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5 **Infections with weakly haemolytic *Brachyspira* species in pigs with**
6 **miscellaneous chronic diseases**

7

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21

22 **Abstract**

23 The prevalence of infections with different *Brachyspira* species was assessed in
24 202 pigs with various chronic herd problems using different methods. Twenty
25 seven pigs (13.4%) were positive for *Brachyspira* spp. with at least one of the
26 methods used. The highest number of positives was identified with mucosal
27 scraping-PCR (23), followed by PET-PCR (22) and bacteriological-biochemical
28 analysis (15). With the exception of three cases of *B. pilosicoli* infections, only
29 weakly pathogenic *Brachyspira* species were identified. The majority were
30 *B. murdochii*, followed by *B. innocens* and *B. intermedia*. Concurrent infections
31 with two or more *Brachyspira* species were common and accounted for 37.1% of
32 the total. Presence of weakly haemolytic *Brachyspira* was associated with wasting
33 and diarrhoea in a number of cases.

34 This investigation shows that infections with weakly haemolytic *Brachyspira* spp.
35 may contribute to colonic pathology in pigs with chronic herd problems and that
36 mixed infections seem to occur more frequently than previously noticed .

37

38 Keywords: *Brachyspira*, NADH-oxidase gene, PCR, pig, prevalence, weakly
39 haemolytic

40

41

42 1. Introduction and Objectives

43

44 Diarrhoeic diseases caused by *Brachyspira* spp. in pigs are widespread and cause
45 considerable economic problems in the swine production (Jacobson et al., 2003).

46 Of the currently known porcine *Brachyspira* species *B. hyodysenteriae* and *B.*
47 *pilosicoli* are well known pathogens which cause swine dysentery and
48 spirochaetal colitis/intestinal spirochaetosis, respectively (Moxley and Duhamel,
49 1999). The pathogenic importance of *B. intermedia*, *B. murdochii* and *B. innocens*
50 was estimated low or non-existent in the past (Taylor and Trott, 1997). Recent
51 observations by several authors (Jensen and Boye, 2006, Neef et al., 1994,
52 Weissenböck et al., 2005), however, revealed a potential pathogenicity.

53 The first aim of this study was to gather information on the prevalence of
54 naturally acquired infections with *Brachyspira* spp. in pigs with chronic diseases,
55 such as wasting, respiratory disease and diarrhoea/enteritis in Austria. We
56 comparatively evaluated conventional bacteriological/biochemical methods and
57 molecular methods for the identification and differentiation of *Brachyspira*. With
58 these methods we intended to find out which of the *Brachyspira* species (*B.*
59 *hyodysenteriae*, *B. pilosicoli*, *B. intermedia*, *B. innocens*, *B. murdochii*) were
60 involved in the infection and we tried to evaluate to which degree they were
61 associated with significant colonic pathology. The second aim was to identify
62 potential multiple infections and to evaluate their significance and prevalence. A
63 final aim was to evaluate the sensitivity of PCR from paraffin embedded tissue
64 (PET) samples. This seems to be particularly important for pathologists intending
65 to perform retrospective studies, as there are currently no broadly available other
66 methods of discrimination of *Brachyspira* species in such samples.

67 **2. Material and Methods**

68

69 2.1. Animals, necropsy, histology and sampling

70 Two hundred and two pigs with miscellaneous chronic herd problems, such as
71 diarrhoea, wasting, respiratory and dermatological problems, which had been
72 submitted for diagnostic workup, were investigated. All pigs were subjected to a
73 complete necropsy one to three hours after euthanasia. After macroscopic
74 examination for the presence of gross lesions, tissue samples from the first and the
75 second colonic loop were fixed in 7% neutral buffered formalin and embedded in
76 paraffin wax for histological examination. From the PET-blocks 3 µm thick
77 sections were cut, subjected to haematoxylin/eosin staining and silver
78 impregnation according to Warthin-Starry and examined under a light
79 microscope. For evaluation of lesions the classification scheme according to
80 Herzog et al. (2005) was slightly modified. The degree of the dilatation of the
81 crypt lumina, lymphohistiocytic infiltration of the lamina propria and the presence
82 of crypt abscesses were assessed and the scores of the examination of the first and
83 the second colonic loop were combined. In Warthin-Starry stained sections the
84 spirochaetal load and the quantity of other bacteria were assessed based on the
85 score system in Herzog et al. (2005).

86 In all animals, presence of porcine circovirus type 2 (PCV-2) was investigated by
87 *in situ* hybridization of an inguinal lymph node (according to Rosell et al., 1999).
88 *Lawsonia (L.) intracellularis* infection was assessed by immunohistochemical
89 examination of ileum (according to Jensen et al., 2006).

90 For PCR and bacteriological-biochemical examination, mucosal scraping material
91 from a piece of tissue measuring approximately three times three cm from the first

92 colonic loop was taken. In order to minimise potential PCR inhibition by faecal
93 material all visible intestinal contents was rinsed from the mucosal surface with
94 tap water. The samples were stored at -20 °C until processing for PCR.

95

96 2.2. Bacteriology and biochemical classification

97 To keep the bacteria viable, cotton swabs dipped in mucosal scraping material
98 were transported in Amies medium to be investigated the very same day. Samples
99 were streaked onto Columbia agar (Oxoid, Basingstoke, UK) with 5% sheep
100 blood and MacConkey agar (Oxoid, Basingstoke, UK). Agar plates were
101 incubated at 37°C aerobically and anaerobically (blood agar). For *Salmonella*
102 detection, samples were preenriched in Rappaport-Vassiliadis 10 broth (Oxoid,
103 Basingstoke, UK) and subsequently streaked onto Rambach (Merck, Darmstadt,
104 Germany) and xylose-lysine-desoxycholate (XLD) agar (Oxoid, Basingstoke,
105 UK). Bacteria were identified using a range of standard phenotypic identification
106 protocols (Quinn et al., 1994). For isolation of spirochaetes, samples were plated
107 to Trypticase soy agar (TSA) (Oxoid, Basingstoke, UK) containing 8% (vol/vol)
108 defibrinated ovine blood, spectinomycin (400 µg/ml), colistin (25 µg/ml), and
109 vancomycin (25 µg/ml) and were incubated at 37 °C under anaerobic conditions
110 for at least 8 days (Jenkinson and Wingar, 1981). Spirochaetal growth was
111 recognized by the presence of β-haemolytic areas and confirmed by phase contrast
112 microscopy. When spirochaetal growth was observed, colonies were subcultured
113 to TSA without antibiotics and checked under the microscope to verify the
114 presence of spirochaetes only. Single colonies were also transferred into Kunkle's
115 broth, a prereduced anaerobic Trypticase soy broth containing 2% fetal calf serum
116 and 0.002 % ethanolic cholesterol (Kunkle et al., 1986). The enzymatic reactions
117 of the isolates obtained were tested with the commercial API ZYM system

118 (BioMérieux, Marcy l' Étoile, France). Bacteria were pelleted by centrifugation
119 from Kunkle's broth, resuspended in sterile saline to 10^7 cells/ml, placed in the
120 test cupules, and the strips were read after 4 h of incubation at 37 °C (Hunter and
121 Wood, 1979). To test for indole production, 2 ml of broth culture (10^8 cells/ml)
122 was extracted with 1 ml of xylene and then 4 drops of Kovac's reagent were
123 added. Development of a red or purple colour at the surface indicated a positive
124 culture (Kim et al., 2005). In order to control the results of the biochemical
125 classification all *Brachyspira* isolates were subjected to PCR.

126

127 2.3. PCR

128 2.3.1. Sensitivity testing of the PCR primers

129 Ten μ l bacterial culture suspensions in Kunkle's broth containing defined colony-
130 forming units (cfu) of the type strains of *B. hyodysenteriae* (ATCC 27164), *B.*
131 *innocens* (ATCC 29796), *B. intermedia* (ATCC 51140), *B. murdochii* (ATCC
132 51284), and *B. pilosicoli* (ATCC 51139) were added to 25 mg scraping material.
133 Colony forming units of type strains were determined by dilution on TSA without
134 antibiotics. DNA extraction was done as described above.

135 For validation of the detection limit of the primer sets applied (genus specific:
136 BrNox3 -F/R; species specific: Murd1 -F/R; Innoc1 -F/R; Intermed2004 -F2/R2;
137 Pilo2004 -F3/R3; Hyo1 -F/R) (primer sequences: Weissenböck et al., 2005)
138 dilution series of these extracts were performed in duplicate and subjected to
139 PCR.

140

141 2.3.2. DNA- amplification by PCR

142 Total DNA was extracted from mucosal scrapings, PET-material and bacterial
143 cultures (isolates) using NexttecTM Genomic DNA Isolation Kit for Tissue and
144 Cells (Nexttec GmbH, Leverkusen, Germany). In the case of PET-material, three
145 consecutive, 10 µm thick sections were deparaffinized in xylene, and the pellets
146 were washed in ethanol and dried, before being subjected to DNA extraction.

147 For selective detection of the *Brachyspira* species relevant for pigs, we used the
148 genus- and species-specific primer sets described above, which target portions of
149 the NADH oxidase (*nox*)-gene.

150 For DNA amplification each 25 µl reaction mixture contained 10 µl of
151 HotMasterMix (2.5x) (Eppendorf, Hamburg, Germany), 12 µl distilled water, 1 µl
152 (0.4 µM) of the appropriate forward and reverse primers, and 1µl of template
153 DNA. Following an initial denaturation for 2 min at 94 °C, the reaction mixture
154 was subjected to 40 cycles of heat denaturation at 94 °C for 30 s, annealing
155 temperatures as indicated in Weissenböck et al. (2005) for 30 s, and DNA
156 extension at 72 °C for 1 min, completed by final extension of further 5 min at 72
157 °C. Suspensions of *Brachyspira* reference strains (see above) served as positive
158 controls. Negative PCR controls contained 1µl of laboratory grade water instead
159 of DNA.

160 Following PCR, 10 µl of the amplification products were subjected to
161 electrophoresis in 2% Tris acetate- EDTA- agarose gel. The gel was stained with
162 ethidium bromide, and the bands were visualised under UV light.

163 The efficacy of DNA extraction from mucosal scrapings was controlled by
164 amplification of zinc finger x-encoded and zinc finger y-encoded genes (Aasen
165 and Medrano, 1990).

166

167 **3. Results**

168

169 3.1. Animal data, necropsy and histological findings

170 The 202 pigs investigated in this study came from 70 different farms located in
171 the federal states of Upper Austria, Lower Austria, Styria and Burgenland.

172 Seven males, 107 castrated males and 88 females were investigated. The weight
173 ranged from 4 kg to 113 kg (with the majority between 10 and 30 kg). Data on the
174 age of the pigs were fragmentary and are not shown.

175 In 27 pigs (13.4%) at least one of the applied methods detected *Brachyspira*.

176 These animals originated from 21 different farms (30% of all farms investigated)
177 in 3 different federal states (Upper Austria, Lower Austria, Styria). One hundred
178 and seventy five pigs were negative for *Brachyspira* sp. Relevant data of the
179 positive and negative group are comparatively presented in Table 1. Concerning
180 case history, the groups had comparable prevalences of diarrhoea and respiratory
181 problems. The only pronounced difference was the percentage of pigs with
182 wasting (50.8% in negative animals vs. 81.5% in positive animals).

183 Comparing pathological and other laboratory findings of the two groups showed
184 that the diagnosis colitis was made in 51.8% of the *Brachyspira*-positive animals
185 and only in 25.1% of the negative pigs. Colitis was diagnosed in cases which
186 showed macroscopically mucoid or watery colonic contents and/or histological
187 presence – at least in moderate extent – of mucus- distended crypt lumina and
188 crypt abscesses (Table 2). Among the *Brachyspira*-positive cases, colitis was
189 more frequently found with infections involving *B. pilosicoli*, *B. intermedia* and
190 *B. innocens*. Infections with *B. murdochii* were often without colonic pathology.
191 Concurrent infections with other bacterial agents also increased the likelihood of
192 colitis. In *Brachyspira*-positive cases, a thorough evaluation of the number,

193 localization and morphology of colonic bacteria was performed on Warthin-Starry
194 stained slides (Table 2). This examination revealed bacteria with *Brachyspira*
195 morphology in 25 of 27 cases. The bacteria were predominantly found in the crypt
196 lumina, but also on the colonic surface epithelium and, less frequently, invading
197 the lamina propria. In all positive cases there were dilated crypts containing
198 mucus. The degree of dilatation did not strictly correlate with the degree of colitis
199 or the spirochaetal load. In all *Brachyspira*-positive cases also other bacteria (rod
200 shaped bacteria, cocci) were present in crypt lumina (Table 2).

201 In seven cases a high spirochaetal load was found. In all except one of these cases
202 colitis was present. In three of them a few crypt abscesses were present. The non-
203 spirochaetal bacterial colonization was low to moderate in all these cases.

204 In cases (18) with a low to moderate spirochaetal load, crypt abscesses were
205 observed three times more often than in cases with a high spirochaetal load. Crypt
206 abscesses were rather correlated with the severity of non-spirochaetal colonization
207 than with the quantity of spirochaetes. In two only bacteriologically positive
208 samples no helical shaped bacteria could be seen.

209 In addition to spirochaetes the following (potential) intestinal pathogens were
210 found using specific assays: PCV-2, *L. intracellularis*, different enteral bacteria,
211 such as haemolytic *Escherichia coli*, *Salmonella cholerae suis* and *Clostridium*
212 *perfringens* (Table 2). While the overall percentage of PCV-2 infections was
213 comparable between positive and negative pigs (63% vs. 54.8%), there was a
214 remarkable difference between *L. intracellularis* infections (14.8% of positive
215 cases vs. only 6.8% of negative cases). Concerning infections with "other" enteric
216 bacteria the ratios were reversed. In only 25.9% of the positive animals, but in
217 40% of the negative animals additional enteric pathogens were isolated.

218 In only four of the cases with colitis (among them three with high spirochaetal
219 load) we found no other pathogenic agent than *Brachyspira* (twice: *B. innocens*;
220 *B. innocens* and *B. murdochii*; *B. murdochii*). The other four cases did not show
221 signs of enteritis nor showed an additional infection with other intestinal
222 pathogenic agents.

223 The percentage of pigs with pathologically diagnosed respiratory diseases, such as
224 porcine respiratory disease complex (PRDC), *Actinobacillus pleuropneumoniae*
225 (APP) infection or interstitial pneumonia, was considerably higher in negative
226 (84%) than in positive pigs (55.6%).

227

228 3.2. Bacteriological-biochemical classification

229 From 15 of 202 samples *Brachyspira* were recovered by cultivation. According to
230 the biochemical classification *B. murdochii* was present in all 15 cultures. In three
231 cultures *B. pilosicoli* was additionally detected.

232

233 3.3. PCR

234 3.3.1. Sensitivity testing of the primers:

235 The detection limit of the primer sets applied ranged between 400 and 38000
236 bacteria per ml of sample volume (Details in Table 3).

237

238 3.3.2. PCR of mucosal scrapings

239 Colonic mucosal scrapings from 202 pigs examined with the genus-specific
240 primers (BrNox3) yielded *Brachyspira* amplification products in 23 cases.

241 Of these 23 *Brachyspira*-positive samples PCRs with species-specific primers
242 yielded 16 cases (69.6%) of infections with a single *Brachyspira* species and six

243 cases (26.1%) with mixed infections (five double infections, one triple infection)
244 (Details in Tables 4 and 5). There was no detection of *B. hyodysenteriae* with this
245 method.

246

247 3.3.3. PCR of PET samples

248 In only 18 (78%) of the 23 positive colonic mucosal scraping samples
249 *Brachyspira* were detected with genus-specific primers (Tables 4 and 5).

250 Species-specific PCR, however, detected *Brachyspira* spp. in 21 of the 23 cases
251 (91.2%). Fifteen (65.2%) showed infection with a single *Brachyspira* species. In
252 six cases (26.1%) mixed infections (five double-, one triple infections) were
253 found.

254 Comparative evaluation of PCR results from scrapings and PET revealed an
255 82.7% level of accuracy concerning detection rates of one *Brachyspira* species. *B.*
256 *murdochii* could be detected in 14 of 15 cases (93.3%). *B. innocens* could be
257 detected in only five of nine cases (55.6%). One sample, which showed a weak
258 inconclusive amplification product in PCR on scraping material, and thus was not
259 considered positive, had a clearly positive PET-PCR result. *B. intermedia* was
260 found in mucosal scraping material and in PET material in the same four cases
261 (100 %). In addition to the single detection of *B. pilosicoli* in mucosal scraping
262 this species was found in further three PET samples.

263 The four samples, which yielded a *Brachyspira*-positive result only by the
264 bacteriological investigation but not by PCR of scraping material, were re-
265 examined by species-specific PET-PCR. In only one of these bacteriologically
266 positive samples *B. innocens* could be detected.

267 Also in PET material there was no detection of *B. hyodysenteriae*.

268

269 3.3.4. PCR of bacteriological isolates

270 Fifteen bacteriological isolates were investigated with species-specific PCR. In all
271 of them, *Brachyspira* spp. were detected with genus-specific primers.

272 In eight cases, the PCR of bacterial isolates revealed different or additional
273 *Brachyspira* spp. compared to the PCR of scraping and PET material. (Table 5).

274

275 After compilation of all results the percentage of single infections versus mixed
276 infections shifted only slightly towards mixed infections. There were 62.9% single
277 infections, with the majority of them (40.7%) *B. murdochii* infections. Among the
278 mixed infections, still *B. murdochii*/*B. innocens* infections dominated and there
279 was one more infection with all four *Brachyspira* species (Table 4).

280

281 4. Discussion

282

283 The results of all four methods for determination of the *Brachyspira* species
284 present (scraping-PCR, PET-PCR, biochemical classification, PCR of
285 bacteriological isolates) used in the present study were not corresponding in all
286 cases, a fact which may be explained by several reasons. First, there were cases
287 which yielded a positive result by bacteriological-biochemical investigation, but a
288 negative result with PCR. These seem to be cases with low numbers of bacteria,
289 which could be isolated in culture, but their quantity was below the threshold of
290 detection by PCR. It has been shown that culture has a high sensitivity with a
291 detection limit as low as 140 bacterial cells per gram faeces (Fellström et al.,
292 2001) which is clearly superior to the detection threshold of the PCRs used in the

293 present study. Second, an even higher proportion of cases was positive by PCR
294 but negative by culture. Even although considerable care was taken to preserve
295 the viability of these fastidious bacteria by using adequate transport media, this
296 result reflects an occasional problem encountered with cultural typing of
297 *Brachyspira* sp. (Råsbäck et al., 2006). While in case of *B. hyodysenteriae* culture
298 and biochemical testing is more sensitive than PCR, in case of weakly haemolytic
299 *Brachyspira* and mixed infections PCR has been considered more sensitive. In
300 addition, a proportion of the cases with negative culture might have undergone
301 antimicrobial therapy leading to loss of viability of the bacteria (Nathues et al.,
302 2007). Because utmost care was taken to prevent cross or laboratory
303 contamination of samples it appears unlikely that a contamination event was
304 responsible for the higher number of positives by PCR. Finally, in several cases,
305 especially mixed infections, there was no agreement of the bacterial species found
306 with different methods. PCR investigation – due to its differential sensitivities to
307 different *Brachyspira* species – may miss bacterial species the quantity of which is
308 close to the detection limit and may thus lead to different results concerning the
309 involved bacterial species in samples with varying preparation history, like
310 mucosal scrapings or PET samples. This result also agrees with findings of
311 Råsbäck et al. (2005) who emphasize the difficulties in the detection of mixed
312 infections with weakly haemolytic *Brachyspira* spp.

313 According to the results of this study mixed infections with weakly haemolytic
314 *Brachyspira* seem to occur quite frequently (37.1 % of all positive samples),
315 which is clearly discrepant to Råsbäck et al. (2005) who found only 1.7 - 3.8 %
316 multiple infections. Probably mixed infections are underreported in the literature,
317 because on the one hand the detection of more than one *Brachyspira* sp. in culture
318 can be complicated by the overgrowth of one of the species (Møller et al., 1998)

319 and on the other hand biochemical typing is not designed to identify multiple
320 infections (Fellström et al., 1996, Fossi et al., 2004, Råsbäck et al., 2005).
321 Overgrowth often seems to be the case with *B. murdochii*, which were
322 comparatively easily detected by culture. Other weakly haemolytic *Brachyspira*
323 species, which were found in mixed infections with *B. murdochii* by PCR, but not
324 by culture, are probably more fastidious and were thus never detected by
325 cultivation.

326 Intriguingly, PCR in PET material was largely comparable with PCR from
327 scrapings. So species-specific PCR on PET material detected 91 % of the
328 *Brachyspira* spp. infections achieved by PCR on scraping material. However,
329 genus-specific PCR on PET material was positive for *Brachyspira* in only 78 % of
330 the samples. This is probably due to degeneracy of the primers and the resulting
331 lower sensitivity as well as the longer amplicates generated with the BrNox
332 primer set. Also, the specific primer sets producing short amplicates (*B.*
333 *pilosicoli* and *B. intermedia*) yield more reproducible results if applied to PET
334 than primer sets in use for identification of *B. innocens*, the amplification product
335 of which is longer.

336 Nonetheless the species-specific PCR system described here can be applied to
337 PET material with only very minor loss of sensitivity and is thus well suited for
338 retrospective studies. Together with histopathological examinations (bacterial load
339 and colonic lesions) this method is also an appropriate tool for interpretation of
340 the pathogenicity of involved *Brachyspira*.

341 Infections with weakly haemolytic *Brachyspira* did not cause outbreaks of
342 enterocolitis in the present study. However, they might contribute to wasting and
343 diarrhoea in some cases, as these syndroms were more frequently associated with
344 *Brachyspira*-positive than with *Brachyspira*-negative pigs. Colitis was more

345 frequently associated with *B. pilosicoli*, *B. intermedia* and *B. innocens*, - both in
346 single and mixed infections – than with single infections with *B. murdochii*. Cases
347 of colitis with no obviously involved pathogens other than *Brachyspira* comprised
348 *B. innocens* infections (two single, one mixed) and one *B. murdochii* infection. In
349 these cases also a high spirochaetal load seemed to be responsible for the
350 pathogenicity of the diagnosed *Brachyspira*. These findings are in accordance
351 with Neef et al. (1994), Weissenböck et al. (2005) and Jensen and Boye (2006),
352 who asserted the possibility of the pathogenicity of *B. murdochii* and/or *B.*
353 *innocens*.

354 We could not find significant differences in the colonic pathology in cases of
355 *Brachyspira* multiple infections with and without the involvement of *B. pilosicoli*.
356 This could be explained by a low load of *B. pilosicoli* in these samples, which
357 were thus not capable of inducing lesions compatible with intestinal
358 spirochaetosis or a persistent infection (Duhamel, 2001).

359 In conclusion, this study presents evidence that *B. murdochii*, *B. innocens* and *B.*
360 *intermedia* are most frequently present in routine diagnostic submissions of pigs
361 with miscellaneous chronic diseases. More than a third these cases were double or
362 multiple infections. Association with colonic pathology is more likely when
363 certain *Brachyspira* species (predominantly *B. innocens*) are present in high
364 numbers.

365

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371

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Table 1 Comparison of case histories, pathological and other laboratory findings in *Brachyspira*-positive and *Brachyspira*-negative pigs.

	<i>Brachyspira</i> -positive (n=27)	<i>Brachyspira</i> -negative (n=175)
Case history		
wasting	22 (81.5%)	89 (50.8%)
diarrhoea	16 (59.3%)	81 (46.3%)
respiratory signs	11 (40.7%)	71 (40.6%)
Pathological diagnoses		
colitis	14 (51.8%)	44 (25.1%)
respiratory diseases	15 (55.6%)	147 (84%)
Laboratory findings		
PCV-2 infection	17 (63%)	96 (54.8%)
PMWS	6 (22.2%)	56 (32%)
<i>L. intracellularis</i> infection	4 (14.8%)	12 (6.8%)
other enteric bacteria	7 (25.9%)	70 (40%)

Table 2 Compilation of intestinal necropsy- and histological findings, spirochaetal- and non-spirochaetal colonization, results of bacteriological examinations of colonic mucosa and presence of specified other pathogens or infections in *Brachyspira*-positive cases

No.	Wasting	Necropsy	Histology	Colitis	Warthin Starry	Bacteriology	Other infections
1	W	Fw, Mt	I++, D++, A++	C	S+, b++	E.c., Cl.p.	PIA, PCV2++
2	W	Fp	I++, D+		S+++; b+		PIA, PCV2++
3	W	no lesions	I++, D++		S++, b+		PCV2++
4	-	no lesions	I++, D+, A+		S+, b++		
5	-	no lesions	I+, D+		S++, b+		PCV2+
6	W	Fp	I+, D++		b++		PCV2++
7	W	Fmb; Mth	I+, D+	C	S+, b+		PCV2+++; PMWS
8	W	Fw	I++, D++, A+	C	S+++; b+		
9	-	no lesions	I++, D++		S+, b++		
10	W	no lesions	I++, D+, A+		S+, b++		PCV2+
11	W	no lesions	I++, D+, A++		S++, b++	E.c.	PCV2+
12	W	Fw, Mt	I++, D++, A+	C	S+++; b++		
13	-	no lesions	I++, D+		S++, b++		
14	W	no lesions	I++, D++		S+, b+		PCV2++; PMWS
15	W	Fmb, Mt	I++, D++, A+	C	S+, b++		PCV2+
16	W	Fw	I++, D++, A++	C	S++, b+++		
17	W	Fmb, Mth	I+, D++, A+	C	S++, b++		PCV2+
18	W	Fw, Mth	I++, D+++; A++	C	S++, b+++	haem.E.c.	
19	-	Fmb, Mh	I++, D++	C	S+++; b+		
20	W	Mt	I++, D++, A+		S++, b++	haem.E.c.	PCV2+
21	W	no lesions	I+, D+, A+		S+, b++		PCV2+++; PMWS
22	W	no lesions	I+++; D++, A++	C	b+		PIA
23	W	Fw	I++, D+++; A+		S+, b++	E.c.	PIA
24	W	Fw, Mth	I+++; D+, A+	C	S+++; b++		PCV2+
25	W	Fmb	I++, D++	C	S+++; b+	haem.E.c.	PCV2+
26	W	Fmb, Mth	I+, D++	C	S+++; b++		PCV2+
27	W	Fp, Mh	I+, D+++	C	S+, b+	haem.E.c., Cl.p., S.c.s.	PCV2+++; PMWS

Wasting: W: present case originated from a group with wasting problems

Fw: fecal consistency watery
Fp: fecal consistency pasty
Fmb: fecal consistency mucoid with admixture of blood
Mth: Mucosa thickened (t) and/or hyperaemic (h)
I: lymphohistiocytic infiltration of lamina propria
D: dilated crypts containing mucus
A: crypt abscesses
+: mild; small numbers
++: moderate; moderate numbers
+++: severe; high numbers
C: present case classified as colitis
S: spirochaetal colonization (loosely coiled bacteria in crypt lumina)
b: colonization with other bacteria
PIA: porcine intestinal adenomatosis, *Lawsonia intracellularis* infection
PCV2: porcine circovirus- 2 infection
PMWS: postweaning multisystemic wasting syndrome
E. c.: *Escherichia coli*
haem. E.c.: *haemolytic Escherichia coli*
Cl. p.: *Clostridium perfringens*
S. c.s.: *Salmonella cholerae suis*

Table 3 Detection limits of the genus- and species-specific PCR (expressed as colony forming units of bacteria per ml spiked scraping samples)

Brachyspira species	Bacterial concentration (cfu/ml)	Detection limits of species-specific primers	Detection limits of genus-specific primers
<i>B. hyodysenteriae</i>	9.6×10^7	3.8×10^4	9.6×10^4
<i>B. pilosicoli</i>	1.8×10^7	7.2×10^3	1.8×10^4
<i>B. murdochii</i>	1×10^7	4×10^2	4×10^4
<i>B. intermedia</i>	8.5×10^7	3.4×10^3	3.4×10^4
<i>B. innocens</i>	1.2×10^8	4.8×10^2	4.8×10^4

Dilution series were performed in duplicate

Table 4 Distribution of *Brachyspira* species in PCR positive samples. Compilation of scraping-, and PET- and culture-PCR results. n=27

Detected species	Number of samples	%
mur	11	40.7
inn	4	14.8
int	1	3.7
pil	1	3.7
mur+inn	4	14.8
mur+int	1	3.7
mur+pil	1	3.7
inn+pil	1	3.7
mur+inn+pil	1	3.7
mur+inn+int+pil	2	7.4

mur: *B. murdochii*; inn: *B. innocens*; int: *B. intermedia*; pil: *B. pilosicoli*

Table 5 Comparison of PCR results of scrapings vs. PET vs. isolates and biochemical differentiation

Sample No.	Scraping PCR	PET PCR	Biochemical differentiation	Isolate PCR
1	pil	pil	pil	pil
2	inn	inn	pil	pil + inn
3	neg	inn	mur	mur
4	mur + int	mur + int	pil	int + pil + inn
5	mur	mur	mur	mur
6	neg	neg	mur	mur
7	n.s.	neg	mur	mur
8	mur	mur+inn	mur	mur
9	mur	mur	mur	mur
10	mur	mur	mur	mur
11	mur	mur	mur	mur
12	mur	mur	mur	mur
13	mur	mur	ND*	ND
14	mur	mur	ND	ND
15	int	int	ND	ND
16	inn	inn	ND	ND
17	inn	inn	ND	ND
18	mur + inn	mur	ND	ND
19	inn	inn	mur	mur
20	inn + mur	mur + pil	ND	ND
21	neg	neg	mur	mur
22	neg	neg	mur	mur
23	inn	neg	ND	ND
24	mur + inn + int	mur + int + pil	ND	ND
25	mur	mur + pil	ND	ND
26	mur + inn	mur + inn	ND	ND
27	mur + int	int	ND	ND

n.s.: not specified, only with genus specific primers positive

mur: *B. murdochii*; inn: *B. innocens*; int: *B. intermedia*; pil: *B. pilosicoli*

neg: PCR negative

*: biochemical differentiation not done because of unsuccessful cultivation