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**The examination of intestinal spirochetosis
(brachyspirosis) of the domestic ducks and hens**

PhD dissertation thesis

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INTRODUCTION AND AIMS OF THESIS

The public demand for poultry origin food is increasing from year to year in Hungary. This fact gives big challenge for those who working in the poultry sector, as the poultry experts and the veterinary technicians alike. The genetic progress of the poultry has slowed down in recent decades, this is especially true among the animals kept in intensive conditions, which are producing near their maximum genetic capacity. As a result, the production increase is the primary concern, while other important factors (like animal health) can be relegated to the background.

Keeping large groups of animals together will test the animals' health. Due to the pressure of business policy on the owners and breeders, they are seeking to maximize profit. They are trying to achieve this either by increasing the number of animals or by reducing the expenses. One option for the latter is buying cheaper feed, as the main component of the expenses. As a result, this feed may have no proper nutritional value for the birds, or its quality may be bad (e.g. mycotoxin contaminated). The second option of minimizing the expenses is to neglect cleaning and disinfection, which leads to poor environmental hygiene. It should be also noted that the whole poultry sector – especially among the intensively kept species (chickens and turkeys) – has a very strict breeding session. This means that the receiving of the animals and the slaughtering may be scheduled several months in advance, and the animal density is more than acceptable in hygienic aspect. This may result in accumulation of the pathogens in the environment.

Because of these facts, instead of the well defined pathogens that caused huge losses in the past decades, now the multifactorial diseases are the common cause of serious production losses in the poultry sector.

Among the young poultry enteric diseases we know some, in which one or more viruses play role in the development of diseases (such as runting-stunting syndrome of chickens and the turkeys PEC/PEMS disease). Enteric diseases sometimes occur when the feed quality is not appropriate. This may also lead digestive disorders and intestinal dysbacteriosis. In this case *E. coli* or Clostridium species (colibacillosis, necrotic enteritis) is the direct cause of illness and death. A mycotoxin contaminated feed alone can cause diarrhoea and inflammation in the intestinal tract. Furthermore, spirochete species (*B. hyodysenteriae*, *B. alvinipulli*, *B. intermedia*, *B. pilosicoli*) are able to cause inflammation and diarrhea in adult birds, when predisposing factors are present.

Because of the nature of the multifactorial diseases, the prevention and effective intervention management need a complex approach, taking account into the elimination of the predisposing factor(s) as well. The animal breeders usually think that the vaccination and antimicrobial medication is the only technique against the infectious diseases. Against the multifactorial diseases sometimes there are no vaccines or its application is impractical or not economical. Following the completion of the treatment – if the predisposing factors (e.g., overcrowding, and poor quality feed) are present - often recurrence happens, and in the top of that, this may result resistance against the used antibiotic drugs.

Thanks to the research and development, now there are effective vaccines against certain diseases (Marek's disease, Derzsy disease etc), which threatened the very existence of the poultry sector in the previous decades. These diseases have become sporadic nowadays. In our institute in the Poultry Disease Laboratory we meet multifactorial diseases more frequently.

Avian intestinalis spirochaetosis (AIS) is a disease of birds characterized by colonization of the caecum and colon by *Brachyspira* species and enteric symptoms. The aims of the study are to investigate the domestic ducks' and hens' intestinal spirochetosis' epidemiology, clinicopathology, histopathology and etiology (immunohistochemistry, bacteriology, molecular biology) under natural circumstances and experimental infection. The aims are also to specify the causative agent of the disease, as well as define the *Brachyspira* strains' antibiotic sensitivity. Regarding the disease in domestic duck flocks, there was no publication in the technical literature, and is considered a new result. As broiler breeder flocks, this study gives new findings and observations in Hungary.

The intestinal spirochetosis among the adult, egg producing hens – as described in in the technical literature – can cause persistent diarrhea, mild egg production decrease, but no significant mortality increase. In duck populations however – during the egg production as well – high mortality and severe fibronecrotic typhlocolitis – as in swine dysentery – could be seen. In both poultry species predisposing factors (stress of the egg laying season, and the inappropriate quality of the feed) was also played a role in the pathogenesis of the disease.

MATERIALS AND METHODS

In the course of research samples submitted to our institute – CAO-VDD Budapest, Poultry Disease Laboratory – for routine diagnostic examination were processed. The bacteriology and antimicrobial susceptibility testing performed in the Laboratory Bacteriology, the molecular biological examination were made in the Molecular Biology Laboratory. The experimental infection took place in the animal house of CAO-VDD.

Our investigation took place in the two (A and B) adult primary breeder duck flocks, four (C, D, E, F), four to seven weeks old young duck flocks, and in three (G, H, I) broiler breeder flocks, which was 50, 87, 47 weeks old, respectively

After gross pathological examination, the kidney, liver, caecum, colon and rectum, and in some cases also the ileum, altered arthral wall, spleen, lungs, heart and brain were fixed in 4% buffered formaldehyde solution, embedded in paraffin, cut into 4 µm thick sections, and stained with haematoxylin and eosin.

The colon and caecum of all ducks were examined by immunohistochemistry technique. Detection of spirochetes was performed using a commercially available, fluorescein isothiocyanate labelled rabbit immune serum (National Veterinary Services Laboratories, Ames, USA). This serum was shown to label the *B. alvinipulli* strain isolated from one goose, and the *B. pilosicoli* and *B. intermedia* isolated from diseased ducks from Flock A and B, and prepared on a glass slide. After deparaffination, the sections were heated in citrate buffer solution (pH 6.0) in a microwave oven (750 W, 20 minutes) and subsequently treated in 3% H₂O₂ solution for 10 minutes and then with 2% milk powder solution at 37°C for 10 minutes. Antigen-antibody binding was detected using a test kit containing a horseradish peroxidase labelled polymer (EnVision™ + anti-rabbit HRP, Dako, Glostrup, Denmark). As chromogen, 3-amino-9-ethylcarbazole solution (Sigma Aldrich Co., St. Louis, MO, USA) was used and the sections were counterstained with Mayer's haematoxylin. The positive control was a colon section of a pig that had died of swine dysentery, while the negative control was a section series on which brachyspira-specific antibody had been replaced with phosphate buffer solution.

Culture of bacteria was attempted from heart blood, liver and altered arthral fluid under aerobic conditions and from caecal contents and colorectal contents under aerobic and anaerobic conditions on common agar, 10% sheep blood agar and Drigalski's agar media. Culture of *Brachyspira* species from the affected caecum, colon and rectum was also attempted. After making a 1-2 cm long incision in the affected part of the intestine with sterile scissors, the intestinal mucosa was scraped with a sterile inoculating loop. The material thus

obtained was inoculated onto a selective medium. The media were placed in a jar and incubated with a reagent providing anaerobic conditions at 42°C for 3–6 days. The shape and motility of the bacteria was examined by dark-field microscopy of fresh preparations made from the brachyspira colonies. The biochemical properties of the pure cultures (hippurate hydrolysis, α -galactosidase, α -glucosidase, β -glucosidase) were tested using Rosco tablets (Rosco, Taastrup, Denmark). Indole production of the strains was determined using filter paper impregnated with indole reagent. The minimum inhibitory concentrations of tiamulin, lincomycin and amoxicillin were determined by agar dilution method with a two-fold dilution while those of tetracycline and erythromycin by the E-test (AB BIODISK). Trypticase soy agar (TSA) containing 5% sheep blood was applied in both cases.

The fresh pure cultures of putative *Brachyspira* strains from the affected animals were examined by PCR amplification, sequencing methods and phylogenetic analyses. Total DNA was extracted from the cultured bacteria strains using a modified Guanidine HCl based method. Partial PCR amplification of the nicotinamide adenine dinucleotide reduced oxidase (Nox) gene was performed by using the primers and protocol described by Townsend et al. (2005). Amplified PCR products were purified then sequenced in both directions (Applied Biosystems, Foster City, USA) with the primers used for amplification. Nucleic acid and protein databases were searched using the programs BLASTN and BLASTX at the National Center for Biotechnology Information, Bethesda, Maryland, USA (<http://www.ncbi.nlm.nih.gov>).

Clinically healthy one-day-old pekin ducklings and Babcock chicks were used for experimental infection. At one day of age, the birds were weighed and randomly distributed into groups. The *Brachyspira* strains used for experimental infection had been isolated from the large intestine animals affected by intestinal spirochaetosis, and identified based on their cultural, biochemical characteristics and DNA sequence analysis. Subsequently the ducklings were observed daily and weighed weekly. All statistic calculations were carried out using multi-way ANOVA method with R program (© 2009 The R Foundation for Statistical Computing). Three from each group after euthanasia were exsanguinated weekly at the end of the first, second, third and fourth week, respectively. The animals were necropsied. For histopathological and immunohistochemical examination, samples were taken from the spleen, liver, kidney, thymus, bursa of Fabricius, occasionally from the tongue and from the wall of the ileum, caecum, colon. Bacteria from infected and control birds were reisolated and identified according to the biochemical classification system, as described previously. The humane care of ducklings was ensured during management, treatment and slaughter according to EU-equivalent laws and regulations in force in Hungary (Act No. XXVIII of 1998 on the Protection and Humane Treatment of Animals).

RESULTS AND CONCLUSIONS

Avian intestinal spirochetosis (AIS) is a disease of birds characterized by a pronounced colonization of the caecum and/or rectum with anaerobic intestinal spirochetal bacteria of the genus *Brachyspira*. The investigations identified *B. hyodysenteriae* and *B. pilosicoli* in association with intestinal lesions of the laying ducks and drakes. These ducks had fibronecrotic typhlocolitis and severe pathological changes of the kidney (tubularnephrosis, fibrosis). In the majority of cases, spirochetes were detected in the deep layers of the mucosal lesions and were isolated from the large intestinal lesions. These observations suggest a pathological role of the spirochete in the disease. The clinical signs, gross lesions and histopathological changes of intestinal spirochetosis described in ducks species are similar to those of intestinal spirochetosis occurring in mammals, especially in swine.

The disease in domestic ducks appeared most frequently in one-year-old breeder stocks during (or following) the egg-laying period, however it is proven that the spirochetes can also colonize the large intestine of the one-day-old ducklings. Because of these, the young (several weeks old) duck flocks that were submitted to our laboratory were investigated in term of intestinal spirochetosis. By immunohistochemistry, culturing and molecular biological methods brachyspira-infection of the colonic mucosa could be detectable in four-to-seven weeks old young duck flocks. The spirochetes isolated from different young ducks could be defined as *Brachyspira pilosicoli*. The autopsy and the histopathological examination of animals showed less severe colon lesions (mild fibrinous inflammation) than of the adult ducks case could be seen. However, it seems just before the egg laying season, in the 32-36 weeks old duck flocks – with the effect of predisposing factors – severe fibronecrotic typhlocolitis accompanied by tubularnephrosis – typical lesions of adult ducks AIS – could develop.

Regarding the broiler breeder hen flocks, the first hungarian appearance of the disease was published. The clinical signs – in association with the international literature – were persistent diarrhea, mild egg production decrease and the fecal contamination of the environment. The mortality did not increase significantly. The causative spirochetes could be defined as *B. hyodysenteriae* in five cases and *B. intermedia* in one case.

Epidemiological observations showed that the duck flocks was involved only in the middle region of the country and in the Great Hungarian Plain region (traditionally waterfowl breeding areas of our nation), whilst samples from diseased hen flocks was received from any part of the country. Our investigation suggests that the infection may be present in wild birds as well.

Resistance monitoring of the strains against the most used drugs of this study show that the strains against tetracycline proved to be sensitive the most of the time (24 out of 21 stains susceptible), tiamulin was proved to be effective too (17/24 susceptible). It is recommended to carry out resistance tests against lincomycin (8 / 24 susceptible) or amoxicillin (12/24 susceptible) before the actual treatment. Most likely the erythromycin therapy against AIS will not lead to success (only 5 / 24 strains were susceptible).

Our experimental infection study demonstrated that *B. pilosicoli*, *B. hyodysenteriae* and *B. alvinipulli*, isolated from sick and dead ducks, and orally administered to one-day-old ducklings could be reisolated in a proportion of infected birds. Colonization, however – detected by immunohistochemical reaction – only in groups with *B. pilosicoli* and *B. alvinipulli* strains could be seen. As for the chicken experimental infection, only with congeneric *B. hyodysenteriae* could be cultured from infected birds. No gross pathological or histological lesions were detectable in the intestinal mucous membrane including the colonized intestinal glands could be in connection with the AIS. Further studies are needed to establish whether brachyspira-infection of day-old ducklings and consequent large intestinal colonization plays a role in the aetiology of fibronecrotic typhlocolitis and nephropathy of laying ducks observed by us during the egg-laying season.

NEW SCIENTIFIC RESULTS

- We were the first to publish on the intestinal spirochaetosis of adult domestic ducks in the international technical literature.
- We established that most frequently *Brachyspira hyodysenteriae* and *B. pilosicoli* can be cultured from pathological lesions in adult egg-laying duck stocks.
- We provided observation at first hand about the intestinal spirochaetosis of adult hens in Hungary, and proved that the most frequent causative spirochete in those cases was *B. hyodysenteriae*.
- We performed examinations to establish the frequency of the disease in hungarian duck and hen flocks.
- We provided information about the causative agents' antimicrobial sensitivity.
- We proved that brachyspira infection and the clinical manifestation of the intestinal spirochaetosis is present in young domestic duck flocks.
- We carried out experimental infections using different *Brachyspira* strains, and examined if the bacteria could colonize the large intestinal system of one-day-old ducks and hens.

LIST OF PUBLICATIONS

Scientific publications of the thesis

1. Glávits R., Ivanics É., Nemes Cs., Dán Á., Kaszanyitzky É., Samu P., **Thuma Á.**, Beregszászi A., Simon A., Aladics S., Berta P., Dobos-Kovács M.: Vizsgálatok és megfigyelések a liba, kacsá, és a házityúk intestinalis spirochaetosisáról (brachyspirosisáról) a sertésdysenteriával összehasonlítva. Magyar Állatorvosok Lapja. 2008. 130. 663-670.
2. Ivanics É., Glávits R., **Thuma Á.**, Simon A., Berta P., Kaszanyitzky É., Samu P., Dencső L., Ursu K., Dán Á.: Intestinalis spirochaetosis (Brachyspirosis) hazai tojótyúk-állományokban. Magyar Állatorvosok Lapja. 2009. 131. 323-330.
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5. **Thuma Á.**, Ivanics É., Kaszanyitzky É., Dán Á., Glávits R.: Brachyspira fertőzöttség vizsgálata növendékkacsa-állományokban. Magyar Állatorvosok Lapja. 2011. 133. 464-470

Presentations

1. Glávits Róbert, Ivanics Éva, Nemes Csaba, Dán Ádám, Kaszanyitzky Éva, Samu Péterné, **Thuma Ákos**, Beregszászi Anikó, Simon Anna, Aladics Sándor, Dobos-Kovács Mihály: Vizsgálatok és megfigyelések a liba, a kacsá és a házityúk intestinalis spirochaetosisáról (brachyspirosisáról) a sertésdysenteriával összehasonlítva. 16. Derzsy Napok tudományos konferencia. Zalakaros, 2008. június 5-6.
2. **Thuma Ákos**, Dán Ádám, Juhászné Kaszanyitzky Éva, Dencső László, Fazekas Béla, Glávits Róbert: Naposkacsák kísérletes fertőzése *Brachyspira pilosicolival* illetve *B. alvinipullival*. MTA Akadémiai beszámoló. 2010. jan. 26.
3. **Thuma Ákos**, Ivanics Éva, Dán Ádám, Juhászné Kaszanyitzky Éva, Glávits Róbert: Brachyspira fertőzöttség vizsgálata növendékkacsa-állományokban. MTA Akadémiai beszámoló. 2010. jan. 26.

4. Ivanics Éva, Glávits Róbert, Bistyák Andrea, **Thuma Ákos**, Ping József: Víziszárnyasok bélgyulladásai. 18. Derzsy Napok tudományos konferencia. Siófok, 2010. június 3-4.
5. **Thuma Ákos**, Dán Ádám, Juhászné Kaszanyitzky Éva, Samu Péterné, Glávits Róbert: Naposkacsák és naposcsibék kísérletes fertőzése különböző állatfajokból izolált *B. hyodysenteriae* törzsekkel. MTA Akadémiai beszámoló. 2011. jan. 25.
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