ORIGINAL ARTICLE

First detection of *vanA* positive *Enterococcus faecium* clonal complex 17 in hospital wastewater in Algeria: an epidemiological report

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Abstract

Enterococcus spp. are Gram-positive cocci that are recognised as critical opportunistic pathogens, especially in immunocompromised patients. Vancomycin is considered as the drug of last resort for the treatment of infections caused by *Enterococcus* species, making vancomycin resistance a serious public health concern. In this article, we report the first environmental *vanA* positive *Enterococcus faecium* isolates in Algeria. The strains were selectively isolated from hospital wastewater and then identified using matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry. Antibiotic susceptibility testing was performed using the disc diffusion method. Vancomycin resistance genes were searched for by standard PCR and the clonal relatedness of our isolates was investigated by multilocus sequence typing. A total of five highly vancomycin-resistant Gram-positive bacteria were isolated and identified as *Enterococcus faecium*. The isolates harboured the *vanA* gene and were assigned to the clonal complex 17. Our findings confirm the great potential of hospital wastewater as a reservoir and dissemination pathway of multidrug resistant organisms, and alert to the need for better regulation of hospital waste management in order to reduce their impact on the environment.

Keywords: Algeria, *Enterococcus faecium*, hospital wastewater, *vanA*, vancomycin resistance
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Introduction

Enterococcus spp. are Gram-positive cocci that are ubiquitous in the environment and feature among the normal inhabitants of the gastrointestinal tract of humans and animals [1]. However, they are also recognised as critical opportunistic pathogens, especially in immunocompromised patients, where *Enterococcus faecium* and *Enterococcus faecalis* are the most important species [2]. They can cause a variety of life-threatening infections such as urinary tract and surgical wound infections, bacteraemia, and endocarditis [3]. With the rapid emergence and dissemination of antibiotic resistance among these pathogens, vancomycin is considered as the drug of last resort for the treatment of such infections [4]. Vancomycin is a glycopeptide antibiotic which acts by inhibiting the cell wall biosynthesis of Gram-positive bacteria. This inhibition is based on binding to the terminus of the murein pentapeptide precursors, the D-alanyl-D-alanine, which is the target of glycopeptides [2,5]. However, after thirty years of glycopeptide use, the first vancomycin-resistant *Enterococcus faecium* (VREfm) emerged in 1986 [6]. Acquired vancomycin resistance is mediated by different clusters (*vanA*, *B*, *D*, *E*, *F*, *G*, *L*, *M*, and *N*), with *vanA* being the most clinically

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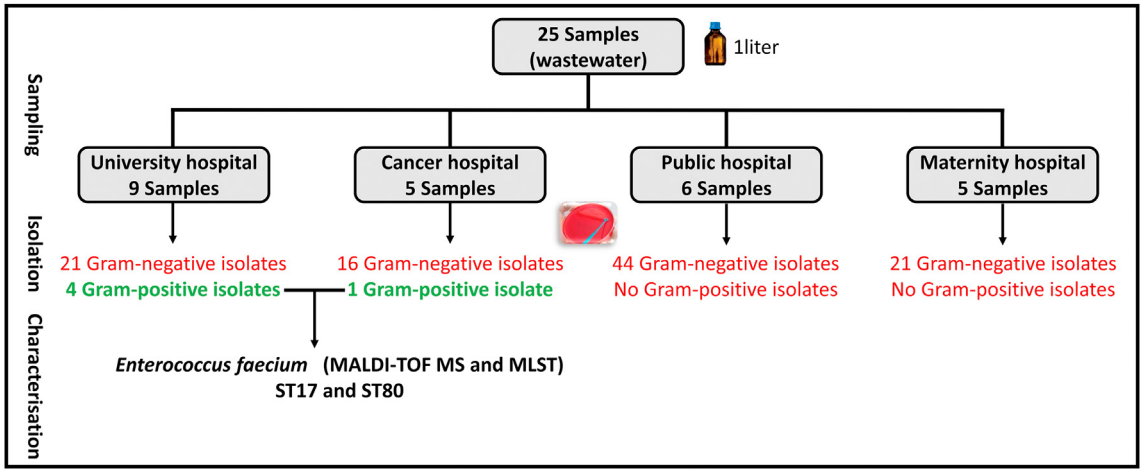

FIG. 1. Method flow chart for the present study.

relevant. These operons are responsible for replacing the final D-alanine of the murein pentapeptide precursor with a D-lactate or a D-serine, resulting in the non-binding or decreased binding of the glycopeptide to its target [7].

Antibiotic-resistant bacteria including VREfm have been widely considered as hospital-associated pathogens. However, in recent years, bacteria which are resistant to last resort antibiotics have been widely detected in community-acquired infections, suggesting the presence of potential reservoirs for such pathogens outside hospitals [8]. Hospitals are hot spots for antibiotic resistant bacteria and resistance genes. These latter, harbored either by bacterial cells, plasmids or phages, are certainly eliminated via wastewater discharge contributing to the widespread dissemination of antibiotic resistance in the environment and possibly impacting human health [9]. Monitoring the occurrence of antibiotic-resistant bacteria in hospital sewage would; therefore, appear to be of crucial importance. In the present study, we report the first environmental *vanA* positive *E. faecium* isolates from Algeria via their isolation from hospital wastewater.

Materials and methods

These isolates were obtained coincidentally during a programme to screen for carbapenem- and colistin-resistant Gram-negative bacteria in hospital sewage between November 2018 and October 2019. Twenty-five wastewater samples were collected in one-litre sterile glass bottles from four hospitals in Batna, Algeria (Fig. 1). Two hundred millilitres of each sample were aseptically centrifuged at 7000 rpm (4° C) for 15 min and then one 10 µL inoculation loop of each pellet was cultured on MacConkey agar plates with different selective antibiotic combinations, each containing 64 µg/mL of vancomycin. At the same time, 2 mL of the pellet was enriched in brain heart infusion broths, each supplemented by 64 µg/mL of vancomycin. After overnight incubation, a 10 µL-inoculation loop from each tube was cultured on MacConkey agar plates with the same antibiotic combinations. Plates were incubated aerobically at 37° C for 24 h. Gram-positive isolates were purified, then identified at the species level using matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS) [10]. Antibiotic susceptibility testing was performed using the disc diffusion method according to the antibiotic committee of the French society for microbiology, and minimum inhibitory concentrations (MIC) of vancomycin were determined using the E-test method. The following antibiotics were tested: penicillin, vancomycin, teicoplanin, erythromycin, clindamycin, gentamycin, doxycycline, minocycline, linezolid, fosfomycin, pristinamycin, rifampicin, and nitrofurantoin.

In addition, genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany), and then vancomycin resistance genes of *vanA* and *vanB* were searched for by standard PCR using previously described primers [11]. Standard PCR conditions were as follows: initial denaturation at 95° C for 15 min; 35 cycles at 95° C for 30 s, 55° C for 50 s, and 72° C for one min; and final incubation at 72° C for 10 min.

The clonal relatedness of our isolates was investigated by multilocus sequence typing (MLST) using PCR amplification and sequencing of the internal fragments of seven housekeeping genes *atpA, ddl, gdh, purK, gyd, pstS,* and *adk*. Sequence types were determined according to the MLST database as previously described [12] https://pubmlst.org/bigsdb?db=pubmlst_efaecium_seqdef&page=profiles&scheme_id=1.

Results and discussion

Altogether, 97 Gram-negative and five Gram-positive isolates were obtained. All Gram-positive strains were identified at the species level as *E. faecium* with an identification score >2. Four of these isolates were from wastewater from the Batna university hospital and one from the wastewater from the anti-cancer hospital. Antibiotic susceptibility testing performed using the disc diffusion method showed that our isolates exhibited different multidrug resistance phenotypes. As shown in Fig. 2, all isolates were resistant to penicillin G, vancomycin, teicoplanin, erythromycin, clindamycin, and gentamycin. Three isolates were intermediate to rifampicin and only one was intermediate to pristinamycin. However, the most effective antibiotics were doxycycline, minocycline, linezolid and fosfomycin. In addition, minimum inhibitory concentrations, determined using the E-test method, confirmed that our isolates were highly resistant to vancomycin with MICs >256 µg/mL. Standard PCR performed to detect *vanA* and *vanB* genes showed that our isolates harboured the *vanA* gene. MLST results showed that four of our isolates were assigned to the ST80 and one to the ST17. These two sequence types belong to the clonal complex 17 (Fig. 2).

In Algeria, only four previous reports have described the detection of vancomycin-resistant *E. faecium* isolates harbouring the *vanA* gene. The first detection, published in 2013, reported the isolation of *vanA* positive *E. faecium* from a surgical wound infection being treated at the University Hospital of Algiers [13]. The second report described a potential outbreak which involved three VREfm clonally related strains (identical pulsotype) isolated over a six-month period at the Batna University Hospital. Interestingly, the three responsible strains were assigned to the sequence type ST80 [14], the same as identified in our study. The third study reported the detection of *vanA* gene in a collection of clinical *E. faecium* isolates from different Algerian regions, also belonging to the CC17 [15]. The last one described a high prevalence of *vanA* positive *E. faecium* isolates in hospitalized patients at the Tlemcen University Hospital [16].

Indeed, it is not surprising that our isolates belonged to CC17, as this is known to be adapted to hospital environment [1]. However, what is of concern is the possible dissemination of such hospital-associated antibiotic resistant pathogens into the environment and the community via wastewater. Recently, *vanA* positive *E. faecium* isolates belonging to CC17 were recovered both from hospital wastewater and surrounding environments in South Africa [17], confirming the suggestion of the possible dissemination of such resistant organisms via aquatic environments.

VREfm is listed by the World Health Organization in the high priority category of pathogens for which new drugs are urgently needed [18]. Several previous studies have reported the isolation of *vanA* positive *E. faecium* from different water environments, including hospital and urban wastewater [19], animal waste [20], and surface water [21]. The detection of such drug-resistant pathogens in hospital sewage is of great interest, considering their potential dissemination into the environment and the community via the water cycle. The persistence of vancomycin resistant *Enterococcus* and the *vanA* gene in surface water affected by wastewater has been previously investigated. The authors confirmed that *vanA* positive *Enterococcus faecium* were cultured from water and sediment for up to three days after a sewage spill. In addition, the *vanA* gene was detectable by quantitative PCR for an additional week [22], confirming the impact of the discharge of wastewater on the environment.

Our findings confirm the great potential of hospital wastewater as a reservoir and dissemination pathway for multidrug resistant organisms, and alert to the need for better regulation of hospital waste management in order to reduce this impact on the environment.

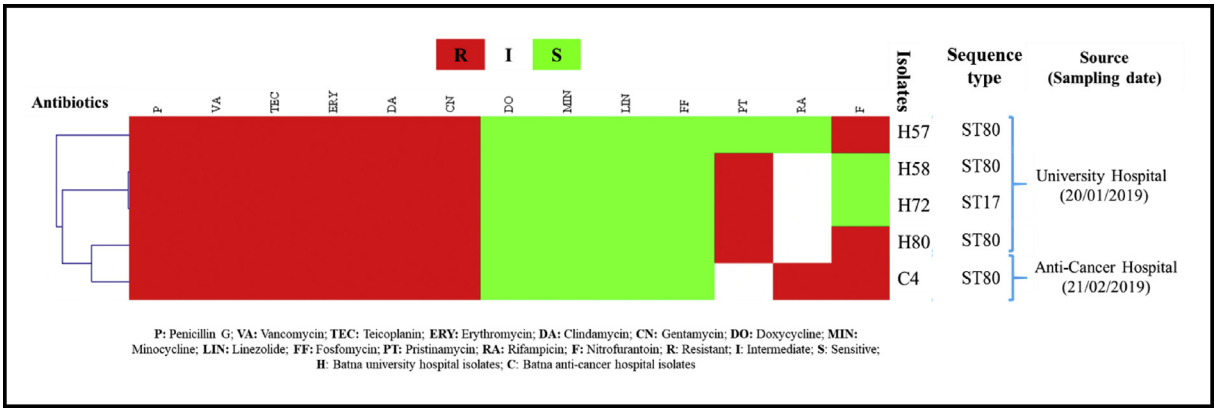

FIG. 2. Antibiogram and MLST results of the *E. faecium* isolates clustered using the MultiExperimentViewer (MEV) software version 4_6.

Transparency declaration

The authors declare no conflicts of interest.

Ethical approval

Not required

Author statement

Zineb Cherak: Methodology, Investigation, Validation, Visualization, Writing - Original Draft. **Esma Bendjama**: Methodology, Investigation, Validation. **Abdelhamid Moussi**: Supervision, Writing - Review & Editing. **Amel Benbouza**: Resources. **Nadia Grainat**: Resources. **Jean-Marc Rolain**: Conceptualization, Writing - Review & Editing. **Lotfi Loucif**: Conceptualization, Methodology, Visualization, Writing-Review & Editing. All authors approved the final manuscript.

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