PhD dissertation thesis

Examination of the pathogenesis of low pathogenic avian influenza virus in poultry

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INTRODUCTION AND OBJECTIVES

In recent years, the regular appearance of the highly pathogenic avian influenza virus (HPAIV) has put the poultry sector in a difficult situation worldwide, including Hungary. In the past, HPAIVs (H5 and H7) introduced by wild birds have mutated from low pathogenic avian influenza viruses (LPAIV) to HPAIV in poultry. Gene reassortment among LPAIVs with high genetic diversity in wild birds has contributed significantly to the diversity of viruses emerging in poultry flocks. For this reason, LPAIVs have been a focus of research over the last 20 years.

Based on its pathogenicity, the H9N2 subtype belongs to the LPAIVs, so the infection may be asymptomatic, but may also result in milder to more severe clinical signs or even death. The H9N2 subtype is noteworthy because it illustrates the potential for the emergence of a human transmissible avian virus without a mammalian vector species (e.g. pig). H9N2 subtype is detectable in a small proportion of human cases caused by AIVs. Its zoonotic significance is mainly due to the fact that in the case of AIVs causing fatal human infections (either H5N1/H5N6 or H7N9 subtypes), the so-called internal genes are at least partly derived from the H9N2 AIV.

H9N2 infections in poultry do not usually occur alone, but in co-infection with other respiratory or immuno-suppressive pathogens (e.g. chicken infectious bronchitis virus, metapneumoviruses, Mycoplasmas, *Ornithobacterium* rhinotracheale), causing clinical signs, mortality and significant economic losses.

The importance and relevance of my research is to gain a better understanding of the pathogenicity of H9N2 LPAIVs, which is important from both zoonotic and animal health (economic) perspectives. This knowledge will contribute to the development of more effective control methods (e.g. vaccines)**.**

The overall aim of my work was to gain a better understanding of the pathogenicity of H9N2 avian influenza viruses, their pathogenesis in infected broiler chickens.

Specific objectives were:

- to assess the pathogenicity, tissue distribution and disease development characteristics of H9N2 virus strains of different geographical origin, and to describe the lesions they cause
- with a given H9N2 strain, to test the objectives of the previous point for different infection routes
- in the case of IBV co-infection, to investigate the course of H9N2 infection, as in practice respiratory pathogens are most often mixed infections and IBV is one of the most common pathogen of the panel.
- investigating H9N2 virus shedding during disease progression
- development of pathological methodology for a more accurate/deeper understanding of H9N2 lesions
- interpretation of results obtained by combining several diagnostic methods: clinical and pathological examination, histopathology, immunohistochemistry, PCR
- developing an infection model to assess the efficacy of vaccination.

MATERIALS AND METHODS

One hundred, 21-day-old broiler chickens (Ross 308 hybrid) included in the study were housed in isolators. The number of broilers (18 birds/infected group) were kept to the minimum that could still be evaluated and a single control group of 10 birds was used in addition to several infected groups at the same time.

Sampling days 5 and 11 were chosen to investigate the acute and chronic phases of disease based on the literature.

We used A/chicken/Middle East/8616/2016 ('A'), A/chicken/Middle East/4531/2016 ('B'), and A/chicken/North Africa/2021/2016 ('C') H9N2 strains, isolated from natural infections and belonging to the G1-lineage. Broiler chickens were infected with 10^8 EID_{50} viruses in 0.2 ml volume for each infection route. The control group was treated with 0.2 ml PBS intranasally & intratracheally (IN&IT).

The pathogenicity of the virus strains was investigated by (a) clinical symptoms, (b) pathological lesions, (c) general histopathology, (d) immunohistochemistry, (e) real-time PCR. To achieve our objectives, we used several study designs: comparing strains from different geographical origins under the same route of infection, then examining tissue tropism of the same strain under different routes of infection (IN&IT versus

IV), and examining tissue tropism of H9N2 virus under concurrent IN&IT infectious bronchitis (IBV) co-infection.

Birds were observed daily to record clinical signs and mortality during the observation period (D0-D11). Oronasal and cloacal swabs were collected from live birds on days 5 and 11 from each group and at each observation day from the strain A infected group. Additional swab samples were collected from air sacs for PCR at necropsy. Trachea, lungs, kidneys, spleen, pancreas and brain samples were collected for histopathology, IHC and PCR tests. Birds died were also sampled in the same way.

During the IHC studies, *the indirect two-step immunoreaction* used for HPAIV *was adapted to H9N2 virus* by modifying the protocol.

We also introduced a new method in the statistical evaluation: in addition to the tests traditionally used in the literature, we used the Continuation Ratio mixed effect (CR) model and the Cumulative link model (also known as the proportional odds model). The use of these is advantageous not only because they are specifically optimised for the analysis of ordinal data, but also because they are more appropriate for modelling of data from repeated measures (not independent observations).

RESULTS

1. General pathology

Clinical signs

In general, respiratory symptoms, sniffling, gurgling, sneezing, wheezing and occasionally dyspnoe were observed in most infected birds.

The disease progression of the groups infected with Middle Eastern strains was almost identical in terms of the severity of clinical symptoms. The North African strain caused more pronounced clinical symptoms, particularly during the acute phase of disease development, which subsided to the same extent as the other strains during the acute phase.

Mortality

One chicken (5.5%) in group A-Middle East 1 and four birds (22%) in group C-North Africa died on days 4-6 post infection (dpi), while no mortality occurred in group B-Middle East 2 (0%). Mortality rates did not show significant differences (Fisher exact test $p = 0.113$).

Gross pathology

Macroscopic lesions in the airways were noted, including reddish or purple-red trachea mucosa, vascularisation of the air sacs and congestion in the lungs, with occasional fibrin plug at the bifurcation.

During necropsy of the birds exterminated at dpi 5 from groups of strain 'A-Middle East 1' and 'B-Middle East 2', we observed flushed trachea and slightly infiltrated air sacs. In the dead birds, we observed a dark red tracheal mucosa along the entire length of the trachea, a veined and opalescent infiltration in the thoracic air sacs, slight infiltration in the abdominal air sacs, and a dark red, sometimes oedematous lungs. In strain C-North Africa, we observed fibrin plug in the trachea of dead birds and fibrin precipitation and congestion in the large airways. In severe cases, venous and opalescent infiltration of the abdominal air sacs and fibrin plug of the intrapulmonary airways were observed. In the euthanized birds, the above described processes were observed to a lesser extent. In 11 dpi, the tracheal and pulmonary lesions disappeared at necropsy, with only veined and opalescent infiltration in the air sacs found in the groups.

Histopathological results

Lesions were observed in the trachea, lungs and kidneys, while no lesions were found in the spleen, pancreas and brain.

The tracheal lesions were characterized by lymphocytic inflammation. The severity of lesions was significantly reduced on 11 dpi compared to 5 dpi (OR (dpi11/dpi5): 262), and significant differences were detected between the 'A-Middle

East 1' and 'C-North Africa' strains (OR (A-Middle East 1/C-North Africa): 8.36, p = 0.0116).

Pulmonary lesions included interstitial pneumonia and bronchopneumonia/bronchitis. The severity of lesions was significantly reduced at 11 dpi compared to 5 dpi (OR (dpi11/dpi5): 281, p <0.0001), but no significant difference was detected between the three strains.

Pathological changes in the kidneys were only observed in samples taken at 5 dpi. The lesions were mainly characterized by tubulonephrosis and glomerulonephritis, with some cases of mild lymphocytic inflammation observed only at 5 dpi. Significant differences were found only for tubulonephrosis in "A-Middle-East 1." and "C-North Africa" strain (OR (A-Middle East 1/ C-North Africa): 31.95, p = 0.0026), and "B-Middle East 2." and "C-North Africa" (OR (B-Middle East 2/ C-North Africa): 248.25, p = 0.0002).

Immunohistochemical results

Viral antigen was identified in 30-50% of trachea, lungs and kidneys samples from infected birds. Positive IHC results were only found in samples taken at 5 dpi from infected chickens, i.e. all control animals were negative on both sampling days and samples taken at 11 dpi. Positive IHC staining appears as a highly contrasting, clearly visible dark red precipitation in the nucleus of epithelial cells deposited in the

trachea, in the alveolar epithelial cells of the lungs and in the cytoplasm of the renal tubular epithelium. In the kidneys, a higher proportion of whole cell staining was observed, whereas in the airways, staining of the nuclei was characteristic. Viral antigen was occasionally found in the spleen in 8% of chickens in all three infected groups. No viral antigen was detected in the pancreas and brain.

PCR results

After IN&IT infection, the virus can be detected from the oronasal swab on 2 dpi. Viral shedding from the upper respiratory tract decreases dramatically after 4 dpi, becoming almost minimal by 8 dpi and negative by 10 dpi. Cloacal shedding was detected from 4 dpi with a low DNA copy number, which increased slightly as the observation period progressed and then ceased from 9 dpi.

Comparison of results obtained with different diagnostic methods

Spearman's rank correlation coefficients (rs) were calculated to compare pathology and IHC and pathology and PCR in trachea, lungs and kidneys. We found a statistically significant ($p < 0.05$) but moderate positive correlation between histopathology and IHC results ($rs = 0.37$ for trachea and lungs; 0.47 for kidneys). Similarly, we found a significant and moderate but negative

correlation between pathology scores and PCR Ct scores (rs = [-0.39] for kidneys, [-0.48] for lungs and [-0.6] for trachea).

2. **Investigating the pathogenesis of strains from different geographical origins**

Clinical signs

Significant differences were observed between strain 'A - Middle East 1' and 'C - North Africa' (p= 0.011), and between strain 'B - Middle East 2' and 'C - North Africa' (p= 0.0055). However, statistical analysis showed no significant difference between strain 'A-Middle East 1' and 'B-Middle East 2' (p= 0.8078). Strain 'C-North Africa' caused the most severe symptoms. The acute (5 dpi) and chronic (11 dpi) phases of disease development also differed markedly in clinical symptoms.

Gross pathology

No significant differences were found between the three strains in terms of trachea lesions.

The air sacs analysis revealed significant differences between the strain 'A-Middle East1' and 'B-Middle East2.'(OR (A/B): 10.34, p = 0.0199) and between the 'A-Middle East1' and 'C-North Africa' strain (OR (A/C): 33.97, p = 0.0003). and 'C-North Africa' strain showed more severe air sac lesions, decreasing to 11 dpi.

Histopathology

When analysing the histopathological lesions, it was not possible to establish a clear ordering between strains.

Immunohistochemistry

Significant differences were found between strains for lungs and kidneys tropism ($p = 0.0065$ and 0.0008). Lung samples from birds infected with strain "C-North Africa" showed an upward shift from strain "B-Middle East 2." ($p = 0.0045$).

In terms of kidney samples, the "C-North Africa" strain was found to be more virulent than strain"A-Middle East 1." and "B-Middle East 2.", as indicated by a significantly higher median IHC score ($p = 0.0008$ and $p = 0.0166$). The median of the overall IHC scores also showed a significant correlation with strain type ($p = 0.0028$), i.e. the amount of viral antigen was higher in birds infected with strain C-North Africa than in birds infected with A-Middle East 1. and "B-Middle East 2." strains ($p=0.0215$ and $p=0.0042$, respectively). No significant differences between strains were detected in trachea and spleen samples.

PCR

Viral shedding from oronasal and cloacal swabs was investigated. All chickens except one ("A-Middle-East 1." group) shed the virus at 5 dpi through the oronasal swab; however, at 11 dpi, virus shedding was significantly reduced (p

<0.0001). A significant difference was observed in the "A-Middle-East 1." and "C-North Africa" strains (p = 0.0175). For the cloacal swab sample, the dpi variable was not significant and no significant difference between strains could be detected. None of the H9N2 virus strains were detected in the brainstem.

The PCR results also show a difference in pathogenicity between the strains, with the "C-North Africa" strain being the most prevalent in organs and swab samples. It is noteworthy that in cloacal swab samples only the "C-North Africa" strain was detected; in spleen and kidney samples the "B-Middle-East 2." and "C-North Africa" strains were detected.

3. Impact of different routes of infection on pathogenesis

Clinical signs

During IN&IT infection, clinical signs worsened slightly over time but remained below score 1. In case of IV infection, clinical signs reached the IN&IT infection score 2 days later, but sooner, and showed higher scores at 7 and 8 dpi, and then declined.

Gross pathology

In the case of pathological lesions, we saw milder lesions at 5 dpi in the air sacs for the IV infection route, while at 11 dpi hardly any lesions were seen.

Histopathology

For IV infection, at 5 dpi, we saw milder pathological lesions in the trachea and lungs, while more severe lesions were seen in the kidneys. No lesions were found in the spleen, brain and pancreas at any infection routes.

At 11 dpi, tracheal lymphocytic inflammation was scored higher in the IV route, while more severe lesions were seen in the lungs in the IN&IT route. This observation provides information on the tissue tropism of the virus: regardless of the route of infection, the H9N2 virus is found in the upper respiratory tract and the lungs.

Immunohistochemistry

IV infection resulted in more antigen detection in the kidneys (50% vs. 18%), spleen (20% vs. 9%), and less in the lungs (20% vs. 36%). The positive rate of trachea samples was quite similar (60% vs. 55%). Despite the numerical trends, the Mann-Whitney U-test showed no significant differences between infection routes ($p > 0.05$ per tissue and in the overall scores).

PCR

In the case of IV infection, the virus can be detected in samples (cloacal swab and spleen) that are not detected in the natural route of infection (IN&IT). In the case of IV infection, the virus is detectable in the air sac swab, trachea and lungs at the same level, and in the oronasal swab at a higher level than in the

IN&IT route. PCR results also confirm that the virus tissue tropism is the upper respiratory tract and lungs. The PCR results of samples taken at 11 dpi show that in the natural route of infection (IN&IT) the virus is almost completely cleared from the body, being detectable only in trachea samples. In the IV route of infection the virus is detectable in the trachea, the oronasal swab and to a lesser extent in the lungs.

4. Impact of IBV co-infection on the pathogenesis of H9N2

Clinical signs

In terms of clinical symptoms, the co-infected group has a higher rate of mild to moderate respiratory symptoms one day later, with a higher rate of symptoms at 7-8, 10 dpi than the H9N2-only infected group. No mortality occurred in the co-infected group.

Gross pathology

At autopsy, air sac lesions in the co-infected group show an increasing trend at 11 dpi compared to 5 dpi. In the H9N2-only infected groups, scoring of air sac lesions shows a decreasing trend to 11 dpi.

Histopathology

In the co-infected group, the lesion was moderate. Evaluation of samples taken at 5 dpi showed that H9N2 infection alone caused more severe lesions in the airways than co-infection. In the kidneys, IBV co-infection showed more severe lesions. In the chronic phase, more severe lesions were seen in the IBV co-infection group in terms of tracheal lymphocytic inflammation and metaplasia and bronchitis.

Immunohistochemistry

With nephropathogenic IBV and "A-Middle-East1." strain showed a significantly higher incidence of AIV in the kidneys (60% vs 18%). Interestingly, less virus was found in the lungs and trachea (30% vs 36% and 20% vs 55%, respectively). Statistical comparison showed that kidney IHC scores were significantly higher in the co-infected with nephropathogenic IBV group than in the H9N2-only group ($p=0.0449$). No significant difference was observed in other tissues.

PCR

Based on the 5 dpi PCR results, the H9N2 virus was detected in the trachea at the same level, in the air sac swab and lungs at a higher level and in the oronasal swab at a lower level compared to the co-infected group. H9N2 was detected in the spleen only in the co-infected group. No H9N2 virus was

detected in the cloacal swab, brain and kidneys in any of the groups.

The PCR results at 11 dpi, which indicate a chronic phase of disease development, show that the H9N2 virus infection has already progressed, with virus found only in the trachea, while in the co-infected group, H9N2 virus was found in the oronasal swab, trachea and lungs, thus the disease progression was prolonged in the presence of co-infection.

CONCLUSIONS

Our observations confirmed a clear tissue tropism of H9N2 LPAIV strains to the respiratory tract and, to varying degrees, to the kidneys. The AIV strains tested did not show replication in the central nervous system. The presence of viruses and their lesions were predominant in the acute phase of infection (5 dpi) and significantly reduced or disappeared in the subacute, chronic phase (11 dpi). In several parameters, statistically significant, clear virulence differences were observed between H9N2 isolates of different geographical origin, belonging to the same subtype (H9N2) and the same lineage (genetic lineage) (G1): the "C-North Africa" strain was found to be significantly more virulent than the Middle Eastern ("A" and "B") strains, which did not show any notable

differences. Further studies are needed to investigate the genetic background that may explain the difference in virulence of H9N2 strains.

IN&IT infection caused less frequent but more severe symptoms and lesions, while IV infection caused a higher frequency of clinical symptoms but with milder manifestations, but these differences were not statistically significant.

Following co-infection with LPAIV and IBV, the disease localised to the airways without significant difference in symptoms and lesions, and is more prolonged in time.

NEW SCIENTIFIC RESULTS

The virulence and tissue tropism of the H9N2 virus were investigated:

1. A statistically significant difference in virulence between the H9N2 LPAIV strains of different geographical origin (Middle East and North Africa) tested. The "C-North Africa" strain is found to be more virulent than the "A-Middle East 1." and "B-Middle East 2." strains.
2. Tissue tropism of H9N2 viruses is primarily associated with the upper respiratory tract and lungs, but also occurs in the kidneys.
3. Our infection model suggested that the H9N2 virus alone can develop severe disease without a predisposing factor, occasionally causing mortality. The clinical symptoms varied widely, ranging from lethargy to asphyxia due to fibrin plug.
4. IHC methodological development: Adaptation of the indirect two-step immune reaction formerly used for HPAIV to the H9N2 LPAI virus.
5. Use of a new statistical method. In addition to/instead of non-parametric tests we used regression models adapted to ordinal variables, which represent a completely new approach to data analysis in influenza virus research.

PUBLICATIONS OF THE RESEARCH

Bóna Márta, Tatár-Kis Tímea, Mándoki Míra, Farsang Attila, Kiss István: **The growing importance of the H9N2 subtype avian influenza virus in the world** Literature summary Hungarian Veterinary Journal 145./19-36. https://doi.org/10.56385/magyallorv.2023.01.19-36

Bóna, M.; Kiss, I.; Dénes, L.; Szilasi, A.; Mándoki M. **Tissue tropism of H9N2 Low Pathogenic Avian Influenza virus in broiler chickens by Immunohistochemistry.** Animals 2023, 13 (6), 1052; https://doi.org/10.3390/ani13061052

Bóna, M.; Földi, J.; Dénes, L.; Harnos A.; Paszerbovics, B.; Mándoki, M. **Evaluation of the Virulence of Low Pathogenic H9N2 Avian Influenza Virus Strains in Broiler Chickens.** Veterinary Sciences 2023, Volume 10, Issue 12, 671 https://doi.org/10.3390/vetsci10120671

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