Effect of changes of vaccination strategies on IBV epidemiology, diagnosis and control: an Italian retrospective study

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Summary
Infectious bronchitis virus (IBV) is among the most impactful poultry pathogens, whose control, based on biosecurity and routine vaccination, is hampered by the existence of countless genetic variants sharing poor cross-protection. A retrospective study was conducted on IBV positive samples collected in Italian broiler farms from 2012 to 2019. In 2015, the adopted vaccination protocol shifted from a Mass and 793B-based vaccines to the administration of Mass and QX vaccines, allowing to study how changes in vaccination strategies may affect IBV epidemiology, control and diagnosis in the field. The most frequently detected lineages were QX (70.3%), 793B (15.8%) and Mass (11.9%). The relative frequencies of QX and 793B detections remained stable throughout the study, while Mass detections significantly increased after the vaccination change. Rather than to an actual growth of Mass population size, this finding may be attributable to different vaccine interactions, with Mass strains being more frequently concealed by 793B vaccines than by QX ones. Based on the obtained results, the two vaccination protocols appear to be similarly effective in fighting IB outbreaks, which in the last decade have been caused primarily by QX field strains in Italy. These results indicate that vaccination strategies may significantly affect IBV epidemiology and diagnosis, and should therefore be considered when choosing and interpreting diagnostic assays and planning control measures.

Introduction

Infectious bronchitis virus (IBV) is one of the most impactful avian pathogens affecting the poultry industry. Currently classified within the species *Avian coronavirus*, genus *Gammacoronavirus*, family *Coronaviridae*, order *Nidovirales*, it is a worldwide distributed ssRNA virus with a complex epidemiology. IBV causes a vast range of symptoms collectively referred to as infectious bronchitis (IB), whose control is usually achieved by combining routine vaccination and stringent biosecurity measures (Jackwood 2012). Prone to both mutation and recombination events, IBV exists in an ever-increasing number of genetic variants, which differ in terms of pathogenicity, tropism and geographic distribution (Bande *et al.* 2017). Multiple variants frequently co-circulate within a given area and the immune response to a certain strain usually does not fully protect against others, complicating the attempts to control the disease (de Wit *et al.* 2011). Different vaccination strategies are adopted, either implementing an "homologous" vaccination based on the same lineage of the circulating field strain or combining multiple "heterologous" vaccines based on different lineages to broaden the protection spectrum (Jordan 2017).

Despite being undisputedly effective in controlling the disease, the routine implementation of live vaccines also has some drawbacks: the circulation of vaccine strains could favour the occurrence of rolling reactions and recombination events, and their unwanted persistence and spread could possibly complicate the diagnostic process (Jackwood and Lee 2017). Additionally, the immunological pressure exerted on field strains by vaccines has been recognized as one of the major driving forces

of IBV evolution (Franzo *et al.* 2019, Jackwood *et al.* 2012). Unfortunately, all these implications are often overlooked when planning IBV control strategies and interpreting epidemiological results.

To fill this gap, a retrospective study was conducted on samples collected in Italian broiler farms over a 7 years timespan, during which a significant change in the applied vaccination protocol occurred, providing an interesting opportunity to better understand how vaccination strategies could affect the circulation of different strains, the occurrence of IB outbreaks and the interpretation of diagnostic results.

Materials and methods

The study was based on convenience sampling, including specimens collected for diagnostic purposes in Italian broiler farms between June 2012 and September 2019 from both healthy and diseased animals, either to confirm the presence of a field strain or for routine monitoring. In the period from 2012 to 2014, the vaccination protocol adopted in the considered farms relied on a vaccine based on lineage GI-I (Mass) administered at 1 day of age (doa) and one based on lineage GI-13 (793B) applied at 14 doa until December 2013, and later at 1 doa. At the end of 2014, the 793B vaccine was replaced by a lineage GI-19 (QX)-based vaccine administered at the hatchery. The samples consisted of either tracheal swabs or renal tissues processed in pools, grouping together specimens collected from a single productive cycle of a single farm. The sampling broadly reflected the different densities of poultry farms in different regions. A minority of the farms were sampled more than once in different production cycles for diagnostic purposes and with no longitudinal consistency.

IBV presence was preliminarily screened by real time RT-PCR (Quantification of Avian Infectious Bronchitis Virus-IBV-kit; Genesig, Southampton, UK) assay, and then positive samples were amplified by RT-PCR (Cavanagh *et al.* 1999) and Sanger sequenced. Genotypization was performed by comparison to a reference database (Valastro *et al.* 2016) based on the third hypervariable region of the S1 gene. Despite the fact that the adopted classification is based on full S1 sequencing, the routine diagnostic process often relies on a smaller target region because it allows for lower costs and a greater detection efficiency (Manswr *et al.* 2018).

In addition to the sequencing results, information about age at sampling and presence of symptoms or lesions possibly ascribable to IB (i.e. respiratory or renal signs or aspecific drops in production) were collected when available.

An attempt to discriminate field and vaccine sequences was made by calculating the p-distance from reference vaccine strains using the MEGA7 software (Kumar *et al.* 2016): sequences were considered vaccine-derived when the p-distance was less than 0.01, and field strains otherwise.

A recombination analysis was performed on the aligned sequences with the RDP, GENECONV, Chimaera and 3Seq methods implemented in the RDP4 software (Martin *et al.* 2015). Recombination events were considered as such only when detected by two or more methods with a significance level lower than 10-5 and adopting Bonferroni correction.

Pearson's Chi-square test with Yates' continuity correction was applied to infer the presence of significant differences in the detection frequency of different lineages, possible IB-related symptoms before and after the vaccination change and also the number of potential IB outbreaks detected in presence of specific lineages. Z-tests were performed to assess the presence of statistically significant differences in the mean age at sampling based on the detected strain. Statistical analyses were conducted using R software, setting the significance level lower than 0.05.

Results

Five hundred and four samples proved positive to the preliminary real-time RT-PCR screening. Two of them were negative to the following RT-PCR, while 23 were positive but it was not possible to retrieve a high quality sequence. Four hundred and seventy-nine samples were successfully sequenced. These samples were collected at a mean age of 36.3 days in farms located mainly in North Italy (338 from Veneto, 62 from Lombardy, 14 from Piedmont, 6 from Friuli-Venezia Giulia, 3 from Emilia Romagna, 1 from Trentino-Alto Adige). Additionally, 32 and 14 samples were collected in the Campania and Molise regions, respectively. For 8 samples no information were available about the location of the farm.

The yearly number of detections of each lineage and symptoms and lesions possibly caused by IBV is listed in Table I.

GI-19 (QX) was the most frequently detected lineage (70.3%), followed by GI-13 (793B) (15.8%) and GI-I (Mass) (11.9%). In addition, strains belonging to lineages GI-16 (Q1), GI-12 (D274) and GII-1 (D1466) were sporadically detected, and two strains were labeled as the result of recombination events between 793B and QX strains. The presence of possibly IB-related symptoms was reported in 232 cases (48.4%). In the samples from diseased animals, 183 QX, 21 Mass, 15 793B and 3 Q1 strains were detected.

Table II enlists the yearly number of QX detections

Table I. *Yearly number of detections of each lineage and symptoms possibly ascribable to Infectious Bronchitis (IB) in broiler farms.*

		GI-1 Mass	GI-12 D274	GI-13 793B	GI-16 Q1	GI-19 QX	GII-1 D1466	Recombinant QX+793B	Total
2012	Total	0	0	2	0	8	0	0	10
	With symptoms	0	0	0	0	6	0	0	6
2013	Total	6	0	15	3	37	0	0	61
	With symptoms	2	0	2	3	21	0	0	28
2014	Total	3	1	3		38	0	0	45
	With symptoms	1	0	1		20	0	0	22
2015	Total	16	0	31	2	140	1	0	190
	With symptoms	5	0	3	0	77	0	0	85
2016	Total	15	0	17	0	81	0	2	115
	With symptoms	7	0	9	0	53	0	0	69
2017	Total	14	0	7	0	16	0	0	37
	With symptoms	5	0	0	0	9	0	0	14
2018	Total	1	0	1	0	6	0	0	8
	With symptoms		0	0	0	3	0	0	3
2019	Total	2	0	0	0	7	0	0	9
	With symptoms	1	0	0	0	4	0	0	5
	Total	57	1	76	5	337	1	2	479
	With symptoms	21	0	15	3	193	0	0	232

Table II. *Yearly number of vaccine and field QX strain detections.* A vaccine origin was presumed when the p-distance from the reference vaccine strain was less than 0.01, otherwise the strains were considered field ones.

QX sequences	Field origin	Vaccine origin	Total
2012	8	0	8
2013	37	0	37
2014	38	0	38
2015	136	4	140
2016	64	17	81
2017	8	8	16
2018	2	4	6
2019	1	6	6

divided by vaccine or field origin based on a p-distance threshold. All the strains belonging to other lineages were labeled as having a vaccine origin.

Discussion

From the obtained results, the predominance of the QX lineage in the Italian territory appears undisputed. Firstly isolated in China in 1993, this lineage is considered one of the main threats in Europe and Asia and is associated with respiratory, renal and reproductive signs (Valastro *et al.* 2016). QX was the most frequently detected lineage during the entire considered period not only in absolute terms, but also in presence of symptoms possibly ascribable to IB (83.2%).

Despite some limitations to the possible presence of other uninvestigated respiratory pathogens and to the impossibility of certainly establish the pathogenic role of an IBV strain solely based on a PCR positivity, this finding is consistent with previous works that reported QX as responsible for the majority of IB outbreaks in Italy (Franzo *et al.* 2016). QX strains were divided into vaccine and field ones based on a p-distance threshold. While no definitive conclusion can be drawn based only on phylogenetic analyses (Jackwood and Lee 2017), similar criteria have been already proposed (Worthington *et al.* 2008, Legnardi *et al.* 2019) and may be useful from a practical standpoint since IBV diagnosis often relies only on molecular assays. The robustness of the proposed criterion is supported by the fact that QX vaccine strains were detected only after the introduction of the homologous vaccination in 2015. While they were initially a small minority, the percentage of QX strains with a vaccine origin has gradually increased in the following years. However, QX field strains appear to still circulate.

All Mass, 793B, D274 and D1466 strains were labeled as vaccine ones. The circulation of 793B strains was demonstrated even after the discontinuation of the homologous vaccination. This finding may be ascribable to 793B vaccine strains persisting within the considered farms or spreading from neighboring facilities that adopts this vaccine, especially in densely populated poultry areas.

Two strains originated from the recombination between QX and 793B strains were also detected, consistently with some previously reported Italian

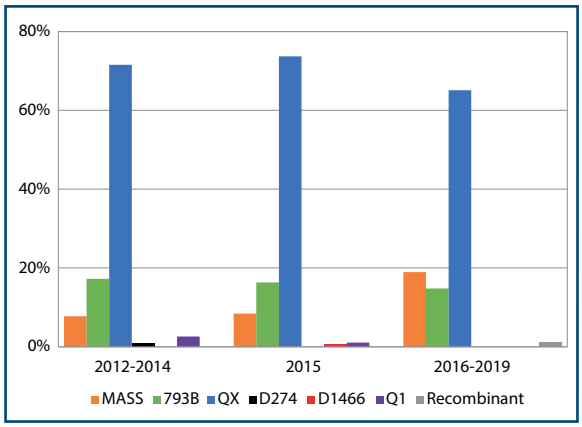

Figure 1. *Relative frequency of detection of different lineages of Infectious Bronchitis virus in broiler farms throughout the three considered periods.*

cases (Moreno *et al.* 2017). These strains were identical to each other and were both detected in Campania in 2016, and probably originated from a single recombination event. The absence of further detections of similar strains in the same territory seems to suggest their poor viability.

Because of the considerable variability in the number of samples taken during each year, the considered timespan was divided into three periods to obtain more consistent outcomes: the first, from 2012 to 2014, when the adopted vaccination comprised Mass and 793B vaccines; the second, limited to 2015, which was considered as a transitional period from a 793B to a QX-based vaccination, since several production cycles may be needed to observe the benefits of a newly implemented protocol (Franzo *et al.* 2016); the third, from 2016 onwards, when the only adopted vaccines were Mass- and QX-based ones.

As showed in Figure 1, while the frequency of QX and 793B detections was comparable throughout the three periods, a statistically significant rise was observed in Mass detections in the period 2016-2019. Since the use of Mass vaccines remained constant, this finding may not be caused by an actual increase in the Mass population size, but it could be the result of the different interactions of Mass-based vaccines with 793B and QX ones. When administered together, 793B vaccines persist at higher titers than Mass ones at a late age (Tucciarone *et al.* 2018), while Mass vaccines replicate more than QX ones after approximately 35 doa when co-administered at 1 doa (Russo *et al.* 2016). Even if multiple strains are present in the same sample, the RT-PCR assay applied in this study allows to amplify and characterize only one of them, usually the predominant one or the one with more affinity for the used primers, possibly conditioning the probability of detecting one or the other strain. Thus, considering that the mean age of sampling was 36.3 days, 793B vaccines may have been detected more easily than Mass ones when they were co-present, while Mass strains could have been able to overcome the QX vaccine competition.

Based on the obtained results, it is unclear whether the introduction of QX-based vaccines has led to significant benefits in fighting the disease. On the one hand, the ratio between QX field strains and vaccine ones has progressively lowered after the introduction of homologous vaccines in 2016, and the number of samples conferred to our laboratory has consistently decreased in the last years, possibly reflecting a similar trend in IB-related problems in the considered poultry farms, thus reducing the need of diagnostic confirmation. However, no significant differences were found between the relative number of potential IB outbreaks reported before and after the change in vaccination protocol. Similarly, the frequency of QX detections in presence of symptoms did not change regardless of the administered vaccines. In addition, the possible reduction of IB outbreaks may also be attributable to the recent trend, in the considered poultry farms and more generally in the whole Italian productive system, towards the administration of multiple IBV vaccines at the hatchery, which seems to guarantee a more standardized administration and a better vaccine coverage (Franzo *et al.* 2016).

Conclusions

The present work confirms that vaccination strategies may have significant and sometimes unpredictable consequences on IBV molecular epidemiology and diagnosis. In particular, the implemented vaccination protocol may influence the outcome of RT-PCR-based assays, leading to the preferential detection of a strain over the others based on the different interactions between vaccines. For this reason, vaccination protocols should always be taken into account when choosing a diagnostic assay and for a proper interpretation of the results. It appears evident that works aimed at assessing how different combinations of vaccines interact would be of great benefit for a more conscious implementation of vaccination.

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