

University of Veterinary Science
Aladár Aujeszky Doctoral School of Veterinary Science

Comparative pheno- and genotypic analysis of
Bordetella avium* and *Ornithobacterium rhinotracheale
strains from wild and domesticated birds

Brief summary of Ph.D. thesis

Réka Szabó

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Supervisor:

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Tibor Magyar, DSc
Institute for Veterinary Medical Research,
Centre for Agricultural Research,
Hungarian Academy of Sciences

Introduction

Respiratory tract infections are causing considerable economic losses in the poultry industry in many areas of the world. Fungi, viruses and bacteria are indicated as causes of respiratory diseases, either alone, or in combination with other microorganisms. Non-infectious factors (e. g. climatic conditions, high levels of ammonia, high stocking density) contribute to the severity of the disease.

Clinical signs caused by *B. avium* and *O. rhinotracheale* are usually mild. *O. rhinotracheale* infection in broilers is most common in 2-8 week-old birds, while it is most prevalent in breeders by the height of egg production. Clinical signs include nasal discharge, coughing, sneezing, and they usually disappear after 5-7 weeks post infection. Mild clinical signs are sometimes followed by dyspnoea, prostration and sinusitis. Bordetellosis is also characterized by mild respiratory signs. Sneezing, coughing, clear nasal and ocular discharge are the most common clinical signs associated with infection. The airways become partially occluded with mucoid exudate, leading to dyspnoea and sometimes suffocation. In the field, other infections and management problems can aggravate the respiratory disease. Heavy economic loss due to poor performance, drops in egg production, decreased hatchability, increased mortality, increased medication costs and increased condemnation rates make these two pathogens significant.

The aim of our study was to isolate *B. avium* and *O. rhinotracheale* from poultry farms in Hungary and use the established strain collection to examine the diversity of strains. Firstly, we characterized the isolates by determining their phenotypical properties. We aimed to investigate whether the possible variety in these features could be interpreted as signs of host adaptation. We further characterized a smaller number of strains by molecular methods.

During the course of our work we planned to establish groups of *B. avium* and *O. rhinotracheale* characterized by their differences in various properties. We also aimed to find a molecular method suitable for the examination of the diversity of isolates and to be used in epidemiological studies.

Materials and Methods

Strains

64 *O. rhinotracheale* and 19 *B. avium* strains, of various host origin, isolated in Hungary or other countries, were included in this study. Strains were either new isolates or strains from the strain collection of IVMR CAR HAS. Strains from both sources were identified by colony morphology and species-specific PCR reactions.

Phenotypic characterization of *B. avium* and *O. rhinotracheale*

Biochemical tests

Isolates were identified and characterized with conventional biochemical tests, including the production of indole, urease, nitrate reductase, and the fermentation of arabinose, dulcitol, glucose, lactose, maltose, sucrose, and sorbitol. Tubes were supplemented with 2% SPF inactivated chicken sera in order to support the growth of *O. rhinotracheale*. Tests were incubated for 4 days.

Growth requirements assay

Growth requirements of the *B. avium* and *O. rhinotracheale* strains were examined at 31, 37 and 41 °C, on Columbia and tryptic soy agar and supplemented with 5% sheep blood. All media were incubated for 48 h.

Haemolytic assay

Although both *B. avium* and *O. rhinotracheale* were originally described as non-haemolytic bacteria, *O. rhinotracheale* isolates showing signs of β -haemolysis were recently reported. Thus, haemolytic properties of strains were examined on Columbia agar supplemented with 5% sheep blood, after 48 h in room temperature following incubation at 37°C for 48 h.

Haemagglutination tests

The adhesion of the isolates was examined by slide haemagglutination tests using red blood cells (RBCs) from sheep, horse, rabbit, cattle, duck and chicken. RBCs were washed in saline, then suspensions containing 10% of RBCs were made with PBS. 20 μ l aliquots of the RBC suspensions were mixed gently with a fresh colony of bacteria on glass slides. The results of the reactions were detected after one minute and evaluated on a five-point scale (0-4).

Serotyping

The serotype of the *O. rhinotracheale* strains was determined in agar gel precipitation test. Antisera against representative strains of the most prevalent serotypes (A–E) were raised in 4-week-old specific-pathogen-free chickens. The field isolates were grown on Columbia agar

enriched with 5% sheep blood, at 37 °C for 48 h. The bacteria were harvested with phosphate-buffered saline, and the turbidity of the suspension was adjusted to 3 McFarland standards. Heat-stable antigens were prepared and were used as the antigens in AGP. The AGP test was incubated in a humid chamber and was evaluated after 24, 48 and 72 h.

Antimicrobial susceptibility tests

19 *B. avium* and 50 *O. rhinotracheale* strains were selected for the antimicrobial susceptibility tests by the disk diffusion method. Colonies grown on Columbia agar plates supplemented with 5 % sheep blood were suspended in Mueller-Hinton broth. Turbidity of the suspension was adjusted to 0.5 McFarland standards. The suspension was streaked on the surface of Mueller Hinton agar enriched with 5 % sheep blood, and antibiotic discs were applied. Results were interpreted based on the breakpoints given either in CLSI document M31-S1 or in CLSI document M100-S21.

The MICs of amoxicillin, doxycycline and erythromycin were determined by broth microdilution, in order to quantify the level of resistance. For the determination of MIC, twofold dilutions of each antibiotic were prepared with final concentrations ranging from 0.03 µg/ml to 64 µg/ml. Wells were inoculated with bacterial suspension of log phase cultures in Mueller-Hinton broth. Plates were read after 24 (*B. avium*) or 48 (*O. rhinotracheale*) hours of incubation at 37 °C.

Genotypic characterization of *B. avium* and *O. rhinotracheale*

Polymerase chain reactions

Primers for molecular identification, amplification of the partial 16S rRNA gene and the ERIC-PCR and RAPD assays were chosen from the literature. DNA templates were prepared by the boiling method from fresh colonies. All amplification reactions were performed in an ESCO Swift Mini thermocycler. The amplified DNA products were resolved by 1.5% (wt/vol) SeaKem agarose with 1×TBE buffer by electrophoresis (9 V/cm). Detection and documentation of the PCR products Kodak Gel Logic 212 Imaging System was used under UV light.

Analysis of ERIC-PCR and RAPD assay results

The presence (1) or absence (0) of different sized bands after each amplification reaction was used to generate a binary matrix. The Dice coefficient was used to estimate the genetic similarity of the *O. rhinotracheale* isolates, and a dendrogram was created with the unweighted pair group method with arithmetic average (UPGMA) method. The robustness of the dendrogram topology was assessed with 100 bootstrap replicates in PyElph version 1.4.

Partial 16S rRNA gene sequence analysis

The partial 16S rDNA nucleotide sequences of *B. avium* and *O. rhinotracheale* strains were determined at a specific region of the gene. The sequencing primers were universal primers 27F and 1492R. Purification of PCR products and sequencing reaction were performed by Macrogen Europe Ltd with the traditional Sanger dideoxy-nucleotide method. Chromatograms were evaluated with Chromas LITE 2.01, sequences were aligned by BioEdit 7.1.3.0 software.

Sequences of our strains were compared with *B. avium* and *O. rhinotracheale* sequences from the GenBank by BioEdit software. The similarities of the nucleic acid sequences were calculated by the Clustal W algorithm. Following the alignment, phylogenetic dendrogram was constructed using the MEGA 6.06 software, with the Neighbor-Joining method with the Jukes-Cantor correction rate. The resultant tree topologies were evaluated by bootstrap analyses with 500 random samplings.

Multi-locus sequence typing

MLST typing of nine *B. avium* strains was carried out by analysing partial sequences of seven housekeeping genes (*adk*, *fumC*, *glyA*, *tyrB*, *icd*, *pepA*, *pgm*). As the primers designed for the typing of other *Bordetellae* did not yield PCR products suitable for further sequence analysis, new primers were designed using the Primer3Plus software for the amplification of the designated gene sequences.

The nucleotide sequences of the PCR products were determined as described above and were compared to those in the database of the *Bordetella* Multi-Locus Sequence Typing website.

Results

Strain identification

Strain identification confirmed the isolation of 13 *O. rhinotracheale* strains from turkeys, three from chickens, two from pigeons one from goshawk and one from a sparrowhawk as well as six *B. avium* strains originating from turkeys. The isolation rate was 6% for *O. rhinotracheale* and 1.8% for *B. avium*. Isolates in the strain collection were positive in the identification tests as well. The size of amplified PCR fragments of the species-specific PCRs were 784 bp for *O. rhinotracheale* and 524 bp amplicon for *B. avium*.

Phenotypic characterization of *B. avium* and *O. rhinotracheale*

Biochemical tests

All the *B. avium* strains gave negative results in biochemical tests. *O. rhinotracheale* isolates gave negative results in the indole and nitrate reduction test, and were positive in the urease test. None of the isolates fermented arabinose, dulcitol, or sorbitol. 56,3% of the *O. rhinotracheale* strains fermented glucose, 65,6% used lactose, 50% used maltose and 21,9% fermented sucrose.

Growth requirements assay

The *B. avium* strains grew uniformly on all four media at all tested temperatures. All *O. rhinotracheale* isolates grew at 37 °C on all four agars. No *O. rhinotracheale* strains grew at 31 °C on the agars not supplemented by blood. 81.8% grew at this temperature on the media containing blood. All *O. rhinotracheale* strains grew at 41 °C on the agars supplemented by blood. 45.5% of the strains did not grow on the media without blood.

Haemolytic assay

None of the *B. avium* isolates showed signs of haemolysis. 39% of the *O. rhinotracheale* were non-haemolytic and 61% showed signs of β -haemolysis after 48 h in room temperature following incubation at 37°C for 48 h.

Haemagglutination tests

The *B. avium* strains showed better haemagglutinating activity than the *O. rhinotracheale* isolates. The *B. avium* strains gave stronger reactions and the lack of agglutination was rarer. The *B. avium* strains did not react with rabbit RBCs, but a large percent of the strains agglutinated the other mammalian RBCs (horse and sheep: 78.9%; cattle: 52.6%) Only one and two strains showed no reaction with duck and chicken RBCs, respectively, and the strongest (level 3) reactions were observed with avian RBCs. Horse, cattle and sheep RBCs were not agglutinated by any of the *O. rhinotracheale* strains. Chicken RBCs were agglutinated by four strains, duck RBCs by nine and rabbit RBCs by nine isolates.

Serotyping

Serotype A was the most prevalent among the field isolates (49 strains; 83%). Two isolates (3.4%) belonged to serotype B, and five isolates (8.5%) to serotype D. Three isolates (5.1%) could not be typed with antisera raised against serotypes A–E.

Antimicrobial susceptibility tests

All *O. rhinotracheale* strains were susceptible to chloramphenicol and spectinomycin and most of them to tilmicosin. A high percent of the strains were resistant to gentamicin, nalidixic acid, sulfamethoxazole-trimethoprim, and polymyxin B and sulphonamids were also among the less effective antibiotics. The strains isolated from goshawk, sparrowhawk and pigeons were susceptible to a higher number of antibiotics than strains isolated from poultry.

The MIC values against *O. rhinotracheale* strains ranged from 0.12 µg/ml to 32 µg/ml in case of amoxicillin and erythromycin, and 0.6 µg/ml to 32 µg/ml for doxycycline. MICs were in the lower range against isolates from wild birds and strains from backyard chickens.

Ciprofloxacin, chloramphenicol, erythromycin, sulfamethoxazole-trimethoprim and oxytetracycline proved also effective although some of the strains showed only moderate susceptibility.

MICs against *B. avium* isolates 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. MICs of all three antibiotics against German strains were in the higher range of the overall values

Genotypic characterization of *B. avium* and *O. rhinotracheale*

ERIC-PCR

Thirteen pattern types were identified among the *O. rhinotracheale* strains included in the study. The most common pattern (type 1) consisted of 29 isolates. ERIC types did not correlate with either the place or time of isolation. However, the ERIC pattern and the host of origin of the isolate showed some correlation: type 2, type 3, and the majority of type 1 (26 strains) were made up of isolates from turkeys. The isolates originating from birds of prey also belonged to type 1. The isolates from chickens were highly variable: the nine chicken isolates belonged to eight ERIC types.

RAPD assays

The RAPD assay with the M13 primer assigned the *O. rhinotracheale* isolates to 10 distinct fingerprint patterns. 42 field isolates and the type strain for serotype A belonged to type 1. Type 2 and 3 consisted of two isolates each, whereas seven isolates showed unique RAPD patterns. No correspondence between the M13 RAPD patterns and either the isolation date, host, geographic origin, or serotype was established.

The OPG11 assay separated the field isolates into two main types. Two isolates belonged to both type 3 and 8 and four field isolates had unique patterns.

The RAPD assay with the OPH19 primer also assigned the isolates to two main types. 57.1% of the isolates belonged to type 1 and 17.5% belonged to type 2. Type 4 was made up by three strains originating from chickens and type 6, differing only by one line from type 2, contained the type strains for serotype D and E. Two strains had unique patterns with this method.

Partial 16S rRNA gene sequence analysis

A *B. avium* strains showed 100% similarity to each other and sequences from GenBank originating from birds. 16S rRNA sequence analysis grouped our 42 *O. rhinotracheale* isolates into two clusters. The majority of the field isolates (37 strains) showed 100% similarity to sequences from GenBank and the type strains of serotypes A, B, and E. Four of the five strains with unique sequences were isolated from chickens and one from a turkey.

Multi-locus sequence typing

As the primers designed for the typing of other *Bordetellae* did not yield PCR products suitable for further sequence analysis, new primers were designed. The new PCR products were fit for sequence analysis. The analysis of the partial sequence of genes *icd*, *pepA* és *pgm* revealed 100% similarity among our isolates and the only other *B. avium* strain in the database, strain 197N. A G→A point mutation in base 119 in the sequence of the *adk* gene was identified in three strains (Ba01, Ba08, Ba18). Similarly, two T→C point mutations in bases 4 and 40 in strain Ba18 in the sequence of the *fumC* gene, a T→C point mutations in base 83 in strain Ba18 in the sequence of the *glyA* gene, a C→T point mutations in base 245 in strains Ba01, Ba07, Ba08 and Ba18 in the sequence of the *tyrB* gene were identified. The other strains showed 100% homology with the respective gene sequences of strain 197N. Thus, strains Ba13, Ba14, Ba15 and Ba16 belong to ST76, the same sequence type as strain 197N. Strains Ba01 and Ba08, Ba07 and BA09 as well as strain Ba18 form three new sequence types.

Conclusions

Due to the variability of *O. rhinotracheale* isolates in carbohydrate fermentation tests, we did not find them suitable for the identification of this bacterium. Results of the indole, nitrate and urea tests, on the other hand, were consistent enough to be part of our strain identification protocol.

Broad temperature range of growth for the organisms could be explained by the fact that temperatures in the airways of birds are not uniform: the temperature is lower in the upper respiratory tract and it gradually gets higher near the lungs.

A haemolysin-like protein is considered to be responsible for the haemolytic properties of *O. rhinotracheale*, its role as a virulence factor, however, is not entirely clear, and further studies are required to fully understand it.

Haemagglutinating activity is an indicative of adhesion in a number of bacterial species, and virulent pathogens generally have stronger adhesive and haemagglutinating capacities. Filamentous hemagglutinin, the adhesin of *B. avium*, is a well-known virulence factor. It can be hypothesised that an adhesin-like structure is responsible for the haemagglutinating activity of *O. rhinotracheale*. Differences in the haemagglutinating activity suggest diversity of the structures responsible for haemagglutination, but further characterization is needed to understand these phenomena.

In harmony with data in the literature, serotype A was found to be the most prevalent among Hungarian *O. rhinotracheale* isolates. Serotyping is the traditional method of distinguishing *O. rhinotracheale* isolates, however, its value is dubious, especially with the recognition of vaccines that are cross-protective against several serotypes, and the lack of difference in the virulence of different serotypes.

Consistent with general expectations, the poultry industry aspire to limit the use of antibiotics. Bacterial diseases, however, are still causing considerable economic losses, thus, antibiotics cannot be completely evaded. At the same time, overuse of antibiotics in veterinary medicine has been suggested to contribute to the selection of resistant pathogens. These facts and the lack of uniform *in vitro* susceptibility of these pathogens underline the importance of the adequate and prudent application of antibiotic therapy based on proper antimicrobial susceptibility testing.

The diversity seen in phenotypical properties of *O. rhinotracheale* and *B. avium* could correspond with unknown or not well-characterized virulence factors, and could be the basis of further research in this area. Furthermore, these results could provide useful information concerning the differences in the spread, prevalence and survival in the environment of these pathogens. However, diversity of these properties cannot be seen as a sign of host adaptation.

Examination of genetic diversity – in contrast to phenotypic properties – provide more accurate information on the phylogenetic relationships among the isolates, and are not influenced by the fastidiousness of the organisms. Whole genome analysis provides the most accurate results, however, it is also the most expensive method. MLST analysis only requires the sequencing of designated genes, and is considered less costly than the former procedure. Rep-PCRs and RAPD analyses are more cost-efficient methods suitable to be used in small laboratories. With sufficient reproducibility in the laboratory, they may be useful as the first step of epidemiological studies, providing fast results that could be backed up by further methods.

The analysis of 16S rRNA gene sequences is routinely used to differentiate organisms at the genus level across all major phyla of bacteria. The sequence variability in this region often allows comparison to be made at the species and subspecies levels. It is routinely used to examine the genetic diversity of *O. rhinotracheale*, however, due to the high levels of similarity in this gene, its discriminatory power is limited when it is used to study the intra-species variability of this pathogen.

In this study, ERIC-PCR proved the most powerful technique for detecting intra-species genetic diversity in *O. rhinotracheale* isolates and may therefore be useful in epidemiological studies of *O. rhinotracheale* outbreaks.

In accordance with data in the literature, the results of both the analysis of 16S rRNA gene sequences and the MLST data suggest that *B. avium* isolates have very limited genetic variability. Our results suggest that primers and PCRs developed for the analysis of *Bordetellae* are not suitable for the MLST analysis of *B. avium*. It could be hypothesised that the MLST data of strain 197N are the result of an *in silico* analysis of its whole genome sequence.

Our new *B. avium* sequence types did not correspond with either the time, place or host origin of the strains. Further additions of *B. avium* data to the database is needed for a better understanding of these sequence types.

New scientific results

1. We first performed a complex, comparative pheno- and genotypic examinations of *B. avium* and *O. rhinotracheale* strains from Hungary.
2. We found Hungarian *B. avium* strains to be uniform and Hungarian *O. rhinotracheale* isolates to be variable in phenotyping tests.
3. We revealed that the variability of *O. rhinotracheale* strains in pheno- and genotyping methods cannot be regarded as a sign of host adaptation.
4. We found serotype A to be the most prevalent among Hungarian isolates of *O. rhinotracheale*. We also described serotype B and D strains among Hungarian isolates. Three isolates could not be typed with antisera against serotypes A–E.
5. ERIC-PCR and the RAPD assay with M13 primer were the most discriminatory methods for investigating the genetic diversity of Hungarian *O. rhinotracheale* isolates, and are suitable methods for epidemiological studies.
6. The *O. rhinotracheale* strains isolated from chickens were found to be more heterogeneous on molecular typing methods than the isolates recovered from turkeys.
7. New PCRs were developed for the analysis of *B. avium* strains for the *Bordetella* MLST. Three new sequence types were identified by MLST analysis of *B. avium* strains. Sequence data of nine *B. avium* strains were analysed.

Publications based on the results of the PhD dissertation

Research papers in peer-reviewed journals

Szabó R., Magyar T.: **A baromfi *Ornithobacterium rhinotracheale* okozta megbetegedése. Irodalmi áttekintés**, Magyar Állatorv. Lapja, 136. 589–597, 2014.

IF: 0,185

Szabó R., Wehmann E., Magyar T.: **Antimicrobial susceptibility of *Bordetella avium* and *Ornithobacterium rhinotracheale* strains from wild and domesticated birds in Hungary**, Acta Vet. Hung. 63. 413–424, 2015.

IF: 0,646

Szabó R., Wehmann E., Makrai L., Nemes Cs., Gyuris É., Thuma Á., Magyar T.: **Characterization of *Ornithobacterium rhinotracheale* field isolates from Hungary**, Avian Pathol. DOI: 10.1080/03079457.2017.1321104.

IF: 1,336

Publications not related to the PhD dissertation

Gyuranecz M., Dénes B., Hornok S., Kovács P., Horváth G., Jurkovich V., Varga T., Hajtós I., Szabó R., Magyar T., Vass N., Hofmann-Lehmann R., Erdélyi K., Bhide M., Dán Á.: **Prevalence of *Coxiella burnetii* in Hungary: screening of dairy cows, sheep, commercial milk samples, and ticks**, Vector Borne Zoonotic Dis., 12. 650–653, 2012.

IF: 2,277

Conference presentations

- Gyuris É., Szabó R., Wehmann E., Magyar T.: Antimicrobial susceptibility of *Riemerella anatipestifer* and *Ornithobacterium rhinotracheale* strains from wild and domesticated birds in Hungary (poszter) [Konferencia absztraktfüzet p. 45.] International Pasteurellaceae Conference 2014. Prato, 2014.05.13–16.
- Szabó R., Gyuris É., Wehmann E., Magyar T.: Antimicrobial susceptibility of *Bordetella avium*, *Ornithobacterium rhinotracheale* and *Riemerella anatipestifer* strains from wild and domesticated birds in Hungary (poszter) [Konferencia absztraktfüzet p. 27.] Med-Vet-Net Association International Scientific Conference. Lyngby, 2013.06.24–25.
- Szabó R., Gyuris É., Wehmann E., Magyar T.: Hazai baromfifajokból és vadmadaraktól izolált *Bordetella avium*, *Ornithobacterium rhinotracheale* és *Riemerella anatipestifer* törzsek antibiotikum-érzékenységének vizsgálata (poszter) [Szerk.: Janda T., ISBN:978-963-8351-41-8, p. 251–253.] II. ATK Tudományos Nap, Velünk élő tudomány. Martonvásár, 2013.11.08.
- Szabó R., Wehmann E., Magyar T.: Isolation and characterisation of *Ornithobacterium rhinotracheale* from wild and domesticated birds in Hungary (előadás) [Acta Microbiol. Imm. Hung. Supplement 58:219] 16th International Congress of the Hungarian Society for Microbiology. Budapest, 2011.07.20–22.

Academic reports

- Szabó R., Wehmann E., Magyar T.: *Ornithobacterium rhinotracheale* törzsek jellemzése. Budapest, 2016. 01. 24.
- Szabó R., Wehmann E., Magyar T.: *Ornithobacterium rhinotracheale* törzsek jellemzése genotipizáló PCR módszerekkel. Budapest, 2016. 01. 26.
- Szabó R., Magyar T.: Hazai baromfifajokból és vadmadaraktól izolált *Bordetella avium* és *Ornithobacterium rhinotracheale* törzsek antibiotikum-érzékenységének vizsgálata. Budapest, 2015. 01. 27.
- Szabó R., Wehmann E., Magyar T.: *Ornithobacterium rhinotracheale* törzsek izolálása és jellemzése. Budapest, 2011. 01. 25.

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