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Original Research Article

# Prevalence and molecular detection of *Klebsiella pneumoniae* isolated from children with infectious diarrhoea in AL-Ramadi city

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ABSTRACT

**Objectives**: Infectious diarrhoea result from a wide range of bacteria, viruses and parasites. This condition is also identified as gastroenteritis, *Klebsiella pneumonia* is a well-known as one of most common bacterial pathogens causing gastroenteritis. This study aims to estimate the incidence and identifying both the phenotypic and genotypic characterization of *Klebsiella pneumonia* causing gastroenteritis in children under the age of five in al-Ramadi Maternity and Children Teaching Hospital.

**Materials and Methods**: Stool samples were collected for 106 children suffering from gastroenteritis, Cultural and microscopical approaches were used for *Klebsiella pneumonia* selection, its characteristic features were confirmed using the Vitek2 compact system, anti-microbial sensitivity test, and biofilm production test. furthermore, DNA extracted, purification and Polymerase chain reaction (PCR) were accomplished for genotypic confirmation.

**Results:** In the presented research, stool samples were collected for 106 children suffering from gastroenteritis, and 100 samples were identified as source for bacterial gastroenteritis. The bacterium under consideration (*Klebsiella pneumonia*) has a percentage of 30%. This isolate revealed resistance to Ceftazidime (80%), nalidixic acid (33%), amikacin (36%), Azithromycin (20%), vancomycin (10%), and Imipenem (6%). Likewise, four genes in *Klebsiella pneumonia* isolate were studied via PCR and the results indicates htrA, iss, Mrka and rmpA were 15 (50%), 9 (30%), 6 (20%), 0 (0%) respectively. The results of biofilm production for *Klebsiella pneumonia* exposed that 3 (10%) were strong, 10 (33%) moderate, 7 (24%) weak, and 10 (33%) non-producers.

**Conclusions**: The presented research displayed the bacterium under consideration (*Klebsiella pneumonia*) has a higher resistance rate to the commonly antibiotics used for bacterial gastroenteritis. In addition, *Klebsiella pneumonia* (under consideration) with high resistance to antibiotics showed resistance genes in PCR, in addition to strong biofilm production.



## 1. Introduction

Diarrheal illnesses represent a significant cause of sickness and death among children under five years old, accounting for approximately 500,000 annual fatalities and ranking third in the global burden of disability-adjusted life-years.[1] Many bacteria, viruses, and parasitic species can produce diarrhea, which is typically a sign of digestive tract illnesses. Worldwide, diarrhea has a wide range of characteristics and a seasonal distribution. Each year, about 1.7 billion individuals suffer from diarrhea illness worldwide. It is considered as a major source of morbidity and mortality among children and represents a worldwide health concern.[1] Regional, social, and economic development, as well as geographic living practices, are intimately linked to the distribution and incidence of diarrheal diseases.[2,3] The

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majority of diarrhea-causing pathogens primarily transmit through the fecal-oral route. However, each pathogen employs unique infection mechanisms and diverse strategies to elicit clinical symptoms of diarrhea.[4–7]

Nevertheless, other research indicated that the predominant pathogenic agents linked to this disease were gut bacterial flora like Escherichia spp., Salmonella spp., Shigella spp., and *Klebsiella pneumoniae*.[8,9] Coastal zones were the sites of more frequent observations of *Vibrio parahaemolyticus*.[10,11]

It is challenging to identify its origin based on clinical symptoms, so laboratory testing is frequently crucial. The "gold standard" for treating bacterial diarrhea is still depends on the standard criteria of microbiology lab techniques for isolating the causative bacteria. However, this is affected by a variety of factors, including bacterial count in the sample, proficiency of lab operators, types of culture media and methods. Furthermore, the isolation will be significantly impacted by antibiotic treatment, producing falsely negative results. The lower estimation of bacteria in comparison with virus among sample obtaining may be due to the variation in the revealing sensitivity related with phenotypic and genotypic based approaches. Reducing turn around times and increasing detection method success rates have been attempted in an attempt to address issues with the medical remedy and epidemiologic assessments connected to diarrhoea infections. Among these efforts has been the creation of suitable assays depends on PCR based finding.[12,13]

In general, molecular finding procedures have a higher sensitivity than the cultural techniques. Prior to strain isolation, it is assumed that sample screening using molecular techniques were more efficient than direct selection of isolate in all samples. This is due to earlier approach which focus on samples that tested positive for isolates. Finding employment costs would be decreased and the rate of positive detection would be raised. When it comes to bacterial diseases that require enrichment prior to isolate, like *Vibrio* and *Salmonella* species. More causative agents among the same samples will discovered through identifying the nucleic acids of supplemented culture, while molecular revealing based on stool nucleic acids may neglect certain samples which have lower bacterial counts.

## 2. Materials and Methods

### *2.1. Collection of stool samples*

All specimens were taken from September 2022 to December 2022. The approval of parental consent for pediatric patients was obtained according to the guidelines of the Research Ethics Committee at Anbar University. Patients' information and history were recorded using a formal sheet questionnaire. Samples were collected from patients who attended al-Ramadi Maternity and Children Teaching Hospital. Samples were isolated from the stool of children under five years of age, and then cultured on media blood agar, MacConkey agar, and S. S agar. Criteria for inclusion and exclusion in the study include factors such as age range of the children, clinical presentation of infectious diarrhea, diagnostic methods used for *Klebsiella pneumoniae* detection, and any comorbid conditions that may affect the study outcomes. This research has been approved by the Scientific Research Ethics Committee at Anbar University on[11,12] with serial number 964783720431.

### *2.2. Isolation and identification of bacterial isolates*

106 samples were collected from the excreta of infected children, and 30 isolates were identified as *Klebsiella pneumoniae*. Its characteristic features were confirmed using the Vitek2 compact system (BioMerieux, France).

### *2.3. Antimicrobial susceptibility test (AST*

The Kirby-Bauer method is a standardized system for testing the efficacy of antimicrobials against micro-organisms. Pure isolated colonies (2-4) were inoculated in brain-heart broth (2ml) to obtain a bacterial suspension, which was compared with a 0.5 McFarland turbidity tube, Muller Hinton agar plates were inoculated using cotton swabs from that prepared suspension and incubated under standard condition. Anti-microbial discs were positioned upon the medium's surface. Then incubated at 37°C for 24 hours. For interpreting the results, the Clinical and Laboratory Institute's standards were followed (Clinical and Laboratory Institute Standards 2022).

### *2.4. Molecular identification*

The PCR approach was used for revealing four virulency genetic factor (*htrA, rmpA, mrkA,* and *iss gene*) in the Klebsiella pneumonia isolates.

### *2.5. Extraction of bacterial DNA*

The bacterial gDNA was recovered from bacterial isolates by the use of gDNA mini bacteria kit according to protocol from Favorgen, Taiwan. The pure gDNA solution was kept in -20 $^{o}$C until use in the PCR.

### *2.6. PCR amplifications*

The PCR amplification was used to identify the virulence genes present in Klebsiella pneumonia isolates; the primers used in PCR were lasted as seen in Table 1.

### *2.7. Biofilm test*

The micro-titter plate method was used to detect the capability of *Klebsiella pneumonia* isolates for biofilm

**Table 1:** The primers used in current study. The key word F: Forward sequences, R: Reverse sequences.

| Gene name | | Primer sequence | Product size | | TM (°C) |
|---|---|---|---|---|---|
| rmpA | F | 5-CGCAGAAGTATTCCGTTGGT-3 | 967 bp | AB289644. | 58 |
| | R | 5-TTTGTTAGCCGTGGATAATGG-3 | | | |
| mrkA | F | 5-AGTGCTTTCACCCCCTCCT-3 | 862 bp | M55912.1 | 59 |
| | R | 5-GAGTGACTGGGGTGAGCAA-3 | | | |
| iss | F | 5-TGTCACATAGGATTCTGCCGTT-3 | 450 bp | NZ_RZLR01001650.1 | 60 |
| | R | 5-TTCACCCTCAGAGAGAGGCT-3 | | | |
| htrA | F | 5-CGTTCTGCCAGGATGGTTCT-3 | 1071 bp | AJ430233.1 | 59 |
| | R | 5-CCCCAATGATGACATCGCCT-3 | | | |

formation according to as following: Firstly, inoculum preparation (Turbidity standard) To obtain 1.5 x $10^8$ CFU for each ml, few drops of freshly overnight broth culture were transferred into 5 ml sterile brain heart infusion medium, 0.5 Mc-Farland standard was used to evaluate the tube turbidity. Secondly, preparation of the 96-well micro plate (ELISA): Biofilm formation assessed as mentioned by,[14] in which 200 $\mu$l of the diluted cultures was loaded into the micro wells using a micropipette (three duplicates of each isolate), then 200 $\mu$l of sterile BHI broth was added to 6 wells as a negative control. The plate was then sealed with a lid and aerobic incubation for eighteen hours at 37 $^o$C. Thirdly, bacterial cultures were removed from all wells and washed twice with a sterile phosphate buffer saline (PBS). The plate was dried on filter paper by inverting the plate upside down. Then, 200 $\mu$l of methanol was added in each well for 10 min that helps fixing the adhered bacterial cells (forming biofilm cells). Fourthly, the washing process was repeated carefully at room temperature to ensure the removal any excess of methanol from the wells. Finally, 200 $\mu$l of the crystal violet stain (0.1 % w/v) was added in each well, the plate was left for 15 min at room temperature, excess stained was removed by washing three times with PBS, late to dry completely.

## 3. Result and Discussion

### *3.1. Isolation and identification of bacteria*

106 samples were collected from the discharge of children with gastroenteritis from al-Ramadi Maternity and Children Teaching Hospital. The samples infected with bacteria were identified and diagnosed using morphological and cultural criteria and the Vitek2 compact automated system, 30 isolates of the *Klebsiella pneumoniae* bacteria were obtained, with a percentage of (30%).

### *3.2. Antimicrobial susceptibility testing*

The result showed a highest resistance of *Klebsiella pneumoniae* to Ceftazidime (80%), then amikacin (36%), nalidixic acid (33%), Azithromycin (20%), vancomycin (10%), Imipenem was found (6%) as shown in Figure 1 and Table 1.

**Table 2:** The antibiotic susceptibility of *Klebsiella pneumoniae*

| Antibiotics | Resistant % | Intermediate % | Sensitive % |
|---|---|---|---|
| Ceftazidime | 80 | 0 | 20 |
| Amikacin | 36 | 0 | 64 |
| Nalidixic Acid | 33 | 0 | 67 |
| Azithromycin | 20 | 0 | 80 |
| Vancomycin | 10 | 0 | 90 |
| Imipenem | 6 | 0 | 94 |

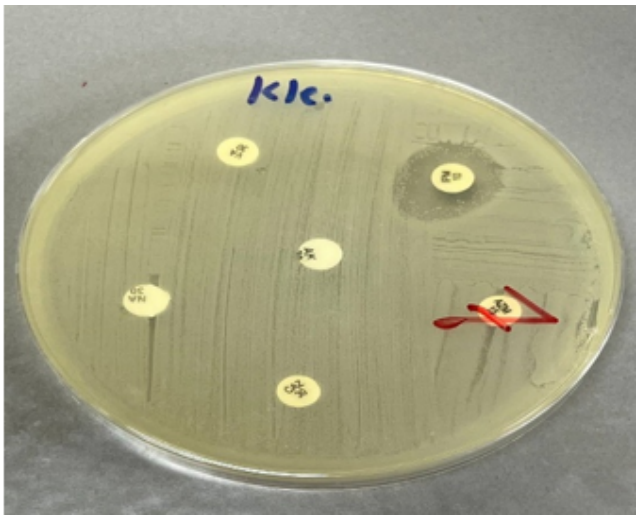


**Figure 1:** Antibiotic resistance of *Klebsiella pneumoniae* on Muller Hinton Agar.

### *3.3. Detecting of rmpA gene*

In this study, none of the isolates showed a result for this gene (0%) as shown in Figure 2. The rmpA gene is a regulator of mucoid phenotype A (RmpA) gene found in *Klebsiella pneumoniae,* This gene is responsible for producing of the well-known capsular polysaccharide (CPS), as it is a sticky substance that helps these microbes to escape from the immunity defense procedure.[15] The rmpA gene has been shown to be important for the virulence of *K. pneumoniae*. Studies have shown that mutants of *K. pneumoniae* that lack the rmpA gene are less virulent in animal models of infection. This is likely due to the fact

that the rmpA gene is required for the production of CPS.[16]


**Figure 2:** Gel electrophoresis for detection of rmpA gene (967 bp). 100-bp DNA ladder. PCR product were electrophoresis using agarose (1.5%) at 70V for 1.5 hr.

### 3.4. Detecting of iss gene

In this study, 9 isolates out of 30 showed results for this gene, at a rate of (30%) as shown in Figure 3. The iss gene is a member of the insertion sequence (is) family of mobile genetic elements, iss elements are little DNA sequences that have the ability to shift chromosomally. They are often found in association with antibiotic resistance genes. This gene is responsible for the insertion of other genetic elements, such as antibiotic resistance genes, into the Klebsiella chromosome. This can contribute to the development of multidrug-resistant (MDR) Klebsiella infections.[17] It is also found in other bacteria for example *E. coli*, *S. enterica*. It is a common element in the genomes of these bacteria and have an important responsibility for bacterial development and alteration.

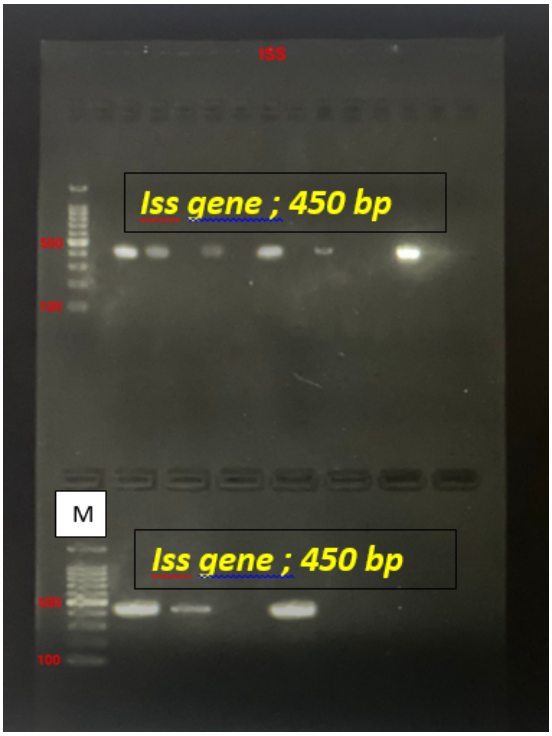


**Figure 3:** Gel electrophoresis for detection of iss gene (450 bp). 100-bp DNA ladder. PCR product were electrophoresis using agarose (1.5%) at 70V for 1.5 hr.

### 3.5. Detection of mrkA gene

In this study, 6 isolates out of 30 showed results for this gene, with a percentage of 20% as shown in Figure 4. The mrkA gene in *Klebsiella pneumoniae* is a one of MarR family members which are known as transcriptional regulators. MarR genes are responsible for regulating of genes collaborate in stress reply and antibiotic resistance. This gene has been shown to control the appearance of numerous genes that are responsible in antibiotic resistance, including the acrAB efflux pump and the ompK36 porin.[18] It has the capability of binding to the promoters' region in the acrAB efflux pump gene and repress it is expression. It is also binds to the promoters' region in the ompK36 porin which activates its expression.[19]

### 3.6. Detecting of htrA gene

In this study, 15 isolates out of 30 showed results for this gene, with a percentage of 50%. as shown in Figure 5. The htrA gene is a protease which has an important function in the bacterium's virulence. It was firstly discovered in 1992 by researchers at the University of California, San Francisco (UCSF). It is a chromosomal gene of *K. pneumoniae*, and it encodes a protein that is involved in the degradation of proteins. It is also involved in the production of bacterial biofilm, which are populational of bacteria that are inserted

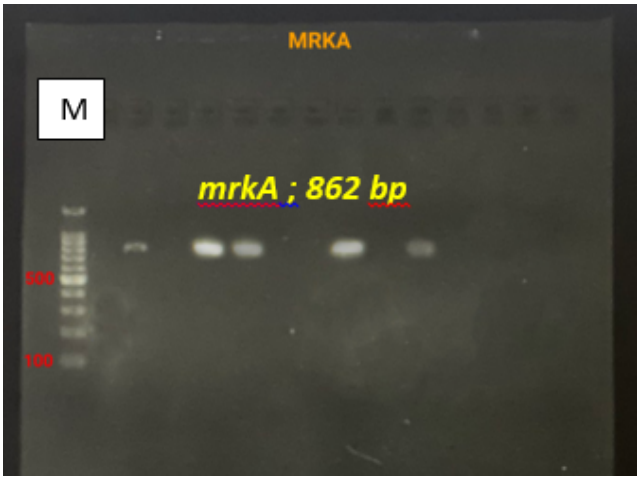


**Figure 4:** Gel electrophoresis for detection ofmrkA gene (862 bp).M: 100-bp DNA Marker. PCR product were electrophoresis using agarose (1.5%) at 70V for 1.5 hr

in the matrix of extracellular polymeric substances (EPS). Bacterial biofilm is an important for the survival of *K. pneumoniae*, as they protect the bacteria from antimicrobial agents and other environmental stresses.[20] The htrA gene is a promising target for the development of new antimicrobial therapies for the treatment of *K. pneumoniae* infections. Inhibition of the htrA protein has been shown to reduce the virulence of *K. pneumoniae* in animal models, and it is likely that inhibitors of the htrA protein would also be effective in treating *K. pneumoniae* infections in humans.[20]

### *3.7. Biofilm Formation ability by Klebsiella pneumoniae*

This study examined *K. pneumoniae* ability to build biofilms. The findings revealed that 3 isolates (10%) had a great capacity for this creation, 10 isolates (33%) had a moderate capacity, 6 isolates (24%) were relatively weak producers of biofilm, and 10 isolates were non producers (33%) as shown in Figure 6. In the current work, *Klebsiella pneumoniae* ability to produce biofilms was assessed using an ELISA technique. Antibiotic resistance of this bacterium is partly due to its capability to construct biofilm on the biotics and the non-biotics surface, such as catheter and another medical means.[21] It is found that the clinical isolates *K. pneumoniae* in the gastric system, isolates associated with pneumonia and urinary tract infections, a number of prerequisites for biofilm formation have been found.[22] On other hand, a biofilm forms when planktonic bacteria adhere to a surface, initiating a series of events. The transition from planktonic to biofilm state is a multifaceted process influenced by genetic and environmental factors. Key genes implicated in *K. pneumoniae* biofilm formation encompass fimbriae, polysaccharides, quorum sensing (QS)

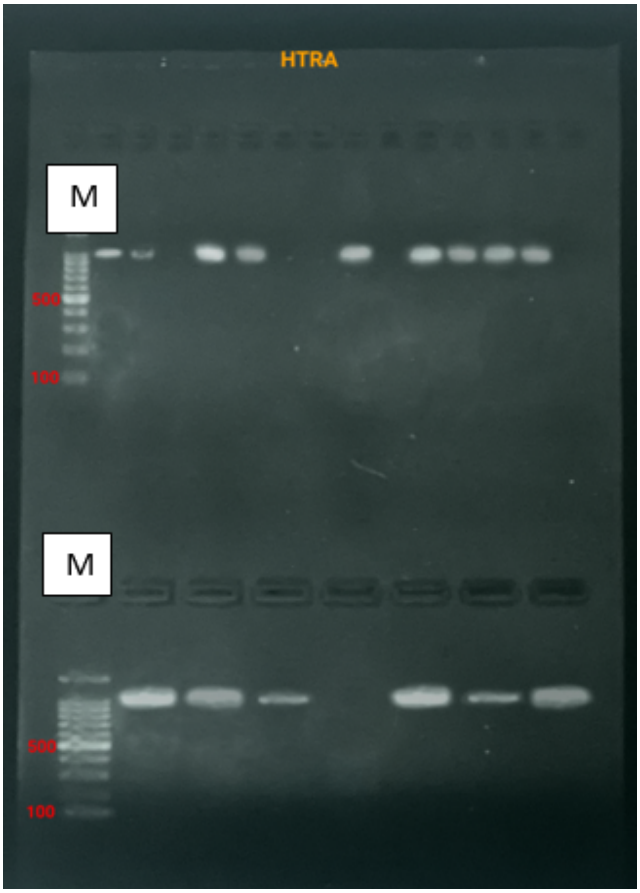


**Figure 5:** Gel electrophoresis for detection ofhtrA gene (1071 bp). M: 100-bp DNA Marker. PCR product were electrophoresis using agarose (1.5%) at 70V for 1.5 hr

systems, efflux pumps, among others.[23,24]

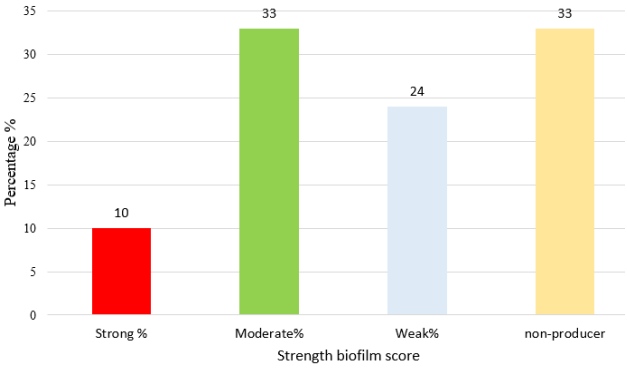


**Figure 6:** Strength of biofilm score

## 4. Conclusions

The research findings reveal that the bacterium under investigation, *Klebsiella pneumoniae*, exhibits elevated resistance levels to commonly prescribed antibiotics for

bacterial gastroenteritis. Moreover, the examined *Klebsiella pneumoniae* strains displaying heightened antibiotic resistance also demonstrated the presence of resistance genes in PCR analysis, along with robust biofilm formation.

## 5. Conflicts of Interest

The authors state that there is no conflict of interest.

## 6. Source of Funding

None.

## 7. Acknowledgments

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## Author biography

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