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1 ***In vitro* susceptibility of bovine digital dermatitis associated spirochaetes to**
2 **antimicrobial agents**

3

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21

22 **Abstract**

23 Bovine digital dermatitis (BDD) is an infectious lameness in cattle, which has a
24 large global impact in terms of animal welfare and cost. The majority of evidence
25 suggests that spirochaetes are the aetiological agent of this disease. The aim of this study
26 was to identify the susceptibility of BDD associated spirochaetes to a range of
27 antimicrobial agents with a view to potential usage *in vivo* to treat this widespread cattle
28 disease. A microdilution method was adapted to determine the *in vitro* susceptibilities of
29 19 UK digital dermatitis spirochaetes (6 *Treponema medium*/*Treponema vincentii*-like, 8
30 *Treponema phagedenis*-like and 5 *Treponema denticola*/*Treponema putidum*-like) to
31 eight relevant antimicrobials. The BDD spirochaetes exhibited the highest susceptibility
32 to penicillin and erythromycin and this information may now be used to aid development
33 of efficacious treatments.

34 This study has also identified that BDD spirochaete T167 is spectinomycin
35 resistant and that the likely biological basis is a point mutation in the 16S rRNA gene.
36 Interestingly, nearly all *Brachyspira* isolate 16S rRNA gene sequences in Genbank have
37 this substitution, suggesting it may be responsible for the characteristic spectinomycin
38 resistance reported for the *Brachyspira* genus.

39

40 **Keywords:** Bovine Digital Dermatitis, *Treponema*, spirochaetes, spectinomycin
41 resistance, *Brachyspira*

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47 1. Introduction

48 Bovine Digital Dermatitis (BDD) is an infectious lameness in cattle, responsible
49 for considerable economic loss as well as being very painful for infected animals
50 (Losinger, 2006; Whay et al., 1997). Whilst this disease is currently prevalent worldwide
51 in most dairy herds, its treatment and control continue to remain poorly understood
52 (Laven and Logue, 2006). Many lines of evidence have suggested that spirochetes, and
53 more specifically treponemes, are the aetiological agents of BDD (Demirkan et al., 1998;
54 Moter et al., 1998). We recently described the isolation of a large number of spirochaetes
55 from BDD lesions and demonstrated that the isolates could be divided into 3 distinct
56 groups using phylogenetic and phenotypic analysis (Evans et al., 2008). These three
57 phylogroups were placed within the genus *Treponema* using 16S rRNA and flagellin
58 gene analysis, were regularly isolated from UK BDD lesions and have also been
59 identified in BDD lesions in Germany and the USA (Choi et al., 1997; Stamm et al.,
60 2002). The three phylogroups; group 1, 2 and 3 have been described by their similarity to
61 nearest relatives as *Treponema medium*/*Treponema vincentii*-like, *Treponema*
62 *phagedenis*-like and *Treponema denticola*/*Treponema putidum*-like respectively.

63 There remains no single BDD treatment regime with proven efficacy. Given that
64 spirochaetes are the probable aetiological agents of BDD, identifying *in vitro*
65 susceptibilities of BDD-associated spirochaetes to a range of antimicrobial agents is of
66 importance. No standard accepted methodology exists for measuring the *in vitro*
67 susceptibility of antimicrobial agents against *Treponema*, the genus that the BDD
68 spirochaetes are reported to be part of. Broth microdilution has been used successfully to
69 determine the minimal inhibitory concentration (MIC) of various spirochaetes from
70 *Leptospira*, *Borrelia* and *Brachyspira* genera (Hunfeld et al., 2005; Karlsson et al., 2003;

71 Murray and Hospenthal, 2004; Ruzic-Sabljić et al., 2005). Hence, this study aimed to
72 design and implement a broth microdilution protocol to determine the MIC and minimal
73 bactericidal concentration (MBC) of the BDD associated spirochaetes to several
74 antimicrobials.

75

76 **2. Materials and methods**

77 2.1. Bacterial isolates.

78 Nineteen isolates (Table 1) were used to investigate the antimicrobial
79 susceptibility of digital dermatitis associated spirochaetes. These included 16
80 spirochaetes isolated during a survey of BDD lesions from various farms across England,
81 UK (Evans et al., 2008) including; five group 1 isolates, eight group 2 isolates and three
82 group 3 isolates. All 16 isolates were from Holstein Friesian cows with typical BDD
83 lesions, except G187, which was from a case of bovine interdigital dermatitis (IDD). A
84 group 1 isolate (T136E), and a group 3 isolate (T136P); both isolated from the BDD
85 lesion of a cow on a Shropshire farm, England, UK were also included to ensure group
86 representatives from different geographical UK regions were tested. A group 3
87 spirochaete (G179), isolated from a case of contagious ovine digital dermatitis (Demirkan
88 et al., 2001) was also included in the study.

89 2.2. Antimicrobial susceptibility testing.

90 Antimicrobial susceptibility testing was performed by a broth microdilution
91 method using sterile 96-well polystyrene flat-bottomed microplates (Appleton Woods,
92 Birmingham, U.K.). In this study, each microplate included positive controls (bacteria
93 inoculated without antimicrobial agents), negative controls (no bacteria or antimicrobial),
94 and serial twofold dilutions of each of eight antimicrobials, all in oral treponeme

95 enrichment broth (OTEB; Anaerobe Systems, CA, USA) inclusive of the respective
96 serum supplement. The respective OTEB serum supplements were 10 % (v/v) rabbit
97 serum (RS) for group 1 strains and 10 % (v/v) foetal calf serum (FCS) for group 2 and 3
98 strains.

99 Microplates were incubated in an anaerobic cabinet overnight prior to use. The
100 group 1, 2 and 3 strain inoculums were taken from cultures grown for six, four and seven
101 days respectively, with all cultures being grown at 36 °C under anaerobic conditions in
102 OTEB supplemented with respective sera. Bacteria counts were determined using a
103 Petroff-Hausser counting chamber (Fisher) under phase contrast microscopy. Trial
104 experiments identified that a 50 µl inoculation volume promoted the most consistent
105 growth in all treponemes cultures from each of the three BDD groups. The stock
106 inoculum of group 1, 2 and 3 isolates contained 8.75×10^7 , 1.14×10^8 and 2.69×10^8
107 treponemal organisms/ml respectively. Prior to treponeme inoculation, each well had a
108 volume of 150 µl resulting in a final volume after inoculation of 200 µl. The microplates
109 were incubated at 36 °C under anaerobic conditions for the required time for MIC
110 measurement. The antibiotics tested were penicillin G, ampicillin, oxytetracycline,
111 gentamicin, lincomycin, spectinomycin and erythromycin (Sigma-Aldrich, Dorset, U.K.);
112 and enrofloxacin (Bayer, Leverkusen, Germany). Antibiotic test ranges are listed in Table
113 2.

114 2.3. Determination of MICs.

115 Preliminary experiments were necessary to identify the late exponential/early
116 stationary phase microdilution time point for each different BDD group, particularly
117 given the known phenotypic differences previously described for each group, especially
118 their growth characteristics (Evans et al., 2008). Growth curves were recorded for each of

119 the BDD associated spirochaete groups by measuring the absorbance of the microplate
120 wells at 540nm every day for up to 10 days using a Multiskan microtitre plate reader
121 (Thermo Scientific, Hampshire, U.K.). The late exponential/early stationary phase time
122 point was identified for each different group as three days for group 3 isolates and four
123 days for group 1 and 2 isolates.

124 The MIC for each antibiotic was taken as the lowest concentration of antibiotic
125 that prevented growth in wells. Cell growth was determined by comparison of the
126 absorbance measurement immediately after inoculation with the absorbance measured at
127 the late exponential/early stationary phase time point specific (and previously
128 determined) to the group that the tested isolate belongs (three days for group 3 isolates
129 and four days for group 1 and 2 isolates). The MIC values were taken as the medians of
130 three experiments performed on different days, with freshly prepared inocula, medium
131 and antibiotics.

132 2.4. Determination of MBCs.

133 For each antibiotic, after MIC determination, 10 μ l of culture from the MIC well
134 and the next three wells of increased antibiotic concentration were sub-cultured to new
135 wells containing 190 μ l OTEB with the respective serum supplement but with no
136 antimicrobials included. Microplates were incubated for a further five days at 36°C under
137 anaerobic conditions. Growth was assessed using phase contrast microscopy. The MBC
138 was defined as the dilution that contained no treponeme cells in the sub-cultured media.
139 The MBC values are reported as the medians of three experiments performed on different
140 days.

141 2.5. Study validation.

142 Control substances included all the above described antibiotics, except
143 spectinomycin and enrofloxacin, and used *Treponema phagedenis* biotype Reiter as a
144 control microorganism for testing. The control microorganism was grown and
145 susceptibility tested under the same conditions as the group 2 (*T. phagedenis*-like) BDD
146 treponeme group. To validate the method described here; the control data produced were
147 then compared to previously described results from treponeme macrodilution MIC testing
148 (Abramson and Smibert, 1971) and a statistical comparison implemented using linear
149 regression analysis.

150

151 **3. Results**

152 The individual *in vitro* susceptibilities of the 19 BDD associated treponemes to
153 eight antimicrobial agents are summarised in Tables 2 and 3. General trends of
154 antimicrobial MICs and MBCs could be observed for each of the three BDD treponeme
155 groups as well as for all three groups as a whole. For example, the group 3 isolates
156 appeared to be more susceptible to gentamicin than the other two groups, whilst group 2
157 isolates were less susceptible to lincomycin than the other two groups. All groups were
158 most susceptible to penicillin and erythromycin whilst not being particularly susceptible
159 to enrofloxacin. The ovine isolate had near identical MIC and MBC values to the group
160 3 BDD associated treponemes.

161 The Group 2 isolate T167 had MIC values above the spectinomycin concentration
162 test range chosen for that group (see Table 2 for group antibiotic test ranges) and testing
163 was repeated with increased spectinomycin concentrations in an attempt to identify the
164 MIC and MBC values. After allowing for the solubility of the antibiotic, the highest
165 concentration tested was 12,288mg/L and the MIC was still not reached, this is an

166 inhibitory concentration of more than 2048 times the value of the other group 2
167 spirochaetes.

168 The MIC microdilution method described in this study was validated by
169 comparing the results for six of the eight compounds against a control microorganism, *T.*
170 *phagedenis* biotype Reiter, to results previously obtained using a macrodilution method
171 (Abramson and Smibert, 1971). The median values obtained from the triplicates for each
172 antibiotic microdilution MIC test matched the previously described macrodilution results
173 for three antimicrobials and were a single dilution different for the remaining three
174 antibiotics. Indeed, comparison of the published data (Abramson and Smibert, 1971) with
175 relevant current data for *T. phagedenis* biotype Reiter using linear regression analysis
176 showed a highly significant correlation ($R=0.9905$; $P<0.0001$) indicating the efficacy and
177 reproducibility of the microdilution method.

178

179 **4. Discussion**

180 The described microdilution method has enabled the antimicrobial sensitivities of
181 several antibiotics against the BDD associated spirochaetes to be identified. Both genus
182 and group specific antibiotic susceptibility trends could be observed. The ovine isolate
183 had near identical MIC and MBC values to the group 3 BDD associated treponemes
184 which agrees with previous genotypic and phenotypic observations that it is in fact a part
185 of the group 3 BDD associated spirochaetes (Evans et al., 2008). When including all the
186 spirochaetes studied, penicillin and erythromycin have the lowest MICs and MBCs and
187 they are therefore likely to be the most effective antibiotics for BDD treatment.

188 BDD treatment is generally implemented on a whole herd basis using footbaths,
189 although some animals may be treated with foot sprays. Oxytetracycline, erythromycin,

190 lincomycin and spectinomycin have all been used in footbaths (Laven and Logue, 2006)
191 although only the use of erythromycin (35mg/L twice within 24 hours) has shown
192 reproducible, moderate efficacy (Laven and Hunt, 2002; Laven and Proven, 2000). This
193 is in good agreement with the data presented here where the *in vitro* tests have identified
194 that the spirochaetes are substantially more susceptible to erythromycin than
195 oxytetracycline, lincomycin or spectinomycin. Individual cases of BDD are treated using
196 the application of topical antimicrobials. Oxytetracycline has been commonly employed
197 as a test agent in individual treatment and has been reported to be efficacious (Blowey
198 and Sharp, 1988; Britt et al., 1996; el-Ghoul and Shaheed, 2001). Whilst the results
199 presented here show that the BDD spirochaetes are somewhat susceptible to
200 oxytetracycline, the results do not suggest this is the most effective agent. The reported
201 oxytetracycline efficacy may be due to the high concentrations used in single topical
202 application such as 20-100g/L in solution (Britt et al., 1996; el-Ghoul and Shaheed, 2001)
203 and with topical application antimicrobial sprays being up to 25 times more concentrated
204 than antibiotic present in the footbath (Laven and Logue, 2006). Systemic antibiotics
205 have also been used to treat BDD. Good results have been reported using penicillin or
206 ceftiofur when administered intra-muscularly (Read and Walker, 1998). In contrast,
207 several small studies have suggested that systemic antibiotics were an ineffective
208 treatment method (Blowey and Sharp, 1988; Rebhun et al., 1980). Penicillin being
209 effective for BDD treatment concurs with it exhibiting the most anti-spirochetal activity
210 in this study. However, the use of systemic penicillin would require farmers to stop
211 milking cows during treatment periods, which would be costly and probably not well
212 received.

213 In addition to this useful data for designing BDD treatment, another observation
214 of relevance to general spirochaete antibiotic resistance mechanisms has been identified.
215 In this study the spectinomycin MIC of T167, a group 2 BDD spirochaete, was at least
216 2048 times that of the other group 2 isolates. Given that T167 is genotypically and
217 phenotypically identical to the other group 2 spirochaetes (Evans et al., 2008); this
218 suggests that this isolate had acquired spectinomycin resistance. A literature and database
219 search for spectinomycin resistance mechanisms identified the most likely cause to be a
220 single nucleotide difference in the 16S rRNA gene of T167 which was absent from the
221 remaining isolates. This same cytosine to thymine transition at position 1192 (using
222 *Escherichia coli* numbering) of the 16S rRNA gene has previously been identified as
223 being responsible for spectinomycin resistance in *Neisseria gonorrhoeae* and *E. coli*
224 (Galimand et al., 2000).

225 Having identified the spectinomycin resistance point mutation in BDD
226 spirochaete T167 a more general search was carried out to locate the presence of this
227 mutation in several genera of clinically significant spirochaetes; *Treponema*, *Leptospira*,
228 *Borrelia* and *Brachyspira*. Genbank was interrogated for all 16S rRNA genes from each
229 of the above genera, sequences aligned and the results are shown in Table 4. No other
230 BDD spirochaetes (six Swedish and five American) present in Genbank or any other
231 identified *Treponema*, *Leptospira* or *Borrelia* had the spectinomycin resistance mutation
232 present. However, this spectinomycin resistance point mutation has been selected *in vitro*
233 in *Borrelia burgdorferi* with no significant fitness cost identified (Criswell et al., 2006).
234 Interestingly, after aligning a total of 242 *Brachyspira* 16S rRNA gene sequences from
235 Genbank we identified that all had the spectinomycin resistance linked thymine present
236 (at position 1192) except *Brachyspira innocens* strains B256 and D7A/10/25 (Genbank

237 accessions M57744 and AB093321). Strains B256 and D7A/10/25 were identified as
238 having a cytosine and a guanine present instead respectively. A common characteristic of
239 *Brachyspira* species is spectinomycin resistance (Ochiai et al., 1997) suggesting this
240 resistance substitution is likely to have occurred prior to the division of many of the
241 species in that genus. Furthermore, the majority of *Brachyspira* isolation methods use
242 spectinomycin as a selection agent (Brooke et al., 2003; Calderaro et al., 2005; Feberwee
243 et al., 2008; Songer et al., 1976). The cytosine to guanine transversion, present in strain
244 D7A/10/25 is reported to allow for bacterial spectinomycin resistance (Galimand et al.,
245 2000). Whilst it is unclear whether B256 was originally isolated with or without
246 spectinomycin in selective medium (Kinyon and Harris, 1979), subsequent data has
247 identified it as susceptible to spectinomycin (Smith et al., 1991) which is in agreement
248 with there being no spectinomycin resistance associated substitution present.
249 Furthermore, in another study, B256 has been grown without the presence of
250 spectinomycin due to its known susceptibility (Milner et al., 1995). Thus, for *Brachyspira*
251 and specifically *Brachyspira innocens*, there may be many isolates which are present in
252 samples but cannot be isolated as they are not spectinomycin resistant. This might
253 suggest the use of spectinomycin for *Brachyspira* isolation needs to be reappraised.

254

255 Here, we have adapted a microdilution susceptibility testing method to obtain
256 relevant antibiotic susceptibility data for UK digital dermatitis spirochaetes. This method
257 may now be applied to isolates from other countries to investigate whether different
258 susceptibility patterns exist and to test future potential treatments. Furthermore, this study
259 has identified the likely biological basis of the characteristic spectinomycin resistance
260 reported for the *Brachyspira* genus.

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267

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- 360
- 361

362 **Tables**

363

364 **Table 1. Spirochaetes tested for susceptibility to antimicrobial agents**

Strain no.	Strain	Isolation date	UK Region	Nearest Relative ^a	Group
1	T184Y	6/10/03	Merseyside	<i>Treponema medium/Treponema vincentii</i>	1
2	T19	7/10/03	Merseyside	<i>Treponema medium/Treponema vincentii</i>	1
3	T54	7/10/03	Merseyside	<i>Treponema medium/Treponema vincentii</i>	1
4	T56	7/10/03	Merseyside	<i>Treponema medium/Treponema vincentii</i>	1
5	T18A	7/10/03	Merseyside	<i>Treponema medium/Treponema vincentii</i>	1
6	T136E	28/1/04	Shropshire	<i>Treponema medium/Treponema vincentii</i>	1
7	T167	8/12/03	Cheshire	<i>Treponema phagedenis</i>	2
8	T257	12/1/04	Cheshire	<i>Treponema phagedenis</i>	2
9	T354B	12/1/04	Cheshire	<i>Treponema phagedenis</i>	2
10	T119A	4/2/04	Shropshire	<i>Treponema phagedenis</i>	2
11	T320A	24/2/04	Merseyside	<i>Treponema phagedenis</i>	2
12	T2721A	24/2/04	Merseyside	<i>Treponema phagedenis</i>	2
13	G187	12/5/04	Gloucestershire	<i>Treponema phagedenis</i>	2
14	G169A	13/5/04	Gloucestershire	<i>Treponema phagedenis</i>	2
15	T354A	12/1/04	Cheshire	<i>Treponema denticola/Treponema putidum</i>	3
16	T136P	4/2/04	Shropshire	<i>Treponema denticola/Treponema putidum</i>	3
17	T3552B	24/2/04	Merseyside	<i>Treponema denticola/Treponema putidum</i>	3
18	G819CB	7/5/04	Gloucestershire	<i>Treponema denticola/Treponema putidum</i>	3
19	G179 (Ovine)	15/3/00	Northern Ireland	<i>Treponema denticola/Treponema putidum</i>	3

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366 ^aAs determined by 16S rRNA gene phylogenetic analysis.

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368 **Table 2. Digital Dermatitis associated spirochaete minimal inhibitory concentrations**
 369 **to eight antimicrobial agents**

Strain no. ^a	Median MIC (mg/L)							
	Penicillin	Oxytetracycline	Ampicillin	Lincomycin	Spectinomycin	Erythromycin	Gentamicin	Enrofloxacin
1	0.0469	0.375	0.0938	6	1.5	0.0117	12	24
2	0.0469	0.375	0.0938	6	1.5	0.0059	24	48
3	0.0469	0.375	0.0938	6	1.5	0.0117	3	48
4	0.0469	0.375	0.0938	6	3	0.0059	12	48
5	0.0469	0.375	0.0938	6	1.5	0.0117	6	48
6	0.0469	0.375	0.0938	12	1.5	0.0117	12	48
7	0.0117	0.375	0.0469	24	>12288	0.0469	12	24
8	0.0235	0.375	0.0938	12	6	0.0235	24	24
9	0.0235	0.375	0.0938	12	6	0.0469	12	96
10	0.0117	1.5	0.0938	24	6	0.0469	24	48
11	0.0235	0.1875	0.1875	24	6	0.1875	48	48
12	0.0235	0.75	0.0938	12	3	0.0469	12	24
13	0.0117	0.375	0.0469	12	6	0.0469	24	48
14	0.0235	0.375	0.1875	24	3	0.0469	24	48
15	0.0469	0.75	0.3750	6	12	0.0235	3	96
16	0.0235	0.375	0.3750	0.75	1.5	0.0117	0.75	12
17	0.0469	0.75	0.3750	6	1.5	0.0117	1.5	48
18	0.0235	0.75	0.1875	6	3	0.0117	1.5	24
19	0.0235	0.375	0.1875	6	3	0.0235	1.5	48
MIC ₉₀ ^b	0.0469	0.75	0.3750	24	12	0.0469	24	96

370 ^a 1-6 are group 1 BDD spirochaetes with antibiotic test ranges (mg/L) of: penicillin
 371 0.003-0.375; oxytetracycline 0.023-3; ampicillin 0.0059-0.75; lincomycin 1.5-192;
 372 spectinomycin 0.375-48; erythromycin 0.003-0.375; gentamicin 0.75-96 and enrofloxacin
 373 3-384. 7-14 are group 2 BDD spirochaetes with the same test ranges as group 1 except for
 374 erythromycin 0.0059-0.75. 15-19 are group 3 BDD spirochaetes with test ranges as group

375 2 except: penicillin 0.0059-0.75; oxytetracycline 0.0469-6; ampicillin 0.0117-1.5;
 376 lincomycin 0.375-48; spectinomycin 0.75-96 and gentamicin 0.1875-24.

377 ^b Cumulative susceptibility results across all spirochaetes tested are expressed as MIC₉₀,
 378 the concentration at which 90% of digital dermatitis associated spirochaetes are inhibited.

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380 **Table 3. Digital Dermatitis associated spirochaete minimal bactericidal**
 381 **concentrations to eight antimicrobial agents**

Strain no. ^a	Median MBC (mg/L)							
	Penicillin	Oxytetracycline	Ampicillin	Lincomycin	Spectinomycin	Erythromycin	Gentamicin	Enrofloxacin
1	0.0938	0.75	0.0938	24	3	0.0235	12	48
2	0.1875	0.75	0.1875	12	3	0.0235	48	96
3	0.0938	0.75	0.0938	12	6	0.0117	24	96
4	0.1875	0.75	0.1875	24	6	0.0235	24	96
5	0.0938	0.75	0.1875	12	3	0.0117	12	48
6	0.0469	0.75	0.1875	12	6	0.0235	48	48
7	0.0469	6	0.375	48	>12288	0.0938	24	96
8	0.0469	3	0.1875	24	24	0.1875	48	96
9	0.0469	1.5	0.375	48	12	0.0938	24	192
10	0.0469	6	0.375	48	12	0.1875	48	96
11	0.0469	1.5	0.375	96	48	0.3750	96	192
12	0.0469	1.5	0.375	48	6	0.0938	96	96
13	0.0469	1.5	0.375	48	12	0.1875	96	96
14	0.0938	3	0.375	24	6	0.1875	48	96
15	0.3750	3	1.5	12	12	0.1875	6	192
16	0.0938	3	0.75	6	6	0.0469	6	48
17	0.1875	6	1.5	12	6	0.0938	3	96
18	0.1875	6	0.75	24	6	0.0469	6	96
19	0.1875	3	0.75	12	6	0.0469	3	96
MBC ₉₀ ^b	0.1875	6	1.5	48	48	0.1875	96	192

382 ^a 1-6, group 1; 7-14, group 2; 15-19, group 3.

383 ^b Cumulative susceptibility results across all spirochaetes tested are expressed as MBC₉₀,
384 the concentration at which 90% of digital dermatitis associated spirochaetes are killed.

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388 **Table 4. Relationship between spectinomycin susceptibility and the 16S rRNA gene**
389 **point mutation C1192T across Spirochaete genera of clinical significance.**

Genus	Base at 1192 ^a	Spectinomycin susceptibility	Substitution at 1192 ^b	New spectinomycin susceptibility	Note
<i>Treponema</i>	C	Susceptible	T	Resistant	1 clinical isolate obtained from a study of 19 (This study).
<i>Borrelia</i>	C	Susceptible	T	Resistant	Two spectinomycin resistance isolates were selected <i>in vitro</i> (Criswell et al., 2006). No clinical strains reported.
<i>Leptospira</i>	C	Susceptible	-	-	-
<i>Brachyspira</i>	T	Resistant	C	Susceptible	All strains isolated are typically resistant. One isolate in Genbank had no substitution and this has been reported to be susceptible (Smith et al., 1991).

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391 ^aNucleotide typically found at 16S rRNA gene position 1192 (using *Escherichia coli*
392 numbering).

393 ^bInstances where an alternative nucleotide appear at 16S rRNA gene position 1192 (using
394 *Escherichia coli* numbering).

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