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DETECTION OF CARBAPENEM AND COLISTIN RESISTANT GENES IN PRESERVED SAMPLES OF *P. AERUGINOSA*

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ABSTRACT

Pseudomonas aeruginosa is a rod shaped, aerobic and gram-negative bacteria that is found in humans, soil as well as water and plants. It has become worldwide threat to immunocompromised patients due to its increasing resistance profile. The aim of present research is to revive Pseudomonas aeruginosa from preserved samples having different sources, i.e. clinical, diabetic foot ulcer, raw milk, chicken embryo and broiler and cockroach samples. This study also targets antibiotic susceptibility profile of P. Aeruginosa against Carbapenem and Colistin for the detection of three resistant genes i.e blaKPC, blaIMP and MCR-1 gene. A total of 150 samples were revived, in which sixty-five samples show growth for P. aeruginosa. All these samples were enriched in Peptone water for 48 hours. All samples were cultured and analyzed on the Cetrimide agar. Among sixty-five, twenty-four (n=24) were Clinical samples, twenty (n=20) were diabetic foot ulcers, seven (n=7) were raw milk samples, ten (n=10) were chicken embryos and broiler and four (n=4) were cockroach samples. P. aeruginosa was identified based on colony morphology subsequently microscopy after the Gram-staining and biochemical tests. After identification, the bacterium was further tested for antimicrobial sensitivity according to the Kirby-Baur method. Antibiotic tests showed 100% resistance against carbapenem (imipenem, meropenem and Doripenem) and 0% Colistin resistance. None of the samples showed Colistin resistance. The DNA of the Pseudomonas aeruginosa was extracted by chemical method and ran on the gel electrophoresis. Furthermore, the DNA bands were visualized under UV Transilluminator. Extracted DNA was further amplified by using the site-specific primer of drug resistance gene blaKPC, blaIMP and mcr-1. The PCR results showed 9.23% positive isolates for KPC gene and 6.15% positive IMP gene. None of these sixty-five isolates contain mcr1 gene. In summary, our study's findings could help with the development of therapeutic strategies and the evaluation of the efficacy of currently being used antibiotic therapies. The study offers insightful details regarding profile of antibiotic resistance. The presence of KPC and IMP genes is important to track the cause of resistance in Pseudomonas aeruginosa.

Keywords: *Pseudomonas aeruginosa, Carbapenem resistance, Colistin resistance, blaKPC and blaIMP genes, mcr-1 gene*

INTRODUCTION

Pseudomonas aeruginosa is a widespread opportunistic pathogen (1), affecting immunocompromised patients worldwide. It is a Gram-negative, aerobic, rod-shaped bacillus. Immunosuppressed patients, the elderly, and those with basic diseases can all contract infections from opportunistic microorganisms. Pseudomonas aeruginosa was listed as having the highest priority on the 2017 global priority list of diseases by the World Health Organization (WHO). It is known that bacteremia caused by P. aeruginosa might result

in up to 40% deaths within 30 days (2). P. aeruginosa has wide genome, which allow its colonization in different habitats such as natural ecosystems and in human hosts. It is often found in soil, water, and humid environments and infects aquatic habitats, animals, and plants. Gessard isolated P. aeruginosa from green pus for the first time in 1882, and Migula published the first description of the genus Pseudomonas in 1894 (3). It is also a member of ESKAPE pathogens, the group of six microorganisms, including Enterobacter spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter faecium. These pathogens have unique characteristics related to rising resistance patterns; in fact, ESKAPE pathogens are growing regarding the amount of resistance rising incidence and in terms of severity due to the development of new resistance mechanisms (4). P. aeruginosa can spread from people to animals and from animals to people, hence it is also known as zoonotic pathogen. P. aeruginosa infections in commercial poultry can result in septicemia, respiratory and intestinal infections, and significant mortality rates in the case of embryo infections that cause death in the shell (5). This pathogen can persist in long-term bacterial infections, where it parasitizes the host's defenses, erodes them, and evolves to stay alive for a longer period (6).P. aeruginosa can grow on human skin, in the respiratory tract (chronic airway inflammation) and in intestinal mucosa. P. aeruginosa is the significant source of chronic bronchial infection (CBI), chronic obstructive pulmonary (COPD), cystic fibrosis (CF), asthma and bronchiectasis, which leads to increased inflammation, both locally and systemically, and become major life threat (7). P. aeruginosa is also abundant in hospitals, causing around 10% of hospital-acquired infections (8). P. aeruginosa prevalence in patients who acquired infection from the intensive care unit (ICU) could reach up to 23% (9), while in intensive care unit, 48.7% patients may have resistant P. aeruginosa (10). The U.S. Centers for Disease Control and Prevention (CDC) stated that in 2017 a total of 32,600 hospitalized patients had multi-drug resistant (MDR) P. aeruginosa infections (11). P. aeruginosa is the frequent cause of endocarditis and diabetic foot infections. It is also prevalent in otitis external and keratitis (12). It becomes a significant source of nosocomial infections including ventilator-associated pneumonia (VAP), intensive care unit infections, central line-related blood stream infections, surgical site infections, urinary tract infections, burn wound infections, keratitis, and otitis media (13). Bloodstream infections caused by P. aeruginosa and VAP can have mortality rates of up to 40% (14). It also causes diseases in immunosuppressed patients that are diagnosed with neonatal infections, cancer, and severe burns (15). This bacterium becomes difficult-to-treat because it has low intrinsic antibiotic susceptibility and extraordinary capacity of becoming antibiotics resistant (16). P. aeruginosa has grown to be a serious hazard to healthcare, which will have a detrimental impact on mortality, morbidity, and expense (17). An important worldwide problem is antimicrobial resistance (AMR). According to the 2019 Centers for Disease Control and Prevention (CDC), resistant bacteria caused over 2.8 million illnesses and over 35,000 deaths every year between 2012 and 2017 (18). P. aeruginosa possesses the potential to create biofilms, intrinsic resistance to numerous drug classes, and above all the speed at which it can develop resistance to continuing therapies has been increasing at an alarming rate. More than 32,000 multidrug resistant (MDR) infections occur annually in the United States because of Pseudomonas aeruginosa's emergence and exponential spread; many of these infections are linked to healthcare settings (HAIs) (19). P. aeruginosa resistance mechanisms may be divided into intrinsic and acquired categories, and the mechanisms differ significantly depending on the region (20). Resistance mechanisms develop resistant strains against important antibiotics such as β-lactams, quinolones, aminoglycosides, and colistin. Resistance can arise due to specific gene mutations or the acquisition of genes resistant to antibiotics. Competency processes such as mobile DNA translocation and Certain components help bacteria develop multidrug resistance (MDR), which can develop extreme drug resistance (XDR) (21). Over time, P. aeruginosa shows greater resistance to the effects of conventional antibiotics (22).

Globally, the prevalence of MDR P. aeruginosa is increasing, ranging from 15% to 30%, including in China (23). P. aeruginosa uses its innate one-to-many drug class resistance as a means of defense (24), the capability to produce biofilm and the ability to quickly develop resistance to continuing therapies (25). Colistin is a medication that works against P. aeruginosa and is mostly used to treat MDR pathogen infections. Its use is controversial because of adverse effects, including an increased risk of nephrotoxicity, and it is not a novel antibiotic (26).

The rise of multidrug resistant (MDR) Pseudomonas aeruginosa is related to inappropriate and excessive intake of antibiotics. Resistance to Carbapenem and Colistin has led to the development of severe pneumonia, wound infections, gastrointestinal tract (GIT) infections, and Urinary tract infections (UTI). The discovery of resistant genes KPC, IMP and MCR-1 provides valuable insights into the mechanism of resistance, guiding the selection of suitable antibiotic therapies.

Today antibiotic resistance is the biggest issue worldwide. Addressing the exact cause of antibiotic resistance in P. aeruginosa is necessary. Identifying resistance genes from preserved samples helps track the emergence and spread of resistance patterns. This knowledge can be effective in development of enhanced treatment approaches, including combination therapy or the creation of new antibiotics tailored to target resistance mechanism. MATERIALS AND METHODS

Study Design and Sample Collection

This study aimed to analyze antimicrobial resistance patterns and resistance genes in Pseudomonas aeruginosa isolated from five different sources. The research was conducted at the University of Haripur in a BSL-2 Microbiology Laboratory following standard biosafety protocols.

A total of 150 samples were revived, of which 65 were confirmed as P. aeruginosa. Confirmed isolates included: raw milk (n=7), clinical samples (n=24), diabetic foot patients from Hazara Division (n=20), chicken embryo and broiler samples (n=10), and cockroaches (n=4).

Isolation and Identification

Samples were preserved at 4°C and processed at room temperature. Enrichment was performed in peptone water at 37°C for 24–48 hours. Selective isolation was carried out on Cetrimide agar. Colonies were identified based on morphology, Gram staining, and biochemical tests including oxidase, catalase, indole, TSI, and citrate utilization tests.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility was determined using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, following CLSI guidelines. The antibiotics tested were imipenem, meropenem, doripenem, colistin, amikacin, and ampicillin. Bacterial suspensions were adjusted to 0.5 McFarland standard. Plates were incubated at 37°C for 24 hours, and zones of inhibition were measured in millimeters. Colistin resistance was further evaluated using the agar dilution method to determine minimum inhibitory concentration (MIC).

DNA Extraction

Genomic DNA was extracted from resistant isolates using the phenol–chloroform method. Extracted DNA was quantified and stored at –20°C until further analysis.

Detection of Resistance Genes

PCR was performed to detect carbapenem resistance genes (blaIMP, blaKPC) and the colistin resistance gene (mcr-1). Amplification reactions were carried out in a 25 µL reaction mixture containing template DNA, primers, master mix, and nuclease-free water. Each run included positive and negative controls. Amplified products were analyzed using standard PCR protocols.

Primers used in the study

Specific primers for KPC, IMP and MCR-1 are used in this study. Following table shows primers sdetails;

Table 1: Primers sequences for PCR and their sizes

Target	Primer name	Sequence	Amplicon size (bp)	Primer conc.	Reference
bla KPC	KPC-F	TCGCTAAACTCGAACAGG	785	0.2	Monteiro et al., 2012
	KPC-R	TTACTGCCCGTTGACGCCCAATCC			
bla IMP	IMP-F	GAGTGGCTTAATTCTCRACC	120	0.2	Monteiro et al., 2012
	IMP-R	AACTACCAATARTAAC			
Mcr-1	MCR1-F	TCCAAAATGCCCTACAGACC	205	0.2	Liu et al., 2020
	MCR1-R	GCCACCACAGGCAGTAAAAT			

Table 2: PCR steps and optimization conditions

Step	Temperature	Time	No. of cycles
Initial Denaturation	94°C	10 minutes	1
Final Denaturation	94°C	30 sec	1
Primer annealing	50°C	45 sec	35
Initial Extension	72°C	30 sec	35
Final extension	72°C	10 minutes	1
Hold	4°C	∞	-

PCR conditions for KPC, IMP and MCR-1 gene are given below.

Table 3: Conditions for KPC, IMP and MCR-1 gene

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	5min	1
Denaturation	95°C	20sec	1
Annealing	55°C	45sec	35
Initial extension	72°C	30sec	1
Final extension	72°C	10min	∞

Gel Electrophoresis

The amplified product was observed by using gel electrophoresis. 2g of agarose gel was prepared which contained 2g of agarose in 100ml of 1X TBE buffer. Placed in microwave oven for 1-2 minutes to dissolve the powder completely. 3µl of ethidium bromide was added in the gel. Comb adjusted in the gel tray then solution transferred to the tray and placed for 30-40 minutes for solidification. After this comb was removed, gel placed in caster which was filled with 1X TBE buffer prepared from the stock solution of 10X TBE buffer (tris 54 g, boric acid 22.5 g, EDTA 4.6 g). DNA, PCR products, ladder, positive and negative control were loaded along with loading dyes in wells. Electric current was supplied at 80V for 50 minutes. Gel was visualized under UV transilluminator.

Table 4: PCR steps and optimization conditions

Chemicals	Quantities per cycle
TBE buffer 1x	35ml
Agarose gel	0.7mg
Ethidium bromide	2µl
PCR Product	5l/well
DNA ladder (100bps)	5l

RESULTS

Sample details

In this study, a total of 65 isolates were revived which were preserved from 2020 to 2022. These isolates have different sources including seven (n=07) samples from raw milk, twenty-four (n=24) clinical (urine, pus, sputum, blood) isolates, twenty (n=20) isolates from diabetic foot ulcer, ten (n=10) isolates from chicken embryo and broiler and four isolates (n=4) from cockroaches.

On cetrimide agar plates, pseudomonas physically appeared as tiny mucoid colonies with a yellow or blue green color and a fruity smell. The incubation period for enrichment was 48 hours and 24 hours for growth on cetrimide agar plates. In microscopy, P. aeruginosa was identified by its purple Gram-negative rods. Under microscope, a change in color from transparent to greenish appeared as purple Gram- negative rods.

Table 5: Sample details from different sources

Number of Sample	Sample ID	Source	Bacteria isolated
07	LR	Raw milk	*P. aeruginosa*
20	LD	Diabetic foot ulcer	*P. aeruginosa*
24	LC	Clinical samples	*P. aeruginosa*
4	LK	Cockroach	*P. aeruginosa*
10	LB	Chicken embryo and broiler	*P. aeruginosa*

Prevalence Details of samples

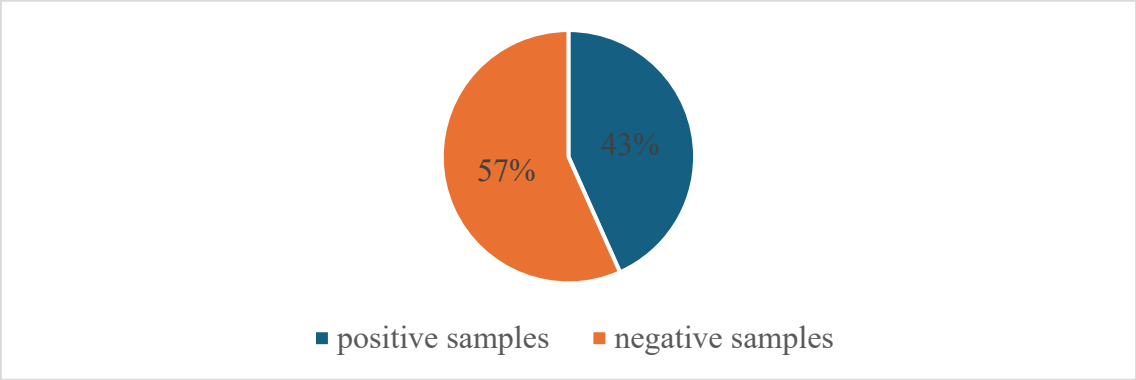

Figure 1: Prevalence of *P. aeruginosa*

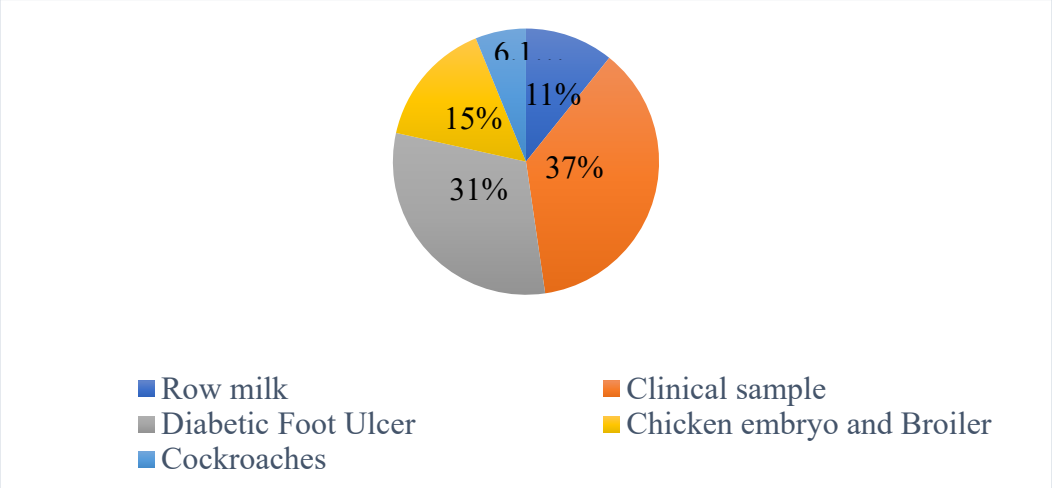

Figure 2: Prevalence of *Pseudomonas aeruginosa* isolated from different sample

Enrichment of preserved samples

The preserved samples were enriched in autoclaved peptone water. Each 100 microlitre sample was enriched in 10 ml peptone water and incubated for 48 hours at 37°C.

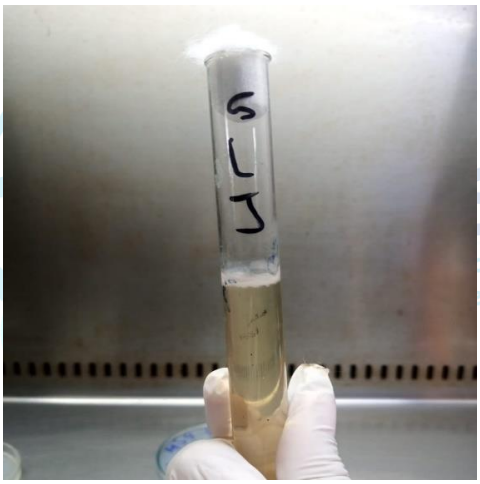

Figure 3: enrichment of sample in peptone water

Colony morphology

Colony morphology shows the visual features of bacterial colony on cetrimide agar plate. Positive samples show Colonies of bright green color on petri plates.

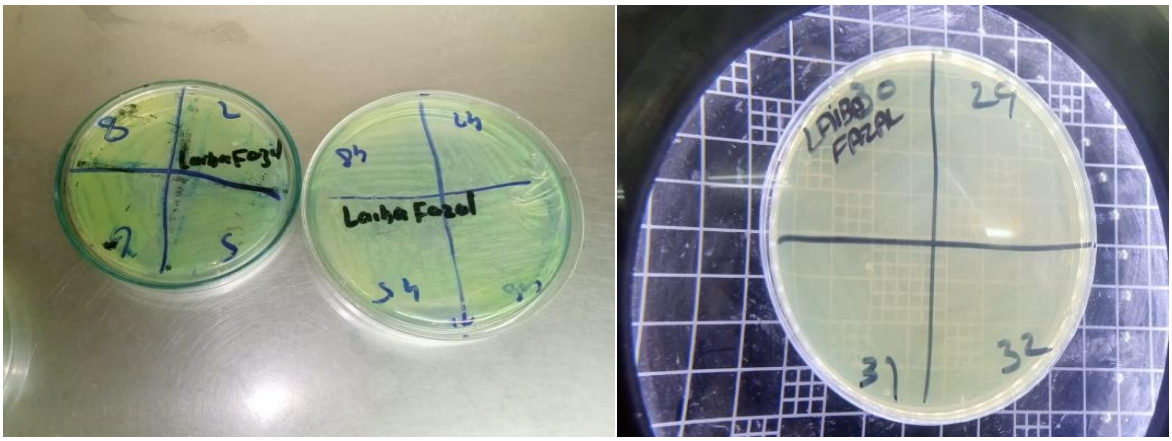

Figure 4: Growth of *Pseudomonas aeruginosa* on Cetrimide agar

Microscopy Results

Under microscope, *P. aeruginosa* appear as pink rods (gram negative). Colonies were picked with sterile loop to smear on glass slide. Slide was visualized under light microscope at 100X lens.

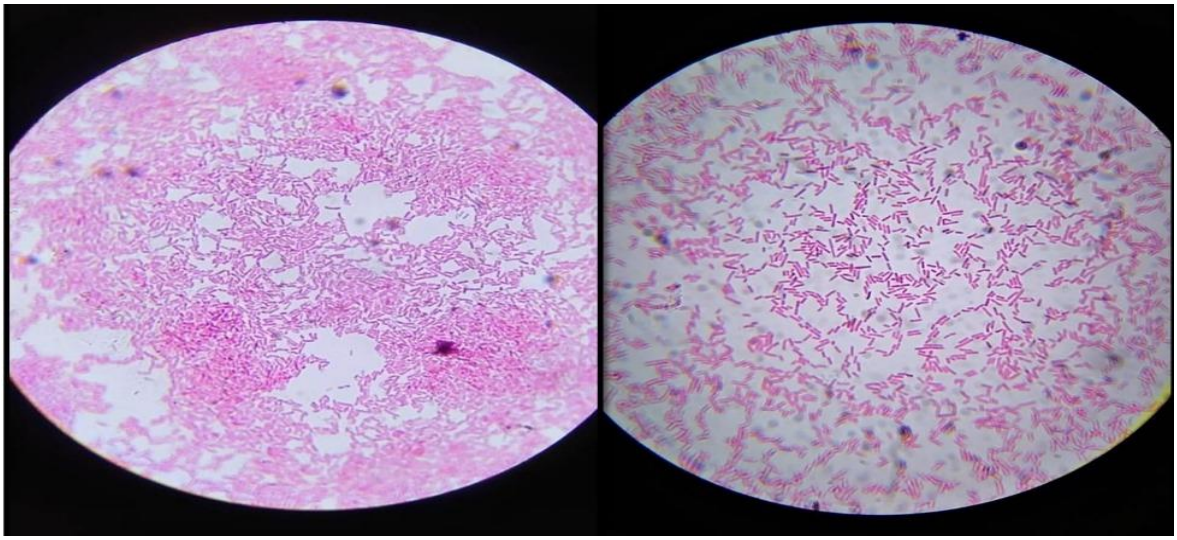

Figure 5: Microscopic appearance of *P. aeruginosa*

Biochemical Tests for *P. aeruginosa* identification

Table 6: Biochemical tests for *P. aeruginosa* identification

Tests	Results of *Pseudomonas*
Catalase	Positive
Oxidase	Positive
Triple sugar	Negative
Citrate	Positive
Indole	Negative

Figure 6: Positive Catalase test

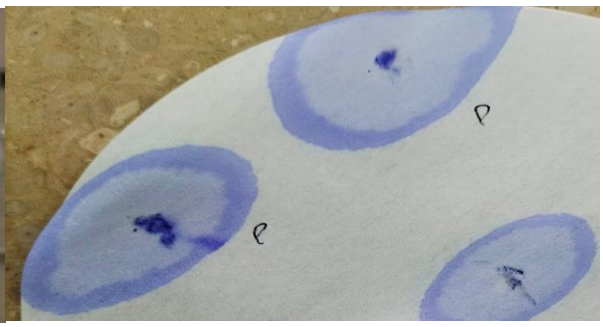

Figure 7: Positive Oxidase test



Figure 8: Negative Indole test Figure 9: Negative TSI test Figure 10: Positive Citrate test

Antibiotic Susceptibility profile

The disc diffusion method was utilized in studies to determine the antibiotic susceptibility of bacterial isolates. Appropriate antibiotics to test bacterial isolates resistance to various antibiotics. The measured zone contains the most potent medications against *Pseudomonas aeruginosa*. Muller Hinton Agar based antibiotic susceptibility pattern was determined for *P. aeruginosa* isolates from raw milk samples, clinical samples, diabetic foot ulcer, cockroach and chicken embryo and broiler. A total of 65 samples were tested to imipenem, meropenem, doripenem, Colistin, ampicillin and amikacin. Antibiotic zones diameter breakpoints of *P. aeruginosa* were determined as per (CLSI) and measured in millimeters that were evaluated as sensitive and resistant.

Antibiotics zones diameter breakpoints of Pseudomonas aeruginosa in millimeter according to CLSI (2024)

A zone diameter value or minimum inhibitory concentration is used to classify an organism as non-susceptible, resistant, intermediate, susceptible, susceptible-dose dependent, or susceptible. Using recognized breakpoints, one can interpret the MIC or zone diameter values obtained from a susceptibility test. Breakpoints are thought to be reliable indicators of expected clinical outcome since they are mostly derived from pharmacologically and clinically rich datasets that are used in vitro and in vivo data. Following table shows MIC for different antibiotics.

Table 7: MIC of different antibiotics used

	Disc content	Sensitive	Intermediate	Resistant
Imipenem	10µg	≥19	16-18	≤15
Meropenem	10µg	≥19	16-18	≤15
Doripenem	10µg	≥19	16-18	≤15
Colistin	10µg	2µg	4-6µg	≥8µg

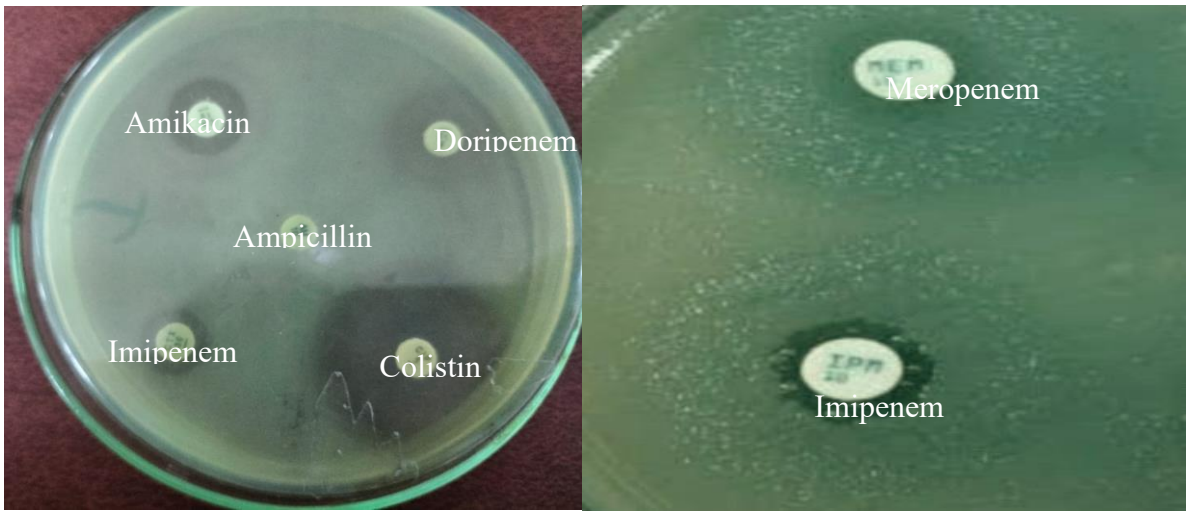

Figure 11: Antibiotic susceptibility testing by disc diffusion method

Table 8: An overview of total sensitive and resistant antibiotics

Antibiotics	Raw milk (n=7)		Clinical (n=24)		Diabetic foot (n=20)		Chicken embryo (n=10)		cockroach (n=4)	
	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)
Imipenem	3	4	4	20	8	12	4	6	2	2
Meropenem	1	6	9	15	5	15	6	4	3	1
Doripenem	5	2	14	10	12	8	10	0	4	0
Colistin	7	0	24	0	20	0	10	0	4	0
Amikacin	3	4	17	7	15	5	7	3	3	1
Ampicillin	3	4	16	8	11	9	8	2	3	1

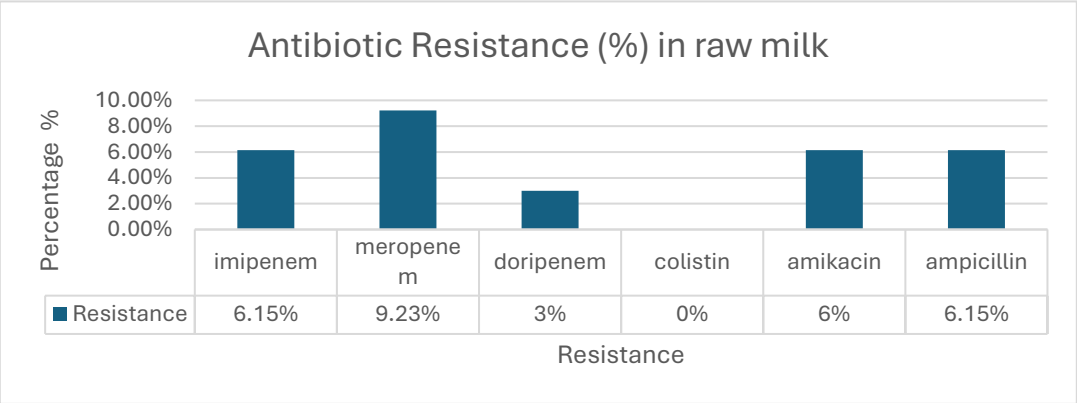

Figure 12: Antibiotic resistance in raw milk

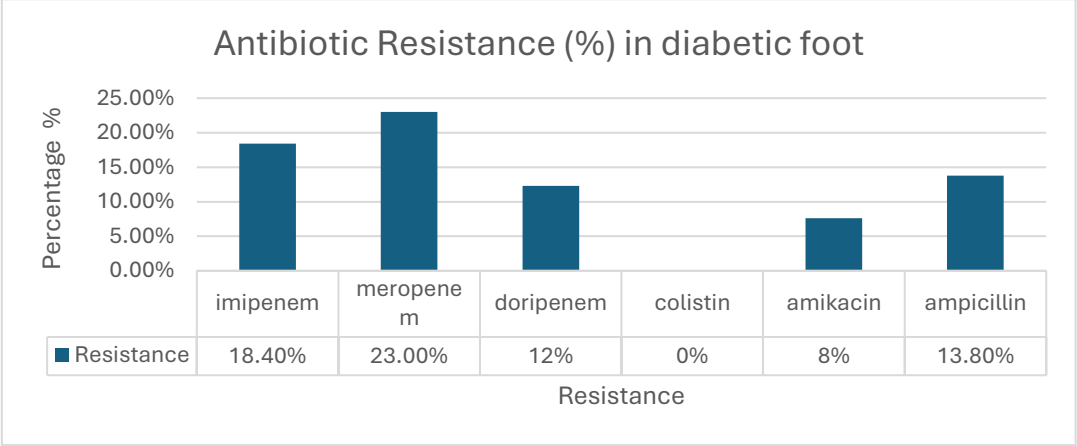

Figure 13: Antibiotic resistance in Diabetic foot patients

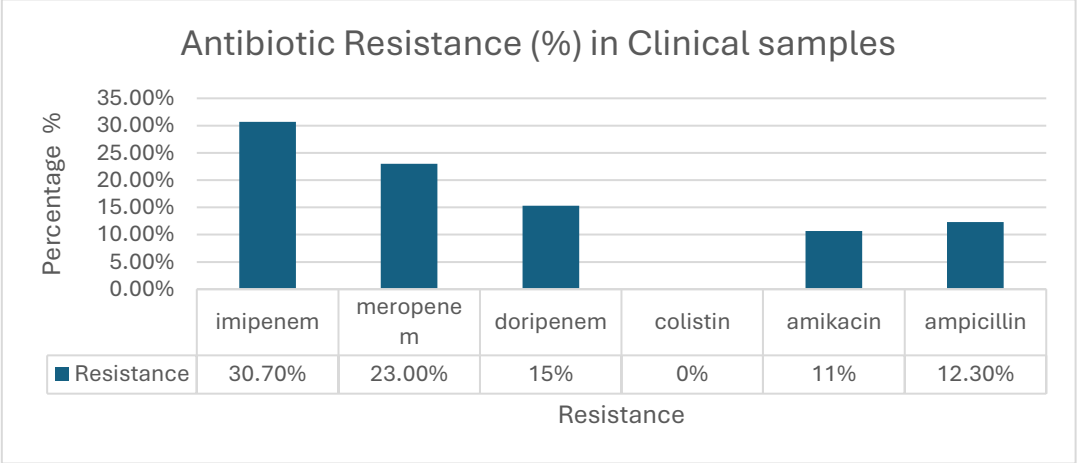

Figure 14: Antibiotic resistance in Clinical isolates

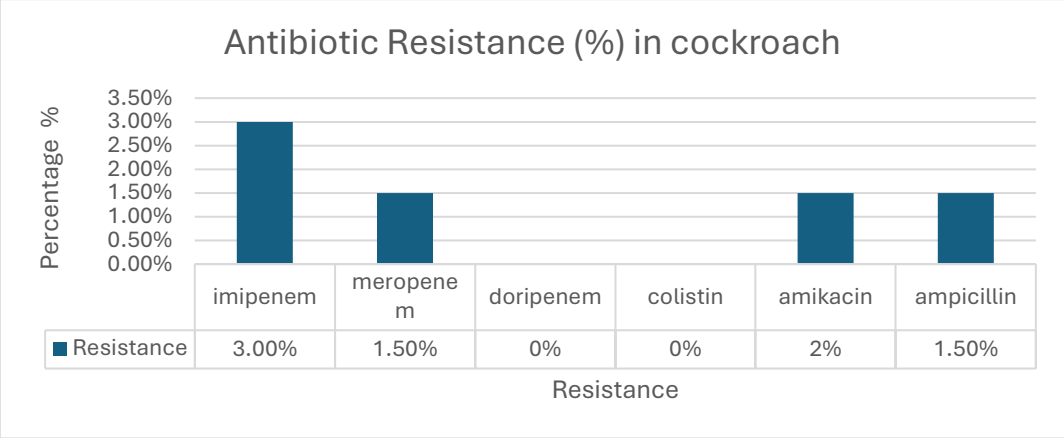

Figure 15: Antibiotic resistance in Cockroach

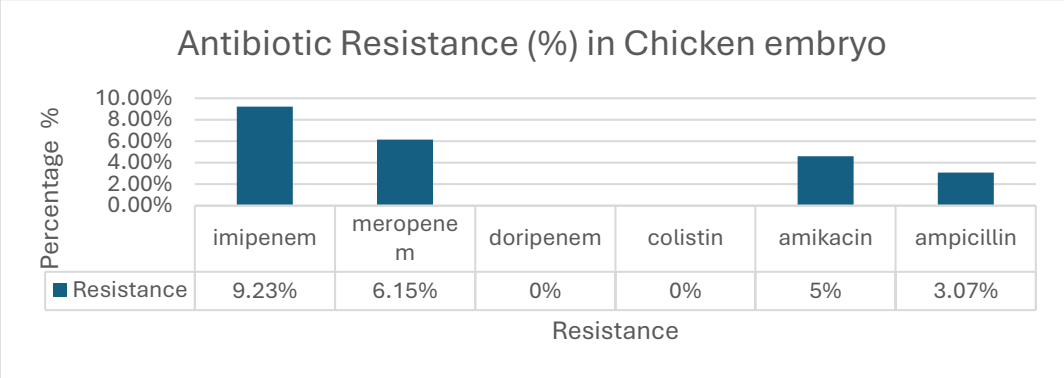

Figure 16: Antibiotic resistance in Chicken embryo and broiler

Comparative Analysis of Resistance in each source

Comparative analysis shows high resistance of imipenem among all the antibiotics.

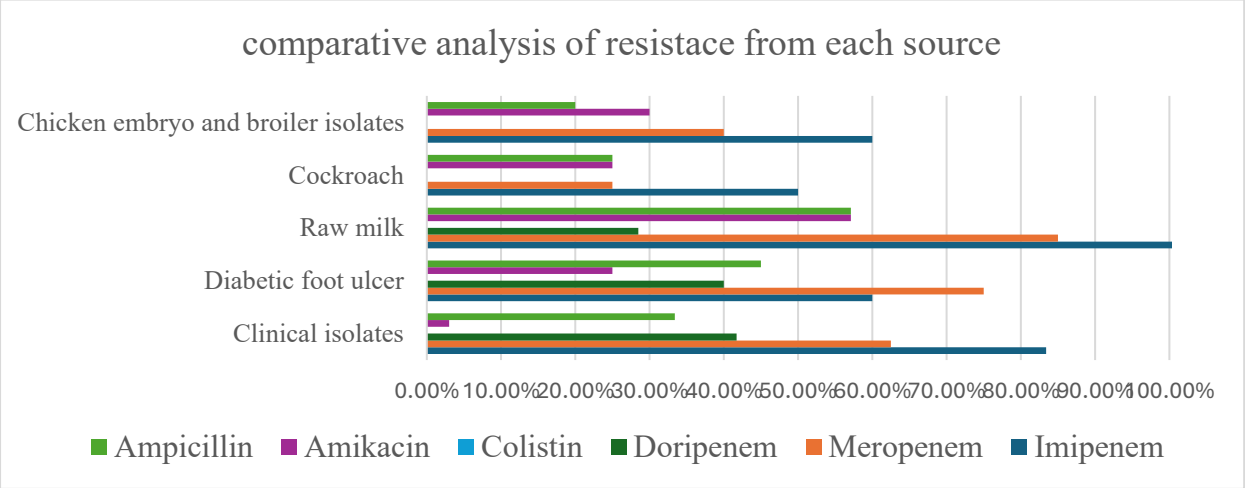

Figure 6: Comparative analysis of resistance in each source

Chi-square Analysis

Results were analyzed using chi-square in SPSS software.

Table 9: Statistical method chi-square for resistance

Chi-square	8.72
Degree of freedom	20
p-value	0.98

We will accept the Null hypothesis because the p-value is large i.e, P > α and 0.98 > 0.05. Hence, concluded that there is no association between antibiotics used and source of sample.

Table 10: Total Resistant isolates from each source

Antibiotics	Clinical isolates (n=24)	Diabetic foot ulcer Isolates (n=20)	Raw milk Isolates (n=7)	Cockroach Isolates (n=4)	Chicken embryo Isolates (n=10)	P value= 0.00290 (≤ .05)
Imipenem	20	12	4	2	6	
Meropenem	15	15	6	1	4	
Doripenem	10	8	2	0	0	
Colistin	0	0	0	0	0	
Amikacin	7	5	4	1	3	
Ampicillin	8	9	4	1	2	

PCR Amplification of Resistant Genes

Specific primers are used for the detection of *bla*KPC, *bla*IMP and mcr1 gene.

Table 11: Total Genes detected from samples

Genes	Positive	Negative
KPC	7	58
IMP	4	61
MCR-1	0	65

Figure 18: DNA extraction of *P. aeruginosa* by chemical method

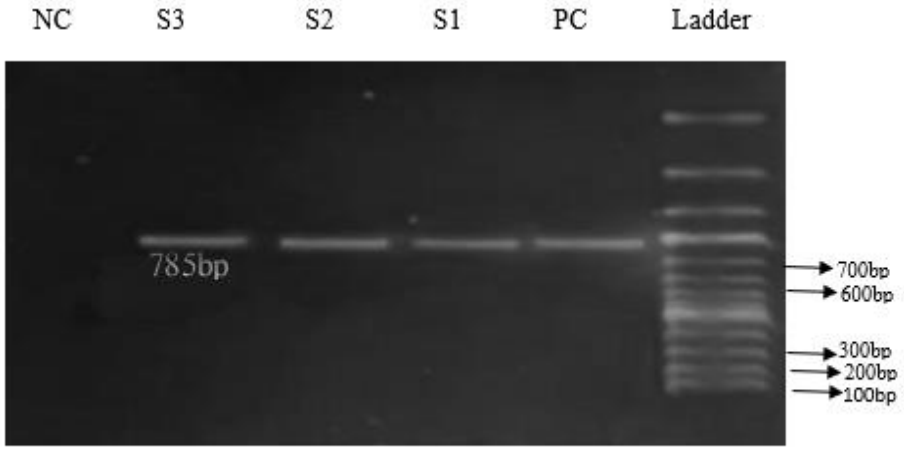

Figure 19: Amplification of *bla* KPC (785bp) through PCR

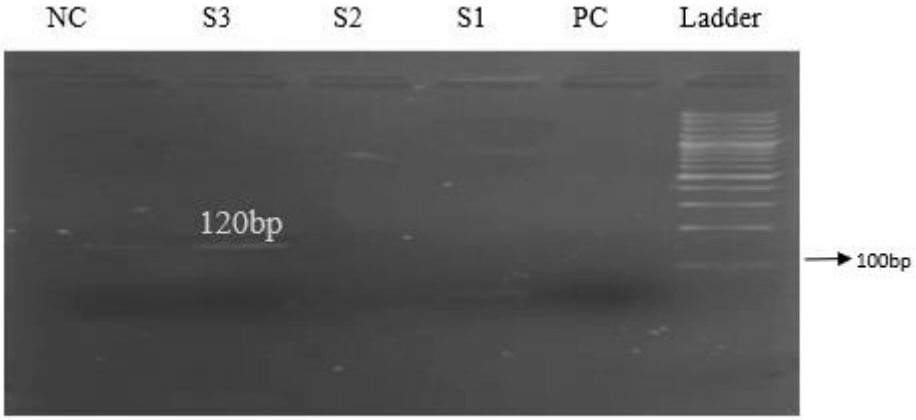

Figure 20: Amplification of *bla*IMP (120bp) gene though PCR

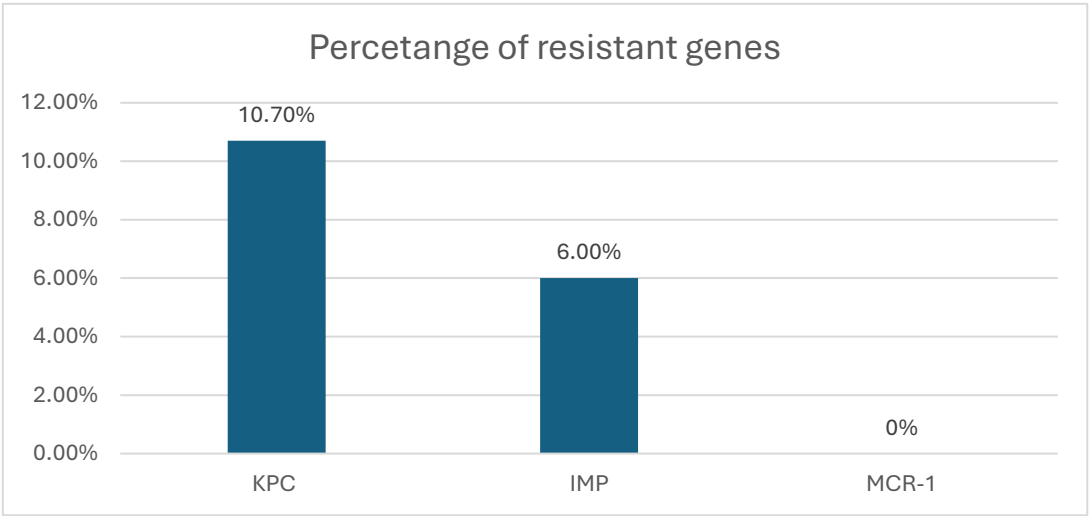

Figure 21: Overall prevalence of resistant genes

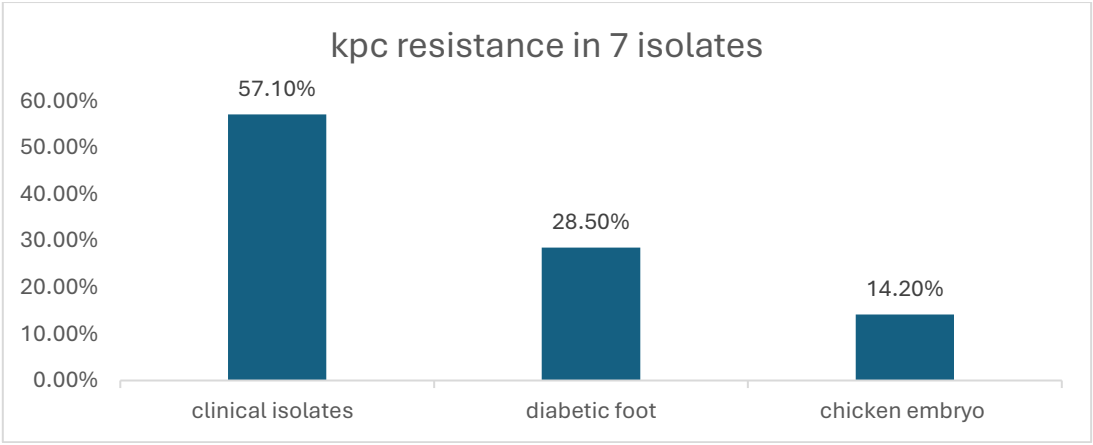

Figure 22: Prevalence of KPC gene in different sources

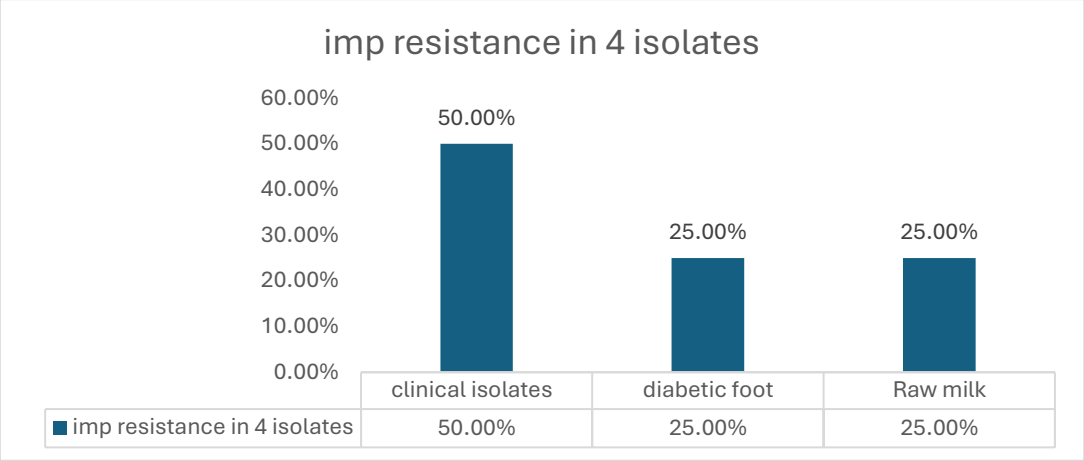

Figure 23: Prevalence of IMP gene in different sources

Discussion

The present study aimed to determine the prevalence of antimicrobial-resistant *Pseudomonas aeruginosa* and to investigate the frequency of carbapenem resistance genes (*blaKPC*, *blaIMP*) and the colistin resistance gene (*mcr-1*) among resistant isolates. *P. aeruginosa* is a ubiquitous Gram-negative opportunistic pathogen belonging to the family Pseudomonadaceae and is well recognized for its intrinsic and acquired resistance mechanisms (27). Its relatively large genome (approximately 5.5–7 Mbp) encodes numerous regulatory proteins, efflux pumps, and metabolic pathways that facilitate environmental adaptation and multidrug resistance (28). This genomic versatility significantly contributes to its persistence in clinical and environmental settings.

In the current study, the overall prevalence of *P. aeruginosa* among revived samples was 43.3% (65/150). This finding indicates substantial circulation of this pathogen across diverse sources, including clinical, food, animal, and environmental samples. Similar prevalence trends have been reported in previous regional and international studies, where *P. aeruginosa* was frequently isolated from hospital settings and environmental reservoirs (29).

Antimicrobial susceptibility testing revealed high resistance rates to carbapenems, particularly imipenem (67.6%) and meropenem (63.07%), followed by doripenem (30.76%). Carbapenem resistance in *P. aeruginosa* is a growing global concern and is often mediated by carbapenemase production, porin loss, and efflux pump overexpression (30). The observed resistance levels in this study align with reports indicating increasing carbapenem resistance in South Asian regions (31). Resistance to amikacin (30.76%) and ampicillin (36.92%) were also detected, reflecting the organism's intrinsic resistance mechanisms and acquired resistance determinants (32). Notably, none of the isolates demonstrated resistance to colistin, suggesting that polymyxins remain effective therapeutic options in the study setting. However, cautious use is recommended due to the global emergence of plasmid-mediated colistin resistance genes (33).

Molecular analysis demonstrated that 10.7% of isolates carried the *blaKPC* gene, while 6.1% harbored the *blaIMP* gene. The *blaKPC*-positive isolates were predominantly obtained from clinical and diabetic foot samples, indicating a higher burden of carbapenemase-producing strains in healthcare-associated infections. Similarly, *blaIMP* was detected in both clinical and non-clinical sources, highlighting the possible dissemination of metallo-β-lactamase genes across environmental and food chains. The presence of these genes is consistent with previous findings reporting the spread of KPC and IMP carbapenemases in *P. aeruginosa* isolates worldwide (34).

Importantly, none of the isolates tested positive for the *mcr-1* gene, which correlates with the observed phenotypic susceptibility to colistin. Although plasmid-mediated colistin resistance remains relatively uncommon in *P. aeruginosa* compared to Enterobacteriaceae, continuous surveillance is essential to prevent its emergence and spread (35).

Overall, the findings highlight a concerning level of carbapenem resistance among *P. aeruginosa* isolates, particularly in clinical sources, and emphasize the importance of molecular surveillance to detect resistance genes. The detection of *blaKPC* and *blaIMP* genes underscores the need for strict antimicrobial stewardship, infection control practices, and continuous monitoring of resistance trends to limit further dissemination of multidrug-resistant strains.

CONCLUSION

Based on our findings, this study demonstrated that *P. aeruginosa* has a high level of resistance in patients suffering from different diseases as well as in chicken, cockroach and food items. The frequency of carbapenem resistance genes *bla* KPC and *bla* IMP depict a positive correlation between resistant antibiotics and resistant genes in *P. aeruginosa*. This study did not find Colistin resistance and similarly, no prevalence of Colistin resistant mcr-1 gene was found in our isolates.

RECOMMENDATIONS

The following recommendations are drawn based on the conclusion of the study.

1. This study will support the urgent need for regular screening and inspection of *Pseudomonas aeruginosa*.
2. It indicates spread of resistance from animals (cockroach and chicken) to food items (raw milk) and into the patients (diabetic and clinical). Thus, this data will support the urgent need for regular screening and inspection of *P. aeruginosa* in all aspects of our life including food items and poultry animals.
3. It may help to design therapeutic strategies and monitor the efficacy of administered antibiotic treatments.

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