RESULTS OF THE RESEARCH

Respiratory tract infections are one of the most important animal health care problems in the poultry industry worldwide. Pathogenic bacteria are often included in the aetiology of these diseases. The main aim of the project was to investigate the prevalence and significant biological properties of three of these bacterial pathogens: *Bordetella avium*, *Ornithobacterium rhinotracheale* and *Riemerella anatipestifer*.

Research on *Bordetella avium* and *Ornithobacterium rhinotracheale*

B. avium and *O. rhinotracheale* are pathogenic bacteria of worldwide distribution that cause respiratory disease in several avian species. The examined strains were identified by species-specific PCR. The *B. avium* strains proved to be uniform in their phenotypic properties, and gave negative in conventional biochemical tests. The *O. rhinotracheale* isolates were negative in the indole and nitrate tests and positive in the urea test. Their ability to use different sources of carbohydrates was variable. The growth requirements of the strains were tested on four different solid media at three different temperatures (31, 37, and 41 °C). Both *B. avium* and *O. rhinotracheale* strains grew at all three temperatures. Agars supplemented with sheep blood proved to be the ideal growth media for the growth of *O. rhinotracheale*. 61% of the *O. rhinotracheale* strains showed signs of β-haemolysis after incubation for 48 hours at 37°C followed by 48 hours at room temperature. The *B. avium* strains showed more intensive haemagglutinating activity than the *O. rhinotracheale* isolates that is most likely an indicator of the presence of adhesin-like structures, as seen in other bacterial pathogens. Differences in phenotypic characteristics of the strains did not correspond to their host origin, thus cannot be regarded as a sign host adaptation to various bird species.

Antimicrobial susceptibility of 19 *B. avium* and 36 *O. rhinotracheale* strains were tested by Kirby-Bauer disk diffusion method, and the minimal inhibitory concentrations (MIC) of amoxicillin, doxycycline and erythromycin were also determined. Most *O. rhinotracheale* strains were resistant to nalidixic acid, sulfamethoxazole-trimethoprim and gentamicin, and were susceptible to ampicillin, chloramphenicol, spectinomycin and tilmicosin. All *B. avium* strains were resistant to ceftiofur and lincomycin and susceptible to doxycycline, gentamicin, polymyxin B, spectinomycin and sulphonamides. The MICs ranged widely with all three antibiotics tested against *O. rhinotracheale* strains, from 0.12 μg/ml to 32 μg/ml for amoxicillin and erythromycin, and 0.6 μg/ml to 32 μg/ml for doxycycline. Concerning *B. avium* isolates, values ranged from ≤0.03 μg/ml to 1 μg/ml for amoxicillin, from ≤0.03 μg/ml to 0.12 μg/ml for doxycycline and from 8 μg/ml to 16 μg/ml for erythromycin. Our findings support the idea that use of antibiotics in a region or a farm may affect antimicrobial resistance and the need for prudent application of antibiotic therapy based on proper antimicrobial susceptibility testing.

Thirty-seven field isolates of *O. rhinotracheale*, collected from various locations in Hungary between 1997 and 2015, were used for further serological and molecular characterization.

To date, 18 different serotypes have been identified (A–R) using agar gel precipitation or enzyme-linked immunosorbent assays. Antisera against representative strains of serotypes A–E were raised in 4-week-old specific-pathogen-free chickens. Heat-stable antigens were

prepared from the examined strains and serotyped. Most of the isolates from turkeys and those from birds of prey belonged to serotype A. Isolates from chickens showed somewhat higher variability: two of them belonged to serotype A, two were serotype D, and one was not typeable with A-E antisera.

In a phylogenetic analysis of the partial 16S rRNA sequences (1334 bp), the *O. rhinotracheale* isolates formed two clusters. The majority of the field isolates (91.9%) showed 100% similarity to sequences from GenBank and the type strains of serotypes A, B, and E. By sequence analysis of partial 16S rRNA sequences (1341 bp) of the *B. avium* strains 100% similarity were observed between them. Thirty seven strains of *O. rhinotracheale* were molecularly characterized by enterobacterial repetitive intergenic consensus (ERIC)-PCR and random amplified polymorphic DNA (RAPD) assays with the OPG11, OPH19, and M13 primers. Thirteen distinct patterns were identified with ERIC-PCR, and the RAPD assay with the M13 primer assigned the isolates to 10 different patterns. The other two RAPD assays were unsuitable for distinguishing and grouping the isolates. Neither ERIC type nor RAPD pattern correlated with the place or year of isolation. However, the strains isolated from chickens and pigeons were more heterogeneous on ERIC-PCR than the isolates recovered from turkeys. Strains isolated from wild birds belonged to the most common pattern type. In this study, ERIC-PCR was the most effective method for investigating the genetic diversity of *O. rhinotracheale* isolates.

Eleven *O. rhinotracheale* strains isolated in Hungary were analysed by the multilocus sequence typing (MLST) method as well. The PCR followed partial sequencing of seven housekeeping genes (*adk*, *aro*E, *fum*C, *gdh*A, *mdh*, *pgi* and *pmi*) was performed as described previously (Thieme, S, Mühldorfer, K, Lüschow, D, Hafez, HM: Molecular characterization of the recently emerged poultry pathogen *Ornithobacterium rhinotracheale* by Multilocus Sequence Typing, PLoS ONE, 11. e0148158, 2016). Sequence type 5 (ST5) was found in two *O. rhinotracheale* isolates from a turkey and a chicken. A new ST was described in an isolate from chicken (OR007), and the partial sequencing data were verified by the whole genome sequencing. The analysis of sequence data of OR007 revealed unique allele types on *adk*, *aro*E, *fum*C, *pgi* and *pmi*. We have identified new unique allele types in further three isolates from chickens and a sparrow hawk. The new allele types were created by new point mutation(s) or combination of point mutation(s). The most varied gene was *fum*C (Fig 1).

Multi-locus sequence typing (MLST) analysis of *B. avium* strains revealed point mutations in four out of the seven examined gene sequences. Three new sequence types were identified, and four strains belonged to the same sequence type as the only other strain in the *Bordetella* MLST database.

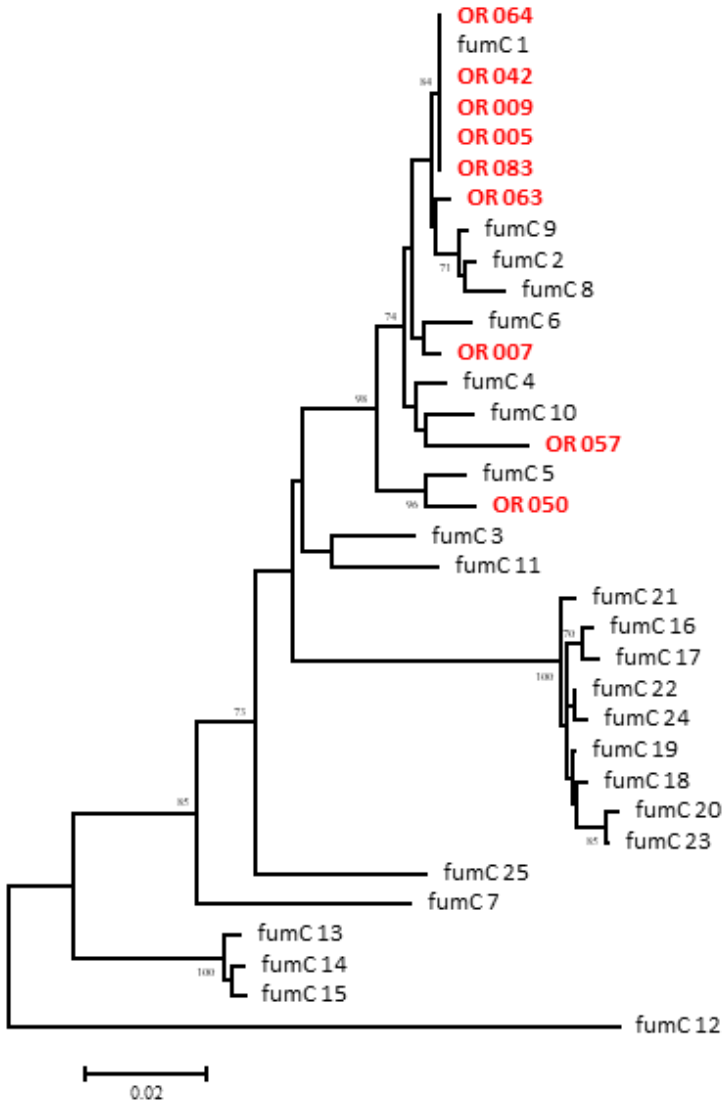

Figure 1.
Phylogenetic tree based on partial nucleotide sequence (489 bp) of *fum*C gene of *Ornithobacterium rhinotracheale* isolates from Hungary and reference allele types. (Neighbor-joining method with Jukes-Cantor correction)

Research on *Riemerella anatipestifer*

R. anatipestifer is a widely distributed bacterial pathogen of birds responsible for remarkable losses to poultry production, especially among waterfowl. The presence of *R. anatipestifer* in flocks of various poultry species (goose, duck, turkey, chicken) from different regions of Hungary, mainly from Bács-Kiskun, Pest, Komárom-Esztergom, Veszprém and Fejér counties, and occasionally from Békés, Borsod-Abaúj-Zemplén, Heves, Jász-Nagykun-Szolnok- and Nógrád counties, was examined. Detailed pathological, histopathological and bacteriological examination were carried out on birds belonging to different species (3732 ducks, 2618 geese, 1838 turkeys and 4382 chickens) and age groups.

The anatipestifer disease was one of the most frequently diagnosed disease in goslings and ducklings between 2010 and 2015. It occurred in 5-day to 17-week old geese and 3 to 6.5-week old ducks, respectively. Enlarged spleen, sero-fibrinous pericarditis, perihepatitis and airsacculitis, catarrhal enteritis, sero-fibrinous meningitis and occasionally catarrhal pneumonia, oedema and hyperaemia of the subcutaneous connective tissue over the cranium, sero-fibrinous arthritis and caseous salpingitis could be seen in both species by pathology and histopathology examination. *R. anatipestifer* caused generally just local lesions, catarrhal pneumonia in 8 to 17-week old geese. On the other hand, it was rather rare among turkeys, it was diagnosed only in seven cases, and it occurred in 12 to 19-week old birds. In two cases, the bacterium caused septicaemia or bacteraemia and similar lesions were seen as described above. In the majority of the cases, *R. anatipestifer* produced only local lesions such as purulent osteomyelitis of the cranium and seropurulent meningitis. Purulent osteomyelitis in the cranium caused by *R. anatipestifer* infection has not been previously reported in turkeys.

Bacteriological samples were taken from different organs, and incubated on Columbia agar supplemented with blood at 37°C. The growth requirements of the strains were examined in aerob condition and in an atmosphere containing 5% CO_2. The growth of the 24 percent of the strains was more abundant with increased CO_2 concentration.

The identification of the *R. anatipestifer* suspect isolates was confirmed by a species-specific polymerase chain reaction (PCR), then the phenotypic properties of the strains were examined in detail. Altogether, we isolated 139 strains from geese, 24 strains from ducks, and 3 strains from turkeys that were added to our existing strain collection. The *R. anatipestifer* strains showed low variability in the biochemical tests. The majority of the strains were negative in the ureum tests, and did not ferment glucose and saccharose, although some strains gave positive or uncertain results.

A total of 185 *R. anatipestifer* strains isolated from geese and ducks between 2000 and 2015 were tested against 13 widely used antibiotics (ampicillin, doxycycline, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, penicillin, spectinomycin, streptomycin, sulphamethoxazole–trimethoprim, sulphonamide compounds, and tetracycline) by the Kirby-Bauer disk diffusion method. The highest resistance rates were observed for flumequine, tetracycline, erythromycin and streptomycin (94%, 91.4%, 75.1% and 71.4% resistance, respectively), and thus the use of these antibiotics is not recommended against *R. anatipestifer* infection. The majority of the strains were susceptible to florfenicol (97.9%), ampicillin (95.1%), penicillin (93%), sulphamethoxazole–trimethoprim (92.4%), and spectinomycin (86.5%). The resistance patterns showed some variation depending on the geographical origin of the strains. The overall resistance rate of the strains has increased in the recent years that is in harmony with the generally reported trend of the spread of antibiotic resistance in bacteria. These findings underline the importance of applying the antibiotics properly, based on an accurate diagnosis and antimicrobial susceptibility testing.

Plasmid profile was defined on 16 selected multidrug resistant (MDR) *R. anatipestifer* isolates that were resistant to seven or more antibiotics. Plasmids were extracted by the alkaline lysis method (Qiagen Plasmid Mini Kit, Qiagen, Germany). Electrophoresis of the DNA was carried out on a 0.8% agarose gel. Thirteen (81.3%) isolates were found to possess plasmid bands. The sizes of the plasmids were approximately 2900 bp (46.2%), 4700 bp (30.7%), and 5700 bp (23,1%), respectively. All of the strains had a single plasmid band. The presence of resistance genes was determined using PCR. PCR conditions were optimized for the amplification of *aac(6')-Ib* and, *aph(3')-IIa* (aminoglycosides), *tet*B, *tet*M (tetracyclines), *erm*F and *mph*

(macrolides). Fifteen isolates (93.8%) were positive for *aac(6')-Ib* that located on plasmid. The aminoglycoside phosphotransferase *aph(3')-IIa* is primarily inactivating kanamycin and neomycin. In this study, five isolates were positive for this locus. The *erm*F gene was detected in 11 isolates (68.8%), while the *mph* gene was carried only by one strain.

Serotyping is currently the most widely practiced method for classifying *R. anatipestifer* isolates since immunity induced by *R. anatipestifer* vaccines appears to be serotype-specific with no cross-protection. The *R. anatipestifer* strains were serotyped using the agar-gel precipitation test. Antisera against representative strains of serotypes 1, 2, 4, 7, and 10 were raised in 16-week-old specific-pathogen-free chickens. These serotypes were supposed to be the most frequent ones in our geographical region. Heat-stable antigens were prepared from 182 field strains and used the test. 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. The rather high incidence of serotype 1,7, a type with multiple antigenic factors, seems to represent a new antigenic combination, and its potential to cause disease or induce protection needs further clarification.

For further analysis of phylogenetic variability, a *rpo*B PCR has been established. The *rpo*B gene is encoding the ß subunit of the bacterial RNA polymerase, and seems to be a useful target to make distinction between the strains to facilitate epidemiological studies.

ERIC-PCR (specific to conservative, repetitive sequences in the bacterial genome) was developed, standardized, and then used to examine the genomic diversity of 166 field isolates of *R. anatipestifer* collected from geese and ducks. The field strains and five reference strains showed 17 distinct patterns consisting of five to 12 bands ranging from approximately150–1800 bp. The majority of the strains belonged to two closely related ERIC-PCR types (A and B), while the other types represented only a few isolates each. There was no association between ERIC-PCR type and host species, place, or year of isolation, however, the ERIC-PCR pattern correlated with serotype for most isolates (Table 1).

Table 1: Distribution of *R. anatipestifer* serotypes and ERIC-PCR types among strains from geese and ducks in Hungary.

Host	Serotype	No. of positive isolates (%)	ERIC-PCR type
All	1	107 (64.5)	A, D, G, L, M, O
	7	8 (4.8)	C
	1,7	28 (16.9)	B
	2	12 (7.2)	F,N
	4	6 (3.6)	E, H, P, Q
	10	2 (1.2)	J, K
	13	1 (0.6)	I
	17	1 (0.6)	I
	18	1 (0.6)	I
	Total no.	166 (100)	

The majority of serotype 1 strains (101/107, 94.4%) belonged to ERIC-PCR type A while the remaining six strains represented five different ERIC-PCR types (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 ERIC-PCR revealing two to four patterns within each serotype. The strains from geese proved to be more diverse than the strains from ducks: twelve ERIC-PCR patterns were found only in strains from this species. These results indicate that ERIC-PCR may be a suitable technique for the molecular identification of RA serotypes, and the detection of subtypes within certain serotypes may aid further epidemiological investigations.

We performed a couple of artificial infection studies to learn more about the pathogenesis of these bacterium species and the possible interaction between them. However, neither mono-infection nor any combinations of these infectious agents resulted in evaluable degree of lesions underlining the multifactorial nature of these diseases. It means that they need predisposition factors that are usually present in various combinations under field conditions.

General comments

The data generated by this research may be useful during epidemiological investigations, and the further characterization of these pathogens utilising our results might increase the possibility of selecting more efficacious vaccine candidates against these economically important diseases.

Two PhD theses were prepared based on the results of this project one of which was successfully defended, and the other is in the process of final preparation.