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Brachyspira hyodysenteriae and other strongly β -haemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*)

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The aims of the current study were to collect intestinal spirochaetes (genus *Brachyspira*) from farmed and wild mallards (*Anas platyrhynchos*) and to identify and classify those isolates that phenotypically resembled *Brachyspira hyodysenteriae*, an enteric pathogen of pigs. The isolation rate of *Brachyspira* spp. was high from both farmed (93 %) and wild mallards (78 %). In wild mallards, it appeared that *Brachyspira* spp. were more likely to be found in migratory birds (multivariate analysis: $RR = 1.8$, 95 % CI 1.1–3.1) than in mallards sampled in a public park. Pure cultures of putative *B. hyodysenteriae* were obtained from 22 birds. All five isolates from farmed mallards and ten randomly selected isolates with this phenotype were used for further studies. All isolates from farmed mallards and two of the isolates from wild mallards were PCR-positive for the *tlyA* gene of *B. hyodysenteriae*. Two isolates from farmed mallards were selected for pulsed field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) analysis. These isolates clustered with the type and reference strains of *B. hyodysenteriae*. 16S rDNA sequence analysis performed on 11 of the strains showed that they were all closely related to each other and to the *B. hyodysenteriae*–*Brachyspira intermedia* cluster. Three of the mallard isolates had 16S rDNA sequences that were identical to those of *B. hyodysenteriae* strains R1 and NIV-1 previously isolated from common rheas (*Rhea americana*). To conclude, the isolates from farmed mallards and two isolates from wild mallards were classified as *B. hyodysenteriae* based on the fact that they could not be differentiated by any of the applied methods from type, reference and field strains of *B. hyodysenteriae*. The remaining isolates could not be assigned irrefutably to any of the presently recognized *Brachyspira* species. These results point to a broader host spectrum of *B. hyodysenteriae* than is generally recognized, and to the presence in mallards of strongly β -haemolytic and indole-producing spirochaetes that possess many, but not all, of the currently recognized characteristics of *B. hyodysenteriae*.

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Abbreviation: RAPD, randomly amplified polymorphic DNA.

The GenBank accession numbers for the new 16S rDNA sequences reported in this study are AY352281–AY352291.

INTRODUCTION

Spirochaetes have long been known to colonize the intestines of some birds (Jansson *et al.*, 2001). Reports on potentially pathogenic *Brachyspira* species in chickens and common rheas, as well as the availability of simple biochemical classification schemes, specific PCR assays and various

DNA fingerprinting methods, have led to increased knowledge of the complexity of intestinal spirochaetes in avian species, especially poultry. Information about intestinal spirochaetes in wild birds is, however, very limited. Wild-living waterbirds in Australia and the United States seem to be commonly infected (Swayne & McLaren, 1997; Oxberry *et al.*, 1998). Waterbirds have been suggested as potential natural reservoirs and transmitters of *Brachyspira pilosicoli* to animals and humans (Oxberry *et al.*, 1998; Duhamel, 2001).

Brachyspira hyodysenteriae is recognized as the aetiological agent of swine dysentery, a mucohaemorrhagic diarrhoeic disease of pigs (Ochiai *et al.*, 1997; Harris *et al.*, 1999). This agent has also been suggested as the cause of necrotizing typhlocolitis in common rheas (*Rhea americana*) (Sagartz *et al.*, 1992; Buckles *et al.*, 1997). Furthermore, *B. hyodysenteriae* may be present in wild rodents on farms with infected pigs (Duhamel, 2001). The natural occurrence of *B. hyodysenteriae* in other mammalian and avian species has not been verified. Interestingly, neither *B. hyodysenteriae* nor any other intestinal spirochaete has been isolated from wild boars in Sweden (Fellström & Jacobsson, 2002).

B. hyodysenteriae is routinely diagnosed by culture, phenotypic traits, which consist of strong β -haemolysis, indole production, the inability to cleave hippurate and α -galactosidase activity (Fellström *et al.*, 1999), and by specific PCR assays (Leser *et al.*, 1997; Ateyo *et al.*, 1999; Fellström *et al.*, 2001). Several atypical *B. hyodysenteriae* isolates of porcine origin have recently been described. These include isolates that do not produce indole (Fellström *et al.*, 1999) and those that are negative for a *B. hyodysenteriae*-specific 23S rDNA-based PCR test (Murray *et al.*, 2003). PFGE, randomly amplified polymorphic DNA (RAPD) and phylogenetic analysis based on 16S rRNA sequence analysis showed that these atypical variants are genetically closely related to typical porcine *B. hyodysenteriae* (Fellström *et al.*, 1999; Murray *et al.*, 2003).

In a previous study, we cultured a putative *B. hyodysenteriae* isolate from a mallard (*Anas platyrhynchos*) on a gamebird farm (Jansson *et al.*, 2001). The present study was designed to confirm this finding, to investigate whether the same spirochaete phenotype was present in wild-living mallards and to identify these isolates. Finally, we wanted to examine whether age, season, sex or migratory status was associated with the presence of *Brachyspira* spp. in wild mallards. We chose the mallard as our target species because it is the most numerous and widespread of all ducks. The mallard is an omnivorous dabbling duck that lives on wetlands, sometimes in close association to human populations. In addition, it is a common game species and is also the ancestor of domestic ducks.

METHODS

Strains and isolates. All strains and isolates of porcine, canine and avian origin that were used for phenotyping, PCR and genetic analyses

are shown in Table 1. One of those isolates from a mallard on a gamebird farm (AN383:2/00) has previously been described phenotypically (Jansson *et al.*, 2001). A further 27 cloacal swabs were obtained in June 2000 from adult mallards on the same farm. The sampling was approved by the Swedish Ethical Committee for Scientific Experiments (protocol C33/99). Samples from wild mallards were collected independently from two separate locations. Between November 2000 and May 2001, 33 wild mallards were sampled in a public park with an ornamental lake in southern Sweden (Pildammsparken, Malmö; 55° 35' N 12° 58' E). These samples were collected as freshly deposited faeces. Additionally, 184 migrating mallards were consecutively caught on an island in south-eastern Sweden during October and November 2002 (Ottenby Bird Observatory, Öland; 56° 12' N 16° 24' E). The birds were caught in a duck funnel trap with the approval of the Swedish Museum of Natural History/Swedish Environmental Protection Agency (protocol 412-1038-02 Nf) and the sampling was approved by the Swedish Ethical Committee for Scientific Experiments (protocol M109/02). The samples were obtained either as cloacal swabs or as fresh faeces. In the latter case, birds were confined individually to boxes on clean newspaper until they defecated, and their faeces were collected. The sex and age of the birds were determined as described previously (Boyd *et al.*, 1975). Birds less than 1 year old were considered as juveniles, and older birds as adults. All the sampled mallards were clinically healthy and showed no signs of diarrhoea. The samples were transported at ambient temperature by surface mail in Amies medium and cultured within 24–48 h at the Department of Bacteriology, National Veterinary Institute, Uppsala, Sweden. Spirochaetes were thereafter isolated and classified according to previous descriptions (Fellström *et al.*, 1999).

PCR analysis. PCR for detection of the *tlyA* haemolysin gene of *B. hyodysenteriae* was used according to previously described protocols (Fellström *et al.*, 2001), with some modifications. Each sample was tested twice. In total, ten type, reference and field strains of porcine origin, one chicken isolate, one isolate from a dog and 15 mallard isolates were analysed by PCR.

PFGE. PFGE was performed as described previously (Fellström *et al.*, 1999). A dendrogram was created with the GelCompar program based on combined gels of *MluI*- and *SalI*-digested DNA, using a matrix of band-matching coefficients by the unweighted pair group method with arithmetic mean (UPGMA) clustering fusion strategy. Ten type, reference and field strains of porcine origin, one chicken isolate, one isolate from a dog and two mallard isolates with a biochemical phenotype consistent with group I spirochaetes were studied by PFGE.

RAPD. Two mallard isolates with a biochemical phenotype consistent with group I spirochaetes, one chicken isolate and ten type, reference and field strains of porcine origin were studied by RAPD, as described previously (Quednau *et al.*, 1998), with a few modifications. The cells were lysed by boiling and the isolates were typed with primers P1254 (5'-CCGCAGCCAA-3') and P73 (5'-ACGCGCCT-3'), separately, which resulted in two sets of banding patterns. Those patterns were combined in the GelCompar program with the combined gels option.

Sequence analysis of the 16S rRNA gene. 16S rDNA sequences of 11 of the selected *Brachyspira* isolates from farmed and wild-living mallards were determined as described by Pettersson *et al.* (1996), with some modifications. The 16S rRNA gene was first amplified with the PCR primers listed in Table 2. The sequencing system (Pettersson *et al.*, 1996) was adapted to the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) with the primers listed in Table 2. Labelled terminators (Big Dye; Applied Biosystems) were used in the cycle sequencing reactions. The primer sequences are given in Table 2. Contigs were created using the ContigExpress program included in the Vector NTI suite (Informax). The 16S rDNA sequences were aligned manually using Genetic Data Environment software (Smith, 1992), to a prealigned set of

Table 1. Biochemical characteristics, PCRs and GenBank accession nos of porcine, canine and avian *Brachyspira* strains included in this studyInd, Indole production; Hipp, hippurate cleavage; α -gal, α -galactosidase activity; NA, not available; ND, not done; +/-, different results were obtained when duplicated.

Strain	Species	Animal of origin	β -Haemolysis	Ind	Hipp	α -Gal	<i>tlyA</i> *	GenBank accession no.	Reference
B78 ^T	<i>B. hyodysenteriae</i>	Pig	Strong	+	–	–	+	U14930	Harris <i>et al.</i> (1972)
B204	<i>B. hyodysenteriae</i>	Pig	Strong	+	–	–	+	U14932	Kinyon <i>et al.</i> (1977)
R1	<i>B. hyodysenteriae</i>	Rhea	Strong†	+†	NA	–†	NA	U23035	Sagartz <i>et al.</i> (1992)
NIV-1	<i>B. hyodysenteriae</i>	Rhea	Strong†	+†	NA	–†	NA	U23036	Jensen <i>et al.</i> (1996)
AN983:90	<i>B. intermedia</i>	Pig	Weak	+	–	–	–	U14933	Pettersson <i>et al.</i> (1996)
PWS/A ^R	<i>B. intermedia</i>	Pig	Weak	+	–	–	–	U23033	Stanton <i>et al.</i> (1997)
C378	<i>Brachyspira murdochii</i>	Pig	Weak	–	–	–	–	U14918	Pettersson <i>et al.</i> (1996)
56-150 ^R	<i>B. murdochii</i>	Pig	Weak	–	–	–	–	AY312492	Stanton <i>et al.</i> (1997); K.-E. Johansson and others (unpublished)
C173	<i>Brachyspira innocens</i>	Pig	Weak	–	–	–	–	U14921	Pettersson <i>et al.</i> (1996)
B256 ^T	<i>B. innocens</i>	Pig	Weak	–	–	+	–	U14920	Kinyon & Harris (1979)
P43/6/78 ^T	<i>B. pilosicoli</i>	Pig	Weak	–	+	+	–	U14927	Pettersson <i>et al.</i> (1996)
AN497:93	<i>B. pilosicoli</i>	Pig	Weak	–	+	+	–	U14927	Pettersson <i>et al.</i> (1996)
C1 ^T	<i>Brachyspira alvinipulli</i>	Chicken	Weak	–	+	+	–	U23030	Swayne <i>et al.</i> (1992); Stanton <i>et al.</i> (1998)
AN4578/01	<i>Brachyspira</i> spp.	Dog	Weak	–	+	–	ND	AY349933	K.-E. Johansson and others (unpublished)
AN383:2/00	<i>B. hyodysenteriae</i>	Mallard ^a ‡	Strong	+	–	–	+	AY352291	Jansson <i>et al.</i> (2001); this study
AN3035:3/00	<i>B. hyodysenteriae</i>	Mallard ^b	Strong	+	–	–	+	ND	This study
AN3049:2/00	<i>B. hyodysenteriae</i>	Mallard ^b	Strong	+	–	+/-	+	ND	This study
AN3060:1/00	<i>B. hyodysenteriae</i>	Mallard ^b	Strong	+	–	–	+	ND	This study
AN3052:1/00	<i>B. hyodysenteriae</i>	Mallard ^b	Strong	+	–	–	+	800 bp§	This study
AN1409:2/01	<i>B. hyodysenteriae</i>	Mallard ^c	Strong	+	–	–	+	AY352281	This study
AN1418:2/01	<i>Brachyspira</i> spp.	Mallard ^c	Strong	+	–	–	–	AY352282	This study
AN3599:2/02	<i>Brachyspira</i> spp.	Mallard ^d	Strong	+	–	–	–	AY352283	This study
AN3624:2/02	<i>Brachyspira</i> spp.	Mallard ^d	Strong	+	–	–	–	AY352284	This study
AN3886:2/02	<i>Brachyspira</i> spp.	Mallard ^d	Strong	+	–	–	–	AY352285	This study
AN3902:2/02	<i>Brachyspira</i> spp.	Mallard ^d	Strong	+	–	–	–	AY352286	This study
AN3907:2/02	<i>B. hyodysenteriae</i>	Mallard ^d	Strong	+	–	–	+	AY352287	This study
AN3930:2/02	<i>Brachyspira</i> spp.	Mallard ^d	Strong	+	–	–	–	AY352288	This study
AN3931:2/02	<i>Brachyspira</i> spp.	Mallard ^d	Strong	+	–	–	–	AY352289	This study
AN3949:2/02	<i>Brachyspira</i> spp.	Mallard ^d	Strong	+	–	–	–	AY352290	This study

*Primer for PCR taken from Fellström *et al.* (2001).†Data from Trott *et al.* (1996).‡Mallards were: *a*, farmed bird, faecal sample; *b*, farmed bird, cloacal swab; *c*, wild bird in a public park, faecal sample; *d*, wild migrating bird, faecal sample.

§Sequence not deposited; preliminary data on 800 bp show close similarity to the sequence of R1 and NIV-1.

Table 2. Primers used for PCR and sequencing of the 16S rRNA gene of *Brachyspira* spp.Positions are numbered according to the *Escherichia coli* 16S rRNA sequence.

Designation (direction)	Position	Sequence (5'–3') or reference	Application
kag-007 (forward)	11–35	GTTTGATYCTGGCTCAGARCKAACG	PCR
kag-009 (reverse)	1496–1520	CTCCGGTACGGMTGCCTTGTACG	PCR
kag-008 (forward)	15–30	GATYCTGGCTCAGARC	Sequencing
584 (forward)	334–352	Johansson <i>et al.</i> (1998)	Sequencing
631 (reverse)	519–534	Johansson <i>et al.</i> (1998)	Sequencing
538 (forward)	800–818	Johansson <i>et al.</i> (1998)	Sequencing
kag-011 (reverse)	922–939	CTTGTGCGGGYCCCGTC	Sequencing
597 (forward)	1175–1193	Johansson <i>et al.</i> (1998)	Sequencing
kag-010 (reverse)	1501–1518	TCCGGTACGGMTGCCTTG	Sequencing

Brachyspira spp. sequences retrieved from the Ribosomal Database Project (Maidak *et al.*, 2001).

Statistical analyses. Relative risks between isolation rate of intestinal spirochaetes and sampling sites, seasons, age groups and sexes were calculated, and the confidence intervals were precision based (Kleinbaum *et al.*, 1982). To examine which of the potential risk factors (age, sex, migratory status and season) were most important for finding *Brachyspira* spp., a log linear model specified as a multivariate generalized linear model, with a Poisson distribution of the error term and log link function, was used. For inclusion in the final model, a *P* value < 0.01 was required.

RESULTS AND DISCUSSION

Strains and isolates

Brachyspira spp. were isolated from 25 of 27 (93%) cloacal swabs collected from mallards on the game farm and from

169 of 217 (78%) samples from wild mallards. The results of spirochaete cultures from wild mallards and relative risks are shown in Table 3. On average, 1.5 spirochaete isolates with different phenotypes were obtained from each infected wild mallard. The multivariate analyses indicated that the presence of *Brachyspira* spp. among the wild mallards examined appeared to be determined by whether the birds were migrating or not (*RR* = 1.8, 95% CI 1.1–3.1).

Isolates with a particular phenotype characteristic of *B. hyodysenteriae* were obtained in pure culture from five of 27 (19%) farmed mallards and from 18 of 217 (8%) wild mallards. Birds with strongly β -haemolytic *Brachyspira* spp. were distributed throughout the sampling period. This spirochaetal phenotype was cultured from birds of both sexes and from both juvenile and adult birds. In most cases,

Table 3. Results of *Brachyspira* spp. cultures in 217 wild mallards

RR, Relative risk; *CI*, confidence interval. Birds of unknown age or sex were not included in the analyses.

Variable	<i>Brachyspira</i> spp.		<i>RR</i> ; 95% <i>CI</i> limits
	Positives/sampled	Prevalence (%)	
Sampling site			
Public park	15/33	45	Baseline
Bird station (migratory birds)	154/184	84	3.3; 2.2–5.1
Sex			
Male	88/121	73	Baseline
Female	79/94	84	2.9; 1.8–4.6
Unknown	2/2	100	
Age			
Adults	27/50	54	Baseline
Juveniles	127/151	84	1.7; 1.0–3.0
Unknown	15/16	94	
Season			
Spring (February to May)	9/18	50	Baseline
Autumn (September to November)	160/199	80	2.6; 1.5–4.4

spirochaetes with strong β -haemolysis were accompanied by growth of weakly haemolytic spirochaetes on the same agar plate.

The overall isolation rate of *Brachyspira* spp. (78 %) in the wild mallards in this study was similar to previous studies (74–88 %) where waterbirds of various species were analysed in Australia and the United States by culture or indirect fluorescent antibody test (Swayne & McLaren, 1997; Oxberry *et al.*, 1998). Taken together, these results and those of previous studies strongly support the conclusion that intestinal spirochaetes are commonly found in at least some wild-living waterbird species. The isolation rate from farmed mallards was even higher than in the wild mallards. The population density is often higher on game farms than in natural habitats, and wild birds frequently fly in and out of game farms. The level of faecal contamination in ponds, lakes and on the ground may be significant.

In selective sampling from autumn-migrating mallards, the aim was to sample birds flying in from different areas in order to avoid the effect of local variations on prevalence. High population densities may be present in some breeding, resting and feeding areas. There was no way of determining whether the migrating mallards that were sampled in this study originated from different areas, or if they had lingered together to breed, feed or rest before being caught at the bird observatory. The isolation rate from these migrating mallards during autumn was almost twice as high as in mallards sampled during spring in the public park. However, of the putative risk factors for *Brachyspira* spp. in wild mallards, it appeared that migratory status was the most important. As migration may temporarily suppress immunocompetence in birds through increased corticosteroid levels (Holberton *et al.*, 1996; Råberg *et al.*, 1998), it cannot be excluded that their susceptibility to spirochaetal infections is increased during migratory seasons, or that the shedding of spirochaetes is promoted by migration. However, young age, sex (females) and season (autumn) could also be important risk factors for finding *Brachyspira* spp. or simply reduce the prominence of migration as a risk factor. The current evidence does not enable a firm conclusion on this issue. Moreover, there were several missing observations concerning age and sex that could distort the results of the statistical analyses. Thus, the inference that *Brachyspira* spp. is associated with migration should be considered indicative rather than conclusive.

PCR assay

The results of the PCR assay are shown in Table 1. All selected isolates from farmed mallards and two isolates from wild mallards (AN1409:2/01 and AN3907:2/02) were PCR-positive for the *tlyA* gene and were therefore classified as *B. hyodysenteriae*. The remaining isolates from wild mallards were negative for the *tlyA* gene and could not be classified by PCR.

PFGE and RAPD

The two isolates that were analysed by PFGE and RAPD

originated from the mallard farm. The dendrogram based on RAPD analysis is shown in Fig. 1. The similarity values for both assays showed that the avian isolates were closely related to the reference strains B78^T and B204 of *B. hyodysenteriae*, thus confirming their classification based on PCR as *B. hyodysenteriae*.

Phylogenetic analysis

The phylogenetic tree based on 16S rRNA sequence analysis is shown in Fig. 2. Accession numbers of the 16S rRNA gene of mallard isolates are given in Table 1. Phylogenetic analysis based on 16S rRNA sequences showed that all of the 11 studied *Brachyspira* strains grouped in the *B. hyodysenteriae*–*Brachyspira intermedia* cluster. The similarity values of the 16S rRNA sequences in this cluster varied from 99.4 to 100 %. This observation, and the fact that there were no coherent subclusters comprising only representatives of the respective species, shows that it is not possible to distinguish between *B. hyodysenteriae* and *B. intermedia* by sequence analysis of 16S rDNA, in accordance with previously published results (Pettersson *et al.*, 1996). Three of the mallard strains (AN383:2/00, AN1409:2/01 and AN3907:2/02) had identical sequences, that were also identical to two strains of *B. hyodysenteriae* (R1 and NIV-1) that have been isolated from common rheas from farms in Ohio and Iowa in the United States (Sagartz *et al.*, 1992; Jensen *et al.*, 1996). The mallard isolates possessing this sequence were from a farmed bird, and from the two wild birds. These two wild birds had been

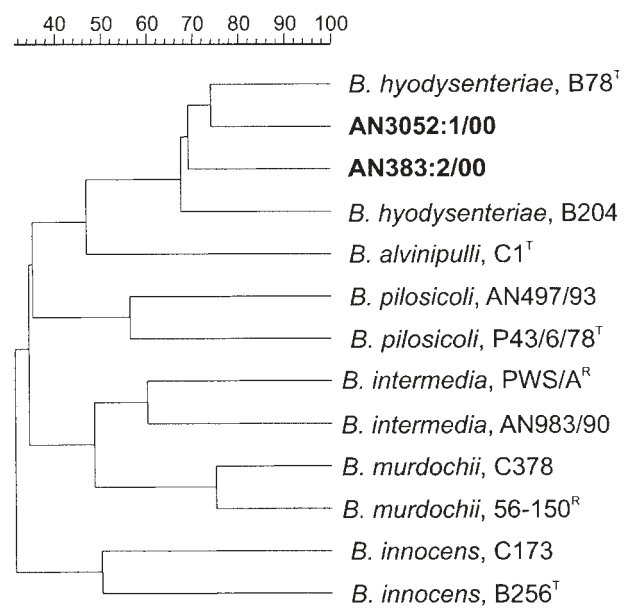


Fig. 1. Dendrogram as deduced from RAPD banding patterns of *Brachyspira* spp. Seven type or reference strains, four porcine field isolates and two isolates from farmed mallards (shown in bold) were studied. The analyses were performed using the UPGMA clustering fusion strategy (Vauterin & Vauterin, 1992).

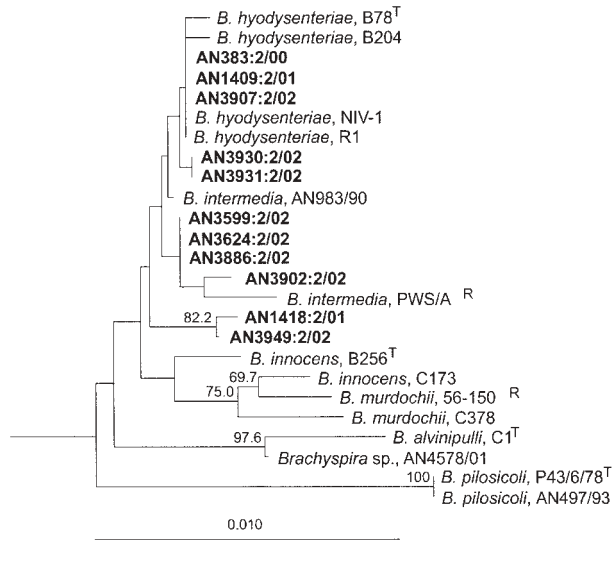


Fig. 2. Evolutionary tree based on sequence analysis of the 16S rRNA gene showing the phylogenetic relations between *Brachyspira* spp. isolated from mallards (shown in bold) and other strains of *Brachyspira* spp. The tree was constructed by neighbour-joining (Saitou & Nei, 1987) from a distance matrix comprising 1399 nucleotide positions, which was corrected for multiple substitutions at single locations by the two-parameter method of Kimura (1980). *Brachyspira aalborgi* was used as outgroup. Bootstrap percentages from 1000 resamplings of the dataset are given for some of the stable nodes. The scale bar shows the distance equivalent to one substitution per 100 positions.

sampled during different seasons, years and at separate locations. We believe that these three mallard isolates and the two previously described isolates from common rheas should be considered as *B. hyodysenteriae*. This specific *B. hyodysenteriae* genotype seems to be genetically rather stable, present on two continents in both farmed and wild-living avian hosts and possibly adapted to birds, as it has not been found in pigs. Two additional strains (AN3930:2/02 and AN3931:2/02) were also identical with respect to their 16S rRNA sequences, and differed from strains R1 and NIV-1 by only one nucleotide. These two latter isolates both originated from wild-living migrating mallards. Three strains from wild mallards (AN3599:2/02, AN3624:2/02 and AN3886:2/02) were identical and related to the sequence of strain AN3902:2/02 and the reference strain PWS/A of *B. intermedia*. Finally, two strains (AN1418:2/01 and AN3949:2/02) formed a cluster with a relatively stable node (82.2%) basal to the *B. hyodysenteriae*–*B. intermedia* cluster. However, this cluster has no unique nucleotides that differentiate it from all other *Brachyspira* strains. Consequently, the negative PCR analysis and phylogenetic analyses of these isolates could not assign them to any *Brachyspira* species; however, they seem to be most closely related to *B. hyodysenteriae* and *B. intermedia*. The phylogenetic tree

(Fig. 2) should therefore be interpreted with caution, because it is based on few sequence differences and many of the nodes are not stable (bootstrap values lower than 67%).

Clinical significance

The clinical significance of *B. hyodysenteriae* in non-porcine species remains somewhat unclear. In common rheas, the infection has been associated with necrotizing typhlocolitis and high mortality (Sagartz *et al.*, 1992; Buckles *et al.*, 1997). On the other hand, challenge infection of juvenile common rheas with the R1 rhea strain of *B. hyodysenteriae* was inconclusive as to the development of lesions, although spirochaetes were seen in histological sections in 75% of the infected birds (Buckles, 1996). In the same study, mallard ducklings were infected by strain R1. Histological evaluation revealed spirochaetes in only 7% of the inoculated ducks and none of the birds developed any lesions (Buckles, 1996). Furthermore, in inoculation experiments of pigs, *B. hyodysenteriae* strains R1 and R358 from common rheas also failed to colonize the animals and they did not develop any lesions (Stanton *et al.*, 1997). In a preliminary challenge infection trial approved by the Swedish Ethical Committee for Scientific Experiments (protocol C17/2), we used one of the mallard isolates of *B. hyodysenteriae* (AN3052:1/00) to inoculate three grower pigs twice orally (data not shown). We were not able to reisolate the bacterium from rectal swabs obtained daily from each pig, except from one pig within 24 h of the inoculation. Spirochaetes did not grow from caecal or colonic samples obtained at necropsy 4 weeks post-inoculation. All pigs remained healthy. In the case of mallards, we have at present no reason to believe that *B. hyodysenteriae* or *B. hyodysenteriae*-like variants are pathogenic. None of the sampled birds in this study showed any signs of disease or diarrhoea. However, we were not able to investigate any of the birds for histological evidence of lesions. This does not necessarily mean that *B. hyodysenteriae* and *B. hyodysenteriae*-like variants are avirulent in mallards; they seem more likely to be opportunistic or apathogenic to the host. Taken together, the current lack of evidence of pathogenicity of avian isolates of *B. hyodysenteriae* in pigs indicates avirulence. Additional studies are needed to confirm this conclusion. Moreover, if these extended challenge trials should confirm the apathogenic nature of avian *B. hyodysenteriae*, these isolates may serve as useful comparative tools in identifying pathogenicity mechanisms in other strains of *B. hyodysenteriae*.

Conclusions

To conclude, we were able to classify the strongly β -haemolytic and indole-positive spirochaete isolates from farmed mallards and two isolates from wild mallards as *B. hyodysenteriae*. This was based on the fact that they could not be differentiated with any of the applied methods from type, reference and field strains of *B. hyodysenteriae* originating from pigs and common rheas. The remaining isolates could not be irrefutably assigned to any of the presently recognized *Brachyspira* species. These results point to a broader host

spectrum of *B. hyodysenteriae* than generally recognized, as well as to the presence of strongly β -haemolytic and indole-producing spirochaetes that possess many, but not all, of the currently recognized characteristics of *B. hyodysenteriae*.

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