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Molecular fingerprinting of pathogenic *Enterococcus* isolates using random amplified polymorphic DNA Analysis

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Abstract

Enterococcus sp. is considered to be one of the most prevalent species in the oral cavity, particularly in endodontic infections. The aim of the present study was to investigate the molecular fingerprinting of *Enterococcus* sp in patient's urine diversity by randomly amplified polymorphic DNA (RAPD-PCR) analysis of *Enterococcus*. Specific results for fingerprinting were obtained by the five primers retained for RAPD analysis produced different fragment patterns with varied number of bands. The primers yielded a total of 71 distinct bands 67.6 % were considered as polymorphic bands and 32.4% were considered as monomorphic bands. The OPA-01 primer has showed the highest polymorphism 76.9 % and total of 15 bands ranged from 300 bp-1700 bp. While, the OPD-05 primer has showed the lowest polymorphism 57.2 % and total of 14 bands ranged from 190 bp-2200 bp. Interestingly, OPA-01 primer was a unique negative marker for isolate 7 and, on the other hand, gives a positive one unique band only with isolate 9. The Dendrogram based on RAPD results grouped the thirteen *Enterococcus* isolates into two different clusters with about 82.2% genetic similarity. Moreover, the genetic distance among *Enterococcus* isolates was relatively low. The smallest genetic distance (0.167) was estimated between E 10 and E 13. Results indicate the presence of sufficient nuclear DNA level variations among studied *Enterococcus* isolates using RAPD markers, and the RAPD data might be a good source of information about the diversity of native *Enterococcus* isolates.

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Introduction

Enterococci are Gram-positive, coccus-shaped bacteria and are common colonizers of different animal hosts, plants, soil, food, and water (Devriese *et al*., 2006; Fisher and Phillips, 2009). Previously, these bacteria were considered as normal commensals of the gastrointestinal tract, oral cavity, and vagina in humans (Klein, 2003; Kayaoglu and Qrstavik, 2004). However, there was recently an increase in nosocomial infections caused by enterococcal species, including urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wound, and endodontic infections, which were largely attributed to the antimicrobial resistance profiles (Sedgley *et al*., 2008; Santagati *et al*., 2012; Lins *et al*., 2013). Therefore, *Enterococci* now rank among the top three nosocomial bacterial human pathogens, and several multidrug-resistant strains have emerged that pose great therapeutic challenges (Souto and Colombo, 2008; Zoletti *et al*., 2011). Among various enterococcal species, Molecular-based methods have shown some promises in genotyping *Enterococci* bacteria strains (Scheidegger *et al*., 2009) and randomly amplified polymorphic DNA polymerase chain reactions (RAPD-PCR) assay has become a rapid and reliable tool in *Enterococci* taxonomy and for intra- and inter- species genomic differentiation of various bacteria (Krawczyk *et al*., 2006; Hassan *et al*., 2014). RAPD techniques specific for LAB have been established and successfully applied (Nieto-Arribas *et al*., 2011; Buhnik-Rosenblau *et al*., 2013). The objectives of this study were to establish a molecular clustering of *Enterococci* isolates that obtained from Taif patient's urine by using RAPD fingerprinting.

Materials and methods

This work was carried out in the Microbial Genetics laboratory, Biotechnology and Genetic Engendering Unit, Scientific Research Center, Taif University, KSA.

Isolation and purification of clinical bacterial isolates

Thirteen *Enterococcus* strains isolated from urine swabs that collected from hospitalized patients in the Taif hospitals, KSA according to Franklin and Matthew (2012). Sterile dry swabs were used for streaking of clinical samples onto sterile Petri dishes containing Nutrient agar media (biolife, USA). Inoculated streaked dishes were incubated at 28°C for 48 h. Single colonies were picked up by sterile inoculation needles and then sloped into cultures of nutrient agar media.

Genomic DNA extraction

The cell pellets from all isolates were used to extract genomic DNA using (Jena Bioscience, Germany) extraction kit following the manufacturer's instructions.

RAPD analysis

For RAPD analysis, five 10-mer random primers were used (supplied by Amersham Pharmacia Biotech. NJ. USA.). Names and the sequences of the primers are illustrated in Table (1). Following the experiments for optimization of component concentrations, PCR amplification of random primers were carried out according to (Williams *et al.,* 1990; Hassan and Ismail 2014) in 25 µl volume containing 1µl (20 ng) of genomic DNA, 12.5µl of Go Taq® Green Master Mix, Promega, USA. 1µl of primer (20 p.mol), deionized distilled water (up to a total volume of 25 µl). For DNA amplification, the C1000TM Thermo Cycler Bio-Rad, Germany, was programmed under the conditions involving denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 Sec, primer annealing at 35°C for 1.5 min and primer extension at 72°C for 2.5 min; final extension step at 72°C for 7 min. Amplified DNA products were analyzed by electrophoresis in 1.5% agarose gel run in TBE. The gels were stained with ethidium bromide (5 µg ml^{-1}). 100 pb. DNA Ladder RTU, (Gene Direx®) was used as a standard. DNA was visualized by UV illumination and then photographed by a Bio-Rad Gel Doc 2000 device.

Data analysis

The amplification products of RAPD-PCR were scored

for the presence "1" or absence "0" and missing data as "9". The genetic associations between isolates were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to cluster analysis by un-weighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated. The computations were performed using the program NTSYS-PC version 2.01 (Rohlf, 2000). The Jaccard 's similarity matrix was subjected to principal component analysis.

Results and disscusion

RAPD and Cluster analysis of Enterococcus isolates from Taif hospitals, KSA Molecular markers are efficient tools for cultivar identification and estimation of relatedness through DNA fingerprinting. RAPD markers where developed by Williams *et al.* (1990).

RAPD technique using single arbitrary 10-mer oligonucleotides primers to amplify discrete fragments of DNA using Polymerase Chain Reaction (PCR) (Ibrahim, *et al.*, 2015).

Table 1. Names and sequences of RAPD primers.

Primers name	Primers sequences
OPA-01	5-CAGGCCCTTC-3'
OPA-06	5-GGTCCCTGAC-3'
OPA-08	5-GTGACGTAGG-3'
OPA-10	5-GTGATCGCAG-3'
OPD-05	5-TGAGCGGACA-3'

Table 2. Polymorphic bands of each genetic primers and percentage of polymorphism in thirteen pathogenic *Enterococcus* isolates.

Primers name	Total Bands	Monomorphic Bands	Polymorphic Bands	% Monomor-phism	% Polymor-phism
OPA-01	13	3	10	23.1	76.9
OPA-06	16	6	10	37.5	62.5
OPA-08	15	4	11	26.7	73.3
OPA-10	13	4	9	30.7	69.3
OPD-05	14	6	8	42.8	57.2
Total	71	23	48	32.4	67.6

This technique has been used extensively in many different applications and in different bacterial species because of its simplicity (Hassan *et al.*, 2014).

Genomic diversity of *Enterococcus* isolates was investigated by RAPD analysis.

Table 3. Genetic similarity among thirteen pathogenic *Enterococcus* isolates based on the five RAPD-PCR primers**.**

	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13
E1	0.000												
E2	0.414	0.000											
E3	0.414	0.000	0.000										
E4	0.339	0.497	0.497	0.000									
E5	0.454	0.304	0.304	0.304	0.000								
E6	0.304	0.643	0.643	0.178	0.339	0.000							
E7	0.178	0.542	0.542	0.304	0.414	0.208	0.000						
E8	0.414	0.591	0.591	0.591	0.454	0.542	0.304	0.000					
E9	0.454	0.643	0.643	0.643	0.591	0.591	0.497	0.238	0.000				
E10	0.375	0.375	0.375	0.643	0.497	0.698	0.591	0.238	0.414	0.000			
E11	0.414	0.497	0.497	0.339	0.304	0.454	0.454	0.339	0.542	0.454	0.000		
E12	0.375	0.375	0.375	0.178	0.270	0.414	0.414	0.542	0.698	0.591	0.304	0.000	
E13	0.454	0.454	0.454	0.304	0.414	0.414	0.339	0.542	0.497	0.824	0.375	0.339	0.000

The RAPD results illustrated in Table (2 and 3) and Fig. (1, 2, 3, 4, 5 and 6) showed polymorphic numbers of the genetic bands, which were the electrophoretic products of PCR for *Enterococcus* isolates. RAPD-PCR reactions were performed on thirteen *Enterococcus* isolates collected from Taif hospital of urine patient's samples using eight different 10-mer primers, which were pre-selected for their performance with *Enterococcus* isolates DNA. Out of the eight primers five retained for RAPD analysis produced different fragment patterns with varied number of bands. The primers yielded a total of 71 distinct bands (RAPD markers), 67.6 % were considered as polymorphic bands and 32.4% were considered as monomorphic bands.

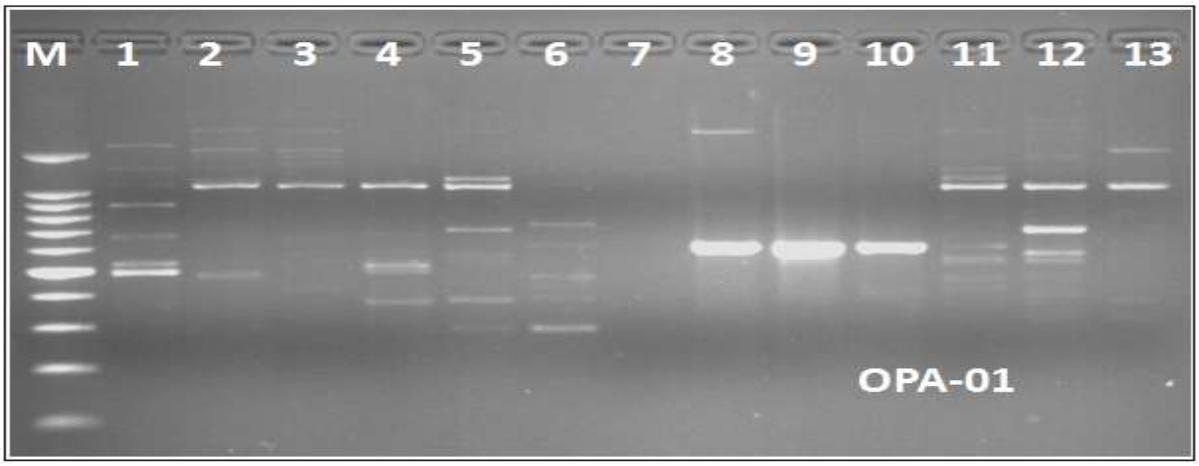

Fig. 1. RAPD profile of thirteen pathogenic *Enterococcus* isolates generated by primer OPA-01.

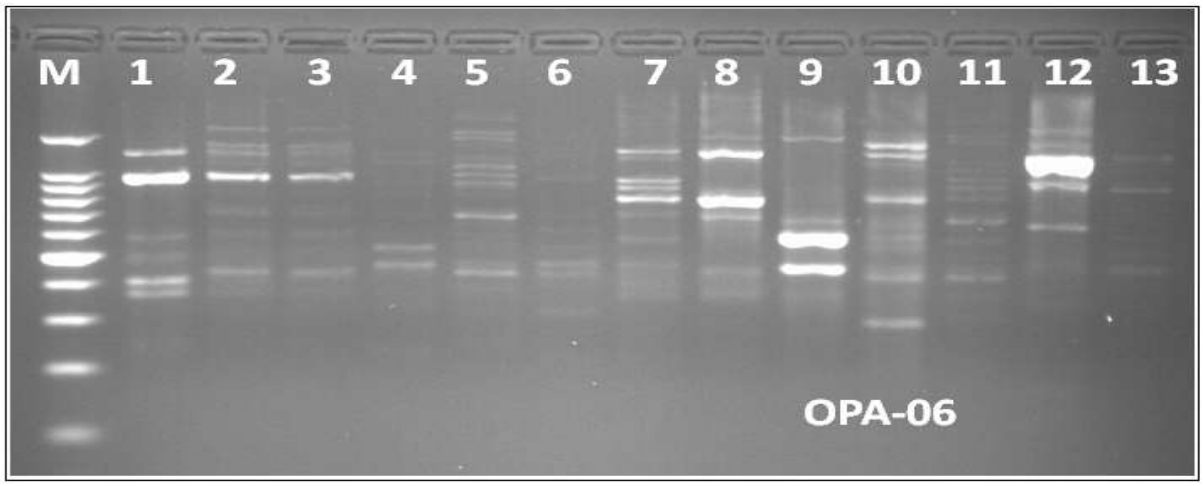

Fig. 2. RAPD profile of thirteen pathogenic *Enterococcus* isolates generated by primer OPA-06.

Table (2) record the number of amplified fragments scored for each *Enterococcus* isolates. The amplified products were highly polymorphic among the *Enterococcus* isolates. A total of 71 fragments from all analysis were used for identification and the evaluation of genetic similarities and designing the phylogenetic tree for these thirteen *Enterococcus* isolates. The total number of bands as shown in Table (2) varied from 16 bands with primer OPA-06 (Figure 2) to 13 bands with primer OPA-01 and OPA-10 (Figures 1 and 4). The total of monomorphic amplicons was 23 and the total of polymorphic amplicons was 48. It can be concluded from our study that RAPD markers are effective in detecting similarity between *Enterococcus* isolates and they provide a potential tool for studying the inter-strain

genetic similarity and the establishment of genetic relationships. The RAPD-PCR results using primer (OPA-01) has showed the highest polymorphism, a total of 13 bands in these *Enterococcus* isolates. Also, this primer give a unique negative band with strain 7, while, the same primer give a positive unique band with strain 9. Three common bands were observed in all isolates which exhibited about 23.3 % monomorphism, while the other fragments have showed 76.9% polymorphism (Table 1). On the other hand, primer OPD-05 showed the lowest polymorphism. The molecular size of the amplicon products ranged from 190 bp-2200 bp.

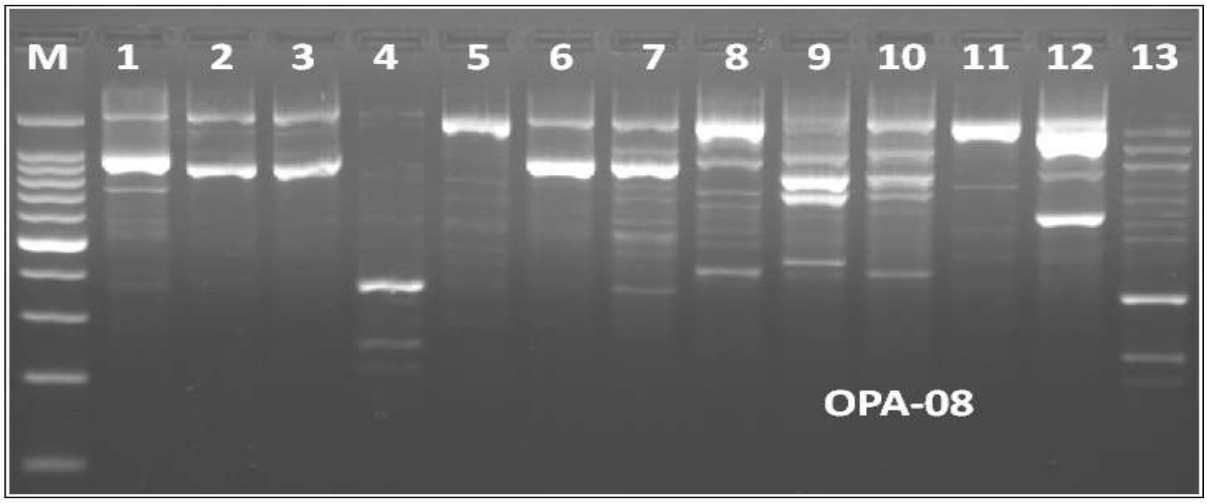

Fig. 3. RAPD profile of thirteen pathogenic Enterococcus isolates generated by primer OPA-08.

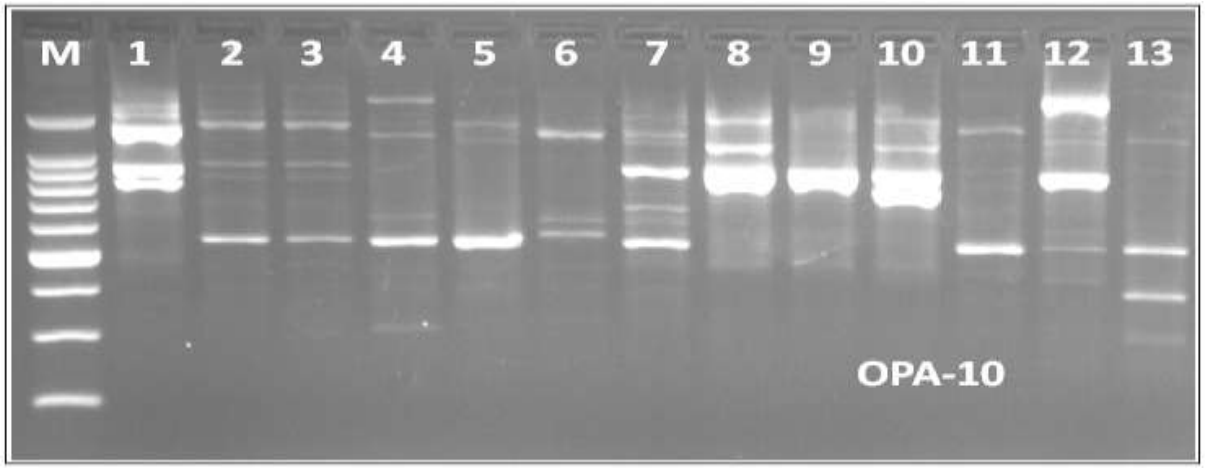

Fig. 4. RAPD profile of thirteen pathogenic *Enterococcus* isolates generated by primer OPA-010.

According to genetic similarity and intra-species differentiation, the thirteen *Enterococcus* isolates were grouped into two different clusters with about 82.2% genetic similarity. Ten *Enterococcus* isolates (E1, E2, E3, E4, E5, E6, E7, E11, E12 and E13) were grouped in the first cluster and three *Enterococcus* isolates E8, E9 and E10 were grouped in the second cluster (figure 6). Finally, we can say that RAPD markers found sufficient nuclear DNA level variations among *Enterococcus* isolates that obtained from Taif hospitals, Saudi Arabia.

The RAPD data presented here might be a good source of information about the diversity of native *Enterococcus* isolates. RAPDs were proved to be useful as genetic markers in antibiotic resistance bacteria fingerprinting.

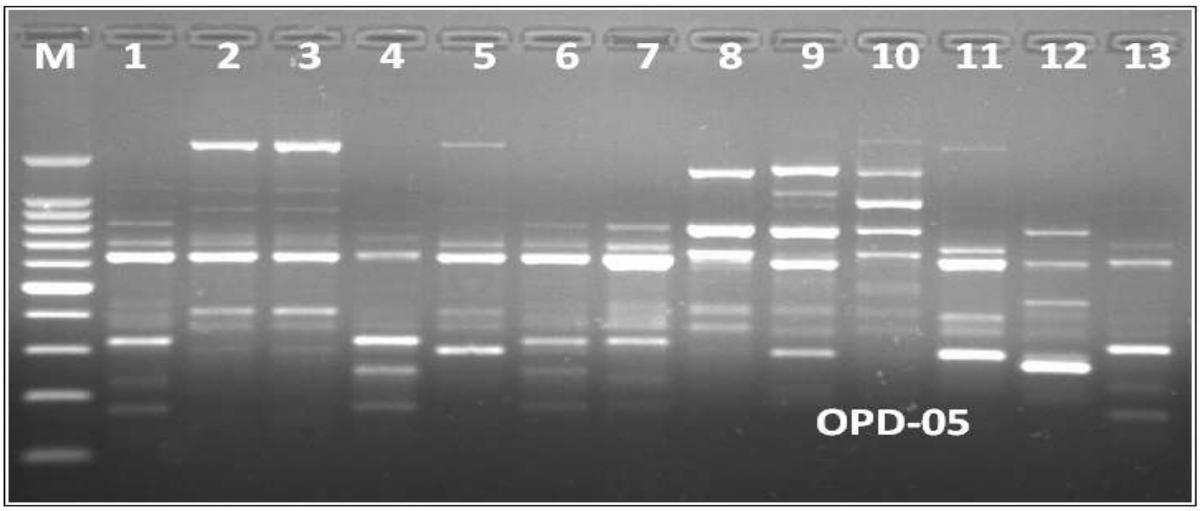

Fig. 5. RAPD profile of thirteen pathogenic *Enterococcus* isolates generated by primer OPD-05.

Although major bands from RAPD reactions are highly reproducible, minor bands can difficult to repeat due to random priming nature of this PCR reaction and potential confounding effects associated with co-migration with other markers (Yoke-Kqueen *et al.*, 2006; Manakant and Tanaya, 2012). The use of multiple primer sets in RAPD analysis can be used as a rapid method for preliminary biotyping of multidrug resistant strains. In a previous study using different Operon primers, the discriminatory power of RAPD and its ability to characterize strains was demonstrated. Operon primers also have been used in several previous studies and demonstrated to powerfully discriminate epidemiologically related isolates (Lin *et al.*, 1996; Sahilah *et al.*, 2014).

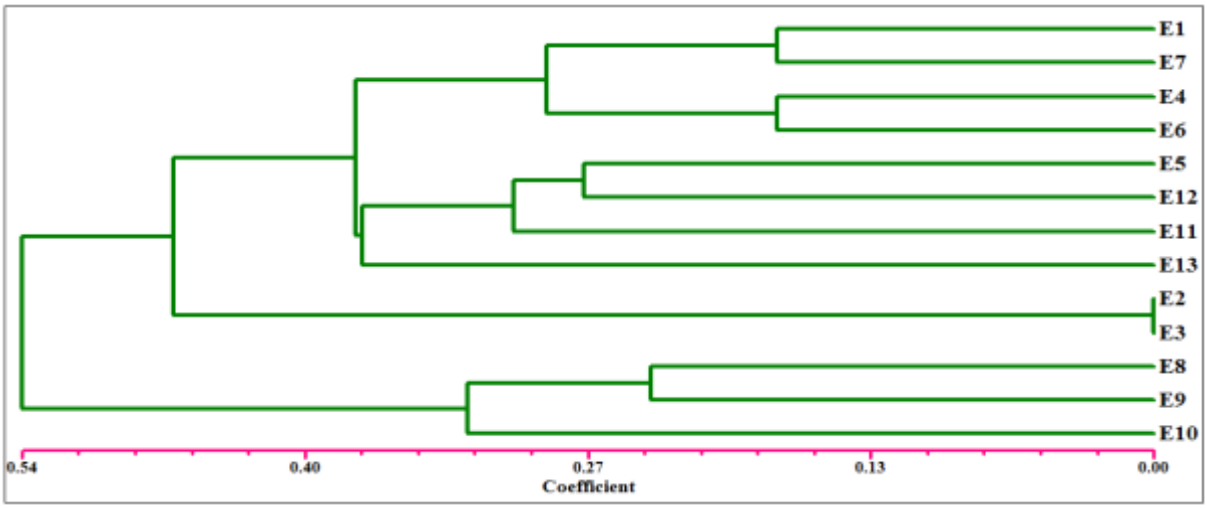

Fig. 6. Dendrogram analysis among the pathogenic *Enterococcus* isolates collected from Taif hospital in Saudi Arabia based on the five RAPD primers.

Conclusions

The RAPD applicability in bacterial studies and the establishment of genetic relationships demonstrated with this study. It is important to mention the fact that data results from RAPD assays can be extended to further dissect traits in a more refined way to exactly knowledge on specific genes and genetic pathways using other molecular methodologies. There is also the opportunity and need to study sequences of specific polymorphic bands, to

determine some genes detected by RAPD experiments.

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