Research Article

Evaluation of Antimicrobial Resistance Profiles of Respiratory Microbiota in Diseased Dogs in Pakistan

Zarreen Sajjad[1]*, Muhammad Abubakar[1], Muhammad Fayaz[1], Shumaila Manzoor[1], Fatima Sajjad[2], Eidnawaz[1], Muhammad Masood Tahir[3]

[1]National Veterinary Laboratory, Islamabad, Pakistan; [2]University of Agriculture, Faisalabad, Pakistan; [3]Pioneer Pets Hospital, Rawalpindi, Pakistan.

Abstract | Bacterial resistance is shown to be an inevitable side effect due to the excessive use of antibiotics, becoming a significant concern worldwide. The aim of this study was the retrospective evaluation of the antimicrobial resistance profile of bacteria isolated from companion animal infections in the region of Rawalpindi. A total of 228 dogs were used in the study and their clinical samples were analyzed using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for bacterial identification. *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were the most frequently detected pathogens. *E. coli* was identified in 154 samples, while *Klebsiella* and *P. aeruginosa* were found in 21 and 45 samples, respectively. Co-infections included E. coli with Klebsiella (10 samples), *E. coli* with *P. aeruginosa* (14 samples), and *Klebsiella* with *P. aeruginosa* (4 samples). Antibiotic susceptibility testing using the Kirby-Bauer method revealed that 43% of *E. coli* isolates (77/178) exhibited Extended Spectrum Beta-lactamase activity. ESBL production was also detected in 20% of *K. pneumoniae* (7/35) and 5% of *P. aeruginosa* (3/63) isolates. 128 isolates of *E. coli*, 13 isolates of *K. pneumoniae* and 11 isolates of *P. aeruginosa* were found MDR. A high prevalence of ESBL-producing *E. coli* (43%) was observed, significantly greater than in *K. pneumoniae* (20%) and *P. aeruginosa* (5%) ($p < 0.05$), aligning with previous studies. *E. coli* also showed the highest multidrug resistance (72%), particularly to commonly used antibiotics like Sulfamethoxazole-Trimethoprim and Ampicillin. Carbapenems remained effective across all species. This study highlights high prevalence of antimicrobial resistance in companion animals poses a serious One Health threat, linking animal infections to human and environmental health risks.

Keywords | Antimicrobial resistance, Extended spectrum beta lactamase-producing bacteria, Kirby-bauer, Matrix-Assisted laser desorption ionization-time of flight mass spectrometry, Multidrug resistance, prevalence

Received | June 17, 2025; **Accepted** | July 14, 2025; **Published** | July 26, 2025
***Correspondence** | Zarreen Sajjad, National Veterinary Laboratory, Islamabad, Pakistan; **Email:** Zarreensajjad927@gmail.com
Citation | Sajjad Z, Abubakar M, Fayaz M, Manzoor S, Sajjad F, Eidnawaz, Tahir MM (2025). Evaluation of antimicrobial resistance profiles of respiratory microbiota in diseased dogs in Pakistan. Res J. Vet. Pract. 13(3): 50-56.
DOI | https://dx.doi.org/10.17582/journal.rjvp/2025/13.3.50.56
ISSN | 2308-2798

INTRODUCTION

Antimicrobial resistance (AMR) is a growing global health concern that transcends human medicine and significantly impacts veterinary health and One Health initiatives (McEwen and Collignon, 2018). In recent years, increasing attention has been directed towards companion animals, particularly dogs, as potential reservoirs and disseminators of antimicrobial-resistant bacteria. Among the many microbial ecosystems in dogs, the respiratory microbiota represents a critical but often underexplored niche, particularly in the context of AMR (Mitchell *et al.*, 2017).

The respiratory tract of dogs, like that of humans, harbors a complex community of microorganisms, including bacteria

that can exist in a commensal, symbiotic, or pathogenic relationship with the host (Souza *et al*., 2020). Chronic rhinitis and nasal neoplasia are the most common causes of nasal discharge in dogs. Canine chronic rhinitis (CCR) is characterized by lymphoplasmacytic or mild neutrophilic infiltrates in the nasal mucosa causing nasal discharge, sneezing, and coughing (Windsor and Johnson, 2006; Reagon and Sykes, 2020).

In healthy dogs, the respiratory microbiota plays a role in maintaining immune homeostasis and preventing colonization by opportunistic pathogens (Mitchell *et al*., 2017). However, during disease states such as bacterial pneumonia, kennel cough, or secondary infections following viral illness, there is often a disruption in the normal microbial balance also called as dysbiosis. This disruption can lead to the overgrowth of microorganisms, many of which may harbor antimicrobial resistance genes (ARGs), complicating treatment outcomes (Daodu *et al*., 2017; Maboni *et al*., 2019).

In recent years, Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a rapid, accurate, and cost-effective tool for the identification of bacterial pathogens. This allows identification within minutes, significantly improving the speed and reliability of diagnostic microbiology. Its application has proven valuable in AMR studies, where timely and accurate identification of resistant organisms is critical for effective treatment and surveillance (Hrabak *et al*., 2013; Florio *et al*., 2020).

Respiratory infections in dogs are commonly treated with broad-spectrum antibiotics, often without culture and sensitivity testing, leading to an increased likelihood of resistance (Lappin *et al*., 2017; Weese *et al*., 2019). Resistant pathogens such as *E. coli, Pasteurella multocida,* and *Bordetella bronchiseptica* have been frequently isolated from dogs with respiratory diseases (Viitanen *et al*., 2015; Fastres *et al*., 2019). Evaluating the AMR profiles will not only enhance our understanding of microbial dynamics but will also inform better strategies in veterinary medicine (Bourély *et al*., 2019; Vientos-Plotts *et al*., 2023). Given the interdependent human, animal, and environmental dimensions of AMR, it is logical to take a One Health approach when addressing this problem (McEwen and Collignon, 2018).

The specific objectives of this study include identifying respiratory microbiota of both healthy and diseased dogs and assessing their resistance to a range of common antibiotics. Through these objectives, the research will contribute to a deeper understanding of how AMR develops in the respiratory tract of dogs and provide valuable insights for better management practices, including the use of targeted antimicrobial therapies and stewardship strategies to mitigate the rise of AMR in companion animals.

MATERIALS AND METHODS

Study design

This study was a cross-sectional observational investigation aimed at identifying respiratory microbiota and their AMR profiles in dogs presenting with respiratory symptoms to a veterinary outpatient department (OPD) in Rawalpindi from January, 2025 to April, 2025. Nasal swab samples were collected from dogs on random basis presenting with respiratory symptoms (e.g., coughing, sneezing, nasal discharge). Bacterial identification was conducted through culture-based methods and MALDI-TOF. AST followed CLSI guidelines using standard techniques like the Kirby-Bauer method (Hudzicki, 2009) and analyzed statistically to determine prevalence and resistance trends, with ethical approval and informed owner consent obtained prior to study commencement.

Sample collection

Sterile cotton-tipped swabs were used to collect samples from the anterior nares of each dog. Swabs were immediately placed into sterile transport media (LB broth) and transported to the microbiology laboratory under refrigerated conditions (4˚C) for processing (Singh *et al*., 2015).

Bacterial isolation and culture

Upon arrival at the laboratory, broth enriched samples were incubated at 37˚C for 24 hours. Then a loopful culture was streaked onto selective media (MacConkey agar). Plates were incubated aerobically at 37˚C for 24 hours. Morphologically distinct colonies were sub-cultured to obtain pure isolates for further analysis (Narang et al., 2023).

Bacterial identification by MALDI-TOF MS

Pure bacterial colonies were identified using MALDI-TOF MS (SmartfleX® by Bruker, Germany). A small portion of each colony was transferred onto a MALDI target plate and overlaid with matrix solution. After air drying, the samples were analyzed using Bruker Biotyper system. Bacterial species identification was performed based on the mass spectral fingerprint and compared to a reference database. Identification scores ≥ 2.0 were considered reliable for species-level identification (Singhal *et al*., 2015).

Antimicrobial susceptibility testing (AST)

AST of the confirmed bacterial isolates was performed using the standard Kirby-Bauer method in accordance

Table 1: List of antibiotic discs used and their classification.

Abbreviation	Full name	Antibiotic Class/ Group
CAZ	Ceftazidime	Beta-lactams (3rd-gen Cephalosporins)
AZM	Azithromycin	Macrolides
CN	Gentamicin	Aminoglycosides
CTX	Cefotaxime	Beta-lactams (3rd-gen Cephalosporins)
TE	Tetracycline	Tetracyclines
NA	Nalidixic Acid	Quinolones
FEP	Cefepime	Beta-lactams (4th-gen Cephalosporins)
SXT	Sulfamethoxazole-Trimethoprim	Sulfonamides (Folate synthesis inhibitors)
MRP	Meropenem	Beta-lactams (Carbapenems)
CIP	Ciprofloxacin	Fluoroquinolones
AMP	Ampicillin	Beta-lactams (Aminopenicillins)
IMP	Imipenem	Beta-lactams (Carbapenems)
C	Chloramphenicol	Amphenicols
CAL	Ceftazidime + Clavulanic Acid	Beta-lactams (Combination)
CTL	Cefotaxime + Clavulanic Acid	Beta-lactams (Combination)

with Clinical and Laboratory Standards Institute (CLSI) guidelines. Bacterial suspensions were prepared in sterile saline and adjusted to 0.5 McFarland turbidity standards. The inoculum was uniformly spread onto Mueller-Hinton agar plates (Hudzicki, 2009).

Commercial antibiotic-impregnated discs representing commonly used antibiotics in veterinary and human medicine were placed on the agar surface as shown in Table 1. Plates were incubated at 37 °C for 24 hours. The diameters of inhibition zones were measured in millimeters, and results were interpreted as susceptible or resistant based on CLSI breakpoints (Hudzicki, 2009).

Data analysis

Isolate frequency, distribution, and antimicrobial resistance profiles were documented. Descriptive statistics were used to analyze the prevalence of different bacterial species and their resistance patterns. Comparisons between different species and their sensitivity to different antimicrobial groups were made using appropriate statistical test (Chi-square test), with a significance level set at $p < 0.05$ (Liu *et al*., 2025).

Informed consent

Informed consent has been obtained for client-owned animals included in this study.

RESULTS

Microbiological diagnosis of bacterial isolates

A total of 228 clinical samples were subjected to analysis comparing the proteome profile of the bacteria obtained by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) with a database. *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were the most predominant bacteria isolated. Several samples yielded more than one species of bacteria. Upon MALDI-TOF analyses, alone *E. coli* was identified in 154 samples out of 228 total samples. Similarly, alone *Klebsiella* and *P. aeruginosa* were isolated in 21 and 45 samples respectively. 10 isolates were found positive for both *E. coli* and *Klebsiella*. *P. aeruginosa* was present along with *E. coli* in 14 samples. Only 4 samples were found positive for both *Klebsiella* and *P. aeruginosa* summarized in Table 2.

Table 2: Frequency of bacterial isolates identified by MALDI-TOF.

	E. coli	*K. pneumoniae*	*P. aeruginosa*
No. of isolates	178	35	63
Percentage (%)	64	13	23

Antimicrobial susceptibility testing

All isolates were subjected to antibiotic susceptibility testing using Kirby-Bauer method. According to results, 77 *E. coli* isolates out of total 178 isolates positive for *E. coli* were identified as having Extended Spectrum Beta-lactamase (ESBL) activity which means they were sensitive against CTL (Cefotaxime + Clavulanic Acid) and CAL (Ceftazidime + Clavulanic Acid) and resistant against CTX and CAZ alone. Whereas ESBL activity by *K. pneumoniae* and *P. aeruginosa* was recorded in 07 out of 35 and 03 out of 63 respectively. 43% of 178 *E. coli* showed ESBL activity, whereas ESBL activity of *K. pneumoniae* and *P. aeruginosa* was found in 20% and 5% of their respective isolates as shown in Table 3.

Table 3: Prevalence of ESBL bacteria in the clinical isolates from dog respiratory tract.

Bacterial spp.	No. of isolates	ESBL	ESBL (%)	ESBL ± SE (%)
E. coli	178	77	43	43 ± 3.71[a]
K. pneumoniae	35	07	20	20 ± 6.75[b]
P. aeruginosa	63	03	05	5 ± 2.68[c]

ESBL = Extended Spectrum Beta-Lactam, S. E. = Standard Error; (p value < 0.05).

Chi-square statistical analyses revealed, significant difference in the proportions of ESBL-positive isolates among *E. coli*, *K. pneumoniae* and *P. aeruginosa* (p-value < 0.05). This proves that there exist a significant difference in the prevalence of ESBL activity between these bacterial species.

128 isolates of *E. coli*, 13 isolates of *K. pneumoniae* and 11 isolates of *P. aeruginosa* out of 178, 35 and 63 respectively, were found multidrug resistant (MDR) given in Table 4.

Table 4: Prevalence of MDR strains in the clinical isolates of dog respiratory tract.

Bacterial spp.	No. of isolates	MDR	MDR (%)	MDR ± S.E (%)
E. coli	178	128	72[a]	72 ± 3.34[a]
K. pneumoniae	35	13	37[b]	37 ± 8.16[b]
P. aeruginosa	63	11	17[c]	17 ± 4.78[c]

MDR = Multidrug Resistant, S. E. = Standard Error. (p value < 0.05).

These results were subjected to statistical analyses using Chi-Square test and a statistically significant difference was calculated in proportions of multidrug-resistant isolates between the three bacterial species (*E. coli, K. pneumoniae*, and *P. aeruginosa*) at the 5% significance level (p-value < 0.05). These results truly depict that there exist a significant different among the prevalence of MDR bacteria among all these bacterial species as summarized in Figure 1 also.

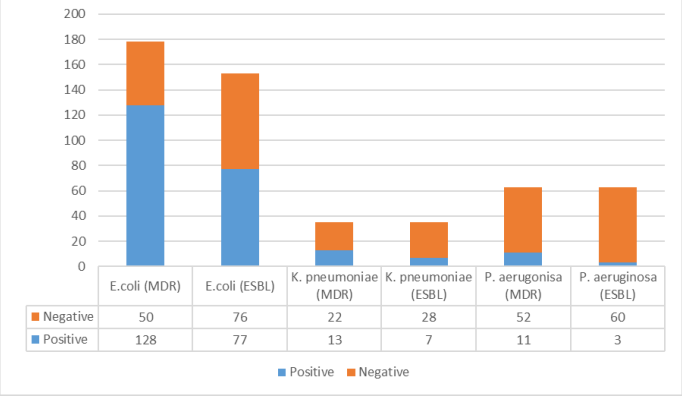

Figure 1: Prevalence of ESBL and MDR strains of clinical isolates of dog respiratory tract.

According to CSLI guidelines, *E. coli* showed highest resistance by 76% isolates against Sulfamethoxazole-Trimethoprim (SXT), 65% by Ampicillin (Amp) and 48% by Nalidixic Acid (NA). Whereas Cefotaxime (CTX), Ceftazidime (CAZ) and Ciprofloxacin (CIP) suffered resistance against 47%, 41% and 39% isolates of E. coli, respectively. However, *E. coli* was found most sensitive against Carbapenems with both Imipenem (IMP) and Meropenem (MRP) exhibiting 98% sensitivity each (2% resistance). In the similar way, Antibiograms of *K. pneumoniae* and *P. aeruginosa* are explained in Table 5 (percentage) and Figure 2.

Table 5: Antibiotic resistance (%) of *E. coli, Klebsiella* and *Pseudomonas* isolates determined by AST.

Antibiotic	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
CAZ	41%	43%	21%
AZM	30%	62%	27%
CN	19%	04%	23%
CTX	47%	29%	35%
TE	29%	25%	25%
NA	48%	08%	54%
FEP	06%	37%	31%
SXT	76%	08%	25%
MRP	2%	04%	20%
CIP	39%	33%	19%
AMP	65%	21%	17%
IMP	2%	00%	6%
C	25%	33%	10%
CAL	05%	04%	6%
CTL	02%	00%	2%

(p value < 0.05).

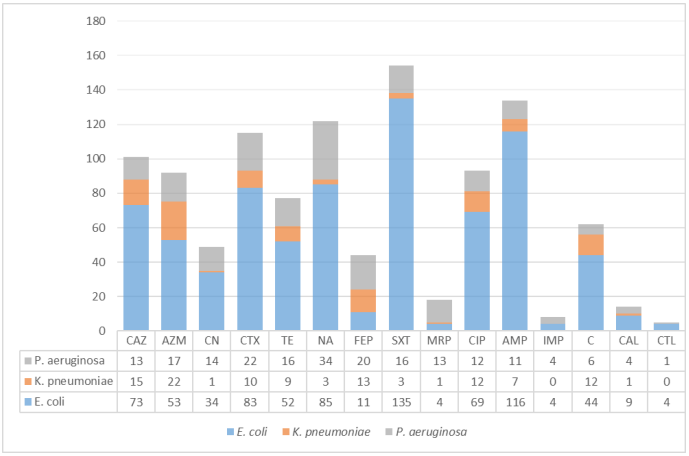

Figure 2: No. of resistant isolates of *E. coli*, *K. pneumoniae* and *P. aeruginosa* against different antibiotics.

DISCUSSION

Chronic rhinitis and nasal neoplasia are the most common

causes of nasal discharge in dogs (Cohn, 2020). CCR is characterized by lymphoplasmacytic or mild neutrophilic infiltrates in the nasal mucosa causing nasal discharge, sneezing, and coughing (Windsor and Johnson, 2006). The etiology of CCR is unknown, and although infectious, immune-mediated, and allergic mechanisms have been suggested, dogs may respond poorly to antimicrobials, glucocorticoids, and antihistamines, making these etiologies unlikely (Hakansson *et al.*, 2018). In dogs, carcinoma accounts for up to 66% of nasal neoplasia, with adenocarcinoma being the most common subtype (Cohn, 2020).

The present study investigated the prevalence and antimicrobial resistance profiles of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* isolated from canine respiratory tract samples, highlighting the concerning trends in extended-spectrum beta-lactamase (ESBL) activity and multidrug resistance (MDR) among these pathogens. *E. coli* emerged as the predominant isolate, consistent with previous reports identifying it as a common opportunistic pathogen in both human and veterinary settings. The use of MALDI-TOF mass spectrometry enabled rapid and accurate identification of bacterial species, proving to be an efficient diagnostic tool for clinical microbiology as briefed in Figure 3.

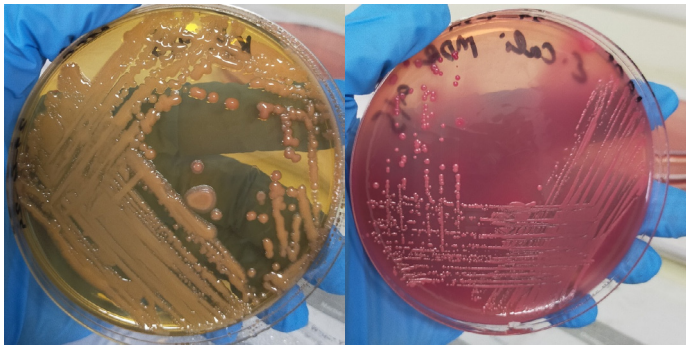

Figure 3: Growth of *E. coli* (Right) and *K. pneumoniae* (Left) on MaCconkey Agar.

Our findings reveal a notably high prevalence of ESBL-producing *E. coli* (43%) compared to *K. pneumoniae* (20%) and *P. aeruginosa* (5%), with statistically significant differences among the species ($p < 0.05$) and these findings are very much consistent with those reported by Husna *et al.* (2023). This suggests that *E. coli* has a greater capacity for acquiring and disseminating ESBL genes, likely driven by selective pressure from overuse or misuse of antibiotics. These enzymes confer resistance to a broad range of beta-lactam antibiotics, rendering many commonly used therapies ineffective.

Similarly, the MDR profile showed *E. coli* to be the most resistant, with 72% of isolates showing resistance to three or more antibiotic classes. In contrast, *K. pneumoniae* and *P. aeruginosa* demonstrated 37% and 17% MDR rates, respectively. These significant differences ($p < 0.05$) underscore the need for species-specific surveillance and targeted antimicrobial stewardship strategies. Similar results were observed in the study conducted by Alali *et al.* (2022).

Antibiotic susceptibility testing further highlighted the resistance burden. *E. coli* exhibited highest resistance to Sulfamethoxazole-Trimethoprim (76%), Ampicillin (65%), and Nalidixic Acid (48%), which are frequently used antibiotics in veterinary practice and these findings align with findings of Vranic and Uzunovic (2016). However, Carbapenems (Imipenem and Meropenem) remained highly effective, with only 2% resistance, suggesting they may still serve as effective last-resort options. Similarly, *K. pneumoniae* showed high resistance to Azithromycin (63%) and moderate resistance to Ceftazidime (43%) and Cefepime (37%), while maintaining sensitivity to Carbapenems and Gentamicin. *P. aeruginosa*, known for intrinsic resistance mechanisms, demonstrated highest resistance to Nalidixic Acid (54%), but retained susceptibility to Clavulanic acid combinations and Imipenem. Number of resistant isolates of *E. coli*, *K. pneumoniae* and *P. aeruginosa* against 15 different antibiotics are illustrated in the Figure 4.

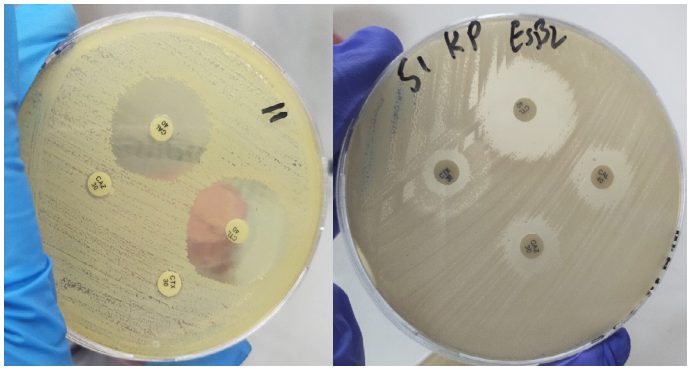

Figure 4: ESBL activity of *E. coli* (Left) and *K. pneumoniae* (Right) on Muller-Hinton Agar.

These results collectively reflect a worrying rise in antimicrobial resistance among bacterial respiratory pathogens in dogs, with potential zoonotic implications. The presence of ESBL and MDR strains, particularly in *E. coli*, underscores the urgent need for prudent antibiotic use, routine susceptibility testing, and continuous monitoring to inform effective treatment and containment strategies.

Study limitations

One of the key limitations of the study is its retrospective design. The focus on a single region (upper respiratory tract) and species (dogs) also limits its generalizability. Another limitation is the small number of certain bacterial isolates for example *P. aeruginosa* which reduces its statistical power. Over reliance on phenotypic identification methods

without molecular confirmation may affect accuracy in resistance detection.

ACKNOWLEDGEMENT

This is the first study from Pakistan to assess antimicrobial resistance patterns in respiratory microbiota of diseased dogs. It fills a key gap in veterinary AMR data and highlights the potential role of companion animals in the spread of resistant pathogens.

NOVELTY STATEMENT

The authors gratefully acknowledge the support of National Veterinary Laboratory, Pakistan for providing the necessary laboratory facilities and resources to conduct this research. We also extend our thanks to the Pioneer Pets Hospital and pet owners who participated and cooperated in sample collection.

AUTHOR'S CONTRIBUTION

ZS carried out the design, data collection, experiment, statistical analyses and interpretation. MA conceived of the study and participated in its design. MF contributed in data collection. SM participated in analyses and interpretation. FS contributed in coordination, literature search and sequence alignment. Eidnawaz contributed in experiment and interpretation. MMT carried out the write up and drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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