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Identification of vancomycin-susceptible major clones of clinical *Enterococcus* from Algeria

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ABSTRACT

The main objectives of this study were to characterize clinical strains of *Enterococcus* spp. isolated from Algerian inpatients and outpatients, to investigate their susceptibility to antibiotics and to analyse their phylogenetic relatedness. A total of 85 non-duplicate *Enterococcus* spp. isolates collected between 2010 and 2013 from various clinical samples, including urine, vaginal swab, pus, blood and semen, from Algerian inpatients ($n = 62$) and outpatients ($n = 23$) were identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS). Antibiotic susceptibility testing was performed using disk diffusion and Etest methods. Clonal relatedness was analysed using multilocus sequence typing (MLST). *Enterococcus faecalis* was the most predominant species (75.3%), followed by *Enterococcus faecium* (21.2%), *Enterococcus gallinarum* (2.4%) and *Enterococcus casseliflavus* (1.2%). High-level resistance to aminoglycosides was significantly more prevalent in hospitalized patients than in outpatients. None of the *E. faecalis* and *E. faecium* isolates were resistant to vancomycin. High genetic diversity was observed among the *E. faecalis* isolates, with the identification of a new clonal complex (CC256), as well as the detection of *E. faecalis* ST6 and *E. faecium* lineages ST17, ST18 and ST78 associated with hospital isolates. This is the first report of *E. faecalis* ST6 and *E. faecium* ST17 and ST18 in Algeria. Although acquired vancomycin resistance was not observed among the enterococcal strains, there is a continued need to monitor the level of antibiotic resistance among enterococci as well as the evolution of the *E. faecalis*/*E. faecium* ratio.

1. Introduction

Enterococci are part of the commensal flora of the intestinal tract of humans and animals; they also colonize the skin, oral cavity and genital tract and are widespread in the environment [1]. Despite their commensal lifestyle, enterococci may cause serious and even fatal human infections and have become one of the main pathogens causing nosocomial and extrahospital infections [1,2]. *Enterococcus faecalis* has been the most frequently species encountered in human pathology [3,4]. However, currently there has been an increase in clinical cases caused by *Enterococcus faecium* reported in the USA and Europe [4–7]. The rise of *E. faecium* hospital-acquired infections is essentially due to the increasing use of vancomycin and broad-spectrum antimicrobials in hospital settings [5]. The *E. faecium* species is characterized by its remarkable genome plasticity, making it capable of acquiring multiple resistance genes, colonizing patients and persisting in hospital settings [2,5,6,8].

The ability of enterococci to grow and persist in hostile conditions and their transmission through hand contact ensures their survival in hospital environments and increases the species reservoirs [1]. Enterococci are inherently resistant to certain

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antibiotics such as cephalosporins, clindamycin and low concentrations of aminoglycosides [1,9]. However, on the other hand, acquired resistance to penicillin, chloramphenicol, erythromycin and tetracycline as well as high levels of resistance to aminoglycosides have already been reported [9,10]. Also, glycopeptide resistance in enterococci has been extensively described [10–13]. In Algeria, two clinical cases of vancomycin-resistant enterococci (VRE) have thus far been reported in Algiers (*E. faecalis* in 2008 and *E. faecium* in 2013) [14,15]. Usually *vanA* and *vanB* genes confer resistance to vancomycin both in *E. faecalis* and *E. faecium* [16]. It should be noted that VRE are mainly represented by *E. faecium* strains belonging to the former clonal complex 17 (CC17) [5,17] that is associated with hospital-acquired *E. faecium* infections [5,7,17,18]. The intrinsic resistance to certain antibiotics among enterococci and the genetic transfer of resistance genes are the major causes for the failure of treatment in enterococcal infections [3].

Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) has emerged as a new technology in clinical microbiology laboratories for the rapid identification of bacterial strains at the species and subspecies levels [19,20]. Currently, multilocus sequence typing (MLST) is the most efficient tool used for clonality analysis and studying the genetic relatedness of enterococcal strains [6,21].

The main objectives of this study were to identify *Enterococcus* clinical strains isolated from hospitalized patients and outpatients in Algeria by MALDI-TOF/MS, to investigate their susceptibility to antibiotics and to analyse their phylogenetic relationships.

2. Materials and methods

2.1. Bacterial strains and species identification

Eighty-five clinical strains of *Enterococcus* from various clinical specimens were collected from three different hospitals in three cities in north-eastern Algeria (Annaba, Guelma and El-Kala) between 2010 and 2013. These human isolates included strains collected from hospitalised ($n = 62$; 72.9%) and non-hospitalized ($n = 23$; 27.1%) individuals. Identification at the genus level was done using standard microbiological tests [22], whilst species identification of enterococci was performed using MALDI-TOF/MS (microflex™; Bruker Daltonik GmbH, Bremen, Germany) with the flex control and Biotyper 3.0 software (Bruker Daltonik) as previously described [23].

2.2. Antibiotic susceptibility testing

Isolates were screened for phenotypic resistance to amoxicillin (10 μg), high-level (HL) aminoglycosides [HL gentamicin (500 μg) and HL kanamycin (1000 μg)], vancomycin (30 μg), linezolid (30 μg), ciprofloxacin (5 μg), tigecycline (15 μg), nitrofuran (300 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg) (all from i2a, Montpellier, France) using the disk diffusion method on Mueller–Hinton agar (bioMérieux, Craponne, France) according to the recommendations of the Comité d'antibiogramme de la Société Française de Microbiologie (CA-SFM) 2013 recommendations. Minimum inhibitory concentrations (MICs) were determined using the Etest method for vancomycin and tigecycline according to CA-SFM guidelines 2013.

2.3. Detection of vancomycin resistance genes

Screening for the presence of *vanC* resistance genes was performed on the isolates presenting vancomycin MICs of ≥4 μg/mL using PCR amplification and sequencing. The primers used are shown in Table 1.

2.4. Clonality analysis

The genetic relationship among the clinical isolates was investigated by MLST using seven housekeeping genes (*gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt* and *yiqL* for *E. faecalis* and *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS* and *adk* for *E. faecium*) as previously described [24,25]. Phylogenetic analysis among *E. faecalis* isolates was performed using eBURST software as previously described [17,21].

2.5. Statistical analyses

Statistical analyses were performed using Epi Info software v.7 according to the recommendations of the US Centers for Disease Control and Prevention (Atlanta, GA) (http://www.openepi.com/Menu/OE_Menu.htm) using a two-sided Pearson's χ^2 test or Fisher's exact test, as appropriate. Differences were considered statistically significant at a P-value of <0.05.

3. Results

3.1. Bacterial strain identification

Identification of strains using conventional microbiological methods showed that all of the isolates belonged to the *Enterococcus* genus. Microbiological characterization of the genus was followed by MALDI-TOF/MS identification at the species level (Fig. 1) and classified 64 (75.3%) *E. faecalis*, 18 (21.2%) *E. faecium*, 2 (2.4%) *Enterococcus gallinarum* and 1 (1.2%) *Enterococcus casseliflavus*, with an average identification score of 2.29.

3.2. Epidemiological features of the Enterococcus sp. strains

Antibiotic susceptibility test results of the *Enterococcus* spp. strains as well as classification of the patients infected by each strain analysed are presented in Table 2. Infection sites were classified according to the samples from which each enterococci strain was isolated, including 32 (37.6%) from urinary tract infections (UTIs), 18 (21.2%) from pus, 18 (21.2%) from vaginal samples, 16 (18.8%) from bacteraemia and 1 strain (1.2%) from a semen sample.

3.2.1. Antimicrobial susceptibility patterns

Antimicrobial susceptibility testing showed that *E. faecium* isolates were more resistant to high-level aminoglycosides and ciprofloxacin than *E. faecalis* (Table 2). On the other hand, resistance to amoxicillin and nitrofuran was detected only among *E. faecium* strains, with resistance percentages of 83.3% and 38.9%,

Table 1
Oligonucleotide primers used for *vanC* genes.

Primer name	Length (nucleotides)	Primer sequence	Amplicon size (bp)	Reference
VanC-F	25	GAGGCAATTCACCGGAATACACCGT	957	This study
VanC-R	23	GCCATCATGGCAGGATAGCGGGA		This study
VanC1-F	26	TGTGATCCAAGCTATTGACCCGCTGA	922	This study
VanC1-R	24	CGTAGGATAACCCGACTTCCGCCA		This study