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Monitoring the Burden of the Antibiotic Resistance and Virulence Genes in Pet Animals Suffering from Bacterial Otitis

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ABSTRACT

The etiology of otitis in dogs and cats is multifactorial and complex, involving bacterial and fungal pathogens. This study aimed to describe microbiological features and susceptibility profiles of bacterial pathogens associated with 212 cases of external otitis, comprising 118 dogs and 94 cats. Ear swabs were processed to identify bacterial etiologies following standard microbiological methods, followed by antibiotic sensitivity profiling of each obtained isolate and PCR screening for a list of virulence and resistance genes. The overall resistance incidence for the obtained isolates against amoxicillin was 68.38%, amoxicillin/clavulanic acid was 55.4%, ceftriaxone was 26.6%, ceftazidime was 27.5%, cefoperazone (22.9%), enrofloxacin (1.6%), gentamicin (9.9%), amikacin (7.2%), linezolid (92.8%), tylosin was 76%. PCR screening of isolated bacteria revealed that *E. coli* and *K. pneumoniae* harbored ESBL genes like *bla*TEM, *bla*CTX-M, and other resistance genes *aad*B and *qnr*B. Likewise, the other Gram-negative isolates of *P. aeruginosa* and *P. mirabilis* were also positive for the same resistance genes. Gram-positive *S. aureus* harbored *bla*Z and *nor*A resistance genes. Current work provides priceless surveillance value in monitoring resistant bacteria in pet animals as Customized monitoring programs can help identify certain AMR frameworks, aid clinicians in making logical treatment choices, and restrict the selection and spread of AMR within the population.

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INTRODUCTION

Otitis externa is a regular disorder seen by veterinarians; up to 20% of canines and 6% of felines can experience this problem **(Angus, 2004).** It is an inflammation of the external auditory canal, which can affect the entire length extending from the external meatus passing by both vertical and horizontal parts up to the level of the tympanic membrane, including the ear pinna, or just an inflammation of a specific part. It can be unilateral, bilateral, acute, or chronic if recurrence or persistence occurs for three months or longer. Acute uncomplicated inflammation is often easy to deal with and can be easily treated, unlike chronic or recurrent inflammation, which is much more challenging **(Bajwa, 2019).** Otitis is a complex condition with multiple contributing factors. The primary, secondary, predisposing, and perpetuating (PSPP) system is a commonly used framework to identify those factors in each case **(Nuttall, 2023).**

Primary factors are factors that promote ear canal inflammation by a direct route, such as foreign bodies, parasites, allergy, and hypersensitivity, which is the most common primary cause, followed by aural neoplastic lesions including ceruminous gland adenoma, squamous cell carcinoma, sebaceous adenocarcinoma, and cholesteatoma, as well as endocrinopathies chiefly hypothyroidism and hyperadrenocorticism, in addition to autoimmune diseases like pemphigus foliaceus and mucous membrane pemphigoid **(Paterson and Matyskiewicz, 2018).**

Infectious agents, including bacteria and fungi, were historically considered perpetuating factors for ear problems. However, recently they have been reclassified as secondary factors, which can only lead to pathology in abnormal ear environments or when combined with other predisposing factors. By cytology and culture, relatively few bacterial genera, including *Staphylococcus* spp. and *Bacillus* spp., have traditionally been identified as normal inhabitants of healthy dogs' normal ear canal microflora. The overgrowth of these bacteria, or proliferation of other opportunistic pathogenic microbes in the ear, can worsen the inflammation, paving the way for the perpetuation of inflammation **(Borriello *et al*., 2020).**

Predisposing factors do not solely initiate the problem, but they contribute to altering the ear canal environment and increasing the likelihood of otitis externa. Physical features of ears such as pendulous or V-shaped dropped pinna, hairy ears narrow ear canals, and excessive earwax buildup. These features lead to the trapping of moisture and heat inside the ear, favoring the environment for bacterial and fungal growth. Owner-related approaches like frequent ear cleaning, using a wet paper cloth during cleaning, and misusage of antimicrobial ear cleaners. Swimming and changes in temperature and humidity all significantly increase the threat of developing otitis externa **(O'Neill *et al.,* 2021).**

Perpetuating factors are the histopathological chronic acquired changes in the ear canal, including epidermal and glandular hyperplasia, stenosis, failure of epidermal cell migration, calcification, and ossification of the auditory canal. Such changes exacerbate the flare-up of infection and make it more frequent and aggravated. This can also result in tympanic membrane rupture, allowing the infection to spread in the middle ear **(Huang *et al.,* 2009).**

Dogs with otitis may develop one or more of the subsequent clinical signs: redness, pruritus, discharge with an offensive odor, head shaking, self-injury, alopecia, ulceration, and ear hematoma **(Pye, 2018).** Extension of infection from the external to the middle ear canal can cause pain during food chewing or swallowing because of temporomandibular joint affection and Horner syndrome due to cranial nerve impairment. Accumulation of fluids inside the middle ear or cochlear damage results in hearing deterioration and the spreading of infection to the internal ear, causing nervous signs such as head tilting, nystagmus, and ataxia **(Bruyette, 2020).**

Otitis externa is a condition that affects dogs and has a significant negative impact on their welfare. A recent study conducted by primary veterinary care ranked otitis externa as the second most severe disorder among eight common canine ailments (Summers *et al.,* 2019). It doesn't only affect animals' welfare but also affects the quality of life of their owners **(Noli *et al.,* 2011).**

The microflora of the normal canine ear canal revealed the dominance of *Staphylococcus* spp., *Bacillus* spp., *Malassezia* spp., *Corynebacterium* spp., *Streptococcus* spp., Gram-negative rods, and *Micrococcus* spp. **(Lyskova *et al.,* 2007).** Dysbiosis of the ear canal is described by the overabundance of bacteria or yeast as *Malassezia* spp. increasing the susceptibility to ear infection, although shifting from healthy to diseased ears does not have a clear pattern; it may also differ depending on the canine breed **(Secker *et al.,* 2023).**

Family pets act as a swimming pool for various species of bacteria and resistance genes of clinical importance for human beings. The close contact between humans and companion animals through licking, petting, and physical injury provides favorable conditions for transmitting antimicrobial-resistant bacteria. It has been reported that adults and their puppies frequently share their skin microflora, highlighting the importance of the contact role **(Bhat, 2021).**

However, little is known regarding the ear carriage of antibiotic resistant and virulent bacterial pathogens in pet animals suffering from otitis which poses a serious zoonotic risk for humans. Thus, the main purpose of the current work was to investigate the occurrence of multidrug resistance and virulence genes in dogs and cats suffering from bacterial otitis.

MATERIALS AND METHODS

Ethics statement

All dogs and cats incorporated in this study were swabbed (ear swabs) during routine examination by professional veterinarians either at the private veterinary clinics or from shelters in the Greater Cairo Area (GCA) as well as the veterinary hospital of Cairo University. Samples were collected upon the approval of the owners in a laboratory routine investigation protocol based on the request of each case-responsible veterinarian. All animal-incorporated procedures are categorized as "noninvasive, no-pain-causing procedures". The animal research and reporting of *in vivo* experiments (ARRIVE) guidelines have been completely followed and covered.

The legal requirements or guidelines in Egypt for the care and use of animals have been followed and the institutional animal care and use committee

(IACUC) of the Faculty of Veterinary Medicine, Cairo University, Egypt guidelines have been fully covered. The ethical approval was obtained from the IACUC, approval number Vet CU131020241033.

Sampling and specimens' collection

A total of 212 ear swab samples were collected pets along as period (September 2023-2024), samples include 118 dogs and 94 cats, both genders, different ages, and breeds during their visits to different private veterinary clinics and from shelters in Greater Cairo Area (GCA) as well as the university veterinary hospital of Cairo University.

All animals had no record of previous hospitalization, and all were clinically diagnosed as otitis cases showing one or more clinical signs as headshaking, odor, redness of the skin, swelling, scratching, increased discharge, and scaly skin. Dogs and cats who had previously received topical or systemic treatment with antibiotics or anti-inflammatory drugs 10 days before sample collection were excluded from the study.

Samples of ear exudate were collected by inserting single-use commercially available sterile cotton-tipped swabs into the horizontal ear canal and then rolling it out after completing a full 360-degree rotation **(Choi *et al*., 2018).** Extreme caution was carried out to avoid surface contamination. Such samples were immediately transported to the laboratory in a cool condition until being further processed in the Microbiology Laboratory of the Faculty of Veterinary Medicine, Cairo University.

Isolation and identification

Although there are several anaerobic bacterial causes of otitis as *Bacteroides, Fusobacterium, Actinomyces, Clostridium,* and *Peptostreptococcus*, the current work was directed toward the most common bacterial causes of otitis in animals with a high incidence of resistance gene harboring. Swabs were inoculated in nutrient broth and were incubated for 24 hr at 37°C. A loopful of culture was streaked on MacConkey agar, Mannitol salt agar (MSA), nutrient agar, and blood agar plates, and then they were incubated at 37°C for 24 hr. Lactose fermenter and non-lactose fermenter colonies on MacConkey agar, mannitol fermenter and non-mannitol fermenter colonies on MSA, and exo-pigment producer and endo-pigment producer colonies on nutrient agar were picked up, then sub-cultured on 5% sheep blood agar (Himedia®, India) and were incubated to test the hemolytic pattern of the developed colonies.

Alpha, gamma, and beta-hemolytic colonies on blood agar were picked up to prepare bacterial microscopic films and were Gram stained to ensure the presence of either Gram-negative or Gram-positive bacterial cells, which were further described as cocci, bacilli, or coccobacilli. MacConkey cultures that showed Gram-negative short bacilli or coccobacilli were then tested for oxidase, urease, triple sugar iron (TSI), indole, methyl red, Vogus Proskauer, and citrate (IMViC) according to methods described by **Kao *et al*., 2016).** MSA cultures that showed Gram-positive cocci were then tested for catalase, coagulase, and sugar fermentation according to methods described by **Kao *et al*., 2016).** PCR was used as a molecular confirmation to complete the biochemical identification.

Antimicrobial susceptibility testing of the recovered isolates

The obtained isolates were subjected to antimicrobial susceptibility testing using Mueller-Hinton agar by the Kirby-Bauer disc diffusion method. All antibiotic discs used were supplied by the Himedia® company, India. The antibiotic discs used were amoxicillin (AX, 10µg), amoxicillin/clavulanic acid (AMC, 20/10µg); ceftriaxone (CTR, 30µg), ceftazidime (CAZ, 30µg), cefoperazone (CPZ, 75µg), enrofloxacin (EX, 5µg), gentamicin (GEN, 10µg), amikacin (AK, 30µg), linezolid (LZ, 30 µg. Diameters of the inhibitory zone obtained around the antibiotic discs were measured after incubation at 37°C for 24 hr and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2022).

Molecular identification of the obtained isolates and resistance genes detection

DNA extraction

DNA was extracted from all obtained isolates using a QIAamp DNA Mini Kit (Qiagen®, Germany) following the manufacturer instructions.

Molecular detection of the antibiotic resistance and virulence genes from the recovered isolates

According to the kit instructions, the QIA amp DNA mini kit was used for bacterial extraction. Emerald amp GT PCR master mix was used as it contains the precursors and enzymes necessary to run a PCR assay and the condition for each gene is illustrated in detail in **Table (1)**.

E. coli isolates were tested for *pho*A, *tsh*, *iss*, *bla*TEM, *bla*CTX-M, *aad*B, and *qnr*B. *K. pneumoniae* were tested for 16S, 23S, ITS, *mrk*A, *rmp*A, *bla*TEM, *bla*CTX-M, *aad*B and *qnr*B. *P. mirabilis* isolates were tested for *atp*D, *rsb*A, *zap*A, *bla*TEM, *aad*B, and *qnr*B. *P. aeruginosa* samples were tested for 16S rRNA, *tox*A, *pel*A, *bla*TEM, *bla*CTX-M, and *qnr*B. *S. aureus* samples were tested for 23S rRNA, *ica*D, *hla, bla*Z, and *nor*A. Oligonucleotide primers were used as they have specific sequences and amplify a specific product as shown in **Table (2)**.

Table (1): The thermal cycler condition used in the detection of each tested gene.

Target	Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
K. pneumoniae	16S, 23S, ITS	94˚C, 5 min	94˚C, 30 sec	55˚C, 30 sec	72˚C, 30 sec	35	72˚C, 7 min
	*mrk*A	94˚C, 5 min	94˚C, 30 sec	55˚C, 40 sec	72˚C 45 sec	35	72˚C, 10 min
	*rmp*A	94˚C, 5 min	94˚C, 30 sec	50˚C, 40 sec	72˚C 45 sec	35	72˚C, 10 min
E. coli	*pho*A	94˚C, 5 min	94˚C, 30 sec	55˚C, 40 sec	72˚C 45 sec	35	72˚C, 10 min
	Tsh	94˚C, 5 min	94˚C, 30 sec	54˚C, 40 sec	72˚C 45 sec	35	72˚C, 10 min
	iss	94˚C, 5 min	94˚C, 30 sec	54˚C, 30 sec	72˚C 30 sec	35	72˚C, 7 min
S. aureus	23S rRNA	94˚C, 5 min	94˚C, 30 sec	55˚C, 1 min	72˚C 1.2 min	35	72˚C, 12 min
	*bla*Z	94˚C, 5 min	94˚C, 30 sec	50˚C, 40 sec	72˚C 50 sec	35	72˚C, 10 min
	*nor*A	94˚C, 5 min	94˚C, 30 sec	50˚C, 40 sec	72˚C 45 sec	35	72˚C, 10 min
	*ica*D	94˚C, 5 min.	94˚C, 30 sec.	49˚C, 40 sec	72˚C, 40 sec	35	72˚C, 10 min
	hla	94˚C, 5 min	94˚C, 30 sec	53˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min
P. aeruginosa	16S rDNA	94˚C, 5 min	94˚C, 30 sec	52˚C, 40 sec	72˚C, 1 min	35	72˚C, 10 min
	*tox*A	94˚C, 5 min	94˚C, 30 sec	54˚C, 40 sec	72˚C, 40 sec	35	72˚C, 10 min
	*pel*A	94˚C, 5 min	94˚C, 30 sec	60˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min
P. mirabilis	*atp*D	94˚C, 5 min	94˚C, 30 sec	58˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min
	*rsb*A	94˚C, 5 min	94˚C, 30 sec	58˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min
	*zap*A	94˚C, 5 min	94˚C, 30 sec	59˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min
Gram negative bacteria	*bla*TEM	94˚C, 5 min	94˚C, 30 sec	54˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min
	*qnr*B	94˚C, 5 min	94˚C, 30 sec	55˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min
	*aad*B	94˚C, 5 min	94˚C, 30 sec	58˚C, 30 sec	72˚C, 30 sec	35	72˚C, 7 min
	*bla*CTX-M	94˚C, 5 min	94˚C, 30 sec	54˚C, 40 sec	72˚C, 45 sec	35	72˚C, 10 min

Table 2: The forward and reverse pair of primers used in the detection of each tested gene.

Gene	Sequence	Amplified product	Reference
K. pneumoniae			
16S, 23S, ITS	ATTTGAAGAGGTTGCAAACGAT	130 bp	Turton *et al*., 2010
	TTCACTCTGAAGTTTTCTTGTGTTC		
*mrk*A	CGGTAAAGTTACCGACGTATCTTGTACTG	475 bp	Alcántar-Curiel *et al.,* 2018
	GCTGTTAACCACACCGGTGGTAAC		
*rmp*A	ACTGGGCTACCTCTGCTTCA	535 bp	Yeh *et al.,* 2007
	CTTGCATGAGCCATCTTTCA		
E. coli			
*pho*A	CGATTCTGGAAATGGCAAAAG	720 bp	Hu *et al.,* 2011
	CGTGATCAGCGGTGACTATGAC		
tsh	GGTGGTGCACTGGAGTGG	620 bp	Delicato *et al*., 2003
	AGTCCAGCGTGATAGTGG		
iss	ATGTTATTTTCTGCCGCTCTG	266 bp	Yaguchi *et al*., 2007
	CTATTGTGAGCAATATACCC		
S. aureus			
23S rRNA	AC GGAGTTACAAAGGACGAC	1250 bp	Bhati et al., 2016
	AGCTCAGCCTTAACGAGTAC		
*bla*Z	TACAACTGTAATATCGGAGGG	833 bp	Bagcigil *et al.* 2012
	CATTACACTCTTGGCGGTTTC		
*nor*A	TTCACCAAGCCATCAAAAAG	620 bp	Pourmand *et al*., 2014
	CTTGCCTTTCTCCAGCAATA		
*ica*D	AAACGTAAGAGAGGTGG	381 bp	Ciftci *et al*. 2009
	GGCAATATGATCAAGATA		
hla	GAAGTCTGGTGAAAACCCTGA	704 bp	Fei *et al*., 2011
	TGAATCCTGTCGCTAATGCC		
P. aeruginosa			
16S rRNA	GGGGGATCTTCGGACCTCA	956 bp	Spilker *et al*., 2004
	TCCTTAGAGTGCCCACCCG		
*tox*A	GACAACGCCCTCAGCATCACCAGC	396 bp	Matar *et al*., 2002
	CGCTGGCCCATTCGCTCCAGCGCT		
*pel*A	CATACCTTCAGCCATCCGTTCTTC	786 bp	Ghadaksaz *et al*., 2015
	TCCCTACCTCAGCAGCAAGC		
P. mirabilis			
*atp*D	GTATCATGAACGTTCTGGGTAC	595 bp	Bi *et al*., 2013
	TGAAGTGATACGCTCTTGCAG		
*rsb*A	TTGAAGGACGCGATCAGACC	467 bp	Pathirana *et al*., 2018
	ACTCTGCTGTCCTGTGGGTA		
*zap*A	ACCGCAGGAAAACATATAGCCC	540 bp	Pathirana *et al*., 2018
	GCGACTATCTTCCGCATAATCA		
All Gram-negative isolates			
*bla*TEM	ATCAGCAATAAACCAGC	516 bp	Colom *et al*., 2003
	CCCCGAAGAACGTTTTC		
*qnr*B	GATCGTGAAAGCCAGAAAGG	469 bp	Robicsek *et al*., 2006
	ACGATGCCTGGTAGTTGTCC		
*aad*B	GAGCGAAATCTGCCGCTCTGG	319 bp	Frana *et al*., 2001
	CTGTTACAACGGACTGGCCGC		
*bla*CTX-M	ATGTGCAGYACCAGTAARGTKATGGC	593 bp	Archambault *et al.*, 2006
	TGGGTRAARTARGTSACCAGAAYCAGCGG		

RESULTS

In dogs, the total number of collected samples was 118 samples, 70 were males and 48 were females. Concerning the ear type 76/118 (64.4%) dogs had pendulous ears while 42/118 (35.6%) had erect or semi-erect ears. 79 were individually owned dogs, and 39 were shelter dogs. The number of samples that showed positive bacterial growth was (83%, *n*= 98/118) and the remaining (17%, *n*=20/118) were negative for bacterial growth. Throughout the positive samples, (84.7%, *n*=83/98) grew a single type of bacteria and (15.3%, *n*=15/98) grew two types of bacteria making the total number of bacterial isolates 113 isolates 67 from individually owned dogs and 46 from shelter dogs. In individually owned dogs, the number and percentage of bacterial isolates were (31%, *n*=21/67) *S. aureus*, (27%, *n*=18/67) *P. aeruginosa*, (19%, *n*=13/67) *K. pneumoniae*, (14%, *n*=9/67) *E. coli,* and (9%, *n*=6/67) *P. mirabilis*. While in shelter dogs (37%, *n*=17/46) *K. pneumoniae*, (26%, *n*=12/46) *E. coli*, (17%, *n*=8/46) *S. aureus*, (11%, *n*=5/46) *P. aeruginosa*, and (9%, *n*=4/46) *P. mirabilis* as shown in **Table 3**.

Table 3: The number and percentage of isolated bacteria from canine and feline with otitis.

Bacteria	Canine (*n*=113)				Feline (*n*=109)			
	Household *n* (%)	Shelter *n* (%)	Total no.	*P* value	Household *n* (%)	Shelter *n* (%)	Total no.	*P* value
S. aureus	21 (31)	8 (17)	29	0.09	21 (38) *	6 (11)	27	0.002
P. aeruginosa	18 (27) *	5 (11)	23	0.03	13 (23)	11 (21)	24	0.75
E. coli	9 (14)	12 (26)	21	0.08	8 (14)	9 (17)	17	0.69
K. pneumoniae	13 (19)	17 (37) *	30	0.03	11 (20)	22 (42) *	33	0.01
P. mirabilis	6 (9)	4 (9)	10	0.9	3 (5)	5 (9)	8	0.41
Total	67 (59.3)	46 (40.7)	113		56 (51.4)	53 (48.6)	109	

* indicates significance at P. value <0.005 in the same row.

Regarding cats' samples, the total number of collected samples was 94, 55 males and 39 females; 48 were household cats and 46 were shelter cats. The number of samples that showed positive bacterial growth was (91.5%, *n*=86/94), and the remaining (8.5%, *n*=8/94) were negative for bacterial growth. Throughout the positive samples, 72% (*n*=62/86) grew a single type of bacteria and 28% (*n*=24/86) grew two types of bacteria making the total number of bacterial isolates 109 isolates: 56 from household cats and 53 from shelter cats. In household cats, the number and percentage of bacterial isolates were (38%, *n*=21/56) *S. aureus*, (23%, *n*=13/56) *P. aeruginosa*, (20%, *n*=11/56) *K. pneumoniae*, (14%, *n*=8/56) *E. coli,* and (5%, *n*=3/56) *P. mirabilis*. While in shelter, cats (42%, *n*=22/53) had *K. pneumoniae*, (17%, *n*=9/53) *E. coli*, (11%, *n*=6/53) *S. aureus*, (21%, *n*=11/53) *P. aeruginosa*, and (9%, *n*=5/53) *P. mirabilis,* as shown in **Table 3**.

Both correlation between the cats and dogs ages and the most common bacterial isolates is illustrated and summarized in **Table 4**.

Table 4: The prevalences of each recovered bacterial agent in correlation with age of dogs and cats

Age	Number		Most common bacteria
	Canine	Feline	
0 to 1 year	11	27	*S. aureus*
1 to 5 years	60	54	*E. coli* and *K. pneumoniae*
>5 years	47	13	*P. aeruginosa* and *P. mirabilis*

Regarding the isolation-breed correlation in dog isolates were as follows: Retrievers 32, Cocker spaniel 19, mongrel 16, German shepherd 15, Beagle 11, Griffon 10, Rott willer 5, French bulldog 4, Pug 3, York shier 1, Shih tzu 1, and poodle 1 as shown in **Table 5**. Regarding the isolation-breed correlation in cat isolates were as follows: Persian 20, Himalaya 23, Siamese 5, British short hair 2, Mixed 26, Mongrel 18 as shown in **Table 5**.

Table 5: Correlation of the otitis incidence with the age and breeds of cats and dogs.

Dogs			
Breed	0-1 year	1-5 years	>5 years
Retrievers	0 (0%)	15 (12.7%)	17 (14.4%)
Cocker	0 (0%)	13 (11%)	6 (5.1%)
Mongrel	3 (2.5%)	12 (10.2%)	1 (0.8%)
German	2 (1.7%)	4 (3.4%)	9 (7.6%)
Beagle	2 (1.7%)	4 (3.4%)	5 (4.2%)
Griffon	3 (2.5%)	3 (2.5%)	4 (3.4%)
Rott willer	0 (0%)	4 (3.4%)	1 (0.9%)
French bulldog	0 (0%)	2 (1.7%)	2 (1.7%)
Pug	0 (0%)	2 (1.7%)	1 (0.8%)
Poodle	1 (0.8%)	0 (0%)	0 (0%)
Shih tzu	0 (0%)	0 (0%)	1 (0.8%)
York shire	0 (0%)	1 (0.8%)	0 (0%)
Cats			
Breed	0-1 year	1-5 years	>5 years
Persian	6 (6.4%)	11 (11.7%)	3 (3.2%)
Himalaya	7 (7.4%)	14 (14.9%)	2 (2.1%)
Siamese	0 (0%)	3 (3.2%)	2 (2.1%)
British short hair	0 (0%)	2 (2.1%)	0 (0%)
Mixed	5 (5.3%)	17 (18.1%)	4 (4.3%)
Mongrel	9 (9.6%)	7 (7.45%)	2 (2.1%)

The antibiotic sensitivity results in dogs are detailed in **Table 6**. Coagulase-positive *Staphylococcus* strains were sensitive to most of the antibiotics used in this study; the highest sensitivity was towards ceftriaxone and amikacin at 100%, and the highest resistance was against linezolid at 86%. In Gram-negative Enterobacteriaceae, firstly, *E. coli* strains were susceptible to amikacin (91%, *n*=19/21), gentamicin (86%, *n*=18/21), and enrofloxacin (76%, *n*=16/21), while showing a high resistance (62%, *n*=13/21) regarding amoxicillin clavulanic acid, ceftriaxone, and cefoperazone. *K. pneumoniae* isolates were 100% resistant to amoxicillin and amoxicillin-clavulanic acid and less resistant to amikacin (10%, *n*=3/30) and gentamicin (17%, *n*=5/30). *P. aeruginosa* isolates showed 0% resistance against ceftazidime and cefoperazone and 74% (*n*=17/23) against amoxicillin. *P. mirabilis* isolates were 50% (*n*=5/10) and 80% (*n*=8/10) resistant to amoxicillin and lincomycin, respectively and 0% were sensitive to tylosin and linezolid. All *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates were 100% resistant to lincomycin, tylosin, and linezolid antibiotics. The percentage of antibiotic resistance in dogs is shown in **Fig.1**.

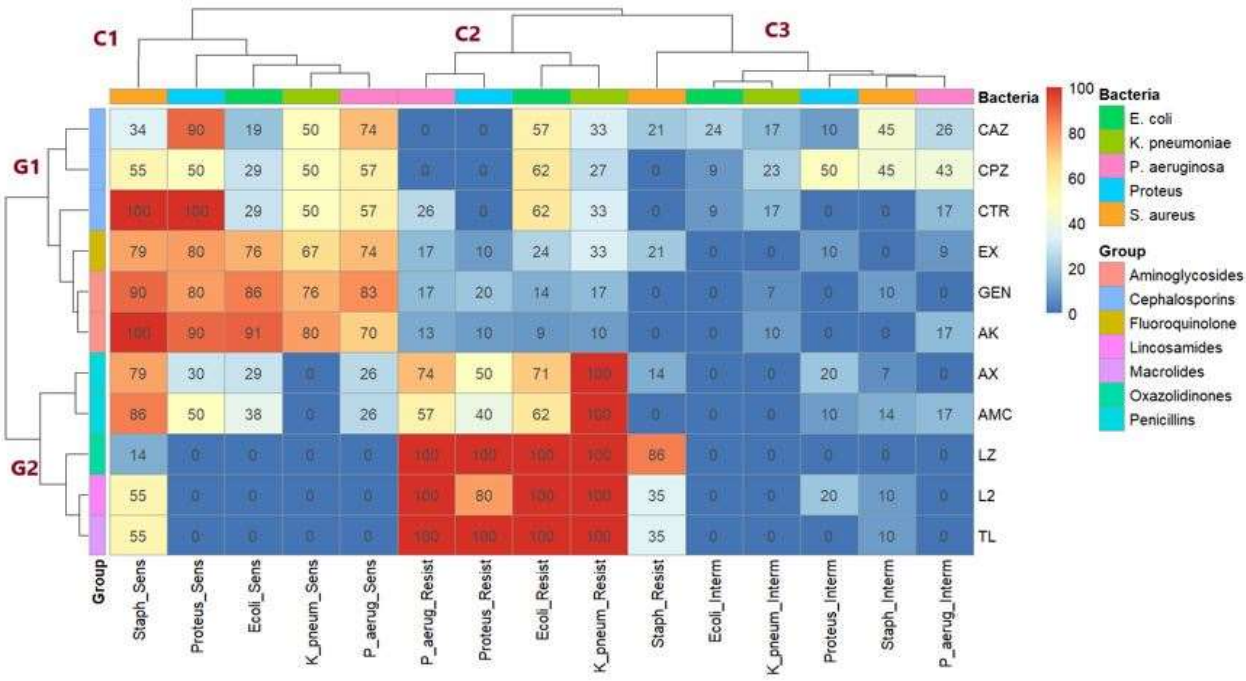

Fig.1: Heatmap illustrating the antibiotic susceptibility profiles of the dog isolates

Table 6: Collective antibiotic susceptibility profile of the obtained isolates from dog cases.

Drug	*S. aureus* (*n*=29)			*E. coli* (*n*=21)			*K. pneumoniae* (*n*=30)			*P. aeruginosa* (*n*=23)			*P. mirabilis* (*n*=10)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
	n (%)														
AX	23 (79)	2 (7)	4 (14)	6 (29)	0 (0)	15 (71)	0 (0)	0 (0)	30 (100)	6 (26)	0 (0)	17 (74)	3 (30)	2 (20)	5 (50)
AMC	25 (86)	4 (14)	0 (0)	8 (38)	0 (0)	13 (62)	0 (0)	0 (0)	30 (100)	6 (26)	4 (17)	13 (57)	5 (50)	1 (10)	4 (40)
CTR	29 (100)	0 (0)	0 (0)	6 (29)	2 (9)	13 (62)	15 (50)	5 (17)	10 (33)	13 (57)	4 (17)	6 (26)	10 (100)	0 (0)	0 (0)
CAZ	10 (34)	13 (45)	6 (21)	4 (19)	5 (24)	12 (57)	15 (50)	5 (17)	10 (33)	17 (74)	6 (26)	0 (0)	9 (90)	1 (10)	0 (0)
CPZ	16 (55)	13 (45)	0 (0)	6 (29)	2 (9)	13 (62)	15 (50)	7 (23)	8 (27)	13 (57)	10 (43)	0 (0)	5 (50)	5 (50)	0 (0)
EX	23 (79)	0 (0)	6 (21)	16 (76)	0 (0)	5 (24)	20 (67)	0 (0)	10 (33)	17 (74)	2 (9)	4 (17)	8 (80)	1 (10)	1 (10)
GEN	26 (90)	3 (10)	0 (0)	18 (86)	0 (0)	3 (14)	23 (76)	2 (7)	5 (17)	19 (83)	0 (0)	4 (17)	8 (80)	0 (0)	2 (20)
AK	29 (100)	0 (0)	0 (0)	19 (91)	0 (0)	2 (9)	24 (80)	3 (10)	3 (10)	16 (70)	4 (17)	3 (13)	9 (90)	0 (0)	1 (10)
LCM	16 (55)	3 (10)	10 (35)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	30 (100)	0 (0)	0 (0)	23 (100)	0 (0)	2 (20)	8 (80)
TL	16 (55)	3 (10)	10 (35)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	30 (100)	0 (0)	0 (0)	23 (100)	0 (0)	0 (0)	10 (100)
LZ	4 (14)	0 (0)	25 (86)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	30 (100)	0 (0)	0 (0)	23 (100)	0 (0)	0 (0)	10 (100)

Antibiotic sensitivity results of cats are detailed in **Table 7**. As in dogs, most coagulase-positive *Staphylococcus* strains isolated from cats were also sensitive to most antibiotics used in the study; according to amikacin and gentamicin, all isolates were 100% sensitive; in linezolid, the resistance was 56% (*n*=15/27). Gram-negative *E. coli* isolates were resistant to amoxicillin (88%, *n*=15/17), amoxicillin-clavulanic acid (71%, *n*=12/17), and enrofloxacin (59%, *n*=10/17) while highly sensitive to gentamicin and amikacin (83%, *n*=14/17). *K. pneumoniae* isolates isolated from cats are highly resistant to amoxicillin (91%, *n*=30/33), amoxicillin-clavulanic acid (88%, *n*=29/33), and cefoperazone (54%, *n*=18/33). On the other hand, isolates were highly sensitive to amikacin and gentamicin (91%, *n*=30/33) and (88%, *n*=29/33), respectively. In *P. aeruginosa,* 100% of isolates were resistant to lincomycin and linezolid; furthermore*, 92%* (*n*=22/24) *of* isolates were resistant to amoxicillin, amoxicillin-clavulanic acid (79%, *n*=19/24), tylosin (71%, n=17/24), enrofloxacin (63%, *n*=15/24), and ceftriaxone (50%, *n*=12/24). The highest sensitivity was to amikacin and gentamicin (88%, *n*=21/24) and (84%, *n*=20/24), respectively. *Proteus* isolates were sensitive to most antibiotics, especially ceftazidime; all isolates were 100% sensitive. *E. coli*, *K. pneumoniae*, and *P. mirabilis* isolates were all found to be 0% sensitive to lincomycin, tylosin, and linezolid. The percentage of resistance to antibiotics is shown in **Fig.2**.

Table 7: Collective antibiotic susceptibility profile of the obtained isolates from cat cases

Drug	*S. aureus* (*n*=27)			*E. coli* (*n*=17)			*K. pneumoniae* (*n*=33)			*P. aeruginosa* (*n*=24)			*P. mirabilis* (*n*=8)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
	n (%)														
AX	19 (70)	3 (11)	5 (19)	2 (12)	0 (0)	15 (88)	2 (6)	1 (3)	30 (91)	2 (8)	0 (0)	22 (92)	6 (75)	0 (0)	2 (25)
AMC	21 (78)	4 (14)	2 (7)	5 (29)	0 (0)	12 (71)	2 (6)	2 (6)	29 (88)	5 (21)	0 (0)	19 (79)	7 (87)	0 (0)	1 (13)
CTR	24 (89)	3 (11)	0 (0)	12 (71)	2 (12)	3 (17)	9 (27)	11 (33)	13 (40)	5 (21)	7 (29)	12 (50)	7 (87)	1 (13)	0 (0)
CAZ	15 (56)	8 (29)	4 (15)	10 (59)	2 (12)	5 (29)	15 (46)	2 (6)	16 (48)	20 (84)	2 (8)	2 (8)	8 (100)	0 (0)	0 (0)
CPZ	11 (44)	16 (59)	0 (0)	5 (29)	4 (24)	8 (47)	13 (40)	2 (6)	18 (54)	12 (50)	8 (33)	4 (17)	6 (75)	2 (25)	0 (0)
EX	24 (89)	0 (0)	3 (11)	7 (41)	0 (0)	10 (59)	15 (46)	7 (21)	11 (33)	9 (37)	0 (0)	15 (63)	6 (75)	0 (0)	2 (25)
GEN	27 (100)	0 (0)	0 (0)	14 (83)	0 (0)	3 (17)	29 (88)	1 (3)	3 (9)	20 (84)	3 (12)	1 (4)	7 (87)	0 (0)	1 (13)
AK	27 (100)	0 (0)	0 (0)	14 (83)	0 (0)	3 (17)	30 (91)	1 (3)	2 (6)	21 (88)	1 (4)	2 (8)	7 (87)	1 (13)	0 (0)
LCM	21 (78)	0 (0)	6 (22)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	33 (100)	0 (0)	0 (0)	24 (100)	0 (0)	1 (13)	7 (87)
TL	24 (89)	3 (11)	0 (0)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	33 (100)	2 (8)	5 (21)	17 (71)	0 (0)	0 (0)	8 (100)
LZ	4 (15)	8 (29)	15 (56)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	33 (100)	0 (0)	0 (0)	24 (100)	0 (0)	0 (0)	8 (100)

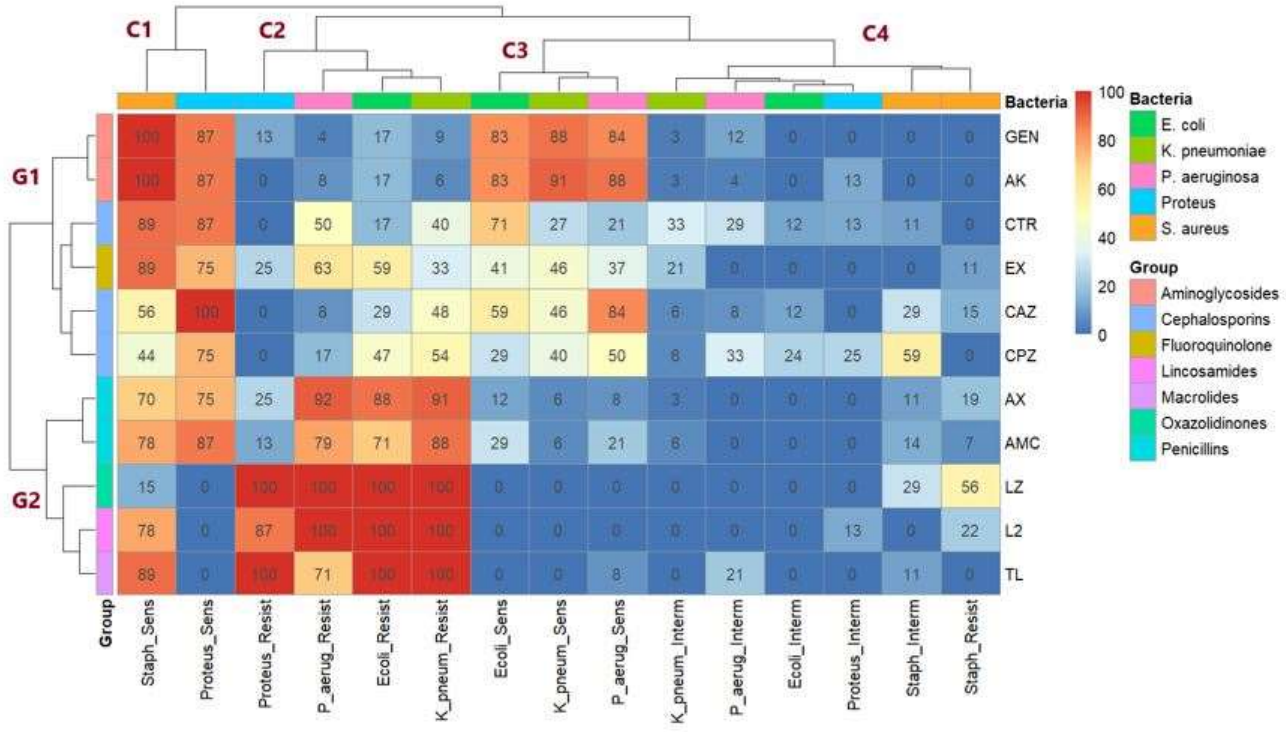

Fig. 2: Heatmap illustrating the antibiotic susceptibility profiles of the cat isolates.

PCR screening of isolated bacteria revealed that *E. coli* and *K. pneumoniae* harbored ESBL genes like *bla*TEM, *bla*CTX-M, and other resistance genes *aad*B and *qnr*B. Likewise, the other Gram-negative isolates of *P. aeruginosa* and *P. mirabilis* were also positive for the same resistance genes. Gram-positive *S. aureus* harbored *bla*Z and *nor*A resistance genes. Detection, virulence, and resistance genes of screened bacterial isolates are detailed in **Fig 3, 4 and 5** and **Table 8**.

Table 8: Collective tabulation of the detection, virulence, and resistance genes of screened bacterial isolates.

Bacterial strains	Detection genes	Virulence genes	Resistance genes
E. coli	-	*pho*A, *tsh,* and *iss*	*bla*TEM, *bla*CTX-M, *aad*B, and *qnr*B
K. pneumoniae	16S, 23S, ITS	*mrk*A and *rmp*A	*bla*TEM, *bla*CTX-M, *aad*B, and *qnr*B
P. aeruginosa	16S rDNA	*tox*A and *pel*A	*bla*TEM, *bla*CTX-M, and *qnr*B
P. mirabilis	-	*atp*D, *rsb*A, and *zap*A	*bla*TEM, *aad*B, and *qnr*B
S. aureus	23S rRNA	*ica*D and *hla*	*bla*Z and *nor*A

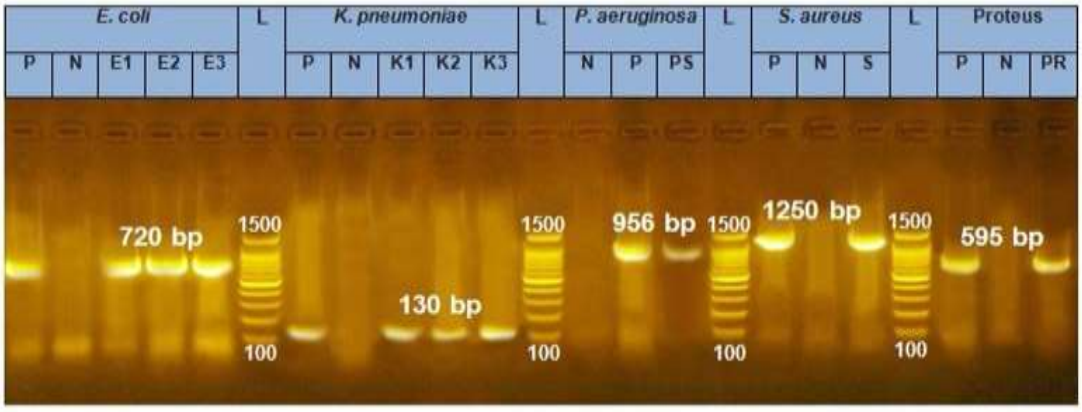

Fig. 3: Gel electrophoresis pictures; showing the detection of the species-specific identifying gene of different obtained isolates. 720bp gene for *E. coli*, 130bp gene for *K. pneumoniae*, 956bp gene for *P. aeruginosa*, 1250bp gene for *S. aureus*, and 595bp gene for *P. mirabilis* L; ladder, P; positive control, N; negative control, K1-3; *K. pneumoniae* isolates, E1-3; *E. coli* isolates, PR; *P. mirabilis* isolate, and PS; *P. aeruginosa* isolate.

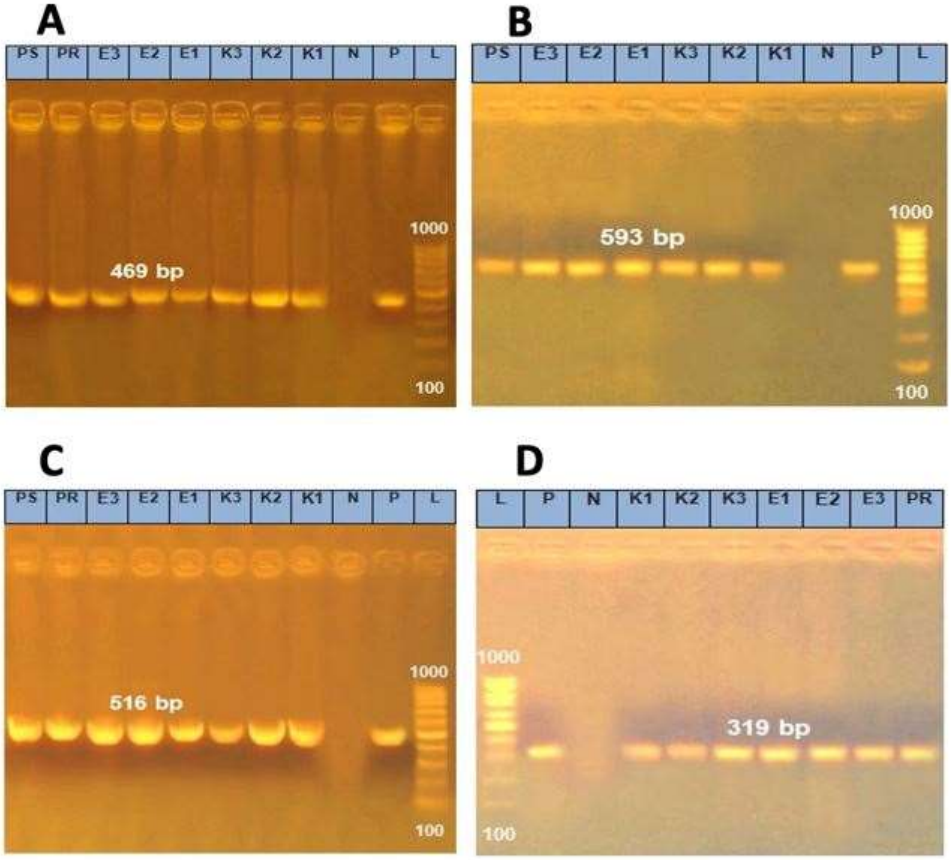

Fig. 4: Gel electrophoresis pictures; A) showing the detection of the 469bp *qnr*B gene in different obtained isolates, B) showing the detection of the 593bp *bla*CTX gene in different obtained isolates, C) showing the detection of the 516bp *bla*TEM gene in different obtained isolates, and D) showing the detection of the 319bp *aad*B gene in different obtained isolates. L; ladder, P; positive control, N; negative control, K1-3; *K. pneumoniae* isolates, E1-3; *E. coli* isolates, PR; *P. mirabilis* isolate, and PS; *P. aeruginosa* isolate.

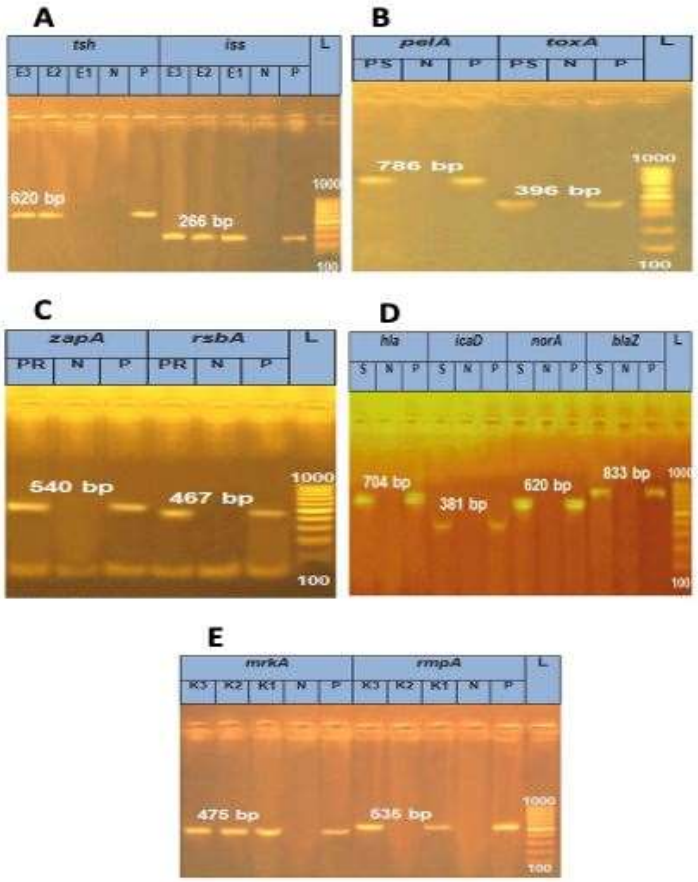

Fig.5: Gel electrophoresis pictures; **A)** showing the detection of the 620bp *tsh* gene and 266bp *iss* gene in obtained *E. coli* isolates, B) showing the detection of the 786bp *pel*A gene and 396bp *tox*A gene in obtained *P. aeruginosa* isolates, **C**) showing the detection of the 540bp *zap*A gene and 467bp *rsb*A in obtained *P. mirabilis* isolates, and **D)** showing the detection of the 704bp *hla* gene, 381bp *ica*D gene, 620bp *nor*A gene, and 833bp *bla*Z gene in obtained *S. aureus* isolates. **E**) showing the detection of the 475bp *mrk*A gene and 535bp *rmp*A in obtained *K. pneumoniae* isolates. L; ladder, P; positive control, N; negative control, S; *S.aureus* isolates, E1-3; *E. coli* isolates, PR; *P. mirabilis* isolate, PS; *P. aeruginosa* isolates, K1-3; *K. pneumoniae* isolates.

DISCUSSION

Dogs and cats are considered on the top of the list of the most worldwide owned pet animals. Increasing the human-pet animals' companionship is associated with increased worldwide risk of emerging zoonosis. With the increase of microbial antibiotic resistance mechanisms, the associating zoonotic risk became a serious hidden crisis which has been indicated and highlighted in several studies in Egypt **(Hassanien *et al.,* 2021; Moussa *et al.,* 2021; Fathi *et al.,* 2022; Ataya *et al.,* 2023; Aboul-Ella *et al.*, 2025);** Africa **(Maruve and Essack 2022),** and in different parts of the world **(Smith *et al*., 2018).**

The current study provides information on the occurrence of bacteria associated with otitis in dogs and cats and highlights alarming rates of multidrug-resistant bacteria. Otitis is commonly diagnosed in clinics using routine clinical examination, leading to empiric treatment based on the examination results. Therefore, the current work compared the overall susceptibility results between Gram-positive and Gram-negative bacterial groups, and our analysis showed that susceptibilities for amoxicillin, amoxicillin/clavulanic acid, cephalexin, erythromycin, and rifampicin were considerably different between the two groups of bacteria as shown in Table (3). These results reinforce the importance of correct identification and antimicrobial susceptibility testing, mostly in challenging and multidrug-resistant cases.

Family pets function as a swimming pool for various species of bacteria and resistance genes of clinical importance for human beings. The close contact between humans and companion animals through licking, petting, and physical injury provides favorable conditions for transmitting antimicrobial-resistant bacteria. It has been reported that adults and their puppies frequently share their skin microflora highlighting the importance of the contact role (Bhat, 2021). Antimicrobial resistance (AMR) is regarded by the World Health Organization (WHO) as one of humanity's top ten threats to global public health. It is

predicted that by 2050, the number of deaths caused by antibiotic resistance could increase from the present estimate of 700,000 deaths per year to ten million annually **(Brogan and Mossialos 2016).** The misuse and overuse of antibiotics is the primary cause of the AMR threat **(Wintersdorff *et al*., 2016).** Another concerning research declared that plenty of pet owners attempted the treatment of their pets with antibiotics before going to veterinarians **(Chipangura *et al*., 2017).** According to a study in 2023 in Italy, 21% (23/111) of pet animal owners admitted to giving their pets oral antibiotics without consulting a veterinarian through using antibiotic leftovers, buying them from pharmacies without a prescription, or using an old one. Among the studied, 17.1% (19/111) declared that they had stopped giving the antibiotic before completing the recommended course of treatment due to several reasons like the existence of side effects, disappearance of symptoms, finishing the pack before ending the treatment, and other unexplained reasons **(Candellone *et al*., 2023).**

A previous study performed in Egypt in 2023 discussing the zoonotic transmission of bacterial resistance originating from pet animals' urinary tract infections **(Ataya *et al.,* 2023).** The overall bacterial incidence in feline UTIs was 64% (82/128). Also, the bacterial incidences of UTIs were nearly equal in both the cat and dog 'groups, despite being a little bit higher in dogs than in cats. When comparing animal species, the incidence in cats was 63.7% (72/113) and in dogs was 66.6% (10/15). Also, the results indicate higher incidences in both animal species. Concerning the incidence of bacterial isolates from UTIs in different animal species; the common bacterial isolates and their incidence in dogs were *E. coli* and *Staphylococcus spp.* (26% and 40%, respectively).

A one-year study carried out in the United Kingdom in 2016 found that the prevalence of otitis in specific canine breeds characterized by pendulous or droopy ear pinnae has a great incidence of otitis; it was found to be 1.76 and 1.84 times higher in comparison with other dogs having erect ears **(O'Neill *et al*., 2021).** According to the findings of the current study, dogs with droopy, pendulous ears are 1.88 times more likely to develop otitis than dogs with erect ears, indicating that dogs with droopy ears are almost twice as likely to develop ear inflammation as their counterparts with prick ears. This information can be useful for veterinarians to identify and prevent ear infections in dogs with droopy ears. There is no doubt that shelters have an important vital role in society as they house millions of animals every year and support the ownership of pets. Despite that, shelters are considered a main illustration of biological instability. It is common for shelters to have a diverse population of animals; these animals often have not received adequate health care in the past, and some have had to scavenge or hunt to survive. Poor shelter conditions and overcrowding can lead to stress within individuals, species, and between varied species, which increases the likelihood of exposure to many pathogens. All these factors can contribute to the arising of new pathogens or changes in the virulence of existing ones **(Pesavento and Murphy, 2014).**

A recent study conducted in South Korea has indicated that *Klebsiella* infection in companion animals can manifest on different sites. The study found that the highest distribution of *Klebsiella* was found in the feces of diarrheic animals, at 25.6%, followed by ear canal infections at 23.3%, as well as in urine, nasal cavity, skin, and genitalia. The study also observed that 65.1% (28/43) of companion animals were having *K. pneumoniae* infection. Out of 28 *K. pneumoniae* strains (*n*=10), 35.7% carry various types of CTX-M genes. This suggests that among clinical isolates of *K. pneumoniae,* there is a particularly high probability of ESBL carriage **(Lee *et al*., 2021).** In the present study, all tested *K. pneumoniae* samples were positive for the CTX-M resistance gene. A study was done for the screening of *K. pneumoniae* and its virulence and resistance genes from companion animals and humans suffering from urinary tract infections. The majority of *K. pneumoniae* strains, 80% from companion animals and 30% from humans were MDR and showed resistance to more than five different antibiotic classes. Among the screened *qnr* genes, *qnr*B was the most prevalent, and it was considerably more common in *K. pneumoniae* that was not susceptible to fluoroquinolones. For the virulence factors, *K. pneumoniae* samples were all positive for *ent*B, *mrk*D, and *fim*H-1. Additionally, *kpn*, *kfu*, and *ycf*M were also frequently occurring. This accordingly emphasizes the role of pet animals as major resistance determinant reservoirs, which is a significant issue in veterinary medicine regarding *K. pneumoniae*, as previous research has demonstrated its susceptibility to nosocomial transmission **(Marques *et al*., 2019).**

ESBL provides resistance against third and fourth-generation cephalosporins, which are used for the treatment of infections caused by Enterobacteriaceae such as *E. coli* and *K. pneumoniae*. The main genes of ESBL isolated from animals and humans were the groups CTX-M, SHV, and TEM **(Bush and Jacoby 2010).** Here in the present study, all tested samples of *E. coli, K. pneumoniae*, and *P. aeruginosa* were found to be positive for CTX-M and TEM resistance genes. Additionally, the *P. mirabilis* isolate was positive for the TEM gene. It has been documented that *K. pneumoniae* colonizes pet animals, causing infections of the urinary and respiratory tracts, pyometra, as well as bloodstream infections (Martin, *et*

al., 2016). In a study in China to investigate the MDR *K. pneumoniae* from clinically diseased dogs and cats, it was found that 82.9 % were resistant to amoxicillin-clavulanic acid, *bla*CTX-M gene was the second most common ESBL gene in dogs (22.9%) and cats (28.6%). Among the quinolone resistance genes, *qnr*B and *qnr*S were 31.4% *aac(6')-Ib-cr* was 45.7%, and *oqx*AB was 100% **(Zhang *et al*., 2023).**

E. coli is frequently subjected to antibiotic pressure as it is the most prevalent bacteria in the intestinal microflora. The researchers discovered that 86.64% of *E. coli* were resistant to antibiotics and displayed a wide range of antibiotic resistance **(Caneschi *et al*., 2023),** greater than findings from another research **(Hata *et al*., 2022; Sevilla-Navarro *et al*., 2022).** Reliance on β-lactam antibiotics as an affordable and easily accessible treatment for pet infections may be the cause of the high Gram-negative bacterial resistance against them. However, decreased levels of resistance to other medications, particularly aminoglycosides, may be due to their infrequent use because of their expensiveness or adverse effects **(Khalifa *et al*., 2021).** Male dogs may have a higher susceptibility because androgen hormones favor the increase in sebum production, which may be a risk factor for latent infection flare-ups, while female estrogens produce the opposite effect **(Kumar *et al*., 2014).** According to **Topala *et al*., (2007) and Barua *et al*., (2021),** the difference between male and female predisposition to otitis was not significant in dogs. On the other hand, **Fernandez *et al.*, (2006)** noted that otitis occurs more frequently in female dogs than in male dogs. Also in cats, the studied cases revealed that there were no significant differences between queens and tom cats **(Lefkaditis *et al*., 2009; Waly and Sayed 2013).**

Despite the great value of the data obtained from the current work, it still needs further prospective investigations. First, the number of samples from dogs and cats was not homogeneous since we relied on the availability of samples from private clinics, shelters, and university veterinary hospitals, which were mostly from dogs (118/212) compared to cats (94/212). An important consideration is that diagnostic sample populations still reflect a limited group of animals; hence, they may not necessarily answer questions of broader relevance. Second, as this is laboratory-based surveillance, we could not truly differentiate colonization versus clinical infection; however, all samples were collected from animals presenting characteristic clinical signs of otitis. Moreover, only heavy growth of a single bacterial species in mixed cultures or light-heavy growth in pure culture was considered potentially associated with otitis. We could not determine the minimum inhibitory concentration of antimicrobials, given that the data collected is part of the diagnostic work-up, and disk-diffusion testing is the suitable way to report the antimicrobial susceptibility profile of the obtained isolates. However, these data still provide priceless value in monitoring multidrug-resistant bacteria associated with otitis in small animals.

CONCLUSION

The present work's conclusion sought to emphasize the need for more focus on companion animals' roles in AMR epidemiology, particularly given that the antimicrobials utilized are also used in human medicine. Facilities like veterinary hospitals should be encouraged to create internal microbiological monitoring strategies to gather and exchange data, given the lack of collective control programs for companion animals in the European Union (EU). Customized monitoring programs can help identify certain AMR frameworks, aid clinicians in making logical treatment choices, and restrict the selection and spread of AMR within the population. Surveillance may also focus only on specific specimens or also bacterial species such as the *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species (ESKAPE) group, which are extremely important from one health perspective, to prevent and address potential emerging health threats at the human–animal–environment interface.

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Conflict of interest

The authors declare that they have no conflict of interest

Authors Contributions

The study was performed by Mayson Hamdy, designed and supervised by Sherif Marouf, Soliman Mohamed, and Haitham Farghali, while Hassan Aboul-Ella and Mayson Hamdy contributed to the implementation of the work, analysis of the results, and writing the manuscript.

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