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Phenotypic and Genotypic differences in the expression of virulence factors in antimicrobial resistance of *Enterococcus faecalis* clinical strains.

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There is no information on investigations comparing phenotypic and genotypic differences in MDR and MDS *E. faecalis* strains in literature. The study included 70 clinical *E. faecalis* isolates from human origin. None of the isolates tested demonstrated any *in vitro* sensitive to vancomycin, with MIC values of all the strains for vancomycin of ≥23 µg/mL. High-level resistances to ampicillin and streptomycin were detected by the disc diffusion method in 77% and 80% of the isolates, respectively. The MDR *E. faecalis* strains which were investigated produced significantly amounts of extracellular material binding Congo red and gelatinase quantity in culture supernatants when compared with MDS strains. The capacity to produce lipase and DNase activity were not found in any of the isolates investigated. Cytolysin activity was detected in 70% of MDR *E. faecalis* strains, where in only 20% of MDS *E. faecalis*. MDR strains adhered to polystyrene to a more extent than MDS strains. The presence of virulence genes (*esp*, *cylA*, *gelE* and *EF3314*) were investigated by PCR. MDR *E. faecalis* strains showed more detection of these genes than MDS *E. faecalis* strains. The presence of these genes did not correlate completely with their phenotypic expression. In conclusion, the MDR *E. faecalis* strains have impaired virulence when compared to MDS strains.

Key words: ***Enterococcus faecalis*, multidrug resistant, multidrug sensitive, virulence factors.**

Enterococci are a normal inhabitant of the gastrointestinal tract of humans and animals, and are commonly found in food and in the environment (Kühn et al., 2005; Teuber et al., 2003). Enterococci are becoming an important cause of nosocomial infections, including bacteraemia, endocarditis and surgical wound infections (Lester et al. 2008). Enterococci have become the third most common cause of nosocomial infections in hospitals (CDC, 2003), with *E. faecalis* as the most predominant (80%) followed by *E. faecium* (15–20%) (Morrison et al. 1997). Moreover, treatment of these infections has become more and more difficult due to the increasing number of antibiotic resistant enterococci (Malani et al. 2002). The increasing incidence of antibiotic resistant enterococci is due to the massive use of antibiotics both in agriculture (as animal growth promoters) and in the human health care system, according to Teuber (2001). The emergence of resistance to antimicrobial agents during therapy threatens the successful treatment of several infections and risks the further spread of resistant organisms to other patients and to the community.

Antimicrobial resistance (AR) enterococci strains have been isolated all over the world from clinical specimens with increasing frequency (Sephard and Gilmore 2002; McDonald 2006; Freitas et al. 2011). Currently, there are no perspectives for introducing new drugs that could be used against this nosocomial pathogen (Landman et al. 2002), therefore investigations into its

physiology are justified as they may lead to the discovery of new therapeutic opportunities. It seems that acquiring genes which determine antibiotic resistance usually comes at a biological cost of impaired bacterial physiology (Andersson 2006), regardless whether the genes had been acquired in vivo (Schrag and Perrot 1996) or in vitro (Sanchez et al. 2002; Deptuła and Gospodarek 2010). Correlation between antibiotic resistance and virulence expression is not a new issue; it seems to be a task quite rarely undertaken when compared with those concerning antibiotic resistance, biofilm formation and epidemiology of infections caused by enterococci. From the evolutionary standpoint, the selection of AR has been taking place for quite a short time—since the 1940s. Experimental studies have proved that AR microorganisms, when in the presence of selective agents preserving the antibiotic resistance genes, are able to recover their previous levels of fitness during several hundreds of passages (Schoustra et al. 2006).

No information on investigations comparing phenotypic and genotypic differences in the expression of virulence factors in MDR and MDS *E. faecalis* clinical strains can be found in literature.

MATERIALS AND METHODS

Clinical isolates of enterococci and study location

Consecutive clinical enterococci isolates from patients' specimens submitted to the Clinical Microbiology Laboratory at Prince Abdulrahman Al Sudairy Hospital, Sakaka, Al Jouf, Saudi Arabia were studied. Only one isolate per patient was included in this study. All isolates were stored in brucella glycerine broth at -20°C until tested.

Growth and storage conditions

Isolates were grown on Brain Heart Infusion Agar (BHIA) (Scharlau, Spain) at 37 ^{o}C for 24 h and stored at -80 ^{o}C in Brain Heart Infusion Broth (BHIB) containing 30% (v/v) of glycerol (Sigma, Steinheim, Germany), and sub-cultured twice before use in assays.

Identification of enterococcal isolates

Identification of enterococci used standard conventional and commercial tests. These included the Gram stain reaction, growth on bile-aesculin agar, growth in the presence of 6.5% NaCl and absence of catalase. The identification to species level used API-20 Strep system (bio Merieux, Cedex, France) and the software supplied by the manufacturer.

Antimicrobial susceptibility test

Antimicrobial susceptibility of enterococcal strains towards the 10 selected antibiotics was determined using the disk diffusion method described by NCCLS (2001). Disk diffusion assays were performed on Mueller-Hinton Agar (MHA) (Oxoid) and inhibition zone diameters were measured in millimeters using a calliper. Strains were classified as resistant, susceptible and intermediate according to the criteria from NCCLS (for ampicillin, amoxicillin/clavulanic acid, chloramphenicol, ciprofloxacin, erythromycin, rifampicin, streptomycin, teicoplanin, tetracycline and vancomyci) (NCCLS 2001) and bioMe´rieux (for gentamicin) (bioMe´rieux 1996). The strains *E. faecalis* ATCC 29212 and ATCC 51299 were used as sensitive and resistant controls respectively. The Etest (AB Biodisk, Solna, Sweden) was used to determine (MIC) of each isolate against vancomycin. Stored enterococcal isolates were revived and subcultured at least twice prior to testing to ensure viability and purity. The inoculum, equivalent to 0.5 Mc Farland turbidity standards, was used to streak the surface of the Mueller–Hinton agar. The plates were incubated at 35°C in ambient air for a full 24 h. Reading and interpretation of the MICs from the Etest followed the manufacturer's instructions and the NCCLS interpretative standards. The MIC breakpoint for the antimicrobial agents tested were interpreted as susceptible (S), intermediate susceptible (I) and resistant (R), were 5≤ mg/l, 8–16 mg/l and ≥32 mg/l, respectively for vancomycin.

Virulence factors

Production of hydrolytic enzymes

Gelatinase activity was assessed according to Tiago et al. (2004). Lipase activity was assessed according to Tiago et al. (2004). A positive reaction was indicated by a clear halo around the colonies. DNase test. DNase activity was tested as described by Ben Omar et al. (2004) by using the medium DNase agar (Scharlau, Spain) with 0.05 g/l of methyl green (Sigma). A clear halo around the colonies was indicative of a positive result.

Cytolysin production

Production of hemolysin was determined by streaking enterococcal isolates on sheep and human blood agar plates (Psoni et al., 2006). Sheep blood agar was prepared using Azide Blood agar base (ABAB) (Scharlau, Spain) with 5% defibrinated sheep blood (Liofilchem, Roseto degli Abruzzi-TE, Italy). Human blood agar was prepared using ABAB with 5% of human blood from types A and O (both from voluntary donors). The presence or absence of zones of clearing around the colonies was interpreted as β-hemolysis (positive hemolytic activity) or γ-hemolysis (negative hemolytic activity), respectively. When observed, greenish zones around the colonies were interpreted as α-hemolysis and taken as negative for the assessment of hemolytic activity (Semedo et al. 2003a).

Biofilm Formation

Biofilm formation was detected by inoculating the isolates into trypticase-soy broth [TSB] with 0.5% glucose and incubated at 37ºC (Jayanthi et al. 2008). After overnight incubation, the culture was diluted 1:40 in fresh TSB-0.5% glucose. Two hundred microliters of the diluted solution was added to flat-bottomed polystyrene microtiter well and incubated for 48 hours at 37ºC. Wells were gently washed three times with distilled water. After drying the plates in an inverted position at room temperature for 1 hour, the adherent biofilm was stained with 0.1% safranin and allowed to stand for 20 minutes at room temperature. Absorbance of the biofilm on the bottom surface of each well of the dried plates was determined at 490 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Test was carried out in triplicate and the average of the three optical density (OD) values was taken. Culture medium without organism was taken as blank. Mean OD value of positive control was taken as standard. Those values above 0.2 were considered as high biofilm producers. Values below 0.081 were categorized into low or non-biofilm producers. OD values above the standard but within 0.081 and 0.2 were taken as moderate biofilm producers.

Virulence genes

Enterococcal DNA was prepared by suspending a loop of overnight colonies in a tube that contained 500 µl sterile distilled water, boiling for 10 min and then centrifuging at 14 000 g for 5 min. An aliquot of the supernatant (5 µl) was used as the template in a final volume of 25 µl PCR mixture, which contained: 13 PCR buffer, 2 mM $MgCl_2$, 200 µM each dNTP, 400 nM each primer and 0.25 U *Taq* DNA polymerase (Life Technologies). Samples were amplified on a DNA thermal cycler (MJ Research) by heating for 5 min at 95 ^{o}C, followed by 30 cycles of 95 ^{o}C for 60 s, 58 ^{o}C for 60 s (52 ^{o}C for *gelE* and 63 ^{o}C for *esp*, as indicated by Shankar et al., 1999) and 72 ^{o}C for 60 s, and a final step of 72 ^{o}C for 10 min. PCR products were analysed by gel electrophoresis in 0.8% (w/v) agarose gel (Life Technologies). Oligonucleotides were synthesized by a custom primer service (Life Technologies) and are described in Table 1.

Statistical analysis

The correlation between the occurrence of antibiotic resistant and virulence genes was calculated using the 2 or Fisher's exact test. The tests were performed using the SPSS statistical package. A p-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

The bacteria belonging to the genus Enterococcus are normal inhabitants of the gastrointestinal tract of many animals and men. They have controversial properties, as opportunistic pathogenic enterococci are able to cause human infections (Hunt 1998). Most strains of *E. faecalis* were isolated from stool. Properties of 70 enterococci isolates have been analyzed in our work. The biochemical identification was carried out by a commercial kit (bio Merieux, Cedex, France), which includes a panel of 40 biochemical substrates. The susceptibility patterns of all the strains tested by disc diffusion method are presented in Table 2. None of the isolates tested demonstrated any *in vitro* sensitive to vancomycin and teicoplanin, with MIC values of all the strains for vancomycin of ≥23 µg/mL. High-level resistances to ampicillin and streptomycin were detected by the disc diffusion method in 77% and 80% of the isolates, respectively. Variable (1–39%) resistance rates were found for the other drugs tested except for glycopeptides. Unexpectedly, isolates showed high-level resistance to vancomycin. Among resistant isolates, 45 were found highly resistant (MIC, ≥32 mg/l) to vancomycin Fig. 1.

The distribution of virulence factor

Table 1 PCR primers and products for detection of *E. faecalis* virulence determinants

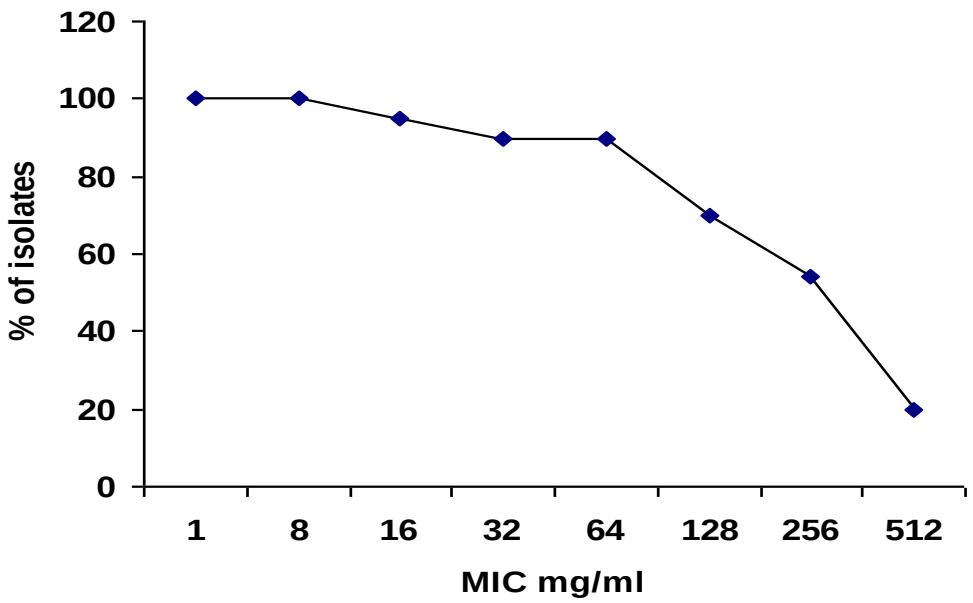
Gene	Sequence (5'-3')	Product size (bp)	GenBank accession no.	Position	Reference
16S rRNA	TGGCATAAGAGTGAAAGGCGC GGGGACGTTCAGTTACTAACGT	290	AF070224	179 468	Baldassarri et al. (2001b)
esp	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGA	932	AF034779	1217 2149	Shankar et al. (1999)
gelE	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	405	M37185	762 1163	Eaton & Gasson (2001)
cylA	GACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTTAC	688	AD1CLYL	6656 7344	Creti et al. (2004)
EF3314	AGAGGGACGATCAGATGAAAAA ATTCCAATTGACGATTCACTTC	566	NC_004668	35 601	Creti et al. (2004)

Table 2 Susceptibility rates of *E. faecalis* strains.

Antibiotic	Zone diameter breakpoint (mm) [a]	Susceptibility rate (%)	
		S	R
Ampicillin	8-10	23	77
Amoxicillin/clavulanic acid	ND	99	1
Chloramphenicol	ND	90	10
Ciprofloxacin	ND	100	0
Erythromycin	ND	90	10
Gentamicin	ND	85	15
Rifampicin	ND	100	0
Streptomycin	ND	20	80
Teicoplanin	16	100	0
Tetracycline	ND	61	39
Vancomycin	12	0	100

[a] European Committee on Antimicrobial Susceptibility Testing (2011)
S sensitive, R resistant, ND not determined

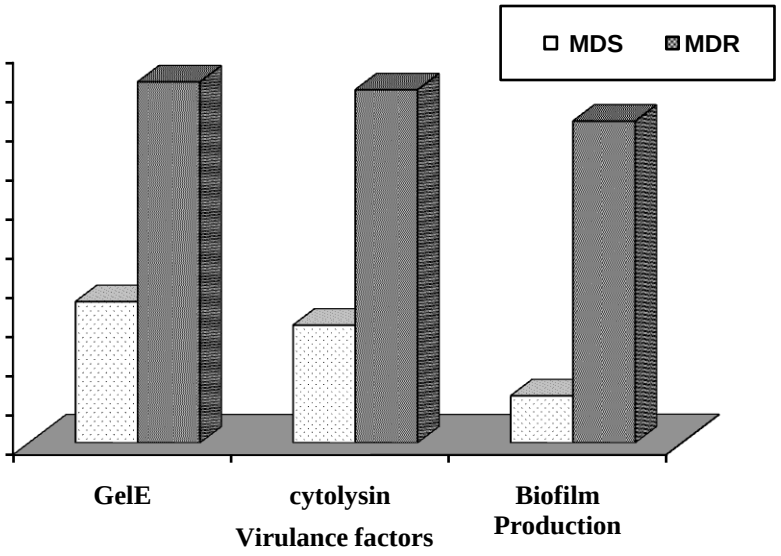
Figure.1 Distribution *of E. faecalis* isolates based on their MIC values to Vancomycin.



results for MDS and MDR *E. faecalis* strains, by origin. Several virulence factors, such as *GelE*, enterococcal surface protein (*Esp*), AS, cytolysin, lipase, and haemagglutinin, are possibly associated with the colonisation and pathogenesis of enterococci (Chow et al. 1993). *GelE* is a protease produced by *E. faecalis* that is capable of hydrolysing gelatine, collagen, casein, haemoglobin, and other peptides (Kreft et al. 1992). It might play an important role in the severity of systemic disease, as shown in several independent studies (Chow et al. 1993; Eaton & Gasson 2001). *GelE* was also shown to be enriched in clinical isolates in some studies (55%-100%) versus 27%-66% in stool isolates from healthy volunteers (Eaton & Gasson 2001). Archimbaud et al. (2002) also found that 17 (58.6%) of 29 *E. faecalis* strains isolated from clinical isolates were *GelE*-positive. In the present study, *GelE* was detected in 92% of MDR *E. faecalis* and 36% to MDS *E. faecalis* (Fig. 2). The capacity to produce lipase and DNase activity were not found in any of the isolates investigated. Lipase and DNase as virulence factors in enterococci seems to be their importance reduced (Semedo et al. 2003a). *GelE* was found to be the most common factor in *E. faecalis* strains. It was concluded that *GelE*, AS, and cytolysin could not be considered important virulence factors by themselves.

In this study, Cytolysin is a cytolytic protein capable of lysing human, horse, and rabbit erythrocytes (Ike et al. 1987). Cytolysin is thought to have an important role in human infections, which is produced in 11%-70% of strains (Eaton & Gasson 2001), compared to 0%-25% in stool isolates (Eaton & Gasson 2001). Ike et al. (1987) reported that 60% of clinical *E. faecalis* isolates were cytolysin-positive in Japan. These findings suggest that this factor may play an important role in human infections. Similar results were found in the study by Coque et al. (1995). Archimbaud et al. (2002) reported that 5 (17.2%) of 29 *E. faecalis* strains isolated from clinical isolates were positive for cytolysin. Eaton and Gasson (2001) observed that 44% of *E. faecalis* strains from food showed cytolysin activity, whereas none of the *E. faecium* strains did. In the present study, cytolysin activity was detected in 90% of MDR *E. faecalis* strains, where in only 30% of MDS *E. faecalis*. Bacterial adherence to host cells appears to be a multifactorial phenomenon involving specific and non- specific interactions. The rates of adherence activity of MDR (82%) strains examined in this study were higher than those in MDS (12%) strains (Fig. 2). Some authors have indicated that MDR *A. baumannii* isolates strongly adhered

Fig. 2 The distribution of virulence factors of MDS and MDR *E. faecalis* strains.



to polystyrene and epithelial cells (Lee et al. 2008). Investigations over MDR *Klebsiella pneumoniae* strains show strong adhesion to intestinal epithelial cells (Di Martino et al. 1997). The adhesive properties depended on the antibiotic resistance profile of these strains.

The presence of genes that encode surface protein (*esp*), gelatinase (*gelE*), cytolysin activator (*cylA*), collagen-binding protein (*ace*) and biofilm-associated proteins (*EF3314*) was investigated by PCR (Table 3).

Table 3 Occurrence of the virulence genes among MDS and MDR *E. faecalis* strains

Gene	MDS *E. faecalis*	MDR *E. faecalis*
esp	24	33
gelE	12	27
cylA	29	90
EF3314	47	3

The *esp* gene was detected in 33% and 24% MDR and MDS *E. faecalis* isolates respectively. Coque et al. (1995) and Shankar et al. (1999) found the *esp* gene exclusively in *E. faecalis* strains. The production of *esp* is proposed to promote the primary attachment to biotic and abiotic surfaces and to be involved in hiding the protein from the immune system (Toledo-Arana et al., 2001). The *cylA* genes were detected in 90% of MDR as compared 28% of MDS *E. faecalis*. Eaton and Gasson (2001) described a higher percentage of *cylA* genes among clinical strains compared with food strains. Production of cytolysin appears to be a major risk factor associated with pathogenic enterococci: a fivefold increased risk of death of patients within 3 weeks of bacteraemia caused by β-hemolytic enterococci, compared with bacteraemia caused by non- β -hemolytic strains was reported by Huycke et al. (1991). In the present study, only the presence of *gelE* gene was investigated. The 419 bp fragment of *gelE* gene was amplified for 27% of MDR isolates and 12% of MDS *E. faecalis*. The presence of the *gelE* gene was, however, not strictly correlated with its expression. Silent *gelE* genes have been observed both in *E. faecalis* isolates from food and in clinical strains, with a higher incidence of silent genes in the latter group (Eaton & Gasson, 2001). *EF3314* was chosen because of its significant similarity (47% in MDR isolates) with biofilm-associated proteins (Cucarella et al., 2001). The PCR survey indicated that this gene is always present and specific for *E. faecalis*; its role as a possible novel *E. faecalis*-restricted antigen is currently under investigation. In our hands, results obtained by phenotypic tests always revealed a lower percentage of strains that produced haemolysin, gelatinase or aggregation substance and biofilm production compared to genotypic characterization. This

may be due to the presence of silent genes that are expressed only under *in vivo* conditions, to the presence of undetected gene mutations or to the fact that detection by PCR of a single gene inside an operon, as is the case of *cylA* for haemolysin production, may overlook the absence of other genes that are necessary for phenotypic expression. Techniques such as RT-PCR may provide useful information on the level of expression of the target DNA.

In conclusion, we found that when compared with MDS strains, the MDR *E. faecalis* strains have impaired virulence *in vitro* study mainly as a result of slow growth and reduced expression of some exoenzymes, cytolysin and biofilm production. The results of this study also indicate that *E. faecalis* strains possess distinctive patterns of potential virulence factors, with a larger number of genes that encode potential virulence factors. Further investigations are needed to evaluate the expression of such factors, which may not be revealed by *in vitro* phenotypic tests during the course of infection.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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