Spirochetes are a unique group of bacteria with a common ancestry and they comprise a very early branch of the evolutionary tree of living organisms. Taxonomically, spirochetes belong to the order Spirochaetales, which contains the genus Brachyspira, amongst other genera [23]. Seven species of Brachyspira have been described: B. hyodysenteriae [11], B. intermedia [31], B. mucedo [31], B. innocens [16], B. pilosicoli [36], B. aalborgi [32] and B. aalborgi [33]; two other yet unconfirmed species: B. pulli [33] and B. canis [6] have been suggested.

Members of the genus Brachyspira are pathogenic for animals (pigs, dogs, wild rodents, birds, guinea pigs, opossums), humans and non-human primates; where they cause gastrointestinal disorders such as chronic diarrhea and rectal bleeding, clinically defined as intestinal spirochitosis [35]. The significance and consequences of Brachyspira infections have been recognized worldwide for decades. B. hyodysenteriae and B. pilosicoli are major causative agents of diarrheal diseases in pigs and a variety of animal species [34]. B. pilosicoli is frequently isolated from humans, and can probably be zoonotic [2] because the canine isolate, obtained from the feces of a dog with diarrhea and classified as B. pilosicoli, was an electrophoretic type similar to that of the spirochetes isolated from the feces of Australian Aboriginal children with diarrhea living in the same community and adult individuals with diarrhea living in a distant community [6]. Infections in dogs are due to three species, namely B. hyodysenteriae, B. pilosicoli and B. canis [2, 8, 25]. A number of antimicrobial agents are active against Brachyspira species [5, 14, 18] with the most commonly used classes of agents being the macrolides, lincosamides, pleuromutilins, and nitroimidazoles. Nevertheless, acquired resistance to these agents is increasing, thus limiting their usefulness.

Generally, macrolides act by inhibiting protein synthesis through binding to the 50S ribosomal subunit and stimulating the dissociation of the peptidyl-tRNA molecule from the ribosome during elongation [37], which results in chain termination and a reversible stoppage of protein synthesis. The clinical usefulness of macrolides is limited by the acquisition of resistance by sensitive isolates. The first mechanism of macroline resistance described is the post-transcriptional modification of the 23S RNA by the adenine-N6 methyltransferases. These enzymes add one or two methyl groups to adenine at the N6 position of adenine (A2058 in Escherichia coli), which greatly reduces the affinity of ribosomes to all macrolides [37]. Genes encoding these methylases had been designated erm (erythromycin ribosome methylolation), with some exceptions, especially in antibiotic-producing organisms [27]. The 50S ribosomal target site of erythromycin overlaps the binding site of newer macrolides, as well as structurally unrelated lincosamides and streptogramin B antibiotics [37]; target site modification by methylases results in resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS₉). The RNA methylase are the best studied among MLS₉ resistance mechanisms [27]. Besides target site modification by methylases, a variety of other mechanisms, including mutations, inactivation, and efflux have been described [15, 37]. Mutation of A2058 to G, C, or U in E. coli and other bacterial species also reduces the affinity of ribosome for macrolides [15, 29]. Inactiva-
tion of MLSβ antibiotics by esterases, phosphotransferases, acetyltransferases, hydrolases, and nucleotidyltransferases [27, 37] confers resistance to structurally related antibiotics only. Efflux, mediated by either a pump with narrow or one with broad specificity, is responsible for intrinsic resistance to antibiotics [37].

In Japan, there is scanty information on the current susceptibility of intestinal spirochetes to antimicrobial agents. We undertook this study to provide such information. Specifically, common antimicrobial agents were tested for their susceptibility against field isolates of Brachyspira species obtained from dogs; the genetic basis of the resistances was also investigated. Furthermore, the genetic changes associated with tylosin resistance in *in vitro* selected tylosin-resistant mutants of some reference strains and tylosin-sensitive canine isolates were studied.

**MATERIALS AND METHODS**

**Sample collection:** Colonic mucosa and large intestinal contents were obtained from 128 stray dogs at Kasama City, Ibaraki Prefecture, Japan between 1999 and 2000. The dogs were euthanized prior to sample collection; samples were taken to the laboratory for subculture immediately after collection.

**Reference strains and antimicrobial agents:** Reference strains used were *Brachyspira hyodysenteriae* ATCC 27164T, *B. innocens* ATCC 29796T, and *B. pilosicoli* ATCC 51139T; they were maintained on TSA containing 5% sheep blood. Antimicrobial agents were supplied by Pfizer Co., Tokyo, Japan (carbadox); Novartis Animal Health Co., Tokyo, Japan (tiamulin); Shionogi & Co., Ltd., Osaka, Japan (tylosin), Rhône-Poulenc Japan Chemical Co., Ltd., Tokyo, Japan (diametridazole), Dainippon Pharmaceutical Co., Ltd., Osaka, Japan (erythromycin), The Pharmacia Upjohn Japan Co., Tokyo, Japan (lincomycin), and Upjohn, U.S.A. (spectinomycin). Stock solutions containing 1,000 µg/ml of each agent were prepared with appropriate solvents, and stored at 4°C or used immediately.

**Isolation and identification of organisms:** Samples were serially diluted ten-fold from 10⁻⁴ to 10⁻⁵ in trypticase soy broth (TSB; Beckton Dickenson, U.S.A.) and poured onto trypticase soy agar (TSA; Beckton Dickenson, U.S.A.) containing 5% sheep blood and 400 µg/ml spectinomycin [30]. Inoculated plates were incubated anaerobically at 37°C for 72 hr by means of a GasPak system (BBL, U.S.A.) with prewarmed vents, and stored at 4°C.

**Biochemical tests:** Carbohydrate fermentation and indole production were carried out as previously described [21].

**Antimicrobial sensitivity assay:** The minimum inhibitory concentration (MIC) of each of the agents against the isolates was determined by agar dilution with a two-fold dilution from 1,000 to 1 or 0.0625 µg/ml final concentration of the agents in TSA containing 5% sheep blood as described by Kitai et al. [17].

**Analysis of extra-chromosomal DNA:** The association of resistances in the canine isolates with extra-chromosomal DNA (plasmids) was investigated. Plasmids were extracted by the hot alkaline method and separated on 0.8% agarose by using the tris-acetate EDTA buffer system based on Kado and Liu [13].

Genetic analysis of tylosin resistance: Tylosin-resistant isolates and *in vitro* selected resistant mutants of both reference strains and tylosin-sensitive field isolates (obtained after three successive subculture on TSA-5% sheep blood-1 µg/ml of tylosin at interval of three days) were analyzed for mutations in their 23S rDNA. InstaGene™ Purification Matrix (BIO-RAD, U.S.A.) was used to extract and purify genomic DNA from the isolates; the DNA was used as template for 23S rDNA sequencing as described previously [29]. 23S rDNA was sequenced using a capillary sequencer (ABI, U.S.A.) and BigdyeTM Terminator Ver. 3.0 sequencing standard (ABI PRISM®, Applied Biosystems, U.S.A.); base alignment was determined in both directions with an automate sequencer (ABI PRISM®, 3100 Genetic Analyzer, Applied Biosystems, Hitachi, Japan).

**RESULTS**

**Biochemical identity of isolates:** The twenty-nine canine isolates were weak β-hemolytic on blood agar, indole-negative, and fermented glucose, galactose, lactose, trehalose, fructose, maltose, mannose and raffinose, but not arabinose, inositol, rhamnose or sorbitol.

**Genetical identification of canine isolates:** The DNAs extracted from 9 (D6b/6/15, D7b/6/15, D1a/8/31, D3a/8/31, D1a/10/15, D7a/10/25, D1a/11/9, D2a/11/9, and D4a/11/9) out of 29 isolates were amplified by NOX1 primers. The DNAs from 8 (D1a/10/5, D2a/10/25, D3b/10/25, D4b/10/25, D7b/10/25, D8d/10/25, D9d/10/25, and D5a/11/9) out of 29 isolates were amplified by the primers designed by the sequence of 16SrDNA gene of *B. pilosicoli*. The DNAs of 12 (D1d/6/15, D2a/6/15, D3d/6/15, D4a/6/15, D5d/6/15, D9d/6/15, D10a/6/15, D12a/6/15, D2a/8/31, D2a/10/5, D5a/10/25, and D3a/11/9) out of 29 isolates were amplified by NOX4 primer. All the isolates were genetically confirmed as belonging to the genus *Brachyspira*.

**Electron microscopical observation of the spirochetes:** The morphological properties were compared among the isolates and are shown in Fig. 1. There was no difference among the isolates. The cell was about 0.27 to 0.5 µm in
diameter and was 5.5 to 17 µm in length (Fig. 1-A). The end of a cell is a point type with 8 to 14 periplasmic flagella at each end of the cell and the flagella were originally from periplasmic membrane (Fig. 1-B).

Susceptibility testing: The susceptibility (based on MIC) of the field canine isolates to six antimicrobial agents commonly used to treat intestinal spirochetosis is shown in Table 1. Carbadox was the most active, with MIC of < 0.00625 µg/ml for more than 90% of the isolates. Both tylosin and erythromycin had MICs of 3.13 µg/ml for more than 90% of the isolates, but different MIC ranges of 0.78-3.13 and 0.2-6.25 µg/ml respectively. By the interpretation criteria used [28], which suggested a breakpoint MIC for resistance as > 4 µg/ml [28], more than 90% of the isolates were resistant to tiamulin. Lincomycin and dimetridazole were also active, with low MICs of 1.56 and 0.2 µg/ml respectively, for more than 90% of the isolates.

Extra-chromosomal DNA: No plasmids were extracted from any of the isolates.

Table 1. Minimum inhibitory concentrations (MIC) of six commonly used antimicrobial agents against 29 canine intestinal spirochetes in Japan

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>MIC values (µg/ml)</th>
<th>Range</th>
<th>For ≥ 90% of the isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoloxines</td>
<td></td>
<td>&lt;0.00625</td>
<td>&lt;0.00625</td>
</tr>
<tr>
<td>Carbadox</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrolides</td>
<td></td>
<td>0.78–3.13</td>
<td>3.13</td>
</tr>
<tr>
<td>Tylosin</td>
<td></td>
<td>0.2–6.25</td>
<td>3.13</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td>0.2–12.5</td>
<td>6.25</td>
</tr>
<tr>
<td>Pleuromutins</td>
<td></td>
<td>0.39–1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>Tiamulin</td>
<td></td>
<td>≤0.1–0.39</td>
<td>0.2</td>
</tr>
<tr>
<td>Lincomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimetidazole</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. In vitro selection of tylosin-resistance in sensitive field canine isolates and reference strains

<table>
<thead>
<tr>
<th>Strain/Isolate</th>
<th>MIC (µg/ml)</th>
<th>Parent</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. hyodysenteriae</em> ATCC 27164</td>
<td>6.25</td>
<td>&gt; 200</td>
<td></td>
</tr>
<tr>
<td><em>B. pilosicoli</em> ATCC 51139</td>
<td>6.25</td>
<td>&gt; 200</td>
<td></td>
</tr>
<tr>
<td>D1a/8/31</td>
<td>3.13</td>
<td>&gt; 200</td>
<td></td>
</tr>
<tr>
<td>D2a/11/9</td>
<td>3.13</td>
<td>&gt; 200</td>
<td></td>
</tr>
<tr>
<td>D5a/10/25</td>
<td>3.13</td>
<td>&gt; 200</td>
<td></td>
</tr>
</tbody>
</table>

> , Higher than the value of 200 µg/ml.
<, Lower than the value of 0.00625 µg/ml.
≤, MIC value of 0.1 µg/ml and lower.

Strain/Isolate

breakpoint MIC for resistance of > 4 µg/ml [28], more than 90% of the isolates were resistant to tiamulin. Lincomycin and dimetidazole were also active, with low MICs of 1.56 and 0.2 µg/ml respectively, for more than 90% of the isolates.

Extra-chromosomal DNA: No plasmids were extracted from any of the isolates.

In vitro selection of tylosin-resistance: Tylosin-resistant mutants of three sensitive field isolates (D1a/8/31, D2a/11/9 and D5a/10/25) and two reference strains (ATCC27164 and ATCC51139) were selected by successive subculture on blood agar. Their susceptibilities to tylosin are shown in Table 2. MICs of mutants were about 65 times higher than that of the parent strains.

Genetic analysis of tylosin resistance: The nucleotide base sequences of 23S rDNA in both mutants and parent strains, corresponding to nucleotide 2050 and 2070 base positions of 23S rDNA in *E. coli* are as shown in Table 3. The mutations in the 23SrDNA were observed at positions 2058 in mutants of reference strains (A→G transition) and 2062 in mutants of the field isolates (A→C transversion).
### DISCUSSION

Antimicrobial drug resistance in *Brachyspira* is widespread; strains resistant to antimicrobials, namely carbadox, tylosin, erythromycin, tiamulin, lincomycin, and dimetridazole, in common use for the treatment of intestinal spirochetosis have been reported [4, 5]. The present state of knowledge on the distribution of resistance in *Brachyspira* is insufficient, and more work is required to continuously monitor the development of resistance in this genus.

The results of this study indicated variable in vitro sensitivities of the Japanese canine isolates to the tested antimicrobials. The exceptional activity of carbadox was noteworthy, even though the activity could have been enhanced to this level by the anaerobic incubation conditions employed in this study [26]. This is in agreement with two previous studies from Japan [17, 18], and elsewhere in the world [5, 14]. This drug is exclusively used in veterinary medicine, usually as an anti-infective agent and growth promoter in pigs [5, 19]. Although carbadox is neither mutagenic nor carcinogenic [38], its genotoxic potential has caused its usefulness to be questioned [20].

As in the case of carbadox, none of the isolates were resistant to tylosin. This is also an interesting observation, which indicated the potential for clinical effectiveness. There is the possibility that these isolates are from a clone (population) that has not been previously exposed to the drug or at least not frequently. This drug has not been widely used at sub-inhibitory concentrations in dogs as a growth promoter as it has been the case in pigs, poultry and cattle. Some of the isolates were, however, resistant to erythromycin, an observation that confirms the fact that erythromycin-resistant bacteria do not cross-resist towards tylosin but resistance to tylosin is crossed towards erythromycin [7]. Structural differences between tylosin and erythromycin can be used to explain this observation. A disaccharide branch extends from C5 of tylosin (as in other 16-membered macrolides), whereas a monosaccharide occupies similar position in erythromycin (14-membered) and 15-membered macrolides. Ribosomes can still form short peptides in the presence of 14- or 15-membered but not larger macrolides [24]. So that, the potential for bacteria to resist erythromycin is much higher. Recently though, some in vitro selected erythromycin-resistant mutants cross-resistant tylosin [9]. Although no tylosin resistant field isolate was isolated in this study, this outcome should be treated with caution, given that the sample size was small, and it was not a representative of the entire country. Tylosin resistance in *Brachyspira* is widespread [4, 28], owing to its widespread use both as a therapeutic agent and a growth promoter in animal feeds in many countries [19].

As against reports from other countries, more than 90% of our isolates were resistant to tiamulin, a pleuromutilin derivative. Tiamulin has been reported to be effective against some species of *Brachyspira* [5, 14]; but resistant clones have also been isolated [5]. The activity of lincomycin is high, consistent with a previous report on porcine isolates from Japan [18]. Lincomycin-resistant isolates have also been reported elsewhere [4, 5]. Dimetridazole is a nitro-imidazole derivative, and showed good activity against the field isolates. All the isolates did not harbor extra-chromosomal DNA. Plasmids were reported previously in *B. hyodysenteriae* [1, 4], but no relationship between carriage of these plasmids and resistance was established [4].

Genetic analysis of in vitro selected tylosin-resistant mutants has revealed mutations at different base positions of 23S rDNA in the reference and field strains. Tylosin is a 16-membered macrolide, which is naturally produced by the actinomycete *Streptomyces fradiae*. It exerts its antimicrobial action by binding in the polypeptide exit tunnel of the bacterial 50S ribosomal subunit, adjacent to the peptidyltransferase center, where it inhibits protein synthesis by interfering with peptide bond formation as well as blocking the passage of nascent peptide chain through the tunnel [10, 37].

Genetic mutation as a mechanism of resistance to macrolides is not new. Mutation of A2058 to G, C, or U in bacteria reduces the affinity of ribosome for macrolides [29]. Recently, Karlsson et al. [15] described, in *Brachyspira hyodysenteriae*, an A2058 to T transversion and A2058 to G transition mutations in the nucleotide base sequences of 23S rDNA of clinical tylosin-resistant isolates and in vitro-selected tylosin-resistant mutants of sensitive strains, respectively. This present study reports a new transversion mutation (A→C) at the 2062-nucleotide base position of 23S rDNA in mutants of sensitive field isolates of *Brachyspira* species, and it also confirms the A2058 mutation in the mutants of *Brachyspira* reference strains.

In conclusion, this study has described the antimicrobial sensitivity of, and a new mutation associated with tylosin resistance in, canine isolates of Japanese origin. Although in a limited number of isolates, these findings will provide help in the proper choice of antimicrobials for the treatment...
of Brachyspira infections in the dog population, it will also motivate a more extensive study of a similar nature in the nearest future. The new mutation reported herein, will add to current understanding of the mechanisms of macrolide resistance.

ACKNOWLEDGMENTS. We thank Pfizer Co., Tokyo, Japan, Novartis Animal Health Co., Tokyo, Japan, and Shionogi & Co., Ltd., Japan for providing the antimicrobial agents. We also thank Mr. Y. B. Ngwai for his comments on this manuscript.

REFERENCES


Int. Pig Veterinary Society Cong.


