Performance of culture media for the isolation and identification of *Staphylococcus aureus* from bovine mastitis


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Rapid isolation and identification of pathogens is a major goal of diagnostic microbiology. In order to isolate and identify *Staphylococcus aureus*, a number of authors have used a variety of selective and/or differential culture media. However, to date, there are no reports comparing the efficacy of selective and differential culture media for *S. aureus* isolation from bovine mastitis cases using the 16S rRNA (*rrs*) gene sequence as a gold standard test. In the present study, we evaluated the efficacy of four selective and/or differential culture media for the isolation of *S. aureus* from milk samples collected from cows suffering from bovine mastitis. Four hundred and forty isolates were obtained using salt–mannitol agar (SMA, Bioxon), Staphylococcus-110 agar (S110, Bioxon), CHROMAgar Staph aureus (CSA, BD-BBL) and sheep’s blood agar (SBA, BD-BBL). All bacterial isolates were identified by their typical colony morphology in the respective media, by secondary tests (for coagulase and *b*-haemolysis) and by partial 16S rRNA (*rrs*) gene sequencing as a gold standard test. Sensitivity, positive predictive and negative predictive values were higher for SMA (86.96, 52.63 and 95.95 %, respectively) compared with S110 (70.00, 23.73 and 90.91 %, respectively), CSA (69.23, 28.13 and 95.74 %, respectively) and SBA (68.75, 37.93 and 89.58 %, respectively) while specificity values were similar for all media. Data indicated that the use of culture media for *S. aureus* isolation combined with determination of coagulase activity and haemolysis as secondary tests improved accuracy of the identification and was in accordance with *rrs* gene sequence-analysis compared with the use of the culture media alone.

**INTRODUCTION**

The importance of *Staphylococcus aureus* as a successful pathogen resides in its wide genetic diversity and host range and the different pathologies associated with infection. *S. aureus* is associated with hospital-acquired and community-acquired infections and with human carriers (Miller & Diep, 2008) as well as livestock associated infections (Graveland et al., 2011), for which meticillin-resistant *S. aureus* (MRSA)
and meticillin-sensitive *S. aureus* (MSSA) isolates are important pathogens (Hata *et al.*, 2010). In the present study, we investigated the identification of *S. aureus* isolates associated with bovine mastitis in backyard farms. The production system is prevalent in rural milk production in México and constitutes a major source of income for the owners (Sánchez & Solorio, 2004). Primary isolation and presumptive identification of the pathogen is crucial for microbiological diagnosis and epidemiological surveillance. In order to isolate and identify *S. aureus* a number of authors have used a variety of selective and/or differential culture media. Among the most common media used are salt–mannitol agar (SMA) and Staphylococcus-110 agar (S110), which are selective and differential media that take advantage of the tolerance of *S. aureus* to 7.5% (w/v) NaCl as well as its metabolic activities, such as mannitol fermentation in SMA or gelatinase activity in S110 media. Sheep's blood agar (SBA) is a rich medium in which the β-haemolytic activity of *S. aureus* isolates may be clearly differentiated (Brown, 1919). Baird–Parker agar, which is a moderately selective and differential medium, is used to detect tellurite tolerance and lecithinase activity of *S. aureus* (Baird-Parker, 1962); incubation for up to 48 h is required prior to identification of the specific colony morphology. For this medium, the incubation time is a great disadvantage for the rapid identification of *S. aureus*.

The United States National Mastitis Council (NMC) in 1999 suggested the use of SBA for the isolation of bovine mastitis pathogens and the presumptive identification of *S. aureus*. This proposal was later supported by Boerlin *et al.* (2003) who combined the detection of haemolytic and coagulase activities of *S. aureus* isolates to increase the sensitivity and specificity of the microbiological identification up to 99.4% and 100%, respectively. This approach has been successfully used for the isolation and primary identification of *S. aureus* isolates from food and human clinical samples. Recently, alternative selective and differential culture media, such as CHROMAgar Staph aureus (CSA, BD-BBL) and CHROMAgar MRSA (BD-BBL), chrom ID (bioMérieux), MRSASelect (Bio-Rad) and Brilliance MRSA (Oxoid), all of which are based on the use of specific chromogenic substrates, were developed for the isolation of *S. aureus* and MRSA from clinical samples (Boyce & Havill, 2008; Gaillot *et al.*, 2000; Nahimana *et al.*, 2006). CHROMAgar Staph aureus showed high sensitivity (95.5%) and specificity (99.4%) for the identification of *S. aureus* isolated from human clinical samples and has been widely used in microbiological analysis of samples of clinical (Perry & Freydière, 2007), food (Ritter *et al.*, 2009) and animal (Wang *et al.*, 2008) origin.

To our knowledge, to date, there are no reports comparing the performance of selective and differential culture media for *S. aureus* isolation from bovine mastitis. Specifically, comparisons of the performance of selective and differential culture media have not yet been validated using partial sequencing of 16S rRNA (*rrs*) gene as a gold standard test. The *rrs* gene sequence is a powerful tool for the precise and rapid identification of bacterial pathogens, which has been used as a gold standard test for the validation of ID32 Staph and Vitek identification systems based on phenotypic profiles (Becker *et al.*, 2004). Therefore, in this study, we have evaluated the efficacy of SMA, S110, CSA and SBA for the rapid primary identification of bovine *S. aureus*, isolated from cows living in backyard farms in the central region of the State of Michoacán in México.

**METHODS**

**Study design.** Sample collection was performed according to a random model of proportional distribution (Espinoza *et al.*, 2004; Sánchez *et al.*, 2004; Sánchez & Solorio, 2004), with which a representative number of farms to be sampled in each locality was calculated as a function of the prevalence of bovine mastitis per farm in the region (65%), considering a confidence level of 95% and precision of 10%. Sampling by conglomerates was applied to each selected farm. Samples were collected from February 2010 to September 2011. Sixteen backyard farms were selected based on the following features that are typical of backyard, non-technified dairy milk production systems: 6–30 cows in each herd feeding with seasonal forages; manual milking; poor hygiene conditions; manpower constitutes members of the family and the main income is from the sale of milk in villages nearby the backyard farms. Milk samples (~25 ml) were collected and evaluated for the presence of subclinical mastitis by using the California mastitis test (CMT, Rice, 1997). Samples scoring from 1+ to 4+ using the CMT were considered as positive in the absence of signs of udder inflammation (Bhutto *et al.*, 2012). One hundred and forty-four quarters from 96 cows were positive in the CMT. When clear signs of udder inflammation and reduction in milk quality were observed, samples were considered as positive for clinical mastitis. All samples were stored on ice for transportation. Upon arrival to the lab, each of the milk samples was simultaneously inoculated onto all of the four test culture media by using cotton swabs soaked in the sample. A routine of soaking and inoculation was established in order to reduce variations due to sample loading.

**Culture media, bacterial growth conditions and biochemical identification.** SMA and S110 media from Bioxon were prepared following the manufacturer’s instructions. CSA and SBA plates were purchased from BD-BBL. Inoculated plates were incubated aerobically at 37°C for 18 to 24 h. Criteria for the identification of *S. aureus* in the selective or differential culture media were in accordance with the colony morphology expected for each culture medium as follows: for SMA, whitish to yellow colonies that changed the colour of phenol red to yellow, indicating mannitol fermentation; for S110, cream-coloured to golden or yellow colonies having or not having a halo of gelatin hydrolysis and positive for mannitol fermentation after the addition of bromothymol blue; for CSA, mauve colonies; and for SBA, whitish to yellow colonies, presenting the typical clear halo of β-haemolysis. *S. aureus* ATCC 27543 (an isolate of bovine origin) and *S. aureus* NCTC 8325 (an isolate of human origin) were used as reference strains. All colonies were subcultured in the rich medium Luria–Bertani agar (LBA) to ensure their purity before further identification tests were performed and for short-term storage. Identification of *S. aureus* and other staphylococci was performed by using the following tests: morphology following Gram staining, catalase activity, mannitol fermentation, gelatin hydrolysis, mixed sugar fermentation by methyl red test, Voges–Proskauer test for acetoin production, coagulase activity and β-haemolysis. The level
of contamination was calculated as the percentage of samples in which fungi or yeasts were found in SBA (Ericsson Unnerstad et al., 2009).

**Coagulase activity and β-haemolysis tests.** Coagulase activity and β-haemolysis were determined in all the 440 colonies, irrespective of the culture medium where they were isolated, their identification by biochemical tests or final identification by rrs gene sequence analysis. Coagulase activity was determined by using a Coagulase Plasma Rabbit kit (BD-BBL), following the supplier’s instructions and considering a final reading after 24 h of incubation with the plasma. β-Haemolytic activity was determined in SBA plates (BD-BBL).

**Sequencing of the variable region 3 of the 16S rRNA (rrs) gene.** To confirm the identity of all 440 bacterial isolates, sequencing of the variable region 3 of the 16S rRNA (rrs) gene was performed (Lee et al., 1996) and was considered as the gold standard test for the identification of the isolates. Sequencing was performed at the Laboratorio Nacional de Genómica para la Biodiversidad (LANGEBIO, Irapuato, México). Similarity searches were performed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Information (NCBI). The partial rRNA gene sequences analysed in this study have been deposited in the GenBank database with accession numbers JQ511370-JQ511809. Sequences with similarity values of 98% or higher were considered to belong to the same species (Stackebrandt & Goebel, 1994; Staley, 2006).

**Data and statistical analysis.** To evaluate the ability of the tested culture media to isolate *S. aureus*, frequency of isolation (FI) was analysed. FI was calculated as the total number of *S. aureus* true positives, (Tp) and false negatives (Fp), confirmed by rrs gene sequencing, divided by the total number of isolates (n). To evaluate their ability to correctly identify *S. aureus*, comparisons of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were performed. For culture media comparison (Table 1), positive criteria for each isolate were defined as the identification of colony morphology as follows. Tp were considered those isolates correctly identified from colony morphology and rrs gene sequence analysis. False positives (Fp) were only suggestive of *S. aureus* by colony morphology. True negatives (Tn) were considered negative for both culture-based and molecular identification approaches. Fn were negative in culture medium but positive in rrs gene sequence analysis. For phenotypic identification of *S. aureus* isolates using colony morphology in the tested culture media in combination with the presence of coagulase and haemolytic activities as confirmatory tests (Table 2), positive criteria for each isolate were considered as the expected colony morphology in the respective culture medium tested and positive coagulation of plasma and β-haemolysis in SBA, both after 24 h of incubation. Tp fulfilled positive criteria in the phenotypic tests and positive identification with the rrs gene sequence analysis. Fp fulfilled the positive criteria in the phenotypic test but not the rrs gene sequence test. Tn did not fulfill either of the two criteria. Fn did not fulfill the positive criteria in phenotypic evaluation but were present in rrs gene sequence analysis. Efficacy of the medium to isolate *S. aureus* was calculated as the FI of *S. aureus* in each medium, which was calculated as FI= (Tp + Fp)/n × 100, where n is the total number of isolates. Sensitivity (St) was calculated as St= (Tp/ Tp + Fp) × 100. Specificity (Sp) was calculated as Sp= (Tn/ Tn + Fn) × 100. PPV was calculated as PPV= (Tp/Tp + Fp) × 100. NPV was calculated as NPV= (Tn/Tn + Fn) × 100.

The concordance between the identification of isolates in the selective/differential culture media and the rrs gene sequencing was estimated with the kappa index of concordance (Landis & Koch, 1977). Confidence interval (CI) limits were set to 95%. Comparisons for statistic calculations were performed against SMA in Table 1 or against SMA + coagulase + β-haemolysis as they were the medium or the combination of medium plus secondary tests with the best performance. Statistically significant differences were calculated after comparing treatment proportions with the Yates’ correction of the χ², using the MedCalc software v12.3.0.0 (MedCalc Software bvba). A P<0.05 was considered statistically significant. Agreement between the two identification approaches was considered in accordance to kappa index values as follows (Landis & Koch, 1977): 0, no agreement; 0–0.2, non-significant; 0.2–0.4, low agreement; 0.4–0.6, moderate agreement; 0.6–0.8, good agreement; 0.8–1.0, very good agreement.

**RESULTS**

**Performance of culture media**

Prevalences of subclinical and clinical mastitis were 60.41% (58/96) and 4.16% (4/96), respectively. Seventy-two out of 440 isolates grown on the different culture media tested were confirmed to be *S. aureus* by rrs gene sequence analysis. The total number of isolates in each medium is shown in Table 1. The total number of isolates was lower for SBA than for the other media because of the concomitant isolation of bacteria showing extensive growth or filamentous fungi, which in some cases did not allow us to properly describe the colony morphology or to isolate the colony; therefore, only colonies that were clearly isolated were considered for this study. Considering all of the culture media tested, *S. aureus* was the species most frequently identified (16.36%), while the following staphylococcal species were detected with less frequency: *S. epidermidis* (8.41%), *S. haemolyticus* (7.05%), *S. equorum* (6.36%), *S. xylosus* (5.91%), *S. saprophyticus* (5.91%), *S. sciuri* (4.77%), *S. succinus* (4.09%), *S. hominis* (3.64%), *S. intermedius* group (2.50%), *S. devriesi* (2.05%) and other species of the genus *Staphylococcus* (12.51%). Bacteria different from the genus *Staphylococcus* were also isolated in these culture media and were found at the following frequencies: *Aerococcus viridans* (2.27%), *Bacillus pumilus* (1.59%), *Bacillus subtilis* (1.36%), *Arthrobacter sulfureus* (1.36%), *Aerococcus urinaeaequi* (1.14%), *Bacillus cereus* (0.91%), *Paenibacillus macquariensis* (0.91%) and others (10.91%). The level of contamination of our samples was 13.0%.

The evaluation of the efficacy of culture medium to isolate *S. aureus* is shown in Table 1. Since SBA is a rich, non-selective culture medium, it is considered the gold standard for *S. aureus* isolation (NMC, 1999). FI of *S. aureus* was similar for SMA and SBA and was lower for S110 and CSA. CSA was the only medium that showed a statistically significant difference against SMA (P=0.0467). The ability of each medium to correctly identify *S. aureus* isolates was also analysed. Sensitivity and specificity were determined for each culture medium (Table 1). SMA showed the highest sensitivity to detect *S. aureus* compared with the other culture medium (Table 1). S110, CSA and SBA showed similar but low sensitivity values. The four media tested showed similar specificity values, with SBA having a
slightly lower value than the other medium. To further evaluate the ability of the culture media to discriminate for *S. aureus* or other species, positive and negative predictive values, as well as the kappa index of concordance were calculated (Table 1). Higher PPVs were obtained for SMA and SBA compared with the values calculated for S110 and CSA. NPVs were similar among the four culture media tested. Agreement between the use of selective media and *rrs* gene sequence analysis (gold standard test) was determined by calculating the kappa index of concordance. The kappa index value for SMA and CSA resulted in moderate agreement with the *rrs* gene sequencing, whereas SBA and S110 showed a low agreement. Global concordance between the four culture media tested in this study resulted in a global kappa index of 0.49, which indicates a moderate agreement among them.

Table 1. Performance of culture media for the isolation of *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Tp</th>
<th>Fn</th>
<th>Tn</th>
<th>Fp</th>
<th>% FI (95% CI)</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA (n=112)</td>
<td>20</td>
<td>3</td>
<td>71</td>
<td>18</td>
<td>20.53</td>
<td>(18.46–22.54)</td>
<td>86.96</td>
<td>79.78</td>
<td>52.63</td>
<td>95.95</td>
</tr>
<tr>
<td>S110 (n=125)</td>
<td>14</td>
<td>6</td>
<td>82</td>
<td>23</td>
<td>16.00</td>
<td>(14.31–17.69)</td>
<td>70.00*</td>
<td>78.14</td>
<td>23.73</td>
<td>90.91</td>
</tr>
<tr>
<td>CSA (n=126)</td>
<td>9</td>
<td>4</td>
<td>90</td>
<td>23</td>
<td>10.32*</td>
<td>(9.24–11.38)</td>
<td>69.23*</td>
<td>79.65</td>
<td>28.13</td>
<td>95.74</td>
</tr>
<tr>
<td>SBA (n=77)</td>
<td>11</td>
<td>5</td>
<td>43</td>
<td>18</td>
<td>20.78</td>
<td>(18.76–22.84)</td>
<td>68.75*</td>
<td>70.49</td>
<td>37.93</td>
<td>89.58</td>
</tr>
</tbody>
</table>

*Statistically significant difference (P<0.05) compared with SMA.

Table 2. Combined use of culture media and secondary test

<table>
<thead>
<tr>
<th>Culture medium + confirmatory tests</th>
<th>Tp</th>
<th>Fn</th>
<th>Tn</th>
<th>Fp</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA + Positive coagulase 24 h (n=112)</td>
<td>23</td>
<td>0</td>
<td>86</td>
<td>3</td>
<td>100</td>
<td>(97.58–100)</td>
<td>96.63</td>
<td>88.46</td>
<td>0.98</td>
</tr>
<tr>
<td>SMA + α-haemolysis (n=112)</td>
<td>21</td>
<td>2</td>
<td>84</td>
<td>5</td>
<td>91.3*</td>
<td>(88.88–93.72)</td>
<td>94.38</td>
<td>80.77</td>
<td>0.87</td>
</tr>
<tr>
<td>SMA + Positive coagulase 24 h + α-haemolysis (n=112)</td>
<td>23</td>
<td>0</td>
<td>89</td>
<td>0</td>
<td>100</td>
<td>(97.58–100)</td>
<td>100</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>S110 + Positive coagulase 24 h (n=125)</td>
<td>18</td>
<td>2</td>
<td>97</td>
<td>8</td>
<td>90.00*</td>
<td>(87.58–92.42)</td>
<td>92.38</td>
<td>69.23</td>
<td>0.84</td>
</tr>
<tr>
<td>S110 + α-haemolysis (n=125)</td>
<td>13</td>
<td>7</td>
<td>103</td>
<td>2</td>
<td>65.00*</td>
<td>(62.58–67.42)</td>
<td>98.10</td>
<td>86.67</td>
<td>0.85</td>
</tr>
<tr>
<td>S110 + Positive coagulase 24 h + α-haemolysis (n=125)</td>
<td>19</td>
<td>1</td>
<td>105</td>
<td>0</td>
<td>95.00*</td>
<td>(92.72–100)</td>
<td>100</td>
<td>99.06</td>
<td>0.98</td>
</tr>
<tr>
<td>CSA + Positive coagulase 24 h (n=126)</td>
<td>12</td>
<td>1</td>
<td>106</td>
<td>7</td>
<td>92.31*</td>
<td>(89.89–94.73)</td>
<td>93.81</td>
<td>63.16</td>
<td>0.87</td>
</tr>
<tr>
<td>CSA + α-haemolysis (n=126)</td>
<td>10</td>
<td>3</td>
<td>98</td>
<td>15</td>
<td>76.92*</td>
<td>(74.50–79.34)</td>
<td>86.73*</td>
<td>40.00</td>
<td>0.72</td>
</tr>
<tr>
<td>CSA + Positive coagulase 24 h + α-haemolysis (n=126)</td>
<td>12</td>
<td>1</td>
<td>109</td>
<td>4</td>
<td>92.31*</td>
<td>(89.89–94.76)</td>
<td>96.46</td>
<td>75.00</td>
<td>0.92</td>
</tr>
<tr>
<td>SBA + Positive coagulase 24 h (n=77)</td>
<td>14</td>
<td>2</td>
<td>55</td>
<td>6</td>
<td>87.50*</td>
<td>(85.08–89.92)</td>
<td>90.16</td>
<td>70.00</td>
<td>0.79</td>
</tr>
</tbody>
</table>

*Statistically significant difference (P<0.05) compared with SMA + coagulase + β haemolysis.
Combined use of culture media and secondary test

As the use of differential and/or selective culture media for the isolation and primary identification of \textit{S. aureus} isolates is not enough to ensure the identity of the isolates, a secondary test is needed to precisely identify the isolates as \textit{S. aureus}. Determinations of coagulase and $\beta$-haemolysis activities are easy and affordable confirmatory techniques to identify \textit{S. aureus}. When one or both of these confirmatory tests were used in combination with each selective medium, they improved the ability to correctly identify \textit{S. aureus} isolates (Table 2). For SBA, only the combination with coagulase test was presented because the criterion of $\beta$-haemolysis has been already considered in the positive colony morphology description, although $\beta$-haemolysis was confirmed again after primary isolation. All isolates were $\beta$-haemolytic. The use of coagulase at 24 h as a secondary test of identification improved sensitivity, specificity, PPV and NPV in combination with the respective culture medium. However, SMA and coagulase showed higher values of sensitivity and PPV compared with the other culture media. Using $\beta$-haemolysis as secondary test of identification also increased specificity, specificity and PPV when combined with the respective culture medium. However, most media had low PPV values, thus increasing the probability of obtaining false positives.

Using both coagulase and $\beta$-haemolysis as secondary tests for identification of \textit{S. aureus} increased sensitivity, specificity, PPV and NPV of the identification when combined with the respective culture medium, mainly in SMA, for which all calculated values reached 100% and maximum concordance with \textit{rsr} gene sequencing.

For an identification process, the use of the selective/differential media in combination with both secondary tests, determination of coagulase and haemolytic activities results in the highest values for the kappa index of concordance against the gold standard test, \textit{rsr} gene sequencing.

**DISCUSSION**

Validation on the use of selective and/or differential culture media for the primary isolation and presumptive identification of \textit{S. aureus} has been reported previously (Han et al., 2007; Kateete et al., 2010; Ollis et al., 1995). In all of these reports, commercially available series of biochemical tests or immunological methods have been used as gold standard tests for the identification of \textit{S. aureus}.

Previous publications have reported better sensitivities for CSA in samples of human origin than with other culture media, contrary to our observations. Specificity and sensitivity values were close to 100% (Boyce & Havill., 2008; Carricajo et al., 2001; Gaillot et al., 2000; Kateete et al., 2010; Perry et al., 2003; Perry & Freydière, 2007). In these reports, the gold standard tests for the identification of \textit{S. aureus} were based on biochemical tests (API ID 32 Staph System, VITEK 2 ID-GP), hybridization probes (Accuprobe) or real-time PCR (BD Gene Ohm MRSA). Becker et al. (2004) showed that identification tests commonly used for \textit{S. aureus} and other staphylococci that are based on biochemical tests such as ID 32 Staph and VITEK 2 failed to correctly identify 23.6% and 34.5% of isolates, respectively. Stutz et al. (2011) have also shown that Staphaurex, an identification test based on the detection of relevant surface virulence factors, failed to correctly identify \textit{S. aureus} due to genetic polymorphisms present in the cognate encoding genes (\textit{spa}, \textit{clfA} and \textit{fnbA}).

CSA was originally designed and tested for the isolation of \textit{S. aureus} from human clinical samples and has been used to culture bovine milk samples from cows affected with mastitis (Wang et al., 2008). Those isolates were further identified with an \textit{S. aureus}-specific PCR assay (Martineau et al., 1998). When the present report was in preparation it was brought to our attention that a selective chromogenic medium (CHROMAgar Mastitis GP; bioMérieux) would soon become available for the isolation of bovine mastitis Gram-positive pathogens. The principle for the identification of \textit{S. aureus} in this new medium seems to be the same as that for CSA, since criteria for identification of \textit{S. aureus}-related colonies are the same for both media. It would be interesting to independently evaluate the performance of this new chromogenic medium for the presumptive identification of mastitis pathogens, but using a highly precise gold standard test for \textit{S. aureus} identification based on gene sequence analysis.

Alternatives to the phenotypic identification of \textit{S. aureus} isolates are molecular approaches. An example of these approaches is the PCR-mediated identification of the ubiquitous \textit{tuf} gene, encoding elongation factor Tu (EF-Tu). This gene is amplified with genus-specific primers and then hybridized with a species-specific capture hybridization probe to identify \textit{S. aureus} isolates (Martineau et al., 2001). An approach using real-time PCR and a fluorescence resonance energy transfer-probe specific for \textit{S. aureus} has also been reported (Sakai et al., 2004). Recently Pletinckx et al. (2012) compared chromogenic media with different salt concentrations to isolate MRSA from diverse anatomical sampling sites in pigs. The sensitivity value (85.5%) was similar for ChromID MRSA (bioMérieux) to that reported in this study for CSA (86.96%), but lower than that reported by Gaillot et al. (2000) for human samples (95.5%). The gold standard test for \textit{S. aureus} identification in Pletinckx’s report was a multiplex PCR assay for the detection of an \textit{rsr} gene region specific to \textit{S. aureus}, the \textit{mecA} gene related to meticillin resistance and the \textit{nuC} gene encoding a thermonuclease. Combined real-time PCR detection of these \textit{S. aureus}-specific genes may act as a better gold standard test than biochemical-based identification tests, thus explaining the reduced sensitivity values for ChromID MRSA.
The difference in effectiveness estimated for use of SBA and the coagulase test between Boerlin et al. (2003) and our report resides again in the use of ID 32 STAPH kit or rrS gene sequence analysis as gold standard tests for the identification of S. aureus. In fact, Boerlin et al. (2003) also used rrS sequence analysis, but only to confirm doubtful S. aureus identifications.

A major advantage of rrS or other gene sequencing approaches is that a particular isolate may be correctly identified despite variations in its phenotypic behaviour. Sequencing of the rrS gene has been validated for the identification of known species of the genus Staphylococcus (Takahashi et al., 1999). rrS sequencing cannot distinguish between S. intermedius group-related species (Ghebremedhin et al., 2008), but it was able to distinguish S. aureus from other species of the genus. Furthermore, rrS gene sequencing is not able to distinguish between S. aureus and S. simiae; however, both species are easily distinguishable because S. simiae is negative in the coagulase test and has, so far, been isolated only from chimpanzees (Pantúcek et al., 2005).

An essential complementation of the use of a selective or differential culture medium for primary isolation in the veterinary microbiology laboratory is the use of a secondary test that helps to identify the presumptive S. aureus isolates. Due to their low cost and simplicity, coagulase activity and haemolysis have been extensively used to identify S. aureus isolates. When both of these tests were combined to identify the isolates obtained in SMA, the concordance kappa index raised to 1.0, suggesting a very good agreement with rrS sequencing. These data are in accordance with those reported by Boerlin et al. (2003), for which sensitivity and specificity values were raised close to 100% when both haemolysis and coagulase (at 4 h) were used as identification tests for S. aureus from bovine origin. Our samples were inoculated in plates using cotton swabs soaked in the milk samples, which may introduce variation in the number of isolates obtained in each culture medium. Despite this, significant differences among culture media performance were detected by the statistical analysis. Also, there is a coincidence in our sensitivity values with previous reports using other molecular approaches. SBA as a rich medium may also allow the isolation of microbial species that contaminate the samples. For bovine mastitis diagnosis it is widely recognized that the teat channel hosts a large amount of microbes, thus presenting high contamination levels compared with human clinical samples (Braem et al. 2012; Zadoks et al., 2011). Ericsson Unnerstad et al. (2009) reported sample contamination levels of 4.5% while Persson et al. (2011) described contamination levels of 18%. Our sample contamination level (13%) is between these values. Differences in the level of contamination may be attributed to the particular production system and hygiene practices in the farms under study. Sample contamination was measured as the frequency of yeasts and fungi present on SBA medium. The presence of opportunistic yeasts in bovine mastitis cases has been previously reported (Scaccabarozzi et al., 2011; Zaragoza et al., 2011; Seker, 2010); therefore, we could not discount the role of isolated yeasts in mastitis cases.

In summary, due to its efficacy in isolating S. aureus, its sensitivity, specificity and predictive values, alone or in combination with secondary identification tests, and by its concordance with rrS gene sequence analysis, SMA was the culture medium that was best for isolating and identifying S. aureus from cases of bovine mastitis followed by S110, CSA and SBA. Further evaluation of bacterial isolation or identification strategies, such as new chromogenic media or biochemical tests, should consider the use of rrS or other gene sequence analysis as a better standard for proper and robust validation.

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