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3 **Differentiation of *Staphylococcus aureus* and *Staphylococcus epidermidis* by**

4 **PCR for the fibrinogen binding protein gene (*fib*)**

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6 **R. Sunagar[*], S. N. Deore[*], P. V. Deshpande[†], A. Rizwan[†], A. D. Sannejal[†], S.**

7 **Sundareshan[†], D. B. Rawool[*], S. B. Barbuddhe[‡], M. K. Jhala[§], A. S. Bannalikar[#], D. M.**

8 **Mugalikar[∥], V. J. Kumari[¶], K. Dhanalakshmi[¶], Y. N. Reddy[¶], P. P. Rao[*], C. Babra[^], J. G.**

9 **Tiwari[^], T. K. Mukkur[^], P. Costantino[^], J. D. Wetherall[^], S. Isloor[†], N. R. Hegde[*]**

10 [*]Ella Foundation, Genome Valley, Turkapally, Shameerpet Mandal, Hyderabad, India;

11 [†]Veterinary College, Karnataka Veterinary, Animal and Fisheries Sciences University,

12 Bengaluru, India; [‡]ICAR Research Complex for Goa, Old Goa , India; [§]Veterinary College,

13 Anand Agricultural University, Anand, India; [#]KNP College of Veterinary Sciences,

14 Maharashtra Animal and Fishery Sciences University, Shirwal, India; [∥]Bombay Veterinary

15 College, Maharashtra Animal and Fishery Sciences University, Mumbai, India; [¶]College of

16 Veterinary Science, Sri Venkateswara Veterinary University, Hyderabad, India; [^]School of

17 Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth,

18 Australia

19

20 **Correspondence**:     Nagendra R. Hegde

21                         Genome Valley, Turkapally

22                         Shameerpet Mandal

23                         Hyderabad – 500078, India

24                         Email: hegden@ellafoundation.org

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26 **Interpretive Summary**: Rapid and accurate identification of bacteria is important for

27 understanding pathogenesis as well as to implement control or eradication programmes for

28 mastitis. Subclinical mastitis is frequently caused by staphylococci of which *S. aureus* and *S.*

29 *epidermidis* are involved to a significant extent. This study describes the application of a

30 multiplex polymerase chain reaction for the identification and differentiation of *S. aureus* and

31 *S. epidermidis*. Although the test was moderately sensitive, it could accurately and

32 reproducibly detect the two species in milk under controlled laboratory conditions even when

33 present among a mix of several other bacteria.

34

**ABSTRACT**

Mastitis is one of the most common and burdensome disease afflicting dairy animals. Among the infectious causes, staphylococci are frequently associated with clinical and subclinical mastitis. Whereas *S. aureus* is the predominant species involved, *S. epidermidis* and other coagulase-negative staphylococci are increasingly being isolated from cases of bovine mastitis. Although *S. aureus* and *S. epidermidis* can be easily differentiated based on their biochemical properties, such phenotypic identification is time consuming and laborious. With the aim to rapidly identify *S. aureus* and *S. epidermidis*, a multiplex PCR was developed, during which process, we found that a single gene encoding the adhesin fibrinogen binding protein (*fib* gene) could be used to identify and differentiate the two species. Incorporating this observation, a multiplex reaction combining a triplex PCR for *S. aureus* and a duplex PCR for *S. epidermidis* was standardized, first using bacterial cultures, and then with pasteurized milk spiked with live organisms or DNA extracted from the organisms. The test could specifically detect *S. aureus* and *S. epidermidis* even in the presence of a dozen other organisms. The limit of detection for detecting *S. aureus* and *S. epidermidis* separately was 10-100 cfu/mL for simplex PCR and $10^4$ cfu/mL for multiplex PCR. On the other hand, the limit was $10^6$ cfu/mL by multiplex PCR for the simultaneous detection of both the organisms when spiked into culture medium or pasteurized milk. Overnight enrichment enhanced the assay sensitivity by 100-fold. The assay had a high diagnostic sensitivity and specificity. The application of the test was verified on 602 field isolates of staphylococci which had been earlier characterized by phenotypic methods. Importantly, 25 coagulase negative isolates were identified as *S. aureus* by the multiplex PCR. The test can be adapted for use in clinical diagnostic laboratories.

**Keywords:** bovine mastitis, staphylococci, detection and differentiation, multiplex PCR

## INTRODUCTION

Bovine mastitis is one of the most important diseases affecting dairy industry. The disease costs around Rs. 72 billion (~US$ 1.4 billion) per annum (Bansal and Gupta, 2009) to India, where livestock sector contributes 5-6% to the gross domestic product. Speedy diagnosis of mastitis, particularly that of subclinical cases, and rapid identification of the causative agent(s) is crucial for designing appropriate therapy and management to control mastitis.

Bacteria account for most of the intramammary infections (**IMI**). During mastitis, multiple pathogens, variable shedding, inhibitors from both the host and the microbes, antibiotic residues, presence of inflammatory cells, and the persistence of inflammation even after the clearance of bacteria produce a dynamic state of udder infection and inflammation (Cremonesi et al., 2005; Dinsmore et al., 1992; Gillespie and Oliver, 2005; Koskinen et al., 2009; Phuektes et al., 2001; Pyorala and Mattila, 1987; Sears et al., 1990; Suojala et al., 2008; Taponen et al., 2009). These and other factors such as unfavorable conditions during transport of samples and during enrichment (Dinsmore et al., 1992; Koskinen et al., 2009), incubation or freezing before culturing, inoculum volume, and enrichment conditions (Dinsmore et al., 1992; Gillespie and Oliver, 2005; Schukken et al., 1989; Taponen et al., 2009) can influence isolation and identification of bacteria. Further, phenotyping methods are time consuming, have low sensitivity, lack inter-laboratory standardization, and are not highly reliable (Phuektes et al., 2001; Pitkala et al., 2005; Pyorala and Mattila, 1987; Sears et al., 1990; Suojala et al., 2008), necessitating the development of better tests.

Among the known and potential pathogens that account for most IMI, only a few species of staphylococci predominate (Tenhagen et al., 2006; Wilson et al., 1997). Staphylococci can establish infection rapidly, and frequently persist and remain undetected for long periods particularly in subclinical mastitis. Phenotypic methods remain the gold

86  standard for the identification of staphylococci, and several culture-based systems have been

87  commercialized. However, 30-50% of mastitic milk samples may not show any bacterial

88  growth under conventional culture conditions (Bradley et al., 2007; Dinsmore et al., 1992;

89  Makovec and Ruegg, 2003; Olde Riekerink et al., 2008; Taponen and Pyorala, 2009).  In

90  addition, phenotypic methods have poor discriminatory power as they frequently misidentify

91  species due to overlapping phenotypes (Giammarinaro et al., 2005; Morot-Bizot et al., 2004;

92  Pitkala et al., 2005; Renneberg et al., 1995; Rhoden and Miller, 1995; Rossi et al., 2001;

93  Zadoks and Watts, 2009).

94      Polymerase chain reaction has several advantages over phenotypic methods,

95  including: (a) direct detection and identification without culturing, (b) rapid turnaround time,

96  (c) high throughput capability, (d) high accuracy, reproducibility and sensitivity and/or

97  specificity, and (e) objective user-independent interpretation.  Single as well as multiplex

98  PCR using both end-point and real-time methodologies, either alone or in combination with

99  other techniques, have been applied for the identification of staphylococci to genus and

100  species level as well as to predict the ability of the organisms to express toxins or virulence

101  determinants or resistance to antibiotics (Chiang et al., 2012a; Giammarinaro et al., 2005;

102  Hirotaki et al., 2011; Jukes et al., 2010; Kondo et al., 2007; Lee et al., 2008; Martineau et al.,

103  2001; Mason et al., 2001; Mehrotra et al., 2000; Morot-Bizot et al., 2004; Nihonyanagi et al.,

104  2012; Oh et al., 2009; Sabat et al., 2006; Sasaki et al., 2010; Shome et al., 2011;

105  Strommenger et al., 2003; Tristan et al., 2003; Valvatne et al., 2009). Various genes that have

106  been probed either independently or in combination include *16S rRNA*, *23S rRNA*, *clfA*, *coa*,

107  *cpn60*, *femA/B*, *gap*, *gla*, *groESL*, *hsp60*, *ITS*, *nuc*, *orfX*, *rdr*, *rpoB*, *sodA*, *spa*, *tuf*, as well as

108  the Sa442 and Se705 fragments (Chiang et al., 2012a; Couto et al., 2001; Drancourt and

109  Raoult, 2002; Edwards et al., 2001; Ghebremedhin et al., 2008; Goh et al., 1996; Hamels et

110  al., 2001; Heikens et al., 2005; Hirotaki et al., 2011; Jukes et al., 2010; Lee et al., 2008;

111 Martineau et al., 2001; Martineau et al., 1996; 1998; Mason et al., 2001; Poyart et al., 2001;

112 Sasaki et al., 2010; Shome et al., 2011; Vannuffel et al., 1999; Yugueros et al., 2000).  Several

113 molecular diagnostic kits have also been commercialized.

114   *Staphylococcus aureus* is the most important species among the mastitis-associated

115 staphylococci (Jain, 1979; Wilson et al., 1997).  This is followed by a group of coagulase

116 negative staphylococci (**CoNS**) (Pyorala and Taponen, 2009), most importantly *S.*

117 *epidermidis*, besides *S. chromogenes*, *S. simulans* and *S. haemolyticus*. In a recent pilot study,

118 we found that the frequency of isolation of *S. aureus* was more in back yard setting and that

119 CoNS were more frequent in organized dairy sector in a small region in South India (Hegde

120 et al., 2012). We reasoned that the pathobiology of mastitis caused by *S. aureus* or CoNS

121 could be different, and that rapid identification and differentiation of *S. aureus* and *S.*

122 *epidermidis* could assist in designing differential intervention strategies.  During

123 bioinformatics analyses to develop PCRs for simultaneous detection of virulence

124 determinants and differentiation of mastitis-associated *S. aureus* and *S. epidermidis*, we found

125 that the gene encoding the fibrinogen binding protein (***fib***) can differentiate the two species.

126 We report the development in a single reaction of a multiplex PCR assay for concurrent

127 detection and differentiation of *S. aureus* and *S. epidermidis*.  The results indicate that these

128 assays could be adapted for the detection of these organisms in milk.

129

130             **MATERIALS AND METHODS**

131 *Bioinformatics Analyses*

132   Complete or partial genomes of *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S.*

133 *saprophyticus* were aligned using ClustalW program.  Unique primers were designed for

134 fibrinogen binding (*fib*) and thermonuclease (***nuc***) genes using the PrimerQuest programme

135 (Integrated DNA Technologies, Coralville, Iowa, USA).

136

### *Bacterial Strains and Biochemical Methods*

All media and reagents for microbiological assays were obtained from HiMedia Laboratories (Mumbai, India). Reference strains of bacteria were obtained from Microbial Type Culture Collection, Chandigarh, India. Field isolates of staphylococci were obtained from clinical and sub-clinical bovine mastitis samples. Species identification was performed by colony and microscopic morphology, sugar fermentation, and coagulase, phosphatase and thermonuclease activities (Sundareshan et al., 2012). The identified *S. aureus* and *S. epidermidis* strains were maintained at −80ºC in brain heart infusion (**BHI**) broth supplemented with 20% glycerol (v/v).

### *Extraction of DNA, Primers, and PCR Conditions*

For routine PCR, bacterial genomic DNA was extracted from overnight culture of a single colony, by using RealGenomics DNA isolation kit (Real Biotech Corporation, Chennai, India). For limit of detection experiments, one mL of overnight culture was used without dilution or serially diluted ten-fold to one mL in PBS. Genomic DNA was prepared by heating the culture or diluted sample in boiling water for 10 min, harvesting the supernatant and extracting DNA (Rawool et al., 2007). For experiments without or with enrichment, known cfu of bacteria were inoculated into milk or milk plus BHI (1:1) and DNA extracted following overnight incubation at 37ºC.

All PCR related materials including primers were obtained from Sigma-Aldrich (Bengaluru, India). Primers were selected based on published information or designed through bioinformatics analyses (Table 1). PCR was carried out as follows: initial 5-min denaturation step at 94ºC, followed by 30 cycles of 30 s of denaturation at 94ºC, 30 s of annealing at 60ºC, and 1 min of extension at 72ºC, with 10 min final extension at 72ºC. The specificity of each pairs of primers was verified by testing against DNA of standard strains.

161 Multiplex PCR was standardized by varying the combination of primer pairs, annealing

162 temperature, and concentrations of primers, dNTPs and enzyme.

163 *Analytical and Diagnostic Parameters*

164 Limit of detection (**LOD**) was determined by serially diluting ($\log_{10}$-fold) known cfu's

165 of *S. aureus* and/or *S. epidermidis* in PBS or pasteurized milk, and subjecting to multiplex

166 PCR. LOD was determined to be the final $\log_{10}$ dilution of the template in which the product

167 was clearly visible after agarose gel electrophoresis.

168 True and false positives and negatives for multiplex PCR (test) were deduced by

169 comparison with culture and biochemical identification (standard). Samples corroborating

170 with the standard in positive and negative pool were identified as true and those that did not

171 conform to the results of the standard were identified as false. From the resulting data, the

172 following parameters were calculated as indicated and converted to percentages.

173 Analytical sensitivity = LOD

174 Analytical specificity (accuracy) = [No. test positive]/[No. standard positive]

175 Diagnostic (clinical) sensitivity = [No. true positive]/[No. true positive + No. false negative]

176 Diagnostic (clinical) specificity = [No. true negative]/[No. true negative + No. false positive]

177 Positive predictive value = [No. true positive]/[No. true positive + No. false positive]

178 Negative predictive value = [No. true negative]/[No. true negative + No. false negative]

179

180 **RESULTS**

181 *Standardization of Simplex PCR*

182 While comparing 15 *S. aureus* and two *S. epidermidis* genomes to select primers for

183 virulence genes, we identified short stretches within the sequences of *fib* to be able to

184  differentiate the two species.  Primers for both the species as well as for differing lengths of

185  *FnBp* and *nuc* fragments were designed to suit multiplexing (Table 1).

186     Initial amplification of individual fragments was standardized using reference strains.

187  *23S rRNA* (1250 bp), *nuc* (441 bp) and *Sa-fib* (200 bp) fragments were observed with *S.*

188  *aureus* templates, whereas fragments of *Se-fib* (700 bp) and *Se-124* (124 bp) were produced

189  from *S. epidermidis* templates (Fig. 1, left of marker). Each amplicon was verified by

190  nucleotide sequencing. Combinations of primer pairs also confirmed the amplification of *23S*

191  *rRNA*, *nuc* and *Sa-fib* from *S. aureus* templates, and of *Se-fib* and *Se-124* from *S. epidermidis*

192  templates (Fig. 1, right of marker).

193  ***Standardization of Multiplex PCR***

194     The multiplex PCR was optimized to ensure that all the target gene sequences were

195  amplified to reasonably equivalent extent in a single reaction. However, amplification of *23S*

196  *rRNA* was inhibited whenever primers for *FnBp* were also used in the same reaction mixture

197  (Table 2).  Hence, multiplex PCR was then standardized with primers for *Sa-fib* (Fig. 1),

198  which could differentiate between *S. aureus* and *S. epidermidis* (see below). In addition, the

199  eubacterial *16S rRNA* primers inhibited the amplification of not only the species-specific *23S*

200  *rRNA* gene but also that of *nuc* (Table 2, and data not shown).  Therefore, the multiplex PCR

201  included amplification of *23S rRNA*, *nuc*, *Sa-fib*, *Se-fib* and *Se-124* fragments whereas the

202  amplification of eubacterial *16S rRNA* was performed in a separate tube.

203     Multiplex PCR was able to detect *S. aureus* and/or *S. epidermidis* in a mixture of the

204  two. Fragments corresponding to *S. aureus* or *S. epidermidis* were amplified with the triplex

205  or duplex primer pairs, respectively, and a mixture of the templates produced all the five

206  amplicons (Fig. 1, right, and Fig. 2, lanes 1-3). To verify the suitability of the test in the

207  presence of other organisms, two different experiments were performed. First, each CoNS

208  and non-staphylococcal species as well as *S. aureus* and *S. epidermidis* were cultured and

8

209 template DNA prepared separately, and equal quantity of each template was mixed together.

210 As expected, when templates from six different species of CoNS and five different species of

211 non-staphylococci were spiked into the multiplex reaction, no inhibition of amplification of

212 any of the five amplicons was observed (data not shown). However, since this does not

213 approximate a mixed infection or culture, a second experiment was performed where all

214 CoNS and non-staphylococcal species were cultured in a single tube with or without *S.*

215 *aureus* and *S. epidermidis*, and DNA extracted. Even under these circumstances, no inhibition

216 of amplification was observed (Fig. 2, lane 4). The absence of amplification with CoNS and

217 non-staphylococcal species was not attributable to PCR inhibitors or to the inadequacy of the

218 PCR protocol, because the control fragment of *16S rRNA* (240 bp) was amplified from all the

219 bacterial species listed in Table 3, and the results for some of these are shown in Fig. 2 (lanes

220 5-10). Further, the presence of *16S rRNA* fragment in the DNA extracted from individual

221 cultures of *Staph. hemolyticus*, *Staph. saprophyticus*, *Staph. chromogenes*, *E. coli*, *Salmonella*

222 *typhi* or *Streptococcus. agalactiae* (Fig. 2, lanes 5-10) or *Staph. warneri*, *Staph. sciuri*, or

223 *Stapyh. xylosus* (data not shown), indicated the presence of template DNA in the first set of

224 experiments.

225 *Application of Multiplex PCR to Field Isolates*

226 In order to validate the multiplex PCR, the assay was applied to 602 bovine mastitis-

227 associated isolates, which were previously identified as belonging to *Staphylococcus* genus

228 and then to a particular species by culturing followed by various biochemical tests such as

229 sugar fermentation, coagulase activity on rabbit plasma, production of phosphatase, and

230 thermonuclease activity (manuscript in preparation).

231 Whereas the biochemical method identified 242 isolates as *S. aureus* and 22 isolates

232 as *S. epidermidis*, the respective number of isolates identified by multiplex PCR was 267 and

233 22 (Table 4). Therefore, the analytical specificity of multiplex PCR was 100%, meaning all

234 the 242 isolates identified by phenotypic method were identified as *S. aureus* by multiplex

235 PCR. However, multiplex PCR could identify 25 CoNS isolates as *S. aureus*. These results

236 show that the multiplex PCR had diagnostic sensitivity and specificity of 100% and 93.5%

237 for *S. aureus*, and 100% and 100% for *S. epidermidis*, respectively. The positive and negative

238 predictive values were 90.64% and 100% for *S. aureus*, and 100% and 100% for *S.*

239 *epidermidis*, respectively.

240 Since the multiplex PCR was performed with overnight cultures, which yield

241 abundant bacteria, it was important to compare the different fragments when amplified singly

242 or in the multiplex reaction. The LOD of simplex and multiplex PCR assays were determined

243 with DNA extracted from ten-fold serial dilutions of individual cultures of *S. aureus* and *S.*

244 *epidermidis*. The detection limit of simplex PCR for *S. aureus* with *23S rRNA* was $10^1$

245 cfu/mL, whereas it was $10^2$ cfu/mL with *Sa-fib* and *nuc*. For *S. epidermidis*, the detection with

246 *Se-124* and *Se-fib* was $10^1$ and $10^2$ cfu/mL, respectively. By contrast, the LOD of *S. aureus*

247 and *S. epidermidis* with triplex (*23S rRNA*, *nuc*, *Sa-fib*) or duplex (*Se-fib*, *Se-124*) PCR,

248 respectively, was $10^4$ cfu/mL for each organism when the bacteria were grown overnight in

249 mixed cultures. The detection limit dropped to $10^6$ cfu/mL for simultaneous detection of *S.*

250 *aureus* and *S. epidermidis* when all five sets of primers were used together (Table 5). In order

251 to simulate conditions in milk, the LOD studies were repeated using known cfu's of cultures

252 or equivalent DNA diluted in pasteurized milk. The results did not differ from those

253 described above (data not shown). We further investigated whether overnight enrichment

254 culture would enhance the LOD. In these experiments, the analytical sensitivity was

255 observed to be two $\log_{10}$ better for duplex, triplex or multiplex reactions (Table 5). Thus, the

256 multiplex PCR had an analytical sensitivity of $10^6$ cfu/mL irrespective of whether buffer or

257 pasteurized milk was used for dilution.

258 In order to explore whether *fib* gene could be exploited to differentiate other

259  staphylococci, sequences available in GenBank were aligned along with those of *S. aureus*

260  and *S. epidermidis*. The only *fib* sequences available were those of *S. haemolyticus* and *S.*

261  *saprophyticus*. Indeed primers unique to these organisms could be designed (Supplementary

262  Fig. 1). Accordingly, PCR would be predicted to amplify unambiguous fragments of 350 and

263  620 bp for *S. hemolyticus* and *S. saprophyticus*, respectively, which are easily separable from

264  fragments of *S. aureus* (210 bp) and *S. epidermidis* (720 bp) by agarose gel electrophoresis.

265

266                                                  **DISCUSSION**

267            Several studies have highlighted the feasibility of using PCR for the detection of

268  bacteria in milk from clinical and subclinical mastitis (Cressier and Bissonnette, 2011;

269  Gillespie and Oliver, 2005; Hirotaki et al., 2011; Koskinen et al., 2009; Phuektes et al., 2003;

270  Phuektes et al., 2001; Ramesh et al., 2002; Shome et al., 2011; Taponen et al., 2009).

271  Because of the higher frequency of isolation (Oliveira et al., 2006; Pearson and Mackie,

272  1979; Shome et al., 2011; Thorberg et al., 2006), *S. aureus* and *S. epidermidis* have been

273  targeted (Edwards et al., 2001; Hirotaki et al., 2011; Kilic and Basustaoglu, 2011; Martineau

274  et al., 1996; 1998; Mason et al., 2001; Morot-Bizot et al., 2004; Pereira et al., 2010; Phuektes

275  et al., 2001; Shome et al., 2011; Taponen et al., 2009). However, only a few studies have

276  explored the simultaneous detection and differentiation of *S. aureus* and *S. epidermidis* but

277  have targeted different genes in combination, or by using a single gene in real-time PCR

278  assays (Chiang et al., 2012a; 2012b; Josefson et al., 2011; Kilic and Basustaoglu, 2011;

279  Morot-Bizot et al., 2004; Shome et al., 2011). On the other hand, a few studies have explored

280  the detection of multiple genes within a single species or a group of organisms (Costa et al.,

281  2005; Cremonesi et al., 2005; Jukes et al., 2010; Lin et al., 2009; Mason et al., 2001; Morot-

282  Bizot et al., 2004; Nihonyanagi et al., 2012; Sabat et al., 2006; Shome et al., 2011; Tristan et

283  al., 2003). Since deletion in the target region of one gene can produce a false negative result

284    (Ghebremedhin et al., 2008; Horakova et al., 2008), we attempted a multiplex PCR to detect

285    at least two genes in each of *S. aureus* and *S. epidermidis*. Our results show that multiplex

286    PCR using *23S rRNA, nuc, fib* and *Se-124* can be used for the identification and

287    differentiation of *S. aureus* and *S. epidermidis*. One must, however, note that mastitis is a

288    consequence of polymicrobial infection and colonization of the mammary gland, and our

289    assay can only detect two of the causative agents.

290          The only genes which by themselves have been shown so far to differentiate *S. aureus*

291    and *S. epidermidis* are *16S rRNA*, *nuc* and *femA* (Edwards et al., 2001; Hirotaki et al., 2011;

292    Jukes et al., 2010). Whereas differentiation with *16S rRNA* (Edwards et al., 2001) and *femA*

293    (Jukes et al., 2010) was accomplished by melting curve analyses, that with *nuc* (Hirotaki et

294    al., 2011) was achieved with conventional PCR. In addition, studies with *16S rRNA* and *nuc*

295    used isolates grown in culture, and did not conduct spiking studies, while those with *femA*

296    applied the test to blood samples. Importantly, none of these groups performed LOD

297    analyses. We have now shown that sequences of *fib* can also be used to differentiate between

298    the two species under a variety of scenarios, including mixed DNA, mixed culture, when

299    spiked into milk, and when spiked and enriched. This is the first and the only colonization-

300    associated gene described so far for the differentiation of *S. aureus* and *S. epidermidis*. In

301    addition, it may also be possible to differentiate *S. haemolyticus* and *S. saprophyticus*, and the

302    availability of complete gene sequences of other CoNS may reveal the possibility to

303    differentiate various other species as well.

304          One could argue that PCR detects the mere presence of nucleic acids may not be

305    useful to assess bacterial load in milk because even dead and degraded organisms could be

306    detected. Indeed, it is possible that pasteurization will not eliminate DNA, and certain

307    regions of the bacterial genome, particularly those that are thermoresistant, could be

308    amplified by PCR even with a zero total plate count. The pasteurized milk that was used in

309 this study was negative by simplex PCR for both *S. aureus* and *S. epidermidis* (data not

310 shown). Since the PCR could detect DNA equivalent to 10-100 cfu, it is safe to assume that

311 remnants of DNA in pasteurized milk can be ruled out. It is, however, important to perform a

312 quantitative analysis of the detectability of nucleic acid in comparison with cfu. This detailed

313 and complicated analysis was beyond the scope of this study.

314       The multiplex PCR could identify *S. aureus* and *S. epidermidis* among a mixed culture

315 containing more than a dozen staphylococci and non-staphylococci. However, these

316 experiments did not analyze competitive growth advantage, if any, of any of the organisms.

317 It is possible that certain organisms could grow better than others and hence may reduce the

318 LOD of targeted species. Nonetheless, even though the LOD was lower when mixed cultures

319 were used, it was clear that the LOD was equivalent between *S. aureus* and *S. epidermidis*,

320 suggesting equal growth under mixed culture conditions. The LOD by PCR for *S. aureus* or

321 *S. epidermidis* was $10^4$ cfu/mL whereas that for simultaneous detection of both the organisms

322 was 100-fold lower ($10^6$ cfu/mL) when mixed templates or cultures were used. This could be

323 attributed to PCR drift or competitive inhibition between primers (Hsih and Tsen, 2001;

324 Kosters et al., 2002; Lopes et al., 1999; Morot-Bizot et al., 2004). Under laboratory

325 conditions, the LOD for staphylococci ranges from $10^0$ to $10^5$ cfu/mL. In various clinical

326 specimens, including milk, the LOD ranges from $10^0$ to $10^9$ cfu/mL (Boving et al., 2009;

327 Chiang et al., 2006; Chiang et al., 2012a; 2012b; Cremonesi et al., 2005; Cressier and

328 Bissonnette, 2011; Gillespie and Oliver, 2005; Jaffe et al., 2000; Kilic and Basustaoglu, 2011;

329 Kilic et al., 2010; Lee et al., 2008; Lin et al., 2009; Mason et al., 2001; Morot-Bizot et al.,

330 2004; Peles et al., 2007; Phuektes et al., 2001; Ramesh et al., 2002; Shome et al., 2011).

331 Apparently healthy cow's raw milk has been shown to contain up to $10^7$ cfu/mL of bacteria

332 (Dan et al., 2008; Lingathurai and Vellathurai, 2010; Peles et al., 2007; Ramesh et al., 2002).

333 It is conceivable that the bacterial load will be much higher, albeit variable, during mastitis.

334 An extrapolation of the results of *in vitro* testing to milk would be difficult as the biology of

335 the mammary gland and the physicochemical properties of the milk strongly influence

336 microbial growth. In addition, pathogenic bacteria may outgrow non-pathogenic bacteria *in*

337 *vivo* because of the virulence factors and invasive strategies utilized by the pathogens.

338 Indeed, staphylococci can account for 50-70% of the microbial load of milk in case of

339 mastitis (Leonard and Markey, 2008; Lingathurai and Vellathurai, 2010; Mekibib et al.,

340 2010). Thus, it may be possible to apply PCR not just for qualitative diagnosis but also to

341 evaluate the extent of microbial load. In this context, advancements like pit-stop multiplex

342 PCR (Lopes et al., 1999) or real-time PCR could more accurately predict bacterial load once

343 correlations are established.

344 In our study, 25 clinical isolates which were coagulase negative biochemically and

345 hence classified as species other than *S. aureus* were PCR positive for *S. aureus* markers. On

346 further investigation, these isolates were found by PCR to carry at least a fragment of the

347 coagulase (***coa***) gene (Sundareshan, unpublished observations). This suggests that the *coa*

348 gene is not functional in these 25 isolates. A similar finding of mutated *coa* has been reported

349 (Phonimdaeng et al., 1990). Together with the observations that proper identification of

350 CoNS species by phenotypic methods not only requires a combination of phenotypic and

351 molecular assays (Akineden et al., 2011), one could argue that PCR-based assays are more

352 accurate than biochemical assays.

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359 encouragement.

360

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618

619    **Table 1. Oligonucleotide primers used in the present study**

| Primer | Sequence (5'-3') | Length (bp) | Reference |
|--------|------------------|-------------|-----------|
| *23S rRNA* | ACGGAGTTACAAAGGACGAC (For) | 1250 | Straub et al. 1999 |
|  | AGCTCAGCCTTAACGAGTAC (Rev) |  |  |
| *FnBp* | AGCACAAGGACCAGTCGAGGAAAT (For) | 750 | This study |
|  | TCTTCTTTGGCAGGTGGTACTGGT (Rev) |  |  |
| *Sa-fib* | AATTGCGTCAACAGCAGATGCGAG (For) | 210 | This study |
|  | GGACGTGCACCATATTCGAATGTACC (Rev) |  |  |
| *Nuc* | GTGCTGGCATATGTATGGCAATTGT (For) | 441 | This study |
|  | TCTTTGACCTTTGTCAAACTCGA (Rev) |  |  |
| *Se-fib* | AGTACAGAACCGTTATGCCTGGCT (For) | 720 | This study |
|  | TGATGAGTCAATTCGTGCTCCCGT (Rev) |  |  |
| *Se-124* | ATCAAAAAGTTGGCGAACCTTTTCA (For) | 124 | Martineau et al. 1996 |
|  | CAAAAGAGCGTGGAGAAAAGTATC (Rev) |  |  |
| *Eubac* | GGAGGAAGGTGGGGATGACG (For) | 240 | Martineau et al. 1996 |
|  | ATGGTGTGACGGGCGGTGTG (Rev) |  |  |

620    [*]The eubacterial *16S rRNA* primers were used separately to confirm the presence of genomic DNA.

**Table 2. Standardization for selection of primers for multiplex PCR**

| Primer combination used | | | | | | | Products amplified | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *23S* | *Nuc* | *FnBp* | *Sa-fib* | *Se-fib* | *Se-124* | *E16S* | *23S* | *Nuc* | *FnBp* | *Sa-fib* | *Se-fib* | *Se-124* | *E16S* |
| + | | | | | | | + | | | | | | |
| + | + | | | | | | + | + | | | | | |
| + | | + | | | | | - | | + | | | | |
| | + | + | | | | | | + | + | | | | |
| + | + | + | | | | | ± | + | - | | | | |
| + | + | | + | | | | + | + | | + | | | |
| + | + | | + | + | | | + | + | | + | + | | |
| + | + | | + | + | + | | + | + | | + | + | + | |
| + | + | | + | + | + | + | - | - | | + | + | + | + |

*23S = 23S rRNA*; *E16S* = Eukaryotic *16S rRNA*

623 **Table 3. Bacterial strains used and the observed reactivity in PCR for various genes**

| Bacterial species (reference or isolate identification number) | Species-specific marker targeted | | | | | |
|---|---|---|---|---|---|---|
| | *Eu16S* | *23S* | *Sa-fib* | *Nuc* | *Se-124* | *Se-fib* |
| *Staphylococcus aureus* (MTCC96) | + | + | + | + | - | - |
| *Staphylococcus epidermidis* (MTCC3382) | + | - | - | - | + | + |
| *Staphylococcus epidermidis* (MTCC3615) | + | - | - | - | + | + |
| *Staphylococcus saprophyticus* (MTCC6155) | + | - | - | - | - | - |
| *Staphylococcus haemolyticus* (MTCC3383) | + | - | - | - | - | - |
| *Staphylococcus warneri* (MTCC3050) | + | - | - | - | - | - |
| *Staphylococcus sciuri* (MTCC6154) | + | - | - | - | - | - |
| *Staphylococcus xylosus* (MTCC7441) | + | - | - | - | - | - |
| *Staphylococcus chromogenes* (MTCC3545) | + | - | - | - | - | - |
| *Streptococcus agalactiae** (S55) | + | - | - | - | - | - |
| *Micrococcus luteus* (MTCC106) | + | - | - | - | - | - |
| *Escherichia coli* (MTCC739) | + | - | - | - | - | - |
| *Psudomonas aeruginosa* (MTCC2453) | + | - | - | - | - | - |
| *Salmonella typhi* (MTCC743) | + | - | - | - | - | - |

624 **S. agalactiae* was isolated from milk sample obtained from a clinical mastitis case

625 **Table 4. Comparison of conventional and PCR identification of staphylococci**

| | | |
|---|---|---|
| **No. of staphylococcal isolates tested** | | 602 |
| **No. of coagulase-negative staphylococci (CoNS)** | | 317 |
| **No. of *S. epidermidis*** | **Biochemical identification** | 22 |
| | **PCR identification** | 22 |
| **No. of *S. aureus*** | **Biochemical identification** | 242 |
| | **PCR identification** | 267 |

626

627

**Table 5. Limit of detection (LOD) of simplex and multiplex PCR using mixed culture of**

*S. aureus* **and** *S. epidermidis*.

| Gene fragment amplified | | | | | Cfu/mL under indicated culture condition | |
|---|---|---|---|---|---|---|
| *23S* | *Nuc* | *Sa-fib* | *Se-124* | *Se-fib* | Non-enriched | Enriched |
| + | | | | | $10^1$ | $10^1$ |
| | + | | | | $10^2$ | $10^2$ |
| | | + | | | $10^2$ | $10^2$ |
| | | | + | | $10^1$ | $10^1$ |
| | | | | + | $10^2$ | $10^2$ |
| + | + | + | | | $10^4$ | $10^2$ |
| | | | + | + | $10^4$ | $10^2$ |
| + | + | + | + | + | $10^6$ | $10^4$ |

**Figure Legends**

631

632 **Figure 1. Standardization of simplex and multiplex PCR.** Products amplified by simplex

633 or multiplex PCR on *S. aureus* and *S. epidermidis* isolates were subjected to agarose gel

634 electrophoresis. Lanes: 1, *23S rRNA*; 2, *Nuc*; 3, *Sa-fib*; 4, *Se-fib*; 5, *Se-124*; M, DNA marker;

635 6, multiplex PCR of *S. aureus* with *23S rRNA, Nuc and Sa-fib* specific primers; 7, duplex

636 PCR of *S. epidermidis* with *Se-fib and Se-124* and 8, multiplex PCR with all primers using *S.*

637 *aureus* and *S. epidermidis* templates.

638

639 **Figure 2. Simplex and multiplex PCR on mixed cultures.** Products amplified by simplex

640 or multiplex PCR on mixed cultures of *S. aureus* and *S. epidermidis* isolates were subjected

641 to agarose gel electrophoresis. Lanes: 1, *S. aureus*; 2, *S. epidermidis*; 3, *S. aureus* and *S.*

642 *epidermidis;* 4, mixed template of *S. aureus, S. epidermidis,* CoNS and non-staphylococcus

643 species listed in Table 3*;* M, DNA marker; 5, *S. haemolyticus*; 6, *S. saprophyticus*; 7, *S.*

644 *chromogenes*; 8, *E. coli*; 9, *Salmonella typhi* and 10, *Streptococcus agalactiae.*

645

**Figure 1**                                               **Sunagar et al.**
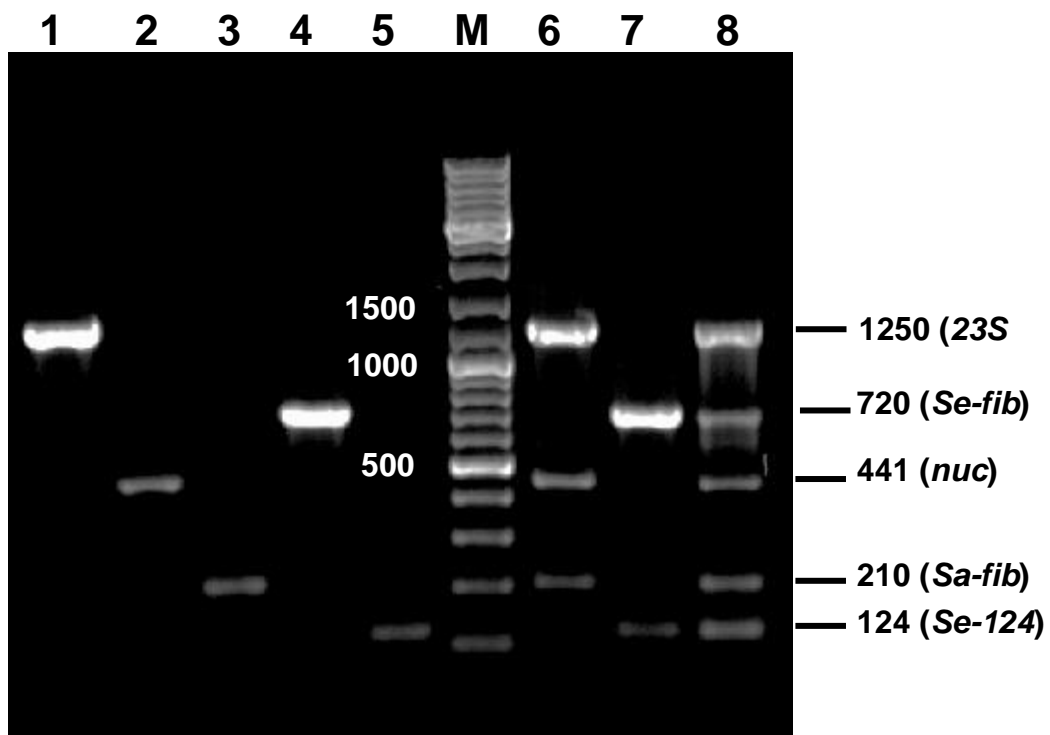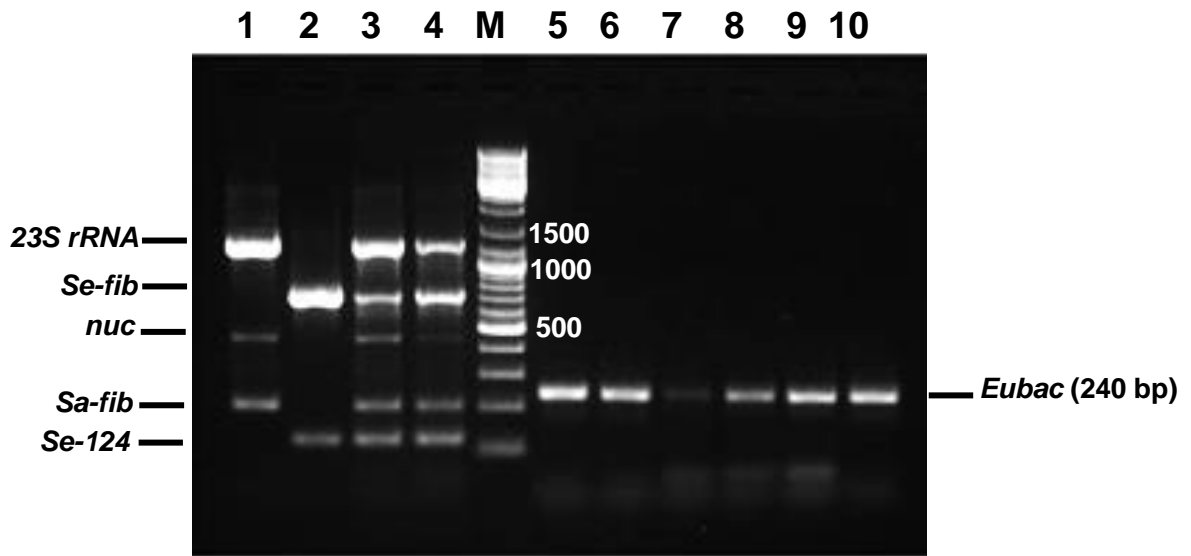
**Figure 2**

652 **Supplementary Figure 1. Sequence alignment and prediction of primers for _S._**
653 **_haemolyticus_ and _S. saprophyticus_.** Sequences of _fib_ gene of _S. epidermidis_ (Epi1, Epi2), _S._
654 _saprophyticus_ (Sapro), _S. aureus_ (NCTC, MW2, RF122), and _S. haemolyticus_ (Hae) were
655 aligned by using ClustalW, and potential primers were predicted for differentiating the
656 organisms. For each organism, the location of the forward and reverse primers is highlighted
657 and in bold. Forward primers are in upper case and the reverse complements of the reverse
658 primers are in lower case.

```
659  Epi1    ATGGCATATGATGGCTTATTTACTAAAAAAATGATTGAATCATTACAAGGTTTAGTCTCA 60
660  Epi2    ATGGCATATGATGGCTTATTTACTAAAAAAATGATTGAATCATTACAGGGTTTAGTCTCA 60
661  Sapro   ATGGCATATGATGGTTTATTTACAAGAAAATGGTGGAATCGTTACAATTTCTTGTAGAC 60
662  NCTC    ----------ATG--------AAAAATAAATTGATAG---------------------- 19
663  MW2     ----------ATG--------AAAAATAAATTGATAG---------------------- 19
664  RF122   ----------ATG--------AAAAATAAATTGATAG---------------------- 19
665  Hae     ---GTGTATGTTAATCCG--TTAAGATATACAGCTAA--------------------- 32
666
667  Epi1    GGCCGAATTCATAAAATAAATCAACCAGAAAATGACACAATAATTATTGTAATAAGACAA 120
668  Epi2    GGCCGAATTCATAAAATAAATCAACCAGAAAATGACACAATAATTATTGTAATTAGACAA 120
669  Sapro   GGAAGAATCCATAAAATAAATCAACCAGAAACGACACGCTTCTTGTCGTTATTCGACAA 120
670  NCTC    ------------------------------CAAAATCTTTATT-------AACAT 37
671  MW2     ------------------------------CAAAATCTTTATT-------AACAA 37
672  RF122   ------------------------------CAAAATCTTTATT-------AACAA 37
673  Hae     ------------------------------CAATACAGAAGTTACAAT-CAACGG 56
674
675  Epi1    AATCGTAAAAATCATCAACTACTTCTATCGATTCACCCTAGTTTCTCTCGTTTACAATTA 180
676  Epi2    AATCGTAAAAATCATCAACTACTTCTATCGATTCATCCTAGTTTCTCTCGTTTACAATTA 180
677  Sapro   AACCGAAAAAATCATCAGCTTCTACTTTCTATTCATCCTAGCTTTGCTAGAATGCATTTA 180
678  NCTC    TAGCGGCAATAGGTATTACTACAACTA------------------------------ 64
679  MW2     TAGCGGCAATAGGTATTACTACAACTA------------------------------ 64
680  RF122   TAGCGGTAATTGGTATTACTACAACTA------------------------------ 64
681  Hae     TACTGGACC**AAACGGTAGC-ACAATTATCGA**TG------------------------ 88
682
683  Epi1    ACTACTAAAAAATACGACAATCCATTTACCCCACCAATGTTTGCAAGAGTTTTCCGTAAA 240
684  Epi2    ACTACTAAGAAATACGACAATCCATTTACACCACCAATGTTTGCAAGAGTTTTCCGTAAA 240
685  Sapro   ACAAATAAAAAATATGATAATCCATTTGATCCACCCATGTTTGCACGCGTTTTCCGTAAA 240
686  NCTC    --------------------C**AATTGCGTCAACAGCAGATGCGAG**CG----------- 91
687  MW2     --------------------C**AATTGCGTCAACAGCAGATGCGAG**CG----------- 91
688  RF122   --------------------C**AATTGCGTCAACAGCAGATGCGAG**CG---------- 91
689  Hae     --------------------ATAGTACTGAAATTAAAATTTATAAAG---------- 115
690
691  Epi1    CACCTTGAGGGAGGCTTTATAAAAGCAATTCGACAGGTTGGTAACGATAGACGTATAGAG 300
692  Epi2    CACCTTGAGGGAGGCTTCATAAAAGCAATTCGACAGGTTGGTAACGATAGACGTATAGAG 300
693  Sapro   CATATAGAAGGTGGTATCGTTAAAGCAGTAAGACAAATTGGGAATGATAGACGTGTAGAA 300
694  NCTC    -------------------------------------AAGGATACGGTCCAAG------- 107
695  MW2     -------------------------------------AAGGATACGGTCCAAG------- 107
696  RF122   -------------------------------------AAGGGTACGGTCCAAG------- 107
697  Hae     -------------------------------TTGCAGATGATCAATAT------ 132
698
699  Epi1    ATTGATATCGAAAGTAAAGATGAAATTGGTGATACAATATATAGAACTATCATATTGGAA 360
700  Epi2    ATTGATATCGAAAGTAAAGATGAAATTGGTGATACAATATATAGAACTATTATATTGGAA 360
701  Sapro   ATTGATGTTCAAAGCAAAGATGAAATTGGAGATACAATATATAGAACTATAATTTTAGAA 360
702  NCTC    -----------AGAAAAGAAACCAGTG--------------------------AGTA 127
703  MW2     -----------AGAAAAGAAACCAGTG--------------------------AGTA 127
704  RF122   -----------AGAAAAGAAACCAGTG--------------------------AGTA 127
705  Hae     -----------CTACCAGACAGCAATA--------------------------GAA 151
706
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34

```
707   Epi1    ATAATGGGTAAACATAGTAACCTTATCCTTGTCGATGATCAACGTAAAATCATCGAAGGG 420
708   Epi2    ATAATGGGTAAACATAGTAACCTTATCCTTGTCGATGATCAACGTAAAATCATCGAAGGG 420
709   Sapro   ATTATGGGTAAACACAGTAACTTAATATTAGTTGATGATCAGCGTAAAATCATTGAAGGC 420
710   NCTC    TTAAT-------CACAATATCGTAG-------------------AGTACAATGATGG- 158
711   MW2     TTAAT-------CACAATATCGTAG-------------------AGTACAATGATGG- 158
712   RF122   TTAAT-------CACAATATCGTAG-------------------AGTACAATGATGG- 158
713   Hae     TTTATGATTATTCTCAATATGAGAATG------------------TTACTAATGATTAT 192
714
715   Epi1    TTTAAACATCTAACTCCTAACACAAAT--C**AGTACAGAACCGTTATGCCTGGCT**TCAAAT 478
716   Epi2    TTTAAACATCTAACTCCTAACACAAAT--C**AGTACAGAACCGTTATGCCTGGCT**TCAAAT 478
717   Sapro   TTTAAGCATTTAACACCGAATACCAAT--CAA**TATCGCACAGTAATGCCTGGCT**TTCAAT 478
718   NCTC    --------------TACTTTTAAATAT--CAATCTAGACCA---------------AAAT 187
719   MW2     --------------TACTTTTAAATAT--CAATCTAGACCA---------------AAAT 187
720   RF122   --------------TACTTTTAAATAT--CAATCTAGACCA---------------AAAT 187
721   Hae     --------CCAATTTCTGTTAATGGTGATAATACTGCCCC----------CATTAATT 232
722
723   Epi1    ATGAAGCACCACCAAATCAAAATAAAATCAATCCTTATGAATTAACAGGACAAGAGGTAT 538
724   Epi2    ATGAAGCACCACCAAATCAAAATAAAATCAATCCTTATGAATTAACAGGGCAAGAGGTAT 538
725   Sapro   ATGAAACACCACCAACTCAAAATAAAATTAATCCTTTTGAAATAACTGGTAATAAAGTCA 538
726   NCTC    TTAACTCAACACCTAAATATATTAAATTCAAACAT--------------------GACT 226
727   MW2     TTAACTCAACACCTAAATATATTAAATTCAAACAT--------------------GACT 226
728   RF122   TTAACTCAACACCTAAATATATTAAATTCAAACAT--------------------GACT 226
729   Hae     TTGGAGATATAAATACACATTACATTATCAAAGTA--------------------GTTA 271
730
731   Epi1    TAAAATATATAGACTTTAATGCTGGAAAGATTGCCAAACAATTACTTAATATATGTGAGG 598
732   Epi2    TAAAATATATTGACTTTAATGCTGGAAAGATTGCCAAGCAATTACTTAATATATGTGAGG 598
733   Sapro   TACAATATATCGATTTCAATAAGGGCAAAATTGCACGTCAACTTCTTGATCATTTCGAAG 598
734   NCTC    ATAATATTTTAGAATTTAACGAT**gg**--------------------------------- 251
735   MW2     ATAATATTTTAGAATTTAACGAT**gg**--------------------------------- 251
736   RF122   ATAATATTTTAGAATTTAACGAT**gg**--------------------------------- 251
737   Hae     GTAAATATCAGCTTGATTCAGAAGG--------------------------------- 296
738
739   Epi1    GCTTTAGTCCGCTAATCGCTAATGAAATTGTGAATCGTCGTCAATTTATGACTCAAGAAA 658
740   Epi2    GCTTTAGTCCACTAATCGCTAATGAAATTGTGAATCGTCGTCAATTTATGACTCAAAAAA 658
741   Sapro   GCTTTAGTCCATTAATTACAAATGAAATTGTGAATCGCAAACGATTTATGACAAATGATA 658
742   NCTC    ---------------**tacattcgaatatggtgcacgtcc**ACAATTTAATAAACCAGCAG 295
743   MW2     ---------------**tacattcgaatatggtgcacgtcc**ACAATTTAATAAACCAGCAG 295
744   RF122   ---------------**tacattcgaatatggtgcacgtcc**ACAATTTAATAAACCAGCAG 295
745   Hae     --------------TAACGTAAATATCCAACAATGGGCATCTATG-GTTACAACTAATA 340
746
747   Epi1    CATTACCTGATGCTTTTGATGAAGTCATGGCCGAAACAAAATCATCACCTACCCCTGTCT 718
748   Epi2    CATTACCTGATGCTTTTGATGAAGTCATGGCCGAAACCAAATCGGCATCTACTCCTGTCT 718
749   Sapro   CGTTACCAGAAGCATTTGATGAAGTCATCAATGAAATTAACCATACACCTACACCCGTTT 718
750   NCTC    CGAAAACTGATGCAACT-------------------------------------------A 313
751   MW2     CAAAAACTGATGCAACT-------------------------------------------A 313
752   RF122   CGAAAACTGATGCAACT-------------------------------------------A 313
753   Hae     AATATTATGGTACTGATGACACG-----------------------------------G 364
754
755   Epi1    TTCATAAAAATCATGAAACAGGTAAAGAGGACTTCTATTTCATGAAACTTAATCAATTCT 778
756   Epi2    TTCATAAAAATCATGAAACAGGTAAAGAGGATTTCTATTTCATGAAACTTAATCAATTCT 778
757   Sapro   TTCATAAAAATCACGAGACGGGTAAAGAAGATTTTTACTTTATGAAATTAAATCAATTTT 778
758   NCTC    TTAAAAAAGAACAA-----------------------------AAATTGATTCAAGCTC 343
759   MW2     TTAAAAAAGAACAA-----------------------------AAATTGATTCAAGCTC 343
760   RF122   TTAAAAAAGAACAA-----------------------------AAATTGATTCAAGCTC 343
761   Hae     CTAAATATGGTAAC-----------------------------AACATAACACTTGCTA 394
762
763   Epi1    ATGATGATA-TAGTTGAGTATCATTCACTTCATGAACTATTGGATCGCTTTTATGATGCA 837
764   Epi2    ATGATGATA-TAGTTGAGTATCATTCACTTCATGAACTATTGGATCGCTTTTATGATGCA 837
765   Sapro   ATGATGATG-TAACAGAATATGACTCATTACATGATTTATTAGACCGTTATTATGATGCA 837
766   NCTC    AAAATCTTG-TGAGAGAATTTGAAAAAACACAT--ACTGTCAG------------TGCA 387
767   MW2     AAAATCTTG-TGAGAGAATTTGAAAAAACACAT--ACTGTCAG------------TGCA 387
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768   RF122   AAAATCTTG-TGAGAGAATTTGAAAAAACACAT--ACTGTCAG-------------TGCA 387
769   Hae     CTagtaatggtaacggaaatgGTGACGATACTG--ACAGTGATGCCG--------ACGCA 444
770
771   Epi1    CGTGGAGAGAGAGAAAGAGTCAAACAACGTGCAAATGATTTAGTCAGATTTGTACAACAG 897
772   Epi2    CGTGGTGAGAGAGAAAGAGTAAAACAACGTGCAAATGATTTAGTCAGATTTGTACAACAG 897
773   Sapro   CGTGGAGAGCGAGAGCGTGTAAAACAACGTGCAAATGATTTAGTGAAATTTGTACAACAA 897
774   NCTC    C-----------ACAGAAAAGCACAAAAGGCAGTCAACTTAGTTTCGTTTG-------- 427
775   MW2     C-----------ACAGAAAAGCACAAAAGGCAGTCAACTTAGTTTCGTTTG-------- 427
776   RF122   C-----------ACAGAAAAGCACAAAAGGCAGTCAACTTAGTTTCGTTTA-------- 427
777   Hae     G-----------ATGCTGACGCCGATGCCGATAGTGAAAAGAAAATAATG-------- 484
778
779   Epi1    CAACTGCAAAAACAACAAAATAAATTGAGTAAACTTATCGATGAGTATGAAAGTGCTAAA 957
780   Epi2    CAACTGCAAAAACAACAAAATAAATTGAGTAAACTTATCGATGAGTATGAAAGTGCTAAA 957
781   Sapro   CAATTGCATAAATTTCAAAATAAATTAAATAAATTGATTGATGAACAAGAAGGTACAAAA 957
782   NCTC    ----------AATACAAAGTGAAGAAAATGGTCT----------------------- 451
783   MW2     ----------AATACAAAGTTAAGAAAATGGTCT----------------------- 451
784   RF122   ----------AATACAAAGTTAAGAAAATGGTCT----------------------- 451
785   Hae     ---------AATTACCAGGTACTGGTAGTGATGA----------------------- 509
786
787   Epi1    AACAAAGAAACTCAGCAATTATATGGAGAGTTGATTACAGCTAATATATATCGTATTCAA 1017
788   Epi2    AACAAAGAAACTCAGCAATTATATGGAGAGTTGATCACAGCTAATATATATCGTATTCAA 1017
789   Sapro   GAAAAGAATTACAACAATTGTATGGGGAACTTATCACTGCTAACATTTATCGTATTAAA 1017
790   NCTC    TACAAG-----------------------------AGCGAATTGATAATGTATTAAA 479
791   MW2     TACAAG-----------------------------AGCGAATTGATAATGTATTAAA 479
792   RF122   TACAAG-----------------------------AGCGAATTGATAATGTATTAAA 479
793   Hae     -AAAAA-----------------------------ATGGAGTGATTTTAGGATCATT 536
794
795   Epi1    CAAGGTGACGATTCTGTTATAGCGTTGAATTACTATACTGGTGAAGAAGTAAAGATTCCA 1077
796   Epi2    CAAGGTGACGATTCTGTTATAGCGTTGAATTACTATACTGGTGAAGAAGTAAAGATTCCA 1077
797   Sapro   CAAGGTGACAAATCGGTCACAGCCTTAAATTATtacatcggtgaagaagtcacTATACCG 1077
798   NCTC    ACAAG----GATTAG--------TTAAATAA------------------------- 498
799   MW2     ACAAG----GATTAG--------TTAAATAA------------------------- 498
800   RF122   ACAAG----GATTAG--------TAAAATAA------------------------- 498
801   Hae     ATTTGCTGCAATTGG---------AACATTACTATTAGGTAAGAATCGTAG------- 578
802
803   Epi1    CTTAACCCTACAAAATCGCCATCAACTAACGCTCAATATTATTATAAACAATATAACCGT 1137
804   Epi2    CTTAACCCTACAAAATCGCCATCAACTAACGCTCAATACTATTATAAACAATATAACCGT 1137
805   Sapro   CTTAACCCTACAAAGGCACCAGCAGTGAATGCACAAAATTATTACAAACAGTATAATAAA 1137
806   NCTC    ------------------------------------------------------------
807   MW2     ------------------------------------------------------------
808   RF122   ------------------------------------------------------------
809   Hae     ------------------AAAAATTAATGATAAAAAATAA----------------- 600
810
811   Epi1    ATGAAAACacgggagcacgaattgacgcatcaGATTAAACTTACTAAAGAGAATATTGAT 1197
812   Epi2    ATGAAAACacgagagcacgaattgacgcatcaGATTAAACTGACTAAAGAGAATATTGAT 1197
813   Sapro   TTAAAGACAAGAGAACATGAATTGCACCATCAAATCGATTTAACCAAAGAAAACATTAAT 1197
814   NCTC    ------------------------------------------------------------
815   MW2     ------------------------------------------------------------
816   RF122   ------------------------------------------------------------
817   Hae     ------------------------------------------------------------
818
819   Epi1    TACTTTGATAATATTGAACAACAGTTAAAGCACATTACCGTTGATGATATCGATGATATT 1257
820   Epi2    TACTTTGATAATATTGAGCAACAGTTAAAGCACATTACCGTTGATGATATCGATGATATT 1257
821   Sapro   TATTTTGAAAGTATAGAACAACAACTTGCTCATATTTCAGTCAATGATATTGATGACATT 1257
822   NCTC    ------------------------------------------------------------
823   MW2     ------------------------------------------------------------
824   RF122   ------------------------------------------------------------
825   Hae     ------------------------------------------------------------
826
827   Epi1    CGCGATGAACTTGCTGAACAGGGATTCATGAAGCAAAGAAAACAAAGTAAAAAGAAAAAA 1317
828   Epi2    CGCGACGAACTTGCTGAACAGGGATTCATGAAGCAAAGAAAACAGAGTAAAAAGAAAAAG 1317
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829   Sapro   CGCGATGAATTAGCCGAGCAAGGGTATATGAAACAACGTAAACAGAGTAAGAAGAAAAAG 1317
830   NCTC    -----------------------------------------------------------
831   MW2     -----------------------------------------------------------
832   RF122   -----------------------------------------------------------
833   Hae     -----------------------------------------------------------
834
835   Epi1    CAGTCTAAAATTCAACTTCAAACGTATCATTCTACTGATGGAGATACGATATTAGTAGGT 1377
836   Epi2    CAGTCTAAAATTCAACTTCAAACGTATCATTCTACTGATGGAGATACGATATTAGTAGGT 1377
837   Sapro   AAACAAACTATGCAGTTACAAGAATACGTATCTACAGATGGAGATACAATCATGGTTGGT 1377
838   NCTC    -----------------------------------------------------------
839   MW2     -----------------------------------------------------------
840   RF122   -----------------------------------------------------------
841   Hae     -----------------------------------------------------------
842
843   Epi1    AAAAATAATAAACAAAATGATTATCTTACCAATAAAAAGGCACGTAAAAATCATTTATGG 1437
844   Epi2    AAAAATAATAAACAAAATGATTATCTTACCAATAAAAAGGCACATAAAAATCATTTATGG 1437
845   Sapro   AAAAACAATAAACAAAATGATTATTTAACGAATAAATTAGCTAAGAAACATCAACTTTGG 1437
846   NCTC    -----------------------------------------------------------
847   MW2     -----------------------------------------------------------
848   RF122   -----------------------------------------------------------
849   Hae     -----------------------------------------------------------
850
851   Epi1    TTTCATACAAAGGATATTCCAGGATCTCATGTTGTCATACTCAATGAGAGTCCAAGCGAA 1497
852   Epi2    TTCCATACAAAAGATATTCCAGGATCTCATGTTGTCATACTCAATGAGAGTCCAAGCGAA 1497
853   Sapro   TTCCATACGAAAGATATTCCCGGATCACATGTCGTCATATTAAGTGATGATCCGAGTGAA 1497
854   NCTC    -----------------------------------------------------------
855   MW2     -----------------------------------------------------------
856   RF122   -----------------------------------------------------------
857   Hae     -----------------------------------------------------------
858
859   Epi1    ACAACGATTAAAGAAGCGGCAATGCTAGCTGGGTACTTCTCTAAAGCTGGGAGTTCAGCT 1557
860   Epi2    ACAACGATTAAAGAAGCGGCAATGCTAGCTGGGTATTTCTCTAAAGCTGGTAGTTCAGCT 1557
861   Sapro   GAAACAATTAAAGAAGCGGCCATGTTATCTGGTTACTTCTCAAAAGCAGGTAGTTCAGCT 1557
862   NCTC    -----------------------------------------------------------
863   MW2     -----------------------------------------------------------
864   RF122   -----------------------------------------------------------
865   Hae     -----------------------------------------------------------
866
867   Epi1    CAGATTCCTGTAGATTATACAGAAATTAGACACGTTCATAAACCTTCTGGAGCAAAACCT 1617
868   Epi2    CAAATTCCTGTAGATTATACGGAAATTAGACACGTTCATAAACCTTCTGGGGCAAAACCT 1617
869   Sapro   CAAATTCCAGTTGATTTTACTGAAATTAAACACGTGCATAAGCCTTCAGGTGCAAAACCA 1617
870   NCTC    -----------------------------------------------------------
871   MW2     -----------------------------------------------------------
872   RF122   -----------------------------------------------------------
873   Hae     -----------------------------------------------------------
874
875   Epi1    GGATTCGTGACATATGACAACCAAAAGACCCTATTTGCAACACCTGATTATGATCATATT 1677
876   Epi2    GGATTCGTGACATATGACAACCAAAAGACCCTATTTGCAACACCTGATTATGATCATATT 1677
877   Sapro   GGTTTTGTAACTTATGATAACCAAAAAACACTTTATGCAACACCCGATTATGATCACATT 1677
878   NCTC    -----------------------------------------------------------
879   MW2     -----------------------------------------------------------
880   RF122   -----------------------------------------------------------
881   Hae     -----------------------------------------------------------
882
883   Epi1    CAACAAATGAAAGAATCATAA 1698
884   Epi2    CAACAAATGAAAGAATCATAA 1698
885   Sapro   CAAAAAATGAAAGTAAAGTAA 1698
886   NCTC    --------------------
887   MW2     --------------------
888   RF122   --------------------
889   Hae     --------------------
```