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A case study of a *Riemerella anatipestifer* infection on a commercial turkey farm in Croatia

Fallstudie zur *Riemerella anatipestifer* Infektion in einer kommerziellen Putenfarm in Kroatien

L. Lozica, **M. Mazić** and **Ž. Gottstein***

University of Zagreb, Faculty of Veterinary Medicine, Zagreb, Croatia

*Correspondence: gottstei@vef.hr; zgottstein@gmail.com

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Abstract

In this study, *Riemerella anatipestifer* was isolated in a commercial turkey farm in Croatia. The flock showed signs of respiratory distress, lethargy and elevated mortality. In total twenty poults were examined and the most frequent pathomorphological findings were polyserositis and fibrinous airsacculitis. *R. anatipestifer* strains were isolated from the air sacs, lungs and spleen from three different birds from the same poultry house. The isolates were compared based on the 16S rRNA gene sequences, and the results of the phylogenetic analysis indicated the infection was caused by serotype 6. The flock received an antibiotic therapy via the drinking water consisting of enrofloxacin, after which the symptoms ended without recurrent infections. This is the first report of *Riemerella* infection found on a commercial turkey farm in Croatia.

Key words

Riemerella anatipestifer; turkey; clinical signs; necropsy; 16S; phylogeny

Zusammenfassung

In dieser Studie wurde *Riemerella anatipestifer* in einer kommerziellen Putenfarm in Kroatien isoliert. Die Herde zeigte Anzeichen von Atemnot, Lethargie und erhöhter Sterblichkeit. Insgesamt wurden 20 Puten untersucht. Die häufigsten pathomorphologischen Befunde waren Polyserositis und fibrinöse Luftsacculitis. *R. anatipestifer*-Stämme wurden aus den Luftsäcken, Lungen und Milz von drei verschiedenen Tieren aus demselben Geflügelstall isoliert und die Isolate anhand der 16S-rRNA-Gensequenzen verglichen.

Die Ergebnisse der phylogenetischen Analyse zeigten, dass die Infektion wahrscheinlich durch den Serotyp 6 verursacht wurde. Die Herde erhielt eine Antibiotikatherapie über das Trinkwasser, nach der die Symptome ohne wiederkehrende Infektionen endeten. Es handelt sich hier um den erster Bericht über eine *Riemerella*-Infektion auf einer kommerziellen Putenfarm in Kroatien.

Stichworte

Riemerella anatipestifer; Puten; klinische Anzeichen; Autopsie; 16S; Phylogenie

Introduction

Riemerella anatipestifer (R. anatipestifer) infection is a disease occurring primarily in domestic ducks and geese, although it has been isolated from other domestic and wild birds (Hess et al., 2013; Omaleki et al., 2020; Ruiz and Sandhu, 2020). The infection has the most significant impact on duck production, where it causes major economic losses because of high mortality, weight loss, condemnations and treatment costs (Ruiz and Sandhu, 2020). Usually, the most frequently observed clinical symptoms are respiratory and neurological signs, and diarrhoea (Rubbenstroth et al., 2009; Hess et al., 2013), although the bacteria were also isolated from clinically healthy birds (Ryll et al., 2001). The disease is highly contagious and occurs through the respiratory system and skin wounds (Chang et al., 2019; Ruiz and Sandhu, 2020). This study describes a clinical case of *R. anatipestifer* infection on a commercial turkey farm in Croatia. To our knowledge, *Riemerella* infection has not been reported in Croatia to date, and this study presents a rare mild case with low mortality rates and no recurrent infections.

Material and methods

Case history

Ten 24-day-old poults were submitted to the Department of Poultry Diseases with Clinic (Faculty of Veterinary Medicine, University of Zagreb) for clinical and pathomorphological examination. The majority of the flock showed respiratory symptoms and growth retardation. After 10 days, another 10 poults from the same flock were submitted for examination due to prolonged signs of infection. The farm has a floor production system in 8 detached houses, which are located on the same site with 13.000 poults per house. In case of enteritis, a phytogenic mixture based on garlic, oregano and thyme is added to the feed (300 ppm) in order to raise antibiotic-free turkeys. The immuno-prophylaxis program includes vaccination against Newcastle disease (ND) and Turkey rhinotracheitis (TRT) by coarse spray in the hatchery, and later revaccination of ND via drinking water at 35 d of age (Ministry of Agriculture, 2013; 2020).

Sampling and laboratory testing

After clinical examination of the submitted poults, blood samples were collected for serological examination using ELISA, and the birds were then euthanised to perform the necropsy. Afterwards, swabs were taken from the infraorbital sinuses, conjunctiva, trachea, air sacs, lungs, peritoneum and other macroscopically changed organs upon indication, and subjected to bacteriological examination.

The swab samples were plated on Brilliant Green agar (Oxoid, Basingstoke, UK), UTI Brilliance Clarity Chromogenic agar (Oxoid, Basingstoke, UK) and Columbia agar (Oxoid, Basingstoke, UK) enriched with 5% sheep blood (Biognost, Zagreb, Croatia), and later they were tested for *Chlamydia psittaci* using qPCR method as described by Ménard et al. (2006). All the plates were aerobically incubated at 37°C for 24 h, while blood agar plates were additionally incubated with 5% CO_2. Identification based on the morphological characteristics and biochemical analyses was not successful, so the bacterial colonies were identified using Bruker Microflex LT matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). The bacterial strains were stored in Brain Heart Infusion broth (Oxoid, Basingstoke, UK) with 50% glycerol at –20°C until further analysis.

The blood samples were transferred to tubes and allowed to clot for approximately 1 h. Afterwards, sera were separated and transferred to clean tubes, and kept at –20°C until further analysis. Commercially available ELISA antibody test kit for *Mycoplasma synoviae* (BioChek, Ascot, UK) was used according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility test was done for three *R. anatipestifer* strains using disk diffusion assay on Columbia agar (Oxoid, Basingstoke, UK) enriched with 5% sheep blood (Biognost, Zagreb, Croatia). The inoculum was prepared by suspending the bacterial colonies in sterile saline solution adjusted to turbidity of 0.5 McFarland standards. The assay was performed with the following antibiotic discs- 5 µg of enrofloxacin, 30 µg of flumequine, 10 µg of doxycycline, 10 µg of amoxicillin, 10 µg of lincomycin, 300 µg of sulphonamides compound, and 109 µg of lincomycin/spectinomycin (Oxoid, Basingstoke, UK). The inhibition zone diameters were interpreted according to the Clinical and Laboratory Standards Institute guidelines (Wayne, 2015).

DNA extraction, amplification and purification

DNA of the three *R. anatipestifer* strains was extracted using Chelex 100 (BioRad, Hercules, CA, USA) according to the manufacturer's instruction. Amplification was done through polymerase chain reaction using 16S rRNA gene primers- 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT). The reaction mixture was composed of 25 µl GoTaq G2 Hot Start Green Master Mix (Promega, Madison, WI, USA), 2 µl each of 10 pmol primers, 5 µl of DNA and 16 µl of Nuclease- Free Water (Promega, Madison, WI, USA) (total volume = 50 µl). The cycling parameters for amplification included: 95°C for 5 min and then 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 40 s; and final elongation at 72°C for 7 min. PCR products were visualised using 1% agarose gel electrophoresis, stained with Midori Green Advance (Nippon Genetics Europe GmbH, Düren, Germany). The expected amplicon size was 1400 bp. Purification of the amplicons from gel slices was done using ReliaPrep™ DNA Clean-up Concentration System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Afterwards, the isolates were submitted to Sanger (dideoxy) sequencing using 16S rRNA primers- 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Macrogen Inc., Amsterdam, The Netherlands).

Phylogenetic analysis

Sequences were edited using BioEdit Sequence Alignment Editor version 7.0.5.3. (Hall, 1999). Afterwards, they were aligned using ClustalW, cut to equal lengths and analysed using Molecular Evolutionary Genetics Analysis software (MEGA X) version 10.0.5. (Kumar et al., 2018). The data was analysed using Maximum likelihood method and Kimura 2-parameter model with a discrete Gamma distribution (Kimura, 1980). The queries were made using sequences through the basic local alignment search tool (BLAST) of the National Center of Biotechnology Information (NCBI, USA) in order to search for genetically similar microorganisms based on the data available in the GenBank. Sequences available under accession numbers AY871817, AY871820-AY871825, AY871831, AY871832, AY871835, EF641575 and EU715011 were used for the construction of the phylogenetic tree as they showed highest similarities to our sequences, while *Riemerella columbina* (GU903273.1) was used as an outgroup. *R. anatipestifer* sequences analysed in this study are available in the GenBank under accession numbers MW290517-MW290519.

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors and all research was conducted in an ethical and responsible manner. Permission for sampling was granted by the farm.

Results

Clinical and pathomorphological examination

The birds were noticeably stunted for their age. Clinical symptoms included sneezing, gasping, foamy conjunctivitis, lethargy and ataxia, while several birds had unilateral eye swelling. The most common gross pathology observations were peritonitis and airsacculitis. In case of peritonitis, peritoneal serosa and mesentery were covered with foamy, white exudate, and occasionally fibrinous exudate (Figure 1). Based on the observed clinical signs and *post-mortem* lesions, differential diagnoses included chlamydiosis, mycoplasmosis, bordetellosis, pasteurellosis, colibacillosis and *Ornithobacterium rhinotracheale* infection.

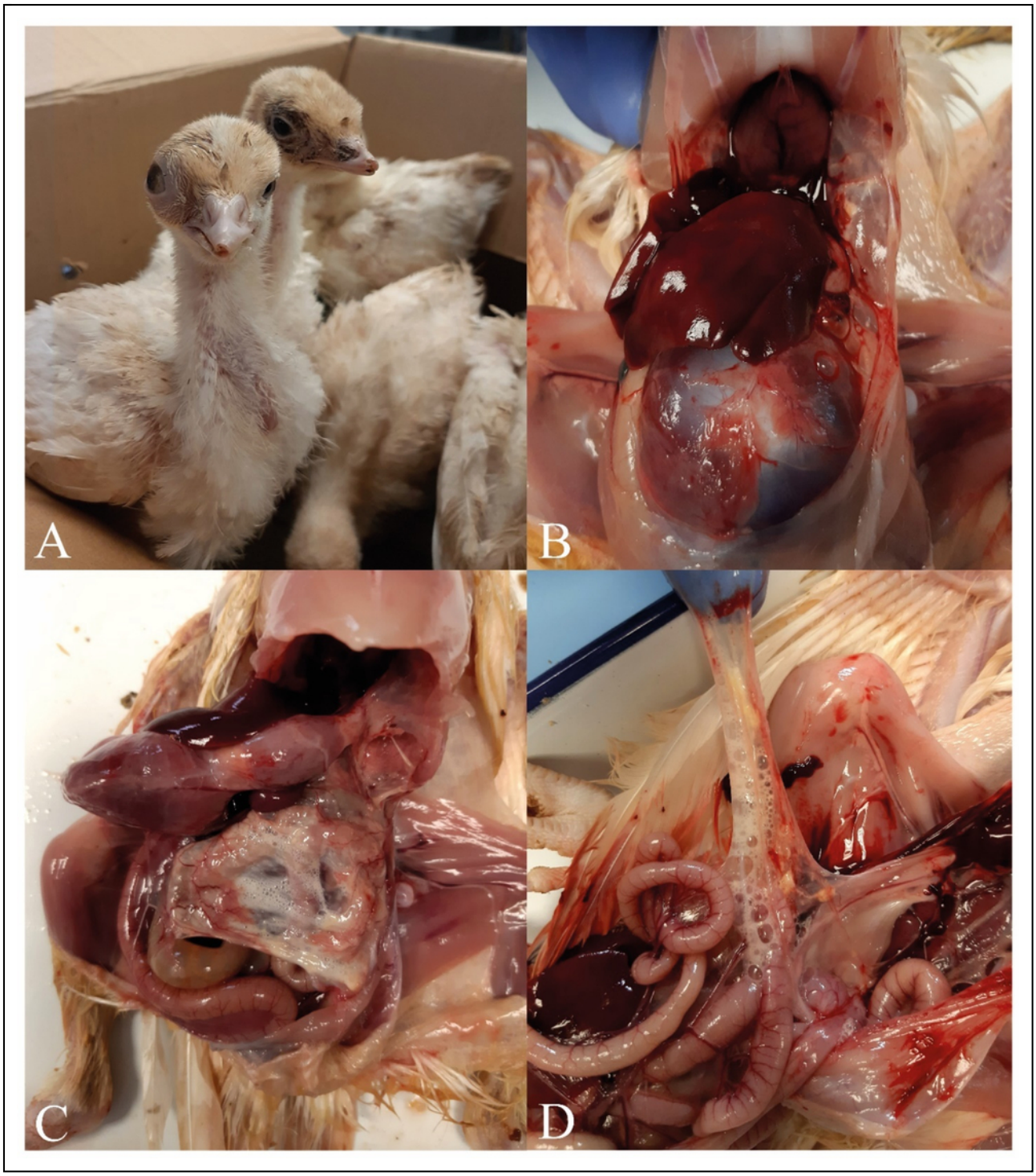

Figure 1. The most common clinical and pathomorphological examination findings. (A) Unilateral eye swelling. (B) Fibrinous airsacculitis. (C) Foamy exudate on the mesentery. (D) Foamy and fibrinous exudate on the mesentery.

Die häufigsten klinischen und pathomorphologischen Untersuchungsergebnisse. (A) Einseitige Augenschwellung. (B) Fibrinöse Luftsakkulitis. (C) Schaumiges Exsudat auf dem Mesenterium. (D) Schaumiges und fibrinöses Exsudat im Mesenterium.

Laboratory findings

R. anatipestifer was detected in the air sacs, lungs and spleen of three different birds. The first strain was isolated from a 24-day-old poult, while the other two were isolated from two 34-day-old poults, which were submitted to the examination 10 days after the initial examination. The samples were negative for *Chlamydia psittaci, Mycoplasma synoviae, Bordetella avium, E. coli, Pasteurella multocida* and *Ornithobacterium rhinotracheale*. The results of the antimicrobial susceptibility testing showed the strains were resistant to flumequine and lincomycin (Table 1).

Table 1. Antimicrobial susceptibility of the *R. anatipestifer* isolates.

Antimikrobielle Empfindlichkeit der *R. anatipestifer*-Isolate.

	Isolates		
Antimicrobial agent	**1363**	**1401**	**1402**
Amoxicillin	S	I	I
Doxycycline	S	S	S
Enrofloxacin	S	S	S
Flumequine	R	R	R
Lincomycin	R	R	R
Lincomycin/spectinomycin	S	S	S
Sulphonamides compound	S	I	I

*S = susceptible, I = intermediate, R = resistant

Phylogenetic analysis

The phylogenetic analysis confirmed the identification of the bacteria. The isolates showed 100% identity match with *R. anatipestifer* serotype 6 according to BLAST (Figure 2).

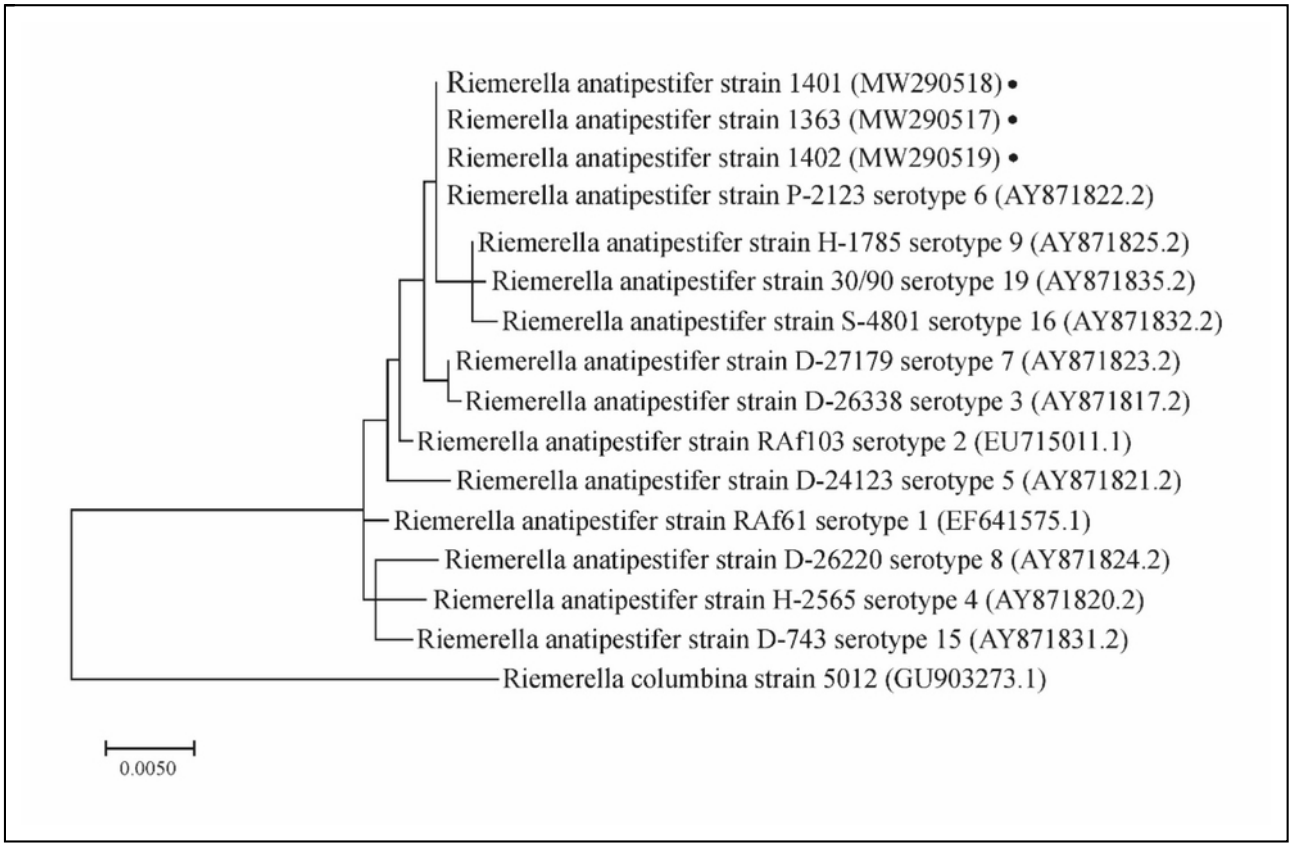

Figure 2. Phylogenetic tree of the *R. anatipestifer* isolates based on the 16S rRNA gene sequences. The sequences marked with dots were analysed in this study.

Phylogenetischer Baum der *R. anatipestifer*-Isolate basierend auf den 16S-rRNA-Gensequenzen. Die mit Punkten markierten Sequenzen wurden in dieser Studie analysiert.

Discussion

In *R. anatipestifer* infection the most common lesion is fibrinous polyserositis, particularly pericarditis and airsacculitis (RUIZ and SANDHU, 2020). In the present investigation, serous foamy exudate was more prevalent, although fibrinous airsacculitis was also detected in several birds. However, conjunctivitis and serous nasal discharge were the most prominent symptoms in all birds submitted to the examination. The mortality rate in the poultry house with sick birds was 3.1%, which indicates a less severe infection since the typical mortality rates in *Riemerella* infections vary from 5 to 75% (TANG et al., 2018; RUIZ and SANDHU, 2020). The infections are also often recurring due to their high contagiousness and presence of persisters (i.e. dormant or non-growing bacterial cells) which have an important role in the emergence of drug resistance (TANG et al., 2018; GOLLAN et al., 2019). The flock was treated with enrofloxacin via drinking water (10 mg/kg) for five days. The symptoms ceased after therapy, and there have been no recurrent infections. As the disease is easily transmitted and often occurs as a result of poor zoohygienic conditions, inadequate ventilation or other ongoing infections, improvement of the biosecurity and management practices was recommended. In case such outbreak repeats, implementation of the autogenous *R. anatipestifer* vaccine is advised.

This report presents a case of a mild *Riemerella* infection with a low mortality rate and no further recurrences or spreading to other flocks on the farm. The antibiotic therapy was very efficient, which is probably a result of the continuous use of herbal supplements and minimal use of antimicrobials.

Authors' contribution

Ž.G. and L.L. performed the clinical and post-mortem examination. M.M. and L.L. performed the laboratory analyses. L.L wrote the manuscript with the support from Ž.G.

Conflict of interest

The authors have declared no conflict of interest.

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