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ORIGINAL ARTICLE

TEM and SHV Genes in *Klebsiella pneumoniae* Isolated from Cockroaches and Their Antimicrobial Resistance Pattern

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Abstract
Objectives: *Klebsiella pneumoniae* is a gram-negative rod bacterium, a known cause of community-acquired bacterial pneumonia and is an important hospital-acquired pathogen that causes severe morbidity and mortality. The aim of this study was to identify the TEM and SHV genes in *K. pneumoniae* isolated from cockroaches obtained from hospitals.
Methods: In this study, 250 cockroaches were collected from different hospitals in the province of Chaharmahal Va Bakhtiari, which is located in southwest Iran. The samples were examined for the presence of *K. pneumoniae* by plating onto a combination of culture media, and the antimicrobial susceptibility patterns of isolated *K. pneumoniae* from samples were evaluated using the disk diffusion test. In addition, from the culture, genomic bacterial DNA was extracted, and sequence-specific targets (TEM and SHV genes) were amplified using the polymerase chain reaction (PCR) method.
Results: Out of 250 cockroach samples collected from various hospitals, 179 samples (71.60%) were positive for *K. pneumoniae*. PCR reaction was performed using specific oligonucleotide primers (TEM-F, TEM-R and SHV-F, SHV-R) for the amplification of each gene, and amplified products were visualized on 1% agarose gel electrophoresis. Of all the specimens amplified by PCR in this research, 32 samples (17.87%) were positive for TEM and 15 samples (8.37%) were positive for SHV.
Conclusion: Detection of TEM and SHV genes using molecular methods and their pattern of antimicrobial resistance can provide useful information about the epidemiology of and risk factors associated with *K. pneumoniae* infection.

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1. Introduction

Studies done in the 19th century have proven that insects have an important role in the transmission of diseases to humans, one of which are cockroaches [1]. Most cockroaches are infected with pathogenic bacteria causing leprosy, dysentery, bubonic plague, pimples, abscesses, and food poisoning. Cockroaches also have a symbiotic relationship with more than 100 bacteria species, 60 yeast species, 90 protozoa species, and 45 parasite worm species [2]. Important carriers of bacteria include the German cockroach (*Blattella germanica*) and the American cockroach (*Periplaneta americana*). German cockroaches, one of the most commonplace pests that carry pathogenic bacteria, have been reported many years ago [3]. The bacteria contaminant could be from air, water, food, or contact with vectors harboring the pathogens. Cockroaches stay in filthy environments in households, shops, and even hospitals where both clinical and environmental samples are kept [4,5].

Moreover, the medical importance of cockroaches is rather substantial. They are among the medically important pests in urban environments that cause serious public health problems, and they have been especially associated with an outbreak of dysentery [6]. Most pathogenic bacteria have been recently isolated from cockroaches, such as *Salmonella* spp., *Campylobacter* spp., *Shigella* spp., *Pseudomonas aeruginosa*, and *K. pneumoniae* [7,8].

Klebsiella was first discovered by Karl Friedlander at 1882 [9]. *Klebsiella* consists of seven types, of which *K. oxytoca* and *K. rhinoscleromatis* have been demonstrated in human clinical specimens. *K. pneumoniae* is a gram-negative, rod-shaped, lactose-fermenting, nonmotile, and encapsulated bacillus of the Enterobacteriaceae family [10,11]. It is an opportunistic pathogen found in water, soil, and plants; it also exists as a normal flora in mucosal surfaces such as the intestines, pharynx, mouth, and skin in mammals. This bacterium was also recognized as a community-acquired pulmonary pathogen, mainly among patients with a history of chronic alcoholism [10,12,13]. In humans, *K. pneumoniae* can colonize the gastrointestinal tract, bowel, bladder, skin, or pharynx, which may cause various clinical outcomes, including pneumonia, thrombophlebitis, bacteremia, and urinary tract infection [13]. Almost 160 variants of the TEM-1 and TEM-2 penicillinases have been described, many of which exhibit activity against extended-spectrum cephalosporins [14]. Class A enzymes are mainly plasmid encoded, and the first to be described at amino-acid sequence level were the enzymes TEM-1 and TEM-2 [15].

The SHV family has been derived from *Klebsiella* spp. SHV-1 is universally found in *K. pneumonia* [16], evolved as a chromosomal gene in *Klebsiella* spp., and was later incorporated into a plasmid, which has spread to other enterobacterial species [17]. A total of 40 types of SHV have already been reported [18].

In this research, we identified the presence of TEM and SHV genes in *K. pneumoniae* isolated from cockroaches from Shahrekord hospitals using the polymerase chain reaction (PCR) technique and their antibiotic resistance pattern.

2. Material and methods

2.1. Sampling

Cockroaches were randomly collected for 6 months (January−June 2013), from six hospitals located in Chaharmahal Va Bakhtiari province (Kashani Shahrekord, Imam Ali Farrokhshahr, Shohada Farsan, Imam Reza Lordegan, and Valiasr Boroujen). A total of 250 cockroaches were collected from different parts of the hospitals by handlers who wore sterile gloves. Live cockroach specimens were immediately carried to the Biotechnology Research Center of the Islamic Azad University and killed using chloroform-soaked cotton. The tubes containing the samples were filled with 70% ethanol for 5 minutes to decontaminate their external surface and then allowed to air dry. Next, cockroaches were washed with sterile normal saline to remove the residue ethanol. Finally, their viscera were removed with sterile forceps under a dissecting microscope, and the instruments were sterilized after every dissection. Cockroach gut was kept in 2 mL sterile normal saline for 5 minutes to produce a homogenate specimen.

2.2. Bacterial isolation

After the samples were isolated and placed in test tubes containing normal saline, the gut homogenate was kept in buffered peptone water, after which the samples were inoculated on blood agar and Mac-Conkey agar (MCA), then incubated for 24 hours at 37°C. For the growth of gram-negative bacteria such as *K. pneumonia*, BPW was used that was inoculated in seven primary media (Sheep Blood Agar, Chocolate Agar, Mac-Conkey, Deoxycholate Citrate Agar, SS agar, Mannitol Salt Agar, and Xylose Lysine Deoxycholate). In addition, identification of gram-negative bacteria was achieved by use of standard methods (API System; bioMerieux, Marcy-l'Étoile, France). The biochemical reagents and test used to identify *K. pneumoniae* included triple sugar iron agar, simmons citrate, indole, urease, motility, and H_2S. The biochemical characteristics of *K. pneumoniae* identified were as follows: positive citrate utilization test, negative methyl red test, negative indole test, positive urease test, positive Voges−Proskauer test, sucrose, acid and abundant gas production from glucose, lactose, mannitol sugar fermentation tests, and maltose.

2.3. Picking up the cultivation of susceptibility

Antimicrobial susceptibility profiles were determined using the dilution method on Mueller–Hinton agar, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The antimicrobial agents tested included ceftazidime, nalidixic acid, cefixime, gentamicin, amikacin, cephalexin, and imipenem. The CLSI breakpoints were used for the interpretation of minimum inhibitory concentrations (CLSI 2012). The results were interpreted after 24 hours of incubation at 37°C, as sensitive, intermediately sensitive, and resistant according to the zone diameter around each antibiotic disk.

2.4. DNA extraction and PCR

K. pneumoniae genomic DNA was extracted using the DNA extraction kit (DNP Kit; CinnaGen, Tehran, Iran) according to the manufacturer's recommendation. The extracted DNA was quantified with spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell [19].

To detect the TEM and SHV genes, PCR reactions were performed in a total volume of 25 µL containing 2 µL of the DNA sample, 1 µM of each primers, 2 mM Magnesium chloride ($MgCl_2$), 5 µL of 10 × PCR buffer AMS, 200µM dNTPs, and 1 unit of *Taq* DNA polymerase (CinnaGen Co., Tehran, Iran). The PCR assay was performed at 95°C for 5 minutes and then for 32 cycles of 94°C for 1 minute, 58°C for 40 seconds, 72°C for 40 seconds, and a final extension at 72°C for 5 minutes, with a final hold at 10°C in a thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany). For SHV amplifications, conditions for thermal cycling remained the same except for the annealing temperature (55°C). The primer sequences for TEM and SHV are shown in Table 1.

The amplified products were run on 1% agarose gel and staining with ethidium bromide (0.5 mg/ml) in a dark room. The electrode buffer used was Tris-borate-EDTA (TBE), which consists of Tris-base 10.8 g 89 mM, boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 mL of 0.5 M EDTA (pH 8.0) (all components were combined in sufficient H_2O and stirred to dissolve). A 100-bp ladder molecular weight marker (Roche, New Jersey, USA) was used to measure the molecular weight of the amplified products. Aliquots (14 µL) of PCR products were applied to the gel. A constant voltage of 84 V for 20 minutes was used for product separation. The images of ethidium bromide stained DNA bands were digitized using a UVItec documentation system (UVItec, Paisley, UK).

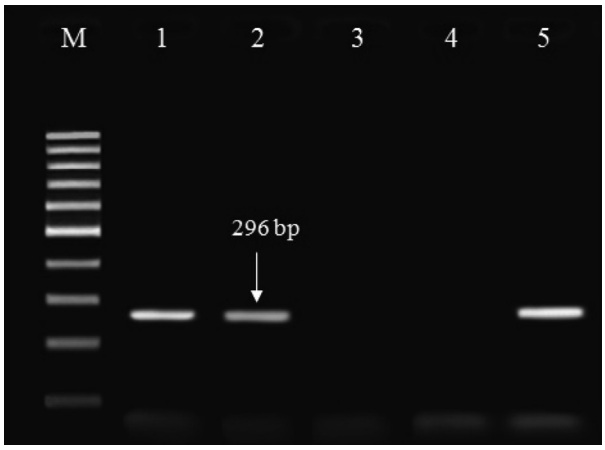

Figure 1. Agarose gel electrophoresis of the products amplified with polymerase chain reaction (PCR) using the specific primers for *Klebsiella pneumoniae* TEM gene samples. Lane M: 100-bp DNA ladder (Fermentase, Leon-Rot, Germany); lanes 1, 2, and 5: PCR products of the positive samples; lanes 3 and 4: negative samples.

2.5. Statistical analysis

All data were analyzed by using MS Excel 2007 and SPSS software (Version 17, SPSS Inc., Chicago, USA), and the p value was calculated using Chi-square and Fisher's exact tests to find any significant relationship. A p value of <0.05 was considered statistically significant.

3. Results

The quality of extracted DNA from samples was examined by electrophoretic analysis through a 1% agarose gel. Out of 250 cockroach samples collected from various Iranian hospitals, 179 samples (71.60%) were infected with *K. pneumoniae*. The TEM and SHV genes were successfully amplified with the TEM-F and TEM-R and SHV-F and SHV-R primers. The agarose gel electrophoresis of the PCR-amplified products for

Table 1. Primers for identification of *Klebsiella pneumoniae* TEM and SHV genes.

Gene target	Primer name	Sequence	Annealing temperature	Amplicon size (bp)
TEM	TEM-F	5′-TCCGCTCATGAGACAATAACC-3′	58	296
	TEM-R	3′-ATAATACCGCACCACATAGCAG-5′		
SHV	SHV-F	5′-TACCATGAGCGATAACAGCG-3′	60	450
	SHV-R	3′-GATTTGCTGATTTCGCTCGG-5′		

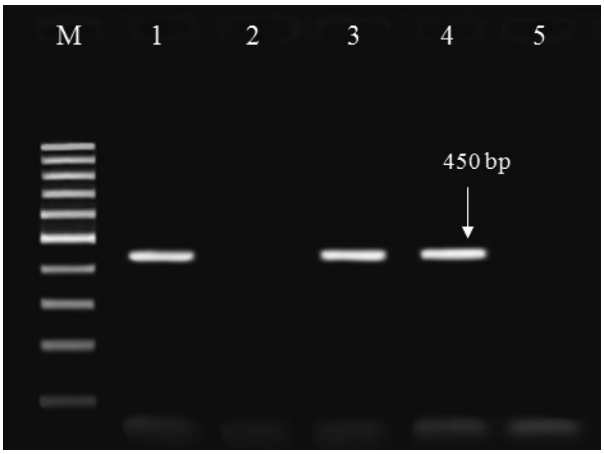

Figure 2. Agarose gel electrophoresis of the products amplified with polymerase chain reaction (PCR) using the specific primers for *K. pneumoniae* SHV gene samples. Lane M: 100-bp DNA ladder (Fermentase, Leon-Rot, Germany); lanes 1, 3 and 4: PCR products of the positive samples; lanes 2 and 5: negative samples.

TEM and SHV genes is shown in Figures 1 and 2, respectively. From the samples that were assayed by PCR in this research, only 32 samples (17.87%) were positive for the TEM gene and 15 samples (8.37%) were positive for the SHV gene (Table 2). Moreover, Table 3 shows the antimicrobial susceptibility pattern of *K. pneumoniae* TEM and SHV genes. The overall susceptibility of isolated *K. pneumoniae* strains to antimicrobial agents was 64.3% for nalidixic acid, 65.4% for cephalexin, 69.8% for cefixime, 73.50% for gentamicin, 83.2% for ceftazidime, 85.1% for amikacin, and 100% for imipenem. According to these results, imipenem, amikacin, and ceftazidime were the most effective agents against isolated *K. pneumoniae.*

4. Discussion

Klebsiella consists of seven types—*K. pneumoniae*, *K. planticola*, *K. terrigena*, *K. rhinoscleromatis*, *K. ozaenae*, *K. ornithinolytica*, and *K. oxytoca*—and two of these species, *K. oxytoca* and *K. rhinoscleromatis*, have been demonstrated in human clinical specimens [10,11]. *K. pneumoniae*, one of the most common causes of gram-negative sepsis, usually inhabits the human and animal intestinal tract [10,20]. Some gram-negative bacilli such as *K. pneumoniae* and *Escherichia coli* strains of particular types produce β-lactamases. The Enterobacteriaceae family produces β-lactamases, which are encoded by plasmids. Among the most important β-lactamases are SHV and TEM. For the first time, TEM-1 from a blood culture (Temonera) in Greece was isolated from *E. coli* [21,22]. One of the first reported β-lactamases is TEM-1, which is encoded by plasmids; however, other bacteria such as *Vibrio cholerae* and *Haemophilus* strains of *P. aeruginosa* and *Neisseria* also have the ability to produce it. Today, reports indicate the prevalence of TEM-1 β-lactamase in certain parts of the world, suggesting that this type of β-lactamase is a global problem [23,24]. According to a 2011 study by Shebani et al [24], out of 70

Table 2. Number of *K. pneumoniae* TEM and SHV genes.

Hospitals	Number of samples	Positive for *Klebsiella*	Positive for TEM gene	Positive for SHV gene
Imam Reza Lordegan	60	39	9	0
Valiasr Boroujen	60	52	11	7
Shohada Farsan	40	19	0	2
Imam Ali Farokhshahr	30	21	3	1
Kashani Shahrekord	60	48	9	5
Total	250	179	32	15

Table 3. Antibiotic resistance patterns of *K. pneumoniae* TEM and SHV genes.

Antimicrobial agent	Sensitive	Half-resistant	Resistant	Total
Nalidixic acid	64.30	0	35.70	100
Cephalexin	65.40	2.9	31.70	100
Cefixime	69.80	0	30.20	100
Gentamicin	73.50	0	26.50	100
Ceftazidime	83.20	1.80	15	100
Amikacin	85.10	6.30	8.60	100
Imipenem	100	0	0	100

samples of *E. coli*, 27 (38.58%) were ESBL (extended spectrum beta-lactamase) positive and 43 samples (61.42%) were ESBL negative, and that there were only 10 samples (37.04%) with *TEM-1* β-lactamase gene and 17 samples (62.96%) were free of the *TEM-1* β-lactamase gene [24]. TEM has been responsible for several unrelated outbreaks in the United States [25] and a recently reported outbreak in Europe with the same frequency [26]. A study from Korea revealed that SHV is the most common ESBL found in Korea [27]. SHV (especially SHV-5) is commonly encountered and reported worldwide [27,28]. In another study in India, the reported frequency of ESBL-producing *Klebsiella* spp. was between 6% and 87% [29−31].

In conclusion, our results suggest that the PCR method can be used for the specific, rapid, simple, and highly sensitive detection of *K. pneumoniae* in samples. Furthermore, isolated *K. pneumoniae* from cockroaches collected from various hospitals has more resistance to nalidixic acid. Imipenem seems to be the only antimicrobial agent that showed 100% sensitivity and may be used as the drug of choice for this type of infection.

Conflicts of interest

The authors declare no conflicts of interest.

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