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Phylogenetic Analysis and Molecular Identification of *Staphylococcus aureus* Isolated from Skin Lesions of Cats

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Abstract | This study aims to investigate the nature and dynamics of *Staphylococcus aureus* (S. aureus) in the skin lesions of cats. For this purpose, a total of one hundred skin swabs samples were collected from cats and were inoculated onto mannitol salt agar, sheep blood agar, and STAPH 110 agar for bacterial isolation. The isolates were further characterized using catalase, oxidase, and coagulase tests. Based on these conventional culture methods, 52 out of 100 skin lesion samples were confirmed to be S. aureus. To further characterize and diagnose S. aureus, VITEK® system was applied. However, based on this analysis, only 12 out of 52 isolates (23%) were detected. Molecular identification of *S. aureus*, achieved by amplifying a target region of approximately 350 bp within the *23S rRNA* gene using specific primers, identified 47 (90.3%) isolates as positive. The sequence analysis of 23S *rRNA* gene of *S. aureus* isolates was performed and are available under accession numbers PQ476659.1, PQ476660.1, and PQ476661.1. The phylogenetic of the *23s rRNA* gene of *S. aureus* showed a separate clade due to diverse genetic relationship. Isolates characterized here showed a distinct clustering patterns compared to isolates characterized previously. Taken together, there are limitations to conventional methods for detecting *S. aureus*. Therefore, merging the conventional and molecular diagnostics tools is an important consideration for accurate identification of *S. aureus* in veterinary practices.

Keywords | Molecular detection, Genetic characterization, *23s rRNA*, *S. aureus,* Skin, Bacterial isolation

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INTRODUCTION

Feline dermatology includes many skin disorders that effect the health of cats (Noli *et al*., 2023). *Staphylococcus aureus* is an opportunistic pathogen in cats (Bannoehr and Guardabassi, 2012; Krapf *et al*., 2019). Also, *Staphylococcus* species have important role in feline skin disease. *Staphylococcus* species can convert to pathogenic when host defense mechanisms are compromised, causing conditions such as pyoderma and abscesses (Faccin *et al*., 2023).

Staphylococcus aureus is Gram-positive bacteria, which may cause various types of infections that depends on virulence factors. Therefore, *S. aureus* can be isolated from different sources of samples (Jafaar and Kandala, 2025). The development of antimicrobial resistance and virulence genes is an emerging concern around the globe (Phumthanakorn *et al*., 2022). *Staphylococcus aureus,* as MRSA, is documented as a major pathogen in human skin infections (Mustapha *et al*., 2014). However, MRSA strains have been commonly isolated from healthy cats (Hanselman *et al*., 2009) and

may act as reservoirs of *Staphylococcus* spp (Woudstra *et al*., 2023). It has previously been identified that silver nanoparticles and gentamicin are promising candidates in controlling the antimicrobial resistance of *S. aureus* in mastitis in animals (Al-Dujaily and Mahmood, 2022).

Polymerase chain reaction (PCR) offers quick and accurate methods for detecting *S. aureus* in veterinary medicine. Owing to its high-sensitivity and specificity, it offers confirmatory and gold standard for molecular testing (Saraiva *et al*., 2018). A study conducted on feline otitis externa confirmed infection of *Staphylococcus aureus*. This study revealed the infection rate to be 5.8%according to the results of *23S rRNA* of *S. aureus* (Abbas and Rady, 2023a; Abbas and Rady, 2023b). Radhy (2023) has reported that *S. aureus* was isolated from 10% of eye infections in cats. Ahmed and Yousif (2021a) have isolated *S. aureus* from mastitis in ewes and molecular identification was performed using *23srRNA* genes and investigated antimicrobial resistance genes (acc-aph) frequency, On the other hands, Ahmed and Yousif (2021b) have reported the presence mecA gene in S. aureus with a rate 16.32%. The present study aims to identify and characterize S. aureus infections in cats and offer to contribute in investigating the *Staphylococcus* spp. and related antimicrobials resistance genes in the variable cases of skin lesions in the cats.

MATERIAL AND METHODS

Samples Collection

A total of 100 skin lesions swab samples were collected from 100 individual cats from Baghdad province during November 2023 to June 2024. These cats were examined clinically for detecting skin lesions. There were a total of 59 females and 41 males, whereas 43 cats were less than one year and 57 cats were equal to or more than one year in age. The distribution of breed include: 68 Crossbreed, 13 Persians, 9 Scot, and 10 Himalayans. The skin swabs were collected by sterile cotton swab and transported with icebox to the bacterial laboratory of high education of the Clinical Pathology of the Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad.

Bacteriological Identification of *S. Aureus*

All swabs were streaked firstly on mannitol salt agar and incubated at 37°C for 24-48 hours. Then cultured on 5% sheep blood agar with an incubation period of 24-48 hours at 37°C, to detect a hemolysis around the colony. The selective media (STAPh110) were used for staphylococcus identification at 37°C for 24 hours (Markey *et al*., 2014). The Gram stain was performed according to the instructions from the commercial kit. The biochemical tests were performed in the present study as per earlier reports (Markey *et al*., 2014), including catalase, oxidase, and coagulase tests. To confirm identification of *Staphylococcus aureus*, VITEK® system was used. The Staphylococcal isolates were stored at -20° brain heart infusion broth with 10% glycerin (Al-Rasheed *et al*., 2022).

Molecular Diagnosis of *S. Aureus*

The DNA was extracted from the bacterial suspensions at concentrations up to $1x10^9$ CFU/ml after cultivation on nutrient broth with an incubation period of 24 hours at 37°C according to the instructions of the manufacturer of the kit (Presto mini gDNA bacteria kit, Geneaid, Taiwan), and the final elute of the DNA was stored at -20 °C for conducting molecular assays. The target area of the *23s rRNA* gene of *S. aureus*, about 350 bp, was identified by two primers, F-TCGGAATCTGGGAGGACCAT and R-AATCGTAAGTCGGTTCGGTCC (Gatta and Al-Graibawi, 2021).

The total volume of the PCR reaction was 25 µL, including 12.5 µL of master mix (50 units/mL of Taq DNA polymerase at pH 8.5, 3 mM $MgCl_2$, 400 µM each of dNTP), and 1 µL for each forward and reverse primer (10 picomol), and completed the volume into 25 µL by Nuclease-free water according to the instructions of Promega (USA). The protocol of thermocycles is documented in the Table 1. The PCR products were loaded on electrophoresis with red-safe stain (ABM, Canada), then visualized under a gel documentation system (ACCURIS/ USA).

Table 1: The protocol of thermocycles for amplifying *23s rRNA* gene of *S. aureus*.

Steps	Temperature (C°)	Time	Cycles
Initial denaturation	94	10 min.	1
Denaturation	94	45 sec.	35
Annealing	58	45 sec.	
Extension	72	45 sec.	
Final extension	72	7 min.	1
Hold	4	24 hr.	1

The sequence data of the *23s rRNA* gene of *S. aureus* were submitted to National Centre for Biotechnology Information (NCBI) for three samples under accession numbers PQ476659.1, PQ476660.1, and PQ476661.1. The sequences of *S. aureus* were aligned with sequence data that were available in the NCBI of the same species. The phylogenetic tree was constructed using MEGA6 software and depended on the evolutionary distances and number of base substitutions per site (Tamura et al., 2013).

Statistical Analysis

A One-way ANOVA test was used for detecting the significant difference of the mean of the values in the study,

while the odds ratio confidence interval (CI) was used for estimating the risk factors of the data in the study. Chi-square and two-proportion Z-Test were used to detect differences between datasets. Differences were considered significant at ($P < 0.05$) (SPSS, version 20).

RESULT AND DISCUSSION

Bacteriological Identification

The main aim of the study was the recovery of *S. aureus* isolates from feline skin infections cases, which was conducted by conventional methods such as culture, morphological appearances under microscope, biochemical tests. Analysis of the cultures indicated *Staphylococcus aureus* produced yellow colonies, on the Mannitol Salt Agar (MSA) indicating mannitol fermentation (Figure 1). While *Staphylococcus aureus* on blood agar produced colonies that were surrounded by beta-hemolysis, which is a clear hemolytic zone, *S. aureus* on STAPH110 media appeared as yellow golden colonies (Figure 2). Gram- stained smears of *Staphylococcus aureus* were conducted and revealed that Gram-positive single or in grouped cocci.

Figure 1: The colonies of *Staphylococcus aureus* on mannitol salt agar (yellow in color), after incubation 37°C for 24-48 hours.

The *Staphylococcus aureus* biochemical profile indicated that results were positive for catalase and coagulase tests and negative for oxidase test, as expected. Beta hemolysis was observed on blood agar, and mannitol fermentation in MSA media. The total positive culture isolates were 52 out of 100 skin lesions identified as *S. aureus* by the conventional culture method. To confirm identification of *Staphylococcus aureus*, VITEK® system was used. A total of 12 *Staphylococcus aureus* cultures out of 52 isolates were confirmed by VITEK examination.

Figure 2: The colonies of *Staphylococcus aureus* on STAPH 110 (golden yellow), after incubation 37°C for 24-48 hours.

Table 2: Infection rate of *Staphylococcus aureus* according to category of diagnosis.

Category of diagnosis	Infection rate	Statistical value	
Confirmation by Vitek	12/52 (23%)	Chi-square	47.98
Confirmation by PCR	47/52 (90.3%)*	p-value	0.0043

Molecular Detection of *S. aureus*

All bacterial isolates (52 out of 100 skin lesion samples) were identified by using conventional methods. However, only 47 out of 52 (90.3%) isolates were confirmed by conventional PCR as *S. aureus* (Table 2), which produced a specific targeting fragment of 350 bp of the *23s rRNA* gene (Figure 3). When the diagnosis was confirmed by the Vitek system, only 12 out of 52 isolates (23%) were detected, indicating low sensitivity of Vitek method in *S. aureus* diagnosis of skin samples and high sensitivity of PCR method for detecting *S. aureus* (Table 2).

A larger proportion of *Staphylococcus aureus* was detected using conventional methods. However, using the VITEK® system, only a total of 12 S. aureus isolates were confirmed. These results display the importance of molecular methods for bacterial identification, but conventional methods remain widely used because of low cost, especially in non-specialist laboratories (Rajapaksha et al., 2019). However, these methods must be required to distinguish *S. aureus* from other *Staphylococcus* species in animals through biochemical profiling (Karmakar *et al.*, 2016). The VITEK® system can produce inaccurate results of some bacteria identification because of the use of low range of biochemical panel through the tests, and increase the risk of contamination (Funke and Funke-Kissling, 2005).

Identification of only 12 out of the 52 isolates suggests that there is relatively low significance to identify *S. aureus* using classical methods. This also indicate that the necessity of used method in diagnostic bacteriology for accuracy, especially with isolates from animal sources. Moreover, misdiagnosis of *Staphylococcus* species had negative impact on clinical practice, treatment, and antimicrobial susceptibility testing (Ryman *et al*., 2021). Therefore, combination of conventional and molecular methods can provide a highly reliable result and diagnostic confidence to staphylococcal identification.

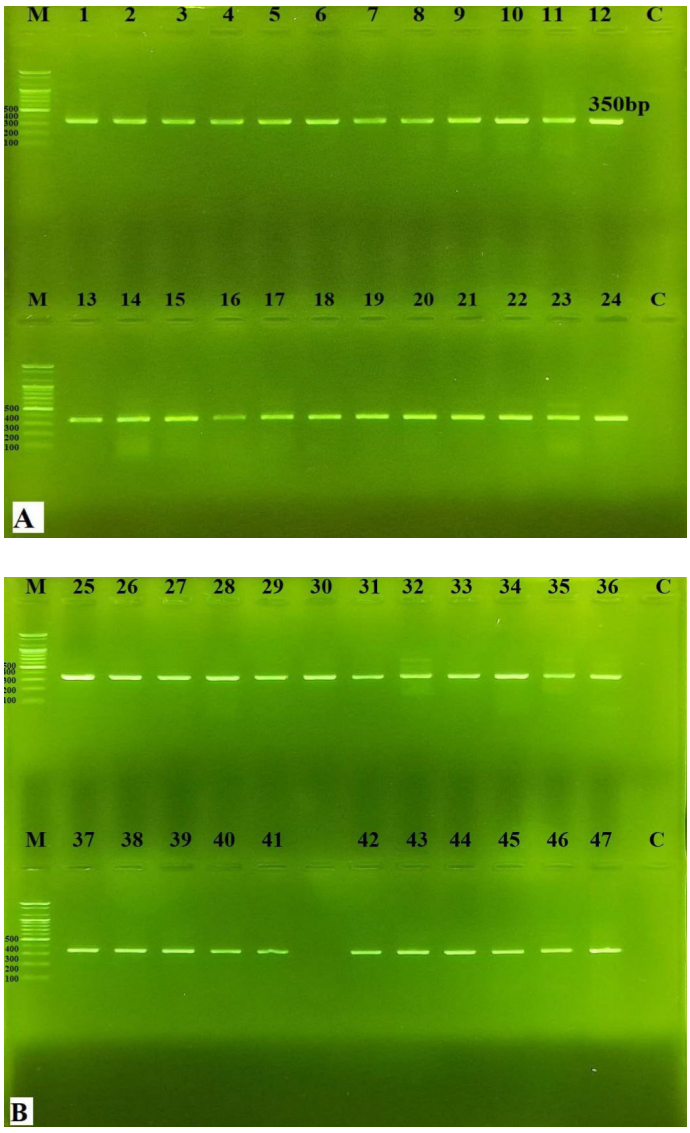

Figure 3: Amplification of 350bp fragment of *23srRNA* of *Staphylococcus aureus* (Agarose gel electrophoresis 1.2%); showing. M: ladder 100 sizers, 47 lanes positive cases. Lane C negative control under blue light documentation.

Clinical and Physical Examination

The present study analyzed clinical, physical examination and vital parameters between non-infected and infected cats with *S. aureus*. The body temperature and respiratory rate in the infected cats were significantly increased compared to the non-infected cats. In contrast, the heart rate was not significant differences between the infected and non-infected cats (Table 3).

Table 3: Vital signs of non-infected and infected cats with *S. aureus (*confirmed based on PCR).

Parameters	Non infected (53)	Infected cats (47)	P value
Temperature °C/minute	38.65 ± 0.05	39.29 ± 0.05*	0.00001
Respiratory rate/minute	25.02 ± 0.40	30.21 ± 0.41*	0.00001
Heart rate / minute	176.13 ± 2.97	179.68 ± 2.71	0.3839

Range and M± SE, *significant differences at (P<0.05).

The most common and documented clinical lesions of *Staphylococcus* skin infections in cats were pus formation in wounds or post-operative sites and hair loss and redness on the abdomen. While less frequently observed lesions included ophthalmia and periocular hair loss, omphalitis in kittens, and external ear crusting. Statistical analysis revealed that there are no significant differences between lesion type and *Staphylococcus* infection, indicating that the presence of a specific skin lesion does not significantly predict infection status 0f *Staphylococcus* (Table 4).

Table 4: Infection rate of *Staphylococcus aureus* confirmed based on PCR and according to category of skin lesions.

Lesion Type	Total Number	Infected cats	Infection Rate (%)
hair loss and redness on the abdomen	28	13	46.43%
Crusting of external ear	3	1	33.33%
Ophthalmitis and hair loss around the eyes	4	2	50.0%
Omphalitis in kitten	8	4	50.0%
Pus formation in wound and post-operation	57	27	47.37%

*Chi-square= 0.275; p-value= 0.991. No statistically significant association between skin lesion type.

The fever in the infection significantly occurred due to stimulation in the hypothalamic thermoregulatory center, and by mediated endogenous pyrogens (Dinarello, 2004). The elevated respiratory rate could be attributed to the systemic inflammatory response and the associated metabolic demand in small animals with bacterial dermatitis (Faccin *et al*., 2023). However, the heart rate was not significantly different between groups of cats. This may suggest that the compensatory cardiovascular response was variable in cats, and needed to adaptation of the cats (Abbott, 2005). The skin lesions were variable in this study. Pus formation indicating that suppurative dermatitis with *S. aureus* due to its enzymes and toxins productions (Del Giudice, 2020). Similarly, hair loss and erythema were related to folliculitis

or dermal invasion (Faccin *et al*., 2023). Also, ear lesion in cat can be associated to multifactorial etiology of includes bacteria, mites, fungi, and environmental allergens (Nardoni et al., 2014).

Distribution of the Infection According to the Gender and Age

Infection rates of *Staphylococcus* confirmed based on the PCR results, was classified to gender and age of cats. Analysis indicated female cats were 39.02%, male cats were 52.54%. Cats with less than one year were 65.12%, and aged one year or older were 33.33%. Although the infection rate was higher in males, no statistically significant association was established. However, the difference was significant in less than one-year cats. The infection rate of *Staphylococcus* according to PCR results was not statistically and significantly higher in male than female cats, this finding is consistent with Backel and Cain (2017), where authors have identified that male cats may be more susceptible to the skin infections because of their history of fight. More notably, the infection rate was significantly higher in young cats and susceptibility to *Staphylococcus* infection because of young cats may have immature immune systems, and high susceptible to opportunistic pathogens, and increasing risk of bacterial colonization and infection (Faires *et al*., 2009). The season and physiological statues in cats were significantly affected and will require further investigations (AL-Ani *et al*., 2019).

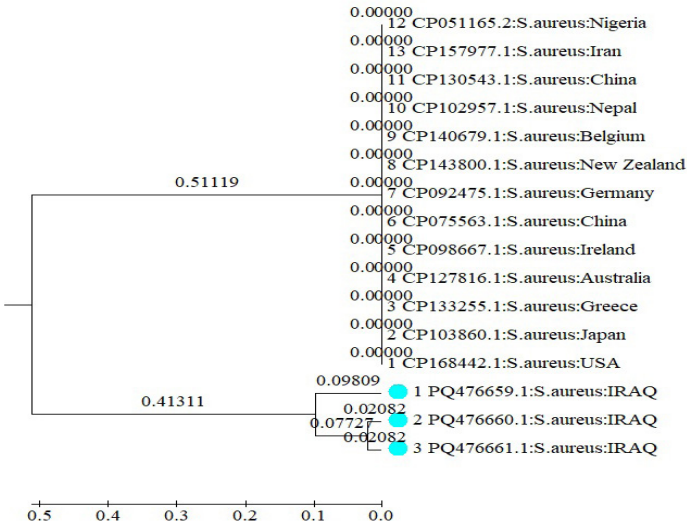

Figure 4: Phylogenetic tree of *Staphylococcus aureus* (*23s rRNA* gene) characterized in this study.

Phylogenetic Study of *23S RRNA* of *S. Aureus*

The phylogenetic analysis of the *23s rRNA* gene of *Staphylococcus aureus* isolated from skin infection was conducted with reference strains from other geographical countries, including USA, China, Iran, Germany, Japan, Nigeria and others (Figure 4). The isolates characterized in this study (PQ476659.1, PQ476660.1, PQ476661.1) formed as separate clade, because of distinct genetic relationship, and were separated from other reference strains including Iranian strains (CP157977.1). The nearest world clade had zero genetic distance between them.

The difference between the Vitek system and PCR in detection of bacterial infection revealed limitations of conventional diagnostic methods. These results are consistent with findings of Luteijn *et al*. (2011) who have noted higher sensitivity and specificity of PCR methods in the bacterial identification. Vitek systems depended on metabolic profiles and phenotypic characteristics, which can be produced intervening between coagulase-positive and coagulase-negative *Staphylococcus spp*., leading to false negative or even misidentification (Vinshia *et al*., 2024). In contrast, the PCR targets specific sequences of specific gene of bacteria, allowing rapid and accurate identification (Clifford *et al*., 2012). In veterinary skin infection, where samples can contaminate, or chronic infections may impair bacterial culture. These support that the molecular diagnostics can be used in routine identification of *S. aureus* in skin infections in cats for accurate identification and antimicrobial therapy use to control he spread of this pathogen.

The current study was conducted on skin lesions of 100 cats, with an infection rate of 47% of *S. aureus*. The analysis of *23S rRNA* of *S. aureus* from ears of cats revealed the infection rate to be 5.8% and association with feline otitis (Abbas and Rady, 2023a; Abbas and Rady, 2023b). Radhy (2023) has reported that *S. aureus* was isolated from 10% of eye infections in cats whereas Ahmed and Yousif (2021a) have isolated *S. aureus* from mastitis in ewes and molecularly characterize them with *23s rRNA* genes. Moreover, Hayyawi (2012) has studied many bacterial isolates from external ears in sheep and the most pathogens were *S. aureus* with a rate of about 30.76 %. Saher *et al*. (2009) have identified *S. aureus* with a rate of 21.8 % from dogs with tumor tissue's samples. The culturing of the samples was conducted from nose, throat and ear of human patients and they have identified *S. aureus* as the most common pathogen (81.6%) (Al-Hamadany, 2013). The differences in the percentage between the present study and other studies in Baghdad may be attributed to selection of one organ of cats in the current study. All previous studied have highlighted the ability of 23*srRNA* genes for investigating *S. aureus* in animals and human's samples. The isolates reported in this study clustered within a distinct clade, and close genetic relationship may indicate a localized adaptation of certain *S. aureus* in the feline host. The host-specific evolutionary patterns and limited diversity of *S. aureus* has been demonstrated (Monecke *et al*., 2011). However, Iranian strain did not group with the Iraqi cluster due to host specific factors and due to the fact that the Iranian isolates

was isolated from urine of human.

CONCLUSIONS AND RECOMMENDATIONS

This study concluded that there are limitations to conventional methods for identifying *S. aureus*. Phylogenetic analysis of the *23s rRNA* gene of *S. aureus* showed Iraqi isolates formed a distinct cluster, suggesting that there was local adaptation to feline hosts in Iraq, while the Iranian isolate was genetically distant. Epidemiologically, young cats were more susceptible to *S. aureus* infection. Merging the conventional and molecular diagnostics tools is an important consideration for accurate identification of *S. aureus* in veterinary practices.

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NOVELTY STATEMENTS

The novelty of this study is included the molecular detection and phylogenetic analysis of *23s rRNA* gene of *S. aureus*, which isolated from skin lesions in cats.

AUTHOR'S CONTRIBUTIONS

Mawj Firas Abdulameer collected and cultured the samples and completed other diagnostic tests. Ayman Barzan Abdulgafor designed the study, interpreted the results, and reviewed the manuscript.

Conflict of Interest

The authors have no conflict of interest to disclose.

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