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Antimicrobial susceptibility of *Brachyspira* spp. isolated from commercial laying hens and free-living wild mallards

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Short Title: MICs of avian *Brachyspira* spp.

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Abstract

In vitro antimicrobial susceptibility to tylosin, valnemulin, tiamulin, doxycycline, lincomycin and ampicillin was investigated by broth dilution in 48 *Brachyspira* spp. isolates from commercial laying hens (*n*=30) and free-living wild mallards (*Anas platyrhynchos*) (*n*=18). Presumed pathogens (*B. alvinipulli*, *B. intermedia*, *B. pilosicoli*), commensals (*B. murdochii*, *B. innocens*, "*B. pulli*"), and isolates of undetermined species affiliation were included. The laying hens had not been exposed to therapeutic levels of antimicrobials for at least 50 weeks before sampling, and low levels of environmental antimicrobial exposure were presumed in mallards. No isolates with decreased susceptibility to tylosin, valnemulin, tiamulin or doxycycline were found. Decreased susceptibility to lincomycin (MIC 16 µg/ml) was detected in two isolates (*Brachyspira* sp.) from laying hens. Five isolates showed decreased susceptibility to ampicillin (MIC 16->32 µg/ml), including two "*B. pulli*" and one *B. alvinipulli* from laying hens, and isolates of *B. pilosicoli* and "*B. pulli*" from mallards. Decreased susceptibility to ampicillin was associated with *β*-lactamase activity in 4 isolates. A new variant of a class D *β*-lactamase gene designated $bla_{oxa\text{-}192}$ was identified in a *B. pilosicoli* isolate of mallard origin. This is the first time the genetic basis for antimicrobial resistance is described in *Brachyspira* spp. from a free-living wild bird. Isolates displaying decreased susceptibility to ampicillin were accompanied by fully susceptible isolates of the same species or other genotypes within three laying hen flocks. This underlines the need of performing antimicrobial susceptibility tests on single clones/genotypes, and to analyze multiple isolates from the same flock.

Introduction

Colonization with intestinal spirochaetes of genus *Brachyspira* commonly occurs in mammals, e.g. pigs, dogs, rodents and humans, and in domestic and free-living wild birds, especially gallinaceous and anseriform species. In chickens, a wide range of potentially pathogenic and commensal species may be found, including *B. alvinipulli, B. intermedia* and *B. pilosicoli* that have been linked to diarrhoea, wet litter, faecal staining of egg shells and impaired laying performance in adult chickens (Hampson and McLaren, 1999; Stanton *et al*., 1998; Stephens and Hampson, 2002). Among the remaining recognized and proposed species found in chickens, *B. murdochii*, *B. innocens* and "*B. pulli*" are presumed non-pathogenic species, and the swine dysentery agent *B. hyodysenteriae*, although an unusual finding in chickens (Feberwee *et al*., 2007), has not yet been linked to disease. Free-living wild mallards host a wide range of *Brachyspira* spp. including known pathogens of livestock, i.e. *B. hyodysenteriae* (Jansson *et al*., 2004), "*B. suanatina*" (Råsbäck *et al*., 2007), *B. pilosicoli*, *B. intermedia* and *B. alvinipulli*, the presumed commensal "*B. pulli*", and isolates that cannot presently be identified to species level (Jansson *et al*., unpublished observation). It is not known whether colonization of mallards is associated with enteric disease.

Antimicrobial agents, especially pleuromutilins, macrolides and lincosamides are widely used to control swine dysentery in pig herds. In *B. hyodysenteriae* of porcine origin, widespread resistance has emerged to tylosin in the USA, Denmark, Australia, Finland, Belgium, Sweden and Spain (Kinyon and Harris, 1980; Rønne *et al*., 1990; Smith *et al*., 1991; Honkanen-Buzalski *et al*., 1999; Hommez *et al*., 1998; Bengtsson *et al*., 2010; Hidalgo *et al*., 2010). Tiamulin resistance has been described in recent years from Hungary, the United Kingdom, the Czech Republic, Germany, the Netherlands, Spain and Japan (Molnár, 1996; Gresham *et al*., 1998;

Lobova *et al*., 2004; Rohde *et al*., 2004; Duinhof *et al*., 2008; Hidalgo *et al*., 2010; Ohya and Sueyoshi, 2010). Compared to the situation in pigs, there is little information on antimicrobial susceptibility in *Brachyspira* spp. of avian origin. Attempts to treat chicken flocks colonized by intestinal spirochaetes have met with limitations mostly because of lack of approved products and application of long withdrawal times for eggs. Moreover, chickens are likely to get reinfected from environmental sources, especially when housed on litter, which may lead to recurrence of clinical signs (Smit *et al*., 1998; Stephens and Hampson, 1999). More recently, Burch *et al*. (2006) reported improved egg production and reduced mortality in chickens colonized by *B. pilosicoli* that were treated with tiamulin.

The objective of this study was to investigate antimicrobial susceptibility of *Brachyspira* spp. isolates from laying hens with a history of low antimicrobial use and, for comparison, isolates from free-living wild mallards were included.

Materials and Methods

Spirochaete isolates. All analyzed isolates (Table 1) originated from commercial Swedish laying hens (*n*=30) or free-living wild mallards (*Anas platyrhynchos*) (*n*=18) that had been sampled in Sweden. The isolates have been investigated by phenotypic and molecular tests, and identified to species level when possible (Jansson *et al*., unpublished results). Information on sampling is available elsewhere (Jansson *et al*., 2004). The laying hens were approximately 65 wks of age at sampling, and they had not been exposed to therapeutic levels of antimicrobials during the production period, i.e. approximately 50 weeks prior to sampling. No information was available on antimicrobial use during the rearing period. The environmental exposure to antimicrobials of

the sampled mallards was assumed to be low. Following selective culture, the isolates had been subcultured once, and if necessary subcultured to pure genotypes by 10-fold serial dilution as described (Jansson *et al*., 2008). The isolates were stored in liquid nitrogen (Fellström and Gunnarsson, 1995) in the strain collection of the National Veterinary Institute (SVA), Uppsala, Sweden until further use.

Antimicrobial susceptibility tests. Thawed isolates were grown on fastidious anaerobe agar supplemented with 10% equine blood (FAA) (SVA) and subcultured twice. The purity of all isolates was assessed by phase contrast microscopy. The MICs of tiamulin, valnemulin, doxycycline, lincomycin, tylosin, and ampicillin were determined by broth dilution in VetMIC™ Brachy panels (SVA) as described previously (Karlsson *et al*. 2003). The medium for the susceptibility tests was brain heart infusion broth (Difco, BD, Sparks, Maryland, USA) supplemented with 10% fetal calf serum (SVA). The MIC was read as the lowest concentration of the antimicrobial agent that prevented visible growth. Strains B78^{T} (*B. hyodysenteriae,* ATCC 27164) and Wichita (*Staphylococcus aureus* subsp. *aureus*, ATCC 29213) were used as controls.

***β*-lactamase detection.** Nitrocefin disks (AB Biodisk, Solna, Sweden) were used to determine *β*-lactamase activity of isolates AN3382/1/03, AN304/04, AN1268/3/04, AN3635/3/02 and AN6052:2/1/00. The disks were moistened with phosphate buffered saline and two 1 µl loops of bacterial cells from 3-day old cultures from FAA plates were smeared on the disks and incubated for 30 min at room temperature. *β*-lactamase activity was detected by observing colour change from yellow to red after 5 and 30 min. Strain B78^{T} (*B. hyodysenteriae,* ATCC 27164) was used as negative control and strain Rosenbach 1884 (*Staphylococcus aureus* subsp. *aureus*, CCUG 15915) as positive control.

Amplification and sequencing of the *β*-lactamase and 23S rRNA genes. PCRs were performed to attempt to detect a *β*-lactamase gene of isolates AN3382/1/03, AN304/04, AN1268/3/04, AN3635/3/02 and AN6052:2/1/00. PCR amplification of the 23S rRNA gene (870 nucleotides, positions 1864-2733 *E. coli* numbering, acc. no. J01695) of isolate AN1828/02 was performed to identify possible point mutations that could explain the decreased susceptibility to lincomycin displayed by this isolate. Chromosomal DNA was prepared from 3-day old cultures on FAA plates as previously described (Råsbäck *et al*., 2006). Primers used for PCR amplification and sequencing of *β*-lactamase and 23S rRNA genes are listed in Table 2. Primers for the *β*-lactamase gene were designed based on sequences deposited in the GenBank database, acc. nos. AY619003 (*B. pilosicoli*, strain BM4442) (Meziane-Cherif *et al*., 2008), CP002025 (*B. pilosicoli*, strain 95/1000) (Wanchanthuek *et al*., 2010), and AY227054 (*Fusobacterium nucleatum* subsp. *polymorphum*, strain N161) (Voha *et al*., 2006). PCRs were performed under the following conditions: 3 min at 94°C, 30 cycles with 30 s denaturation at 94°C, 40 s annealing at 59°C and extension at 72 for 54 s, followed by 10 min final extension at 72°C. Amplicons were analyzed by electrophoresis in 1.5% agarose gels. Water was used as negative control and *B. hyodysenteriae* strain B78^{T} (ATCC 27164) was used as positive control for amplification of the 23S rRNA gene. PCR amplicons were purified by the illustra GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Uppsala, Sweden). Sequencing reactions were performed using the BigDye® Terminator v3.1 kit (Applied Biosystems) in an ABI PRISM® 2700 Genetic Analyzer at Uppsala Genome Center, Uppsala University, Uppsala, Sweden [http://www.genpat.uu.se/node462]. Sequences were edited and translated using the CLC bio Main Workbench (CLC bio, Århus N, Denmark). A Neighbour joining tree of class D *β*-lactamase genes of *B. pilosicoli* was constructed from a high fidelity nucleotide alignment made

by the algorithm included in the CLC Sequence Viewer free software, version 6.4 using default parameters.

Results

Antimicrobial susceptibility and *β*-lactamase activity. The MICs of tested antimicrobial agents are presented in Table 1. No isolates with decreased susceptibility to tylosin, valnemulin, tiamulin or doxycycline were found. Two isolates of *Brachyspira* sp. (AN1828/03 and AN1831/03) from the same organic laying hen flock (A, Table 1) showed decreased susceptibility to lincomycin (MIC 16 µg/ml), and five isolates showed decreased susceptibility to ampicillin (MIC 16->32 µg/ml). The latter isolates included two "*B. pulli*" (AN3382/1/03 and AN304/04) and one *B. alvinipulli* (AN1268/3/04) from three separate laying hen flocks (flocks C, Q and R, Table 1), and a *B. pilosicoli* (AN3635/3/02) and a "*B. pulli*" (AN6052:2/1/00) isolate from free-living wild mallards. Four of these isolates (AN3382/1/03, AN304/04 AN1268/3/04 and AN3635/3/02) and the positive control strain Rosenbach 1884 produced *β*-lactamase that hydrolysed the chromogenic cephalosporin nitrocefin, in less than five minutes, and the fifth isolate (AN6052:2/1/00) showed a weak colour change after 30 min. For *B. hyodysenteriae* B78^T no colour change was observed after 30 minutes. Susceptible isolates as well as isolates with decreased susceptibility to ampicillin were found when several isolates from the same laying hen flock (flocks C, Q and R, Table 1) were analyzed. Based on previously published phenotypic and molecular analyses (Jansson *et al*., 2008), isolates originating from the same chicken flocks represented different species (flock C, Q and R, Table 1) or different genotypes of the same species (flock R). These isolates originated either from a single faecal sample (flocks C, and R)

or from different faecal samples from the same flock (flocks C, Q and R). Similarly, susceptible *Brachyspira* isolates (AN3635/2/02 and AN3635/5/02) were found together with an isolate with decreased susceptibility (AN3635/3/02) in a sample from a mallard.

Amplification and sequencing of *β*-lactamase and 23S rRNA genes. For isolates AN3382/1/03, AN304/04, AN1268/3/04, and AN6052:2/1/00 no amplicons of predicted sizes were detected by the *β*-lactamase gene PCRs, but for isolate AN3635/3/02 amplicons of predicted sizes were obtained with primer combinations BlaF1/BlaR3, BlaF2/R3, BlaF3/R3 and BlaF3/BlaR4 (Table 2). A complete *β*-lactamase gene sequence was obtained from the sequenced and assembled amplicons of primer combinations BlaF1/BlaR3 and BlaF3/BlaR4, and sequence information is available in the GenBank database (acc. no. JF273470). Comparison of the predicted amino acid sequence in the GenBank database revealed closest similarity to class D *β*-lactamase genes from *B. pilosicoli* human isolate H/A1 (OXA-136 gene, acc. no. ABW76138) (260/269 amino acid identities, E value 3e-147), *B. pilosicoli* porcine strain 95/1000 (OXA-136 gene, acc. no. YP_003785827, 262/269 amino acid identities, E value 2e-140), *B. pilosicoli* human isolate Gap 51.2 (OXA-136 gene, acc. no. ABW76137, 261/269 amino acid identities, E value 4e-140), and *B. pilosicoli* human isolate BM4442 (OXA-63 gene, acc. no. AAU88145, 248/269 amino acid identities, E value 6e-130). The new gene variant described in this paper was designated $bla_{\text{oxa-192}}$. A tree showing the genetic relationship of this gene to previously published class D *β*-lactamase genes of *B. pilosicoli* and to the gene $bla_{\text{OXA-85}}$ of *Fusobacterium nucleatum* subsp. *polymorphum*, strain N161 is shown in Fig. 1. No mutations were identified within the 23S rRNA gene fragment of isolate AN1828/02, compared to the wild type sequence of *B. hyodysenteriae*.

Discussion

Reports on antimicrobial susceptibility of *Brachyspira* spp. isolates from avian hosts are few and have focused on the enteropathogenic species *B. alvinipulli*, *B. intermedia* and *B. pilosicoli* of chickens and *B. hyodysenteriae* from farmed common rheas (Trampel *et al*., 1999; Hampson *et al*., 2006). Trampel *et al*. (1999) investigated MICs of 11 antimicrobial agents by agar dilution test for four isolates each from chickens (*B. pilosicoli*, *B. alvinipulli*) and common rheas (*B. hyodysenteriae*) of US origin. All isolates were susceptible to lincomycin, carbadox and tiamulin, whereas the MICs of chlortetracycline, oxytetracycline, bacitracin, erythromycin, tylosin, neomycin, penicillin and streptomycin varied between isolates. Notably, the MICs of penicillin ranged from 17.0-170 IU/ml in chickens but were higher in isolates from common rheas; 1.700 IU/ml. In another study, Hampson *et al*. (2006) investigated MICs of tiamulin, lincomycin, tylosin, metronidazole and tetracycline by agar dilution tests for 42 isolates of *B. intermedia* and *B. pilosicoli* from chickens, of which the majority were of Australian origin. In total, 22 isolates, represented by both *B. intermedia* and *B. pilosicoli*, showed reduced susceptibility to tylosin, and the MICs of ampicillin for two isolates of *B. pilosicoli* from one farm were >32 mg/l. Neither of these studies supplied information on prior exposure to antimicrobials. The isolates investigated in the present study came from chickens that had not been exposed to antimicrobials at least 50 wks prior to sampling (Jansson *et al*., 2008) and also the overall use of antimicrobials in Swedish poultry is low (Bengtsson *et al*., 2010). For many years, only one product has been approved for laying hens (oxytetracycline) and its long withdrawal period for eggs (7 days) poses an important obstacle to its use. Further, it can be assumed that the environmental exposure to antimicrobials in *Brachyspira* spp. originating from free-living wild birds is low.

Brachyspira colonization is common among commercial Swedish laying hens, particularly in non-caged systems indoors and in free-range birds (Jansson *et al*., 2008), but only a few farmers report health problems or production losses in *Brachyspira* colonized flocks. However, severe production losses, diarrhoea and faecal smears on egg shells in association with *Brachyspira* spp. colonization have been encountered (D Jansson, unpublished observations). To the best of the authors' knowledge no laying hen flocks have so far been treated for intestinal spirochaete colonization in Sweden. In this study it was therefore assumed that the investigated *Brachyspira* isolates represent a more or less naive spirochaetal population in terms of selective pressure on resistance determinants, which explains the fewer findings of decreased susceptibility among the isolates from chickens in this study compared to previous reports.

Although most investigated isolates displayed high susceptibility to all tested antimicrobials, a few isolates showed decreased susceptibility to ampicillin and lincomycin. *β*-lactam antibiotics are among the most widely used antibiotics in both humans and livestock. Decreased susceptibility to *β*-lactam antibiotics has been reported in *Brachyspira* isolates from pigs (Dassanayake *et al*., 2005; Mortimer-Jones, 2008), humans (Tompkins *et al*., 1987; Brooke *et al*., 2003; Dassanayake *et al*., 2005; Meziane-Cherif *et al*., 2008; Mortimer-Jones, 2008), and chickens (Hampson *et al*., 2006). Except for the early paper by Tompkins *et al*. (1987), in which isolates were not identified to species level, all the isolates displaying decreased susceptibility to *β*-lactam antibiotics were identified as *B. pilosicoli*. Chromosomally located class D *β*-lactamase genes, i.e. $bla_{\text{OXA-63}}$, $bla_{\text{OXA-136}}$ and $bla_{\text{OXA-137}}$, have been identified in *B. pilosicoli* isolated from humans and pigs (Meziane-Cherif *et al*., 2008; Mortimer-Jones, 2008). The extensive use of *β*-lactam antibiotics in both humans and pigs to treat a wide range of bacterial infections may have selected for resistance in *B. pilosicoli* as a side effect. However, the finding in this study of a closely related *β*-lactamase gene in an isolate of *B. pilosicoli* from a free-living wild mallard (Fig.

1) with presumed low level of exposure to β-lactam antibiotics suggests alternative explanations. A β-lactamase gene could have been acquired by horizontal gene transfer within the genus *Brachyspira* or from an unrelated species as suggested by Mortimer-Jones *et al*. (2008). Alternatively, the isolate may have been exposed to β-lactam antibiotics when present in another niche. *Brachyspira pilosicoli* has a broad host range that includes humans (Trivett-Moore *et al*., 1998), a variety of domestic animals (e.g. pigs, dogs, and poultry) (Trott *et al*., 1996; McLaren *et al*., 1997; Duhamel *et al*., 1998) and free-living feral and wild birds (Oxberry *et al*., 1998; Jansson *et al*., unpublished observation), and the fact that is may be found in faecally contaminated surface waters (Oxberry *et al*., 1998) lends some support to this hypothesis. Further, isolates of *B*. *pilosicoli* may cross species barriers by experimental inoculation (Sacco *et al*., 1997; Trott and Hampson, 1998) and zoonotic spread of this species has been suggested (Hampson, 2006). In this study we also found β-lactamase activity in the chicken enteropathogen *B*. *alvinipulli* and in presumed non-pathogenic "*B*. *pulli*" isolates from chickens and a mallard, but we failed to amplify any β-lactamase gene(s). Thus, our results suggest that β-lactamase genes may be more broadly distributed among members of the genus *Brachyspira* than currently recognized.

Several mutations that cause decreased susceptibility to lincosamides and macrolides in other bacteria are located within domain V of the 23S ribosomal RNA gene (Vester and Douthwaite, 2001). In *B. hyodysenteriae* an A2058T 23S rRNA mutation is associated with both macrolide and lincosamide resistance (Karlsson, *et al.* 1999). However, no mutations were found when a DNA fragment covering domain V was sequenced for one of the isolates with decreased lincomycin susceptibility (AN1828/02) in this study. Additionally this isolate and AN1831/03 were both macrolide susceptible, which makes this antimicrobial susceptibility phenotype unusual.

Despite selective culture and repeated subculture a common problem when studying avian *Brachyspira* spp. is the occurrence of isolates that contain more than a single species or genotype (Jansson *et al*., 2008). This is caused by the intrinsic motility and diffuse growth pattern of *Brachyspira* spp. on solid agar media. Definitive separation of species and genotypes from such isolates often requires 10-fold serial dilution and subculture. In this paper we show that chicken flocks as well as individual chickens and mallards, can be simultaneously colonized by fully susceptible and *Brachyspira* spp. isolates with decreased susceptibility. If antimicrobial susceptibility tests are performed on isolates that consist of several species or genotypes with different susceptibility patterns, the result of the test will reflect the number of cells in the sample and their relative growth rate which may lead to incorrect MICs.

In conclusion, the results obtained in this study showed that a majority of *Brachyspira* spp. isolates from commercial laying hens were susceptible to the antimicrobials that may be of therapeutic value in cases of intestinal spirochaete colonization, given a low level of antimicrobial exposure of the target population. We also showed that antimicrobial susceptibility tests should only be performed on isolates consisting of a single species and genotype, and that more than one isolate from a chicken flock should be investigated before treatment is initiated. Further, decreased susceptibility to amoxicillin was found in an isolate of *B. pilosicoli* from a mallard. To the best of our knowledge this is the first report of a *β*-lactamase gene of *Brachyspira* spp. found in an avian host, and also in a free-living wild animal species. The *β*-lactamase gene described here ($bla_{\text{OXA-192}}$) was similar but not identical to previously described *β*-lactamase genes in *B. pilosicoli* of human and porcine origin. The finding of *β*-lactamase activity in "*B. pulli*" and "*B. alvinipulli*", points to a broader occurrence of *β*-lactamase genes within the genus *Brachyspira* than previously known. Further work is required to characterize these genes and to determine their occurrence.

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Figure legend

Figure 1. *Neighbour joining tree showing the genetic relationship among class D β-lactamase genes (793 nucleotide positions) of* Brachyspira pilosicoli *strains originating from humans (BM4442, Wes-B, H/A1, H/A2, H/A3 and Gap 51.2), pigs (Cof-10 and 95/1000) and an isolate from the present study (free-living wild mallard, isolate AN3625/3/02, shown in bold). Strain and β-lactamase gene designations and GenBank database accession numbers are shown in the figure. The* $bla_{OXA\text{-}85}$ *class D β-lactamase gene from* Fusobacterium nucleatum *subsp.* polymorphum *strain N161 was used as outgroup. The scale bar represents the distance equivalent to 18 substitutions per 100 nucleotide positions.*

Table 1. In vitro *activities (MICs) of six antimicrobial agents for isolates of Brachyspira spp. of avian origin tested by broth dilution*

Table 2. *Primers used for PCR and DNA sequencing*

Table 1

Isolates[a]	*Brachyspira* spp.[b]	Species of origin	Farm/sampling location[c,d]	Housing conditions	MIC (µg/ml)					
					Tiamulin (0.063->8)	Valnemulin (0.031->4)	Doxycycline (0.125->16)	Lincomycin (0.5->64)	Tylosin (2->128)	Ampicillin (0.5->32)
AN1828/03	*Brachyspira* sp.	Chicken	A	Free-range, organic	0.25	0.25	0.25	**16**	≤2	2
AN1831/03	*Brachyspira* sp.	Chicken	A	Free-range, organic	0.25	0.125	0.25	**16**	≤2	2
AN2929/1/03	*"B. pulli"*	Chicken	B	Free-range, organic	0.125	0.063	≤0.125	1	≤2	1
AN2929/2/03	*B. intermedia*	Chicken	B	Free-range, organic	0.125	0.125	0.25	1	≤2	1
AN3370/03	*B. intermedia*	Chicken	C	Free-range, organic	≤0.063	≤0.031	0.25	≤0.5	8	2
AN3382/1/03	*"B. pulli"*	Chicken	C	Free-range, organic	0.25	0.25	0.25	≤0.5	4	**>32**
AN3382/2/03	*B. alvinipulli*	Chicken	C	Free-range, organic	≤0.063	≤0.031	0.25	≤0.5	≤2	≤0.5
AN3536/03	*B. intermedia*	Chicken	D	Noncage, indoor	≤0.063	≤0.031	0.25	≤0.5	≤2	1
AN3541/03	*B. intermedia*	Chicken	D	Noncage, indoor	≤0.063	≤0.031	0.25	≤0.5	≤2	1
AN3549/1/03	*B. murdochii*	Chicken	D	Noncage, indoor	≤0.063	≤0.031	≤0.125	≤0.5	≤2	≤0.5
AN5102/11/03	*B. intermedia*	Chicken	E	Free-range, organic	0.5	0.25	0.25	1	≤2	1
AN5112/03	*B. intermedia*	Chicken	E	Free-range, organic	0.125	0.063	0.25	≤0.5	≤2	1
AN2004/1/04	*B. intermedia*	Chicken	F	Free-range, organic	0.25	0.125	0.25	1	≤2	2
AN1780/3/03	*B. murdochii*	Chicken	G	Free-range, organic	0.125	≤0.031	0.25	≤0.5	8	≤0.5
AN2538/1/03	*B. murdochii*	Chicken	H	Noncage, indoor	0.25	0.125	≤0.125	1	4	≤0.5
AN2540/1/03	*B. innocens*	Chicken	H	Noncage, indoor	≤0.063	≤0.031	≤0.125	≤0.5	4	≤0.5
AN3165/2/03	*B. innocens*	Chicken	I	Noncage, indoor	≤0.063	≤0.031	≤0.125	≤0.5	4	≤0.5
AN3172/1/03	*Brachyspira* sp.	Chicken	I	Noncage, indoor	≤0.063	≤0.031	≤0.125	≤0.5	≤2	1
AN4113/03	*B. innocens*	Chicken	J	Noncage, indoor	0.25	0.125	0.5	≤0.5	≤2	≤0.5
AN4323/4/03	*B. innocens*	Chicken	K	Noncage, indoor	≤0.063	≤0.031	0.25	≤0.5	4	1
AN4341/03	*B. innocens*	Chicken	L	Furnished cage	≤0.063	≤0.031	0.25	≤0.5	4	1
AN4737/03	*B. murdochii*	Chicken	M	Noncage, indoor	0.125	0.125	≤0.125	1	8	≤0.5
AN5156/03	*B. innocens*	Chicken	N	Noncage, indoor	≤0.063	0.063	≤0.125	≤0.5	4	1
AN64/1/04	*B. innocens*	Chicken	O	Noncage, indoor	≤0.063	≤0.031	0.25	≤0.5	4	1
AN181/1/04	*B. murdochii*	Chicken	P	Noncage, indoor	≤0.063	≤0.031	≤0.125	≤0.5	4	≤0.5
AN304/04	*"B. pulli"*	Chicken	Q	Noncage, indoor	0.125	0.125	0.25	≤0.5	≤2	**>32**
AN315/04	*B. innocens*	Chicken	Q	Noncage, indoor	≤0.063	0.063	0.25	≤0.5	4	≤0.5
AN1263/2/04	*B. alvinipulli*	Chicken	R	Free-range, organic	≤0.063	≤0.031	≤0.125	≤0.5	≤2	≤0.5
AN1268/3/04	*B. alvinipulli*	Chicken	R	Free-range, organic	0.125	0.063	≤0.125	≤0.5	≤2	**>32**
AN1268/7/04	*Brachyspira* sp.	Chicken	R	Free-range, organic	≤0.063	≤0.031	0.5	≤0.5	≤2	≤0.5
AN6043:2/1/00	*Brachyspira* sp.	Mallard	Public park	Free-living, wild	0.5	0.25	≤0.125	2	4	≤0.5
AN6045/1/00	*"B. pulli"*	Mallard	Public park	Free-living, wild	0.25	0.125	≤0.125	1	4	1
AN6052:2/1/00	*"B. pulli"*	Mallard	Public park	Free-living, wild	0.25	0.125	≤0.125	≤0.5	≤2	**16**
AN6041:1/1/00	*B. alvinipulli*	Mallard	Public park	Free-living, wild	0.5	0.5	≤0.125	1	4	1
AN6050:2/1/00	*B. alvinipulli*	Mallard	Public park	Free-living, wild	≤0.063	≤0.031	≤0.125	≤0.5	4	≤0.5
AN3649/1/02	*B. alvinipulli*	Mallard	Bird station	Free-living, wild	≤0.063	≤0.031	≤0.125	≤0.5	4	≤0.5
AN3940/1/02	*B. alvinipulli*	Mallard	Bird station	Free-living, wild	≤0.063	≤0.031	≤0.125	≤0.5	≤2	≤0.5
AN3495:4/1/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	≤0.063	≤0.031	≤0.125	≤0.5	≤2	1
AN3597/1/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	≤0.063	≤0.031	≤0.125	2	≤2	1
AN3600/1/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	0.25	0.25	≤0.125	1	4	1
AN3617:2/1/02	*Brachyspira* sp.	Mallard	Bird station	Free-living, wild	0.5	0.25	≤0.125	1	≤2	1
AN3635/2/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	0.125	0.063	≤0.125	≤0.5	≤2	1
AN3635/3/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	0.5	0.25	0.25	1	≤2	**>32**
AN3635/5/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	≤0.063	≤0.031	≤0.125	≤0.5	≤2	1
AN3875/1b/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	0.125	≤0.031	≤0.125	1	≤2	2
AN3608:1/1/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	≤0.063	≤0.031	≤0.125	≤0.5	4	1
AN3617:1/1b/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	0.25	0.25	≤0.125	≤0.5	4	2
AN3927:3/2/02	*B. pilosicoli*	Mallard	Bird station	Free-living, wild	≤0.063	≤0.031	≤0.125	≤0.5	4	1

[a]The first four numbers is the unique serial number of a sample.

[b]References to species designations: Jansson *et al*., 2008 (isolates from chickens), Jansson *et al*., unpublished results (isolates from mallards).

[c]Farm designations as in Jansson *et al*., 2008.

[d]Public park: Pildammsparken, Malmö, Sweden (55° 35' N, 12° 59' E); Ottenby bird station, Öland, Sweden (N 56° 12', E 16° 24') (GDS 84).

Table 2

Gene	Primer designation	Primer direction	Position	Primer sequence (5'-3')	Reference
β-lactamase	Bla F1	Forward	442-460[a]	AAA CTT GCT CAC CGT GCG T	This study
β-lactamase	Bla F2	Forward	619-641[a]	TTA GAA ATT CTG TAA GCA ATA CT	This study
β-lactamase	Bla F3	Forward	810-829[a]	CTA ATA GCA ATG CTG AAG GA	This study
β-lactamase	Bla R1	Reverse	1548-1567[a]	TTC ACC GTG CGG TTA ATA GA	This study
β-lactamase	Bla R2	Reverse	1578-1600[a]	TGA ATT GCT AAT TAA TTT ACT GA	This study
β-lactamase	Bla R3	Reverse	1436-1455[a]	GCA AGG TCA TCT GAA TTG AT	This study
β-lactamase	Bla R4	Reverse	1545-1564[a]	ACC GTG CGG TTA ATA GAG TA	This study
β-lactamase	Bla R5	Reverse	1472168-1472187[b]	ATT ATA ACG CAC GGT AAG CG	This study
β-lactamase	Bla R6	Reverse	1704-1723[a]	TCA AAC TAT AAA CTC CTA GT	This study
23S rRNA	2058Fo	Forward	1858-1879[c]	GAG AGG TTA GCG TAA GCG AAG C	Karlsson *et al*., 1999
23S rRNA	Spiro2 Re	Reverse	2745-2767[c]	GCT TCC CAC TTA GAT GCR TTC AG	Pringle *et al*., 2004

[a]Position according to *B. pilosicoli* strain BM4442 class D β-lactamase (OXA-63) gene and flanking regions, GenBank acc. no. AY619003.

[b]Position according to *B. pilosicoli* strain 95/1000 class D β-lactamase (OXA-136) gene, GenBank accession no. CP002025.

[c]Position according to *E. coli*, 23S rRNA gene.