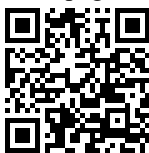


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Title: Phenotypic Characterization of Multidrug Resistant *Klebsiella Spp.* Isolated from Pediatric Patients

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Phenotypic Characterization of Multidrug Resistant *Klebsiella* Spp. Isolated from Pediatric Patients

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ABSTRACT

Background. Multidrug resistant (MDR) *Klebsiella* has become a global problem and its infections are difficult to eradicate due to the presence of β -lactamases. The current study aimed to identify multidrug resistant isolates in children due to their high prevalence in Lahore, Pakistan.

Methodology. In this study, 150 samples of blood, urine, pus, ascitic fluid, pleural fluid, Bronchoalveolar lavage (BAL), and CSF were collected from patients admitted in the University of Child Health Sciences, The Children's Hospital, Lahore from November 2021 to April 2022. Then, different microbiological techniques including culturing, Gram staining, identification of different species by using API, antibiotic susceptibility testing as well as molecular techniques including PCR, were used to identify MDR *Klebsiella* spp. positive samples.

Results. Out of the 150 samples, 58 were found positive for *Klebsiella* spp. while 92 were negative for *Klebsiella* spp. Among these 58 strains, 48 came from urine samples, 8 from pus samples, and 2 from blood samples. Furthermore, 34 were collected from male and 24 from female patients. The age group of the sampled children was 6 to 10 years. Afterwards, these 58 isolates were screened for extended spectrum β -lactamase (ESBL), metallo β -lactamase (MBL) and AmpC. It was found that out of these 58 isolates, 12 were ESBL positive (20.68%), 11 were MBL positive (18.9%) and 20 were AmpC positive (34.48%). Among β -lactamase genes, NDM -1 (72.7 %), SHV (41.6%), TEM (8.3%) and CTX-M (25%) were detected.

Conclusion. The study concluded that the rate of MDR *Klebsiella* infection is high in children and it may be complicated for the clinicians to treat them.

Keywords: extended spectrum β -lactamase (ESBL), *Klebsiella* spp., multidrug resistance (MDR), pediatric patients, polymerase chain reaction (PCR)

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Highlights

- Collection of Samples from The Children's Hospital for the isolation of *Klebsiella* strains.
- Screening by using microbiological techniques for microbe identification.
- Antimicrobial sensitivity testing on all *Klebsiella* positive samples. ESBL, MBL, and AmpC were phenotypically characterized.
- Identification of enzymes related to resistance on gene level.

1. INTRODUCTION

The prevalence of β -lactamases is increasingly being reported worldwide. β -lactams are commonly used broad-spectrum antibiotics with a high efficacy, cost-effectiveness, easy delivery, and low adverse effects. Irrational use and overuse of antibiotics has caused new strains of bacteria to emerge with multidrug resistance (MDR) patterns. *Klebsiella* spp. is a β -lactamase-producing bacteria causing respiratory infections and post-surgical complications in immunocompromised patients [1].

Klebsiella spp. is a gram-negative, rod-shaped, encapsulated bacteria that belongs to the family Enterobacteriaceae. It is a non-motile bacteria and can ferment lactose. About 78 serotypes of *Klebsiella* spp. have been determined [2]. High antibiotic intake has caused the rise of resistance among organisms, while multiple antimicrobial resistance has turned into a significant medical problem [3]. The epidemiology of resistant bacteria depends upon the geographical region, as well as population susceptibility. Occasionally, it changes from one population center to another within the same country due to the variability in the pattern of antibiotic usage and changes in the way of life [4].

Three major groups of β -lactamases including cephalosporin (AmpC), extended spectrum β -lactamases (ESBL) and

carbapenamases such as metallo- β -lactamase (MBL) are of great concern in healthcare settings. The presence of ESBL, AmpC, and MBL in a single bacterial species lessens the efficacy of β -lactam containing antibiotics, β -lactamase inhibitor and carbapenams, collectively [5]. The main function of ESBL is to degrade aztreonam and oxymino-cephalosporins even in the presence of β -lactamase inhibitors. ESBL is derived from point mutation in genes like *blaSHV* and *blaTEM* and identified after the emergence of third-generation cephalosporins [6].

Bacterial species producing AmpC and MBL enzymes show significant resistance to cephamycins and carbapenams, respectively [7]. Carbapenamases can be found in Enterobacteriaceae in various forms including expanded-spectrum oxacillinases and clavulanic-acid inhibitors (β -lactamases or metallo- β -lactamases). Carbapenams (imipenem and meropenem) are considered as first line therapy for the treatment of severe infections caused by ESBLs producing Enterobacteriaceae [8].

The influence of antimicrobial agents leads microbes to constantly seek new protective strategies known as "resistive mechanisms". For instance, some Enterobacteriaceae can release ESBLs. ESBL compounds separate and annihilate a few normally used antibiotics including penicillins and cephalosporins, making these medications insufficient to treat

diseases [9, 10]. Rather than taking oral antimicrobial agents at home, patients with these diseases may require hospitalization and intravenous carbapenem therapy [11, 12].

According to Gerardo Alvarez-Uria, cephalosporin-resistant *K. pneumoniae* shows the greatest yearly rise in clean water accessibility related to AMR. AMR is associated with poor sanitation conditions and non-availability of clean water [13, 14]. As per previous studies, *Klebsiella* has the tendency to be resistant anywhere around the globe. So, there is a need to address the widespread use of antibiotics and follow the guidelines of WHO to mitigate the crisis related with beta-lactam-resistant *Klebsiella* spp. and focus on new interventions regarding its treatment [15, 16].

There is a huge difference in the resistance mechanism of children and the adult population. The resistance rates to other antimicrobial agents, such as ceftazidime, amikacin, imipenem, ciprofloxacin, and aztreonam are also different in adults and children [17, 18]. Studies on children are scarce. So, the prevalence of MDR *Klebsiella* and the detection of β -lactamase genes in children in Lahore is focused in the current study.

2. MATERIALS AND METHODS

2.1. Study Design

This cross-sectional study was conducted from November 2021 to April 2022 at the Department of Microbiology, University of Child Health Sciences, The Children's Hospital, Lahore, Pakistan in collaboration with the Lahore Garrison University. The isolates of *Klebsiella* spp. were included, while the isolates of other bacterial spp. were excluded.

2.2. Sample Collection

Clinical samples of blood, urine, pus, and other fluids were received from The Children's Hospital, Lahore, Pakistan. The samples were collected from different departments of the hospital including its surgical ward, neurosciences ward, as well as hematology, oncology, gastroenterology, cardiology, OPD, emergency and medical units. Among these samples, 73 were urine samples, 32 were blood samples, and 45 were pus swabs and other fluids. These samples were sent to the microbiology section of the hospital for analysis.

2.3. Isolation of *Klebsiella* Spp.

The collected samples were processed by using different culture media such as blood, MacConkey and cysteine lactose electrolyte deficient (CLED) agar. The cultures were kept overnight in an incubator at 37°C for the propagation of bacterial cultures. These bacterial cultures were grown on different media and identified by colony morphology, Gram staining, analytical profile index (API) testing and other biochemical tests. Pink, mucoid lactose fermenting colonies on MacConkey agar and yellow mucoid colonies on CLED agar were processed for further analysis.

The respective API test kit was selected and the sample was inoculated in each well of the kit. Each well was used to perform a specific test. After adding the sample and reagents in the strip, it was incubated. Post incubation, the results were checked and every test was assigned a numeric code. Then this code was matched with the API manual guidebook or through other API documentation software. In this way, the identity of the bacteria was determined and reported.

2.4. Antibiotic Susceptibility Testing

Kirby-Bauer disc diffusion method was used to determine the susceptibility and resistance profile of *Klebsiella* spp. Muller-Hinton agar media plates were used for the sensitivity test. McFarland 0.5 turbidity standard was followed to prepare a bacterial suspension and an equally distributed lawn of bacteria was made on the agar plate. Two agar plates were subjected to antibiotic discs using a disc dispenser (OXOID) and kept in incubator at $35\pm 1^\circ\text{C}$ overnight. The antibiotics discs used in the current study were cefoxitin, ceftazidime, cefepime, ceftriaxone, cefuroxime, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, cefotaxime, piperacillin-tazobactam, amoxicillin-clavulanate acid, cefoperazone, sulfamethoxazole, tobramycin, and levofloxacin. The zones of inhibition created by these discs were measured in mm. The isolated *Klebsiella* spp. were declared as resistant or susceptible according to the manual of Clinical and Laboratory Standards Institute (CLSI) 2009.

2.5. Phenotypic Screening of ESBL, MBL and AmpC

The clavulanate-induced activity enhancement of cefotaxime or ceftazidime indicated the presence of ESBL production. To detect ESBL production, CLSI confirmatory tests were performed by placing ceftazidime and cefotaxime alone and in combination with ceftazidime-clavulanate and cefotaxime-clavulanate respectively on inoculated Muller-Hinton Agar plates. For carbapenamases, the zones of inhibition produced by imipenem-EDTA discs and imipenem alone were compared after 16 to 18 hours of incubation at 35°C . The isolates were considered as MBL producers if the zone of inhibition created

by imipenem + EDTA disc was measured as ≥ 5 mm. AmpC production was detected by 3-aminophenyl Boronic acid (APB) disk potentiation test, while $5\mu\text{l}$ of APB stock solution was added to cefotaxime (CTX), ceftazidime (CTZ), and cefoxitin (FOX) disks. An increase of ≥ 5 mm in the zone of CTX and/or CTZ and/or FOX disk, either alone or in combination with APB was considered as a sign of AmpC production.

2.6. Identification of β -lactamase Genes

Molecular identification of *Klebsiella* spp. was done by using polymerase chain reaction (PCR) test to detect ESBL, MBL and AmpC genes.

2.7. Statistical Analysis

Descriptive statistics were calculated and SPSS software was used.

3. RESULTS

In the current study, a total of 150 samples were processed in the Microbiology Laboratory of The Children's Hospital, Lahore. On culturing, 150 strains were positive to bacterial infection. Of the 150 isolates, 58 (38.7%) were *Klebsiella* positive and the remaining 92 (61.3%) were positive to *Escherichia coli*, *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Enterobacter* spp. Gender-wise distribution indicated that 58.7% samples were collected from male and 41.3% were collected from female patients. Age-wise, *Klebsiella* infection was the most common in the age range of 6-10 years.

3.1. Macroscopic and Microscopic Characteristics of *Klebsiella* Spp.

The texture of the growth was examined. There were large, grey-white, mucoid and non-hemolytic colonies on blood agar. While, yellow to bluish mucoid colonies were observed on CLED agar and

pink colonies were visible on MacConkey agar (Figure 1).

3.2. Gram Staining

In Gram staining, the microscopic picture showed Gram-negative, rod shaped (bacilli), singly arranged and pink colour bacteria (Figure 2).



Figure 1. Colonies of *Klebsiella* spp. on Blood Agar, MacConkey Agar and CLED Agar

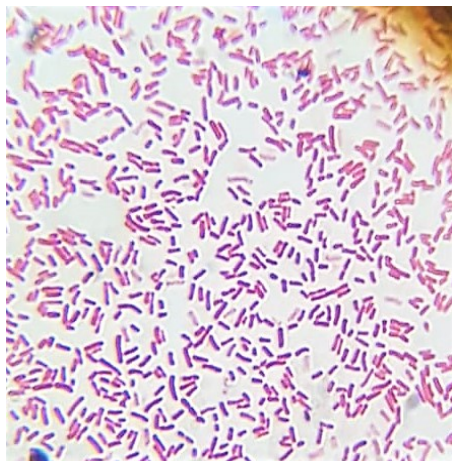


Figure 2. Gram Staining of *Klebsiella* spp. at 100X.

3.3. Biochemical Characteristics of *Klebsiella* Spp.

Biochemical testing is the second step used by the scientists for the rapid identification of microorganisms based on their color production. The results of

Simmon's citrate test, urease and triple sugar iron (TSI) tests were positive, while the result of the indole test was mostly recorded as negative, although it may be positive for a few species of the bacteria (Figure 3).

3.4. API (Analytic Profile Index) Testing

Further specie-level identification of the bacterial colonies was performed by using the API test method. The scores for positive test results were added and the resulting seven-digit code was entered in an online API software. The picture below shows 20E API results for *Klebsiella* spp. Each mini-well of the API kit was incubated at 36°C. After incubation, the results were observed and a code was generated based on the biochemical reactions. Then, the code was noted for the identification of bacterial species. The identification manual or the API software was used for the confirmation of concern specie (Figure 4).



Figure 3. (A) Simmon's Citrate Shows Positive Reaction (Blue Colour). (B) TSI (Triple Sugar Iron) Shows Positive Reaction (Yellow Colour). (C) Urease Test Shows Positive Reaction (Pink Colour). (D) MIO (Motility Indole Ornithine) Shows Negative Reaction After Adding 1 Drop of Kovac's Reagent (No Pink-Coloured Ring Formation).



Figure 4. Positive Reactions of *Klebsiella* spp. on API Testing

3.5. Antibiotic Susceptibility Testing

The 58 samples of *Klebsiella* spp. were observed to show resistance as 95.5% against amoxicillin-clavulanate acid, 98.2% against ciprofloxacin, 3.44% against gentamicin, 3.44% against tobramycin, 1.8% against meropenem, 0% against imipenem, 1.8% against amikacin, 96.6%

against ceftriaxone, 98.2% against piperacillin-tazobactam, 98.2% against levofloxacin, and 100% against ceftazidime, cefotaxime, sulfamethoxazole, cefoperazone, and cefepime (Figure 5). The antibiogram susceptibility pattern is shown below in Table 1.

Table 1. Antibiogram susceptibility pattern of *Klebsiella* spp.

Target Bacteria	Antibiogram Disc	Total No. of Isolates	Disc Potency	%			M±SD	V
				R	I	S		
<i>Klebsiella</i> spp.	Ciprofloxacin	58	5µg	98.2	1.8	0	33.26 (55.18)	2104.38
	Amoxicillin-clavulanate acid		10/20 µg	95.5	1.5	0	32.22 (54.7)	2993
	Gentamicin		10µg	3.44	8.62	82.75	31.60 (44.36)	1968.6
	Tobramycin		10µg	3.44	0	96.6	33.34 (54.8)	3003.6

Target Bacteria	Antibiogram Disc	Total No. of Isolates	Disc Potency	%			M±SD	V
				R	I	S		
	Meropenem		10µg	1.8	3.44	94.8	33.34 (53.22)	2833
	Imipenem		10µg	0	0	100	0 (0)	0
	Amikacin		10µg	1.8	0	98.2	33.33 (56.18)	3156.5
	Ceftriaxone		30µg	96.6	3.41	0	33.33 (54.81)	3004.5
	Cefotaxime		30µg	100	0	0	0(0)	0
	Piperacillin-tazobactam		100/10µg	98.2	1.8	0	33.33 (56.18)	3156.5
	Ceftazidime		30µg	100	0	0	0 (0)	0
	Sulfamethoxazole		23.75µg	100	0	0	0 (0)	0
	Cefepime		30µg	100	0	0	0 (0)	0
	Levofloxacin		5µg	98.2	1.8	0	33.33 (56.18)	3156.5
	Cefoperazone		30µg	100	0	0	0 (0)	0

Note. V= Variance Coefficient, R= resistant, I= intermediate, S= sensitive

3.6. ESBL Confirmatory Test

To detect ESBL production, CLSI confirmatory tests were performed by placing ceftazidime and cefotaxime on inoculated Muller-Hinton agar plates. The

CLSI test, performed using a combination of discs, was interpreted as ESBL positive when the zone of inhibition was observed as ≥ 5 mm. A total of 12 (20.7%) isolates were positive for ESBL and 46 (79.3%) isolates were negative (Figure 6).

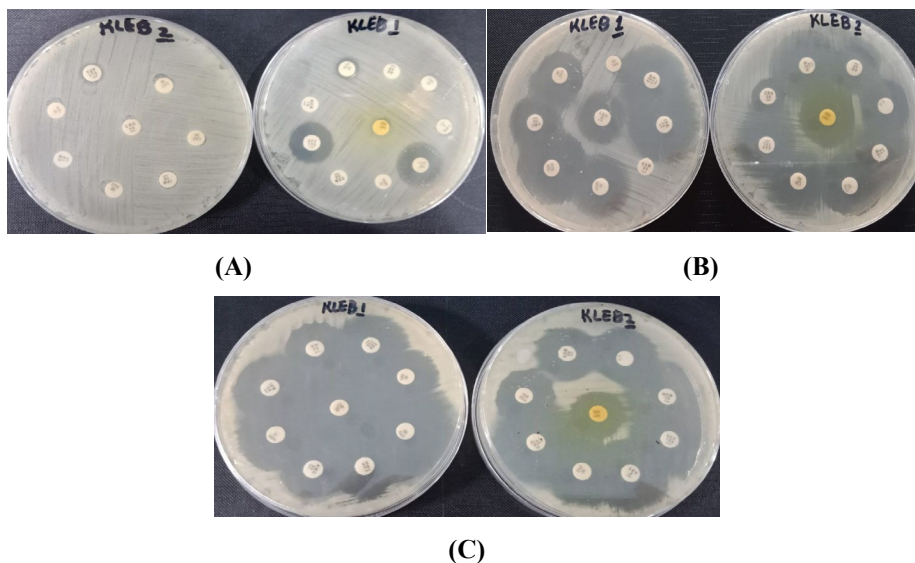


Figure 5. (A) Antimicrobial Susceptibility Testing of *Klebsiella* spp., in Which All the Antibiotics are Resistant (B) In Which Some Antibiotics are Resistant and Some are Sensitive. (C) In Which All the Antibiotics are Sensitive



Figure 6. ESBL Confirmatory Test. A Key-Hole Effect is Produced

3.7. MBL Confirmatory Test

The zones of inhibition created by imipenem-EDTA discs and imipenem alone were observed and compared after 16 to 18 hours of incubation at 35°C. The isolates were presumed as MBL producers if the zone of inhibition created by the imipenem + EDTA disc was measured as ≥ 5 mm, as shown in Figure 7. A total of 11 (19.0%) isolates were positive for MBL and 47 (89.0%) isolates were negative.

3.8. AmpC Confirmatory Test

An increase in the diameter of the zone of inhibition of about ≥ 5 mm for cefoxitin (FOX) disk alone and in combination with APB was documented and considered as indicative of AmpC production, as shown in Figure 8. A total of 20 (34.5%) isolates were positive for AmpC and 38 (65.5%) isolates were negative.

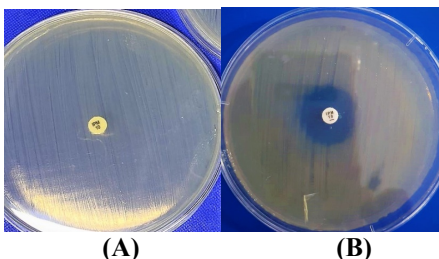


Figure 7. (A) Imipenem Alone, the Zone is

Resistant. (B) Imipenem + EDTA, the Zone Size Increases upto ≥ 5 mm.

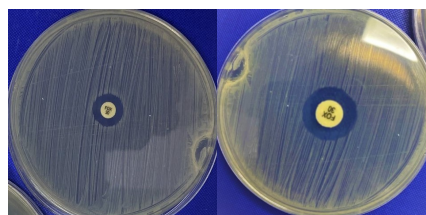


Figure 8. (A) Cefoxitin Alone, the Zone is Resistant. (B) Cefoxitin + APB (3-Aminophenyl Boronic Acid), the Zone Size Increases upto ≥ 5 mm

3.9. Identification of β -lactamase Genes

Multigene optimax thermal cycler (Labnet, USA) was used to perform the PCR test to detect the drug resistant gene in the isolated DNA. All the *Klebsiella* spp. samples were processed at the Department of Microbiology, University of Child Health Sciences, The Children's Hospital, Lahore. The results showed the presence of the resistive gene. The desired samples were analyzed for the detection of ESBL, MBL and AmpC genes with primers shown in Table 2.

Table 2. Sequences of different genes

Primers	Sequences	Temp. (°C)	Amplicon Size (bp)
NDM	ATGGAATTGCCCAATATTATG CACTCAGCGCAGCTTGTCTGGC	66	813
TEM	GAGTATTCAACATTTCCGTGTC TAATCAGTGAGGCACCTATCTC	62	800
SHV	ATGCGTTAT ATTCGCCTGTG TTAGCGTTGCCAGTGCTC	60	867
CTX-M	GCGTGATAACCACTTCACCTC TGAAGTAAGTGACCAGAATC	60	300

4. DISCUSSION

In this study, 58 strains of *Klebsiella* species were isolated from 150 clinical samples and tested for the production of different types of β -lactamases, such as ESBL, MBL, and AmpC enzymes. Out of these 58 isolates, 43 (74.1%) were found to be resistant because of β -lactamase enzymes, while 15 (25.9%) were not resistant. The same study conducted at Babol University of Medical Sciences revealed a slightly lower prevalence of resistant strains with β -lactamase enzymes equivalent to 64.4% of the *Klebsiella* spp. isolates [19]. In the year 2010, the prevalence of the resistant strains of *Klebsiella* because of β -lactamase was reported as 64.1%, which increased to 74.1% in 2021, highlighting the increasing trend of AMR globally.

The high prevalence of this resistant strain emphasizes the upcoming threat in terms of difficulty in treating patients. The presence of resistant genes detected on a molecular level in this study confirmed its alignment with the global trends. The prevalence of resistant genes suggests the limited options left in terms of antibiotic selection for treatment, which is a growing challenge in the management of diseases.

This study highlights the need to focus

on the prevention of medication overuse, self-medication and the reduction of environmental factors involved in exacerbating the spread of AMR. It also highlights other genetic factors involved in the horizontal transfer of AMR, all of which eventually contribute in the persistence and dissemination of resistance gene within the community. Moreover, to reduce or combat the increasing threat of AMR, surveillance and monitoring mechanisms are needed to track the evolution and spread of resistant genes along with the rising trend of antibiotic consumption.

The greater presence of *Klebsiella* spp. in urine than blood or pus corresponds with the findings of previous studies. These studies showed that the pathogenic bacteria target sites are specific and the probability to find the pathogen exceeds if the right sample is collected from the right place.

In the current study, phenotypic detection showed the frequency of ESBL gene equal to 12 (20.7%), MBL gene equal to 11 (19.0%) and AmpC gene equal to 20 (34.5%), among all positive cultures. A similar study conducted at the Aga Khan Hospital on ESBL containing AMR showed 7.2% ESBL containing *Klebsiella* among all the positive samples of enterobacteriaceae. While the current study found 27.9% ESBL among all positive

samples of *Klebsiella* antimicrobial resistant strains. In another study conducted in Lahore, 26.1% of the clinical samples tested positive for ESBL-producing *Klebsiella* spp. This shows a significant prevalence of the antibiotic-resistant bacteria in the area, which is a cause for concern in terms of healthcare and antimicrobial resistance [20].

In the current study, the co-existence of enzymes was checked. It was found that 2 (4.6%) isolates co-produced MBL+ESBL, followed by 1 (2.3%) that co-produced AmpC+ESBL, and 1 (2.3%) that co-produced AmpC+MBL. All of the three enzymes were present in 1 (2.3%) *Klebsiella* isolate. Another study also confirmed the co-production of these enzymes in 20.87% of enterobacteriaceae isolates. It mentioned that among the isolates tested, approximately 20.87% were found to produce all three types of enzymes simultaneously [21]. This co-existence of multiple antibiotic resistance mechanisms in the same bacterial isolate is a matter of concern because it might contribute in increasing the resistance of these particular bacteria, limiting treatment options and increasing the risk of infections that are difficult to control. It also emphasizes the importance of prudent antibiotic use and continued research into new antibiotics and alternative treatment strategies. In most of the studies, the co-production of ESBL and AmpC was noticed [22, 23].

Various genes in β -lactamases were detected on the basis of the molecular analysis that showed the three genes of ESBL, namely SHV (41.6%), CTX-M (25.0%), and TEM (8.3%). These results are comparable with previous studies. A study conducted in Iraq showed a high prevalence of SHV gene (86.04%) for ESBL production [24]. The presence of genes in AmpC producing *Klebsiella* spp.

strains from another study indicates, 35.2% of strains had the CIT gene and 61.7% had the EBC gene. It's important to note that the prevalence of specific genes in bacterial populations can vary based on several factors, including geographic location, time period, and the specific methods used for detection and analysis [25].

Another study found the gene NDM-1 β -lactamase producing bacteria in environmental samples. There are implications of this finding for the health of people relying on public water and public sanitation facilities. The prevalence of NDM-1 β -lactamase in this study also highlights the probability of finding these bacteria in environmental samples, including public water and sanitation facilities. Furthermore, international surveillance of clinical isolates and environmental samples also needs to be conducted [26].

4.1. Conclusion

In developing countries like Pakistan, antibiotic resistance has reached alarming levels. The effect of antibiotics is inhibited by various resistance mechanisms developed by the bacteria. This study revealed clinical, molecular, laboratory, and demographic features of MDR *Klebsiella* spp., the co-existence of enzymes and the presence of various β -lactamases, all of which is helpful for clinicians to better judge the clinical scenarios and make recommendations for the selective use of antibiotics. A significant prevalence of MDR *Klebsiella* among children highlights the poor dose management of antibiotics by patients and a weakened immune system. Hence, there is a need to improve the hygiene of children and encourage the lesser use of medication for minor complaints. To prevent the spread of resistant strains, specific targeted

antimicrobial agents need to be used. Out of the 43 MDR *Klebsiella* samples, 5 showed more than 1 enzyme's activity. This indicates the potential complexity of resistance mechanisms, highlighting the need of stringent monitoring and a targeted therapeutic line of action.

4.2. Policy Implications

Collaboration between healthcare facilities, researchers, and policymakers is necessary to develop and implement strategies that can help mitigate the antibiotic resistance crisis. This may include promoting antibiotic stewardship programs, investing in research for new antibiotics, and enhancing infection prevention and control measures in healthcare settings. For the successful treatment of patients, β -lactamases should be detected and reported as early as possible. Moreover, a proper surveillance system is also needed for impeding antibiotic resistance.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

All data is provided in the manuscript.

FUNDING DETAILS

No funding has been received for this research.

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