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Molecular characterization of low-level vancomycin-resistant enterococci found in coastal water of Thermaikos Gulf, Northern Greece

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ABSTRACT

Enterococcus includes species that may pose emerging health risks and has been used as biomarkers for environmental contamination while little is known concerning their occurrence in marine water. Classification of enterococci in environmental samples can be problematic and requires polyphasic taxonomy. In this study, we investigated the presence of vancomycin-resistant enterococci (VRE) in the inner bay of Thermaikos Gulf in Northern Greece. Based on physiological and biochemical criteria, 121 presumptive enterococcal strains were identified. High-level VRE were undetectable in seawater and only 35 vancomycin gene-negative strains possessed low-level vancomycin resistance. Genotyping by pulsed field gel electrophoresis (PFGE) proved to be more reliable for marine enterococcal discrimination and revealed distinguished characteristics of the seawater enterococci, indicating high genetic diversity. Random amplified polymorphic DNA-PCR (RAPD-PCR) was unable to separate distinct species analyzed in this study. This study indicates the need of polyphasic taxonomy for seawater enterococcal species' identification and provides information for future biomonitoring programs of Thermaikos Gulf.

1. Introduction

Enterococci are Gram-positive bacteria that live as part of natural flora in the human and animal gastrointestinal tract. Because of their abundance in feces of warm-blooded animals and their long survival in the environment, they have been traditionally used as indicators of fecal contamination in the aquatic environment (Del Mar Lleo et al., 2005; Manero et al., 2002). Certain species are considered to be opportunistic pathogens for humans and over the last decade have emerged as a significant cause of hospital infections due to their ability to acquire high- or low-level resistance to glycopeptides such as vancomycin and teicoplanin (Iversen et al., 2002; Gholizadeh and Courvalin, 2000). Six phenotypes named vanA–vanE and vanG have been isolated according to their glycopeptide resistance (Woodford, 2001).

The competence of vancomycin-resistant enterococci (VRE) to spread in different environments and to transfer antibiotic-resistance genes among different species, indicates their release to the environment as a major matter of concern

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(Novais et al., 2005). Although a number of studies report a high prevalence of VRE in aquatic habitats including crude inflow, treated effluent, surface water and sludge from urban and hospital wastewater treatment plants, much less is known about the survival, growth and occurrence of VRE in natural waters such as marine water (Harwood et al., 2001).

Thermaikos Gulf is the most important gulf in Northern Greece and is a natural harbor of the city of Thessaloniki. However, Thermaikos Gulf is considered to be one of the most polluted coastal zones in Greece. It is the final receptor of both municipal and industrial secondary-treated wastewaters from the city of Thessaloniki (Nikolaidis et al., 2006). Furthermore, two heavily polluted rivers, Axios and Aliakmon, with pollutant loads that include agricultural runoff, animal husbandry, industrial effluents and potential fecal pollution indicators (Simeonov et al., 2003; Arvanitidou et al., 2005) are discharging into the gulf. In addition, VRE isolates have been reported from Thessaloniki city hospital units (Gikas et al., 2005; Sofianou et al., 2004; Pournaras et al., 2000). It was of interest to examine the hygienic status of seawater by investigating whether VRE strains are present in the proximal coastal zone of Thessaloniki city harbor. Additionally, the aim of this study was to determine the diversity of *Enterococcus* strains present in marine water from the above gulf.

2. Methods

2.1. Seawater sampling and isolation of enterococci

Sampling was carried out once a month during the years 2003 and 2004. Water samples were obtained from 12 different defined regions of the gulf costal zone, at a distance of 300–500 m from the coast (Fig. 1) in sterile bottles, 30 cm below the surface by standard methods as recommended in section 9030 of Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1998).

Bacteriological examination was carried out using the multiple tube technique in a series of 10-fold dilutions, with five tubes per dilution and quantified using the most probable number. Azide dextrose broth (Merck KGaA, Germany) was used as a presumptive test followed by the confirmation test in kanamycin azide (KA) agar (Merck KGaA, Germany). For each sample, 5–6 morphologically different colonies were isolated and stored at −80 °C in a medium containing 15% glycerol.

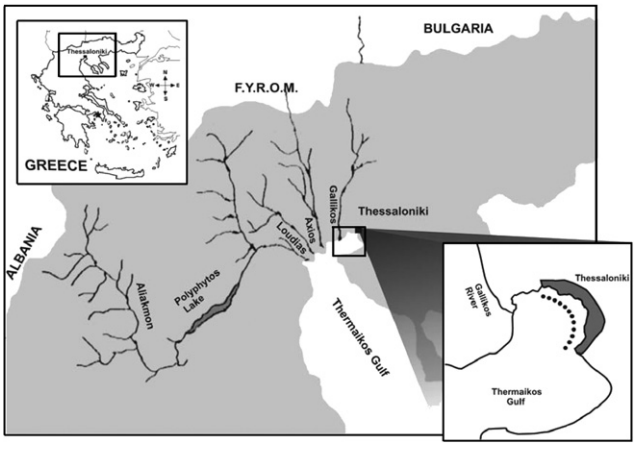

Fig. 1 – Thermaikos Gulf map showing the rivers (Simeonov et al., 2003) discharged into. Sampling locations in Thessaloniki inner bay are indicated by filled circles.

2.2. Phenotypic characterization of isolates

Seawater isolates were subjected to preliminary tests to determine their membership in the genus of *Enterococcus*, including Gram staining, catalase activity, aesculin hydrolysis and growth in the presence of 40% (v/v) bile, in brain heart infusion broth (Merck KGaA, Germany) with 6.5% NaCl (37 °C), at pH 9.6 (37 °C), and growth at 45 °C. Identification to the species level was performed with standard biochemical tests that were prepared in-house. Biochemical tests included carbohydrate fermentation with 1% L-arabinose, D-sorbitol, lactose, D-raffinose, ribose, sorbose, sucrose and methyl-α-D-glucopyranoside, arginine hydrolysis, pyruvate utilization and pigment production. The results of the above tests were interpreted using published standard biochemical identification charts (Domig et al., 2003; Manero and Blanch, 1999; Facklam and Collins, 1989). Motility was detected as described by Van Horn et al. (2002) using isolates that were inoculated in trypticase soy broth and incubated at 30 °C for 2 h. The direct wet mount method was then used to detect motility by dark-field microscopy. In addition, enterococcal strains were isolated from blood cultures of patients admitted to 'Sotiria' General Hospital, Athens, Greece. Blood cultures were processed by automated system Bactec 9240 (Beckton Dickinson). The strains isolated were identified by ApiStrep (Biomerieux) and the automated system Vitek2.

2.3. Determination of minimum inhibitory concentration (MIC)

Susceptibility of seawater enterococcal isolates to vancomycin was examined by the broth dilution method, according to the National Committee for Clinical Laboratory Standards recommendations (NCCLS, 1998). The vancomycin concentration used to determine the MICs ranging from 2 to 64 μg/ml. Seawater enterococcal strains with resistance to MIC were further analyzed for genotypic characterization and detection of the resistance genotypes.

Sensitivity testing for human enterococcal isolates was performed by the Kirby–Bauer method, according to NCCLS directions and MIC by automated system Vitek2 (Biomerieux). Human enterococcal strains were further analyzed for detection of the genotypes resistant to vancomycin by PCR amplification.

2.4. PCR amplification for the determination of van-resistant strains

PCR amplification was performed as described previously (Depardieu et al., 2004) to determine vancomycin resistance genes in enterococcal isolates with vancomycin MICs ranging from 2 to 8 μg/ml. Seven different primer pairs corresponding