PFGE analysis of enterococci isolates from recreational and drinking water in Greece

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ABSTRACT

Biotyping and DNA fingerprinting by pulsed-field gel electrophoresis were applied to a collection of enterococci recovered from recreational and drinking water, in order to identify possible genetic relationships. Clinical strains of hospital origin were compared to the environmental isolates. A total of 104 enterococci were isolated from 128 recreational water (94 marine and 34 river water) and 470 drinking water supplies (440 municipal and 30 natural spring water samples). Sixty-two isolates were characterised as *Enterococcus faecium* recovered from all sources, 32 *E. faecalis* (from all sources), 4 *E. durans* (from marine, river and municipal water), 4 *E. gallinarum* (from marine water) and 2 *E. avium* (from marine and municipal water). Biotypes, determined with API20Strep, among *E. faecium* were correlated with certain environmental sources, while antibiotypes, determined with Etest, did not reveal any relationship to the sample origin. Even though genetic diversity was observed among the studied strains, common clonal types were also identified in different sources, suggesting a possible common origin of the enterococci. Cluster analysis revealed a genetic relationship between certain environmental *E. faecium* and clinical strains.

Key words | biotypes, DNA fingerprinting, *Enterococcus* spp, water supplies

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INTRODUCTION

Enterococci have emerged as important nosocomial and community-acquired pathogens in recent years (Mundy *et al.* 2000). It is also demonstrated that they have the capacity to exchange genetic information by conjugation, a process known to take place in the gastrointestinal tract, and thus acquiring virulence and antimicrobial resistance factors (Mundy *et al.* 2000). They are members of the natural intestinal flora of animals and humans and are being released into the environment directly or via sewage outlets. These characteristics, in addition to their ability to persist longer in the environment than other bacteria, have made them good indicators of faecal contamination in water quality studies (Godfree *et al.* 1997).

The Greek Ministry of Health set the limits of pollution for enterococci in marine and river waters (recreational waters) to 100 cfu/100 ml, while for drinking water supplies the limit is zero. The US Environmental Protection Agency (USEPA) advised different limits for marine water and freshwater samples (104 cfu/100 ml for marine water and 61 cfu/100 ml for freshwater) (Office of Science and Technology, US Environmental Protection Agency 2000). Several studies of both recreational and drinking water samples suggested that enterococci are more relevant indicators than faecal coliforms and *Escherichia coli* of water quality (Godfree *et al.* 1997; Kinzelman *et al.* 2003). Epidemiological studies have demonstrated a direct relationship between the density of enterococci in surface waters and the risk of swimmer-associated gastroenteritis (Kinzelman *et al.* 2003). Furthermore, the risk posed to humans is greater for human-derived waste than for animal-derived waste (Wiggins *et al.* 1999).

Multi-resistant and especially glycopeptide-resistant enterococci (GRE) were previously characterised from

doi: 10.2166/wh.2006.007

human and non-human sources (Woodford *et al.* 1998; Borgen *et al.* 2002; Iversen *et al.* 2002). The presence of GRE isolates in the environment is possibly due to the spread of hospital strains or the use of avoparcin as a feed additive in animals (Iversen *et al.* 2002). Comparison of antibiotic resistance patterns has been applied for the identification of enterococci isolated from different sources (Wiggins *et al.* 1999). Even though application of the multiple antibiotic resistance index successfully discriminated different types of faecal streptococci (Wiggins *et al.* 1999), genetic fingerprinting by the application of pulsed-field gel electrophoresis (PFGE) might be a more suitable method for the identification of individual or clonally related strains (Hagedorn *et al.* 1999; Borgen *et al.* 2002).

In this study we have identified the enterococcal species isolated from different environmental sources in south-western Greece and we have characterised their biotypes, antibiotic resistance patterns and PFGE types. We have investigated the possible relation of biotypes and clonal types to the sources of isolated enterococci. In order to determine a possible relationship of the environmental to human isolates, comparison with clinical strains of nosocomial origin was also performed.

METHODS

One hundred and twenty-eight recreational water samples (94 marine and 34 river water) and 470 drinking water supply samples (440 municipal and 30 natural spring water samples) were collected in the Achaia Prefecture (south-western Greece) during a six-month period (May – December 2002). All were placed on ice and bacteria isolation was processed within 6 h by the application of the membrane filter technique (Slanetz & Bartley 1957; ISO 7899-2 2000). Enterococci were characterised by Gram stain, catalase production, growth in 6.5% NaCl, bile esculin hydrolysis and additional biochemical tests by the API 20Strep system (bioMerieux, SA Lyon, France). Biotypes were determined according to the analytical profile index provided by the manufacturer.

Three clinical *Enterococcus faecium* strains resistant to glycopeptides (GRE) were included in the genotypic experiments as controls, representing three already identified endemic clones in the University Hospital of Patras.

Minimal Inhibitory Concentrations (MICs) of the antibiotics penicillin G (pG), ampicillin (amp), amoxicillin with clavulanic acid (amc), oxacillin (ox), ceftriaxone (cro), gentamicin (gm), erythromycin (em), ciprofloxacin (cip) and vancomycin (va) were determined by the Etest (BIODISK, Solna, Sweden) in Mueller-Hinton agar and the results were interpreted according to NCCLS criteria (NCCLS 2001). Beta-lactamase production was tested by nitrocefin disks (Difco Laboratories, Detroit, MI) according to the manufacturer's instructions.

Total DNA was extracted from all isolates and embedded in agarose disks according to previously published protocols (Morrison *et al.* 1999). Clonal types were identified by pulsed-field gel electrophoresis (PFGE) of *Sma* I DNA digests in a CHEF DR III apparatus (Bio-Rad Laboratories, Hercules, CA). One to six band differences defined a PFGE subtype and seven or more band differences defined a distinct PFGE type characterised by capital letters for *E. faecium* strains and by small letters for the rest of the species (Tenover *et al.* 1995). The three patterns of GRE clinical strains were used as reference. PFGE types were evaluated by visual analysis followed by computer analysis using Whole Band Analyzer version 3.3 (BioImage, Ann Arbor, MI) software for a UNIX SparcStation 4 running under the SunOS version 5.5.1 operating system. Cluster analysis of electrophoretic band patterns was performed using SPSS for Windows software, version 10.0.1. Similarity levels were calculated by the Dice coefficient and clustering was achieved by the unweighted-pair group method using arithmetic average algorithms (UPGMA). The cut-off value was determined based on the criteria of Tenover *et al.* (1995).

RESULTS AND DISCUSSION

A total of 104 Gram-positive cocci that grew in the presence of 6.5% NaCl and hydrolysed esculin, being identified as enterococci, were isolated from 69 recreational water samples (60/94 or 64% positive marine samples and 9/34 or 26.4% river water samples) and 35 drinking water samples (30/440 or 6.8% positive municipal water and

5/30 or 16.6% spring water samples). All positive drinking water samples exceeded the zero limit for enterococci set by the Greek Ministry of Health. Moreover 8 recreational water samples were contaminated with levels higher than the 100 cfu/100 ml standard for marine and river waters. No monthly difference was observed in the occurrence of enterococci in the water samples during the study period. Faecal coliforms and *E. coli* were identified in 44 out of the 60 positive for enterococci marine water samples (73.3%), while 12 samples harboured both coliforms and enterococci. All 9 enterococci-positive river water samples and 5 natural spring water samples also harboured coliforms and *E. coli*. Among the municipal water samples 2 (6.7%) were positive only for enterococci, 6 (20%) contained both coliforms and enterococci, while 22 (73.3%) contained all three indicators (coliforms, *E. coli* and enterococci). It has

Table 1 | Enterococcal species isolated from different environmental sources

		Recreational water (128)[a]		Drinking water supplies (470)		
Species	**Biotypes[b]**	**Marine water (94)**	**River water (34)**	**Municipal water (440)**	**Natural spring water (30)**	**Total**
E. faecium (62)	7157711	11 (18%)[d]	–	6 (20%)[d]	3 (60%)[d]	20 (19%)[d]
	7357711	9 (15%)	–	–	–	9 (8.5%)
	7157511	7 (11.6%)	–	–	–	7 (7%)
	5157771	4 (7%)	–	–	–	4 (4%)
	5157711	4 (7%)	–	–	–	4 (4%)
	7357611	–	–	9 (30%)	–	9 (8.5%)
	5356771	–	–	5 (16.7%)	–	5 (5%)
	5157511	–	2 (22.2%)[d]	–	–	2 (2%)
	7157771	–	2 (22.2%)	–	–	2 (2%)
E. faecalis (32)	5143711	10 (17%)	2 (22.2%)	4 (13.4%)	2 (40%)	18 (17%)
	7143711	8 (13%)	2 (22.2%)	3 (10%)	–	13 (12%)
	7153311	–	–	1 (3.3%)	–	1 (1%)
E. durans (4)	7157401	1 (1.6%)	1 (11.2%)	–	–	2 (2%)
	7353411	1 (1.6%)	–	1 (3.3%)	–	2 (2%)
E. gallinarum (4)	7357573	2 (3.3%)	–	–	–	2 (2%)
	7357572	2 (3.3%)	–	–	–	2 (2%)
E. avium (2)	5142710	1 (1.6%)	–	1 (3.3%)	–	2 (2%)
Total	17	60 (64%)[c]	9 (26.4%)[c]	30 (6.8%)[c]	5 (16.6%)[c]	104 (100%)

[a]Number of samples.
[b]The code number of biotypes from the analytical profile index of API Strep (bioMerieux).
[c]Percentage of positive samples for enterococci.
[d]Percentage of isolates characterized in each biotype among the enterococci.

been reported that swimming-associated gastrointestinal disease presents a higher association with recreational waters containing high densities of *E. coli* and enterococci than those containing only faecal coliforms (Bartram & Rees 2000).

Characterization at the species level classified the enterococci into 17 biotypes (Table 1). The majority of the isolates were *E. faecium* (62 or 59.6%), followed by *E. faecalis* (32 or 30.8%), *E. durans* (four or 3.8%), *E. gallinarum* (four or 3.8%) and *E. avium* (two or 2%). The most prevalent species among all sample sources was *E. faecium* followed by *E. faecalis*. In north Greece among 1670 bathing water samples, the most frequently isolated enterococcal species were *E. avium*, *E. raffinosus* and *E. faecium* (Arvanitidou *et al.* 2001). Budnick *et al.* (1996) have isolated 32 enterococci, among 138 marine and freshwater bathing samples, that were characterised as *E. casseliflavus* or *E. gallinarum* (18/32), *E. faecium* (11/32), *E. casseliflavus* (1/32), *E. gallinarum* (1/32) and *E. durans* (1/32).

Biotyping of *E. faecium* revealed that one biotype (7157711) was isolated from marine, spring and municipal water samples, while four more biotypes were exclusively found in isolates from marine waters (7357711, 7157511, 5157771 and 5157711), two more in isolates from municipal waters (7357611 and 5356771) and another two from river water isolates (5157511 and 7157771) (Table 1). This finding was not observed for the rest of the enterococcal species and it is reported in the literature for the first time. In more detail, 18 *E. faecalis* belonging to biotype 5143711 were recovered from all sources, whereas 13 more isolates of biotype 7143711 were recovered from marine, river and municipal waters. Four *E. gallinarum* characterized into two biotypes (7357573 and 7357572) were isolated only from marine waters. *E. avium* and *E. durans* isolates, including one and two biotypes, respectively, were recovered from marine, river and municipal waters.

Antibiotic resistance patterns of enterococci have been successfully analysed by several investigators and contributed to the correct classification of isolates into four types, according to the isolation source (human, cattle, poultry and wild) (Wiggins *et al.* 1999). In another study, comparison of antibiotypes of well-known isolates resulted in a correct classification of enterococci from different sources (Hagedorn *et al.* 1999). In the present study, no association between the antibiotic resistance patterns and the source of isolates was found (data not shown). Figure 1 shows the percentage of resistant strains to the antibiotics used, among the enterococcal species. Most enterococci expressed low-level resistance to gentamicin (MIC$\leq$500 mg/L), except five *E. faecium* that had MICs $\geq$1024 mg/L. The majority were also resistant to oxacillin and ceftriaxone, while several strains expressed additionally resistance to erythromycin and ciprofloxacin. A considerable percentage of erythromycin and ciprofroxacin-resistant enterococci isolated in bathing waters were detected in a previous study in north Greece (Arvanitidou *et al.* 2001). Multi-resistant enterococci were also isolated from animal sources (Aarestrup *et al.* 2000). In this collection, no isolate was found to produce beta-lactamase and no glycopeptide-resistant *Enterococcus* spp. was detected. This is in contrast to Iversen *et al.* (2002) who reported the presence of GRE isolates in the environment.

The application of PFGE analysis of *Sma*I DNA fragments in our collection resulted in the characterization of 18 clonal types among *E. faecium*, 14 in *E. faecalis*, two in *E. durans*, three in *E. gallinarum* and one in *E. avium* (Table 2). Common clonal types were identified in strains isolated from different sources (types A, B, C, D, E, H, I, K, M, Y among *E. faecium* and types a, b, c, d, e, k, i and m among *E. faecalis*). The presence of certain clonal types among *E. faecium* and *E. faecalis* from different sources indicates the possible common origin of some strains. The identification of closely related strains in different sources may, in part, be explained by a possible transmission of

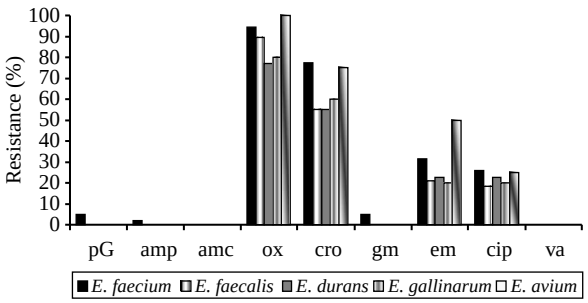

Figure 1 | Antibiotic resistance patterns of all enterococcal species. pG: penicillin G, amp: ampicillin, amc: amoxicillin with clavulanic acid, ox: oxacillin, cro: ceftriaxone, gm: gentamicin, em: erythromycin, cip: ciprofloxacin and va: vancomycin.

Table 2 | PFGE types of enterococci in relation to samples' origin

Bacterial species	Sample	Number of strains	PFGE patterns and number of strains included
E. faecium ($n = 62$)	Marine water	35	A = 2, B = 2, C = 4, D = 6, E = 5, F = 3, G = 2, H = 1, I = 2, K = 2, L = 1, M = 2, O = 1, V = 1, Y = 1
	Municipal water	20	A = 2, B = 4, C = 2, D = 4, E = 2, I = 1, J = 1, K = 2, R = 1, Y = 1
	River water	4	C = 1, D = 1, H = 1, M = 1
	Spring water	3	D = 1, E = 1, N = 1
E. faecalis ($n = 32$)	Marine water	18	a = 2, b = 2, c = 2, d = 2, e = 2, f = 1, g = 1, h = 2, i = 1, k = 1, m = 1, o = 1
	Municipal water	8	a = 1, b = 1, c = 1, d = 2, e = 2, m = 1
	River water	4	a = 1, j = 1, i = 1, k = 1
	Spring water	2	c = 1, r = 1
E. durans ($n = 4$)	Marine water	2	t = 1, y = 1
	Municipal water	1	t = 1
	River water	1	t = 1
E. gallinarum ($n = 4$)	Marine water	4	q = 2, s = 1, v = 1
E. avium ($n = 2$)	Marine water	1	w = 1
	Municipal water	1	w = 1
Total		104	38 PFGE types

n: number of strains.

isolates from natural spring waters to the rivers and finally to marine water, since these samples were collected in the same area. A circulation of *E. faecium* from hospitalised patients to hospital sewage and surface water was proposed by Iversen *et al.* (2004) in a study where the isolates were identified as genetically related by PFGE.

Diversity and the identification of one cluster including seven GRE strains were observed in a study including enterococci from sewage in Sweden (Iversen *et al.* 2002). It has been reported that *E. faecium* strains causing infections in human or hospital epidemics are genetically distinct from animal-derived strains (Willems *et al.* 2000). The comparison of PFGE types of the three main clones (type A clinical strain GRE120, type B clinical strain GRE130 and type C clinical strain GRE17) that were identified during an outbreak of glycopeptide-resistant *E. faecium* in our University Hospital during 2000–2001 (Kolonitsiou *et al.* 2004) showed a genetic relationship with certain vancomycin-sensitive environmental *E. faecium* (Figure 2). This result indicates that, even though no GRE isolate was identified in the environment, our isolates were genetically related and since *E. faecium* has a greater ability to acquire drug resistance we cannot exclude the future emergence of GRE in the environment.

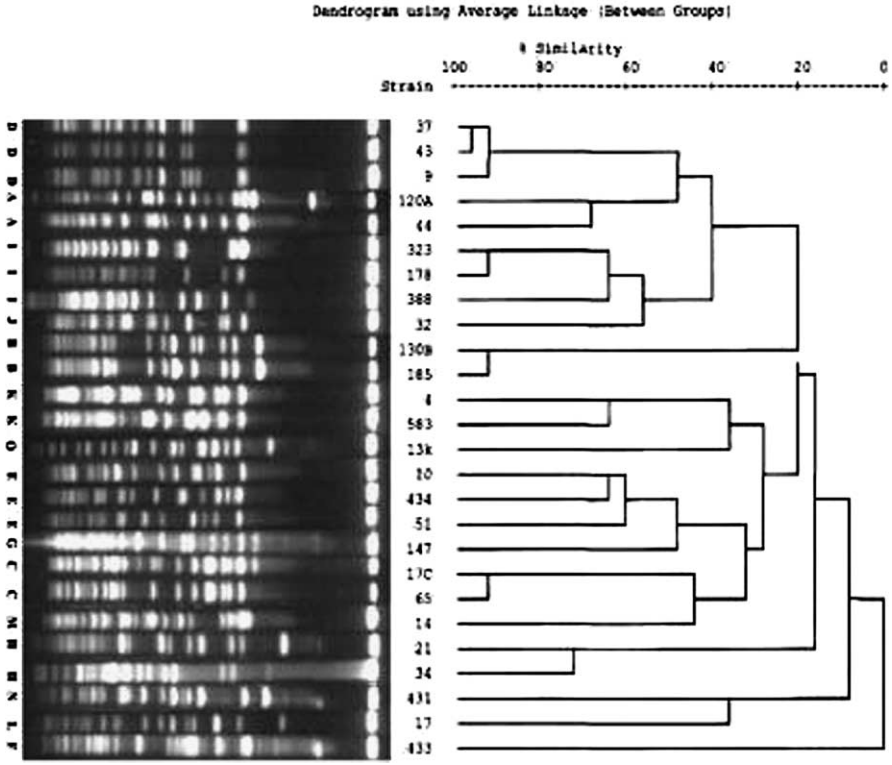

Figure 2 | PFGE of *Sma*I macrorestriction fragments of *E. faecium* representative isolates and the resulting dendrogram. Letters in bold indicate PFGE types. Type D: strains 37, 43 and 9, from marine, municipal and spring water; type A: clinical strain 120, PFGE type A and strain 44 from marine water; type I: strains 323, 178 and 388 from marine and municipal water; type J: strain 32 from municipal water; type B: clinical strain 130 PFGE type B and strain 185 from municipal water; type K: strains 4 and 583 from marine and municipal water; type O: strain 13k from marine water; type E: strains 10, 434 and 51 from marine, municipal and spring water; type G: strain 147 from marine water; type C: clinical strain 17, PFGE type C and strain 65 from river water; type M: strain 14 from river water; type H: strains 21 and 34 from marine and river water; type N: strain 431 from spring water; type L: strain 17 from marine water and type F: strain 433 from marine water.

CONCLUSIONS

Biotyping could successfully classify *E. faecium* from different sources. DNA fingerprinting by PFGE revealed polyclonality among the studied strains, but also a possible common origin of some strains from different sample sources that belonged to the same genotype. The genetic relationship of certain *E. faecium* with GRE of hospital origin indicates that molecular typing methods should regularly be applied, both in clinical and environmental isolates. It is important, besides doing routine microbial analysis, to identify the origin of isolates by molecular fingerprinting methods.

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