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Random amplified DNA polymorphism of *Klebsiella pneumoniae* isolates from Mansoura University Hospitals, Egypt

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***Klebsiella pneumoniae* is one of the major causes of nosocomial infection. Understanding the route and dynamics of dissemination in the outbreaks of infections relies on using accurate typing methods. This is achieved through molecular typing using random amplified polymorphic DNA (RAPD) analysis technique in correlation to the infection source and antibiotic resistance. In this study, a total of 300 clinical isolates were collected from different clinical sources among Mansoura University Hospitals, Dakahlia governorate, Egypt. Ninety six isolates were identified as *K. pneumoniae*. The antimicrobial susceptibility test showed high resistance to the majority of tested antimicrobials, especially to β-lactams. *K. pneumoniae* isolates were categorized into 24 different antimicrobial susceptibility patterns (A1-A24). Furthermore, RAPD analysis was applied as a molecular typing method using two individual primers, AP3 and OPA13. RAPD typing showed 51 distinct patterns (R1-R51) verified into 5 groups (A, B, C, D, and E). The most common patterns were D11, D18 and D19 included in groups B and C. On the other hand, more molecular variable isolates were distributed in groups D and E. Correlation between RAPD analysis and antibiotying established that specific RAPD pattern D11 was associated with multidrug resistant isolates. This confirms that cross acquisition can play an important role in the epidemiology of nosocomial colonization and infection with *K. pneumoniae* at Mansoura University Hospitals. In conclusion, this study emphasized the need for appropriate monitoring of *K. pneumoniae* infections, by using both traditional and molecular methods. RAPD proved to be effective technique in discriminating *K. pneumoniae* isolates.**

Key words: *Klebsiella pneumoniae*, random amplified polymorphic DNA (RAPD), antimicrobial susceptibility, typing, dendrogram.

INTRODUCTION

Klebsiella pneumoniae is an important nosocomial pathogen that causes severe morbidity and mortality worldwide, with immune compromised patients, diabetic, elder and pediatric patients. *K. pneumoniae* predominantly causes respiratory and urinary tract infections as well as surgical wounds infection (Cao et al., 2014).

Extensive use of antibiotics such as aminoglycosides, extended-spectrum cephalosporins and carbapenems was associated with multidrug resistance to *K. pneumoniae* (Souli et al., 2010). *K. pneumoniae* is an important cause of nosocomial infections in many parts of the world, especially in intensive care units (Abdel-Hady et al., 2008). Epidemiological characterization of *K. pneumonia*e is highly significant to monitor the spread of its infection and assists in controlling their resistance and pathogenicity.

Infection control efforts aim to identify the source of the infection and the mode of the transmission (de Sauza et al., 2005).

Traditional techniques for typing *K. pneumoniae* based on phenotypic characteristics including biotyping, antibiogram typing, O-serotyping, bacteriocin and phage typing are insensitive for typing and differentiation between isolates (Bricker, 2011).

Molecular methods such as plasmid-profile analysis, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism, gene sequencing and pulsed-field gel electrophoresis (PFGE) are more perceptive typing techniques (Bricker, 2011). Polymerase chain reaction (PCR)-based typing techniques, such as RAPD analysis is fast and easy to perform (de Sauza et al., 2005).

RAPD typing has been applied successfully for epidemiological investigations of many bacterial and fungal outbreaks (RAPD is based on PCR amplification of a set of fragments by using short arbitrary primers 6-12 bp that target several unspecified genomic sequences (Williams et al.,1990). The resulting amplified fragments function as polymorphisms for DNA fingerprinting. It has been widely used for typing and characterization of bacterial isolates in cases of outbreaks including *K. pneumoniae* (Ben-Hamouda et al., 2003), *Staphylococcus aureus*, *Staphylococcus intermedius*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus rattus* (Truong et al., 2000) and *E. coli* (Lin et al., 2010). Monitoring the spread of specific strains is of epidemiological importance in order to detect the source of infection and control bacterial pathogenicity. *K. pneumoniae* has been associated with nosocomial infection worldwide (Lin et al., 2010). Also, *K. pneumoniae* had been detected in outbreak of infection especially extended spectrum resistant isolates in Egypt (Abdel-Hady et al., 2008). However, no data on the genetic characterization of *K. pneumoniae* isolates by RAPD profile in Egypt was reported to date.

The aim of this study was the epidemiological characterization of molecular variability among *K. pneumoniae* isolates from various clinical sources at Mansoura University hospitals. This was achieved through molecular typing using RAPD analysis technique in correlation to the infection source and antibiotic susceptibility pattern.

MATERIALS AND METHODS

Isolation and identification of *K. pneumoniae* clinical isolates

A total of 96 clinical isolates of *K. pneumoniae* were isolated from 300 different patients distributed among Mansoura University Hospitals, Dakahlia governorate, Egypt in the period between January 2012 to April 2012. The isolated strains were purified and identified according to Crichton (1996). All the purified isolates were maintained in glycerol 30% at -80ºC.The experimental protocol conducted in the study complies with the ethical guidelines and the principals of care, use and handling of human subjects in medical research adopted by "The research Ethics Committee, Faculty of Pharmacy, Mansoura University, Egypt which is in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki involving use and handling of human subjects).

Antimicrobial susceptibility testing

Susceptibility to antibiotics was determined by the disc-diffusion method using Mueller–Hinton agar (Becton, Dickinson and company, USA) according to the Clinical and Laboratory Standards Institute (CLSI) 2011. Ten antimicrobial discs were used including; amikacin (AK, 30 µg) (aminoglycosides), amoxicillin/clavulanic acid (AMC, 30 µg) and ampicillin/sulbactam (SAM, 20 µg) (beta-lactam beta-lactamase inhibitor), cefepime (FEP, 30 µg) (fourth generation cephalosporin), ceftazidime (CAZ, 30 µg) and ceftriaxone (CRO, 30 µg) (third generation cephalosporin), cefuroxime (CXM, 30 µg) (second generation cephalosporin), ciprofloxacin (CIP, 5 µg) and levofloxacin (LEV, 5 µg) (quinolone) and meropenem (MEM, 10 µg) (carbapenem); all discs were supplied from Oxoid products, UK.

Genotypic analysis

Genomic DNA extraction

The chromosomal DNA of all isolates of *K. pneumoniae* was prepared using Thermo Scientific GeneJet Genomic DNA purification Kit #K0721 (Thermo Fisher Scientific, European Union), according to manufacturer instructions. Genomic DNA was eluted by adding 50 µl EB buffer (10 mMTris-HCl, pH 9, 0.5 mM EDTA) and visualized by electrophoresis on horizontal gels containing 1% agarose and stained with ethidium bromide.

RAPD-PCR amplification

DNA typing was carried out by RAPD analysis according to Williams et al. (1990) with some modifications. RAPD analysis was performed using oligonucleotides AP3; 5`…TCACGATGCA…3' (Green et al., 2011) and primer; OPA13; 5'…CAGCACCCAC…3' (Rodrigues et al., 2008). PCR was performed in a 20 µl reaction volume containing 100 ng of genomic DNA, 20 µM of the used primer, 1.5U of FlexiTaq DNA polymerase, 5x GoTaq® Flexi buffer and supplied by manufacturer (Promega, USA). The negative control without DNA was included in each PCR run.

The amplification was performed in thermocycler machine, FPROGO2D, Tchne, LTD, Duxford Cambridge, UK. The amplification protocol consisted of the following steps: initial denaturation 95°C for 5 min., followed by 40 cycles of denaturation at 95°C for 30 sec, annealing temperature at 30ºC for 30 sec, extension at 72°C for 2.5 min. and final extension at 72°C for 10 min. Amplified PCR products were mixed with 6X loading buffer, separated using 1% agarose gels and visualized by UV transillumination. DNA fingerprints were compared by visual inspection.

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Reproducibility of the amplification patterns was confirmed by the repetition of the PCR reactions. Electrophoresed agarose gels were analyzed visually and scored using a binary code. The binary code was analysed using DendroUPGMA: A dendrogram construction utility, Universitat Rovira i Virgili (URV), Tarragona, Spain. The dendrogram was constructed based on Jaccard coefficient and unweighted-pair group method with arithmetic mean clustering method (UPGMA) (de Sauza et al., 2005).

RESULTS

A total of 300 clinical isolates were collected from different clinical sources among Mansoura University Hospitals, Dakahlia governorate, Egypt. Ninety six isolates were identified as *K. pneumonia*e. The sampling information of all isolates including clinical sources, dates and places of isolation are illustrated in Table 1.

Antimicrobial susceptibility and resistance pattern

As determined by disc-diffusion antimicrobial susceptibility testing, a high resistance percentage was observed for most antimicrobials where 76.04% were resistant to cefuroxime (second generation cephalosporins), 73.9% were resistant to ceftazidime and ceftriaxone (third generation cephalosporins), 67.7% were resistant to amoxicillin/clavulanic acid and ampicillin/sulbactam (beta-lactam/beta-lactamase inhibitor combinations) and 56.25% were resistant to ciprofloxacin and levofloxacin (quinolone). On the other hand a lower resistance was noticed for cefepime (fourth generation cephalosporin) (36.45%), amikacin (aminoglycoside) (27.04%) and meropenem (carbapenem) (9.37%). Among isolates, 16 were considered multiple drug resistant (MDR) (resistant to more than two classes of antibiotics) named 8, 9, 10, 13, 15, 17, 21, 22, 23, 24, 37, 57, 71, 72, 88 and 94 (Table 2).

The tested *K. pneumoniae* isolates were distributed into 24 different antimicrobial susceptibility patterns (A1-A24) according to their resistance to different antimicrobial groups (Table 2). Most patterns shared resistance to cephlosporines and beta-lactam/beta-lactamase inhibitor. The most predominant pattern A2 involved 13 isolates showing resistance to all tested antimicrobials except amikacin (aminoglycoside) and meropenem (carbapenem). On the other side, pattern A22 involved 13 isolates with susceptibility to all antimicrobials. The second common pattern was A1 representing 12 isolates showing resistance to all tested antimicrobials except cefepime, amikacin and meropenem (carbapenem). Strain number 94 was the most resistant isolate as it was resistant to all tested antimicrobials.

RAPD analysis

K. pneumoniae isolates were typed by RAPD technique to investigate the patterns of *K. pneumoniae* infection at Mansoura University Hospitals. Two individual primers, AP3 and OPA13 were used. The primer OPA13 could amplify variable amplicons ranging in size from 350 to about 3000 bp. Most isolates (44%) shared a common amplicons size 400, 450, 550, 750, 1350 bp. RAPD analysis using AP3 primer showed amplified bands ranging in size from 200 to about 3000 bp with most common amplicon size 350-850 bp. RAPD profile of all isolates using OPA13 and AP3 primers are represented in Figures 1 and 2 respectively.

Cluster analysis of RAPD profile of both primers AP3 and OPA13 classified 96 *K. pneumoniae* isolates into 5 groups (A, B, C, D, and E) with distinct patterns (D1-D51) representing the 96 isolates (Figure 3). Isolates considered with the same pattern if the level of similarity was ≥70%. The most common patterns were D11, D18 and D19 included in groups B and C. Pattern D18 represented eleven isolates; isolates 7 and 73 were isolated from blood samples from pediatric hospital and isolates 14, 49, 60, 75, 77, 84, 89, 90 and 96 were obtained from urine samples. Samples 14, 49, 60, 84, 89, 90 and 96 were taken from the Kidney center and samples 75 and 77 were isolated from the Digestive center. Also, the pattern D11 included ten isolates (8, 9, 10, 13, 15, 16, 18, 20, 21 and 22), with percentage similarity 100% and all of them were obtained from urine samples except isolate 8 from endotracheal tube (Figure 3). On the same instance, isolates number 8, 9, 10, 13, 15 and 22 had the same antibiotype (A5) (Table 2).These isolates were mainly obtained from kidney center indicating the possibility of cross infection. Also, isolates 33, 85, 91, 93 and 94 chaired the same pattern D21 and they were all obtained from urine samples of kidney center except isolate number 33 from blood. On the other hand, groups D and E included more variable isolates. Thirty five isolates were distributes in groups D and E with 27 variable patterns D24-D51 (Figure 3).

DISCUSSION

The extensive use of antibiotics leads to high incidence of resistance among bacterial populations associated with an ultimate change in the susceptibility profile of microorganisms (Cao et al., 2014). The outbreaks of *K. pneumoniae* infection (Ben-Hamouda et al., 2003) raised the attention in hospitals. Epidemiological investigations are important for successful microbial control to minimize and eliminate the source of infection. Characterization of the organisms in the infection outbreak is very important aseptic in the control of nosocomial infection (Bricker, 2011). In the present study, the antibiotyping of 96 *K. pneumoniae* was investigated. The isolates established different antibiotic sensitivity patterns (A1-A24) indicating the diversity in their susceptibility to the tested anti-microbials (Table 2). Likewise, in the study of Ben-Hamouda et al., 2003 thirteen different antibiotic susceptibility patterns (A1-A13) were detected among 49

Table 1. List of isolates, their clinical sources, isolation centers and dates of isolation.

Isolate number	Clinical source	Isolation center	Date of isolation
1	Sputum	Digestive System Center	1/1/2012
2	Throat	Digestive System Center	1/1/2012
3	Sputum	University Hospital	2/1/2012
4	Nose	University Hospital	2/1/2012
5	Endotracheal aspirate	Emergency Hospital	5/1/2012
6	Sputum	Tumor Hospital	5/1/2012
7	Blood	Pediatric Hospital	5/1/2012
8	Endotracheal aspirate	Emergency Hospital	12/1/2012
9	Urine	Tumor Hospital	12/1/2012
From 10 to 17	Urine	Kidney Center	14/1/2012
From 18 to 26	Urine	Kidney Center	21/1/2012
27	Blood	Pediatric Hospital	26/1/2012
28	Endotracheal aspirate	Emergency Hospital	26/1/2012
29	Blood	Pediatric Hospital	2/2/2012
30	Urine	University Hospital	5/2/2012
31	Blood	Pediatric Hospital	9/2/2012
32	Endotracheal aspirate	University Hospital	12/2/2012
33	Blood	Pediatric Hospital	16/2/2012
34	Sputum	University Hospital	19/2/2012
35	Heart Tube	Pediatric Hospital	23/2/2012
36	Blood	Pediatric Hospital	23/2/2012
37	Urine	Pediatric Hospital	23/2/2012
38	Throat	Digestive System Center	23/2/2012
39	Urine	Kidney Center	25/2/2012
40	Endotracheal aspirate	Emergency Hospital	27/2/2012
41	Urine	Kidney Center	28/2/2012
42	Urine	Pediatric Hospital	1/3/2012
43	Urine	Digestive System Center	1/3/2012
From 44 to 50	Urine	Kidney Center	3/3/2012
51	Endotracheal aspirate	University Hospital	4/3/2012
52	Urine	Digestive System Center	5/3/2012
53 and 54	Urine	Kidney Center	6/3/2012
55	Blood	Pediatric Hospital	8/3/2012
From 56 to 61	Urine	Kidney Center	10/3/2012
From 62 to 70	Urine	Kidney Center	13/3/2012
71	Diabetic foot	University Hospital	18/3/2012
72	Sputum	University Hospital	18/3/2012
73	Blood	Pediatric Hospital	19/3/2012
74	Urine	Digestive System Center	19/3/2012
75	Urine	Digestive System Center	19/3/2012
76	Sputum	University Hospital	21/3/2012
77	Urine	Digestive System Center	22/3/2012
78	Urine	University Hospital	25/3/2012
79	Urine	Digestive System Center	26/3/5012
80	Wound	University Hospital	28/3/2012
From 81 to 86	Urine	Kidney Center	31/3/2012
From 87 to 91	Urine	Kidney Center	7/4/2012
From 92 to 96	Urine	Kidney Center	14/4/2012

Table 2. Source and antibiotic sensitivity patterns of 96 *K. pneumoniae* clinical isolates.

Antibiotype	Isolate	Source of isolate	Resistance phenotype
A1	1 35 11, 12, 14, 18, 26, 39, 62, 67, 78, 91	Sputum Heart tube Urine	-2ndceph, 3rdceph, Q, β-β/lactamase
A2	3, 76 4 38 5, 40 69, 74, 83, 84, 86, 95, 96	Sputum Nose swab Throat swab ETA Urine	-2ndceph, 3rdceph, 4thCeph, Q, β-β/lactamase
A3	6 73 54, 75, 93	Sputum Blood Urine	-2ndceph, 3rdceph, 4thCeph, β-β/lactamase
A4	56 7,36	Urine Blood	-2ndceph, 3rdceph, 4thCeph , AG, β-β/lactamase
A5	8 9, 10, 13, 15, 22, 23, 24, 88	ETA Urine	-2ndceph, 3rdceph, Q, AG, β-β/lactamase
A6	71 17, 21 37,57 72	Diabetic foot Urine Sputum	-2ndceph, 3rd ceph,4thCeph, Q, AG, β-β/lactamase
A7	19,89	Urine	-2ndceph, β-β/lactamase
A8	34 20, 48, 52, 70, 82, 85, 90	Sputum Urine	-Q
A9	25, 41, 42, 92	Urine	-2ndceph, 3rdceph, β-β/lactamase
A10	27	Blood	-2ndceph
A11	28	ETA	-2ndceph, 3rdceph, 4thCeph , Cap, Q, β-β/lactamase
A12	29 51 47, 63, 64, 65	Blood ETA Urine	-2ndceph, 3rdceph
A13	31	Blood	-3rdceph, 4thCeph, Cap, AG, β-β/lactamase
A14	32	ETA	-2ndceph, 3rdceph, Cap, Q, β-β/lactamase
A15	33	Blood	-2ndceph, 3rdceph, 4thCeph, AG, β-β/lactamase
A16	43 , 79	Urine	-2ndceph, 3rdceph, 4thCeph, Cap, AG, β-β/lactamase
A17	45	Urine	-2ndceph, 3rdceph, Cap, β-β/lactamase
A18	46	Urine	-2ndceph, 3rdceph, AG, β-β/lactamase

Table 2. Contd.

A19	55 80	Blood Wound	-2nd ceph, 3rd ceph, 4th Ceph, Cap, Q, β-β/lactamase
A20	59	Urine	-2nd ceph, 3rd ceph, Q
A21	61	Urine	-2nd ceph, 3rd ceph, AG
A22	2 16, 30, 44, 49, 50, 53, 58, 66, 68, 77, 81, 87	Throat Swab Urine	-Sensitive to all
A23	94	Urine	-Resistant to All
A24	60	Urine	-AG

AG, aminoglycosides; β-β/lactamase; beta-lactam, beta-lactamase inhibitor; 2nd ceph, second generation cephalosporin; 3rd ceph, third generation cephalosporin; 4th Ceph, fourth generation cephalosporin; Cap, carbapenem; Q, quinolone; ETA, endotracheal aspirate.

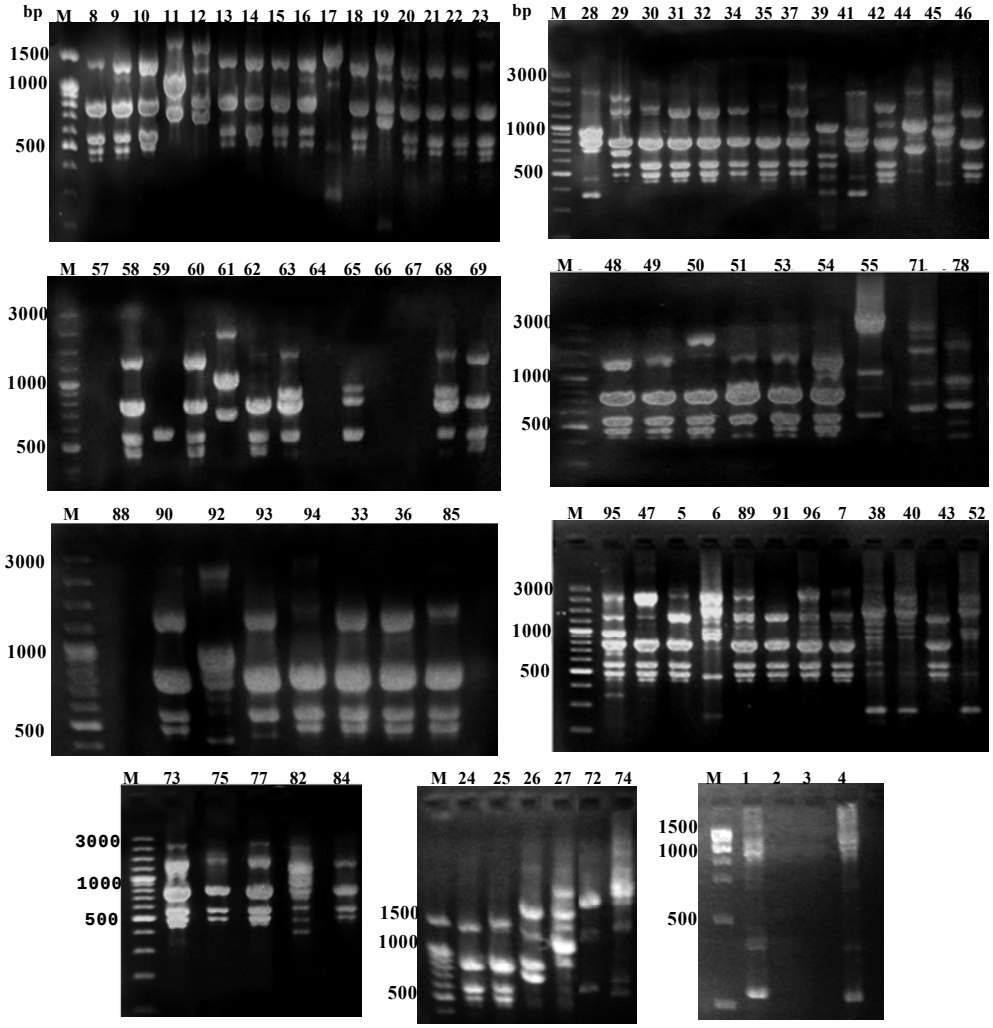

Figure 1. Random amplified polymorphic DNA fingerprinting of clinical isolates of *K. pneumoniae* using (OPA13) primer (Lane M was DNA marker).

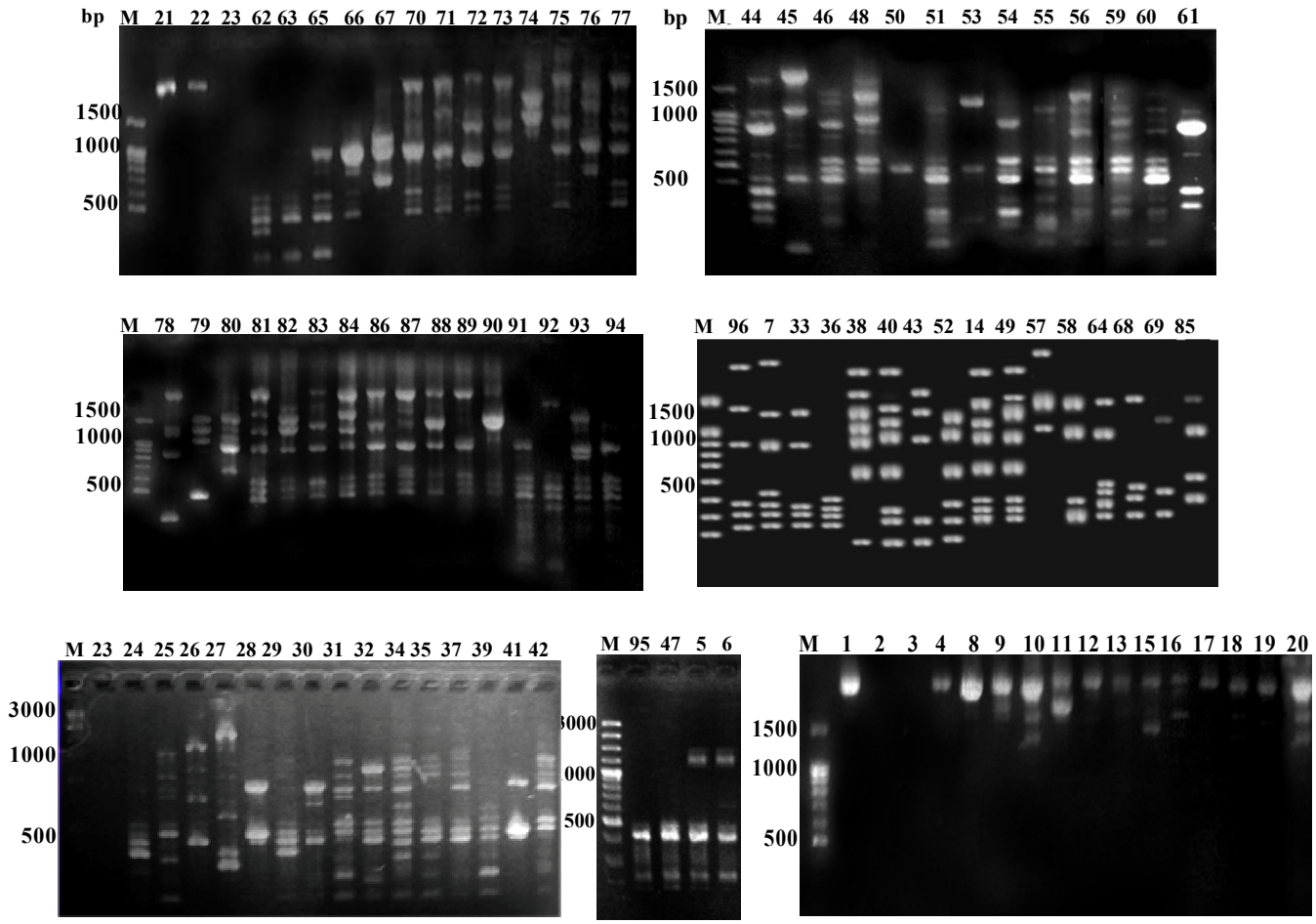

Figure 2. Random amplified polymorphic DNA fingerprinting of clinical isolates of *K. pneumoniae* using (AP3) primer (Lane M was DNA marker).

K. pneumoniae isolates.

In the present study, we also detected that the majority of *K. pneumoniae* isolates were resistant to cephalosporins and beta-lactam/beta-lactamase inhibitor and 16.66% of the isolates were MDR. Also, high incidence of MDR *klebsiella* isolates was established in nosocomial out breaks (Cao et al., 2014). The widespread use of antibiotics especially the expanded-spectrum cephalosporins, was associated with *K. pneumoniae* producing extended-spectrum beta-lactamase in different localities in Egypt (Abdel-Hady et al., 2008) and all over other countries; in Tunisian (Ben-Hamouda et al, 2003), Greece (Souli et al., 2010) and Taiwan (Lin et al., 2010). In contrast, most of the isolates were susceptible to meropenem and amikacin. Similarly, 97% of *K. pneumoniae* isolates were susceptible to carbapenem in the study of lin et al. (2010) and 87.2% of the isolates were susceptible to amikacin in the study of Das et al. (2006).

However, the antibiotic susceptibility patterns did not show enough strain-to-strain variation to discriminate between different isolates (Ben-Hamouda et al, 2003). Differentiation between isolates with slight variations in resistance profiles requires genetic based methods. Therefore, genotypic methods, including plasmid typing, RAPD analysis (de Sauza et al., 2005), PCR fingerprinting and PFGE of chromosomal DNA restriction fragments (Cao et al., 2014) have been used to investigate nosocomial outbreaks of *K. pneumoniae*.

RAPD-PCR is a genotypic identification and characterization system. RAPD profile proves specificity and sensitivity to define bacterial isolates (de Sauza et al., 2005). RAPD analysis has a great ability to type a wide variety of bacterial species and detect the genetic differences between isolates (Lin et al., 2010). It was used in typing various organisms and detection outbreak of nosocomial infections of *K. pneumoniae* at Tunisian neonatal ward (Ben-Hamouda et al., 2003) and Barazil (de Sauza et al., 2005). RAPD analysis is used in identification of phylogenetic diversity and genetic structure of both *K. pneumoniae* and *K. oxytoca* of 120 clinical isolates collected from 22 European hospitals (Brisse and Verhoef,2001).

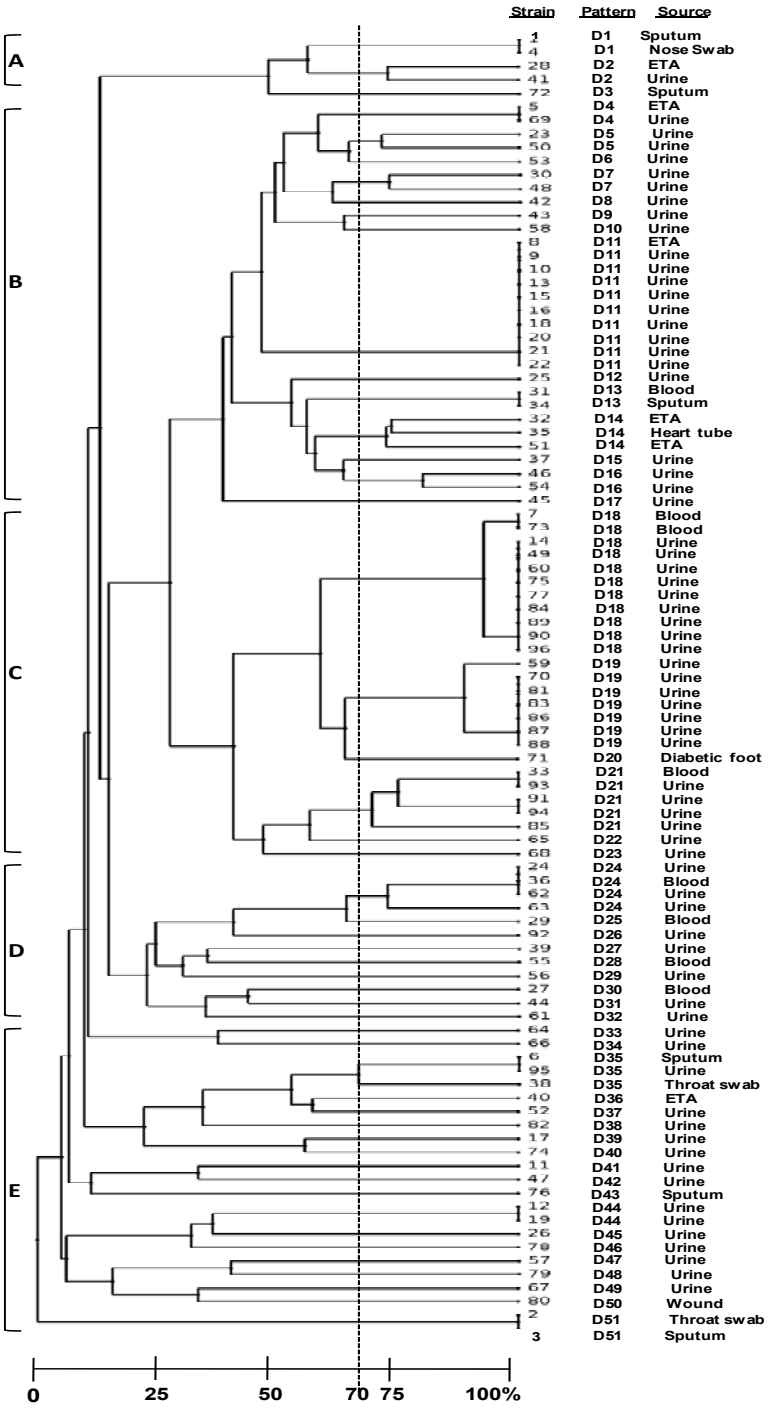

Figure 3. Dendrogram representing random amplified polymorphic DNA analysis of the *K. pneumoniae* isolates with polymorphism percentage (53%) and 52 alleles. The scale indicates similarity percentage. The respective clinical source and the antibiotic sensitivity pattern of each isolate.

However, molecular characterization of *Klebsiella* infection using RAPD profile in Egypt is limited. In the present study the molecular typing of 96 *K. pneumoniae* was performed using RAPD analysis with both primers OPA13 and AP3. RAPD distinguished *K. pneumoniae* into different subtypes based on the numerous fingerprints generated (Figure 3). A specific RAPD patterns D11, D18 and D19 were the most common among *K. pneumoniae* isolates. The main source of these isolates was urine samples obtained from Kidney Center/Mansoura University, ruling

the possibility of the cross infection. In addition, through application of RAPD technique we could detect genetic homogeneity between different isolates obtained from various sources. This can be useful to monitor this pathogen in nosocomial infections. Also, high molecular diversity of the remaining strains (70.83%) could discriminate between isolates on molecular level. Heterogeneous *K. pneumoniae* pathogenic isolates were also observed by de Sauza et al. (2005).

The correlation between antibiotyping and RAPD analysis was also assessed. Two isolates 5 and 69 showed the same antibiotype (A2) and have the same RAPD pattern D4. They were isolated from endotracheal aspirate and urine respectively. In addition six isolates 8, 9, 10, 13, 15 and 22 had the same antibiotype (A5) and the same RAPD pattern D11. Also, the urine isolates 84 and 96 had the same antibiotype (A2) and the same RAPD pattern D18. This confirms that cross acquisition can play an important part in the epidemiology of nosocomial colonization and infection with *K. pneumoniae* at Mansoura University hospitals.

The relationship between RAPD patterns and multiple drug resistant isolates was also analyzed. MDR isolates marked 8, 9, 10, 13, 15 and 22 established the same antibiotype (A5) and the similar RAPD patterns (D11). These isolates represent 37.5% of the total MDR. Horizontal spread has been well-known as the major mechanism for the emergence and maintenance of extended spectrum beta lactamase (ESBL) producing *K. pneumoniae* outbreaks as reported by Paterson et al. (2004). Outbreaks caused by ESBL producing *K. pneumoniae* have been related to the cross transmission. It is predominate in areas with excessive antibiotic use where the potential for patient to patient transmission of organisms is the greatest, such as neonatal units, intensive care units and surgical units (Randrianirina et al., 2009).

In the following study, the outbreak of MDR *K. pneumoniae* among urine samples obtained from Kidney center was monitored by RAPD analysis highlights the need of infection control measures and adoption of new antibiotic policies. Also, improving hospital hygiene and application of stuff training programs should be applied for controlling the spread of microbial infection.

Conclusion

The present work showed a high resistance profile of the studied clinical isolates of *K. pneumoniae.* RAPD analysis showed high molecular diversity among *K. pneumoniae* isolates indicating its discrimination capability in molecular typing. Moreover, this method showed a good potential to identify clonal lineages, as it recognized identical genotypes among *K. pneumoniae* isolates. The present study highlights the need for appropriate epidemiological monitoring of *K. pneumoniae* infections, by using both traditional and molecular methods. As well, monitoring of resistance to broad-spectrum antimicrobials in hospital environment is necessary to control antimicrobial use.

Conflict of interest

There is no conflict of interest.

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