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Original Article

Molecular Detection of *bla TEM* and *bla CTX-M* Genes in Clinical and Food-Borne *Klebsiella pneumoniae* Isolates

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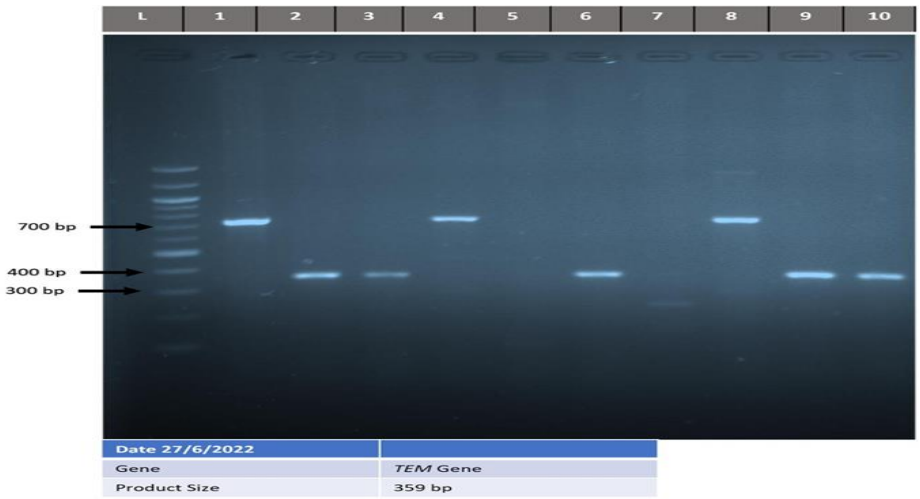
ABSTRACT

To isolate *Klebsiella pneumoniae,* two hundred clinical and food samples were cultivated on differential and selective culture media. 120 bacterial isolates were isolated from the food and clinical samples. On MacConkey agar, 81 isolates of lactose fermenters (67.5%) and 39 isolates of non-lactose fermenters (32.5%) were found. Out of 120 bacterial isolates discovered in clinical and food sources, 32 were *K. pneumoniae* isolates, including 14 from food sources and 18 from clinical sources. *K. pneumoniae* was determined to be the species of bacteria isolated based on biochemical testing, morphological characteristics, and cultural identities. This conclusion was confirmed by the Vitek system. The findings of this investigation, which used phenotypic methods, revealed a high frequency of isolates of *K. pneumoniae* that produce Extended Spectrum B-Lactamase (ESBL) from patients in Iraq and a low frequency of isolates from food sources. After bacterial DNA was isolated, pure DNA samples with concentrations between 76 and 91 ng/ml were produced. Utilizing particular primers for the genes *bla TEM* and *bla CTX-M*, this work carried out the molecular detection of two ESBL-coding genes. According to the findings of this study, *bla TEM* was only found in clinical isolates at a detection rate of 27.77% and *bla CTX-M* at 38.88%. Isolates from food sources tested negative for *bla TEM* and *bla CTX-M.*

GRAPHICAL ABSTRACT



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Introduction

Due to the misuse of antibiotics, the incidence of multidrug-resistant (MDR) human pathogenic microorganisms and their genetic determinants has grown dramatically in clinical and environmental contexts. As a result, infection treatment choices become restricted, especially when MDR bacteria possess genes encoding extended range antibiotic resistance. One of the most important resistance mechanisms among Enterobacteriaceae is the production of β-lactamases, including extended-spectrum- and AmpC-lactamases [1]. According to an epidemiological research, Enterobacteriaceae that produce extended-spectrum-lactamase (ESBL), particularly *Escherichia coli* and *Klebsiella pneumoniae,* are the leading sources of community-acquired illnesses [2]. *CTX-M, TEM*, and *SHV* genes are the most common and clinically relevant ESBL genes, with CTX-M enzymes emerging as the most common. CTX-M is classified into five groups based on their amino acid identities*: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9*, and *CTX-M-25*. *K. pneumoniae* typically generates all three kinds of enzymes, but the advent of carbapenemase-producing *K. pneumoniae* and colistin-resistant *K. pneumoniae* has caused the most recent public health worry [3]. Extended-spectrum beta lactamases (ESBLs) have played a major role in the clinical area in recent decades in the wide-ranging and complicated world of B-lactamases. Their significant stems the fact that they greatly broaden the range of the earlier B-lactamases, allowing them to hydrolyze all penicillins, cephalosporins (excluding cephamycins) and aztreonam. Furthermore, most ESBL genes are located on plasmids that confer antibiotic resistance to many types of antibiotics and are easily transferred between and among bacterial pathogens [4]. Extended spectrum lactamase, is a Gram-negative bacterium enzyme imparting resistance to penicillins, cephalosporins, and monobactams, provides a unique therapeutic problem. In *Enterobacteriaceae* from retail food, extended range TEM-, SHV-, and CTX-M type enzymes have been found [5].

Materials and Methods

Bacterial isolation

The collected clinical and food samples were cultured on selective and differential media to isolate *Klebsiella pneumoniae*. One hundred clinical and one hundred food samples were all inoculated on MacConkey agar, and incubated at 37 °C for 24 hours and the grown colonies were further investigated.

Bacterial identification

Bacterial isolates were identified to the genus level using both microscopic and macroscopic characteristic on selective and differential media, according to MacFaddin [6]. The species identity was verified by the Vitek system.

DNA extraction

The Geneaid Genomic DNA Mini Kit's gram-negative bacteria extraction method was used to extract DNA (Thailand).

Molecular detection

PCR Amplification of ESBL coding genes

The primers used to detect ESBL coding genes in *K. pneumoniae* were designated in this study; PCR technique was adopted to investigate the presence of these genes in the isolated clinical and food origin *K.pneumoniae* (Table 1).

Table 1: Designated Primers that used for the amplification of ESBL coding genes

Primer Name	Sequences (′5 ⟶ ′3)	Size (bp)
bla TEM-F	TATGTGGTGCGGTATTATCC	359bp
bla TEM-R	AGTTAATAGTTTGCGCAACG	
bla CTX-M-F	GCTTTCTGCCTTAGGTTGA	543bp
bla CTX-M-R	AATCAGCGAGTTGAGATCAA	

The form of these primers was lyophilized. A 25-L total volume of PCR mixture was employed, consisting of 12.5 L of PCR premix, 1.5 L of each primer, and 4 L of template DNA. With sterile de-ionized distilled water, the remaining volume was finalized, and then it was vortexed. Template DNA was not present in the negative control, thus distilled water was added in its place. Before placing them in the thermo-cycler PCR instrument, the PCR reaction tubes were quickly centrifuged to mix and brought the contents to the bottom of the tubes. The following programs were adopted to amplify *blaTEM* and *blaCTX-M* (Tables 2 and 3).

Determination of PCR product specificity

To determine the specificity of PCR results, agarose gel electrophoresis was used. To recognize the PCR specificity, agarose gel was prepared in a 1.5% concentration by boiling 1.5 g of agarose powder in 100 ml of 1X TBE buffer, cooling the agarose to between 50 and 60 °C, adding 5 L of red safe dye while mixing, pouring the agarose into a jar, and cooling the jar to 20 °C. A few wells were carefully formed with a comb at one side of the agarose gel when it was poured, about 5-10 mm from the gel's end. The comb was then removed after the gel's final solidification. The jar was placed in the tank for electrophoresis. The first left or middle well of the agarose electrophoresis gel was filled with six microliters of the 100 bp DNA ladder, and the remaining wells were filled with ten microliters of each PCR product. After that, the electrophoresis tank was sealed with its unique top and an electric current of 75 volts was matched for an hour [7]. Gel documentation system was used to visualize the red safe stained bands in gel.

Results and Discussion

Klebsiella pneumoniae isolation and identification

Two hundred clinical samples were cultured on selective and differential culture media to isolate *Klebsiella pneumoniae*. The results revealed the isolation of 120 bacterial isolates from clinical and food samples. Two types of colonies appeared on MacConkey agar; 81 isolate of lactose fermenters (67.5%) and 39 isolate of non-lactose fermenters (32.5%). MacConkey agar is selective for Gram-negative *Enterobacteriaceae* and differential between lactose fermenter appeared as pink colonies and non-lactose fermenter appeared as pale colonies [8]. The lactose fermenters were of two principal types of colonies; small pink flattened colonies subjected to another culture step on eosin methylene blue agar to confirm the identification and the results revealed the appearance of colonies with green metallic sheen.

Table 2: Program used to amplify *blaTEM*

Stage	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	1 min	1
Denaturation	95	30 sec	35
Annealing	49	45 sec	
Extension	72	30 sec	
Final extension	72	7 min	1

Table 3: Program used to amplify *bla CTX-M*

Stage	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	1 min	1
Denaturation	95	30 sec	35
Annealing	48	45 sec	
Extension	72	30 sec	
Final extension	72	7 min	1

Large mucoid convex pink colonies which were suspected to be *Klebsiella* subjected to further identification steps such as Chromogenic media.

Out of 120 bacterial isolates isolated from clinical and food sources; thirty-two *K. pneumoniae* isolates, eighteen isolates from clinical sources, and fourteen isolates from food sources. The isolated bacteria were identified depending on biochemical tests as well as morphological and cultural characteristics.

Vitek system was used to confirm the identification of the isolated bacteria to species level and detect the antimicrobial sensitivity. Results revealed that 32 *K. pnemoniae* were isolated in this study.

Regardless of the growing role of *K. pneumoniae* sensu stricto (Kp1) in human infections linked to high levels of antibiotic resistance and virulence and revival in *K. pneumoniae* species complex (KpSC) epidemiology [9]. It is unknown how nonclinical sources, such as food products, contributed to the current rise of KpSC. This is mainly because there aren't any defined techniques for identifying and isolating *K. pneumoniae* strains in samples from the environment, food, or animals. For *Klebsiella spp*., many selective culture mediums have already been created. MacConkey inositol-carbenicillin agar is one of the most popular [10]. Over the course of time, bacteria have developed resistance to different, conventionally used antibiotics [11, 12]. Antimicrobial resistance is a serious issue that has unintentionally arisen as a result of modern healthcare. In both healthcare and community settings, Enterobacteriaceae that produce ESBLs continue to be the most frequent source of expanded-spectrum cephalosporin resistance in these infections. It will continue to be crucial that we keep a close look out for ESBLs in patient isolates and during surveillance because they are now endemic in clinical isolates of Enterobacteriaceae [13].

This point is worth noting, as it potentially could lead to treatment failure, prolonged diseases, and elevated morbidity and mortality rates. The transmission of resistance is a plasmid-mediated genetic determinant, may be attributed to the development of MDR among these isolates [14]. Studies have shown a rising pattern in the incidences of *K. pneumoniae* isolates with multiple antibiotic resistance. It has also been reported that infections with multiple antibiotic resistance attributes have a negative impact on the treatment of bacterial infections, especially in elderly, children, and immune-compromised individuals [15]. The use of available antibiotics in appropriate combinations is the only promising alternative technique to treat MDR bacterial infections [16]. This increased in the β-lactam antibiotic resistance isolates among *Klebsiella* strains can be explained in most cases to the production of β-lactamase enzyme that destroyed the β-lactam ring and inactivated it and this enzyme was encoded by plasmid that easy to transfer among strains [17].

ESBL phenotypic detection

The results of double disc synergy method appeared the high prevalence of ESBL producers among *K. pneumoniae* isolated from Iraqi patients and less frequency among the food origin isolates.

Results of molecular study

DNA concentration and purity

Following DNA extraction using the Genomic DNA Mini Bacteria Kit, DNA concentration ranged between 76 and 91 ng/ml, while purity ranged between 1.89 and 1.99. DNA is typically considered as "pure" when the ratio is between 1.8 and 2.0. If the ratio is noticeably lower than the required ratio, it may suggest the presence of protein, phenol, or the other pollutants that absorb heavily at or near 280 nm [18].

Result of ESBL coding genes detection by PCR bla CTX-M gene

The results obtained by PCR technique for bla *CTX-M* gene (543 bp) are displayed in Figure 1.

In addition to the companion animals, the environment, food, and livestock, isolates encoding CTX-M have been found in nosocomial and community settings [19]. Antimicrobial resistance is a serious issue that has unintentionally arisen as a result of modern

healthcare. In both healthcare and community settings, Enterobacteriaceae producing ESBLs continue to be the most frequent source of the expanded-spectrum cephalosporin resistance in these infections. It will continue to be crucial that we keep a close look out for ESBLs in patient isolates and during surveillance because they are now endemic in clinical isolates of Enterobacteriaceae [13].

The bla TEM

The results obtained by PCR technique for bla *TEM* gene (359 bp) are depicted in Figure 2.

Early in the 1960s, the TEM-1 plasmid-mediated beta lactamase was discovered [20]. This enzyme comes in different forms called TEM-type ESBLS. This enzyme was given its name because it was initially discovered in an isolate of *Escherichia coli* taken from a blood culture of a Greek patient by the name of Temoneira [21].

Over the course of time, bacteria have developed resistance to different, conventionally used antibiotics [11]. The gene *bla CTX-M* was detected as 38.88%, while *bla TEM* 27.77% in clinical isolates only. Food origin isolates were negative for both *bla* TEM and *bla* CTX-M.

In a previous study, there were 92% and 76%, respectively, more *blaCTX-M* and *blaTEM* genes in ESBL strains. These findings showed that the majority of the ESBL-producing bacteria in the outpatients sent to Al-Zahra Hospital had *CTX-M* and *TEM* enzymes. The most common genes were *bla CTX-M*, over *bla TEM*, according to our data, which were in agreement with findings from the earlier research carried out in various regions [22].

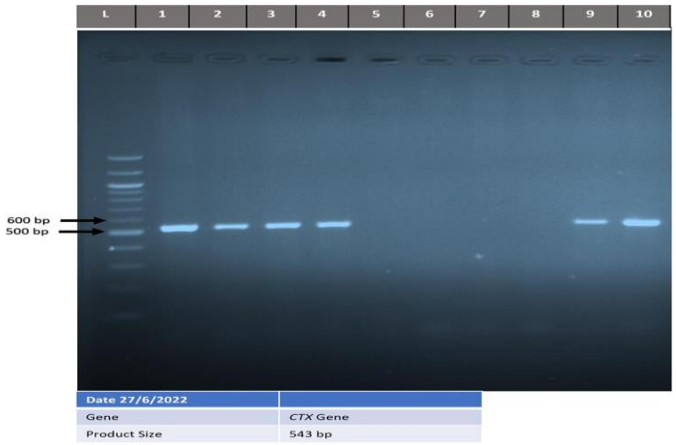

Figure 1: Agarose gel electrophoresis of PCR product of *ctx-m* (543bp), 1.5% agarose, red safe stain, TBE buffer, 75 volt, 1hr L: DNA lader, lan1: K1, lan2:K4 lan3:K5, lan4:K6 , lan5:K7 , lan6:K8, lan7 :K9, lan8:K10, lan9:K11, and lan10:K13

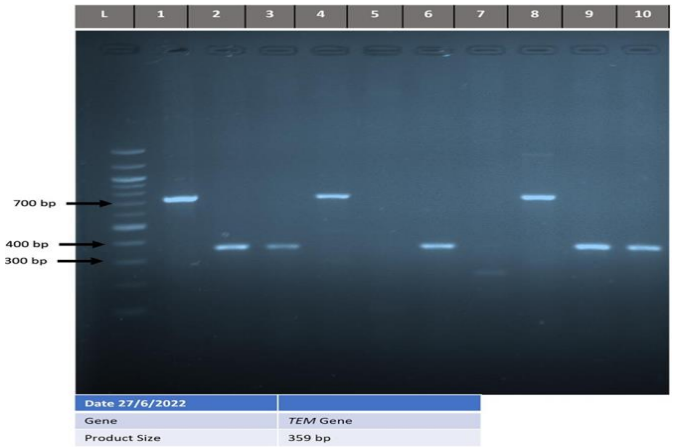

Figure 2: Agarose gel electrophoresis of PCR product of *bla TEM* (359bp), 1.5% agarose, red safe stain, TBE buffer, 75 volt, 1hr L: DNA lader, lan1: K1, lan2:K4 lan3:K5, lan4:K6 , lan5:K7 , lan6:K8, lan7 :K9, lan8:K10, lan9:K11, and lan10:K13

In a study from the Eastern Province of Saudi Arabia, *blaCTX-M* (97.4%) was more common in Enterobacteriaceae than *blaSHV* (23.1%), *blaTEM* (0.0%), and *blaTEM*. Similarly, the additional investigations have shown that the Eastern region's ESBL-producing Gram-negative bacteria tend to be of the *blaCTX M* type. Numerous ESBL resistance genes produced by Gram-negative bacteria have been documented in research conducted worldwide. For instance, *blaCTX-M*, followed by *blaSHV* and *blaTEM*, was the most common type in the Asian Pacific area. The most common ESBL resistance genes in Burkina Faso's Enterobacteriaceae were *blaCTX-M* (40.1%), *blaTEM* (26.2%), and *blaSHV* (5.9%). Together with the current findings, these data demonstrated that there are geographical and local variations in the frequency of different ESBL gene types [23]. The geographical expansion of *CTX-M*-type ESBLs to Australia, Belgium, Turkey, and South Africa is perhaps the report's most significant contribution. There have been no more reports of *CTX-M*-type-lactamases being found in these countries. Furthermore, in our analysis there were more *CTX-M*-type ESBLs than *TEM*-type ESBLs [24], noted that the *CTX-M* genes are the most common and that *CTX-M-1* genes make up their majority. However, the Brazilian investigation discovered that a*TEM* and *CTX-M* type ESBL genes had the highest incidence rates [25]. Three K. pneumonia isolates producing ESBLs were found to co-carry one or two ESBL-encoding genes, such as *blaTEM* and *blaCTX-M,* in this investigation. According to a different study by Zhou *et al.*, the extended spectrum beta lactamase produced by *E. coli* encoding the *CTX-M* gene was discovered in a giant panda maintained in a cage in Shanghai, China. Therefore, the primary ESBL-encoding genes in captive giant pandas may be *blaCTX-M.* Our findings were consistent with those of other investigations, where the two primary ESBL families were *TEM*- and *CTX-M*-types [26]. Our research found no correlation between the TEM, SHV, and CTX-M -lactamase genes' presence with the production of -lactamases or the ESBL phenotype. The expression of chromosomal or plasmid-mediated AmpC -lactamases can conceal the ESBLs presence. In addition, AmpC -lactamases-producing ESBL-producing bacteria can result in a false negative when trying to detect ESBL. More crucially, the presence of the gene as detected by PCR does not necessarily mean that the gene is expressed because the expression of -lactamase genes depends on the environmental factors including the use of antibiotics [27-31].

Conclusion

Klebsiella pneumoniae is a frequent pathogen contaminating foods from local sources in Iraq. The prevalence of bla CTX-M gene in *K. pneumoniae* is a clinical source. In this study, food-origin K. pneumoniae did not carry bla CTX-M or bla TEM.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

The author declared that they have no conflict of interest.

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