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Full Length Research paper

Antimicrobial susceptibility of *Enterococcus faecalis* and a novel *Planomicrobium* isolate of bacterimia

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During nucleotide sequences of *16SrDNA* gene, *Enterococcus faecalis* was predominant in 322 samples; 11 isolates (100%) were from outpatients' vaginal swabs and 3 isolates (75%) from inpatients' blood samples. The three isolates from the blood showed two frame shift mutations "Deletion" at sequence positions 21 and 1076 bp to be 100% similar with type strain OG1RF. The isolates totally showed no resistance to gentamycin, streptomycin and erythromycin whereas, there was high resistance to amoxicillin/clavulanic acid (71.4%), methicillin and vancomycin (92.8%, for each). All isolates from the blood were completely resistant to penicillin and ampicillin. The study also detected *Planomicrobium mcmeekinii* from an inpatients' blood case (containing Gene or Point mutation type Transversion at position 358 bp resulting to 99% sequence identity to ATCC 700539) as first reporting and revealing it to be a new subspecies or species.

Key words: *Enterococcus faecalis, Planomicrobium mcmeekinii*, 16SrDNA and sequencing.

INTRODUCTION

In past years, Enterococci have rapidly emerged as important nosocomial and community acquired pathogens (Kapoor et al., 2005). These organisms can cause series of invasive infection including endocarditis, meningitis, urinary tract infection and bacterimia (Guardado et al., 2006; Nallapareddy et al., 2006; Peters et al., 2007; Singh et al., 2007). *Enterococcus faecalis* is responsible for 90% of enterococcal infection (Shepard and Gilmore, 2002). Furthermore, it is among the most common pathogens isolated from infected surgical sites, urinary tract infection, blood-stream and vagina (Dupre et al., 2003; Jahic et al., 2006). However, *E. faecalis* is associated with 6% mortality rate in early onset septicemia which rises to 15% in late-onset infection; in general, it is implicated in 7 to 50% of fatal cases (Jahic et al., 2005). Enterococci are considered as an important difficult-to-treat pathogens due to their intrinsic resistance to a wide range of antibiotics that most notably include beta-lactams and aminoglycoside frequently used to treat infections with Gram-positive cocci (Kapoor et al., 2005). In addition, enterococci have the ability to acquire resistance to antimicrobial agents through plasmid transferring, transposons and chromosomal exchange or mutations (Mundy et al., 2000; Tiwari and Sen, 2006). Moreover, management of vancomycin resis-tant enterococci infections poses a clinical challenge as these organisms may be resistant to several antimicrobials with unique mechanisms of action (Wong et al., 2000).

Detection and infection of microorganisms involved cultivation-based techniques, which has been a challenging task due to the mixed nature of the infection and the diverse physiological and nutritional requirements for culture. 16SrDNA-PCR-based diagnosis of *E. faecalis* has been reported by many workers (Simonsen et al., 2003; Carvalho et al., 2004; Sedgley et al., 2006).

Planomicrobium mcmeekinii a Gram-positive, rod-shaped bacterium (Reddy et al., 2002), is one of the five *Planococcus* species within the family planococcaceae (Tow and Cowan, 2003). *Planococcus* spp. have been isolated from a variety of marine environments, fermented seafood and some Antarctic lakes. In general many of these species have been reported to produce an orange or yellow pigment (Sheridan and Brenchley, 2000; Engelhardt et al., 2001). On the basis of the phenotypic and phylo-genetic data and the genomic distinctiveness, Yoon et al. (2001) proposed that *Planomicrobium*, a new

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genus belonging to the family planococcaceae, contains two previously assigned planococcal species, namely *P. mcmeekinii* and *Planomicrobium* okeanokoites.

The present study was undertaken to speciate and determine *E. faecalis* strains in blood and vagina with their antimicrobial susceptibility. Also to confirm the isolation of a new species or subspecies of *P. mcmeekinii* for the first time from blood samples.

MATERIALS AND METHODS

Source of isolation

Two hundred and thirty three samples were collected from an outpatients' vaginal swab (n = 101) and inpatients' blood sample (n = 132) from Al-Sadr Teaching Hospital, Al-Basrah General Hospital and Al-Basrah Hospital for birth and Child, from Basrah city, Iraq. These samples were enrolled from April 2006 to July 2006. Swabs of vagina and 2 ml of blood samples were added in sterile tubes of Brain Heart Infusion Broth (HIMEDIA) and streaked on Mannitol Salt Agar (ALPHA). Colonies grown after incubation were Gram stained and cultured into Nutrient Agar (ALPHA) for testing (Talan et al., 1989).

Genomic DNA extraction

Deoxyribonucleic acid (DNA) extraction was done according to Sambrook et al. (1989), Al-Badran (2003) and Japoni et al. (2004). 5 ml of Tryptic Soy Broth (ALPHA) was inoculated with tested bacteria and incubated at 37°C for 18 h. The grown bacteria were re-washed three times by Phosphate Buffer Saline (Oxoid). The washed bacteria was resuspended in 500 ml of Tris-EDTA buffer, 30 µl of 10% Sodium Dodecyle Sulphate and 30 µl of 25 mg/ml solution of Proteinase K (Promega) and then incubated for 1 to 3 h at 37°C. 100 µl of 5 M NaCl solution was added and incubated at 65°C for 10 min. DNA was purified by two extraction with phenol: chloroform: isoamyl alcohol (24:25:1) and precipitated with 70% chilled ethanol. The DNA was resuspended in 50 µl of Tris-EDTA buffer as stock. To check for DNA, the samples were loaded in 0.8% agarose gel 1 × TBE (54 g Tris-base, 0.5 M EDTA, 1- L distilled water, PH = 8, then diluted with 400 ml of distilled water) and electrophoresed at 60V for 30 min.

16SrDNA gene (PCR)

The 16SrDNA was detected by thermocycler apparatus (BioRAD Co.) according to the procedure and materials of MWG Biotech AG Co. Barker et al. (2005) used the primers: 27Forward 5'-AGAGTTTGA TCM TG GC TCAG-'3 and 1492Reverese 5'-TACGGYTACCTTGTTACG-'3 (Stackebrandt and Goodfellow, 1991). The polymerase chain reaction (PCR) is a mixture of the final volume of 50 µl containing 37.7 µl sterilized millipore water, 10 µl Taq-dNTPs buffer mix (5X), 2.5 µl of each primer (5 pmol), 2 µl $MgCl_2$ (50 mM), 1 µl DNA template and 0.3 µl of Taq DNA polymerase.

The PCR program involved initial denaturation at 95°C for 5 min, 30 cycle (denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min) and final extension at 72°C for 10 min, then soaking at 4°C to indefinite. The amplified PCR mixtures were resolved by electrophoresis through 1% agarose gel at 60V for 1.5 h prepared in 1 × TAE (242 g Tris-base, 57.1 ml Glacial acetic acid, 100 ml EDTA 5 M, 1-L distilled water, PH = 8, then 10 ml of this solution diluted with 490 ml of distilled water) containing 1 µl ethidium bromide in 100 ml agarose solution. Products were viewed under ultraviolet (UV) light system (UVi Co.). The band of 1000 to 1500 bp was indicative to 16SrDNA gene.

16SrDNA gene sequences

The 16SrDNA products sequencing and its preparation was according to MWG Biotech AG Co. with the procedure of Barker et al. (2005). For the purification DNA product, 60 µl of 20% polyethylene glycol applied Biosystem (ABI) was added to 30 µl of PCR product (*16SrDNA* gene) and mixed by vortex (Whirlimixer) and incubated at 4°C overnight after centrifugation (Scotlab) at 1200 rpm for 20 min (twice). The pelleted DNA was mixed with 0.5 ml of 70% chilled ethanol and the recentrifuged product was dried in a vacuum drier (Thermo) for 30 min. The DNA was resuspended in 15 µl of Millipore sterilized water and left overnight at 4°C and then send to the MWG Biotech AG Co. for sequencing.

Identification of bacteria

All bacterial species were identified (using the DNA sequencing products) in "BLAST" provided by the National Center for Biotechnology Information Service (NCBI)" http://www.ncbi.nlm.nih.gov" (Kerbauy et al., 2011).

Phylogenetic tree

The sequences data obtained from the present study (n = 15) and from type strains (n = 25) by GenBank (Sung et al., 2006) were aligned and concatenated at 1346 bp and compared to assign the differences using "CLUSTALW" http://www .ebi. ac.uk / clustalw/ (Kerbauy et al., 2011), then a phylogenetic tree by Neighbour Joining method was viewed by http://www.phylogeny. Fr/version2_ cgi/index.cgi/ (Dereeper et al., 2008).

Detection of *mecA* gene by PCR

The PCR mixture and procedure depend on Geha et al. (1994). The primers for *mecA* gene (Forward 5'-GTAGAAATGACTGAACGTCCGATGA-'3 and Reverse 5'-CCAATTCCACATTGTTTCGGTCTAA-'3) with the size of 300 to 310 bp and PCR mixture was 12.5 µl of Go Taq Green Master Mix.2x (Promega), 1 µl of each primer (100 pmol), 5 µl DNA template, 5.5 µl nuclease-free water and 25 µl mineral oil (FisherBiotech). A DNA thermocycler was programmed with the initial denaturation at 94°C for 4 min, 30 cycles (denaturation at 94°C for 45 s, annealing at 56°C for 45 s and extension at 72°C for 2 min. The PCR products electrophoresed by agarose (2%) was added to 1 × TBE buffer; the band of suitable size was indicative of *mecA* gene.

Detection of *vanA* and or *vanB* gene(s) by PCR

All PCR mixture and procedures have been discussed previously (above) in *mecA* gene with the exception of primers for *vanA* (Forward 5'-CATGAATAGAATAAAAGT TGCAATA-'3 and Reverse 5'- CCCCTTTAACGCTAATACGACGATCAA-'3) and *vanB* (Forward 5'-GTGACAAACCGGAGGCGAGGA-'3 and Reverse 5'-CCG CCA TCCTCCTGCAAAAAA-'3) genes with the size of 300 bp for each. A DNA thermocycler was programmed with initial denaturation at 94°C for 10 min, 30 cycles (denaturation at 94°C for 30 s, annealing at 50°C for 45 s and extension at 72°C for 30 s) and

extension at 72°C for 10 min. The PCR product was electrophoresed and visualized as previously.

Vancomycin resistance by agar screen method

From overnight culture, 2 pure colonies approximately 10^5 colony forming unit (CFU) were grown as straight lines in Muller Hinton (Bioanalyse) plates supplemented with 4 mcg/L, 8 to 16 mcg/L and 32 mcg/L vancomycin, then incubated at 37°C for 48 h. Any visible growth indicated vancomycin resistance (CLSI, 2007; Al-Hadithi and Abd Al-Abbas, 2003).

Disc diffusion method

Disc diffusion (Bioanalyse) of gentamycin (10 mcg), streptomycin (10 mcg), erythromycin (15 mcg), chloramphenicol (30 mcg), penicillin (10 mcg), ampicillin (25 mcg), amoxicillin/clavulanic acid (20/10 mcg), and 1 mcg of oxacillin (methicillin) was tested by spreading 0.1 of 1.5 ml BHIB cultured with bacteria (18 h) into mannitol salt agar. Each isolate was tested for growth with all antibiotic discs (NCCLS, 2000).

RESULTS

PCR amplification with 16SrDNA eubacterial primers (27 Forward and 1492 Reverse) showed the gene bands at 100 to 1500 bp for all bacterial isolates (n = 15). However, the sequencing of 16SrDNA genes (1346) with "BLAST" revealed that *E. faecalis* was a predominant (93.3%) enterococcal species (n=14) from infected specimens (n = 15) including 11(100%) from the vagina (n = 11) and 3 (75%) the blood (n = 4), whereas only one axenic isolate of *P. mcmeekinii* from the blood (Figure 1). The rooted neighbour joining phylogenetic tree showed the distribution and relationship between the tested bacteria (No.1 to 15) and the reference strains (n = 25) from the Gene Bank. All the *E. faecalis* isolates (No.1 to 11) from the vagina were 100% sequence identity with ATCC51299, while the other three identical *E. faecalis* isolates (No. 12, 13 and 14) from the blood were different from strain ATCC51299 in two positions of nucleotide sequences; the first one was a frame shift mutation (deletion of base A) from the sequence position 21 bp (Figure 2) and the second frame shift mutation (deletion of base A) was at 1076 bp (Figure 3). However, these three strains (No. 12, 13 and 14) were identical with the OG1RF strain (ATCC47077). Moreover, the single isolate of *P. mcmeekinii* was closely related with strain *P. mcmeekinii* ATCC700539 (99% sequences similarity) due to the single Gene or Point mutation (Transversion) of base C instead of A at position 358 bp (Figure 4) changing the amino acid Lys (AAA) to Gln (CAA), therefore, from our knowledge, the present report describes a first isolation of *P. mcmeekinii* from blood sample as an axenic culture, and according to the 16SrDNA sequences (1346bp), this isolate is revealed to be a new subspecies or species.

In general, all *E. faecalis* isolates showed no resistance to gentamycin, streptomycin and erythromycin (Table 1) and there were no differences in prevalence between vagina and blood *E. faecalis* isolates. In contrast, one isolate was resistant to chloramphenicol (7.1%) and 3, 4, 10 and 13 isolates were determine as resistant to penicillin (21.4%), ampicillin (28.5%), amoxicillin/clavulanic acid (71.4%) and oxacillin (92.8%), respectively with high prevalence in resistance to penicillin from blood isolates than vagina. According to agar screening method, vancomycin resistance depend on the concentration in the plate since all isolates were resistant in 4 and 16 µ/ml (100% for each), while one isolate was sensitive in 32 µ/ml (92.8%). The presence of the intrinsic *mecA* gene (92.8%), *vanA* and/or *vanB* genes (92.8%) were confirmed by PCR.

DISCUSSION

Conventional biochemical tests and commercial identi-fication system as well as phenotypic variants are not included in the level of subspecies and often miss identified (Seifert et al., 2003). In contrast, the high-quality of 16SrDNA sequence database provides excellent identifica-tion at the species and subspecies levels; furthermore, it can lead to the recognition of novel pathogens and non-cultured bacteria (Clarridge, 2004; Mellmann et al., 2006). In the present study, primers F27 and R1294 were used to amplify the 16SrRNA gene for all bacteria species to prevent losing of any species. *E. faecalis* is a predominant (93.3%) enterococcal species in the vagina (100%) and blood (75%) (Figure 1). *E. faecalis* is more frequent among the enterococcal species isolated from clinical sample especially vaginosis and bacteremia (Cetinkaya et al., 2000; Shepard and Gilmore, 2002; Karlowski et al., 2004; Jahic et al., 2005). Thus, any wound in the infected vagina might be the route to the bloodstream, since *E. faecalis* including proteases may help them to break down the normal barriers between the tissue and bloodstream (Huycke et al., 2002). Furthermore, enterococci have emerged as a major cause of nosocomial infection either locally or systematically including wounds, bacteremia and endocarditic infections (Nallaparedd et al., 2006; Peters et al., 2007) and it cause inflammation of the heart valves (Gilmore, 2002). Moreover, the three *E. faecalis* (No.12, 13 and 14) isolates from the blood were muted (Figures 2 and 3). This may be due to the foreignness of the abnormal environment "blood" (Satyanarayana et al., 2005); at the same time, they were identities with the *E. faecalis* strain OG1RF containing 3 Ebp (encoding for the endocarditis biofilm-associated pili) operons for producing surface pili which is used for attachment to the host surface and are antigenic in human during endocarditis (Nallapareddy et al., 2006). But, from a medical standpoint of pathogenicity, this species has the capacity to acquire a wide variety of antimicrobial resistance

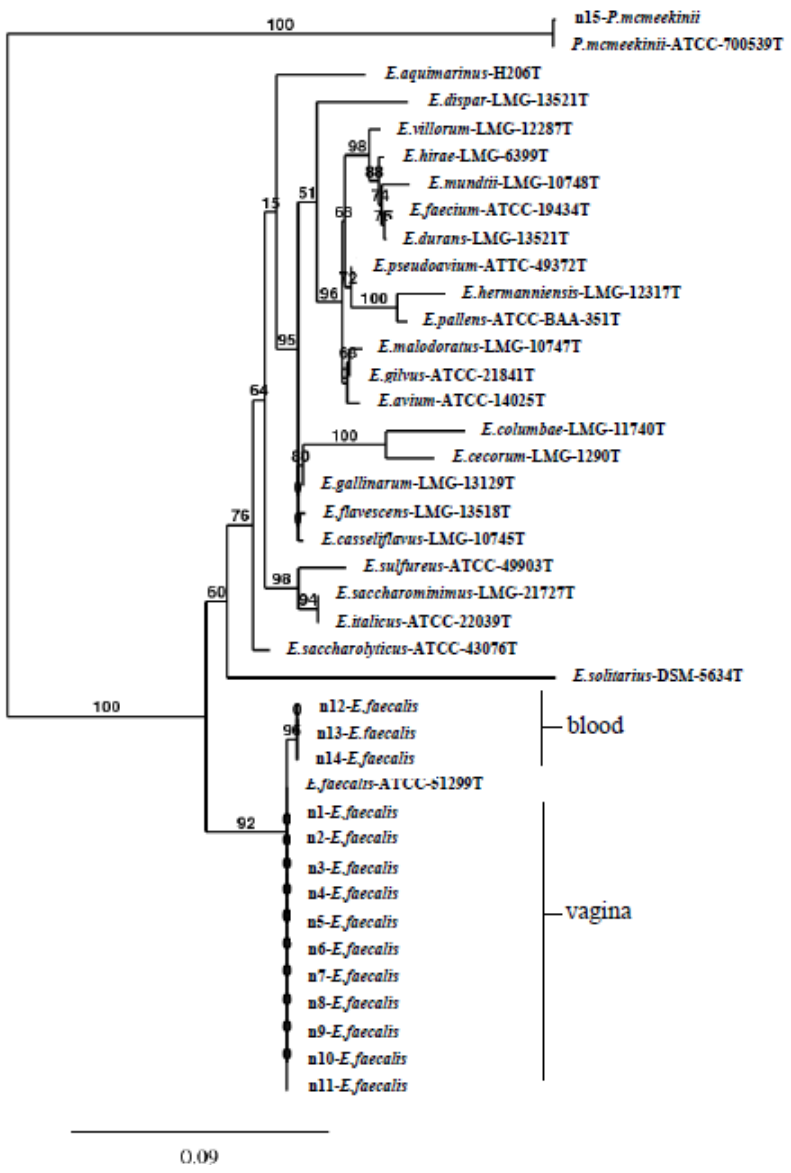

Figure 1. Rooted Neighbour-Joining tree based on 16SrDNA gene sequences (concaten- ated sequences at 1346bp.) showing the phylogenetic relationships of *E.faecalis* between the isolates (No. 1 to 14) and the reference strains (T) from GeneBank isolates. The tree has been rooted with *P.mcmeekinii*.
T: type strain, **ATCC** (American Type Culture Collection), **LMG** (Gent-Laboratorium voor Mirobiologie) and **DSM** (Deutsche Sammlung von Mikroorganismen and Zellkulturen) German Collection of Microorganisms and Cell Culture.

factors (Aakra et al., 2010), which present serious problems in the management of patients (Gilmore, 2002).

Table 1 shows a high resistance of *E. faecalis* to vancomycin (92.8%), oxacillin (92.8%) and amoxicillin/ clavulanic acid (71.4%) without different prevalence between in-(blood) and out-(vagina) patients. However, treatment should comprise a bacterial synergic combination of an aminoglycoside and a cell-wall active agent, such as vancomycin (Pupin et al., 2007). In general, enterococcal isolates with lowered susceptibility to vancomycin can be categorized as *vanA*, *vanB* and *vanC* genes (Chi et al., 2007). These genes lead to the production of an alternative structure D-alanine-D-lactate, instead of D-alanine-D-alanine found in the cell wall of susceptible bacteria (Courvalin, 2006). Furthermore, *vanA* and *vanB* are the most carried on plasmid and are readily transferable, thus *E. faecalis* can transfer these plasmids by conjugation (Cook et al., 2011).

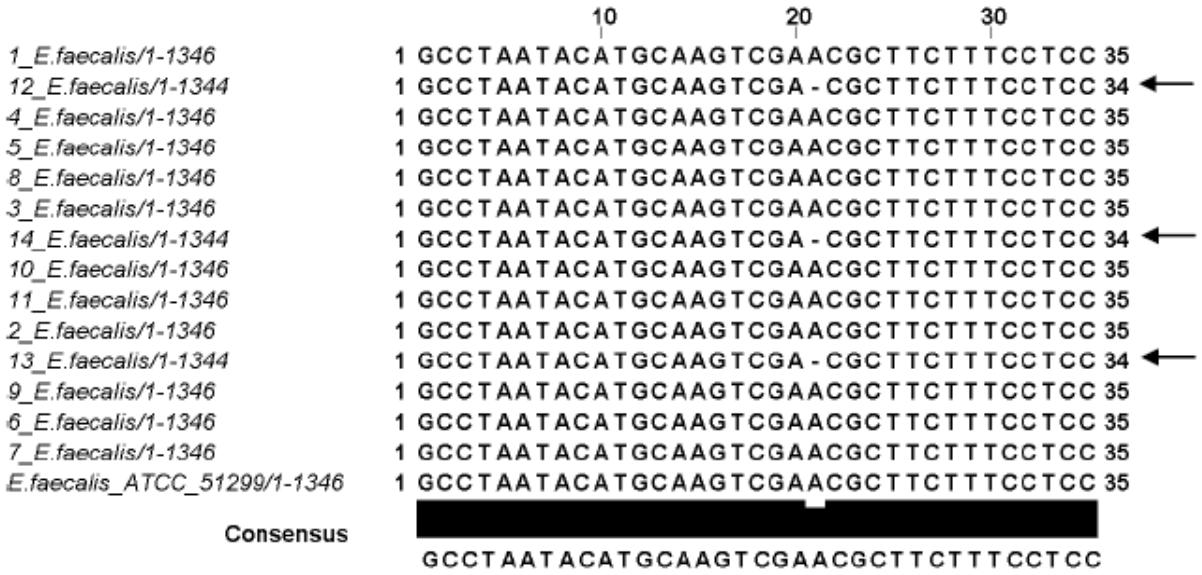

Figure 2. CLUSTALW for comparison of nucleotide sequences alignment (1346bp) for 16SrDNA gene of *E.faecalis* (No. 1 to 14) and ATCC51299. Isolates No. 12, 13 and 14 show Frame Shift Mutation (deletion nucleotide A) at the position 21bp.

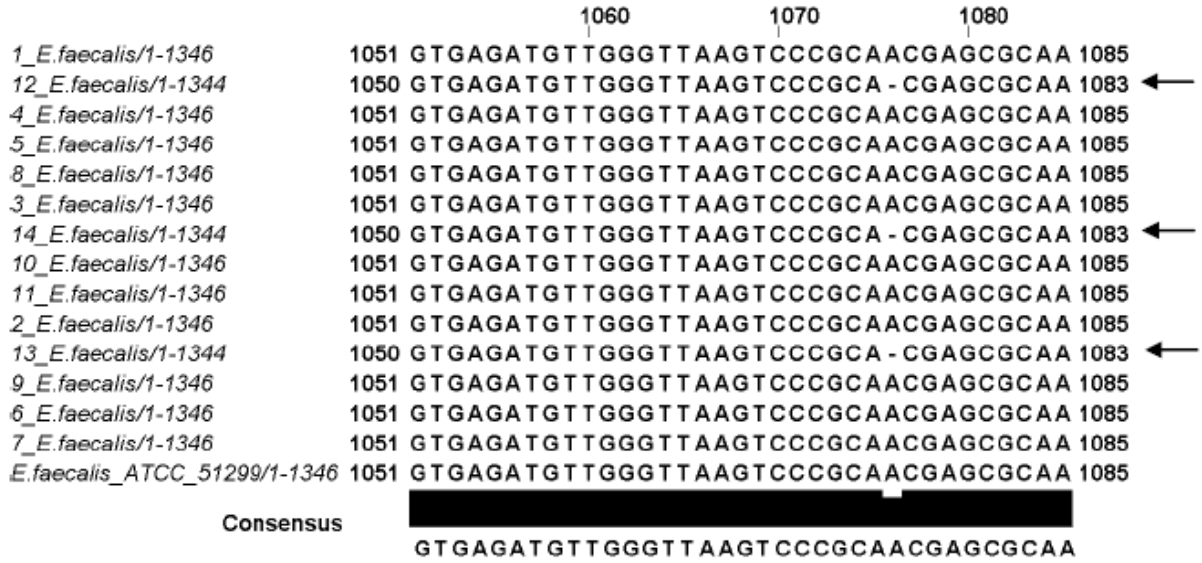

Figure 3. CLUSTALW for comparison of nucleotide sequences alignment (1346 bp.) for 16SrDNA gene of *E.faecalis* (No. 1 to 14) and ATCC51299. Isolates No. 12, 13 and 14 show Frame Shift Mutation (deletion nucleotide A) at the position 1076bp.

Although, the levels of resistance increase with the duration of exposure, but there is no methicillin or vancomycin using Basrah city. Therefore, the high resistance could be due to the plasmid transferring. Moreover, antimicrobial resistance is not a phenomenon restricted to a specific class of antimicrobials because of cross-resistance due to overlapping targets of different antimicrobials or co-selection related to genetic linkage between resistance genes (Simonsen et al., 2003).

Even the resistance to penicillin (21.4%) and ampicillin (28.5%) were low, but there was difference between the resistance of blood and vaginal isolates for both antimicrobial discs. However, the high susceptibility of gentamycin, streptomycin, erythromycin, chloramphenicol

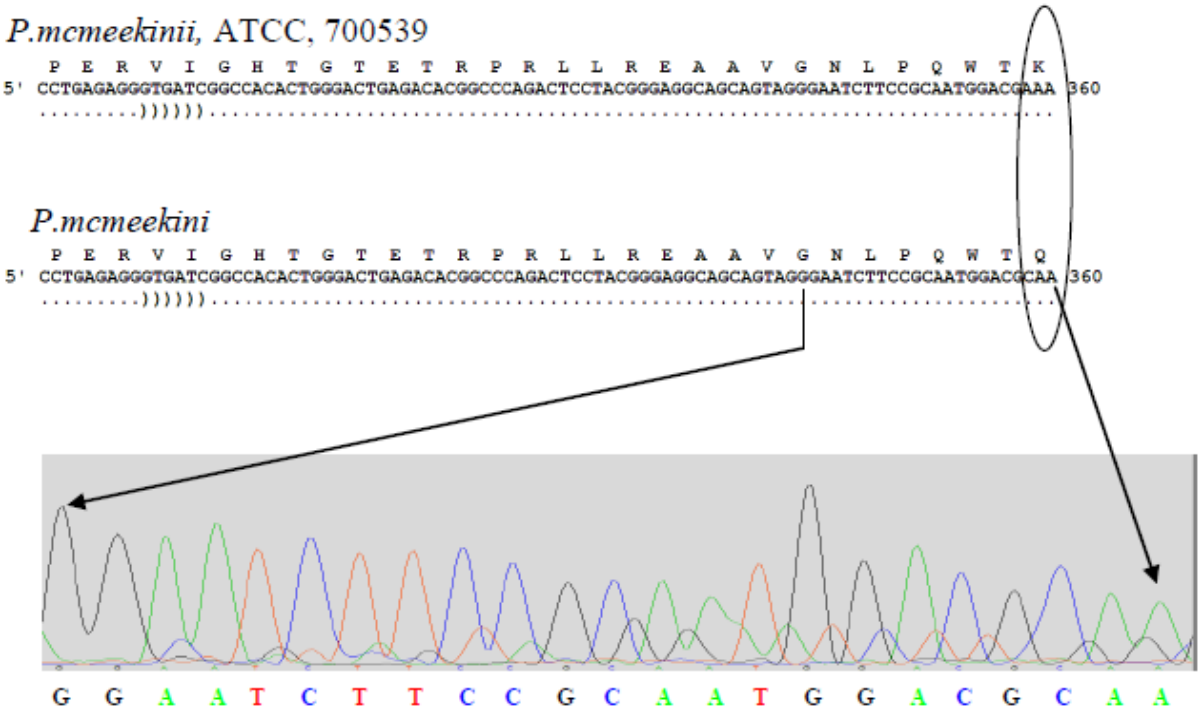

Figure 4. Comparison of 16SrDNA nucleotide sequences gene (1346bp.) for the isolate *planomicrobium mcmeekinii* (with peaks) and strain ATCC700539. A Gene or Point Mutation type Transvertion (C instead A) at the position 358bp. changing the amino acid Lys (AAA) to Gln (CAA).

col, penicillin and ampicillin (vaginal isolates) were in accor-dance with the results of Zhamel et al. (2003), Carvalho et al. (2004) and Aakra et al. (2010). The prevalence of resistance varied widely among laboratories (Simonsen et al., 2003); therefore, to detect a suitable antimicrobial drug, the susceptibility should be tested yearly. On the other hand, the present study appeared fortuitously a single isolate of *P. mcmeekinii* (1% difference in sequence of reference strain ATCC 700539) from blood patient which could be reported either a new separated species or subspecies. Since, according to some guidelines, a range of about a 0.5% to 1% difference (99.5 to 99% similarity) is often used for classification (Song et al., 2003). Bosshard et al. (2003) used ≥ 99% similarity to define species and ≥ 95 to < 99% to define a genus whereas, Hall et al. (2003) adopted a distance score of 0.00 to less than 1% as the criterion for species identity. While, Tang et al. (1998, 200), suggested a 0.5% difference as the limit for species designation. Furthermore, a strain with a small genotypic difference (less than 0.5%) has been considered as subspecies (Chen et al., 2002). When there is a clear phenotypic uniqueness, genogroups with less than 1% differences in sequence have in fact been named as a new species (Kattar et al., 2001; Roth et al., 2003; Tortoli, 2003). However, a comparison of sequences for several subspecies shows differences from 1 to 14 bp (Clarridge, 2004). Some of these variations among the researchers could be due to the fact, the percent difference can vary if it is calculated using only the first 500 bp or all 1500 bp of 16SrDNA gene sequences, and can also vary with the program used for calculations. Likely, the total sequence of *P. mcmeekinii* was 1346 bp after concate-nated. However, Clarridge (2004) appeared that isolates with a small genotypic difference (0.4 to 0.9%) but a definite phenotypic difference have been considered either separate species or subspecies. All the new species have been detected since 1990, most of which were grown from clinical samples and are potentially pathogenic, many of them differ from another by only a few base pairs, but seem to be correlated with unique phenotypic charac-teristics, clinical significance and niche, sometimes called sequevars (Tortoli, 2003; Takagi and Shin-ya, 2011). Because new sequences are found in almost all studies of clinical mycobacterial strains, the prospect is that many more sequevars will be detected, swelling the numbers of potential subspecies clades (Clarridge, 2004).

In conclusion, *E. faecalis* was observed as the predo-minant isolate from enterococcal bacteremia and vagina. The high resistance of *E. faecalis* to amoxicillin, methicillin and vancomycin revealed an alarming mark for uncon-trollable of these bacteria in future. Therefore, importance or rational use of antimicrobials in patient

Table 1. Antimicrobial susceptibility of *Enterococcus faecalis* isolated from vaginal outpatients' swab and blood inpatients' sample.

Source of sample	No.	Antimicrobial resistance / Intermediate										VN		
		CN	ST	E	C	P	AM	AMC	mecA	OX	vanA+B	ml/µ4	ml/µ16	ml/µ32
		10 mcg	10 mcg	15 mcg	30 mcg	10 mcg	25 mcg	20/10 mcg		1 mcg				
Vagina	1	S	S	S	S	S	S	R	+	R	+	R	R	R
	2	S	S	S	S	S	S	S	+	R	+	R	R	R
	3	S	S	S	S	S	S	R	+	R	+	R	R	R
	4	S	S	S	S	S	S	S	+	R	+	R	R	R
	5	S	S	S	S	S	R	R	+	R	+	R	R	R
	6	S	S	S	S	S	S	S	+	R	+	R	R	R
	7	S	S	S	S	S	S	R	+	R	+	R	R	R
	8	S	S	S	R	S	S	R	+	R	+	R	R	R
	9	S	S	S	S	S	S	R	+	R	+	R	R	R
	10	S	S	S	S	S	S	R	+	R	+	R	R	R
	11	S	S	S	S	S	S	S	+	R	-	R	R	S
Blood	12	S	S	S	S	R	R	R	+	R	+	R	R	R
	13	S	S	S	S	R	R	R	-	S	+	R	R	R
	14	S	S	S	S	R	R	R	+	R	+	R	R	R
	%	0	0	0	7.1	21.4	28.5	71.4	92.8	92.8	92.8	100	100	92.8

negative; **CN**, gentamycin; **ST**, streptomycin; **E**, erythromycin; **C**, chloramphenicol; **P**, penicillin**; AM**, ampicillin; **AMC**, amoxicillin/clavulanic acid; **,** resistant; **+,** positive; **- ,**Sensitive; **R ,S *mecA***, methicillin resistant gene; **OX,** oxacillin(methicillin); ***vanA+B***, vancomycin resistant *gene A* and *B*; **VN**, vancomycin.

management and infection control is needed. *P. mcmeekinii* was isolated from bacteremia case to appear in a new separate species or subspecies.

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