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Identification of Klebsiella Pneumonia Isolates from Sewage Water of Some Hospitals in Wasit Province

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Abstract: The specific objective of the study was to validate the identification of Klebsiella pneumoniae isolates and to identify the presence of two virulence-associated genes namely mrkD and rmpA using Polymerase Chain Reaction (PCR) method. Thirty (30) K. pneumoniae were isolated and recovered in March and April 2024 by sampling sewage samples of different hospitals in the Wasit Province along with the respective metabolism feature. The identification of isolates was done with the API 20E system and the VITEK 2 automated platform. The disk diffusion method of antimicrobial susceptibility testing was performed on 12 different antibiotics. The strongest resistant rates were with respect to Ampicillin and Amoxicillin-Clavulanic acid (100 percent), the lowest resistance was during Imipenem (5 percent). Through the PCR analysis, 14 isolates out of the 30 (47 percent) were positive with the mrkD gene and 10 of 30 (34 percent) with the rmpA gene. To sum up, it is evident that the prevalence of K. pneumoniae in sewage samples is extremely high in hospital settings, which demonstrates the danger of environmental contamination and once again

explains why execution of effective waste water-treatment and infection-control methods, such as in healthcare institutions, is of paramount priority.

Keywords: Klebsiella pneumoniae, polymerase chain reaction, virulence factors

Introduction

Klebsiella is a clinically notable genus in the family of the Enterobacteriaceae; out of which Klebsiella pneumoniae was found to be the most common species that cause pneumonia, and to a minor degree, K. oxytoca [1]. Gram-negative, facultative anaerobes Members of this genus are Gram-negative, nonmotile, facultative anaerobes. They have a rod shape (about 0.311, 1m wide and 0.6, 6m long), and can be found as singular, or paired, or short-chain. Such bacteria are identifiable because of the heavy concentration of the capsular polysaccharide (CPS) layer on culture plate, making them appear mucoid [2]. On MacConkey plate, colonies of Klebsiella are big and mucoid with a reddish pigmentation as a result of lactose fermentation and acid production.[3]

K. pneumoniae sources of virulence include its capsule, lipopolysaccharides (LPS) fimbriae and siderophores that assist adhesiveness, colonization and invasion during infection. Other mechanisms that have been shown to be involved in virulence, transmembrane proteins on the outer membrane, porins, efflux systems, iron dependence systems, and all allantoin metabolism pathways have also been noted in recent studies [4]. The strain-specific polysaccharides corresponding to the capsular antigens (K antigens) assist the bacterium in evading the phagocytosis. Of the 77 capsular types, capsular type K1 and K2 have strong associations with severe infections in human beings [3]. Serotype specific genes have also been implicated in pyogenic liver abscesses; magA which is serotype specific to the K1 type and k2A which is serotype specific to the K2 type [5 7]. Moreover, rmpA gene can be found on plasmids and chromosomes which regulates the production of CPS and is linked mucoid aspect of virulent strains.[9,10]

Siderophores are low-molecular-weight molecules that aid Klebsiella pneumoniae to store iron in the host iron-binding proteins, thus enhancing the survival and growth of the bacterium in the setting that is limited in terms of iron. Siderophores The most common siderophores secreted by *K. pneumoniae* are enterobactin, which is coded by entB gene [3]. Production of various siderophores enables the pathogen to avoid sequestration mechanisms by the host. Interestingly it has been shown that YbtS gene is one of the downstream genes that make up the yersiniabactin pathway and is highly up regulated in respiratory tract isolates against those in blood, urine and faeces patients [12]. Individually and collectively, all the virulence genes contribute to the different steps of the infection process, such as colonization, invasion, dissemination, and pathogenic outcome in general of K. pneumoniae. Based on this, the current study will focus at isolating *K. pneumoniae* in samples of sewage and screening of some of the virulence-related genes related to its pathogenicity.

2. Material and methods:

2.1. Bacterial Isolates

This research study was carried out in the microbiology laboratory in the College of Science, University of Wasit. One hundred and forty-eight sewage samples were taken in February to April 2024 in diverse hospitals in Wasit province. Sterile containers were used to make the samples and smear loops placed with each sample under anaerobic conditions in MacConkey agar and blood agar (Oxoid, UK) plates; subsequently incubated at 370 C temperature in 24-hour

time. For initially identifying bacterial isolates a morphological identification was followed by the use of a suite of biochemical tests, Gram staining, growth on MacConkey, catalase, oxidase, urease formation, motility, indole production, methyl red reaction, Voges-Proskauer reaction, Simmons citrate utilization and triple sugar iron (TSI) [13]. Verification of presumptive Klebsiella pneumoniae was carried out after confirmation of some isolates with the API 20 E identification plan and VITEK 2(R) auto identification scheme (BioMrieux(R) France).

2.2. Mucoviscosity Test:

All the *Klebsiella pneumoniae* isolates were inoculated onto blood agar plates then incubated at 37 ℃ in the use of 24 hours. The string test in order to evaluate mucoviscosity entailed grazing a bacterial colony with the tip of an inoculating loop and raising it vertical causing a mucous string to appear. The hypermucoviscous phenotype was determined as positive when over 5mm of the hypermucoviscous string was produced by the isolate [14]..

2.3. Antibiotic Sensitivity Test:

The Kirby Bauer disk diffusion was used as the method of antimicrobial susceptibility testing, described by [15]. Antibiotic discs were selected according to the recommendations offered by the Clinical and Laboratory Standards Institute (CLSI).[16]

2.4. DNA Extraction:

The extraction of the genomic DNA was done by boiling method as reported earlier by [17], but in slight modifications. In short, loopfuls of bacterial cultures that had been grown in brain heart agar (BHA) media 24 hours later were suspended in 1 mL of sterile 1X TE buffer (pH 8.0) replacing sterile distilled water. The cell lysis was therefore performed by boiling the bacterial suspension at 95 ℃ in a water bath over 10 minutes. After lysis, the mixture was then centrifuged 5 minutes at 10 000 rpm. The crude DNA containing supernatant was thoroughly collected and aliquoted in 100 L and placed at -20 ℃ until further analysis.

2.5. Primers Preparation:

Table 1 shows the primers that were used to detect the presence of mrkD and rmpA genes. The synthesis of these primers was carried out by Macrogen (Korea) and it was then provided as a lyophilized form. Primers were reconstituted in nuclease-free water according to the instructions of the manufacturer in order to obtain a stock solution of 100 pmol/ml. In order to obtain working solution 10 ml of stock solution was added to 90 ml of nuclease free water and the final concentration was 10 pmol/mL.

Table 1: Primers that used in Polymerase Chain Reaction Technique

Primers	Sequence 5’ – 3’	Product size (bp)
mrkD-F	AAGCTATCGCTGTACTTCCGGCA	340
mrkD-R	GGCGTTGGCGCTCAGATAGG	
rmpA-F	CATAAGAGTATTGGTTGACAG	622
rmpA-R	CTTGCATGAGCCATCTTTCA	

2.6. Polymerase Chain Reaction Technique:

The polymerase Chain Reaction (PCR) assays were carried out in a total volume of 20-m L per reaction tube. And sterile double-distilled water (ddH 2 O) was added to the reaction mixture to bring it up to the set volume. A negative control (no-template control) was also included in each amplification and this contained all the PCR components minus the template DNA which was substituted with sterile water. All of the reaction mixes contained 10 L of GoTaq18 Green Master Mix (Promega, USA), 1 L of both primers (working solution at 10 3/L), and 2 L of bacterial DNA template. A thermal cycler device (Techne, USA) was used in PCR amplifications. PCR products were separated by electrophoresis on a 2.5% agarose gel stained

with ethidium bromide (0.5 µg/mL) and visualized under ultraviolet (UV) light.

2.7. Statistical Analysis:

Chi square was used to search for differences in the distributions of the studied determinants. [19] The statistical significance was described as a P value of less than 0.05.

3. Results and Discussion:

3.1.*K. pneumoniae* isolates

The results of the analysis revealed that among the 148 total specimens of sewage water conducted in the different locations of the hospital, 79 specimens were reported having Gram-negative bacteria, and 45 specimens have reported Gram-positive bacteria. The rest 24 specimen specimens did not indicate bacterial growth in the culture conditions applied. These samples were obtained in hospitals as well as in other places so as to make the sampling representative.

3.2. Mucoviscosity Test Results:

All specimens of *Klebsiella pneumoniae* were grown on blood agar plate containing 5% defibrinated sheep blood. Of the 30 isolates resulted, 8 (27%) were positive screened as it gave a positive result with the string test that yielded a viscous string of about 6.5 cm. The rest of the isolates exhibited moderate or weak degree of mucoviscocity as explained in Table 2.

Table 2. Mucoviscosity test of Klebsiella pneumoniae

Mucoviscosity character	Total of Isolates	Percentage
Hypermucoviscosity	8	27%
Moderate	6	20%
Few	16	53%

3.3. Biochemical Reactions Results:

All the bacterial isolates were allowed to be tested in a detailed battery of biochemical tests to determine their biochemical profile. The results of these tests are outlined in Table 3

Table (3): Biochemical tests for differentiation and identification of Klebsiella isolates

No.	Biochemical tests	Klebsiella pneumoniae isolates
1	Oxidase test	Negative (-ve)
2	Indole test	Negative (-ve)
3	Motility test	Negative (-ve)
4	Urease test	Positive (+ve)
5	Triple sugar Iron test	yellow slant, yellow butt
6	Simmon's Citrate Agar test	Positive (+ve)

3.4. Antibiotic Susceptibility Results:

The disk diffusion method was used to carry out susceptibility testing to antimicrobials. The findings showed that the highest sensitivity level was Imipenem (85 percent), Meropenem (79 percent), Gentamicin (74 percent), Tobramycin (66 percent), and Trimethoprim/sulfamethoxazole (63 percent) with the *Klebsiella pneumoniae* isolates. On the contrary, the isolates were highly-resistant to some antibiotics such as; Ampicillin and Amoxicillin Cavulanic acid (100%), Piperacillin (82%), Cefotaxime (66%) and Ceftriaxone (55%). Table 4 shows results in details

Table 4: Antimicrobial Susceptibility K. pneumoniae isolates

Antibiotics sensitivity pattern	Resistant	Sensitive
Trimethoprim\sulfamethoxazole	36.5%	63%
Gentamicin	17.5%	74%
Cefotaxime	66%	6%
Ceftriaxone	55%	12.5%
Imipenem	5%	85%
Meropenem	24%	79%
Piperacillin	82%	5%
Amoxicillin + Clavulanic acid	100%	0%
Ampicillin	100%	0%
Tobramycin	20%	66%
Amikacin	25%	17.5%
Tetracyclin	40%	37%

3.5. Genomic DNA Extraction Results:

The bacterial genomic DNA which extracted from all samples showed integrity band with concentration 6µg/ml,Figure 1.

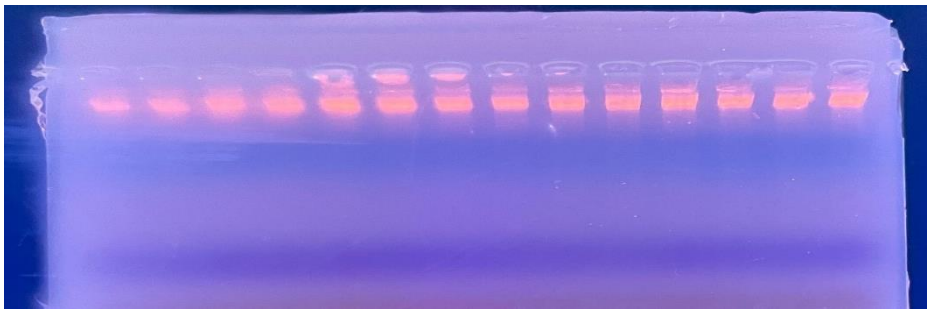

Figure (1): Gel electrophoresis of genomic DNA. The sample was migrated on 0.1% agarose gel at 70 Volt for 1 hour.

3.6. Polymerase Chain Reaction Results:

Polymerase Chain Reaction (PCR) was done among 30 isolates of *Klebsiella pneumoniae* to see the two virulence-related genes; mrkD and rmpA. The findings showed that 14 isolates (47%) were mrkD positive and 10 isolates (34%), were rmpA positive. The PCR amplification pattern of the said genes was observed in Figures 2 and 3 respectively showing the result of mrkD and rmpA on agarose gel electrophoresis.

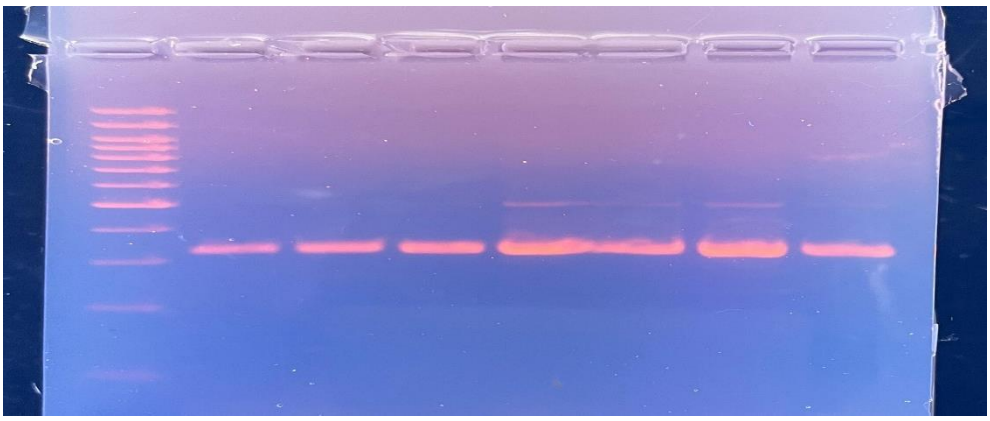

Figure 2: Gel Electrophoresis for PCR products (340 pb) of *mrkD* gene *K. pneumoniae*. Electrophoresis was performed on 2.5 % agarose gel and run with a 80 volt for 120 min. Lane M is a (100 bp) ladder, Line 1,2,3,4,5,6 and 7 are a positive results of *mrkD* .

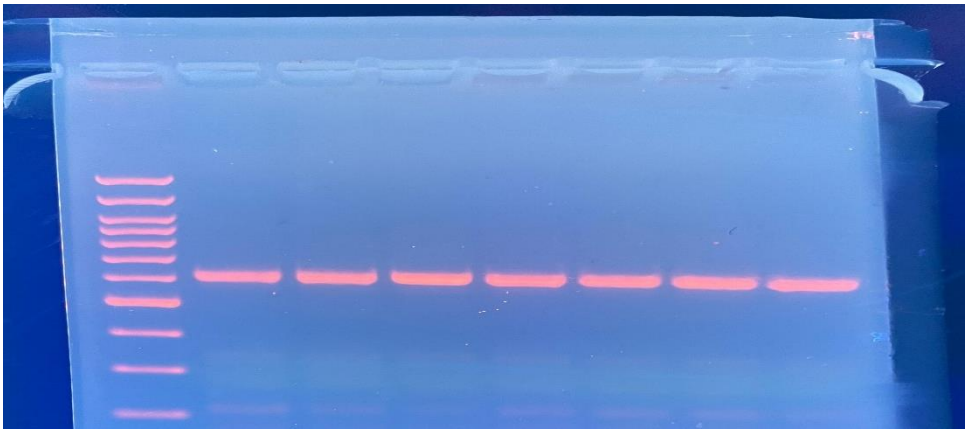

Figure 2: Gel Electrophoresis for PCR products (622 pb) of *rmpA* gene. Electrophoresis was performed on 2.5 % agarose gel and run with a 80 volt for 120 min. Lane M is a (100 bp) ladder, Line 1,2,3,4,5,6 and 7 are a positive results of *rmpA*

4. Discussion

Klebsiella pneumoniae **is a major pathogen that can cause nosocomial and community acquired infections. This pathogen harbors numerous virulence factors, which help this microorganism to cause infections. The isolates of *K. pneumoniae* had *Klebsiella pneumoniae* is a serious opportunistic pathogen which causes both hospital acquired and community acquired diseases. It has diverse variety of virulence factors, mainly ascribed to its pathogenicity, as these factors assist it in colonization, invasion, and to evade the hosts immune-related responses.** A high resistance level of K. pneumoniae isolates to various antimicrobial agents was observed in this study, most of which were multidrug-resistant (MDR) and this trend could be due to the inconsistencies in the use of antibiotics in a medical unit [20]. Outer membrane porin gene, among the virulence related factor, has greatly influenced the flow of antibiotic into the bacterium outer membrane, examples include, OmpK 35 and OmpK 36.

According to Jaber et al. [21], the effect of the mutations inherited in OmpK36 is the minimization of membrane permeability by a crack, hence limiting the entry of antibiotics into the cell and thus resulting in resistance. These indeed are mutations especially in OmpK35 and OmpK36 which can be the representation of an evolutionary process of antibiotic resistance [22]. In past studies, prevalence of MDR K. pneumoniae has been reported up to 84 per cent [23]. Transmission of the resistance genes is highly promoted by mobile genetic elements including plasmid, transposons, and integrons which enhance the rapid dispersion of antimicrobial resistance across the bacteria population.[24]

The rmpA is one of the leading virulence genes and it is plasmid located and is significantly linked with hypermucoviscosity. It is an intracellular capsular polysaccharide regulator that boosts the resistant capacity to phagocytosis and environmental stress of the bacterium, as indicated by Rivero et al. [25]. In the current research, the rmpA gene was identified in 40 percent of isolates, a report that agrees with Mirzaie et al. [26], who established it in 48 percent of isolates. Further, the known correlation of the presence of rmpA, with aerobactin, has reported that 96 per cent of rmpA-positive isolates reproducibly co-produce aerobactin [27]; the same trend was held in our study, with 40 per cent also producing aerobactin. Also, mrkD gene of type 3 fimbrial adhesion that peripherally attacks epithelial attachments was found in 60 percent of the isolates which support the findings of Chen et al. about colonization and virulence [28].

Kotay et al. [29] find that sewage water is an environment with all essential nutrients that favor the survival and multiplication of pathogenic and non-pathogenic species of bacteria. This is because of its complicated structure which has different organic and inorganic compounds necessary in growing microbes. Among the infection reservoirs are the hospital sinks and shower drains which may contain strains of the bacteria being genetically similar or identical to those found on the patients [30]. It is believed that the main pathway in which the *Klebsiella pneumoniae* transmits through these contaminated surfaces to hospital sewage systems is through droplet transmission during water usage on sinks and shower.[31]

It is noteworthy that contamination of sinks and showers can become nonlocal. It has been reported that *Gram-negative bacteria* have the potential to travel along the interlinked pipe systems of wastewater systems and infect the nearby plumbing systems which include the other drains [32]. This could be because bacteria injected upon normal use of sinks or showers could be absorbed into existing biofilms where they maintain their stay and overtime multiply to levels that could be detected. Alternatively late occurring and unattributable contamination events could also add to such dissemination patterns [33]. Recently Hopman et al. documented an environmental outbreak of a deadly carbapenemase-carrying Pseudomonas aeruginosa of carbapenemase-producing origin [34] that was also due to a comparable source of contamination.

Conclusions:

The result of the present study is comparable to the previous reports that show the spread of Gram-negative pathogens through hospital plumbing system [29]. More so, this piece of work gives supportive evidence surrounding the spread of carbapenemase-producers Enterobacterales (CPE) through the path of infected sewage water. This contamination cannot be considered to stay in one place and it can be allowed to spread the resistant strains to other parts of the hospital infrastructure. Poor treatment of the hospital sewage water is also a dangerous threat, which can become a source of letting the multidrug-resistant bacteria and resistance genes into the external environment. In turn, this is a significant threat to the healthy population of a country as it can result in the pollution of the natural sources of water and propagation of antimicrobial resistance factors in the environment.

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