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Revised version

## Isolation of Spirochetes of Genus *Treponema* from Pigs with Ear Necrosis

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“2002” has been added to Stamm et al.

The recently published reference has been updated to:

Stamm, L.V., Walker, R.L., Read, D.H., 2008. Genetic diversity of bovine ulcerative mammary dermatitis-associated *Treponema*. *Vet. Microbiol.* 136, 192-196.

It is not clear to us were the “Walker et al. 1995” reference should be included in the text. We have not used the same method for culturing and all DD DNA sequences mentioned in the manuscript are from later publications.

Accepted Manuscript

## 1 **Abstract**

2 Various ear lesions, often caused by ear biting, are common in pigs. Some herds  
3 have a high frequency of ear necrosis, a syndrome characterized by necrotic  
4 lesions along the rim of the pinna, often bilateral and sometimes resulting in loss  
5 of the entire ear. In samples from such lesions spirochetes have been observed  
6 microscopically but never isolated or identified. In this study two herds with  
7 periodic outbreaks of ear necrosis among weaners were investigated. Samples  
8 were collected from ear lesions and from the gingiva of the pigs. Spirochetes were  
9 observed in silver stained histological sections and by phase contrast microscope  
10 in scrapings from the necrotic lesions. From an ear lesion a pure spirochete isolate  
11 was obtained and identified as a yet unnamed species of genus *Treponema*,  
12 closely related to spirochetes found in digital dermatitis in cattle. From the oral  
13 samples two pure isolates were obtained. One of these isolates was identified as  
14 the same species as in the ear lesion and one as *Treponema socranskii*. Species  
15 identification was based on 16S rRNA gene sequences.

16

17 **Keywords:** Ear necrosis; Ear biting; *Treponema*; Spirochete; Pig

18

## 19 **1. Introduction**

20

21 Ear necrosis in pigs is a scantily studied malady. Other names used are necrotic  
22 ear syndrome and ulcerative spirochetosis of the ear (Cameron, 2006). In a case  
23 study (Harcourt, 1973) the lesions were reported to appear at around 8-9 weeks of

24 age with the presence of bilateral small reddened areas close to the junction of the  
25 lower margin of the ear with the neck. The lesions then progressed to necrosis and  
26 in severe cases involved the entire length of the margin of the pinna. On  
27 histopathological examination numerous spirochetes were found together with  
28 coccoid bacteria. In several other skin disorders of pigs such as facial necrosis in  
29 suckling pigs, wound infections and foot-rot spirochetes have been observed since  
30 the early 1900's (Cleland, 1908; Dodd, 1906; Gilruth, 1910; Hindmarsh, 1937;  
31 Neitz and Canham, 1930; Osborne and Ensor, 1955). Coccoid bacteria found in  
32 ear necrosis lesions in pigs have been identified as *Staphylococcus hyicus* and  
33 beta-hemolytic streptococci (Cameron, 2006). In a recent large-scale study of the  
34 prevalence of clinical signs in Danish finisher pigs, ear necrosis was the most  
35 common finding. About 30% of the recorded clinical signs were ear necrosis,  
36 defined as: open wound(s) or crust(s) at the tip or base of one or both ears  
37 (Petersen et al., 2008). Ear lesions defined as ear biting were also a common  
38 clinical observation (28.3%) in a study from the Netherlands (Blocks et al., 1994).  
39  
40 To the best of our knowledge the spirochetes observed in smears and histological  
41 sections from various skin diseases in pigs have never been isolated. Species  
42 names as *Borrelia suilla* and *Borrelia suis* have been used, however without  
43 references to an original source (Cameron, 2006; Penny et al., 1971).  
44  
45 In this study, two pig herds with periodic outbreaks of ear necrosis among  
46 weaners were investigated. The aims were to isolate and identify spirochetes and

47 spirochetal DNA from the necrotic ear lesions, and because ear biting has been  
48 discussed as a cause of ear necrosis, from the gingiva of the pigs.

49

## 50 **2. Materials and methods**

51

### 52 *2.1. Pig herds investigated and clinical signs*

53

54 Ear ulcers were observed at several occasions during two years in two organic pig  
55 herds, A and B, in Sweden. Herd A was a piglet producing herd with 180 sows  
56 employing batch production (~24 sows/batch) with all in-all out procedures  
57 practiced in all units except for the dry sow unit. The farrowing unit had  
58 individual pens with partially slatted floors. When the piglets were two weeks old,  
59 sows and piglets were moved to a barn with four group pens (12 sows +  
60 piglets/pen) with deep straw bedding and access to a small outdoor surface. Pigs  
61 did not have direct contact to herd mates in adjacent pens but the airspace was  
62 shared for two pens. Pigs were weaned at 6 weeks of age, fed with dry feed with  
63 constant access ad libitum, and kept in the group pens until sale. Batches of pigs  
64 were sold at approximately 30 kilos of weight to the finishing herd B. The first  
65 signs of ear ulcers were noted right before or after weaning, although bites or  
66 scratches on the pigs' ears were minimal. Ulcers were bilateral and situated close  
67 to the junction of the lower margin of the ear. Over the course of several weeks,  
68 ulcers increased in size and spread along the rim of the ear and became necrotic.  
69 In the most advanced cases sloughing of the necrotic tissue occurred, resulting in

70 a total loss of the ear. During the two year period the number of pigs with ear  
71 ulcers in each group varied, with the highest prevalence observed during the  
72 winter period, when 50-70% affected pigs were commonly noted. Pigs were not  
73 medically treated due to ear necrosis. General health and growth rate were  
74 seemingly unaffected. At 60-80 kilos of weight the lesions had healed, leaving  
75 affected ears with a normal appearance or a portion of the ear missing.

76

### 77 *2.2. Histology*

78

79 In three pigs from herd A sent for necropsy, bilateral lesions at the ear base and  
80 the ear pinna were observed. Samples for histology were taken from the margin of  
81 the lesions. After fixation and embedding in paraffin by standard methods 5-7 µm  
82 sections were cut for hematoxylin and eosin (HE) and Warthin-Starry silver  
83 staining.

84

### 85 *2.3. Sampling for bacteriological cultures and culture conditions*

86

87 Samples were collected from live pigs after cleaning the ear lesions with water of  
88 drinking quality. Scalpel blade scrapings were taken from the margins of the  
89 lesions. The scrapings were transported in tubes with FAB (fastidious anaerobe  
90 broth, LAB 71, LabM, Lancashire, UK). From the gingiva, cotton swab samples  
91 were taken and transported in Amies medium with charcoal (COPAN, Brescia,  
92 Italy). The samples were transported directly to the lab for culturing. Four ear

93 lesion samples were taken from both herds and four gingival samples from herd  
94 A. Flasks with FABSA (FAB with 25% fetal calf serum, S 0115, Biochrom AG,  
95 Germany; 10% rabbit serum, R4505 Sigma, Sigma-Aldrich Sweden AB; 10 µg/ml  
96 rifampicin Sigma, Sigma-Aldrich Sweden AB, and 10 µg/ml enrofloxacin Fluka,  
97 Sigma-Aldrich Sweden AB,) and OTEBSA (oral treponeme enrichment broth  
98 (OTEB), Anaerobe Systems, Morgan Hill, CA, USA 25% fetal calf serum, S  
99 0115, Biochrom AG, Germany; 10% rabbit serum, R4505 Sigma, Sigma-Aldrich  
100 Sweden AB; 10 µg/ml rifampicin Sigma, Sigma-Aldrich Sweden AB, and 10  
101 µg/ml enrofloxacin Fluka, Sigma-Aldrich Sweden AB) were inoculated with  
102 scrapings and swabs and incubated in 37 °C, in anaerobic jars on a shaker (90  
103 rpm). The samples from herd A were inoculated only in FABSA and the samples  
104 from herd B in both FABSA and OTEBSA. The heavily contaminated gingival  
105 cultures were purified through a membrane filter with pore size 0,22 µm. The  
106 filter was placed on FAA plates (fastidious anaerobe agar with 10% horse blood,  
107 National Veterinary Institute, Uppsala, Sweden) and inoculated with a drop of  
108 broth culture. The filter was removed after 2-3 days and the growth under the  
109 filter was checked through phase contrast microscopy.

110

#### 111 *2.4. Amplification and sequencing of 16S rDNA and ISR2*

112

113 DNA was prepared from the broth cultures and from three ear lesion scrapings  
114 from herd B. The material was washed twice in PBS (phosphate buffered saline,  
115 pH 7.3), boiled for 10 min in sterile water and cell debris removed by

116 centrifugation. Primers trepF 5'-GTTTGATCCTGGCTCAGAACG-3' and trepR  
117 5'-CGGTACGGCTACCTTGTTACG-3' were designed for amplification and  
118 DNA sequencing of the 16S rRNA gene. Internal primers used for sequencing  
119 have been described previously (Johansson et al., 2004). For amplification of the  
120 16S-tRNA<sup>Ile</sup> region of the intergenic spacer region 2 (ISR2) a protocol developed  
121 by Stamm et al. was followed (Stamm et al., 2002). The sequence from the ISR2  
122 PCR was determined using the PCR primers as sequencing primers. Amplified  
123 fragments were purified (illustra GFX PCR DNA and Gel Band Purification Kit,  
124 GE Healthcare) and sequenced on an ABI PRISM 3100 Genetic Analyzer  
125 (Applied Biosystems). Sequences were edited using ContigExpress, Vector NTI  
126 Advance 10 (Invitrogen).

127

### 128 **3. Results**

129

#### 130 *3.1. Gross pathology*

131

132 In all three pigs from herd A sent for necropsy, bilateral chronic active necrotizing  
133 dermatitis situated close to the junction of the lower margin of the ear pinna and  
134 ear bases was observed. The lesions consisted of irregular bleeding margins at the  
135 surface, a layer of necrosis and underlying this, irregular zones showing different  
136 degrees of blackish grey and red discoloration. The crusts were flat to thick,  
137 irregular, and slightly raised above the epidermis. Both ears were thickened and  
138 the lesions were frequently associated with mild edema and erythema.

139

140 *3.2. Histology*

141

142 The main histological features were of chronic ulcerative and superficial pustular  
143 dermatitis with severe acanthosis and hyperkeratosis (Fig. 1). Adjacent to the  
144 ulcers the surviving epidermis was hyperplastic with numerous rete ridges  
145 extending deep into the dermis. The epidermis was covered by a thick layer of  
146 crust consisting of necrotic epidermal cells, degenerated inflammatory cells, and  
147 proteinaceous exudates. Inflammatory changes were a prominent part of the  
148 overall reaction and neutrophils formed a main proportion of the cells involved.  
149 There was vasculitis in the arterioles and venules characterized by hyaline  
150 degeneration, medial hyperplasia and thrombosis.

151

152 Silver stained sections from all three pigs showed numerous spirochetes, often  
153 aligned in bundles within bands of connective tissue at the junction of the necrotic  
154 and granulation tissues (Fig. 2). Coccoid or cocco-bacillary bacteria were  
155 frequently seen both within the thick layer of exudates on the surface of early  
156 lesions and deep within the tissues of the more severely ulcerated lesions.

157

158 *3.3. Isolation of spirochetes*

159

160 From herd B spirochetes were observed through phase contrast microscopy in two  
161 of four scrapings and one of these samples resulted in a pure isolate of spirochetes

162 designated as T A4. In one of four scrapings from herd A, a low number of  
163 spirochetes were observed through phase contrast microscopy. It was not possible  
164 to obtain a spirochete culture from this sample. Isolation of T A4 was made in  
165 OTEBSA but growth of the pure isolate was supported equally well by FAB with  
166 fetal calf serum and after subculture a fully grown culture was obtained in 4-5  
167 days. From the four gingival samples obtained from herd A two spirochete  
168 isolates, designated T M1 and T M4, were purified through filter on agar and  
169 colony material was transferred as agar cylinders, with a pasteur pipette, to new  
170 broth cultures. On FAA plates all three isolates formed pinpoint colonies. Isolates  
171 T M1 and T A4 had distinct hemolysis zones but no hemolysis was observed for T  
172 M4.

#### 174 3.4. 16S rRNA gene sequences from cultures

175  
176 A 16S rRNA gene fragment of 1466 nucleotides was obtained for T A4 and T M1  
177 and of 1455 nucleotides for T M4 (deposited in GenBank with accession numbers  
178 FJ805835-FJ805837). The sequences for T A4 and T M1 were identical except for  
179 two positions (one in each isolate) that had polymorphic nucleotide positions,  
180 134<sub>R</sub> in T A4 and 199<sub>Y</sub> in T M1 (*Escherichia coli* numbering). These two  
181 positions were polymorphic with two nucleotides in 50% representation and were  
182 present in sequences from both strands. Except for the polymorphism the 16S  
183 rRNA gene sequences for T A4 and T M1 were also identical to a deposited  
184 sequence for a yet unnamed *Treponema* sp. (GenBank accession number

185 EF061268) isolated from digital dermatitis in cattle in the UK (Evans et al., 2008).  
186 The T M4 isolate was most closely related to *Treponema socranskii* subsp.  
187 *paredis* (GenBank accession number AY369254) with 1451 identical nucleotides  
188 out of 1455 (Correia et al., 2003).

189

### 190 3.5. ISR2 sequence from tissue scraping

191

192 For two of the three DNA preparations from the lesion scrapings from herd B the  
193 ISR2 PCR resulted in a single band at the size of approximately 300 bp (A3 and  
194 A4). The third sample was negative. The amount of product was very low in  
195 sample A3 but the product from sample A4, which was the sample that also  
196 yielded a spirochete culture, was purified and sequenced. The chromatogram  
197 showed a clearly dominating sequence but with traces of contaminating  
198 nucleotides. ISR fragments from *Treponema* from digital dermatitis, isolate 9-  
199 3143 (AF179259), and ulcerative mammary dermatitis, clone C1BT2-3  
200 (AY342044), in cattle in USA were the closest related deposited sequences in  
201 GenBank (Stamm et al., 2002; Stamm et al., 2008). When the sequence from the  
202 present study was compared to the AF179259 sequence 313 out of 317  
203 nucleotides were identical and for AY342044, which is shorter, 205 out of 209  
204 were identical. Both comparisons covered the highly variable region between 16S  
205 rRNA and tRNA-Ile.

206

## 207 4. Discussion

208

209 While production losses associated with ear necrosis have not been documented  
210 the condition should be considered an animal welfare problem. In this study  
211 samples from necrotic ear lesions were investigated regarding the presence of  
212 spirochetes. Spirochetes were observed in ear lesion samples from both of the  
213 studied herds. One *Treponema* sp. isolate was obtained from herd B and silver  
214 impregnated histological sections from herd A showed spirochetes in the  
215 epidermis. Besides that *Treponema* spp. are fastidious and difficult to culture in  
216 competition with fast growing bacteria in the samples, other reasons for only  
217 obtaining one isolate could be that scrapings may be too superficial or that the  
218 spirochetes were unevenly distributed in the lesions. Biopsy samples from the  
219 margin of the lesions would probably increase the chance of isolating spirochetes.  
220 The ISR2 sequence directly from a tissue sample from a pig in herd B was  
221 obtained with primers developed to be universal for the genus *Treponema* (Stamm  
222 et al., 2002). ISR2 amplicons from digital dermatitis samples from cattle claws  
223 usually contain fragments from several species of *Treponema* and need to be  
224 cloned before sequencing. However from the sample from herd B it was possible  
225 to obtain a readable DNA sequence without cloning, indicating that a single  
226 species was dominating. Only a limited number of ISR2 sequences from  
227 *Treponema* spp. are deposited in GenBank. However two of these, from skin  
228 diseases in cattle, shared a high similarity to the sequence from the ear lesion,  
229 especially considering that intergenic spacer region between 16S rRNA and  
230 tRNA-Ile is highly variable. There are two deposited 16S rRNA sequences from

231 the digital dermatitis isolate from USA, 9-3143, clone 5 and clone 7 (AF109070-  
232 71) and these belong to the same 16S rRNA phylotype as the digital dermatitis  
233 isolate from the UK (EF061268) and T A4. This indicates that the dominating  
234 phylotype in the ear lesion of one pig in this study also is the isolated.

235

236 In addition to the spirochetes, numerous cocci could be seen both through direct  
237 microscopy of the samples and in the histological sections. *Staphylococcus hyicus*  
238 and beta-hemolytic streptococci were identified in the samples through  
239 conventional methods (data not shown).

240

241 Ear biting is commonly suggested as the cause of ear necrosis in pigs. In the  
242 investigated herds sometimes as much as 70% of the pigs were affected, and as in  
243 the herd described by Harcourt, the lesions were always bilateral and pigs of a  
244 similar age involved (Harcourt, 1973). The anatomically consistent localization  
245 and the epidemic spread in the herd followed by healing are in our view difficult  
246 to explain solely by opportunistic bacterial infection. Further the *ad libitum*  
247 feeding regime practiced in herd A should have minimized the risk for ear biting.

248

249 Several species of genus *Treponema* have been isolated from the mouth of  
250 humans, cats and dogs and are associated with periodontal disease (Holt and  
251 Ebersole, 2006; Nordhoff et al., 2008; Riviere et al., 1996; Valdez et al., 2000).

252 The spirochetal flora in the mouth of the pig has not been investigated before. In  
253 this study the 16S rRNA gene sequences from two gingival isolates showed that

254 the pig oral flora can include *T. socranskii* and the *Treponema* sp. found in the ear  
255 lesion. As for the oral flora of dogs and humans it is likely that analyzing many  
256 oral samples from pigs would reveal several other *Treponema* spp. Isolates T M1  
257 and T A4 are obviously not the same clone since both have a polymorphism in the  
258 16S rRNA gene but at different positions. Despite this, the findings of the same  
259 phylotype at both sampling sites indicate a possible relationship between ear  
260 necrosis and oral spirochetes in pigs. Additionally, the 16S rRNA gene sequences  
261 of T A4 and T M1 were identical (except for the polymorphisms) to the sequence  
262 deposited for an isolate from digital dermatitis in cattle (Evans et al., 2008) and  
263 moreover, only differed five nucleotides from an isolate from ovine foot rot  
264 (GenBank accession number AF363634) (Demirkan et al., 2001). This yet  
265 unnamed phylotype of *Treponema* occurs in different skin disorders in three  
266 different animal species and in the oral flora of the pig, suggesting both a  
267 pathogenic potential and a broad host range.

268

269 Spirochetes have been observed in other different skin lesions in pigs for example  
270 in foot-rot, castration wounds and ulcers in the mouth (Blandford et al., 1972;  
271 Gilruth, 1910; Osborne and Ensor, 1955). Isolation and identification of such  
272 spirochetes is of interest both for comparison between the different conditions in  
273 pigs and for comparison with skin diseases caused by *Treponema* spp. in other  
274 animals including humans.

275

276 **5. Conclusion**

277

278 Spirochetes of genus *Treponema* can be isolated from ear necrosis lesions and  
279 from the gingiva in pigs. The *Treponema* phylotype isolated from the ear lesion  
280 was closely related, but not identical, to one of the oral isolates. Increased  
281 understanding of the role of the spirochetes in porcine skin disorders and the route  
282 of infection would be helpful both for choice of treatment of acute lesions and to  
283 be able to recommend preventive measures.

284

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382

### 383 **Figure legends**

384

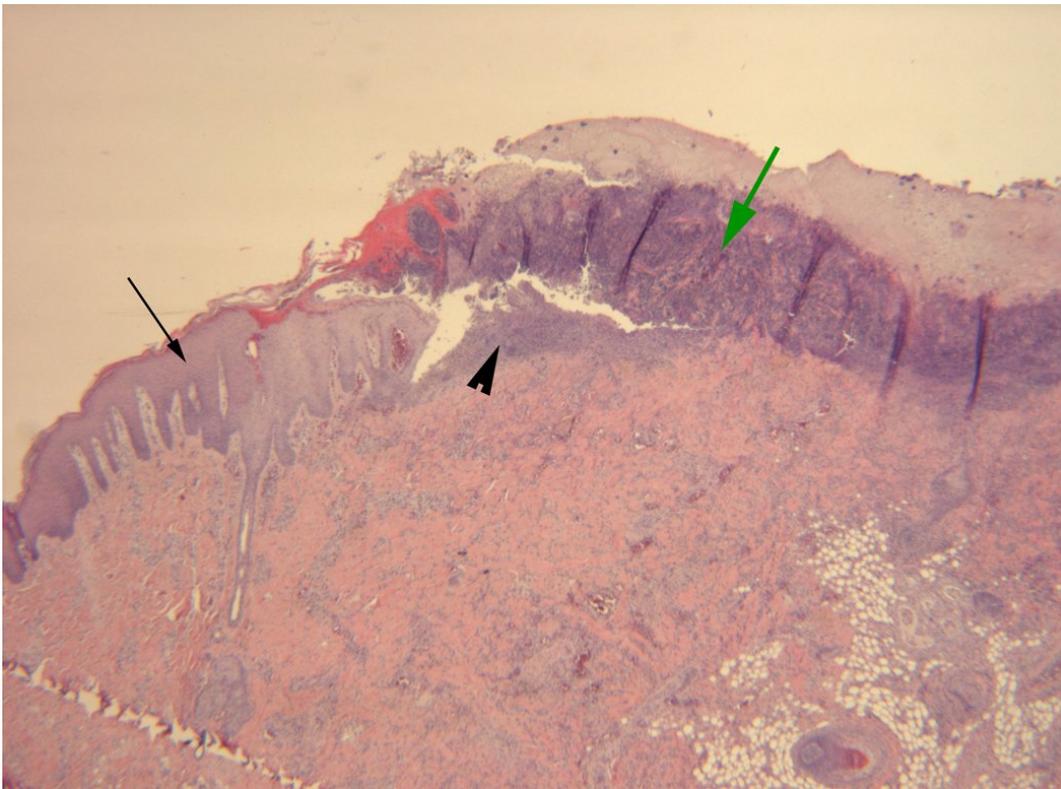
385 **Fig. 1.** Histological section of an ear necrosis lesion from herd A. The epidermis  
386 is covered by thick crusts (green arrow). Below the crusts there is an ulcer with  
387 abundant degenerated inflammatory cells (arrow head). Epidermis shows severe  
388 thickening (acanthosis) with rete ridges (black arrow). Hematoxylin and eosin;  
389 (original magnification 10 X 0.63 X 25).

390

391 **Fig. 2.** Silver impregnated sections of an ear necrosis lesion from herd A.  
392 Numerous spirochetes are visualized at the junction of the necrotic and  
393 granulation tissues. The arrows show bundles of spirochetes aligned with  
394 connective tissue. Warthin-Starry; (original magnification 10 X 100).  
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1 Figure 1.



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14 Figure 2.

