Research Article

Molecular Analysis of *Staphylococcus* Species Isolates from Buffalo Mastitis in Baghdad City

Naseir Mohammed Badawi*, Jinan Sahib Abdulnabi Al-Naseri, Amanee Mohammed Radhy

Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

Abstract | The study aimed to perform molecular characterization of *Staphylococcus* spp. isolated from cases of subclinical mastitis in buffaloes. Milk samples were collected from 70 apparently healthy water buffaloes in Baghdad Province, Iraq and screened with California mastitis test (CMT). The samples were cultured on Mannitol Salt Agar (MSA), where coagulase-positive *Staphylococcus* isolates were identified by a color change from red to yellow. DNA was extracted from the isolated *Staphylococcus* species, and polymerase chain reaction (PCR) was performed for molecular analysis. Out of 70 buffalo milk samples, 19 (27.1%) tested positive using the CMT. Among these, nine isolates were molecularly confirmed as *Staphylococcus* species by targeting a 350 bp region of the *23S rRNA* gene. Sequence analysis revealed that *Staphylococcus aureus* accounted for 31.5% and *Staphylococcus intermedius* for 15.7% of the isolates causing subclinical mastitis among the 19 buffaloes. The phylogenetic analysis of *S. aureus* revealed that some Iraqi isolates were closely related to each other. One isolate clustered closely with an *S. aureus* strain previously isolated from a case of cat otitis in Iraq. Additionally, three isolates showed global relatedness, clustering with methicillin-resistant *S. aureus* (MRSA) strains from the USA, Ireland, and Pakistan. The phylogenetic tree of *S. intermedius* indicated that the Iraqi isolate was closely related to *S. intermedius* strains from humans in the Netherlands. Other reference isolates in the tree belonged to *S. pseudintermedius* and were derived from canine skin lesions, with origins in Iraq, Grenada, Kenya, Australia, South Korea, Thailand, USA, and Germany. The study concluded that *S. aureus* is a major etiological agent of subclinical mastitis in buffaloes, while *S. intermedius* appears to have an emerging role in the disease. Phylogenetic analysis suggests that *Staphylococcus* spp. possess zoonotic potential, with pets possibly serving as reservoirs for these pathogens. These findings raise important public health concerns regarding the contamination of buffalo milk and its potential implications for human health.

Keywords | Molecular analysis, *S. aureus*, *S. intermedius*, Buffalo, Mastitis

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***Correspondence** | Naseir Mohammed Badawi, Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq; **Email:** naseir.m@covm.uobaghdad.edu.iq, naseir.badawi78@gmail.com

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INTRODUCTION

Water buffalo (*Bubalus bubalis*) are important animals for the dairy industry, especially in peri-urban and rural regions. The milk of buffalo, which contains a high level of fat and protein contents, is essential and major source of food security and rural livelihoods (Chiariotti *et al.*, 2025). In Iraq, the average milk production of buffalo was about 9.7 kg/ day, reflecting that buffalo is an important dairy animal (Avadesian, 2012). Mastitis is mammary gland inflammation and a significant productivity disease in buffalo. Mastitis causes economic losses because of decreased milk production and quality, increased veterinary efforts, and the culling rate of infected

buffalo. *Staphylococcus* species, particularly *S. aureus*, are the common pathological agents of mastitis, representing about 65% of bacterial isolates in some reports (Preethirani *et al*., 2015; Hoque *et al*., 2022).

Mastitis in cattle is one of the most important diseases causing significant economic losses, with *Staphylococcus* species being the primary causative agents in Iraq (Al-Rasheed *et al*., 2022). The present study was conducted on cows from various regions in Baghdad. Findings revealed that *Staphylococcus aureus* was the most prevalent etiological agent, accounting for 36.15% of the bacterial isolates. The incidence of subclinical mastitis was 30.47%, compared to 10.0% for clinical mastitis, out of a total of 120 animals examined (Yass *et al*., 1994). *S. aureus* is etiological cause of mastitis in both dairy buffaloes and cows, especially due to its biofilm formation ability, resistance to the immune system, and tendency to induce chronic mammary gland inflammation. *S. aureus* is responsible for poor prognosis rates and increased chronic infection, especially in older animals, and when extended lactating periods (Wójcik and Siwicki, 2022; Kerro-Dego and Vidlund, 2024).

Moreover, *S. intermedius* as coagulase-positive *staphylococci*, is recognized in some animals such as dogs in the previous (Kikuchi *et al*., 2004). The phenotypic characteristics of *S. intermedius* are similar to those of *S. aureus* and it is occasionally isolated from cases of bovine mastitis. Therefore, molecular analysis is required to accurately distinguish *S. intermedius* from *S. aureus*. *S. intermedius* has been reported to represent approximately 12% of *Staphylococcus* isolates in mastitis cases in dairy cows (Pascu *et al*., 2022).

On the other hand, coagulase-negative *staphylococci*, especially *S. epidermidis*, are becoming more identified in subclinical mastitis in recent years. *S. epidermidis* frequently isolated in mastitis of dairy cows. Although typically less virulent, *S. epidermidis* can produce biofilms and exhibit antimicrobial resistance, which interferes with production processes and complicates control programs (Ruiz-Romero and Vargas-Bello., 2023; Kløve *et al*., 2025). Traditional methods of diagnosis of mastitis in dairy cows, such as California mastitis test, somatic cell count, and bacterial isolation, are restricted diagnostic methods because of their low accuracy and sensitivity degree, although these methods were still important for guiding treatments and hygienic conditions (Stanek *et al*., 2024). *Staphylococcus* species are characterized by bacterial traditional methods, including Gram stain, catalase test, coagulase test, selective media such as mannitol salt, blood agar for identification of hemolysis type (Fernandes *et al*., 2021).

Molecular diagnostic methods like polymerase chain reaction (PCR) are extremely sensitive and specific methods and early and accurate in the detection of the bacteria strains. The targets genes, such as intergenic spacer region between *16S rRNA* and *23S rRNA* have been especially useful for *Staphylococcus* species identification (Gatta and Al-Graibawi, 2021; Gumaa *et al*., 2021; Guccione *et al*., 2014), and *S. aureus* genotyping in mastitis outbreaks (Guccione *et al*., 2014). Moreover, the *23S rRNA* gene of *Staphylococcus* sequencing is essential method for the identification of multidrug resistant *S. aureus* strains (Salauddin *et al*., 2020). Also, *16S rRNA* sequencing has been used in the diagnosis of *Staphylococcus* species in milk of buffalo for control on mastitis (Salman *et al*., 2023). In Iraq, methicillin-resistant *Staphylococcus aureus* (MRSA) was detected at 33.4% in raw milk. This investigation highlight the presence of MRSA in animal's products and needed for regular monitoring of mastitis in animals to protect public health (Kanaan and Al-Shammary, 2013).

The investigation of subclinical mastitis in buffaloes from Iran, which detected *Staphylococcus* isolates in 48.55% out of 51 milk samples include 14% *S. aureus* (Beheshti *et al*., 2011). Subclinical mastitis in buffaloes was 66%, and *Staphylococcus* species were the most predominant bacteria about 34% in buffaloes from northwest of Pakistan (Ali *et al*., 2021). In Iraq, subclinical mastitis in buffaloes was 23.8%, the most commonly diagnosed bacteria included *S. lentus*, *S. epidermidis*, *S. uberis*, *S. sciuri*, *S. thoraltensis*, *S. aureus*, *S. haemolyticus*, *S.* chromogens, *S. agalactiae*, *S. xylosus*, *S. equi* and *S. canis* using the VITEK technique (Al-Zainy and Al-Jeburii, 2015). Molecular characterization studies on *Staphylococcus* isolates in buffalo mastitis are limited. The aim of the current study is isolation of *Staphylococcus* species from the milk of Iraqi dairy buffalo for molecular and phylogenetic identification of the *23S rRNA* gene of *Staphylococcus* spp. for investigating the molecular identification of the pathogens, such as *S. aureus* and *S. intermedius*.

MATERIAL AND METHODS

Animals and milk samples collection

Milk samples were collected from 70 apparently healthy water buffaloes (*Bubalus bubalis*) from different regions of Baghdad Province, Iraq (Taji, Fudhailiyah, and Rashidiya), from December 2024 to January 2025. Buffaloes used in the study were lactating and aged between 4 and 6 years. The teats of Buffaloes were properly cleaned with drinking water and dried, disinfected with 70% ethanol. The initial udder milking was discarded, and about 8–10 mL of the milk was collected in the sterile tube from each buffalo. All tests were conducted in the Microbiology Laboratory and Polymerase Chain Reaction Laboratory, College of Veterinary Medicine.

Isolation and initial identification of bacteria

The milk samples initially screened with the California

Mastitis Test (CMT) (Ramuada *et al*., 2024). Then a loopful of each positive sample by CMT was streaked onto Mannitol Salt Agar (MSA) for 24–48 hours at 37°C, coagulase-positive *Staphylococcus* isolates were identified by color change on MSA from red to yellow, and coagulase test was done by incubating the bacterial suspension with rabbit plasma at 37°C for 24 hours. All bacterial isolates were identified using conventional method and stored in brain heart infusion agar at −20°C until molecular analysis (Kateete *et al*., 2010).

Molecular assays

Extracted DNA from isolated *Staphylococcus* species was done according to the instructions of the Presto™ Bacteria Genomic DNA Kit (Geneaid Biotech Ltd., Taiwan). The eluted DNA samples were stored at −20°C until PCR test. Polymerase Chain Reaction for amplification target regions of 350 bp of *23S rRNA* gene of *Staphylococcus* spp. were amplified by universal primers that designed specifically for *Staphylococcus* spp. by Gatta and Al-Graibawi (2021). PCR reaction (25 µL for each sample) included 12.5 µL of Master Mix reagent of Promega (Corporation, USA), 1 µL of forward primer: TCG-GAA-TCT-GGG-AGG-ACC-AT (10 pmol/µL), 1 µL of reverse primer: AAT-CGT-AAG-TCG-GTT-CGG-TCC (10 pmol/µL), 2 µL of extracted DNA, and 8.5 µL of nuclease-free water. The thermal cycling conditions were 94°C for 5 minutes (1cycle), 94°C for 40 seconds, 58°C for 40 seconds, and 72°C for 40 seconds (35 cycles), and 72°C for 7 minutes (1 cycle). All amplicons were loaded on electrophoresis in 1.5% agarose gel with ethidium bromide. The DNA ladder 100 bp DNA sizer was used as a marker. Electrophoresis was set at 120 volts, 80 mA for 45 minutes, and the gel was imagined under ultraviolet light system.

Sequencing and phylogenetic analysis

The PCR-positive products of amplicons of target areas of *23S rRNA* gene of *Staphylococcus* spp. were sent for sequencing at Macrogen Inc. (South Korea) with forward primer (16 pmol/µL). The phylogenetic tree was created by MEGA 11 version. The nucleotide sequences were aligned and selected with close sequences of NCBI. The evolutionary relationship of isolates was determined using the UPGMA method (Kaur and Kaur, 2024). Evolutionary distances were estimated by per-site rate of substitution. All positive *Staphylococcus* species isolated from buffalo milk in Iraq were submitted to the NCBI GenBank database (Table 1).

RESULT AND DISCUSSION

Molecular detection

Out of 70 milk samples collected from apparently healthy water buffaloes, 19 buffaloes (27.1%) were positive for subclinical mastitis according to CMT results. The positive samples exhibited 9 isolates that were diagnosed as *Staphylococcus* depending on cultural and biochemical tests. Further confirmation tests included PCR amplification of fragments about 350 bp of the *23S rRNA* gene (Figure 1). All positives samples according to PCR results were sequenced and aligned to reference sequences in GenBank to determine *Staphylococcus* species. Six of the isolates were classified as *Staphylococcus aureus* and were recorded in GenBank under accession numbers PV628203.1, PV628204.1, PV628205.1, PV628206.1, PV628207.1, and PV628208.1. Three isolates were classified into *Staphylococcus intermedius* with accession numbers PV628211.1, PV628212.1, and PV628213.1.

Table 1: The accession numbers of *Staphylococcus* species isolated from milk of buffaloes.

Buffalo isolate ID	*Staphylococcus* species	Accession number
Iraq1	*S. aureus*	PV628203.1
Iraq2	*S. aureus*	PV628204.1
Iraq3	*S. aureus*	PV628205.1
Iraq4	*S. aureus*	PV628206.1
Iraq5	*S. aureus*	PV628207.1
Iraq6	*S. aureus*	PV628208.1
Iraq1	*S. intermedius*	PV628211.1
Iraq2	*S. intermedius*	PV628212.1
Iraq3	*S. intermedius*	PV628213.1

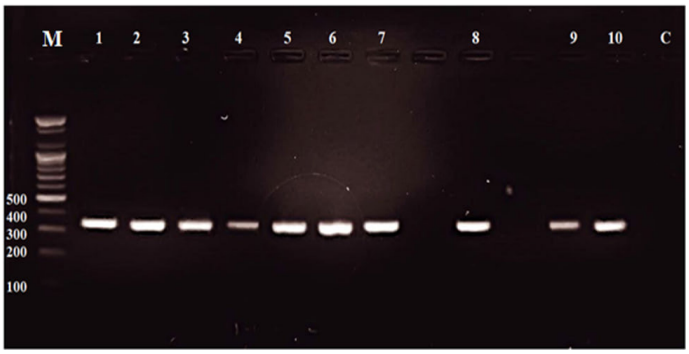

Figure 1: The figure showed agarose gel electrophoresis (1.5%) with ethidium bromide. Lane 1 – 7, 8, 9: positive samples of *23S rRNA* of *Staphylococcus* spp. about 350bp. Lane C: Negative control, lane 10: positive control, and M: DNA ladder.

The positive percentage of mastitis cases in CMT in our study (27.1%) was aligned with observations of some researchers that reported subclinical mastitis rates 23.8% in buffalos from Iraq (Al-Zainy and Al-Jeburii, 2015), 23.85% in dairy buffaloes from India (Sharma et al., 2018), and a little bit higher (34.82%) in buffalos from Iran (Beheshti *et al*., 2011), and 53% in buffaloes from Pakistan (Akhtar, 2012). Subclinical mastitis is a very important form in dairy cattle due to unapparent clinical signs but its significant effect on milk quality and production amount. In buffaloes, this form of mastitis has been hardly reported,

despite the economic losses and the need for a regular screening program (Zeryehun *et al*., 2013). The variation in the infection rates of mastitis may refer to different in animal managements, environment contamination, and diagnostic methods. Additionally, the most studies depend on conventional techniques of bacterial identification. The limitation of conventional bacterial identification methods such as culture purity, and variability in biochemical tests could lead to misdiagnosis of some bacteria, while the molecular detection of the *rRNA* genes of bacteria is stander and gold technique (Al-Mozan, 2023).

Nine isolates out of 19 buffaloes with subclinical mastitis were molecularly confirmed as *Staphylococcus* species by targeting an area of about 350 bp of the *23S rRNA* gene. This gene was used as a molecular marker for recognizing *Staphylococcus* species because this interspace region between *23S rRNA* and *16S rRNA* genes contained sufficient variability for species recognition (Kosmeri *et al*., 2024).

Sequence analyses revealed that 31.5% of isolates of *S. aureus* and 15.7% of isolates of *S. intermedius* caused subclinical mastitis out of 19 buffaloes. *S. aureus* is a common pathogen associated with subclinical mastitis in both bovines and buffaloes (Nigam, 2015). Moreover, the prevalence rates of *S. aureus* with subclinical mastitis in buffaloes differ among countries: in Bangladesh from 37.4% to 72.7% (Hoque *et al*., 2022; Chowdhury *et al*., 2025), in Pakistan 56.12% (Ijaz *et al*., 2023), and in Egypt 26.9% (El-Ashker *et al*., 2015). The differences in the percentage of the infection rates among studies may depend on the type of diagnostic method (Leeflang *et al*., 2009).

S. intermedius group, which includes *S. intermedius*, *S. pseudintermedius*, and *S. delphini*. The infection of this group of bacteria primarily occurred in dogs (Bannoehr and Guardabassi, 2012), although *S. intermedius* group was documented in 5.7% of mastitis isolates from buffalo in Turkey (Pamuk *et al*., 2012). In the conducted study, 15.7% of isolates were *S. intermedius* because of this study designed to identify coagulase-positive *Staphylococcus*.

Phylogenetic analysis

Sequence analyses of nine *Staphylococcus* isolates revealed that 31.5% and 15.7% isolates were recognized as *S. aureus* and *S. intermedius*, respectively. The phylogenetic tree of *S. aureus* (Figure 2) revealed that the Iraqi isolates, Iraq 1 (PV628203.1) and Iraq 2 (PV628204.1), are closely related and have genetic distance about 0.0075. Also, Iraq 4 (PV628206.1) closely clustered to isolate CP141506.1 (human MRSA strain from Kenya) with a low genetic distance and substitution per site (0.0073), suggesting that this isolate had a zoonotic potential and antibiotics resistant capability, and *S. aureus* strain (CP134620.1) of cow mastitis from Spain. Also, Iraq1 and Iraq2 *S. aureus* isolates were close to human MRSA isolates from Pakistan. The Iraqi *S. aureus* isolates Iraq 5 (PV628207.1) and Iraq 3 (PV628205.1) are clustered in same clade with CP140671.1 (the mupirocin-resistant variant from Belgium), ON909005.1 (otitis in cat from Iraq), and CP102952.1 (wild animals in Nepal) with a genetic distance of approximately 0.0069. The isolate PV341350.1 from sheep milk in Iraq is very genetically similar to Egyptian isolate MG372745.1 (cow mastitis), forming a unique lineage when compared to Iraqi isolates from buffaloes.

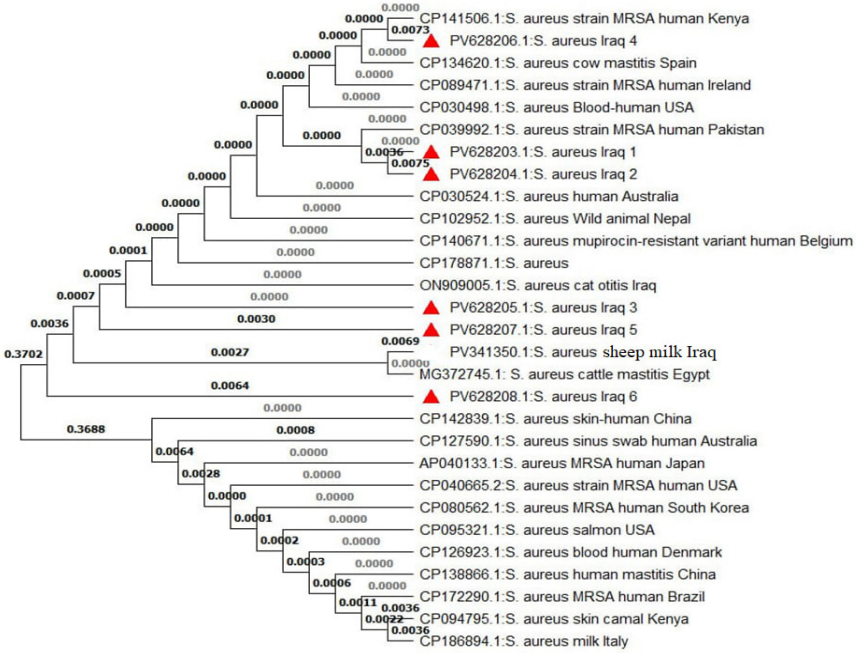

Figure 2: Phylogenetic tree of the partial sequence of the *23s rRNA* gene of *S. aureus* in buffaloes, showing genetic distance and substitution per-site between isolates.

The phylogenetic tree reconstruction depends on a substitution-per-site model. Two isolates of *S. aureus* in buffaloes, Iraq 1 and Iraq 2 with 0.0075 genetic distance, reflected that these isolates originated from the same clonal lineage. This result is consistent with other finding documenting that *S. aureus* may adapt as a dominant clone in animals or humans (Howden *et al*., 2023). Also, some isolates in the present study was genetically close to Iraqi isolate from cat otitis. This result reflected that cats may act as reservoirs for *S. aureus*, and can spread these strains to the livestock (Khairullah *et al*., 2023). Moreover, some Iraqi isolate of *S. aureus* in buffaloes clustered with subclade of strains from human, wild animal, and cow mastitis. This result suggests the broad host adaptability of *S. aureus* (Matuszewska *et al*., 2020). Also, some Iraqi isolates of buffaloes in the conducted study were close to MRSA isolates from human, Selim *et al*. (2025) revealed that the 61.9% out of 168 *S. aureus* isolates were recognized as MRSA according molecular confirmation.

The phylogenetic tree of *S. aureus* in the conducted study showed some isolates close with strains from wild animals in Nepal (Monecke *et al*., 2022). This finding highlights that wildlife may serve as a reservoir for *S. aureus* and the potential for zoonotic transmission. The genetic clustering between some Iraqi and the Egyptian *S. aureus* isolates indicated the regional circulation of *S. aureus* lineages in the Middle East (Montelongo *et al*., 2022). This finding suggests that there are similar genetic and pathogenic characters for *S. aureus* for both cattle and buffalo, and possibly this bacterium crosses the border.

The phylogenetic analysis of *S. intermedius* Iraqi isolates and global strains (Figure 3) revealed that *S. intermedius* Iraq 1 (PV628211.1) and Iraq 2 (PV628212.1) were clustered together in the same clade with no genetic distance between them (0.0000). While *S. intermedius* Iraq 3 (PV628213.1) is forming a separate branch with a substitution rate of 0.0074 when compared to *S. intermedius* Iraq 1 and Iraq 2. Interestingly, all three Iraqi isolates were close to same area of Netherlands *S. intermedius* strains MF678886.1 and MK015768.1 from human, which represent *16S-23S intergenic spacer sequences*. Most other reference isolates in the phylogenetic tree belong to *S. pseudintermedius* of canine sources with various lesions in the ear, wound, and skin, and various countries (e.g., Grenada, Kenya, Australia, South Korea, Thailand, USA, and Germany). Also, one Iraqi *S. pseudintermedius* isolate, PQ390244.1, from another study was clustered with other *S. pseudintermedius* strains. The association between Iraqi strains in the current study with human Netherlands strains supported the human source of *S. intermedius* in buffalo in Iraq.

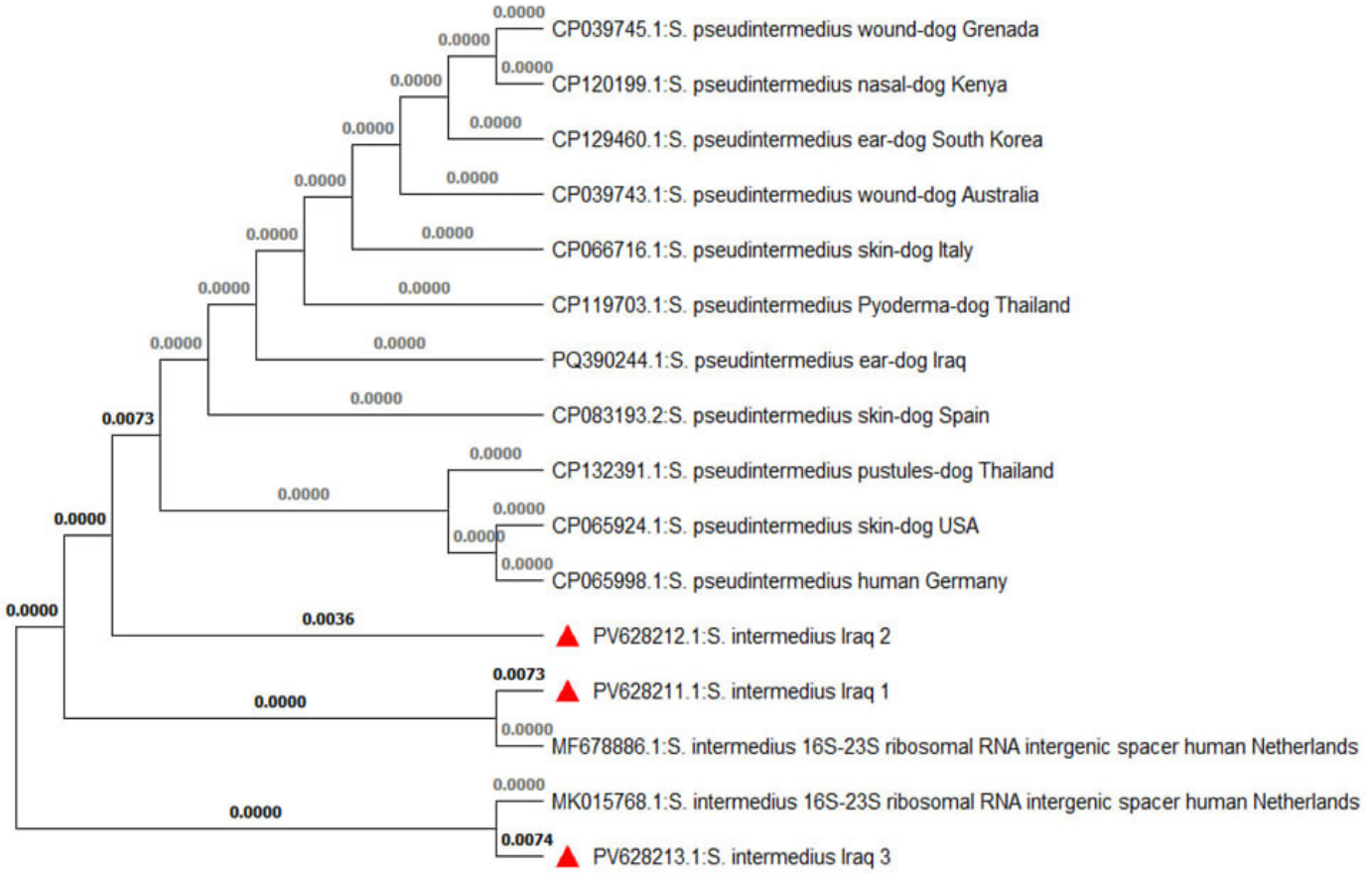

Figure 3: Phylogenetic tree of the partial sequence of the *23s rRNA* gene of *S. intermedius* in buffaloes showing genetic distance and substitution per-site between isolates.

Pathogenic *S. intermedius* may be a primary cause of mastitis in humans and cattle on dairy farms. Out of 290 lactating cows in Gondar City, Addis Ababa, *S. intermedius* was isolated from 33.3% of milk samples, and the pathogenic role of *S. intermedius* and its contribution to the mastitis occurrence were established (Getahun *et al*., 2025).

Staphylococcus intermedius in the phylogenetic tree of the conducted study clustered with strains from canine and human sources. This result indicates that dogs may act as a source of infection to buffaloes. Many studies have demonstrated that dogs serve as a reservoir for *S. intermedius* (Štempelová *et al*., 2022; Hisirová *et al*., 2025). Iraq 3-*S. intermedius* isolate grouped with strains of human origin. This result of Iraq 3 isolate exhibited host specialization, as it originated from human hosts. *S. intermedius* rarely infects to humans but, when it developed virulence factors, becomes a serious infection (Yarbrough *et al*., 2018; Moses *et al*., 2023). *S. intermedius* in buffalo milk could represent emerging public health concerns; further studies and epidemiological investigations are important for understanding these pathogens.

CONCLUSION AND RECOMMENDATIONS

Staphylococcus spp. are a major cause of subclinical mastitis in buffaloes, particularly *S. aureus*. *S. intermedius* has an emerging role in buffalo mastitis. Phylogenetic analysis showed a genetic relationship between buffalo and human or pet strains from other countries, indicating zoonotic potential or the role of the pets as a reservoir. Detection of *S. intermedius* raises concerns about the impact of buffalo milk contamination on public health. Based on the results of the study, it is recommended to use the molecular assays in buffalo farms for accurate diagnosis of mastitis-causing pathogens, especially *Staphylococcus* species. Furthermore, the zoonotic potential and antimicrobial resistance, and virulence genes of *Staphylococcus* species in dairy buffaloes should be investigated.

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NOVELTY STATEMENT

The novelty of this study is rooted in the molecular characterization of *Staphylococcus* spp. isolated from subclinical mastitis in buffaloes and highlight the concerns about the impact of buffalo milk contamination on public health.

AUTHOR'S CONTRIBUTION

Naseir Mohammed Badawi completed all diagnostic tests, designed the study, and reviewed the manuscript. Jinan Sahib Abdulnabi Al-Naseri collected the milk samples and bacterial identification. Amanee Mohammed Radhy interpreted the results and prepared the manuscript.

Generative AI and AI-assisted technology statement

The authors declare that were no used artificial intelligence or AI technologies in the creating of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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