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Full Length Research Paper

Gelatinase production, antimicrobial resistance and pheromone response of conjugative plasmids of *Enterococcus faecalis* isolated from Egypt

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Published data concerning correlation between virulence factors and resistance markers is relatively scarce. The aim of this study was to determine the relationship between antibiotic resistance, gelatinase production and pheromone response in the *Enterococcus faecalis* pathogens isolated from Egypt. Out of the 19 *E. faecalis* clinical isolates, 5 were able to produce gelatinase enzyme. These virulent isolates were multidrug resistant and showed high level vancomycin resistance. They were tested for mating ability. They cotransfered pheromone response genes together with vancomycin resistance determinants and gelatinase production to sensitive recipient strains. The *gel E* gene was detected in all donor isolates and their corresponding transconjugants phenotypically and genotypically. The PCR amplicons of a heavy gelatinase producing isolate and its transconjugants were subjected to sequencing. Significant homology was detected with OG1RF strain following sequence search with a GenBank database. Statistical analysis revealed significant positive direct correlation between vancomycin- and chloramphenicol-resistance, and gelatinase production in *E. faecalis* isolates.

Key words: Correlation, *Enterococcus faecalis*, gelatinase, antimicrobial resistance, conjugation.

INTRODUCTION

Enterococci have emerged as very important nosocomial pathogens, and this is attributed, among other factors, to their broad natural and acquired resistance to antimicrobial agents, including glycopeptides, vancomycin and teicoplanin (Cetinkaya et al., 2000; Gold, 2001;Oskoui and Farrokh, 2010). Although a lot of *Enterococcus* species have been identified, only two are responsible for the majority of human infections, that is, *Enterococcus faecalis* and *Enterococcus faecium*. The most common nosocomial infections produced by these organisms are urinary tract infections (associated with instrumentation and antimicrobial resistance), followed by intra-abdominal and pelvic infections. They also cause surgical wound infections, bacteraemia, endocarditis, neonatal sepsis and rarely meningitis. A major reason why these organisms sms survive in hospital environment is the intrinsic resistance to several commonly used antibiotics and, perhaps more importantly, their ability to acquire resistance to all currently available antibiotics, either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons. The emergence of vancomycin-resistant enterococci (VRE) is a cause of concern, as once established, it is very difficult to control (Sood et al., 2008). There is initial, usually asymptomatic colonization of gastrointestinal tract by enterococcal strains possessing various traits, such as antibiotic resistances, cytolytic toxin genes, or possibly aggregation substance or the protease gelatinase upon hospital admission. (Morris et al., 1995). A report by Kuhnen et al. (1988) showed that the most frequent isolates from two intensive

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care units were *Enterococcus* (formerly Streptococcus) *faecalis* subsp. liquefaciens (the subspecies named liquefaciens are, by definition, gelatinase producers).

This article highlights gelatinase production by enterococci, along with their antimicrobial resistance. We focused on the probability of cotransfer of pheromone responses as well as some antimicrobials from resistant gelatinase producing *E. faecalis* isolates to plasmidless strains.

MATERIALS AND METHODS

Test organisms

A total of 19 *E. faecalis* isolates were recovered from 23 stool specimens, collected from the clinical pathology laboratory of Tanta University Hospitals during summer season. Purity and identity of the clinical isolates were confirmed (Koneman et al., 1992; WHO, 2003). An isolate of *Escherichia coli* L99 used as a plasmid molecular weight marker was obtained from Department of Microbiology, Faculty of Pharmacy, Tanta University. *Staphylococcus aureus* (RN4220) and *E. faecalis* (JH2-2) were used as recipient strains in this study. Both recipients were obtained from Department of Clinical Analysis and Toxicology, Faculty of Pharmaceutical Science, University of Sao Paulo, Brazil.

Chemicals and culture media

All chemicals used were purchased from Sigma, USA while culture media were purchased from Oxoid, UK. Dream green PCR master mix, 2X, (Fermentas, USA), DNA ladder (100 pb) and nuclease free water (Fermentas, USA) were used. Forward and reverse primers, used for amplification, were synthesized by a custom primer service (Fermentas, USA). All primers were reconstituted with nuclease free water to obtain a final concentration of 100 picomle/µl (stock solution).

Detection of gelatinase producing *E. faecalis* isolates

Phenotypic detection

Production of gelatinase was determined on nutrient agar containning 3% gelatin. Single colonies were streaked onto plates, grown overnight at 37°C, then 1% tannic acid was poured on the surface of the medium to precipitate the unhydrolyzed gelatin, leaving a transparent halo around the colonies indicating positive result (Eaton and Gasson, 2001; Lopes et al., 2006).

Genotypic detection of the virulence plasmid

PCR detection of *gel E* gene located on the virulence plasmid in the tested isolates was carried out according to Creti et al. (2004). The primers that were used to check for the presence of this gene were: *gel E* genes, F- 5'-ACCCCGTATCATTGGTTT-3'-, 5'-ACGCATTGCTTTTCCATC-3'. The strain used as a negative control was: *E. faecalis* (JH2-2). When the green master mix was brought to a final volume of 25 µl, each reaction contained 0.05 units/µl of Taq DNA polymerase, PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP and the plasmid DNA were used. All PCR amplifications were performed in a TC-3000G Thermocycler (TECHNE, UK) using the following PCR programme: 5 min at 95°C; 30 cycles of 60 s at 95°C, 60 s at 52°C, 60 s at 72°C and 10 min at 72°C. The PCR products were run on a 0.9% A 9539 agarose gel (Sigma, USA). The amplicon size was 405 bp (Creti et al., 2004).

Susceptibility of the virulent isolates to different antimicrobial agents

Susceptibility of gelatinase producing *E. faecalis* isolates to different antimicrobials was performed using break point concentration of agar diffusion technique according to the procedure described by the Clinical and Laboratory Standard Institute guidelines (CLSI, 2010).

Plasmid DNA study

Plasmid DNA analysis

Plasmids of selected *E. faecalis* isolates were extracted by modified alkaline lysis method (Alebouyeh et al., 2005) and they were purified using the QIAprep Spin Miniprep kit (Qiagen). Profiles were compared after electrophoresis on 1% agarose gel. The plasmid sizes were measured by comparing their mobility with those of plasmids of known size, like the ones from *E. coli* (L99).

Mating study

A plasmid-free, esculin fermentor, rifampicin- and fusidic acid-resistant (F^-, Esc^+, RIF^r, FUS^r) strain of *E. faecalis* (JH2-2) was used as a recipient strain. *S. aureus* (RN4220) was also used as another recipient strain in this study and it is a plasmid-free, mannitol fermentor, chloramphenicol-resistant (F^-, Man^+, CHL^r) strain. Filter mating was performed using a 1:1 donor-recipient mixture. Briefly, 0.5 ml of each overnight culture of the gelatinase producing isolates (donors) and one of the recipients in brain heart infusion (BHI) broth were mixed and harvested using 0.45 µm-pore-size filters and incubated on BHI agar plates at at37°C for 24 h. Cells were harvested and diluted in BHI broth and grown for 2 days at 37°C on BHI agar plates. For selection of transconjugants, enterococcal transconjugants were selected on bile esculin azide agar supplemented with one of the antimicrobials to which the donor strain is resistant and 100 µg/ml of rifampicin and 25 µg/ml of fusidic acid (to which the JH2-2 strain is resistant). Staphylococcal transconjugants were selected on mannitol salt agar supplemented with one of the antimicrobials to which the donor strain is resistant and 100 µg/ml of chloramphenicol (to which the RN4220 strain is resistant). The frequency of transfer was expressed relative to the number of donor cells (Davis et al., 1980; Lyon et al*.,* 1983; Birnboim and Doly, 1979).

Detection of virulence factor and antimicrobial resistance markers in transconjugants

The transconjugant colonies were picked with tooth pick and cultured onto the selective media containing break points of each of the tested antimicrobials and incubated overnight. Transconjugants were also tested for gelatinase production as described before. Plasmid analysis of the *S. aureus* as well as *E. faecalis* transconjugants was carried out as described earlier. Gelatinase producing donors and their transconjugants were all tested for their ability to aggregate when exposed to a cell free culture filtrate of JH2-2. This method tests for any pheromone response as described by Ike and Clewell (1984).

Curing of plasmids

Traits to eliminate plasmids of the transconjugants were done using ethidium bromide (E Br) at elevated temperature 42°C. Two fold serial dilution of E Br were prepared in Luria Bertani (LB) broth. Aliquots of 100 µl of 10^7 CFU/ml suspension of the test transconjugant were inoculated into the serial E Br dilutions. The tubes were gently shacked and incubated at 42°C for 24 h. After overnight incubation, the tubes were inspected for the presence of turbidity.

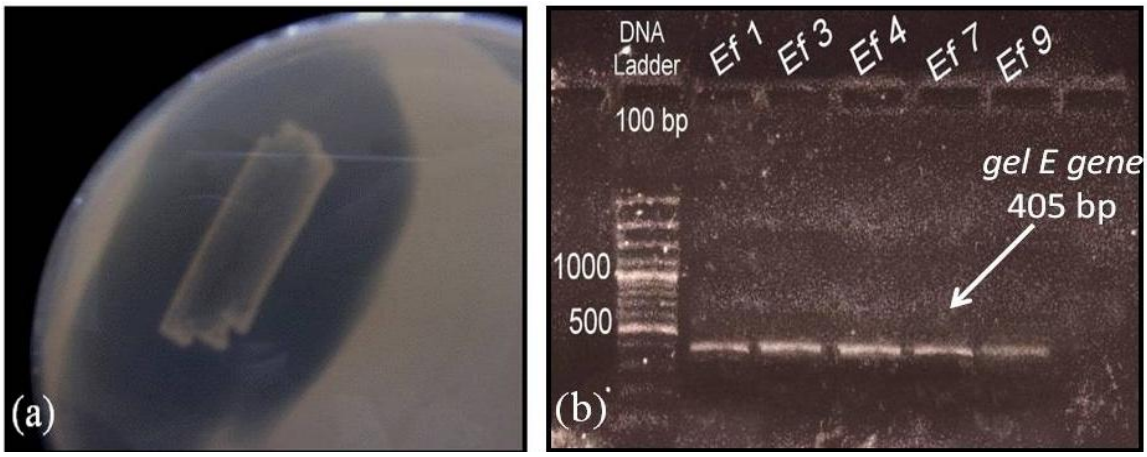

Figure 1. Detection of gelatinase production by *E. faecalis* isolates, (a) gelatin agar showing clear halo zone around the colonies, (b) electrophoregrams of the amplified PCR products of *gel E* gene.

The subinhibitory concentration of E Br was taken as the highest concentration showing turbidity. Hundred microliter from subinhibitory concentration for each isolate were subcultured by aseptically spreading on the surface of LB agar plates. After overnight incubition at 37°C, each of the resultant colonies was picked with tooth pick and subcultured on LB agar plates containing the break point concentration of the tested antimicrobials. Control plate containing LB agar without antimicrobial agent was simultaneously subcultured. Colonies which grew on the control plates but failed to grow on antimicrobial agent containing plate were detected as the cured cells. Detection of the virulence factors was carried out on the cured derivatives as previously mentioned. The plasmids of each cured strain were prepared, electrophoresed and photographed as mentioned before (Darfeuille-Michaud et al., 1992; Mandal et al., 2003).

Nucleotide sequencing and analysis

Nucleotide sequence data was obtained by stepwise sequencing. Initially, forward primers of *gel E* gene were used for sequencing. As new sequence data became available, customized synthetic primers sequencing reaction products were analyzed with the Applied Biosystems model 373A DNA sequencer (Applied Biosystems, Inc.). Sequencing was performed at laboratories of the City of Scientific Research and Technological Science at New Borg ELarab, 21934 Alexandria, Egypt; using the Big dye terminator v.3.0 sequencing kit and a 3700 DNA analyzer (Applied Biosystem, Foster City, CA, USA). Nucleotide sequence analysis & searches for homologous DNA sequences in the GeneBank database libraries were performed with the program BLASTN 2.2.26+ as well as BLASTX 2.2.26+ (http://www.ncbi.nlm.nih.gov/BLAST/Blast). The GeneBank accession number CP002621.1 was assigned to the nucleotide sequences of *gel E* gene of the selected donor and their transconjugants. G blocks were determined for phylogenetic analysis http://www.phylogeny.fr/version2_cgi/simple id=653280cc12fd1fb407ea532380ae4864&tab_index=6.

Statistical analysis

The data was analyzed with SPSS version 15 statistical software package SPSS (SPSS, Inc., Chicago, IL). Correlations between virulence factor and resistance markers were evaluated using the Pearson's *r* coefficient.

RESULTS

Detection of gelatinase production by *E. faecalis* isolates

Five out of 19 (26.3%) *E. faecalis* isolates were gelatinase producers. Production of gelatinase was detected as a clear zone around the growth of the test isolates as shown in Figure 1a. PCR experiment was carried out on the plasmid DNA of gelatinase producing *E. faecalis* isolates. Bands with molecular weight 405 bp were detected in the electrophoregram shown in Figure 1b, indicating the presence of *gel E* gene.

Susceptibility of the virulent clinical isolates to different antimicrobial agents

As shown in Table 1, all gelatinase producing *E. faecalis* isolates were resistant to SSS and VAN. Moreover, all the 5 isolates showed vancomycin resistance (VAN A type) according to the MIC results (data not shown). On the other hand, AMP, IMP, STR and TET were active on all the tested isolates as shown in Table 1.

Analysis of plasmid profiles of gelatinase producing *E. faecalis* isolates

Plasmid profiles and resistance patterns of selected isolates

The plasmid profiles of the 5 gelatinase-producing *E. faecalis* isolates are presented in the electrophoregram as shown in Figure 2. Four plasmid sizes were detected among the tested *E. faecalis* isolates including 140, 106, 1.6 and 1.2 MDa. The high molecular weight plasmids 140 and 106 MDa were present in all the studied isolates.

Table 1. Incidences of antimicrobials resistance among gelatinase producing *E. faecalis* isolates.

AMA* break point** (µg/ml)	*E. faecalis* (n = 5)
AMP ≥ 32	0
IMP ≥16	0
RAD ≥64	4
CTX ≥64	2
FEP ≥32	2
STR ≥64	0
TET ≥16	0
CHL ≥32	4
RIF ≥4	4
ERY ≥ 8	2
AZ ≥ 2	2
SSS ≥512	5
TRI ≥ 16	2
SXT ≥ 80	2
VAN ≥ 32	5
NOR ≥ 16	2
CIP ≥ 4	2
ENX ≥ 4	3
MOX ≥8	2

*AMA: Antimicrobial agents, AMP: Ampicillin, IMP: Imipenem, RAD: Cephradine, CTX: Cefotaxime, FEP: Cefepime, STR: streptomycin, TET: Tetracycline, CHL: Chloramphenicol, RIF: Rifampicin, ERY: Erythromycin, AZ: Azithromycin, SSS: Sulfamethoxazole, TRI: Trimethoprime, SXT: Sulfamethoxazole /Trimethoprime, VAN: Vancomycin, NOR: Norfloxacin, CIP: Ciprofloxacin, ENX: Enoxacin, MOX: Moxifloxacin.
**Break points were done according to CLSI, 2010.

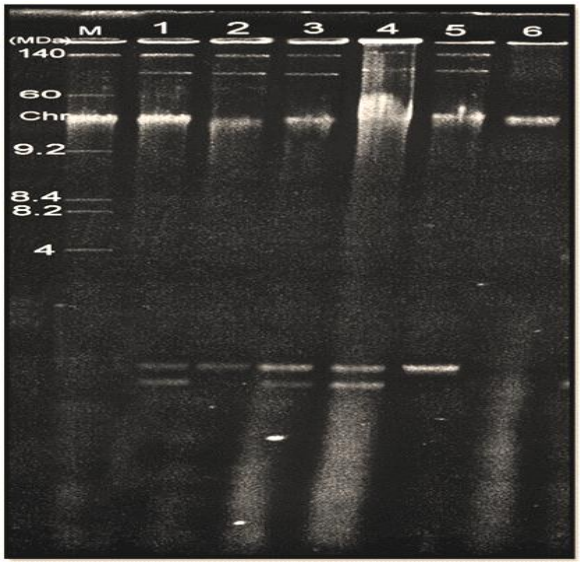

Figure 2. Electrophoregrams of virulent *E. faecalis* isolates. Lanes 1-5: gelatinase producing isolates (Ef 3, 1, 4, 7 and 9, respectively); Lane 6: JH2-2, a negative control of *E. faecalis* strain; Lane M: molecular weight marker.

Conjugal transfer of the plasmids of gelatinase producing isolates

Three multiresistant gelatinase producing *E. faecalis* isolates were selected, one from each resistance pattern, and subjected to conjugation and curing experiments. The plasmid profiles of the donors, transconjugants and the cured derivatives are presented in the electrophoregrams shown in Figure 3.

As shown in Table 2, all the donors plasmids were transferable except for 140 MDa. It was noted that 106 MDa plasmid was the most common one transferred to all transconjugants. Conjugation failed when dEf 9 was incubated with *S. aureus* RN4220 recipient strain (Table 2).

As recorded in Table 2, the resultant transconjugants acquired some resistance markers from the donors including RAD, VAN, CHL, TRI or SSS. All these resistance markers were lost after curing except for cEf 7. The data shown in Table 2 revealed also that gelatinase production, which is a virulence factor presented by the tested donors of *E. faecalis* isolates, was transferred to the corresponding transconjugants. All gelatinase-producing donors and the corresponding transconjugants exhibited a clumping response upon exposure to a cell free solution of pheromones comprising an *E. faecalis* JH2-2 culture filtrate.

Detection of gelatinase transfer in dEf 3 and tEf 3

Being heavy gelatinase producers, dEf 3 isolate and its transconjugants, tEf 3 and tS 3, were selected for the PCR experiment and sequence analysis.

PCR amplification of gel E gene

All of the dEf3, tEf3 and tS3 were selected and subjected to the PCR experiment. Positive amplicons of *gel E* gene were detected at 405 bp as shown in Figure 4. The resultant amplicons were subjected to sequencing.

Analysis of the obtained sequence

Single sequence analysis: Analysis of nucleotide sequences of dEf 3, tEf 3 and tS3 amplicons revealed that these sequences belong to *E. faecalis* (OG1RF strain). Moreover, the OG1RF strain is gelatinase producer (Gulhan et al., 2006). The percentage of identity of the donor sequence to that of OG1RF strain was 86%, while those of the transconjugants (tEf 3 and tS3) were 90 and 89%, respectively. In addition, significant E- values were recorded for the donor ($5e^{-69}$) and also for the trans-conjugants ($2e^{-108}$ & $3e^{-99}$), respectively, as recorded in Tables 3, 4 and 5. The program BLASTX 2.2.26+ was used to translate

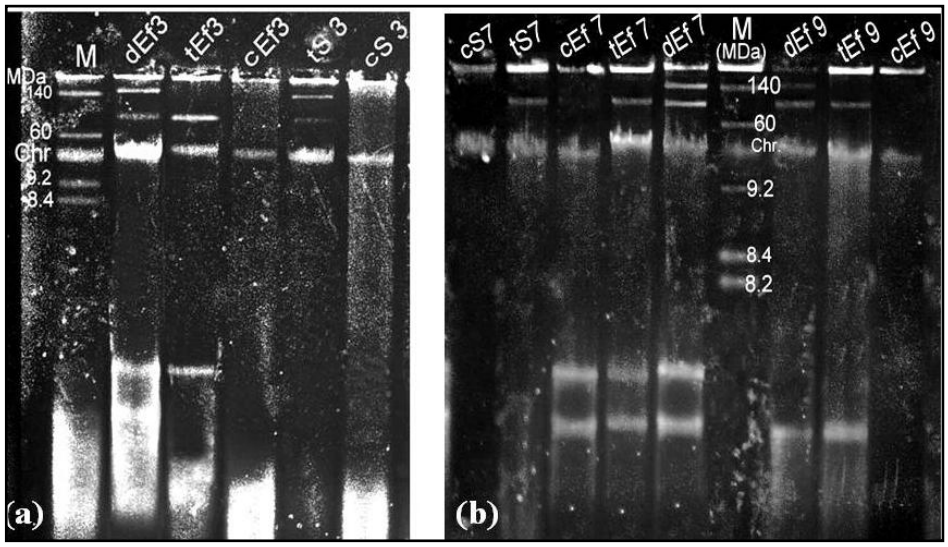

Figure 3. Electrophoregrams showing the plasmid profiles of the donors, transconjugants and selected cured derivatives of (a) Ef 3 isolate, (b) Ef 9 and dEf 7 isolates of gelatinase producing *E. faecalis*. Lane M; molecular weight marker.

Table 2. Gelatinase production, pheromone response, plasmid profiles and resistance patterns of donors, transconjugants and cured derivatives of selected *E. faecalis* isolates.

Isolate* code	Gelatinase production	Pheromone response	Plasmid** profiles	Resistance patterns
dEf 9	+	+	140- **106**- 1.2	ENX- SSS-VAN
tEf 9	+	+	**106** – 1.2	VAN
cEf 9	-		--	--
dEf 7	+	+	140- **106**- 1.6- 1.2	RAD-VAN-CHL-TRI-SSS-SXT
tEf 7	+	+	**106**- 1.6- 1.2	RAD-VAN-CHL-TRI-SSS
cEf 7	-	-	1.6- 1.2	TRI-SSS
tS 7	+	+	**106**	VAN-CHL
cS 7	-	-	--	--
dEf 3	+	+	140- **106**- 1.6	RAD-CTX-FEP-VAN-NOR-CIP-ENX-MOX-CHL-ERY-AZ-SSS
tEf 3	+	+	**106**- 1.6	VAN -CHL-SSS
cEf 3	-	-	--	--
tS 3	+	+	**106**	VAN-CHL
cS 3	-	-	--	--

*dEf: Donor of *E. faecalis*; tEf:transconjugant of *E. faecalis*; cEf: cured *E. faecalis*; tS: transconjugant of *S. aureus*; cS: cured *S. aureus.* ***Bold** number refer to the common plasmid transfered.

the nucleotide sequence of the donor to protein and to detect its function. It was found that the sequence belong to gelatinase enzyme, gluzincin superfamily and peptidase M-4. Putative. It was found that the sequence belong to gelatinase enzyme, gluzincin superfamily and peptidase M-4. Putative conserved domains of this enzyme are identified within the input sequence starting from nucleotide 217 to nucleotide 360 for the donor amplicon. A representative graphic showing the position of each conserved domain of the donor isolate was shown in Figure 5.

Multiple sequence analysis:

DNA alignments were carried out according to the phylogenitic software of MUSCLE (G blocks). Figure 6 shows an alignment between the homologous sequences of

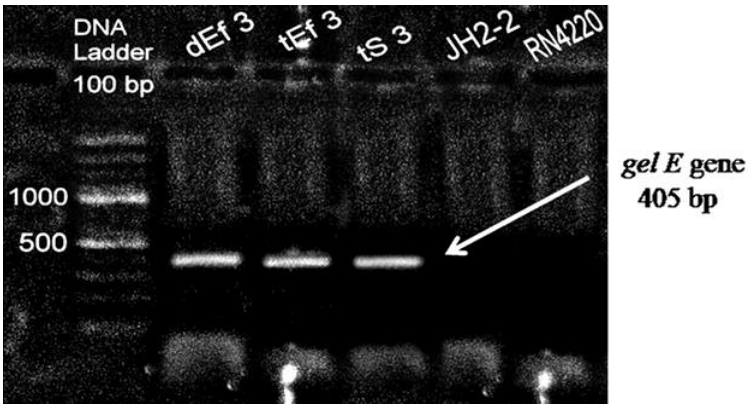

Figure 4. Electrophoregrams of *gel E*- amplicons of donor (dEf 3) of *E. faecalis* isolates, and its transconjugants (tEf 3 and tS3). Lane JH2-2 and RN4220: negative controls.

Table 3. A hit list from a BLAST search with gelatinase producing *E. faecalis* (dEf 3) as the query against the nr database. Black arrow points to the most significant subject in the database.

Accession	Description	Maximum score	Total score	Query coverage (%)	E-value	Maximum identity (%)
CP002621.1	*Enterococcus faecalis* OG1RF, complete genome	270	270	56	5e-69	86
M37185.1	*Enterococcus faecalis* gelatinase (*gelE*) gene, complete cds	270	270	56	5e-69	86
JN246675.1	*Enterococcus faecalis* strain LN68 Fsr and gelE-sprE operons, complete sequence	265	265	56	3e-67	85
HE574483.1	*Enterococcus faecalis sprE, gelE, fsrC, fsrB, fsrD* and fsrA genes, strain LN68	265	265	56	3e-67	85
CP002491.1	*Enterococcus faecalis* 62, complete genome	265	265	56	3e-67	85
EF105504.1	*Enterococcus faecalis* GM gelatinase (*gelE*) gene, complete cds	265	265	56	3e-67	85
D85393.1	*Enterococcus faecalis* gelatinase (*gelE*) DNA, complete cds	265	265	56	3e-67	85
EU862241.3	*Enterococcus faecalis* strain H81 gelatinase (*gelE*) gene, partial cds	259	259	56	1e-65	85
AE016830.1	*Enterococcus faecalis* V583, complete genome	254	254	56	5e-64	85
FP929058.1	Enterococcus sp. 7L76 draft genome	243	243	56	1e-60	84
FJ858146.1	*Enterococcus faecium* strain QSE32 fsr operon, complete sequence; and GelE (*gelE*) and SprE (*sprE*) genes, complete cds	243	243	56	1e-60	84

Table 4. A hit list from a BLAST search with gelatinase producing *E. faecalis* (tEf 3) as the query against the nr database. Black arrow points to the most significant subject in the database.

Accession	Description	Maximum score	Total score	Query coverage (%)	E-value	Maximum identity (%)
CP002621.1	*Enterococcus faecalis* OG1RF, complete genome	401	401	82	2e-108	90
M37185.1	*Enterococcus faecalis* gelatinase (*gelE*) gene, complete cds	401	401	82	2e-108	90
JN246675.1	*Enterococcus faecalis* strain LN68 Fsr and gelE-sprE operons, complete sequence	396	396	82	7e-107	89

Table 4. Contd.

HE574483.1	*Enterococcus faecalis sprE, gelE, fsrC, fsrB, fsrD* and *fsrA* genes, strain LN68	396	396	82	7e-107	89
EF105504.1	*Enterococcus faecalis* GM gelatinase (*gelE*) gene, complete cds	396	396	82	7e-107	89
D85393.1	*Enterococcus faecalis* gelatinase (*gelE*) DNA, complete cds	396	396	82	7e-107	89
CP002491.1	*Enterococcus faecalis* 62, complete genome	390	390	82	3e-105	89
EU862241.3	*Enterococcus faecalis* strain H81 gelatinase (*gelE*) gene, partial cds	379	379	82	7e-102	88
AE016830.1	*Enterococcus faecalis* V583, complete genome	379	379	82	7e-102	88
FP929058.1	Enterococcus sp. 7L76 draft genome	374	374	82	3e-100	88
FJ858146.1	*Enterococcus faecium* strain QSE32 fsr operon, complete sequence; and GelE (*gelE*) and SprE (*sprE*) genes, complete cds	374	374	82	3e-100	88

Table 5. A hit list from a BLAST search with gelatinase producing *S. aureus* (tS3) as the query against the nr database. Black arrow points to the most significant subject in the database.

Accession	Description	Max score	Total score	Query coverage (%)	E-value	Maximum identity (%)
→ CP002621.1	*Enterococcus faecalis* OG1RF, complete genome	370	370	99	3e-99	89
M37185.1	*Enterococcus faecalis* gelatinase (*gelE*) gene, complete cds	370	370	99	3e-99	89
JN246675.1	*Enterococcus faecalis* strain LN68 Fsr and gelE-sprE operons, complete sequence	364	364	99	2e-97	89
HE574483.1	*Enterococcus faecalis sprE, gelE, fsrC, fsrB, fsrD* and *fsrA* genes, strain LN68	364	364	99	2e-97	89
EF105504.1	*Enterococcus faecalis* GM gelatinase (*gelE*) gene, complete cds	364	364	99	2e-97	89

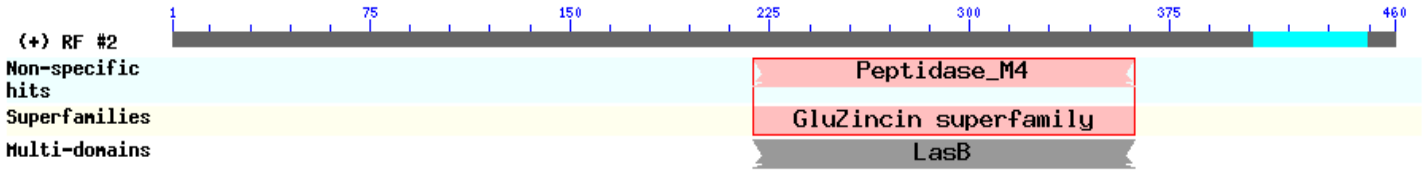

Figure 5. Graphical summary for conserved domains of seq. 11 (dEf 3).

nucleotides from gelatinase producing *E. faecalis* donor (dEf 3), tEf 3 and tS 3. The percentage of identity of the 3 sequences was 66%. Six selected G blocks were determined. Flank positions of these blocks are [63 75] [77 172] [186 252] [254 289] [293 374] [377 388].

Phylogenetic tree:

To investigate the genetic relationships between the donor *E. faecalis* dEf 3 (seq. 11), the *E. faecalis* transconjugants tEf 3 (seq. 12) and the *S. aureus* transconjugants tS 3 (seq. 15), an amplified plasmid DNA of *gel E* gene was used. The phylogenetic tree derived from these data (Figure 7) shows that the transconjugants belong to genetic group different from that of the donor. Moreover, it was noted that both transconjugants belong to the same genetic group (carry the same genetic characteristics). In addition, *E. faecalis* transconjugants (seq. 12) is closer to the donor (seq. 11), than *S. aureus* transconjugants (seq. 15) as shown Figure 7.

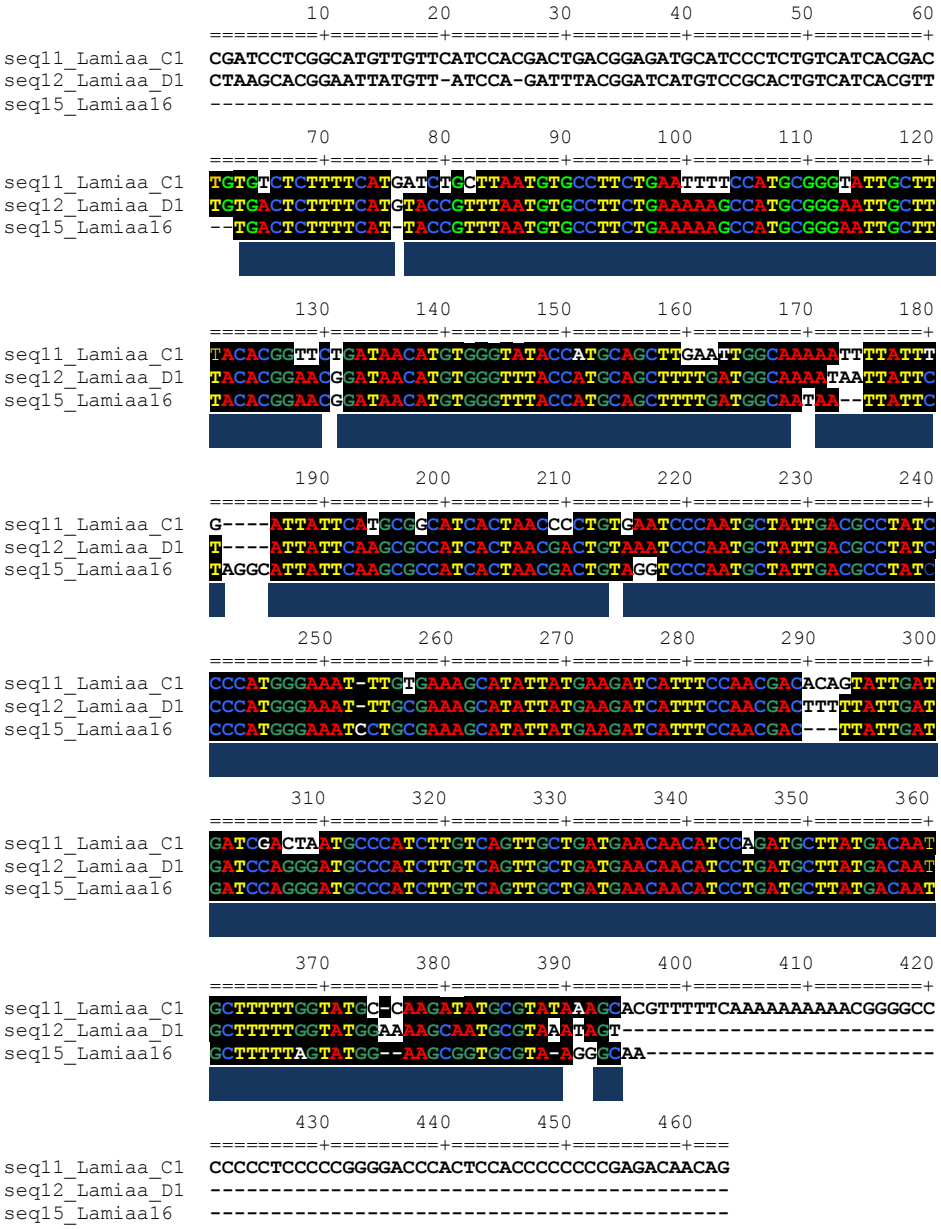

Figure 6. Multiple sequence alignment using MUSCLE software of the donor (dEf3), enterococcus transconjugant (tEf3), and staphylococcus transconjugant (tS3). Appropriate use of color can highlight positions that are either identical in all the aligned sequences or share common physicochemical properties.

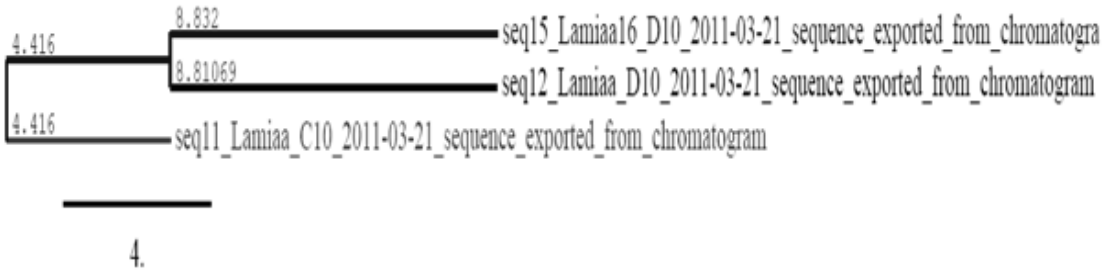

Figure 7. Phylogenetic tree of dEf3, tEf3 and tS3 showing the branch length. It is based on genes that do not match organismal phylogeny, suggesting horizontal gene transfer has occurred.

Table 6. Pearson correlation coefficient (*r*-values) between virulence factors and resistance markers.

Resistant marker	*E. faecalis* (*gel E*)
RAD	0.389
CTX	0.00
FEP	0.00
STR	0.00
TET	0.00
CHL	0.601*****
ERY	0.00
AZ	0.00
SSS	-0.433
TRI	-0.321
SXT	-0.121
VAN	0.673******
NOR	0.00
CIP	0.00
ENX	0.00
MOX	0.00

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Correlation between gelatinase production, pheromone responses, resistance markers and plasmid profiles of the tested *E. faecalis* isolates

The inability of the 140 MDa plasmid to transfer by conjugation to the corresponding transconjugant tEf 9 was accompanied by loss of resistance to ENX and SSS. On the other hand, conjugation and curing experiments revealed that loss of 106 and 1.2 MDa plasmids from cEf 9 was accompanied by loss of resistance to VAN. Moreover, cEf 9 became unable to produce gelatinase enzyme and lost its ability to respond to pheromone. It was found that dEf 7 isolate carried 4 plasmids with molecular sizes 140 MDa, 106 MDa, 1.6 MDa and 1.2 MDa. Conjugation experiment revealed that that *E. faecalis* transconjugant tEf 7 gained106 MDa, 1.6 MDa and 1.2 MDa plasmids and became resistant to RAD, VAN, CHL, TRI and SSS while *S. aureus* transconjugant (tS 7) gained only 106 MDa plasmid that conferred resistance to VAN and CHL. The inability of the 140 MDa plasmid of the isolate dEf3 to transfer by conjugation was accompanied with the loss RAD, CTX, FEP, NOR, CIP, ENX, ERY, and AZ of resistance makers in the corresponding transconjugant while the transfer of 106 MDa and1.6 MDa plasmids was accompanied with gaining resistance to VAN, CHL and SSS, the production of gelatinase enzyme and response to pheromone by tEf 3. When the conjugation and curing experiments were done using RN4220 recipient strain, it was found that tS 3 gained 106 MDa plasmid and also it became resistant to VAN and CHL. In addition, the corresponding transconjugant produced gelatinase enzyme and exhibited a clumping response upon exposure to a cell free solution of pheromones comprising to an *E. faecalis* JH2-2 culture filtrate.

Statistical analysis of the correlation between gelatinase production and resistance markers of the *E. faecalis* isolates

Table 6 shows Pearson correlation coefficient between gelatinase production and resistance markers. Gelatinase production by *E. faecalis* isolates showed significant positive direct correlation with CHL and VAN.

DISCUSSION

The pheromone system plays an important role in the horizontal spread of genes between strains of *E. faecalis*, including those genes encoding antibiotic resistance and virulence traits (Woodford et al., 1993; Wirth, 1994). Several virulence factors, such as gelE, enterococcal surface protein (Esp), aggregation substance (AS), cytolysin, lipase and haemagglutinin are possibly asso-ciated with the colonisation and pathogenesis of enterococci. GelE is a protease produced by *E. faecalis* that is capable of hydrolysing gelatine, collagen, casein, haemoglobin and other peptides. It might play an important role in the severity of systemic diseases (Gulhan et al., 2006). In the present study, 19 *E. faecalis* isolates were recovered from stool samples. Five out of 19 (26.7%) were identified as gelatinase producers using phenotypic and genotypic techniques. Gulhan et al. (2006) reported that 68% of blood culture isolates, and 27% of community-acquired faecal isolates were gelE-positive. Also, Strzelecki et al. (2011) reported that 140 *E. faecalis* isolates (91% of the group) harbored the *gelE* gene, but only 81 isolates (53%) produced active gelatinase.

Palazzo et al. (2005) reported that fifty-one vancomycin-resistant enterococci samples isolated from different geographic regions in Brazil harbored the *vanA* gene as demonstrated by PCR analysis, and in a majority of strains, the gene was associated with a transferable plasmid of 70 kb (106.4 MDa). In the present study, 4 plasmid sizes were detected among the tested *E. faecalis* isolates including 140, 106, 1.6 and 1.2 MDa. The high molecular weight plasmids 140 and 106 MDa were present in all the studied isolates.

The glycopeptide vancomycin is the “first choice” alternative to penicillin-aminoglycoside combination for treatment of systemic enterococcal infections. The frequency of vancomycin resistant enterococcus (VRE) isolates had increased worldwide. The frequency of VRE isolates among nosocomial infections in USA was only 1% in 1989, but increased to 7.9% by 1993 (Center for Disease Control and Prevention, 1995). High (16.8%) detected rate of enterococcal urinary tract infections was reported in Canada (Low et al., 2001). Moreover, Al-Jarousha et al. (2008) reported that 66.6% of the enterococcus isolates detected in Gaza were vancomycin resis-

tant. In our study, 5 out of 19 (26.3%) *E. faecalis* isolates were vancomycin resistant and also gelatinase producers. High level vancomycin resistance (VanA) was also detected as determined by MIC test (data not shown). Similar finding was detected by Courvalin (2005), Henrique et al. (2008) and Oskoui and Farrokh (2010) who reported that VanA and VanB are widespread globally and confer the most prevalent glycopeptide resistance phenotype.

In the present study, while the 5 gelatinase producing *E. faecalis* isolates were multidrug resistant, 4 out of them were sensitive to AMP. These results were in agreement with the study of Radu et al. (2001) who reported that all the tested *E. faecalis* and *E. faecium* isolates were multidrug resistant. They showed resistance to kanamycin, nalidixic acid, VAN, CTX, ERY, CHL and STR although sensitive to AMP which was the most active agent against these isolates. This finding is of great importance since these 2 species have been associated with human infection and that ampicillin is therefore the drug of choice in the treatment of enterococcal infections. Although, penicillinase production is common in *S. aureus*, it is very rare in enterococci. Enterococci are intrinsically resistant to cephalosporins because of the presence of penicillin binding proteins (PBP) with low affinities for these agents (Woodford, 2005). The linkage between a β-lactam resistant PBP and vancomycin resistance does not appear to have occurred yet in *E. faecalis*, which may account for the sporadic detection of vancomycin resistant *E. faecalis* (Sood et al., 2008).

Enterococci possess a variety of mechanisms for transferring antibiotic resistance determinants to susceptible recipients. The pheromone-mediated conjugation systems of several plasmids have been studied including those of pDA1, pCF10, pPD1 and pAM373 (Wirth, 1994; Heaton and Handwerger, 1995; Ike and Clewell, 1984; Fujimoto et al., 1995). In the present study, tS3 and tS7 acquired a single pheromone responsive plasmid (106 MDa) together with VAN A and CHL resistance which indicates that the pheromone responsive plasmids in our hospitals frequently carried antimicrobial resistance determinants. Several other pheromone responsive plasmids have been reported previously (Wirth, 1994; Pournaras et al., 2000).

In our study, a homologous-nucleotide search with a GenBank database revealed significant sequence identities between the gelatinase encoding gene of Ef 3, tS3 and tEf3 and that of OG1RF strain. Alignment comparisons of the gelatinase of tEf3, or Ef3 tS3 with that of OG1RF, indicate 86, 89 and 90% homologies at positions 11588137-1588397, 588143-1588442 and 1588123-1588442, respectively. These regions of high homology belong to the positions of the gelatinase enzymatic active site. It was found that the nucleotides sequences of the 3 strains belong to gelatinase enzyme, gluzincin superfamily, peptidase M-4. Putative conserved domains of this enzyme are identified within the input sequence starting from nucleotide 217 to nucleotide 360 for the donor amplicon. Mäkinen et al. (1989) Su et al. (1991) and Qin et al. (2000) reported that a homologous-protein search revealed significant amino acid sequence similarities between the gelatinase of *E. faecalis* and neutral proteases from *Bacillus thermoproteolyticus*, and elastase from *Pseudomonas aeruginosa*. The amino acid sequence matrix plots of the gelatinase versus thermolysin of *B. thermoproteolyticus* and versus elastase of *P. aeruginosa* showed that each point reflects at least 40% homology within an 8-amino-acid alignment of the corresponding proteins.

In the present study, the phylogenetic tree derived from the data showed that the transconjugants belong to genetic group different from that of the donor. Moreover, it was noted that both transconjugants belong to the same genetic group (carry the same genetic characteristics). In addition, *E. faecalis* transconjugant (seq. 12) is closer to the donor (seq. 11) than the *S. aureus* transconjugant (seq. 15). It was deduced that horizontal gene transfer from an ancestor of seq.11 to the ancestor of seq.12 and seq. 15 occurred because this would most simply explain the results. The results shown of this study are similar with those reported by Baxevanis and Ouellette (2001).

It was found that these gelatinase producing isolates and their transconjugants were positive in clumping experiments and contained pheromone responsive conjugative plasmid weighing 106 MDa suggesting that the pheromone system possibly contributed to vancomycin and/or chloramphenicol resistance in our setting. The statistical analysis revealed significant positive and direct correlation between gelatinase production and resistance to vancomycin or chloramphenicol (transfer of chloramphenicol resistance in *S. aureus* was detected by MIC but data not shown). Lata et al. (2009) reported that a significant correlation was observed in the distribution of multiple-antimicrobial resistance (erythromycin-rifampicin-gentamicin, methicillin and vancomycin-gentamicin-streptomycin; $r = 0.9747$; $p = 0.0083$) and multiple-virulence factors (gelE+ esp+; $r = 0.9747$; $p = 0.0083$; gelE+ efaA+; $r = 0.8944$; $p = 0.0417$) among different Enterococcus spp.

To our knowledge, this is the first report in Egypt on the relationship between gelatinase production, vancomycin resistance and pheromone response in *E. faecalis* isolates. In conclusion, the present study showed that enterococci served as reservoir for drug resistance genes. Therefore, the conjugal transfer of gelatinase production, pheromone response, as well as VanA type of vancomycin resistant genes from enterococci to other Gram-positive bacteria like *S. aureus* isolates pose a threat to public health. This gives rise to concern that such transfer in humans under natural conditions could be feasible.

New approaches are needed to control antimicrobial resistance with respect to the use of antibiotics. The era where acute or chronic bacterial infections were treated

with ‘‘antibiotics-only’’ appears to have come to an abrupt end. It is time to intensify attention on search for compounds that can block the expression of genes encoding virulence factors or even inhibit the conjugation process. Formulations containing antimicrobial agents combined with such conjugation inhibitory compounds would be a potential solution for the problem. Finally, data of our work strongly recommend periodic evaluation of both virulence factors as well as resistance markers in *E. faecalis* isolates otherwise spread of resistance combined with virulence genes among bacterial pathogens would leave clinicians with fewer treatment options.

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