Antibiotic resistance and virulence-associated genes profile analysis of some *Klebsiella pneumoniae* isolates

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ABSTRACT

The emergence of the extended spectrum β-lactamase (ESBL) that produced by Klebsiella pneumonia is a major problem worldwide due to transfer of resistance gene from one microbe to another. A total of 134 samples were used to isolate twenty three Klebsiella pneumoniae isolates from various clinical specimens along six months. They were identified by microbiological methods as Klebsiella pneumonia and confirmed with 16S rDNA sequencing ana lysis. The antimicrobial susceptibility of Klebsiella isolates was determined. The amplification of bla_{TEM}, bla_{SHV} , bla_{CTX-M} and OmpK35 genes was performed by PCR. The ESBL phenotype was detected in 16 (69.5%), that showed the presence of the three ESBL genes (bla $_{CTX-M}$, bla $_{SHV}$ and bla $_{TEM}$). The ESBL K. pneumoniae isolates were more resistant than the others because they showed resistance to multiple resistance spectrum antibiotics β-lactams, Cephalothin (91.4%), Cefuroxime (91.4%), Cefoxitin (47.8%), Ceftazidime (78.3%), Ceftriaxone (87%) and Cefepime (78.3%). For rep-PCR, 73 bands were resulted from the rep-PCR primers. Out of them, 36 bands were monomorphic with a monomorphism average of 49.3%, while 37 bands were polymorphic bands with a polymorphism average of 50.7 %. The number of total bands for each primer varied from 12 to 17 bands, and the bands size ranged from 210 to 2400 bp. These data provide new epidemiological information about the clonal nature of K. pneumoniae isolated from hospital patients in Taif, Saudi Arabia.

Key words: *ESBL genotypes, bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. K. pneumonia.*

INTRODUCTION

Gram-negative bacteria are potential causes of both infections acquired in hospital and community. Multiple resistance to broad spectrum antibiotics β - lactams is one of the most important Features. Antibiotic resistance of *K. pneumoniae* is accompanied mainly by expanded production of spectrum β-lactamases (ESBLs) that hydrolyze the third generation antibiotic, but can be inhibited by clavulanic acid (Aarestrup *et al*., 2008; Pitout and Laupland, 2008 and Alzahrani *et al*., 2016). In recent years, the rapid proliferation of ESBL isolates has been reported in many parts of the world, and the CTX-M enzymes are more dominant ESBLs. (Bonnet, 2004 and Morosini *et al*., 2006). Of particular concern is the emergence and diffusion of ESBLs CTX-M family between *K. pneumoniae* within the community (Pitout and Laupland, 2008). The CTX-M enzymes are a type of molecular proportions of extended-spectrum b-lactamases (ESBLs) active against oxyiminocephalosporins and monobactams, with a general preference for

cefotaxime and ceftriaxone (Poirel *et al*., 2012). Several studies have repeatedly used a PCR vertical component (rep-PCR), which targets (REP), (ERIC), or BOX elements, to compare diversity of bacterial genome (Hassan *et al*., 2014b). Furthermore, we use rep-PCR technique to differentiate between *K. pneumoniae* strains. In rep-PCR DNA fingerprinting, PCR amplification is achieved among heterogeneous contiguous elements to obtain DNA fingerprints that can be analyzed easily using a software program to identify patterns. Previous studies chose the rep-PCR because it is a simple, differentiate and cheap technique (Lessa *et al*., 2012 and Alharthi *et al*., 2016). The rep-PCR has been used successfully to characterize *K. pneumoniae* and *E. coli* isolates (Priscilla *et al*., 2011). In the present study, molecular detection of ESBL genes by PCR is an attractive alternative (Xu *et al*., 2008 and Gazin *et al*., 2012). The 16S rDNA sequence contains characteristics that make it suitable methods as a global indicator of evolution. In addition, the 16S rRNA gene sequence is a useful method for bacterial identification, where nucleotide sequences are identified in this region and compared to the available sequences of databases to obtain homogeneous matches, allowing bacterial identification of the target samples. (Salman *et al*., 2012; Sabir *et al*., 2013; Chandran and Mazumder, 2015 and Alsanie *et al*., 2018). The aim of this study was to screen antibiotic susceptibility of *K. pneumoniae* from patients from King Faysal hospital in Taif, Saudi Arabia, detect the common ESBL genes bla_{TEM}, bla_{SHV} and bla_{CTX-M} and DNA fingerprinting of these isolates using rep-PCR technique.

MATERIALS AND METHODS

K. pneumoniae strains

Of 134 bacterial isolates, twenty three *K. pneumoniae* isolates were obtained from inpatients of King Faisal Hospital in Taif, Saudi Arabia. The bacterial species were identified using the fully automated VITEK-2 COMPACT microbiology system (BioMérieux, Inc., Durham, NC, USA). *K. pneumoniae* (ATCC 700603) ESBL-producing strains were used as controls for the antimicrobial susceptibility tests.

Antibiotic susceptibility testing

Using the recommended clinical standard (CLSI), World Health Organization method, the susceptibility of *K. pneumoniae* against 16 antibiotics was determined (Hassan *et al*., 2014a).

The 16S rDNA gene sequencing

The genomic DNA was isolated using DNA extraction kit (Gena Bioscience, Germany) from all *K. pneumoniae* isolates according to the manufacturer's instructions. For each isolate one fragment of the DNA (about 1465 pb) was amplified from the 16S rDNA gene (Alsanie et al., 2018). The pieces were punctuated using the QIA quick PCR purification kit (QIAGEN, Valencia, CA, USA) and sequenced in DNA Analyzer 3146 Applied Bioscience (Applied Biosystems, USA). The sequencing texts were edited and compiled using DNASTAR software (Laser gene, Madison, WI, USA). Using the NCBI server, BLAST searches were performed (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table (1): Primer sequences and amplicon sizes of virulence genes in K. pneumonia.

Primers	Primer sequence (5′→3′)	Product size (bp)
CTX-MF	ACC GCC GAT AAT TCG CAG AT	588
CTX-MR	GAT ATC GTT GGT GGT GCC ATA A	
SHV-F	TAC CAT GAG CGA TAA CAG CG	450
SHV-R	GAT TTG CTG ATT TCG CTC GG	
TEM-F	TCC GCT CAT GAG ACA ATA ACC	296
TEM-R	ATA ATA CCG CAC CAC ATA GCA G	
OmpK35-F	CTC CAG CTC TAA CCG TAG CG	241
OmpK35-R	GGT CTG TAC GTA GCC GAT GG	

Table (2): Virulence genes patterns among pathogenic K. pneumonia isolates.

Isolates	Presence or absence of virulence genes			
	CTX-MF	***SHV***	***TEM***	***OmpK35***
Klebsiella-1	–	+	+	+
Klebsiella-2	+	+	+	+
Klebsiella-3	–	+	+	+
Klebsiella-4	+	+	+	+
Klebsiella-5	+	+	+	+
Klebsiella-6	+	+	+	+
Klebsiella-7	+	+	+	+
Klebsiella-8	+	+	+	+
Klebsiella-9	+	+	+	+
Klebsiella-10	+	+	+	+
Klebsiella-11	–	+	+	+
Klebsiella-12	+	+	+	+
Klebsiella-13	+	+	+	+
Klebsiella-14	–	+	+	+
Klebsiella-15	+	+	+	+
Klebsiella-16	–	+	+	+
Klebsiella-17	+	+	+	+
Klebsiella-18	+	+	+	+
Klebsiella-19	+	+	+	+
Klebsiella-20	+	+	+	+
Klebsiella-21	+	–	+	+
Klebsiella-22	–	+	+	+
Klebsiella-23	+	+	+	+

Polymerase chain reaction test of extended-spectrum beta-lactamase bacteria

Four genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *OmpK35*) in ESBL-producing *K. pneumoniae* were detected according to Alzahrani *et al*. (2016). Primer sequences and PCR conditions for each gene are described in Table (1). The PCR was performed with the Go Taq® Green Master Mix, Promega, USA. Expected sizes of the amplicons were ascertained by electrophoresis in 1.5 % agarose gel with an appropriate molecular size marker (100-bp DNA ladder, MBI, Fermentas, Lithuania, USA).

Rep-PCR analysis

Five repetitive sequence primers (BOX-A1, GTG-5, M-13, Rep-10 and Rep-18) were used to amplify genomic DNA of the *K. pneumoniae* isolates according to Gaber *et al*. (2015).

Statistical analysis

The similarity matrix was subjected to cluster analysis by an unweighted pair group method for arithmetic mean and a dendrogram was generated using the program NTSYS-PC version 2.01 (Rohlf, 2000).

Table (3): Polymorphic bands of each genetic primers and percentage of polymorphism in twenty three K. pneumonia isolates based on the five rep-PCR primers.

Primers	Total bands	No. of monomorphic bands	No. polymorphic bands	Monomorphic bands %	Polymorphic bands %
BOX-A1	17	6	11	35.3	64.7
GTG-5	14	6	8	42.8	57.2
M-13	12	5	7	41.7	58.3
rep-10	14	13	1	92.8	07.2
rep-18	16	6	10	37.5	62.5
Total	73	36	37	49.3	50.7

RESULTS AND DISCUSSION

Antimicrobial susceptibility

Twenty three *K. pneumoniae* isolates obtained from patients at King Faisal Hospital in Taif, Saudi Arabia were tested for their antibiotic resistance against 20 antibiotics. Results are shown in Fig. (1). Large percentage of the *K. pneumoniae* were resistant to Cephalothin (91.4%), Cefuroxime (91.4%), Cefoxitin (47.8%), Ceftazidime (78.3%), Ceftriaxone (87%), Cefepime (78.3%), Aztreonam (86.9%), Ampicillin (95.7%), Amox-Clav (91.4%) and Trim-Sulf (82.6%), but moderate susceptible to Tigecycline (69.6%), Amikacin (56.6%), Gentamicin (52.2%), Ertapenem (52.2%), Imipenem (56.5%) and Meropenem (65.3%). Antibiotics also play an important role in reducing the disease or death caused by bacterial infections in humans and animals. However, the use of antibiotics at random was the main cause of the emergence and spread of antibiotic-resistant bacterial strains (Aarestrup *et al*., 2008 and Krishnamurthy *et al*., 2013). The main source of *K. pneumoniae* is the elemntre canal of animals. Poor handling of farm animals can cause contamination of drinking water and injure the person when drinking this water (Alzahrani *et al*., 2016).

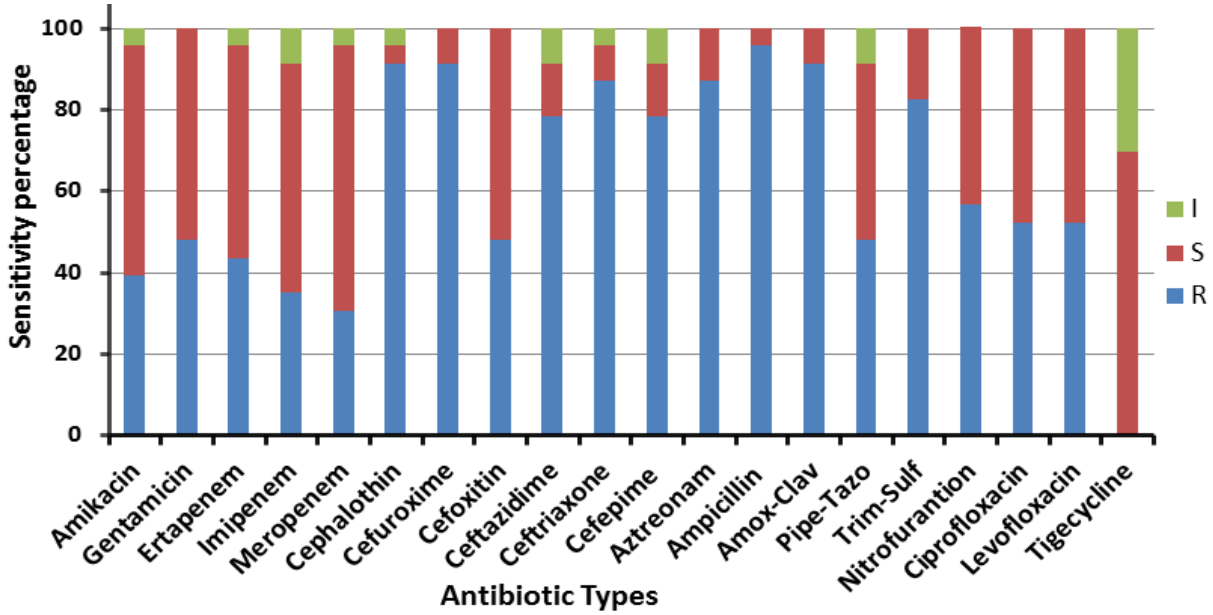

Fig. (1) : Antimicrobial resistance profiles of twenty three K. pneumonia isolates against twenty antibiotics. R= resistance, S= sensitive and I= intermediate.

Detection of beta-lactamases genes in *K. pneumoniae*

Among 23 *K. pneumonia* isolates 16 (69.5%) showed the presence of the three ESBL genes (*bla* $_{CTX-M}$, *bla* $_{SHV}$ and *bla* $_{TEM}$). The other seven *K. pneumoniae* isolates showed the presence of at least one ESBL gene and showed the absence of *bla* $_{CTX-M}$ gene (Table 2 and Figure 2). Six *K. pneumoniae* isolates that showed positive *bla* $_{CTX-M}$, *bla* $_{SHV}$ and *bla* $_{TEM}$ genes were more resistant than the other *K. pneumoniae* isolates because they showed resistance to Cephalothin, Cefuroxime, Cefoxitin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Ampicillin, Amox-Clav and Trim-Sulf. Negative PCR results do not exclude the possibility of a *bla* $_{CTX-M}$ in studied isolates. Because of the increasing complexity of β-lactam resistance in different bacteria, the key to control the spread of antibiotics is to reduce the giving of an antibiotic without bacterial culture analysis (Asgar, 2007a and Krishnamurthy *et al*., 2013). ESBL producers typically carry plasmid resistors. In this study six isolates *K. pneumoniae* showed higher resistance than others. The resistant plasmid usually habitat ESBL producing bacteria. Similar results have been obtained by Yazdi *et al*. (2012), Yuan *et al*. (2012) and Alzahrani *et al*. (2016). The majority of ESBLs-portable genes on plasmids are derived from penicillinases that belong to families of TEM and SHV genes. While the CTX-M group is a new family of ESBL (Xiong *et al*., 2002; Pitout and Laupland, 2008 and Alzahrani *et al*., 2016).

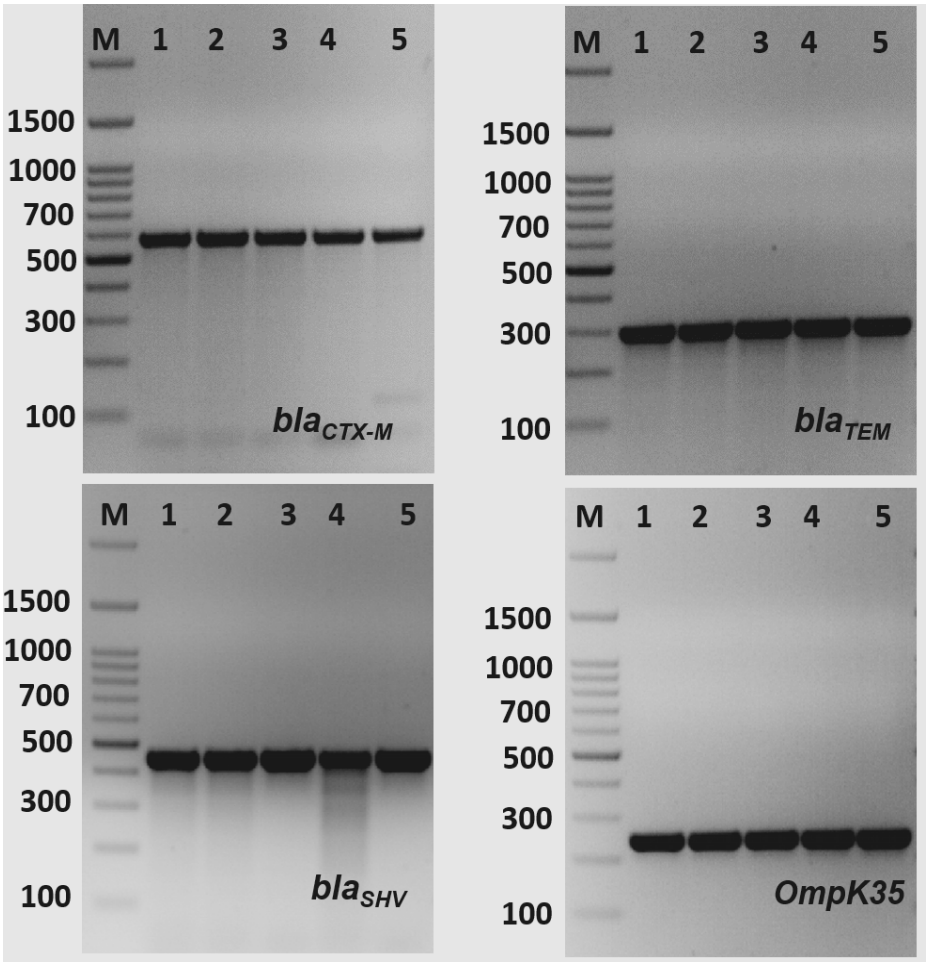

Fig. (2): Amplification of some specific genes produced by some K. pneumonia isolates by single PCR. (A) $bla_{CTX\text{-}M}$ gene with size of 588 bp. (B) bla_{TEM} gene with size of 269 bp. (C) bla_{SHV} gene with size of 450 bp. (D) OmpK35 gene with size about of 241 bp. First lane on each panel is 100 bp molecular weight markers.

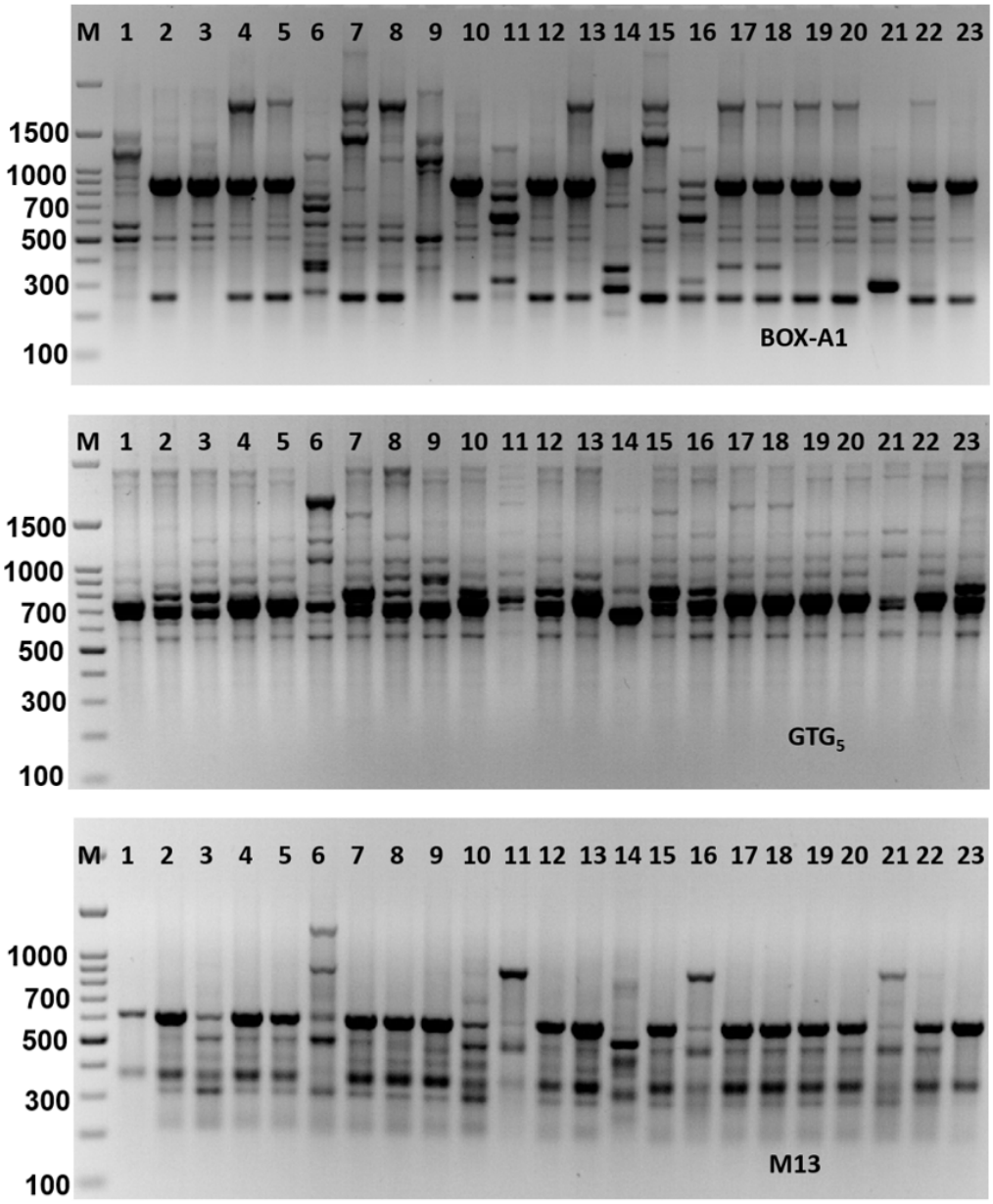

Fig. (3): Rep-PCR profile of 23 K. pneumoniae isolates generated with three rep primers, BOX-A1, GTG5and M13, respectively. First lane on each panel is 100 bp molecular weight markers

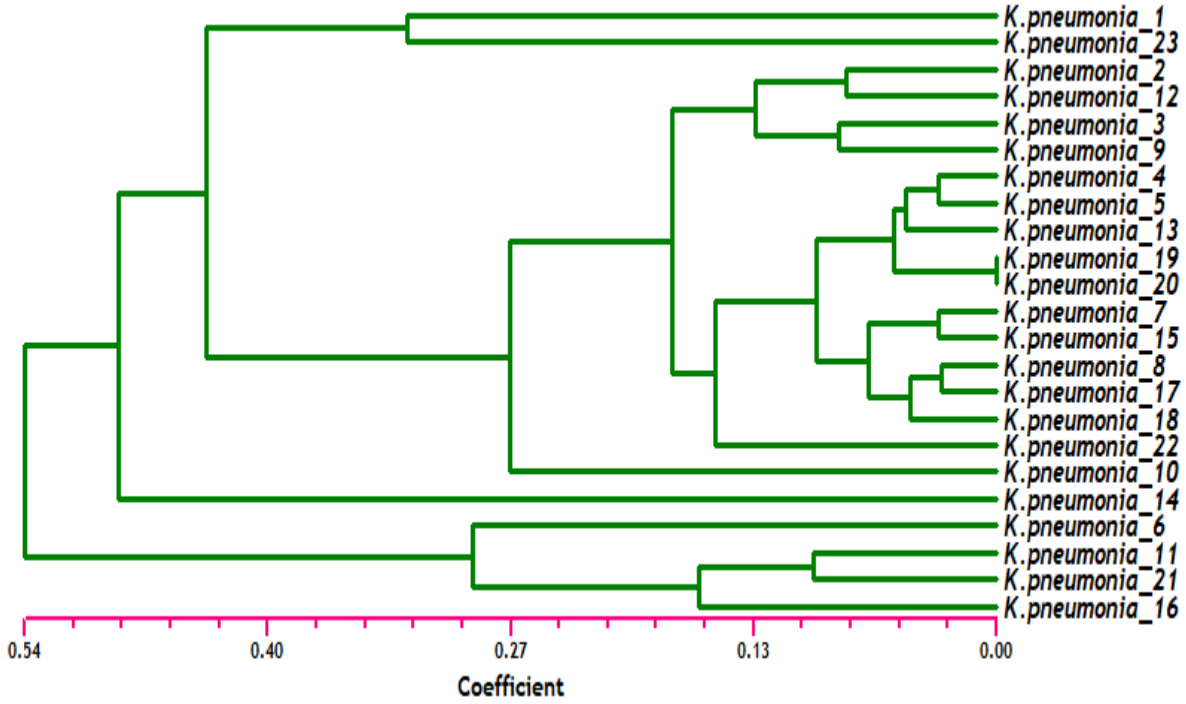

Fig. (4): Dendrogram analysis among twenty three K. pneumoniae isolates generated with five rep-PCR primers.

Rep-PCR analysis

Molecular markers are effective tools for molecular characterization and correlation estimation through DNA fingerprinting. Beta-lactam *K. pneumoniae* were characterized using Rep-PCR markers. The rep-PCR results are summarized in Table (3) and shown in Fig. (3). The polymorphic and monomorphic bands were produced from the PCR amplification. Seventy four bands were resulted from the rep-PCR primers. Out of them, 36 bands were monomorphic with a monomorphism average of 49.3%, while 37 bands were polymorphic bands with a polymorphism average of 50.7 %. The number of total bands varied from 12 to 17 bands, with primers M-13 and BOX-A1, and the bands size ranging from 210 to 2400 bp. The highest polymorphism among *K. pneumoniae* isolates was revealed by BOX-A1 primer (64.7%), followed by that revealed by rep-18 primer (62.2%). However, the lowest polymorphism was 7.2% resulted from application of rep-10 primer.

Genetic distances and the cluster dendrogram

A total of 73 fragments from all rep-PCR analysis was enough for determination of genetic similarities and designing the phylogenetic tree for these Beta-lactam *K. pneumoniae* isolates. According to dendrogram constructed drowned using UPGMA based on Jaccard's similarity coefficients, genetic similarity and intra-species differentiation ranged from 0.00 to 0.74 (Fig. 4), the *K. pneumoniae* isolates were

grouped into two different clusters with about 46% genetic similarity. The first cluster contained the most *K. pneumoniae* isolates, while the second cluster contained *K. pneumoniae* isolates K6, K11, K16 and K21. The first cluster contained two sub-clusters, the first sub-cluster contained *K. pneumoniae* isolates K1, K2, K3, K4, K5, K7, K8, K9, K10, K12, K13, K15, K17, K18, K19, K20, k22 and K23, while the second sub-cluster contained K14 only. The dendrogram showed the highest genetic similarity between K19, K20, while the lowest relationship was between K1 and K6. K11 and K21were in the same sub-cluster and appeared more similar to each other than K16.

CONCLUSION

The results of PCR detection b-lactamase genes confirm that there is an increase in the prevalence of ESBL-producing organisms among *K. pneumoniae* isolates. Many of these *K. pneumoniae* isolates showed resistance to many antibiotics, which are likely to contain one or more plasmids carrying bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. The increased incidence of such microbes carrying b-lactamase genes increases their spread among the healthy peoples and is difficult to control. Further studies are need to focus on the means of infection and how to control.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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الملخص العربي

مقاومة المضادات الحيوية ومقارنة الجينات المرتبطة بالفوعة لبعض عزلات الكلبسيلا الرئوية

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أن ظهور عزلات من بكتريا الالتهاب الرئوي *klebsiella* متحملة للـ β-lactamase مشكلة رئيسية في جميع أنحاء العالم بسبب نقل جينات المقاومة بين العزلات المختلفة وبعضها. الهدف الاساسي لهذه الدراسة هو التوصيف الجزيئي لسلالات بكتريا الكلبسيلا المعزولة من المرضى في محافظة الطائف، بالمملكة العربية السعودية وكذلك التأكد من وجود بعض جينات الفوعة في هذه السلالات البكتيرية. تم عزل ١٣٤ عزله بكتيريا ممرضة من مرضي مستشفيات محافظة الطائف خلال فترة ستة أشهر كان من بينهم ثلاث وعشرين عزلة كلبسيلا *klebsiella*. تم التعرف علي العزلات بالطرق الميكروبيولوجية وعرفت كعزلات كلبسيلا وقد تم تأكيد تعريف هذه العزلات باستخدام تحديد توالي جين 16S rDNA . تم قياس حساسية هذه العزلات للعديد من المضادات الحيوية و كذلك تم عمل تحديد وجود جينات bla_{TEM} و bla_{SHV} و $bla_{CTX\text{-}M}$ في تلك العزلات بواسطة تفاعل البلمرة المتسلسل . تم الكشف عن وجود النمط المظهري ESBL في ١٦ عزلة والتي تمثل (٦٩.٥ ٪) من مجموع العزلات ، والتي أظهرت وجود جينات ESBL الثلاثة bla_{TEM} و bla_{SHV} و $bla_{CTX\text{-}M}$ و كانت عزلات الكلبسيلا من النوع ESBL أكثر مقاومة من غيرها لأنها أظهرت مقاومة لمضادات حيوية ذات العلاقة بـ β-lactams والتي أظهرت مقاومة لكل من Cephalothin (91.4٪) و Cefuroxime (91.4%) و Cefoxitin (47.8٪) و Ceftazidime (78.3٪) Ceftriaxone. (87%) و Cefepime (78. ٪) . بالنسبة لـ rep-PCR ، تم إنتاج حوالي ٧٤ شظية من بادئات rep-PCR. من بينها ٣٦ شظية كوحيدة الاشكال المظهرية بمعدل ٤٩.٣ ٪ ، في حين كانت ٣٧ شظية متعددة الأشكال المظهرية بمعدل ٥٠.٧ ٪ من اجمالي عدد الشظايا الكروموسومية وقد تراوح عدد الشظايا لكل بادئ من ١٢ إلى ١٧ شظية ، ويتراوح حجم الشظايا بين ٢١٠ و ٢٤٠٠ قاعدة نيتروجينية. قد توفر هذه النتائج بعض المعلومات الوبائية الجديدة حول طبيعة انتشار العدوى بعزلات الكلبسيلا الرئوية المعزولة من مرضى المستشفيات في الطائف بالمملكة العربية السعودية.