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1 **Association of *Treponema spp.* with canine periodontitis**

2

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30 **Abstract**

31 To evaluate the association of oral *Treponema* (*T.*) spp. with severity of canine periodontitis,
32 subgingival plaque samples of dogs of various breeds undergoing surgery were investigated.

33 A wide range of oral *Treponema* spp. was analysed by a molecular and culture independent
34 approach applying DNA-DNA dot blot hybridization analysis and fluorescence in situ
35 hybridization using *Treponema* specific oligonucleotide probes specific for phylogenetic
36 groups I to VII of oral treponemes as well as probes specific for *T. socranskii* and *T.*
37 *denticola*.

38 To assess the periodontal status of affected dogs clinical parameters were measured and the
39 periodontal status was classified from grade 0 (physiological periodont) to 3 (severe
40 periodontitis). The periodontal status correlated significantly with an increasing concentration
41 of volatile sulphur compounds (VSC, $r = 0,854$) determined with a Halimeter, indicating a
42 positive correlation between the presence of VSC-producing bacteria and periodontitis.

43 In this study *Treponema* spp. of phylogenetic groups III, V, VI, and VII were not detected in
44 any sample, whereas *T. denticola*-like treponemes were found only in 2 of 51 animals.
45 However, treponemes belonging to phylogenetic groups I, II and IV of oral treponemes or
46 *T. socranskii* were found in up to 64.84% of the dogs. The detection rate of *Treponema* spp.
47 was significantly associated with an increased periodontal status. Treponemes present in
48 periodontal lesions were also visualized by fluorescence in situ hybridization of gingival
49 biopsies showing *Treponema* spp. not only in the microbial biofilm but also within the
50 gingival tissue.

51 The data presented here indicate that oral *Treponema* spp. are associated with canine
52 periodontitis. Similar to human periodontitis, treponemes of groups I, II, IV and *T. socranskii*
53 were found more frequently the higher the degree of periodontitis was.

54

55 **Key words:** canine periodontitis, *Treponema* spp., VSC, Halimeter

56 **Introduction**

57 Periodontal disease is a common problem in pets, especially in dogs, and in the past, various
58 medically important bacterial pathogens have been described in these animals. Most of these
59 studies have been performed in dogs utilizing culture-based methodology (Allaker et al.,
60 1997; Elliott et al., 2005; Hardham et al., 2005; Hennes and Harvey, 1991). In general, these
61 studies assumed that bacterial pathogens in dogs are identical or very similar to those found in
62 human patients (Riviere et al., 1996; Valdez et al., 2000). Besides several well-known gram-
63 negative anaerobic genera like *Prevotella*, *Tannerella*, and *Porphyromonas*, the genus
64 *Treponema* gained much interest, as it is of particular importance in humans (Chan and
65 McLaughlin, 2000; Moter et al., 2006; Rocas et al., 2001; Sela, 2001). In recent studies,
66 treponemes similar to *T. denticola*, *T. socranskii*, *T. vincentii*, *T. maltophilum*, *T. medium* and
67 *T. pectinovorum* have been reported in dogs and cats (Riviere et al., 1996; Valdez et al.,
68 2000). Treponemes are particularly difficult to cultivate or even as yet-uncultured, therefore
69 cultivation results only partly reflect the occurrence and diversity of treponemes in particular
70 settings (Choi et al., 1994). Therefore during the last decade, non culture-based methods have
71 been developed to get a deeper understanding of ecological niches like the oral cavity
72 (Sakamoto et al., 2005). In pets, so far no attempts were made to systematically investigate
73 the occurrence of *Treponema* spp. in periodontal disease, utilizing non culture-based
74 methodology to detect single phylogenetic groups of these pathogens.

75 The aim of the present study was therefore to investigate the occurrence of *Treponema* spp.
76 via 16S rRNA targeting a dog cohort undergoing surgery. Based on methodology from human
77 dentistry we defined the periodontal status of four teeth and sampled DNA from two well
78 defined sites of two teeth each. Furthermore we determined volatile sulphur compounds
79 (VSC) in an attempt to monitor other bacterial pathogens, as VSC concentrations are known
80 to be correlated with the appearance of periodontal pathogens, being able to reduce sulphate

82 The data presented here show that like in human medical patients the occurrence of
83 treponemes is associated with the degree of periodontitis. In addition, the spectrum of
84 *Treponema* spp. detected in this study seems to be basically the same as in human
85 periodontitis.

86

87 **Materials and methods**

88 **Study population**

89 The study population consisted of 101 dogs, which underwent surgery for numerous reasons.
90 All animals were withheld feed for 12 hours and received water ad libitum. Animals received
91 no antibiotic treatment for at least 3 months prior to sampling or, where treated, antibiotics
92 were administered no earlier than 12 h before examination and sample collection. The animals
93 were sedated as described previously (Kellermeier et al., 2004). Because PCR amplification
94 and therefore subsequent detection of treponemes failed in samples of 10 dogs these dogs
95 were excluded in following analyses resulting in a total number of 91 dogs.

96

97 **Clinical measurements**

98 *Halitosis assessment through VSC determination*

99 Volatile sulphur compounds (VSC) concentrations in snout air were determined using a
100 sulfide monitor (Halimeter®, Interscan RH17K, USA). This apparatus, originally constructed
101 for use in human medicine, was slightly modified to be useful for dogs by replacing the straw
102 with the middle part of a 5ml syringe. Concentrations were determined in triplicate and a
103 mean value was calculated. Measurement of VSC's was always performed in the morning.

104 In addition, halitosis was determined organoleptic by one single investigator, by lifting the
105 upper lip and smelling malus odor in a distance of approx. 2 cm. Four grades were defined, no
106 odor (0), odor, but not unpleasant (1), intensive bad odor (2), and intensive odor, even before

107

108 *Periodontal status*

109 To assess periodontal status the gingival index (GI), calculus index (CI), plaque index (PI),
110 pocket depth (PD), mobility index (MI) and furcation index (FI) were evaluated from the
111 canine tooth (tooth 204) and the fourth premolar (tooth 208) of the left maxilla as well as
112 from the first incisivus (tooth 401) and the first molar (tooth 409) of the right mandible as
113 previously described (Rateitschak et al., 2003). GI, CI, PI and PD were measured at the
114 fascial side of the teeth. MI was calculated manually by moving the tooth in orofascial
115 direction. FI was determined by probing the tooth with a furcation probe from the fascial side.
116 The periodontal status was then calculated by the mean value of the sum of the periodontal
117 indices.

118

119 **DNA extraction and amplification**

120 Samples were obtained principally as previously described (Moter et al., 1998). In brief,
121 subgingival plaque samples were obtained by probing the fascial sulci of the fourth premolar
122 of the left maxilla (tooth 208) and the first molar of the left mandible (tooth 409) with sterile
123 paper points for 10 seconds. Subsequently paper points were transferred in 450 µl PBS, pH
124 7.4. After vortexing paper points were removed and 50 µl lysis buffer (500 mM Tris-HCl, pH
125 9.0, 20 mM EDTA, 10 mM NaCl, 1% sodium dodecylsulfate [SDS]) were added and no
126 additional DNA purification was done. For PCR one microliter of bulk DNA was added to the
127 PCR reaction mixture. PCR was repeated three times at least to obtain positive amplification
128 products.

129 For PCR amplification of approximately 550 bp of 16S rRNA gene the universal primers
130 TPU-1 (5'-AGAGTTTGATCMTGGCTCAG-3', position 8-27 in *Escherichia coli* 16S rRNA
131 gene) and the universal reverse primer RTU-3 (5'-GWATTACCGCGGCKGCTG-3', position
132 518-535 in *Escherichia coli* 16S rRNA gene) were used. Amplification of the partial 16S

133 rDNA was performed using a final volume of 50 µl containing 2.5 mM MgCl₂, 0.2 mM

134 dNTPs, 25 pmol of each primer and 2,5 U *Taq* polymerase. Samples were preheated at 94 °C
135 for 5 min followed by amplification with 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s. A
136 total of 30 cycles was carried out with a final elongation step at 72 °C for 10 min. Successful
137 amplification was verified by agarose gelelectrophoresis.

138

139 **Dot blot hybridization**

140 Dot blot analyses were carried out principally as previously described (Ehmke et al., 2004;
141 Moter et al., 1998). In brief, PCR products were spotted and cross-linked on a nylon
142 membrane. Oligonucleotide probes specific for the seven phylogenetic groups of oral
143 treponemes TRE I-VII as well as probes specific for *T. socranskii* (TSOC) and *T. denticola*
144 (TDEN) were used.

145 All probes have been deposited in ProbeBase, an online resource for 16S rRNA-targeted
146 oligonucleotide probes where probe difference alignments are available (Loy et al., 2003).

147 Probes were labelled nonisotopically using the DIG labelling system (Boehringer Mannheim,
148 Germany). All hybridization steps were performed at temperatures between 54 °C and 59 °C
149 depending on the probe used. Stringency washes were optimized for each probe by varying
150 the washing buffer containing 5x SSC and 0,1 % SDS, 2x SSC and 0,1 % SDS or 0,1x SSC
151 and 0,1 % SDS and the temperatures ranging from 56 °C to 62 °C. Chemiluminescence
152 detection was performed with alkaline phosphatase labelled anti-DIG antibody and CSPD
153 according to the manufacture's instructions (Boehringer Mannheim, Germany). After
154 exposure to X-film (X-OMAT AR, Kodak, Germany) from 2 to 12 h the membrane was
155 stripped with buffer containing 0,2 M NaOH and 0,1% SDS.

156 16S rRNA amplification products of representative *Treponema* strains were used as controls
157 in dot blot analyses as listed below.

158

159

160 Fluorescence *in situ* hybridization (FISH)

161 Biopsies were fixed in PBS, pH 7.4, with 4% formaldehyde (v/v) and embedded in Technovit
162 8100 (Heraeus Kultzer, Germany) according to the manufacture's instructions. Histological
163 sections (4 µm) were mounted on Superfrost® slides (Roth, Germany) and stored at 4°C.

164 For FISH all probes were synthesized and 5'-labelled with Cy3 (indocarbocyanine) for the
165 treponeme specific oligonucleotide probes TRE I, and TRE IV while FITC (fluorescein
166 isothiocyanate) was used for labelling the eubacterial probe EUB 338 (Thermo Hybaid,
167 Interactiva division, Germany).

168 FISH was performed principally as described previously (Moter et al., 1998; Sunde et al.,
169 2003). In brief, hybridization was performed at 46 °C in a humid chamber for 4 h using 20 µl
170 hybridization buffer for each section (20% deionised formamide, 0,45 M NaCl, 20 mM Tris-
171 HCl pH 7.4, 0,01% SDS) and 100 ng of the probes. After incubation sections were washed
172 with distilled water, dried and mounted with ProLong® Antifade reagent (Molecular Probes,
173 The Netherlands).

174 For each FISH applications control strain were included as listed below. Strains were fixed in
175 3.7% (v/v) formaldehyde/PBS, pH 7.4 and stored at -20 °C. For FISH fixed cells were spotted
176 onto teflon-marked slides (Paul Marienfeld, Germany), air-dried and dehydrated in 50%, 80%
177 and 96% (v/v) ethanol.

178

179 Fluorescence microscopy

180 FISH analyses were performed using an epifluorescence microscope (DMBL, Leica)
181 equipped with a 100 W high pressure mercury lamp and a digital camera (DC300F, Leica) for
182 taking photomicrographs. Filters used for detection of FITC and Cy3 signals were L5 resp.
183 Y3 (Leica, Germany).

184

185

186 **Control strains**

187 To ensure specificity the following strains were included as controls for dot blot hybridization
188 and FISH: *T. vincentii* ATCC 35580, *T. denticola* (ATCC 33521^T), *T. maltophilum* (ATCC
189 51939^T), *T. lecithinolyticum* (ATCC 700332^T), *T. amylovorum* (ATCC 700288^T),
190 *T. phagedenis* (Biotype Reiter), *T. brennaborensis* (DSM 12168^T), *T. socranskii* ssp.
191 *socranskii* (ATCC 35536^T), *T. socranskii* ssp. *buccale* (ATCC 35534^T), *Treponema*
192 *socranskii* ssp. *paredis* (ATCC 35535^T), *T. pectinovorum* (ATCC 33768^T).

193 Additionally, for dot blot hybridization 16S rRNA controls from yet uncultivated *Treponema*
194 phylotypes were included from clones harbouring partial 16S rRNA gene sequences
195 belonging to oral phylotype groups TRE I-TRE VII as described previously (Choi et al., 1994;
196 Moter et al., 2006)

197

198 **Statistical analysis**

199 SPSS[®] was used for statistical analysis. Box- and scatterplots were used to show differences
200 in the distribution of the dogs involved in this study. Boxplots show quartiles of the
201 distribution indicated as a box with the corresponding median. Samples scored outside of the
202 boxplots were posed solitary by indicating the number of the sample. The periodontal status
203 between groups positive for treponemes was compared by the Mann-Whitney-U-test.

204 Because no PCR products were obtained from plaque samples of ten dogs due to technical
205 problems these dogs were not included in this study.

206

207 **Results**

208 **Clinical measurements**

209 First clinical parameters were assessed to monitor the periodontal status of the dogs involved
210 in this study. As shown in Fig. 1, subjective and halimeter determination of halitosis

211

212 clinical periodontal status. In Fig. 2, VCS concentrations, organoleptic grades and the
213 periodontal status are given, clearly showing that with an increasing periodontal status VSC
214 concentration as well as organoleptic grading increased.

215 Because of this association, the study cohort was divided to their periodontal status. 23 of the
216 animals had a normal periodont (status 0), 53 showed minor (status 1), 23 medium (status 2),
217 and 2 major (status 3) periodontitis.

218

219 **Microbiological investigation**

220 After these initial clinical measurements, samples from the facial sulcus of teeth 208 and 409
221 were taken from 101 dogs for subsequent PCR amplification. Although a total of three repeats
222 of every sample was conducted to obtain PCR products of bacterial 16S rRNA genes for
223 further dot blot hybridization assays, samples from ten dogs remained negative in PCR
224 amplification at all, most probably due to the presence of inhibitory substances rather than
225 due to degradation of the samples (Matto et al., 1998).

226 From the remaining 91 dogs, DNA was amplified successfully with eubacterial primers TPU1
227 and RTU3. All amplicons were further processed for DNA-DNA dot blot analysis with 9
228 different oligonucleotide probes for the specific detection of *Treponema* phylotype groups
229 I-VII, *T. socranskii* or *T. denticola*. As the probe detecting 16S rDNA of *T. denticola* in the
230 initial testing of 51 dogs revealed positive results only in 2 dogs (3.92%), this probe was not
231 used further on.

232 Probes specific for *Treponema* phylogenetic group I (TRE I) gave positive results in 17 dogs
233 (18.68 %), TRE II in 40 (43.96 %) and TRE IV in 54 (57.45 %) animals, while *T. socranskii*
234 was detected in 22 (24.18 %) of the dogs. In contrast, TRE III, TRE V, TRE VI and TRE VII
235 were not detected at all (Tab.1).

236 In total, 59 (64.84%) dogs gave positive results for at least one probe. 43 (47.25%) dogs were

237

238 *Treponema* groups detected by dot blot analysis was TRE II and TRE IV in 17 (18.68%) dogs
239 followed by the combination of TRE I, TRE II and TRE IV and TRE I, TRE II, TRE IV and
240 *T. socranskii*, each in 8 (8.79%) dogs. The combination of TRE II, TRE IV and *T. socranskii*
241 was detected in 6 (6.59%) dogs while the combination of TRE IV and *T. socranskii* was found
242 in 3 (3.29%) dogs. The combination of TRE I, TRE II and *T. socranskii* were found only in 1
243 (1.09%) dog at the same time.

244 Interestingly, in those animals harbouring treponemes, the periodontal status was in general
245 higher than in those reacting negative as substantiated by further statistical analysis. The
246 statistical analysis revealed a significant association between periodontal status and detection
247 of *Treponema* spp. (TRE I, II, IV and *T. socranskii*: $p = 0.001$), suggesting a role of
248 treponemes in the aetiology of periodontitis. Fig. 3 summarizes these results by showing the
249 association between periodontal status and the detection rate of *treponemes* of group I, II and
250 IV respectively *T. socranskii*.

251 To visualize the morphology and localization of treponemes in plaque of the dogs, gingival
252 biopsies taken from two dogs with periodontal status 3 were further investigated by
253 fluorescence in situ hybridization (FISH). Hybridization of gingival tissue sections with
254 probes TRE I and TRE IV showed the typical morphology of spirochetes (Fig. 4). FISH using
255 the bacterial probe EUB 338 revealed, that also spirochetes, next to other bacteria, were
256 present in high numbers. FISH results also indicate that treponemes are not only present
257 within the plaque biofilm but also within the gingival tissue.

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264 **Discussion**

265 From a clinical point of view, VSC measurement in humans seems to be a simple and rapid
266 chair-side method to detect shifts in the resident microbiological flora, indicating an increase
267 in possible periodontal pathogens or contributors to disease pathogenesis (Socransky et al.,
268 1998). Measurement of VSC can be performed both, organoleptic or using a halimeter.
269 Although organoleptic measurement is a more subjective technique and a quite simple clinical
270 method for scoring halitosis these data correlate well with those data gained from
271 measurements using a halimeter (Fig. 1).

272 Increase of VSC is due to VSC-producing bacteria and correlates with an increasing number
273 of periodontopathogenic bacteria including black-pigmented bacteria like *Porphyromonas*
274 spp. or *Fusobacterium* spp., *Prevotella* spp. and *Treponema denticola* which have been shown
275 to be potent producers of VSC and an increasing grade of periodontitis (Awano et al., 2002;
276 Fukamachi et al., 2005; Pavolotskaya et al., 2006; Torresyap et al., 2003). The correlation
277 between VSC and periodontal status is reflected in our data as increasing VSC concentrations
278 are associated with an increasing grade of periodontitis (Fig. 2). Therefore, measurement of
279 VSC concentrations and the coverage of different periodontal indices are reliable markers to
280 record the oral health status in dogs.

281 After assessing an objective scoring of the oral health status we then examined if the
282 prevalence of treponemes is correlated with an increasing degree of periodontitis. To our
283 knowledge, this is the first time the association of treponemes with periodontal status in dogs
284 was investigated systematically. So far, mostly conventional cultivation procedures have been
285 used to screen for putative periodontal pathogens in dogs (Elliott et al., 2005; Hardham et al.,
286 2005; Sarkiala et al., 1993; Valdez et al., 2000). However, for human periodontitis it has been
287 shown that the culture dependant approach misses a high proportion of species that are
288 fastidious or yet-to-be cultured (Choi et al., 1994; Kumar et al., 2003; Paster et al., 2001;

290 have a high prevalence in human periodontitis (Moter et al., 1998; Paster et al., 2001). We
291 therefore used culture-independent techniques which already have been applied in human
292 periodontitis to assess the association of treponemal phylotypes with severity of periodontitis
293 in dogs (Choi et al., 1994; Moter et al., 1998; Moter et al., 2006; Sakamoto et al., 1999; Willis
294 et al., 1999).

295 By this approach treponemes were detected more frequently in dogs with a higher periodontal
296 status. In 57.45% of the dogs treponemes belonging to group IV were detected, while
297 treponemes of group TRE I, TRE II and *T. socranskii* were detected in 18.68%, 43.96% and
298 24.18% of the dogs, respectively. As shown by box plots in Fig. 3 the prevalence of these
299 treponemes correlates clearly with the grade of periodontitis. Therefore, our data imply that
300 treponemes are associated with periodontitis in dogs and may play a role in the pathogenesis
301 of canine periodontitis together with other oral pathogens like black-pigmented anaerobe
302 bacteria (BPAB) (Hardham et al., 2005). This assumption was further supported by FISH
303 visualizing spirochetes in periodontal biofilms on sections from gingival biopsies (Fig.4). In
304 these sections treponemes are not only located in the plaque biofilm but also within the tissue
305 implicating their invasive and pathogenic character. These results are also in line with
306 previous immunohistochemical analyses of canine plaque samples detecting oral treponemes
307 more frequently in dogs suffering from periodontitis than in healthy ones (Riviere et al.,
308 1996). Similar to our results treponemes related to *T. maltophilum*, a cultivable representative
309 of oral group IV treponemes, were frequently detected in a cultivation based study (Valdez et
310 al., 2000).

311 Likewise to human periodontitis (Ehmke et al., 2004; Moter et al., 1998; Moter et al., 2006)
312 we found in our study that treponemes associated with canine periodontitis belong to group
313 TRE I, II and IV or to *T. socranskii*. At least these phylotypes are likely to be important
314 diagnostic markers for the diagnosis of canine periodontitis until pathogenicity has not be

315

316 VII were detected in these samples. Interestingly only 2 of the 51 dogs harboured *T.*
317 *denticola*-like treponemes belonging to group TRE II although *T. denticola* is considered a
318 major oral pathogen in human periodontitis being present at high frequencies while
319 phylogenetic group TRE II was prevalent in 43.96% of the dogs (Sela, 2001). Our preliminary
320 results therefore imply that treponemes belonging to group TRE III, TRE V, TRE VI and
321 TRE VII as well as *T. denticola*-like treponemes seem to be hardly ever associated with
322 canine periodontitis.

323 In this study three different *Treponema* phylotype groups as well as *T. socranskii* could be
324 detected by screening 91 dogs. Our data also indicate that dogs suffering from periodontitis
325 also exhibit a high diversity of different *Treponema* spp.. Only 4.39 % of the dogs were
326 positive just for *T. socranskii* resp. 13.18 % for TRE IV while all other *Treponema*-positive
327 dogs exhibited at least two different groups of treponemes as listed in Tab. 1.

328 To proof similarity of treponemes present in dogs and humans and to extend our knowledge
329 of treponemal diversity in dogs additional and more discriminatory phylogenetical approaches
330 are necessary to assess a more comprehensive and detailed knowledge on this topic as it has
331 been already shown in human periodontitis (Dewhirst et al., 2000).

332 Summarizing, the detection of treponemes by dot blot hybridization in gingival plaque of
333 dogs provides strong evidence for an association of these bacteria in canine periodontal
334 disease, a finding similar to that in human medical area. The same phlotypes that are
335 associated with human periodontitis are also abundant in dogs and might serve as diagnostic
336 markers for canine periodontitis. Fluorescent *in situ* hybridization demonstrated tissue
337 invasiveness of the treponemes suggesting a pathogenic role in periodontitis. However, not all
338 *Treponema* spp. involved in human periodontitis were present in dogs. Further studies are
339 necessary to assess deeper insights on the diversity and epidemiology of *Treponema* spp. in
340 dogs to evaluate the exact function of *Treponema* spp. in canine periodontitis.

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442 **Legends to Tables and Figures**

443

444 **Table 1:** Distribution of oral treponemes in plaque samples by dot blot hybridization using
445 group- and species-specific oligonucleotide probes. A total of 91 dogs were
446 screened for treponemes. While 32 dogs remained negative at all, treponemes
447 could be detected in 59 dogs in different frequencies and combinations.

448 * only 51 dogs were screened for *T. denticola*-like treponemes

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451 **Figure 1:** Concentrations of VSC in ppb measured by a halimeter and organoleptic results
452 scoring from 0 = no halitosis, 1 = slight halitosis, 2 = pronounced halitosis and 3 =
453 very strong halitosis.

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456 **Figure 2:** Association of VSC values, organoleptic results and the periodontal status
457 determined by clinical indices.

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460 **Figure 3:** Detection of phylogenetic groups TRE I, TRE II and TRE IV resp. *T. socranskii*
461 detected by dot blot hybridization depending on the periodontal health status.
462 The frequency of detection is significantly ($p = 0,001$) associated with an
463 increasing periodontal health status.

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466 **Figure 4:** FISH analyses of gingival bioplates from sites with periodontitis hybridized with
467 (a) TRE 1-cy3 (yellow) resp. (c) TRE IV-cy3. Same sites, (b) and (d), hybridized
468 with EUB-FITC (green) showing the total bacterial count. Bars 10 μm .

469

Oligonucleotide probe	no. of positive dogs (%)	combinations of <i>Treponema</i> spp	no. of positive dogs (%)
<i>Group-specific probes</i>			
TRE I	17 (18.68%)	TSOC	4 (4.39%)
TRE II	40 (43.96%)	TRE IV	12 (13.18%)
TRE III	0	TRE IV-TSOC	3 (3.29%)
TRE IV	54 (57.45%)	TRE II-TRE IV	17 (18.68%)
TRE V	0	TRE II-TRE IV-TSOC	6 (6.59%)
TRE VI	0	TRE I-TRE II-TSOC	1 (1.09%)
TRE VII	0	TRE I-TRE II-TRE IV	8 (8.79%)
<i>Species-specific probes</i>			
TSOC	22 (24.18%)	TRE I-TRE II-TRE IV-TSOC	8 (8.79%)
TRENT*	2 (2.00%)		







