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Phenotypic and Molecular Evaluation for Accurate Differentiation of Staphylococcus Aureus and Staphylococcus Xylosus Under Standardized Laboratory Conditions

Salman Nawaz*[1], Arsalan Ahmad[2], Mustaqeem Khan[2], Idowu Taiwo Oluwafemi[3], Muhammad Ismail[2]

[1] Department of Biology, Ecology and Earth Sciences, DiBEST, University of Calabria, Italy, Corresponding Author's Email: salmannawazsahir@gmail.com,
[2] Sarhad Institute of Allied Health Sciences, Sarhad University of Science & IT, Peshawar, Pakistan, Email: arsalantechnologist@gmail.com, pathologist.a1@gmail.com, muhammadismail17004@gmail.com
[3] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Italy, Email: idowutaiwo994@gmail.com

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Abstract

The genus *Staphylococcus* comprises a diverse group of Gram-positive bacteria that include both major human pathogens and commensal species. Among them, *Staphylococcus aureus* is a clinically significant pathogen, whereas *Staphylococcus xylosus* is a coagulase-negative species that is generally non-pathogenic. Due to shared phenotypic traits and occasional ambiguity in molecular assays, accurate discrimination between these species remains challenging in routine microbiological diagnostics. This study aimed to comparatively evaluate phenotypic and molecular methods for reliable differentiation of *S. aureus* and *S. xylosus* under standardized laboratory conditions. Under strictly controlled laboratory conditions, eleven *S. aureus* reference strains and three *Staphylococcus xylosus* isolates were examined using a tiered diagnostic approach. Phenotypic characterization was performed on Columbia blood agar, assessing colony morphology, pigmentation, and hemolysis. Species confirmation was evaluated using latex agglutination targeting clumping factor and Protein A, followed by molecular confirmation via endpoint PCR targeting the *femB* gene. PCR results were interpreted based on the presence of a clear ~120 bp amplicon reproducible across duplicate runs. Diagnostic performance of phenotypic methods was assessed using *femB* PCR as the reference criterion, calculating sensitivity, specificity, predictive values, accuracy, exact 95% confidence intervals, and Cohen's kappa for inter-method agreement. All *S. aureus* strains produced uniformly golden-pigmented, β-hemolytic colonies, whereas all *S. xylosus* isolates were non-pigmented and non-hemolytic. Latex agglutination yielded strong positive reactions in all *S. aureus* strains (11/11) and remained negative for all *S. xylosus* isolates (0/3). *femB* PCR generated strong, reproducible ~120 bp amplicons in all *S. aureus* strains, while *S. xylosus* isolates exhibited only very faint, non-reproducible bands near the expected size, interpreted as non-specific amplification or partial sequence homology with related CoNS genes. Both colony morphology and latex agglutination demonstrated 100% sensitivity and 100% specificity relative to PCR classification, with perfect agreement ($\kappa = 1.00$). When applied under standardized laboratory conditions, colony morphology and latex agglutination provide highly reliable phenotypic discrimination between *S. aureus* and *S. xylosus*, while *femB* PCR serves as a robust confirmatory tool. The observation of faint, non-reproducible *femB*-like signals in *S. xylosus* highlights the importance of cautious molecular

interpretation and supports an integrated phenotypic-molecular diagnostic strategy for accurate species-level identification.

Keywords: Staphylococcus Aureus; Staphylococcus Xylosus; Coagulase-Negative Staphylococci; Colony Morphology; Hemolysis; Latex Agglutination; femb PCR; diagnostic Accuracy; Cohen's Kappa; Standardized Conditions

Introduction:

The genus *Staphylococcus* was first described by Ogston in 1883 and later classified by Rosenbach in 1884 on the basis of colony pigmentation and pathogenic potential (Götz et al., 2006). Members of this genus are Gram-positive, non-motile, catalase-positive cocci that occur singly, in pairs, or in irregular clusters resembling bunches of grapes (Becker, Ballhausen, et al., 2014). They are facultative anaerobes widely distributed in nature and form part of the normal microbiota of human and animal skin and mucous membranes (Becker, Ballhausen, et al., 2014). More than sixty *Staphylococcus* species have been identified to date, most of which exist as commensals, though several can act as opportunistic or pathogenic organisms under favorable conditions (Battaglia & Garrett-Sinha, 2023) (Becker, Ballhausen, et al., 2014). On the basis of their ability to clot plasma, *Staphylococcus* species are divided into coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS) (Becker, Heilmann, et al., 2014). Among these, *Staphylococcus aureus* is the most significant CoPS species and a major human pathogen, whereas *Staphylococcus xylosus* represents one of the non-pathogenic or opportunistic CoNS species (Thomson et al., 2022) (Touaitia et al., 2025). *S. aureus* is among the most clinically important bacterial pathogens, responsible for a broad range of infections ranging from superficial skin abscesses to life-threatening diseases such as pneumonia, osteomyelitis, endocarditis, and septicemia (Touaitia et al., 2025) (De Visscher et al., 2014). The organism's pathogenic success is attributed to a combination of virulence factors that facilitate colonization, immune evasion, and tissue invasion, including Protein A, coagulase, hemolysins, and leukocidins, as well as a diverse repertoire of toxins and enzymes (Balaban & Rasooly, 2000). The global emergence of methicillin-resistant *S. aureus* (MRSA) further emphasizes the importance of its rapid and reliable identification in diagnostic laboratories and infection-control programs (De Visscher et al., 2014).

On blood agar, *S. aureus* generally produces golden-yellow, smooth, circular colonies surrounded by clear zones of β-hemolysis (M, 2018). However, hemolytic activity and pigmentation can vary significantly among strains and are influenced by environmental and nutritional factors (Becker, 2023). Under certain conditions, the bacterium may form small colony variants (SCVs), a slow-growing, non-pigmented, and often non-hemolytic phenotypes associated with persistent infections and diagnostic ambiguity (Becker, 2023). Such variability complicates species confirmation when laboratories rely solely on morphology and traditional biochemical characteristics. *Staphylococcus xylosus* is a coagulase-negative species belonging to the *S. saprophyticus* cluster group (Kloos et al., 1976). It occurs as a common commensal on animal skin and mucosa and is also frequently isolated from fermented foods such as sausages, cheeses, and meat products, where it contributes to nitrate reduction and flavor formation (Vela et al., 2012) (Leroy et al., 2017). Although generally regarded as non-pathogenic, *S. xylosus* has occasionally been implicated in opportunistic infections, including bovine mastitis, wound infections, and prosthetic-device contamination, particularly in immunocompromised hosts (Leroy et al., 2017). Despite clear differences in pathogenic potential, *S. aureus* and *S. xylosus* share several phenotypic traits such as Gram-positive coccal morphology, catalase positivity, tolerance to high NaCl concentrations, and facultative anaerobic growth (Quintieri et al., 2018) (Becker, Ballhausen, et al., 2014). Because of these similarities, phenotypic overlap may lead to diagnostic confusion, especially in mixed cultures or environmental samples (Vela et al., 2012).

Accurate differentiation between *S. aureus* and *S. xylosus* is critical for patient management, epidemiological surveillance, and quality control in food and veterinary microbiology. Classical identification methods such as colony morphology, pigmentation, hemolysis pattern, and biochemical reactions remain widely used but are often insufficient for conclusive identification (M, 2018) (Becker, 2023) (Vela et al., 2012). Variations in culture media, incubation conditions, or strain physiology can produce inconsistent results (Proctor et al., 2006) (Hale, 1947) (Becker, 2023). Some *S. aureus* isolates show weak or delayed coagulase activity, while certain *S. xylosus* strains may yield false-positive coagulase reactions, further complicating the diagnostic process (Proctor et al., 2006) (Hale, 1947) (Becker, 2023).

The latex agglutination test is one of the most practical and rapid phenotypic tools for confirming *S. aureus* identity. This assay detects clumping factor (bound coagulase) and Protein A on the bacterial surface, both of which are typically abundant in *S. aureus* but absent in most coagulase-negative species (Essers & Radebold, 1980) (França et al., 2021). When present, these surface molecules cause visible clumping within seconds, providing a strong positive result. Despite its convenience, false-negative results have been documented in certain MRSA strains due to reduced antigen expression (Davies et al., 2008), Conversely, weak or non-specific agglutination has occasionally been observed among CoNS, including *S. xylosus*, highlighting the necessity for molecular confirmation (Becker, Heilmann, et al., 2014). Molecular identification techniques based on polymerase chain reaction (PCR) offer superior specificity and reproducibility compared to phenotypic assays (Moraes et al., 2013). Several genes have been investigated as diagnostic targets for *S. aureus* including *femA* and *femB* (Rohrer et al., 1999). The *femB* gene, which encodes a factor essential for peptidoglycan synthesis and methicillin resistance expression, is highly conserved among *S. aureus* isolates but absent in most coagulase-negative staphylococci (Rohrer et al., 1999). PCR amplification of *femB* yields a distinct band that serves as a reliable marker for *S. aureus* identification. When used alongside phenotypic tests, *femB* PCR significantly increases diagnostic confidence and reduces the likelihood of misclassification (Davies et al., 2008) (Jonas et al., 2002). Although these diagnostic methods are well established, few studies have systematically compared the performance of phenotypic and molecular assays under strictly standardized laboratory conditions. Most available data originate from clinical isolates that vary in morphology, antigen expression, or genetic stability, introducing uncontrolled variability (Bernardy et al., 2020) (Burford-Gorst & Kidd, 2024) (Gómez-González et al., 2010). The use of authenticated reference strains minimizes such inconsistencies and enables the evaluation of true diagnostic specificity and reproducibility. Reference-strain-based benchmarking is especially valuable for validating protocols and establishing performance standards across laboratories (*Certified Reference Materials*, n.d.).

The present study was therefore conducted to evaluate the diagnostic reliability of standard phenotypic and molecular techniques including colony morphology, latex agglutination, and *femB*-targeted PCR for confirming *S. aureus* identity and excluding *S. xylosus* under controlled laboratory conditions. Eleven *S. aureus* reference strains and three *S. xylosus* reference isolates were examined under identical experimental parameters. The study aimed to: (i) characterize phenotypic differences between *S. aureus* and *S. xylosus* reference strains; (ii) assess the diagnostic specificity of latex agglutination; and (iii) validate *femB* PCR as a confirmatory molecular test. By integrating phenotypic and molecular evaluations, this study provides a standardized comparison of diagnostic accuracy and inter-method concordance. The findings are expected to support diagnostic standardization and enhance the reliability of *S. aureus* identification in both clinical and research microbiology laboratories.

Materials and Methods

Bacterial Strains and Culture Conditions

A total of fourteen *Staphylococcus* isolates were analyzed in this study, comprising eleven *Staphylococcus aureus* and three *Staphylococcus xylosus* strains. The *S. aureus* isolates consisted of well-characterized laboratory reference strains such as COL, USA300, SA113, SH1000, HG001, NCTC 8325 RNI, DSM20491, DSM20372, Newman, T002, T003 obtained from established reference collections. In contrast, the *S. xylosus* isolates were not sourced from reference collections; instead, they were recovered from laboratory mice housed at the Institute of Medical Microbiology and Hospital Hygiene, University of Marburg, Germany.

All isolates were routinely cultured on Columbia blood agar (Becton Dickinson GmbH) supplemented with 5% defibrinated sheep blood. The plates were aerobically incubated for 24-48 hours at 37 °C. Following incubation, colony morphology including size, pigmentation, margin, elevation, and hemolysis type was recorded for each strain.

Colony Morphology and Hemolysis Observation

Morphological differentiation between *S. aureus* and *S. xylosus* was assessed based on colony color, size, opacity, and hemolytic characteristics on blood agar. After incubation, bacterial colonies displaying characteristic morphology of *Staphylococcus aureus* and *Staphylococcus xylosus* were carefully selected for subsequent analyses. Each batch, consisting of one clinical *S. xylosus* batch and a batch of eleven reference *S. aureus* strains, was processed independently to ensure experimental consistency and accuracy. Each strain was sub cultured twice before final evaluation to ensure phenotypic consistency.

Latex Agglutination Test

Latex agglutination testing was performed using the StaphAurex™ Latex Kit (Bio-Rad Laboratories, USA), following the manufacturer's instructions. The assay detects two surface antigens specific to *S. aureus*, clumping factor (bound coagulase) and Protein A through visible agglutination of latex particles. A single drop (approximately 25 µL) of the latex reagent was placed on a sterile, clean agglutination card. Using a sterile inoculating loop, 2–3 well-isolated colonies from freshly incubated Columbia Blood Agar plates were transferred and thoroughly mixed with the reagent drop to form a uniform suspension. The card was gently rocked for 30 seconds, and the reaction was observed against a white background under adequate illumination. A visible clumping reaction within 30 seconds was recorded as positive, while smooth, milky suspension without clumping was recorded as negative. A known *S. aureus* reference strain (COL) served as the positive control, and *S. xylosus* was used as the negative control. To ensure reproducibility, every assay was run in duplicate.

Genomic DNA Extraction

Genomic DNA was extracted directly from freshly grown colonies collected from Columbia Blood Agar plates incubated at 37 °C, following standard *staphylococcal* DNA extraction procedures. For the mechanical disruption method, sterile tubes were prepared by adding 0.1-millimeter glass beads and 750 µl of sterile nuclease-free water. Bacterial colonies were collected using a sterile loop and suspended by swirling in the bead-containing solution. The tubes were then placed in a MagNALyzer and subjected to mechanical lysis at a speed of 70,000 rpm for 2 minutes. Following lysis, the samples were centrifuged at 8000 rpm for ten minutes to separate the lysate from the debris. Approximately 500 µl of the supernatant was transferred into freshly labeled tubes and stored at 4°C. DNA concentration and purity were determined spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (Peqlab GmbH). Before being used again, all DNA extracts were kept at -20 °C.

PCR Amplification of the *femB* Gene

Species confirmation of *S. aureus* was performed through PCR targeting the *femB* gene, using primer sequences described previously by (Klotz et al., 2003).

No.	Name	Sequence (5' → 3')
1	*FemB* fw	AATTAACGAAATGGGCAGAAACA
2	*FemB* rev	TGCGCAACACCCTGAACTT

Table:1. *Overview of the primers used*

PCR reactions were carried out in a total volume of 25 µL containing 2.5 µL of 10× PCR buffer, 2.5 µL $MgCl_2$, 0.5 µL each dNTP, 2.5 µM of each primer, 0.25 µL of Taq DNA polymerase (New England BioLabs), and 5 µL of DNA template. The final volume was achieved by adding Nuclease-free water. Amplifications were performed in an Eppendorf Mastercycler Gradient thermocycler under the following conditions:

Step	Temp	Time
Denaturation	94°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	1 min
Number of Cycles	35	--
Final Extension	72°C	10 min
Hold	4°C	∞

Table: 2. *PCR Thermocycling Programs for Target Gene Fragments*

Amplicons were analyzed by electrophoresis on 1.5% (w/v) agarose gels prepared in 1× TAE buffer and run at 120 V for 45–60 minutes. Gels were stained with GelRed™ during preparation and visualized under UV transillumination. A 100-bp DNA ladder (Thermo Fisher Scientific) was used as the molecular-weight marker. The expected *femB* amplicon size was approximately ~120 bp. Positive controls (*S. aureus* COL) and non-template controls (*water*) were included in each PCR run. All PCR assays were repeated twice to verify consistency of results.

Data Interpretation and Statistical Analysis

Diagnostic outcomes were recorded for each strain as categorical variables: (i) colony phenotype classified as *S. aureus*-like versus CoNS-like morphology, (ii) latex agglutination positive or negative, and (iii) *femB* PCR band interpretation. For *femB* PCR, a reaction was considered positive only when a clear band at ~120 bp was observed and reproducible across duplicate PCR runs. Very faint, non-reproducible signals near ~120 bp were classified as non-specific or indeterminate and were not considered true-positive amplification, consistent with possible partial homology or non-specific primer binding in CoNS. Colony morphology and latex agglutination were each evaluated as diagnostic screening tests using PCR classification (strong, reproducible *femB* amplification) as the reference classification criterion. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy were calculated for both phenotypic methods, with exact (Clopper–Pearson) 95% confidence intervals (CI).

Agreement between phenotypic methods and PCR classification was assessed using Cohen's kappa (κ). Statistical analyses were performed using Microsoft Excel (Microsoft Corp., USA).

Results

Colony Morphology and Phenotypic Differentiation

All *Staphylococcus aureus* reference strains (n = 11) exhibited consistent and characteristic colony morphology on Columbia blood agar following 24–48 hours of incubation at 37 °C. Colonies were uniformly circular, convex, smooth-edged, and showed the typical golden-yellow pigmentation attributed to staphyloxanthin production. Hemolysis assessment revealed clear and complete β-hemolytic zones surrounding the colonies for all *S. aureus* strains tested, confirming their hemolytic phenotype under standardized culture conditions (Shown in Figure 1). In contrast, the *Staphylococcus xylosus* reference isolates (n = 3) produced colonies that were substantially smaller (0.5–1.5 mm), smooth, and either off-white or translucent. No hemolysis was observed for any of the *S. xylosus* isolates, consistent with a γ-hemolytic profile typical of CoNS species (Shown in Figure 1). These observations clearly differentiated the species at the phenotypic level, with the absence of pigmentation and hemolysis serving as defining traits for *S. xylosus* under identical incubation conditions.

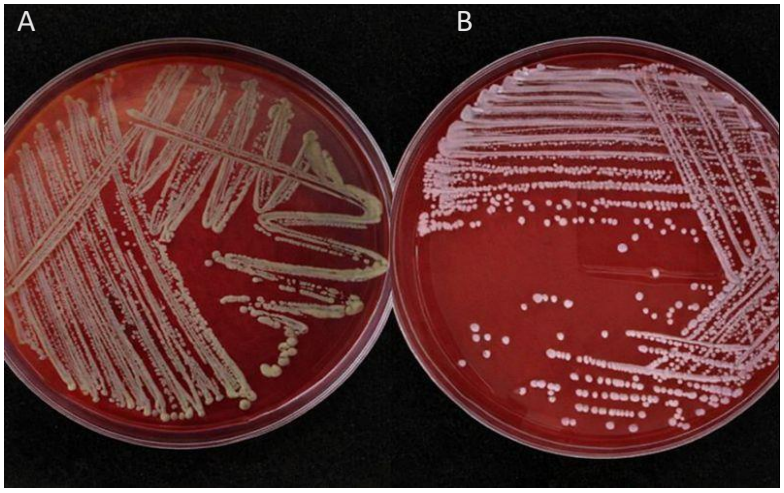

***Fig: 1**. Blood agar culture of Staphylococcus aureus and Staphylococcus xylosus strains.*

A) S. aureus reference strain HG001 showing golden-yellow pigmentation and ß hemolysis. B) Clinical S. xylosus strain showing off-white coloration and absence of hemolysis.

Across all replicate cultures, neither species exhibited morphological variability. The *S. aureus* group displayed complete consistency in colony size, pigment intensity, and β-hemolysis pattern, whereas *S. xylosus* consistently produced non-pigmented, non-hemolytic colonies.

Using predefined morphological criteria, colony morphology correctly classified all strains under standardized conditions. Morphology demonstrated 100% sensitivity (11/11; 95% CI: 71.5–100%) and 100% specificity (3/3; 95% CI: 29.2–100%) for distinguishing *S. aureus* from *S. xylosus*. PPV, NPV, and overall accuracy were each 100%**,** and agreement between morphology and PCR classification was perfect (Cohen's κ = 1.00).

Latex Agglutination Assay Performance

Latex agglutination testing using the StaphAurex™ kit provided a rapid and highly discriminatory phenotypic confirmation method. All eleven *S. aureus* reference strains yielded strong, rapid, and distinct agglutination reactions, visible within 10–15 seconds of mixing, indicating the presence of Protein A and clumping factor, two hallmark surface antigens of *S. aureus*. In contrast, each of

the three *S. xylosus* isolates produced no agglutination, resulting in 0% positivity. All negative controls also remained non-reactive, confirming the absence of nonspecific clumping and supporting the assay's high specificity under the test conditions. Latex agglutination demonstrated 100% sensitivity (11/11; 95% CI: 71.5–100%) and 100% specificity (3/3; 95% CI: 29.2–100%) for discrimination of *S. aureus* from *S. xylosus* under standardized conditions. PPV and NPV were both 100% (PPV 11/11; 95% CI: 71.5–100%; NPV 3/3; 95% CI: 29.2–100%), and overall accuracy was 100% (14/14; 95% CI: 76.8–100%). Agreement between latex agglutination and PCR classification was perfect (Cohen's $\kappa = 1.00$).

DNA Extraction Yield and Quality

Mechanical disruption using the MagNA Lyser system produced high DNA yields for all reference *S. aureus* and *S. xylosus* strains. DNA concentrations for *S. aureus* reference strains ranged widely but consistently achieved acceptable yields (e.g., COL 317.4 ng/µL, USA300 456.6 ng/µL, HG001 478.5 ng/µL) as shown in Table 3.

Strain	**Colony Size**	**DNA (MagnaLyser) Extraction Yield [ng/µl]**
COL	Large	317.4
USA300	Large	456.6
SA113	Large	255.3
SH1000	Large	468.2
HG001	Large	478.5
Strain	**Colony Size**	**DNA (MagnaLyser) Extraction Yield [ng/µl]**
8325 RN1	Large	336.2
DSM20491	Large	349.6
DSM20372	Large	243.8
Newman	Large	346.7
T002 LT3391-02 Rhein hessen	Large	419.4
T003 LT2981-04 Rhein hessen	Large	388.3

Table:3. *DNA Concentration of Reference Staphylococcus aureus Strains Extracted by Mechanical Disruption*

Similarly, *S. xylosus* isolates yielded DNA concentrations between 243.8 ng/µL and 475.4 ng/µL, comparable to *S. aureus* yields and suitable for downstream molecular assays as shown in Table 4.

Strain	Isolation Site	DNA (MagnaLyser) Extraction Yield [ng/µl]
S.xylosus 1	Nasal	243.8
S.xylosus 2	Gut	472.7
S.xylosus 3	Nasal	475.4

Table 4: *DNA Concentration of Staphylococcus xylosus Clinical Isolates Extracted by Mechanical Disruption*

Across *S. aureus* reference strains (n=11), DNA yields had a mean ± SD of 369.1 ± 81.0 ng/µL (median 349.6; range 243.8–478.5 ng/µL). *S. xylosus* isolates (n=3) yielded DNA concentrations with a mean ± SD of 397.3 ± 132.9 ng/µL (median 472.7; range 243.8–475.4 ng/µL). These yields were sufficient for standardized template input (50 ng/µL) across all PCR reactions.

***femb* PCR Amplification for Species Confirmation**

PCR amplification targeting the *femB* gene yielded distinct differentiation between the two species. All eleven *S. aureus* reference strains produced strong, sharp bands of approximately 120 bp, consistent with the expected amplicon size and confirming the presence of *femB* (Shown in Fig 2). No non-specific products or primer-dimer artifacts were observed in these reactions, indicating high primer specificity and robust template quality. By contrast, all three *S. xylosus* isolates exhibited only very faint bands near the 120 bp region. These faint bands were significantly weaker than those from *S. aureus* and were not consistently reproducible. No-template controls remained negative throughout all runs, excluding contamination as a source of these signals (Shown in Figure 2). These faint bands may be due to non specific primer binding or partial homology with related CoNS genes.

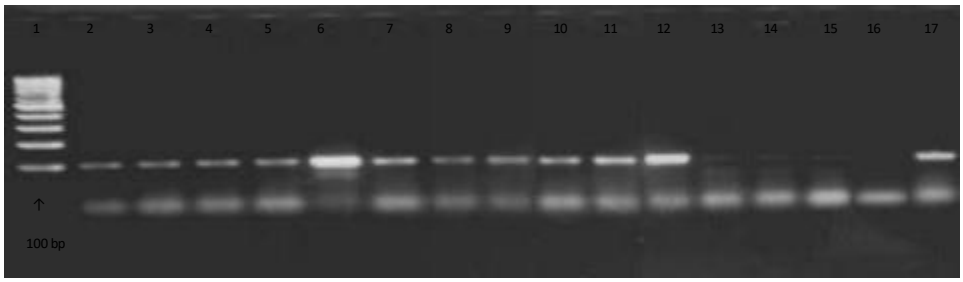

Fig: 2.: The gel image shows the amplicon of the femB subunit of S. aureus and S. xylosus

Gel electrophoresis image of the femB amplicons obtained by PCR from Staphylococcus aureus reference strains (lanes 2–12) and Staphylococcus xylosus (lanes 13–15). A 100 bp DNA ladder was applied as a size standard (lane 1). Sterile H_2O was used as a negative control (lane 16), and a clinical S. aureus strain served as the positive control (lane 17). The bands of the femB subunit are located near the 120 bp marker.

Using the predefined interpretation criterion (clear ~120 bp band reproducible across duplicate PCR runs), *femB* PCR showed 100% positivity in S. aureus (11/11) and 0% reproducible positivity in S. xylosus (0/3). Although faint ~120 bp signals were observed in all *S. xylosus* isolates, these were weak and not consistently reproducible, and no-template controls remained negative, supporting non-specific amplification consistent with partial homology or non-specific primer binding.

Overall Diagnostic Concordance

A combined assessment of colony morphology, latex agglutination, and *femB* PCR demonstrated complete concordance across all 14 strains. All *S. aureus* strains produced golden β-hemolytic colonies, strong latex agglutination, and strong reproducible *femB* amplicons. All *S. xylosus* isolates produced non-pigmented, non-hemolytic colonies, no latex agglutination, and no reproducible *femB* amplification, although very faint, inconsistent bands near ~120 bp were occasionally observed. Collectively, the integration of phenotypic screening with confirmatory molecular criteria provided clear and reproducible species-level discrimination under standardized laboratory conditions.

Discussion

Accurate differentiation between *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) remains a central priority in diagnostic microbiology, with significant implications for patient management, epidemiological tracking, and laboratory quality assurance. In this study, we evaluated three routinely used diagnostic approaches, colony morphology, latex agglutination, and *femB* PCR using eleven *S. aureus* reference strains and three *S. xylosus* strains under standardized laboratory conditions. The findings demonstrate that when applied together, these assays provide highly reliable species-level identification. However, each method also carries limitations that warrant consideration, particularly when strains exhibit atypical phenotypes or borderline molecular responses. These outcomes align with previously published literature regarding staphylococcal identification challenges and support continued integration of phenotypic and molecular tools for robust species confirmation (Becker, 2023c) (Karmakar et al., 2016) (Becker, 2023). The phenotypic assessment performed in this study confirmed the classic macroscopic traits attributed to *S. aureus*. All eleven reference strains displayed uniformly golden-yellow pigmentation and complete β-hemolysis when cultured on blood agar, consistent with descriptions by earlier taxonomic and clinical reports (M, 2018). The stability of these traits under controlled conditions reiterates the phenotypic homogeneity of *S. aureus* and highlights the value of colony morphology as an initial screening tool. In sharp contrast, all *S. xylosus* isolates formed smaller, off-white to translucent colonies lacking hemolysis, characteristic of a γ-hemolytic CoNS profile. These results support observations in previous studies that *S. xylosus* rarely produces pigments or hemolysins and typically forms small, non-hemolytic colonies (Vela et al., 2012). Such a clear phenotypic distinction in reference strains reinforces the diagnostic utility of colony morphology when strains grow under optimal conditions.

Nevertheless, although the reference strains in this study showed no phenotypic variability, the broader literature documents numerous cases where *S. aureus* does not express typical pigmentation or hemolysis. Environmental stress, nutrient limitation, and prolonged incubation may cause reduced pigment production or atypical colony size (Becker, 2023). Furthermore, small colony variants (SCVs), not included in this section of the study are particularly known for their slow growth, absence of pigment and reduced hemolysis (Becker, 2023). Therefore, while morphology proved reliable for the reference strains tested here, real-world conditions demand careful interpretation, especially when colonies exhibit atypical or mixed traits.

Latex agglutination provided a second, rapid confirmation tool and performed with exceptionally high accuracy in distinguishing the two species. All eleven *S. aureus* isolates produced strong, rapid agglutination, whereas all *S. xylosus* isolates remained negative. These findings directly reflect the biological basis of the assay, which detects clumping factor and Protein A, surface antigens abundantly expressed by *S. aureus* but absent from most CoNS (Essers & Radebold, 1980). The complete concordance observed here mirrors previous reports demonstrating near-perfect specificity and sensitivity in reference strains and clinical isolates with normal phenotypes (Niskanen et al., 1991). The lack of any weak or ambiguous agglutination in *S. xylosus* also

supports the assay's discriminatory power when applied under ideal conditions. Despite this strong performance, latex agglutination is known to exhibit limitations in certain scenarios. Several studies have documented false-negative results in MRSA strains due to undetectable amounts of clumping factor and protein A that reduce antigen availability (Davies et al., 2008). SCVs, again excluded from this analysis, may also exhibit weak or absent clumping due to down-regulation of surface protein expression (Sifri et al., 2006). Conversely, occasional weak agglutination has been reported in some CoNS species (Blake & Metcalfe, 2001). Some coagulase negative Staphylococcus spp. isolated from buffalo can be misidentified as Staphylococcus aureus by phenotypic analysis although such events were not observed in *S. xylosus* in this study. These limitations reinforce the need for complementary molecular testing, especially when phenotypic assays produce borderline or unexpected outcomes. Nonetheless, the complete species-level separation observed in this study strongly validates latex agglutination as a rapid and reliable diagnostic tool for *S. aureus* identification when used in controlled laboratory environments.

Molecular confirmation through *femB* PCR provided a third layer of diagnostic resolution and further supported the species-level distinction between the two test groups. All eleven *S. aureus* strains yielded strong and distinct ~120 bp *femB* amplicons, consistent with the gene's known conservation among *S. aureus* strains and its essential role in peptidoglycan biosynthesis. This high reproducibility is in line with established molecular diagnostic studies that consider *femB* one of the most reliable species-specific markers for *S. aureus* identification (Rohrer et al., 1999). The absence of non-specific amplification in the *S. aureus* reactions indicates both high primer specificity and good DNA template quality, which was ensured by the use of mechanical lysis for all extractions. The faint amplification bands observed in all *S. xylosus* isolates represent a more nuanced finding. Their significantly reduced intensity and inconsistent visibility suggest likely non-specific primer binding or partial sequence homology with related CoNS genes. Previous studies have noted that certain staphylococcal species share limited conserved regions near *fem* loci, which may contribute to such weak off-target amplification under low-stringency conditions (Vannuffel et al., 1999), (Alborn et al., 1996). Importantly, no-template controls were consistently negative, ruling out contamination as the source of these faint signals. This reinforces the idea that molecular cross-reactivity, though minimal, may still occur when targeting conserved structural genes. Given these findings, *femB* PCR can be considered a highly sensitive and reliable marker for confirming *S. aureus*, but its exclusivity may require cautious interpretation in rare ambiguous cases. In diagnostic laboratories, such faint or borderline amplicons especially in the absence of agglutination and characteristic morphology should prompt confirmatory sequencing or the use of additional molecular markers such as *nuc* or *femA* (Meng et al., 2020). Nevertheless, within the controlled reference context of this study, *femB* PCR correctly classified all *S. aureus* isolates and did not yield any strong false-positive signals in *S. xylosus*.

Taken together, the combined results from morphology assessment, latex agglutination, and *femB* PCR demonstrated complete concordance for all fourteen reference strains tested. Each method independently distinguished *S. aureus* from *S. xylosus* with high reliability, and none of the assays produced contradictory results under standardized conditions. This supports the utility of a tiered diagnostic approach in which morphology serves as the primary screen, latex agglutination provides rapid confirmation, and *femB* PCR establishes molecular certainty. Such a multimodal workflow aligns with the diagnostic rationale and is widely recommended in clinical microbiology guidelines (Brown et al., 2005). In conclusion, the data presented here demonstrate that colony morphology, latex agglutination, and *femB* PCR collectively provide a robust, reproducible, and complementary diagnostic framework for distinguishing *S. aureus* from *S. xylosus*. When applied under standardized laboratory conditions, these methods deliver high diagnostic accuracy and complete inter-method concordance, validating their continued use in routine microbiological identification. The study also underscores the importance of integrating phenotypic and molecular

tools to ensure precise species-level differentiation, especially in settings where accurate identification holds clinical or epidemiological significance.

Conclusion

This study demonstrates that the combined use of colony morphology, latex agglutination, and *femB*-targeted PCR provides a highly reliable and reproducible framework for differentiating *Staphylococcus aureus* from *Staphylococcus xylosus* under standardized laboratory conditions. All eleven *S. aureus* reference strains consistently expressed characteristic phenotypic traits, including golden pigmentation and β-hemolysis, while all *S. xylosus* isolates produced small, non-pigmented, non-hemolytic colonies. Latex agglutination showed complete specificity, yielding strong positive reactions only with *S. aureus* and no agglutination with any *S. xylosus* strains. Molecular confirmation through *femB* PCR further validated species identity, producing strong amplicons in all *S. aureus* strains and only faint, non-specific bands in *S. xylosus*.

The complete concordance observed between phenotypic and molecular assays highlights the diagnostic robustness of this three-tiered approach. These findings reinforce the importance of integrating classical and molecular methods, particularly when identifying staphylococcal species that share overlapping characteristics. By establishing clear diagnostic performance under controlled reference conditions, this study provides a valuable baseline for laboratory standardization, quality control, and method validation. The workflow presented here can be effectively applied in clinical, veterinary, and research laboratories to ensure accurate and consistent identification of *S. aureus* where precise species discrimination is essential.

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