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Screening municipal wastewater effluent and surface water used for drinking water production for the presence of ampicillin and vancomycin resistant enterococci

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ABSTRACT

The emergence of clinical enterococcal isolates that are resistant to both ampicillin and vancomycin is a cause of great concern, as therapeutic alternatives for the treatment of infections caused by such organisms are becoming limited. Aquatic environments could play a role in the dissemination of antibiotic resistant enterococci. This study investigated the presence of ampicillin and vancomycin resistant enterococci in the treated effluent of six wastewater treatment plants (WWTPs) and in surface water used as a source for drinking water production in the Netherlands. Membrane filtration in combination with selective media with ampicillin or vancomycin was applied to determine the presence of ampicillin resistant *Enterococcus* (ARE) and vancomycin resistant *Enterococcus* (VRE) species. Ampicillin resistant *Enterococcus faecium* (minimal inhibitory concentration (MIC) >16 μg/mL; n = 1033) was observed in all studied WWTP effluents. In surface water used for drinking water production (intake locations), no ARE or VRE were observed. At both types of location, intrinsic vancomycin resistant *Pediococcus* spp., *Leuconostoc* spp. and *Lactobacillus* spp. were isolated with the vancomycin medium. The ampicillin resistant *E. faecium* (ARE*fm*) isolates (n = 113) did not contain the *vanA* or *vanB* gene, but MIC testing for vancomycin showed intermediate vancomycin resistance (2–8 μg mL^{-1}) to occur in these ARE*fm* strains. This study documents the discharge of ampicillin resistant *E. faecium* strains with intermediate vancomycin resistance by the WWTPs into the surface water, but no presence of these strains downstream at intake locations for drinking water production.

1. Introduction

Enterococcus species are part of the natural intestinal flora of both humans and animals. Because of their abundance in the faeces of warm-blooded animals and their long-term survival in the environment, they have traditionally been used as indicators of faecal contamination in the aquatic environment, including sewage, rivers and coastal areas (Anonymus, 1986; Haach et al., 2003; Nishiyama et al., 2015; Shibata et al., 2004), where they are ubiquitously detected (Lleo et al., 2005; Murray, 1990).

Some of the *Enterococcus* species have also been widely reported as opportunistic pathogens causing infections of the urinary tract, the bloodstream or skin wounds of immunocompromised persons (Jett et al., 1994) in healthcare settings. Ampicillin and vancomycin are important antibiotics in the treatment of those infections. In infections with ampicillin resistant enterococci (ARE), vancomycin can still be used, but that has led to the development of *Enterococcus* strains that are not susceptible to vancomycin; these are known as vancomycin resistant enterococci (VRE). Thus, therapeutic options for ARE and VRE infections are becoming limited.

It has been discovered that the phenotypic association of ampicillin and vancomycin resistance is often due to a genetic linkage and co-transfer of determinants responsible for resistance to both antibiotics (Shepard and Gilmore, 2002). A polyclonal outbreak of VRE, 95% of which were *Enterococcus faecium*, in several hospitals in northeast Ohio led to the identification of transferable ampicillin

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and vancomycin resistance among many of the isolates (Donskey et al., 1999; Shepard and Gilmore, 2002). An analysis of several of the VRE strains isolated during the outbreak revealed the presence of a novel Tn*916*-like transposon (Tn*5382*) encoding the *vanB* resistance. The transposon was integrated within a larger transferable element that also contained a gene encoding an alternate PBP5 with decreased affinity for binding to ampicillin (Carias et al., 1998; Shepard and Gilmore, 2002).

Enterococci possesses a natural, low-level intrinsic resistance to β-lactam antibiotics (including ampicillin), which is due to the low affinity of their penicillin binding proteins (PBPs) for the β-lactam agents (Kak and Chow, 2002). This natural resistance was mainly found in *E. faecium* and *Enterococcus faecalis*, but was also described in *Enterococcus raffinosus* strains (Facklam et al., 2002). On top of the intrinsically present resistance to beta-lactams and aminoglycosides, hospital-derived *E. faecium* has acquired resistance to high levels of aminoglycosides and beta-lactams (including ampicillin) through a combination of mutations and horizontal gene transfer (Top et al., 2008a).

VRE infections are caused mostly by *E. faecalis* and *E. faecium* (Cetinkaya et al., 2000; Jett et al., 1994; Marothi et al., 2005). Nine gene clusters associated with vancomycin resistance have been identified in *Enterococcus* species: *vanA* to *vanN* (Table 1).

VanA and *vanB* are clinically the most important genotypes (Arias and Murray, 2013; Cetinkaya et al., 2000; Klare et al., 2003). The *vanA* resistance operon is acquired through the Tn*1546* transposon, and the *vanB* resistance operon is acquired through the exchange of transposons Tn*1547* and/or Tn*5382* (Kak and Chow, 2002). Vancomycin intrinsically resistant species do not cause the same infection control concerns as *E. faecium* or *E. faecalis* VRE, as their resistance is chromosomal rather than plasmid mediated (Griffin et al., 2012).

The transfer of resistant bacteria from environmental compartments to humans may occur through contaminated food (Perreten et al., 1997), manure (if used as a fertilizer) and contaminated surface water used for irrigation or as recreational water. Wastewater and sludge from municipal sewage water treatment plants have been reported as favourable environments, consisting of variable mixtures of bacteria, nutrients and antimicrobial agents, for both survival and gene transfer (Lindberg et al., 2004), spreading resistant bacteria in both aquatic and terrestrial environment (Iversen et al., 2004). An additional concern is the possible presence of resistant enterococci in surface water used as a source for the production of drinking water.

The presence of a large reservoir of VRE in the environment could pose a threat for the transmission of vancomycin resistant bacteria to humans, either of enterococcal strains harbouring vancomycin-resistance genes, or via the horizontal spread of the genetic elements. This study investigated the presence of enterococci that are resistant to ampicillin and vancomycin in effluent from wastewater treatment plants (WWTPs) and in the surface water used for drinking water production in the Netherlands, applying membrane filtration and Slanetz and Bartley agar (SBA) complemented with ampicillin or vancomycin.

2. Material and methods

2.1. Sampling and sampling locations

In September 2014, 1-L sample was collected in sterile bottles at six WWTP effluent locations and at four locations (one river, two canals and one lake) where surface water is used as a drinking water production source (intake locations). All sampling points were located in the western part of the Netherlands. At the municipal WWTPs, the amount of the influent wastewater and the amount of treated wastewater that was discharged were similar. The flow rates at the WWTPs 1–6 were 2×10^3 m^3 d^{-1}, 3×10^3 m^3 d^{-1}, 8×10^3 m^3 d^{-1}, 8×10^3 m^3 d^{-1}, 1.3×10^4 m^3 d^{-1} and 2.8×10^4 m^3 d^{-1} respectively. Beside domestic waste water and storm water, WWTPs 2 and 4 also received hospital waste water. Treatment steps in these WWTPs are comparable and consist of: bar screens, grit chambers, primary sedimentation, aeration, activated sludge and a second sedimentation step. At the WWTPs, samples were collected over a 24-h period, harvesting 45 ml every 60 min. All samples were transported at 4 °C to the laboratory and analysed within 24 h after sampling.

2.2. Isolation, enumeration and identification

Filtration was performed (ISO 7988-2:2000) using a nitrate membrane filter (0.45 μm, Sartorius, Germany) and Slanetz and Bartley agar (SBA, Oxoid, England). Additionally, SBA with the addition of 16 μg mL^{-1} of ampicillin (Sigma Aldrich, A9393-5G, USA) or the addition of 16 μg mL^{-1} of vancomycin (Sigma Aldrich, 75423-5VL, USA) were used for the detection and enumeration of ARE and VRE, respectively. The concentrations of the antibiotics were based on clinical breakpoints indicated by the Clinical and Laboratory Standards Institute (CLSI, 2014). In order to provide the correct enumeration of the colonies in 1 L, sub-samples of 50–100 ml were filtered. Petri dishes of the three different media were incubated for 48 h at 37 °C. After incubation, filters with pink, red, maroon or brown colonies were removed from the SBA agar and placed on Bile-esculin-azide agar (BEAA, Merck, Germany) and incubated for another 2 h at 44 °C. After incubation, dark brown to black colonies were considered as enterococci. Ten percent of the isolates obtained on ampicillin-SBA for each location and the selection of isolates obtained on vancomycin-SBA (all colonies found at intake locations and a few morphologically different colonies per WWTP) were freshly cultured on SBA without supplement and subsequently identified (n = 1333) using matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS, Software version 3.0, Microflex series, Bruker Daltonics Inc., Germany), following the manufacturer's instructions. Score values of ≥2.0 were considered as reliable identifications. Ampicillin and vancomycin resistant *E. faecium* strain (AVRE) was used as a positive control, and ampicillin and vancomycin sensitive *E. faecalis* strain (ATTC 27270) was used as a negative control. The AVRE strain was kindly provided by the microbiology laboratory of the University Medical Centre Groningen (UMCG).

2.3. Antibiotic susceptibility testing

As a quality control, the selectivity of SBA supplemented with ampicillin was tested first. The MICs of two randomly chosen isolates per WWTP location (in total 12 isolates) were analysed by the Vitek 2 system (version 6.01, bioMérieux, France). In short, a 0.5 McFarland (McF) bacterial suspension was prepared using fresh colonies and then the AST-P586 card (bioMérieux, France) was used for susceptibility measurements according to the manufacturer's instructions. An MIC of ≥16 μg mL^{-1} was considered as resistant (CLSI guidelines, 2014). Secondly, 20 randomly chosen ARE isolates per WWTP were investigated for their ability to grow on SBA supplemented with vancomycin and the MIC values were determined using the Epsilometer test (Etest) (bioMérieux, France). Selected isolates were streaked on SBA supplemented with vancomycin and incubated for 48 h at 37 °C. In order to determine the MIC, first a suspension of 2.0 McF in 0.45% saline solution was prepared by emulsifying freshly grown colonies using a sterile swab. Brain–heart infusion (BHI) agar (Oxoid, England) was used as medium for Etest. It was inoculated with the 2.0 McF suspension and the Etest strip vancomycin (range of 0.016–256 μg mL^{-1}) was

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