Seroprevalence and prevalence of Infectious Bronchitis Virus in broilers, laying hens and broiler breeders in Spain

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ABSTRACT Infectious Bronchitis Virus (**IBV**) is one of the most important viral diseases which causes important economic losses in poultry industry. This study aimed to assess the seroprevalence, prevalence, and variants of IBV in broilers, layers, and broiler breeders´ farms of *Gallus gallus* species in Eastern Spain. Thus, 29, 16, and 14 flocks of broilers, layers and broiler breeders, respectively were analyzed. To assess seroprevalence, sera samples were analyzed by ELISA. Tracheal swabs and tissue samples were tested by PCR to know the prevalence and detect specific variants. An IBV seroprevalence of 100% was detected in the 3 productive orientations. According to PCR results, a prevalence of 38% in broilers, 44% in layers and 43% in broiler breeders was obtained. The variant-specific RT-PCR analysis showed that 4/91, Massachusetts, QX, Italy-02 and D274 strains were present in commercial flocks in eastern Spain, being 4/91 the most prevalent in all the productive orientations. In layers 100% of QX prevalence, 14% of Italy 02 and 14% of D274 was detected. Regarding broilers, a prevalence of 18% of Massachusetts strain was also detected. In contrast, in broiler breeders´ farms only 4/91 strain was found. In conclusion, our findings showed the presence of IBV in eastern Spain and the changing situation of the IBV variants´ prevalence, being different according to the productive orientation. The continuous emergence of new variants emphasizes the importance of continuous IBV monitoring in order to optimize vaccination strategies.

Key words: IBV, prevalence, seroprevalence, ELISA, RT-PCR

INTRODUCTION

Avian Infectious Bronchitis Virus (**IBV**) is a highly contagious viral disease that is considered as responsible of significant economic losses in poultry industry worldwide (Moreno et al., 2017). The etiological agent is a virus which belongs to the genus *gamma Coronavirius* of the family *Coronaviridae* of the order *Nidovirales* (Cavanagh et al., 2007). This disease is primarily characterized by upper respiratory symptoms in birds; however, can also affect other organs like kidneys and reproductive tract (Jackwood and de Wit, 2013) which results in airsacculitis, proventriculitis, nephritis, enteritis (Yu et al., 2001; Sjaak de Wit et al., 2011; Cook et al., 2012), decrease in production, poor egg quality and significant mortality (Moreno et al., 2017).

IBV is worldwide distributed and it exist as many serotypes (Jackwood and de Wit, 2013). The process of quick molecular evolution is due to the capacity of antigenic variation by mutation or recombination events allowing the adaptation to changes under selection pressure (Kusters et al., 1990). Due to this variability, a lack of protection on the applied vaccine protocols could occur, so identifying the present strains´ genotype in farms is necessary to optimize control programs and the epidemiological knowledge of this pathogen. Nowadays, some different techniques are used routinely to control of IBV exposure that can detect antibody responses in sera samples like ELISA (2018). Moreover, many reverse-transcriptase polymerase chain reactions (**RT-PCR**) based techniques have been developed to identify IBV and some IBV genotypes. These molecular methods are highly sensitive and specific compared with other diagnostic methods (Alhatami et al., 2020).

Due the continuous e25ergence of new IBV serotypes, constant surveillance is essential to know the prevalence of IBV and the circulating strains in order to adjust appropriate control programmes to mitigate the low degree of cross-protection of commercial vaccines among

different serotypes (Jones et al., 2004). In this context, the aim of this study was to assess the seroprevalence, prevalence and variants of IBV in broilers, layers and broiler breeders' farms of *Gallus gallus* species, located in eastern Spain.

MATERIALS AND METHODS

This study was carried out on 71 poultry farms located in eastern Spain over a period of 18 mo. The productive categories of commercial poultry included in this study were broilers (29 farms), layer hens (19 farms), and broiler breeders (14 farms). The main regional poultry companies were involved in the study. All the samples were analyzed at the Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana, (**CECAV**) located in Spain.

Selection of Target Population

Firstly, to find the target population, all the active farms and flocks located in eastern Spain during previous 12 mo were identified. During this period, 421 active farms were found for broilers, 56 active farms for laying hens and 16 active farms for broiler breeders. A sample size of 29, 23, and 14 flocks of broilers, layers and broiler breeders, respectively, were calculated (Table 1). The flock's selection was done by random simple sampling using a number generator without repetition (http://nosetup.org/), giving a correlative number to each unit.

To calculate the sample size for the study, the active flock was taken as an epidemiological unit. A 95% of confidence level was considered and a prevalence of 10% was expected. The active flocks previously described were taken as population size. The sample size was calculated as follows:

$$n = \left(1 - \propto^{1/d}\right) \times \left(N - \frac{d-1}{2}\right)$$

Where n = sample size; $\propto$ = type I error = 1-95% confidence level; d = expected prevalence and N = population size.

Sampling Procedures

Broiler farms were sampled once at more than 42 d of life to discard maternal and vaccine antibodies against IBV. Laying hens and broiler breeders were sampled 3 times at different ages, when it was possible. The first sampling was carried out at 26 to 28 wk for layers and at 30 to 32 wk for broiler breeders, coinciding with the laying peak. The

second sampling was made 10 wk later for both productive orientations in order to observe potential antibodies oscillation. And the last sampling was performed at 60 wk for layers and at 50 wk in broiler breeders, to increase the probability of finding a field strain (Barberis et al., 2018). At each sampling time, for laying hens and broiler breeders 14 serum samples and 10 tracheal swabs with sterile aluminium swabs (Deltalab, Barcelona, Spain) were collected from live birds selected randomly at each flock. For broilers, 14 serum samples from live birds were collected. Tissue samples of trachea and kidney were collected at each sample time from 5 broilers, with a total of 10 samples per flock (5 tracheas and 5 kidneys). All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (Spain, 2013). Collected samples were transported to the laboratory under refrigeration conditions between 0°C and 4°C. Animals were checked daily to detect symptoms related to IBV.

Serology Analysis

Serum samples were processed according to Garcia et al. (2016). During the analysis, the sera samples were maintained under refrigeration conditions at 0°C to 4°C. The serological analysis was performed by ELISA using a commercial test (BioChek IBV ELISA kit; BioChek, ER Reeuwijk, The Netherlands) designed to detect IBV antibodies in serum. Titers were calculated as described by the manufacturer. Each sample test was diluted (1:500) in sample diluent reagent according the manufacturer's instructions. Briefly, 100 μL of diluted sample (1:500) were added to the appropriate well. Each sample was run in a single well. The plated was covered and samples were incubated at room temperature 22 to 27°C for 30 min. Each well was then washed with 350 μL of wash buffer (4 washing times). Then, 100 μL of conjugate reagent was added into the appropriate wells. The wells were covered and incubated at room temperature 22 to 27°C for 30 min. Each well was then washed as previously described and 100 μL of substrate reagent was added to each well. The plate was again covered and incubated for 15 min. Each reaction was quenched with 100 μL of stop solution. Absorbance was measured at 405 nm. Sera with s/P-values above the cut-off level of 0.2 (titer $\geq$ 834) were considered positive.

Detection of IBV by RT-PCR Method

Sample Processing All the swabs were stored at −20°C until the PCR analysis. The ten trachea swabs taken in each farm for layers and broiler breeders were divided into 2 pools of 5 swabs. For each broiler flock,

Table 1. Flock sampling size in broilers, layers, and breeders according to IBV expected prevalence.

	Expected prevalence (%)	Active farms	Estimated sample size (number of flocks)	Sample size used[1] (number of flocks)
Broilers	10	421	29	29
Laying hens	10	56	23	16
Broiler breeders	10	16	14	14

[1]Due to production and biosecurity issues, some layers farms did not participate in the study.

the 5 tracheas were analyzed as a pool. Likewise, the 5 kidneys collected were also processed as a pool. The flock was considered positive if at least one of the 2 pools tested were positive

RNA Extraction. RNA was extracted from each pool using the QIAmp cador Pathogen Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. First, tissue samples pools were ground and diluted in 1 mL of PBS (OXOID, Hampshire, UK). Then these samples were mixed by pulse vortexing and briefly centrifuged at $6,000 \times g$ for 1 min. 200 μL of supernatant were collected of each pool and were mixed with 20 μL of proteinase K and 100 μL of lysis buffer. On the other hand, swabs pools were diluted in 200 μL of PBS (OXOID) which were mixed by pulse vortexing with 20 μL of proteinase K, 1 μL of Carrier and 100 μL of lysis buffer. After incubation for 15 min at 20 to 25°C all the samples (tissue and swab samples) were briefly centrifuged to remove drops. Then, 350 μL of Buffer ACB were added and mixed thoroughly by pulse-vortexing to adjust the binding conditions for RNA purification. The mixture was put on a 2 mL collection column and centrifuged at $6,000 \times g$ for 1 min. The collection tube was washed with 600 μL wash buffer (AW1) and centrifuged as described above. After a second wash step with 600 μL wash buffer (AW2) and centrifugation at $20,000 \times g$ for 2 min, the RNA was eluted from the column by addition of 100 μL elution buffer (AVE) and incubated at room temperature for 1 min. After incubation, the RNA was centrifuged at $20,000 \times g$ for 1 min. The RNA extracted was then collected in sterile microtube and preserved at −80°C after PCR analysis.

Reverse transcriptase polymerase chain reactions (**RT-PCR**). A first RT-PCR was performed in all the RNA samples extracted to detect the presence of IBV with the Kylt IB-aCoV (AniCon Labor GmbH, Emstek, Germany), following the manufacturer's recommendations. This RT-PCR was performed in QuantStudio 5 Thermocycler (ThermoFisher scientific).

If this first RT-PCR is positive, variant-specific RT-PCR methods were performed to detect IBV variants using AniCon Labor GmbH kits following the manufacturer's protocols. Hybridization probe-based chemistry was used with the following primers: Kylt IBV-Variant 02, Kylt IBV-Variant 4/91 (793b), Kylt IBV-Variant Arkansas, Kylt IBV-Variant D1466, Kylt IBV-Variant D274, Kylt IBV-Variant Italy02, Kylt IBV-Variant Massachusetts, Kylt IBV-Variant Q1, Kylt IBV-Variant QX, and Kylt IBV-IB80 (AniCon Labor GmbH). A QuantStudio 5 Thermocycler (Thermo Fisher Scientific, Waltham, MA) was used according to the following conditions: 1 cycle of 50°C for 10 min and 95°C for 1 min, then 42 cycles of 95°C for 10 s, 60°C for 1 min.

RESULTS

Due to production and biosecurity issues, some layers selected farms did not participate in the study, so the final number of layer flocks analyzed was 16.

Table 2. Positive samples detected by ELISA and PCR.

		ELISA		PCR	
	N	n	%	n	%
Broilers	29	29	100	11	38
Laying hens	16	16	100	7	44
Broiler breeders	14	14	100	6	43

N: sample size; n: positive farms.

Seroprevalence

Based on ELISA results, the farms were divided in 2 groups: negatives and positives. The positive group was composed of farms without reported vaccination and with 10% or more positive reactions and vaccinated farms with a suspect titer of infection (Titers ≥12,000 and Vaccination Index [**VI**] ≥400) (Biocheck Interpretation and Application of Results Manual, Biocheck 2018). Veterinary services of vaccinated farms reported that vaccination was carried out with different variant strains or combination of them, as 4/91, Ma5, H120, 1/96, D274 and M41 variant strains. The level of vaccination reported for broilers farms was 62% (18/29), 78% for layers (11/16) and for breeders' farms was 100% (14/14).

The ELISA applied on sera samples from the analyzed flocks demonstrated a high presence of antibodies against to IBV. Table 2 shows the results obtained from samples collected without considering vaccination, showing 100% of IBV seroprevalence of the 3 productive orientations.

Prevalence

The IBV detection was made by PCR from samples of the positive groups according to ELISA results. The IBV prevalence was 38% in broilers farms (11 /29), 44% in layers farms (7/16), and 43% in broiler breeders (6/14; Table 2).

IBV Variant Strains

Variant-specific RT-PCR method to detect IBV variants was carried out from the 24 PCR positive samples (11 from broilers; 7 from layers hens; 6 from broiler breeders; Table 3). In broilers' farms the strains detected were 4/91 (100%) and Massachusetts (18%). In 2 farms, the 2 strains were found at the same time. In layers' farms the strains were Qx (100%), 4/91 (43%), Italy-02 (14%), and D274 (14%). In some of these farms (43%), up to 2 different variants were found (QX with D274, 4/91 or Italy-02). On the other hand, in broiler breeders' farms only the 4/91 strain was found (100%).

DISCUSSION

IBV is a major threat for intensively raised poultry, causing direct losses due to animal mortality, reduced daily weight gain and decreased egg production and

Table 3. IBV detected strains according to productive categories.

	N	4/91		Mass		Qx		Italy		D274	
		n	%	n	%	n	%	n	%	n	%
Broilers	11	11	100	2	18	-	-	-	-	-	-
Layers	7	5	43	-	-	7	100	1	14	1	14
Broiler breeders	6	6	100	-	-	-	-	-	-	-	-

N: sample size; n: positive farms to the IBV strain.

quality (Cavanagh, 2007). IBV has been diagnosed in Spain since the early seventies by virus isolation and serological techniques (Dolz et al., 2008). The present study was conducted to monitoring the seroprevalence and prevalence of IBV in poultry industry in eastern Spain. The seroprevalence observed in our study in all the productive orientations (100%) is closed to that reported in Ethiopia (94%) and Nigeria (84%) (Hutton et al., 2017; Bhuiyan et al., 2018). Moreover, the seroprevalence found in our study seems higher compared to that found by other researchers in Algeria (78.25%) (Barberis et al., 2018), Pakistan (67%) (Ahmed et al., 2007), and France (61%) (Auvigne et al., 2013). The seroprevalence observed in this study may be due to higher vaccination level (Jackwood and deWit, 2013). Also, the samples were collected at advanced ages in all the flocks being consistent with the results observed by Javed et al. (1991) and Barberis et al. (2018) who reported that the seroprevalence of IBV increased with age because of the log period of exposure to field viruses.

Concerning prevalence, we found a 44% in layers, 43% in broiler breeders and 38% in broilers. In previous years, Worthington et al. (2008) showed similar rates (57%) to our research of commercial poultry in Spain. However, Giner et al. (2017) reported higher rates (78.4−94.4%) between 2012 and 2016. Other researchers as Andreopoulou et al., in 2019, detected higher rates (83.96%) of IBV in layer and broilers flocks in Greece. Similarly, in a study conducted by Roussan et al. (2009) in Jordanian, overall of 92.9% of commercial flocks were positive for IBV in PCR test.

The variant-specific RT-PCR analysis showed that the 4/91, Massachusetts, QX, Italy-02 and D274 strains are present in commercial flocks in Easter Spain.

The most common variant found was 4/91, present in 100% of broilers and broiler breeders and 43% of layers flocks. These rates agreed with the rates found by 9Roussan et al., 2009 in Jordan who shown that broilers, layers and breeders´flocks exhibited the 100, 78, and 61% of 4/91 prevalence, respectively. Lower rates were found in Spain by Giner et al. (2017) (10−48%) and Worthington et al. (2008) (25.9%), nevertheless the overall rates found in Western Europe are in agreement with our findings.

The second variant most commonly found was QX, present in 100% of layer flocks. Previous surveys in Spain (Worthington et al., 2008) did not found the QX type, but the circulation of this variant in different European countries like France, Belgium and Germany have been reported. Giner et al. (2017) observed a significant increase of this variant during 2016, where the rate was 81.6%.

Regarding broilers, Massachusetts variant was found in 18% of flocks. Similar prevalence was found in Spain between 2002 and 2006, where 50% of the variants were identical to vaccines (Worthington et al., 2008). Also, Giner et al. (2017) showed rates from 5 to 20% in Spain. This was not surprising considering the extensive use of 4/91 and Massachusetts variants in live vaccines (Worthington et al., 2008). Recent works has confirmed that live IBV vaccines have been found to persist in poultry for many weeks after administration (Naqui et al., 2003).

Of the IBV genotypes, D274 was detected in 18% of layers farms. This finding agrees with the reported results in Spain, where the D274 variant was detected in the 17.6% of farms investigated (Worthington et al., 2008). Similar rates were found in Belgium (22.8%), however, in other European countries the D274 prevalence was lower, such as UK (7%), Holland (6.9%), or Germany (10%) (Worthington et al., 2008).

In this study, Italy 02 was detected only in 18% of layers farms. This genotype was described by Dolz et al., 2006 and Worthington et al. (2008) in Spain, being the most frequently IBV strain detected in contrast to the low prevalence found in our research. However, recent studies (Giner et al., 2017) reported a decrease in the prevalence of this variant in Spain and match with Moreno et al. (2017) that this serotype has been replaced by other serotypes such as QX and 793B (4/91). This hypothesis confirms the low detection of this genotype in our study and explains the increased prevalence of QX and 4/91.

More than 1 IBV variants were found in 18% of broilers and 43% of layers flocks analyzed in this study. This is in agreement with previous observations showing that flocks may be infected simultaneously with several types of IBV (Cavanagh et al., 1999). Unlike most sequencing methods, the RT-PCR technique allows detecting the different strains present in the sample and not just the predominant strain (Giner et al., 2017).

In conclusion, this study describes the seroprevalence and prevalence of IBV in poultry industry in eastern Spain. Our findings illustrated the value of continued surveillance of IBV due the emergence of new variants and changing prevalence. Due to the pathogenicity of some emerging serotypes, epidemiological information is important to adjust vaccination plans to protect poultry farms.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Alhatami, A. O., F. alaraji, H. M. Abdulwahab, and Y. I. Khudhair. 2020. Sequencing and phylogenetic análisis of infectious bronchitis virus variant strain from an outbreak in egg-layers flocks in Baghdad, Iraq. Vet. World 13:1358–1362.

Ahmed, Z., K. Naeem, and A. Harmeed. 2007. Detection and seroprevalence of infectious bronchitis virus strains in commercial poultry in Pakistan. Poult. Sci. 86:1329–1335.

Andreopoulou, M., G. Granzo, C. M. Tucciarone, Z. Prentza, K. C. Koutoulis, M. Cecchinato, and I. Chaligianni. 2019. Molecular epidemiology of infectious bronchitis virus and avian metapneumovirus in Greece. Poult. Sci. 98:5374–7384.

Auvigne, V., S. Gibaud, L. Léger, X. Malher, R. Currie, and A. Riggi. 2013. A longitudinal study of the incidence of Avian Infectious Bronchitis in France using strain-specific haemagglutination inhibition tests and cluster analysis. Revue Méd. Vét. 164:417–424.

Barberis, A., N. Alloui, A. Boudaoud, O. Bennoune, and A. Ammar. 2018. Seroprevalence of Infectious Bronchitis Virus in broiler farms in Batna, East Algeria. Int. J. Poult. Sci. 17:418–422.

Bhuiyan, Z. A., M.d. Giasuddin, and Z. U. M. Khan. 2018. Seroprevalence of infectious bronchitis virus in different types of chicken in Bangladesh. Asian J. Med. Biol. Res. 4:132–136.

Biocheck. 2018. Biocheck interpretation and application of results manual. Accessed Nov. 2021. https://www.biochek.com/wp-content/uploads/2018/01/BIOCHEK-INTERPRETATION-Manual.pdf?x14571.

Cavanagh, D., K. Mawditt, P. Britton, and C. J. Naylor. 1999. Longitudinal field studies of infectious bronchitis virus and avian pneumorirus in broilers using type-specific polymerase chain reactions. Avian Pathol. 28:593–605.

Cavanagh, D. 2007. Coronavirus avian infectious bronchitis virus. BMC Vet. Res. 38:281–297.

Cook, J. K. A., M. Jackwood, and R. C. Jones. 2012. The long view: 40 years of infectious bronchitis research. Avian Pathol. 41:239–250.

Dolz, R., J. Pujols, G. Ordoñez, R. Porta, and N. Majó. 2008. Molecular epidemiology and evolution of avian infectious bronchitis virus in Spain over a fourteen-year period. Virology 274:50–59.

Dolz, R., J. Pujols, G. Ordóñez, R. Porta, and N. Majó. 2006. Antigenic and molecular characterization of isolates of the Italy 02 infectious bronchitis virus genotype. Avian Pathol 35:77–85.

Garcia, C., J. M. Soriano, and P. Catala-Gregori. 2016. Development of a seroprevalence map for Mycoplasma gallisepticum in broilers and its application to broilers from Comunidad Valenciana (Spain) over the course of two years (2009-2010). Vet. Med. 61:136–140.

Giner, A., F. Blond, T. Bru, and R. Vila. 2017. Bronquitis infecciosa aviar: genotipos identificados en España del 2012 al 2016. LIV Symposium Científico de Avicultura. Accessed Nov. 2021 https://www.wpsa-aeca.es/articulo.php?id_articulo=4535.

Hutton, S., J. Bettirdge, R. Christley, T. Habte, and K. Ganapathy. 2017. Detection of infectious bronchitis virus 793B, avian metapneumovirus, Mycoplasma galisepticum and Mycoplasma synoviae in poultry in Ethiopia. Trop. Anim. Health Prod. 49:317–322.

Kusters, J. G., E. J. Jager, H. G. M. Niesters, and B. A. M. van der Zeijst. 1990. Sequence evidence for RNA recombination in field isolates of avian coronavirus infectious bronchitis virus. Vaccine 8:605–608.

Moreno, A., G. Franzo, P. Massi, G. Tosi, A. Blanco, N. Antilles, M. Biarnes, N. Majo, M. Nofrarías, R. Dolz, D. Lelli, E. Sozzi, A. Lavazza, and M. Ceccinato. 2017. A novel variant of the infectious bronchitis virus resulting from recombination events in Italy and Spain. Avian Pathol. 46:28–35.

Jackwood, M. W., and S. deWit. 2013. Infectious bronchitis. Pages 139−160 in Diseases of Poultry. D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez and V. Nair, eds. Wiley-Blackwell Publishing, Ames, IA.

Javed, T., M. Siddique, and A. Hameed. 1991. Persistence and morpho-pathological studies on infectious bronchitis in chickens in Pakistan. Asslut. Vet. Med. J. 25:216–228.

Jones, R. C., C. E. Savage, C. J. Naylor, J. K. A. Cook, and M. A. El-Houadfi. 2004. Possible North African progenitor of the major European infectious bronchitis variant (793B, 4/91, CR88). Proceedings of the IV International Symposium on Avian Corona and Pneumovirus Infections.

Naqui, S., K. Gay, P. Patalla, S. Mondal, and R. Liu. 2003. Establishment of persistent avian infectious bronchitis virus infection in antibody-free and antibody positive chikens. Avian Dis. 47:594–601.

OIE. 2018. Terrestrial manual: avian infectious bronchitis. 2018. Accessed Nov. 2021. https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.02_AIB.pdf

Roussan, D. A., G. Y. Khawaldeh, and I. A. Shaheen. 2009. Infectious bronchitis virus in Jordanian chickens: seroprevalence and detection. Can. Vet. J. 50:77–80.

Sjaak de Wit, J. J., J. K. Cook, and H. M. van der Heijden. 2011. Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol. 40:223–235.

Spain. 2013. Royal Degree 53/2013, 1st of February, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. Bolet. Off. Estado 44:318–335.

Worthington, K. J., R. J. W. Currie, and C. Jones. 2008. A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. Avian Pathol. 37:247–257.

Yu, L., Y. Jiang, S. Low, Z. Wang, S. J. Nam, W. Liu, and J. Kwang. 2001. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis. 45:416–424.