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Isolation and molecular detection of *Staphylococcus aureus* from feline otitis in Baghdad

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Abstract

This study was conducted to isolate and identify *Staphylococcus aureus* from cats with otitis externa in Baghdad province. The ear swabs were collected from cats (n: 120), processed using bacteriological isolation, and confirmed by conventional PCR for the (23S rRNA) gene. The isolation was done by culturing on blood agar, mannitol salt agar, and Hi Chrom agar. Further diagnosis and confirmation for the suspected colony was done by Gram stain and biochemical tests. Of the 120 ear swabs, 30 (25%) were positive for *S. aureus*. The isolates of *S. aureus* showed a high rate of resistance to Ampicillin 100 and 80% for Erythromycin, Penicillin, and Methicillin. In comparison, most isolates were sensitive to Vancomycin 66.6%, Ciprofloxacin, and Oxacillin 60%. The molecular assay revealed that all the *S. aureus* isolates were positive for specific primers (23S rRNA) gene, giving a particular band at 350 bp. The infected cats showed infectious form; a high infection rate was recorded with the unilateral form 33.3%, while the most frequent form was the acute form 40% of feline otitis externa. The infection rate increased in females 26.3%, and one-year cats showed the highest isolation percentage of *S. aureus* 25.9%. Stray cats recorded a significantly higher infection rate 34.3% with feline otitis externa. While there were substantial differences in isolation rates among the months of study, the highest percentage was reported in January 80% and December 70%. *S. aureus* causes feline otitis, particularly in the cold months.

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Introduction

The term otitis externa is used when the outside canal's inflammation is affected (1). Feline otitis is clinically considered a complex, challenging disease, unilateral or bilateral, and acute or chronic. Typical signs of otitis externa include head shaking, aural itching, pain, and discomfort during ear palpation (2). The primary causes of the disease are usually unnoticed clinically; one of the most important factors is the change in the ear environment. Bacteria, fungus, over-cleaning, and yeast overgrowth are the leading causes of secondary otitis externa. The predisposing factors include excessive local moisture, trauma, and ear canal obstruction (2,3). Many bacterial species play an essential role in otic diseases, and most of them are classified as opportunistic infections; the most bacterial isolated from cases of otitis externa are *Staphylococcus spp., Pseudomonas spp., Escherichia spp., Proteus spp.*, and *Enterococcus spp.* (4-7). *S. aureus* is a gram-positive bacterium that constitutes part of the commensal microbiota in humans and animals' skin, nose, and respiratory tracts. *S. aureus* is highly pathogenic, causing various diseases, including skin infections, osteomyelitis, septic arthritis, septicemia, and food poisoning. It is mainly caused by *S. aureus* thermo-stable enterotoxins that can resist the pasteurization temperature (8). The production of golden pigment characterizes it, and the ability of *S. aureus* to cause disease is associated with a large number of virulence factors,

including *S. aureus* enterotoxins (SEs), Panton-Valentine leucocidin (PVL), hemolysis (Hlα and HIβ), most of which are involved in the adherence, colonization, and tissue invasion (9). These bacteria usually resist most routinely used antimicrobial medications, including beta-lactams, aminoglycosides, and macrolides (10-12). *Staphylococcus species* were the most commonly isolated bacterial organisms from different animals with otitis externa (4,13). The resistance of the antibiotics of these bacteria can result in therapeutic failure, increased medical expenses, and mortality (14). *S. aureus* is becoming a significant threat to public health due to the close physical contact (licking, stroking, and petting) between companion animals and their owners, which can result in infection with these pathogenic bacteria (15). Pets have been shown to serve as reservoirs for bacteria resistant to antimicrobials, and there have been reports of human-to-animal transfer. Symptoms of a *Staphylococcus* contagion of the skin usually include pus, crusting, erythema, and sensitivity in the skin near the irritant or wound (16). Recently, many PCR-based molecular methods have been developed as an alternative way to accurately identify bacterial infection (17-20). Careful design and protocol are necessary for a PCR assay to detect, quantify, and identify microorganisms in clinical specimens (21,22). Analysis of PCR results is also a critical issue in diagnosis, particularly for chronic inflammatory diseases (23-25). Recent data on virulence gene profiles and information on antibiotic resistance determinants are essential to develop effective disease-relieving methods. The 23 S rRNA gene has proven helpful in identifying *S. aureus* pathogens (26-28). This study was designed to identify and isolate *S. aureus* from feline otitis externa.

Materials and methods

Ethical approve

This study was approved by the ethical and research committee of collage of Veterinary Medicine, University of Baghdad, Ministry of High Education and Scientific Research.

Bacteriological examination

One hundred and twenty cats of both genders and different ages were used to collect ear samples. All animals were housed in Veterinary hospitals and pet clinics in Baghdad province from August 2021 to July 2022. All culturing procedures, Gram stain, and biochemical tests (9). The ear swabs were streaked on blood agar; the suspected *Staphylococcus* colonies were subculture on selective media mannitol salt agar and Hi-chrome agar and incubated aerobically at 37 C° for 24 hours. The growing colonies were visually inspected for size, shape, and color. The suspected colonies were examined by Gram stain and biochemical tests (catalase, oxidase, DNAse, coagulase, and gelatin liquefaction).

Antimicrobial susceptibility test

Antimicrobial susceptibility test of *S. aureus* isolates was performed using Mueller-Hinton agar as described by Kirby-Bauer's disk diffusion method (9). The inhibition zone diameter for each antibiotic disc was measured by using Vernier and then compared the result with the standard diameter of the antibiotic inhibition zone as mentioned in Clinical and Laboratory Standards Institute (29).

Extraction of DNA and PCR assay

The PCR assay was done on the 30 isolates of *S. aureus* by the following methods. First, bacteria were exposed to DNA extraction using resto™ Mini g DNA Bacteria Kit (Geneaid. USA). Second, the purity and concentration of extracted DNA were measured using Nanodrop (ActGene, USA). One primer set was used in this study, the 23S rRNA primer, which has (F-5'TCGGAATCTGGGAGGACCAT-3') and (R-5'AACGTAAGTCGGTTCGGTCC-3') sequence this primer gives an amplicon size reach of 350pb (26). PCR master mix reaction was prepared by using GoTaq® Green Master Mix from Promega, USA. The PCR tubes containing an amplification mixture were transferred to a thermal cycler and started the program for amplification (Table 1). The final PCR-based products were evaluated with 1% gel electrophoresis, ethidium bromide, and UV imager examination (Cleaver Scientific, UK).

Table 1: PCR program for detection of 23S rRNA gene

Step	°C	Time	Cycles
Initial denaturation	94	7min	1
Denaturation	94	45sce	
Annealing	58	45sce	35
Extension	72	45sce	
Final extension	72	7min	1
Hold	4	24	-

Statistical analysis

The SAS program was used to determine the effect of different factors in this study. The Chi-square test was used to assess the significance of the comparison between percentages.

Results

Clinical study of cats

The infected cats with otitis externa showed multi-infection form; a higher infection rate was recorded in the unilateral form 33.3% than in the bilateral form, while the most frequent form was the acute form 40% than the chronic form of feline otitis externa (Table 2).

Table 2: The infection rate of cats with different infection forms

Clinical sign	No. of infected cats	%	X^{2*}	P*	OR*	95%CI*
Chronic form	26/120	21.6%	9.45	<0.01	2.41	1.36-4.25
Acute from	48/120	40%				
Unilateral form	40/120	33.3%	3.79	0.05	0.56	0.32-1.00
Bilateral form	27/120	22.5%				

* X^2 = Chi- square value, *P = Probability, *OR = Odds ratio, *95%CI = 95% confidence interval.

Bacteriological examination

Out of 120 ear swabs, 30 (25%) were positive for *S. aureus*; the *S. aureus* colonies appeared on blood agar as round and opaque colonies with β-hemolysis, while on the selective Mannitol salt agar the colonies were mucoid, rounded, smooth and change the color of media to yellow color and on Hi-chrome agar, the *S. aureus* isolates appeared as green colonies (Figure 1). The Gram stain of suspected *S. aureus* colonies revealed a positive, cocci, grape-like cluster. The isolates were positive for catalase, gelatinase, DNase, and coagulase tests.

Antimicrobial susceptibility testing

The isolates of *S. aureus* showed a high resistance to Ampicillin 100% and 80% erythromycin, penicillin, and methicillin. While the isolates were sensitive to Vancomycin 66.6%, ciprofloxacin and oxacillin 60 % (Table 3).

Molecular detection of *S. aureus*

The total genomic DNA of *S. aureus* 30 isolates was successfully extracted, and this DNA produced sharp, clear, and pure bands using gel electrophoresis (Figure 2). The purity and concentration of extraction DNA was between 1.5-2 and 40-90.5 Nanogram/microliter, respectively. The study revealed that all *S. aureus-positive* bacteriological isolates gave positive results when tested with 23S rRNA primer and gave a specific primer amplicon size 350bp (Figure 3).

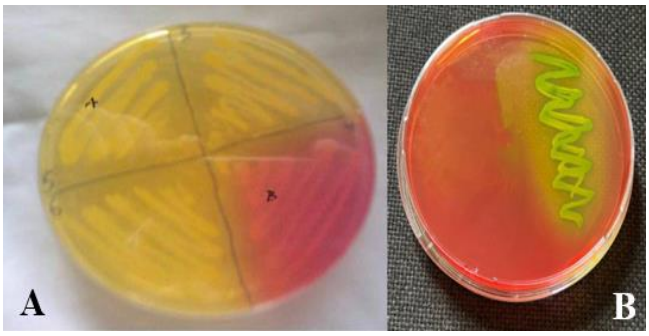

Figure1: Appearance of *S. aureus* isolate on A: mannitol salt agar and B: Hi-chrome agar.

Percentage of *S. aureus* in feline otitis externa according to gender and age

The results showed a non-significant difference between the isolation rates of *S. aureus* according to the gender and age groups of cats with otitis externa. The current study showed that higher infection rates were recorded in females 26.3% than males 22.9% (Table 4). In contrast, according to age, the highest percentage of isolation of *S. aureus* appeared in cats more than one year 25.9% (Table 5).

Table 3: The percentage of isolates of *S. aureus* to antibiotics

Antibiotic	Isolation	Sensitive	Intermediate	Resistant
Erythromycin	30	4 (13.33%)	2 (6.66%)	24 (80%)
ciprofloxacin	30	18 (60%)	0	12 (40%)
Amikacin	30	16 (53.33%)	8 (26.66%)	6 (20%)
Oxacillian	30	18 (60%)	0	12 (40%)
Teteracylin	30	6 (20%)	6 (20%)	18 (60%)
Neomycin	30	10 (33.33%)	0	20 (66.66%)
Penicillin	30	6 (20%)	0	24 (80%)
Methicillin	30	6 (20%)	0	24 (80%)
Vancomycin	30	20 (66.66%)	4 (13.3%)	6 (20%)
Ampicillin	30	0	0	30 (100%)
Cefotaxim	30	16 (53.33%)	2 (6.66%)	12 (40%)
X^{2*}		68.40	42.85	87.41

* X^2 = Chi- square value

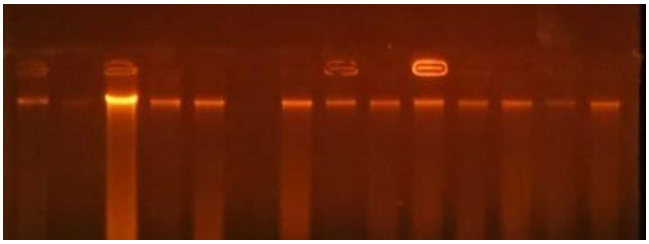

Figure 2: Agarose gel electrophoresis shows bands of total genomic DNA extracted from *S. aureus* isolates.

Percentage of *S. aureus* in feline otitis externa according to breeds and months

The present results revealed no significant difference among cats according to breeds and months of study. The stray 34.3% and Scottish 28.5% cat breeds had the highest rates of infection with otitis externa (Table 6). Significant differences appeared in isolation rates among the months of study; the highest percentage was reported in (cold months) January 80%, November 50%, and December 70%, while negative results were reported in April, May, June, and July (Table 7).

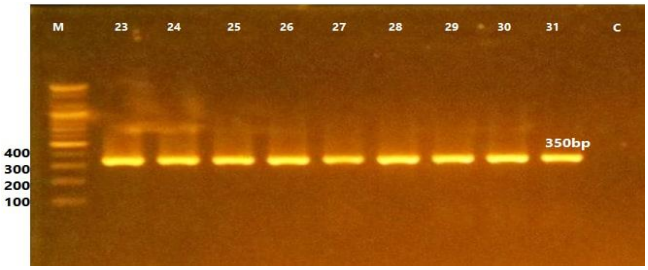

Figure 3: Amplification of 23S rRNA gene of *S. aureus*, the positive bands showed at 350bp (lanes 1-31), M: DNA ladder, C: control negative, the gel electrophoresis was sitting on 100 V 80 m A for 30 minutes.

Table 4: The isolation percentage of *S. aureus* according to the sex of cats

Sex	No. tested	No. +ve	%	X^2	P	OR	95%CI	P
Male	48	11	22.9%	0.18	0.67	1.20	0.51-2.82	0.66N.S*
Female	72	19	26.3%					

* X^2 = Chi- square value, *P = Probability, *OR = Odds ratio, *95% CI = 95% confidence interval, *N.S = Not significant.

Table 5: The isolation percentage of *S. aureus* according to the age groups of cats

Age (year)	No. tested	No. +ve	%	X^2	P	OR	95%CI	P
≤1	39	9	23%	0.11	0.73	1.17	0.47-2.85	0.73N.S*
>1	81	21	25.9%					

* X^2 = Chi- square value, *P = Probability, *OR = Odds ratio, *95% CI = 95% confidence interval, *N.S = Not significant.

Table 6: The infection rate with *S. aureus* in different breeds of cats

Bread	No. tested	No. +ve	%	X^2	P	OR	95%CI	P
Chinchilla	19	2	10.5%	4.09	0.36	Reference=1	-	-
Shirazi	30	8	26.6%			3.09	0.58-16.48	0.18
Himalaya	25	5	20%			2.12	0.36-12.38	0.40
Stray	32	11	34.3%			4.45	0.86-22.87	0.07
Scottish	14	4	28.5%			3.40	0.52-22.02	0.19
Total	120	30	25%					

* X^2 = Chi- square value, *P = Probability, *OR = Odds ratio, *95% CI = 95% confidence interval.

Table 7: The isolation of *S. aureus* according to the months of study

Month	No. tested	No.+ve	%	X^2	P	OR	95%CI	P
1st - 2nd	20	10	50%	37.33	<0.0001	41.00	2.18-770.11	0.01
3rd - 4th	20	2	10%			5.54	0.24-123.08	0.27
5th - 6th	20	0	0			Ref=1	-	-
7th - 8th	20	1	5%			3.15	0.12-82.16	0.48
9th - 10th	20	4	20%			11.18	0.56-222.99	0-11
11th - 12th	20	13	65%			73.80	3.88-1401.64	0.004
Total	120	30	25%					

* X^2 = Chi- square value, *P = Probability, *OR = Odds ratio, *95% CI = 95% confidence interval.

Discussion

The infection with *S. aureus* in pet animals was associated with several clinical conditions, such as otitis (30). The results showed that otitis externa was detected in 30 out of 120 (25%) cat ear samples. The present findings were lower than the percentage recorded in South Africa 34.5%; this may be due to the variation in the number of samples taken from cats in the present study (31). This study agreed with Moriello 2013 who classified otitis externa clinically according to the severity of the case into acute or chronic and unilateral or bilateral (32,33). In Libya, the bilateral form was recorded with a high infection rate 75% as compared with the unilateral form 25%; the differences are owing to the low number of examination cats compared to the number of cats used in the present study. Our *S. aureus* isolates were highly resistant to Ampicillin 100%, Erythromycin, Penicillin, and Methicillin in equal percentages 80%. Increased antibiotic resistance may be due to improper use of antimicrobial drugs, resulting in positive selective resistance to *S. aureus* isolates, which poses severe risks to human and animal health (34). This study affected all breeds and ages, but some groups, such as stray cats, are considered at higher risk. Kennis (1) revealed that some breeds of cats are more susceptible to otitis externa than others due to the excessively ceruminous, seborrhea, and high moisture levels in canal ears. According to the sex, non-significant variations were recorded in the present study; this was in agreement with the results of a previous study in Belgian (35). Our findings conflicted with other studies (30-33), which reported significantly higher rates of *staphylococcus* isolates in females than males. The current study found non-significant differences between the age groups of cats; these results were similar to that noted by Bollez *et al.* (35). On the other hand, Bierowiec *et al.* (30) mentioned a significant increase in the isolation rates of *Staphylococcus spp.* According to age groups of 7-36 months than the others. The kittens are more likely to acquire ear infections from small animal clinics and hospitals where other species in contact with the (birds and rabbits) could be the source of infection (36). The seasonal changes in the environment, relative humidity, and temperature are associated closely with increasing the incidence rate of the feline otitis externa and the other primary infections. In a previous study, Cafarchia *et al.* (37) mentioned that the cold month, especially winter, is a predisposing season for ear infections in cats. Also, a previous study in Northern Italy found that the winter season was a risk factor in the incidence of otitis in cats, especially with the other causes (38). In the current study, a PCR assay was applied to characterize *S. aureus* based on the amplification of 23S rRNA. The present findings were compatible with other studies in Iraq, which detected *S. aureus* isolates harboring the 23S rRNA gene in dogs (39) and from ewe's milk samples (40).

Conclusion

The present study demonstrated the critical role of *Staphylococcus aureus* as a cause of feline otitis externa. The indiscriminate use of antibiotics results in the emergence of multi-resistant bacteria for more than one antibiotic. The PCR technique based on the 23S rRNA gene is an excellent diagnostic tool for detecting *Staphylococcus aureus.*

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Conflict of interest

The authors declare no conflict of interest

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العزل والكشف الجزيئي للمكورات العنقودية الذهبية من التهاب الأذن في بغداد

لبنى عبد الكريم عباس و أماني محمد راضي

فرع الطب الباطني والوقائي البيطري، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

هذا الدراسة أجريت لعزل والتعرف على بكتريا المكورات العنقودية الذهبية في القطط المصابة بالتهاب الأذن في محافظة بغداد. جمعت المسحات القطنية من أذن القطط (العدد ١٢٠) وقد تم أجراء العزل البكتيري وتأكيدها بواسطة تقنية تفاعل البلمرة المتسلسل التقليدي لجين *SrRNA23*. أجرى العزل من خلال الزرع على وسط الدم، وسط ملح المنتول ووسط الكروم الخاص بالمكورات العنقودية وتم تأكيد تشخيص المستعمرات باستخدام صبغة الكروم والاختبارات الكيميوحيوية. من ١٢٠ مسحة أذن كانت عدد العزلات الموجبة للمكورات العنقودية الذهبية ٣٠. أظهرت عزلات المكورات العنقودية الذهبية مقاومة عالية لامبسلين ١٠٠% وبنسبة ٨٠% لكل من الاثيرميسين، البنسلين والمشلين، بينما اغلب العزل كانت حساسة للفانكوميسين ٦٦,٦% ولكل من سبروفلوكساسين والاوكساسيلين بنسبة ٦٠%. كشفت نتائج الفحص الجزيئي بان جميع عزلات المكورات العنقودية الذهبية كانت موجبة البادئات الخاصة لجين *SrRNA23* بحجم ٣٥٠ كيلودالتون. أظهرت القطط المصابة علامات متعددة، سجل اعلى معدل للإصابة بالنوع الأحادي الجانب ٣٣,٣%، بينما كان النوع الحاد الأكثر تكرارا ٤٠% لالتهاب الأذن الخارجية. قد ارتفعت معدلات الاصابة في الإناث ٢٦,٣% وقد ظهرت أعلى نسبة عزل للمكورات العنقودية الذهبية في القطط التي أعمارها أقل من سنة ٢٥,٩%. وقد ارتفع معدل الاصابة معنويا بالتهاب الأذن الخارجية في القطط السائبة ٣٤,٣%. بينما لوحظ وجود اختلافات معنوية في معدلات العزل بين أشهر الدراسة، حيث سجلت اعلى نسبة في شهر كانون الثاني ٨٠% ولكانون الأول ٧٠%. المكورات العنقودية الذهبية تتسبب بالتهاب الأذن في القطط وخاصة في الأشهر الباردة.