Vaccine or field strains: the jigsaw pattern of infectious bronchitis virus molecular epidemiology in Poland

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ABSTRACT Infectious bronchitis (**IB**), caused by infectious bronchitis virus (**IBV**), account for severe economic losses in the poultry industry. The continuous emergence of a multitude of IBV variants poses many challenges for its diagnosis and control, and live attenuated vaccines, despite their routine use, still plays a significant role in driving IBV evolution, further complicating the epidemiological scenario. Unfortunately, the impact of different vaccination strategies on IB control, epidemiology, and diagnosis has rarely been investigated.

This work presents the results of a large-scale diagnostic survey performed in Poland to study IBV molecular epidemiology and how vaccination may affect the viral circulation in the field. To this purpose, 589 samples were collected between May 2017 and January 2019, tested by reverse transcription-PCR for IBV and sequenced. Vaccine and field strains were discriminated based on genetic and anamnestic information.

The most commonly detected lineages were 793B (79%) and variant 2 (17.4%), with sporadic detections of QX, Mass, and D274-like strains. Most of the detected strains had a vaccine origin: 46.3% matched one of the applied vaccines, while 36.5% were genetically related to vaccines not implemented in the respective protocol. Besides their practical value for the proper planning of vaccination protocols in Poland, these results suggest that only a fraction (17.2%) of the circulating strains are field ones, imposing a careful assessment of the actual IBV field menaces. Moreover, phenomena like vaccine spreading and persistence seem to occur commonly, stressing the need to further study the epidemiological consequences of the extensive use of live vaccines.

Key words: infectious bronchitis virus, Poland, molecular epidemiology, vaccine spreading, variant 2

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INTRODUCTION

Infectious bronchitis virus (**IBV**) is a worldwide distributed enveloped ssRNA+ virus that belongs to the species *Avian coronavirus*, genus *Gammacoronavirus*, family *Coronaviridae*. It is the causative agent of infectious bronchitis (**IB**), a highly contagious disease that affects the respiratory, reproductive, and renal systems of chickens of all ages and types (De Wit et al., 2011). Its economic impact on the poultry industry is second only to highly pathogenic avian influenza viruses, and IB is estimated to account for the third highest losses among all livestock diseases (TAFS-Forum, 2011).

IBV control relies on routine vaccination, along with proper biosecurity measures (De Wit et al., 2011). However, this approach is hindered by the high genetic variability of the virus, due to both mutation and recombination events, resulting in the continuous emergence of new variants with generally poor cross-protection (Bande et al., 2015). Multiple vaccines, either live attenuated or inactivated, are usually combined in the attempt to obtain a proper protection: the protection against a certain variant may be achieved by administering either a single vaccination based on a strain of the same lineage (homologous vaccination) or multiple vaccines based on different lineages to broaden the protection spectrum, following the so-called "protectotype" concept (heterologous vaccination) (Cook et al., 1999; Jordan, 2017).

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Despite essential to a proper control of the disease, the extensive use of IBV vaccines has some well-known drawbacks. The impact of vaccination protocols on circulating strains, which include field, live-vaccine and also "vaccine-derived" strains, proved remarkable and some lineages even disappeared after the discontinuance of homologous vaccination (Franzo et al., 2014, 2016). Live vaccine strains may spread to unvaccinated flocks, regain virulence because of rolling-like reactions or recombine with other circulating strains, possibly resulting in the emergence of new variants (Matthijs et al., 2008; Jackwood and Lee, 2017; Moreno et al., 2017). In addition, their use has been suggested to increase IBV mutation rate by applying an immunologic pressure on the field population (Jackwood et al., 2012). The persistence of vaccine strains also complicates IBV diagnosis, since many detected strains are usually closely related or identical to the applied vaccines (Jackwood and Lee, 2017). Therefore, clearly vaccination protocols must be taken into account to delineate an accurate picture of IBV epidemiological context within a given area.

In this work, a large scale diagnostic survey was conducted over a 20 mo timespan in Poland, the European leading country in poultry meat production (http://www.fao.org/faostat), to obtain updated information about circulating strains and commonly administered vaccines and study how vaccination choices may affect IBV epidemiology.

MATERIALS AND METHODS

Samples were collected from broiler farms in Poland and consisted of pools of 10 swabs, either tracheal or cloacal. Information about collection date, birds age, applied vaccination, and presence of symptoms was also recorded. Pooled swabs were eluted in 2 mL of 1% PBS solution, and RNA extraction was performed with High Pure RNA Isolation Kit (ROCHE, Basel, CH). Samples were tested for IBV by RT-PCR using SuperScript III One-Step RT-PCR System with Platinum TaqDNA Polymerase kit (Thermo Fisher, Waltham, MA), amplifying a 464 bp hyper-variable region of the S1 gene with XCE1+ and XCE2- primers described by Cavanagh et al. (1999). Positive samples were Sanger-sequenced using the same primers. The chromatograms were evaluated with FinchTV software (http://www.geospiza.com) and consensus sequences were assembled with CromasPRO 1.5 software (http://technelysium.com.au/wp/chromaspro/). Sequences were then aligned using the MUSCLE method implemented in MEGA7 (Kumar et al., 2016) and analyzed for strain genotypization and characterization by comparing them to a database including a set of commonly applied vaccine strains and Valastro et al. (2016) reference sequences.

The detected strains were classified as vaccines when they were identical or closely related (p-distance <0.01) to one of the administered vaccine (Worthington et al., 2008). The other strains were considered: a field strain, if the p-distance was higher than 0.01 compared to known vaccines or a vaccine-derived one, if they were closely related (p-distance <0.01) to known vaccines not matching the ones applied in the farm.

Pearson's Chi-squared test with Yates' continuity correction was applied to infer non-casual distributions of categorical variables. All statistical analyses were performed using R software, setting the significance level to $P < 0.05$.

RESULTS

A total of 589 samples were collected during the period from May 2017 to January 2019. Samples consisted of tracheal (5.3%) or cloacal (44.5%) swabs or a combination of both (50.2%); mean age at sampling was 35.7 D of age (**doa**). In 26 cases, respiratory and/or renal symptoms possibly due to IB were reported; in all the other cases, samples were taken for IBV routine monitoring.

More than 70 different vaccination protocols against IBV were implemented, based solely on live attenuated vaccines and differing in applied vaccines and/or administration schedule. 83.4% of these were based on the combined administration of at least 2 IBV vaccines at 1 doa; 2 or more vaccines were administered at different time points in 9.7% of the cases, while 6.6% of the protocols relied on a single IBV vaccine. No IBV vaccination was performed in 2 cases. The most frequently adopted protocol was based on Mass (lineage GI-I) and 793B (lineage GI-13) vaccines administered at day 1 (225 out of 589 samples, 38.2%). Mass vaccines were implemented in 575 cases (97.6%), while 490 flocks were vaccinated with 793B ones (83.2%): in detail, vaccines based on 4/91, 1/96, and CR88 strains were applied 101 (17.1%), 352 (59.8%) and 37 (6.3%) times, respectively. Other adopted vaccines were based on variant 2, D274, and QX strains, and were implemented in 98 (16.6%), 57 (9.7%), and 9 (1.5%) farms respectively.

A total of 501 samples (85%) tested positive for IBV, and 482 high-quality sequences were retrieved. The sequencing results are presented in Table 1. 793B was the most frequently detected one (382 out of 482 sequences; 79.3%): 224 of the 793B sequences (58.6%) were identical or closely related (i.e., less than 1% genetic distance) to strain 4/91, 125 (32.7%) to strain 1/96 and 5 (1%) to strain CR88, while 28 strains (7.3%) were classified as 793B field strains. A total of 84 strains (17.4%) belonged to variant (lineage GI-23), while QX (GI-19), Mass (GI-I), and D274-like (GI-12) were identified in 9 (1.9%), 5 (1%), and 2 (0.4%) samples, respectively. The remaining 88 samples were negative, including the two samples taken from unvaccinated flocks. Among the different matrixes (tracheal and cloacal swabs or a combination of both), no significant differences in terms of frequency of the identified lineages were detected.

When the correspondence between vaccination strategy and obtained sequence was evaluated, one of the

Table 1. Sequencing results characterized by lineage and vaccine or field origin. Strains were classified as vaccine strains, when genetically identical or closely related (p-distance < 0.01) to one of the applied vaccines, vaccine-derived strains, when genetically identical or closely related to a vaccine not administered in the respective flock, or field strains when not closely related (p-distance > 0.01) to any known vaccine.

Lineage	Total detected strains	Vaccine strains		Vaccine-derived strains		Field strains
G1–1 (Mass)	5	5		/		/
G1–12 (D274-like)	2	2		/		/
G1–13 (793B)	382	1/96 4/91 CR88	117 65 5	1/96 4/91 CR88	8 159 /	28
G1–19 (QX)	9	3		2		4
G1–23 (Variant 2)	84	26		7		51
Total	482	223		176		83

applied vaccines was detected in 46.3% (223/482) of IBV positive samples. As for the remaining 259 strains, 176 (36.5%) were classified as vaccine-derived and 83 (17.2%) as field ones. In all 93.6% of 1/96-like detections occurred when a homologous vaccine was applied, as opposed to 4/91-like (27.4%), Variant 2 (30.9%) and QX (33.3%) detections. All Mass, CR88-like, and D274 detections occurred when a homologous vaccination was implemented. Regarding the 83 field strains, 28 were classified as 793B, 4 as QX, and 51 as variant 2. The frequency of vaccine and field strains detections showed no significant differences among different matrixes.

Considering only the 26 samples taken from symptomatic flocks, 5 of the detected strains (19.2%) were vaccines, 10 (38.4%) were classified as 4/91-like vaccine-derived strains, and 11 (42.4%) were field ones. Among the field strains, 7 were variant 2, 3 793B, and 1 QX.

DISCUSSION

The present study depicts a complex and intricate epidemiological scenario, entangled by the simultaneous circulation of field and vaccine strains, the latter being the vast majority (82.8%). Remarkably, the evaluation of the applied vaccination strategies revealed a surprisingly intricate scenario, involving more than 70 different protocols. The most frequent approach for combining multiple IBV vaccines was to apply them simultaneously at 1 doa (83.4%). Despite the fact that separate administrations of 2 or more IBV vaccines are traditionally considered more effective, possibly because of the booster effect and respiratory epithelium recovery between the applications (Cook et al., 1999; De Wit et al., 2010), nowadays the combined administration at 1 doa is commonly adopted in different countries (Franzo et al., 2016; Jordan, 2017). In fact, hatchery vaccination allows a higher standardization and broader vaccine coverage than field vaccination (Franzo et al., 2016) and at the same time eliminates the need of a second administration, thus reducing the costs. The present study also confirms this trend in Poland. Unfortunately, the limited number of farms applying separate vaccinations and the negligible number of symptomatic flocks prevented the comparison of the efficacy of the 2 strategies. Nonetheless, the relatively low amount of detected field strains (17.2%) and clinical outbreaks (4.4%) seems to support the efficacy of widespread vaccination, regardless of the adopted strategy.

The greater part of the obtained sequences (79.3%) was classified as 793B. Since 92.7% of them were of vaccine origin (4/91, 1/96, or CR88-like), the high prevalence of this lineage may be due to the vast application of 793B vaccines, administered in 83.2% of the sampled flocks. Interestingly, although Mass vaccines were provided even more frequently (97.6% of the times) and often together with the 793B ones, Mass strains were only found in 5 samples. The more frequent detection of 793B strains may be explained by 793B vaccines persisting longer and/or reaching higher titers than the Mass ones in field conditions (Cavanagh et al., 1999; Tucciarone et al., 2018).

The other detected strains were classified as D274-like, QX, and variant 2 strains. While D274-like strains were found only twice in D274-vaccinated flocks, the relevance of QX and variant 2 as field lineages is well known. QX was detected for the first time in China in 1996 and, as of today, it is probably the most important field lineage in both Asia and Europe (Franzo et al., 2017a). It is responsible for severe economic losses, causing respiratory and renal symptoms and is also associated to the "false-layer syndrome" in breeding and laying hens (De Wit et al., 2011). Variant 2 is an emerging lineage that, after staying confined to Israel for nearly a decade following its first detection in 1998 (Callison et al., 2001), spread firstly to the whole Middle East, then to Turkey and Eastern Europe (Ganapathy et al., 2015; Yilmaz et al., 2016; Franzo et al., 2017b). Poland is the first European country where this nephropathogenic variant was detected in 2015, and the threat of its remarkable clinical impact has elicited the introduction of a homologous vaccine (Lisowska et al., 2017). Of the 9 QX detections, 3 vaccine strains were detected in QX-vaccinated flocks, 2 were labeled as vaccine-derived strains, and 4 as field ones. Variant 2 strains were detected in 84 samples: 26 were classified

as vaccine, 8 as vaccine-derived, and 51 as field strains, highlighting the persistence of this lineage in Poland in the considered period. Therefore, the relevance of these lineages in Poland appears to be modest, especially from a clinical perspective.

The large number of vaccine-derived strains (36.5% of the detections) certainly raises some questions on the capability of live vaccines to spread and persist, even in farms where they are not adopted. Notably, 89.3% of the detected vaccine-derived strains were 4/91-like ones. In all 72.8% of the 4/91 detections occurred in farms not adopting this vaccine, as opposed to 6.4% and none of the 1/96 and CR88 detections: the significant difference (P-value < 0.001) suggests a higher spreading capability of the 4/91 vaccine compared to the 1/96-based one, as previously reported (Franzo et al., 2014; Pellattiero et al., 2018). Several causes could justify the observed scenario, including a higher vaccine shedding or longer persistence. Although unlikely, an underling association between farm management and employed vaccine, favoring the dispersion of specific vaccines, cannot totally be excluded.

A possible explanation for the several discrepancies between sequencing results and administered vaccines might reside in the old age of the sampled birds. When Mass and/or 793B vaccines are administered at 1 doa, their titers are expected to peak approximatively at 10 to 14 doa, way before the average age of sampling in this study (i.e., 35.7 doa), and then gradually decrease (Tucciarone et al., 2018). This was confirmed by the fact that one of the applied vaccines was detected 90% of the times when birds were sampled before 16 doa, as opposed to 45.6% of the times when they had 16 or more doa. Therefore, it seems plausible that field and vaccine-derived strains could penetrate in a farm after the applied vaccines decline. Since most of discordances between applied and detected vaccines occurred within the 793B lineage, further efforts should be directed to investigate the causes behind the limited protection from infection even between homologous vaccines.

A strain not matching with any of the applied vaccines was detected in 73.3% of the symptomatic flocks, as opposed to 46.3% in healthy flocks: the difference was statistically significant (P-value $= 0.032$), thus supporting the possible causative role of the detected strains. These strains were classified as 793B (13 detections, of which 10 4/91-like vaccine-derived strains and 3 field ones), Variant 2 (7 field strains) and QX (1 field strain). In fact, while a link between symptoms and detections of variant 2, QX, and 793B field strains can easily be justified, the detection of 4/91-like strains in symptomatic animals should draw attention to the possible pathogenic role of vaccine-derived strains. The number of vaccine-derived and field strains detected in symptomatic flocks is substantially equal, and this should stimulate a discussion on the advantages and disadvantages of a widespread, poorly-planned vaccine application. However, these findings are not conclusive. Even when overt clinical signs are observed, it is practically impossible to certainly determine based solely on molecular analyses if the detected strain is a truly pathogenic one or a virus of vaccine origin incidentally detected in presence of other causative pathogens (Jackwood and Lee, 2017). Moreover, no genetic markers are currently known to consistently distinguish between vaccine and field strains (Jackwood and Lee, 2017). Although a combination of phylogenetic and epidemiological criteria was adopted in the present study to confidently classify sequenced strains, the presence of some misclassification due to the circulation of actual field strains closely related (at least in the considered genomic region) to vaccine viruses cannot be excluded and will deserve more extensive evaluations, possibly based on the entire S1 gene sequence. Nonetheless, besides the potential direct cost of vaccine-induced clinical signs, the endemic circulation of vaccine strains has other effects on IBV epidemiology, increasing the likelihood of recombination events and the genesis of strains with unpredictable features. As demonstrated in the present study, their persistence also severely complicates the interpretation of the epidemiological scenario and thus the planning of effective control strategies.

According to the obtained results, the impact of IBV on poultry health in Poland appears scaled down, likely because of the widespread vaccination. Field strains (belonging to lineages variant 2, 793B, and QX) accounted for a limited part of the total detected strains and were seldom detected in presence of symptoms. To cope with the relatively limited IBV challenge, an outstanding high number of different vaccination protocols were observed. This could be imputable to the organization of the poultry industry in Poland, where vertical integration is quite loose, allowing a certain plasticity of the managerial decisions on vaccination mainly based on the farm history and requirements.

This work may be of great practical help, providing updated information about the strains actually circulating in Poland. Given the extreme heterogeneity of vaccination plans applied in this country, an effort to homogenize them would be hugely beneficial and facilitate the understanding of IBV epidemiology, allowing a more efficient use of limited resources, a maximization of cost-benefit ratio and farm profitability and a reduction of the consequences that uncontrolled circulation of vaccine-derived strains circulation can cause on IBV diagnostics and evolution.

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