Biofilm synthesis and presence of virulence factors among enterococci isolated from patients and water samples

Matthaios Papadimitriou-Olivgeris,[1] Sevasti Filippidou,[2]† Eleanna Drougka,[3] Fotini Fligou,[4] Fevronia Kolonitsiou,[3] Vasiliki Dodou,[5] Markos Marangos,[1] Evangelos D. Anastassiou,[3] Apostolos Vantarakis[2] and Iris Spiliopoulou[3]

Correspondence
Iris Spiliopoulou
spiliopl@upatras.gr

[1]Division of Infectious Diseases, School of Medicine, University of Patras, Patras, Greece

[2]Environmental Microbiology Unit, Department of Public Health, School of Medicine, University of Patras, Patras, Greece

[3]Department of Microbiology, School of Medicine, University of Patras, Patras, Greece

[4]Department of Anaesthesiology and Intensive Care Medicine, School of Medicine, University of Patras, Patras, Greece

[5]Intensive Care Unit, General Hospital 'Saint Andrew', Patras, Greece

The goal of this study was to compare biofilm synthesis among enterococci recovered from clinical samples (infection or colonization) of patients as well as environmental samples in order to determine possible virulence factors and clonal relationship. During a two-year period, clinical samples (blood, catheter tips, bronchial secretions, wounds, peritoneal fluid, urine) and rectal swabs collected from hospitalized patients as well as environmental water samples were tested for the presence of *Enterococcus faecalis* and *Enterococcus faecium*. Antibiotic susceptibility testing was performed by the disc diffusion method and Etest. Strains were tested for the presence of *vanA*, *vanB*, *esp*, *ace* and *asp* genes by PCR. Clones were identified by PFGE (*Sma*I). From infected patients, 48 strains were identified: 24 *Enterococcus faecium* (10 *vanA*-positive, 14 vancomycin-susceptible) and 24 *Enterococcus faecalis* (one *vanA*-positive, 23 vancomycin-susceptible). Among 143 colonizing isolates, 134 were *Enterococcus faecium* (58 *vanA*-positive, 11 *vanB*-positive, 65 vancomycin-susceptible) and nine *Enterococcus faecalis* (three *vanA*-positive, two *vanB*-positive, four vancomycin-susceptible). Among 167 environmental water samples, 51 *Enterococcus faecalis* and 19 *Enterococcus faecium* isolates, all glycopeptide-susceptible, were recovered. In total, 64 strains produced biofilm, whereas 34 were *esp*-positive, 64 *asp*-positive and 54 *ace*-positive. Biofilm production was associated with the presence of *esp* ($P<0.001$) and *ace* genes ($P=0.021$), being higher in infecting ($P<0.001$) and water (P 0.005) isolates as compared with colonizing ones. Clones of environmental water-strains were different than the patients' clones. The differences found in the incidence of antibiotic resistance, virulence factors and clones suggest that hospital and water enterococci are of different origin.

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INTRODUCTION

Enterococci are bacteria that have developed mechanisms to colonize and survive mainly in the gastrointestinal tract of humans. They constitute an important cause of nosocomial infections, the most common species being *Enterococcus faecalis* and *Enterococcus faecium* (Abriouel *et al.*, 2008; Comerlato *et al.*, 2013; Papadimitriou-Olivgeris *et al.*, 2014). Their ability to produce biofilm has been implicated in the induction of infections (Upadhyaya *et al.*, 2011). The *Enterococcus* surface protein (Esp), encoded by the *esp* gene, acts as an adhesin and is involved in the formation of biofilms (Upadhyaya *et al.*, 2011), although this association has not been not confirmed by other investigators (Comerlato *et al.*, 2013). Many proteins have been implicated in enterococcal virulence (Elsner *et al.*, 2000). The most commonly implicated are the

†Present address: Laboratory of Microbiology, University of Neuchatel, Emile-Argand 11, CH2000 Neuchatel, Switzerland.

Abbreviations: CI, confidence interval; ICU, intensive care unit; OR, odds ratio; VRE, vancomycin-resistant enterococci.

aggregation substance (*asp* gene) enhancing the adherence to matrix proteins and the collagen-binding protein encoded by the *ace* gene (Duprè *et al.*, 2003; Vankerckhoven *et al.*, 2004).

Nowadays, a great proportion of nosocomial enterococcal infections are caused by vancomycin-resistant enterococci (VRE) strains (Comerlato *et al.*, 2013). The most commonly identified genes conferring resistance to glycopeptides are *vanA* and *vanB* genes, whereas the majority of VRE are *Enterococcus faecium* (90 %) (Papadimitriou-Olivgeris *et al.*, 2014).

Enterococci, as well as *Escherichia coli*, are frequently used as indicators of faecal contamination of fresh or marine water quality worldwide, since their presence is highly correlated with swimming- or drinking-associated gastrointestinal illness (Thevenon *et al.*, 2012). *Enterococcus faecium* isolates from wastewater, marine or river water are drug-resistant and belong to clonal complex 17 (CC17), which is associated with hospital strains (Sadowy & Luczkiewicz, 2014). In Greece, previous studies showed that environmental *Enterococcus faecalis* and *Enterococcus faecium* isolates exhibit low resistance rates (<10 %) for ampicillin, gentamicin and vancomycin (Grammenou *et al.*, 2006). A high proportion of enterococci from water samples often produce biofilm and carry virulence genes, which have been implicated in the induction of clinical infections. (Abriouel *et al.*, 2008; Ahmad *et al.*, 2014; Comerlato *et al.*, 2013) The presence of such isolates in river or coastal waters poses an important public health problem since they can be a relevant source of resistance and virulence spread to humans.

The goal of this study was to investigate biofilm synthesis, clonality and presence of virulence determinants among enterococci recovered from clinical samples (infecting or colonizing isolates) of hospitalized patients and environmental water samples collected from nearby seashores in order to determine potential strains' clonal and virulence dissemination associated with any of the aforementioned sources.

METHODS

Bacterial strains from hospitalized patients. The study was conducted from January 2010 to December 2011 in the Medical Microbiology Laboratory at the University of Patras. A collection of 24 *Enterococcus faecalis* and 24 *Enterococcus faecium* isolates recovered from clinical samples (blood, catheter tips, bronchial secretions, wounds, peritoneal fluid, urine) of infected adult patients as well as nine *Enterococcus faecalis* and 134 *Enterococcus faecium* colonizing isolates was investigated. Patients were hospitalized at the University General Hospital of Patras, a tertiary-care hospital with 700 beds and approximately 100 000 annual admissions (including one-day admissions).

Bacterial strains from water samples. One hundred and sixty-seven water samples were collected from environmental water sources of the Achaia Prefecture (south-western Greece). Samples were analysed according to ISO standard methods for the detection and enumeration of *Enterococcus* spp. (ISO 7899-02 : 2000). In total, 19 *Enterococcus faecium* and 51 *Enterococcus faecalis* isolates were further investigated.

Identification and antimicrobial susceptibility testing. Enterococci were identified phenotypically by Gram stain, catalase production and the Vitek 2 Advanced Expert System (bioMérieux). Susceptibility to ampicillin, erythromycin and chloramphenicol was determined by the disc diffusion method, whereas, MICs of vancomycin, teicoplanin, linezolid, daptomycin, tigecycline and gentamicin (high-level) were determined by a gradient method (Etest, bioMérieux). Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines.

Biofilm synthesis and presence of virulence factors. All isolates were tested for biofilm formation, as described by Extremina *et al.* (2011). Briefly, a 1 : 40 dilution of overnight bacterial cultures in tryptic soy broth (TSB; Becton Dickinson) with 0.5 % glucose was used to inoculate wells in a microtitre polystyrene plate. All biofilm assays were performed in triplicate. After 48 h of incubation at 37 °C, plates were washed three times with dH_2O. They were then dried for 1 h at 60 °C in an inverted position, followed by addition of 200 µl 0.1 % safranin into each well. Wells were washed three times with dH_2O. Safranin bound to biofilm was solubilized in 200 µl dH_2O and absorbance was measured at 490 nm with an automated microplate reader. Reference strains *Staphylococcus epidermidis* ATCC 35984 (RP62A) and ATCC 12228 were used as positive and negative controls, respectively. Biofilm formation was considered to be moderate or high when absorbance was >0.150 OD_{490} and weak or absent when $OD_{490} \leq 0.150$ (Extremina *et al.*, 2011).

The presence of *vanA* and *vanB* genes (conferring resistance to vancomycin), as well as *esp*, *asp* and *ace* (encoding enterococcal surface, aggregation substance and collagen-binding proteins, respectively), was investigated by PCR (Bell *et al.*, 1998; Mannu *et al.*, 2003; Vankerckhoven *et al.*, 2004).

Genotypes of isolates. Clones were identified by PFGE of chromosomal *Sma*I DNA digests, interpreted according to already established criteria (Tenover *et al.*, 1995). PFGE types of *Enterococcus faecalis* were identified by capital letters and those of *Enterococcus faecium* by lower-case letters.

Statistical analysis. SPSS version 19.0 software was used for data analysis. Categorical variables were analysed by using the Fisher exact test or χ^2 and continuous variables with the Mann–Whitney U test, as appropriate. Univariate analysis was used to determine those risk factors associated with biofilm production. Backward stepwise multiple logistic regression analysis used all significant variables from the univariate analysis with a $P<0.08$ in order to determine which ones were independently associated with biofilm synthesis. Odds ratios (ORs) and 95 % confidence intervals (CIs) were calculated to evaluate the strength of any association. All statistical tests were two-tailed and $P<0.05$ was considered statistically significant.

RESULTS

Infecting and colonizing strains

Forty-eight infecting strains (one strain per patient) were included; 21 recovered from patients hospitalized in medical wards, 13 in surgical wards and 14 in intensive care units (ICUs). Sixteen were isolated from primary bacteraemias, 11 from urinary tract infections, eight from intra-abdominal infections, six from skin and soft-tissue infections, five from catheter-related bloodstream infections and two from

pneumonias. Twenty-four (50 %) were *Enterococcus faecium* (10 *vanA*-positive and 14 vancomycin-susceptible) and 24 *Enterococcus faecalis* (one *vanA*-positive and 23 vancomycin-susceptible). Among the 48 isolates, biofilm synthesis was observed in 26 (54 %), while 17 (35 %), 14 (29 %) and nine (19 %) isolates were positive for the presence of *esp*, *asp* and *ace* genes, respectively. Bloodstream infections (primary or catheter-related), as compared with the other sources of infection, were associated with the presence of *esp* (52 % vs 12 %, $P=0.008$). No other association among biofilm or virulence genes and specific infection source was observed. *Enterococcus faecium* isolates were classified into four PFGE types, a (14 strains), b (six), d (three) and e (one), whereas *Enterococcus faecalis* were classified into three pulsotypes, A (20 strains), D (one) and E (three).

Among the 143 colonizing isolates, 33 were recovered from patients hospitalized in the medical wards, 22 from the surgical wards and 88 from the ICUs. The majority (134, 94 %) were *Enterococcus faecium* (58 *vanA*-positive, 11 *vanB*-positive and 65 vancomycin-susceptible) and nine *Enterococcus faecalis* (three *vanA*-positive, two *vanB*-positive and four vancomycin-susceptible). Biofilm synthesis was observed in 18 isolates (13 %), whereas eight (6 %), four (3 %) and six (4 %) strains were positive for the presence of *esp*, *asp* and *ace* genes, respectively. *Enterococcus faecium* strains were classified into nine PFGE types with the majority of strains belonging to type a (89 strains, 66 %), followed by types d (12), b (10), e (seven), h (seven), g (five), c (three) and i (one). *Enterococcus faecalis* isolates were classified into four types, A (five strains), D (one), E (two) and F (two).

Water sample isolates

Among 70 strains recovered from 167 water samples, 19 were *Enterococcus faecium* (27 %) and 51 *Enterococcus faecalis* (73 %), all vancomycin-susceptible. Twenty isolates (29 %) were positive for biofilm formation, whereas nine (13 %), 46 (66 %) and 39 (56 %) carried the *esp*, *asp* and *ace* genes, respectively. *Enterococcus faecium* isolates were classified into 13 PFGE types, with five strains belonging to type j and three to type k, whereas the remaining 11 isolates represented 11 pulsotypes. *Enterococcus faecalis* isolates were classified into 19 PFGE types, with 24 strains belonging to type G and six to type H, whereas the remaining 21 represented 17 pulsotypes. PFGE types identified among strains from the water samples were different from those recovered from hospitalized patients. Table 1 depicts the characteristics of the human (colonizing, infecting) and water strains.

Comparison of *Enterococcus faecium* and *Enterococcus faecalis* strains

Enterococcus faecium was the predominant species among the colonizing (94 %) isolates, whereas, only 27 % of

Table 1. Resistance rates, presence of virulent genes and biofilm synthesis of human (colonizing and infecting) and water origin isolates

AM, ampicillin; E, erythromycin; C, chloramphenicol; HL-GM, high-level gentamicin; VA, vancomycin; TEC, teicoplanin; DPC, daptomycin; LN, linezolid; TGC; tigecycline.

Characteristic	Antibiotic or gene	All $n=261$ (%)	Colonizing $n=143$ (%)	Infecting $n=48$ (%)	Water $n=70$ (%)	P*	P†	P‡
Enterococcus faecium*/*Enterococcus faecalis		**177/84**	**134/9**	**24/24**	**19/51**	**<0.001**	**<0.001**	**0.019**
Antibiotic-resistant isolates	AM	122 (47)	101 (71)	17 (35)	4 (6)	<0.001	<0.001	<0.001
	E	223 (85)	127 (89)	37 (77)	59 (84)	0.056	0.384	0.345
	C	47 (12)	17 (12)	28 (40)	2 (11)	0.602	<0.001	<0.001
	HL-GM§	106 (18)	76 (53)	25 (52)	5 (7)	1.000	<0.001	<0.001
MIC (mg l^{-1}) by Etest	VA	112.5	256	256	3	0.132	0.026	0.078
	TEC	13.5	48	32	0.75	0.024	<0.001	<0.001
	LN	1.2	2	1.5	1.5	<0.001	<0.001	0.073
	DPC	1.4	3	2	1.5	0.563	<0.001	<0.001
	TGC	0.3	0.75	0.25	0.75	<0.001	0.187	<0.001
Presence of genes by PCR	*vanA*	72 (28)	61 (43)	11 (23)	0 (0)	0.016	<0.001	<0.001
	vanB	13 (5)	13 (9)	0 (0)	0 (0)	0.041	0.011	–
	asp	64 (25)	4 (3)	14 (29)	46 (66)	<0.001	<0.001	<0.001
	ace	54 (21)	6 (4)	9 (19)	39 (56)	0.003	<0.001	<0.001
	esp	34 (13)	8 (6)	17 (35)	9 (13)	<0.001	0.103	0.006
Biofilm synthesis (%)		64 (25)	18 (13)	26 (54)	20 (29)	<0.001	0.007	0.007

*Comparison between human colonizing and infecting strains.
†Comparison between human colonizing and water strains.
‡Comparison between water and human infecting strains.
§HL-GM resistance was assessed by Etest.

water isolates belonged to this species. Moreover, *Enterococcus faecium* showed higher resistance rates to most antibiotics. *Enterococcus faecalis* as compared with *Enterococcus faecium* prevailed in biofilm synthesis (37 % vs 19 %, $P=0.002$) and the presence of *esp* (20 % vs 10 %, $P=0.029$), *asp* (61 % vs 7 %, $P<0.001$) and *ace* (62 % vs 1 %, $P<0.001$).

Comparison among biofilm producers and non-producers

In total, 64 strains produced biofilm (18 colonizing, 26 infecting and 20 water isolates). Table 2 depicts the factors associated with biofilm synthesis. Multivariate analysis among all strains revealed that biofilm production was associated with the presence of the *esp* gene, susceptibility to vancomycin and infecting strains.

DISCUSSION

In the present study, biofilm production among enterococci from human and environmental sources was investigated. Infecting and colonizing strains from hospitalized patients and those recovered from water samples were compared for clonal relationship and virulence determinants.

Vancomycin resistance of nosocomial isolates was more common in *Enterococcus faecium* carrying the *vanA* gene, as was also shown by previous studies (Abriouel *et al.*, 2008; Duprè *et al.*, 2003; Papadimitriou-Olivgeris *et al.*, 2014). No enterococcal water isolate was VRE. In the study of Schwartz *et al.* (2003), VRE were recovered from all wastewater biofilms collected near the Hospital of Mainz, Germany, but they were not found in drinking water samples. The rates of resistance to ampicillin, erythromycin and gentamicin of water enterococcal isolates in this study were remarkably higher than those recovered from water samples collected from the same region in 2002 (Grammenou *et al.*, 2006). This finding, combined with the characterization of different PFGE types among hospital and water enterococci, suggests that multi-resistant water isolates may derive from other origins such as animal sources. High resistance rates and *vanA*-positive enterococci from livestock farms and poultry slaughterhouses in northern and central Greece have been characterized (Tzavaras *et al.*, 2012).

Even though the percentage of biofilm production from infecting isolates (54 %) is comparable to that reported in previous studies (38–93 %) (Comerlato *et al.*, 2013; Duprè *et al.*, 2003; Gozalan *et al.*, 2015; Ira *et al.*, 2013; Tsikrikonis *et al.*, 2012), biofilm synthesis from colonizing isolates (13 %) was low (Johansson & Rasmussen, 2013; Tsikrikonis *et al.*, 2012). Furthermore, Tsikrikonis *et al.* (2012) found that 70 % of colonizing isolates were biofilm producers, whereas, in the study of Johansson & Rasmussen (2013), biofilm synthesis was more common in normal flora isolates as compared with those recovered from infective endocarditis. The percentage of biofilm-producers among water isolates in our study was similar to

Table 2. Comparison between biofilm producers and non-producers among all enterococcal strains

HL, high level.

	Univariate analysis			Multivariate analysis	
	Non-biofilm-producing enterococci ($n=197$)	Biofilm-producing enterococci ($n=64$)	*P*	*P*	OR (95 % CI)
Enterococcus faecalis	53 (27 %)	31 (48 %)	0.002		
Category of strain					
Human colonizing	125 (64 %)	18 (28 %)			
Human infecting	22 (11 %)	26 (41 %)	<0.001*	0.002*	3.3 (1.6–7.1)*
Water	50 (25 %)	20 (31 %)			
esp-positive	8 (4 %)	26 (41 %)	<0.001	<0.001	12.8 (5.1–31.9)
asp-positive	43 (22 %)	21 (33 %)	0.094		
ace-positive	34 (17 %)	20 (31 %)	0.021		
Vancomycin-susceptible†	124 (63 %)	52 (81 %)	0.009	0.017	2.7 (1.2–6.1)
HL gentamicin resistance	85 (42 %)	21 (30 %)	0.187		
Linezolid MIC (mg l^{-1})	1.1	0.8	<0.001		
Daptomycin MIC (mg l^{-1})	1.4	1.0	0.001		
Tigecycline MIC (mg l^{-1})	0.3	0.3	0.716		

*Comparison of infecting strains with both colonizing and water strains.
†Defined as the absence of *vanA* or *vanB* genes.

that published by Ahmad *et al.* (2014) (30 %). Biofilm was also associated with *Enterococcus faecalis* (48 % vs 19 % in *Enterococcus faecium*), a finding similar to that published by Comerlato *et al.* (2013).

The role of Esp protein in provoking infection is supported by the work of Vankerckhoven *et al.* (2004) and Shankar *et al.* (1999). In our study, the presence of the *esp* gene was more common in infecting (17, 35 %) as compared with colonizing (eight, 6 %) or water isolates (nine, 13 %). The percentage of *esp*-positive infecting clinical isolates was comparable with that reported in other studies, where 41 % and 30 % of enterococci carried this gene (Gozalan *et al.*, 2015; Upadhyaya *et al.*, 2011). Even higher rates of *esp* carriage have been reported, ranging from 52 % to 77 % (Comerlato *et al.*, 2013; Ruiz-Garbajosa *et al.*, 2006; Tsikrikonis *et al.*, 2012). This diversity in *esp* presence may be due to the variability of infection sites from which enterococci were isolated, verified also in our collection, since strains recovered from primary or catheter-related bloodstream infections more commonly carried the *esp* gene, as compared with strains isolated from other infection sites. A low percentage of *esp*-positive colonizing strains, comparable with that published by Upadhyaya *et al.* (2011)(7%), was detected in this study (6%). This low percentage may be explained by the fact that Esp is not essential for intestinal colonization or translocation of *Enterococcus faecium* and *Enterococcus faecalis* isolates, as shown in murine models (Heikens *et al.*, 2009). However, Raad *et al.* (2005) and Ruiz-Garbajosa *et al.* (2006) have reported higher percentages of *esp* carriage among colonizing isolates as compared with infecting ones. Contradictory results have also been published, comparing *esp* incidence among VRE and VSE (Billström *et al.*, 2008; Duprè *et al.*, 2003; Vankerckhoven *et al.*, 2004; Woodford *et al.*, 2001). In our study, no difference was detected among VRE and VSE on *esp* carriage (11 % vs 14 %, $P > 0.05$).

Water enterococcal isolates may derive from different sources, other than human faeces, making it difficult to find a single host-specific indicator (Gilmore *et al.*, 2014). It has been suggested that enterococcal isolates carrying the *esp* gene may be human-specific (Scott *et al.*, 2005). In our study, only 13 % of water isolates harboured the *esp* gene, which is lower than those reported by Ahmad *et al.* (2014) (22 %) and Thevenon *et al.* (2012) (>90 %), indicating that our water isolates were from non-human sources. This finding is reinforced by the fact that clonal analysis by PFGE revealed high genetic diversity among water isolates, with different types from the human ones. Asmat *et al.* (2014) found that 30 % of enterococci isolated from recreational beach water produced biofilm, a result similar to our water isolates (29 %). In that report, the presence of the *esp* gene in beach water enterococci was highly associated with biofilm production, since 83 % of biofilm producers carried the gene (Asmat *et al.*, 2014). This result is higher than our finding (40 %). This lower percentage indicates that Esp is not the only factor necessary for biofilm production.

The most common virulence factor observed was *asp* (64 strains, 25 %) followed by *ace* (54, 21 %), whereas, their presence was statistically higher in *Enterococcus faecalis*. Even though some studies failed to detect these genes in *Enterococcus faecium* (Duprè *et al.*, 2003; Mannu *et al.*, 2003), in our study, 13 *Enterococcus faecium* (5 %) carried *asp* and two (1 %) the *ace* determinants. Our results are in agreement with the literature concerning higher incidence of virulence genes (*esp*, *asp*, *ace*) among *Enterococcus faecalis* (Abriouel *et al.*, 2008; Duprè *et al.*, 2003; Elsner *et al.*, 2000). Abriouel *et al.* (2008) reported lower incidence of *ace* gene in enterococci from water samples as compared with clinical isolates, a finding contradicting our results, since 39 (56 %) water isolates were *ace*-positive, while only 15 (8 %) clinical isolates carried the gene.

Among the 64 biofilm-producing strains, 26 (41 %) carried *esp*, whereas, eight additional *esp*-positive isolates were biofilm-negative. A strong association between the presence of the *esp* gene and biofilm synthesis was found, which is consistent with most studies (Tsikrikonis *et al.*, 2012; Upadhyaya *et al.*, 2011), even though other investigators failed to find such an association (Comerlato *et al.*, 2013; Ira *et al.*, 2013). The identification of 38 biofilm-producing *esp*-negative strains suggests that even though *esp* is important to biofilm production, there are other factors that may affect biofilm formation. This is reinforced by the fact that the collagen-binding protein, encoded by the *ace* gene, was found to be statistically associated with biofilm synthesis, a fact that is reported for the first time.

Both *Enterococcus faecium* and *Enterococcus faecalis* showed genetic diversity, and the presence of virulence determinants as well as biofilm production was not associated with the main clones identified. The fact that PFGE types from hospitalized patients are distinct from those of water samples strongly indicates no clonal spread among the different sources, as shown also by Abriouel *et al.* (2008), reinforcing that nosocomial VRE do not contaminate river or coastal waters. In another study from the Gulf of Gdansk, Poland, clones associated with resistant nosocomial strains were discovered in wastewater and marine environments indicating the importance of monitoring wastes for the presence of nosocomial isolates (Sadowy & Luczkiewicz, 2014).

In conclusion, biofilm synthesis was more frequent in the infecting isolates as compared with colonizing and water ones, associated with the presence of *esp* and *ace* genes. Virulence determinants such as *esp*, *ace* and *asp* aid enterococci to overcome the host barriers and provoke clinical infections. The significant differences found in the incidence of antibiotic resistance, virulence factors and PFGE types suggest a distinctive origin of patients' and water isolates.

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REFERENCES

Abriouel, H., Omar, N. B., Molinos, A. C., López, R. L., Grande, M. J., Martínez-Viedma, P., Ortega, E., Cañamero, M. M. & Galvez, A. (2008). Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. *Int J Food Microbiol* **123**, 38–49.

Ahmad, A., Dada, A. C., Usup, G. & Heng, L. Y. (2014). Occurrence of *Enterococcus* species with virulence markers in an urban flow-influenced tropical recreational beach. *Mar Pollut Bull* **82**, 26–38.

Asmat, A., Dada, A. C. & Gires, U. (2014). Biofilm formation, gel and *esp* gene carriage among recreational beach enterococci. *Glob J Health Sci* **6**, 241–253.

Bell, J., Turnidge, J., Coombs, G. F. & O'Brien, F. (1998). Emergence and epidemiology of vancomycin-resistant enterococci in Australia. *Commun Dis Intell* **22**, 249–252.

Billström, H., Lund, B., Sullivan, A. & Nord, C. E. (2008). Virulence and antimicrobial resistance in clinical *Enterococcus faecium*. *Int J Antimicrob Agents* **32**, 374–377.

CLSI (2012). *Performance Standards for Antimicrobial Susceptibility Testing*, 20th Informational Supplement M100–S20. Wayne, PA: Clinical and Laboratory Standards Institute.

Comerlato, C. B., Resende, M. C., Caierão, J. & d'Azevedo, P. A. (2013). Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin. *Mem Inst Oswaldo Cruz* **108**, 590–595.

Duprè, I., Zanetti, S., Schito, A. M., Fadda, G. & Sechi, L. A. (2003). Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *J Med Microbiol* **52**, 491–498.

Elsner, H. A., Sobottka, I., Mack, D., Claussen, M., Laufs, R. & Wirth, R. (2000). Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium* blood culture isolates. *Eur J Clin Microbiol Infect Dis* **19**, 39–42.

Extremina, C. I., Costa, L., Aguiar, A. I., Peixe, L. & Fonseca, A. P. (2011). Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates. *J Microbiol Methods* **84**, 167–173.

Gilmore, M. S., Clewell, D. B., Ike, Y. & Shankar, N. (editors) (2014). *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston, MA: Massachusetts Eye and Ear Infirmary.

Gozalan, A., Coskun-Ari, F. F., Ozdem, B., Unaldı, O., Celikbilek, N., Kirca, F., Aydogan, S., Muderris, T., Guven, T. & other authors (2015). Molecular characterization of vancomycin resistant *Enterococcus faecium* strains isolated from carriage and clinical samples in a tertiary hospital, Turkey. *J Med Microbiol* **64**, 759–766.

Grammenou, P., Spiliopoulou, I., Sazakli, E. & Papapetropoulou, M. (2006). PFGE analysis of enterococci isolates from recreational and drinking water in Greece. *J Water Health* **4**, 263–269.

Heikens, E., Leendertse, M., Wijnands, L. M., van Luit-Asbroek, M., Bonten, M. J., van der Poll, T. & Willems, R. J. (2009). Enterococcal surface protein Esp is not essential for cell adhesion and intestinal colonization of *Enterococcus faecium* in mice. *BMC Microbiol* **9**, 19.

Ira, P., Sujatha, S. & Chandra, P. S. (2013). Virulence factors in clinical and commensal isolates of *Enterococcus* species. *Indian J Pathol Microbiol* **56**, 24–30.

Johansson, D. & Rasmussen, M. (2013). Virulence factors in isolates of *Enterococcus faecalis* from infective endocarditis and from the normal flora. *Microb Pathog* **55**, 28–31.

Mannu, L., Paba, A., Daga, E., Comunian, R., Zanetti, S., Duprè, I. & Sechi, L. A. (2003). Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *Int J Food Microbiol* **88**, 291–304.

Papadimitriou-Olivgeris, M., Drougka, E., Fligou, F., Kolonitsiou, F., Liakopoulos, A., Dodou, V., Anastassiou, E. D., Petinaki, E., Marangos, M. & other authors (2014). Risk factors for enterococcal infection and colonization by vancomycin-resistant enterococci in critically ill patients. *Infection* **42**, 1013–1022.

Raad, I. I., Hanna, H. A., Boktour, M., Chaiban, G., Hachem, R. Y., Dvorak, T., Lewis, R. & Murray, B. E. (2005). Vancomycin-resistant *Enterococcus faecium*: catheter colonization, *esp* gene, and decreased susceptibility to antibiotics in biofilm. *Antimicrob Agents Chemother* **49**, 5046–5050.

Ruiz-Garbajosa, P., Cantón, R., Pintado, V., Coque, T. M., Willems, R., Baquero, F. & del Campo, R. (2006). Genetic and phenotypic differences among *Enterococcus faecalis* clones from intestinal colonisation and invasive disease. *Clin Microbiol Infect* **12**, 1193–1198.

Sadowy, E. & Luczkiewicz, A. (2014). Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiol* **14**, 66.

Schwartz, T., Kohnen, W., Jansen, B. & Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol Ecol* **43**, 325–335.

Scott, T. M., Jenkins, T. M., Lukasik, J. & Rose, J. B. (2005). Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. *Environ Sci Technol* **39**, 283–287.

Shankar, V., Baghdayan, A. S., Huycke, M. M., Lindahl, G. & Gilmore, M. S. (1999). Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infect Immun* **67**, 193–200.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., B, E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.

Thevenon, F., Regier, N., Benagli, C., Tonolla, M., Adatte, T., Wildi, W. & Poté, J. (2012). Characterization of fecal indicator bacteria in sediments cores from the largest freshwater lake of Western Europe (Lake Geneva, Switzerland). *Ecotoxicol Environ Saf* **78**, 50–56.

Tsikrikonis, G., Maniatis, A. N., Labrou, M., Ntokou, E., Michail, G., Daponte, A., Stathopoulos, C., Tsakris, A. & Pournaras, S. (2012). Differences in biofilm formation and virulence factors between clinical and fecal enterococcal isolates of human and animal origin. *Microb Pathog* **52**, 336–343.

Tzavaras, I., Siarkou, V. I., Zdragas, A., Kotzamanidis, C., Vafeas, G., Bourtzi-Hatzopoulou, E., Pournaras, S. & Sofianou, D. (2012). Diversity of *vanA*-type vancomycin-resistant *Enterococcus faecium* isolated from broilers, poultry slaughterers and hospitalized humans in Greece. *J Antimicrob Chemother* **67**, 1811–1818.

Upadhyaya, G. P., Lingadevaru, U. B. & Lingegowda, R. K. (2011). Comparative study among clinical and commensal isolates of

Enterococcus faecalis for presence of *esp* gene and biofilm production. *J Infect Dev Ctries* **5**, 365–369.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D. & Goossens, H. (2004). Development of a multiplex PCR for the detection of *asa1, gelE, cylA, esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J Clin Microbiol* **42**, 4473–4479.

Woodford, N., Soltani, M. & Hardy, K. J. (2001). Frequency of *esp* in *Enterococcus faecium* isolates. *Lancet* **358**, 584.