PhD THESIS

Serological and molecular identification of avian infectious bronchitis virus strains in Romania

(SUMMARY OF Ph.D. THESIS)

Ph.D. Student: **Valentin Tudor**

Scientific coordinator: **Prof. Dr. Marina Spînu**

ABSTRACT

Infectious avian bronchitis (BI) is one of the most important avian diseases, declarable at the OIE, which causes massive losses from an economic point of view in the poultry industry. A highly contagious disease caused by a coronavirus (IBV), avian bronchitis has an acute clinical course, affecting the upper part of the respiratory tract in chickens, but also in other bird species.

The virus (IBV) is present worldwide, being transmitted by inhalation or direct contact with sick birds or contaminated manure, equipment or any other materials that have come into contact with the virus. Vertical transmission of the virus through embryos has not been identified, but the virus may be present on the surface of the hatching eggs after its removal from the oviduct or digestive tract (Lukert, 1965).

The high rate of virus replication results in the appearance of genetic mutations or recombination of the genome, which leads to the emergence of new viral genotypes (currently over 50)(Khataby et al., 2011, 2020). This phenomenon is worth considering in the management of the disease outbreaks but also in the prevention of subsequent pathological events. Although live attenuated vaccines have proven to be very effective, inducing strong immunity, protection is provided only in the case of the intervention of the genotype present in the vaccine actually used (Dhinaker and Jones, 1997).

In addition to lack of cross-protection, the advance of prevention and control programs, as well as the design of new vaccines, should take into account the potential risk of vaccine strain pathogenesis, the risk of inadequate vaccination protocols due to maternal antibody interference, or potential recombinations that may occur (Bands et al., 2015, 2016, Cavanagh et al., 1998, 1999, Mockett et al., 1987, Mondal and Naqi, 2001). Despite the genetic similarities reported between IBV and SARS-CoV-2 (Al-Jameel and Al-Mahmood, 2020), avian infectious bronchitis virus (IBV) does not present recognized risks to human health (Ignjatovic and Sapats, 2000).

In young chicks, IBV causes upper respiratory tract damage. In addition, there may be a reduction in feed consumption and a decrease in its conversion rate, thus affecting broiler production. Moreover, the infection creates a breach for opportunistic, secondary infections, which can cause aerosaculitis, pericarditis or perihepatitis (Lambrechts et al., 1993, Loomis et al., 1950, Lucio and Fabricant, 1990, Rosenberger and Gelb, 1987, Tottori et al. ., 1997).

In breeding flocks or laying hens, the infection can lead to a decrease in egg production by up to 70% or affect the quality of the eggshell. The virus can replicate in the oviduct and cause permanent lesions in immature and young birds with the effect

of reducing egg production over a long period of time or stopping production altogether, causing the appearance of "false laying" birds. Eggs from birds that produce pigmented shell will be discolored (Crinion, 1972), with an aqueous consistency of the egg white. Egg production may recover but may also remain low in highly susceptibile flocks (Otsuki et al., 1990).

The ever-changing geographical distribution of IBV genotypes supports the importance of early certainty diagnosis in poultry breeding units. Thus, for example, strains that appeared in China spread, leading to significant economic damage in the rest of the Asian continent, in the Russian Federation, but also in Europe (Mahmood et al., 2011, Bochkov et al., 2006, Beato et al., 2005, Worthington et al., 2008, Irvine et al., 2010, Sigrist et al., 2012).

The paper consists of the addition of published studies, included in the list of publications from the thesis.

The purpose of this study was to obtain IBV isolates from the field, from various commercial farms accounting for losses on the technological flow, clinically associated with signs of avian infectious bronchitis. The aims were:

1. Molecular (by RT-PCR test) and serological (by neutralization virus test and ELISA test) identification of circulating avian infectious bronchitis virus genotypes in Romania and their inclusion in a phylogenetic tree.
2. Phylogenetic analysis of isolates to determine the affinity relationships between isolated and reference strains.
3. Identifying the possible distinct serotypes that may be present on the Romanian territory and establishing the pathotype to which they belong.
4. Carrying out a geno-geographic map of the avian infectious bronchitis virus genotypes identified in Romania.
5. Evaluation of the level of protection induced by the available vaccines against infection with the mainstream serotypes identified in Romania.

The studies presented in the thesis were performed during the doctorate period (2013-2017), at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, Department of Infectious Diseases and also GD Animal Health Laboratory, Deventer, Netherlands.

The thesis entitled “Serological and molecular identification of avian infectious bronchitis virus strains in Romania” is structured in two main parts: the Current state of knowledge covering two chapters and the Personal contribution comprising 8 chapters. The thesis extends over 114 pages, including 17 tables, 25 figures and 245 bibliographical references.

The first part of the thesis (Current state of knowledge) summarizes information from the literature on the characteristics of the avian infectious bronchitis

virus, on the economic impact and pathogenesis of the disease, as well as on the diagnosis and control strategies of the disease. **Chapter 1** contains information on the history of IBV, etiology and taxonomy, general characteristics of coronaviruses, IBV interaction with the immune system, clinical signs and gross lesions, as well as immunological and biochemical diagnostic techniques. **Chapter 2** presents data from the literature regarding the genetic diversity of IBV circulating strains on different continents and in different countries.

The second part (Personal contribution) is structured in 8 chapters and includes the motivation, objectives and study model (**Chapter 3**), followed by the General Research Methodology (**Chapter 4**) and the research itself described in Chapters 5-8. The general conclusions and recommendations are presented in **Chapter 9**, and the originality and innovative contributions in Chapter 10. The cited bibliographic sources are represented by 245 titles.

If in neighboring countries studies have shown both the presence of major genotypes and specific genotypes have been identified, the same sequence of events is possible to occur in Romania, where the poultry industry is highly developed. The application of modern direct diagnostic tests (virus identification) and indirect tests (identification of IBV antibodies) is essential for the correct implementation of short, medium and long term disease prevention and control protocols.

Chapter 3 presents the study model that includes the research conducted, presented in subsequent chapters. The model includes the general objectives of the study, clarified by implementing the sampling protocol and serological/molecular diagnostic tests (Fig.1). The application of this model allowed the circumscription of the work stages and the establishment of the interrelation between them, with the continuous monitoring of the effectiveness of the preventive and control measures during the entire research.

Sampling methodology, the data obtained being comprehensive for the entire territory of Romania, was described in Chapter 4. The appropriate samples for the identification of IBV were collected as close as possible to the onset of the clinical signs. The samples were taken within three consecutive years (2013-2015) from Romanian farms that faced episodes of infectious avian bronchitis.

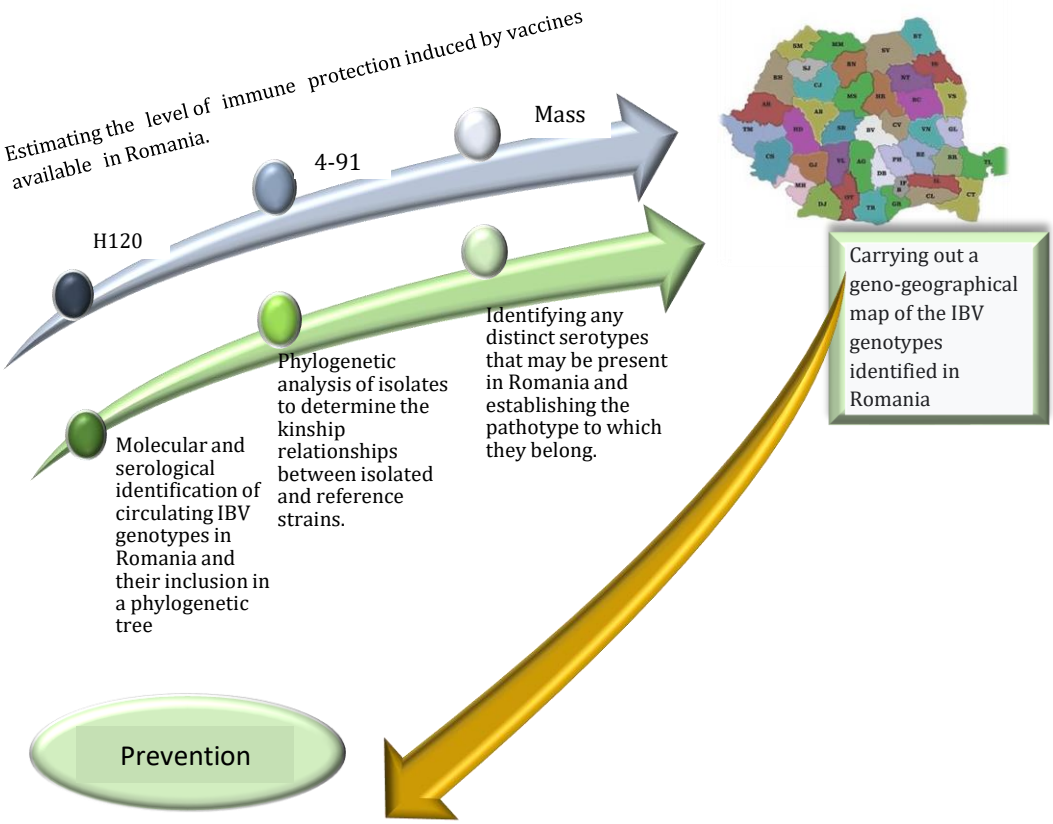

Fig. 1. Integrative model of IBV genotypes circulating in Romania

Subsequently, the research was resumed in 2017. To facilitate data processing and comparative assessment of IBV distribution in the territory, four areas were considered in this doctoral research, as follows: Q1-North West, Q2- North East, Q3-South East and Q4-South West (Fig. 2). A total of 5903 samples were processed.

Cloacal, tracheal and kidney swabs were collected from both chickens and adult birds, located in the four regions on 33 farms. Farms differed in age of the birds at the time of sampling and the breeding technology applied. On all these farms, clinical signs such as weezing, sneezing, and bronchial rales, weight loss, decreased egg production, changes in the eggshell were observed in sick birds. In some cases, the symptoms were atypical, requiring additional laboratory investigations in the direction of potential associated bacterial or viral agents.

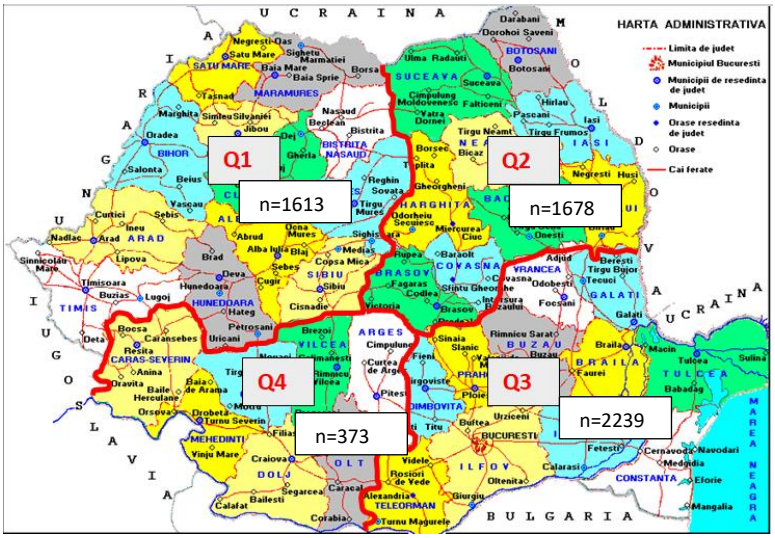

Fig.2. Major sampling zones including farms with clinical infectious brinchitis

Chapter 5 describes the regional distribution of infectious bronchitis virus strains in Romania. For the purposes of this experiment, samples were taken in 2017, from the entire territory of Romania, divided in the four main regions previously described: Q1-North West (n = 78), Q2-North East (n = 68), Q3-South East n = 173) and Q4-South West (n = 53). Cloacal, tracheal, and kidney swabs were collected from both chickens and adult birds. The farms accommodated differnt age groups, both broilers and layers. Clinical signs typical for IBV were observed in all locations, including quantitative and qualitative changes in egg production.

The samples were processed by RT-PCR technique. Common samples (each 5 grouped) were taken and they were transferred and then processed for diagnosis at the GD Animal Health Laboratory, Deventer, the Netherlands. The nucleic acid was extracted by use of High Pure Viral Nucleic Acid kit (Roche Diagnostics, USA) according to the manufacturer's instructions.

Two reverse transcriptase assays were used for the actual RT-PCR assay and subsequent sequencing: a genotype-specific RT-PCR for D1466 and a general RT-PCR - that resulted in the generation of a fragment of approximately 350 base pairs (bp) of the S1 gene, with primers XCE1 + (CACTGGTAATTTTTCAGATGG) and XCE3- (CAGATTGCTTACAACCACC) (Cavanagh and Davis, 1992, Cavanagh et al., 2007). S1 amplicons were separated in a 1% agarose gel and visualized by staining with ethidium bromide (0.50 µg mL-1) and translumination in UV light.

Purified amplicons were sequenced (BaseClear, Leiden, Netherlands) using both XC1 + and XCE3- primers. The data obtained by sequencing were aligned using a

Bio Numerics software computer (Applied Maths, Sint-Martens-Latem, Belgium) (Tudor et al., 2017, Saadat et al., 2017).

Simultaneously, specific bacteriological tests were performed on the samples taken from the growing units, to verify the presence of agents from the families *Enterobacteriaceae,* and *Mycoplasmataceae*, genera *Salmonella* and *Mycoplasma,* respectively.

The preparation of the database and the processing of the preliminary results were performed in the Microsoft® Office - Excel program. Variables with normal distribution were characterized by mean and standard deviation. To assess the statistical significance of differences between regions in the prevalence of different variants of identified strains, the Student's t test was used. Prevalence scores were allocated to IBV variants isolated by regions (Q1-Q4), to facilitate the mathematical interpretation of the results. Thus, the most frequently identified type was assigned a score of 4, the most rarely identified, 1, and the absence was marked with 0.

The correlation coefficient between regions was calculated using the Excel program. To verify the direct or reverse proportionality between the variables, the degree of their interdependence, the Pearson correlation coefficient was used. The significance of the differences was interpreted according to the table of critical values for the Pearson correlation.

Bacteriological tests indicated the simultaneous presence of the genera *Salmonella* and *Mycoplasma*, most likely of the species *S. gallinarum / pullorum* and *M. gallisepticum*, whose involvement in avian pathology is recognized, with egg-laying disorders usually occurring on layer farms. In the NW area of Romania (Q1, n = 78) 33 samples (42.3%) were positive for the presence of IBV. Overall seropositivity rates in Q1 indicated that IBV strains 4-91 / 793B (n = 9) (27.27% / 33 positive samples; 11.54% / 78 total samples) and 1/96 (n = 6) (18 , 18% / 33 samples; 7.7% / 78 samples) had the highest incidence.

Out of the total of 18 positive samples at RT-PCR, 9 showed 100% homology with strain 4-91 / 793B, 4 samples showed 99.4% homology with strain 4-91 / 793B and 5 samples 98.5% homology with 4-91 / 793B. The prevalence of QX strain (D388) was 96.0% (n = 5). The incidence of the IBV Massachusetts strain (M41) was 99.1%.

In the NE area of Romania (Q2) 42, the samples tested for IBV were 100% positive. The combined overall prevalence of IBV 4-91 / 793B was 100%.

Similarly, in the SE area of Romania (Q3), 35 of the tested samples were positive for the presence of IBV, registering a prevalence of 100% (n = 5, 14.28% / 35 samples; 2.9% / 173 samples), but for strain 1/96. Six of the samples showed 99.7% homology with IBV 4-91 / 793B (17.14% / 35 samples; 3.47% / 173 samples) and another 6 samples 99.1% homology with IS / 1494/06 (17.14% / 35 samples; 3.47% / 173 samples). The prevalence of IBV Massachusetts was 97.6% (n = 7) (20% / 35 samples; 4.05% / 173 samples).

In the SW area of Romania (Q4), out of a total of 53 samples, 58% (n = 31 samples) were positive for the presence of IBV. Ten samples showed 100.0% homology with type 4-91 / 793B, 8 99.7% homology samples with 4-91 / 793B, 5 mixed samples: 97.0% homology with 4-91 / 793B and 99.4% homology with QX, while 5 samples showed homology of 100.0% with 1/96 and 5 samples, homology of 98.3% with Xindadi (Table 1). The correlations calculated between areas were shown in Table 2.

Tabel 1. The distribution of identified strains by region

Regions	Pooled samples	Total P	Total N	P%	N%	IBV strains identified
Q1 (NW)	78	33	45	43%	57%	QX; 4-91/793B; 1/96; Mass
Q2 (NE)	42	42	0	100%	0	4-91/793B
Q3 (SE)	173	117	11	68%	32%	Mass, 4-91/793B; 1/96; IS/1494/06
Q4 (SW)	53	30	20	58%	42%	4-91/793B; 4-91+QX;1/96; Xindadi

Tabel 2. Statistical significance of correlation coefficients r between isolated strains, by regions

Q	Q1Q2	Q1Q3	Q1Q4	Q2Q3	Q2Q4	Q3Q4
R	0.7**	0.15*	0.66**	0.29*	0.52*	-0.56**

*-0.01, **-0.001

By applying two types of RT-PCR and evaluating the strains from 33 farms, the dominance of type 4/91 was found.

In Chapter 6, the alterations of the pathological patterns were evaluated by the intervention of different IBV pathotypes in Romanian chickens. For this, the biological material came from several chicken farms, where the birds were vaccinated with the H120 strain. In these locations, macroscopic lesions revealed at necropsy were atypical for IBV infection, including aerosaculitis and congestion of the intestinal mucosa, congestive lungs, cystic oviduct, kidney damage, ovarian involution, and increased mortality. The samples were investigated by the neutralization virus test according to the OIE Diagnostic Manual, as well as by RT-PCR testing, as described above (**Chapter 5**).

The virus neutralization test indicated the presence of antibodies against IBV M41, a common pathogenic strain on the North American continent, and IBV 793B (4/91), common to Europe, while antibodies IBV 1/96, D388 and Italia-02 below the

positive limit. The RT-PCR test identified the presence of vaccine strains M41 and 793B (4/91), a recombinant IBV strain, located between IBV 4/91 velogenic and IBV QX, as well as mass homology with Xindadi. It has been suggested that the identified strain IS / 1494/06 originates in Turkey.

Following the dynamics of the isolates by areas and by years of study, it could be observed that for the Q1 area, in 2014, the only one where PCR was performed, a homology as high as 98-99% of the isolates was observed with the classical pathogenic strain and the vaccine strain 793B (4/91).

Albeit IBV was not detected by PCR in the first year (2013) in the Q2 area, in the following year a homology of 99% with the pathogenic strain 4/91, 98% with the pathogenic strain Massachusetts and 99% with the pathogenic strain D274 was observed, indicating a potential cause for the variety of symptoms recorded. The profile of the strains encountered changes in the last year, the place of the aforementioned strains being taken over by the Chinese IB-Q1 velogenic strain.

For zone Q3, similar to zone Q2, the first year was characterized by the absence of IBV isolates, while in the second year a multitude of strains were present with homology of: 97-99% with vaccine strain D388, not detected by VN, 100% with strain vaccine 4/91 and 99% with Massachusetts vaccine strain. The strain circulation was modified in the third year of the study, homology of 99-100% with the H120 vaccine strain and of 98% with the Clone D274 vaccine strain, respectively, being identified. In the Q4 area, the PCR test was not performed during the study period.

Post-vaccination research has indicated the failure of the H120 vaccine strain used in some circumstances to provide effective cross-protection against pathogenic strains of the disease detected in the area and highlighted the need for further studies on the potential correlations of different diagnostic tests with the atypical clinical picture.

In **Chapter 7**, the prevalence of IBV pathotypes / prototypes on a chicken farm in northwestern Transylvania was investigated in a case study. The samples were collected from a broiler farm with a production of 2.5 million chickens.

The vaccination against IB was carried out using for a period of approximately 4.5 years before the present study, the Ma5 and 4/91 vaccine strains administered on the first day of life, with good results in the protection of vaccinated birds. For economic reasons, the vaccine strains selected by the farmer were modified, which triggered subsequent pathological events.

During the disese episode described in this chapter, the birds showed inconclusive clinical signs for a particular infectious disease and therefore resorted to a complex diagnosis, including testing for IBV, *Salmonella enteritidis, S. typhimurium* and *S. gallinarum, Mycoplasma synovie*. and *M. gallisepticum*, Gumboro disease virus, Newcastle disease virus and reovirus. RT-PCR and sequencing for the detection of IBV,

as well as virus neutralization by beta method, already described in this study, were applied as diagnostic tests (**Chapter 6**).

ELISA for *M. synovie* and *M. gallisepticum* (Idexx Mg / Ms Ab Test), reovirus (Idexx Reo Ab Test), Gumboro disease (Idexx Ibd Ab Test), *Salmonella enteritidis*, *S typhimurium* and *S. gallinarum* ((Salm Gp D) Salmonella Group D Antibody test kit, BioCheck, UK)) were used to detect other potential secondary infection agents. For the detection of antibodies to ND virus infection, the haemagglutination inhibition reaction was used. PCR was also used to detect *M. synovie*, *M. gallisepticum* and salmonella, respectively.

The results of the virus neutralization indicated the presence of IBV M41, a pathogenic strain common to the North American continent and IBV 4/91, common to Europe and also a vaccine strain, while IBV anti-D388 and Italy-02 antibodies were below the positive limit, the value of 7.

In the case of IBV 4/91, the titers were higher for 8 samples than for the previous strains and similar to those for two others, for IB D388 and those for IB Italy 02. Although IBV 4/91 was previously used as a vaccine strain on the farm, the time elapsed post-vaccination did not justify its presence during the study.

All types encountered could be considered pathotypes rather than protectotypes, the vaccine strain used on the farm being different from the isolated serotypes, while suggestive clinical signs were also present. RT-PCR did not detect IBV D1466 or others in the pooled samples. The PCR test performed on the same samples that were used for VN provided negative results for the pooled samples for IBV detection. The IBV strains were compared with the phylogenetic tree provided by Mark Jackwood (http://www.infectious-bronchitis.com/phylogenetic-tree.pdf). The presence of a large number of associated pathogens was also obvious, leading to complication / aggravation of the clinical picture and gross pathological lesions recorded during the episode. Thus, *Salmonella enteritidis, S. typhimurium and S. gallinarum, M. synovie* were present together with a reovirus, Newcastle disease virus and Gumboro disease virus. However, *M. gallisepticum* was absent both in the ELISA test and in the molecular PCR technique. The pathotypes present in the studied flock were not overlaping the potential protectotypes, but more likely they were represented by the wild serotypes, against which the applied vaccination did not ensure an effective protection.

The last research chapter of the thesis, **Chapter 8**, aimed to investigate the real prevalence of IBV on two farms, in chickens and broiler parents, where health problems seemed to be subsequent to a complex of various causes. The research was performed in two steps, the first (A) including a broiler farm, where vaccination was performed with the combination of Ma5 and 4/91 strains, and the second, step (B), a laying hen farm, where vaccination was performed with IB Primer, IB Ma5, IB 4/91,

monovalent inactivated anti-IBV vaccine during growth and anti-IBV vaccination every 2 months during production period. The results were interpreted on both farms based on clinical examinations and gross pathology, consistent with PCR results to confirm microbial causes and their sequence in the morbid episode and to develop the optimal personalized control program for each of the two farms.

On farm A, the lesions were atypical for IB, consisting of signs of different gravity in certain birds (inflammation of the air sacs, from a slight congestion of the intestine to catarrhal and hemorrhagic enteritis, etc.). In some of the birds, there were secondary infections expressed by macroscopic lesions translated by more or less abundant deposits of fibrin, usually found in *E. coli* infection (Smith et al., 1985), dystrophic kidney lesions and respiratory disorders. Repeated virological and PCR examinations eventually led to the identification of a recombinant strain of IBV, resulting in a cross between velogenic IBV 4/91 and IBV QX.

On Farm B, the results of clinical examinations indicated a sudden decrease in egg production by about 10 to 15% in all age groups (30 weeks, 50 weeks and 68 weeks), with changes in the appearance and consistency of the eggshell. The PCR test indicated the presence of an IBV strain that had 96% homology to the IB QX strain.

On the investigated farms, against the background of vaccination protocols applied according to the specific exploitation technology, the complex pathological changes that appeared included as a microbial cause a wild strain of IBV virus (QX), supporting its versatility and potential recombinations that occur despite the uniqueness of the strains affiliated to different geographical areas.

Based on the results obtained, it could be concluded (**Chapter 9**) that the IBV variants detected by RT-PCR in Romania were spread throughout the territory, with the dominance of type 4/91. Useful information was obtained to improve disease prevention and control measures, individualized for each area. Similarly, customized vaccination protocols on farms, depending on the region where they were located, allowed the upgrading of global disease control strategies at national level. Although strain 4/91 was the prevalent one, pathotypes such as Xindadi - previously not found in Romania and probably imported from Turkey, or type D1466, mentioned in other European countries, did not overlap with the vaccine strains used in Romania. It was established that the newly detected pathotypes in Romania lead to a clinical expression with different degrees of gravity of the disease, supporting the versatility of IBV and potential recombination in progress, despite the geographical "affiliation" of some variants.

The identification of multiple vaccine or pathogenic strains in the investigated territory, with annual changes in the structure of the IBV virus, dependent on the area (Q1-Q3), stood for the association of atypical and variable clinical picture with the present pathotypes, wild strains inducing a more serious pathology. In the clinical

study, subsequent to laboratory tests, the etiology proved to be much more complex, multimicrobial, including the genera *Salmonella, Mycoplasma* and *Escherichia*. Post-vaccination research has indicated the failure of the H120 vaccine strain to provide effective cross-protection against pathogenic strains of the disease detected in that area and the pathotypes responsible for clinical expression were not overlaping potential protectotypes, but were more likely to be wild serotypes (Q), against which the vaccination applied did not provide effective protection.

These changes in IBV dynamics jeopardize the outcome of vaccinations and should involve the development of appropriate and personalized prevention protocols for each type of herd.

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