



HAL
open science

Phenotypic and molecular characterization of spp. isolated from laying hens in different housing systems

D.S. Jansson, C. Fellström, T. Råsbäck, I. Vågsholm, A. Gunnarsson, F.
Ingermaa, K.-E. Johansson

► To cite this version:

D.S. Jansson, C. Fellström, T. Råsbäck, I. Vågsholm, A. Gunnarsson, et al.. Phenotypic and molecular characterization of spp. isolated from laying hens in different housing systems. *Veterinary Microbiology*, 2008, 130 (3-4), pp.348. 10.1016/j.vetmic.2008.02.010 . hal-00532398

HAL Id: hal-00532398

<https://hal.science/hal-00532398>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Phenotypic and molecular characterization of *Brachyspira* spp. isolated from laying hens in different housing systems

Authors: D.S. Jansson, C. Fellström, T. Råsbäck, I. Vågsholm, A. Gunnarsson, F. Ingermaa, K.-E. Johansson



PII: S0378-1135(08)00066-7
DOI: doi:10.1016/j.vetmic.2008.02.010
Reference: VETMIC 3964

To appear in: *VETMIC*

Received date: 23-12-2007
Revised date: 11-2-2008
Accepted date: 14-2-2008

Please cite this article as: Jansson, D.S., Fellström, C., Råsbäck, T., Vågsholm, I., Gunnarsson, A., Ingermaa, F., Johansson, K.-E., Phenotypic and molecular characterization of *Brachyspira* spp. isolated from laying hens in different housing systems, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.02.010

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1
2 Phenotypic and molecular characterization of *Brachyspira* spp.
3 isolated from laying hens in different housing systems
4
5

6 **D.S. Jansson^{a, b*}, C. Fellström^b, T. Råsbäck^c, I. Vågsholm^d, A. Gunnarsson^c, F.
7 Ingermaa^c, K-E. Johansson^{c, e}**
8

9 ^a*Department of Pigs, Poultry and Ruminants, National Veterinary Institute (SVA), SE-751 89*
10 *Uppsala, Sweden*

11 ^b*Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), P.O.*
12 *Box 7018, SE-750 07 Uppsala, Sweden*

13 ^c*Department of Bacteriology, National Veterinary Institute (SVA), SE-751 89 Uppsala,*
14 *Sweden*

15 ^d*Department of Antibiotics, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden*

16 ^e*Department of Biomedical Sciences and Veterinary Public Health, P. O. Box 7009, Swedish*
17 *University of Agricultural Sciences (SLU), SE-750 07 Uppsala, Sweden*

18 *Corresponding author Tel.: +46 18 674000; fax: +46 18 309162. E-mail address:

19 desiree.s.jansson@sva.se (D. S. Jansson).
20

21 **Keywords:** Avian intestinal spirochaetes; *Brachyspira*; Chicken; Housing system; PCR;
22 Phenotype; 16S rRNA gene sequencing
23

24 **Short title:** *Brachyspira* spp. in laying hens

25 **Abstract**

26 Several species of intestinal spirochaetes, *Brachyspira* (*B.*) *alvinipulli*, *B. intermedia* and *B.*
27 *pilosicoli*, may cause reduced egg production and faecal staining of eggshells in chickens. The
28 aim of this study was to characterize potentially pathogenic and presumably non-pathogenic
29 *Brachyspira* spp. from commercial laying hens. Selective culture, phenotyping, PCR and 16S
30 rRNA gene sequencing were used and clinical data were collected. Phenotypic profiles were
31 obtained for 489 isolates and 351 isolates obtained after subculture, and 30 isolates were
32 selected for molecular characterization. Seven isolates were positive by a *B. intermedia*-
33 specific PCR based on the *nox* gene, and two were positive in a *B. hyodysenteriae*-specific
34 23S rRNA gene based PCR. By comparative phylogenetic analysis in combination with PCR
35 and phenotyping, seven isolates were identified as *B. intermedia*, eight isolates as *B. innocens*,
36 five as *B. murdochii*, and three isolates each as *B. alvinipulli* and “*B. pulli*”. The remaining
37 four isolates could not be assigned to any presently recognized species. Co-infection with
38 several species or genetic variants of *Brachyspira* spp. were detected in some flocks and
39 samples, suggesting a high level of diversity. Organic flocks with access to outdoor areas
40 were at higher risk (RR=2.3; 95% CI 1.5-3.6) for being colonized than chickens in other
41 housing systems. No significant differences between colonized and non-colonized flocks were
42 found regarding clinical parameters i.e. mortality, egg production, faecally contaminated
43 eggshells, and wet litter. Our results show that a combination of traditional laboratory
44 diagnostics, molecular tests and phylogeny is needed for identification of *Brachyspira* sp.
45 from chickens.

46 1. Introduction

47 The genus *Brachyspira* currently consists of seven recognized species; *B. aalborgi* (Hovind-
48 Hougen et al., 1982), *B. alvinipulli* (Swayne et al., 1995; Stanton et al., 1998), *B.*
49 *hyodysenteriae* (Taylor and Alexander, 1971; Ochiai et al., 1997), *B. innocens* (Stanton, 1992;
50 Ochiai et al., 1997), *B. intermedia* (Stanton et al., 1997; Hampson and La, 2006), *B.*
51 *murdochii* (Stanton et al., 1997; Hampson and La, 2006) and *B. pilosicoli* (Taylor et al., 1980;
52 Trott et al., 1996; Ochiai et al., 1997). Two additional species have been officially proposed;
53 “*B. canis*” (Duhamel et al., 1998) and “*B. suanatina*” (Råsbäck et al., 2007), and the name “*B.*
54 *pulli*” has been assigned to a distinct group of chicken isolates based on data from multilocus
55 enzyme electrophoresis (MLEE) (Stephens and Hampson 1999; Stephens et al., 2005) and
56 16S rRNA gene sequence data (Phillips et al., 2005). All these genotypes except *B. aalborgi*,
57 and “*B. canis*” have, so far, been isolated from at least one bird species. In chickens, *B.*
58 *alvinipulli*, *B. intermedia* and *B. pilosicoli* are considered as potentially pathogenic species
59 causing reduced egg production, delayed start of lay, increased water content in faeces and
60 faecal staining of eggshells, while *B. innocens*, *B. murdochii* and “*B. pulli*” are presumed to
61 be non-pathogenic species. Intestinal spirochaetes isolated from chickens are, with occasional
62 exceptions, weakly haemolytic, and their growth pattern on agar plates does not allow reliable
63 differentiation between species. Presently, differentiation between potentially pathogenic and
64 presumably non-pathogenic *Brachyspira* spp. of chicken origin require molecular diagnostic
65 methods such as PCR, MLEE, pulsed field gel electrophoresis (PFGE), *nox* restriction
66 fragment length polymorphism (RFLP) and/or sequencing of the 16S rRNA gene.

67 The aim of the current study was to characterize representative isolates of *Brachyspira*
68 spp. from commercial laying hens, and to increase the understanding of *Brachyspira* sp.
69 diversity among laying hens in different housing systems.

70

71 **2. Materials and methods**

72 *2.1. Study population and sampling*

73 The population under study in May 2003 to June 2004 consisted of five to six million
74 Swedish commercial laying hens (number estimated by J. Bengtsson, Swedish Board of
75 Agriculture, and J. Yngvesson, Swedish Welfare Agency, pers. commun.). Due to welfare
76 concerns and national legislation the industry was in the process of replacing conventional
77 battery cages with furnished cages (cages with perches, nests and litter boxes), single-tiered
78 floor systems (with litter area, perches and manure bin or a manure removal system), multi-
79 tiered aviary systems (with litter area, perches and manure removal system) and organic
80 production (litter-based housing indoors, organic feed, comparatively low stocking density
81 and access to outdoor pens and pasture) (Tauson, 2005).

82 In the spring of 2003, 104 commercial laying hen farms and one flock on each of these
83 farms were randomly selected from data sets of the Swedish Board of Agriculture and
84 KRAV[®] Incorporated Association (the Swedish certification body for organic production) to
85 include the different housing systems in use except conventional battery cages which were
86 being phased out. An equal proportion of flocks representing each housing system were
87 included. Between May 2003 and June 2004 twenty individual and undisturbed samples of
88 caecal droppings were collected from each flock. To minimize age and seasonal effects, the
89 sampling was carried out when selected chickens reached the approximate age of 65 weeks.
90 Depending on the housing system, droppings were collected from the litter area or from
91 furniture and equipment such as manure conveyor belts, tiers, perches and/or on and under
92 slats. The samples were transported in Amies medium (Venturi Transystem[®], Copan
93 innovation, Italy) at ambient temperature by surface mail. The following data on the sampled
94 flocks and farms were collected from the producers at time of sampling; layer hybrid(s),
95 cumulative mortality (number of dead and euthanized chickens from day of transfer to the

96 laying hen house at approximately 14 to 16 weeks of age until day of sampling/original
97 number of transferred hens*100), egg production (no. of eggs laid per day on day of
98 sampling/no. hens on that day*100), and occurrence of faecal staining of eggshells (cut-off
99 point 5%), wet litter and whether the flock had been treated with antimicrobials.

100

101 2.2. Culture, phenotyping and selection of isolates

102 The samples were cultured within 24 h at the Department of Bacteriology, National
103 Veterinary Institute (SVA), Uppsala, Sweden according to a previously described protocol
104 (Fellström and Gunnarsson, 1995). From each selective agar plate a single isolated haemolytic
105 centre of suspected spirochaete growth was collected. Pure spirochaetal growth on fastidious
106 anaerobe agar (FAA) plates was assessed by phase contrast microscopy. If contamination
107 with non-spirochaetal bacteria was present, the isolate was excluded from the study.

108 Phenotypic characterization included intensity of haemolysis and biochemical tests, i.e. spot
109 indole, hippurate, α -galactosidase and β -glucosidase tests. All indole and hippurate positive
110 isolates, and also, at least two isolates per flock of all other phenotypes were stored in liquid
111 nitrogen in the strain collection of SVA. A preliminary selection of isolates to be further
112 characterized was made to include all detected phenotypic profiles, all housing systems, and
113 approximately half of the colonized flocks. In particular, isolates possessing phenotypes of
114 potentially pathogenic species were selected for further study.

115 Isolates were subcultured whenever a mixture of spirochaetal genotypes were shown to be
116 present in the isolate by 16S rRNA gene sequencing, or suspected to be present based on a
117 phenotypic profile not complying with the classification scheme of porcine *Brachyspira* spp.
118 (Fellström et al., 1999). Subculturing was also made when there was consistently more than
119 one distinct growth pattern present on FAA plates, or in some cases when several different
120 phenotypic profiles had been detected from separate samples from the same flock.

121 Approximately 10^8 bacterial cells from the FAA plate were subjected to tenfold serial dilution
122 in brain heart infusion (BHI) broth (National Veterinary Institute, Uppsala, Sweden). Eight
123 samples of 0.1 ml broth, which were expected to contain between 1 and 100 bacterial cells
124 each, were seeded on FAA plates and were cultured anaerobically at 42°C for 48 to 72 h
125 depending on their growth rate. Bacteria within at least five well demarcated haemolytic
126 centres were then transferred to new FAA plates, and were cultured for at least three days
127 before phase contrast microscopic examination and phenotyping were performed. Isolates
128 representing all different phenotypes that were obtained after subculture were stored in the
129 strain collection of SVA. A final selection of presumed non-mixed spirochaete isolates for
130 molecular characterization was done according to the same criteria as for the preliminary
131 selection.

132

133 2.3. PCR amplification, 16S rRNA gene sequencing and phylogenetic analysis

134 PCRs targeting the NADH-oxidase (*nox*) gene of *B. hyodysenteriae* (Atyeo et al., 1999) and
135 *B. intermedia* (Atyeo et al., 1999, and Phillips et al., 2005 with a modified forward primer (N.
136 Phillips, Murdoch University, pers. commun.), the 23S rRNA gene of *B. hyodysenteriae* and
137 *B. intermedia* (Leser et al., 1997), the *tlyA* gene of *B. hyodysenteriae* and the 16S rRNA gene
138 of *B. pilosicoli* (the latter two as a duplex PCR system) (Råsbäck et al., 2006) were performed
139 two to four times each on the final selection of isolates. The almost complete 16S rRNA gene
140 sequences (1433 to 1434 nts) were determined as previously described (Pettersson et al.,
141 1996; Johansson et al., 2004). Primers used for the PCR systems and for PCR amplification of
142 the 16S rRNA gene sequencing are listed in Table 1. Almost complete 16S rRNA gene
143 sequences (1433 nts) were also determined for the type strains of *B. alvinipulli* (C1^T) and *B.*
144 *intermedia* (PWS/A^T) obtained from the American Type Culture Collection (ATCC[®]) because
145 previously deposited sequences (GenBank accession numbers U23033 and U23030)

146 contained ambiguities. Accession numbers of 16S rRNA gene sequences retrieved from the
147 GenBank database that were used in the phylogenetic analysis, pertaining to type, reference
148 and field strains, and the new sequences for type strains C1^T and PWS/A^T are presented in
149 Table 2. The 16S rRNA gene sequences of type strains of *Borrelia burgdorferi* and
150 *Treponema denticola* (GenBank accession numbers X98228 and AF139203) were used as
151 outgroup for the phylogenetic analysis. The tree was constructed by the neighbour-joining
152 method from a distance matrix comprising approximately 1220 nucleotide positions as
153 previously described (Johansson et al., 2004).

154

155 2.4. Statistical analysis

156 The relative risk for *Brachyspira* spp. colonization in organic flocks vs. other housing systems
157 was assessed by chi square tests and the 95% confidence interval was estimated. Egg
158 production and cumulative mortality was compared for colonized and non-colonized flocks
159 using ANOVA. For differences in faecally contaminated eggshells and wet litter between
160 colonized and non-colonized flocks, the chi square procedure was used. For significant
161 differences a significance level of 5% was required. The data were analyzed by the Proc GLM
162 and FREQ procedures from SAS[®] (Statistical Analysis Systems) Institute (SAS Institute Inc.,
163 SAS/STAT Software version 8, SAS Institute, Cary, NC, USA).

164

165 3. Results

166 3.1. Study population and sampling

167 Samples ($n=1840$) were submitted from 92 of 104 selected flocks, representing 732.000
168 chickens. In one case, the samples were excluded from the study as they arrived after the pre-
169 defined sampling period. In another case, the flock was euthanized prior to sampling due to
170 salmonellosis. The other farmers gave lack of time as the main reason for not submitting

171 samples. None of the flocks had received antimicrobials prior to sampling. The farms were
172 located in 19 of 21 counties, with 68 farms in the southern region of Sweden (Götaland), 17
173 farms in the middle region (Svealand) and seven farms in the northern region (Norrland), in
174 line with the general distribution of the laying hen farms in Sweden. Several different hybrids
175 were represented among the flocks; Lohman Selected Leghorn (LSL) $n=52$, Lohmann Brown
176 (LB) $n=7$, LSL and LB $n=7$, Hy-Line White W-36 and W-98 $n=23$ and Hy-Line Brown $n=3$.
177 The proportions of housing systems differed between regions and hybrids varied between
178 housing systems.

179

180 3.2. Culture and phenotypes

181 Weakly haemolytic intestinal spirochaetes were isolated from 525 of 1840 samples. Culture
182 positive samples originated from 37 of the 92 sampled flocks. The phenotypic profiles of 489
183 isolates are given in Table 3 and 36 cultures that were shown by phase contrast microscopy to
184 be contaminated with non-spirochaetal bacteria were discarded. Between one and five
185 phenotypic profiles were identified within individual flocks (Table 4). In 19 of 37 colonized
186 flocks only one phenotype was detected. Based on the pre-set selection criteria 164 isolates
187 were selected for storage, and 37 of these isolates were preliminary selected for molecular
188 tests as representing all detected phenotypes and 20 of the 37 colonized flocks. The isolates
189 represented flocks with one single phenotype as well as those colonized by several
190 phenotypes. Sequencing of the 16S rRNA gene showed that 12 of the 37 preliminary selected
191 isolates were of non-mixed 16S rRNA sequence types, and the remaining 25 cultures were
192 under suspicion of being of mixed genotypes. This was based on the finding of multiple
193 sequence ambiguities, or in some cases based on non-consistent phenotypic profiles after
194 repetitive phenotypic testing or on morphologic traits on FAA plates. These 25 remaining
195 isolates were subcultured after tenfold serial dilution. Table 5 shows the phenotypic results of

196 351 isolates produced after subculture of the 25 isolates. Up to six phenotypic profiles, which
197 differed from the profile of the subcultured isolates, were produced. The phenotype of 194 of
198 the 351 isolates obtained after subculture, differed from the original phenotype, and two of the
199 phenotypic profiles could not be retrieved as presumably non-mixed spirochaete cultures i.e.
200 w++++ and w+++ (designations explained in Tables 3 to 6). In several cases, isolates
201 obtained after subculture, were phenotypically identical but showed different growth patterns
202 on FAA plates. The final selection of 30 isolates for molecular tests consisted of 12 non-
203 subcultured isolates and 18 isolates obtained after subculture (Table 6). The selected isolates
204 represented 18 of the 37 colonized flocks, and some flocks were represented by more than one
205 isolate (flocks A, B, C, D, E, H, I, Q, and R in table 6).

206

207 3.3. Species specific PCR, sequencing of the 16S rRNA gene and phylogenetic analysis

208 PCR results are presented in Table 6. Resequencing of type strains C1^T and PWS/A^T
209 identified all nucleotides previously deposited as ambiguities (seven and six nucleotides,
210 respectively). Additionally, the new sequences for strains C1^T (GenBank accession number
211 EF455559) and PWS/A^T (GenBank accession number EF455560) differed in three positions
212 each from the earlier deposited sequences (C1^T: A to G in position 223, C to T in position 887
213 and G to A in position 1298; PWS/A^T: A to G in position 61, A to T in position 673 and C to
214 T in position 760; positions as given in new sequences). Accession numbers to the 16S rRNA
215 gene sequences of the resequenced type strains and the selected chicken isolates are given in
216 Tables 2 and 6. An insertion of thymidine in the polyT region following position 973 was
217 detected in isolate AN5156/03.

218 A phylogenetic tree representing the evolutionary relationships among 16S rRNA gene
219 sequences is shown in Fig. 1. All nine of the 30 characterized isolates with the phenotype of
220 the potentially pathogenic species *B. intermedia* (+--+ in Table 6) clustered monophyletically

221 in the phylogram together with type, reference and field strains of *B. intermedia* (from pigs
222 and chickens), *B. hyodysenteriae* (from pigs, mallards and rheas) and “*B. suanatina*” (from
223 mallards and a pig). Based on a combination of phenotype, PCR and 16S rRNA gene
224 sequence data we propose that seven of the nine isolates represent *B. intermedia*, while the
225 remaining two could not be unequivocally assigned to any of the presently recognized
226 *Brachyspira* spp. A second monophyletic cluster consisted of two sublineages and one single
227 species line. The single species line was represented by the type strain of *B. innocens*. One
228 sublineage contained 13 Swedish chicken isolates and the type strain of *B. murdochii*. None
229 of the Swedish chicken isolates belonging to this group were positive by the PCR-assays, and
230 phenotypically they lacked indole production and hippurate cleavage capacity, possessed β -
231 glucosidase activity and some also had α -galactosidase activity, which is consistent with a
232 classification of presumably non-pathogenic *B. innocens* and *B. murdochii*, respectively. The
233 other sublineage of the second cluster contained three Swedish chicken isolates
234 (AN2929/1/03, AN3382/1/03, AN304/04) from three separate flocks, two Australian chicken
235 strains provisionally designated “*B. pulli*”, and a canine strain which in an earlier study on
236 canine brachyspiras showed a position in a phylogenetic tree which was termed the CN2
237 subcluster within the “*alvinipulli* cluster” (Johansson et al., 2004). A third monophyletic
238 cluster contained three Swedish chicken isolates (AN3382/2/03, AN1263/2/04, AN1268/3/04)
239 from two flocks, a Swedish canine strain (AN4578/01) and the type strain of *B. alvinipulli*
240 (C1^T). The Swedish chicken strains as well as the previously reported canine strain were
241 negative in the indole spot test, had hippurate cleavage capacity, and possessed no α -
242 galactosidase activity, which corresponds to the phenotypic profile of the type strain of *B.*
243 *alvinipulli* (C1) (Stanton et al., 1998). The remaining two chicken isolates could not be
244 assigned to any of the presently known *Brachyspira* spp. Isolate AN3172/1/03 formed a
245 subbranch to the *B. alvinipulli* cluster, and the other unidentified isolate (AN1268/7/04)

246 branched off from the cluster which contained Australian chicken isolates provisionally
247 termed the “*B. pulli*” cluster. The observed phenotypes of these isolates (Table 6) have
248 hitherto not been assigned as characteristic or indicative of any known or proposed species
249 within genus *Brachyspira*. None of the selected isolates clustered with the reference strain of
250 *B. pilosicoli* in the phylogram (Fig. 1), nor did any of them possess the signature sequence of
251 *B. pilosicoli* (hexa-T region at the positions homologous to positions 208 to 211 in
252 *Escherichia coli*).

253 In several flocks more than one *Brachyspira* sp. or genotype originated from the same or
254 from different samples (flocks B, C, D, E, H, I, Q, and R). For instance, different *Brachyspira*
255 species were found in subcultures from the same sample in flock B (*B. intermedia* and “*B.*
256 *pulli*” in AN2929/2/03 and AN2929/1/03, respectively) and in flock C (“*B. pulli*” and *B.*
257 *alvinipulli* in AN3382/1/03 and AN3382/2/03, respectively). Yet another species was found in
258 another sample from flock C (*B. intermedia* in AN3370/03). In other flocks we detected
259 different genotypes of the same species in different samples; such as in flock E, which was
260 colonized by two genotypes of *B. intermedia* (isolates AN5102/11/03 and AN5112/03) and
261 flock R in which two genotypes of *B. alvinipulli* were detected (isolates AN1263/2/04 and
262 AN1268/3/04). Another subculture of sample AN1268/04 could not be identified to species
263 level (AN1268/7/04). On the other hand, isolates with identical 16S rRNA gene sequences
264 were found in some cases when several samples were examined from the same flock (eg. *B.*
265 *intermedia* isolates AN1828/03 and AN1831/03 in flock A and AN3536/03 and AN3541/03
266 in flock D). Also in flock E, all isolates possessed the same phenotype but two different
267 growth patterns on FAA plates, which by sequencing were shown to be different species; *B.*
268 *innocens* (AN315/04) and “*B. pulli*” (AN304/04).

269

270 *3.4. Statistical and geographical analysis*

271 A summary of collected clinical data and culture results with regard to housing systems are
272 shown in Table 7. In this study, organic laying hen flocks were at higher risk (RR=2.3; 95%
273 CI 1.5-3.6) for being colonized by *Brachyspira* spp. than laying hens in other housing
274 systems. No significant differences were found regarding cumulative mortality, egg
275 production, presence of faecally contaminated eggshells or wet litter between colonized and
276 non-colonized flocks. Also, the six flocks that were colonized by potentially pathogenic
277 *Brachyspira* spp. were not significantly different with regard to clinical parameters to the
278 other colonized flocks. Culture positive flocks were identified from 13 of 19 counties and
279 from the southern and middle region (Götaland 41% and Svealand 53%) with potentially
280 pathogenic *Brachyspira* spp. present in five counties and both regions.

281

282 **4. Discussion**

283 Previously published molecular studies of *Brachyspira* spp. from individual chicken flocks or
284 isolates selected from strain collections have shown that avian *Brachyspira* communities are
285 probably more diverse than their porcine counterparts, but diagnostic difficulties have
286 hampered the advance in this field of research. In this study, the use of traditional laboratory
287 diagnostics, i.e. anaerobic culture and phenotypic tests, was combined with molecular tests
288 and phylogeny to characterize and investigate the diversity of *Brachyspira* spp. from
289 commercial laying hens.

290 Phenotypic tests are useful for initial characterization of porcine *Brachyspira* isolates
291 (Fellström et al., 1999). However, the utility of such tests for *Brachyspira* isolates of chicken
292 origin has not been fully investigated, and their value as diagnostic tools has been questioned
293 (Stephens et al., 2005; Townsend et al., 2005). In the present study, phenotypic results were
294 sometimes inconsistent after storage in liquid nitrogen (results not shown). Lack of
295 reproducibility was most probably caused by mixed spirochaetal isolates despite the fact that

296 great care was taken to select bacteria from a single, well demarcated haemolytic centre from
297 each selective agar plate. Different growth rates of various genotypes or even loss of a
298 genotype during storage may have caused phenotypic variability. Subculturing after serial
299 dilution was often necessary to obtain presumed non-mixed isolates for molecular analyses.
300 When applied to presumably non-mixed spirochaete cultures the phenotypic tests produced
301 not only very consistent results, but were also in full or very close agreement with previously
302 published phenotypic results of each *Brachyspira* sp. and corresponded with phylogenetic
303 results. Currently, reliable diagnosis to species level within genus *Brachyspira* cannot be
304 achieved by using solely phenotypic traits. However, our results suggest that phenotyping of
305 avian *Brachyspira* spp. is a useful tool for screening and preliminary characterization, but it
306 needs to be further evaluated in terms of reproducibility. An alternative approach for selecting
307 isolates for molecular analyses is randomization, but this may lead to failure to identify low-
308 prevalent genotypes.

309 The available species-specific PCRs used for this study allowed identification of a subset of
310 the isolates to species level. One of the PCRs specific for *B. intermedia* (Phillips et al., 2005
311 with a modified primer) identified seven of nine isolates with a phenotype consistent with *B.*
312 *intermedia*, while the other two PCRs, which have been developed for identification of
313 porcine *B. intermedia*, identified two of nine (Leser et al., 1997) or none of these isolates
314 (Atyeo et al., 1999). One of these PCRs has previously been shown to lack sensitivity when
315 applied to avian *B. intermedia* strains (Atyeo et al., 1999), which complied with the results of
316 this study. The other PCR (Leser et al., 1997) was reported to give 100% sensitivity but only
317 94.3% specificity when applied to a collection of *Brachyspira* spp. from several different
318 animal species including chickens (Suriyaarachchi et al., 2000) which is in contrast with our
319 results. This is a point of concern for diagnostic laboratories, which sometimes rely solely on
320 PCR for species identification. Furthermore, the remaining two of the nine isolates with a

321 phenotype consistent with *B. intermedia* were positive by the *B. hyodysenteriae*-specific 23S
322 rRNA gene based PCR and were not identified by the three PCRs specific for *B. intermedia*.
323 Notwithstanding these PCR results, the two isolates were not identified as *B. hyodysenteriae*
324 based on weak haemolysis and failure to react in the other PCR systems specific for *B.*
325 *hyodysenteriae*. Also, another problem with PCR is that there is no assay currently available
326 for *B. alvinipulli* and “*B. pulli*”. Clearly, reliable diagnostic PCR systems for all avian
327 *Brachyspira* spp. are needed, and they should be applied in combination with other tests until
328 fully evaluated.

329 The phylogenetic analysis showed that the nine chicken isolates with a phenotype consistent
330 with *B. intermedia* formed several sublineages within the monophyletic cluster containing
331 type, reference and field strains of *B. hyodysenteriae* and *B. intermedia*, and isolates of the
332 recently proposed new species “*B. suanatina*” (Fig. 1). As previously shown, phylogeny
333 based on the 16S rRNA gene does not allow species identification within this cluster
334 (Pettersson et al., 1996). Most interestingly, two of the isolates in this cluster (AN1828/03 and
335 AN1831/03 from flock A) were very closely related phylogenetically to isolates of “*B.*
336 *suanatina*” isolated from pigs and mallards. However, they differed phenotypically from “*B.*
337 *suanatina*” in being weakly haemolytic and also they were PCR positive for the 23S rRNA
338 gene of *B. hyodysenteriae*. This finding supports the fact that there are no strict border lines in
339 nature. The more strains that are analyzed from closely related bacterial species, the greater is
340 the chance to find strains that cannot be unambiguously assigned to any of these species.

341 Based on the sequence data presented in this study we propose that the canine isolate CN2,
342 the Australian chicken spirochetes provisionally termed “*B. pulli*” and the three closely
343 related Swedish chicken isolates (second sublineage of the second monophyletic cluster)
344 belong to a single species, and that these should all provisionally be designated “*B. pulli*”. “*B.*
345 *pulli*” was originally unofficially proposed because it formed a separate group in phenograms

346 constructed from MLEE data (McLaren et al., 1997, Stephens and Hampson, 1999, Stephens
347 et al., 2005), and later, sequence data have shown that these isolates form a distinct cluster in
348 phylograms based on the partial 16S rRNA gene sequence (Phillips et al., 2005). Isolates of
349 “*B. pulli*” are phenotypically indistinguishable from *B. innocens*, but as no representatives of
350 these two species have yet been shown to possess pathogenic properties, routine diagnostic
351 laboratories should focus primarily on discrimination of this group from potential pathogens.

352 The three chicken isolates (AN3382/2/03, AN1263/2/04, AN1268/3/04) and the canine
353 strain (AN4578/01) in the third monophyletic cluster most likely represent *B. alvinipulli*. The
354 type strain of *B. alvinipulli* (C1^T) was originally isolated from a commercial laying hen flock
355 suffering from diarrhoea in the United States (Swayne et al., 1992). Further characterization
356 and pathogenicity tests in SPF-chickens resulted in the description of a new spirochete
357 species, *B. alvinipulli* with enteropathogenic potential for chickens (Stanton et al., 1998). Few
358 isolates of *B. alvinipulli* have been reported after the original description. These include
359 isolates C2 and C3, which originate from the same flock as the type strain (Phillips et al.,
360 2005), isolate 805 from an Australian bird (Townsend et al., 2005) based on partial sequence
361 data of the 16S rRNA gene (1380 bp) and the *nox* gene (893 bp), and isolates from Hungarian
362 geese based on biochemical characteristics and partial sequence data of the 16S rRNA gene
363 (approximately 560 bp) (Nemes et al., 2006). Recently, preliminary findings of *B. alvinipulli*
364 were simultaneously reported from laying hens in Sweden, the Netherlands and the United
365 Kingdom at the 4th International Conference on Colonic Spirochaetal Infections in Animals
366 and Humans, Prague, Czech Republic 2007 (Jansson DS, National Veterinary
367 Institute/Swedish University of Agricultural Sciences, Uppsala, Sweden, Feberwee et al.,
368 Animal Health Service, Deventer, Netherlands and J. R. Thomson, Scottish Agricultural
369 College, Veterinary Services, Edinburgh, United Kingdom, pers. commun). The finding of *B.*
370 *alvinipulli* was later confirmed from chickens (Jansson et al., 2007; Feberwee et al., 2007a;

371 Feberwee et al., 2007b) and from anseriform wild birds (Jansson et al., 2007). The few
372 reports, and the fact that the identification of *B. alvinipulli* relies mainly on sequencing
373 results, seem to indicate that the occurrence of this species may be underestimated among
374 avian hosts.

375 The reason for the apparent absence of *B. pilosicoli* among the isolates in this study, which
376 differs from the situation in Australia (Phillips et al., 2005), remains unclear. The other two
377 potentially pathogenic species were only found in organic production (five flocks) and in a
378 flock housed in single-tiered floor system (Table 6). Furthermore, organic flocks and flocks
379 housed in single-tiered floor systems were more often concurrently colonized by *Brachyspira*
380 spp. with different phenotypes (Table 4), which in several cases were shown to represent
381 different species or genotypes of the same species. Also, in this study, laying hens in organic
382 production were at higher risk for colonization by *Brachyspira* spp. than laying hens in other
383 housing systems. Together, these findings may indicate that there is an environmental source
384 of *Brachyspira* spp. and/or that there are differences between housing systems regarding on-
385 farm biosecurity.

386 In contrast with earlier studies no significant differences in clinical parameters were found
387 between colonized and non-colonized laying hen flocks in this study. A likely explanation is
388 that the flocks were randomly chosen regardless of gastrointestinal disease signs. Possible
389 confounding factors were present, such as hybrid, housing system and presence of other
390 pathogenic microorganisms, and the duration of *Brachyspira* spp. colonization was not
391 known. Furthermore, egg production data were obtained as per cent egg production on the day
392 of sampling. Total produced egg mass or number of eggs from start of lay would possibly
393 have produced more reliable data, but these were not available from the majority of flocks.
394 The absence of significant differences in clinical parameters between flocks colonized by

395 potentially pathogenic spirochaetes and the other flocks may be due to the low number of
396 flocks in this group.

397 The current study clearly demonstrates that there may be a high level of phenotypic and
398 genetic diversity among brachyspiras in commercial laying hens, not only on population level,
399 but also in individual flocks (Table 4), in individual faecal samples (results 3.1. and Table 5)
400 and probably in individual chickens. The phenotypic diversity of *Brachyspira* spp. isolated
401 from individual chicken flocks, and sometimes even the same sample, was confirmed on
402 genetic level by 16S rRNA gene sequencing (Table 6 and Fig. 1). Interestingly, considerable
403 genetic variation of the 16S rRNA gene of *B. aalborgi* has been observed in two human
404 patients (Pettersson et al., 2000). The present study indicates that an analogous situation may
405 exist in chickens, but further investigations of caecal samples from individual chickens are
406 needed. Our results also emphasize the need to apply great care in laboratories to ensure that
407 isolates used for phenotyping and molecular tests consist of non-mixed spirochaete cultures.
408 As shown in Table 5, phenotypic tests may lead to diagnostic mistakes when applied to mixed
409 cultures. One such example in this study was that several isolates possessing the phenotype of
410 *B. innocens* proved to be mixtures of *B. innocens*, and/or *B. murdochii* and the potentially
411 pathogenic species *B. intermedia* (Table 5). It is also very important to recognize that
412 available molecular diagnostic tests may not be fully relevant for avian isolates. A future
413 approach should be to find reliable and distinguishing characters to improve the identification
414 of chicken spirochetes, in particular those that are of clinical significance. Possible synergistic
415 effect between *Brachyspira* spp. regarding colonization and disease also needs to be
416 evaluated.

417

418 **Acknowledgements**

419 We thank Ulla Zimmermann, Lena Lundgren, and Marianne Persson, Dept. of Bacteriology,
420 SVA for excellent technical assistance. The study was funded by grants from the Swedish
421 Farmers' Foundation for Agricultural Research and the Swedish Research Council for
422 Environment, Agricultural Sciences and Spatial Planning.

423

424 **References**

- 425 Atyeo, R.F., Stanton, T.B., Jensen, N.S., Suriyaarachichi, D.S., Hampson, D.J., 1999.
426 Differentiation of *Serpulina* species by NADH oxidase gene (*nox*) sequence comparisons
427 and *nox*-based polymerase chain reaction tests. Vet. Microbiol. 67, 47-60.
- 428 Duhamel, G.E., Trott, D.J., Muniappa, N., Mathiesen, M.R., Tarasiuk, K., Lee, J.I., Hampson,
429 D.J., 1998. Canine intestinal spirochetes consist of *Serpulina pilosicoli* and a newly
430 identified group provisionally designated "*Serpulina canis*" sp. nov. J. Clin. Microbiol. 36,
431 2264-2270.
- 432 Feberwee, A., Hampson, D.J., Phillips, N.D., La, T., van der Heijden, H.J.F., Wellenberg,
433 G.J., Landman, W.J.M., 2007a. Survey of *Brachyspira* spp. in Dutch poultry and the
434 isolation of a *Brachyspira hyodysenteriae*-like spirochaete. Proc. XV Congress of the World
435 Veterinary Poultry Association (WVPA), 10-15 September, Beijing, China. WVPA2007-
436 03-052, p. 260.
- 437 Feberwee, A., Hampson, D.J., Phillips, N.D., La, T., van der Heijden, H.M.J.F., Wellenberg,
438 G.J., Dwars, M., Landman, W.J.M., 2007b. Identification of *Brachyspira hyodysenteriae*
439 and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea
440 and/or reduced production. J. Clin. Microbiol. DOI code: 10.1128/JCM.01829-07.
- 441 Fellström, C., Gunnarsson, A., 1995. Phenotypical characterization of intestinal spirochaetes
442 isolated from swine. Res. Vet. Sci. 59:1-4.

- 443 Fellström, C., Karlsson, M., Pettersson, B., Zimmermann, U., Gunnarsson, A., Aspan, A.,
444 1999. Emended description of indole negative and indole positive isolates of *Brachyspira*
445 (*Serpulina*) *hyodysenteriae*. Vet. Microbiol. 70, 225-238.
- 446 Hampson, D.J., La, T., 2006. Reclassification of *Serpulina intermedia* and *Serpulina*
447 *murdochii* in the genus *Brachyspira* as *Brachyspira intermedia* comb. nov. and *Brachyspira*
448 *murdochii* comb. nov. Int. J. Syst. Evol. Microbiol. 56, 1009-1012.
- 449 Hookey, J.V., Barrett, S.P., Reed, C.S., Barber, P., 1994. Phylogeny of human intestinal
450 spirochaetes inferred from 16S rDNA sequence comparisons. FEMS Microbiol. Lett. 15,
451 345-349.
- 452 Hovind-Hougen, K., Birch-Andersen, A., Henrik-Nielsen, R., Orholm, M., Pedersen, J.O.,
453 Taglbjaerg, P.S., Thaysen, E.H., 1982. Intestinal spirochetosis: morphological
454 characterization and cultivation of the spirochete *Brachyspira aalborgi* gen. nov., sp. nov. J.
455 Clin. Microbiol. 16, 1127-1136.
- 456 Jansson, D.S., Johansson, K-E., Olofsson, T., Råsbäck, T., Vågsholm, I., Pettersson, B.,
457 Gunnarsson, A., Fellström, C., 2004. *Brachyspira hyodysenteriae* and other strongly β -
458 hemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*). J.
459 Med. Microbiol. 53, 293-300.
- 460 Jansson, D.S., Ingermaa, F., Fellström, C., Johansson, K-E., 2007. Isolation of the chicken
461 enteropathogen *Brachyspira alvinipulli* from laying hens and wild ducks. Proc. XV
462 Congress of the World Veterinary Poultry Association (WVPA), 10-15 September, Beijing,
463 China. WVPA2007-03-065, p. 552.
- 464 Jensen N.S., Stanton T.B., Swayne DE., 1996. Identification of the swine pathogen *Serpulina*
465 *hyodysenteriae* in rheas (*Rhea americana*). Vet. Microbiol. 52, 259-269.
- 466 Johansson, K-E., Duhamel, G.E., Bergsjö, B., Olsson Engvall, E., Persson, M., Pettersson, B.,
467 Fellström, C., 2004. Identification of three clusters of canine intestinal spirochaetes by

- 468 biochemical and 16S rDNA sequence analysis. J. Med. Microbiol. 53, 345-350.
- 469 McLaren, A.J., Trott, D.J., Swayne, D.E., Oxberry, S.L., Hampson, D.J., 1997. Genetic and
470 phenotypic characterisation of intestinal spirochetes colonizing chickens, and allocation of
471 known pathogenic isolates to three distinct genetic groups. J. Clin. Microbiol. 35, 412-417.
- 472 Leser, T.D., Møller, K., Jensen, T.K., Jorsal, S.E., 1997. Specific detection of *Serpulina*
473 *hyodysenteriae* and potentially pathogenic weakly beta-haemolytic porcine intestinal
474 spirochetes by polymerase chain reaction targeting 23S rDNA. Mol. Cell Probes 11, 363-
475 372.
- 476 Nemes, C.S., Glávits, R., Dobos-Kovács, M., Ivanics, É., Kaszanyitzky, É., Beregszászi, A.,
477 Szeredi, L., Denczö, L., 2006. Typhlocolitis associated with spirochaetes in goose flocks.
478 Avian Pathol. 35, 4-11.
- 479 Ochiai, S., Adachi, Y., Mori, K., 1997. Unification of the genera *Serpulina* and *Brachyspira*,
480 and proposals of *Brachyspira hyodysenteriae* Comb. Nov., *Brachyspira innocens* Comb.
481 Nov., and *Brachyspira pilosicoli* Comb. Nov. Microbiol. Immunol. 41, 445-452.
- 482 Pettersson, B., Fellström, C., Andersson, A., Uhlén, M., Gunnarsson, A., Johansson, K-E.,
483 1996. The phylogeny of intestinal porcine spirochetes (*Serpulina* species) based on
484 sequence analysis of the 16S rRNA gene. J. Bacteriol. 178, 4189-4199.
- 485 Pettersson, B., Wang, M., Fellström, C., Uhlén, M., Molin, G., Jeppsson, B., Ahrné, S., 2000.
486 Phylogenetic evidence for novel and genetically different intestinal spirochetes resembling
487 *Brachyspira aalborgi* in the mucosa of the human colon as revealed by 16S rDNA analysis.
488 Syst. Appl. Microbiol. 23, 355-363.
- 489 Phillips, N.D., La, T., Hampson, D.J. 2005. A cross-sectional study to investigate the
490 occurrence and distribution of intestinal spirochetes (*Brachyspira* spp.) in three flocks of
491 laying hens. Vet. Microbiol. 105, 189-198.
- 492 Råsbäck, T., Fellström, C., Gunnarsson, A., Aspán, A., 2006. Comparison of culture and

- 493 biochemical tests with PCR for detection of *Brachyspira hyodysenteriae* and *Brachyspira*
494 *pilosicoli*. J. Microbiol. 66, 347-353.
- 495 Råsbäck, T., Jansson, D.S., Johansson, K-E., Fellström, C., 2007. A novel enteropathogenic,
496 strongly haemolytic spirochaete isolated from pig and mallard, provisionally designated
497 “*Brachyspira suanatina*” sp. nov. Environ. Microbiol. 9, 983-991,
- 498 Stanton, T.B., 1992. Proposal to change the genus designation *Serpula* to *Serpulina* gen. nov.
499 containing the species *Serpulina hyodysenteriae* comb. nov. and *Serpulina innocens* comb.
500 nov. Int. J. Syst. Bacteriol. 189-90.
- 501 Stanton, T.B., Fournié-Amazouz, E., Postic, D., Trott, D., Grimont P.A., Baranton, G.,
502 Hampson, D.J., Saint Girons, I., 1997. Recognition of two new species of intestinal
503 spirochetes: *Serpulina intermedia* sp. nov. and *Serpulina murdochii* sp. nov. Int. J. Syst.
504 Bacteriol. 47, 1007-12.
- 505 Stanton, T.B., Postic, D., Jensen, N.S., 1998. *Serpulina alvinipulli* sp. nov., a new *Serpulina*
506 species that is enteropathogenic for chickens. Int. J. Syst. Bacteriol. 48, 669-676.
- 507 Stephens, C.P., Hampson, D.J., 1999. Prevalence and disease association of intestinal
508 spirochaetes in chickens in eastern Australia. Avian. Pathol. 28, 447-454.
- 509 Stephens, C.P., Oxberry, S.L., Phillips, N.D., La, T., Hampson, D.J., 2005. The use of
510 multilocus enzyme electrophoresis to characterise intestinal spirochetes (*Brachyspira* spp.)
511 colonising hens in commercial flocks. Vet. Microbiol. 107, 149-157.
- 512 Suriyaarachchi, D.S., Mikosza, A.S.J., Atyeo, R.F., Hampson, D.J., 2000. Evaluation of a 23
513 rDNA polymerase chain reaction assay for identification of *Serpulina intermedia*, and strain
514 typing using pulsed-field gel electrophoresis. Vet. Microbiol. 71, 139-148.
- 515 Swayne, D.E., Bermudez, A.J., Sagartz, J.E., Eaton, K.A., Monfort, J.D., Stoutenburg, J.W.,
516 Hayes, J.R., 1992. Association of cecal spirochetes with pasty vents and dirty eggshells in
517 layers. Av. Dis. 36, 776-781.

- 518 Swayne, D.E., Eaton, K.A., Stoutenburg, J., Trott, D.J., Hampson, D.J., Jensen, N.S., 1995.
519 Identification of a new intestinal spirochete with pathogenicity for chickens. *Infect.*
520 *Immun.* 63, 430-436.
- 521 Tauson, R., 2005. Management and housing systems for layers – effects on welfare and
522 production. *Worlds Poult. Sci. J.*, 61, 477-490.
- 523 Taylor, D.J., Alexander, T.J.L., 1971. The production of dysentery in swine by feeding
524 cultures containing a spirochaete. *Br. Vet. J.* 127, 58-61.
- 525 Taylor, D.J., Simmons, J.R., Laird, H.M., 1980. Production of diarrhoea and dysentery in pigs
526 by feeding pure cultures of a spirochaete differing from *Treponema hyodysenteriae*. *Vet.*
527 *Rec.* 106, 326-332.
- 528 Townsend, K.M., Ngan Giang, V., Stephens, C., Scott, P.T., Trott, D., 2005. Application of
529 *nox*-restriction fragment length polymorphism for the differentiation of *Brachyspira*
530 intestinal spirochetes isolated from pigs and poultry in Australia. *J. Vet. Diagn. Invest.* 17,
531 103-109.
- 532 Trott, D.J., Stanton, T.B., Jensen, N.S., Duhamel, G.E., Johnson, J.L., Hampson, D.J., 1996.
533 *Serpulina pilosicoli* sp. nov., the agent of porcine intestinal spirochetosis. *Int. J. Syst.*
534 *Bacteriol.* 46, 206-215.

535 **Figure legend**

536 **Fig 1.** Evolutionary tree based on sequence data (approximately 1220 nucleotides) of the 16S
537 rRNA gene of *Brachyspira* spp. isolates and subcultures from Swedish laying hens, of type
538 and reference strains and of selected field strains. The bar shows the distance equivalent to 1
539 nucleotide substitution per 100 positions. Strains of *Borrelia burgdorferi* (GenBank accession
540 number X98228) and *Treponema denticola* (GenBank accession number AF139203) were
541 chosen as outgroup. Isolates from the present study are shown in bold. Suggested species
542 designations of the selected isolates from chickens are given in Table 6.

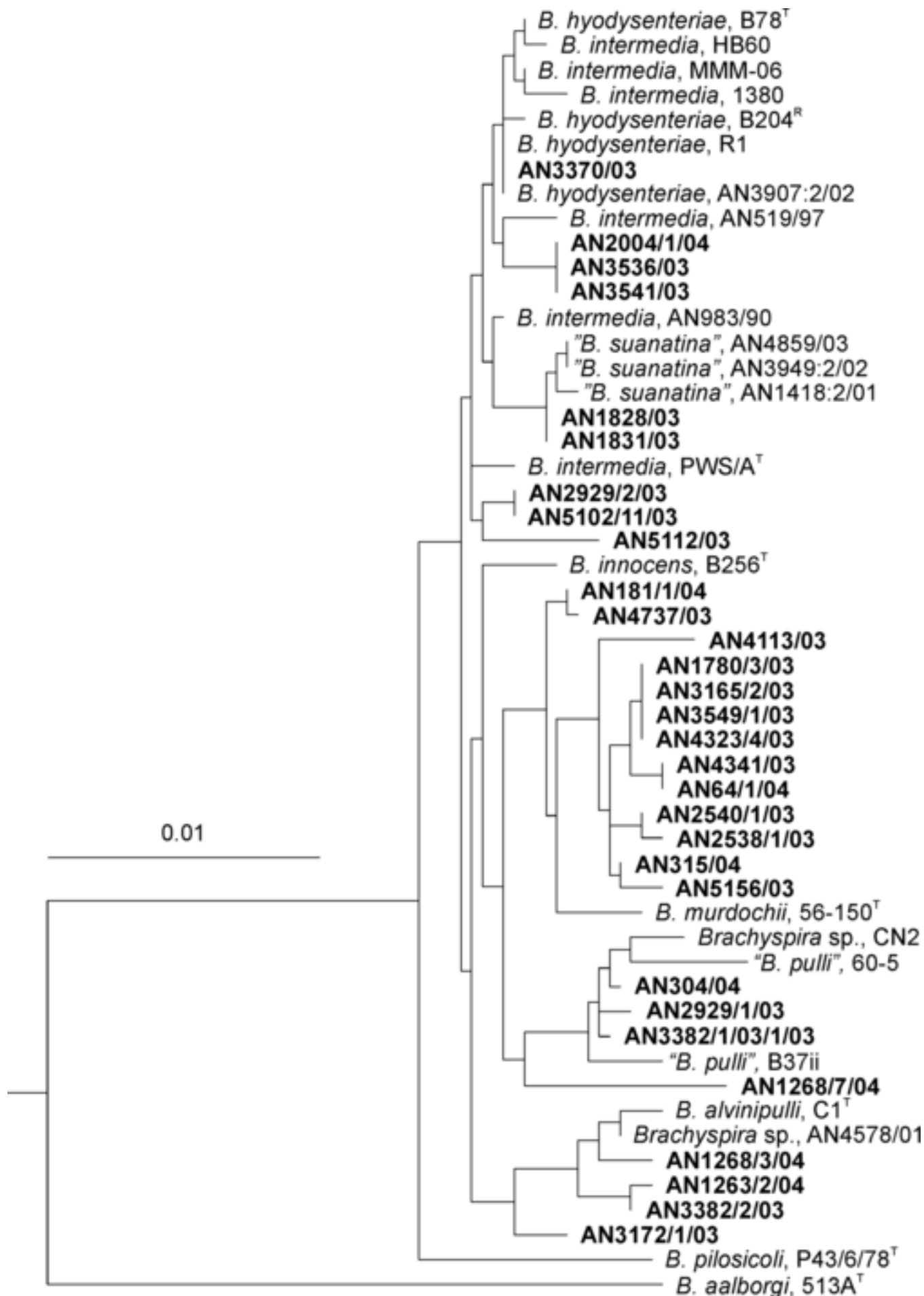


Table 1. List of primers used for PCR and sequencing of the 16S rRNA gene of *Brachyspira* spp.

Application	Gene	Position	Primer sequence (5'-3'), direction and designation	Reference
PCR <i>B. intermedia</i>	23S rRNA	1088-1106 ^{1,2}	CCG TTG AAG GTT TAC CGT G (forward, S-II _f)	Leser et al., 1997
		2098-2114	CGC CTG ACA ATG TCC GG (reverse, S-II _r)	
PCR <i>B. intermedia</i>	<i>nox</i>	158-175 ³	GTC CTG AAA GCT TAA AAA (forward, SINTF1)	Atyeo et al., 1999
		1145-1162	CTA ATA AAC GTC CAG TAT (reverse, SINTR1)	
PCR <i>B. intermedia</i>	<i>nox</i>	516-1073 ⁴	AGAGTTGAAGACACTTATGAC (forward, Bint-nox-f-new) ATAAACATCAGGATCTTTC (reverse, Int2)	N. Phillips, pers. commun. Phillips et al., 2005
PCR <i>B. hyodysenteriae</i>	<i>tly A</i>	514-533 ⁵ 1021-1040	GCA GAT CTA AAG CAC AGG AT (forward, Bh <i>tly A</i> F) GCC TTT TGA AAC ATC ACC TC (reverse, Bh <i>tly A</i> R)	Råsbäck et al., 2006
PCR <i>B. hyodysenteriae</i>	<i>nox</i>	1064-1085 ⁴ 1398-1415	TTA AAA CAA GAA GGA ACT ACT (forward, SHF) CTA ATA AAC GTC TGC TGC (reverse, SHR)	Atyeo et al., 1999
PCR <i>B. hyodysenteriae</i>	23S rRNA	103-120 ⁶ 1387-1403	CGG TAA GTG ATG TAC TTG (forward, S-If) AGC CTC AAC CTT AAA GA (reverse, S-Ir)	Leser et al., 1997
PCR <i>B. pilosicoli</i>	16S rRNA	154-179 ⁷ 1060-1083	CAT AAG TAG AGT AGA GGA AAG TTT TT (forward, Bp 16S F) CTC GAC ATT ACT CGG TAG CAA CAG (reverse, Bp 16S R)	Fellström et al., 1997
PCR for sequencing <i>Brachyspira</i> spp.	16S rRNA	11-35 ⁸ 1496-1520	GTT TGA TYC TGG CTC AGA RCK AAC G (forward, kag-007) CTT CCG GTA CGG MTG CCT TGT TAC G (reverse, kag-009)	Johansson et al., 2004
Sequencing of	16S rRNA	15-30 ⁸ 334-352 519-534 800-818 922-939 1175-1193 1501-1518	GATYCTGGCTCAGARC (forward, kag-008) CCARACTCCTACGGRAGGC (forward, 584) ATTACCGCGGCKGCTG (reverse, 631) GTAGTCCACCGTAAACG (forward, 538) CTTGTGCGGGYCCCGTC (reverse, kag-011) GAGGAAGGYGRGGATGAYG (forward, 597) TCCGGTACGGMTGCCTTG (reverse, kag-010)	Johansson et al., 2004

¹ According to the *B. intermedia* 23S rRNA gene sequence (GenBank accession no. U72700).² The three last nucleotides (TAT) have been added to the original primer sequence.³ According to the *B. intermedia nox* gene sequence (GenBank accession no. AF060811).⁴ According to the *B. hyodysenteriae nox* gene sequence (GenBank accession no. U19610).⁵ According to the *B. hyodysenteriae tly A* gene sequence (GenBank accession no. X61684).⁶ According to the *B. hyodysenteriae* 23S rRNA gene sequence (GenBank accession no. U72699).⁷ According to the *B. pilosicoli* 16S rRNA gene sequence (GenBank accession no. J01695).⁸ According to the *E. coli* 16S rRNA gene sequence (GenBank accession no. J01695).

Table 2. *Brachyspira* sp. strains used for phylogenetic comparison with the strains reported in this study. Sequence data for type strains PWS/A^T (*B. intermedia*) and C1^T (*B. alvinipulli*) were determined as part of this study. Sequence data for all other strains and isolates were obtained from the GenBank database.

Strain/isolate	<i>Brachyspira</i> species	Origin: species, country	GenBank accession no.	Reference (sequence data)
B78 ^T	<i>B. hyodysenteriae</i>	Pig, USA	U14930	Pettersson et al., 1996
B204 ^R	<i>B. hyodysenteriae</i>	Pig, USA	U14932	Pettersson et al., 1996
R1	<i>B. hyodysenteriae</i>	Rhea, USA	U23035	Stanton et al., 1996
AN3907:2/02	<i>B. hyodysenteriae</i>	Mallard, Sweden	AY352287	Jansson et al., 2004
AN4859/03	" <i>B. suanatina</i> "	Pig, Sweden	DQ473575	Råsbäck et al, 2007
AN1418:2/01	" <i>B. suanatina</i> "	Mallard, Sweden	AY352282	Jansson et al., 2004
AN3949:2/02	" <i>B. suanatina</i> "	Mallard, Sweden	AY352290	Jansson et al., 2004
PWS/A ^T	<i>B. intermedia</i>	Pig, UK	EF488166	This study
AN983/90	<i>B. intermedia</i>	Pig, Sweden	U14933	Pettersson et al., 1996
AN519/97	<i>B. intermedia</i>	Pig, Finland	EF517536	Råsbäck et al, unpublished
1380	<i>B. intermedia</i>	Chicken, The Netherlands	AY745526	Phillips et al., 2005
HB60	<i>B. intermedia</i>	Chicken, Australia	AY745532	Phillips et al., 2005
MMM-06	<i>B. intermedia</i>	Chicken, Australia	AY745523	Phillips et al., 2005
B256 ^T	<i>B. innocens</i>	Pig, USA	U14920	Pettersson et al., 1996
56-150 ^T	<i>B. murdochii</i>	Pig, Canada	AY312492	Johansson et al., 2004
CN2	<i>Brachyspira</i> sp.	Dog, Norway	AY349934	Johansson et al., 2004
60-5	" <i>B. pulli</i> "	Chicken, Australia	AY745541	Phillips et al., 2005
B37ii	" <i>B. pulli</i> "	Chicken, Australia	AY745542	Phillips et al., 2005
C1 ^T	<i>B. alvinipulli</i>	Chicken, USA	EF455559	This study
AN4578/01	<i>Brachyspira</i> sp.	Dog, Sweden	AY349933	Johansson et al., 2004
P43/6/78 ^T	<i>B. pilosicoli</i>	Pig, UK	U14927	Pettersson et al., 1996
513A ^T	<i>B. aalborgi</i>	Human, Denmark	Z22781	Hookey et al., 1994

Table 3. Phenotypic profiles of 525 weakly haemolytic isolates of *Brachyspira* spp. from 37 Swedish laying hen flocks.

Phenotype ^{1,2}	Biochemical group ³	N	%
+-- ⁴	II	18	3.4
--- ⁵	IIIa	137	26.1
--++ ⁶	IIIbc	262	49.9
----	-	1	0.2
++++	-	7	1.3
+--+	-	54	10.3
-+++	-	10	1.9
-+-+	-	0	0
Contaminated cultures ⁷	-	36	6.9
TOTAL	-	525	100

¹+ = positive reaction; - = negative reaction

²Biochemical results given in the following order: indole production, hippurate cleavage capacity, α -galactosidase activity and β -glucosidase activity.

³Classification according to Fellström et al, 1999.

⁴Phenotype consistent with *B. intermedia* (Fellström et al., 1999).

⁵Phenotype consistent with *B. murdochii* (Fellström et al., 1999).

⁶Phenotype consistent with *B. innocens* (Fellström et al., 1999).

⁷Cultures contaminated by non-spirochaetal bacteria.

Accepted Manuscript

Table 4. Distribution of phenotypic profiles of weakly haemolytic *Brachyspira* spp. isolated from 37 Swedish laying hen flocks.

Phenotypic profiles within flocks ^{1,2}	No. of flocks	Housing systems ⁶	Flock designation
+-- ³	1	Organic (1)	A
--- ⁴	7	FC (1), STFS (1), MTAS (2), Organic (3)	M, P, S, Y, AA, AI, AK
--+ ⁵	11	FC (3), STFS (2), MTAS (4), Organic (2)	J, K, L, N, Q, U, W, AB, AD, AG, AH
+-- ³ / --- ⁴	1	Organic (1)	G
--- ⁴ / --+ ⁵	8	FC (2), STFS (1), MTAS (2), Organic (3)	O, V, T, Z, X, AE, AJ, AL
--+ ⁵ / +-++	1	STFS (1)	AF
--+ ⁵ / +++	1	STFS (1)	I
--- ⁴ , --+ ⁵ / +-++	1	Organic (1)	B
+-- ³ / --+ ⁵ / +-++	1	Organic (1)	C
--- ⁴ / --+ ⁵ / ---	1	STFS (1)	H
+-- ³ / --- ⁴ / --+ ⁵ / +-++	2	STFS (1), Organic (1)	F, D
+-- ³ / --+ ⁵ / +-++ / ++++	1	Organic (1)	E
+-- ³ / --+ ⁵ / +-++ / -+++ / ++++	1	Organic (1)	R

¹+ = positive reaction; - = negative reaction

²Biochemical results given in the following order: indole production, hippurate cleavage capacity, α -galactosidase activity and β -glucosidase activity.

³Phenotype consistent with *B. intermedia* (Fellström et al., 1999).

⁴Phenotype consistent with *B. murdochii* (Fellström et al., 1999).

⁵Phenotype consistent with *B. innocens* (Fellström et al., 1999).

⁶FC=Furnished cages; STFS=Single-tiered floor system; MTAS=multi-tiered aviary system; Organic=organic egg production.

Table 5. Subculturing of weakly haemolytic *Brachyspira* spp. isolates produced up to five phenotypic profiles that differed from that of the subcultured isolate. (Phenotype of subcultured isolate shown in column 1 and phenotypes of isolates obtained after subculture are shown in columns 4 to 13). Grey cells show when the phenotypes of the subcultured isolates corresponded to those of the isolates obtained after subculture. One phenotypic profile (-+--, column 13) that was not present among the subcultured isolates emerged. The table is based on subculture of 25 isolates and ten subcultures.

Phenotypes of subcultured isolates ^{1,2}	Flock identity	N ³	Phenotypes of isolates obtained after subculture ^{1,2}									
			+--+ ⁴	---+ ⁵	--++ ⁶	----	++++	+--+	-+++	-+-+	++-+	-+--
+--+ ⁴	E, F, R	38	27	8	0	0	0	1	0	1	1	0
---+ ⁵	G, H, P, S, T	45	0	45	0	0	0	0	0	0	0	0
--++ ⁶	D, E, H, I, K, O, R	110	15	25	68	2	0	0	0	0	0	0
----	H	8	0	0	0	8	0	0	0	0	0	0
++++	E, R	17	1	0	6	0	0	1	2	6	1	0
+--+	B, C, D, E, R	72	21	12	22	0	0	15	0	2	0	0
-+++	I, R	20	0	0	6	0	1	4	4	2	1	2
-+-+	E, R	23	0	17	0	0	0	0	0	6	0	0
++-+	R	18	1	6	0	0	0	0	0	7	1	3
		351	65	113	102	10	1	21	6	24	4	5

¹+ = positive reaction; - = negative reaction

²Biochemical results given in the following order: indole production, hippurate cleavage capacity, α -galactosidase activity and β -glucosidase activity.

³Total number of isolates obtained by subculture that were phenotypically evaluated.

⁴Phenotype consistent with *B. intermedia* (Fellström et al., 1999).

⁵Phenotype consistent with *B. murdochii* (Fellström et al., 1999).

⁶Phenotype consistent with *B. innocens* (Fellström et al., 1999).

Table 6. List of weakly haemolytic *Brachyspira* spp. isolates from laying hens selected for characterization.

Isolate	BrachyStrain database ²	Housing system ³	Farm identity	Phenotype ^{4,5}	PCR	PCR	PCR	PCR	PCR	PCR	PCR	Proposed species ¹⁴	GenBank Accession no.
					<i>B. int</i> 23S ^{4,7}	<i>B. int nox</i> ^{4,8}	<i>B. int nox</i> ^{4,9}	<i>B. hyo tly A</i> ^{4,10}	<i>B. hyo nox</i> ^{4,11}	<i>B. hyo</i> 23S ^{4,12}	<i>B. pilo</i> 16S ^{4,13}		
AN1828/03	34	Organic	A	+++	-	-	-	-	-	+	-	<i>Brachyspira</i> sp.	EF164962
AN1831/03	35	Organic	A	+++	-	-	-	-	-	+	-	<i>Brachyspira</i> sp.	EF164963
AN2929/2/03 ¹	36	Organic	B	+++	-	-	+	-	-	-	-	<i>B. intermedia</i>	EF164964
AN3370/03	37	Organic	C	+++	+	-	+	-	-	-	-	<i>B. intermedia</i>	EF164965
AN3536/03	38	STFS	D	+++	-	-	+	-	-	-	-	<i>B. intermedia</i>	EF164966
AN3541/03	39	STFS	D	+++	-	-	+	-	-	-	-	<i>B. intermedia</i>	EF164967
AN5102/11/03 ¹	40	Organic	E	+++	+	-	+	-	-	-	-	<i>B. intermedia</i>	EF164968
AN5112/03	41	Organic	E	+++	-	-	+	-	-	-	-	<i>B. intermedia</i>	EF164969
AN2004/1/04 ¹	42	Organic	F	+++	-	-	+	-	-	-	-	<i>B. intermedia</i>	EF164970
AN1780/3/03 ¹	43	Organic	G	+++	-	-	-	-	-	-	-	<i>B. murdochii</i>	EF164971
AN2538/1/03 ¹	44	STFS	H	+++ ⁶	-	-	-	-	-	-	-	<i>B. murdochii</i>	EF164972
AN2540/1/03 ¹	45	STFS	H	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164973
AN3165/2/03 ¹	46	STFS	I	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164974
AN3549/1/03 ¹	47	STFS	D	+++	-	-	-	-	-	-	-	<i>B. murdochii</i>	EF164975
AN4113/03	48	MTAS	J	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164976
AN4323/4/03 ¹	49	STFS	K	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164977
AN4341/03	50	FC	L	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164978
AN4737/03	51	STFS	M	+++	-	-	-	-	-	-	-	<i>B. murdochii</i>	EF164979
AN5156/03	52	MTAS	N	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164980
AN64/1/04 ¹	53	STFS	O	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164981
AN181/1/04 ¹	54	MTAS	P	+++	-	-	-	-	-	-	-	<i>B. murdochii</i>	EF164982
AN315/04	55	STFS	Q	+++	-	-	-	-	-	-	-	<i>B. innocens</i>	EF164983
AN2929/1/03 ¹	56	Organic	B	+++	-	-	-	-	-	-	-	" <i>B. pulli</i> "	EF164984
AN3382/1/03 ¹	57	Organic	C	+++	-	-	-	-	-	-	-	" <i>B. pulli</i> "	EF164985
AN304/04	58	STFS	Q	+++	-	-	-	-	-	-	-	" <i>B. pulli</i> "	EF164986
AN3382/2/03 ¹	59	Organic	C	+++	-	-	-	-	-	-	-	<i>B. alvinipulli</i>	EF164987
AN1263/2/04 ¹	60	Organic	R	+++	-	-	-	-	-	-	-	<i>B. alvinipulli</i>	EF164988
AN1268/3/04 ¹	61	Organic	R	+++	-	-	-	-	-	-	-	<i>B. alvinipulli</i>	EF164989
AN3172/1/03 ¹	62	STFS	I	+++	-	-	-	-	-	-	-	<i>Brachyspira</i> sp.	EF164990
AN1268/7/04 ¹	63	Organic	R	+++	-	-	-	-	-	-	-	<i>Brachyspira</i> sp.	EF164991

¹Designation refers to an isolate obtained from subculturing after serial ten-fold titration.²Strain number in BrachyStrain database. See Strain database at <http://www.brachyspira.se>³FC=Furnished cages; STFS=Single-tiered floor system; MTAS=Multi-tiered aviary system; Organic=Organic egg production.⁴+ = positive reaction; - = negative reaction⁵Biochemical results given in the following order: indole production, hippurate cleavage capacity, α -galactosidase activity and β -glucosidase activity.⁶Isolate obtained by subculture that originally was classified and selected as possessing the phenotypic profile ----, later repeatedly shown to possess a weak β -glucosidase activity.⁷PCR targeting *B. intermedia* based on the 23S rRNA gene (Leser et al., 1997).⁸PCR targeting for *B. intermedia* based on the NADH oxidase (*nox*) gene (Atyeo et al., 1999).⁹PCR targeting *B. intermedia* based on the NADH oxidase (*nox*) gene (Phillips et al., 2005 (reverse primer); and N. Phillips, Murdoch University, Perth Australia, pers. commun. (modified forward primer)).¹⁰PCR targeting *B. hyodysenteriae* based on the *tlyA* gene (Räsback et al., 2006).¹¹PCR targeting *B. hyodysenteriae* based on the NADH oxidase (*nox*) gene (La et al., 2003).¹²PCR targeting *B. hyodysenteriae* based on the 23S rRNA gene (Leser et al., 1997).¹³PCR targeting *B. pilosicoli* based on the 16S rRNA gene (Räsback et al., 2006).

¹⁴Proposed species based on biochemical phenotype, PCR-results and 16S rRNA gene sequence.

Accepted Manuscript

Table 7. Summary of *Brachyspira* spp. culture results and clinical data reported at sampling.

Housing system	Flocks Colonized	flocks	Incidence	Egg production¹	Mortality²	Wet litter	Faecal staining³
	(no.)	(no.)	(%)	(mean % (range))	(mean % (range))	(no. of flocks)	(no. of flocks)
Enriched cages	22	6	27	83 (74-92)	4 (1-7)	NA	5
Single-tiered floor system	26	8	31	78 (56-89)	10(1.4-60)	1	10
Multi-tiered aviary system	23	8	35	79 (70-89)	10 (0.8-67)	1	3
Organic farming	21	15	71	79 (70-86)	7 (0.03-20)	2	4
All flocks	92	37	40	80 (56-92)	8 (0.03-67)	4	22

NA=data not available

¹No. eggs laid per day at day of sampling/no. hens on that day x 100.

²Cumulative mortality from transfer of the hens to the laying hen facility at approximately 16 weeks of age until the day of sampling.

³Faecal staining of at least 5% of eggs on day of sampling.