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1 **Species Identification of Coagulase-Negative Staphylococci: Genotyping is Superior**
2 **to Phenotyping**

3

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21

21 **Abstract**

22

23 Coagulase-negative staphylococci (CNS) are isolated commonly from bovine
24 milk and skin. Their impact on udder health and milk quality is debated. It has been
25 suggested that sources and consequences of infection may differ between CNS species.
26 Species-specific knowledge of the impact and epidemiology of CNS intramammary
27 infections is necessary to evaluate whether species-specific infection control measures are
28 feasible and economically justified. Accurate measurement of impact, sources, and
29 transmission mechanisms requires accurate species level identification of CNS. Several
30 phenotypic and genotypic methods for identification of CNS species are available. Many
31 methods were developed for use in human medicine, and their ability to identify bovine
32 CNS isolates varies. Typeability and accuracy of typing methods are affected by the
33 distribution of CNS species and strains in different host species, and by the ability of test
34 systems to incorporate information on new CNS species into their experimental design
35 and reference database. Generally, typeability and accuracy of bovine CNS identification
36 are higher for genotypic methods than for phenotypic methods. As reviewed in this paper,
37 DNA sequence-based species identification of CNS is currently the most accurate species
38 identification method available because it has the largest reference database, and because
39 a universally meaningful quantitative measure of homology with known species is
40 determined. Once sources, transmission mechanisms, and impact of different CNS
41 species on cow health, productivity and milk quality have been identified through use of
42 epidemiological data and accurate species identification methods, appropriate methods
43 for routine use in research and diagnostic laboratories can be proposed.

44 **Key Words:** Genotyping, phenotyping, bovine, coagulase negative staphylococcus,

45 mastitis

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46 1. Introduction

47

48 The role of coagulase-negative staphylococci (CNS) as etiological agents of
49 bovine mastitis has not been fully elucidated and previous studies in this area have
50 yielded contradictory results. For example, some researchers regard CNS as an important
51 cause of bovine mastitis (Pyörälä and Taponen, 2008), while others consider them minor
52 pathogens with limited impact on milk quality and udder health (Schukken et al., 2008).
53 Presence of CNS is associated with clinical mastitis and with somatic cell counts (SCC)
54 that are, on average, higher than those in culture-negative quarters (Schepers et al., 1997;
55 Kudinha and Simango, 2002). Increased SCC is generally associated with decreased milk
56 production (Seegers et al., 2003) but subclinical intramammary infections by CNS have
57 been associated with increased milk production (Wilson et al., 1997). By contrast, clinical
58 CNS mastitis was linked to decreased milk production (Gröhn et al., 2004) and increased
59 risk of culling (Gröhn et al., 2005). Mere detection of CNS in a milk sample was not
60 associated with an increased risk of treatment or culling for mastitis (Reksen et al., 2006).
61 In addition to debate about the impact and relevance of CNS infections, there is debate on
62 whether or not specific CNS species are associated with the outcome of infection. A
63 Finnish study showed an association between CNS species and severity of clinical
64 symptoms (Honkanen-Buzalski et al., 1994), but a different study from the same country
65 did not show such an association (Taponen et al., 2006). One CNS species,
66 *Staphylococcus chromogenes*, is thought to protect the udder from intramammary
67 infection (Matthews et al., 1990a; De Vliegher et al., 2003), whereas *Staphylococcus*
68 *hyicus*, a closely related species, does not have this effect. CNS have been categorized

69 into human and animal-associated species (Watts and Owens, 1989). Human-associated
70 CNS species, specifically *Staphylococcus epidermidis*, are thought to be more likely to
71 invade and infect the udder than animal-associated species (Devriese and De Keyser,
72 1980; Watts and Owens, 1989). Prevalence of *Staphylococcus epidermidis* may be
73 associated with herd management factors. Specifically, *S. epidermidis* is more common in
74 herds that use linear dodecyl benzene sulphonic acids for postmilking teat disinfection
75 than in herds that use iodine (Hogan et al., 1987; Watts and Owens, 1989). Whether
76 species specific transmission routes and control strategies exist for other CNS species is
77 largely unknown. Because little species-specific information on control of CNS mastitis
78 is available, identification to the group level, possibly supplemented with antimicrobial
79 susceptibility testing, is currently sufficient for most therapeutic and management
80 decisions. The defining characteristic of CNS as a group is the lack of coagulase
81 expression, which is a phenotypic trait. To evaluate whether species-specific infection
82 control measures are feasible and economically justified, species-specific knowledge of
83 the impact and epidemiology of CNS infections is necessary. Accurate measurement of
84 the impact, sources, transmission mechanisms and control options for individual CNS
85 species requires accurate species level identification of CNS (Thorberg and Brändström,
86 2000; Heikens et al., 2005; Sivadon et al., 2005). In this contribution, merits of
87 phenotypic and genotypic methods for CNS species identification are compared with
88 special consideration of identification of CNS isolated from bovine milk.

89

89 **2. The Species Concept**

90

91 Interpretive criteria for the definition of bacterial genera and species are not
92 consistent in the literature and may differ between species, genera and authors (Freney et
93 al., 1999; Lan and Reeves, 2001; CLSI, 2007). In fact, the whole concept of what defines
94 a bacterial species is a matter of debate (Lan and Reeves, 2001). Standards for description
95 of new staphylococcal species were last defined by the Subcommittee on the taxonomy of
96 staphylococci and streptococci of the International Committee on Systematic
97 Bacteriology in 1999 and were based largely on phenotypic criteria (Freney et al., 1999).
98 Nowadays, combinations of phenotypic and genotypic methods are used to define new
99 species, such as biochemical profiling, gas chromatographic analysis of cellular fatty
100 acids, ribotyping, sequencing of the 16S rRNA gene and sequencing of additional
101 housekeeping genes (Becker et al., 2004; Carretto et al., 2005). For many species, there is
102 only a single or a limited number of type strains, and their phenotype and genotype
103 defines the species (Becker et al., 2004; Shah et al., 2007). The aim of this paper is not to
104 discuss the definition of species, but the accurate designation of species names to clinical
105 isolates. Given that species and type strains exist, our task as diagnosticians and scientists
106 is to determine to which species the CNS isolates that we study belong.

107

108 **3. Phenotypic Identification of CNS**

109

110 Phenotypic identification methods are based on evaluation of the expression of
111 genetically encoded characteristics by bacterial isolates. Phenotypic traits include

112 morphology, growth characteristics, ability to metabolize substrates, antimicrobial
113 resistance, and other features that result from DNA-expression but that are not based on
114 detection of the bacterial DNA itself. Over the years, many phenotypic methods have
115 been developed for the identification of staphylococci in diagnostic laboratories. Methods
116 include commercial test systems such as the API 20 Staph system (bioMérieux), API ID
117 32 Staph (bioMérieux), Staph-Zym (Rosco), the Vitek system (bioMérieux) and other
118 combinations of biochemical tests, which may not be available in commercial formats
119 (Bannerman et al., 1993; Devriese et al., 1994; Watts and Yancey, 1994; Ieven et al.,
120 1995).

121 An inherent weakness of phenotypic methods is that there is variability in
122 expression of phenotypic characteristics by isolates belonging to the same species
123 (Bannerman et al., 1993; Ieven et al., 1995; Heikens et al., 2005). Furthermore, the
124 interpretation of phenotypic tests can be subjective (Carretto et al., 2005). Variability in
125 the expression and interpretation of phenotypic characteristics limits the reproducibility
126 of tests, i.e. the ability to generate the same results every time the tests are used. In
127 addition to reproducibility, the typeability and accuracy of phenotypic testing are
128 imperfect. Typeability is the proportion of isolates that are assigned a type by a typing
129 system (Struelens et al., 1996). An increase in the number of tests that is included in a
130 system generally improves typeability. For example, a study of human CNS isolates with
131 an API-system based on 20 biochemical reactions showed a typeability of only 37%
132 (Carretto et al., 2005) whereas a system that included 32 reactions had a typeability of
133 85% (Maes et al., 1997).

134 Accuracy does not have a single standard definition. The concept can be
135 interpreted in two major ways. First, accuracy can denote the level of certainty assigned
136 by a test to its own results. The statement "isolate 13 was identified to *S. cohnii* by API
137 Staph with 99.1% accuracy" (from Heir et al., 1999) would be an example of this
138 interpretation. Second, accuracy can denote the level of agreement between a method and
139 a reference method, i.e. the correctness of the identification. The statement "Isolate 13,
140 identified to *S. cohnii* by API Staph with 99.1% accuracy was identified as *S.*
141 *caseolyticus* by 16S rRNA gene sequence analysis " (based on Heir et al., 1999) shows
142 that the 99.1% "accuracy" assigned to the phenotypic test score did not reflect correct
143 species identification. In the same study, a second isolate was not identified by API Staph
144 typing, and identified as *S. cohnii* by 16S rRNA gene sequencing and tDNA-ILP (tDNA
145 intergenic spacer length polymorphism) (Heir et al., 1999). Results for the second isolate
146 provide an example of limited typeability of the phenotypic method, but without incorrect
147 or inaccurate species identification. The acceptable level of accuracy for phenotypic
148 methods is subjective. In one study, 111 of 122 CNS isolates were identified by the Vitek
149 GPI system, with 29% of those at more than 90% probability of accuracy (Lee and Park,
150 2001). The typeability of this system could be calculated as $111 \text{ of } 122 = 91\%$, if all
151 isolates for which a species name was generated were considered typeable, or as $32 \text{ of } 122 = 26\%$, if only results with more than 90% probability of accuracy were considered
152 acceptable for species identification. In a study on CNS isolates from ovine milk between
153 81.4% and 96.5% typeability was reported for three phenotypic species identification
154 methods, but only 29% of isolates were identified in the same manner by all three
155 methods (Burriel and Scott, 1998). This casts considerable doubt on the accuracy of the
156

157 species identification obtained with each method. In our opinion, high apparent accuracy
158 resulting in incorrect species identification is a bigger concern than limited typeability.
159 The latter alerts the user to the fact that the correct species identity was not determined by
160 the test whereas the former gives a false sense of security. Other authors have also
161 expressed concerns about false rather than ambiguous identification of CNS species by
162 phenotypic systems (Sivadon et al., 2004).

163 Past identification errors or changes in taxonomy may contribute to incorrect
164 species identification by phenotypic methods (Carretto et al., 2005). For example, *S. felis*
165 was named in 1989 (Igimi et al., 1989) and it is thought that many supposed *S. simulans*
166 isolates from cats that were identified before that time were really *S. felis* isolates
167 (Lilenbaum et al., 1999). It is unfair to penalize a system retroactively for
168 misidentification of isolates prior to species reclassification, but once species
169 reclassification occurs, test design, isolate identification and species distributions from
170 studies preceding the reclassification may need to be reinterpreted. Until 1986, *S.*
171 *chromogenes* and *S. hyicus* were not recognized as different species but seen as two
172 subspecies, i.e. subspecies *chromogenes* and subspecies *hyicus*, of the species *S. hyicus*
173 (Hajek et al., 1986). In studies conducted after 1986, *S. chromogenes* was often the most
174 common CNS species found in milk (Table 1). Before 1986, these isolates would have
175 been classified as *S. hyicus*, which would be incorrect under current species definitions.
176 Errors in identification are not limited to the species level, but may occur at the genus
177 level. For example, some versions of the Vitek 2 system erroneously classified human
178 clinical CNS isolates as *Kocuria* species (Ben-Ami et al., 2005; Boudewijns et al., 2005).
179 The distinction between *Staphylococcus* and *Kocuria* is not just of academic interest.

180 CNS isolates are viewed as pathogens and as an indication for treatment, while *Kocuria*
181 species are considered contaminants that do not warrant treatment. Misclassification of
182 *Staphylococcus* as *Kocuria* could result in withholding treatment from a patient, with
183 potentially damaging consequences (Ben-Ami et al., 2005). The limitations of phenotypic
184 methods are not unique to CNS. Similar concerns have been reported for other bacterial
185 genera, such as *Enterococcus* and *Lactobacillus* in foods (Huys et al., 2006) and
186 *Streptococcus* and *Enterococcus* isolates of animal origin (Watts and Yancey, 1994;
187 Hudson et al., 2003; Loch et al., 2005).

188 Phenotypic methods are usually considered less expensive than genotypic
189 methods. Whether or not this is true depends in part on turnover, which affects overhead
190 costs and opportunities and needs for automation. In some clinical laboratories,
191 phenotypic tests are used with such high frequency that an investment in automation of
192 reading and interpretation of tests is profitable (Ieven et al., 1995). In other laboratories,
193 test frequency may be so low that expiration of reagents and the costs of replacing them
194 are a concern. Regardless of test volume, additional testing may be needed to obtain final
195 results from phenotypic methods. This increases cost and turn-around-time of
196 phenotyping testing, thereby narrowing or eliminating the cost and time differences
197 between phenotypic and genotypic identification methods (Ieven et al., 1995; Thorberg
198 and Brändström, 2000). When comparing the cost of phenotypic and genotypic methods,
199 the costs of obtaining inaccurate results must also be considered.

200

200 4. Genotypic Identification of CNS

201

202 Genotypic methods use DNA as the basis for identification. Genotypic methods
203 are used for identification to the species level and for strain typing, i.e. differentiation of
204 isolates at the subspecies level. Genotypic methods used for identification of CNS species
205 include amplified-fragment length polymorphism (AFLP) analysis (Taponen et al., 2006,
206 2007), ribotyping (Carretto et al., 2005), tDNA-ILP analysis (Maes et al., 1997; Heir et
207 al., 1999; Lee and Park, 2001; Rossi et al., 2001; Stepanović et al., 2005), and DNA-
208 sequencing (Heir et al., 1999; Sivadon et al., 2004; Heikens et al., 2005; Sivadon et al.,
209 2005). Across bacterial genera the most common target for DNA-sequencing is the 16S
210 rRNA gene (Lan and Reeves, 2001). Many CNS species are closely related, and 16S
211 sequence based typing may not have sufficient discriminatory power to differentiate all of
212 them (CLSI, 2007; Shah et al., 2007). Therefore, species identification systems based on
213 the housekeeping genes *cpn60* (chaperonin or heat-shock protein 60) (Kwok et al., 1999),
214 *dnaJ* (heat-shock protein 40) (Shah et al., 2007), *rpoB* (beta subunit of RNA polymerase)
215 (Drancourt and Raoult, 2002; Mellmann et al., 2006), *sodA* (superoxide dismutase A)
216 (Poyart et al., 2001; Sivadon et al., 2004; Heikens et al., 2005; Sivadon et al., 2005), and
217 *tuf* (elongation factor Tu) (Heikens et al., 2005) have been developed and implemented
218 for CNS identification.

219 In general, genotypic methods have higher discriminatory power, reproducibility
220 and typeability than phenotypic methods. For example, automated ribotyping identified
221 166 of 177 (94%) of CNS isolated from humans (Carretto et al., 2005). tDNA-ILP
222 identified 157 of 161 (97.5%) *S. sciuri* group isolates from human, animal and

223 environmental sources (Stepanović et al., 2005), 162 of 163 (99%) of human clinical
224 CNS isolates from Belgium (Maes et al., 1997) and 114 of 122 (93%) of human clinical
225 isolates from Korea (Lee and Park, 2001). DNA-sequencing of CNS from bone and joint
226 infections identified 211 of 212 (99%) of isolates (Sivadon et al., 2005). An advantage of
227 DNA sequence-based methods is that they allow for recognition of previously unreported
228 sequences from novel species (Sivadon et al., 2004; CLSI, 2007).

229 Genotypic methods, like phenotypic methods, use cut-off values for acceptable
230 levels of similarity to identify bacterial species. For example, banding pattern similarity
231 of 90% or more is used as a cut-off value to consider isolates members of the same
232 bacterial species in automated ribotyping of CNS with restriction enzyme *EcoRI*
233 (Carretto et al., 2005). Categorization of banding patterns is often based on automated
234 analysis followed by visual inspection and manual correction, introducing some
235 subjectivity in the interpretation (Carretto et al., 2005) Automated data analysis is also
236 used for interpretation of tDNA-ILP results, but thresholds for species identification are
237 not defined clearly (Maes et al., 1997). Because tDNA-PCR is based on banding patterns
238 generated by PCR and separated by electrophoresis, results are somewhat susceptible to
239 PCR and electrophoresis conditions (Maes et al., 1997; Lee and Park, 2001). For analysis
240 of sequence data, interpretation criteria are gene-specific. For the highly conserved 16S
241 rRNA gene, 98% or 99% has been used as cut-off value (Nelson et al., 2003; Gill et al.,
242 2006; CLSI, 2007). Other housekeeping genes, such as *sodA* and *tuf*, are less conserved,
243 which allows them to be used in sequence-based strain typing methods (Zadoks et al.,
244 2005). For these genes, homology values of 97% or more are considered acceptable
245 (Heikens et al., 2005; Sivadon et al., 2005). Within-species heterogeneity of

246 housekeeping genes differs between bacterial species (Loch et al., 2005). Thus, both
247 within species-variability and between-species variability of sequence data may need to
248 be considered to decide on appropriate cut-off values for homology (Lan and Reeves,
249 2001). Some authors specifically include criteria for difference in sequence identity from
250 the next closest species, e.g. 5% or more, in guidelines for interpretation of DNA
251 sequence data of CNS species (Sivadon et al., 2005).

252 When genotypic methods were first developed, they tended to be more labor-
253 intensive than phenotypic methods, more expensive, or both. While some methods, such
254 as automated ribotyping, are still costly, other methods are not necessarily more
255 expensive than phenotypic methods. Affordability and feasibility of use of genotypic
256 methods differs between laboratory settings, just like affordability and feasibility of use
257 of phenotypic methods. Some laboratories have easy access to an automated RiboPrinter,
258 and other laboratories have in-house AFLP or tDNA-ILP facilities. DNA sequencing is a
259 highly portable method because material for sequencing can be shipped to specialized
260 laboratories by mail, and sequence data are routinely provided in electronic format for
261 remote downloading and analysis. There is no need for physical proximity of the
262 investigator or diagnostician to the sequencing facility. In one of our laboratories, species
263 identification of CNS isolates for diagnostic purposes is currently done by DNA
264 sequencing rather than phenotypic methods. Turnover of commercial phenotypic test kits
265 for identification in that laboratory is low. As a result, reagents tend to expire, and the
266 cost of replacing them increases the cost of phenotypic typing. By contrast, PCR and
267 DNA-sequencing are performed routinely so that reagents are fresh and CNS

268 identification fits into the workflow easily. Hands-on time per isolate is similar for
269 phenotypic and DNA sequence-based methods in this laboratory.

270

271 **5. Databases**

272

273 For interpretation of results from phenotypic or genotypic assays, comparison
274 with reference data is essential. Most phenotypic species identification methods were
275 developed for microbial isolates obtained from humans (Watts and Yancey, 1994).
276 Common CNS species in humans include, among others, *S. epidermidis*, *S. haemolyticus*,
277 *S. hominis*, *S. simulans*, *S. xylosus* and *S. capitis* (Maes et al., 1997; Lee and Park, 2001;
278 Carretto et al., 2005). Results of eight studies from seven countries and three continents
279 indicated that the most common CNS species isolated from bovine milk are *S.*
280 *chromogenes*, *S. hyicus*, *S. simulans*, *S. epidermidis* and *S. xylosus* (Table 1).
281 *Staphylococcus chromogenes* and *S. hyicus*, the two most common CNS species from
282 bovine milk, were not recognized by an early version of the Staph-Zym system, while the
283 latter three species, all of which are also important in human medicine, were identified
284 correctly (Watts and Washburn, 1991). This is an example of the fact that most
285 commercial systems are developed for identification of human rather than animal
286 pathogens, and that the systems may not identify animal pathogens accurately. Early
287 versions of the Vitek and API Staph system also showed limited ability to identify *S.*
288 *chromogenes* and *S. hyicus* (Matthews et al., 1990b). Recent work in our laboratory
289 showed that API 20 Staph and BBL Crystal tests had low sensitivity and specificity in the
290 detection of *S. chromogenes* and *S. hyicus* isolates, respectively, when analyzing 82

291 isolates obtained in the USA from heifers around calving (unpublished data). In a second
292 dataset, encompassing 172 CNS isolates from lactating animals in The Netherlands,
293 StaphZym and API 20 Staph testing also showed limited typeability and accuracy. For
294 example, all *S. epidermidis* isolates were identified correctly but many isolates of *S.*
295 *chromogenes* and *S. hyicus* were not (Sampimon et al., in preparation). Thorberg and
296 Brändström (2000) reported more favorable results for StaphZym analysis, i.e. 94%
297 accuracy across bacterial species. They suggest that cost may be the main impediment to
298 routine use of the method. Considering, however, that accuracy of detection of *S.*
299 *chromogenes* was only 86%, even after additional tests were performed, and that *S.*
300 *chromogenes* is the most common species isolated from bovine milk (Table 1), the value
301 of the test for routine diagnostics can be questioned.

302 Strain differences between bacterial isolates from different host species may
303 compound species identification problems (Watts and Yancey, 1994). For some Gram-
304 positive bacteria that are commonly found in humans and in bovine milk, specifically
305 *Streptococcus agalactiae* and *Staphylococcus aureus*, host-species specific groups of
306 strains have been identified (Smith et al., 2005; Sukhnanand et al., 2005). To our
307 knowledge, strain level comparisons of CNS isolates from humans and cattle have not
308 been reported. If host-species associated strains exist within CNS species, phenotypic
309 methods that were developed using human isolates may not identify bovine strains
310 correctly, even if the bacterial species is common to both host species.

311 The problem of a CNS-species distribution that differs from the one found in
312 humans is not unique to bovine CNS. CNS populations in other animal species and in
313 foods also differ from those in humans (Table 2). As a result, phenotypic methods that

314 were developed for identification of human CNS may also fail to identify CNS from
315 other animal species accurately. Some CNS species are common to many hosts, including
316 humans, e.g. *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. simulans*, and *S.*
317 *xylosus*. Other CNS species, such as *S. caprae*, *S. chromogenes*, *S. felis*, *S. gallinarum*
318 and *S. sciuri* are the most common species in small ruminants (Deinhofer and Pernthaner,
319 1995; Pengov 2001), cattle (Table 1), cats (Igimi et al., 1994; Lilenbaum et al., 1999),
320 chickens (Awan and Matsumoto, 1998; Aarestrup et al., 2000) and treefrogs (Slaughter et
321 al., 2001), respectively, while they are rare in other host species. Species that are rarely
322 isolated from human clinical samples, e.g. *S. equorum*, *S. pasteurii* and *S. capitis*
323 (relatively common in birds and horses, Table 2), are not included in the API 20 Staph
324 database (Ieven et al., 1995; Carretto et al., 2005) or, in the case of *S. fleuretti* and *S.*
325 *vitulinus*, in any commercial system (Stepanović et al., 2005). *Staphylococcus equorum*
326 was the fifth-most common species isolated from milk in the Netherlands in the
327 aforementioned study (Sampimon et al., in preparation). Because this species is not
328 recognized by API 20 Staph testing, and because many studies of bovine CNS use API
329 testing (Chaffer et al., 1999; Matthews et al., 1990b; Sampimon et al., 2007) the
330 prevalence of *S. equorum* in milk may be underestimated. In contrast to the API 20 Staph
331 test, the API Staph ID 32 test does have the ability to identify *S. equorum* from milk
332 samples (Taponen et al., 2006).

333 Keeping databases up to date is a major challenge. In 1995, 31 *Staphylococcus*
334 spp. had been named (Ieven et al., 1995). By 2003, this number had increased to 38
335 (Spergser et al., 2003). By 2007, the NCBI Taxonomy database
336 (<http://www.ncbi.nlm.nih.gov/>) listed 43 named *Staphylococcus* spp. and more than 50

337 unnamed *Staphylococcus* spp. These numbers do not include subspecies within named or
338 unnamed species. Recently named CNS species include *Staphylococcus nepalensis*,
339 which was first isolated from a goat in the Himalayas and identified as a new species in
340 2003 (Spargser et al., 2003), and *Staphylococcus fleuretti*, which was first named in 2000
341 (Vernozy-Rozand et al., 2000). Among CNS isolates from bovine milk that were
342 characterized in our laboratory, both *S. nepalensis* and *S. fleuretti* have been identified
343 using DNA sequencing and comparison with on-line databases. Phenotypic systems for
344 routine diagnostic use cannot be updated every time a new bacterial species is identified
345 or every time strain level differences between isolates from different host species are
346 recognized. By contrast, it is extremely easy to add sequences from new strains or species
347 to a reference DNA database such as GenBank shortly after detection. The availability of
348 reference data affects the typeability and accuracy of phenotypic as well as genotypic
349 methods. Like phenotypic methods, genotypic methods that have been used for
350 identification of human CNS but not bovine CNS may suffer from limited typeability
351 when bovine isolates are first characterized. For example, AFLP failed to identify 19 of
352 99 isolates (19%) upon its first use with a reference database of 39 staphylococcal species
353 and subspecies (Taponen et al., 2006). In a subsequent study using a reference database
354 of 48 species and subspecies, including *S. equorum* and *S. fleuretti*, only 11 of 120
355 isolates (9%) could not be identified (Taponen et al., 2007). Although isolate collections
356 differed between the two studies (Taponen et al., 2006, 2007), it seems reasonable to
357 infer that typeability increased when the reference database of the system was expanded.
358 Initial comparison of DNA sequence data with tDNA-PCR data showed only 75%
359 typeability of bovine CNS by tDNA-PCR and 73% agreement with DNA sequence data.

360 With use of sequence-based species identification, previously unidentified tDNA-PCR
361 patterns could be named and added to the tDNA-PCR database to improve typeability
362 (Supré et al., in preparation).

363 Because the use of DNA-sequencing for CNS identification is relatively new,
364 interpretive criteria are still under development (CLSI, 2007). Some genes are so
365 conserved that differentiation of species or subspecies based on sequence data is not even
366 possible. For example, 16S rRNA gene sequencing failed to distinguish *S. caprae* from *S.*
367 *capitis*, whilst automated ribotyping was able to differentiate the two species (Carretto et
368 al., 2005). Interpretation of sequence data for genes that are less conserved than 16S can
369 be a challenge too. When we first started to use *rpoB* sequencing for identification of
370 bovine CNS in our laboratory in 2005, many *S. hyicus* isolates could not be identified
371 with certainty based on *rpoB* data alone, because the homology between *rpoB* sequence
372 data available in GenBank and those obtained from milk isolates was 94%, which is
373 below the 97% homology criterion that was suggested at the time for housekeeping genes
374 other than 16S (Heikens et al., 2005; Sivadon et al., 2005). The next best match in the
375 *rpoB* database showed 89% homology, which did not meet the criterion of 5% difference to
376 the next bacterial species (Sivadon et al., 2004). Sequencing of additional housekeeping
377 genes, i.e. the 16S rRNA and *cpn60* genes, confirmed the isolates as *S. hyicus* with more
378 than 99% homology. It appears that within the species *S. hyicus*, considerable sequence
379 diversity exists within the *rpoB* gene. Once a larger variety of *rpoB* alleles is added to the
380 on-line database, identification of *S. hyicus* based on this gene should no longer be a
381 problem. In the mean time, Mellmann et al. (2006) have proposed to use 94% homology
382 as the cut-off value for *rpoB* homology in CNS species, underscoring that there is

383 considerable within-species heterogeneity in the DNA sequence of this gene. By this
384 criterion, our original *rpoB* sequence data would have been sufficient to categorize all
385 potential *S. hyicus* isolates as such. Whether the rule that the difference in sequence
386 homology to the next best match should be 5% (Sivadon et al., 2004) should be
387 maintained is doubtful. For some species, e.g. *S. fleuretti* vs. *S. pulveri* or *S. haemolyticus*
388 vs. *S. croceolyticus*, the difference in DNA sequence homology is routinely 4%,
389 exemplifying that the difference between within-species and between-species homology
390 may be more relevant than a single pre-defined cut-off value. As the uptake by
391 laboratories of DNA sequencing for microbial identification increases, more reference
392 data and standard protocols for generating and interpreting of sequence data will become
393 available (CLSI, 2007).

394 One undeniable advantage of DNA sequence-based methods over all phenotypic
395 and genotypic methods is that it provides a quantitative measure, down to the last base
396 pair of the genetic code, of the certainty with which an isolate has been identified (CLSI,
397 2007). For other genotypic methods, such as automated ribotyping and tDNA-ILP, some
398 of the limitations of phenotypic methods apply, i.e. similarity coefficients can be
399 calculated but there is no universally meaningful quantitative measure of the genetic
400 relatedness of isolates.

401

402 **6. Discussion**

403

404 Efforts are underway to compare species identification of bovine CNS by
405 genotypic and phenotypic methods, as documented in references cited in this paper, or in

406 manuscripts that were still in preparation at the time this paper was written. Using DNA-
407 sequencing, over 99% of CNS isolates from bovine milk can be identified. So far, other
408 genotypic methods and phenotypic methods have lower typeability and accuracy, which
409 may manifest in missing, incorrect, or ambiguous results. As databases grow, other
410 methods may become more accurate, although database updates may not keep pace with
411 the increase in number of described *Staphylococcus* species and subspecies.

412 For diagnostic work, the choice of typing methods should be determined by a
413 number of considerations, such as the goal of isolate identification, speed, ease of use,
414 cost, availability of equipment and trained personnel, etc. In the case of CNS
415 identification in the context of mastitis control, the best method would be a fast, simple
416 and cheap method that provides a relevant level of differentiation. The amount of
417 information regarding clinical relevance, treatment or management of CNS mastitis that
418 is based on accurate species level identification is limited. Until proven otherwise, CNS
419 may be the most relevant level of identification for mastitis diagnostics, and this level is
420 achieved through phenotypic methods. If CNS species of specific importance to udder
421 health and mastitis control are identified, simplified phenotypic or genotypic methods
422 targeting this subset of CNS may be of value for routine diagnostics. Simplified
423 phenotypic diagnostic methods have been developed for identification of clinically
424 significant CNS species in hospitals (Ieven et al., 1995). Such simplified methods can be
425 of great utility if they are rapid and inexpensive, and if they do not misidentify isolates
426 belonging to species that are not covered by the simplified method. For example, in the
427 hypothetical situation that *S. chromogenes* and *S. epidermidis* were shown to have
428 positive (Matthews et al., 1990a; De Vliegher et al., 2003) and negative (Devriese and De

429 Keyser, 1980; Watts and Owens, 1989) impact on udder health, respectively, whereas
430 other CNS species are merely innocent bystanders, it would suffice to have a system that
431 classified CNS isolates as *S. chromogenes*, *S. epidermidis*, or "other CNS species". This
432 would be similar to existing diagnostics for streptococci from bovine milk, which are
433 commonly differentiated into *Strep. agalactiae*, *Strep. dysgalactiae*, or *Strep. uberis* with
434 the remaining group comprised of other species of streptococci and enterococci.
435 Unidentified isolates from rest groups can be identified by additional testing as necessary.
436 Simplified schemes for routine diagnostics could be phenotypic or genotypic. A
437 simplified phenotypic scheme for identification of CNS from bovine mastitis has already
438 been proposed (Devriese et al., 1994). If clinically relevant, a simplified genotypic
439 scheme, such as a multiplex PCR for a limited number of clinically relevant CNS species,
440 could be developed, as has been done for the most clinically relevant *Streptococcus* spp.
441 (Phuektes et al., 2001). In light of the discovery of new species, such as *S. equorum*,
442 which was in the top-5 of most common species in our laboratory, and in changes in
443 identification methods, existing simplified diagnostic schemes may need to be re-
444 evaluated and updated. When evaluating simplified identification schemes, it is important
445 to assess sensitivity, i.e. the ability to recognize known members of the species, and
446 specificity, i.e. the ability to exclude isolates that are not members of the species. The
447 latter is not always done (Devriese et al., 2002). As for any other diagnostic test, the
448 positive and negative predictive value of a test result will not only depend on the
449 sensitivity and specificity of the test, but also on the composition of the bacterial
450 population. This composition may differ significantly between dairy cattle, humans and

451 other host species, and a test system that is useful in one diagnostic or research setting
452 may not be as useful in a different setting.

453

454 **7. Conclusion and Outlook**

455

456 DNA sequence-based species identification of CNS is currently the most accurate
457 species identification method available because it has the largest reference database, and
458 because a universally meaningful quantitative measure of homology with known species
459 is determined. DNA sequence-based species identification could therefore be considered
460 the Gold standard and should be used as the reference identification methodology. If a
461 diagnostic laboratory does not have access to a reference method, or a method that has
462 been validated through comparison with a reference method, characterization of CNS
463 isolates is best limited to actual observations, such as coagulase reaction, novobiocin
464 resistance, etc. Reporting as "coagulase-negative *Staphylococcus* species" may be more
465 appropriate than reporting of more detailed but potentially inaccurate results. At present,
466 species-specific recommendations for management and control of CNS mastitis in dairy
467 herds have not been formulated, and identification of CNS as such will suffice for routine
468 diagnostics. In research, genotypic methods for species identification are to be preferred
469 over phenotypic methods. Once the source, transmission mechanisms, and impact of
470 different CNS species on cow health, productivity and milk quality have been identified
471 through use of epidemiological data and accurate species identification methods,
472 appropriate identification methods for routine use in research and diagnostic laboratories
473 can be proposed.

474

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475

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478

479

480 **Conflict of Interest**

481

482 Neither one of the authors (R.N. Zadoks, J. L. Watts) has a financial or personal

483 relationship with other people or organizations that could inappropriately influence or

484 bias the paper entitled "Species Identification of Coagulase-Negative Staphylococci:

485 Genotyping is Superior to Phenotyping". J. L. Watts is an employee of Pfizer Animal

486 Health, Kalamazoo, MI.

487

487 Table 1

488 Distribution of coagulase-negative staphylococcal species (in % of isolates tested) in
 489 bovine milk. Based on phenotypic and genotypic methods as specified in Matthews et al.,
 490 1990b; Watts and Washburn, 1991; Devriese et al., 1994; Waage et al., 1999; Thorberg
 491 and Brändström, 2000; Kudinha and Simango, 2002; Taponen et al., 2006; Sampimon et
 492 al., 2007.

Country ¹	Belgium	Finland	NL	Norway	Sweden	USA	USA	Zim	Total ³
State ²						TN	LA		
Isolates	65	99	108	149	77	105	94	131	828
<i>S. auricularis</i>	0	0	0	1	0	0	0	0	0
<i>S. capitis</i>	0	0	7	1	0	1	0	0	1
<i>S. caseolyticus</i>	0	0	0	0	0	0	0	2	0
<i>S. chromogenes</i>	12	27	42	15	17	40	11	24	24
<i>S. cohnii</i>	0	0	0	0	8	0	0	0	1
<i>S. epidermidis</i>	9	5	7	0	13	5	12	23	9
<i>S. equorum</i>	0	1	0	0	0	0	0	0	0
<i>S. fleuretti</i>	0	1	0	0	0	0	0	0	0
<i>S. haemolyticus</i>	0	3	7	2	8	4	1	0	3
<i>S. hominis</i>	3	0	0	0	0	4	7	18	4
<i>S. hyicus</i>	5	2	6	15	12	12	35	8	12
<i>S. intermedius</i>	0	0	0	0	0	0	3	0	0
<i>S. kloosii</i>	0	0	0	0	0	0	0	1	0
<i>S. lentus</i>	0	0	0	0	0	0	0	3	0
<i>S. muscae</i>	0	0	0	0	0	0	0	1	0
<i>S. saprophyticus</i>	0	0	5	0	3	1	0	4	2
<i>S. sciuri</i>	0	2	0	1	6	6	3	2	2
<i>S. simulans</i>	34	36	10	54	16	13	3	1	22
<i>S. warneri</i>	25	3	0	1	5	5	7	0	4
<i>S. xylosus</i>	12	0	16	3	13	9	17	7	9
unknown	0	19	0	8	0	1	0	7	5

493

494 1) NL = the Netherlands, USA = United States of America, Zim = Zimbabwe.

495 2) TN = Tennessee, LA = Louisiana. Two states were included to reflect some of the
 496 geographic diversity within the USA.

497 3) Total calculated as weighted average of results for each studied, where weight
 498 equals the number of isolates per study.

499 Table 2

500 Distribution of coagulase-negative staphylococcal species from various host species and
 501 food. Based on phenotypic and genotypic methods as specified in Cox et al., 1988; Igimi
 502 et al., 1994; Deinhofer and Pernthaner, 1995; Madsen and Christensen, 1995; Maes et al.,
 503 1997; Awan and Matsumoto, 1998; Lee et al., 1998; Lilenbaum et al., 1999; Aarestrup et
 504 al., 2000; Lilenbaum et al., 2000; Pengov, 2001; Slaughter et al., 2001; Carretto et al.,
 505 2005 and in Table 1.

CNS species	Avian	Bovine	Canine	Caprine	Equine	Feline	Human	Ovine	Sausage	Treefrog
<i>S. capitis</i>	X	-	-	-	X	-	-	-	-	-
<i>S. caprae</i>	-	-	-	X	-	-	-	X	-	-
<i>S. carnosus</i>	-	-	-	-	-	-	-	-	X	-
<i>S. chromogenes</i>	-	X	-	-	-	-	-	-	-	-
<i>S. cohnii</i>	X	-	-	-	-	-	-	-	-	-
<i>S. epidermidis</i>	-	-	X	X	-	X	X	X	-	-
<i>S. felis</i>	-	-	-	-	-	X	-	-	-	-
<i>S. gallinarum</i>	X	-	-	-	-	-	-	-	-	-
<i>S. haemolyticus</i>	-	-	-	-	X	X	X	-	-	-
<i>S. hyicus</i>	X	X	-	-	-	-	-	X	-	-
<i>S. lentus</i>	X	-	-	X	X	-	-	-	-	-
<i>S. saprophyticus</i>	-	-	-	-	-	-	-	-	X	-
<i>S. sciuri</i>	-	-	-	-	-	-	-	-	-	X
<i>S. simulans</i>	X	X	-	X	-	X	-	X	X	-
<i>S. xylosus</i>	X	-	X	-	X	-	-	-	X	X

506

507

507

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508

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