location of a gene in an intrinsically resistant strain does not necessarily prevent transfer to other species, thereby contributing to species diversity. Furthermore, the vanC1-type vancomycin resistance gene was encoded on the chromosome. Our data indicate that vanC1-type E. faecium strains could be detected in humans. Even if the strain was phenotypically susceptible to vancomycin, the fact that E. faecium are able to naturally acquire vanC1 from the bacterial community is a cause for concern because the possibility that complete gene clusters and functional genes will be transferred and expressed cannot be ruled out. This study underlines that E. faecium are very potent resistance gene collectors and possibly donors (Schwaiger et al. 2012). We should always monitor enterococci in not only human clinical isolates, but also in the commensals from diverse habitats.
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