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Original article

Antibiotic resistance profile and random amplification typing of β-lactamase-producing Enterobacteriaceae from the local area of Al-Taif and nearby cities in Saudi Arabia

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Background: The emergence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* constitutes a serious problem because of the transfer of resistance genes from one organism to another.

Objectives: To screen the antibiotic susceptibility of *E. coli* and *K. pneumoniae* from inpatients at King Abdul-Aziz Hospital, Al-Taif, Saudi Arabia and to detect common ESBL genes, and random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) genotyping of the Enterobacteriaceae.

Methods: We analyzed 43 bacterial strains isolated from clinical samples of sporadic cases for their reactivity to different antibiotics. Strains that showed resistance by monoplex PCR were screened for ESBL TEM- and CTX-M-1-, -2-, and -9-encoding genes. Random amplifications were used for typing the resistant strains.

Results: The majority of the ESBL containing strains were sensitive to meropenem, amikacin, gentamicin, and tigecycline. PCR detection using ESBL gene-specific primers showed that 17 of 43 strains harbored genes for ESBL TEM, CTX-M-1, or CTX-M-2. RAPD typing revealed marked variation among the ESBL-producing *E.coli* in relation to each other.

Conclusions: Considerably high incidence of ESBL-producing bacteria was present patients from the local area of Al-Taif and nearby cities in Saudi Arabia with TEM and CTX-M subtypes being the most commonly detected variants. There was evidence of a polymorphic genetic pattern among ESBL-producing bacteria.

Keywords: Antibiotic resistance, *E.coli*, *Klebsiella pneumoniae*, RAPD typing, Saudi Arabia

Gram-negative bacteria are potential causes of both nosocomial and community-acquired infections. Multiple antibiotic resistance to broad-spectrum β-lactams are considered one of the most important traits. Antibiotic resistance of Enterobacteriaceae is mainly accompanied by the production of extended spectrum β-lactamases (ESBLs) that hydrolyze third-generation cephalosporins and aztreonam, but can be inhibited by clavulanic acid [1–3].

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ESBLs are primarily produced by gram-negative organisms of the Enterobacteriaceae family, especially *Escherichia coli* and *Klebsiella pneumoniae* [4, 5]. The Ambler classification, clusters the enzymes into 4 molecular classes (A–D) according to their amino acid sequences [6]. ESBLs are class A β-lactamases that cleave oxyimino-cephalosporins and monobactams, but not cephamycins or carbapenems. Although there are many genotypes of ESBLs, SHV, TEM, and CTX-M types are considered the most common [7]. CTX-M-type ESBLs, which are non-TEM and non-SHV derivatives, represent a new and rapidly growing family of molecular class-A ESBLs

[8]. According to their amino acid sequence similarities, they have been classified into five groups: 1, 2, 8, 9, and 25/26, and, to date, more than 40 CTX-M β-lactamases have been reported.

ESBL-producing bacteria are among the common microorganisms that produce severe diarrhoea, postoperative abdominal wound sepsis, urinary tract infections, and respiratory tract infections [2]. Cephalosporins commonly used in patients with septic infections are ineffective in the case of ESBL-producing bacteria. ESBL-producing bacteria can acquire resistance to antimicrobials such as the aminoglycosides, cotrimoxazole, tetracyclines, trimethoprim, and quinolones with the potential of the development of multidrug resistant microbes [9–12].

There is a shortage of the new antibiotics especially for gram-negative bacteria that produce ESBLs [13]. Delays in laboratory diagnosis and the use of inappropriate antibiotic therapy are among factors that increase the severity of the disease with a subsequent increase in mortality [14, 15]. Accordingly, rapid detection of ESBL-producing bacteria may aid in selecting the appropriate antibiotic with a subsequent improvement in the antibacterial outcomes [16]. Rapid detection is also necessary to screen patients and subsequently improve hospital infection control policies, avoid misuse of antibiotics, thus prolonging the efficacy of the currently available antibiotic armamentarium [17, 18].

Current techniques for detecting ESBL producers are based on the determination of susceptibility to expanded-spectrum cephalosporins followed by the inhibition of the ESBL activity, mostly by the use of clavulanic acid or tazobactam [19]. The double-disk synergy test and the ESBL "E-test" were proposed for that purpose. Sensitivities and specificities of the double-disk test and of the E-test are good, ranging from 80% to 95% [20]. Automated bacterial identification and antibiotic susceptibility testing are also used in the detection of ESBL-producing organisms. The performances of those systems differ depending on the species investigated, with much higher sensitivity (80%–99%) than specificity (50%–80%) [19, 20].

Those tests require overnight growth, meaning that up to 24 to 48 h can elapse, before ESBL production is detected once the isolate has grown [19, 20]. This may result in a delay in the initiation of appropriate antibiotic therapy [17]. Molecular detection of ESBL genes by PCR is an attractive alternative [19–21]. In the current study, we intended to conduct antibiotic susceptibility screening of *E. coli* and *K. pneumoniae* from inpatients and detect the common ESBL genes. Meanwhile, we intended to genotype the ESBL positive bacteria with random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR).

Materials and methods

Bacterial culture and identification

Our study protocol was approved by Taif University Medical Ethics Review Board (project No. 3364-435-1) in accordance with the guidelines for the protection of human subjects. Samples were collected for clinical purposes from inpatients from the local area of Al-Taif and nearby cities at King Abdul-Aziz Hospital, Al-Taif, Saudi Arabia between February 1st and August 30th 2015 after patient consent documented on standard hospital forms. The clinical samples were from various origins: 38 were from urinary tract infections, and 5 were from suppurative wounds in the perineum, sepsis of various postoperative wounds, and the liver. We received 43 bacterial isolates anonymized by coding linked to patient identities from the clinical laboratory, which were derived from the samples originally collected for clinical purposes. The isolates were subcultured on selective media including blood agar and MacConkey agar [22] and gram-negative rods were selected for further identification [23].

Bacterial isolates confirmation and antimicrobial susceptibility tests

Cultured bacteria were resuspended in 0.45% saline and matched to the required McFarland units. Two milliliters of bacterial suspension were automatically loaded into a VITEK 2 microbial identification system (bioMérieux, Durham, NC, USA) for identification with gram-negative bacilli and antimicrobial susceptibility testing-GN04 cards. Reference strains including *E. coli* American Type Culture Collection 25922 were used as controls. Criteria used to characterize ESBL are those of the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards) [24]. The gram-negative bacilli were tested for their susceptibility to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefalothin, cefoxitin, ceftazidime, ceftriaxone, cefepime, imipenem, meropenem, amikacin, gentamicin,

ciprofloxacin, tigecycline, nitrofurantoin, and trimethoprim/sulfamethoxazole.

Molecular characterization of beta-lactamases in Enterobacteriaceae

DNA extraction

DNA was extracted from ESBL-positive isolates using a DNA extraction kit (Koma Biotech, Seoul, Korea). Briefly, 1 mL of bacterial suspension was centrifuged for 5 min at 3000 rpm. The supernatant was discarded and the pellet was resuspended in 200 µL lysing solution and 20 µL proteinase K at 60°C for 30 min and then further incubated at 95°C for 15 min. Then, 200 µL of ethanol was added to each sample, which was vortexed and loaded onto an XPTG mini column. After centrifugation at 13,000 rpm for 1 min, the bound DNA was washed twice then the excess ethanol was discarded by centrifugation for 3 min at 13,000 rpm. Elution of the DNA was completed by adding 100 µL of sterile double-distilled water onto the membrane bound DNA, and centrifuged at 13,000 rpm for 2 min.

Polymerase chain reaction for detection of the ESBL-genes

PCR was conducted for individual TEM, CTX-M group 1, group 2 and group 9 DNA was using 0.4 pmol/µL of each primer (**Table 1**) [25]. The PCR amplification was conducted using a PCR master mix (Solis BioDyne, Tartu, Estonia) as follows: initial denaturation at 95°C for 5 min; 40 cycles of 95°C for 40s, 60°C for 40s, and 72°C for 1 min; and a final elongation step at 72°C for 10 min. Amplicons were visualized after separation of amplicons in 1.5% gel electrophoresis in Tris-acetate EDTA and staining with ethidium bromide. A 100 bp DNA ladder (Solis BioDyne) was used to provide size markers.

Table1. Oligonucleotide sequences used to identify genes encoding important β-lactamase (*bla*) genes and random primers used for genotyping of the bacteria

Primer name	Primer Sequence 5′–3′	Position	Amplicon size
TEM-1 and TEM-2			
MultiTSO-T_forward	CATTTCCGTGTCGCCCTTATTC	13–34	800
MultiTSO-T_reverse	CGTTCATCCATAGTTGCCTGAC	812–791	
CTX-M group 1, group 2 and group 9			
MultiCTXMGp1_forward	TTAGGAARTGTGCCGCTGYA	61–80	688
MultiCTXMGp1-2_reverse	CGATATCGTTGGTGGTRCCAT	748–728	
MultiCTXMGp2_forward	CGTTAACGGCACGATGAC	345–362	404
MultiCTXMGp1-2_reverse	CGATATCGTTGGTGGTRCCAT	748–728	
MultiCTXMGp9_forward	TCAAGCCTGCCGATCTGGT	299–317	561
CTXMGp9_reverse	TGATTCTCGCCGCTGAAG	859–842	
Random primers			
OP-A1	CAGGCCCTTC		
OP-A3	AGTCAGCCAC		
OP-A4	AATCGGGCTG		
OP-A5	AGGGGTCTTG		
OP-A6	GGTCCCTGAC		
OP-A7	GAAACGGGTG		
OP-A8	GTGACGTAGG		
OP-A9	GGGTAACGCC		
OP-A10	GTGATCGCAG		
OP-B7	GGTGACGCAG		
OP-D5	TGAGCGGACA		

Bacterial genotyping using RAPD analysis

For RAPD analysis, 11 different 10-mer random primers, which were preselected for their performance with DNA from isolates of Enterobacteriaceae (**Table 1**) were used (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Following experiments to optimize component concentrations, PCR amplification of random primers was conducted according to published methods [26, 27]. Briefly, 1 μL (20 ng) of genomic DNA, 12.5 μL of Go Taq Green Master Mix, Promega, USA, 1 μL of primer (20 pmol), and deionized ultrapure distilled water up to a total volume of 25 L were used. For DNA amplification, an initial denaturation at 94°C for 5 min was conducted, and followed by 94°C for 30 s, 35°C for 1.5 min, and 72°C for 2.5 min for 40 cycles and a final elongation step at 72°C for 7 min. DNA amplicons were separated by electrophoresis in 1.5% agarose gel in TBE. The gels were stained with ethidium bromide (5 μg ml^{-1}). A 100 bp DNA ladder (with 1500 bp and 500 bp reference bands) was used to provide molecular weight markers suitable to determine DNA fragment sizes (AccuRuler 100 bp DNA RTU Ladder, Ready-To-Use Cat. No. 02001-500; Maestrogen, Las Vegas, NV, USA). DNA was visualized by illumination with ultraviolet light and then photographed using a Gel Doc 2000 device (Bio-Rad Laboratories, Hercules, CA, USA).

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 similarity coefficient for pairwise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to cluster analysis by an unweighted pair group method for arithmetic mean and a dendrogram was generated. The computations were performed using the program NTSYS-PC version 2.01 [28]. Jaccard's similarity matrix was subjected to principal component analysis.

Results and Discussion

There is a current increase in ESBL-producing bacteria worldwide mainly *E. coli*. Among the 43 tested samples, 27 isolates were *E. coli* and 16 were *K. pneumoniae* (**Table 2**).

ESBL-producing bacteria were detected in 17/43 isolates (39.5%): 14 strains were *E. coli* and 3 strains were *K. pneumoniae*. All *E. coli* isolates and 2/3 of *K. pneumoniae* were positive for bla_{TEM} (**Table 3**).

Only 2/14 *E coli* and 2/3 *K. pneumoniae* showed resistance to nitrofurantoin. Ten of 14 *E. coli* and 3/3 *K. pneumoniae* ESBL strains were resistant to ciprofloxacin. ESBLs confer resistance to the oxyimino-cephalosporins (i.e., cefotaxime, ceftriaxone, ceftazidime) and monobactams (i.e., aztreonam) [29]. ESBLs do not hydrolyze the cephamycins (e.g., cefoxitin and cefotetan), or the carbapenems (imipenem and meropenem), and their hydrolytic activity can be inhibited by several β-lactamase inhibitors such as clavulanic acid and tazobactam [29].

Table 2. Antibiotic sensitivity of the examined samples (*Escherichia coli* and *Klebsiella pneumoniae*).

Antimicrobial	Resistant strains						Nonresistant strains					
	E. coli			*K. pneumoniae*			*E. coli*			*K. pneumoniae*		
	R	S	I	R	S	I	R	S	I	R	S	I
Ampicillin	14	0	0	3	0	0	12	1	0	10	3	0
Amoxicillin/clavulanic acid	2	8	4	1	1	1	1	8	4	7	6	0
Piperacillin/tazobactam	5	8	1	2	0	1	2	11	0	7	6	0
Cefoxitin	2	9	3	1	1	1	0	11	2	7	6	0
Ceftazidime	14	0	0	3	0	0	0	13	0	7	6	0
Cefepime	14	0	0	3	0	0	0	13	0	7	6	0
Imipenem	0	13	1	0	3	0	0	13	0	7	6	0
Meropenem	0	13	0	0	3	0	0	13	0	7	6	0
Amikacin	0	14	0	1	2	0	0	13	0	7	6	0
Gentamicin	4	10	0	1	2	0	2	11	0	8	5	0
Ciprofloxacin	10	4	0	3	0	0	2	11	0	8	5	0
Tigecycline	0	14	0	0	2	1	0	13	0	7	5	1
Nitrofurantoin	2	11	1	2	0	1	0	13	0	10	0	3
Trimethoprim/Sulfamethoxazole	9	5	0	3	0	0	7	6	0	8	5	0

Two of 14, *E. coli* and 1/3 *K. pneumoniae* showed resistance to cefoxitin and clavulanic acid inhibition, while all the tested strains were sensitive to imipenem and meropenem (**Table 3**). In a Russian study, 39.7% of *E. coli* isolates and 21.8% of *K. pneumoniae* isolates, which were ESBL-producing strains were resistant to ciprofloxacin [30]. Unfortunately, ESBL-producing organisms often also possess resistance determinants to other important antibiotic groups, such as aminoglycosides and fluoroquinolones, leaving an extremely limited range of effective agents [31]. Only four *E. coli* strains were found to be resistant to gentamicin, while all *E.coli* strains were sensitive to amikacin. A single *K. pneumoniae* strain was found to be resistant to both gentamicin and amikacin.

In the present study, the majority of the bla_{TEM}-type ESBLs were predominant (16/17) followed by bla_{CTX-M} group 1, 3/3 *K. pneumoniae*, and 2/14 *E. coli* isolates were positive for bla_{CTX-M} genes from CTX-M group 1. Only a single *E. coli* isolate was positive for the CTX-M-group 2, and none were positive results for the CTX-M group 9 gene (**Table 3**). *K. pneumoniae* isolate 5 (negative for TEM positive for CTX-M-1) was more resistant than the other two *K. pneumoniae* (positive to TEM and CTX-M-1) because they showed resistance to amikacin and gentamicin. The negative PCR results in this report do not negate the possibility that modified bla_{CTX-M} were present in these isolates. Because of the increased complexity of β-lactam resistance in gram-negative organisms, the key to effective surveillance is the use of both phenotypic and genotypic analyses in concert [32].

Molecular markers are efficient tools for molecular identification and estimation of relatedness through DNA fingerprinting. RAPD markers were developed by Williams et al. [26]. RAPD technique using single-arbitrary-10-mer oligonucleotides was used to amplify discrete fragments of DNA using PCR. This technique has been used extensively in many different applications and in different bacterial species because of its simplicity [27]. Genomic diversity of *K. pneumoniae* and *E. coli* isolates (n = 17) was investigated by RAPD analysis. The RAPD results showed polymorphic numbers of the amplicons in different *E. coli* (n = 14) and *K. pneumoniae* (n = 3) isolates described in **Table 4** (**Figure 1**).

The random primers yielded 207 distinct bands, 142 (68.6%) were considered as polymorphic and 65 (31.4%) were considered as monomorphic (**Table 4**). The number of amplified fragments scored for each isolate was recorded. The amplified products among the *E. coli* and *K. pneumoniae* isolates were polymorphic.

A phylogenetic tree was constructed for the 14 different *bla E. coli* strains compared with an ESBL-negative *E. coli* control that showed a different pattern of DNA fragments with the different primers (data not shown). The total number of bands varied from 22 with primer OPA-10, to 15 with primer OPA-09 (**Table 4**). The RAPD-PCR results using OPA-06-primer showed the highest polymorphism, the 16 fragments showed 100% polymorphism (**Table 4**).

The molecular size of the amplicon products ranged from 75 bp to 1450 bp. These findings denote that RAPD markers are effective in detecting similarity between *E. coli* strains and provide a potential tool for studying the interstrain genetic similarity and the establishment of genetic relationships.

According to genetic similarity and intraspecies differentiation, the 14 *E. coli* strains and an ESBL-negative *E. coli* control strain were grouped into different clusters with about 54% genetic similarity. E17 was clustered in a separate tree branch far distant from other isolates. E6 and E9 formed another cluster while the rest were clustered in two main subclusters: the first one included (E1, E3, E4, and E7), while the second included (E8 and E10–15). E17, and to lesser extent E6 and E9, were found distant from the other tested ESBL-positive *E. coli* strains (**Figure 2**).

RAPDs proved to be useful as genetic markers in bacteria fingerprinting as previously described [33]. Although major bands from RAPD reactions are highly reproducible, minor bands can be difficult to reproduce because of the random priming nature of this PCR reaction and potential confounding effects associated with comigration with other markers. The use of multiple primers sets in RAPD analysis can be used as a rapid method for preliminary biotyping of the *E. coli*.

Pairwise genetic distance and homogeneity tests were performed to determine the relatedness between the different bacterial strains. Smaller genetic distance between *E. coli* reflected the geographical proximity between them and the results supported the hypothesis that the geographical distance is an important factor influencing the genetic relatedness of *E. coli* strains [34, 35]. A previous study used fingerprinting for resistant different bacteria in Al-Taif in determining

Table 3. Results of polymerase chain reaction of genes encoding important β-lactamases (*bla*) genes in selected bacteria and relevant antibiotic sensitivity

Sample Number	Isolated bacteria	[a]TEM	CTX-M group			[b]Amp	[c]Antibiotic sensitivity											
			CTX-1	CTX-2	CTX-9		Am/Cla	Pip/Taz	Cefo	Cefta	Cefe	Imi	Mer	Amk	Gen	Cip	Tig	Nit	Tri/Sulf
1	*E. coli*	+	–	–	–	R	R	I	R	R	R	S	S	S	S	R	S	S	R
2	*K. pneumoniae*	+	+	–	–	R	R	I	R	R	R	S	S	S	S	R	S	I	R
3	*E. coli*	+	–	–	–	R	S	R	S	R	R	S	S	S	S	R	S	S	R
4	*E. coli*	+	–	–	–	R	I	R	S	R	R	S	S	S	S	R	S	S	S
5	*K. pneumoniae*	–	+	–	–	R	I	R	I	R	R	S	S	R	R	R	I	R	R
6	*E. coli*	+	–	–	–	R	S	S	S	R	R	S	S	S	S	R	S	S	R
7	*E. coli*	+	–	–	–	R	I	S	I	R	R	S	S	S	R	R	S	I	R
8	*E. coli*	+	–	+	–	R	S	R	S	R	R	I	S	S	R	R	S	S	S
9	*E. coli*	+	+	–	–	R	S	S	S	R	R	S	S	S	S	S	S	S	S
10	*E. coli*	+	–	–	–	R	I	S	S	R	R	S	S	S	S	R	S	R	R
11	*E. coli*	+	–	–	–	R	R	R	S	R	R	S	S	S	S	R	S	S	R
12	*E. coli*	+	–	–	–	R	S	S	S	R	R	S	S	S	S	S	S	S	R
13	*E. coli*	+	–	–	–	R	S	S	I	R	R	S	S	S	R	S	S	S	R
14	*E. coli*	+	+	–	–	R	S	S	R	R	R	S	S	S	S	R	S	S	S
15	*E. coli*	+	–	–	–	R	S	S	I	R	R	S	S	S	R	S	S	S	R
16	*K. pneumoniae*	+	+	–	–	R	S	R	S	R	R	S	S	S	S	R	S	R	R
17	*E. coli*	+	–	–	–	R	I	R	S	R	R	S	S	S	S	R	S	R	S

[a]TEM variants including TEM-1 and TEM-2. [b]Amp (ampicillin), Am/Cla (amoxicillin/clavulanic acid), Pip/Taz (piperacillin/tazobactam), Cefo (cefoxitin), Cefta (ceftazidime), Cefe (cefepime), Imi (imipenem), Mer (meropenem), Amk (amikacin), Gen (gentamicin), Cip (ciprofloxacin), Tig (tigecycline), Nit (nitrofurantoin), Tri/Sul (trimethoprim/sulfamethoxazole), *E. coli* = *Escherichia coli* and *K. pneumoniae* = *Klebsiella pneumoniae*. [c]R (resistant), S (sensitive), I (intermediate).

pairwise genetic distance and homogeneity [27]. However, including strains from different genera of bacteria in the same tree did not provide clear picture.

We concluded that a considerably high incidence of ESBL-producing bacteria was present in patients from Al-Taif and surrounds in Saudi Arabia. bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ were the most commonly detected genes. There was evidence for the existence of polymorphic genetic patterns among ESBL-producing bacteria.

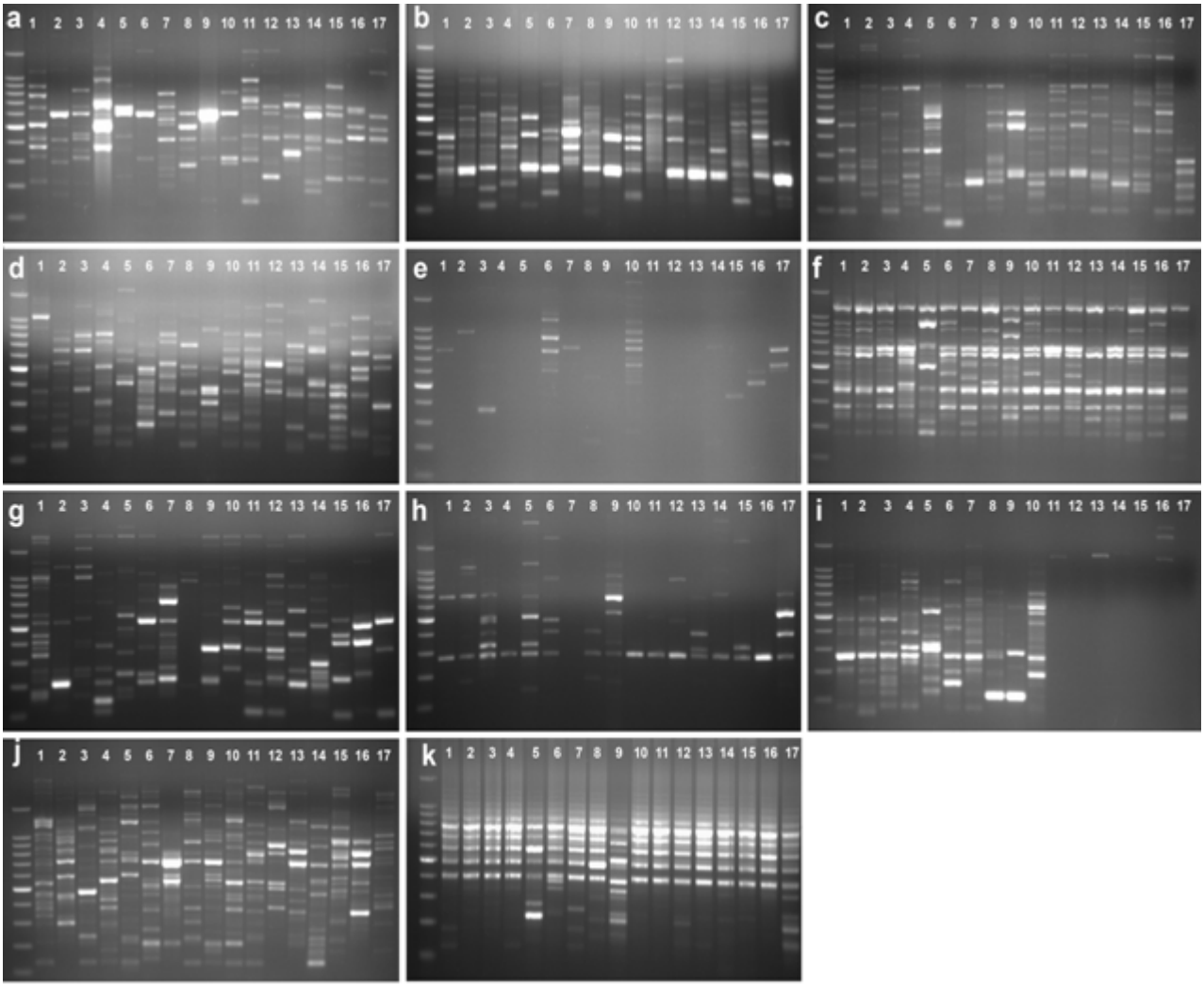

Figure 1. Agarose gel (1.5%) electrophoresis of random amplified polymorphic DNA profiles of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae.* All isolates are *E. coli* except Nos. 2, 5, and 16 as shown in Table 3 (in lanes 2, 5, 16 respectively) are *K. pneumoniae.* In the first left hand lane of each gel a 100 bp DNA ladder was used to provide discrete DNA molecular weight markers (1500 bp reference band, then 1000 to 100 bp in 100 bp steps with a 500 bp reference band) to determine DNA fragment sizes (AccuRuler 100 bp DNA RTU Ladder; Maestrogen, Las Vegas, NV, USA). Primers (a) OPA-01, (b) OPA-03, (c) OPA-04, (d) OPA-05, (e) OPA-06, (f) OPA-07, (g) OPA-08, (h) OPA-09, (i) OPA-10, (j) OPB-07, and (k) OPD-05. An ESBL-negative *E. coli* control showed a different pattern of DNA fragments with the different primers (data not shown).

Table 4. Polymorphic bands of each genetic primers and percentage of polymorphism in the extended spectrum β-lactamase-producing bacterial isolates

Primers	Total Bands	No. of Monomorphic Bands	No. Polymorphic Bands	% Monomorphic bands	% Polymorphic bands
OPA-01	19	4	15	26.3	73.7
OPA-03	20	6	14	30.0	70.0
OPA-04	20	5	15	25.0	75.0
OPA-05	21	5	16	23.8	76.2
OPA-06	16	0	16	0.00	100
OPA-07	18	10	8	55.5	44.5
OPA-08	19	6	13	31.5	68.5
OPA-09	15	1	14	6.67	93.3
OPA-10	22	4	18	18.2	81.8
OPB-07	17	9	12	52.9	47.1
OPD- 05	20	15	5	75.0	25.0
Total	207	65	142		

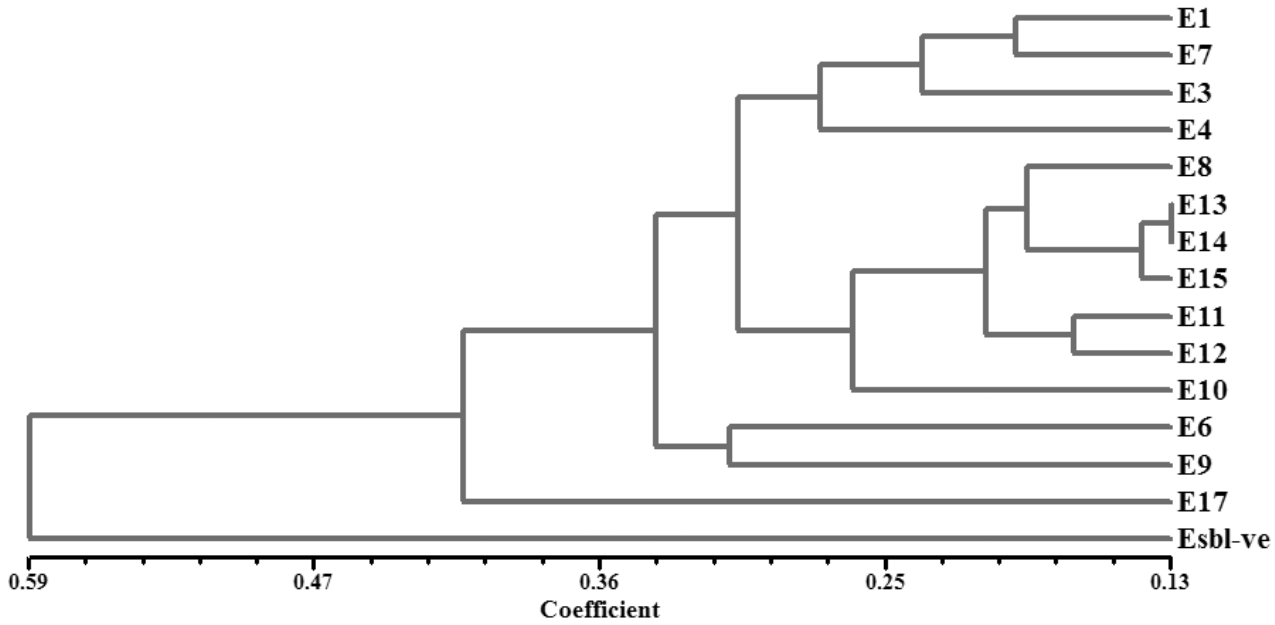

Figure 2. Dendrogram of the 14 *Escherichia coli* isolates collected from inpatients at King Abdul-Aziz Hospital, Al-Taif, Saudi Arabia based on the 11 random amplified polymorphic DNA primers compared with an extended spectrum β-lactamase-negative strain (from our laboratory)

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Conflict of interest statement

The authors declare that there is no conflict of interest in this research.

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