CHARACTERIZATION OF BRACHYSPIRA SP. STRAINS ISOLATED FROM FLOCK OF HENS WITH DIARRHOEA

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Abstract

Spirochetes were isolated from 6 out of 12 diarrhoeic faecal samples obtained from hens. Biochemical identification of the isolated strains was confirmed by PCR analysis specific for Brachyspira pilosicoli, B. intermedia, B. innocens and B. murdochii. Positive PCR for B. intermedia were obtained in 2 strains and there was only 1 positive reaction for B. innocens. There were no positive results in the PCR analysis for B. pilosicoli. The identification to the species level based on PCR results was not possible in 4 cases. Basing on phenotypic properties, Brachyspira sp. isolated from hens has been found to be heterogeneous group of microorganisms.

Key words: chickens, Brachyspira, spirochetosis.

Colonization of the gastrointestinal tract by spirochetes has been documented in a variety of bird species demonstrating different clinical symptoms (4-6, 8, 11, 12, 15). Spirochetes have been isolated from laying hens, broiler breeders, broiler chickens, turkeys, rheas, zoological birds, waterfowl and game birds. Avian intestinal spirochetes, which have been isolated and identified belong to Brachyspira sp., i.e.: B. alvinipulli, B. pilosicoli, B. intermedia, B. innocens, B. murdochii and B. hydysenteriae. Three species, B. pilosicoli, B. intermedia and B. alvinipulli, are connected with avian intestinal spirochetosis (AIS). There is also a large group of Brachyspira sp., which has been isolated from birds, but has not yet been identified to the species level (5, 8). This group of spirochetes is heterogeneous and may form a new Brachyspira species.

Avian intestinal spirochetosis has been reported from the United States, Australia and Europe (4, 6, 8, 12). This intestinal disorder (diarrhoea may be present in 5-20% of the birds of a flock) may cause production problems, such as: reduced egg production, pasty vents, faecal staining of egg shells, poor shell quality, pale egg yolks, increased feed consumption, slower growth and poor feed digestion (4, 8, 12). Post-mortem examination does not usually reveal any gross lesions except pale coloured, foamy caecal contents (4, 12, 15).

It is suspected that AIS in poultry remains unrecognized, because clinical symptoms are often mild or subtle. Another reason may be that Brachyspira sp. can be isolated only on selective media and under anaerobic conditions, which are not routinely used in diagnostic laboratories. Avian intestinal spirochetes have been isolated using the same or similar media and conditions as used for porcine spirochetes.

The crucial biochemical reactions for the identification of spirochetes are indole production and hippurate hydrolysis (2). API ZYM tests are useful for enzyme profiling of isolated strains. Identification can be made using molecular biology methods, like: multilocus enzyme electrophoresis – MEE (8), pulse field gel electrophoresis – PFGE (13), sequencing of rRNA genes (6), PCR specific for 16S rRNA gene of B. pilosicoli (9, 10), 23S rRNA gene of B. intermedia (7) or NADH oxidase genes of B. innocens and B. murdochii (1).

This paper reports the isolation of Brachyspira sp. from laying hens with diarrhoea. The isolates were defined on the basis of phenotypic characterization. Identification to species level was attempted using PCR technique. This report is the first documented case in Poland of diarrhoea in birds associated with Brachyspira infection.

Material and Methods

Bacteriological examination. The study was conducted using samples from one flock of laying hens housed in natural conditions. The birds were 36 weeks old. The flock consisted of 50 birds and the birds were kept free-range on the farm. There was also a herd of pigs on the farm and there was possible contact between chickens and pigs.

Twelve samples of hen faeces, collected directly from the ground, have been examined. All the samples have been described as diarrhoeic, due to significant amount of wet contents. Blood and mucus
were not observed. Direct microscopy smears, stained by Gram method or Victoria blue, were prepared from the samples. The smears were examined under light and phase-contrast microscopes. The samples were cultured on the following bacteriological media: blood agar, Mac Conkey agar and selenite F broth (BioMérieux). They were incubated at 37°C for 24 h in aerobic atmosphere. The samples were also cultured on selective trypticase soy agar (BioMérieux) supplemented with 5% defibrinated ovine blood, 800 µg/ml of spectinomycin (Sigma) and 25 µg/ml of vancomycin (Sigma). The plates were incubated under anaerobic conditions generated by BBL™ GasPak system (Becton Dickinson) for 4 to 7 d at 40°C.

Biochemical properties of Brachyspira isolates. Isolated strains of spirochetes were tested for indole production. The indol test was performed as described by Sutter and Carter (14). Other biochemical properties of the strains were determined by API ZYM (BioMérieux) test, according to the manufacturer’s instructions.

Identification of the isolates by PCR technique. Brachyspira isolates were grown on trypticase soy agar (BioMérieux) supplemented with 5% defibrinated ovine blood. Chromosomal DNA for PCR was obtained using InstaGene™ Matrix (Bio-Rad) and 20 µl of supernatant was used as template DNA, according to the manufacturer’s instructions.

### Table 1
Characterization of PCR tests

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene amplified in PCR</th>
<th>Thermal cycling conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial denaturation</td>
<td>Denaturation</td>
</tr>
<tr>
<td><strong>B. intermedia</strong></td>
<td>23S rRNA</td>
<td>1 min at 94°C</td>
<td>40 s at 92°C</td>
</tr>
<tr>
<td><strong>B. innocens/B. murdochii</strong></td>
<td>NADH oxidase</td>
<td>1 min at 95°C</td>
<td>30 s at 95°C</td>
</tr>
<tr>
<td><strong>B. pilosicoli</strong></td>
<td>16S rRNA</td>
<td>4 min at 94°C</td>
<td>1 min at 94°C</td>
</tr>
</tbody>
</table>

Three pairs of primers were used for the identification of the strains. One was specific for *B. pilosicoli*, one for *B. intermedia* and one for both *B. innocens* and *B. murdochii*. Fragments of genes amplified in PCR technique, product size, reaction conditions and references are presented in Table 1.

Some modifications were introduced in the identification of *B. innocens* and *B. murdochii*. PCR based on NADH oxidase gene amplification is specific for both spirochetes (1) and gives products of the same size – 729 bp. Products specific for *B. innocens* are digested with *Hind III* (Fermentas) in one place giving two fragments: 333 bp and 396 bp. Products specific for *B. murdochii* are not digested with *Hind III*. Only digestion of PCR products allow to distinguish among *B. innocens* and *B. murdochii*. The PCR products were analysed in 1.5% agarose gel with Versa Doc system (Bio-Rad).

### Results

**Bacteriological examination.** Approximately 25% of the birds in the flock suffered from chronic diarrhoea. No other clinical signs have been observed.

Spirochetal bacteria were observed in direct smears in each of 12 samples of faeces. Weakly β-haemolytic *Brachyspira* sp. were isolated from 6 out of 12 samples. Pathogenic aerobic bacteria were not isolated.

Biochemical properties of *Brachyspira* isolates. Biochemical properties of the isolated Brachyspira strains are presented in Table 2.

Identification of the isolates by PCR technique. Results of PCR tests are presented in Table 2. Two positive results specific for *B. intermedia* and one specific for both *B. innocens* and *B.murdochii*, were obtained (Fig. 1). There were no positive results in PCR amplification of the 16S rRNA gene of *B. pilosicoli*.

One PCR product, obtained from the amplification of NADH oxidase gene of *B. innocens* and *B. murdochii*, was digested with *Hind III*. The digestion resulted in two fragments: 333 bp and 396 bp (Fig. 1). Basing on the result of the digestion this strain was recognized as *B. innocens*.

Positive result for both *B. intermedia* and *B. innocens* were obtained in one of the samples. We were not able to separate those strains. Identification to the species level based on PCR results was not possible in 4 isolates.
Discussion

The presented results confirm that *Brachyspira* sp. can be isolated from gastrointestinal tract of poultry. Spirochetal bacteria were observed in each of 12 faecal samples from hens with diarrhoea. There were 6 samples, from which strains of *Brachyspira* were isolated. *Brachyspira* sp. have fastidious anaerobic growth requirements and the sensitivity of culture depends on the number of organisms present in the sample. This may be the reason why in all 12 samples of spirochetal bacteria were observed and only 6 isolates have been obtained. Another interesting aspect to be considered is that chickens 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 *Brachyspira* strains. In general, *Brachyspira* sp. are more often isolated from birds with diarrhoea than from healthy ones. Dwars et al. (3) reported intestinal spirochetes in 37 of 134 (27.6%) samples from flocks with diarrhoea or reduced production and only 2 of 45 (4.4%) samples from flocks with no signs of disease.

Phenotypic characterization does not always allow the identification to the species level. In our study all the isolated strains were positive in the indole spot test. In 1 out of 6 positive in culture samples mixed infection was identified, which was proved by API ZYM enzyme profiles and PCR. We were not able to separate those strains. In another sample, *B. intermedia* was identified in API ZYM test and confirmed by PCR. For the other isolated strains obtained only identification of the genus *Brachyspira* was possible.

McLaren et al. (8) described heterogeneous groups of avian *Brachyspira* strains, which were not identified to the species level. Four of our isolates had the same or very similar API ZYM codes to those reported by McLaren et al. (8). The pathogenicity of McLaren’s isolates remained unknown, however, most of them were isolated from flocks with production problems.

*B. intermedia* strains isolated from birds belong to diverse group of microorganisms (8, 13), as shown by the following examples: a PCR assay based on the NADH oxidase genes (*nox*) of *B. intermedia* correctly identified 10 porcine strains and amplified DNA only from 4 of 10 strains from chickens (1). Another PCR based on 23S rRNA gene of *B. intermedia* correctly identified all the *B. intermedia* strains, but generated 11 false positive reactions, giving a test sensitivity of 100% and a test specificity of 94.3%. Eight of the false positive reactions were generated from porcine strains and only 3 from chicken strains (13). Results of our research allow to conclude, that we have isolated either unclassified avian *Brachyspira* isolates or atypical *B. intermedia* isolates. Based on our study and two examples given above, it seems that rRNA gene sequence analyses are necessary to confirm the taxonomic positions of the isolated strains.

Recognition of *B. intermedia* in 2 samples indicates that spirochetes may be transmitted between birds and pigs. The flock of hens was housed in natural conditions and occasional contacts with pigs were possible. More detailed research, including isolation of *Brachyspira* sp. from the pigs and its comparison to those isolated from hens is necessary to confirm this conclusion.

To our knowledge, this is the first report in Poland describing the isolation and characterization of *Brachyspira* sp. from hens with diarrhoea. Basing on phenotypic properties, the isolates have been found to be heterogeneous group of microorganisms.
Table 2
Results of biochemical reactions and PCR tests obtained for spirochete strains isolated from hens

<table>
<thead>
<tr>
<th>No. of faecal sample</th>
<th>Biochemical reactions</th>
<th>PCR specific for</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>indole</td>
<td>alkaline phosphatase</td>
<td>leucine aminopeptidase</td>
</tr>
<tr>
<td>1.</td>
<td>+ + + + + - - - - - - - + + + + - + + + - - - -</td>
<td>14.0.12.11.3</td>
<td>+ + -</td>
</tr>
<tr>
<td>2.</td>
<td>+ + + + + - - - - - - - - + + - + - - - + - - - -</td>
<td>14.0.12.2.1</td>
<td>+ - -</td>
</tr>
<tr>
<td>3.</td>
<td>+ + + + + - - - - - - - + + + + - + + - - - -</td>
<td>14.0.13.11.1</td>
<td>- - -</td>
</tr>
<tr>
<td>4.</td>
<td>+ + + + - - - - - - - - - + - - - - - - - -</td>
<td>14.0.12.2.1</td>
<td>- - -</td>
</tr>
<tr>
<td>5.</td>
<td>+ + + + + - - - - - - - + + + + - - + + + +</td>
<td>14.0.12.3.3</td>
<td>- - -</td>
</tr>
<tr>
<td>6.</td>
<td>+ + + + - - - - - - - - - - + + + + - - + + + +</td>
<td>14.0.13.11.1</td>
<td>- - -</td>
</tr>
</tbody>
</table>

* positive reaction
- negative reaction
* PCR specific for both B. innocens and B. murdochii
References


