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1 **Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes***

2

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4

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12

13

13 **Abstract**

14

15 An experimental infection model was developed to study host response to
16 intramammary infection in cows caused by *Staphylococcus chromogenes*. CNS intramammary
17 infections have become very common in modern dairy herds, and they can remain persistent in the
18 mammary gland. More information would be needed about the pathophysiology of CNS mastitis,
19 and an experimental mastitis model is a means for this research. Six primiparous Holstein-Friesian
20 cows were challenged with *S. chromogenes* four weeks after calving. One udder quarter of each
21 cow was inoculated with 2.1×10^6 cfu of *S. chromogenes*. All cows became infected and clinical
22 signs were mild. Milk production of the challenged quarter decreased on average by 16.3% during 7
23 d post challenge. Cows eliminated bacteria in a few days, except for one cow which developed
24 persistent mastitis. Milk indicators of inflammation, SCC and N-Acetyl- β -D-Glucosaminidase
25 (NAGase) returned to normal within a week. Milk NAGase activity increased moderately, which
26 reflects minor tissue damage in the udder. Concentrations of serum amyloid A (SAA) and milk
27 amyloid A (MAA) were both elevated at 12 h PC. MAA was affected by the milking times, and was
28 at its highest before the morning milking. In our experimental model, systemic acute phase protein
29 response with SAA occurred as an on-off type reaction. In conclusion, this experimental model
30 could be used to study host response in CNS mastitis caused by the main CNS species and also for
31 comparison of the host response in a mild intramammary infection and in more severe mastitis
32 models.

33

34 **Key words:** bovine, mastitis, coagulase-negative staphylococci, experimental mastitis model, serum
35 amyloid A, milk amyloid A

36

37 1. Introduction

38

39 An experimental mastitis model represents a means to study both the host response
40 and virulence characteristics of bacteria. In mastitis research, mice and small ruminants have been
41 used as experimental animals (Brouillette and Malouin, 2005). However, the mammary gland of a
42 modern dairy cow differs considerably from that of small experimental animals. Experimental
43 bovine mastitis models have been developed for many major mastitis pathogens including
44 *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus uberis* (Grönlund et al., 2003; Jacobsen
45 et al., 2005; Schukken et al., 1999; Schukken et al., 1999; Pedersen et al., 2003). Studies on
46 experimental models using less common mastitis pathogens, for example *Pseudomonas aeruginosa*
47 and *Arcanobacterium pyogenes*, were also published (Bannerman et al., 2005; Hirvonen et al.,
48 1996).

49 The significance of CNS among mastitis pathogens has increased and in many
50 countries they have become the predominant bacterial species (Pitkälä et al., 2004; Tenhagen et al.,
51 2006). CNS mastitis is usually mild clinical or subclinical mastitis. Infection can be transient and
52 disappear spontaneously or remain persistent in the mammary gland (Chaffer et al., 1999; Davidson
53 et al., 1992; Taponen et al., 2007). Persistent CNS infection may cause economical losses to the
54 farmer by decreasing the milk yield and quality of the milk (Oliver et al., 2003). A number of CNS
55 species have been isolated in bovine mastitis, the most common being *S. chromogenes*, *S. simulans*
56 and *S. epidermidis* (Taponen et al., 2006; Matthews et al., 1991; Aarestrup et al., 1995). Recent
57 studies have shown some differences in the pathogenesis of mastitis caused by different CNS
58 species (Almeida and Oliver, 2001; Zhang and Maddox, 2000).

59 Infection dynamics of bovine CNS mastitis have not been studied using experimental
60 infection models. Reports on experimentally induced CNS intramammary infection in sheep are
61 available. In sheep, experimental CNS mastitis was shown to cause mild clinical or subclinical

62 mastitis. Infection persisted in some animals over the whole study period, which ranged from 6 d to
63 10 wks after inoculation (Winter and Colditz, 2002; Winter et al., 2003; Burriel, 1997).

64 The aim of this pilot study was to investigate the host response in bovine
65 intramammary infection caused by *S. chromogenes*, using an experimental model. Concentrations
66 of serum amyloid A in blood and milk, as well somatic cell count (SCC), and N-acetyl- β -D-
67 Glucosaminidase (NAGase) activity in the milk were determined. Bacterial elimination rates and
68 clinical signs were also investigated.

69

70 2. Materials and Methods

71

72 2.1. Study animals

73

74 Six primiparous Holstein-Friesian cows were used as experimental animals. They
75 were 30 months old (from 27 to 31 months) at parturition. One udder quarter of each cow was
76 experimentally infected by *S. chromogenes* four weeks after calving. One cow was excluded from
77 the study due to *S. aureus* mastitis in another quarter. The cows were kept in a tie-stall barn and fed
78 with silage and concentrates according to Finnish feeding recommendations. At the beginning of the
79 study all the cows were clinically healthy, except for one cow that had mild laminitis. Udder
80 quarters of all cows had a low somatic cell count (SSC) in their milk (<100 000 cells/ml) and they
81 were free from bacterial growth in two subsequent samplings before the experimental infection. The
82 cows did not receive any medical treatment during the study. The Ethics Committee of Helsinki
83 University approved the study protocol.

84

85 2.2. *Inoculation procedure*

86

87 The *S. chromogenes* strain was isolated from a case of clinical mastitis in a dairy cow.
88 The strain was identified with the API Staph ID 32 test. The strain was stored at -80°C (Protect
89 Bacterial Preservers®) and cultured on TSH-blood agar (bioMérieux, France) at 37 °C for 18 h.
90 Two colonies were transferred to Müller-Hinton broth and cultured at 37 °C for 18 h. The density of
91 the bacterial suspension was determined with a spectrophotometer (Stasar, Gilford Instrument
92 Laboratories Inc, Ohio) at 550 nm and using application of McFarland standard (bioMérieux,
93 France). The bacterial culture was pelleted by centrifugation and washed with phosphate buffered
94 saline (PBS) several times. The suspension was diluted in saline to 300 000 cfu (colony forming
95 units)/ml. The inoculate contained 2.1×10^6 cfu in 7 ml of saline. The suspension was cultured on a
96 blood agar plate in a dilution series and colonies were assessed to determine the final inoculum
97 dose.

98 The infection dose used was based on a preliminary study in two cows with different
99 doses of the same *S. chromogenes* strain. The aim of the study was to induce clinical mastitis. The
100 first preliminary testing with a dose of 50 000 cfu did not provoke any clinical signs and bacteria
101 were eliminated from the quarters within 6 h post inoculation. In the final experiment, one udder
102 quarter of each cow was used as the experimental quarter and another quarter as a control quarter.
103 The quarters were infused through the teat canal within 30 min of the morning milking, using a
104 blunt cannula. Prior to infusion, the teat end was disinfected with chlorhexidin. After the infusion,
105 the teat was gently closed with the fingers and the inoculation dose massaged upwards.

106

107 2.3. *Milk and blood samples*

108

109 Milk samples were taken from the experimental and control quarters for
110 bacteriological culturing, SCC, and determination of NAGase (N-Acetyl- β -D-Glucosaminidase)
111 activity and milk amyloid A (MAA). Aseptic milk samples were collected 2 h before the challenge
112 and then at 8, 12, 22, 30, 34, 46, 54, 72, 78, and 96 h, and 7th and 14th day after the challenge. A
113 volume of 100 μ l milk was cultured on blood-esculin agar (TSH-agar) and several dilutions of the
114 milk samples cultured for bacterial counting; the detection limit for bacterial growth was 10 cfu/ml.
115 Colonies were identified as CNS using standard procedures (Hogan et al., 1999) and in unclear
116 cases additionally with the API Staph ID 32 test.

117 SCC was determined by a fluoro-optical method using the Fossomatic-instrument in
118 Valio Ltd Laboratories, Finland. Milk samples were stored frozen at -80°C for later determinations
119 of milk NAGase activity and MAA. Milk NAGase activity was measured by fluorogenic method
120 (Kitchen et al., 1978) using an in-house microplate modification developed by Mattila and
121 Sandholm (1985). Using this method, NAGase activity of normal milk (SCC below 100 000
122 cell/ml) is 0.049-0.062 pmol 4-MU/min/ μ l of milk at 25°C. Inter-assay and intra-assay CV for the
123 NAGase activity were <4.8% for the high control and <6.6% for the low control.

124 Blood samples were collected 2 h before the challenge and at 12, 22, 30, 34, 46, 54,
125 72 and 96 h PC. Serum was separated and serum samples stored frozen at -80°C for later
126 determination of SAA. Concentrations of SAA and MAA were determined using a commercial kit
127 (Tridelta Development, Wicklow, Ireland). The detection limit of the kit was 0.005 mg/ml. Serum
128 and milk samples were initially diluted 1:500 and 1:50 respectively. Dilutions 1:1000 and 1:100
129 were used if results were over the range of the standard curve (75 mg/l and 7.5 mg/l respectively).
130 The inter-assay and intra-assay CV for the SAA and MAA analyses were <10 % and <5 %.

131

132 *2.4. Clinical observations*

133

134 The cows were examined clinically at every sampling. Clinical status consisted of
135 general attitude of the cow, appetite, body temperature, rumen function, consistency of the udder
136 and milk appearance. Signs were divided into three groups: systemic signs, local signs and milk
137 appearance. The scoring system was adapted from Anderson et al. (1986) with slight modifications
138 (scoring from 1 to 3, half numbers also used). Signs were scored according to their severity (1 = no
139 signs or changes and 3 = severe signs or changes).

140

141 2.5. Statistical methods

142

143 Descriptive statistics were performed using SPSS 13.0 software. Results are presented
144 as mean (\pm SEM) and median (range) of the variables. A Wilcoxon Signed Ranks test was used to
145 test the statistical significance of the differences between the challenged and control quarters.

146

147 3. Results

148

149 3.1. Clinical signs

150

151 All cows became infected with *S. chromogenes* and developed mastitis. Clinical signs
152 were mild. The body temperatures of all the cows were normal during the entire study period. One
153 cow had poor appetite at 30 and 54 h PC (post challenge) and her systemic signs were scored from
154 1.5 to 2. The other cows did not show any systemic signs (score 1). All cows had mild local signs in
155 the challenged quarter at 12-78 h PC (scores 1.5-2); these included swelling, increased firmness and
156 heat of the quarter. Two cows had very mild local signs (maximum score 1.5), and only some heat

157 and slight firmness was recorded. Only one cow exhibited mild milk changes (clots, colour
158 changes) 22 h PC.

159 No significant differences in the daily total milk yields during the experiment were
160 recorded. The milk yields of the challenged quarters were slightly decreased at 30 and 46 h PC
161 (consecutive milking times) in all cows compared with the control quarters. The milk yield ratio,
162 calculated as the challenged quarter milk yield per the control quarter milk yield, was 1.02 before
163 challenge. The decrease in the milk yield ratio was on average 0.17 (range 0.08-0.33). Based on the
164 ratio, the milk yield decrease in the challenged quarter was on average 16.3% of the milk yield
165 before challenge (range 7.4%-27.3%). At the end of the observation period the milk yields were
166 close to that before the challenge (0.942). No changes were found in the control quarters at any time
167 points.

168

169 3.2. Bacterial counts

170

171 Bacterial growth peaked in the quarters at the first sampling 8 h PC (220–19500
172 cfu/ml). Bacteria were eliminated fast and at 46 h PC no bacteria were isolated from the milk
173 samples (Fig 1). One cow (number 11) developed persistent mastitis. After the first peak only few
174 colonies of bacteria were isolated from this quarter, but at 7 d PC the number of bacteria increased
175 to 1300 cfu/ml.

176

177 3.3. Indicators of inflammation in the milk

178

179 The SCC increased to $>1.2 \times 10^6$ cells/ml in the milk of all infected quarters. The
180 mean SCC value exceeded 0.15×10^6 cells/ml at 8 h PC. SCC peaked at 30 h PC (from 0.93×10^6

181 to 7.29×10^6 cell/ml; median 2.39×10^6 cell/ml) and then started to decrease to under 0.15×10^6
182 cell/ml at day 7 (Fig.1). SCC in the infected quarter of the cow with persistent infection (11) did not
183 rise at the time when the bacterial count started to increase again.

184 NAGase activity of the milk first decreased and then started to increase (Fig 2),
185 peaking at 22-46 h PC (0.15-0.3 pmol 4-MU/min/ μ l). In the cow with persistent infection, changes
186 in the NAGase activity in the milk were similar to those in SCC. By the seventh day PC the
187 NAGase activity had returned to the basic level in all cows (0.05-0.11 pmol 4-MU/min/ μ l).

188 MAA concentrations in the milk before challenge were under the detection limit.
189 MAA was slightly increased at 22 h after the challenge and then increased during the next 3 d
190 (Fig.3). MAA peaked at 54 h PC (13.94 mg/ml). MAA fluctuated between milking times and was
191 highest before the morning milking. Two cows (7 and 10), had a low concentration of MAA
192 compared with the others (maximum value 2.83 mg/l); these cows also eliminated the infection fast.

193 Before the challenge the serum concentration SAA was 0.3-10.1 mg/l (median 2.41
194 mg/l). The concentration of SAA increased in serum after the challenge, peaking at 46 h PC (Fig.
195 4).

196

197 **4. Discussion**

198

199 This study describes an experimental model for bovine CNS mastitis for the first time.
200 The disease was mild, only one cow showing systemic signs. The local signs seen in the infected
201 udder quarters were mild in all cows. All cows except one eliminated infection from the challenged
202 quarters within the follow-up period of 14 d. The challenge dose used in the present study was high
203 as compared with doses used to induce *S. aureus* or *E. coli* mastitis (Schukken et al., 1999;
204 Hyvönen et al., 2006). This may cause a rapid immune response that enhances the elimination of
205 bacteria. However, in the preliminary challenge test we failed to infect cows with lower doses of

206 this CNS. It seems that a high dose is required to induce CNS mastitis and it may be difficult to
207 induce clinical CNS mastitis. The number of cows included in this study was low and only one
208 species of CNS was used, so our results should be considered as preliminary.

209 *S. chromogenes* was selected for the experiment because it is one of the most common
210 species of CNS isolated from bovine mastitis. Furthermore, some *in vitro* studies indicated *S.*
211 *chromogenes* to be more virulent than other CNS species (Zhang and Maddox, 2000). In this study
212 *S. chromogenes* caused hardly any systemic signs, which is typical of CNS mastitis. Local signs
213 were also mild, but some damage to the udder quarter was present as milk yield in the challenged
214 quarters decreased on average by 16.3%. All cows spontaneously eliminated the infection, except
215 one which developed persistent mastitis. In all three studies on experimental ovine mastitis with
216 CNS (*S. epidermidis*), only 40% of the sheep eliminated infection (Winter and Colditz, 2002;
217 Winter et al., 2003; Burriel, 1997). The difference may be attributable to the different animal
218 species or differences in the virulence of the bacterial species. Winter and Colditz (2002) reported
219 increasing content of cytokines IL-1 β , IL-6 and IL-8 in milk following a *S. epidermidis* challenge,
220 which was induced with an equal inoculum dose as in our study on dairy cows.

221 Somatic cells invade the milk after alarm of the immune system. SCC of the infected
222 quarter can be relatively low in CNS mastitis compared with mastitis caused by major pathogens
223 (Djabri et al., 2002). In the Finnish mastitis survey, SCC was >300 000 cells/ml in 18% of the
224 quarters infected by CNS only (Pitkälä et al., 2004). In our experiment, the SCC curve was of a
225 similar shape although much lower than in experimental studies with major pathogens (Bannerman
226 et al., 2005). Milk SCC did not rise in the quarter with persisting infection at the time when the
227 bacterial growth started to increase, which may indicate that the infection did not trigger any
228 immune response.

229 NAGase is a lysosomal enzyme and reflects udder tissue damage due to inflammation.
230 In our experimental model, milk NAGase activity increased only moderately, which indicated the

231 mild nature of CNS intramammary infection. In more severe infections, such as mastitis due to *E.*
232 *coli*, NAGase activity in the milk can be 9-10 times as high as in the normal milk, suggesting
233 considerable tissue damage (Hyvönen et al., 2006).

234 Acute phase proteins (APP) are involved in early state response to infection.
235 Stimulated by pro-inflammatory cytokines, SAA is excreted from the liver, but is also produced
236 locally in the mammary epithelial cells (Weber et al., 2006). SAA has been suggested to have many
237 immunological roles: it activates leucocytes by chemotaxis, increases phagocytosis and is able to
238 enhance leukocyte adhesion to the endothelial cells (Suffredini et al., 1999). In this study,
239 concentrations of SAA and MAA were both elevated at 12 h PC. MAA was affected by milking
240 times, and was at its highest before the morning milking, reflecting the longer milking interval. This
241 could probably be seen in this mild infection model due to the relatively low rise of MAA, but was
242 not noticed in a more severe experimental mastitis model with *E. coli* (Jacobsen et al., 2005;
243 Hyvönen et al., 2006). The time of sampling in relation to milking should perhaps be taken into
244 account when interpreting low MAA concentrations. Grönlund et al. (2005) for example reported
245 great variation in MAA concentrations in spontaneous subclinical mastitis; sampling time could
246 have had some confounding effect in their study.

247 In our study MAA concentration continued to increase in the milk, even though
248 bacteria had already been eliminated from the quarters. Milk SCC started to decrease before MAA,
249 which continued to fluctuate for much longer. Compared with MAA and SAA concentrations in
250 experimental *E. coli* mastitis determined in the same laboratory with the same assay (Hyvönen et
251 al., 2006), MAA was 100 times lower and SAA three times lower in *S. chromogenes* mastitis. In
252 two cows SAA remained at the basic level over the whole experimental period; one of them was the
253 cow which developed persistent mastitis. This could be linked to the very mild local signs in the
254 udder seen in these two cows. It is possible that local proinflammatory cytokine response in the
255 affected quarters was not high enough to provoke systemic production of SAA. Different patterns

256 could be recognized in systemic and local SAA response. Two cows with the highest and clearest
257 systemic response hardly showed local MAA reaction and eliminated bacteria quickly (cows no. 7
258 and 10). On the contrary, two cows (no. 9 and 11) with no systemic reaction exhibited moderate
259 local MAA response and the other developed persistent infection. It might indicate that quality of
260 the inflammatory response could play an important role for the outcome of infection. The possible
261 role of an on-off type of systemic inflammatory response is interesting and merits further study.

262 In conclusion, with this infection model, *in vivo* virulence of different CNS species
263 and host response of the mammary gland to CNS infection could be investigated. One interesting
264 aspect to study would be the development of persistent intramammary infection and possible host or
265 pathogen factors involved in that. CNS mastitis model is mild and it can also be used for
266 comparison with more severe mastitis models.

267

268 **Conflict of interest**

269

270 None of the authors (H. Simojoki, T. Orro, S. Taponen, S. Pyörälä) has a financial or
271 personal relationship with other people or organizations that could inappropriately influence or bias
272 the paper entitled “Host response in bovine mastitis experimentally induced with *Staphylococcus*
273 *chromogenes*”.

274

275 **References**

276

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358 Figure 1

359 Mean (\pm SEM) SCC ($\times 10^6$ cells/ml; \blacklozenge) and mean (\pm SEM) bacterial growth (log cfu/ml; \blacklozenge) in the
360 milk after experimental intramammary induction with *S. chromogenes*.

361

362 Figure 2

363 Mean (\pm SEM) NAGase activity in milk in infected quarters (\blacklozenge) and control quarters (\blacklozenge) after
364 experimental intramammary induction with *S. chromogenes*.

365

366 Figure 3

367 Milk amyloid A (MAA) concentrations in milk of five cows after experimental intramammary
368 induction with *S. chromogenes*.

369

370 Figure 4

371 Serum amyloid A (SAA) concentrations in serum of five cows after experimental intramammary
372 induction with *S. chromogenes*. Cow number 11 developed persistent infection.







