Original Article

Molecular Characterization of Antibiotic Resistance Genes in Pathogenic Bacteria Isolated from Patients in Taif Hospitals, KSA

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ABSTRACT

This study was carried out to detect the distribution of antibiotic-resistant genes in multi-antibiotic resistant bacteria isolated from Saudi Arabian patients in Taif city. Hence, simple methods were followed herein to isolate and characterize the antibiotic resistant bacteria by the common phenotypic, morphological, biochemical and molecular characters. Out of 200 cultures tested, 60 multidrug resistant bacteria isolates were randomly chosen for isolating the antibiotic resistance genes. About 47% of antibiotic resistant tested bacteria were isolated from urine samples and 53% from stool. The study further aimed to analyze antibiotic resistance rates against commonly used antibiotics among bacterial population of urine and stool samples. These bacterial isolates were identified and categorized into eight species, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Citrobacter freundi*, *Enterobactur sakazakii*, *Salmonella* sp. and *Shigella* sp. The isolates exhibited resistance in decreasing order for Clindamycin (83%), Penicillin G (69.6 %), Rifampin (64.7%), Cefotaxime (53.6%), Cefaclor (51.7%), Ceftriaxone (47.2%), Nitrofurantoin (44.2%), and Norfloxacin (39.7%). Maximum resistance to extended-spectrum β-lactam antibiotics occurred in 11.3% of isolates and the production of extended spectrum β-lactamase was achieved by 3.5% of isolates. Multiple resistances to three or more antimicrobial agents were documented. PCR method was used to isolate the antibiotic resistance genes for analyzing the molecular classification of these isolates. It was based on *CTX-M1*, *CTX-M2* and *mecA* genes which were used for rapid assignment of bacteria into genera and species.

The results indicate that all isolates harbor one or more of antibiotic resistance genes and that the PCR technique is a fast, practical and appropriate method for determining the presence of antibiotic-resistance genes. Moreover, the similarity matrix and differentiation between these isolates was studied dependent on RAPD pattern using 8 different primers.

Keywords: Antibiotic resistant bacteria, *CTX-M1*, *CTX-M2*, *MecA* genes, RAPD.

INTRODUCTION

Antibiotics have been used in clinical practice for about 80 years and, throughout that period the problems posed by resistant bacteria have escalated at a pace that has forced near continuous development of new antibacterial drugs. A large variety of antibiotics are currently being used in human and veterinary medicine, but their efficacy has been threatened by microbial resistance. Currently, there is concern over the possible spread of resistance determinants to antimicrobials[1]. Antibiotic resistant bacteria are an increasing threat to public health, as highlighted by a recent estimate that in the US methicillin-resistant *Staphylococcus aureus* (MRSA) may contribute to more deaths than HIV[2]. Methicillin-resistant strains of *S. aureus* were initially documented in the 1960s[3] and have been associated with higher mortality rates than their drug-sensitive counter-parts[4,5]. The prevalence of multi-antibiotic resistant bacteria in recent decades makes an interest to search for new alternative and effective antibiotics. The antibiotic resistant bacteria are identified that are not killed by commonly used antibiotics. When bacteria are exposed to the same antibiotics over and over, the bacteria can change and are no longer affected by the drug due to changing of bacterial membrane, secretion of enzymes by target organisms, modification of site receptors and/or due to genetic reasons[6]. Antibiotic resistance can result also from large genomic changes, such as the acquisition of entire plasmids or mobile elements encoding resistance factors[7,8]. To study the antibiotic resistance ability, the key steps are the isolation and purification of the antibiotic resistant strains. Identification of the isolated antibiotic resistant organism using molecular techniques such as 16 S rRNA, Random Amplification of Polymorphic DNA (RAPD) and plasmid profile causing infectious processes is usually essential for effective antimicrobial and supportive therapy[9]. Initial treatment may be empiric, based on the microbiologic epidemiology of the infection and the patient symptoms[8]. However, the identification of the infectious organism guides the physician in treatment of the disease, because the necessary data on the causal pathogen including its phenotypic and biochemical characters facilitate the choice of suitable and effective antibiotic[10].

The aim of the present study was identified and detected of antibiotic resistance genes in multi-antibiotic resistant bacteria isolated from hospitalized patients in the Taif hospitals, KSA during the 2013-2014 years. Additionally, the present study was targeted to assess the genetic diversity among the different bacterial species by molecular (RAPD-PCR) markers.

MATERIALS AND METHODS

Sample collection and growth

About 200 clinical samples of urine and stool swabs were collected from hospitalized patients in the Taif hospitals, KSA during the period from September, 2013 till January, 2014. Hospitalized patients were asked to undergo screening for nasal carriage of *antibiotic resistance* strains. They were randomly selected from patients at these hospitals. Clinical samples were taken in aseptic conditions and were transported immediately to the microbial genetics laboratory at Biotechnology and Genetic Engineering Unit, Scientific Research Center, Taif University, KSA.

Isolation and purification of clinical bacterial isolates

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 slope into cultures of nutrient agar media.

Antibiotic sensitivity test

About eight types of antibiotics, Ceftriaxone (CRO), Norfloxacin (NOR), Clindamycin (DA), Penicillin G (P), Cefotaxime (CTX), Nitrofurantoin (F), Cefaclor (CEC) and Rifampin (Ra) were used for disc diffusion bioassay according to Ehinmidu[11]. Clinical bacterial isolates suspensions were spread by sterile glass rods on the surface of nutrient agar media. Then antibiotic discs (Bioanalyse®) were placed onto the surface of the inoculated nutrient agar plates. The plates were then incubated at 28°C for 48 h and then inhibition zones were observed.

Biochemical characteristics of antibiotic resistant bacteria

The cultural, morphological and biochemical criteria of the 60 antibiotic resistant bacteria isolates were used as a taxonomic criteria. Gram stain was the key step and was carried out according to Harrigan and McCance[12]. Also, cell morphology was recorded using oil immersion lense, motility test, catalase test and oxidase test according to Cappuccino and Sherman[13].

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-PCR

RAPD analysis was performed according to Moschetti, *et al.,*[14] using different eight operon primers (Table 2). The RAPD-PCR amplification reactions were performed in C1000™ Thermo Cycler Bio-Rad, using the following PCR program: 1cycle at 94°C, 4 min; 35 additional cycles consisting of 94°C for 45 s, 36°C for 60 s and 72°C for 45 s. Amplified DNA products were analyzed by electrophoresis in 1.5% agarose gel run in TBE buffer. The gels were stained with ethidium bromide (5 µg ml^{-1}). Gene Ruler™ 100 pb. DNA Ladder (Fermentas) was used as a standard marker. DNA was visualized by UV illumination and was photographed by a Bio-Rad Gel Doc 2000 device.

Detection of antibiotic genes

PCR amplification for detection the three antibiotic genes *CTX-M1*, *CTX-M2* and *mecA* was carried out according to Alireza *et al*.,[15] and Fallah *et al*.[16]. PCR amplification of *CTX-M1* was carried out using the following primers: 5`- GGT TAA AAA ATC ACT GCG TC-3` (forward) and 5`-

TTG GTG ACG ATT TTA GCC GC-3` (reverse) with an amplicon size of 860 bp. Primers used for *CTX-M2* were: 5`- ATG ATG ACT CAG AGC ATT CG-3` (forward) and 5`- TGG GTT ACG ATT TTC GCC GC-3` (reverse) with an amplicon size of 890 bp. Primers used for mec A were:5`-TCCAGATTACAACTTCACCAG-3` (forward) and5`-CAATTCATATCTTG TACCG-3`(reverse) with an amplicon size of 162 bp. PCR mixtures (25 µL) contained 1 µL of DNA template, 12.5 µL master mix (Promega) and 1 pM of each primer and 9.5 µL sterilized distilled water. PCR amplifications were performed in C1000^{TM} Thermo Cycler Bio-Rad using the following program for *bla*$_{IMP}$: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s of annealing at 54 °C, and 1 min of extension at 72 °C, with a final extension of 7 min at 72 °C. For *CTX-M1*and *CTX-M2*, amplification was carried out with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 1 min at 95 °C, 1 min of annealing at 60 °C, and 1 min of extension at 72 °C, with a final extension of 10 min at 72 °C. For mecA, amplification was carried using CTX-M1 program with annealing at 58 °C for 45 s. . PCR products were run on 1.5% agarose gels, stained with ethidium bromide and visualized by UV illumination and were photographed by a Bio-Rad Gel Doc 2000 device.

Data analysis

In order to determine the genetic relationship among studied bacteria, RAPD data were scored for presence (1) or absence (0) of the bands using Gene Tools software from Syngene. A simple matching coefficient was estimated by means of the Jaccard's coefficient to construct a similarity matrix. Cluster analysis and dendrogram were produced on the basis of the un-weighted average pair group method (UPGMA) using the NTSYS-PC Statistical Package[17].

RESULTS AND DISCUSSION

Isolation of antibiotic resistant bacteria

About 200 clinical samples of urine, wound and stool swabs were collected from Saudi Arabian patients in Taif city and were then analyzed for presence of pathogenic bacteria on nutrient agar media. All samples showed obvious bacterial growth that was surveyed for presence of multidrug resistant bacteria. Out of 200 cultures tested, 60 multidrug resistant bacteria isolates were randomly chosen for isolating the antibiotic resistance genes. These isolates were given a code number UR-1 to UR-28 for the urine isolates, W-55 for the wound isolate and ST-29 to ST-60 for the stool isolates.

Biochemical characteristics of multidrug resistant bacteria

Based on cultural, morphological and biochemical characteristics given in Table (1), the 60 multidrug resistant bacteria isolates were classified into 8 different groups as follows.

Group 1

This group includes 15 isolates, all of them were gram negative bacteria, rod shaped and motile cells. They showed positive results regarding oxidase and catalase biochemical tests. On MacConkey agar, those 15 isolates grew well and produced rose pink to red colonies. By surveying bacterial characteristics, these isolates could be identified as belonging to *Escherichia coli*.

Group 2

Six isolates were belonging to this group and they were gram positive short coccui bacteria and non motile cells. They showed positive results with regard to

catalase and oxidase tests. Other biochemical tests showed negative results. They grew on mannitol salt agar and gave golden yellow colonies. These isolates were identified as belonging to *Staphylococcus aureus*.

Group 3

This group includes nine isolates and they were gram negative rod shaped and motile cells. They grew well on nutrient agar, they produced positive results with regard to catalase and oxidase tests. These isolates were showed heavily and green colonies pigment in medium, and they were identified as belonging to the *Pseudomonas aeruginosa*.

Group 4

This group includes eight isolates. They were gram negative, rod shaped and non motile bacterial cells. They produced positive results with regard to catalase testes and negative results regarding to oxidase test. These bacterial isolates were identified as strains of *Klebsiella pnemoniae*. For confirmation and to preserve those isolates, round and large undulate white translucent mucoid colonies were produced on MacConkey agar medium.

Group 5

Nine isolates were belong to this group and they were gram negative, rod shaped and motile bacterial cells. Regarding biochemical tests, these bacterial isolates gave positive results against oxidase and catalase tests. These bacterial isolates were identified as strains belonging to *Citrobacter freundii*.

Group 6

This group contains five isolates and they were gram negative, rod shaped and motile bacterial cells. Bacterial isolates of this group gave positive results in view of oxidase and catalase tests. These bacterial isolates were identified as strains belonging to *Enterobactur sakazakii*.

Group 7

Five isolates were belonging to this group. They were gram negative bacteria, rod shaped and motile cells. They showed positive results regard to catalase and biochemical tests. These bacterial isolates of this group were identified as belong to *Salmonella* sp.

Group 8

This group contains three isolates and they showed identical cultural and morphological results like that obtained by bacteria of group No. 7. They also gave similar results regarding biochemical results like bacterial isolates No. 7 except for oxidase reaction which was negative. These bacterial isolates were identified as belonging to *Shigella* sp. The identification of the infectious organism guides the physician in treatment of the disease, because the necessary data on the causal pathogen including its phenotypic and biochemical characters facilitate the choice of suitable and effective antibiotic[10].

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed by the Bioanalyse disc diffusion method using nutrient agar plates, according to the Clinical and Laboratory Standards Institute guidelines[18]. The antimicrobial agents tested and their corresponding concentrations were as follows: Nitrofurantoin (300 mg/disk), Cefaclor (30 mg/disk), Rifamin (5 mg/disk), Cefotaxime (30 mg/disk), Clindamycin (2 mg/disk), Penicillin G (10 U/disk), Norfloxacin (10 mg/disk), Ceftriaxone (30 mg /disk). After incubating the inoculated plates aerobically at 28 °C for 48 h, the susceptibility of the 60 isolates to each

antimicrobial agent was detected using Combination Disk Diffusion Test (CDDT)[19] and the results were interpreted in accordance with criteria provided by CLSI[18]. All the 60 isolates were resistant to one or more antimicrobial agent. For example, the CDDT showed that among the 60 isolates strain number W-55 was resistance to seven of eight antimicrobial agents, while isolate UR-17 was sensitive to all tested antibiotics (Figure 1). The resistance pattern of the all 60 isolates to the eight antimicrobial agents tested in this study are summarized in Figure 2. 48 of the isolates were found to be the most common finding to the resistance to the Clindamycin (83%), followed by 42 bacterial strains were found to be resistance to Penicillin G (69.6%). Cefotaxime resistance was found in about 36 bacterial strains (53.6%). The percentage values of other antimicrobial agent like Cefaclor, Ceftriaxone, Nitrofurantoin, and Norfloxacin were 51.7, 47.2, 44.2 and 39.7 %, respectively. In recent years *CTX-M* enzymes as the most common extended-spectrum β-lactamase have been reported, especially in Europe and North America[2]. Different types of this enzyme have been identified and reported. Therefore, in this study we evacuated the prevalence of this type of β-lactamase enzyme. This enzyme derives from an hospetal bacterium and shows higher activity against Cefotaxime than Ceftazidime[1,20].

PCR analysis for CTX-M and mecA genes

The PCR amplification products of *mecA, CTX-M1* and *CTX-M2* genes producing in *Staphylocuccus aureus* and *E. coli* isolates were show in Figure 3 and 4. Our results showed that the three genes were identified in some isolates. Six isolates of *Staphylocuccus aureus* found to be carried *mecA* gene (Figure 3), that in some aspects is similar to the report from Saudi Arabia[21]. While, *CTX-M1* gene was observed in three *E. coli* isolates and *CTX-M2* in five *E. coli* isolates. Interestingly, isolate number 40 carried both *CTX-M1* and *CTX-M2* genes (Figure 3). Surprisingly, when PCR was carried out to isolate *CTX-M2* gene, isolates numbers 40, 44 and 58 showed different band size in harboring the *CTX-M2* gene (around 580 bp) than isolates numbers 32 and 34 which have the actual size of the *CTX-M2* gene (890 bp). It may be possible that these isolates harbor same gene with different molecular weight. This could be due to that the isolates numbers 40, 44 and 58 are different type than isolates numbers 23 and 34. Therefore in the nearest future, we will try to identify these isolates using 16s rRNA and also we will try to identify the sequence of *CTX-M2* gene in isolates numbers 40, 44 and 58. The gene involved in the production of this enzyme was placed on Mobile elements called ISEcp1, which can exist in different regions of the genome of the bacterium. *E. coli* is the most dominant pathogen carrying CTX-M enzyme gene. The strains which carry this gene are often isolated in Bacteremia or Gastroenteritis infections[15,20].

RAPD-PCR analysis

The PCR-based RAPD fingerprinting method, utilizing arbitrary oligonucleotides is particularly a powerful tool for genetic studies and it is useful as a screening genotyping method[22]. RAPD can generate various fingerprint patterns with unlimited number of primers[23]. In this study, 8 RAPD primers were used for estimating of genetic diversity of antibiotic resistance bacteria. RAPD reactions were performed in duplicate and all amplification products were found to be reproducible (Table 2). The RAPD-PCR results using primer (OPA-10) has showed a total of 20 bands in these 60 used antibiotic resistance bacteria ranged from 250 bp-1900 bp. Three common bands were observed in all isolates which exhibited about 15% monomorphism, while the other 17 fragments have showed 85% polymorphism (Table 2).

This primer detected a unique fragment, over 1.5 kbp, specific to isolate No. 45 (Data not shown). In case of OPA-03 primer, a total of sixteen fragments have showed 100% polymorphism among the 60 isolates (Figure 5). The molecular size of the amplicon products ranged from 200 bp-1950 bp. Also, this primer recognized different unique fragments at 250 bp specific to isolate No. 13. According to genetic similarity and intraspecies differentiation, the 60 isolates were grouped into four different clusters (A, B, C and D) with about 86% genetic similarity. Most isolates were grouped in clusters A and B. 25 isolates, mostly isolated from urine samples were grouped in cluster A (Figure 6). On the other hand, 22 isolates, mostly isolated from stool samples, were grouped in cluster B (Figure 6). 11 of 60 isolates, immediate between urine and stool samples, were attributed into third major cluster C. Interestingly, cluster D accounted for only two unique isolates (Figure 6). RAPDs were proved to be useful as genetic markers in antibiotic resistance bacteria fingerprinting. 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[24,25]. 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[9,26,27].

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Table 1. The morphological and biochemical characterization of antibiotic resistance bacteria

Proposed species	Source	Morphology	Gram Stain	Motility	Oxidase	Catalase
Escherichia coli	Stool	Rod shaped	-	+	+	+
Staphylococcus	Urine	Short coccui	+	-	+	+
Pseudomonas	Wound	Rod shaped	-	+	+	+
Klebsiella	Stool	Rod shaped	-	-	-	+
Citrobacter	Urine	Rod shaped	-	+	+	+
Enterobactur	Urine	Rod shaped	-	+	+	+
Salmonella	Stool	Rod shaped	-	+	+	+
Shigella	Stool	Rod shaped	-	-	-	+

Table 2. Sequences and polymorphism level detected by the eight decamer arbitrary primers that have been used for RAPD analysis

Primers name	Primer sequences	Total Bands	Monomorphic Bands	Polymorphic Bands	% Monomor-phism	% Polymor-phism
OPA-01	5'-CAGGCCCTTC-3'	16	3	13	18.7	81.3
OPA-03	5'-AGTCAGCCAC-3'	16	0	16	00.0	100
OPA-04	5'-AATCGGGCTG-3'	17	2	15	11.7	88.3
OPA-05	5'-AGGGGTCTTG-3'	16	2	14	12.5	87.5
OPA-09	5'-GGGTAACGCC-3'	14	2	12	14.2	87.8
OPA-10	5'-GTGATCGCAG-3'	20	3	17	15.0	85.0
OPA-11	5'-CAATCGCCGT-3'	17	4	13	23.5	76.5
OPD-05	5'-TGAGCGGACA-3'	17	2	15	11.7	88.3
Total		133	18	115	13.5	86.5

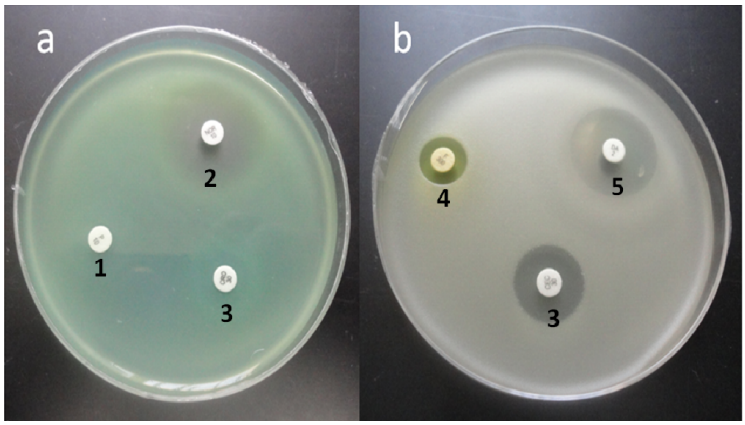

Figure 1. The combination disk diffusion test of the strains number a) W-55 and b) UR-17. 1: Penicillin G, 2: Norfloxacin, 3: Cefaclor, 4: Nitrofurantoin and 5: Clindamycin

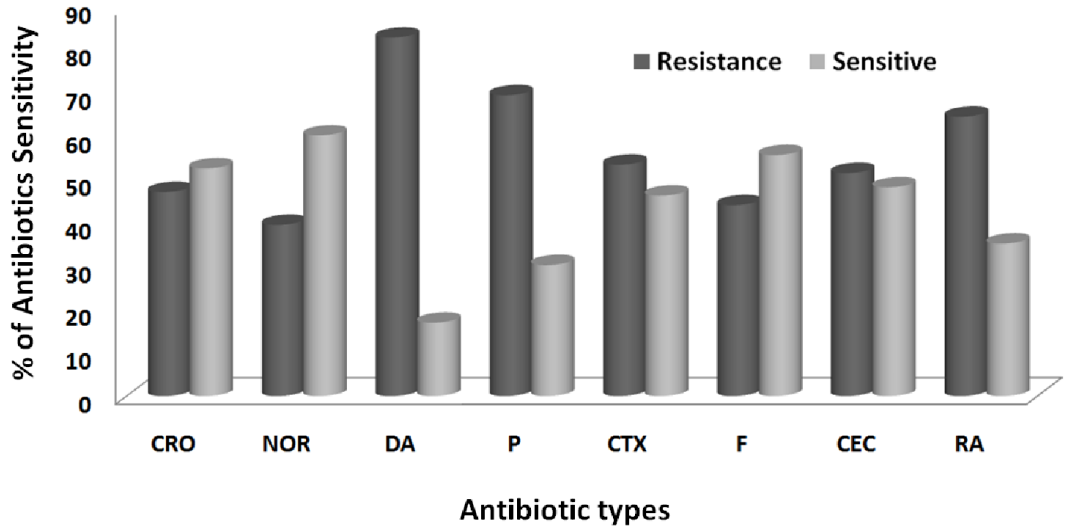

Figure 2. Antimicrobial resistance profiles of sixty isolates. Whereas, CRO = Ceftriaxone, NOR = Norfloxacin, DA = Clindamycin, P = Penicilin G, CTX =Cefotaxime, F = Nitrofurantoin, CEC = Cefaclor and Ra = Rifampin

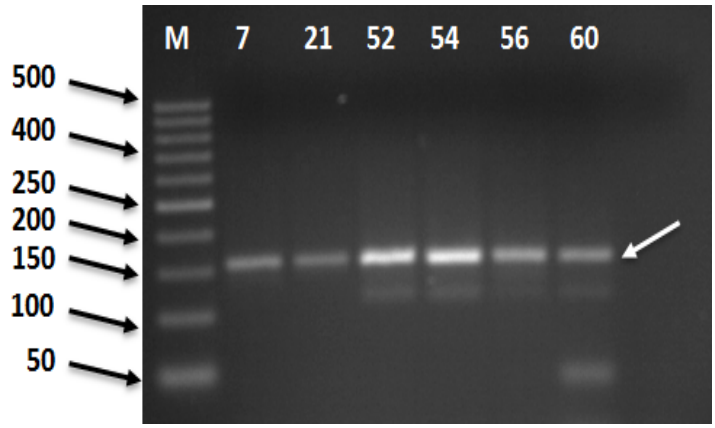

Figure 3. Amplification of *MecA* gene producing in *Staphylocuccus aureus* isolates by single PCR. M; 50-bp DNA ladder. *mecA* gene were found in isolates number 7, 21, 52, 54, 56 and 60

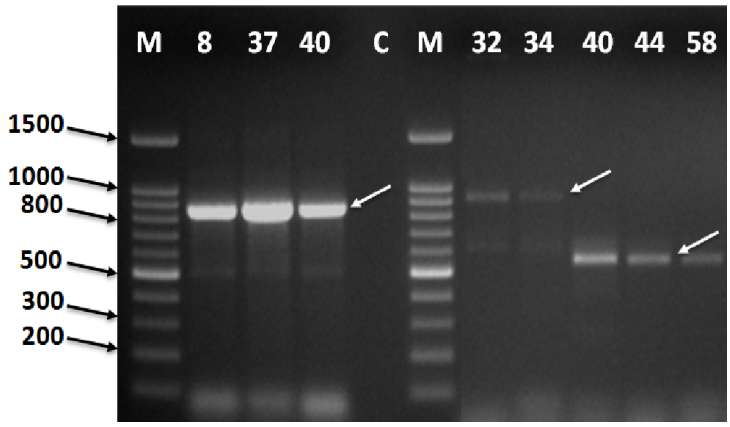

Figure 4. Amplification of *CTX-M1 and CTX-M2* genes producing in *E. coli* isolates by single PCR. M; 100-bp DNA ladder. *CTX-M1* gene were found in isolates number 8, 37 and 40, while, *CTX-M2* were found in isolates number 32, 34, 40, 44 and 58

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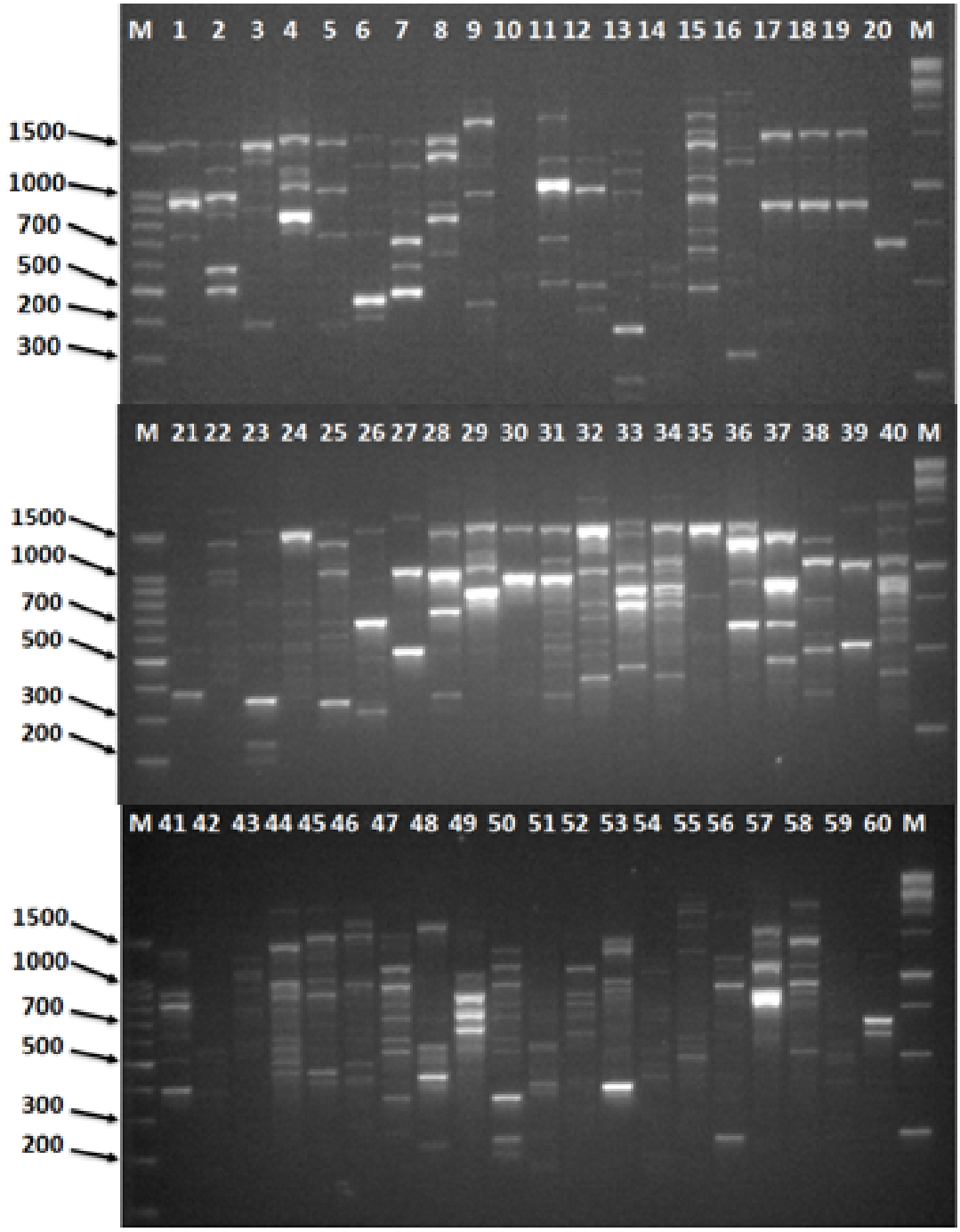

Figure 5. RAPD profile of the sixty antibiotic resistance isolates generated with OP-A3 random primers. M: is 100 bp DNA ladder

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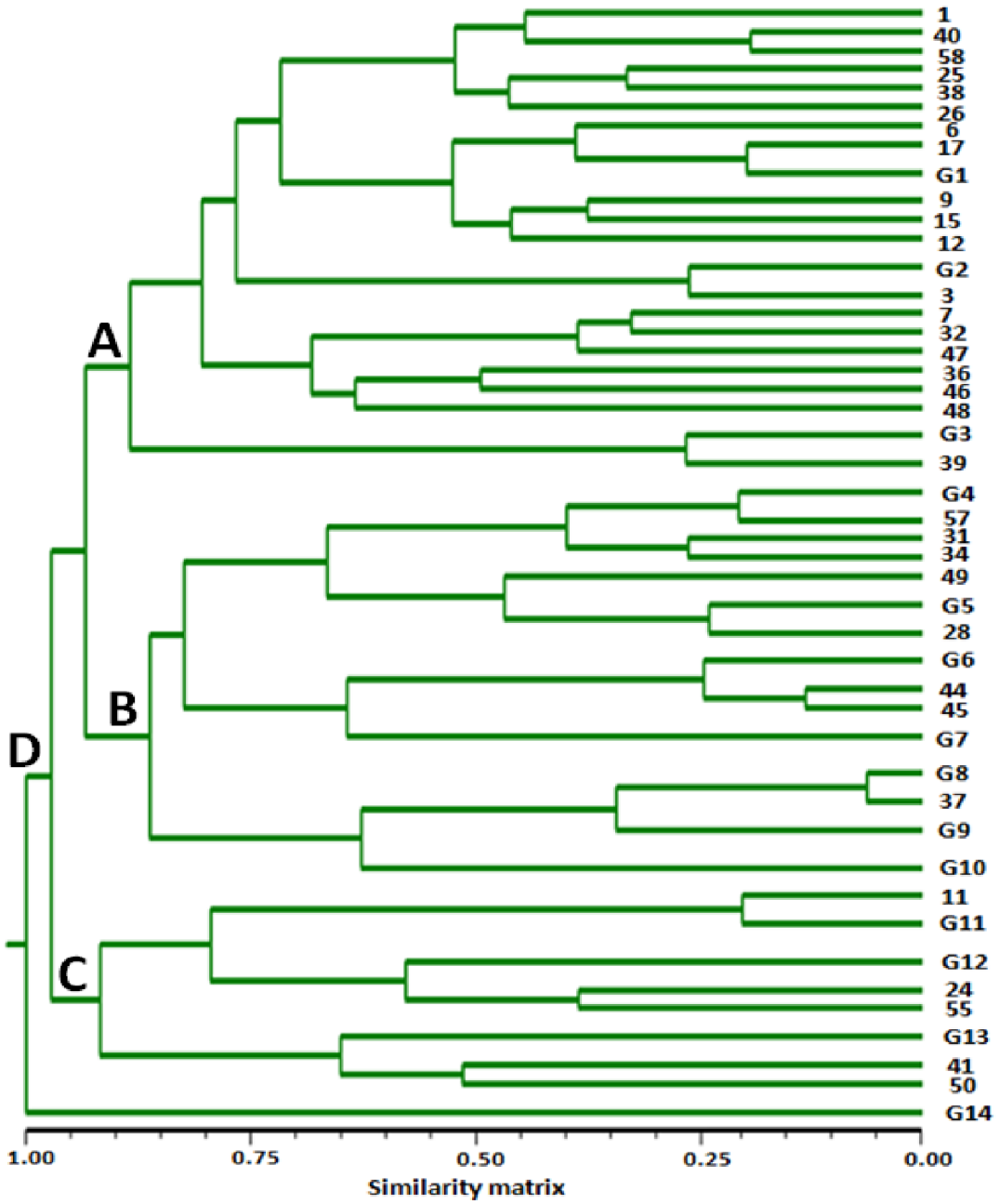

Figure 6. Dendrogram analysis among the 60 antibiotic resistance isolates based on the eight RAPD primers. Whereas, G1=isolates 18, 19; G2=2, 51; G3 =13, 30; G4=4, 42; G5=27, 60; G6= 5, 20; G7=33, 54; G8= 8, 59; G9= 14, 53; G10= 10, 29, G11=16, 21; G12= 22, 35; G13=23, 43 and G14= 52, 56