

## Infectious bronchitis virus infections of chickens in Belgium: an epidemiological survey

*Infectieuze bronchitisvirus-infecties bij kippen in België: een epidemiologisch onderzoek*

<sup>1</sup>P. De Herdt, <sup>2</sup>M. De Gussem, <sup>1</sup>S. Van Gorp, <sup>3</sup>R. Currie

<sup>1</sup> MSD Animal Health, Lynx Binnenhof 5, 1200 Brussels, Belgium

<sup>2</sup> Degudap, Sasstraat 8, 8870 Izegem, Belgium

<sup>3</sup> X-Ovo Limited, Thomson Cooper 3 Castle Court, Dunfermline KY11 8PB, United Kingdom

peter.de.herdt@merck.com

### ABSTRACT

Between April 2012 and July 2015, cloacal and/or tracheal swab samples were collected from four hundred and twenty-four Belgian chicken broiler, breeder and layer flocks. All flocks were kept for production purposes and presented clinical signs suggestive of an infectious bronchitis virus (IBV) infection. The samples were analyzed by real-time polymerase chain reaction (RT-qPCR) to detect the presence of ribonucleic acid (RNA) of IBV. When positive, approximately four hundred base pairs (bp) encoding for the hypervariable region of the IBV S1 protein were sequenced. Sequencing results, cycle threshold (Ct) values and vaccination history were used as criteria to try and distinguish vaccine strains from field strains.

Of all samples examined, 22.4% was negative. In 16.4% of the samples that did contain RNA from IBV, the genotype could not be determined. In most cases, this was due to the recovery of RNA quantities below the lower limit of detection of the sequencing PCR. The remaining positive submissions predominantly revealed RNA from IBV strains that belonged to the 4/91–793B (46.8%), D388–QX (25.2%), D274–D207 (5.8%) and Massachusetts (4.0%) genotypes. Estimations indicated that approximately 58%, 11%, 37% and 46% of these detections, respectively, were vaccine strains. Infections with types CK/CH/Guandong/Xindadi/0903, Ukr/27/2011, NGA/295/2006 and Q1 were observed sporadically.

The results indicate that IBV infections are highly prevalent in Belgian chickens and that at least eight different IBV types were circulating during the monitored period. This underlines the necessity of providing flocks with a strong and broad protective immunity against IBV.

### SAMENVATTING

Tussen april 2012 en juli 2015 werden swabs genomen uit de cloaca en/of trachea bij vierhonderdvierentwintig tomen Belgische kippen, zowel vleeskuikens, ouderdieren als leghennen. Alle tomen werden voor commerciële doeleinden gehouden en ze vertoonden klinische symptomen die konden wijzen in de richting van een infectieuze bronchitisvirus (IBV)-infectie. De monsters werden onderzocht via “real-time polymerase chain reaction” (RT-qPCR) om de aanwezigheid van ribonucleïnezuur (RNA) van IBV na te gaan. Wanneer ze positief bleken, werd een sequencerij uitgevoerd van ongeveer vierhonderd baseparen (bp) die coderen voor de hypervariabele regio van het S1-proteïne. De gevonden sequenties, de “cycle threshold” (Ct)-waarden en het toegediende entschema werden gebruikt om de veldstammen en vaccinstammen van elkaar te kunnen onderscheiden.

Van alle onderzochte monsters was 22,4% negatief. In 16,4% van de monsters waarin RNA van IBV aanwezig was, kon het genotype niet bepaald worden. Dit was vooral omdat de hoeveelheid RNA lager was dan de onderste detectiegrens van de sequenceringsPCR. In de overige positieve monsters werd vooral RNA van IBV-stammen die behoorden tot de genotypes 4/91–793B (46,8%), D388–QX (25,2%), D274–D207 (5,8%) en Massachusetts (4,0%) aangetroffen. Schattingen duiden erop dat respectievelijk ongeveer 58%, 11%, 37% en 46% van de aangetoonde virussen, vaccinstammen waren. Infecties met types CK/CH/Guandong/Xindadi/0903, Ukr/27/2011, NGA/295/2006 en Q1 werden occasioneel gevonden.

De resultaten tonen aan dat IBV-infecties bij kippen in België frequent voorkomen en dat in de gemonitorde periode minstens acht IBV-types circuleerden. Dit bevestigt de noodzaak om kippentomen te voorzien van een sterke en breed beschermende immuniteit tegen IBV.

## INTRODUCTION

Infectious bronchitis virus (IBV) is the cause of a very common and economically important disease in chickens. In young birds, IBV mostly leads to respiratory problems although some strains are nephropathogenic. Mortality in infected flocks may rise to 30%. In layer and breeder chickens, egg production and quality declines are observed.

IBV was first recorded in 1931 in Massachusetts (Schalk and Hawn, 1931). This original IBV was therefore called the Massachusetts type. Being a single stranded RNA virus, IBV appeared highly susceptible to mutation. Variations in the S1 spike protein, which is localized on the surface of the virus particles, have led to the emergence of numerous variants worldwide (Jackwood and de Wit, 2014). Vaccines may afford variable protection against variants, which may hamper control of the disease by vaccination. Formerly, variants were identified on the basis of virus neutralization assays (serotyping) but nowadays, these tests have been replaced by molecular techniques (genotyping).

The situation with respect to IB prevalence and IBV types involved in infection may easily change over time (de Wit et al., 2011a). As such, epidemiological surveys in the Belgian poultry sector demonstrated a clear shift from types that occurred during a first period of monitoring between 1986 and 1995 (Meulemans et al., 2001) and a second monitored period from 2002 to 2006 (Worthington et al., 2008). More recent information has not been well collated. Therefore, it was the aim of the present study to examine the current prevalence of IB in Belgian chicken flocks and to identify the IBV types involved.

## MATERIALS AND METHODS

### Flock data and sampling

From April 2012 to July 2015, veterinary practitioners submitted samples from 181 broiler, 139 broiler breeder and 104 layer chicken flocks. All flocks were kept for production purposes and exhibited signs that might indicate an IBV infection, such as respiratory disorders, wet litter, drops in egg production and egg-shell deformities. Submissions were always accompanied by a completed questionnaire gathering detailed information on the date and site of sampling, the type and age of sampled birds, the clinical signs and lesions observed and the vaccination history (products used, age and route of administration and dosing).

For each flock, ten dry swabs were taken from the cloaca and/or trachea of the birds, put in individual plastic tubes and sent to the diagnostic laboratory. The interval between collection of samples and laboratory examination was approximately two weeks. Except during transport, the samples were stored between 2 and 8 °C.

### Molecular analyses

In order to confirm the presence of RNA from IBV (regardless of genotype) in the submitted samples, the real-time polymerase chain reaction (RT-qPCR) method described by Callison et al. (2006) was applied to ten swab sample pools. Up to 35 cycles were run. The cycle threshold (Ct) value was noted in positive samples that were further analyzed using universal primers specific for the S1 region of the IBV genome as well as an additional primer pair appropriate

**Table 1. Results of screening for the presence of IB viruses from April 2012 to July 2015 in Belgian chicken flocks exhibiting clinical signs, through RT-qPCR and sequencing analyses.**

	Complete collection of	Samples obtained from flocks of		
	samples	Broilers	Breeders	Layers
<b>Number of flocks tested</b>	424	181	139	104
<b>Result of RT-qPCR analysis</b>				
% IBV positive	77.6	84.0	74.8	70.2
% IBV negative	22.4	16.0	25.2	29.8
<b>Genotype distribution (%) of positive samples</b>				
4/91 – 793B	46.8	56.6	37.5	39.7
D388 – QX	25.2	32.9	11.5	28.7
D274 – D207	5.8	2.6	14.4	0
Massachusetts	4.0	3.3	4.8	4.1
CK/CH/Guandong/Xindadi/0903	0.9	0	1.9	1.4
Ukr/27/2011	0.3	0	0	1.4
Q1	0.3	0	1.0	0
NGA/295/2006	0.3	0	1.0	0
Untypeable	16.4	4.6	27.9	24.7

**Table 2. Proportion (%) of RT-qPCR positive samples that showed either 100%, between 99 % and 100% or less than 99 % identity with commercially available vaccine strains, on the basis of sequencing analyses of the hypervariable region encoding the IBV S1 protein.**

IBV genotype	Sequence identity to homologous vaccine strains		
	100 %	99 % – 100 %	< 99 %
4/91 – 793B	64.3	17.5	18.2
D388 – QX	10.8	54.2	35.0
D274 – D207	31.6	52.6	15.8
Massachusetts	38.5	38.5	23.0

**Table 3. Overview of presumed field strains of IBV demonstrated in Belgian chicken flocks between April 2012 and July 2015 (\*).**

IBV genotype	Percentage of total number of isolates	Number of isolates obtained from		
		Broilers	Breeders	Layers
D388 – QX	44.8 %	42	11	21
4/91 – 793B	40.0 %	14	27	25
D274 – D207	7.3 %	1	11	0
Massachusetts	4.2 %	1	4	2
CK/CH/Guandong/Xindadi/0903	1.8 %	0	2	1
Ukr/27/2011	0.6 %	0	0	1
Q1	0.6 %	0	1	0
NGA/295/2006	0.6 %	0	1	0

(\*). Presumed to be field strains on the basis of their S1 sequence homology, PCR Ct values and vaccination history.

for the detection of the D1466 genotype (Cavanagh et al., 1999; Worthington et al., 2008). This generated a PCR product of approximately 400 base pairs (bp) encoding the hypervariable region of the S1 protein for sequencing. Sequencing was conducted according to Worthington et al. (2008), with slight modifications. Obtained sequences were compared with those available in the NCBI Genbank nucleotide database in order to determine the IBV genotype involved.

### Distinction between field strains and vaccine strains

Up to present, an in vitro method that allows the unambiguous distinction between field viruses and vaccine viruses of the same genotypes, does not exist. Live vaccine strains often used in Belgian poultry belong to the genotypes Massachusetts, 4/91 - 793B (Cook et al., 1996; Gough et al., 1992), D388 – QX (Beato et al., 2005; de Wit et al., 2011b; Landman et al., 2005; Toffan et al., 2011; Yu et al., 2001) and D274 – D207 (Davelaar et al., 1984; Jordi et al., 1989). In order to estimate the proportion of field viruses in samples that contained RNA from these genotypes, multiple criteria were used.

First, the sequence of the detected viruses was compared to corresponding sequences in an internal database built by examining vials of all common commercial vaccines through next generation sequencing (X-Ovo Limited, unpublished). Sequences were also

compared to those obtained in vaccinated flocks in other countries where no field strains equivalent to the vaccine genotype were known to circulate. The criteria proposed by Worthington et al. (2006) were used for evaluation of the results. For viruses with 100% sequence homology to the vaccine strains, it was considered most probable they were indeed vaccine strains. Less than 99% homology may have been a field challenge while the intermediate range was considered questionable.

As a second criterion, obtained Ct values were compared to Ct values established during serial monitoring in vaccinated and infected flocks under field and experimental conditions. When the measured Ct value was significantly lower than expected, this could constitute an additional argument for the involvement of a field virus. Especially Ct values below 20 - demonstrating a large amount of viral RNA in the samples - were considered indicative for a field virus infection.

Also the vaccination history was taken into account; the identity of the vaccines used as well as the eventual interval between their administration and the sampling of the birds contributed to the evaluation. Finding the sequence of a genotype that was not included in the vaccination schedule or finding a sequence in vaccinated flocks at a much later moment than could be expected from experiments and field experiences, were considered possible indicators of a field infection.

After evaluating the degree of evidence provided

by the outcome of the three criteria, the detected viruses were classified as presumed field strains or presumed vaccine strains.

## RESULTS

The results of molecular screening in 424 Belgian chicken flocks from April 2012 to July 2015 are summarized in Table 1, Table 2 and Table 3.

Overall 22.4 % of the 424 examined flocks did not reveal any RNA from IBV and was therefore considered negative. The remaining 77.6% of the samples appeared IBV positive (Table 1).

In 16.1 % of the positive samples, it was impossible to classify the detected viruses into a specific genotype. Untypeable strains were found mostly in samples with a very high Ct, indicating they contained only a very small quantity of RNA, insufficient for appropriate sequencing. In the remaining 83.9 % of IBV positive samples, the detected viruses could be attributed to eight different genotypes (Table 1).

Strains belonging to the genotypes 4/91 - 793B, D388 – QX, D274 – D207 and Massachusetts were most prevalent, accounting for approximately 98% of all typeable detections. For these genotypes, 64.3%, 10.8%, 31.6 and 38.5% of the strains, respectively, showed 100% identity with homologous vaccine strains (Table 2).

The variant type 4/91 - 793B was found in 46.8 % of the IBV positive samples. It was estimated that this type was involved in approximately 40% of the IBV field infections. Presumed field strains were found most often in breeder and in layer flocks.

D388 – QX strains of IBV represented 25.2 % of the IBV-positive cases. They were considered responsible for approximately 45% of all IBV field infections, predominantly in broiler flocks.

Variant type D274 – D207 strains accounted for 5.8 % of the positive samples, approximately 37% of which were vaccine. Presumed field strains were demonstrated almost exclusively in breeder flocks.

Classical Massachusetts strains were found in 4 % of the IBV positive flocks; rather even proportions were derived from broilers, breeders and layers. Presumed field infections occurred especially in breeder flocks and sporadically in layer birds.

Other genotypes were found sporadically. Three strains belonged to the variant type CK/CH/Guandong/Xindadi/0903 (Ji et al., 2011); it was obtained twice from breeders and once from layers. The variant type UKR/27/2011 (Ovchinnikova et al., 2012) was found only once in a layer flock. Variants NGA/295/2006 (Ducatez et al., 2009) and Q1 (Toffan et al., 2011; Liu and Kong, 2004; Yu et al., 2001) were also just found once, both in breeders.

Comparing the amino acid composition of the examined part of the S1 protein between possible field strains and homologous vaccine strains (when avail-

able), an average homology of 97.5% was found. Only 2 % of the detections showed less than 90% homology, extending as low as 72% in one QX-type strain.

## DISCUSSION

Molecular techniques are appropriate to run epidemiological studies on IBV infections in chickens, including the identification of IBV types involved (Worthington et al., 2008), on a large scale. Additional advantages are that they can be performed on dry swab samples that are taken in a non-invasive way from live birds and that these can be sent to the lab without taking special precautions to storage and/or shipment conditions (Cavanagh et al., 1999; Worthington et al., 2008). These techniques therefore were suitable to the purpose of the present study.

In order to obtain clear insights in the epidemiological situation, field strains have to be distinguished from live homologous vaccine strains. No standardized method for achieving this differentiation of strains exists (Jackwood and de Wit, 2014; Worthington et al., 2008). In an attempt to distinguish field and vaccine strains, some authors have compared the sequence of genes encoding the S1 protein from obtained isolates with the sequence of vaccine strains (Worthington et al., 2008). However, this only gives unambiguous results in case of extensive sequence differences. Live IBV vaccines are usually obtained after serial passaging of IBV field strains *in vitro*. This procedure leads to attenuation but not necessarily to significant differences in the S1 composition, meaning that some vaccine strains may show virtually 100% homology to field strains for the composition of the S1 protein (Huang and Wang, 2007). Further, vaccine strains might –just as it is observed in field strains– sporadically undergo mutation for the S1 protein under field circumstances. Isolates with slight S1 deviations compared to vaccine strains thus do not necessarily indicate field viruses. This should be kept in mind, especially when encountering isolates that are different from the vaccine strains in only one or a few nucleotides. Thus, although sequencing is often appropriate for the classification of isolates into field or vaccine strains, it cannot attribute all isolates in an undisputed way. In order to enhance the reliability of the classification method, in this study, additional evaluation criteria were used, namely the Ct value of the samples in RT-qPCR and the vaccination history of the birds. Although it cannot be excluded that in some cases vaccine strains and field strains have been confused, this way of working was suitable to provide insights in the overall IBV situation of chickens in Belgium. A basic difference between field and vaccine strains is their virulence. An *in vitro* test determining virulence of IBV strains would therefore be a useful tool for distinguishing field and vaccine strains, especially when they are having a very high degree of S1 homology.

Virulence mechanisms in IBV are however poorly understood (Jackwood and de Wit, 2014). Armesto et al. (2009) have demonstrated experimentally that activity of the replicase gene may be of importance but more research in this field is needed.

At the time this study was initiated, published data on IBV infections in Belgian chicken flocks were scarce. Data obtained by Meulemans et al. (2001) related to the period 1986-1995 and the situation in broilers only. Worthington et al. (2008) organized a more recent study between 2002 and 2006 but it is unclear whether or not their examinations related to all categories of chickens. Despite some limitations, comparing these authors' results with the present ones showed clear changes over time. Indeed, as Massachusetts and D274-D207 were the most prominent types up to the mid-nineties, their prevalence has continuously declined to become rather occasional findings at present. The B1648 variant (Meulemans et al., 1987), which accounted for 11% of the isolates collected between 1986 and 1995, was not recovered in the subsequent surveys. On the other hand, the prevalence of the 4/91 – 793B type, which was observed originally in less than 1% of the cases, has continuously increased to become involved nowadays in approximately 40 % of the IBV field infections in Belgium, especially in breeders and layers. The type D388 – QX first appeared in Belgium in 2004 (personal observations) and quickly became the primary IBV type causing problems at present, especially in broilers.

Besides the major types of IBV mentioned above, several others may sporadically occur in Belgium. As such, Worthington et al. (2008) reported the Italy02 (Jones et al., 2005) and D1466 (Davelaar et al., 1984) variants in 1.9% and 4.4% of the cases, respectively. These strains were not encountered in the present survey. CK/CH/Guandong/Xindadi/0903, Ukr/27/2011, Q1 and NGA/295/2006 variants were documented but none of them accounted for more than 2% of the isolates. A possible explanation for the fact that these variants do not expand could be that currently used vaccination schemes provide protection against them. This can be supported by the fact that infected flocks showed only low virus loads, most probably indicating that the infections were not significantly related to the problems in the flocks and thus were coincidental findings. Further, experimental challenge data (de Wit et al., 2014 ; Massi et al., 2006) have demonstrated a high level of protection against Q1 and Italy02 variants in chickens vaccinated according to the protectotype approach proposed by Cook et al. (1999), a vaccination concept which is often practiced in Belgium. Briefly, improved protection can be achieved against many IBV strains by using a combined vaccination programme incorporating two antigenically different IBV vaccines, as it has been amply elaborated for Massachusetts and 4/91 vaccine strains (Cook et al., 1999 ; Cook et al., 2001 ; de Wit et al., 2014 ; Terregino et al., 2008).

In the past, IBV strains were classified through serotyping. This was done by examining the reaction pattern of isolates with reference antisera in virus neutralization tests. Nowadays, serotyping has largely been abandoned and replaced by genotyping. Genotypes are defined mostly on the basis of genome sequences encoding the IBV S1 protein. For most isolates, serotyping and genotyping lead to the same outcome but exceptions may occur, as demonstrated by Majó et al. (2004). It has been suggested (Jackwood and de Wit, 2014) that different results in serotyping and genotyping are found especially when the level of S1 amino acid homology of an isolate compared to the reference strain, drops below 90%. In the present study, the percentage of homology between field strains and reference vaccine strains was below 90% in 2 % of the cases, at least for the examined S1 fragment. For one of the strains, the homology reached only 72%. It would be interesting to examine these strains further through serotyping and in experimental challenge trials as this could indicate whether mutations relevant to protective immunity building are present.

The results of the present study indicate that IBV infections are highly prevalent in Belgian commercial chicken flocks. At least eight types of IBV appeared to be circulating in the past three years. This underlines the necessity of providing chickens with a strong vaccinal immunity against IBV. A broad protection against a wide range of IBV types can be obtained by applying vaccines according to the protectotype concept (Cook et al., 1999).

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