

REGIONAL DISTRIBUTION OF INFECTIOUS BRONCHITIS VIRUS STRAINS IN ROMANIA

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Abstract

The study aimed at investigating the presence of various variants of the IBV by RT-PCR in Romania, the territory being divided into four main regions: Q1-North West (n=78), Q2-North East (n=68), Q3-South East (n=173) and Q4-South West (n=53) to provide information for improvement of vaccination protocols and IBV infection overall control strategies. Two types of RT-PCR were applied to characterise the strains from the 33 farms. Numerous variants of the IBV virus were identified in different farms of the four major regions of Romania, with the obvious dominance of 4/91. Nevertheless, the identified pathotypes, of which some not mentioned in Romania, like Xinandi which might have been "imported" from Turkey or D1466, mentioned elsewhere in Europe, did not overlap the vaccine strains, leading to the clinical expression of the disease with different degrees of severeness, which stands for IBV versatile character and potential re-combinations occurring in spite of affiliation of certain variants to various geographical areas.

Key words: infectious bronchitis virus, PCR, ELISA, mapping, Romania.

INTRODUCTION

One of the most economically impacting disease in poultry production is the infectious bronchitis, since the causative *Gamma Coronavirus* (Cavanagh et al., 2007, 2008), is spreading fast, it is highly infectious and persists for long periods of time on the farms. The economic loss is further increased by costs for disease control and for implementing specific bio-security conditions (Custura et al., 2012) as well as consumer safety insurance (Lelieveld, 2012), due to various zoonotic bacteria (ie, *Salmonella*) associated to the virus during some episodes (Tudor et al., 2017 a and b; Sato et al., 2017).

All ages are susceptible, chicks as well as layers, and the disease has regularly a very severe course. The morbidity and mortality differ from farm to farm, the clinical signs being expressed by poor weight gain or weight loss, respiratory symptoms and nephropathy (Ignjatovic et al., 2002; Ignjatović and Sapats, 2000; Chousalkar et al., 2007) as well as reduction in the egg production and its quality (Awad et al., 2016; Cavanagh, 2003, 2007). Mutations at the virus level were indicated to be quite frequent and more pathogenic variants

appeared (Ignjatovic et al., 1991). Over the years, numerous variants emerged, most of them of regional importance, not necessarily connected with severe disease (Chen et al., 2015) (ie, Italian 02, It-02). Others, such as 4/91 (known as 793B) were correlated with severe disease, sometimes in previously vaccinated flocks, and led to research and development of vaccines to control the outbreaks and losses (Sjaak et al., 2011).

Novel strains of infectious bronchitis continue to emerge in the field (Bru et al., 2017).

The IBV variants, considered to be induced by genetic recombination and point mutations in S1 gene, impaired the vaccination efforts, due to lack of cross-protection (Cavanagh and Gelb, 2008; Liu et al., 2003).

Since major changes occurred at S1 gene level, its analysis was considered the best strategy to differentiate IBV genotypes and serotypes, allowing to also select appropriate vaccines for disease prevention in various regions (Jackwood, 2012).

Nevertheless, aspects of the immune response and protection against the disease remain unclear (Chhabra et al., 2015; Moreno et al., 2017) and in case of regular vaccinations and simultaneous occurrence of various wild strains

in the same flock, the clinical outcome could be difficult to interpret (Tudor et al., 2017b).

This study aimed at investigating the presence of various variants of the IBV by RT-PCR in Romania to provide information for improvement of vaccination protocols and IBV infection overall control strategies.

MATERIALS AND METHODS

Sampling was performed from the entire Romanian territory divided into four main regions: 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 sampled from both chickens and adult hens from 33 farms. On all farms, clinical signs such as panting, sneezing and bronchial rales, weight loss, decreased egg production, changes in egg shell were encountered. Bacteriology was positive for *Salmonella* and *Mycoplasma* genera. Each five samples were pooled and sent for diagnosis to GD Animal Health Laboratory, Deventer, Netherlands.

Nucleic acid extraction. The viral RNA was extracted from cloacal, trachea and kidney swabs using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, USA) according to the manufacturer's instructions.

IBV RT-PCR and sequencing. Two reverse transcriptase PCRs were used, a genotype specific RT-PCR for D1466 and a general RT-PCR generating a fragment of about 350 bp of the S1 gene with the primers XCE1+ (CACTGGTAATTTTTCAGATGG) and XCE3- (CAGATTGCTTACAACCACC) (Cavanagh et al., 2007).

Agarose gel electrophoresis. The S1 amplicons were separated on a 1% agarose gel, and visualized with 0.50 µg mL⁻¹ ethidium bromide staining and an ultraviolet light transilluminator.

PCR product purification. The purified amplicon was sequenced (BaseClear, Leiden, Netherlands) using both XC1+ and XCE3-

primers. The sequence data were aligned by using computer software Bio Numerics (Applied Maths, Sint-Martens-Latem, Belgium) (Tudor et al., 2017; Saadat et al., 2017).

Statistical analysis. Student's t test was used to estimate the statistical significance of the differences between regions in prevalence of the variants. Prevalence scores were allocated to the isolated IBV variants in the regions (Q1-Q4), meaning the most frequently identified variant was scored 4, the least, 1 and the absence of a variant, 0. The correlation between the regions was calculated using the Excel program. The statistical significance of r was interpreted according to the table of critical values for Pearson correlation.

RESULTS AND DISCUSSIONS

The emergence of numerous IBV variants in the last decades, which is still an ongoing process (Sjaak et al., 2011; deGroot et al., 2012), impeded the design of vaccines/vaccination protocols and implementation of control strategies to prevent severe economic loss and preserve welfare of the birds (Khataby et al., 2016).

Differences in techniques used by different research groups looking at various parts of the S1 region of the S gene lead to difficulties in comparison of the isolates, therefore standardised methods are needed. Genetic peculiarities of the variants conducted to a different antigenic stimulation, therefore there is no overlap between serology and molecular methods in evaluating the cross-protection (Sjaak et al., 2011; Khataby et al., 2016)

In case of IBV, nucleotide sequencing of the S1 glycoprotein (Ignjatović and Sapats, 2000; Moreno et al., 2017) represents the most valuable tool in identifying the variants occurring in a certain area. In Europe, numerous variants were identified: Mass, D207, D212, D3128 D389S, D1466, PL-84084-France, AZ23.74/Italy, B1648-Belgium, UK/91B/67, UK6/82, UK/142/86, UK793/B, 624/Italy.

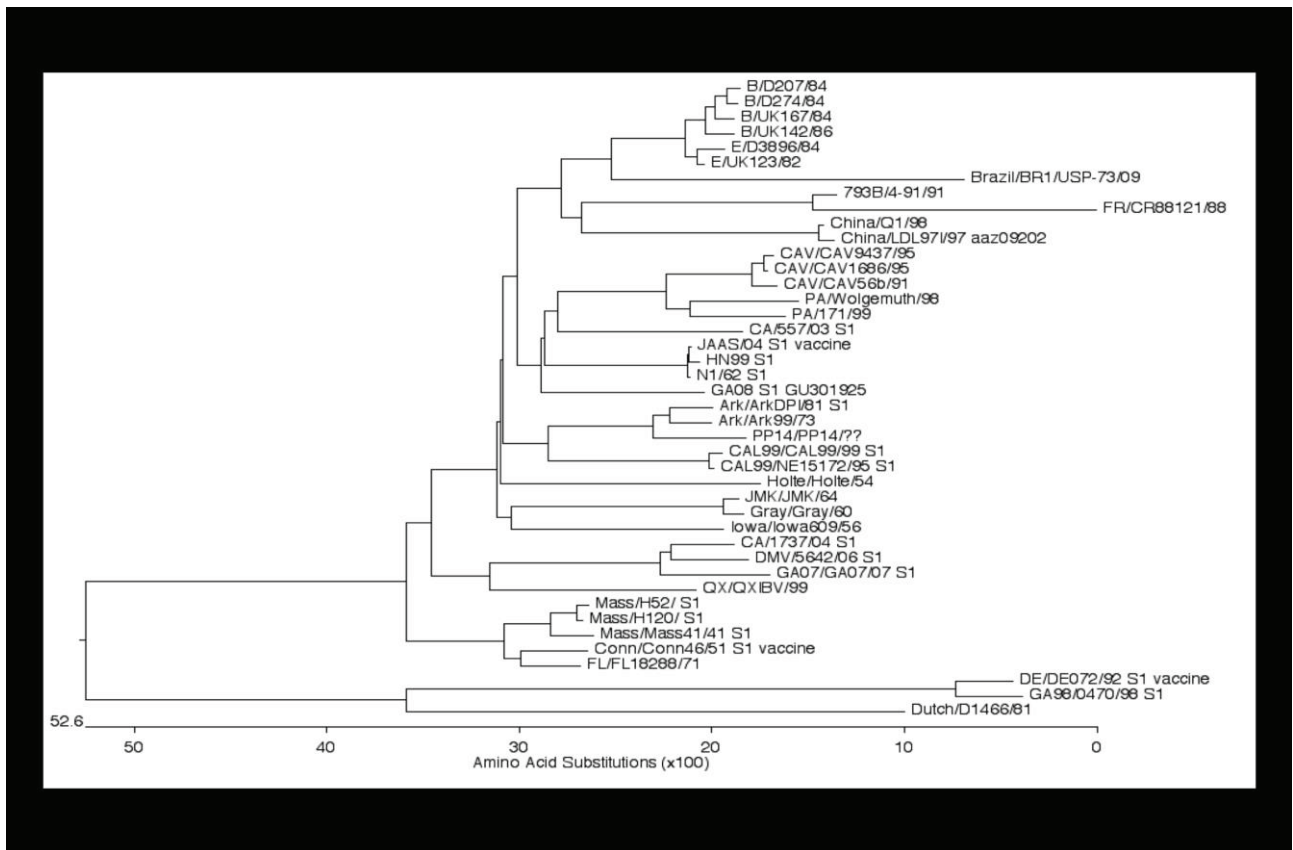


Figure 1. Phylogenetic tree of IBV (Phylogenetic tree provided by Jackwood, <http://www.infectious-bronchitis.com/phylogenetic-tree.pdf>)

Key: Conn= Connecticut, Fla=Florida, Mass= Massachusetts, Beau= Beaudette, Variant 1 and Variant 2 are from Israel, CAV= California variant, Ark= Arkansas, CU-T2= Cornell University, GAV= Georgia variant, D274, D207, and D1466= Dutch isolates, UK/167/84= United Kingdom isolate, DE072= Delaware isolate.

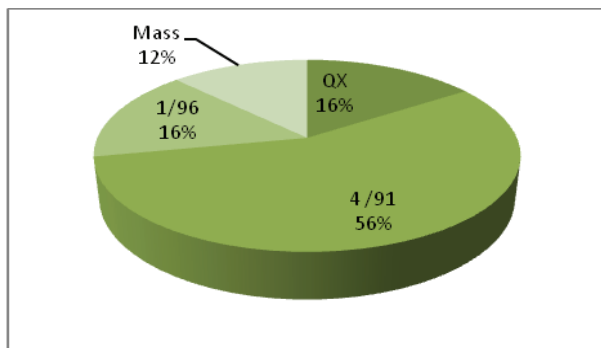


Figure 2. Prevalence of IBV variants in NW Romania

Two new variants emerged, D388 (QX) and the variant Italian-02 (initially considered non-pathogenic - Sjaak et al., 2011). Variants such as D1466, also identified in this study in Romania in less than 1% of the samples, caused lately financial loss on chicken farms.

In Q1 (n=78) 33 samples were positive for IBV. The overall Q1 sero-positivity percentages indicated that the IBV strain 4-91/793B (n=9) and 1/96 (n=6) had the highest incidence (100%). From a total of 18 samples,

9 samples presented 100% homology with 4-91/793B, 4 samples 99.4% homology with 4-91/793B and 5 samples 98.5% homology with 4-91/793B. The prevalence of QX (D388) was 96.0% (n=5). The incidence of the Massachusetts (M41) IBV strains was 99.1%. In Q2 42 samples tested for IBV were 100% positive. The overall combined prevalence for IBV 4-91/793B was 100%.

In Q3, 35 samples were positive for IBV. The prevalence of IBV 1/96 was 100% (n=5), 6 samples presented 99.7% homology with IBV 4-91/793B and 6 samples 99.1% homology with IS/1494/06.

The prevalence of IBV Massachusetts was 97.6% (n=7). In Q4 out of a total of 53 samples 58% were positive for IBV. Ten samples indicated a 100.0% homology with 4-91/793B, 8 samples 99.7% homology with 4-91/793B, 5 samples mix: 97.0% homology with 4-91/793B and 99.4 homology with QX5 samples 100.0% homology with 1/96 and 5 samples 98.3%

homology with Xindadi (Table 1, Figures 2, 3 and 4).

The most frequently observed variant, independently on the vaccination protocol performed on the farm, was 4/91. Since the symptoms of the disease were present and the strains used for vaccination as well, it was difficult to discern concerning the aetiology of the episode.

Table 1. Distribution of identified strains by regions

Romania	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

The overall percentages of the isolated variants were indicated in Table 2.

Table 2. Percentages of isolated variants

	4/91	QX	1/96 and Xindadi	Mass	IS/1494	Total
No	80	10	15	11	6	122
%	65.57	8.20	12.30	9.02	4.92	100.00

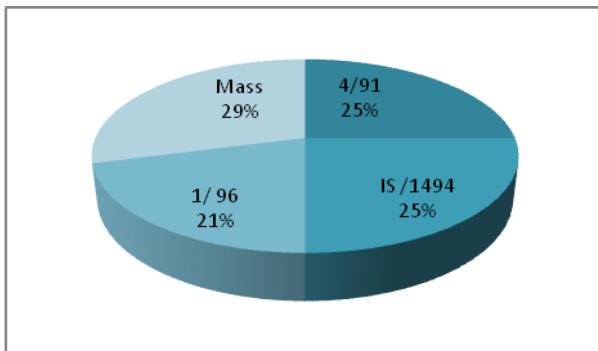


Figure 3. Prevalence of IBV variants in SE Romania

The highest percentage of positive samples (Q2) did not match the highest variety of the strains (Q3 and 4). This might be related to the raising technology and also the reciprocal commercial relationships between the various farms from different areas of the country.

The correlation between the different areas, statistically significant at a level of $p < 0.01$, in terms of prevalence of the variants was the strongest between NW and NE, thus indicating

a strong uphill positive relationship, while the correlation between SE and SW is moderate downhill relationship.

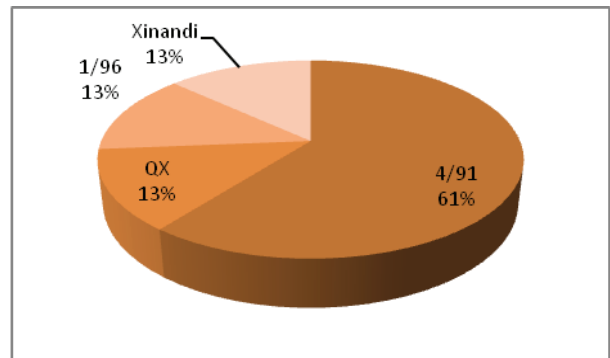


Figure 4. Prevalence of IBV variants in SW Romania

These relationships could be related to the spreading of different variants across the territory (Table 3).

Table 3. Statistical significance of the correlation coefficient for the isolated variants by region

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

*-0.01, **-0.001

No relationship concerning cross-protection could be established based on the isolated IBV variants and vaccine strains used in various locations, which further complicated the establishment of the *in situ* control programs (OIE manual, Smialek et al., 2017).

CONCLUSIONS

Numerous variants of the IBV virus were identified in different farms of the four major regions of Romania, with the obvious dominance of 4/91. Nevertheless, the identified pathotypes, of which some not mentioned in Romania, like Xindadi which might have been “imported” from Turkey or D1466, mentioned elsewhere in Europe, did not overlap the vaccine strains, leading to the clinical expression of the disease with different degrees of severeness, which stands for IBV versatile character and potential re-combinations occurring in spite of affiliation of certain variants to various geographical areas.

ACKNOWLEDGEMENTS

We acknowledge partial financial support from Project PN II PCCE 61/2012.

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