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Molecular Detection of *tem* Gene in *Klebseilla pneumoniae* Isolated from Children and Buffaloes in Basrah Province

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Abstract

Beta-lactam antibiotics represent a group of antibiotics that are widely prescribed. They have a wide range of therapeutic uses. Since their introduction, they induced a wide step in fighting infections. Some bacteria exhibited resistance to this group mostly via extended-spectrum-lactamase enzymes which hydrolyze penicillins and cephalosporins. Antimicrobial resistance is higher in areas of the hospital where extended-spectrum-lactamases produce gram-negative rods. *Klebsiella pneumoniae* (KP) was involved in severe infections and it represents one of the very important causes of nosocomial infections. In the current study, isolation of *K. pneumonia* was achieved in the stool and faecal samples. In the Ibn-Ghazwan hospital, from outpatient wards, Basrah, Iraq were collected 125 stool samples were collected from children and another 125 samples were collected from local buffalos. The result was found that seventy-one isolates out of 175 were doubtful *K. pneumoniae,* which analysed 40.57% of all isolates were 100% resistant to cephalothin, amoxicillin, ceftazidime and 90% resistance to cefotaxime. In this research, depending on the kind of *β-lactamase* genes, the *Tem* gene in *K. pneumoniae* was 83.3% in humans and 76% in animals.

Keywords

Klebseilla pneumonia, tem Gene , PCR , Antibiotic resistance

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Introduction

Klebsiella pneumoniae (KP) is linked to serious illnesses and is one of the most significant causes of infections that happen in nosocomial (Podschun and Ullmann, 1998). KP is the third most common bloodstream infection in children, according to worldwide surveillance data, and the of antibiotic-resistant KP's prevalence has been increased during the recent years (Diekema *et al.*, 2019). In China, the incidence of CRKP has been steadily increasing from the rate registered in 2005 (2.9%–3%) to the rate registered in 2018 (25%–26.3%), and sometimes it increased to 35.8% in newborn patients. This is very concerning (Qiu *et al.*, 2021).

One of the most often prescribed medication groups, beta-lactam antibiotics have a wide range of therapeutic uses. Their introduction, which began in the 1930s of the 20th century, fundamentally altered the struggle against bacterial infectious illnesses. These days, it has been found that the annual cost of such antibiotics might reach $15 billion USD. This expenditure accounts for 65% of the market for antibiotics (Thakuria and Lahon, 2013).

The ring structure enables dividing this group into four subgroups. However, they are listed into the common groups of: penicillins, cephalosporins, carbapenems, monobactams, and beta-lactamase inhibitors (Kotra *et al*., 2002; Walsh, 2003).

An emerging health threat is linked to antibiotic resistance (Amaya *et al*., 2011). Bacteria that express extended-spectrum -lactamases enzymes that hydrolyze penicillins and cephalosporins may be resistant to some of these antibiotics. Antimicrobial resistance is higher in extended-spectrum -lactamases producing Gram-negative rods than in other areas of the hospital (Paterson and Bonomo, 2005).

Members of the Enterobacteriaceae family, particularly E. coli and K. pneumonia, are the main producers of extended-spectrum-lactamases, which aggressively hydrolyze oxyiminocephalosporins and confer resistance against cephalosporins of the third-generation (ex. Ceftriaxone, aztreonam, cefotaxime and ceftazidime). When they are plasmid mediated, they are simply passed from one Enteobacteraceae member to another. They also contain the genes for quinolone and aminoglycoside resistance, which helps to propagate resistance to -lactamases (Perez et al., 2007). SHV-type beta lactamases came their names from sulfhydryl variables (Paterson and Bonomo,

2005). SHV-1 is classified as a member of group A (Chaves et al., 2001). One of the most significant subtypes of ESBL detected in clinical isolates is SHV type β lactamases. (Paterson and Bonomo, 2005). These can show effective hydrolysis of cefotaxime and ceftazidime (Bradford, 2001).

Given the infections brought on by these bacteria have been recorded more often than ever before in the previous ten years, ESBL-producing Enteobacteriaceae may pose a serious threat to human health (Rodriguez-Villalobos,*et al*,2010).

Methods

K. pneumonia was isolated from stool and faecal samples in the current study. In the outpatient wards of the Ibn-Ghazwan hospital in Basra, Iraq, were collected 125 stool samples were collected from children and another 125 samples were collected from local buffalos.

Ethical Approval

The ethical approval was offer acceptance and approval of research and development center, the Ministry of Health.

Culturing of *K. pneumonia*

Samples of loopy feces were inoculated onto MacConkey agar plates and incubated at 37C for a whole night. Lactose fermentation showed isolates of *Klebsiella spp*. where mucoid colonies on *MacConkey agar* were grown (Elmer, 2006; Gerald collee, 2012).

Identification of *K. pneumonia*

By the technique of McFadden, 2000, K. pneumonia isolates were identified. Traditional cultural features, gram staining, the indole test, the simmon's citrate test, and the triple sugar iron test were used to tentatively identify each isolate (Hi-Media, India). The Api 20E System Identification made ultimate identification up to kind level (Pioneer, Germany).

Antimicrobial Sensitivity Testing

All positive isolates underwent antimicrobial testing in accordance with (NCCLS) (2000). Utilizing 10 different antibiotic discs at the following concentrations, using

a modified disc-diffusion technique: amoxicillin (25µg), amikacin (30µg), cephalothin (30µg), cefotaxime (30µg), ciprofloxacin (30µg), gentamicin (10µg), caphalothin (300µg), ceftazidime (5µg), imipenem (20µg) and tetracycline (30 µg), using (NCCLS, 2000and Kirby *et al.*, 1969]. Each *K. pneumonia* strain's inoculum was placed on Mueller-Hinton (MH) agar plates from Difco Laboratories in Detroit, USA (turbidity equivalent to that of a 0.5 McFarland Standard). The widths of the inhibition zone were measured after 24, and then 48 hours of incubation (NCCLS, 2000).

Molecular detection of *tem* gene by PCR assay

According to the German firm Qiagen's recommendations, the DNA was cleaned up and removed. The Tc-312Techne (UK) DNA thermal cycler was used to do the PCR amplifications. As previously indicated in Table (1), the primer design was carried out. The mixture of PCR reaction contained 12.5µl of green master mix of *Taq* DNA polymerase derived from bacteria, $MgCl_2$, dNTPs and buffer at an optimal concentration for amplification of DNA templates by PCR. 2.5 µl of template DNA, 1 µl of each forward and reverse primer, and then the volume was completed to 25 µl by distilled water of free nuclease. The vortex then shook all tubes violently for 10 seconds.

According to a particular protocol for each gene, the PCR tubes were moved to the thermal cycler to begin the amplification process. Table 2 shows the PCR program.

Table (1): Oligonucleotide primer sequences used for PCR amplification of *tem* gene.

Gene	Primer Sequence		Size* (bp)	Reference
Tem	**F**	**5'-TCG CCG CAT ACA CTA TTC TCA GAA TGA-3'**	**445**	**(Monstein et al., 2007)**
	R	**5'-ACG CTC ACC GGC TCC AGA TTT AT-3'**		

*: Size: PCR product (bp).

Table 2: PCR amplification program for *Tem* gene detection according to (Monstein*et al*. (2007))

Stage		Steps	Temperature (C°)	Time	No. of cycles
First		Initial Denaturation	95	5 min	1
Second	I	Denaturation	94	30 s	30
	II	Annealing	50	1	
	III	Extension	72	2	
Third		Final Extension	72	10	1

The PCR results (1/10 volume) were examined using 1% agarose gel electrophoresis in TBE buffer (0.04 M Tris-OH, 0.002 M EDTA [pH 8.5]). The PCR products were seen under UV light after the gels had been stained with ethidium bromide. For the products that were amplified using a single primer set, just one band was seen.

Sequencing of PCR products for *tem* gene

Ten *K. pneumoniae* Tem gene isolate PCR products were chosen, and the forward and reverse primers for the gene were forwarded to macrogen facilities in Korea for sequencing.

The basic local alignment search tool analysis (BLAST)

By blast algorithm (www. ncbi. nlm. nih. gov/BLAST) has been analyzed the Blast sequence.

Multipl Sequence Alignment (MSA)

Using the ClustalW program, multiple sequence alignment of the genes for *K. pneumoniae* was performed (www.ebi.ac.uk/clustalw/).

Statistical Analysis

Data were fed into SPSS, version 24 for tabulation and analysis and the numerical data

.

Results

From 250 samples, 71 isolates were suspected *K. pneumonia*, (38 children and 33 buffaloes). Table 3

Table 3 Prevalence of *Klebsiella pneumonia* that isolated on MacConkey agar.

Source	No. of *Klebsiella* isolates	%
Human	38	53.5
Animal	33	46.4
Total	71	100%

Antimicrobial susceptibility of *K. pneumoniae*

All isolates were susceptible to imipenem 75% to ciprofloxacin but resistant to cephalothin, amoxicillin, and ceftazidime. Ninety percent of them had gentamicin and cefotaxime resistance. Fifty percentof the isolates lacked ceftriaxone resistance. Isolates exhibited ceftriaxone resistance of 75%.

Table 4 Antimicrobial susceptibility of *K. pneumoniae*

TYPE OF ANTIBIOTIC	SENSITIVE (%)	INTERMEDIATE (%)	RESISTANCE (%)
Gentamycin	**0**	**10**	**90**
Cephalothin	**0**	**0**	**100**
Imipenem	**100**	**0**	**0**
Ciprofloxacin	**75**	**25**	**0**
Amoxicillin	**0**	**0**	**100**
Amikacin	**10**	**0**	**90**
Tetracyclin	**0**	**10**	**90**
Ceftazidime	**0**	**0**	**100**

Ceftriaxone	**50**	**0**	**50**
Cefotaxime	**0**	**10**	**90**

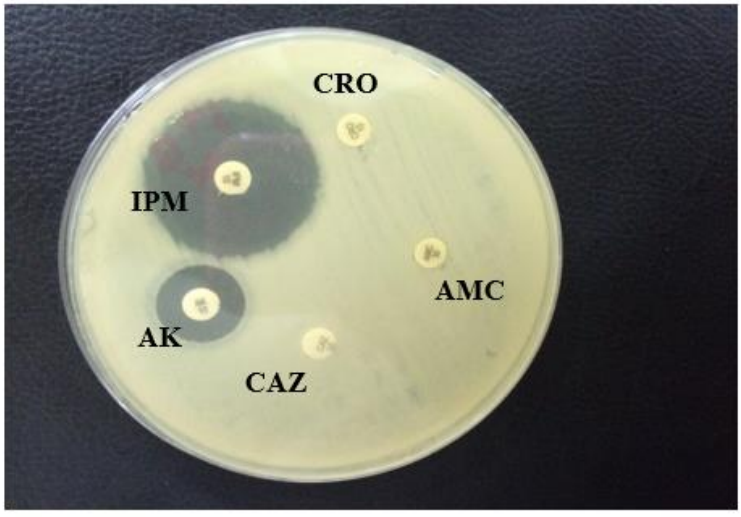

Fig. 1 Antimicrobial susceptibility test.

Molecular study

Distribution of PCR Positive for *Tem* genes in *K. pneumonia*

The outcomes of PCR expansion carried out on the DNA isolated from all of an isolates were ascertained using the electrophoresis analysis. Specific oligonucleotide primers for (*tem*) genes and DNA isolated from isolates successfully bound to form the DNA strands. When examined under ultraviolet light, this successful binding appears as a separate band for each gene using the particular DNA stain ethidium bromide. 44 *Klebsiella* isolates were reported to have PCR products matching to tem (445 bp) based on DNA marker (100 bp DNA ladder) electrophoresis values, Figure 2.

Table (5): Distribution *Tem* and *Shv* genes in *K. pneumoniae* isolates

Source	*PCR+ve*	*Tem In Klebseilla*	*%*
Human	**30**	**25**	**83.3%**

Animal	**25**	**19**	**76%**

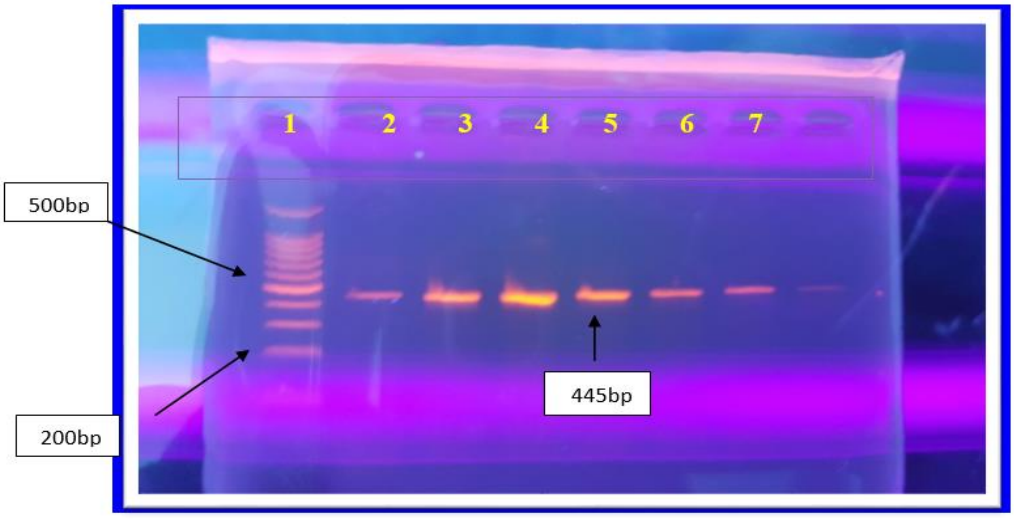

Fig. 2: PCR amplification of *K. pneumonia Tem* gene (445 bp).

Table (6): Different method for isolation, identification and presence of B-lactamases genes of *K. pneumonia*.

Type of test	Total No. of sample	Total of positive sample	%
Culture of test	**175**	**104**	**59.40**
Bio chemical test	**104**	**93**	**89.4**
Api 20	**12**	**10**	**83.3**
PCR	**10**	**6**	**60**
Sorbitol	**104**	**10**	**9.6**
Chi-sq = 64.29 , p= 0.0000001; df =4			

Discussion

Various infections have been linked to various *Klebsiella* species. The significance of *Klebsiella* as a pathogen, however, lies in its ability to ail hospitalized people. Almost usually, the strains involved are *Klebsiella pneumoniae* members with characteristic biochemical profiles (Patrick and Murray, 2005). Because patients often use broad-spectrum antibiotics, multidrug-resistant bacteria like ESBLs may develop. (Archana *et al*., 2011). It was challenging to eradicate multidrug-resistant strains of nosocomial severe infections and community-acquired illnesses with conventional antibiotics.

K. pneumoniae isolates cultured on MacConkey agar medium revealed big, spherical, pink, mucoid lactose-fermenting colonies upon morphological analysis.

The percentage of suspected *K. pneumoniae* in 71 of the 175 isolates was 40.57%, which is comparable to the percentage of *K. pneumoniae* isolated from 56 of the 155 isolates in Darweesh (2000), which was 36.16%.

Antimicrobial resistance is the growth and spread of microorganisms that are resistant to drugs and have progressing novel resistance mechanisms. The fast worldwide proliferation of "superbugs," or multi- and pan-resistant bacteria, which produce diseases untreatable by currently available antimicrobial medications like antibiotics, is particularly concerning. (www.who.in 2021)

The most varied and widely used antibiotic from the large group is *β*-lactams, which make up more than half of all systemic antibiotics now in use (Agrawal *et al*., 2008). With the discovery of penicillin, the antibiotic era started and lasted for 65 years. Antibiotic resistance to *β-lactam* started long before penicillin was invented (Bradford, 2001).

According to (Zoppi, 2022) there are documented instances of people passing these antibiotic resistance genes to cattle, pets, and wildlife.

The strain that was resistant to antibiotics was mostly found in cattle excrement. If it's discharged into the environment, it may contaminate food and water, which might be harmful to human health (Chiu et al.,2004).

During the average person's lifetime, antibiotic resistance has grown to be a significant clinical and public health issue (Levy, 2002). One of the numerous causes of this issue is the overuse of antibiotics (Webster, 2002) along with chromosomal alterations and

genetic material interchange through plasmids and transposons that helps spreading drug impedance among dangerous bacteria (Levy,1999).

All *K. pneumoniae* isolates had 100% cephalothin, amoxicillin, and ceftazidime resistance as well as 90% cefotaxime resistance. The findings of the current investigation corroborated those of Parveen et al. (2010), who found that 73.3% of *Klebsiella* isolates that were meropenem-resistant were also imipenem-resistant. Additionally, according to Shah et al., 86.96% of *Klebsiella* isolates exhibited imipenem resistance (Shah and Desai, 2012). Compared to *K. pneumonia*, *E. coli* was more resistant to the antibiotics utilized in this investigation. The explanation for this may be because of that gene of resistance in *E. coli* is better tightly controlled than the *K.P* resistance gene.

This research bacteria demonstrated a significant level of resistance to the antibiotics Amoxicillin and Gentamycin, which were often used in hospitals.

Detection of *Tem* Gene by PCR Assay.

Outbreaks of food poisoning pose a danger to the public's health and are challenging to treat. The buildup of multidrug-resistant EHEC in the food supply is a source of communicable diseases and a sign that giving antibiotics to cattle as a preventative measure or to feed them leads to a high rate of resistance to these drugs (Rossi, 2001).

Worldwide reports of the extended spectrum β-lactamases have been made. However, even in closely related areas, the prevalence varies greatly. (Yusha et al., 2010) It is hard to get a clear picture of how often ESBLs happen because they are hard to spot and testing and reporting methods vary.

According to the kind of β-lactamase genes used in this investigation, the Tem gene in *K. pneumoniae* was found in 83.3% of people and 76% of animals. By far the most common gene is the *tem* gene, whose origin is unknown. (Widemann *et al*., 1989). TEM 1 can break down penicillins and cephalosporins, which is the cause behind 90% of *E. coli* bacterial resistance against ampicillin, according to Bradford (2001). Extended spectrum beta-lactamases, however, were growing many years ago, which were aggressive monobactams and many newer cephems (Bradford, 2001). Bradford in 2001 stated that such enzymes are mostly mutant of (TEM- 1) and (TEM- 2) such (TEM- 3,

TEM- 4, TEM- 10, TEM- 27, TEM- 92). Many of the bacteria that generate these enzymes shown sensitivity or moderate sensitivity to some or all of these drugs in vitro, yet infected individuals displayed significant impedance (Paterson and Bonomo, 2005).

Tem gene multiple sequence alignment in *K. pneumoniae*.

The BLASTN program was used to compare the *tem gene* sequences that were uploaded to the National Center for Biotechnology Information (NCBI) data bank. Using ClustalW software, partial *Tem gene* sequences were aligned between and with the *blaTem* gene sequence of plasmid pCIB4431 based on the findings of the database analyses.

In the current study, there were similarities in *tem* gene sequences in the isolates of *K. pneumonia* to sequences in strains of related *K. pneumoniae*. The tem gene's similarity to the K. pneumonia strain (NG 050233.1) was determined to be 99%, 98% with *K. pneumonia* strain (NG_050208.1), and 99% with *K. pneumoniae* strain (NG_050241.1). The results of the study are perhaps linked to the fact that the tem gene has other subtypes, which makes the pathogenicity of different *K. pneumoniae* strains different.

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Conclusion

1- *Klebsiella pneumoniae* were highly recovered in children with diarrhea, but only moderately recovered in buffalo feces.

2- At least seven of the antibiotics that were used on each isolate were determined to be futile. All of these isolates were thus thought to be multidrug resistant.

3- The frequency of ESBL-producing isolates is high.

4- Tem genes are present in the great majority of *Klebsiella pneumoniae* isolates that produce ESBLs.

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