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Research Note

Emergence of *Enterococcus gallinarum* carrying *vanA* gene cluster displaying atypical phenotypes

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Abstract. Motile enterococci such as *Enterococcus gallinarum* has the ability to acquire and transfer antibiotic resistance genes to other enterococci. Even though infections caused by *E. gallinarum* are rare, the discovery of this bacteria in food sources and in clinical environments is disturbing. Here, we report the isolation and identification of *E. gallinarum* from the wound of a hospital in-patient. The isolate was identified using 16S rDNA sequencing. Isolate 146 harboured the *vanA* and *vanC1* gene clusters, was vancomycin-susceptible, and displayed resistance to ampicillin, penicillin, erythromycin and teicoplanin. This isolate also showed intermediate resistance to linezolid and sequencing of the 23S rRNA peptidyl transferase region did not unveil any known mutations associated to the conferment of linezolid resistance. The presence of *vanA* did not confer resistance to vancomycin. Structural analyses into the Tn*1546* transposon carrying the *vanA* gene revealed distinct genetic variations in the *vanS*, *vanY* and *vanS-vanH* intergenic region that could be associated to the atypical antibiotic resistance phenotypes of isolate 146. Finding from this study are suggestive of the occurrence of interspecies horizontal gene transfer and that similarities in genotypic characteristic may not necessarily correlate with actual antibiotic resistance pattern of *E. gallinarum*.

The *Enterococcus* species has emerged as a recalcitrant nosocomial pathogen mainly due to its inclination to easily acquire antibiotic resistance genes (Praharaj *et al.*, 2013). It is unsurprising therefore, that infections caused by this bacteria has become increasingly difficult to treat. The situation is made even more alarming with motile enterococci such as *Enterococcus gallinarum* which has been shown to possess the ability to acquire resistance genes and transferring them to other *Enterococcus* species (Praharaj *et al.*, 2013). *E. gallinarum* is usually found in the environment (Getachew *et al.*, 2009; Praharaj *et al.*, 2013) but it can also be found from food sources (Getachew *et al.*, 2009). Cases of nosocomial infection involving *E. gallinarum* are rare and not well studied. An earlier study found two *E. gallinarum* isolates that were susceptible to linezolid (Praharaj *et al.*, 2013). One isolate which carried the *vanA* and *vanC1* genes was characterized as having VanA phenotype. It was highly resistant to vancomycin and teicoplanin. The other isolate characterized as having VanC phenotype carried only the *vanC1* gene. This isolate showed low level vancomycin resistance (MIC, 8 µg/ml) and is susceptible to teicoplanin (Praharaj *et al.*, 2013). Here,

we describe a novel *E. gallinarum* isolate of clinical origin carrying the *vanA* and *vanC1* genes, yet displaying susceptibility to vancomycin. Analyses of the Tn*1546* transposon revealed several genetic variations suggested to contribute to the antimicrobial resistance phenotype. The *E. gallinarum* isolate (Isolate 146) in this study was isolated in May 2011 from the wound infection specimens of a hospital in-patient.

Isolate 146 could only be identified down to the *Enterococcus* genus level using API 20 Strep (bioMérieux, Marcy l'Etoile, France). In order to determine its species identity, we attempted DNA sequencing of the 16S rDNA gene. Bacterial nucleic acid was extracted from a single colony suspended in nuclease-free water using NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. 16S rDNA PCR was performed according to previously published protocols (Misbah *et al.*, 2005) using 2X h-Taq PCR Smart mix (SolGent Co., Daejeon, South Korea). Amplified gene fragments were purified and sequenced in the sense and antisense directions. Upon alignment and editing of the 16S rDNA sequences using Sequencher 4.10.1 (Gene Codes, Ann Arbor, MI, USA), the resulting sequences were subjected to BLAST. Multiplex PCR was then performed for genotyping the isolate for the presence of glycopeptide resistance genes (Dutka-Malen *et al.*, 1995). Nucleic acid amplification of the Tn*1546* transposon, which carries the *vanA* gene cluster was performed to examine the structural and genetic variations in comparison to the Tn*1546* transposon of the prototype *E. faecium* strain BM4147 (Huh *et al.*, 2004). A set of PCRs to amplify known enterococcal virulence factors (collagen-binding protein; *ace*, aggregation substance; *asa*, cytolysin activator; *cylA*, endocarditis antigen; *efaA*, extracellular surface protein; *esp* and gelatinase; *gelE*) (Sedgley *et al.*, 2005) were also performed.

For antibiotic susceptibility testing, disc diffusion assays were performed with ampicillin, erythromycin and penicillin (BBL™ Sensi-Disc™, Becton, Dickinson and Company, New Jersey, USA). Minimum inhibitory concentrations (MIC) to linezolid, teicoplanin and vancomycin were determined using M.I.C. Evaluator Strips (Oxoid, Thermo Fisher Scientific, Cheshire, UK) on Mueller-Hinton agar incubated at 35 ± 2 °C under ambient conditions for 24 h. Results for disc diffusion assay and MIC were interpreted according to guidelines by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2014). The disc diffusion and MIC tests were repeated once to verify the earlier findings. *Staphylococcus aureus* ATCC 25923 and *E. faecalis* ATCC 29212 were used as controls.

DNA sequencing revealed that the 16S rDNA sequences of isolate 146 (accession number LN829114) highly matched those of *E. gallinarum*. Isolate 146 harboured the *vanA* and *vanC1* gene clusters (accession numbers LN829112 and LN829113). Analyses of the Tn*1546* transposon exposed point mutations in the *vanS* (T148G, G160C, A207T and A527T), *vanY* (G229C) and *vanS-vanH* intergenic region (G77A), and a deletion at position 20 in the *vanY*. Sequences of the *vanS*, *vanY* and *vanS-vanH* intergenic region were deposited into GenBank with the accession numbers LN871824-LN871826. The Tn*1546* transposon of isolate 146 has identical length to the prototype *E. faecium* strain BM4147. The nucleic acid amplifications for the virulence factors however, were negative for all the virulence genes. Using disc diffusion assays, the isolate displayed resistance to ampicillin, penicillin and erythromycin (Table 1). This isolate however, was vancomycin-susceptible and teicoplanin-resistant by MIC testing. It also showed an intermediate resistance to linezolid (Table 1). The surfaces of laboratory worktops and biosafety cabinets used for specimen processing were thoroughly decontaminated to prevent cross-contamination with other enterococci strains.

The glycopeptide antibiotics, vancomycin and teicoplanin are generally reserved for the treatment of infections with organisms resistant to common antibiotics (Novotna *et al.*, 2012). Vancomycin-resistant *E. gallinarum* presenting the *vanA* and

Table 1. Antibiotic susceptibility testing on *E. gallinarum* isolate 146 using disc diffusion assays and M.I.C. Evaluator Strips (Oxoid, Thermo Fisher Scientific, Cheshire, UK)

Disc diffusion assay (mm)			MIC (µg/mL)		
Penicillin (10 U)	Erythromycin (15 µg)	Ampicillin (10 µg)	Vancomycin	Teicoplanin	Linezolid
9	8	11	0.03	32	4

vanC1 genes were previously reported in Malaysian broiler chickens (Getachew *et al.*, 2009). For the current isolate, acquisition of *vanA* did not confer resistance towards vancomycin. A similar observation has been reported earlier (Ribeiro *et al.*, 2007). The previous study suggested that the lack of a resistant phenotype was due to incomplete and non-functional Tn*1546* mobile genetic element (Ribeiro *et al.*, 2007) crucial in the conferment of antibiotic resistance. The exact mechanism of teicoplanin resistance however, is not understood. Nevertheless, teicoplanin contains a membrane-anchoring lipid side chain in its structure which is absent in vancomycin (Novotna *et al.*, 2012). It is possible that the peculiar teicoplanin resistance phenotype of isolate 146 mimics the capability of teicoplanin-resistant *E. faecium* to degrade peptidoglycan precursors with D-alanyl-D-alanine terminal (Arthur *et al.*, 1996). Otherwise, these antibiotic resistance phenotypes could very likely be caused by the genetic variations found within the Tn*1546* transposon similar to the observation on *E. faecalis* isolated from the same hospital (Loong *et al.*, 2016). The fact that the *E. faecalis* isolate which carried the *vanA* and *vanC1* gene clusters was discovered later (Loong *et al.*, 2016) suggests the possible emergence of interspecies horizontal gene transfer. The genetic variations in the *vanS*, *vanY* and *vanS-vanH* intergenic region could possibly interrupt the regulation of specific proteins crucial in the expression of vancomycin resistance since degradation of the peptidoglycan precursors requires at least seven enzymes (VanA, H, X, Y, Z, R and S) (Hollenbeck & Rice, 2012).

Linezolid, the oxazolidinone antibiotic was first introduced in 2000 for therapeutic use against gram-positive cocci (Bonora *et al.*, 2006; Praharaj *et al.*, 2013). The disturbing finding of linezolid resistance, despite it being considered as intermediate (Bonora *et al.*, 2006) in isolate 146 suggests that increasing resistance could limit the clinical use of linezolid against resistant Gram-positive bacteria. Resistance is most frequently conferred by a G2576U mutation in the 23S rRNA gene and higher levels of resistance can be achieved with multiple mutated copies of the gene (Hollenbeck & Rice, 2012). Besides that, other mutations in the 23S rRNA peptidyl transferase region (T2500A, G2505A, C2512U and G2513U) have also been associated with linezolid resistance in methicillin-resistant *S. aureus* (Meka *et al.*, 2004) and vancomycin-resistant enterococci (Prystowsky *et al.*, 2001). Analysis of the 23S rRNA sequences (Tsiodas *et al.*, 2001) however, showed that isolate 146 did not possess these mutations. Therefore, it was possible that the intermediate resistance phenotype observed in isolate 146 was caused by an undetectable mutated copy of the 23S rRNA gene, gradually inducing linezolid resistance because nearly all bacteria possess multiple copies of this gene (Morales *et al.*, 2010). The phenotypic differences between *E. gallinarum* isolate 146 and the ones reported in the earlier study (Praharaj *et al.*, 2013) suggest that genotypic similarities do not necessarily correlate with the antibiotic resistance properties. Interestingly, other virulence factors that would promote survival and adaptation in adverse environments and, enable the secretion of toxins and proteases (Sedgley *et*

al., 2005) were absent in the isolate 146. This suggests that the isolate has not been exposed to strains harbouring such virulence factors, hence they were not acquired via horizontal transfer.

The finding of enterococci in animals bred as food sources and humans (Getachew *et al.*, 2009) is distressing. Even though infections by *E. gallinarum* are rare, the ability of this species to acquire antibiotic resistance determinants and virulence factors warrant closer attention. And this include further investigation into the significance of the genetic variations found within the Tn*1546* transposon to understand the molecular basis of these phenotypic differences. Findings from this study suggests that genotypic characteristic similarities may not necessarily correlate with actual antibiotic resistance pattern of *E. gallinarum*. This could affect patient treatment in hospitals that rely on genome amplification of bacteria antibiotic resistance genes.

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