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Genetic and Phenotypic Characterization of Intestinal Spirochetes Colonizing Chickens and Allocation of Known Pathogenic Isolates to Three Distinct Genetic Groups

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Infection with intestinal spirochetes has recently been recognized as a cause of lost production in the poultry industry. Little is known about these organisms, so a collection of 56 isolates originating from chickens in commercial flocks in Australia, the United States, The Netherlands, and the United Kingdom was examined. Strength of β-hemolysis on blood agar, indole production, API ZYM enzyme profiles, and cellular morphology were determined, and multilocus enzyme electrophoresis was used to analyze the extent of genetic diversity among the isolates. The results were compared with those previously obtained for well-characterized porcine intestinal spirochetes. The chicken isolates were genetically heterogeneous. They were divided into 40 electrophoretic types distributed among six diverse genetic groups (groups b to g), with a mean genetic diversity of 0.587. Strains in two groups (groups d and e) may represent new species of *Serpulina*, and the groups contained only strains isolated from chickens. Three genetic groups contained isolates previously shown to be pathogenic for chickens. These corresponded to the proposed species "*Serpulina intermedius*," to an unnamed group (group e), and to *Serpulina pilosicoli*. Two of the chicken isolates (one "*S. intermedius*" and one *S. pilosicoli* isolate) were strongly β-hemolytic, two (both "*S. intermedius*") had an intermediate level of β-hemolysis, and the rest were weakly β-hemolytic. Fourteen isolates of "*S. intermedius*" produced indole, as did one isolate from group d. Isolates identified as *S. pilosicoli* resembled porcine isolates of this species, having four to six periplasmic flagella inserted subterminally in a single row at each end of the cell, and had tapered cell ends. All other spirochetes were morphologically similar, having seven or more periplasmic flagella and blunt cell ends. The identification of three genetic groups containing pathogenic isolates provides an opportunity for more detailed epidemiologic studies with these pathogens and for the development of improved diagnostic tests.

A variety of poorly characterized spirochetes inhabit the gastrointestinal tracts of birds. For example, in an early study with chickens, three distinct morphological types were isolated, all of which failed to induce disease in experimental infections (10). Subsequently, spirochetes were observed in nodules in the cecal walls of chickens, turkeys, and pheasants (17). Oral inoculation of turkeys with these bacteria produced cecal nodules, but intravenous inoculation of day-old chicks failed to produce infection.

Interest in intestinal spirochetal infections in chickens has intensified since the mid-1980s, following the publication of a series of reports implicating these bacteria as a cause of diarrhea, reduced egg production, and/or fecal staining of eggshells in layer flocks in The Netherlands (2, 6), England (8), and the United States (25, 28). Infection appears to be common. For example, Dwars et al. (6) demonstrated intestinal spirochetes in 37 of 134 (27.6%) samples from European layer flocks with diarrhea or reduced production, although they found intestinal spirochetes in only 2 of 45 (4.4%) samples from flocks with no signs of disease. In a more recent study in Western Australia (WA), fecal samples from 16 of 30 (53.3%) broiler breeder and 13 of 37 layer flocks (35.1%) contained spirochetes, and again, those flocks with a history of either diarrhea or reduced production were significantly more likely to contain birds infected with spirochetes than were apparently healthy flocks (18).

Several intestinal spirochetal isolates from chickens have been used in experimental infections of day-old chickens, resulting in diarrhea and reduced weight gain (2, 3, 26). Laying hens experimentally infected with intestinal spirochetes also developed diarrhea, and showed reduced egg production and reduced egg weight (4, 26).

Despite the potential clinical importance of these isolates, only two appear to have been studied in detail. Swayne et al. (26) compared the morphology, biochemical characteristics, and rRNA gene restriction patterns of chicken isolates C1 and C2, isolated from the same flock, with those of the type strains of *Serpulina hyodysenteriae* (strain B78), the etiologic agent of swine dysentery (22), and *Serpulina innocens* (strain B256), a commensal organism of swine (12). In the same study, multilocus enzyme electrophoresis (MEE) was used to compare these two strains with 188 porcine intestinal spirochetes. The morphology of the chicken strains was very similar to that of the porcine *Serpulina* strains, and they were identical to $B256^T$ in the biochemical tests conducted, being weakly β-hemolytic and indole negative and lacking α-galactosidase and α-glucosidase activities. However, they differed in their rRNA gene restriction patterns, and the results of the MEE analysis showed that they belonged to their own distinct genetic group (26). A separate study in which MEE and 16S rRNA gene sequence analysis was used also placed C1 in its own genetic group in the genus *Serpulina*, with sequence similarities of 98.9 and 98.6% with $B78^T$ and $B256^T$, respectively (23).

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The purpose of the current study was to characterize a large collection of intestinal spirochetes isolated from commercial chicken flocks in Australia, the United States, and Europe to determine their diversity and, where information was available, the association of specific genetic groups with disease. The isolates examined included a number previously shown to be pathogenic in experimental infections. The WA isolates were selected as representative isolates of those collected in a prevalence survey of intestinal spirochetes in WA poultry flocks (18), while the other isolates were selected on the basis of their availability in culture collections. The results of MEE analysis, biochemical tests, and transmission electron microscopy with these isolates are described.

MATERIALS AND METHODS

Spirochetes. The intestinal spirochetes examined in this study were isolated either from fecal samples, cecal mucosal scrapings, or cloacal swabs from chickens in commercial flocks. Fifty-six intestinal spirochetes were analyzed: 39 from WA, 3 from Queensland, 7 from The Netherlands, 6 from the United States, and 1 from the United Kingdom (Table 1). Thirty of the 39 (77%) WA isolates were recovered from birds in flocks with either diarrhea (which caused problems of wet litter and/or fecal staining of eggshells) or reduced egg production (18), as were all of the isolates from the United States (26, 28). Strain 1380 was isolated from a bird with diarrhea in a broiler flock in The Netherlands (5). No information was available concerning the disease status of the flocks from which the other strains from The Netherlands, the strains from Queensland, or the strain from the United Kingdom were isolated. Isolates from The Netherlands were provided by H. Smit, Trepo Ltd., Wilnis, The Netherlands; isolates 308.93 and 42167 from the United States were provided by N. S. Jensen, National Animal Disease Center, Ames, Iowa; the isolates from Queensland were provided by C. P. Stephens, Regional Veterinary Laboratories, Toowoomba, Queensland, Australia; and the isolate from the United Kingdom was provided by R. Sellwood, Institute for Animal Health, Compton, England.

Primary isolation of spirochetes was either on Trypticase soy agar (BBL, Becton Dickinson) plates, designed initially for the isolation of porcine intestinal spirochetes (2, 18), or on brucella agar (25). These media contained 5% (or 10% for the isolates from the United States) defibrinated ovine blood, 400 μg of spectinomycin (Sigma Chemicals) per ml, and, for the isolates from WA and the United Kingdom, 25 μg (each) of colistin and vancomycin (Sigma Chemicals) per ml. The plates were incubated for up to 5 days either at 37°C in an atmosphere of 94% N_2–6% CO_2 or at 42°C in an atmosphere of 80% H_2–20% CO_2 (2, 18, 25).

For use in phenotypic tests, spirochetes were grown in prereduced anaerobic Trypticase soy broth (BBL, Becton Dickinson) containing 2% fetal calf serum and 1% ethanolic cholesterol, as described previously (13).

MEE. The culture of isolates, preparation of cells, and MEE analysis of the cells were done by the procedures described previously for porcine and human intestinal spirochetes (14–16). Briefly, 500 ml of broth cultures in the late log phase were harvested by centrifugation at 10,000 × *g* for 20 min, washed in phosphate-buffered saline (pH 7.2), resuspended in 0.5 ml of sterile distilled water, and lysed by three 30-s cycles of sonication at 50 W (Labsonic 1510). Supernatants containing the constitutive enzymes were collected following centrifugation of the lysed suspension at 20,000 × *g* for 20 min and were stored at −70°C.

The electrophoretic mobilities of 15 constitutive enzymes were determined by electrophoresis of the thawed cell supernatants in 11.4% horizontal starch gels (21). The enzymes examined were acid phosphatase, alcohol dehydrogenase, adenylate kinase, alkaline phosphatase, esterase, fructose-1,6-diphosphatase, glucose phosphate isomerase, guanine deaminase, glutamate dehydrogenase, hexokinase, mannose phosphate isomerase, nucleoside phosphorylase, L-leucylglyclglycine peptidase, phosphoglucomutase, and superoxide dismutase. Enzymes were localized by the addition of suitable specific substrates under appropriate buffer conditions, as described elsewhere (15). Differences between isolates in the electrophoretic mobility of a given enzyme were considered to reflect the presence of different alleles at the corresponding structural gene locus. Isolates were characterized by their alleles at each enzyme locus, with isolates having the same alleles at all 15 loci considered to belong to the same electrophoretic type (ET). Genetic diversity (h) was estimated on the basis of the relative frequencies of the different alleles at each enzyme locus, being calculated as $h = (1 - \Sigma p_i^2)\ (n/n - 1)$, where p_i is the frequency of the ith allele and n is the number of ETs (16). The genetic distance between ETs was calculated as the proportion of loci at which different alleles occurred, with the unweighted pair group method of arithmetic averages clustering strategy being used to create a phenogram from this information (16). Figure 1 was created by combining the results of this study with previous results for porcine intestinal spirochetes (15).

Phenotypic characterization. The strength of β-hemolysis for each isolate was determined by stab inoculating spirochetes into Trypticase soy agar supplemented with 5% defibrinated ovine blood and incubating the plates anaerobically for 3 days at 37°C. The strength of β-hemolysis was then compared to that of known strongly β-hemolytic *S. hyodysenteriae* type strain B78 inoculated onto the same plate. To test for indole production, 2 ml of log-phase broth culture was extracted with 1 ml of xylene and 4 drops of Kovács reagent was added. Development of a red or purple color at the surface indicated a positive test. Enzyme profiles were determined with the commercial API ZYM kit (API, Montalieu-Vercieu, France), as described previously (11).

The cell dimensions, number of periplasmic flagella, and shape of the cell ends for 11 representative isolates, selected from the major genetic groups identified by MEE (see Fig. 1), were determined by transmission electron microscopy of negatively stained preparations. The results were compared with those previously published for strains $B78^T$ and $B256^T$ (15), C1 (26), and 308.93 (28). Growth from 1 ml of a log-phase broth culture was centrifuged at 10,000 rpm for 30 s in a microcentrifuge, and the cells were resuspended in 300 μl of 0.01 M sodium phosphate buffer (pH 7.0). Fifty microliters of the cell suspension was mixed with an equal volume of 3.0% phosphotungstic acid (pH 7.0 for isolates belonging to group g in Fig. 1 and pH 6.0 for all other isolates), and the mixture was allowed to stand for 20 s. Formvar-coated grids were placed in the drops for 20 s and were then removed and allowed to dry. The grids were examined in a Phillips CM100 transmission electron microscope.

RESULTS

MEE. The 56 chicken isolates were divided into 40 ETs (ETs A1 through A40), which were distributed through six of the seven major genetic groups apparent on the phenogram (Fig. 1). No chicken spirochetes belonged to MEE group a (corresponding to the species *S. hyodysenteriae*). The mean genetic diversity per enzyme locus for all chicken isolates included in this study was 0.587.

MEE group b (consisting of seven ETs containing porcine spirochetes, and ETs A1 to A10) contained 16 chicken spirochetes from WA, including strongly β-hemolytic strain HB60, as well as strain 1380 from The Netherlands and strain B230 from the United Kingdom. This group corresponded to the proposed species "*Serpulina intermedius*," containing indole-positive, weakly β-hemolytic porcine isolates (15).

MEE group c corresponded to the species *S. innocens*. Only two chicken isolates were in this group, and both were from Australia.

MEE group d is a newly identified genetic group which contained only spirochetes isolated from chickens and did not correspond to any of the genetic groups of porcine isolates previously identified by Lee et al. (15). It was the largest group, with 22 isolates from WA, 1 from Queensland and 2 from The Netherlands. It was most closely related to groups b and c, which contained porcine and chicken isolates, and was separated from them by a genetic distance of 0.686.

MEE group e was another distinct genetic group represented by a single ET containing three isolates (isolates C1, C2, and C4), all from a single flock in the United States. Swayne et al. (26) previously showed that this group is distinct. It was most closely related to groups a to d, being separated from them by a genetic distance of 0.715.

There were only two chicken isolates in group f, one from The Netherlands (isolate 2726) and one from the United States (isolate C5). This group apparently corresponds to the proposed new species "*Serpulina murdochii*" (14), although C5 was relatively distantly related to the other isolates in the group (separated by a genetic distance of 0.630).

Group g corresponded to the new species *Serpulina pilosicoli* (32) and contained three chicken isolates from The Netherlands, two from the United States, and one from Queensland. The strongly β-hemolytic isolate 13316 from The Netherlands was located in this group.

Phenotypic characterization. The results of tests to determine the strength of β-hemolysis on blood agar, indole production, and the API ZYM profiles of the isolates are presented in Table 1. Two isolates (isolates HB60 and 13316) were strongly β-hemolytic, two isolates (isolates E2 and A7) had an

TABLE 1. Electrophoretic type, origin, disease association, strength of β-hemolysis on blood agar, indole production, and API ZYM profiles of the 56 avian intestinal spirochetes examined

ET[a]	Isolate	Origin	Disease association[b]	β-Hemolysis[c]	Indole production[d]	API ZYM profile[e]
A1	B37iii	WA	NA	W	−	ND
A2	22-5	WA	W	W	+	ND
A2	22-6	WA	W	W	+	ND
A2	22-8	WA	W	W	+	14.0.4.2.1
A2	3B-1	WA	L/W	W	+	14.0.4.2.1
A2	P3	WA	NA	W	+	ND
A3	1380	The Netherlands	D	W	+	10.0.4.10.1
A4	E2	WA	L/W	I	+	10.0.13.10.1
A4	A7	WA	L/W	I	+	10.0.4.10.1
A5	Abb60-9	WA	N	W	+	10.0.14.10.1
A5	B52iii	WA	NA	W	+	ND
A6	histo 6	WA	L	W	+	10.0.4.10.1
A6	APWG 33	WA	L	W	ND	ND
A7	B230	United Kingdom	NA	W	+	10.0.4.10.1
A8	2A-10	WA	L/W	W	+	10.0.4.10.1
A9	HB60	WA	W	S	+	10.0.4.10.1
A10	histo 5	WA	L	W	ND	ND
A10	APWG 34	WA	L	W	ND	ND
A11	2A-20	WA	W	W	−	ND
A12	94-0354.03	Queensland	NA	W	−	14.0.12.3.0
A13	C38iv	WA	NA	W	−	ND
A14	H4	WA	L	W	−	ND
A14	H6	WA	L	W	−	14.0.13.11.1
A15	Rag Heal	The Netherlands	NA	W	−	14.0.15.11.1
A16	A34iii	WA	NA	W	−	ND
A17	PHB-11	WA	W	W	−	14.0.13.11.1
A17	PHB-14	WA	W	W	−	ND
A18	94-01966.2	Queensland	NA	W	−	14.0.15.11.1
A19	PHB-8	WA	W	W	−	ND
A20	PHB-3	WA	W	W	−	ND
A21	IV 3	WA	W	W	−	14.0.13.11.1
A21	IV 5	WA	W	W	−	ND
A21	IV 9	WA	W	W	−	ND
A22	IV 4	WA	W	W	−	ND
A23	2B-13	WA	W	W	−	14.0.13.3.1
A24	B67iv	WA	NA	W	−	ND
A25	B37ii	WA	NA	W	−	ND
A26	B37iv	WA	NA	W	−	ND
A27	APWG 35	WA	L	W	ND	ND
A28	60-5	WA	W	W	−	14.0.13.15.1
A29	DH7926	The Netherlands	NA	W	−	14.0.4.13.1
A30	A5	WA	L	W	+	14.0.12.3.1
A30	A6	WA	L	W	−	ND
A31	2A-15	WA	W	W	−	ND
A32	PHB-9	WA	W	W	−	14.0.12.3.1
A33	C1	Ohio	D	W	−	14.0.4.3.1
A33	C2	Ohio	D	W	−	14.0.4.3.1
A33	C4	Ohio	D	W	−	ND
A34	C5	Ohio	D	W	−	14.0.12.7.1
A35	2726	The Netherlands	NA	W	−	6.0.12.6.1
A36	1772	The Netherlands	NA	W	−	14.0.12.3.0
A36	42167	Iowa	D/L	W	−	14.0.4.3.0
A37	308.93	Iowa	D/L	W	−	14.0.4.3.0
A38	4742	The Netherlands	NA	W	−	14.0.12.3.0
A39	13316	The Netherlands	NA	S	−	14.0.13.11.8
A40	QU-1	Queensland	NA	W	−	14.0.12.3.0

[a] ETs were determined by MEE (Fig. 1). ETs A1 to A10, MEE group b; ETs A11 and A12, group c; ETs A13 to A32, group d; ET A33, group e; ETs A34 and A35, group f; ETs A36 to A40, group g.

[b] Signs of disease in flock from which the strain was isolated. W, wet litter; L, low production; D, diarrhea; N, no disease signs; NA, no information on disease signs available.

[c] S, strong β-hemolysis; I, intermediate β-hemolysis; W, weak β-hemolysis.

[d] +, indole produced; −, indole not produced; ND, not determined.

[e] Biochemical profiles with the API ZYM test kit (11). Strains with profiles ending with 10.1 have α-glucosidase but not β-galactosidase activity; those with profiles ending with 3.0 lack β-glucosidase activity.

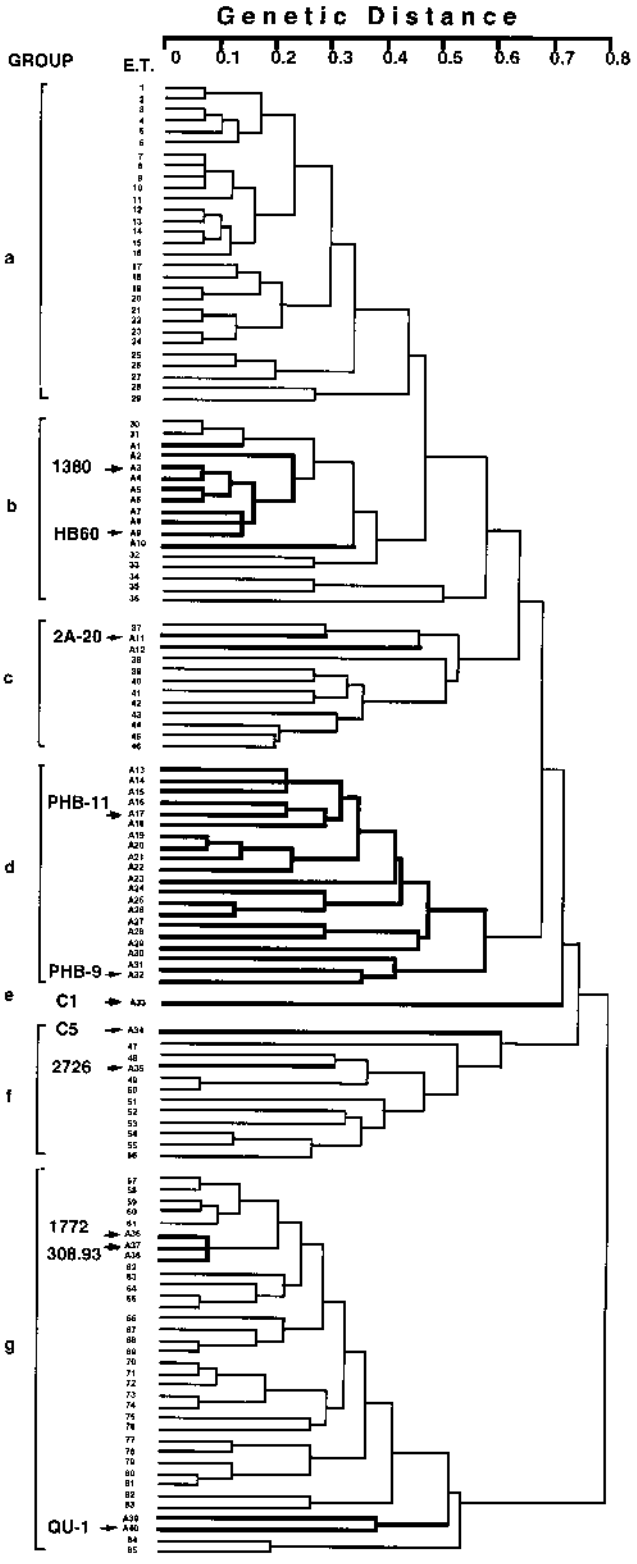

FIG. 1. Phenogram of genetic distance among 40 ETs containing intestinal spirochetes isolated from chickens, outlined in bold, and comparison with ETs containing porcine intestinal spirochetes. Group a corresponds to the species *S. hyodysenteriae*, group b corresponds to the proposed species "*S. intermedius*," group c corresponds to the species *S. innocens*, groups d and e are newly identified, unnamed groups, group f corresponds to the proposed species "*S. murdochii*," and group g corresponds to the species *S. pilosicoli*.

intermediate level of β-hemolysis, and all other isolates were weakly β-hemolytic. Fifteen isolates produced indole, with 14 of these being located in group b ("*S. intermedius*") in Fig. 1. The other isolate was in group d.

The morphologic characteristics of representative chicken intestinal spirochetes from the different genetic groups identified by MEE (Fig. 1) are presented in Table 2. The number of periplasmic flagella per cell end was variable, even for strains belonging to the same MEE genetic group. However, in all cases these were inserted subterminally in a single row at each end of the cell. The chicken intestinal spirochetes belonging to group g (*S. pilosicoli*) had features typical of those described for porcine and human strains of *S. pilosicoli* (31, 32). They had four to six periplasmic flagella at each cell end, although cells of strain 1772 were occasionally seen to have seven flagella. All isolates in this group had tapered cell ends, compared to the blunt ends possessed by the other *Serpulina* isolates.

DISCUSSION

In this study, 56 intestinal spirochetes isolated from chickens in different countries were shown to be genetically heterogeneous. However, on the basis of their grouping relative to those of well-characterized porcine strains, all appeared to belong to the genus *Serpulina* (22). They were divided into 40 ETs belonging to six distinct genetic groups (Fig. 1), four of which (groups b, c, f, and g) have been considered to represent the distinct species "*S. intermedius*," *S. innocens*, "*S. murdochii*," and *S. pilosicoli* (formerly "*Anguillina coli*"), respectively, previously described for porcine isolates (15). The other two groups (groups d and e) are new and only contained isolates from chickens.

Three of the six groups (groups b, e, and g) contained isolates that have previously been shown to be pathogenic in experimentally infected chicks. Group b ("*S. intermedius*") contained 18 of the 56 (32%) isolates examined, including isolate 1380 from The Netherlands, which has been shown to be pathogenic in experimental infections of day-old broiler chickens (3). Also in this group were the strongly β-hemolytic isolate HB60, isolated from a WA layer flock with diarrhea, and histo 6, isolated from a WA broiler breeder flock which was being culled due to poor production (18). All but 1 of the 15 chicken spirochetes tested in group b produced indole, and 9 of 11 isolates tested had α-glucosidase but not α-galactosidase activity (Table 1). These activities are characteristics previously reported for porcine isolates of "*S. intermedius*" (15) and may be useful for identifying chicken isolates belonging to this potentially pathogenic group. Group b was made up of two distinct subgroups of ETs. The first subgroup comprised ETs A1 to A10, which contained all of the chicken spirochetes in this group, together with ETs 30 and 31, containing porcine spirochetes. The second subgroup comprised ETs 32 to 36, which contained only porcine isolates. This subgrouping appeared much less pronounced in a previous study which examined only porcine strains (15). Further DNA-DNA reassociation studies are required to clarify the extent and significance of this subgrouping of organisms which otherwise have phenotypic properties consistent with those of "*S. intermedius*." Strains of this group are relatively uncommon in pigs, where they have been associated with a condition called spirochetal colitis (9), but they are comparatively common in chickens in WA and are present in The Netherlands and the United Kingdom.

Only two isolates from chickens, both from Australia, belonged to the *S. innocens* group (group c). This species is commonly isolated from pigs (15), and such strains are con-

TABLE 2. Morphologic characteristics of chicken intestinal spirochetes selected as representatives of six genetic groups identified by MEE and comparison with the porcine strains *S. hyodysenteriae* B78^T and *S. innocens* B256^T

Strain	MEE group[a]	No. of periplasmic flagella	Cell ends	Length (μm)	Diam (μm)	Wavelength (μm)
S. hyodysenteriae						
B78^T	a	7–10	Blunt	8.3–9.8	0.23–0.45	ND[b]
1380	b	9–14	Blunt	8.69–18.0	0.41–0.50	2.88–4.38
A7	b	10–13	Blunt	10.88–13.83	0.32–0.43	3.58–5.06
histo 6	b	11–14	Blunt	7.55–13.94	0.41–0.43	2.44–3.00
2A-10	b	8–12	Blunt	7.27–12.00	0.25–0.50	2.80–3.64
HB60	b	11–15	Blunt	7.87–11.22	0.17–0.43	2.33–3.62
S. innocens						
B256^T	c	7–10	Blunt	7.4–14.1	0.31–0.40	ND
2A-20	c	8–15	Blunt	8.75–12.38	0.19–0.35	2.19–3.38
PHB-11	d	8–12	Blunt	11.08–26.62	0.27–0.39	2.56–4.38
PHB-9	d	5–7	Blunt	10.69–17.46	0.32–0.39	2.54–4.08
C1	e	8	Blunt	8.76 ± 0.78	0.32 ± 0.02	3.31 ± 0.52
2726	f	9–13	Blunt	8.44–11.31	0.29–0.47	3.96–6.23
308.93	g	5	Tapered	3.4–7.5	0.3–0.4	2.2–3.0
1772	g	5–7	Tapered	9.08–11.00	0.23–0.37	3.00–4.00
QU-1	g	4–6	Tapered	8.25–17.28	0.23–0.56	2.47–4.08

[a] Genetic group by MEE (Fig. 1).
[b] ND, not determined.

sidered to be harmless commensal organisms (12). Porcine isolates of *S. innocens* have failed to colonize experimentally infected chicks (30), and these two isolates probably have no clinical significance.

The new genetic group d contained 25 of the 56 (45%) chicken isolates examined. All but three were from WA, with two from The Netherlands and one from Queensland. This group is not homogeneous since some ETs were separated from each other by genetic distances of up to 0.580, and thus they may not all belong to the same species. To date, the pathogenicity of isolates in this group is unknown, and hence their significance is uncertain. Most were isolated from flocks with production problems. We found no consistent phenotypic features that we could use to identify them.

Group e was represented by only three isolates, all from the United States (Fig. 1). It was most closely related to groups a, b, and c at a genetic distance of 0.715 and, as pointed out previously (26), therefore probably is another new species of *Serpulina*. This group contained strain C1, which was isolated from a chicken in a flock with diarrhea and which has been shown to be pathogenic in day-old chicks and adult layers (26). Isolates C2 and C4 came from birds in the same flock. This should therefore be considered the second group of avian intestinal spirochetes with known pathogenic potential. They were not represented among the isolates from WA or Europe, and their distribution and prevalence in the United States are not known.

Only two strains (strains C5 and 2726) were located in the "*S. murdochii*" group (group f). However, strain C5, which was isolated from the same flock as the isolates in group e, was only distantly related to the other members of the group. The pathogenic potential of these relatively uncommon isolates is not known, although porcine isolates of "*S. murdochii*" are generally considered to be commensal organisms (15).

Six chicken strains of *S. pilosicoli* were identified (group g). These were isolated in Queensland, the United States, and The Netherlands. Human and porcine strains of this species are associated with a condition termed "intestinal spirochetosis," which is characterized by diarrhea and end-on attachment of spirochetes to the colonic epithelium (32). Human and porcine strains of this species have been used to experimentally infect day-old chicks, resulting in diarrhea and a reduced growth rate (30). Five of the six chicken strains were β-glucosidase negative (Table 1), as has previously been described for porcine isolates of this species (7). As well as having β-glucosidase activity, strain 13316 was unusual in being strongly β-hemolytic. The three representative isolates in this group had typical *S. pilosicoli* morphology (31, 32), with four to six (and for one isolate, occasionally seven) periplasmic flagella at each cell end and tapered cell ends (Table 2). In addition, when tested in a PCR-based amplification of a 16S rRNA gene sequence with a primer specific for this species (20), all of these isolates gave a positive reaction (1). Two of these isolates, isolates 308.93 and 42167, were isolated from a layer flock in the United States experiencing diarrhea and a 5% drop in egg production (28). Experimental infection of day-old chickens with the isolate from Queensland (QU-1) resulted in diarrhea and a reduced growth rate, with the characteristic end-on attachment of spirochetes to the cecal epithelium (19). Therefore, *S. pilosicoli* is the third of the genetic groups identified here that has been demonstrated to have pathogenic potential in chickens. Wild birds may also be colonized by strains of this species and therefore pose a risk of infection to chickens. For example, strain S76, which has been identified as *S. pilosicoli* by PCR and MEE (29), was isolated from a chiloe wigeon in the United States. When inoculated into day-old chicks, S76 attached end-on to the cecal epithelium and induced diarrhea (24, 27).

In summary, intestinal spirochetes colonizing chickens are a diverse group of bacteria, some of which may be representative of at least two new species within the genus *Serpulina*. However, DNA-DNA reassociation and 16S rRNA gene sequence analysis studies are necessary to confirm the taxonomic positions of these strains. Chickens also may be colonized by other intestinal spirochetes that are not cultivable under the conditions used in this study, which were based on those developed for porcine spirochetes of the genus *Serpulina*. Of the genetic groups identified, three contained isolates that are known to be pathogenic for chickens: 1380, HB60, and histo 6 in group b

("*S. intermedius*"), C1 in group e (new, unnamed group), and 308.93, 42167, and QU-1 in group f (*S. pilosicoli*). Isolates of "*S. intermedius*" can routinely be identified by their weak β-hemolysis on blood agar and their production of indole, while a PCR for identifying isolates of *S. pilosicoli* is available (21). Distinguishing phenotypic characteristics have not yet been identified for isolates in group e. This study has provided a basis for understanding the diversity and disease associations among a range of intestinal spirochetes infecting chickens. It should now be possible to develop diagnostic techniques to detect and identify the pathogenic species, to study their epidemiology, and to investigate the pathogenic mechanisms involved in infections with these microorganisms.

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