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The incidence of *Riemerella anatipestifer* in Hungary, characterization of the strains by classical and molecular methods

Brief summary of Ph.D. thesis

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Introduction

Riemerella anatipestifer is a widespread pathogen, that occurs in all countries that have intensive duck and goose production, so it is also common in Hungary. *R. anatipestifer* has a broad host spectrum. It causes anatipestifer disease mostly in ducks and geese under eight weeks of age, but can also cause severe losses in turkey flocks.

The disease has been described in chickens, pheasants, partridges, guinea fowl, quails and wild waterfowl, seagulls, swans and various duck species. Predisposing factors are needed, such as young age, inadequate hygiene and nutrition, mycotoxicosis or co-infections.

In acute cases, sick birds are droopy, ocular and nasal discharge, sneezing, greenish diarrhoea and nervous system symptoms, tremor of the head, ataxia can be observed. In chronic cases, lameness, retarded growth can be seen. The most prominent pathologic lesions are sero-fibrinous pericarditis, airsacculitis and peritonitis, enlarged spleen and catarrhal enteritis. Occasionally catarrhal pneumonia, caseous salpingitis and sero-fibrinous arthritis may occur.

Although the consumption of duck and goose meat is less typical to Hungarian consumer habits, millions of our ducks and geese found a good market in Western Europe, bringing significant revenue to the country.

Waterfowl can be utilized in a versatile way, meat, liver and feather can be sold. Anatipestifer disease is of great economic importance, with an estimated 10-75% mortality, further economic losses due to reduced body weight gain, treatment, vaccination costs and slaughterhouse sores.

The aim of the thesis is to summarize the results of clinical, pathological and histopathological examinations of anatipestifer disease in naturally diseased waterfowl and turkeys. On the other hand, we wanted to detect the incidence of anatipestifer disease in domestic geese, ducks and turkeys.

The aim of our studies was to isolate *R. anatipestifer* strains from domestic poultry flocks and to create a collection of strains. In our work, strains of *R. anatipestifer* collected from different geographical locations and from different host species were characterized by various pheno- and genotyping methods. We first determined the phenotypic properties of the isolated bacteria and examined whether the differences found were related to the place or year of isolation or host species. Then we characterized our strains by molecular typing techniques, in order to analyse the genetic diversity of our strains and the resolution of the method. We examined whether the genotypic properties of the strains differ in time, in different geographic area or in different host species.

R. anatipestifer is an intensively studied pathogen, but the number of comprehensive studies on a large number of strains is limited. Our results contribute to a better

understanding of this pathogen and the disease, which, in addition to expanding scientific knowledge, is a prerequisite for the development of more modern and effective diagnostic and prevention methods to reduce the economic impact of anatipestifer disease.

Materials and Methods

Sample collection

The samples were collected from young birds that were submitted to the National Food Chain Safety Office Veterinary Diagnostic Directorate, mainly to the laboratories in Budapest and, to a lesser extent, laboratories in Debrecen and Kaposvár for post mortem examination from domestic goose, duck and turkey flocks in the period of 2010-2014. In one group, 4-10 birds were sent to investigate, and each group represented one case.

A total of 1418 gosling, 411 duckling and 1292 poult cases were received at the laboratories for pathological examination.

In the study period, 141 goose, 31 duck and 7 turkey cases were diagnosed with anatipestifer disease. These cases came from seven counties in Hungary: Bács-Kiskun, Békés, Csongrád, Jász-Nagykun-Szolnok, Pest county, Hajdú-Bihar and Somogy. The diagnosis of anatipestifer disease was only established when *R. anatipestifer* was isolated from the case.

Epidemiological data

Epidemiological data on the diseased flocks (size, age, clinical symptoms, severity of the disease) were obtained partly from the data described in the examination order, partly during the telephone conversation with the field veterinarians.

Pathology and histopathology examinations

The geese, ducks and turkeys submitted for diagnostic examination were examined by the usual routine diagnostic protocol. Body weight was used to determine the development of birds compared to their age and utilization. Then the birds were dissected for diagnosis and samples were taken for additional histopathological, bacteriological, virological and molecular biological tests.

In the detailed pathological examination, organ samples were collected for histopathological examination. Samples were usually taken from the brain and the liver, occasionally from the heart, bursa of Fabricius, lung, spleen, kidney and cranium. Tissue samples were fixed in 10% buffered formaldehyde, 4 μm thick sections were cut after embedding in paraffin and finally stained with haematoxylin-eosin.

Bacteriological examinations

Bacteriological culturing was carried out during the pathological examination from the organ samples taken out for bacteriological examination. Various organs, particularly liver, pericardium, brain ventricle, lung, ovarium, joint, occasionally trachea, conjunctiva, cranium, air sac or peritoneum were sampled. To isolate *R. anatipestifer*, Columbia agar plates supplemented with 5% sheep blood were used. The media were incubated at 37 °C in the presence of 5% CO_2 for 24 hours. Colonies showing suspected colony morphology of *R. anatipestifer* were also plated on Columbia agar medium supplemented with 5% sheep blood and incubated as described above. We performed our tests with the pure cultures obtained.

Bacterial strains

Besides the *R. anatipestifer* strains isolated from birds dying of anatipestifer disease in the cases described above, previously isolated bacterial strains were also included in the study. A total of 165 goose, 29 duck and 3 turkey originated strains were used. The bacteria were identified by colony morphology and species-specific PCRs.

Phenotypic characterization of *R. anatipestifer*

Biochemical tests

Biochemical tests for phenotypic characterization of *R. anatipestifer* isolates were performed. The catalase test was carried out with 3% hydrogen peroxide, which was judged by the presence or absence of effervescence. The isolates were tested for the cytochrome oxidase C enzyme activity by a Bactident Oxydase test strip, which gave a colour reaction. Bacteria were tested in indole, nitrate, urea, glucose, lactose, sucrose probes using conventional broths. The broths were incubated for 2 days at 37 °C.

Growth requirements assay

The ideal culturing conditions for *R. anatipestifer* strains were tested at 37 °C on Columbia agar medium supplemented with 5% sheep under aerobic conditions with elevated, 5% CO2 concentration. The media were incubated for 24 hours.

Haemolytic assay

The haemolytic properties of *R. anatipestifer* strains were tested at 37 °C on a Columbia agar medium supplemented with 5% sheep blood with 5% CO_2. The media were incubated for 24 hours.

Serotyping

The serotype of the isolates was determined by agargel precipitation test (AGP). Serotype-specific antisera were raised in 16-week-old specific-pathogen-free roosters against the most common serotype 1, 2, 4, 7 and 10 strains. The investigated *R. anatipestifer* strains were incubated on Columbia agar medium supplemented with 5% sheep blood for 24 hours at 37 °C, with 5% CO_2,. McFarland grade 3 suspensions were made in PBS, then heat stable antigens were prepared. The slides were incubated in a humid chamber at 37 °C and judged after 48 hours.

Antimicrobial susceptibility tests

Antibiotic susceptibility was tested by Kirby-Bauer disc diffusion method, and 196 *R. anatipestifer* isolates were selected for the assay. Some colonies were suspended in 5 ml of physiological saline, the turbidity of the suspension was adjusted to McFarland 0.5 and streaked onto Mueller-Hinton medium containing 5% sheep blood. The sensitivity of our strains was tested for 13 antibiotics. Based on the breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI 2013, 2015), the tests were evaluated according to the breakpoints developed by the National Food Chain Safety Office, Veterinary Diagnostic Directorate, Bacteriological Laboratory (Budapest).

Genotypic characterization of *R. anatipestifer*

Plasmid isolation

The QIAGEN Plasmid Mini Kit was used for the examination according to the manufacturer's instructions. For detection of plasmids, the products were resolved in 0.5% TopVision agarose gel in 1xTAE buffer with appropriate molecular weight markers of 9 V/cm electric field strength electrophoresis. 15 µl of eluted product and 5 µl of 6 x DNA Loading Dye were measured in the gel pockets. Detection and documentation of the products was performed under UV light with Kodak Gel Logic 212 Imaging System.

Polymerase chain reactions

Primers for *R. anatipestifer* species specific PCRs and ERIC-PCR were selected based on the literature. The DNA template was prepared by the boiling method from fresh colonies, and the reactions were performed in an ESCO Swift Mini thermocycler. The species specific PCR products were resolved in 1.5% SeaKem agarose gel in 1xTBE buffer by electrophoresis at 9 V/cm electric field strength. ERIC-PCR products were checked in 1.5% agarose gel in 1x AccuGENE TAE buffer for 4 hours at 120V. Detection and documentation of the products was made with the help of Kodak Gel Logic 212 Imaging System in UV light.

Analysis of ERIC-PCR assay results

Based on the presence (1) or absence (0) of different sized bands produced by the various ERIC-PCR reactions, a binary matrix was created with PyElph. The Dice coefficient was used to estimate the genetic similarity of the strains and a dendrogram was made with the unweighted pair group method with arithmetic average (UPGMA) method. The robustness of the dendrogram topology was analyzed by 100-repeat bootstrap analysis

Results

Epidemiological data

Anatipestifer disease was diagnosed in 9.9% of gosling cases and 7.5% of duckling cases in 2010-2014. It was rather rare in turkeys, it appeared only in 0.5% of cases. Anatipestifer disease was diagnosed in 5-day-17-week old geese. In ducks, the disease occurred between 3-6.5 weeks of age, while turkey cases were diagnosed at 12-19 weeks of age.

The most common clinical symptoms were lameness, greenish diarrhoea, ataxia, abnormal head position, tremor of the head, side or back lying and flailing. Occasionally, respiratory symptoms, nasal discharge or conjunctivitis were also observed.

In 19% of the goose cases circovirus infection, in 5% of the goose cases the Derzsy's disease was the primary disease. Co-infections were also diagnosed in ducks. In 46% of cases circovirus infection, in 13% mycotoxicosis, and in 8% mycoplasmosis was the primary disease.

In turkeys, *R. anatipestifer* caused septicaemia was diagnosed once, in one case we also saw changes that referred to septicaemia, while in 5 cases the pathogen caused local lesions beside *E.coli* septicaemia.

Pathology examinations

Pathological examinations in geese and ducks

In geese and ducks, sero-fibrinous pericarditis, airsacculitis and peritonitis were the most prominent pathological lesions in anatipestifer disease. We saw a sero-fibrinous exudate on the surface of the liver and in the pericardium. The spleen was usually enlarged, and in the nasal cavity and paranasal sinuses, a muco-purulent exudate has been accumulated, and occasionally catarrhal pneumonia has been observed. We usually saw a catarrhal enteritis in the small intestine. Usually we have also observed the oedema and hyperaemia of the subcutaneous connective tissue over the cranium. Occasionally, we have seen sero-fibrinous arthritis. In some cases, caseous salpingitis has also been observed. We have rarely seen conjunctivitis. Anatipestifer disease was not diagnosed in adult geese or ducks.

Pathological examinations in turkey

In 72% of the cases, *R. anatipestifer* caused only local lesions in turkeys beside *E. coli* septicaemia. Most often, we have seen cranial suppurative osteomyelitis and seropurulent leptomeningitis. Occasionally, in addition to the previous lesions, we also

observed seropurulent ventriculitis and/or seropurulent arthritis caused by *R. anatipestifer*. In the cases described above, although *E. coli* septicaemia was observed, *R. anatipestifer* was always isolated in pure culture from the local lesions.

In one case, the *R. anatipestifer* caused septicaemia, and sero-fibrinous pericarditis, airsacculitis, peritonitis and seropurulent arthritis were observed. In one case, the bacteriological examination did not confirm septicaemia, but *R. anatipestifer* was isolated from the pericardium, air sac, cranium, meninx, ventricules and joint. During pathological examination, sero-fibrinous pericarditis, airsacculitis and peritonitis, cranial suppurative osteomyelitis and seropurulent meningitis and ventriculitis, seropurulent arthritis were seen. Anatipestifer disease was not diagnosed in adult turkeys.

Histopathology examinations

By histological examination, in goose, duck and two turkey cases, sero-fibrinous inflammation was observed in the epicardium of the heart, in the Glisson's capsule of the liver, in the wall of the air sacs and on the serosal surface of the lungs. Serous hepatitis was observed, occasionally in the lower respiratory tract, in the parabronchi of the lung, serous or catarrhal exudate was observed. In case of nervous system involvement, we have seen sero-fibrinous meningitis and ventriculitis in the brain. The leptomeninges of the brain and spine were broaden diffusely, sero-fibrinous inflammation and lymphocytic, histiocytic and heterophil granulocytic infiltration were seen. Similar cellular infiltration occurred in the ventricles of brain, in the central canal of the spine.

In the majority of the turkey cases, signs of purulent osteomyelitis with heterophil granulocyte infiltration was observed in the cranium and seropurulent meningitis or occasionally seropurulent ventriculitis were seen.

Strain isolation and identification

After observation of colony morphology and species-specific PCRs, 136 goose, 24 duck and 3 turkey origin strains were isolated. Molecular identification was performed on all isolates included in the study. The size of amplified PCR fragments of the species-specific PCRs were 546 bp and 338 bp.

Phenotypic characterization of *R. anatipestifer*

Biochemical tests

The *R. anatipestifer* strains were positive in oxidase and catalase tests and negative in indole, nitrate tests and lactose fermentation. The majority of the strains were negative in

urea tests, glucose and saccharose fermentation, but some strains showed positive or uncertain result.

Growth requirements assay

All *R. anatipestifer* strains were grown on Columbia agar medium supplemented with 5% sheep blood at 37 °C and 5% CO_2 concentration. The growth of the 24% of the strains was more abundant with increased CO_2 concentration, under aerobic conditions only small puncture colonies were formed.

Haemolytic assay

98% of the *R. anatipestifer* strains did not haemolyse, while 2% of the strains showed β-hemolysis on Columbia agar medium supplemented with 5% sheep blood, incubated at 5% CO_2 concentration for 24 hours at 37 °C.

Serotyping

Altogether, 83.1% of the strains showed monospecific reaction. On the other hand, 16.9% of the strains reacted equally with serotype 1 and serotype 7 specific sera (serotype 1,7). Two-thirds of the strains (64.5%) belonged to serotype 1 of the presently known 21 serotypes. Fewer strains belonged to serotype 1,7 (16.9%), serotype 2 (7.2%), serotype 4 (3.6%) and serotype 7 (4.8%). Two strains were determined as serotype 10 (1.2%), while serotype 13, 17, and 18 were present in one strain each (0.6%). Some serotypes occurred only in strains from geese or ducks.

Antimicrobial susceptibility tests

The majority of the strains were susceptible to florfenicol (98%), ampicillin/amoxicillin (95.4%), penicillin (93,4%), sulphamethoxazole-trimethoprim (90,8%) and spectinomycin (86,8%). The highest resistance rates were observed for flumequine (93,4%), tetracycline (90,8%), erythromycin (75.5%) and streptomycin (70,9%). The antibiotic susceptibility of the strains to gentamicin, doxycycline, sulphonamide compounds and enrofloxacin was variable.

The resistance rates to florfenicol and spectinomycin from 1993, to erythromycin and streptomycin from 1997, to sulfonamides from 2004 and to doxycycline from 2009 showed an increasing tendency over time. The ratio of resistant strains to the geographical location of the isolation showed a 3-19.5% increase relative to the average. Extensively drug-resistant (XDR) ratios showed an increasing trend over time.

Genotypic characterization of *R. anatipestifer*

Plasmid isolation

59.6% of the strains tested contained a 2900 bp plasmid, while 17% of the strains contained a 4800 bp plasmid. A 5500 bp plasmid was isolated from 6.4% of the strains; at 4.2%, a 6900 bp plasmid was found. 12.8% of the strains showed negative results in plasmid isolation. In all cases, the bacterial strains contained only one plasmid.

ERIC-PCR

The *R. anatipestifer* strains showed seventeen distinct patterns with ERIC-PCR. The majority of the strains belonged to two closely related ERIC-PCR types (type A (60.8%) and B (16.9%)), some strains belonged to type C (4.8%) and F (6%), while the rest of the types contained only a few strains in each. Twelve ERIC-PCR types were found only in strains isolated from geese.

The earliest isolated strains (2000, 2004) are unique and most did not occur later. The goose origin strains showed more diverse patterns than the strains from ducks. A correlation could be seen between ERIC-PCR patterns and serotypes. The majority of serotype 1 strains (94.4%) belonged to ERIC-PCR type A, whereas the remaining six strains represented five different ERIC-PCR types (type D, G, L, M and O). Serotypes 1,7 and 7 corresponded to ERIC-PCR types B and C, respectively. Serotypes 2, 4 and 10 could be subdivided by the ERIC-PCR showing 2–4 patterns within each of these serotypes.

Conclusions

The frequency of anatipestifer disease in goose, duck and turkey was determined on the basis of the examination of the birds arriving for diagnostic examination. In waterfowl, the disease is very common, occurring in the presence of Derzsy's disease, circovirus infection, polyomavirus infection, colibacillosis, or fowl cholera. In case of anatipestifer disease, the great importance of animal health draws attention to proper treatment and prevention.

In goose and duck under 8 weeks of age, in accordance with previous data, we diagnosed the acute septicaemic form of the disease, most often sero-fibrinous pericarditis, airsacculitis and peritonitis was observed. We provided data on the anatipestifer disease in older 9-17 weeks old geese, but most often we found localized lesions such as catarrhal pneumonia, septicaemia mostly due to a more severe predisposing factor.

In turkey, the anatipestifer disease was rare and usually caused only local lesions, cranial suppurative osteomyelitis and seropurulent leptomeningitis, septicaemia occurred only in one case. In turkey, anatipestifer disease is likely to be present only as a result of a more severe immunosuppressive factor. In the literature, we first reported about *R. anatipestifer* caused cranial suppurative osteomyelitis.

Antibiotic susceptibility testing and serotyping help directly in disease control and prevention. The bacterium is a facultative pathogen, anatipestifer disease was a secondary disease in 24% of the goose cases, and in 67% of duck cases, the treatment of primary disease and, if possible, the reduction of predisposing factors are part of the prevention of anatipestifer disease. Our antimicrobial susceptibility tests show that florfenicol, ampicillin/amoxicillin, penicillin and sulfamethoxazole-trimethoprim are mostly effective antibiotics. Enrofloxacin, doxycycline and sulfonamides were less effective. Treatment with flumequin, tetracycline, erythromycin are unlikely successful.

Serotype 1 was the most common serotype among our isolates, serotype 1,7 showed a somehow higher incidence too, and 2, 7 and 4 also occurred in our cases.

We found differences among *R. anatipestifer* strains by phenotypic tests and genotyping methods. The groups separated by the different methods, apart from one exception, were not correlated. The differences revealed by phenotypic studies may be related to virulence factors and contribute to a better understanding of the survival and spread of the pathogen in the environment. In most phenotypic studies, there was no correlation between host species, time or geographical location of isolation. However, a minor host specificity was observed for the less common serotypes. Antibiotic susceptibility testing has also found temporal and spatial differences that are probably related to antibiotic use. Inappropriate use of antibiotics is likely to contribute to the spread of resistance.

Genotyping of the strains gave a more realistic picture of the relationships of the strains, and the environment cannot affect the appearance of that property. The most complete picture can be obtained by complete genome sequencing, but it is the most expensive method, so it is not common in recent everyday practice. In plasmid isolation, a mobile genetic element was investigated, and no correlation was found between the isolated plasmid and the host species, the time or geographical location of isolation. In the ERIC-PCR study, the whole genome was examined, and the discriminatory power of the method made it possible to examine the genetic diversity and grouping of the strains. Seventeen different ERIC patterns have been identified, from which the most of previously isolated strains (2000-2004) were unique. Several ERIC types occurred only in strains isolated from geese, which may be due to host adaptation or, possibly, the less susceptibility of the ducks to the infection. Similarly to the serotypes, there was no difference in the spatial occurrence of the different ERIC types. Probably the strains representing the most frequent ERIC types and serotypes are permanently present in a region, circulating and causing recurring epidemics. In addition, occasionally, some rarer types appeared at different geographical locations. ERIC-PCR is a widespread method because of its speed, simplicity, low cost and equipment requirements. Once checked for reproducibility, it may be suitable for epidemiological investigations.

In our studies, a close correlation was found between the serotypes of the strains and the ERIC-PCR types. With one exception, all ERIC-PCR types represented one serotype. In previous studies, no correlation was found between the serotype of *R. anatipestifer* strains and a genotyping method. According to our results, ERIC-PCR may be suitable for the determination of serotype of *R. anatipestifer* isolates. Namely, serotyping is not a widespread routine method, because only a few specialized laboratories have sera produced against serotype reference strains, which is expensive and requires practice in the implementation and evaluation of the results. In the case of commercially available sera, the nomenclature does not correspond to the names described and accepted in the literature. Determining of the serotype is particularly important for effective control because no convincing cross-protection between different serotypes has been observed.

New scientific results

1. We first reported cranial suppurative osteomyelitis caused by *R. anatipestifer* in turkey.

2. We first performed a comprehensive comparative study on a large number of *R. anatipestifer* strains (197) from different host species in Hungary using several phenotypic and genotypic methods.

3. The first performed a broad antibiotic susceptibility tests of local *R. anatipestifer* strains for 13 antibiotics. The proportion of resistant strains showed an increasing tendency over time.

4. We determined the serotypes occurring in Hungary, determined their prevalence according to host species, time of isolation and geographical origin.

5. We identified 17 different ERIC patterns among our *R. anatipestifer* strains by ERIC-PCR, in which the strains from geese showed a greater diversity (17 types) than the strains from ducks (5 types).

6. We first found association between the serotype of *R. anatipestifer* strains and a molecular typing method, the ERIC-PCR.

Publications based on the results of the PhD dissertation

Research papers in peer-reviewed journals

Gyuris É., Wehmann E., Magyar T.: **A baromfi *Riemerella anatipestifer* okozta megbetegedése. Irodalmi áttekintés**, Magyar Állatorv. Lapja, 138. 3-14, 2016.
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IF: 2,054

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