Szent István University
Postgraduate School of Veterinary Science

Comparison of the pathomechanism of different infectious bronchitis virus strains

PhD dissertation thesis

Written by:
Dr. Benyeda Zsófia

2011

Szent István University
Postgraduate School of Veterinary Science

Supervisor:

Prof. Dr. Miklós Rusvai
Doctor of the Hungarian Academy of Sciences,
university professor
Szent István University, Faculty of Veterinary Science, Department of Pathology and Forensic Veterinary Medicine

Thesis committee members:

Dr. Tamás Bakonyi
PhD, associate professor
Szent István University, Faculty of Veterinary Science, Department of Microbiology and Infectious Diseases

Dr. Róbert Glávits
Candidate of Veterinary Science
Agricultural Office Veterinary Diagnostic Directorate

Dr. Vilmos Palya
Candidate of Veterinary Science, Director
CEVA-PHYLAXIA Vet Bio Co., Scientific Support Directorate

Introduction, aims of the study

The infectious bronchitis of chicken is an infectious disease of viral origin causing acute respiratory symptoms. Nowadays, the disease is still common in poultry flocks worldwide, and the economic consequences, resulting from the morbidity, the mortality, and the indirect losses, are causing serious damage to the poultry industry.

The illness first appeared in the 1930s in North-Dakota (USA), in young chickens. Later the range of the affected age-group and the clinical manifestation extended. Hen- and laying hen flocks became also affected, and besides respiratory symptoms, considerable drop in egg production occurred. Nephropathogen strains were first detected in the 1960s, followed by outbreaks resulting in the affection of different parts of the gastrointestinal tract.

After clarifying the origin of the disease, a huge advance was achieved through the recognition that the virus comprises more than one patho- and serotypes. Today there are more than 65 known serotypes worldwide, but in reality this number is susceptibly much higher. The occurrence of the serotypes in different geographical regions varies greatly, as well as their ability to induce disease and pathologic lesions in different organs. This decreases the possibility of the diagnosis and the prevention of the disease.

The first identified and the most widespread is the Massachusetts (Mass) serotype. Except Australia, where the evolution of the IBV (IB virus) developed independently from the other parts of the world, it is present in every country with large-scale poultry industry (Ignjatovic et al., 2006). Its widespread incidence is partly due to the common use of vaccines including different Mass strains. The numerous known strains of the serotype show a huge variation in virulence and in tissue tropism. The majority of the strains cause only respiratory signs, but in several cases, kidney damage and ‘false layer syndrome’ was also reported.

The second most widespread serotype in Europe is named 793/B or 4/91 serotype, which was first isolated in 1992 in England. Its assumed to appear in 1991, and apart from the usual IB symptoms it was associated with deep pectoral myopathy in layer and parent flocks. Later it turned out, that the same serotype had already been present in France for years. The first strain of this serotype (CR88) was isolated by Picault and his colleagues in 1985. Due to its fast spread in the continent, strains of this serotype were soon included in commercial vaccines of Europe, causing a frequency of occurrence similar to that of the Mass serotype.

The strain belonging to the QX serotype appeared in Europe in 2004 in a layer flock in Holland. This serotype originated from China, where it caused serious losses in outbreaks accompanied by proventriculitis or kidney damage. Similarly to the Chinese experiences, the new serotype spread rapidly in Europe, and its strains appeared one after the other in most West-European countries. Considerable losses occurred in both China and Europe, because the great antigenic difference of QX strains made them able to break through the protection provided by traditional vaccines. In the European cases, kidney damage and the development of ‘false layer syndrome’, was reported besides the explicit respiratory signs.

In Hungary, the strains of the QX serotype were first isolated in 2006. The cases were mainly reported from broiler flocks, causing severe nephroso-nephritis. Further, irreversible damage of the oviduct developed in layer flocks after infection at young age. According to the genetic investigation, the isolates showed 98% similarity with the first isolated Chinese QX strain.

Considering the quick spread of the QX serotype and its strong pathogenic effect compared to the known strains, we found it reasonable to perform a detailed examination of its strains, and to compare them to the reference strains of the two most widespread European serotypes.

In the frame of our work, we isolated strains of QX serotype from clinical cases occurred in five different countries. The behavior of the QX isolates and in comparison the broadly examined Mass and 4/91 reference strains (M41 and 793/B respectively) were investigated after experimental infection of day-old SPF chicks. We followed the clinical signs and pathological lesions as well as the localization of the virus antigen and RNA and the level of the replication in designated organs. In addition to the organs, most often reported to be affected by the QX serotype, we also investigated the possible impairment of the lung, the small intestine, the ovary and the testis. The ability of the serotypes to cause infertility in male gender was also investigated after infection of mature cocks. The antigenic effect of the isolates was followed by the measurement of the increase of humoral antibodies after infection.

With the results of the study, we tried to determine the virulence and the tissue tropism of the QX isolates and the reference strains and to investigate the differences between the three serotypes and within the QX isolates. We also wanted to clarify the reported adverse effect of QX serotype to the glandular stomach, the kidney and the oviduct. The examination of the testis was performed to obtain information about the role of IBV strains in the infertility of cocks.

Materials and methods

Virus strains

In this study we used samples from different pathological condition susceptibility occurred due to IBV infection in China, France, Slovakia, Greece and Hungary in 2005 and 2006.

Viruses were isolated by infecting embryonated SPF chicken eggs with the homogenisated samples. The isolated strains were then determined by RT-PCR (reverse transcription-polymerase chain reaction) followed by sequencing. After the identification and comparison of the sequences, a phylogenetic analysis was performed in comparison with other IBV sequences from the Gene Bank.

Animal study I

Production of the infectious inoculums

After proving that all five isolates belong to the QX serotype, we performed an animal trial, in comparison with the M41 and 793/B reference strains.

The allantoic fluid collected during the virus isolation was used for the production of the infectious inoculums. In case of each sample, allantoic fluids of the embryos, showing IBV specific lesions, were pooled and a 3-step end point dilution cleaning was performed on embryonated SPF chicken eggs. After cleaning of the isolates, the presence of the original virus strain was checked by RT-PCR, followed by sequencing. In order to exclude the presence of other poultry pathogens sterile probe, haemagglutination- and Mycoplasma tests and PCR examinations were performed with the cleaned infectious inoculums. Finally, the cleaned allantoic fluid was titrated and diluted, if necessary, in order to adjust the exact titer of the infectious dose. The M41 and 793/B reference strains arrived from the Weybridge Laboratory (Veterinary Laboratories Agency, Avian Virology Department, Weybridge, UK).

Experimental design

8 groups of day-old chicks were formed in our experiment. In the first 4 groups 50-50 hens were divided and infected by the Chinese, French, Slovakian and Greek isolates. In the groups 5, 6 and 7, 50 cockerels in addition to the 50 hens were infected by the Hungarian isolate and the M41 and 793/B reference strains. The negative control group included 20 hens and 10 cockerels. Chicks in each group were inoculated by intranasal and intraocular installation with 40 µl of 5,7 $\log_{10}$ EID_{50} of the infectious inoculums. After the infection, we followed the clinical signs and pathological changes for 42 days Sample collection from the designated organs was performed 8 times (on days 4, 7, 11, 14, 21, 28, 35 and 42 post infection) for different tests. Five animals per group and per sex were sampled at each occasion. We collected those organs, where virus replication and consequent tissue destruction was expected.

Sampling

First, we took blood from every animal and then we performed pathological examination. After registering the changes we took samples from the following organs: trachea, lung, glandular

stomach, small intestine, ceacal tonsils, kidney, ovary, oviduct and testis (only in case of groups infected by Hungarian QX isolate and the M41 and 793/B strains and the control group). Glandular stomach and small intestine were sampled only between days 4 and 21 post infection, because the results of the test, completed until then, had not justified the collection of further samples. The ceacal tonsils were sampled only for RRT-PCR (Real-Time RT-PCR).

Re-isolation of the infectious strains

From samples taken from the trachea 4 days after infection, we isolated and identified the infectious strains by virus isolation and RT-PCR followed by sequencing.

Cilium activity inhibition test

On 4, 7, 11 and 14 days post infection, we removed the trachea, right after extermination, and placed into a Wasserman tube containing medium. After that we prepared 3-3 round sections from the lower and 4 from the upper part of the organ, possibly including 1 tracheal ring per section. The rings were then placed into the gaps of a 96-well plate, containing medium. We examined the samples under stereomicroscope and scored the activity of the ciliar epithelial cells.

Antibody response

The extent of the humoral immune response induced by IBV was measured by indirect ELISA method. We examined the sera on days 4, 14, 21 and 42 after infection. The antigen used in the reaction was cleaned from the allantoic fluid of embryonated chicken eggs infected by M41 strain. The calibration was performed with a 7-step, double dilution line of a sera with known titer (9,3 virus neutralization titer) and a negative (SPF) sera. The examined sample was considered positive in case of a VN titer ≥3 $\log_2$.

Real-Time RT-PCR (RRT-PCR)

The samples, collected on certain days, were investigated by RRT-PCR analysis to determine and compare the quantity of the viral RNA. First we homogenized the tissue samples, then the homogenates from a given group, sampling day and organ were pooled, RNA was extracted and copied to DNA.

For the determination of the quantity of virus RNA in different organs, TaqMan® probe based, group-specific, real-time PCR was performed. The primers and the probe were designed for the 5'-untranslated region of the M41 strain. Amplifications were performed with serial dilutions (10^{-1} to 10^{-7}) of a reference 4/91 IBV strain with known titre (7,26 $\log_{10}$ EID_{50}/0.2 ml) to assess the quantification assay.

Histopathological examination

The tissue samples removed during the pathological examinations were fixed in 10% buffered formaldehyde solution, embedded in paraffin wax and cut into 4 µm sections. After staining the sections with hematoxylin and eosin, they were examined under light microscope and IBV specific lesions were evaluated and scored between 0 and 3.

Immunohistochemical examinations (IHC)

We performed immunohistochemical staining on 4, 7, 11 and 14 days post infection, when we presumed the presence of the virus antigen. For the detection of the IBV antigen we used DAKO EnVision+ System-HRP AEC kit. Monoclonal antibody against IBV nucleocapsid protein produced in mice was applied as primary antibody.

Animal study II

In order to examine the possible effects of IB virus in increasing infertility of male gender we repeated the infection of cockerels on mature cocks. After checking the IBV negative status of the cocks, the experiment was performed in a simplified and shortened way. We infected by the Hungarian QX isolate and the M41 and 793/B strains again. Clinical findings and pathological changes were followed only for 14 days post infection and we took samples only four times during this study. Sampling was taken from the trachea, the kidney and the testis for histopathological and RRT-PCR processing. Humoral antibody was measured from the blood at every occasion.

The groups were placed in isolated units and cared for by different individual during both studies. The animals were kept in deep litter and supplied with feed and water *ad libitum*. During the clinical observation of the birds, sampling and processing of the tissues, all precautions were taken to avoid cross-infection or contamination between the groups and samples.

Results

Virus strains

During the virus isolation, specific embryo lesions became obvious after 2-3 blind passages and the RT-PCR proved, that the samples from all five clinical cases contain IBV. The partial sequence analysis of the S1 gene confirmed 99% similarity of the French, the Slovakian, the Greek and the Hungarian isolates to the original QX strain, while the Chinese one proved to be 96% identical with it. In case of the two reference strains, this similarity was 79 (M41) and 82% (793/B). Based on the examination of the phylogenetic tree, the samples from each of the five clinical cases could definitely be classify in the group of QX serotype.

Animal infection experiment I

Respiratory form

The respiratory symptoms appeared between 3-8 days after infection. Related to this, hyperaemia and serous-catarrhal inflammation of the trachea, was visible between sampling days 4 and 11. According to the results of the cilium activity inhibition test, the most intensive damage of ciliar movement occurred on the 4th and 7th day, but by the 14th day the regeneration of the ciliar epithelium had been completed. The most severe cilium destruction was induced by the M41, the most moderate by the 793/B strains.

The progression of the lesions with time could be divided into three stages. The degeneration of the tracheal mucus membrane was followed by the explicit proliferation of the inflammatory cells, and finally in the last phase the regeneration of the epithelial layer started. The progression of the changes was identical to that observed at the cilium activity inhibition test, but the regeneration was only completed by the 21st day. The most severe changes were induced by the M41 strain on the 4th day, but at the subsequent samplings they were caused by the QX isolates. Except for the one infected with the Greek isolate, the presence of the virus antigen could be detected in each group. Positivity was seen in the cytoplasm of the epithelial- and inflammatory cells on the 4th and 7th days post infection. With the RRT-PCR, the presence of the 793/B strain could only be detected on the 4th day, contrary to the M41 strain, which was present in a continuously decreasing concentration until the 11th day, while the QX isolates showed a more delayed discharge.

We could not detect any specific pathological lesion in the lungs, but between days 11 and 21 a serous, serous-fibrinous inflammation appeared in the air-sacs. During the histopathological examination, similar but more moderate changes to that described in the trachea, developed in the primer and secondary bronchi. Lympho-histiocytic infiltration detected parabronchially could not always be differentiated from the lymphoid tissue physiologically present in the interstitium of the lungs, therefore these findings can hardly be evaluated on their own. The IHC examination showed the presence of virus antigens in the epithelial cells of the bronchi in case of the French, Chinese and Hungarian QX isolates on the 4th and 7th days post infection. The RNA of the 793/B strain could not be detected in the lungs, concerning the other two serotypes, the amount and discharge of the infectious strains has showed similar results than the ones measured in the trachea.

Enteral form

We tried to determine the development of the enteral form based on the examination of the glandular stomach, the small intestine and the ceacal tonsils.

The samples of the glandular stomach gave negative results with every method. Only the presence of the virus antigen in a single sample indicated the possible replication of the French isolate in this organ.

Inflammatory cell infiltration could be seen in the mucus membrane of the small intestine, although lesions only appeared in limited samples per group. The IHC staining showed the presence of virus antigen only in case of one sample from the group infected by the Chinese isolate. By RRT-PCR, the presence of the two reference strains could be measured in lower quantity at single occasions, while the QX serotype showed a longer persistence and RNA concentration of these isolates were rather high and decreased only by the 21st day of the experiment.

Out of all the examined organs, isolates of the QX serotype replicated in the highest level in the ceacal tonsils. There presence could be detected until the 28th day. Contrary to this, the M41 and the 793/B strains could only be detected in lower quantity and only for a short time.

Nephroso-nephritis

From the 14th day post infection, swelling and paleness of the kidneys could be detected in the groups infected by the QX isolates from China, France, Slovakia and Hungary. In more severe cases urate deposition besides the above changes also occurred. The microscopic findings related to this pathological form were already evident from the 4th day. The inflammatory cell infiltration mostly developed in the interstitium of the kidney, resulting in the necrosis of the tubular epithelial cells in the severe cases. Lympho-histiocytic cells also appeared in the propria of the mucus membrane of the collecting ducts and ureters. According to the mean scores of the microscopic examination, most of the lesions could be connected to the groups infected by the QX isolates during the whole time of the experiment. By the IHC examination we found positivity in the samples of those groups, which were macroscopically also affected. Virus antigen could be seen in the tubular epithelial cells and the epithelial cells of collecting ducts and ureters between day 4 and 14. The results of RRT-PCR confirmed the findings of the morphologic examinations. The RNA of the 793/B strain was hardly measurable, the M41 strain was present in large quantity only in a single occasion, while the QX isolates persisted in higher quantities during the whole experiment.

Genital form

Histopathological changes in the ovary appeared only in moderate forms and only in a few sample per group. In addition to the inflammatory infiltration in the interstitium, the calcification of the primer oocysts was observed in groups infected by the Slovakian and the 793/B strain. In comparison with the other examinated organs, the RNA of the infectious strains was measured in lower quntity, except for the Slovakian isolate, which could be detected in high concentration.

Each of the five QX isolate caused irreversible damage in the oviduct that would lead to the development of ‘false layer syndrome’ in layer flocks. The pronounced dilatation of the wall and the

accumulation of serous like exudates became obvious from the 14th day of the experiment. Histopathological lesions related to this form could be seen from the 11th day, but the changes were rather the consequences than the causes of the macroscopic picture. Besides the flattening of the developing folds of the mucus membrane, the thinning of the wall and in some cases, the forming of inflammatory nodules in the mucus membrane or under the serosal lining could be found. The presence of the QX isolates was detected in a very small amount by RRT-PCR, while the reference strains gave negative results with this method.

In the testis of all the three infected groups, inflammatory cell infiltration developed in the interstitium or between the ductuli of epididymis. Unfortunately, these findings appeared only in few samples per group. Despite of this, RNA of the Hungarian QX isolate was measured in high titer until the 21st day post infection. The presence of the M41 and the 793/B strains was only confirmed in single occasions in lower quantity. Virus antigen could not be detected in the genital organs.

Antibody response

After infection at day-old higher level of antibody titers, indicating wild virus infection, occurred from the 14th day, but homogeneous positivity could only be measured from the 21st day of the experiment.

Animal study II

We detect milder changes and lower virus replication in the designated organs during the repeated examination in mature cocks. This was especially remarkable in case of tracheal samples. In the kidney, the inflammation developed only histopathologically, and the RT-PCR showed the higher replication of the Hungarian QX isolate. Apart from the described alterations in the testis after day-old infection, we could not find any microscopic sign indicating stone formation or the disturbance of the spermatogenesis. Due to the immune maturity of the cocks, the humoral immune response already showed homogeneous positivity on the 7th day.

Discussion

Summarizing the results, the adverse effect of the isolates of the QX serotype was the most explicit in every pathologic form of the disease. Although, the M41 strain generated nearly similar response in the respiratory organs, especially in the trachea, while the 793/B showed more moderate effect. Out of the QX isolates the Chinese one proved to be the most, while the Greek the least pathogenic in these organs.

In our experiment, none of the three serotypes confirmed to be able to induce the development of the clinically manifesting enteral form. Despite of that, the presence of the QX isolates, especially that of the Greek one, could be detected in higher level in the middle and lower part of the gastrointestinal tract. Clinical manifestation of enteral infection develops supposedly only in the presence of certain facultative pathogen agents.

Obvious nephropathogen potential was confirmed in case of the isolates of the QX serotype. Kidney damage developed in the most serious form in the samples of the Slovakian isolate. Although M41 strain could be detected in the kidney, considerable adverse effect was not seen in its samples, which is partly surprising, considering that several strains of the Mass serotype proved to be nephropathogen. It is likely, that certain strains induce pathology only in specified circumstances.

In the ovary we could not reliably prove the direct effect of the investigated strains, but it might be possible that due to the infection, a subsequent neuro-hormonal mechanism, developing without remarkable signs, influences the function of the ovary and reduces the intensity of oocyst maturation. This mechanism was already proved due to the effect of different diseases.

In the oviduct, irreversible alterations resulting in ‘false layer syndrome’ developed in all the five groups infected by the QX isolates, but in the most cases, as the effect of the Chinese one. Although the observed microscopic findings were not informative about the pathomechanism of the evolution of this pathological form and the presence of the virus could not be reliably detected in this organ. Based on these results we can draw the conclusion that the virus does not affect the wall of the oviduct, it is more likely to replicate in the lowest part of the gut, where a subsequent inflammatory reaction can restrict the opening of the oviduct into the coprodeum. The opening is the last step in the development of the Müllerian duct, which assures the connection between the cloaca and the vagina. This occurs in the postembryonal period of the development, normally accomplished by the 7^{th} day of age. This would explain the field and experimental observations, namely that the alteration develops only after infection at very young age, and answer the question why the stricture develops always in the terminal part of the oviduct.

In the testis we have not observed any specific alterations indicating infertility described by other authors. Although the presence of the RNA of the Hungarian QX isolate could be measured in higher quantities in both age-groups, which might cause more serious long-term consequences than those observed in our study.

New scientific results

1. From cases caused by infectious bronchitis virus, occurred in different countries, we isolated 5 strains of the recently appeared QX serotype.
2. We proved the close relationship of each of the five infectious bronchitis virus isolate to the originally identified QX strain and we determined the partial S1 gene sequence of these isolates for the first time.
3. We first reported the appearance of the QX serotype in Slovakia and in Greece.
4. We first performed a comprehensive study about the pathomechanism of the QX serotype in comparison with the two most common European serotypes (Massachusetts and 4/91).
5. After the experimental infection of day-old chicks by the three serotypes we described the clinical signs, the pathological and the histopathological changes.
6. In the designated organs, we compared the severity of evolving lesions, the appearance of virus antigen and the quantity of the virus, by using histopathological, immunohistochemical and molecular biological methods.
7. Between the three serotypes and among the five QX isolates, we could observe obvious differences. QX serotype, especially the Chinese isolate proved to be the most pathogenic and to have the widest tissue tropism.
8. In addition to the explicit respiratory and nephropathogen effect of QX isolates, we were the first to describe the development of irreversible oviduct lesions after experimental infection by this serotype. Besides, we determined that this serotype could be detected in the highest level in the ceacal tonsils.
9. According to our results, the investigated 793/B reference strain proved to be exclusively, while the M41 strain proved to be mainly respiratory types.
10. Considering the findings of the investigated virus strains, we could exclude there potential to induce lesions in the glandular stomach.
11. We presented a new explanation on the evolution of the pathomechanism of the ‘false layer syndrome’.
12. After experimental infection of day-old cockerels and mature cocks, we were the first to prove the appearance of IBV in the testis.

Acknowledgements

I would like to express my thanks to my supervisor Professor Miklós Rusvai, who provided the financial and infrastructural conditions of my work, managed the course of the research, helped me in the interpretation of the results, and in the correction of the manuscripts prepared for publication.

Special thanks to Dr. Vilmos Palya, member of my thesis committee, for mandating me to perform this project. He also provided infrastructural conditions of my work, helped me in the interpretation of the results, and in the correction of the manuscripts prepared for publication.

I would like to acknowledge Dr. Róbert Glávits, member of my thesis committee, who contributed to the evaluation of the histopathological samples and helped me in the correction of the manuscripts prepared for publication.

Thanks for Dr. Tamás Bakonyi, who helped me in the acquirement of the molecular biological methods and in the interpretation of the results.

I also thank to the workers of the former Department of Virological Development of CEVA-PHYLAXIA Vet Bio Co.: Hajnalka Bratu, Edit Fodor, Veronika Kardi, Magdolna Katonáné Lénárd, Dr. Tamás Mató, Dr. Tibor süveges and Máriá Varga, who provided help during the sample collection and processing and in the acquirement of the molecular biological, virological and other laboratory methods.

Thanks to Dr. Levente Szeredi and Ágnes Ráczné Mészáros for the help in performing the immunohistochemical staining and evaluating its results

I would especially thank to my colleagues at the Department of Pathology and Forensic Veterinary Medicine: Dr. Gyula Balka, Dr. Ferenc Baska, Dr. Zoltán Demeter, Dr. Mihály Dobos-Kovács, Dr. János Gál, Dr. Csaba Jakab, Dr. Míra Mándoki, Dr. Elena Alina Palade and Renáta Popp for their advices, help, and support.

Grateful acknowledgements to my family, and friends for their support and for making me possible to focus on my research.