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Influence of Genetic Variations of *Mgrb* and *Pho*Q System on Colistin-resistant in *Klebsiella pneumoniae* Clinical Isolates

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ABSTRACT

Background: Colistin is the only effective treatment for carbapenem-resistant K. pneumoniae, and the rise of antibiotic-resistant strains is a major health concern. **Objective:** The current study aimed to look at the prevalence of colistin resistance among carbapenem-resistant K. pneumoniae in a population of Iraqi patients by conducting a PCR-based molecular screening of the targeted genes (Mgrb and PhoQ system genes) in colistin-resistant Klebsiella pneumoniae isolates.

Patients and methods: A total of 182 clinical samples were collected from Medical City in Baghdad. The VITEK2 technology was used to identified and confirmed *K. pneumonia* isolates, and determine antibiotic sensitivity. The minimum inhibitory concentration (MIC) of colistin-resistant was determined by using E-test. The gene expression of *PhoQ* and *Mgrb* genes was done by using RT-qPCR. **Results:** All 23 Klebsiella spp. were molecularly diagnosed by PCR using a *Rpsl* gene primer, and the E-test showed that 25% were colistin-resistant. The molecular screening for additional genes indicated that 22 isolates (95.7%) contained the *Mgrb* gene and 20 (87.0%) had the *Blashv* and *PhoQ* genes. The *PhoQ* gene increased following colistin exposure, indicating that this mechanism is being employed to compensate for colistin. **Conclusion:** *Mgrb* and *PhoQ* genes were upregulated in response to colistin in particular; when colistin concentration increased the expression of these genes was upregulated.

Keywords: Klebsiella pneumoniae, Colistin, Biofilm formation, Minimum inhibitory concentration, Case series, Baghdad University.

INTRODUCTION

According to a number of different estimations, *Klebsiella pneumoniae*, which is a member of the family Enterobacteriaceae, is the Gram-negative bacterium that is found in hospitals the second most often. Pneumonia, blood poisoning, and urinary tract infections are all common outcomes as a direct result of this factor. In recent years, a multidrug-resistant (MDR) organism, sometimes known as a "superbug" due to its ability to withstand several drugs, has emerged as a major source of worry for the public's health [(1)].

Because Carbapenem-resistant poses such a threat to human health, the World Health Organization (WHO) has designated *K. pneumoniae* as a priority illness. Siderophores, lipopolysaccharide (LPS), capsular polysaccharide (CPS), and adhesion factors are some of the virulence factors that can be found in *K. pneumoniae*. These factors all work together to aid the bacteria in evading the immune system of the host and in spreading to other parts of the body. The production of biofilm is a major factor that contributes to the development of chronic infections. This factor is involved in inflammation as well as the progression of treatment resistance [(2)]. Colistin is the medication of choice for treating infections that are caused by carbapenem-resistant Gram-negative bacteria (GNB). Colistin is a cationic polypeptide antibiotic that works by attaching itself to the negatively charged phosphate group of the LPS found in GNB. This causes the structure of the cell membrane to become disrupted. As the cell's membrane becomes more permeable, the cell itself becomes more porous, the cell's contents leak out, and the cell lyses [(3)]. There are two distinct forms of resistance to the antibiotic colistin; one of these forms is mediated by chromosomes, while the other is mediated by plasmids. Both chromosomes and plasmids include a copy of the phosphoethanolamine transferase gene, which encodes for the mobile colistin-resistant gene *Mcr-1*. Since the first report of *Mcr-1* was made in late 2015, a total of ten distinct Mcr variants have been discovered and assigned the designations *Mcr-1* through *Mcr-10* [(4)].

Due to changes in two-component systems such as *PmrA*/*PmrB* and *PhoP*/*PhoQ*, as well as the *Mgrb* gene, which encodes a negative regulator of *PhoPQ*, this bacterium may acquire resistance to the antibiotic colistin [(5)].

PATIENTS AND METHODS

Collection of the samples:

A total of 182 clinical samples were collected from Medical City in Baghdad. The time period between November 2021 and March 2022 saw the collection of each and every sample for the clinical studies. The samples included faeces, urine, blood, ear swabs, and burn swabs. Additionally, abdominal pus was collected (Baghdad Teaching Hospital and The National Centre for Teaching Laboratories).

Identification of *Klebsiella spp.*:

The analysis of colony morphology led to the discovery of potential isolates. On MacConkey agar and CHROM agar, the general culture features of the colony were used to make the identification of all isolates the vast majority of the time (colour, shape, texture, and size).

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Antimicrobial susceptibility testing

Agar disk diffusion method: Using the single disk Kirby-Bauer method along with the medications colistin and imipenem, we tested 124 Klebsiella isolates to establish their sensitivity to various antibiotics [(6)]. In addition, the breadth of the inhibitory zone was measured, and the results were compared to a chart developed by the Clinical and Laboratory Standard Institute. The VITEK 2 system was used in order to evaluate the efficacy of a variety of new antibacterial chemicals [(7)].

Colistin Minimum Inhibitory Concentration (MIC) **strip testing for *K. pneumoniae* susceptibility (E-test):**

To arrive at this conclusion, a colistin MIC strip was used, and the data were interpreted in accordance with the recommendations made by the manufacturer (Liofilchem, Italy). In this experiment, Mueller Hinton agar was employed, and the minimum inhibitory concentration (MIC) was found by locating the point where two ellipses depicting the incubation process crossed. In the event that the MIC value is discovered to be somewhere in between two different 2-fold dilutions, the higher of the two values should be chosen. Keep in mind the MIC level at which there was no possibility of further development. If the junction on one side of the strip was different from the other, a greater value was assigned to the MIC [(8)].

Biofilm formation (quantitative):

Each Klebsiella *spp*. isolate was shown to produce biofilm utilizing the microtiter plate method. Adherence of bacteria in biofilm formation is defined as the mean optical density (OD) 630 of bacterial samples divided by the mean OD630 of control samples (ODc) [(9,10,11)].

DNA Extraction:

The Norgen DNA extraction Kit was used to extract the whole bacterial genomic DNA. The DNA concentration was determined using The Qubit dsDNA HS (High Sensitivity) assay Kit.

Detection of carbapenem-resistant *K. pneumoniae* and Colistin resistance gene:

target gene amplification *Rpsl* using Klebsiella spp. species-specific primers, including the forward primer 5'-TTTGGAGAACTGC GGACGAC-3' and the reverse primer 5'-CGCTCTGATGGAGTACGACG-3'. Whereas the Colistin-resistance gene *Mgrb* made use of a unique forward primer 5'-AGTGCAAATGCCGCTGAAAA-3' and the reverse primer 5'-GTCATCAT AGCAGGCTGCCT-3' and (*Phoq*) gene forward primer 5'- CTTCCACGAACTCCAGGCAA -3' and the reverse primer 5'-GATCACTTTTGTCGGCGAGC-3' while carbapenem resistance gene (*Blashv*) amplified by using specific forward primer 5'-TAAAGTAGTGCTCTGCGGCG-3' and reverse primer 5'-GCGAGTAGTCCACCAGATCC-3'. The PCR program included initial denaturation in one cycle for 5min at 94°C, and amplification in 35 cycles each of 30 sec. at 94ºC, 45 sec. at 57ºC, and 45 sec. at 72ºC, followed by a final extension cycle for 7min. at 72ºC. To complete the volume for this stage, 12.5 µl of OneTaq (NEB®) master mix, 3 µl of DNA sample, 1 µl (10 pmol/l) of each primer, and 7.5 µl of free-nuclease water were added. By pouring ten microliters of PCR product and DNA ladder into the gel's wells, the samples are loaded. For 80 minutes, the power supply's voltage has largely remained constant at 80V.

Determine the *pho*Q gene expression level:

Here, we employ a proto-script cDNA synthesis kit and a primer specific for each of the *Phoq, Mgrb*, and *Rpsl* genes to synthesize cDNA from RNA. The second part of this process involves picking a cDNA sample from among those that have been treated with colistin (2,32,256 g/ml) and those that have not. Each sample contains three PCR tubes, one for each gene; the quantity of each gene is measured in terms of the Syber Green fluorescent dye. Three microliters of nuclease-free water, five microliters of template DNA, and ten microliters of qPCR master mix made up the reaction mixture. One microliter was devoted to each primer (forward and reverse). One cycle lasting 60 seconds at 95°C was used for initial denaturation; forty to forty-five cycles, each lasting 15 seconds at 95°C and 30 seconds at 60°C, were used for amplification; and one cycle lasting 40 minutes at 60°C to 95°C was used for the melt curve.

Ethical considerations:

The project received approval from the Ethics Committee of Medical City Hospital, Baghdad University's Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, and the Ethics Committee of Medical City Hospital. Everyone present agreed in writing by signing a form. The Declaration of Helsinki, drafted by the World Medical Association, was adhered to in all human studies.

Statistical analysis:

The collected data were coded, processed and analyzed using the SPSS (Statistical Package for Social Sciences) version 22 for Windows® (IBM SPSS Inc, Chicago, IL, USA). Qualitative data were described using number and percent, while quantitative data were described using mean and standard deviation (SD).

RESULTS

Sample Collection:

According to the information that is shown in **Table 1**, a total of 124 (68.13%) distinct Klebsiella species were successfully isolated from 182 samples and identified via the use of colony morphology as well as the technology known as VITEK 2.

It was discovered that the prevalence of *K. pneumoniae* was much higher in females, at 67.7%, than it was in men, at 32.2%. The gap between these two values represents a considerable opportunity for development. This outcome is consistent with the findings of the research, which demonstrated an extremely comparable ratio [(14)]. The percentage was found to be larger than the remainder in 86 of the total samples (69.3%), and this was more prevalent in samples of females than samples of males (p-value 0.05). Between and, there was a significant spike in the number of cases of infections caused by K. pneumoniae (50-69).

Table 1: Gender and age differences in the prevalence of *Klebsiella pneumoniae*.

Gender	Age group					Total
	10-29	30-49	50-69	70-89	> 9	
Male	8	10	18	3	1	40
Female	18	29	26	11	0	84
Total	26	39	44	14	1	124

Antimicrobial Susceptibility of *K. pneumoniae*:

The VITEK 2 system was used to identify whether antibiotics are effective against *K. pneumoniae*, and those results are shown in **Figure 1**.

MIC of Colistin by E-test:

After doing a single-strip diffusion test on each of the 31 isolates, the MIC was calculated using the E-test so that the results could be compared.

The findings of the E-test strip indicate that MIC of colistin for resistant isolates is more than 2 g/ml, while the MIC of colistin for susceptible isolates is between 1 and 2 g/ml (the range of the E-test strip was 0.016 to 256 g/ml).

Table 2 also demonstrated that the disc diffusion test had a much greater margin of error than the E-test, providing validity to the conclusions of a study conducted by [(8)]. The E-test revealed that twelve of the colistin-sensitive isolates were resistant to the antibiotic.

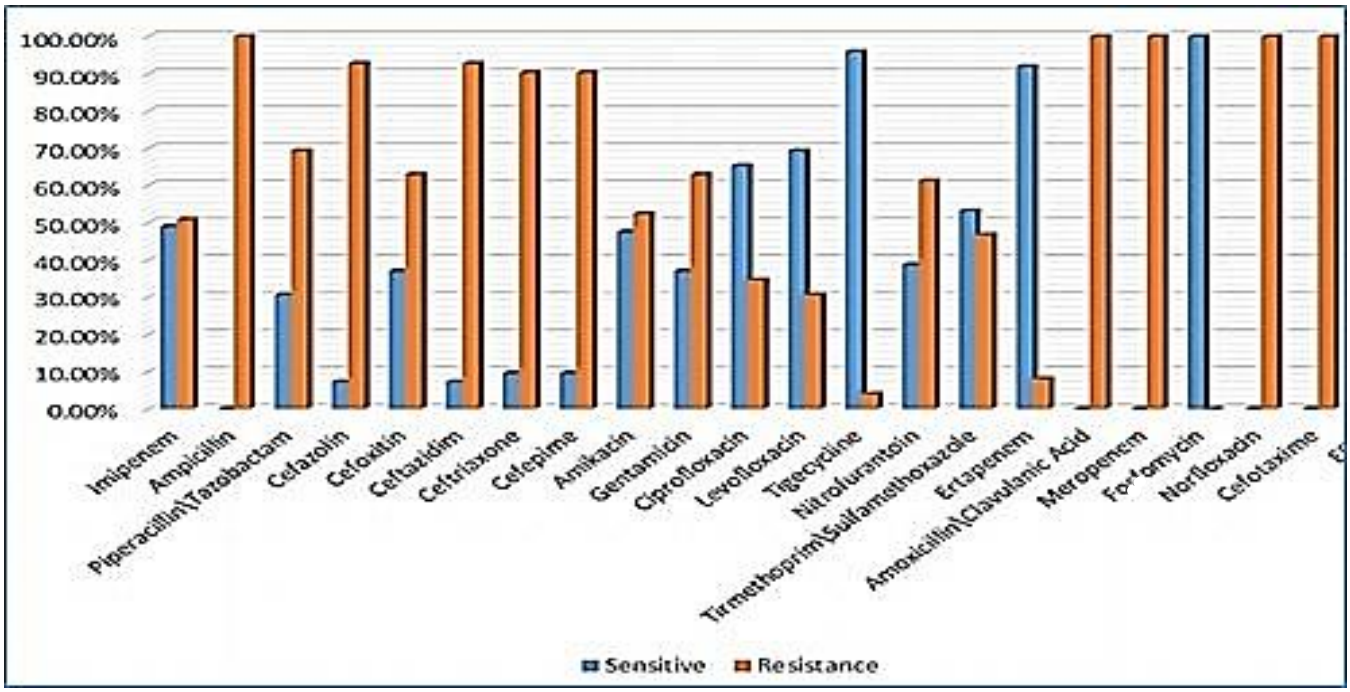

Figure 1: Antibiotic sensitivity of Klebsiella pnemonaie towards selected antibiotics.

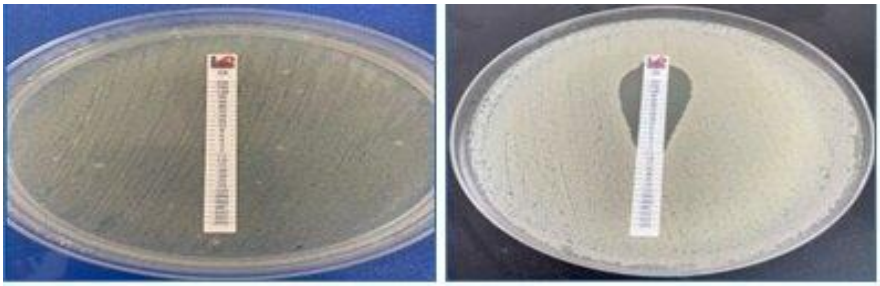

Figure 2: Colistin E test strip to determine MIC for *K. pneumoniae*. Left: Resist isolates. Right: Sensitive isolates.

Biofilm formation among Colistin-resistant *K. pneumoniae* isolates: The strains of Klebsiella pneumoniae that are resistant to colistin are listed in **Table 2**. Colistin-resistant K. pneumoniae was detected in three (5.66%) of the isolates obtained from weak biofilms, which is an increase from the previous finding of colistin-resistant K. pneumoniae in two (3.77%) of the isolates obtained from moderate biofilms. The p-value that was calculated as a consequence was 0.099, which is lower than the significance threshold of 5%. There is no evidence to suggest that the null hypothesis should be rejected; as a result, the Chi2 test is invalid. On the other hand, the findings of a second research [13] called these findings into question by demonstrating that weak biofilm formers (97.1%) were more likely to harbor MDR-isolates than strong biofilm formers (76%). (p = 0.002).

Table 2: The relationship between biofilm formation and Colistin susceptibility

Biofilm Formation	Colistin Susceptibility		Total
	S	R	
Strong	15	24	39
Moderate	7	2	9
Weak	2	3	5
Total	24	29	53

Molecular identification of *Klebsiella spp* DNA Extraction:

Using a method called Norgen®, the genomic DNA of *K. pneumoniae* strains that have been isolated may be removed.

This kit may be used to effectively and quickly extract genomic DNA from Gram-positive and Gram-negative bacteria, which can then be put to use in subsequent applications such as PCR and sequencing.

Using a QubitTM kit, each DNA sample was examined to determine its concentration and purity; the findings indicated a range of 40-60 ng/µl for DNA concentration and a range of 1.8-2 for DNA purity.

Identification of *K. pneumoniae* by PCR:

Using a specific primer for the Rpsl gene that represented *K. pneumoniae* with an amplicon 133 bp product, all Klebsiella spp. isolates that were identified by morphological and culture methods and demonstrated colistin resistance were identified as *K. pneumoniae*.

This was accomplished by determining that the *Rps*l gene represented *K. pneumoniae*.

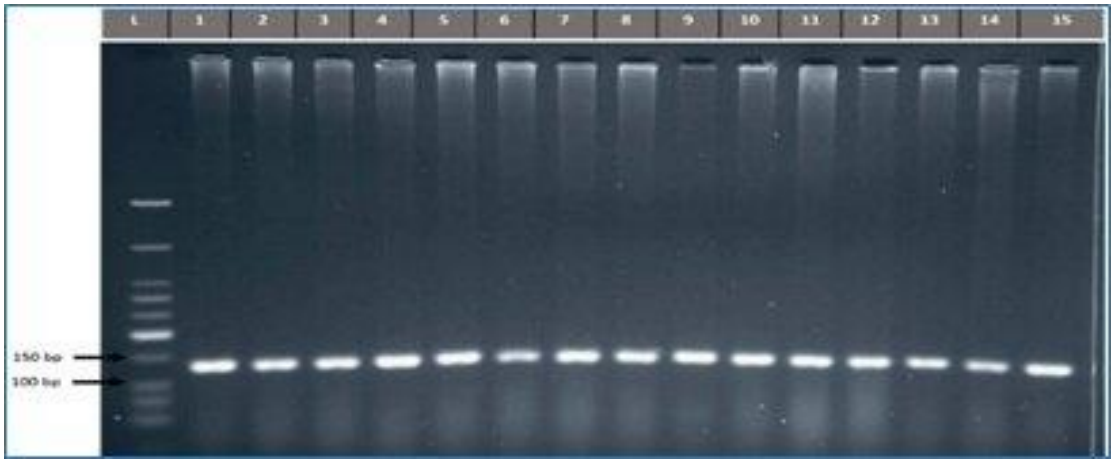

Figure 3: Electrophoretic banding patterns of the amplified products of the *Rpsl* in *K. pneumoniae*. Lane L- 25bp DNA ladder; lanes 1-15: Amplicons of RspI genes (1.8% agarose gel.)

Molecular detection of *colistin* and Carbapenem resistance genes among *K. pneumoniae* isolates:

To look for mutations that are related with resistance to colistin and carbapenem, an amplification primer was employed on the *Mgrb*, *Phoq*, and *Blashv* genes all at the same time. Due to the inactivation of the *Mgrb* gene, which is produced by the overexpression of the lipopolysaccharide modification pathway, carbapenemase-producing *K. pneumoniae* (KPC-KP) strains have developed resistance to the antibiotic colistin. This resistance has allowed these strains to survive treatment with colistin [(5)]. The *Mgrb* gene, which is found in the PhoQ-PhoP (two-component regulatory) system, encodes a negative-feedback regulator.

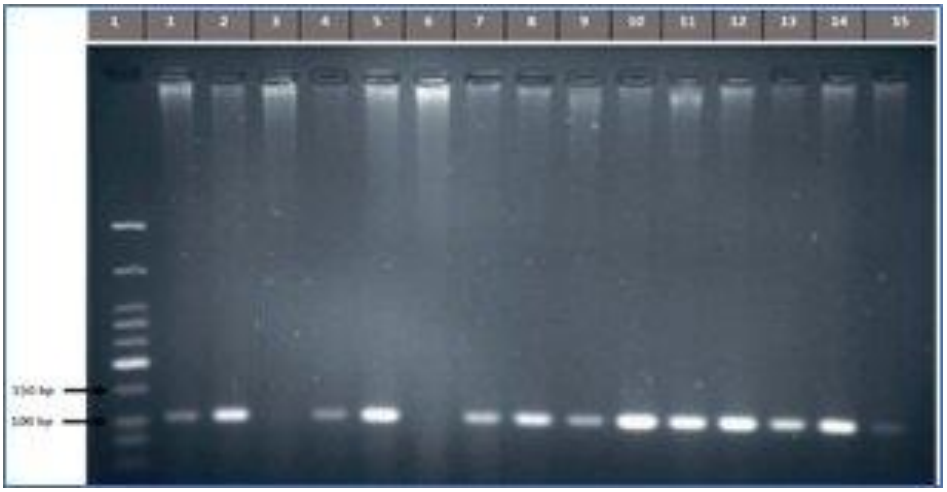

Figure 4: Electrophoretic banding patterns of the amplified products of the *Blashv* -specific Gene for carbapenem resistance (1.8% agarose gel. Lane L- 25bp DNA ladder)

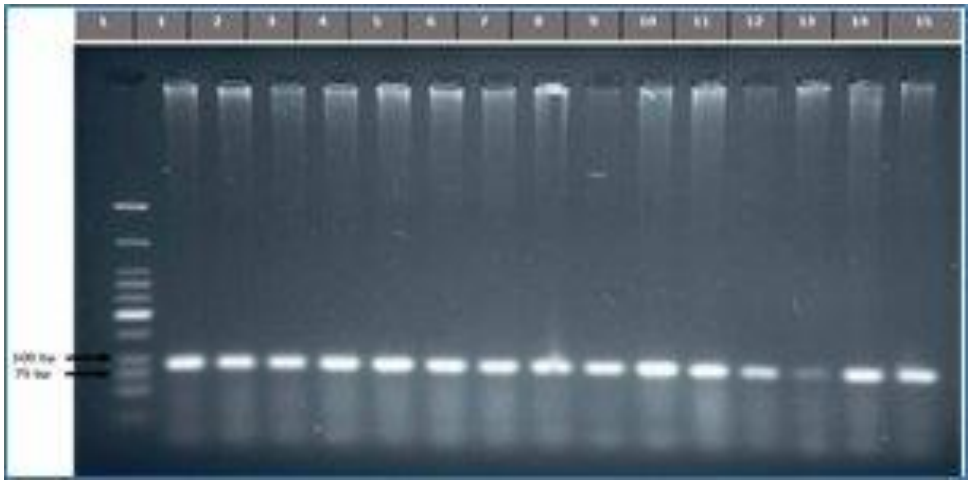

Figure 5: The amplification products of the *Mgrb* gene for colistin resistance in Klebsiella pneumoniae (1.8% agarose gel, other circumstances) show characteristic banding patterns on an electrophoresis gel.

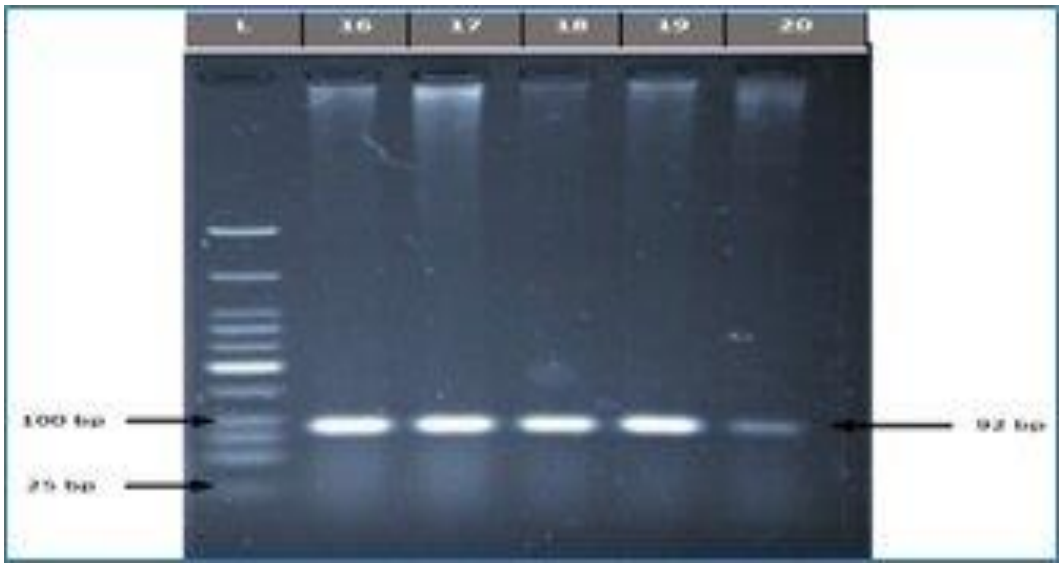

Figure 6: The amplified products of the *PhoQ* Gene for Colistin Resistance in K. pneumoniae exhibit electrophoretic banding patterns on 1.8% agarose gel.

Transcriptional analysis by real-time quantitative PCR:

It can be seen from Table 3, together with Table 4, that there are eight *K. pneumoniae* that had the highest level of colistin resistance, as determined by the E-test and the minimum inhibitory concentration (MIC) using a microtiter plate, were chosen to determine the level of *Mgrb* and *PhoQ* gene expression after being subjected to one of two treatments: first, with low Sub-MIC colistin concentrations (2 and 32) µg/ml, and second, with high Sub-MIC concentration (256µg/ml).

Real-time quantitative polymerase chain reaction, also known as RT-qPCR, was used in conjunction with gene-specific primers to determine the levels of gene expression for the *PhoQ* and *Mgrb* genes.

Table 3: *Mgrb* comparison between 1st colistin treatment and 2nd treatment

***Mgrb* gene**	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**	**P-value**
Fold 1st treatment	8	1.4	0.4	0.14	0.869
Fold 2nd treatment	3	1.3	0.84	0.48	

Table 4. *Phoq* comparison between 1st colistin treatment and 2nd treatment

***Phoq* gene**	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**	**P-value**
Fold 1st treatment	8	1.9	0.94	0.33	0.62
Fold 2nd treatment	3	1.61	0.76	0.44	

DISCUSSION

According to the findings of the study, blood samples contained the highest number of isolated organisms, while urine samples contained the second-highest number. This is as a result of the fact that urinary tract infections (UTIs) are a typical cause for patients to seek medical attention and be given antibiotics in today's medical practice. The higher incidence of UTIs in females as compared to males may be the cause of the greater availability of urine samples from females. This discovery is consistent with the findings of other research that indicated a ratio that is comparable [(12)].

It's possible that the female urethra is to blame for this, given its relatively short length, large diameter, and close proximity to the anus [(13)].

According to the data, the most prevalent age group was adults aged 50–69, which is consistent with the findings of other research showing that people of this age are more vulnerable to infection [(14)]. The findings of the antibiotic susceptibility tests showed a high degree of resistance to the majority of antibiotics used in the study. From these findings, we may conclude that most of the isolates exhibited multi-drug resistance. Resistance to aminoglycosides and cephalosporins was found to be high in *K. pneumoniae* isolates in the present study. Numerous studies conducted at the regional level have shown that these bacteria exhibit high levels of resistance to cephalosporins of the third and fourth generations [(15)].

Antibiotics were not able to make much headway against 31 (25%) of the colistin-resistant isolates. In a similar vein, it was shown that all Gram-negative isolates resistant to colistin were also resistant to Carbapenems [(16)]. Bear in mind that most clinical labs only report colistin tests when the isolates are Carbapenem-resistant. Because resistant bacteria may be selected for treatment with colistin in the future, it is crucial to be aware of the occurrence of colistin-resistant isolates in both patients receiving colistin medication and those who are not. Additionally, the disc diffusion test showed a substantial error when compared to the E-test, which was in agreement with a study by [(8)], outcomes that were able to invalidate the validity of the disk diffusion test. In spite of the fact that 12 of the isolates passed the disk diffusion test, which indicates that they are sensitive to colistin, the E-test demonstrated that them are resistant to the medication because of its low agar diffusion. These conclusions have been questioned despite the fact that it has been established that the majority of colistin-resistant *K. pneumoniae* isolates are multidrug-resistant and biofilm producers [(17)], who found that weak biofilm formers (97.9%) were more likely to harbor multi-drug resistant isolates than strong biofilm formers (76%) (p=0.002). Each isolate had a unique biofilm-forming potential because several variables, including *K. pneumoniae's* physicochemical make-up, the nature of the components' interactions, the surface to which the biofilm adheres, environmental conditions, and so on, modulate that potential [(18)].

Overexpression of the Pmr lipopolysaccharide modification pathway causes colistin resistance in carbapenemase-producing *K. pneumoniae* (KPC-KP) via inactivation of the *Mgrb* gene. The PhoQ-PhoP (two-component regulatory) system is controlled by a negative-feedback regulator that is encoded by the *Mgrb* gene. Results demonstrated that *PhoQ* and *Mgrb* genes were naturally increased in response to colistin exposure, suggesting that this mechanism was being exploited to account for the presence of colistin. It was shown that beneficial connections were present when there was a correlation between an increase in the concentration of the antibiotics and an increase in the expression. The research presented in the aforementioned studies provided conclusive evidence that colistimethate resistance is genetically linked to intricate networks that control modification pathways [(5)].

As a result of what has been said up to this point, it is abundantly evident that it is necessary to conduct tests to determine whether or not colistin is effective against a broad variety of dangerous bacteria and parasites such as *Clostridium perfringens* [(19)], *Brucella melitensis* [(20)], *Proteus vulgaris* [(21,22)], *Staphylococcus aureus* [(23)], *Pseudomonas aeruginosa* [(24,25)] ,and Toxoplasma spp [(26,27)].

In conclusion, females were more likely to have *K. pneumoniae* than men in the 50-69 age group. *K. pneumonia* demonstrated varying resistance to the drugs studied. 31% of 124 *K. pneumoniae* isolates were colistin-resistant. Disc diffusion test error was greater than E-test error (MIC strip). *K. pneumoniae* demonstrated more antibiotic-resistant isolates with robust biofilm development and fewer with poor biofilm formation. RT-qPCR was used to amplify and assess colistin resistance genes *Mgrb* and *Phoq*. These genes were increased in response to colistin, demonstrating that this mechanism accounts for colistin presence. Colistin concentration boosted gene expression, indicating a favourable association.

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Author contribution: Authors are contributed equally in the study.

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