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# DETECTION OF *STAPHYLOCOCCUS AUREUS* AMONG COAGULASE POSITIVE STAPHYLOCOCCI FROM ANIMAL ORIGIN BASED ON CONVENTIONAL AND MOLECULAR METHODS

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## ABSTRACT

The present study aimed to detect *Staphylococcus aureus* (*S. aureus*) among other coagulase positive staphylococci from animal origin by using conventional methods (biochemical tests and latex agglutination) and a molecular method, based on the *nuc* gene, as the gold standard and to assess the usefulness of these methods. For this purpose, total of 344 staphylococcal isolates were collected and analysed. A total of 156 isolates suspicious for *S. aureus* were detected by a conventional biochemical method – 88 from cows, 18 from goats, 7 from pigs, 17 from poultry, 7 from rabbits and 19 from dogs. The majority of *S. aureus* strains gave typical biochemical reactions with the exception of 30 (19.2%) and 25 (16%) that were VP negative and weak positive in fermenting mannitol, respectively. Twelve strains were found to be non-haemolytic (7.7%) and four strains did not ferment trehalose (2.6%). Other staphylococci were identified as *S. pseudintermedius* (n = 103), *S. hyicus* (n = 23) and the rest were coagulase-negative staphylococci. Latex agglutination test resulted in rapid positive reactions with *S. aureus* with exception of 5 strains (3.2%) from cow mastitis milk. Positive agglutination reactions were also established with *S. pseudintermedius*, and *S. hyicus*. PCR confirmed all strains that were preliminary identified as *S. aureus* by amplification of 270 bp fragment of *nuc* gene specific for this species. The atypical reactions in certain strains established in this study have shown that the precise detection of *S. aureus* from animal origin should be done by combination of conventional and molecular methods.

**Key words:** *Staphylococcus aureus*, biochemical method, latex agglutination, *nuc* gene, animals

## INTRODUCTION

*Staphylococcus* spp. are Gram-positive cocci arranged typically in clusters. The majority of them are facultative anaerobes and catalase-positive. Only two species, *Staphylococcus aureus* (*S. aureus*) subsp. *anaerobius* and *Staphylococcus saccharolyticus*, are anaerobes and might be catalase-negative (1).

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Among the forty-three staphylococcal species that have been described so far, seven species are known to exhibit coagulase-positive or variable reactions: *S. aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini* and *S. pseudintermedius* (2). It is accepted that coagulase production correlates well with the pathogenicity of these bacteria and the coagulase-negative staphylococci (CNS) are deemed as "minor pathogens" (3). Staphylococci are commensals in skin and mucous membranes of the upper respiratory and lower urogenital tract in animals and humans (4). Hence, infections caused by them are endogenous but their prolonged environmental storage, despite non-spore forming, suggests indirect infestation as well (5, 6).

*S. aureus* is one of the principal bacterial pathogens in humans causing mild to severe life-threatening conditions such as skin, soft tissue, wound infections, and septicaemia, urinary tract infections, endocarditis

and osteomyelitis as major sequelae (7, 8, 9). *S. aureus* is also an important pathogen in veterinary medicine. It has been reported as etiological agent of various infections in cattle, especially mastitis (10), in horses (11), dogs (12), cats (13), rabbits (14), poultry (15) and reptiles (16). Schaumburg et al. (17) and Monecke et al. (18) have also isolated *S. aureus* from non-human primates in Africa and from diseased hedgehogs in Sweden, respectively.

*S. aureus,* and other coagulase-positive staphylococci produce a wide range of virulence factors, including toxins, enzymes, biofilm and factors that counteract the immune system defense. These determinants are responsible for the pathogenesis of staphylococcal diseases (19). Enterotoxins (A-E) cause food poisoning in humans. SSSS-syndrome in newborns and dogs is due to the exotoxin exfoliatin. Exudative epidermitis in pigs and some skin diseases in humans and dogs are associated with epidermolytic toxins (6). Gangrenous mastitis is due to the α-haemolysin production. The exotoxin, Panton-Valentine leukocidin kills neutrophils and macrophages in cattle, rabbits and humans. The surface protein A of *S. aureus* blocks the specific binding of IgG class immunoglobulins. Enzymes produced by staphylococci include staphylokinase, coagulase, hyaluronidase, lipase, collagenase, proteases, nucleases and urease (6). Some virulence factors are important laboratory tools in detection of staphylococci. Thus, catalase is an enzyme leading to survival of bacteria in macrophages (20) but also the main conventional test for primary differentiation of staphylococci from streptococci. The tube coagulase test categorises staphylococci into two groups differing in pathogenicity as mentioned above. Staphylococcal nuclease hydrolyses DNA and RNA of host cell and thereby facilitates tissue spread. It is also a unique genetic marker for *S. aureus* detection in different clinical samples (21).

The aim of the present study was to detect *Staphylococcus aureus* among other coagulase positive staphylococci from animal origin by using conventional methods (biochemical and latex agglutination tests) and a molecular method, based on the *nuc* gene, as the gold standard and to assess the usefulness of these methods.

## MATERIAL AND METHODS

### *Bacterial strains*

A total of 344 *Staphylococcus* spp. isolates from cows (n=137), goats (n=27), pigs (n=30), birds (n=20), rabbits (n=12) and dogs (n=118) were included in the study. Isolates originated from various pathological conditions – mastitis milk, abscesses, skin lesions, otitis externa, infections on eyes, wounds, joints, urinary tract, rhinitis, oral cavity lesions and vaginitis. Samples were cultured on tripticase soy blood agar (TSA, Fluka, India supplemented with 5% defibrinated sheep blood) and on MacConkey agar (NCIPD, Sofia, Bulgaria). Plates were incubated at 37°C for 24-48 h under aerobic conditions. *S. aureus* ATCC 25922 was used as control strain in all experiments.

### *Biochemical tests*

The following biochemical tests and characteristics of bacterial strains were taken into consideration – Gram staining, catalase and oxidase tests, colony pigmentation, haemolysis, tube coagulase test (rabbit plasma supplied by NCIPD, Sofia, Bulgaria), VP test, ONPG (β-galactosidase, 4 mg disk, HiMedia, India), test with polymyxin B (300 unit disk, Oxoid, UK), acid from mannitol determined on mannitol salt agar (NCIPD, Bulgaria), utilisations of trehalose (NCIPD, Sofia, Bulgaria) and maltose (MkB Test, Rosina, Slovak republic). All tests were carried out according to the manufacturer's instructions and in compliance with the general bacteriology procedures.

### *Latex agglutination*

A Staphylococcus Rapid Latex Kit (Atlas Medical, Cambridge, UK) for the detection of *S. aureus* in cultures was used following precisely the instruction of the company.

### *PCR based on nuc gene*

DNA was extracted without using a commercial kit. Bacterial suspensions were prepared in sterile distilled water, boiled and centrifuged for 5 min at 12,000 g (22).

The concentration and purity of DNA extracts were determined by DNA/RNA spectrophotometer Gene Quant 1300 at A260/A280. The DNA extracts were stored at - 20ºC until the beginning of the trials. PCR was run as described by Brakstad et al. (23) with an expected amplicon size of 270 bp.

## RESULTS

Based on the biochemical methods, a total of 156 *Staphylococcus aureus* strains were presumptively detected among the studied 344 isolates (Table 1). The majority of *S. aureus* originated from cows with mastitis (n=88), 19 strains were isolated from dogs, 18 from goats and 17 from birds (chickens).

**Table 1**. Origin of isolates

| Animals | Origin of isolates (n) | | | | | | | | | | | S. aureus (n) | Other staph. (n) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | | |
| **Cows** | 132 | 5 | | | | | | | | | | 88 | 49 |
| **Goats** | 27 | | | | | | | | | | | 18 | 9 |
| **Pigs** | | 3 | 23 | | | | | | | | 4 | 7 | 23 |
| **Poultry** | | 3 | | | | | 17 | | | | | 17 | 3 |
| **Rabbits** | | 7 | | | | 2 | | | 3 | | | 7 | 5 |
| **Dogs** | | | 36 | 33 | 24 | 6 | | 8 | | 11 | | 19 | 99 |
| **Total** | | | | | | | | | | | | 156 | 188 |

Legend: n = number of isolates; 1 – mastitis milk, 2 – abscess, 3 – skin lesion, 4 – otitis externa, 5 – eye infection, 6 – wound infection, 7 – joint infection, 8 – urinary tract infection, 9 – rhinitis, 10 – oral lesion, 11 – vaginitis

Other staphylococci were determined as *S. pseudintermedius* (n=103), *S. hyicus* (n=23) and CNS (n=62) with biochemical tests (Table 2).

**Table 2.** Distribution of staphylococci other than *S. aureus* in examined samples

| Animals | S. pseudintermedius | S. hyicus | CNS |
|---|---|---|---|
| | (n) | | |
| **Cows** | 4 | | 45 |
| **Goats** | | | 9 |
| **Pigs** | | 23 | |
| **Poultry** | | | 3 |
| **Rabbits** | | | 5 |
| **Dogs** | 99 | | |

Legend: CNS=coagulase-negative staphylococci

Biochemical profiles of *S. aureus* and other important coagulase-positive/variable staphylococci are presented in Table 3. Most of the *S. aureus* strains (114/73%) showed biochemical reactions typical for the species compared to the control strain such as presence of β-haemolysis, positive tube coagulase test, production of acetoin (VP test), negative ONPG test, resistance to polymyxin B, acid from mannitol, trehalose and maltose. A total of 30 strains were VP negative (19.2%), 25 (16%) fermented mannitol weakly and 4 strains did not produce acid from trehalose (2.6%). Twelve strains were found to be non-haemolytic (7.7%). In twenty one strains (13.5%) and in 4 strains (2.6%) two and three atypical reactions were observed, respectively. The majority of S. *pseudintermedius* (58/56.3%) exhibit species-specific biochemical behaviour - β-haemolysis, positive tube coagulase test, negative VP test, positive ONPG test, susceptibility to polymyxin B, fermentation of mannitol, trehalose and maltose. Four *S. pseudintermedius* strains were gamma-haemolytic. Twenty three staphylococcal isolates from pigs were differentiated as *S. hyicus*.

**Table 3.** Key biochemical tests for clinically relevant staphylococci

| Species<br>n | Haemolysis | Coagulase | VP | ONPG | Polymyxin B | Mannitol | Trehalose | Maltose |
|---|---|---|---|---|---|---|---|---|
| *S. aureus n=8* | - • | + | + | - | R | + | + | + |
| *S. aureus n=4* | - • | + | + | - | R | ± • | - • | + |
| *S. aureus n=21* | + | + | - • | - | R | ± • | + | + |
| *S. aureus n=9* | + | + | - • | - | R | + | + | + |
| *S. aureus n=114* | + | + | + | - | R | + | + | + |
| *S. pseudintermedius n=7* | + | + | + • | + | S | ± | + | + |
| *S. pseudintermedius n=58* | + | + | - | + | S | + | + | + |
| *S. pseudintermedius n=4* | - • | + | - | + | S | ± | + | ± |
| *S. pseudintermedius n=23* | + | + | - | + | S | - | + | + |
| *S. pseudintermedius n=11* | + | + | - | + | S | - | + | ± |
| *S. hyicus n=7* | - | + | - | - | R | ± • | + | - |
| *S. hyicus n=7* | - | - | - | - | R | ± • | - • | - |
| *S. hyicus n=9* | - | - | - | - | R | - | + | - |

Legend: + = positive reaction, - = negative reaction, ± = weak positive reaction, atypical reactions are marked with a black dot

The results from the performed latex agglutination test are shown in Table 4. The test led to rapid positive result (up to 10 s) with almost all *S. aureus* strains tested with the exception of 5 strains from cows with mastitis (3.2%). Most of *S. pseudintermedius* strains also gave a rapid agglutination (n=62), 14 strains were positive within 1 min and 27 were negative. Agglutination was observed with some *S. hyicus* strains as well.

**Table 4.** Latex agglutination with coagulase positive/variable staphylococci

| Species | Latex agglutination (n) | | |
|---|---|---|---|
| | Rapid positive | Late positive | Negative |
| *S. aureus* | 151 | - | 5 |
| *S. pseudintermedius* | 62 | 14 | 27 |
| *S. hyicus* | 12 | 4 | 7 |

PCR assay performed with strains biochemically identified as *S. aureus* resulted in amplification of *nuc* gene fragments with an expected molecular size of 270 bp. Specific amplicons with other coagulase positive/variable staphylococci were not observed (Fig. 1).

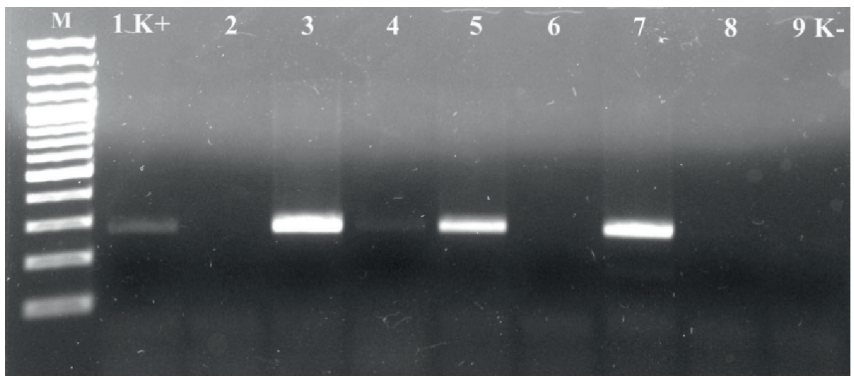


**Figure 1.** PCR for detection of *nuc* gene in *Staphylococcus* strains, M – molecular marker, 100 bp; 1 K+ -reference strain; 2, 6 – *S. pseudintermedius*; 3-5, 7 – *S. aureus* strains; 8 – *S. hyicus*; 9 K- - negative control

## DISCUSSION

The identification of coagulase-positive staphylococci at the species level is essential for the laboratory diagnostic practice. For instance, the interpretation of minimum inhibiting concentrations of oxacillin for detection of methicillin-resistant staphylococci depends on the *Staphylococcus* species (2, 24). The erroneous determination of concentrations could influence the antibiotic drug decision making and hence, the therapy of infections. Therefore, the detection of *S. aureus* among other coagulase-positive staphylococci is an important task for veterinary medicine, as well.

In many laboratories, the *S. aureus* identification is based on conventional biochemical tests, of which the tube coagulase is the main test for differentiating pathogenic staphylococci (25). According to some authors, the differentiation of coagulase-positive staphylococci based on their phenotypic characteristics is uncertain because of the lack of unique species-specific biochemical markers (26). In this study, we selected key biochemical tests for fast and accurate detection of clinically relevant staphylococci from animal origin (6). The panel included tests with established minimum variations in the behaviour of the common pathogenic staphylococci such as detection of free coagulase, presence of haemolysis, VP test for acetoin production, ONPG test to demonstrate β-galactosidase, test with polymyxin B, utilisation of mannitol, trehalose and maltose. The colony pigmentation was taken into account in primary differentiation of the isolates, as well.

The analysis using biochemical tests has undoubtedly determined 114 strains as *S. aureus* which exhibited a typical biochemical profile compared to that of the reference *S. aureus* ATCC 25922 strain and specified in the Clinical Veterinary Microbiology reference book (6). The other 42 strains showed variable behaviour with respect to some parameters and were classified as

suspicious for *S. aureus* until the latex agglutination and PCR tests. Acetoin production is one of the main tests for discrimination of *S. aureus* from *S. pseudintermedius* and *S. hyicus.* An interesting finding in the present study was the detection of 30 (19.2%) acetoin-negative *S. aureus* and 7 (6.8%) VP-positive *S. pseudintermedius* strains. The strains originated from cows with mastitis and dogs affected by otitis externa, respectively. In a study on the phenotype characteristics of 96 *S. aureus* isolates from poultry meat in Spain, Capita et al. (27) demonstrated 100% acetoin production. Similar data about acetoin are established by El-Jakee et al. (5) in *S. aureus* strains from human and animal origin (cattle, dogs, chickens). In the present study, other atypical reactions were observed with regard to fermentation of mannitol, trehalose and to haemolytic activity. Twenty five *S. aureus* fermented mannitol weakly without alteration of mannitol-salt agar colour into yellow, but producing orange-yellow colonies. These strains were isolated from cows with mastitis. Another finding was the isolation of 4 strains with reactions atypical for *S. aureus* to three parameters included in the panel: they originated from dogs with skin lesions and were characterized with lack of haemolysis, weak mannitol fermentation and no acid production from trehalose. Furthermore, the colonies of these isolates were non-pigmented but VP positive, ONPG negative, polymyxin B-resistant and maltose-fermenting – tests useful to distinguish *S. aureus* from other pathogenic staphylococci. Variable responses to ONPG, polymyxin B and maltose were not established which allowed concluding that these tests could be reliable markers in the identification of animal *S. aureus* isolates. Gandra et al. (28) compared two phenotypic tests – ONPG and sensitivity to acriflavine for differentiation of coagulase-positive *S. aureus*, *S. intermedius* and *S. hyicus*, vs PCR of *coa* and *nuc* gene sequences. The authors established similar discriminatory power of both techniques.

Latex agglutination tests for *S. aureus* detection are based on the presence of clumping factor and/or protein A that differentiate staphylococci which do not possess these factors. Only 5 strains isolated from cow mastitis failed in the test. Similar to our results are the findings of Zschöck et al. (29) who evaluated 6 commercial agglutination kits for *S. aureus* detection in bovine mastitis. The authors conclude that slide agglutination methods can provide rapid identification of *S. aureus* from bovine mastitis as well, however the sensitivity and specificity of these tests is lower than those reported for human isolates. Latex agglutination tests may be useful for human *S. aureus* identification in routine diagnostic purposes (30), but concerning other staphylococcal isolates it has to be taken into account that some coagulase-positive/variable staphylococci such as *S. pseudintermedius* and *S. hyicus*, tested in the present study may give rapid agglutination. Therefore, we suggest that these tests alone should not be used for *S. aureus* detection in veterinary medicine.

All strains identified preliminary as *S. aureus* were confirmed by PCR based on *nuc* gene. This gene, coding a thermostable nuclease, seems to be highly specific and a unique genetic marker for *S. aureus* detection confirmed both in our and previous research (21, 31, 32). However, there is a report about *nuc* negative *S. aureus* clade from animals in sub-Saharan Africa (33) harboring a homologue of *nuc* gene. This clade nuc sequence is highly divergent from those of *nuc S. aureus* reference strains. Therefore, it gives false-negative results in standard *nuc* PCR techniques applied alone (33).

*Staphylococcus* spp. show a certain host specificity as the strains isolated from clinical materials differ from species to species, for instance, prevailing species of isolates from ruminants, pigs and dogs are *S. aureus*, *S. hyicus* and *S. pseudintermedius*, respectively (26, 34), which is in line with our results. Although less frequently, coagulase-positive species other than *S. aureus* are also involved in the etiology of bovine mastitis – such as *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* detected in a previous study of ours with BioLog identification system (22). According to Markey et al. (6) *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus* are isolated most commonly from dogs. In the present research, no *S. schleiferi* subsp. *coagulans* was found in canine samples, whereas the occurrence of *S. aureus* from skin lesions and infected wounds was 16.1% (19/118). Comparable results (16%) are reported by El-Jakee et al. (5), about the prevalence of *S. aureus* in samples from dogs. As the other species included in the present work are concerned, our data was comparable to published results (35).

## CONCLUSION

The atypical biochemical behaviour and the absence of clumping factor and/or protein A in some *S. aureus* strains established in this study suggests that the precise detection of *S. aureus*

from animal origin should be done by combination of conventional and molecular methods. In cases where strains show a typical biochemical profile based on the chosen key tests such as β-haemolysis, tube coagulase test, VP test, ONPG test, resistance to polymyxin B, acid from mannitol, trehalose and maltose, the detection of *S. aureus* can be done without PCR confirmation. We believe that the results in this study will add to the clinical microbiology experience.

## CONFLICT OF INTEREST STATEMENT

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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