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### ► To cite this version:

A. Capurro, K. Artursson, K. Persson Waller, B. Bengtsson, H. Ericsson-Unnerstad, et al.. Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and tuf gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis. *Veterinary Microbiology*, 2009, 134 (3-4), pp.327. 10.1016/j.vetmic.2008.08.028 . hal-00532472

**HAL Id: hal-00532472**

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Submitted on 4 Nov 2010

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## Accepted Manuscript

Title: Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and *tuf* gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis



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PII: S0378-1135(08)00354-4  
DOI: doi:10.1016/j.vetmic.2008.08.028  
Reference: VETMIC 4139

To appear in: *VETMIC*

Received date: 15-4-2008  
Revised date: 20-8-2008  
Accepted date: 21-8-2008

Please cite this article as: Capurro, A., Artursson, K., Waller, K.P., Bengtsson, B., Ericsson-Unnerstad, H., Aspán, A., Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and *tuf* gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.08.028

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1 **Comparison of a commercialized phenotyping system, antimicrobial**  
2 **susceptibility testing, and *tuf* gene sequence-based genotyping for species-**  
3 **level identification of coagulase-negative staphylococci isolated from cases**  
4 **of bovine mastitis**

5

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21

21 **Abstract**

22 In order to evaluate the usefulness of some phenotypic and genotypic methods for species  
23 identification of coagulase negative staphylococci (CNS), isolates were obtained from bovine  
24 cases of clinical and sub-clinical mastitis from different geographical areas in Sweden. By  
25 using the Staph-Zym™ test, antimicrobial susceptibility testing, and sequencing of part of the  
26 CNS *tuf* gene and, when needed, part of the 16S rRNA gene we characterized 82 clinical  
27 isolates and 24 reference strains of 18 different species of staphylococci. The genotypic  
28 methods identified nine different species of CNS among the 82 milk isolates. A comparison  
29 with results obtained by *tuf* gene sequencing showed that Staph-Zym™ correctly identified  
30 CNS reference strains to species level more often than bovine milk CNS isolates (83% and  
31 61%, respectively). In addition, tests supplementary to the Staph-Zym™ were frequently  
32 needed in both groups of isolates (50% of reference strains and 33% of milk isolates) to  
33 obtain an identification of the strain. It is notable that Staph-Zym™ judged two isolates as  
34 CNS, although they belonged to other species, could not give a species name in 11% of the  
35 bovine CNS isolates, and gave 28% of the isolates an incorrect species name. The present  
36 study indicates that the studied phenotypic methods are unreliable for identification of CNS  
37 from bovine intra-mammary infections.

38

39 *Keywords:* CNS; mastitis; Staph-Zym™; *tuf* gene sequencing

## 40 1. Introduction

41 Bovine mastitis is often associated with staphylococcal intra-mammary infections. In Sweden,  
42 as in many other countries, *Staphylococcus aureus* (*S. aureus*) is a common finding in bovine  
43 mastitis, but coagulase-negative staphylococci (CNS) are also frequently found (Bengtsson et  
44 al., 2005). CNS have been considered to mainly cause mild sub-clinical mastitis with a slight  
45 raise in milk somatic cell count (SCC) (Honkanen-Buzalski et al., 1994), but have recently  
46 been associated with both clinical and sub-clinical mastitis (Taponen et al., 2006).

47  
48 The understanding and control of CNS mastitis is complicated by the heterogeneity of this  
49 group of bacteria. So far, 16 CNS species have been isolated from bovine mastitis, and despite  
50 some variation between herds and countries, *S. simulans*, *S. chromogenes*, *S. hyicus* and *S.*  
51 *epidermidis* seem to be the most common (Birgersson et al., 1992; Luthje and Schwarz, 2006;  
52 Taponen et al., 2006). Little information is available about species differences in virulence,  
53 but *S. chromogenes* has been reported to cause more severe mastitis than other CNS (Zhang  
54 and Maddox, 2000). Some CNS species are also capable of persisting in the udder for months,  
55 or even throughout the lactation period (Taponen et al., 2007).

56  
57 In spite of the increasing importance of CNS, these bacteria are not routinely identified to the  
58 species level. Conventional procedures are labour-intensive, and most commercial  
59 identification systems vary in accuracy when used on animal strains (Langlois et al., 1983;  
60 Thorberg and Brändström, 2000). However, Staph-Zym™ (Rosco, Taastrup, Denmark) was  
61 considered useful when evaluated on strains from bovine mastitis (Thorberg and Brändström,  
62 2000). Lately, genotypic methods have contributed substantially in the classification of the  
63 genus *Staphylococcus*. Among such methods, sequencing of a part of the *tuf* gene indicates a  
64 high degree of intra-species stability, and excellent sensitivity and specificity among the

65 genus *Staphylococcus* from human isolates (Martineau et al., 2001; Heikens et al., 2005).

66 Differences in antimicrobial susceptibility could also vary between species, and thus be used  
67 for typing of strains (Devriese et al., 2002).

68

69 To identify better diagnostic tools for bovine CNS, the objective of the study was to compare  
70 two phenotypic and one genotypic methods for species identification of CNS isolated from  
71 clinical and sub-clinical cases of bovine mastitis, by using the Staph-Zym™ test,  
72 antimicrobial susceptibility testing, sequencing of part of the CNS *tuf* gene and, when needed,  
73 the 16S rRNA gene.

74

## 75 **2. Materials and methods**

### 76 *2.1. Reference strains and milk isolates*

77 Twenty-four reference strains of 18 different species of staphylococci (Table 1), and 84  
78 presumptive CNS isolates from clinical (n=64) and sub-clinical (n=20) cases of bovine  
79 mastitis were used in the study. Clinical isolates originated from a national Swedish survey on  
80 prevalence of udder pathogens in cases of acute clinical mastitis, conducted in 2002-2003  
81 (Bengtsson et al., 2005). The sub-clinical mastitis isolates were from milk samples sent to the  
82 Mastitis laboratory (National Veterinary Institute, Uppsala, Sweden) during 2003, and  
83 emanated from the central part of Sweden. All milk samples were collected aseptically  
84 according to the method described by Jonsson et al. (1996).

85

86 Milk samples taken from udder quarters with clinical mastitis were directly cultured (10 µl on  
87 5% bovine blood agar plates (Oxoid Ltd, Cambridge, UK)). The agar plates were incubated at  
88 37 °C for 16-24 h, evaluated in accordance with the routine of each field veterinarian, and sent  
89 to the National Veterinary Institute for bacteriological verification. Milk samples taken from

90 udder quarters with sub-clinical mastitis were sent directly to the National Veterinary Institute  
91 for analysis, and 10 µl were cultured on 5% bovine blood agar (Oxoid Ltd) with 0.05%  
92 esculine (Merck, Darmstadt, Germany). The agar plates were incubated at 37 °C for 24 h. The  
93 isolates were identified as CNS by phenotypic appearance and negative coagulase reaction  
94 according to recommendations by the National Mastitis Council (Hogan et al., 1999). Isolates  
95 were stored at – 20 °C in trypticase soy broth (Oxoid Ltd) containing 15% glycerol. After  
96 thawing, the isolates were sub-cultured (over-night at 37 °C) twice on 5% bovine blood agar  
97 (Oxoid Ltd) with 0.05% esculine before use in further studies.

98

### 99 *2.2. Phenotypic identification of CNS species*

100 Phenotypic identification of CNS to species level was performed using the Staph-Zym™  
101 system (A/S Rosco, Taastrup, Denmark) according to the manufacturer's instructions.  
102 Supplementary tests were performed as described by Devriese and Oeding (1975) and  
103 Devriese et al. (1985).

104

105 Isolates were tested for antimicrobial susceptibility by determination of minimum inhibitory  
106 concentrations using a microdilution method (Bengtsson et al., 2008). All isolates were also  
107 examined for β-lactamase production by the "clover-leaf" method (Bryan and Godfrey, 1991).

108

### 109 *2.3. Genotypic identification of CNS species*

110 Bacterial cells were grown overnight on blood agar plates at 37 °C, and the cultures were  
111 checked for purity. A 1 µl loop of cells was suspended in 50 µl lysostaphin (100 µg/ml;  
112 Sigma-Aldrich Corporation, Saint Louis, MO, US) in water, and incubated at 37 °C for 10  
113 min. Then, 50 µl proteinase K (20 mg/ml; Roche, Basel, Switzerland) was added together  
114 with 150 µl 10mM Tris HCl, pH 7.5, and the mixture was incubated at 54 °C for 10 min. The

115 cell lysate was boiled for five min, cooled on ice for 10 min, and briefly centrifuged at 14 000  
116 g to remove cell debris. The supernatant was used as template in the PCR reaction, or was  
117 frozen at  $-20^{\circ}\text{C}$  until further use.

118

119 To prevent contamination, DNA extraction, preparation of reaction mixtures, amplification,  
120 and detection of PCR products were performed in different rooms. Also, aerosol-resistant  
121 filter pipette tips were used throughout all experiments. For the *tuf* gene sequencing, the  
122 primers *tuf-F* and *tuf-R* were used both for PCR amplification and as sequencing primers  
123 (Heikens et al., 2005). The primers used for 16S rRNA PCR amplification and sequencing  
124 were according to Drancourt et al. (1997) and Weisburg et al. (1991). Amplification of part of  
125 the 16S rRNA was carried out in a 50 $\mu\text{l}$  reaction mixture of 50 mM KCl, 10mM Tris-HCl (pH  
126 8.3), 4 mM  $\text{MgCl}_2$ , 0.2 mM of each deoxynucleoside triphosphates, 0.25 mM of each primer  
127 and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems; Foster City, CA, USA)  
128 and 5  $\mu\text{l}$  of extracted DNA as a template. The amplification reaction was started by a heating  
129 step at  $95^{\circ}\text{C}$  for 8 min. A touch-down protocol followed where each cycle involved heating  
130 to  $95^{\circ}\text{C}$  for 15 s, cooling to  $68^{\circ}\text{C}$  for 30 s and heating again to  $72^{\circ}\text{C}$  for 1 min, the annealing  
131 temperature was decreased to  $58^{\circ}\text{C}$  over 20 cycles. After the touch-down cycles, 40  
132 additional cycles of  $95^{\circ}\text{C}$  for 15 s,  $55^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 1 min were performed.  
133 Negative controls with the DNA template substituted to water in the reaction mixture were  
134 included in each PCR run. Amplicons were visualised on 1.5% agarose gels.

135

136 For DNA sequencing, cycle sequencing reactions were carried out with the BigDye  
137 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's  
138 recommendations. Sequencing reaction products were separated and data were collected using  
139 an ABI 3100 automated DNA sequencer (Applied Biosystems). The sequence was fully

140 determined for both strands of each DNA template to ensure maximum accuracy of the data.  
141 Sequences were edited, assembled and analysed using Vector NTI Suite (InforMax, Inc.,  
142 Bethesda, Maryland, US). Sequences were deposited in GenBank (accession numbers  
143 EU570987-EU571092). The *tuf* gene sequences were translated to amino acid sequences and  
144 aligned with ClustalW Multiple Sequence Alignment tool and a rooted phylogenetic tree was  
145 constructed by the use of ClustalW Multiple Sequence Alignment, using default settings. This  
146 was performed via Biology WorkBench 3.2 (<http://workbench.sdsc.edu/>).

147

148 PCR amplification of the *nuc* gene was used to confirm that two strains (ID 021011-023-391  
149 and 021127-019-534) were *S. aureus*, and was performed according to Brakstad et al. (1992).

150

### 151 **3. Results**

#### 152 *3.1. Staph-Zym*

153 Of the 24 reference strains, Staph-Zym™ identified 20 (83%) strains correctly (Table 1).  
154 Supplementary tests were needed for identification of twelve (50%) of the 24 strains. Two  
155 strains could not be identified, as the codes were not found in the Staph-Zym™ code list. The  
156 *S. pulvereri* reference strain was mis-identified as *S. sciuri*. One of the species of the reference  
157 strains (*S. pseudintermedius* CCUG 32915) was not listed in the Staph-Zym™ species list,  
158 and was misjudged as *S. chromogenes*.

159

160 Two of the milk isolates were found to be *Corynebacterium* spp and *Micrococcus luteus* by  
161 16S rRNA gene sequencing were both suggested to be *S. capitis* by Staph-Zym™. These  
162 isolates were excluded from further studies. Of the remaining 82 CNS milk isolates, Staph-  
163 Zym™ suggested a species name for 73 (89%) isolates (Table 2). For nine isolates a “No  
164 match” result was obtained. Supplementary tests were needed for identification of 27 (33%)

165 of the 82 isolates. Two isolates previously identified as CNS were suggested to be *S. aureus*  
166 using *tuf* gene sequencing, and were confirmed as *S. aureus* by a *S. aureus* specific PCR.

167 Those two strains exhibited a negative coagulase reaction, and Staph-Zym™ identified them  
168 as *S. chromogenes*.

169

### 170 3.2. *tuf* gene sequencing

171 A 371 or a 374-nucleotide long fragment of the *tuf* gene was sequenced for all reference  
172 strains and milk isolates. *S. chromogenes* displayed the most diverging *tuf* nucleotide  
173 sequences of the species analyzed, having nucleotide differences at four positions, rendering  
174 all in all seven genogroups. When using the deduced amino acid sequence, it was found that  
175 only one base substitution was non-synonymous. Thus, the deduced amino acid sequence was  
176 used to construct a rooted phylogenetic tree (Figure 1). For 79 milk strains the species was  
177 identified by comparing their partial *tuf* amino acid sequence to sequences obtained for the  
178 reference strains (Table 2). Identification of three milk isolates failed, as their sequences did  
179 not match any of the reference strains, or any sequences deposited in GenBank. For two other  
180 strains no *tuf* gene amplicon was obtained. Therefore, for these five isolates an approximately  
181 1400-nucleotide long fragment of the 16S rRNA gene was sequenced, and sequences were  
182 compared to sequences deposited in GenBank. For two isolates (ID 020902-022-273 and  
183 020703-008-145) the closest match (99.3%) was *S. haemolyticus* (GenBank ID AP006716),  
184 and for one isolate (ID 021107-018-437) the closest match (99.8%) was *S. lentus* (GenBank  
185 ID EF528296). Two isolates were not CNS as judged by their 16S rRNA sequence, but their  
186 closest match was *Corynebacterium* spp and *Micrococcus luteus*, respectively.

187

188 Genotyping identified nine different species of CNS among the 82 strains isolated from cases  
189 of bovine mastitis (Table 2). Overall, *S. chromogenes* and *S. simulans* were the most

190 common. The dominating isolates among clinical and sub-clinical isolates were *S.*  
191 *chromogenes* (29%) and *S. epidermidis* (30%), respectively (data not shown).

192

### 193 3.3. Comparison between *tuf* gene sequencing and Staph-Zym™

194 In comparison with results obtained by *tuf* gene sequencing, only 50 (61%) of the isolates  
195 were correctly identified using Staph-Zym™, and 17 (34%) of these 50 identifications needed  
196 supplementary tests (Table 2). Among the remaining isolates 9 (11%) had no code listed in  
197 the Staph-Zym™ code list (No match), and 23 (28%) were misjudged. Of those 23 cases 10  
198 (43%) needed supplementary tests.

199

### 200 3.4. Antimicrobial susceptibility

201 Antimicrobial resistance was identified in 15 (18%) of 82 milk isolates, and  $\beta$ -lactamase  
202 production was found in 13 of those isolates (1 *S. aureus*, 1 *S. chromogenes*, 5 *S. epidermidis*,  
203 5 *S. haemolyticus*, 1 *S. xylosus*). One *S. epidermidis* isolate was multi-resistant, i.e. resistant to  
204 more than three antimicrobials. This isolate, as well as one additional *S. epidermidis* isolate,  
205 were *mecA* gene positive by PCR. Two *S. chromogenes* isolates did not produce  $\beta$ -lactamase,  
206 but were resistant to trimetoprim/sulfametoxazole. Anti-microbial resistance was not  
207 associated with specific CNS species or *tuf* gene sequences.

208

## 209 4. Discussion

210 One conclusion of this study is that Staph-Zym™ was better at identifying CNS reference  
211 strains to species level than at identifying bovine milk CNS isolates. Supplementary tests  
212 were, however, frequently needed in both groups of isolates. Moreover, Staph-Zym™ could  
213 only identify 61% of the milk isolates correctly, as judged by genotyping methods. The use of  
214 supplementary tests in combination with Staph-Zym™ was not a guarantee for correct

215 identification of bovine milk isolates. Another finding was that variation in antimicrobial  
216 susceptibility was not observed between CNS species, and such testing is therefore not  
217 suitable for CNS strain typing.

218

219 In the literature, limited and diverging information is given on the performance of Staph-  
220 Zym™ and the need for supplementary tests in relation to reference strains (Watts and  
221 Washburn, 1991; Ieven et al., 1995; Burriel and Scott, 1997). We used the same *S.*  
222 *chromogenes* reference strain as Burriel and Scott (1997), and in both studies Staph-Zym™  
223 was unable to give a name (No match) to that strain. In our study, 50% of the reference strains  
224 needed supplementary tests to be identified. One of the strains needing supplementary tests,  
225 the *S. hyicus* reference strain, was also used by Watts and Washburn (1991), but in that study  
226 the strain was identified without the need of supplementary tests. In the study by Thorberg  
227 and Brändström (2000), this reference strain was misidentified by Staph-Zym™ as *S.*  
228 *epidermidis*. Discrepancies between studies in identification of *S. hyicus* may be due to  
229 difficulties in evaluation of the  $\beta$ -glucuronidase and  $\beta$ -galactosidase tests in which *S. hyicus* is  
230 positive and negative, respectively (Bannerman and Peacock, 2007). According to Thorberg  
231 and Brändström (2000), these two tests should be considered positive even when only weakly  
232 coloured. Misidentification of a *S. sciuri* subsp. *sciuri* reference strain as *S. cohnii* subsp.  
233 *cohnii* by Staph-Zym™ has also been reported in human cases (De Paulis et al., 2003). In our  
234 study, the *S. sciuri* reference strain needed supplementary tests for correct identification. The  
235 *S. pulvereri* strain was misidentified as *S. sciuri* by Staph-Zym™. According to the test  
236 instructions *S. pulvereri* (synonymous with *S. vitulus* and *S. vitulinus*) is always mannose  
237 negative, but the reference strain was mannose positive. According to other studies, *S.*  
238 *pulvereri* strains may have variable mannose acidification results (Svec et al., 2004).

239

240 Previous studies on bovine milk isolates, comparing Staph-Zym™ to conventional phenotypic  
241 methods, have reported good agreement (Watts and Washburn, 1991; Thorberg and  
242 Brändström, 2000), but Burriel and Scott (1997) found relatively poor agreement when using  
243 ovine isolates, in spite that the reference method used as gold standard was similar to that  
244 used by Watts and Washburn (1991). In our study, the agreement between the *tuf* gene  
245 identification and Staph-Zym™ was better for *S. simulans* and *S. epidermidis* than for *S.*  
246 *chromogenes*. All *S. chromogenes* correctly identified by Staph-Zym™ needed supplementary  
247 tests. This finding concur largely with those of previous studies (Thorberg and Brändström,  
248 2000; Watts and Washburn, 1991; Burriel and Scott, 1997).

249

250 In the present study, two CNS strains misjudged by Staph-Zym™ as *S. chromogenes* using  
251 supplementary tests were identified as *S. aureus* by *tuf* gene sequencing and PCR  
252 amplification of the *nuc* gene. One of the four supplementary tests performed was the  
253 coagulase reaction in which these two isolates failed to coagulate rabbit plasma. Such *S.*  
254 *aureus* strains have been described previously (Fox et al., 1996). Correct identification of *S.*  
255 *chromogenes* and *S. aureus* is important especially as several studies report *S. chromogenes* to  
256 be the major CNS pathogen isolated in both heifers and primiparous cows (Luthje and  
257 Schwarz, 2006; Taponen et al., 2006).

258

259 Recently, Ghebremedhin et al. (2008) compared phylogenetic analysis of staphylococcal  
260 species based on partial sequencing of different genes, as 16S rRNA sequence comparison,  
261 the most traditional target for bacterial phylogeny and taxonomy, is not considered to have  
262 enough discriminatory power within these genera. They suggested the glyceraldehydes-3-  
263 phosphate dehydrogenase (*gap*) gene to be a suitable target for taxonomical analysis of  
264 *Staphylococcus* species, as compared to *hsp60*, *sodA*, *rpoB* and *tuf* gene sequence analysis.

265 However, they needed to sequence a 931 bp fragment of this gene, as the variable regions  
266 were dispersed over the gene fragment. The method was not applied to clinical staphylococcal  
267 isolates, thus the intra-species variation was not evaluated. We used partial *tuf* gene  
268 sequencing (ca 370bp), after translation to amino acid sequences to identify CNS. In this way,  
269 we obtained complete match between the reference strains used and the bovine mastitis  
270 isolates for *S. simulans*, *S. epidermidis*, *S. hyicus*, and *S. warneri*. The two isolates of *S.*  
271 *xylosus* displayed one amino acid sequence difference; this intra-species sequence variation of  
272 *S. xylosus* is in agreement with the findings of Heikens et al. (2005). *S. chromogenes* also  
273 divided into two groups differing by one amino acid, with five and 17 isolates in each group.  
274 Two isolates that clustered most closely to *S. haemolyticus* (two amino acid differences) also  
275 displayed the closest match by 16S rRNA sequencing to *S. haemolyticus*. For *S. lentus* we did  
276 not have a reference strain to compare with, but this isolate was identified to species by 16S  
277 rRNA sequencing.

278

## 279 **5. Conclusion**

280 Our results support the notion that micromethods such as Staph-Zym™ based on phenotypic  
281 identification may be unable to reliably distinguish between different CNS species, and that  
282 genotypic methods are more reliable for diagnostic purposes in the identification of CNS from  
283 bovine IMI. It is notable that the accuracy of Staph-Zym™ varied widely between CNS  
284 species, and that the test had low specificity in identification of important udder pathogens  
285 such as *S. chromogenes*. There was also a tendency to give an incorrect species name rather  
286 than a 'No match'. Additionally, a drawback of Staph-Zym™ was that coagulase negative *S.*  
287 *aureus* was not identified. Further studies using genotypic methods on field isolates of CNS  
288 species are needed.

289

290 **Acknowledgements**

291 We are very grateful to Sara Frosth for her excellent laboratory support. This project has been  
292 supported by the The Swedish Research Council for Environment, Agricultural Sciences and  
293 Spatial Planning (Formas).

294

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380 Table 1

381 Reference strains (n=24) of the genus *Staphylococcus* used in this study, and their respective  
 382 species identification by Staph-Zym™ (A/S Rosco, Taastrup, Denmark)

Strain	CCUG / ATCC <sup>1</sup>	Staph-Zym™
<i>S. aureus</i>	CCUG 46176	<i>S. aureus</i> <sup>2</sup>
<i>S. aureus</i>	CCUG 41582 / NCTC 8325	<i>S. aureus</i>
<i>S. capitis</i>	CCUG 7326 <sup>T</sup> / ATCC 27840	<i>S. capitis</i> <sup>2</sup>
<i>S. chromogenes</i>	CCUG 4319 <sup>T</sup>	No match <sup>3,6</sup>
<i>S. cohnii</i>	CCUG 35144 <sup>T</sup>	<i>S. cohnii</i>
<i>S. epidermidis</i>	CCUG 46178	<i>S. epidermidis</i>
<i>S. epidermidis</i>	CCUG 21989	No match <sup>4,6</sup>
<i>S. gallinarum</i>	CCUG 28809	<i>S. gallinarum</i> <sup>2</sup>
<i>S. haemolyticus</i>	CCUG 7323 <sup>T</sup>	<i>S. haemolyticus</i>
<i>S. hominis</i>	CCUG 35516 <sup>T</sup>	<i>S. hominis</i>
<i>S. hyicus</i>	CCUG 6509 <sup>T</sup>	<i>S. hyicus</i>
<i>S. hyicus</i>	CCUG 15602 <sup>T</sup> / ATCC 11249	<i>S. hyicus</i> <sup>2</sup>
<i>S. intermedius</i>	CCUG 6520 <sup>T</sup>	<i>S. intermedius</i> <sup>2</sup>
<i>S. intermedius</i>	ATCC 51874	<i>S. intermedius</i> <sup>2</sup>
<i>S. pseudintermedius</i> <sup>5</sup>	CCUG 32915	<i>S. chromogenes</i> <sup>2</sup>
<i>S. pulvereri</i>	CCUG 41685	<i>S. sciuri</i> <sup>2</sup>
<i>S. saprophyticus</i>	CCUG 3706 <sup>T</sup>	<i>S. saprophyticus</i>
<i>S. schleiferi</i>	ATCC 49545	<i>S. schleiferi</i> <sup>2</sup>
<i>S. schleiferi</i>	CCUG 37248 <sup>T</sup>	<i>S. schleiferi</i> <sup>2</sup>
<i>S. sciuri</i>	CCUG 15598 <sup>T</sup>	<i>S. sciuri</i> <sup>2</sup>
<i>S. simulans</i>	CCUG 46177	<i>S. simulans</i>
<i>S. simulans</i>	CCUG 7327A <sup>T</sup>	<i>S. simulans</i>
<i>S. warneri</i>	CCUG 44859	<i>S. warneri</i>
<i>S. xylosus</i>	CCUG 7324 <sup>T</sup>	<i>S. xylosus</i> <sup>2</sup>

383

384 <sup>1</sup>CCUG = Culture Collection, University of Göteborg, Göteborg, Sweden; ATCC = American

385 Type Culture Collection, Manassa, USA. <sup>2</sup>Supplementary tests were needed for identification.

386 <sup>3</sup>The closest Staph-Zym™ codes were *S. hyicus* or *S. chromogenes*. <sup>4</sup>The closest Staph-

387 Zym™ code was *S. epidermidis*. <sup>5</sup>Species not listed in the Staph-Zym™ species list. <sup>6</sup>Code

388 not listed in the Staph-Zym™ code list.

389 Table 2

390 Species identification, using *tuf* gene sequencing, of 82 CNS strains isolated from cases of

391 bovine mastitis, and number of those isolates correctly identified using Staph-Zym™

Species	Identified using <i>tuf</i> gene	Identified by Staph-Zym™ as No match <sup>2</sup>	Correctly identified using Staph-Zym™		Misjudged as	n
			Yes	No		
			n (%)	n (%)		
<i>S. chromogenes</i>	22	4 (18)	13 (59)	5 (23)	<i>S. cohnii</i>	1
					<i>S. haemolyticus</i>	1
					<i>S. simulans</i>	1
					<i>S. warneri</i>	2
<i>S. simulans</i>	20	2 (10)	17 (85)	1 (5)	<i>S. chromogenes</i>	1
<i>S. haemolyticus</i>	12 (2 <sup>1</sup> )	1 (8)	4 (33)	7 (58)	<i>S. capitis</i>	1
					<i>S. chromogenes</i>	1
					<i>S. warneri</i>	4
					<i>S. xylosus</i>	1
<i>S. epidermidis</i>	10	1 (10)	8 (80)	1 (10)	<i>S. chromogenes</i>	1
<i>S. hyicus</i>	9	0 (0)	4 (44)	5 (56)	<i>S. chromogenes</i>	1
					<i>S. haemolyticus</i>	1
					<i>S. warneri</i>	1
					<i>S. xylosus</i>	2
<i>S. warneri</i>	4	0 (0)	2 (50)	2 (50)	<i>S. hyicus</i>	1
					<i>S. simulans</i>	1
<i>S. xylosus</i>	2	1 (50)	1 (50)	0 (0)		
<i>S. aureus</i>	2	0 (0)	0 (0)	2 (100)	<i>S. chromogenes</i>	2
<i>S. lentus</i>	1 <sup>1</sup>	0 (0)	1 (100)	0 (0)		
Total	82	9 (11)	50 <sup>3</sup> (61)	23 <sup>4</sup> (28)		

392 <sup>1</sup> Identified by 16S rRNA sequencing. <sup>2</sup> Code not listed in the Staph-Zym™ code list.393 <sup>3</sup> Seventeen of the isolates (13 *S. chromogenes*, 1 *S. simulans*, 1 *S. haemolyticus*, 1 *S. xylosus*,394 1 *S. lentus*) needed supplementary tests. <sup>4</sup> Ten of the isolates (2 *S. aureus*, 2 *S. haemolyticus*, 2395 *S. hyicus*, 2 *S. chromogenes*, 1 *S. epidermidis*, 1 *S. warneri*) needed supplementary tests.

396 **Figure caption**

397 Fig. 1. A rooted phylogenetic tree was constructed from the *tuf* gene amino acid sequences of  
398 the 24 reference strains (black letters) and 82 strains of CNS (blue letters) isolated from cases  
399 of clinical (CM) or sub-clinical (SCM) mastitis. Information on Staph-Zym™ results is given  
400 within brackets (blue=correct ID; azure blue=species not listed; orange=code not listed;  
401 red=misjudged). Species identification by 16S rRNA is given in grey letters. Green ● =  
402 supplementary tests were needed for Staph-Zym™ identification.

403

