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Molecular characterization of genes generating *beta-lactamase* resistance in Gram-negative bacteria *Klebsiella pneumoniae*

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ABSTRACT

Background: Bacterial resistance to beta-lactam antibiotics represents a major public health challenge that complicates the implementation of treatment programs. Multiple mechanisms contribute to beta-lactamase resistance. A high incidence of ESBL-producing Gram-negative bacteria has been reported in numerous investigations to have that profile. One of the primary triggers of infection of the urinary tract is the invasive bacterium *Klebsiella pneumoniae*. This study aimed to identify beta-lactamase resistance-associated genes in *K. pneumoniae* isolates from Sayed Galal Hospital, Egypt. **Methods:** In this investigation, ten *K. pneumoniae* strains resistant to widely prescribed antibiotics were gathered from various specimens and subjected to traditional PCR analysis to detect the existence of the beta-lactamase resistance *genes* *bla*$_{TEM}$ and *bla*$_{CTX}$ to identify the resistance profile. **Results:** The present investigation revealed a predominance of resistant *K. pneumoniae* strains in vaginal swab specimens from elderly females, followed by pus samples from young females. All isolates exhibited resistance to commonly prescribed antibiotics, highlighting a predominance of ESBL-producing strains. The highest prevalence of *K. pneumoniae* was observed in vaginal swabs (60%, 12 isolates), followed by pus samples (30%, 6 isolates) and urine samples (10%, 2 isolates). Resistant isolates were most prevalent in individuals aged 41–65 years (6 isolates), followed by 0–10 years (2 isolates), and fewer cases in the 11–20 years and 21–40 years age groups (1 isolate each). **Conclusions:** Given the high prevalence of ESBL-producing and antimicrobial-resistant isolates, it is crucial to identify and monitor these bacteria early to prevent future outbreaks. It is also critical for specialists to administer antibiotics appropriately, employing the correct therapeutic approaches, and delivering antibiotics in a proper and logical manner.

Introduction

Klebsiella pneumoniae (*K. pneumoniae)* is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family. This bacterium is frequently isolated from biological specimens during infection [1]. It is a pathogenic enterobacterium that causes a wide range of invasive and societal infections in people, including pneumonia, sepsis, peritonitis, and infections of the genital and urinary tracts [2]. Antibiotic resistance poses a major challenge in treating *K. pneumoniae* infections [3].

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Klebsiella pneumoniae is traditionally considered a hospital-acquired infection affecting immunocompromised hosts; however, hypervirulent and multidrug-resistant (MDR) strains have now been identified due to the existence of a sizable auxiliary genome made up of plasmids and chromosomally expressed genes [4,5]. Given that *K. pneumoniae* strains represent a major global clinical health risk, it will be important to comprehend the variables that contribute to pathogenicity and tolerance as essential factors for patients' therapy [6].

Tolerance to oxyimino cephalosporins is conferred by broad spectrum β-lactamases (ESBLs). The reason behind this is that the common plasmid b-lactamases' genes have undergone mutations that alter the structure of the enzyme at its active location, thereby enhancing the enzyme's ability to hydrolyze oxyimino substances and enhance its affinity [7]. Treatment failure with oxyimino cephalosporins is frequently attributed to the presence of these clinically relevant ESBL-producing pathogens. Particularly *K. pneumoniae* and *E. coli*, *Enterobacteriaceae* are the primary producers of ESBLs [8]. Further limiting the available treatment choices and creating a therapeutic conundrum is the fact that bacteria harboring ESBLs can develop and frequently display greater resistance to various kinds of antibiotics, including monobactams, aminoglycosides, etc. [9].

Vaccination against *K. pneumoniae* is growing as a novel and promising approach to successfully combat this potentially fatal disease. A thorough analysis of various vaccines utilizing experimental animals from 35 studies was carried out by **Ranjbarian et al.** [10]. Significant barriers to vaccination immunotherapy still exist, notwithstanding the efficiency of these efforts: low vaccination reactions in immunocompromised individuals; lengthy and costly procedures; and safety issues [11].

A growing number of reports of GES type ESBLs have been found in *K. pneumoniae* and *Enterobacteriaceae* in particular. The GES-1 type was first identified in a *K. pneumoniae* strain that was obtained in 1998 from France and the Netherlands. There are currently nine distinct variations described. The advent of "pan-resistant" germs may have its roots in GES-2, the first evidence of an ESBL with a spectrum of action that extends to carbapenems by a single mutation [12].

The tolerance rate of *K. pneumoniae* to carbapenem antimicrobial drugs increased eight times between 2005 and 2021 (3.0%–23.8%), based on the China Antimicrobial Surveillance Program [13]. From 2008 to 2016, the percentage of MDR strains that were isolated increased dramatically as well, from 0.3% to 3.5%. Significant regional variations in *K. pneumoniae* tolerance have been noted in China, with East China exhibiting greater isolation rates than other regions [13]. The novelty of this study lies in its specific investigation of beta-lactamase resistance *genes* (*bla*$_{TEM}$ and *bla*$_{CTX)}$ in *K. pneumoniae* strains collected from patients at Sayed Galal Hospital in Egypt. By focusing on a localized setting, the study provides crucial insights into the prevalence and distribution of ESBL-producing *K. pneumoniae* in a specific population, identifying patterns of resistance in distinct sample types (e.g., vaginal swabs, pus samples). This research fills a knowledge gap by revealing a detailed resistance profile in the region, highlighting the importance of tailored strategies to monitor and control the spread of antibiotic-resistant pathogens. Additionally, the study emphasizes the need for precise antibiotic administration, which is critical for curbing the emergence of resistance.

Although MDR-Kp infections usually have a high death rate, there isn't a standardized treatment for these illnesses at the moment that can effectively control them [14]. The aim of this study was to identify the prevalence of beta-lactamase resistance *genes* (*bla*$_{TEM}$ and *bla*$_{CTX}$) in *K. pneumoniae* strains from clinical samples at Sayed Galal Hospital in Egypt, with the goal of understanding the resistance pattern and enhancing strategies for infection monitoring and control.

Materials and Methods

Sample collection

The sample collection process occurred from March 3, 2022, to November 1, 2022. During this period, specimens for bacteriological analysis were received at the microbiology laboratory at Sayed Galal Hospital, as part of the network of 75375 hospitals in Egypt. Specimens included vulvar swabs, pus, and urine samples collected from patients at Sayed Galal Hospital. Each sample was collected following standard aseptic techniques to prevent contamination, and relevant patient data, including gender, birth year, sample source, and

origin, were recorded. Ethical approval for the study was obtained from the International Islamic Center for Population Studies and Research, Al-Azhar University.

Isolation and purification of bacteria

Using the methods described by **Taalab et al.** [15] and **Córdova-Espinoza et al.** [16], *K. pneumoniae* was isolated and identified from specimens. The samples were injected into nutrient broth, and the growing colonies where pure cultures were grown on solid nutrient agar plates [15]. The solidified plates were incubated at 36 °C for 14 to 25 minutes (Sigma, Egypt). The plates were incubated for 24 hours at 36 °C in an aerobic atmosphere [17], and then for an additional 24 hours at 36°C [18]. Gram staining and KOH of the isolated strains were used to show the morphological traits [19]. Laboratory testing was conducted in accordance with **Taalab et al.** [15].

Antibacterial and sensitivity assay

Mueller-Hinton medium was used to assess each isolate's susceptibility to antibiotics in compliance with the Global Standard [20]. The medium was cooled to 45 ˚C before being placed into Petri dishes till it obtained an overall thickness of about 4 mm. The solidified plates were incubated at 36 °C for 14 to 25 minutes to allow the extra moisture to flow out. To inoculate the plates, a sterile swab was immersed into the bacterial suspension. The excess inoculated sample was then eliminated by firmly pressing and rotating the bacterial suspension over the solution level onto the side wall of the tube. After wiping the medium's surface with the inoculated sample and rotating the plate multiple times at a sixty-degree angle between each usage, the swab eventually slid across the edge of the agar layer. After covering the plate, it was left to air dry at room temperature for a short while. Fifteen minutes after inoculation, antibiotic discs were placed on the agar plates, which were then inverted for incubation [21]. Applying sterile forceps, ten discs of antibiotics were selected, and each was gently pressed down to ensure enough interaction with the substrate before being put to each dish. The length of each zone, including the zone of inhibition, was measured in millimeters after a 24-hour incubation period at 37˚C, and it was contrasted to the typical inhibiting area [21].

Genetic identification using 16S ribosomal DNA

Isolates of bacteria were cultivated for twenty-four hours in Luria-Bertani broth (LB). The isolates were centrifuged three times at 12,000 g for 5 minutes and washed in 0.85% NaCl. The Gene JET Genomic DNA Cleaning Kit (Thermo Scientific, USA) was used to isolate genomic DNA in accordance with the manufacturer's instructions. For amplification, two primers were used: forward primer 8F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse primer 1492R (5′-GGG CGG GGT GTACAA GGC-3′). The following parameters were used for the PCR expansion: 4 minutes of preheating at 95°C, 30 seconds of denaturation at 95 °C, 45 seconds of primer annealing at 50 °C, 1 minute of extension at 72 °C, and 10 minutes of post-cycling extension at 72 °C for 35 cycles. A thermal cycler (Applied Biosystems USA) was used to conduct reactions. Agarose was dissolved in 1X TBE buffer, heated to 55 °C, and ethidium bromide was added to the molten gel. After the molten gel was placed into the mini-gel apparatus tray, the comb was put in right away and taken out once the gel had solidified. The gel was subsequently coated with 1X TBE electrophoresis buffer. Each well was filled with 15 µl of dsDNA and 3 µl of 2.5 kbp DNA ladder. The 16S rDNA PCR product was purified using the Promega Wizard Genomic DNA Clean-up Kit [22].

Sequence alignment, phylogenetic analysis, and bioinformatics analysis

After being amplified, the PCR product was cleaned and sequenced. The Finch T.V. 1.4.0 program was used to alter raw sequencing results. The 16S rRNA sequences of the strain were examined using the BLAST program from the National Centre for Genetic Information (NCBI) (MD, USA). Multiple sequence alignment was done using ClustalW 2.1. The phylogenetic trees were produced using MEGA X using the neighbor joining technique [23,24].

Molecular detection of resistant *genes* by PCR from *K. pneumonia*

A: Genetic characterization of the blaTEM *gene* by PCR from *K. pneumoniae*

Using the bla_{TEM}-F(5'TGCGGTATTATCCCGTGTTG-3') and bla_{TEM} -R (5'-TCGTCGTTTGGTATGGCTTC-3'), a 297 bp segment of the bla_{TEM} *gene* was amplified. The PCRs were conducted in 25 µl reaction volumes with 12.5 µl DreamTaq master mix PCR Kit (Thermo Scientific, USA), 0.5 µl of each primer, and 3 µl of DNA templates. using a thermal cycling profile. The denaturing step of the reaction PCR protocol consisted of four minutes at 95°C, followed

by 35 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min. The final extending step took place for ten minutes at 72°C. Following amplification, bla_{TEM} PCR outcomes were numerically evaluated via 1.5% agarose gel electrophoresis [24,14].

b: Genetic determination of the *bla*$_{CTX}$ *gene* by PCR from K. pneumonia

A 217 bp section of the bla_{CTX} *gene* was amplified using bla_{CTX}-F (5'-ACAGCGATAACGTGGCGATG-3') and bla_{CTX}-R (5'-TCGCCCAATGCTTTACCCAG-3'). The PCRs were conducted in 25 µl reaction volumes with 12.5 µl of the DreamTaq master mix PCR Kit (Thermo Scientific, USA), 0.5 µl of each primer, and 3 µl of DNA template under a thermocycling profile. The reaction PCR protocol comprised a denaturation stage lasting four minutes at 95°C, 35 cycles of 45 seconds at 58°C, 30 seconds at 95°C, and one minute at 72°C, and an additional extension phase lasting ten minutes at 72°C. The bla_{CTX} PCR products were amplified, and then they were numerically evaluated using 1.5% agarose gel analysis [25,26].

Statistical analysis

Every test was conducted in triplicate, and statistical evaluations were performed using Mini-Tab software (version 19). For expressing values, utilize the standard deviation, also known as mean ± SD. A statistically significant difference of $p < 0.05$ was considered, unless otherwise stated. Ramachandran plots were used to evaluate the distribution of bla_{TEM} and bla_{CTX} in the specimens.

Results

Isolation and screening of resistant bacterial isolates

The investigation included specimens identified as positive enterobacterial strains, which were confirmed to be *K. pneumoniae*. Twenty of the twenty-eight *Enterobacteriaceae* strains in the current study were *K. pneumoniae*. Among these bacterial isolates, 10 (50%) were resistant to all examined antibiotics, as shown in **figure (1)**.

Distribution of *K. pneumonia* according to gender

All of the bacterial species were taken from Sayed Galal inpatients in Cairo, Egypt. **Table 1** shows the bacterial existence based on the patients' gender. The prevalence of *K. pneumoniae* varies significantly ($p \leq 0.05$) between the sexes, with a higher prevalence in males. Of the isolates, 25% were collected from males, while the majority (75%) came from females, as shown in **table (1)**.

Distribution of *K. pneumonia* according to age

There were four age groups, as shown in Table 2. There was a dramatic difference ($p \leq 0.05$) among most of the age categories where the age range of 21-65 contained the most prevalent bacteria, while the lowest number of bacteria could be seen in the age range of 11-20 as illustrated in **table (2)**.

Distribution of *K. pneumonia* according to type of specimen

Three different types of samples were collected from patients, as illustrated in Table 3. A significant difference was observed in bacterial numbers based on the type of sample ($p \leq 0.05$), with the highest number (60%) of bacteria isolated from vaginal swab specimens, while the lowest number was collected from urine samples, as recorded in **table (3)**.

Distribution of *K. pneumonia* according to gender and antibiotic resistance

The resistant strains of *K. pneumonia* were screened according to the sex of the patients. There is a significant difference ($p \leq 0.05$) in resistant species numbers according to gender, where the most common resistant bacteria were existing among females as seen in **table (4)**.

Distribution of *K. pneumonia* according to age and antibiotic resistance

The resistant strains of *K. pneumonia* were screened according to the age of the patients. There is a significant difference ($p \leq 0.05$) in resistant species numbers according to age, where the most common resistant bacteria were existing among elderly persons with an age range of 41-65 as seen in **table (5)**.

Distribution of *K. pneumonia* according to specimen and antibiotic resistance

Screening of the resistant *K. pneumoniae* strains was done based on the patients' age. There is a significant difference ($p \leq 0.05$) in resistant species numbers according to age, where the most common resistant bacteria were existing among elderly persons with an age range of 41-65 as seen in **table (6)**.

Molecular confirmation for identification of *K. pneumonia*

The identification of a resistant bacterial strain was confirmed using genetic analysis, and it

has taken an accession number of (LC455961) as in the following link: https://www.ncbi.nlm.nih.gov/nuccore/LC455961.1

Molecular detection of the virulent gene by PCR from *K. pneumoniae*

Two DNA samples of *K. pneumoniae* in this study (n = 4) tested positive for the *bla*$_{TEM}$ *gene*, showing the expected PCR amplicon of 297 bp, corresponding to the *bla*$_{TEM}$ gene **(Figure 3)**. While there are five DNA samples of *K. pneumoniae*. Furthermore, in this study (*n* = 4), tested positive for the *bla*$_{CTX}$ *gene* showed a PCR expected amplicon of 217 bp, corresponding to the *bla*$_{CTX}$ *gene* **(Figure 4)**.

The structure of the *bla*$_{TEM}$ and bla$_{CTX}$ proteins could be seen in **figure (5 A, B)**. While the quality structure for both products including *bla*$_{TEM}$ and *bla*$_{CTX}$ proteins where the red color (75%), represented the regions of hypodermin A. Besides, pale yellow (10%) refers to the allowed region. Whil, dark yellow (15%) refer to the unallowed region. This bioinformatic analysis confirmed the PCR results for the subibility of *bla*$_{TEM}$ and *bla*$_{CTX}$ protein for *K. pneumoniae* with high scores of (Z score = 26.721, 24.654; ERRT score = 95.781, 88.524; TM score = 0.791, 0.691), respectively, as described in **figure (5)**.

Table 1. Various percentages of *K. pneumonia* according to the sex.

Bacterial species	**Sex**		**Total**
	Women	**Men**	
K. pneumoniae.	15 (75%)	5 (25%)*	20

* Refers to dramatic difference among groups $p \leq 0.05$

Table 2. Different percentages of *K. pneumonia* according to age.

Age range	***K. pneumoniae***
0-10	5 (25%)[a]
11-20	2 (10%)[b]
21-40	3 (15%)[b]
41-65	10 (50%)[c]
Total	**20**

Different superscript letters refer to dramatic difference among groups $p \leq 0.05$

Table 3. Different percentages of *K. pneumonia* according to the sample collected.

Type of sample	***K. pneumoniae***
Pus	6 (30%) [a]
Urine	2 (10%) [b]
Vaginal swap	12 (60%) [c]
Total	**20**

Different superscript letters refer to dramatic difference among groups $p \leq 0.05$.

Table 4. Different percentages of resistant *K. pneumonia* according to sex.

Bacteria	**Male**	**female**	**Total**
Resistant *K. pneumoniae*	2 [a]	8 [b]	**10**

Different superscript letters refer to dramatic difference among groups $p \leq 0.05$.

Table 5. Different percentages of resistant *K. pneumonia* according to age range.

Age range	Resistant *K. pneumoniae*
0-10	2[a]
11-20	1[b]
21-40	1[b]
41-65	6[c]
Total	**10**

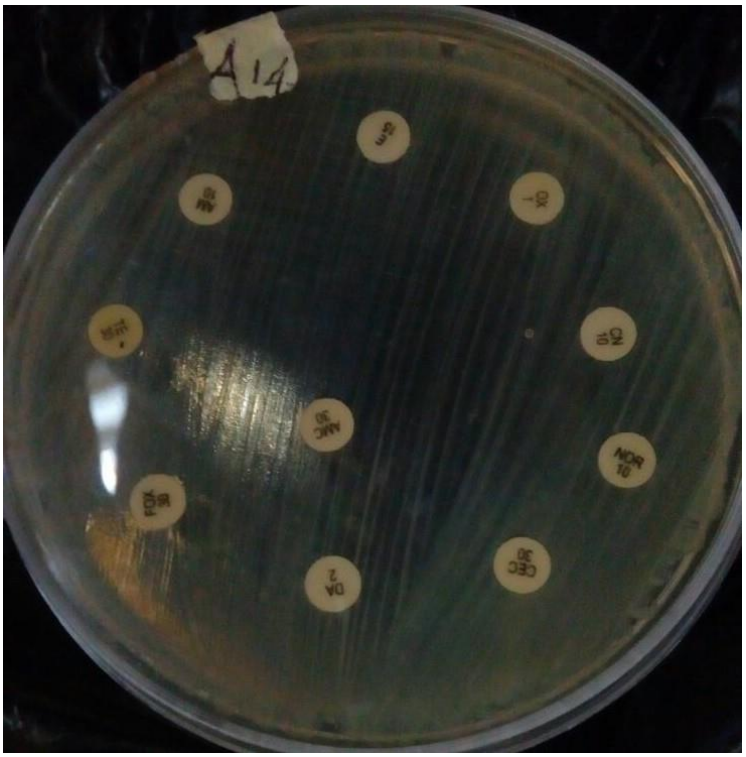
Different superscript letters refer to dramatic difference among groups $p \leq 0.05$.

Table 6. Different percentages of resistant *K. pneumonia* according to type of samples.

Type of samples	Resistant *K. pneumoniae*
Pus	2[a]
Urine	1[b]
Vaginal swap	7[c]
Total	**10**

Different superscript letters refer to dramatic difference among groups $p \leq 0.05$.

Figure 1. A plastic dish contained a strain of *K. pneumoniae* resistant to all common antibiotics.



Key for the used antibiotics: E: Erythromycin, DA: clindamycin, CEC: Cefaclor, NOR: Norfloxacin, AM: Amoxicillin, AMC: Amoxicillin-clavulanic acid, OX: Oxacillin, TE: tetracycline, FOX: cefoxitin, TN: Ciprofloxacin

Figure 2. Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between isolate *K. pneumoniae* strain NRC2-KL and other species belonging to the genus *Klebsiella*. The tree was constructed using the MEGA11 and neighbor-joining method.

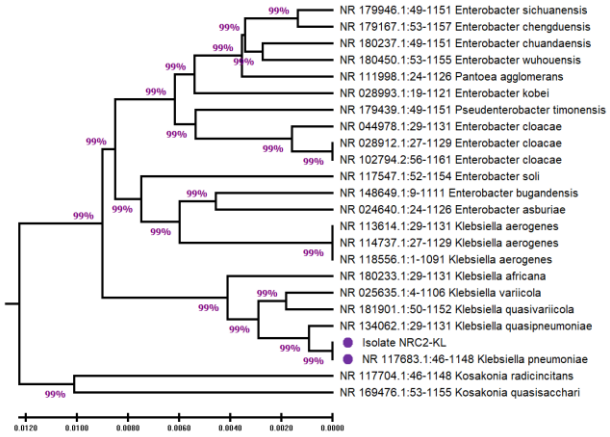


Figure 3. 1.5% gel electrophoresis of PCR for detection of the *bla*$_{TEM}$ gene among *K. pneumoniae*. Lane M: Molecular marker (Thermo Scientific™ Gene Ruler 100 bp DNA Ladder); Lane 1 to 4: *K. pneumoniae* isolates. The expected 297-base pair fragment was amplified corresponding to the *bla*$_{TEM}$ gene.

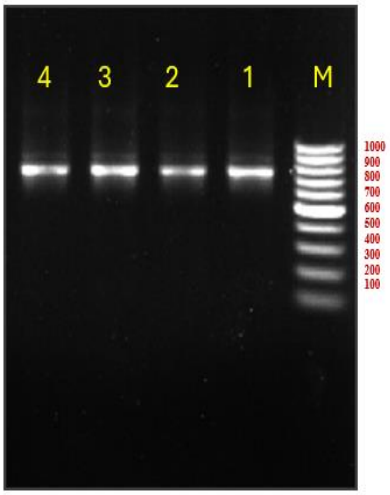


Figure 4. 1.5% gel electrophoresis of PCR for detection of the *bla*$_{CTX}$ gene among *Klebsiella pneumoniae*. Lane M: Molecular marker (Thermo Scientific™ Gene Ruler 100 bp DNA Ladder); Lane 1 to 4: *K. pneumoniae* isolates. The expected 217-base pair fragment was amplified corresponding to the *bla*$_{CTX}$ gene.

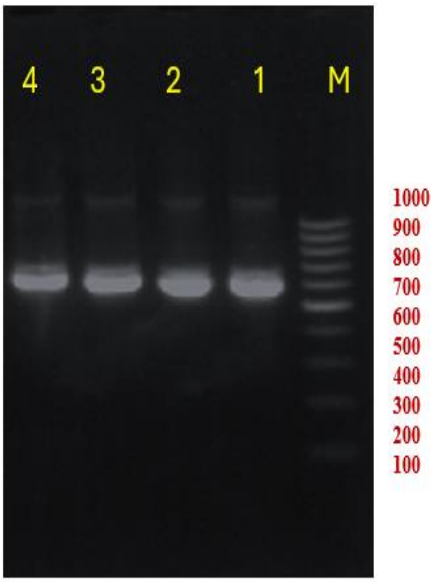
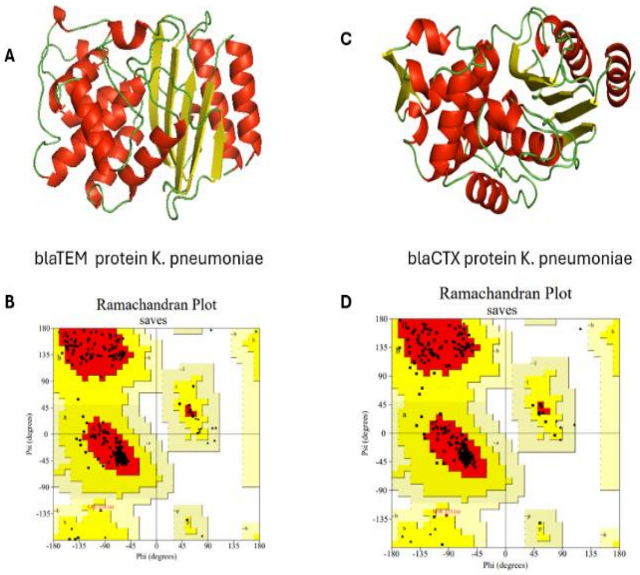


Figure 5. Super imposition of modeled *bla*$_{TEM}$ and *bla*$_{CTX}$ *protein from K. pneumoniae* (predicted model) through cartoon representation. Ramachandran plot *bla*$_{TEM}$ and *bla*$_{CTX}$ protein from *K. pneumoniae* obtained PROCHECK.



Discussion

Multiple nosocomial infections worldwide have been documented to be caused by bacteria from the *Enterobacteriaceae* family [27]. Due to the limited therapeutic options arising from the ongoing increase in antibiotic resistance, infections caused by *Enterobacteriaceae* are increasingly difficult to manage [28]. One of the most widely recognized mechanisms of resistance in Gram-negative bacilli is the production of ESBLs, as reported by **Eichenberger and Thaden**, [29]. ESBLs are a class of enzymes that cause a rise in tolerance towards many prescribed antibiotics [30].

The clinical specimens in the present study showed a significant incidence of MDR isolates of *K. pneumoniae*. The mean occurrence of MDR phenotypes in *K. pneumoniae* isolates was 50%. A notable proportion of *K. pneumoniae* isolates presenting multidrug resistance were ESBL producers. Consistent with the findings of this investigation, a study conducted by **Moradigaravand et al.** [31].

The resistance profiles of each isolate were identified in the current investigation, and the response to antimicrobial test findings against *K. pneumonia* showed that the susceptibilities of the isolated bacteria to the investigated antimicrobials varied according to age, sex, and source of specimen. A plasmid-mediated β-lactamase called prolonged-spectrum beta-lactamase (ESBL), which can hydrolyze and inactivate β-lactam medicines like cephalosporins and monobactams, is one way that *K. pneumoniae* strains can develop resistance to beta-lactam antibiotics [32]. Highly pathogenic and antibiotic-resistant *K. pneumoniae* species are rapidly evolving worldwide [33]. Geographical and population-related factors play a major role in the resistance of bacteria. All types of antibiotics have encountered high resistance levels in *K. pneumoniae* strains [34,35]. Furthermore, the predominant types of *Enterobacterales* from Asia and America are CTX-M ESBLs; however, the recognition rate of CTX-M varies depending on the locale [36-38].

In this work, the resistance isolates from Egypt's *bla*$_{TEM}$ and *bla*$_{CTX-M}$ *genes* were identified using the PCR technique. Research has demonstrated that all bacteria carrying these *genes* had positive ESBL results. Each of the two *genes* under study was shown to be present in ESBL-positive bacteria, which may support their involvement in beta-lactam antibiotic resistance. Numerous articles' evidence suggests that these *genes* have a critical role in the resistance to several antimicrobial agents used for the treatment of infections of human regions in the human body [39-41]. The clonal escape of organisms that manufacture these enzymes in various locations, hospitals, or even wards may be the cause of the stark discrepancies in the abundance of the genes indicated in several Iranian research studies [42]. Besides, it has been demonstrated by **Girmenia et al.** [43] and **Devrim et al.** [44] the rates and types of *Klebsiella* strains isolated differ across nations.

These results emphasize the need for organized national programs in the area that focus on antibacterial monitoring and infection control.

Proven findings from analyses demonstrate that modeled proteins have more favorable areas based on their Ramachandran Plot assessment. In the same line with reports by other investigators [45,46]. Consequently, research is being done around the globe to identify substitute, effective medications that may be able to save the day. For verification of the results from *in silico* methodologies, field studies are still required.

Conclusion

This study highlights the significant impact of antibiotic overuse on the development of bacterial tolerance and the rise in extended-spectrum beta-lactamase producing bacteria, particularly *K. pneumoniae*. The increasing global prevalence of ESBL-producing *K. pneumoniae* underscores the urgent need for diligent monitoring of infections caused by these resistant strains to improve the effectiveness of antibiotic treatments. Among the isolates, 75% were from women (15 isolates), while 25% were from men (5 isolates). Vaginal swabs yielded the highest number of resistant isolates (7 isolates), followed by pus samples (2 isolates) and urine samples (1 isolate). This study's findings emphasize the critical role of the beta-lactamase resistance *genes* bla_{CTX} and bla_{TEM}, which are proliferating at a faster rate than other beta-lactamase-producing *genes*. To curb the spread of resistance genes such as bla_{CTX} and bla_{TEM} in antibiotic-resistant *K. pneumoniae*, hospitals should implement stricter antibiotic protocols, limit unnecessary prescriptions, enhance infection control, and prioritize targeted therapies. Ongoing surveillance and research are crucial to adapt treatment strategies and address emerging ESBL-producing pathogens effectively.

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Conflict of interest

There is no conflict of interest

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