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The Efficiency of Species-Specific Primer Pair to Identify Bacterial Infection in CF Patients in Jeddah, Saudi Arabia by Using PCR-RFLP Protocol

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Abstract: Progressive loss of lung function resulting from the inflammatory response to bacterial colonization is the leading cause of mortality in cystic fibrosis (CF) patients. A greater understanding of these bacterial infections is needed to improve lung disease management. This research was aimed to identify bacteria associated with CF patients by using molecular techniques. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism profiling (PCR-RFLP) and Plasmid profiling to identify and characterize, with prior cultivation, were performed. Four different species were examined in this study; *P. aeruginosa, S. aureus*, *K. pneumoniae and A. baumannii*. Same RFLP bands were found in all *P. aeruginosa* isolates, *S. aureus* and *K. pneumoniae* isolates which had the same bands. Different genotypes were found in *A. baumannii* isolates. Plasmids were detected in 9 (39%) *P. aeruginosa* isolates, 7 (53.8%) in *A. baumannii* isolates, 5 (100 %) *K. pneumoniae* and 4 (50%) *S. aureus* isolates. In conclusion, our results show that CF patients in Jeddah had uncommon species in CF patients. The PCR-RFLP technique has high sensitivity for identifying species associated with infection in CF patients. Different numbers and sizes of plasmids were detected in the isolates and the appearance of the plasmids in the isolates vary among species.

Key words: Species-Specific · Primer · Bacterial · Infection · CF Patients · PCR-RFLP

INTRODUCTION

CF is a common recessive genetic disease which affects the entire body, causing progressive disability and often early death [1]. It results from mutations of the CF transmembrane conductance regulator (CFTR), which functions as cyclic adenosine monophosphate (cAMP) chloride channel at the apical epithelial cell surface [2]. Over 1, 500 mutations have been identified in the CFTR gene [3, 4]. The primary organ systems involved are the lungs [5]. Severity [6] and inflammation [7, 8] are prominent in this disease [9]. The incidence of CF in

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Saudi Arabia was reported to be 1 in 4243 children [10]. Many genetic and epidemiological data have been described in Gulf countries [11]. Respiratory infections are often associated with *P. aeruginosa* [10]. *Staphylococcus aureus* and *Haemophilus influenza* are the most common bacteria isolated from the sputum [12]. Beside the main agents (*P. aeruginosa* and *S. aureus*), Gram-negative non fermentative microorganisms (*Burkholderia cepacia*, *Stenotrophomonas maltophilia, Acinetobacter baumannii*) have been identified in sputum of CF patients [13-15]. Molecular biology techniques for detection and identification of bacteria re now widely used in clinical microbiology and developed for identification of isolates obtained from CF patients. The aim of this study was to determine the bacterial species associated with bacterial infection in CF patients and analyze the efficiency of species-specific primer pair to identify these species by using PCR-RFLP protocol.

MATERIALS AND METHODS

Collection of Patients' Samples: The (CF) patients' samples were collected from King Faisal Specialist Hospital & Research Center (Pediatric Clinic) and the (non-CF) Patients' samples were collected from King Faisal Specialist Hospital research Center and King Abdul Aziz Hospital & Oncology Center from October 2011 until October 2012.

Culturing of Collected Samples: All samples were cultured on the blood sheep agar, MacConkey agar and Chocolate agar. Muller Hinton agar (Merck, Darmstadt, Germany) was used as a test medium for disk diffusion method. All types of media as mentioned above were kindly obtained from Microbiology Laboratory of King Abdul Aziz Hospital. Bacterial identification and antibiotic susceptibility tests were performed as described by Luckett *et al.* [16]. The isolated and identified strains were stored in Luria-Bertani (LB) broth containing 20% glycerol and stored at -20°C.

DNA Extraction: The QIAamp DNA Mini Kit was used to purify bacterial DNA from Gram-negative bacteria. The DNA was extracted by following the manufacturer's instructions. DNA extractions from Gram-positive bacteria were used by the CTAB method [17].

PCR: PCR technique was used to amplify a specific segment of DNA. This technique is sensitive to contamination, so all tips and tubes were autoclaved, the micropipettes and bench top were cleaned with 70% ethanol.

16s rRNA Gene Amplification for *P. aeruginosa*: In order to amplify 612 bp fragment of 16 s rRNA target gene the following primer was used: - PA-F: 5' CGGCCCAGACTCCTACGGG 3'. - PA-R: 5' TTACCGCGGCTGCTGGCAC 3'. Amplification of the target gene was carried out under the following condition: After initial denaturation for 2 min at 95°C, 25 cycles were completed, each consisting of 20 s at 94°C, 20 s at 58°C and 40 s at 72°C. A final extension of 1 min at 72°C was applied. Finally, the reaction could cool at 4°C [18].

16s rRNA Gene Amplification for *S. aureus*: In order to amplify 916 bp fragment of 16 s rRNA target gene the following primer was used: - S.A-F: 5' TAGATGGATCCGCGC 3'. - S.A-R: 5' CTTAATGATGGCAACTAAGC 3'. Amplification of the target gene was carried out under the following condition: initial denaturation for 2 min at 95°C, 25 cycles were completed, each consisting of 20 s at 94°C, 20 s at 58°C and 40 s at 72°C. A final extension of 1 min at 72°C was applied. Finally, the reaction could cool at 4°C [19].

16s rRNA Gene Amplification for *K. pneumoniae*: In order to amplify 1069 bp fragment of 16 s rRNA target gene the following primer was used: - K.P-F: 5' GTAATGTCTGGGAAACTGCC 3'. - K.P-R: 5'CCACCTTCCTCCAGTTTATC 3' Amplification of the target gene was carried out under the following condition: After initial denaturation for 1 min at 94°C, 40 cycles were completed, each consisting of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C. A final extension of 3 min at 72°C was applied. Finally, the reaction could cool at 4°C [20].

16s rRNA Gene PCR Amplification for *A. baumannii*: In order to amplify 1500 bp fragment of 16s rRNA target gene the following primer was used: - ACI-F: 5' TGGCTCAGATTGAACGCTGGCGGC 3'. - ACI-R: 5' TACCT TGTTAC GACTTC A C CCCA 3'. Amplification of the target gene was carried out under the following condition: After initial denaturation for 5 min at 95°C, 35 cycles were completed, each consisting of 45 s at 95°C, 45 s at 48°C and 1 min at 72°C. A final extension of 7 min at 72°C was applied. Finally, the reaction could cool at 4°C [21].

Gel Electrophoresis: Gel electrophoresis was performed to check PCR products after amplification. RFLP for genotyping was carried out after sample digestion by restriction enzymes and to check the presence or absence of the plasmid in the strains. After PCR amplification, the PCR products were analyzed on 2% agarose gel electrophoresis in a TBE buffer containing ethidium bromide at120 v for 1 hour. Amplified products were visualized using a Gel Documentation System by comparison with a molecular size marker (GelPilot 100 bp plus ladder 100 lanes).

RFLP-PCR for *P. aeruginosa* Isolates: The amplified 612 bp PCR product was digested by *Bam HI* enzyme. The enzymatic digestion was performed in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *Bam HI*, 5µl of the bovine serum albumin (BSA) and 11.5 µl of the ddH2O. The reaction was incubated at 37?C for 4 hours then the enzyme was inactivated by incubating at 80 ?C for 20 min. The digestion products were then analyzed by 2% agarose gel electrophoresis at 120 v for 1 hour [22].

RFLP-PCR for *S. aureus* Isolates: The amplified 900 bp PCR product was digested by *Apa I* enzyme. The enzymatic digestion was performed in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *Apa I*, 5µl of BSA and 11.5 µl of the ddH2O. The reaction was incubated at 37°C for 2 hours then the enzyme was inactivated by incubated at 80°C for 20 min. The digestion products were then analyzed by 2% agarose gel electrophoresis at 120 for 1 hour [23].

RFLP-PCR for *A. baumannii* Isolates: The amplified 1500 bp PCR product was digested by *MspI* enzyme. The enzymatic digestion was performed in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *MspI* and 12µl of the ddH2O. The reaction was incubated at 37°C for 1 hour. The digestion products were then analyzed by 2% agarose gel electrophoresis at 120 v for 1 hour [21].

RFLP-PCR for *K. pneumoniae* Isolates: The amplified 1069 bp PCR product was digested by *TaqI* enzyme. The enzymatic digestion was performed in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *TaqI*, 5µl of BSA and 11.5 µl of the ddH2O. The reaction was incubated at 65°C for 1 hour then the enzyme was inactivated by incubating at 80°C for 20 min. The digestion products were then analyzed by 2% agarose gel electrophoresis at 120 v for 1 hour. [20].

Plasmid Patterns of Isolates: GeneJET Plasmid Miniprep Kit (# K0482, Thermo Scientific, USA) was used to purify plasmid DNA. The protocol followed the manufacturer's instructions in the kit. After purification, the products were analyzed by 2.5% agarose gel electrophoresis at 50 v for 2 hours.

RESULTS

Four primer pairs were used in this study. These primers targeted the variable regions in the 16S rRNA gene and every primer pair was specific for each species. PCR assays employing this primer pair produced DNA products of the predicted size (Figs. 1, 2, 3 and 4). By using these primers targeting 16S rRNA gene, results showed an amplified fragment of 612 bp, 900 bp 1500 bp and 1069 bp for *P. aeruginosa*, *S. aureus*, *A. baumannii* and *K. pneumoniae,* respectively.

RFLP among *P. aeruginosa* Isolates: Restriction digestion pattern of 16S rRNA PCR product using *BamHI* enzyme revealed identical digestion pattern among all *P. aeruginosa* isolates i.e. two bands, 400 bp and 120 bp as shown in Fig. 5.

RFLP among *S. aureus* Isolates: Restriction digestion pattern of 16S rRNA PCR product using *ApaI* enzyme revealed identical digestion pattern among all *S. aureus* isolates i.e. two bands, 600 bp and 300 bp as shown in Figure (6).

RFLP among *A. baumannii* Isolates: Restriction digestion pattern of 16S rRNA PCR product using *MspI* enzyme revealed different digestion pattern among the strains. One genotype produces three bands with 900, 500 and 100 bp, while the other genotypes produced four bands with 700, 400, 180 and 120bp as shown in Figure (7).

RFLP among *K. pneumoniae* Isolates: Restriction digestion pattern of 16S rRNA PCR product using *TaqI* enzyme revealed identical digestion pattern among all *K. pneumoniae* isolates i.e., three bands, 600, 240 and 200 bp as shown in Figure (8).

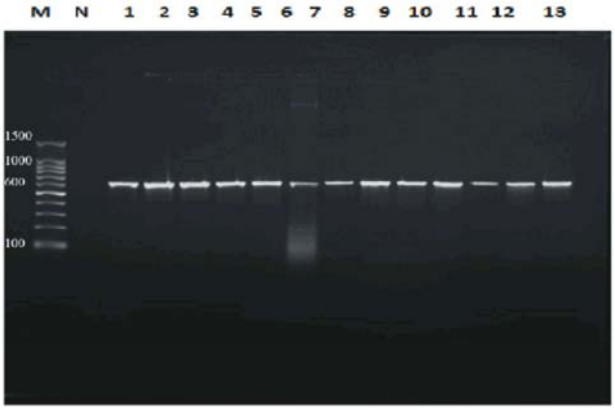

Figure 1 PCR products for 16s rDNA gene of *P.aeruginosa* isolates. PCR products including positive and negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 612bp
Lane 1-6: CF isolates
Lane 7 12: Non CF isolates
Lane13: Positive control (*P.aeruginosa* Atcc 27853)
Lane M: DNA molecuolar marker
Lane N: Negative control (*P.fluorescens*)

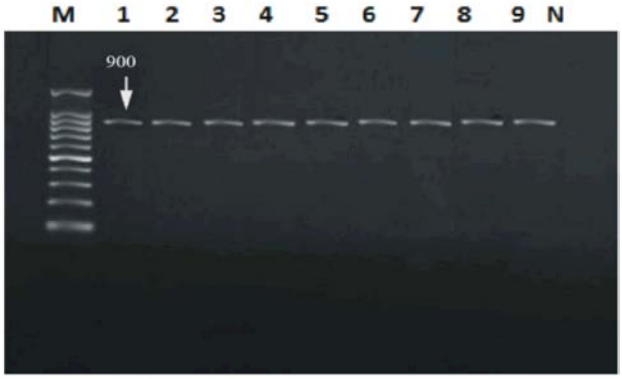

Figure 2 PCR products for 16s rDNA gene of S.aureus isolates. PCR products including positive and negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 900 bp.
Lane 1-4: CF isolates
Lane 5-8: Non CF isolates
Lane9: Positive control (S.aureus Atcc 25923)
Lane M: DNA molecuolar marker
Lane N: Negative control (MRSA)

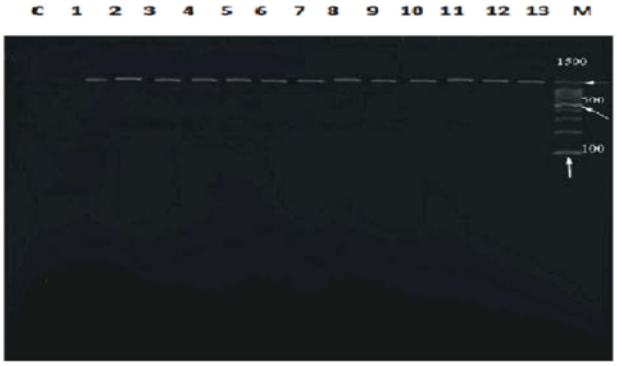

Figure 3 PCR products for 16s rDNA gene of *A.baumannii* isolates. PCR products including negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 1500 bp.
Lane 1-4: CF isolates
Lane 5-13: Non CF isolates
Lane M: DNA molecuolar marker
Lane C: Negative control (*A. haemolyticus*)

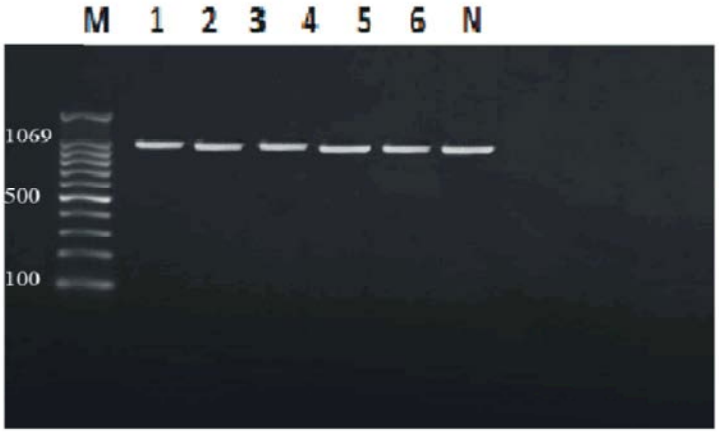

Figure 4 PCR products for 16s rDNA gene of *K.pneumoniae* isolates. PCR products including negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 1069 bp.
Lane 1-3: CF isolates
Lane 4-5: Non CF isolates
Lane M: DNA molecuolar marker
Lane N: Negative control (K. oxytoca)
Lane C: Positive control (*K.pneumoniae* Atcc 700603)

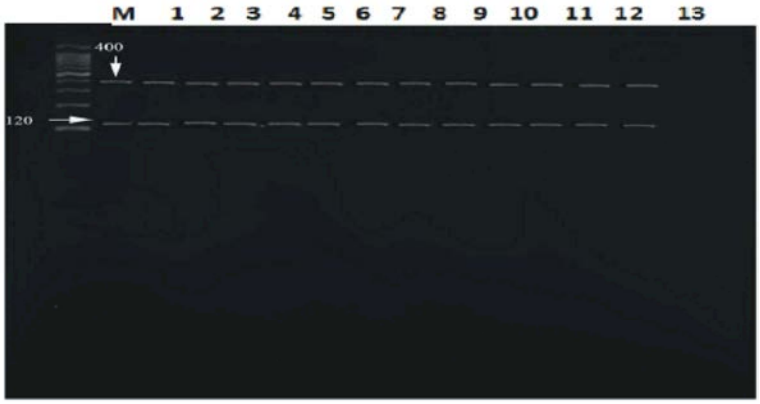

Figure 5 RFLP for *P.aeruginosa* isolates. After digestion with BamHI using 2% agarose gel.
Lane 1-8: CF isolates
Lane 9-13: Non CF isolates
Lane M: DNA molecuolar marker

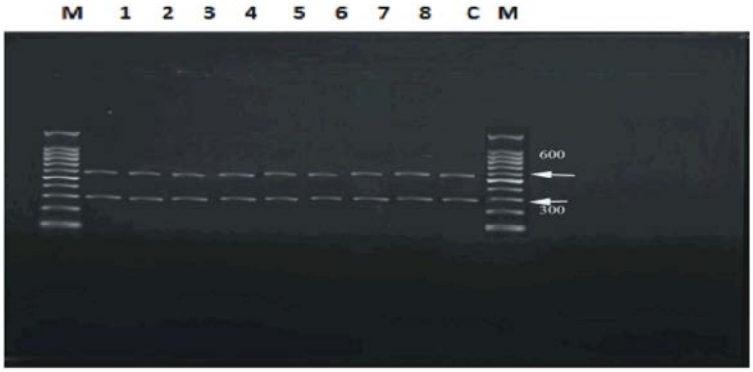

Figure 6 RFLP for *S.aureus* isolates .After digestion with ApaI digestion using 2% agarose gel.
Lane 1-4: CF isolates
Lane 5-8: Non CF isolates
Lane M: DNA molecuolar marker
Lane C: Positive control *S.aureus* (Atcc 25923)

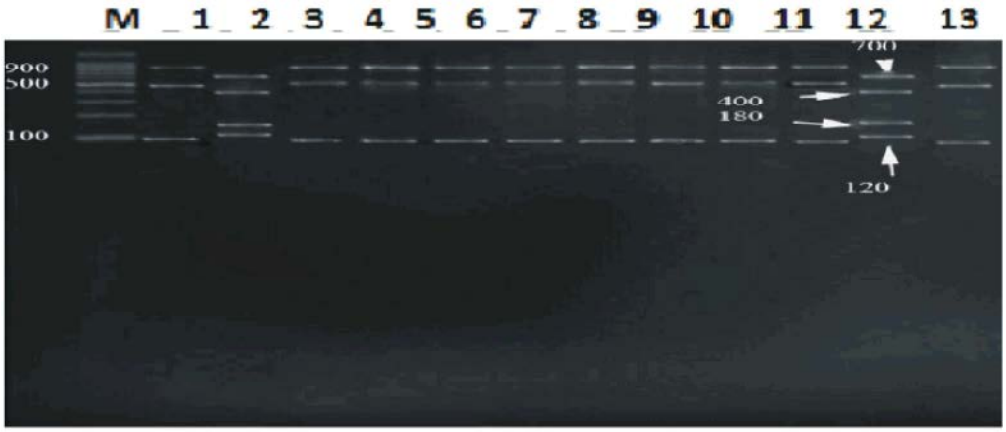

Figure 7 RFLP for *A. baumannii* isolates .After digestion with MspI digestion using 2% agarose gel.
Lane 1-4: CF isolates
Lane 5-12: Non CF isolates
Lane M: DNA molecuolar marker

Figure 8 RFLP for *K.pneumoniae* isolates. After digestion with TaqI using 2% agarose gel.
Lane 1-3: CF isolates
Lane 4-5: Non CF isolates
Lane M: DNA molecuolar marker
Lane C: Positive control *K.pneumoniae* (Atcc 700603)

Figure 9 Plasmid profile of *P.aeruginosa* isolates.
Lane 1-5: CF isolates
Lane 6-12: Non CF isolates
Lane M: Lambda DNA HindIII digest as a marker
Lane C: Positive control (*P.aeruginosa* Atcc 27853)

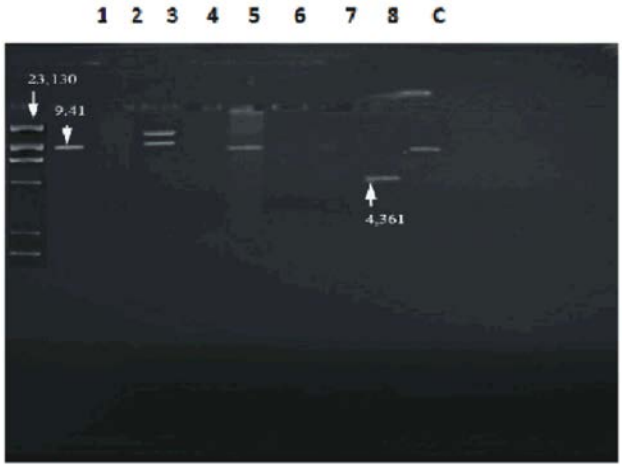

Figure 10 Plasmid profile of *S.aureus* isolates
Lane 1-4: CF isolates
Lane 5-8: Non CF isolates
Lane M: Lambda DNA HindIII digest as a marker
Lane C: Positive control *S.aureus* (Atcc 25923)

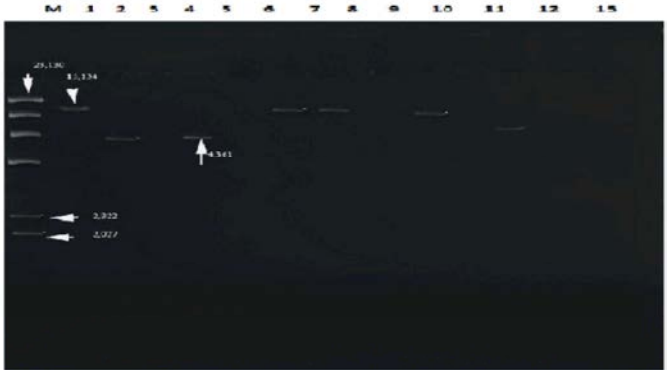

Figure 11 Plasmid profile of *A.baumannii* isolates.
Lane 1-4: CF isolates
Lane 5-13: Non CF isolates
Lane M: Lambda DNA HindIII digest as a marker

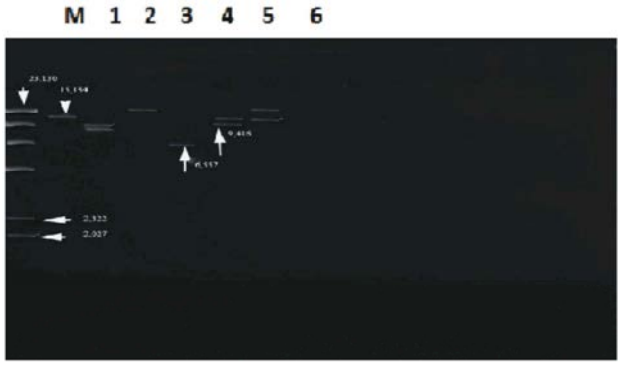

Figure 12 Plasmid profile of *K.pneumoniae* isolates.
Lane 1-3: CF isolates
Lane 4-5: Non CF isolates
Lane M: Lambda DNA HindIII digest as a marker
Lane C: Positive control (*K.pneumoniae* Atcc 700603)

Plasmid Patterns in *P. aeruginosa* Isolates: Plasmids were found in 4 (28.5 %) CF strains and 5 (55.5%) non-CF strains with an average of 2 plasmids per strains; their relative molecular mass ranged from 23, 130 bp to 4, 361 bp. No plasmids were found in 10 (71.4%) CF strains and 4 (44.4%) non-CF strains. Figure (9) shows the profiles of plasmids isolated from strains of *P. aeruginosa*.

Plasmid Patterns in *S. aureus* Isolates: Plasmids were found in 2 (50 %) CF strains and 2 (50%) non-CF strains with an average of 2 plasmids per strains; their relative molecular mass ranged from 20, 000 bp to 4300 bp. No plasmids were found in 2 (50 %) CF strains and 2 (50%) non-CF strains. Figure (10) shows the profiles of plasmids isolated from strains of *S. aureus.*

Plasmid Patterns in *A. baumannii* Isolates: Plasmids were found in 3 (75 %) CF strains and 4 (44.4 %) non-CF strains with 1 plasmid per strains; their relative molecular mass ranged from 15, 130 bp to 4, 361 bp. No plasmids were found in 1 (25 %) CF strains and 5 (55.6%) non-CF strains. Figure (11) shows the profiles of plasmids isolated from strains of *A. baumannii.*

Plasmid Patterns in *K. pneumoniae* Isolates: Plasmids were found in 3 (100 %) CF strains and 2 (100%) non-CF strains with an average of 2 plasmids per strains; their relative molecular mass ranged from 23, 130 bp to 6, 557 bp. Figure (12) shows the profiles of plasmids isolated from strains of *K. pneumoniae.*

DISCUSSION

CF is the most common lethal inherited disease in white persons [24]. Chronic airway inflammation and infection are the greatest causes of morbidity and mortality in CF patients [25] Lungs of CF patients are often colonized or infected in infancy and early childhood with organisms, such as *Staphylococcus aureus* and *Haemophilus influenza*, that may damage the epithelial surfaces, leading to increased attachment of and eventual replacement by P. aeruginosa [26]. Approximately 30, 000 Americans have CF and there are an estimated 1, 000 new cases diagnosed each year (Cystic Fibrosis Foundation www.cff.org.) and there are more than 5, 000 registered CF patients in the UK [27]. The incidence of CF in Saudi Arabia was reported to be 1 in 4243 children [28]. The microbiological analysis of clinical specimens has relied traditionally on cultivation prior to identification have gained widespread acceptance for the study of bacterial communities in samples [29]. This study was conducted to investigate the bacterial pathogens colonizing patients with cystic fibrosis in Jeddah and explored the possibility of amplification of the 16S rDNA followed by restriction analysis to identify 4 different species associated with CF infection.

Database availability of 16s *rDNA* genes for several bacteria species provides fundamental information for comparative studies by using bioinformatics tools, which can predict accurately gene polymorphism among species correlating with phenotypic features. In our study 16s rDNA species-specific primers have been used for identification of all isolates. Selective amplification of *Pseudomonas* 16S rDNA by PCR followed by restriction fragment length polymorphism analysis has been used to detect and differentiate *Pseudomonas* species from clinical and environmental samples [30] used as a conserved region of 16S rRNA primer with a *Pseudomonas* genus-specific primer in a PCR assay to detect *Pseudomonas* DNA in CF sputum. 16S rRNA-based PCR assay provide simple and reliable identification of tested isolates in this study and differentiate them from other phylogenetically closely related species. 16s rRNA PCR assay has 100% sensitivity and specificity for it intended target species. In comparing these results with other studies, we found that the 16S rRNA gene is highly conserved on the *Klebsiella* genus [20]. In the works of [19], the species-specific primers targeting 16S rRNA gene were tested for *K. Pneumoniae* and 65 negative-control organisms, the target sequence was shown to be highly specific for *K. pneumoniae* and, it failed to detect any other bacteria. This work is in-agreement with our results which indicated that the *P. aeruginosa* 16S rRNA– based PCR assay showed highly sensitivity and specificity.

A.baumannii 16s rRNA gene has also exhibited the specificity that gives 100% results for identification of this species [20]. RFLP analysis had shown that it's a powerful taxonomic tool for bacterial identification at the species level and is being used extensively [31, 32]. In this study among the 23 *P. aeruginosa* isolates 1 *Bam HI* RFLP pattern was detected. The low variability of *Pseudomonas* RFLP patterns has been reported earlier [33]. *S. aureus* isolates also have identical *Apa I* RFLP pattern. On the other hand, variability in RFLP pattern was found amongst *A. baumannii* isolates and this was like study by Hernández *et al.* [34]. In order to determine whether the observed multidrug resistance pattern in the isolates was a plasmid or chromosomal mediated, the isolates were

screened for the presence of plasmids. One or more plasmids were isolated from *S. aureus* in this study and this agreed with the study by Piccinini and Zecconi [35]. Plasmid DNA was detected in only 39 % of *P. aeruginosa* isolates in our study. Therefore, it is postulated that most of the resistance genes in *P. aeruginosa* are mostly chromosomal. We can verify this postulate by plasmid curing. This result is like the result obtained by Nikbin *et al.* [36], as they also found the plasmid in 29 % of their *P. aeruginosa* isolates. In *A. baumannii* isolates, plasmid was found in 7 isolates and 6 isolates had no plasmids. Since all isolates, including the 6 isolates that supposedly carry no plasmids, exhibited multidrug resistance to drugs, some of the resistance markers of the *A. baumannii* possibly are chromosomally located, in agreement with previous studies [37-39].

CONCLUSIONS

In Conclusion, results in this investigation showed that CF patients in Jeddah had uncommon species in CF patients (*A. baumannii*. *K. pneumoniae*) also the PCR-RFLP technique has high sensitivity for identifying species associated with infection in CF patients. Different numbers and sizes of plasmids were detected in the isolates and the appearance of the plasmids in the isolates vary among Species.

Conflict of Interest: The authors declare no conflicts of interest.

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