Methods for Identification of *Staphylococcus aureus* Isolates in Cases of Bovine Mastitis

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A total of 272 staphylococcal isolates from cases of bovine mastitis (159 *Staphylococcus aureus*) belonging to 12 different species were identified with ID32 STAPH galleries, and 51 of them were confirmed by 16S rRNA gene (*rrs*) sequencing. The same isolates were examined for their hemolytic activity on sheep blood agar, DNase activity, and coagulase activity and with two rapid identification kits (Slidex Staph Plus kit and RAPIDEC Staph from Bio-Merieux). The results of this study confirm those obtained by other groups with hemolysis, DNase, and coagulase. Only 50% of *S. aureus* isolates from mastitis cases show coagulase activity after 4 h of incubation, and a 24-h incubation is necessary for the full sensitivity of this test. In contrast to results from other studies with human isolates, the Slidex Staph Plus kit was not sensitive enough for the identification of *S. aureus* from bovine mastitis samples. The aurease test of the RAPIDEC Staph kit showed 100% sensitivity and 100% specificity. Used in conjunction with hemolysis patterns, the RAPIDEC Staph kit is therefore very well adapted to rapid, efficient, and cost-effective identification of *S. aureus* in cultures from bovine mastitis samples. Sequencing of *rrs* genes also proved very efficient in identifying the *Staphylococcus* species encountered in these samples and confirming phenotypic identification results with unsatisfactory scores. With continuously improving technologies and decreasing costs, genetic identification methods like *rrs* gene sequencing will soon find a place in routine veterinary diagnostics.

Staphylococcus aureus represents a major agent of contagious bovine mastitis (6). Cumbersome preventive and control measures have to be taken on farms with *S. aureus* mastitis problems, and the treatment of *S. aureus* mastitis is associated with poor success (24, 27), leading to a relatively high culling rate. Reliable and rapid methods for the identification of *S. aureus* from mastitic milk are therefore crucial for the control of this disease and for economically sound udder health management.

Genetic identification methods like sequencing of 16S rRNA genes (*rrs*) allow the identification of many bacterial pathogens (22). These methods are still relatively work-intensive and expensive and have not yet made their way into veterinary routine diagnostic laboratories. Thus, identification of bacterial pathogens still relies mainly on phenotypic criteria. Large numbers of samples are usually analyzed for mastitis under tight financial constraints. Therefore, only simple tests allowing a rough delineation of the most therapeutically and epidemiologically important pathogen classes are used for the identification of bacteria from clinical milk samples.

In the case of the staphylococci, the main clinically relevant factor is the differentiation between the contagious *S. aureus* and other staphylococci. On one hand, selective agars like modified Baird-Parker agar have been used successfully for the detection and identification of *S. aureus* and other coagulase-positive staphylococci (25). However, such agars may not allow the detection of other microorganisms and can be used only for a targeted search of *S. aureus*. On the other hand, several tests are currently used to differentiate *S. aureus* from other staphylococci in primary nonselective cultures.

Testing for the presence and type of hemolysis on blood agar plates represents a first simple and rapid method. Unfortunately, this criterion is not very sensitive (17). Among the three coagulase-positive staphylococci, *S. aureus* is the only one with hemolytic activity that is regularly encountered in clinical milk samples. Therefore, a combination of hemolysis and coagulase activities seems to represent an optimal criterion for the identification of *S. aureus* in cultures from milk samples (17). The presence of a DNase activity is often used as a surrogate marker for the identification of coagulase-positive staphylococci and particularly of *S. aureus* in milk samples. However, the specificity of this criterion or of the more laborious test for thermonucleases is not entirely satisfactory (5).

Several rapid identification tests for *S. aureus* are commercially available and have been extensively validated for use in human medicine (8, 26). They could be very helpful for the identification of *S. aureus* in cultures from milk samples but seem not to be used frequently for this purpose. The majority of these tests are based on slide agglutination. For instance, the Slidex Staph Plus kit from Bio-Merieux is an agglutination test used for the simultaneous demonstration of protein A, clumping factor, and other surface antigens specific for *S. aureus*. A few other tests also make use of enzymatic activities. The aurease activity (a prothrombin activation), for instance, is considered specific for *S. aureus* (12) and is used for the identification of *S. aureus* in the RAPIDEC Staph kit from Bio-Merieux.

Rapid identification kits for *S. aureus* have only rarely been specifically evaluated for mastitis diagnostic (10, 11, 30). However, *S. aureus* populations from cattle and from humans are
different (13). Therefore, separate test validations for the identification of bovine mastitis isolates with the new improved generations of these rapid identification kits would be highly welcome.

The aim of the present study was to evaluate two rapid identification methods for \textit{S. aureus} in mastitis diagnosis and to compare them to other commonly used methods.

**MATERIALS AND METHODS**

**Bacterial isolates.** A total of 252 \textit{Staphylococcus} isolates randomly collected at the diagnostic unit of the Institute for Veterinary Bacteriology of the University of Bern between March and November 2001 were used. Twenty additional \textit{S. aureus} isolates collected during the same period by the diagnostic laboratory Dr. Graub \textit{AG} in Bern were added to this sample. All the isolates were gram-positive, catalase-positive cocci from cases of bovine mastitis. They were all identified with the ID32 STAPH galleries (Bio-Merieux, Marnes-la-Coquette, France).

The isolates with unsatisfactory identification scores were additionally identified by \textit{rS} sequencing as follows. A 1.4-kb fragment of the 16S rRNA gene was amplified by PCR with universal primers (15). The PCR product was purified and sequenced in both directions by use of conserved-region primers (16) with the \textit{dRhodamine} cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Purified sequencing products were run on an ABI 3100 (Applied Biosystems) and edited with Sequencer (Gene Codes, Ann Arbor, Mich.). Identification was achieved by comparison of edited sequences against GenBank with Blast (2). Cluster analysis and tree construction was achieved with the UPGMA method (unweighted pair group method with arithmetic mean) with the program Bioumerics (Applied Maths, Kortrijk, Belgium). The isolates were kept in 2% Trypticase soy agar (Becton Dickinson, Cockeysville, Md.) for the time of the study.

**Diagnostic tests for identification of \textit{S. aureus.}** All the isolates were tested for hemolysis after overnight incubation at 37\(^\circ\)C on sheep blood agar prepared as follows: 15 ml of 5\% sheep blood in Trypticase soy agar (Becton Dickinson) as overlay on 10 ml of blood agar base (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). Hemolysis was recorded as \(\alpha\)-hemolysis, \(\beta\)-hemolysis, double hemolysis (\(\alpha + \beta\)), and negative (no hemolysis). DNase activity was tested on DNase test agar following the manufacturer’s recommendations (Difco Laboratories, Detroit, Mich.). Only strong DNase activities (clearing zone around growth similar to that of the \textit{S. aureus} control strain ATCC 29213) were recorded as positive. Weak DNase activities with clearing zones noticeably smaller than that of the positive control were recorded as negative.

The coagulase test was performed with rabbit plasma following the recommendations of the manufacturer (Bio-Merieux). Results were recorded after 4 and 24 h of incubation at 37\(^\circ\)C. Weak coagulase activities were recorded as positive. The isolates were further tested with the commercial Slide Staph Plus and RAPIDDEC Staph following the instructions of the manufacturer (Bio-Merieux). Weak agglutinations obtained with the Slide Plus kit were recorded as positive. The RAPIDDEC Staph test was performed at the end of the study, and six isolates were no longer available at that time (156 \textit{S. aureus} and 110 non-\textit{S. aureus} isolates were tested with this kit).

**Statistics.** The 95\% confidence intervals for sensitivities and specificities were performed with the NCSS 2000 program (NCSS, Kaysville, Utah) under the assumption of a binomial distribution of the results.

**RESULTS**

**Isolates.** Twelve staphylococcus species were identified (Table 1), and a total of 51 strains were analyzed by 16S rRNA gene sequencing. These included strains with unsatisfactory identification scores by use of ID32 STAPH-galleries as well as control strains with unambiguous identification. A cluster analysis including mastitis isolates and the GenBank entries of 13 \textit{Staphylococcus} type strains (28) revealed a tight clustering of the various isolates around the type strain sequence of the corresponding species for all but three isolates (one \textit{S. lentus} and two \textit{S. haemolyticus}). Each species cluster was clearly separated from the next related species (Fig. 1). This was particularly true for the \textit{S. aureus} isolates, which showed only minor sequence variation within the species. Two strains of the \textit{S. haemolyticus} group clustered separately from the other isolates of this species but still showed 99.2\% sequence similarity to the \textit{S. haemolyticus} type strain.

One isolate phenotypically identified as \textit{S. lentus} showed the best sequence match with \textit{S. pulvereri} (99.3\% identity, compared to 99.1\% with \textit{S. lentus}). However, it was located in the dendrogram on a separate branch showing similar distances to both \textit{S. pulvereri} and \textit{S. lentus}. Overall, the genetic identification was clearly not in agreement with the phenotypic identification in only eight cases (one \textit{S. warneri}, two \textit{S. xylosus}, one \textit{S. saprophyticus}, four \textit{S. haemolyticus}, and one \textit{S. felis}). However, tight genetic clustering of these eight isolates with the corresponding reference strain and a clear genetic separation from the other species demonstrated that their phenotypic identification was not reliable.

**Sensitivity and specificity of the tests.** The isolates of the present study originated from 137 different farms, with one to five isolates per farm (median, one). A subsample of 172 epidemiologically unrelated isolates was drawn from this collection by taking only one isolate of each staphylococcal species per farm. The statistical evaluations of sensitivities and specificities were performed on both the whole collection and the subsample of epidemiologically unrelated isolates. The performances of the different identification tests for \textit{S. aureus} are reported in Table 2. All 159 \textit{S. aureus} isolates tested presented hemolysis on blood agar plates, but 45 of them showed only \(\alpha\)-hemolysis and no detectable \(\beta\)-hemolysis. No species other than \textit{S. aureus} showed \(\beta\)-hemolysis, and 53 non-\textit{S. aureus} isolates (47\%) showed \(\alpha\)-hemolysis (mainly \textit{S. xylosus} and \textit{S. haemolyticus}).

All but two \textit{S. aureus} showed a strong DNase activity (two isolates with weak activities recorded as negative), and 21 isolates from other species also showed a strong DNase activity similar to that of the positive control (17 out of 20 \textit{S. chromogenes}, two \textit{S. hyicus}, and 2 out of 51 \textit{S. xylosus}). However, only three of these isolates simultaneously showed hemolytic activity (one \textit{S. chromogenes} and two \textit{S. xylosus}). The coagulase test was not sensitive after 4 h, and acceptable results were obtained only after 24 h of incubation (Table 2). No species other than \textit{S. aureus} was coagulase positive, and the two \textit{S. hyicus} isolates examined remained coagulase negative despite repeated testing.

**TABLE 1. Staphylococcal isolates examined**

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>No. of epidemiologically independent isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. aureus}</td>
<td>159</td>
<td>101</td>
</tr>
<tr>
<td>\textit{S. xylosus}</td>
<td>53</td>
<td>29</td>
</tr>
<tr>
<td>\textit{S. chromogenes}</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>\textit{S. sciuri}</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>\textit{S. haemolyticus}</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>\textit{S. warneri}</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>\textit{S. simulans}</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>\textit{S. saprophyticus}</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>\textit{S. lugari}</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>\textit{S. epidermidis}</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>\textit{S. felis}</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>\textit{S. lentus}</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>272</td>
<td>172</td>
</tr>
</tbody>
</table>
Twenty-nine isolates from species other than S. aureus (27 S. xylosus, 1 S. sciuri, and 1 S. warneri) could not be tested with the Slidex Staph Plus kit because of positive agglutination with the negative control provided by the manufacturer. This problem was not encountered with any S. aureus isolate, but many isolates from this species were negative with the Slidex kit (Table 2). Two S. haemolyticus isolates were also false-positive in this test. The aurease results of the RAPIDEC Staph kit were all clearcut, and neither false-positive nor false-negative results were encountered with this test.

**DISCUSSION**

A reliable and rapid identification of S. aureus colonies in cultures from milk samples is a cornerstone in the control of S. aureus mastitis. The high specificity and sensitivity of the coagulase test (5) have made it a standard method for the identification of S. aureus in milk. However, only half of the S. aureus isolates are positive in this test after a 4-h incubation, and an overnight incubation is necessary to obtain reliable results (Table 2). This long incubation time represents an important drawback for diagnostic applications, and quicker methods would be preferable. β-Hemolysis also represents an important criterion for rapid presumptive identification of S. aureus in primary cultures. With regard to this criterion, the results of the present study are in complete agreement with previous studies from other veterinary laboratories showing that approximately one fifth to one fourth of the S. aureus isolates from bovine mastitis do not present any detectable beta-hemolytic activity in primary cultures (1, 17). Therefore, beta-hemolytic activity represents a specific but not very sensitive criterion for the identification of S. aureus.

The combination of this test with the 4-h coagulase test may provide a more sensitive and still acceptably rapid identification method (Table 2). Since the proportion of beta-hemolytic S. aureus found in bovine mastitis varies from region to region (19), the performances of this combination in terms of sensitivity may also vary in function of the S. aureus population under investigation and should therefore be interpreted with caution. Because of the frequent presence of a weak DNase activity in many species other than S. aureus (mainly in the alpha-hemolytic S. haemolyticus), the interpretation of DNase tests remains partially subjective and needs some experience. In addition, species other than S. aureus also often present a clear DNase activity (mainly S. chromogenes). This test seems to present acceptable performances only when combined with a test for the presence of hemolytic activity, but under these conditions some potential for wrong identifications remains, for instance, with the pigmented and occasionally alpha-hemolytic S. chromogenes. An additional drawback of this combination is the necessity for overnight incubation.

**FIG. 1.** Cluster analysis of *rrs* sequences from 51 clinical isolates and 13 reference sequences. The scale on the bottom of the figure represents percentages of sequence divergence. Bootstrap values of 500 repeated trees are given at major branches. Except where noted, each sequence is represented by only one isolate. *, isolate identified phenotypically as S. lentus.
Validations of the Slidex Plus test for staphylococcal populations of human origin show very satisfactory sensitivities and specificities of more than 98% (8, 9, 23, 26). Only one study showed a significantly lower specificity (82%) of this test for the identification of *S. aureus* (23). However, the false-positive results in this particular study were all obtained with *S. schleiferi* und *S. lugdunensis* strains. These two species were not encountered in the present study and are mainly isolated from samples of human and canine origin but not from bovine mastitic milk (5, 14). This specificity problem should therefore not be encountered with samples of bovine mastitis, and our study also confirms the relatively high specificity of the Slidex Plus test (Table 2).

In terms of sensitivity, our results are much less satisfactory. The problem of false-negative results was known in human medicine with the earlier generation of Slide tests, which detected only the presence of protein A and clumping factor. The addition of specific antibodies for other surface antigens brought an end to this problem (8). *S. aureus* strains from bovine mastitis belong to a population other than those from human origin (13) and often build a polysaccharide capsule (3, 29). The surface structures and antigens targeted by the Slidex Staph Plus test may possibly be hidden by this capsule or may be different from those of human strains. Further studies would be necessary to confirm this hypothesis and allow the development of improved agglutination tests specifically aimed at populations of bovine origin.

An additional problem with the Slidex Staph Plus kit is the relatively high frequency of “not interpretable” results. This problem was encountered mainly with *S. xylosus* isolates. This species is frequent in milk samples (4, 21) but rare in humans and was not included in validation studies with human isolates. This clearly explains the very low rate of such not-interpretable results from other studies (8, 23). Despite the problems encountered with this kit, a parallel combination of β-hemolysis and Slidex Plus (Table 2) provided acceptable results in the population under investigation. However, for the same reasons as mentioned for the hemolysis-DNase combination, the sensitivity of this combination should also be interpreted cautiously.

In contrast to the Slidex Plus test, the aurease test of the RAPIDEC Staph kit presented a specificity and sensitivity of 100% for the identification of *S. aureus*. These results are similar to or even better than those obtained with staphylococcal populations of human origin (7, 12, 18, 20). In addition, this test is easy to perform, definitive results can be obtained within 2 h, and the costs of the test are compatible with the financial constraints of mastitis diagnosis. The aurease test represents the best of all those examined in the present study and is clearly appropriate for the identification of *S. aureus* in routine diagnosis of bovine mastitis. Its performance was equivalent to or even better than that of the reference method represented by the 24-h coagulase test. Its use in parallel to the β-hemolysis test only on suspect isolates with α- and β-hemolysis activity would allow very quick and efficient identification of all *S. aureus* isolates.

Sequence-based bacterial identification, including the *rrs* genes as targets, is increasingly used in the diagnostic laboratory (22). We used *rrs* sequencing in the present study as a tool to characterize strains that are difficult to identify phenotypically with certainty as well as some control strains with trustworthy phenotypical identification results.

The phylogenetic positions of all the major *Staphylococcus* species have been investigated by Takahashi and collaborators (28). However, knowledge of the sequence variability of clinical isolates is also needed in order to apply *rrs*-based identification for diagnostic purposes. Our study indicates that the *rrs* sequence can be used not only for unambiguous identification of *S. aureus* but also for the identification of other staphylococcal species found in bovine milk samples. Most of the isolates analyzed clearly clustered around the type strain of a species, showed little intraspecies variation, and were clearly separated from the nearest neighbor in the tree (Fig. 1).

Only two situations need further comments. Two *S. haemolyticus* isolates clustered together separately from the other isolates and the type strain of this species. They did not show significant similarity with any other GenBank entry. It is therefore not clear, whether these two isolates represent a phylogenetic subcluster of *S. haemolyticus* or if they form a new, as yet unrecognized *Staphylococcus* species. A limitation of the *rrs*

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**TABLE 2. Sensitivity and specificity of the tests for the identification of *S. aureus***

<table>
<thead>
<tr>
<th>Criteria for identification</th>
<th>All isolates (n = 272)</th>
<th>Epidemiologically unrelated isolates (n = 172)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity (%) (n = 159)</td>
<td>Specificity (%) (n = 113)</td>
</tr>
<tr>
<td>α + β-hemolysis</td>
<td>71.1 (63.4–78.0)</td>
<td>100.0 (96.8–100.0)</td>
</tr>
<tr>
<td>β-Hemolysis</td>
<td>71.6 (64.0–78.5)</td>
<td>100.0 (96.8–100.0)</td>
</tr>
<tr>
<td>Positive DNase</td>
<td>98.7 (95.5–99.8)</td>
<td>81.4 (73.0–88.1)</td>
</tr>
<tr>
<td>Positive coagulase 4 h</td>
<td>60.4 (52.3–68.0)</td>
<td>100.0 (96.8–100.0)</td>
</tr>
<tr>
<td>Positive coagulase, 24 h</td>
<td>99.4 (96.5–100.0)</td>
<td>100.0 (96.8–100.0)</td>
</tr>
<tr>
<td>Positive Slide⁵</td>
<td>62.7 (54.6–70.2)</td>
<td>97.6 (91.6–100.0)</td>
</tr>
<tr>
<td>Positive aurocase (RAPIDEC Staph)</td>
<td>100 (97.7–100.0)</td>
<td>100 (96.7–100.0)</td>
</tr>
<tr>
<td>β-Hemolysis or positive Slide⁵</td>
<td>98.1 (94.6–99.6)</td>
<td>98.2 (93.8–99-8)</td>
</tr>
<tr>
<td>β-Hemolysis or positive coagulase (4 h)</td>
<td>99.4 (96.5–100.0)</td>
<td>100.0 (96.8–100.0)</td>
</tr>
<tr>
<td>α- and/or β-hemolysis and positive DNase</td>
<td>98.7 (95.5–99.8)</td>
<td>97.0 (91.6–99.8)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent 95% confidence intervals.

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approach was encountered with only one S. lentus isolate. This isolate was indisputably identified as S. lentus with the ID32 STAPH gallery but showed the best match with the rrs sequence of S. pulvereri. Cluster analysis positioned this strain at equivalent distances from both the S. lentus and S. pulvereri reference strains. Therefore, the rrs sequence cannot identify this isolate precisely at the species level.

The rrs-based identification approach used as a complementary molecular method to the phenotypic tests gave promising results for the usefulness of this identification technique for staphylococci from cases of bovine mastitis. However, the high costs of this technique are still critical for veterinary diagnostics, especially in mastitis diagnostics. Nevertheless, the cost has already come down massively in recent years. For instance, it is today at a level of approximately 4:1 compared to conventional techniques for mastitis diagnosis in Switzerland. The automation of sample processing, improvements in sequence quality, and especially progress in software tools for automated sequence analysis will certainly cut costs further, opening the door for these techniques in routine veterinary diagnostics in the very near future.

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