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Petra Regenhard, Wolfram Petzl, Holm Zerbe, Helga Sauerwein

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1 +Petra Regenhard<sup>1</sup>, Wolfram Petzl<sup>2</sup>, Holm Zerbe<sup>2</sup>, Helga Sauerwein<sup>1\*</sup>

2

3 <sup>1</sup>Institute of Animal Science, Physiology and Hygiene Group, University of Bonn, Germany

4 <sup>2</sup>Clinic for Ruminants, University of Munich, Germany

5

6 **The antibacterial psoriasin is induced by *E. coli* infection in the**  
7 **bovine udder**

8

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11 **\*Corresponding Author:**

12 Helga Sauerwein

13 University of Bonn

14 Faculty of Agriculture

15 Institute of Animal Science

16 Physiology and Hygiene Group

17 Katzenburgweg 7-9

18 D-53115 Bonn

19 Deutschland

20 Tel.: 0049228732804

21 Fax: 0049228737938

22 E-Mail: sauerwein@uni-bonn.de

23

24 **Abstract**

25

26 Human psoriasin (S100A7) has been described as a member of the family of S100  
27 calcium-binding proteins and is over-expressed in patients suffering from  
28 psoriasis. The bovine homolog was first identified as a cow-derived respiratory  
29 allergen. Human psoriasin as well as its bovine homolog exhibit antibacterial  
30 activity especially against *Escherichia coli*. During *E. coli* mastitis, the host  
31 defence status is a cardinal factor influencing systemic disease severity, and  
32 *Escherichia coli*-mastitis is a common problem in dairy cattle. We therefore  
33 examined by immunoblotting and immunohistochemistry whether and in which  
34 location the bovine protein is expressed in the mammary gland and whether this  
35 expression is inducible by *E. coli*-infection. Additionally, milk samples from the  
36 cows were investigated. To obtain an antiserum, rabbits were immunised with  
37 recombinant bovine psoriasin. Six German Holstein cows in their first lactation  
38 were used; 4 of them were intramammarily infected with an *E. coli* strain isolated  
39 from udder secretions of cows with clinical mastitis, 2 cows served as healthy  
40 control animals. After 24 h, cows were slaughtered and samples were collected  
41 from 3 different locations of the quarters. Psoriasin expression was limited to the  
42 teat cistern of the *E. coli*-infected cows, but was absent in the teat cistern of the  
43 non-infected cows and in the parenchyma of both groups, whereas the expression  
44 on udder skin was demonstrated in both infected and non-infected cows. Psoriasin  
45 thus appears to be a part of the local host defence mechanisms in the udder and  
46 seems to be inducible by infection with *E. coli*.

47 **Keywords**48 *E. coli* mastitis, dairy cows, antimicrobial peptides, psoriasin (S100A7)

49

50 **1. Introduction**

51

52 Mastitis caused by *E. coli* is a common problem in dairy cattle, and the  
53 antimicrobial susceptibility of *E. coli* isolates from clinical bovine mastitis against  
54 antibiotics and chemotherapeutics decreases (Srinivasan et al., 2007). *E. coli*  
55 mastitis causes infection and inflammation of the mammary gland in dairy cows  
56 mainly around parturition and during early lactation. Intramammary infections  
57 with *E. coli* often result in an acute mastitis exhibiting a wide range of disease  
58 severity, which is determined by interactions between the host, the environment,  
59 and the infectious agent (Wenz et al., 2006). Therefore, the host defence status is a  
60 cardinal factor influencing systemic disease severity and outcome of the disease  
61 (Burvenich et al., 2003).

62 Psoriasin (S100A7), a member of the S100 family of calcium-binding proteins, is  
63 in humans abundantly synthesised in keratinocytes of patients suffering from  
64 psoriasis. Gläser et al. (2005) have demonstrated that psoriasin exhibits  
65 antibacterial activity and protects healthy human skin from *E. coli* infections.  
66 From a cDNA library of bovine skin, RAUTIAINEN et al. (1995) isolated a clone  
67 named BDA11 coding for an allergen with a predicted 63.4% amino-acid  
68 sequence identity to human psoriasin. HITOMI et al. (1996) characterised a  
69 calcium-binding protein (CAAF2) abundant in bovine amniotic fluid with the

70 same sequence identity to human psoriasin. The exact physiological function of  
71 psoriasin remains to be elucidated. Recently, bovine psoriasin was expressed  
72 recombinantly, and its antibacterial activity restricted to *E. coli* could be  
73 demonstrated (Regenhard et al., 2009). The aim of this study was to investigate  
74 the protein expression of psoriasin in the bovine udder by performing Western  
75 Blotting and immunohistochemistry in different locations, and to test whether this  
76 expression is inducible by infection with *E. coli*.

77

## 78 **2. Material and Methods**

### 79 **Expression and purification of recombinant bovine psoriasin**

80 Bovine psoriasin was expressed in *E. coli* (BL21) and purified as described  
81 previously (Regenhard et al., 2009).

### 82 **Protein analysis**

83 The purified protein was analysed by SDS-PAGE on a 15 % separation gel and by  
84 subsequent Coomassie staining. The correct mass and the purity of the  
85 recombinant protein were verified by MALDI (Matrix Assisted Laser  
86 Desorption/Ionisation)-TOF mass spectrometric analysis.

### 87 **Protein concentration**

88 Protein concentrations were calculated from UV-absorption spectra in the range of  
89 240-320 nm using the method of WAXMAN et al. (1993).

### 90 **Immunisation of rabbits**

91 Three female rabbits, about 4-months-old and weighing 3 kg, were chosen for  
92 generating antibodies against recombinant bovine psoriasin (rbPs). The animals

93 were kept at the Institute of Animal Science at the University of Bonn, and the  
94 trial was conducted under the approval of the ethics committee of the regional  
95 government (Regional Board, Cologne, 9.33.2.10.54.07.028). A basic  
96 immunisation was performed by injecting 200 µg of pure KLH (Keyhole Limpet  
97 Haemocyanin)-coupled rbPs as immunogen, emulsified with complete Freund's  
98 adjuvant, per animal by a multipoint subcutaneous injection. After 4 weeks, two  
99 enhancing immunisations were carried out as described above except that only  
100 100 µg of pure KLH-coupled rbPs and incomplete Freund's adjuvant were used  
101 instead, with a 4-week interval between each injection. Two weeks after the last  
102 immunisation, the rabbits were sacrificed and serum was collected.

### 103 **Dairy cows**

104 The trial included 6 German Holstein cows in the middle of their first lactation  
105 (aged between 26–30 months, 3 to 5 months post partum) and was conducted  
106 under the approval of the ethics committee of the regional government of upper  
107 Bavaria, Germany (No. 55.2-1-54-2531-108-05). Animals were kept at the  
108 University of Munich in the clinic for ruminants, and never had any signs of  
109 clinical or subclinical mastitis before. Quarter milk samples of these animals were  
110 tested weekly six times pre-trial and on the day of trial to be free from mastitis  
111 pathogens and to have less than 50,000 somatic cells/mL. Daily milk yields were  
112 between 15 and 25 L. Four of 6 animals were infected with *E. coli*, 2 cows served  
113 as healthy control animals.

114 Three weeks before pathogen inoculation, milk yield, California mastitis test  
115 (CMT), food intake, and body temperature of each animal were documented twice

116 daily. Quarter milk samples for determination of the SCC and for bacteriological  
117 examination were collected weekly. The somatic cell count was determined using  
118 the Fossomatic 360 (FOSS Electric, Hillerod, Denmark) cell counter as described  
119 by Schmidt Madsen (1975). Bacterial levels were determined by the lab of the  
120 clinic for ruminants (Munich, Germany) by plating 10  $\mu$ L of quartermilk samples  
121 on Columbia sheep blood agar, Gassner agar, and Edwards agar. After incubating  
122 overnight at 37 °C, the plates were analysed and the number of bacteria was  
123 counted. No animal showed fever, increased SCC, or alteration in milk during this  
124 pre-infection period, and the milk was free from mastitis pathogens.

#### 125 **Preparation of inoculum**

126 The *Escherichia coli* (*E. coli* 1303) strain was isolated from udder secretions of  
127 cows with clinical mastitis. Cultivation of bacteria, storage in glycerol stocks, as  
128 well as their biochemical characterisation were performed in the Institute of  
129 Microbiology, University of Veterinary Medicine (Hannover, Germany) as  
130 described previously (Petzl et al., 2008). Bacteria were kept cryo-conserved for  
131 subsequent infections. Inoculum doses were generated as described by van  
132 Oostveldt et al. (2002).

#### 133 **Experimental infection and tissue sampling**

134 Cows were synchronised by two injections of Cloprostenol-sodium salt  
135 (Estrumate, Prostaglandin-F2  $\alpha$  (PGF2  $\alpha$ )-analogue (Essex, Munich, Germany))  
136 in a 12 day interval, to avoid hormonal influences depending on the sexual cycle.  
137 Animals were inoculated with bacteria three days after the second PGF2  $\alpha$ -  
138 injection during estrus. The 6 cows were split into 2 groups, one of 4 animals and

139 one group of 2 animals each. The first group was inoculated intramammarily with  
140 500 CFU *E. coli* 1303 over 24 h. After application of 20 I.E. oxytocin (i.v.), the  
141 udder was entirely milked out. Teats were cleaned and disinfected with ethanol  
142 (70%). Bacteria were suspended in 2 mL 0.9% sterile pyrogen free saline, and  
143 administered intracisternally through the teat canal. Animals were inoculated in  
144 the hind right quarter with *E. coli*, and in the hind left quarter with 2 mL sterile  
145 saline. The front quarters remain untreated. Every 12 h, udder secretions were  
146 sampled before regular milking. The first 5 mL were discarded; the following 10  
147 mL were used for SCC determination and bacteriology. Cows were milked at the  
148 beginning of the trial, and 12 h, and 24 h after the trial starts. Finally, cows were  
149 killed 24 h after inoculation of the bacteria with a penetrating captive bolt gun  
150 followed by exsanguination. Udder tissue samples were collected aseptically  
151 within 5 to 10 min after killing out of the udder parenchyma 10 cm dorsal of the  
152 milk cistern, and out of the middle of the teat cistern including the udder skin.  
153 Tissue samples were frozen immediately in liquid nitrogen, and then stored at –  
154 80°C until further use.

#### 155 **Non-denaturing protein extraction and determination of protein amount**

156 Protein was extracted under non-denaturing conditions by homogenising udder  
157 tissue samples (parenchyma, epithelium of the teat cistern, teat skin) with a mortar  
158 and pestle under liquid nitrogen in 10 mM HEPES buffer pH 7.4 including the  
159 proteinase inhibitors COMPLETE™ (Roche, Mannheim, Germany) and 1 mM  
160 PMSF (Sigma, Deisenhofen, Germany). The homogenate was centrifuged 10 min  
161 at 14,000 *g* and room temperature, the supernatant was collected, and the total

162 protein amount was determined by using the bicinchoninic acid assay according to  
163 the manufacturer's instructions (Sigma).

#### 164 **Western Blot analysis**

165 Proteins extracts (30 µg total protein per lane), sterile filtered milk samples, and  
166 rbPs (200 ng) as a positive control were separated by electrophoresis in a 15 %  
167 SDS polyacrylamide gel and semi-dry blotted onto a polyvinylidene difluoride  
168 (PVDF) membrane (Amersham, Germany) at 0.8 mA/cm<sup>2</sup> for 55 min. The PVDF  
169 membrane was blocked with Roti®-Block (Roth, Karlsruhe, Germany), diluted 1  
170 to 10 with TBST (Tris-buffered saline: 10 mM TRIS, 150 mM NaCl with 0.05%  
171 Tween 20) for 1 h at room temperature, and subsequently incubated with rabbit  
172 antiserum (1:30,000 in TBST) over night at 4 °C. After washing several times  
173 with TBST, the membrane was incubated with goat anti-rabbit IgG-HRP  
174 (1:40,000 in TBST) (Southern Biotech, Birmingham, AL, USA) for 1 h at room  
175 temperature. After washing, the membrane was then developed using the Super  
176 Signal® West Pico Chemiluminescent Substrate kit (Pierce, Bonn, Germany).  
177 Equal loading was compared after stripping the membrane by a Western Blot  
178 against β-actin.

#### 179 **Immunohistochemistry**

180 The indirect peroxidase method was performed using polyclonal antibodies  
181 against rbPs. Bouin-fixed tissues cut in sections of 8 µm were deparaffinised,  
182 rehydrated, antigen was demasked by boiling the sections in 10 mM citrate buffer  
183 pH 6.0 in a microwave oven, blocked with goat serum, and subjected to the  
184 following protocol: incubation with rabbit anti-psoriasin antiserum (1:3000 in

185 Tris-buffered saline (TBS): 10 mM Tris-HCl, 15.4 mM NaCl pH 7.4) over night  
186 at 4 °C, elimination of endogenous peroxidase activity by treating with 3% H<sub>2</sub>O<sub>2</sub>  
187 in TBS for 30 min at 20 °C, incubation with goat anti-rabbit IgG peroxidase  
188 conjugate (1:800 in TBS, Southern Biotech, Birmingham, UK) for 90 min at 20  
189 °C, staining with 3-amino-9-ethylcarbazole (Biozol, Eching, Germany) as  
190 chromogen. Sections were counterstained in Mayer`s Haematoxylin and mounted  
191 with Kaiser`s Glycerol gelantine (Merck, Darmstadt, Germany). For negative  
192 controls, the primary antibody was replaced with pre-immune rabbit antiserum in  
193 the same dilution (1:3000 in TBS).

194

### 195 **3. Results**

#### 196 **Production of anti psoriasin antibodies**

197 Polyclonal antisera against bovine psoriasin were obtained from rabbits after  
198 immunisation with purified rbPs as immunogen. These antisera were tested to  
199 detect psoriasin in bovine udder tissue samples by immunoblotting.  
200 Simultaneously tested pre-immunisation sera failed to detect bovine psoriasin by  
201 immunoblotting, whereas the psoriasin antisera in an appropriate dilution  
202 delivered a specific signal about 11 kDa. Due to the specificity of the obtained  
203 signal, further purification steps of the antisera were not performed.

204

#### 205 **Intramammary *E. coli* infection and mastitis of dairy cows**

206 We consistently observed that as little as 500 CFU, administered in 2 ml of sterile  
207 saline was enough to elicit a strong cellular response in the inoculated udder

208 quarter within 12 h. Major clinical symptoms, such as udder swelling and elevated  
209 counts of somatic cells in milk were restricted to pathogen inoculated udder  
210 quarters. All animals inoculated with the *E. coli* 1303 suffered from acute mastitis  
211 within 12 h post inoculation. Prior to receiving the inoculum dose, the udder  
212 secretions of all animals have repeatedly been tested negative for major and minor  
213 mastitis pathogens. Quarter milk samples from the animals challenged with *E. coli*  
214 were positive for *E. coli* bacteria at every milking after inoculation whereas  
215 control quarters and all quarters of control animals remained bacteriologically  
216 negative during the observation period.

#### 217 **Body temperature, milk yield, differential blood cell numbers**

218 All cows inoculated with *E. coli* 1303 developed fever ( $>39.2^{\circ}\text{C}$ ) within the first  
219 14 h after infection with maximal values ( $41.1 \pm 0.2^{\circ}\text{C}$ ) between 15 h and 18 h  
220 after inoculation. This was accompanied by a significant leukopenia. Leukopenia  
221 reached its maximum 12 h after pathogen inoculation. Milk yield dropped  
222 increasingly within the 24 h period in all udder quarters of infected cows.

#### 223 **Psoriasin expression in the bovine udder in *E. coli* -infected versus non- 224 infected dairy cows**

225 We examined whether and in which location psoriasin is expressed in the  
226 mammary gland. Western Blot analysis of the teat skin revealed that psoriasin is  
227 expressed in healthy dairy cows as well as in *E. coli*-infected cows (Fig. 1 a). No  
228 psoriasin expression was detectable in the udder parenchyma (Fig. 1 b), neither in  
229 the healthy nor in the infected cows. In the epithelium of the teat cistern, psoriasin  
230 expression is visible only in protein extracts from *E. coli*-infected cows, but not in

231 the healthy controls (Fig. 1 c). Equal loading of every SDS-PAGE was compared  
232 after stripping the PVDF membrane and re-probing the Western blot with an  
233 antibody against beta-actin.

234 Results of Western Blot analysis were verified by performing  
235 immunohistochemistry (Fig. 2), psoriasin expression could not be detected in udder  
236 parenchyma, it was abundantly expressed on teat skin, and expression was up-  
237 regulated in teat cistern epithelium in *E. coli* infected cows in contrast to healthy  
238 cows.

#### 239 **Psoriasin expression in milk samples in *E. coli* -infected versus non-infected** 240 **dairy cows**

241 Psoriasin as a secreted protein was detectable by Western blotting only in milk  
242 samples from *E. coli* -infected dairy cows (24 h after infection), there only in the  
243 inoculated udder quarter, but neither in milk from non-inoculated quarters of  
244 infected animals nor in milk of healthy control animals (data not shown).

245

#### 246 **4. Discussion**

247 Little is known to date about the physiological role of psoriasin in cattle and  
248 particularly in the udder. Other antimicrobial peptides such as beta defensins are  
249 suggested to play a role in local host defence during udder infections and to  
250 counteract mastitis (Roosen et al., 2004). In antimicrobial susceptibility assays,  
251 the inhibitory effect of bovine psoriasin was restricted to *E. coli* strains  
252 (Regenhard et al., 2009). Due to the fact that human psoriasin protects not only  
253 affected, but also healthy human skin from *E. coli* infection (Gläser et al., 2005),

254 and *E. coli* is ubiquitous in the cow's environment, the question was whether an  
255 infection with *E. coli* will induce the expression of psoriasin or, alternatively the  
256 expression in the udder might be constitutive.

257 In addition to its expression in healthy skin, expression of human psoriasin is  
258 described in many other locations. The expression of psoriasin is not limited to  
259 psoriatic skin, it is also expressed in many other altered epithelial cells like  
260 carcinomatous tissues from skin (Alowami et al., 2003), bladder (Celis et al.,  
261 1996, Ostergaard et al., 1999), stomach (El-Rifai et al., 2002), and breast in  
262 humans (Moog-Lutz et al., 1995, Leygue et al., 1996, Al-Haddad et al., 1999,  
263 Enerbäck et al., 2002, Emberley et al., 2003, 2004). Much less is known about the  
264 presence of bovine psoriasin in different tissues. Like human psoriasin, it is found  
265 in the amniotic fluid (Hitomi et al., 1996) and in the skin (Rautianen et al., 1995).  
266 Antimicrobial peptides are localised in specific cells or tissue types in the body  
267 most susceptible to infection, and the udder with its galactogenic infection  
268 pathway is such a location. This is the first report that bovine psoriasin protein is  
269 expressed in the mammary gland and therefore might be a host defence protein  
270 and a part of innate immunity. In udder skin epithelial cells, psoriasin seems to be  
271 expressed due to the contact with *E. coli* in the environment, e. g. the faeces. The  
272 teat canal establishes a border between the environment and the teat cistern and  
273 parenchyma of the udder. No psoriasin expression could be detected in healthy  
274 dairy cows in these locations. In contrast, psoriasin could be detected 24 h after  
275 intracisternal *E. coli* infection in the epithelium of the teat cistern. Therefore,  
276 psoriasin expression was induced by infection with *E. coli* in the teat cistern

277 epithelium. In the parenchyma after 24 h of infection, no psoriasin expression  
278 could be demonstrated, neither in the healthy control nor in *E. coli* infected cows.  
279 Psoriasin expression might be detectable in alveolar tissue after challenges of  
280 more than 24 h, however, parenchyma might indeed rather be excluded as  
281 psoriasin expressing tissue. In human breast cancer, psoriasin is highly and more  
282 frequently expressed in those cancer forms in which the cells concerned have their  
283 origin in milk ducts, not in alveolar cells (Leygue et al., 1996). Psoriasin is  
284 expressed in epithelial cells, and we speculate that the differentiated alveolar cells  
285 in mammary gland parenchyma are restrained from expressing antimicrobial  
286 peptides. Via the galactogenic infection pathway, the teat cistern epithelium is the  
287 first tissue exposed to the pathogenic microorganism and therefore the first line of  
288 defence. Milk ducts were not specifically investigated herein therefore it remains  
289 unknown whether their epithelia are able to express psoriasin and thus continue  
290 the line of defence.

291 In spite of absence of a signal sequence at its amino terminal, psoriasin is known  
292 to be a secreted protein (Madsen et al., 1991). Therefore, we examined whether  
293 psoriasin was secreted into the milk in infected dairy cows. The effect of infection  
294 on the presence of bioactive innate immune proteins in milk is still unclear.  
295 Psoriasin was only detectable in milk samples 24 h after infection with *E. coli*,  
296 only in the inoculated udder quarter. Lutzow et al. (2008) found increased levels  
297 of the S100 calcium-binding protein A12 (S100A12) in milk from dairy cows  
298 challenged with *S. aureus*. S100A12 and S100 A7 belong to the same family of  
299 calcium binding proteins, and Lutzow et al. (2008) were able to demonstrate that

300 recombinant S100A12 also inhibited growth of *E. coli* in vitro. Using a proteomic  
301 approach, Smolenski et al. (2007) identified three members of the S100 calcium-  
302 binding protein family (S100A9, S100A11, and S100A12) only in milk from a  
303 mastitic cow, but not in milk from peak lactation or colostrum. Therefore, the  
304 presence of bioactive innate immune proteins in milk may determine the  
305 susceptibility of the mammary gland and its ability to resolve the infection.  
306 The mode of action even of human psoriasin still remains unclear. Zn<sup>(2+)</sup>-saturated  
307 psoriasin showed diminished antimicrobial activity, suggesting that Zn<sup>(2+)</sup>  
308 sequestration could be a possible antimicrobial mechanism (Gläser et al., 2005).  
309 Thus, psoriasin may be key to the resistance of skin against *E. coli*. Antimicrobial  
310 peptides or proteins may have a direct effect on the microbe, such as by damaging  
311 or destabilising the bacterial membrane or acting on other targets. Regardless of  
312 their actual target of action, all antibacterial peptides must first interact with the  
313 bacterial cytoplasmic membrane. The antimicrobial peptides interact with the  
314 negatively charged phospholipids head groups on the outer surface of bacterial  
315 membranes via electrostatic and hydrophobic interactions. In consequence, the  
316 development of resistance against antimicrobial peptides is less probable (Zasloff  
317 et al., 2002).

318

319

320

## 321 **5. Conclusion**

322 Dairy cattle with clinical mastitis caused by *E. coli* exhibit a wide range of disease  
323 severity. A high degree of genotypic variability is characteristic for *E. coli* strains  
324 and the *E. coli* strains differ in their serotype and virulence. However, Wenz et al.  
325 (2006) were unable to find any correlation between *E. coli* virulence genes and  
326 systemic disease severity. They concluded that specific cow factors might play a  
327 more important role in determining disease severity. Psoriasin expressed in the  
328 mammary gland as part of the local host defence mechanism and with its  
329 antibacterial activity is a new putative candidate for such an additional cow-  
330 specific factor influencing mastitis susceptibility. Due to its inducibility, bovine  
331 psoriasin as an evolutionarily ancient component of host defence is an interesting  
332 target for immune modulation in the udder, and it may be a possible alternative to  
333 conventional antibiotics.

334

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466 **Figure captions**467 **Figure 1:**

468 Western Blot analysis: Detection of psoriasin in teat skin (a), udder parenchyma (b), and the  
469 epithelium of the teat cistern (c) in *E. coli*-infected versus non-infected dairy cows. PC=  
470 Positive Control (rbPs), GS = healthy control animal (Gold Standard), *E. coli* = over 24 h with  
471 *E. coli*-infected dairy cows. Relative loading was compared after stripping with an antibody to  
472 beta-actin.

473

474 **Figure 2:**

475 Psoriasin expression in the udder depends from location and infection status: The location  
476 was assessed with a polyclonal antibody against bovine psoriasin. Figure A, B teat skin,  
477 Figure C, D udder parenchyma, Figure E, F teat cistern epithelium. Figures on the left side  
478 (A, C, and E) are showing healthy control animals, figures on the right side (B, D, and F)  
479 *E. coli* infected animals. The small picture in each left corner is showing the corresponding  
480 negative control stained with pre-immune serum. On teat skin, psoriasin was detectable in  
481 non-infected and infected animals (A and B). Detection of psoriasin expression failed in udder  
482 parenchyma in both groups (C and D). The epithelium of the teat cistern shows positive  
483 staining only in the infected cow (F) in contrast to the control animal (E). Scale bars 50  $\mu$ m.

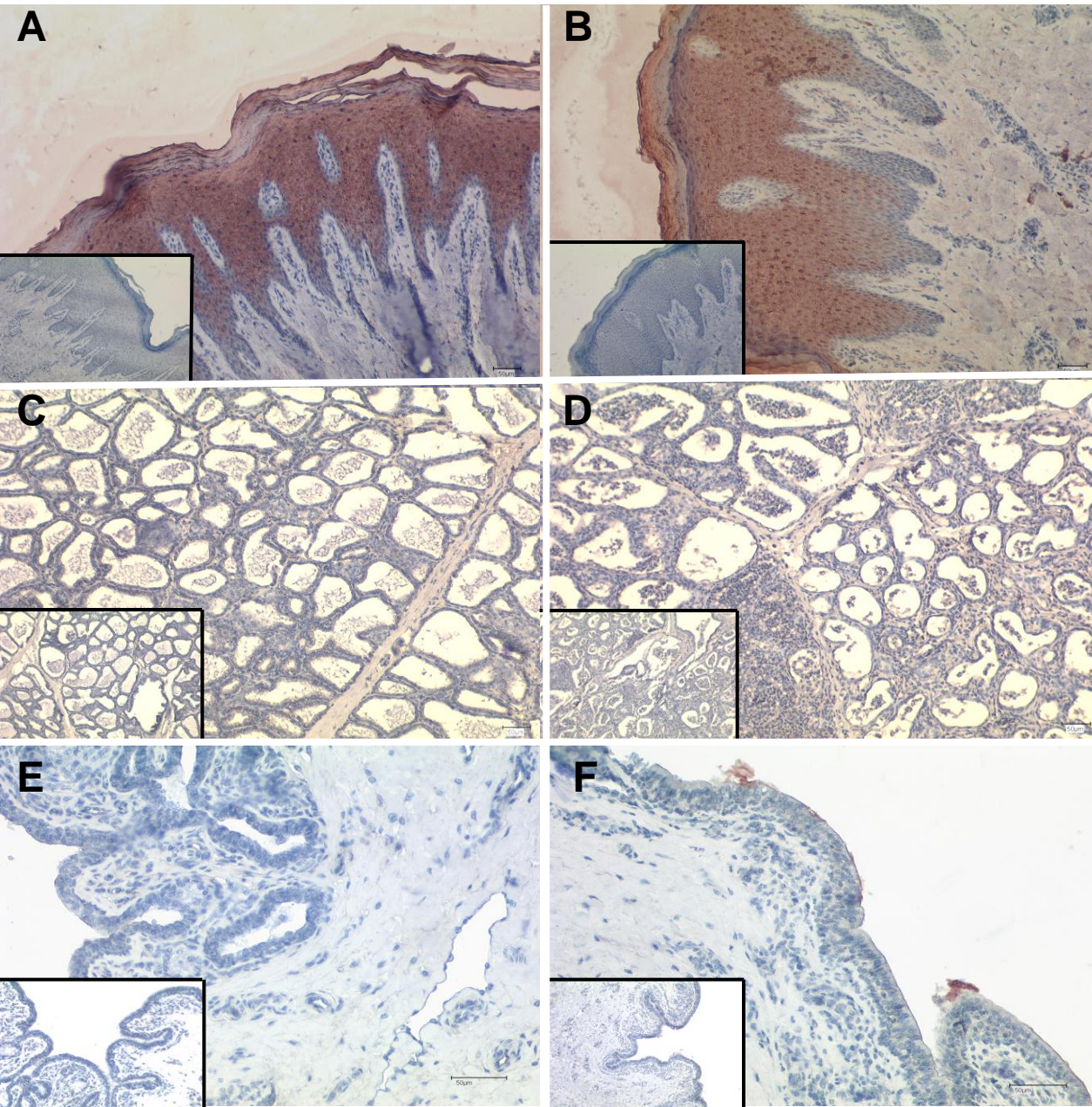


Figure 2