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CICLO XXXIV

Molecular epidemiology of infectious bronchitis virus: insights into the interplay between viral evolution, diagnosis and control

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Coordinatore: Prof. Cecchinato Mattia

Supervisore: Prof. Cecchinato Mattia

Dottorando: Legnardi Matteo

Index

Introduction

The poultry sector plays a pivotal role in the global food industry, both from an economic and a social standpoint. Poultry meat production has been estimated at 337 million tonnes in 2020, more than any other meat type (FAO, 2021), and represents the main growth driver of the meat market in virtually every country, both in low- and high-income regions (OECD/FAO, 2021). Additionally, egg production is steadily increasing and in 2019 it amounted to more than 84 million tonnes (http://www.fao.org/faostat), representing another key source of high-quality protein worldwide.

Poultry production systems are highly diverse, ranging from backyard farming to intensive methods featured by a high level of mechanization and vertical integration. However, infectious diseases represent a constant threat at every level. The proximity of many susceptible hosts, their high turnover rate and genetic similarity, the immunodepression caused by stressful transport and farming conditions are just some of the factors favoring the occurrence and increasing the impact of infectious disease outbreaks in poultry (Espinosa et al., 2020). In addition, the rise of alternative poultry production and the growing pressure to reduce the use of antimicrobials pose additional difficulties in the prevention and control of infectious diseases (Jeni et al., 2021).

Among the most relevant poultry pathogens is avian coronavirus, more commonly known as infectious bronchitis virus (IBV). According to a quantitative analysis conducted by the World Bank (2011), infectious bronchitis (IB) was the third animal disease to cause the highest livestock losses globally between 2006 and 2009, after highly pathogenic avian influenza and echinococcosis. Its economic burden is further confirmed by several studies conducted in different production systems and geographic contexts (Biggs, 1982; Colvero et al., 2015; Jones et al., 2018). IBV relevance is also reflected by the attention it attracts among researchers, which makes this virus the fifth most studied poultry pathogen (Bertran et al., 2020).

Etiology

IBV belongs to the order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae* (https://ictv.global/taxonomy/). Four genera are currently described among *Orthocoronavirinae*: *Alphacoronavirus* and *Betacoronavirus* groups are viruses of mammals, while viral species from *Deltacoronavirus* and *Gammacoronavirus* infect mainly birds. The genus

Gammacoronavirus comprises three subgenera: *Igacovirus*, which includes avian coronavirus as well as other viruses infecting turkeys, guineafowls and other fowls; *Brangacovirus*, whose only representative is a goose coronavirus; and *Cegacovirus*, comprising viruses infecting marine mammals (Zhou et al., 2021). Based on phylogenetic comparisons, it is believed that these marine mammals coronaviruses originated from avian coronaviruses (Woo et al., 2014), with *Brangacovirus* probably representing a transition stage from *Igacovirus* to *Cegacovirus* (Papineau et al., 2019; Zhou et al., 2021).

IBV is an enveloped virus with a round or pleomorphic virion which is approximately 120 nm in size and originates from budding at internal host cell membranes (Cavanagh et al., 2007). It features a single-stranded, positive-sense RNA genome whose length is approximately 28 kb. The genome organization is 5'-untranslated region (UTR)-1a/1ab-S-3a-3b-E-M-5a-5b-N-UTR-3' (Jackwood et al., 2007). The two UTRs at the 5' and 3' ends contain *cis*-acting elements and play a role in RNA transcription and replication by interacting with viral replicases and possibly other host proteins (Yang & Leibowitz, 2015). More than half of the genome is constituted by ORFs 1a and 1ab, largely overlapped through a -1 ribosomal frameshift, that encode polyproteins that are post-translationally cleaved into 15 nonstructural proteins (nsp2-16). These nsps perform a variety of functions, including the formation of the RNA-dependent-RNA-polymerase (RdRp). The 3a, 3b, 5a and 5b ORFs encode for other nsps, whose functions are mostly unknown (Jackwood et al., 2007). However, protein 5b has been proven to be involved in the inhibition of host protein production. This makes up for the fact that, due to a missing cleavage site, gammacoronaviruses do not express nsp1, which is responsible for host shutoff in viruses belonging to the genera *Alphacoronavirus* and *Betacoronavirus* (Kint et al., 2016).

IBV is featured by four structural proteins, namely the Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N) proteins. The S glycoprotein is a type I transmembrane protein which forms club-shaped protrusions on the virion surface, determining the peculiar crown-like appearance of coronaviruses. It is post-translationally cleaved in two subunits, S1 and S2, by a furin-like protease in the Golgi complex (de Haan et al., 2004). The S1 gene forms the head of the spike and plays a crucial role in host receptor binding, containing many key epitopes inducing neutralizing antibodies. Being a major target of the host immune response, the S1 gene is under an intense selection pressure, which results in a remarkable genetic heterogeneity. For these reasons, it represents by far the most studied portion of the genome. On the other hand, the S2 subunit, which forms the stem of the spike, is far more conserved (Belouzard et al., 2012). The S2 subunit is involved in the virus-cell membrane fusion process and is thought

to play a role in determining viral tropism (Bickerton et al., 2018), while the strict host range of IBV seem to be determined by an interplay between the two subunits (Promkuntod et al., 2013). The E protein is implicated in virion budding and promoting the efficient release of IBV by altering the host cell secretory pathway and Golgi pH (Westerbeck & Machamer, 2019). Together with the M protein, which is the most abundant component of the envelope, it also contributes to virion assembly (Wang et al., 2009). Finally, the N protein packages the viral RNA into a ribonucleoprotein complex and participates in RNA replication and transcription (Fan et al., 2005).

Evolution

RNA viruses are known as prone to mutations, which occur at a rate several orders of magnitude higher than those of their DNA counterparts. This high mutation rate is mainly attributed to their reliance on RNA-dependent polymerases, which lack any proofreading activity and are therefore more error-prone than DNA polymerases. This allows for a rapid adaptation to the pressure elicited by the host immune response or therapy (Figlerowicz et al., 2003), making RNA viruses responsible for most emerging diseases (Carrasco-Hernandez et al., 2017). While this is true also for members of *Coronaviridae*, this family represents somewhat an exception. In fact, coronaviruses possess the largest known genomes among RNA viruses, an unsustainable feature if the substitution rate was on par with viruses with significantly smaller genomes (Holmes, 2009). It is hypothesized that coronaviruses might be able to reduce the error rate through some sort of repair mechanisms, possibly attributed to an ExoN domain found in their replicase gene, which is also twice the size of most RNA viruses. This domain, encoding for a 3'-to-5' exoribonuclease, is related to the DEDD superfamily of exonucleases, which are involved in proofreading activities (Minskaia et al., 2006). Nonetheless, there is no doubt that the mutation rate of coronaviruses, estimated at 10^{-4}-10^{-5} substitutions/site/year in the case of IBV (Franzo et al., 2017), is still enough to ensure a noteworthy evolutionary potential.

In addition to mutation, recombination is another relevant source of genetic variability for IBV. This occurs by means of a copy-choice mechanism: coronaviruses are featured by a discontinuous replication process, in which the RdRp and RNA transcript regularly detach from the RNA template; the transcript may therefore join a different template to progress RNA synthesis, thus generating a chimeric RNA molecule with features inherited from both templates (Lai, 1996). In the case of IBV, a multitude of natural recombination events have been identified over the years (Cavanagh et al., 1992; Casais et al., 2003; Moreno et al., 2017; Huang

et al., 2021), likely favored by the frequent co-circulation of multiple IBV strains (Capua et al., 1999; Al-Mubarak et al., 2020).

Classification

IBV marked genetic variability has led to the emergence of countless variants, which differ in terms of antigenicity, pathogenicity, tissue tropism and geographical distribution. Different classification criteria are used to characterize them based on different features, either functional or genomic.

The first classification is based on serotypes and groups together strains that produce reciprocally neutralizing antibodies, assessed by two-way virus neutralization (VN) testing. Despite its usefulness, this approach suffers from some limitations: standardization between different laboratories is difficult, and every serotype requires a separate neutralization test with a dedicated antiserum, resulting in high costs (De Wit, 2000). Hemagglutination inhibition tests (HI) may be used as a simpler alternative, but strains need to be previously treated with neuraminidase and results interpretation is complicated by frequent cross-reactions (Cook et al., 1987). For all these reasons, and due to the increasing occurrence of co-circulations, serotyping is becoming less and less practical, finding little use in routine diagnostic activities (World Organization of Animal Health, 2018).

A second functional classification is based on protectotypes, which group together strains capable of inducing protection against each other (Lohr, 1988; Cook et al., 1999). This method is of great practical value as it directly informs the planning of effective vaccination protocols. However, the cross-immunization tests required for a comprehensive assessment of protection efficacy are laborious and hard to standardize (De Wit et al., 2000). Protectotypes should be therefore intended more as a concept than a coherent, systematic classification.

The last classification, which is the most frequently used nowadays, is based on viral genetic traits. Until recently, its standardization has been hampered by a lack of consensus over the assay to use, the genomic portion to consider and the nomenclature, which could generate confusion from both research and diagnostic perspectives. Most of these issues have been solved by the recent proposal of a comprehensive and robust phylogenetic classification scheme, based on the entire sequence of the S1 gene, which identified a total of six genotypes (from GI to GVI), further divided into 32 lineages (GI-1, GI-2, etc.) and several unique variants (Valastro et al., 2016). As a rule of thumb, different genotypes feature 30% and 31% distances at nucleotide and amino acid level, respectively; for lineages, which should include at least three strains from at

least two different outbreaks to be considered as such, mutual differences should be higher than 13% at nucleotide and 14% at amino acid level. Subsequent works have further expanded this classification (Chen et al., 2017; Jiang et al., 2017; Ma et al., 2019; Domanska-Blicharz et al., 2020), leading to the current recognition of 8 genotypes and 36 lineages (Figure 1).

The three described classification methods do not correlate perfectly. For instance, protectotypes are generally broader than serotypes. This is due to different serotypes sharing some common epitopes, and to the immune response not being limited to the production of neutralizing antibodies (De Wit et al., 2000). As for serotyping and genotyping, the two methods are featured by some degree of overlap, but the difference exhibited by different serotypes at S1 amino acid sequence level may vary from 50% to as low as 2% (World Organization for Animal Health, 2018), resulting in significant exceptions (Mondal & Cardona, 2007; Feng et al., 2017). Finally, the disagreement between genotyping and protectotyping is demonstrated by several *in vivo* challenge studies, which consistently report how vaccines can elicit at least a partial protection against strains belonging to other lineages (Cook et al., 1999; Terregino et al., 2008; Awad et al., 2015, 2016; Toro et al., 2015).

Host range, transmission and clinical manifestations

IBV is highly contagious and causes disease in all types of chickens at every age, albeit the signs are usually more severe in young birds (Bande et al., 2016). IB is traditionally considered a disease of chickens, and there is no evidence it may affect other hosts. However, IBV has been isolated in other species, such as peafowls and teals (Liu et al., 2005); IBV-like viruses have also been found in many species including pheasants, quails, pigeons and wild anatids (Cavanagh, 2005; Cavanagh et al., 2002; Hughes et al., 2009; Domanska-Blicharz et al., 2014; Torres et al., 2017).

IBV is transmitted either by direct or indirect contact through inhalation or ingestion of virus particles, which are excreted in high concentration in aerosol (Jackwood & de Wit, 2020), faeces and possibly uric acid (Dolz et al., 2013). Venereal transmission also seems possible (Gallardo et al., 2011), while the vertical route is not considered relevant, despite IBV being detected in viable embryos and hatched chicks from experimentally infected specific pathogen-free (SPF) layers (Cook et al., 1971; Pereira et al., 2016).

In spite of its denomination, the fact that IBV is not responsible only of respiratory signs is known at least since the 1950s (Cook et al., 2012). IBV can replicate in a range of epithelial cells, including those of the trachea, lungs, kidney, oviducts, testes, bursa of Fabricius and

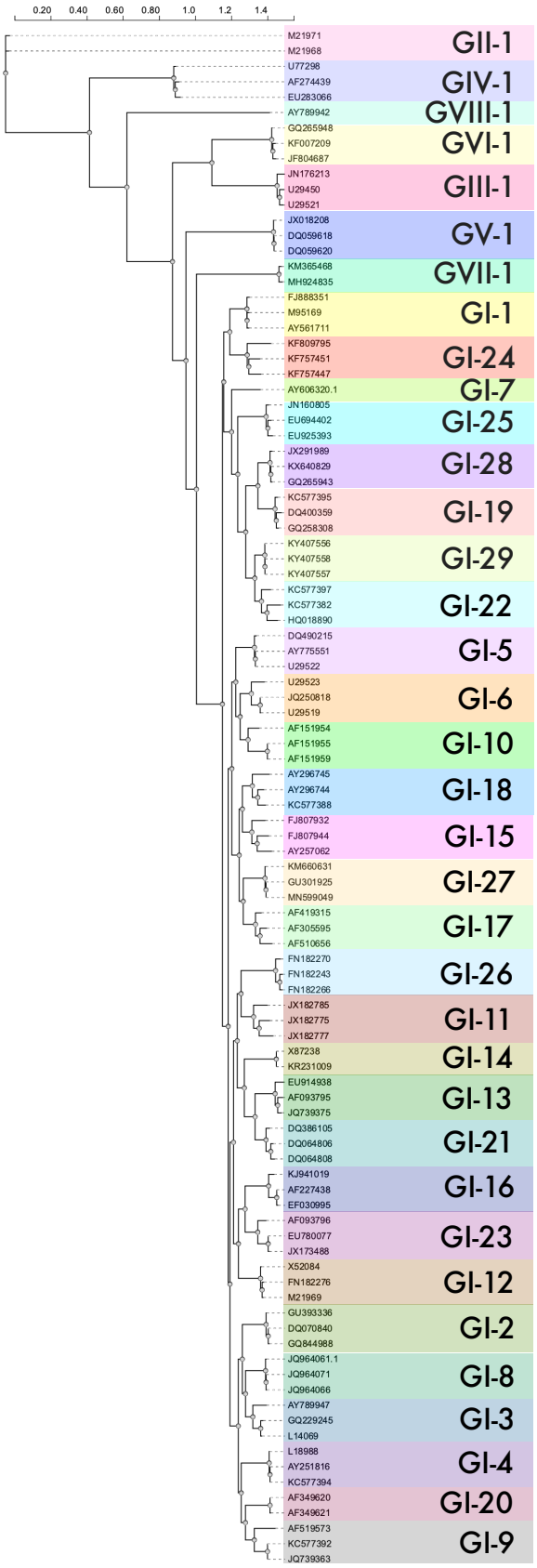

Figure 1. Phylogenetic relations between currently recognized lineages. A total of 101 full S1 reference sequences were aligned with FFT-NS-i in MAFFT online (Katoh et al., 2002), then a tree was built with Maximum Likelihood method (1000 bootstraps) in PhyML (Guindon et al., 2010).

gastrointestinal tract (Cavanagh, 2007). However, significant and well documented differences may be observed in tissue tropism depending on the strain involved (Dhinakar Raj & Jones, 1997; Lee et al., 2002).

Primary infection always occurs at the respiratory level, possibly resulting in signs such as sneezing, rales, nasal discharge, coughing and listlessness, typically after an incubation of 24-48 hours (Cavanagh, 2007). The infection may then spread systemically, resulting in a more complex symptomatology. Nephropathogenic strains cause nonspecific signs such as depression, wet droppings and increased water intake, which are usually observed in broilers (Bande et al., 2016). When laying hens are affected, the involvement of the oviduct may result in a drop in egg production, whose severity may vary from mild to more than 70% of production losses. Egg production may not recover or take weeks or months to get back to pre-infection levels. Egg quality may also be affected, with a frequent occurrence of depigmented, misshapen, soft or rough shells and watery albumens (Jackwood & de Wit, 2020). Additionally, the infection of female chicks soon after hatch may result in the so-called "false layer syndrome", in which the oviduct becomes cystic and non-patent and egg-laying is thus irreversibly impeded (De Wit et al., 2011b; Cook et al., 2012). IBV has also been proposed as a causative agent of proventriculitis (Yu et al., 2001; Pantin-Jackwood et al., 2005), but a clear causality link is yet to be established (de Wit et al., 2011a).

Mortality may vary greatly depending on the strain involved, the age and immune status of the birds and the possible presence of secondary pathogens (Jackwood & de Wit, 2020). In particular, interactions have been observed with pathogens such as *Escherichia coli* (Matthijs et al., 2009), *Mycoplasma spp.* (Ganapathy & Bradbury, 1999; Kleven et al., 1978), *Ornithobacterium rhinotracheale* (Thachil et al., 2009), avian influenza (Belkasmi et al., 2020) and Newcastle disease virus (Nakamura et al., 1992).

Diagnosis

IBV diagnosis relies on a range of different laboratory techniques, each with its advantages and limitations. The main diagnostic tests include viral isolation, serological and molecular assays. Clinical evaluation, necropsies and consultation of zootechnical parameters are useful to retrieve only supporting evidence, due to the absence of pathognomonic signs or lesions.

Viral isolation may be performed on embryonated chicken eggs, by inoculating samples into the allantoic cavity between 8 and 11 days of incubation, or on tracheal organ cultures (TOCs) from SPF embryos (World Organization of Animal Health, 2018). Cell cultures may also be

used but they are considered less efficient, and strain adaptation is often required to induce a cytopathic effect (Darbyshire et al., 1975). Suitable samples may be collected from trachea, kidneys or oviduct. Isolation from cloacal samples is also possible, but the recovery rate is lower. For a successful incubation, it is of the utmost importance to collect samples as close as possible to the beginning of infection, and to keep them under ice until the arrival at the laboratory (Jackwood & de Wit, 2020). Infected embryos show lesions such as dwarfing, curling, urate deposits in the kidney and death, usually between the second and the fourth passage, while ciliostasis is observed in TOCs within 24-48 hours (World Organization of Animal Health, 2018); however, these findings are indistinguishable from the ones caused by other viruses, most notably adenovirus (McMartin, 1993), thus requiring further serological or molecular analyses for confirmation. Viral isolation is still essential for vaccine production and many research purposes, such as to evaluate pathogenicity, assess the protection conferred by vaccines against challenge strains and facilitate whole genome sequencing. On the other hand, it is rarely used in routine diagnostics due to its slow turnaround time and stringent requirements (de Wit et al., 2000).

Several serological tests are available for IBV diagnosis, including VN, HI, ELISA assays and agar gel precipitation test (AGP). As previously mentioned, only VN and HI allow serotypization. VN may be performed by varying the viral dilution and keeping a constant serum concentration (alpha method) or vice versa (beta method); the latter tends to be more widely used due to higher precision (Hesselink, 1991). Despite being considered the gold standard because of its high sensitivity, VN adoption is limited by its cost and laboriousness. In comparison, HI is more prone to cross-reactions, but it still finds some use because of its simplicity and speed (de Wit et al., 2000). AGP is another quite inexpensive technique that also features a high specificity, but it lacks sensitivity and correlates poorly with other serological tests due to short-lived precipitating antibodies (Monreal et al., 1985). ELISA tests are by far the most frequently adopted thanks to the number of commercially available kits, which allow for a quick, standardized and cost-effective titration of antibodies. Several in-house alternatives have also been developed (Karaca & Syed, 1993; Chen et al., 2011; Lei et al., 2017; Yin et al., 2021), many of which are based on serotype-specific monoclonal antibodies, but they are not widely accessible.

Nowadays, molecular tests are employed in most research and diagnostic activities. Biomolecular methods, mainly based on PCR, feature an exceptional sensitivity and a rapid turnaround. Suitable samples may be collected from different districts using different media, and

their storage and shipment usually have few requirements. A multitude of options is available in terms of RT-PCR assays, either commercial or in-house, traditional or real-time, generic or strain-specific (Cavanagh et al., 1999; Jackwood et al., 2003; Worthington et al., 2008; Jones et al., 2011; Tucciarone et al., 2018a). The main feature of molecular tests is the possibility to precisely characterize the detected strain, either through traditional Sanger sequencing, restriction fragment length polymorphism (RFLP) or Next Generation Sequencing (NGS). In addition to strain classification, these techniques serve many purposes, such as the reconstruction of IBV evolutionary history (Zhao et al., 2016), investigation of spreading dynamics (Franzo et al., 2017) and identification of viral subpopulations (Toro et al., 2012). Despite the absence of robust genetic markers, under certain circumstances it is also possible to differentiate vaccines from field strains (Mardani et al., 2006). Other molecular techniques have been described, including tests based on loop-mediated isothermal amplification (LAMP) (Chen et al., 2010), microarrays (Sultankulova et al., 2017) and padlock probes (Ciftci et al., 2019), but they are far less used than PCR-based ones.

Since none of the described assays can be considered fully exhaustive, the best diagnostic approach should rely on multiple tests and be tailored on the epidemiological context and desired outputs (e.g., confirmation of a suspected IB outbreak, routine monitoring, assessment of vaccine coverage). Other practical features to consider are the specifications and requirements of each test in terms of turnaround time, cost and required samples, which may need specific storage and shipping conditions. Another aspect of IBV diagnosis that deserves particular attention is the interpretation of the results. The implemented vaccination protocol is a crucial information to consider: when molecular tests are used, for instance, the replication of vaccine strains may mask the simultaneous presence of field strains (Cavanagh et al., 1999); as for serology, knowing the vaccination details is pivotal to know the immune response levels to expect. When available, data on the local epidemiological scenario and farm clinical history should also be taken into account, along with information on the time elapsed between the beginning of infection and sampling, presence of other pathogens (either suspected or confirmed), breed and productive type and housing conditions.

Control

Because of its ubiquity and impact on all production types, the proper control of IBV is of the utmost importance in every poultry farming system. IBV control mainly relies on mass vaccination, along with strict biosecurity procedures and proper management, such as the

adoption of one-age, all-in/all-out systems, proper cleaning and disinfection protocols (de Wit et al., 2011a). Not only vaccination allows to reduce clinical signs, but it may also help decreasing viral transmission and shedding (de Wit et al., 1998).

Several approaches are adopted, differing in terms of administered vaccine types and strains, administration route and vaccination timing. The broiler industry relies solely on live attenuated vaccines, while layers and breeders are primed with live attenuated vaccines followed by a boost with inactivated vaccines, or again with attenuated vaccines if the infectious pressure is high (Jordan et al., 2017). Despite some partial successes in the development of recombinant (Johnson et al., 2003; Toro et al., 2014; Tan et al., 2019) and subunit vaccines (Yang et al., 2009; Eldemery et al., 2017), all commercially available vaccines are produced with technologies that have remained substantially unchanged for over 50 years (Cook et al., 2012).

Live vaccines have two major advantages over killed ones: they are far more effective and can be mass administered. Mass application is performed either by spray, considered as the gold standard, or drinking water, which instead is becoming deprecated (Jackwood & de Wit, 2020); gel administration has also proved promising in experimental conditions (Tucciarone et al., 2018b), but it is not currently adopted. On the other hand, inactivated vaccines need to be injected individually, but they are inherently safer than attenuated ones, which may revert to virulence (Hopkins et al., 1986; Nix et al., 2000; Abozeid et al., 2017). In addition to rolling-like reactions, the cocirculation of live vaccines and field strains may also facilitate the occurrence of recombination events (Mardani et al., 2010; Quinteros et al., 2016; Moreno et al., 2017; Xu et al., 2019; Huang et al., 2021).

Aside from safety concerns, the main shortcoming of vaccination is related to the absent or partial cross-protection between many IBV subtypes. Despite the low correlation between genetic similarity and cross-protection, homologous vaccines are more likely to induce neutralizing antibodies than heterologous ones (de Wit et al., 2011a). Nonetheless, several challenge studies have demonstrated that protocols based on the combination of multiple heterologous vaccines may also be highly effective (Cook et al., 1999; Terregino et al., 2008; Awad et al., 2015, 2016). While the underlying immune mechanism is still not entirely known, the effectiveness of this strategy may depend on the high levels of cell-mediated immunity elicited at tracheal level (Cook et al., 1999; Smialek et al., 2017; Awad et al., 2016).

The timing of vaccination is another much debated topic, especially for broilers, with different schools of thought backed by partially contrasting evidence. On one hand, early vaccination seems to elicit a suboptimal humoral immune response, possibly due to interference

by maternally derived antibodies (Mondal & Naqi, 2001; Van Ginkel et al., 2015; Saiada et al., 2019). Similarly, when resorting to protocols based on multiple vaccines, the observation of a waiting period of at least two weeks between subsequent administrations induces a stronger humoral response (Cook et al., 1999; de Wit et al., 2010).

On the other hand, current trends at field level do not reflect these recommendations. Nowadays, many live IBV vaccines are marketed as suitable for day-old administration, and hatchery vaccination has become the norm in many production systems (Smialek et al., 2017; Abdul-Cader et al., 2018). Several factors contribute to this discrepancy: first, vaccine efficacy seems to depend more on local tracheal immunity than on humoral response (Awad et al., 2016); secondly, hatchery vaccination is easier to standardize and control compared to farm administration, resulting in an earlier and more uniform coverage (Franzo et al., 2016); thirdly, administering multiple vaccines to day-old broilers is often enough to protect them for the entire cycle, thus eliminating the need for additional administrations and reducing both vaccine-related costs and animal stress (Franzo et al., 2020). Ultimately, the protective efficacy of a given approach does not seem the sole deciding factor for its adoption, and its practical advantages and limitations should be considered equally important.

Aim of the thesis

This thesis collects the results of several studies exploring different aspects of IBV epidemiology. All included works rely on RT-PCR assays, which, as previously explained, are a staple of most IBV research and monitoring activities. However, their usefulness and reliability strongly depend on multiple factors, including a sound assay design, a rational implementation tailored to the epidemiological context of interest, and an attentive result interpretation based on complementary information. Here, the advantages and limitations of molecular methods are thoroughly discussed.

Another relevant topic is IBV control, and particularly vaccination. An updated perspective is provided on current trends in vaccination, which may vary widely between different production systems, and on their long-term, large-scale impact on IBV spread. Additionally, the feasibility of a novel mass administration method based on gel delivery is also evaluated in a field context.

Nonetheless, the main underlying subject of the reported works is probably viral evolution. At the very end, all reported studies deal with this topic, be it by investigating how it shapes genomic composition, reconstructing evolutionary dynamics at various levels, or describing how different strains coexist and interact.

The common thread of this work, however, is to demonstrate that addressing the topics of viral evolution, diagnosis and control separately is not sufficient to fully capture the complexity of IBV epidemiology. In particular, such an approach does not allow to take into account the many practical issues and managerial variables encountered in the field, which could require specific diagnostic or control solutions to be addressed effectively. Instead, one should focus on the many interconnections between these three subjects. While some are fairly well-studied, as the impact of genetic variability on vaccination protocols, others are less obvious and often overlooked. The collected field and experimental results, along with a review of the current literature, are used to corroborate the importance of a holistic approach towards the study of IBV, which, if adopted, could improve both the comprehension and control of this challenging and fascinating pathogen.

Infectious bronchitis virus evolution, diagnosis and control

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Review

Infectious Bronchitis Virus Evolution, Diagnosis and Control

Matteo Legnardi, Claudia Maria Tucciarone *, Giovanni Franzo and Mattia Cecchinato

Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università, 16, 35020 Legnaro, Italy; matteo.legnardi@phd.unipd.it (M.L.); giovanni.franzo@unipd.it (G.F.); mattia.cecchinato@unipd.it (M.C.)

* Correspondence: claudiamaria.tucciarone@unipd.it; Tel.: +39-04-9827-2968

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Abstract: RNA viruses are characterized by high mutation and recombination rates, which allow a rapid adaptation to new environments. Most of the emerging diseases and host jumps are therefore sustained by these viruses. Rapid evolution may also hinder the understanding of molecular epidemiology, affect the sensitivity of diagnostic assays, limit the vaccine efficacy and favor episodes of immune escape, thus significantly complicating the control of even well-known pathogens. The history of infectious bronchitis virus (IBV) fits well with the above-mentioned scenario. Despite being known since the 1930s, it still represents one of the main causes of disease and economic losses for the poultry industry. A plethora of strategies have been developed and applied over time, with variable success, to limit its impact. However, they have rarely been evaluated objectively and on an adequate scale. Therefore, the actual advantages and disadvantages of IBV detection and control strategies, as well as their implementation, still largely depend on individual sensibility. The present manuscript aims to review the main features of IBV biology and evolution, focusing on their relevance and potential applications in terms of diagnosis and control.

Keywords: infectious bronchitis virus; coronavirus; viral evolution; diagnosis; control; vaccination

1. Infectious Bronchitis Virus

Infectious bronchitis virus (IBV) is currently classified within the species *Avian coronavirus*, genus *Gammacoronavirus*, family *Coronaviridae*, order *Nidovirales*. IBV is an enveloped virus with a single-stranded positive-sense RNA genome of approximatively 27 kb, which displays the following gene organization: 5′UTR-1a/1ab-S-3a-3b-E-M-5a-5b-N-3′UTR. The RNA molecule has a 5′ *cap* and a 3′ poly(A) tail and acts as mRNA for translation of the replicase polyproteins, whose coding regions occupy the 5′ two-thirds of the viral genome. The two untranslated regions (UTR) play a key role in virus replication interacting with viral replicases and potentially other host proteins [1,2]. Non-structural proteins (nsp) are encoded by two open reading frames (ORFs) (ORF1a and ORF1ab), which are translated in 2 polyproteins (pp1a and pp1ab) through a -1 ribosomal frameshift. The polyproteins are then cleaved into 15 nsps (nsp2-16) by autoproteolytic activity. The function of each protein is reviewed in Jackwood et al. [3].

The Spike (S) protein is the most described IBV structural protein. This transmembrane protein, organized in trimers, determines the typical crown-like aspect of coronaviruses, observable at electron microscopy [4]. It is post-translationally cleaved in 2 sub-units, S1 and S2. The S1 contains at least 2 domains potentially involved in host receptor binding [5] and is therefore considered the main determinant of host and tissue tropism [4,6]. For the same reason, the most relevant epitopes are located on this protein, including neutralizing ones. The high intensity of the selective pressures driven by the host immune response acting on this region is probably responsible for its remarkable genetic

heterogeneity, both among coronaviruses and within IBV [3,5,7]. Because of strains heterogeneity and linkage with biological features, the Spike-coding gene has been used to classify IBV variants into genotypes for a long time. However, the absence of common classification and nomenclature criteria complicated the understanding of IBV molecular epidemiology until recent years [8]. The more conserved S2 sub-unit contains the transmembrane domain anchoring the S protein to the envelope [9] and is fundamental for viral fusion [10]. The interplay between S1 and S2 subunits might determine the overall avidity and specificity of virus attachment and thus viral tissue and host range [11].

The other surface proteins, Membrane (M) and Envelope (E), are involved in virus morphogenesis and assembly. Besides E and S, the M protein interacts with the Nucleocapsid (N) one, which directly binds genomic RNA to form a helical ribonucleoprotein complex and probably participate in RNA packaging [12,13].

2. Viral Evolution

Mutation is the ultimate source of genetic polymorphism and represents the first fundamental substratum for evolution, since it generates the genotypic (and phenotypic) variants that spread and become fixed through genetic drift or natural selection [14–16].

RNA viruses are known for having an extremely high error rate (approximately 10^{-4} to 10^{-5} misincorporations per nucleotide position) because of the lack of RNA-dependent RNA polymerase (RdRp) proofreading activity coupled with the absence of RNA repair mechanisms [17,18]. This leads to approximately 1 mutation·genome^{-1} replication^{-1}, which is a 10-fold higher rate than retroviruses and 10,000-fold greater than most DNA viruses, probably justifying the limited genome size of RNA viruses. In fact, while the continuous production of mutants favors virus adaptability in the event of environmental changes, the accumulation of multiple mutations in the same viral particle would lead to a dramatic fitness reduction [19].

Coronaviruses, including IBV, with their 30 kb long genome, can be considered an exception within the RNA virus group since their large replicase gene contains an ExoN domain in the nsp14, related to host proteins of the DEDD superfamily of exonucleases and involved in proofreading and repair activity. Nevertheless, the estimated substitution rate of IBV (10^{-4}–10^{-5} substitutions/site/year) is still remarkable [20] and guarantees a noteworthy evolutionary potential. This reflects on the heterogeneity of variants that have emerged all around the world [14,15]. In fact, the current classification, based on the S1 sequencing, includes 6 genotypes, further divided into 32 lineages, exhibiting a pairwise genetic distance of 30% and 13%, respectively [8]. However, the fate of both historical and new variants is largely unpredictable: some circulate at worldwide level for decades, while others show a limited temporal and spatial persistence [14,16]. Experimental evidences revealed the emergence of different mutants even within the same host during infection [21] or within single vaccine lots [22]. Interestingly, vaccines featured by a higher number of subpopulations were associated with higher replication titres within the host and the induction of a stronger immune response, potentially attributable to the replication ability in different tissues and a broader epitopic spectrum [23]. Despite apparently advantageous from certain perspectives, the presence of viral subpopulations can increase the risk of reversion to virulence due to the selection of more fit variants, as suggested by the occurrence of post-vaccine reactions induced by subpopulation-rich vaccines [24,25].

While the random selection of IBV variants (genetic drift) is often implicitly stated in many manuscripts, the actual role of natural selection has been explicitly accounted less frequently. More recently, the relevance of natural selection in shaping IBV evolution has been determined for both vaccine and field strains. In longitudinally monitored birds vaccinated with live-attenuated Ark vaccines, for which subpopulations differed in the S protein sequence, minor viral subpopulations were rapidly selected and mutations emerged during passages in chickens, suggesting that the phenotype of these subpopulations was advantageous and/or favored adaptation to the chicken host [22,24]. Accordingly, when chicken-derived strains were re-isolated and passaged in eggs, at least some of them reverted to the original vaccine sequence in some positions [22]. Minor modifications

in the S protein are likely responsible for the different IBV affinity for receptors located in different tissues, thus affecting both tropism and pathogenicity [4,26].

A study by Gallardo et al., based on S sequence analysis, highlighted that the predominant IBV phenotype contained in the vaccine became a minor population in the host, and also significant differences were detected in the incidence of some distinct IBV populations in the considered tissues and fluids. Therefore, in addition to the host role, the specific tissues could exert a certain pressure, selecting the variants able to replicate better in a particular microenvironment [23].

Closely related strains sometimes demonstrate a relevant heterogeneity in cross-neutralization patterns, highlighting that mutations in specific amino-acid sites are responsible for immune evasion [15]. Accordingly, strain D1466 (G-II) and the recently emerged D181 variant, although being formally classified within the same lineage based on the overall S1 genetic identity, demonstrated only 9% cross-relationship [27].

Immune response is thus expected to be the main selective force acting on IBV evolution, at least in the antigenic regions. Some experimental evidences seem to further support this hypothesis. When field strains were inoculated in vaccinated and unvaccinated chicken groups, the emergence of non-synonymous mutations was described only in some of the vaccinated birds [28]. Evidences of vaccine-driven immune selection were also provided by a recent epidemiological study performed in field conditions, where an increase in the selective forces acting on the S1 protein of QX strains has been documented after the introduction and mass application of a homologous vaccine [7]. The diversification tendency after the vaccination strategy change was especially strong on sites exposed on the Spike surface and particularly in or nearby receptor binding motifs, which can be considered likely target of effective immune response (Figure 1).

However, it should be emphasized that vaccination is not "per se" a risk factor for viral evolution, but improper vaccination can be. Natural selection is expected to predominate when $Ne \cdot S >> 1$ (i.e., effective population size × selection coefficient is much higher than 1). While vaccination clearly imposes a strong selection coefficient, it also drastically reduces viral replication and therefore the population size, preventing the emergence of more fit variants.

On the other hand, improper vaccine selection or administration, as well as factors suppressing the bird immune response, can allow the virus to circulate in a partially immune environment, potentially leading to the emergence, selection and spreading of immune escape variants [29–31].

Current studies are essentially based on the S protein (or part of it), representative of less than 10% of IBV genome, whereas IBV virulence, pathogenesis and tropism are polygenic and also associated with genetic variants outside of the S gene [32–34], especially in, but not limited to, the replicase gene [35,36]. Additionally, most of the currently available studies were based on sequences obtained through Sanger sequencing, which allows to investigate only major subpopulations. Benefits will surely derive from the wider application of Next Generation Sequencing (NGS) technologies to the study of whole-genome IBV dynamics within the host, and by the integration of these data with the evolutionary patterns observed at the epidemiological level. In fact, the relationship between the within-individual and population level fitness is still unknown, as well as the reasons causing some variants to remain restricted to limited geographic areas and time periods, while others are able to persist and spread worldwide.

A second pivotal source of genetic variability is recombination. Coronaviruses are featured by a high recombination rate, mediated by a copy-choice mechanism [37]. In this process, the RdRP switches from one RNA molecule (the donor template) to another (the acceptor template) during the synthesis while bound to the nascent nucleic acid chain, generating a chimeric RNA molecule with mixed ancestry. The strategy adopted by coronaviruses in gene expression regulation facilitates this phenomenon since it depends on discontinuous transcription, which relies entirely on the viral RdRp template-switching property [13,38]. IBV makes no exception: several studies reported recombination events which occurred in field conditions, involving both field and vaccine strains [39–41]. This evidence has risen some concerns on the potential risk of new recombinant strain emergence, especially when

multiple vaccines are administered and/or vaccine strains circulate for a long time. Single detections or small clusters of recombinant strains are typically reported, suggesting the limited fitness of most strains. However, some noteworthy exceptions have been described. A study performed in Italy and Spain reported 4 different recombination events involving QX and 793B strains [42]. While Italian recombinants were only sporadically detected, the Spanish ones emerged among the dominant field strains in the region. Therefore, both positive and negative selection can act on recombinant viruses, likely depending on the peculiar recombination pattern and local epidemiological context.

Besides affecting viral biology, recombination represents a remarkable challenge for IBV classification. As previously mentioned, the current classification is officially based on the S1 gene sequencing [8], even though the hypervariable regions of this segment are usually sequenced because of practical and economic constraints. Little is known about the rest of IBV genetic backbone, where frequent recombination events involving even distantly related strains are likely [43–45]. Therefore, the S1-based classification could be poorly representative of the actual IBV evolutionary history and biological properties. Additionally, recombination analysis is rarely performed, leading to the risk of misclassifying chimeric viruses as new genotypes.

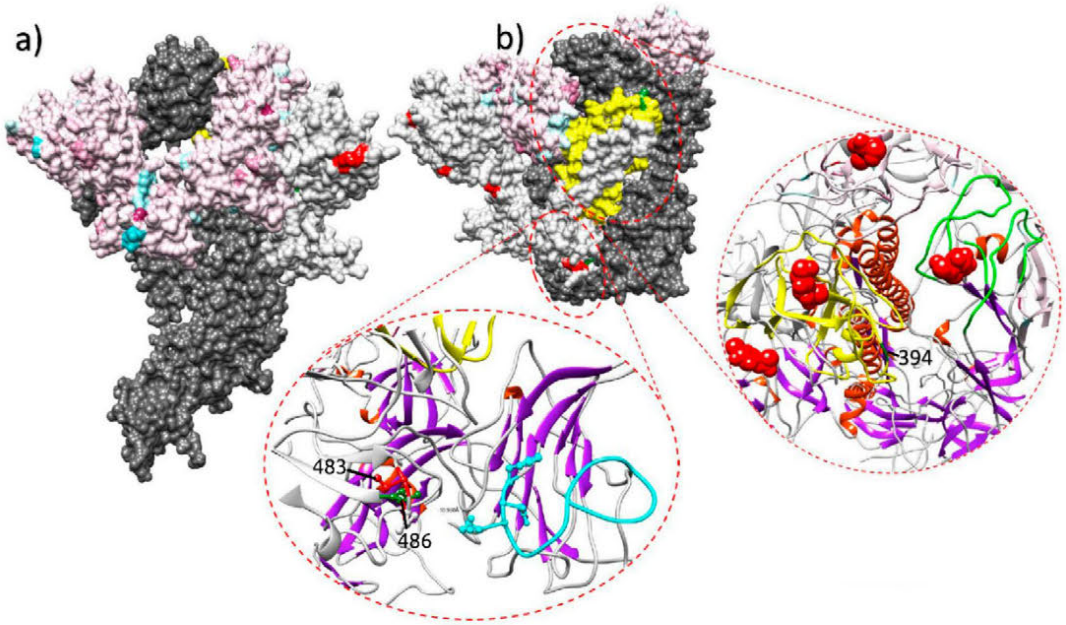

Figure 1. Lateral (**a**) and upper (**b**) view of the quaternary structure of the IBV spike protein. The full spike protein is reported for one monomer only (dark gray), while other monomers have been edited to highlight different selective pressure features. In the color-coded monomer, sites under a stronger selective pressure before the homologous vaccination introduction are reported in light blue, while the ones under a stronger selective pressure after the vaccination change are coded in purple. In the white-colored monomer, sites under episodic diversifying or directional selection in the post-vaccination change period are reported in red and green, respectively. The region demonstrating the stronger diversification tendency after the homologous vaccination introduction is highlighted in yellow. The ribbon visualization of relevant protein domains is reported as inserts. Lower insert reports S1 N-terminal receptor binding motif and the partial ceiling over it (coded in light blue). Sites of the nearby monomer under episodic diversifying and directional selection in the post-vaccination period are reported in red and green, respectively. The right insert displays the S1 C-terminal domain region. Two monomers are represented: one depicting the region with the highest diversification tendency after the homologous vaccination introduction (in yellow) and the other highlighting the corresponding extended putative receptor binding motif loops (in green). Figure from [7].

3. Approaches to IBV Diagnosis

While zootechnical parameters and presence of symptoms are useful as preliminary evidences of infectious bronchitis (IB) outbreaks, IBV-induced signs are not pathognomonic. Laboratory assays are therefore mandatory to detect and characterize IBV strains. The most used diagnostic tests include viral isolation, serological and molecular assays.

3.1. Viral Isolation

When attempting to isolate field viruses, samples should be promptly collected when IB-compatible signs are at first observed, since IBV titres peak in the first week post-infection, possibly before clinical manifestations [46]. Tracheal tissues or swabs are the sample of choice especially in the acute phase, but kidneys or oviduct are also suitable sites, especially in presence of lesions. Isolation may also be attempted from caecal tonsils and cloacal swabs, but viral recovery rate seems lower [46]. To investigate IBV presence at flock or farm level, pooled samples should be taken from both symptomatic and healthy animals. Samples should be carefully stored on ice and rapidly sent to the laboratory to preserve virus viability. Allantoic cavity of embryonated eggs or tracheal organ cultures (TOCs) from specific pathogen-free (SPF) chickens are considered more efficient than cell culture [47]. IBV causes urate deposits in mesonephros, stunting, curling and embryonic death when cultured on embryonated eggs, and ciliostasis on TOCs. Lesions are usually observed within the third passage when using embryonated eggs, while ciliostasis on TOCs usually occurs after the first passage [48]. In either case, the presence of these signs does not allow to ascertain IBV presence. Serological or biomolecular methods should be used to confirm and further characterize the isolated strain [49]. Nowadays, viral isolation is not routinely performed for diagnostic purposes because of its lengthiness and stringent requirements, but it is still useful for several other scopes such as vaccine production, sample enrichment before whole genome sequencing, pathogenicity tests and evaluation of the protection conferred by vaccines against virulent challenges.

3.2. Serology

Methods based on antibody detection are used to study previous IBV circulation or assess the induced immunological response. A range of serological tests is available, including agar gel precipitation (AGP), enzyme-linked immunosorbent assay (ELISA), virus neutralization (VN) and hemagglutination inhibition (HI).

AGP allows for quick and inexpensive responses but is not routinely adopted since it lacks in sensitivity and the main precipitating antibodies (i.e., IgM) are detected only for a few weeks after exposure [46].

Commercial ELISA tests are the most commonly applied for routine serological monitoring because of their cost-effectiveness and rapid turnaround. Antibody titration is useful to monitor both the vaccination response, especially in layers and breeders, and exposure to field virus. For a proper interpretation, baseline values should be established based on a regular monitoring of the local situation since antibody titres depend on many factors like breed, type, age at sampling, vaccination program and schedule [50,51].

The observed results should be compared to the baseline in terms of average titres, within-group uniformity and persistence. Reasonably high, uniform and lasting titres are indicative of a proper vaccination, while low, uneven and short-lived titres suggest a defective vaccination, likely due to an improper administration or poor quality of the vaccine batch. When the observed titres are significantly higher than expected, a field infection should be suspected.

The majority of the available ELISA kits are designed to detect polyclonal antibodies against the whole virion and do not allow serotypization. Several alternatives to commercially available ELISA kits have been developed [52–56], some of which are based on serotype- or strain-specific monoclonal antibodies [57–59], but their use in routine diagnostics is currently limited.

VN and HI tests can identify serotype-specific antibodies and are therefore useful to assess the specific response to both field and vaccine strains. However, only VN should be considered the method of choice for serotyping purposes, because of the more frequent occurrence of cross-reactions using HI [60]. Unfortunately, the adoption of VN for routine monitoring is limited by its laboriousness.

Aside from the described methods, other serological assays have been recently developed, including methods based on Unknown multi-analyte profiling (xMap) [61], microarray [62] and strip-based [63] technologies, but they are not commercially available yet.

3.3. Molecular Techniques

Currently, biomolecular assays are the most used tools for IBV detection, because of their sensitivity and quick response time. Besides viral RNA detection, they allow to characterize the detected strains from a genetic standpoint, allowing to properly plan and evaluate vaccination protocols and assess the presence of specific field strains. It is worth noting that the mere PCR positivity does not imply an active infection at the moment of sampling since genome traces can persist for a relatively long period after viral clearance. Thus, the results should be carefully interpreted.

A multitude of RT-PCR and qRT-PCR-based methods have been validated, either generic and targeting virtually all IBV subtypes, or genotype- or strain-specific [64–68]. The most commonly targeted region is the S1 gene, where the genetic variability featuring IBV variants is concentrated [3]. Due to the vast adoption of live vaccines, which may persist for the whole production cycle in broilers, the majority of samples usually proves positive to generic assays, and further characterization, either by sequencing or a panel of specific assays, is required to obtain meaningful results. Nevertheless, when multiple strains are present, generic RT-PCR followed by Sanger sequencing would detect only one strain, typically the one with the highest titre. However, the differential primers affinity may also bias the results. For this reason, it is not uncommon for different assays to lead to the detection of different strains [69]. On the other hand, the use of strain-specific tests only would limit the diagnostic sensitivity to an *a priori* subset of IBV strains or genotypes. In fact, the ideal diagnostic algorithm should comprise both generic and specific assays selected on the actually circulating variants in the field.

Restriction fragment length polymorphism (RFLP) has been used as an alternative to sequencing and specific probes development. The use of restriction enzymes represents a quick, inexpensive technique, but, unfortunately, not all strains can be differentiated in this way [70], and the adopted enzymes would require periodic updates to keep up with IBV evolution.

Quantitative real-time RT-PCR (qRT-PCR) allows the quantification of the targeted genetic material present in the sample. Real-time assays targeting conserved regions of IBV genome cannot quantify the respective contribution of simultaneously present strains, limiting their usefulness to screening purposes. On the other hand, a properly chosen panel of strain-specific qRT-PCR assays enables their precise differentiation. Field strains quantification may help to discriminate an incidental detection from a disease primarily caused by IBV, while vaccine strain titres can be used to assess vaccine coverage, replication and persistence. The description of vaccine kinetics in longitudinal studies may be of great aid in characterizing different vaccination protocols, replication dynamics of the various vaccines and potential interactions when co-administered [68].

Currently, the main issue of PCR-based methods used for IBV characterization is the lack of consensus on the genetic classification method. The results provided by different laboratories may differ because the considered genomic region differs in size or position, or because the same genetic subtype is referred to with different names by different laboratories [15]. As previously mentioned, the recently proposed classification by Valastro et al. [8] effectively solved this issue. However, historical strains are still referred to using the traditional nomenclature sometimes by researchers and more often in the field. Unfortunately, the use of full S1 sequencing is not routinely used for monitoring because of its cost and sensitivity limits [71].

The absence of clear genetic markers also prevents the reliable discrimination between vaccine and field strains [72], although consistent genetic differences were sometimes demonstrated [73–75].

Anamnestic data like the presence of symptoms and vaccination schedule should thus be taken into account for more confident deductions.

Suitable specimens for PCR testing include dry swabs, tissues and FTA cards. These are paper substrates containing chemicals that protect nucleic acids and inactivate pathogens with no risk of contamination, requiring less stringent conitions for storage and shipment but possibly entailing a loss in sensitivity [71]. Samples are usually collected at tracheal or cloacal level but, depending on the presence of particular symptoms and lesions, they should also be taken from the kidney or oviduct. Different strains may have a different tropism [76], thus samples collected from different districts may lead to different results. Pooled samples are typically considered an acceptable cost/benefit trade-off to obtain information about the epidemiological status of a group of birds as an epidemiological unit, while individual samples should be analyzed to assess the vaccine coverage or prevalence of a field virus within a flock [68].

Besides being vastly adopted for diagnostic purposes, PCR-based methods followed by sequencing are of great use for research studies of IBV epidemiology. For example, phylodynamic projects allow to investigate the evolutionary history and spreading dynamics of the viral population, providing useful information to evaluate and improve IBV control measures on a large-scale perspective [20,77].

Other molecular techniques, such as loop-mediated isothermal amplification (LAMP) and padlock probes (PLPs) combined with rolling circle amplification (RCA), have been validated for IBV detection [78,79]. Additionally, NGS techniques have recently been used to study IBV [80,81]. This approach, despite being currently too expensive and laborious for routine use, has proven useful for research purposes, particularly because it allows to precisely study the presence of subpopulations within a single sample and contextualize IBV within the respiratory disease complex.

4. Diagnostic Approach Selection

No technique can be considered fully conclusive by itself, usually requiring the combination of multiple tests to obtain a complete picture of the infectious status of single animals, flocks or larger populations. Rather than implementing a standard set of assays, the best diagnostic approach should probably be decided on a case-by-case basis to suit the particular demands and peculiarities of the epidemiological context. Different tests have different costs and response times, and they may require different types of samples, which are not always easily collected in certain situations (i.e., samples that require the sacrifice of birds, or whose shipment requires stringent precautions). The purpose of the survey is also crucial to decide which tests to use. For instance, the investigation of a suspected IB outbreak may impose a different approach compared to the assessment of vaccination coverage.

In a typical field situation, the common adoption of multiple live vaccines may complicate the diagnostic process and mask the simultaneous presence of field strains [68,74,82]. For this reason, the implemented vaccination protocol should be taken into account while interpreting the results. Other factors include the timing of sampling, presence and type of symptoms, productive and housing type, and presence (either suspected or confirmed) of other respiratory and immunosuppressive pathogens.

The most suitable procedures for each of the main diagnostic purposes are described in Table 1.

Table 1. Diagnostic guidelines describing the suitable procedures based on the intended purpose.

Diagnostic Aim	Suitable Assays	Sampling Time	Suitable Samples
Confirmation of suspected infection (based on symptomatology)	Viral isolation [a]	As soon as symptoms are observed, up to 10 days post-infection	Well-preserved individual or pooled samples; tracheal specimens are preferable; kidneys and oviduct may be sampled based on symptoms
	Molecular assays [b]: RT-PCR, qRT-PCR	As soon as symptoms are observed	Individual or pooled samples; tracheal/cloacal specimens; kidneys and oviduct may be sampled based on symptoms
	Serology: ELISA, VN [c], HI [c], AGP	From 7 days post-infection (2 weeks for ideal results)	Sera
Surveillance (absence of infection)	Molecular assays [b]: RT-PCR, qRT-PCR	Anytime; if vaccination is implemented, infection occurrence is more probable when vaccines start to fade	Individual or pooled samples; tracheal/cloacal specimens
	Serology: ELISA		Sera
Immunological response to vaccination	Serology: ELISA, VN [c], HI [c]	From 7 days post-vaccination onwards; longitudinal studies may be performed for more informative results	Sera
Vaccination coverage	Molecular assays [b]: RT-PCR, qRT-PCR		Individual samples; tracheal/cloacal specimens
Vaccination kinetics (replication and persistence)	Molecular assays [b]: qRT-PCR		Individual or pooled samples; tracheal/cloacal specimens

[a] IBV presence must be confirmed by other techniques. [b] Do not assess viral viability; coexistent strains may be discriminated by genotypization or using specific assays. [c] Allows for serotypization.

5. Relevant Factors for IBV Control

The remarkable economic impact of IBV on poultry production encourages the implementation of massive vaccination strategies, whose wide adoption requires to settle between ideal application and practical aspects. As for other pathogens, IBV control can be eased by good management, correct bird density, air quality, all-in/all-out period duration, etc. [83]. Nevertheless, even optimally managed IBV-positive flocks were estimated to yield 3% less than IBV-free flocks [84,85].

The first essential barrier against IBV is biosecurity, with the strict implementation of external and internal measures regulating the flow of animals, people, supplies and waste/manure [86,87]. Adequate empty period and disinfecting procedures are necessary to reduce the risk of IBV infection dragging cycle after cycle. Farm density has been associated with a higher risk of viral transmission among farms; however, whethere this is due to airborne transmission eased by proximity or to the sharing of common risk factors (horizontal contacts or environmental conditions) remains to be established [88]. Nevertheless, biosecurity alone rarely guarantees the complete prevention of disease transmission.

To compensate for these aspects, vaccination is the most effective and frequently adopted option: even though it cannot fully prevent the infection, a decrease in clinical signs and infectious pressures can be achieved. For instance, H120-vaccinated animals showed a reduced viral transmission ($R_0 < 1$) and shedding (more than 1 $\log_{10}$ of excreted titre reduction) after homologous challenge in experimental conditions [89].

Broiler vaccination relies on the administration of live attenuated vaccines, obtained from embryonated egg passaging of field strains [90]. These vaccines are commonly adopted for priming in layers and breeders, then inactivated vaccines can be administered for immunity boost [48,91], ensuring adequate maternal antibody transfer and protection of the reproductive tract [92,93]. On the other hand, inactivated vaccines are not as effective as live ones in inducing a proper protection and strong mucosal immunity at the tracheal level [94,95]. Hence, subsequent live attenuated vaccines can be administered between 4 and 6 weeks apart to enhance the protection in layers, depending on the infectious pressure [15]. Attenuated vaccines are most commonly used because of lower administration costs and the availability of mass administration procedures, both at hatchery and in

the field. No individually administered vaccine would be suitable for frequent immunizations, thus limiting research on other types of vaccines, such as recombinant or subunit vaccines [90].

Nonetheless, the development of new vaccines based on reverse genetics [96] and recombination techniques is currently pursued. Although theoretically promising, the results of the available studies show partial protection against strains featured by a Spike gene closely related to the newly inserted one [97,98], but inadequate against heterologous ones [99]. Many of these genetic constructs [100,101] are based on a Beaudette strain (GI-1) backbone, which lacks of pathogenicity and would prevent residual effects of the attenuation process, others focus on the deletion of pathogenicity-associated genes [96], obtaining attenuation without the loss of protection. Recombinant vaccines have been produced also by cloning IBV proteins into the backbone of other viruses (Fowl Adenovirus, FAdV [102,103]; Newcastle disease virus, NDV [104,105]; avian Metapneumovirus, aMPV [106]), achieving a variable degree of protection. However, individual administration was still required for these vaccines, thus limiting their practicability. The choice of the insert region is currently the focus of intense research since it can deeply affect the achieved protection levels [98,99], because of innate protein immunogenicity and the contribution other factors such as conformational constraints, post-translational modifications or interactions with other proteins.

Other options, such as subunit vaccines [107–109], have also been explored, even though the absence of replication compared with attenuated vaccines constitutes a key limitation to the achievable protection, possibly requiring several and repeated administrations [90].

It is long-established that IBV extreme genetic variability impacts on the reliability of vaccine strategies in relation to the local epidemiological scenario and cross-protection among different strains [48]. Two schools of thought have often opposed each other on this topic, some in favour of homologous vaccination, others of heterologous one. This dichotomy is based on the principle that a homologous vaccine to the field strain is more likely to generate neutralizing immunity against it [90], whereas heterologous vaccination usually aims at providing a broader but less specific immunity towards different and potentially unknown circulating strains, in line with the "protectotype" concept [110–113]. Nevertheless, "hybrid" solutions are often applied, including one heterologous strain (e.g., Mass-based vaccine) and one homologous vaccine strain.

Several vaccine combinations have been tested so far in line with the different epidemiological scenarios, with particular attention to Mass-like (GI-1) and 793B-like (GI-13) strains, American strains such as Ark (GI-9), Conn (GI-1), DE072 and GA98 (GIV-1), QX (GI-19), Q1 (GI-16) and Var2 (GI-23), suggesting a variable protection degree depending on the challenge virus [114–118]. The most common combinations, based on Mass-like and 793B-like strains, usually ensure good protection also against new variants [90,119]. A thorough evaluation of the most suitable and already available combinations should thus be performed before developing new variant-specific vaccines.

Although the likelihood of cross-protection is slightly higher among more similar strains, there is a weak correlation between genetic similarity and protection of two strains [15], which hampers conclusions without experimental confirmation. In fact, the level of protection is usually evaluated by challenge infections of vaccinated animals, and the efficacy is assessed on clinical signs, challenge virus detection and ciliostasis, depending on the legal requirements dictated by Food and Drug Administration and/or European Pharmacopoeia [113]. This requires the involvement of animals, personnel, dedicated facilities and a great investment of resources and time, before reaching a reliable assessment of the cross-protection [120]. Serology is also of little help in establishing protection levels, since antibody levels and cross-reactivity lack correlation with protection [121,122]. New methods based on deep learning approaches have been proposed and could aid in predicting the protection levels between a vaccine and a new field strain, starting from the genetic and aminoacidic sequence of the S1 protein [123]. An approach free from clinical trial expenses and lengthiness would certainly help and speed up the response to new IBV strains appearing on the field.

Alternatively, an accessory evaluation of vaccine kinetics [68] and coverage [124,125] can be carried out by molecular assays (i.e., qRT-PCR), which quantify the vaccine titres replicating in the bird

respiratory tract along the productive cycle. Because of the common persistence of the vaccines and the expected competition between vaccine and field strains for cellular receptors [68,82], the vaccine yield could be considered as a proxy of vaccination quality and protection, in addition to classical immunity assessment. Nevertheless, vaccination strategies in the field are often far from coherent, standardized or constant [74,126], even within the same country where the local epidemiological scenario is known. This heterogeneity hinders both the identification of field infections and the monitoring of vaccine intake.

Besides vaccine type selection, the attempt of reducing labour and vaccine supply, inadequate mass administration, the use of fractional doses and wrong timing could significantly affect animal protection, coverage, vaccine-associated virulence and mixed vaccine-field virus circulation [46]. IBV vaccines are commonly administered by spray at the hatchery, by backpack sprayer or drinking water at the farm or, less frequently, by eye-drop application when attenuated, or by injection if killed [125,127]. The accuracy of the administration procedures deeply impacts on the achieved protection [15,125,128]. Vaccine route, dose and titre, temperature and quality of the water used for dilution, size of the sprayed particle, interactions with other vaccines, housing features such as flock size, ventilation and lights, are only some of the variables affecting vaccination efficacy [46,50,129] which need to be taken into account when planning a vaccine strategy.

Mass spray vaccination is an approach that clearly minimizes work whilst maximizing coverage, and it is usually applied at hatchery or farm by spray, which is considered analogous to the gold standard oculo-nasal vaccination in mimicking the entrance of respiratory viruses [120,130], when properly managed. However, improper settings of the diameter of the sprayed droplets can cause a deep penetration of the vaccine virus in the lungs and consequent vaccine reactions [120].

Spray vaccination could raise managerial problems when applied in a hatchery that supplies chicks to several farms requesting different vaccines. Nonetheless, the concern of chick contamination with different administered strains at the hatchery was diminished by Pellattiero et al., proposing that not yet replicating vaccines seem unable to spread to other non-vaccinated animals located in the same environment for several hours [131].

Spray vaccination has been progressively replacing administration via drinking water, which is largely deprecated since it often implies the combination of vaccines and other substances, normally dissolved in the water or to be co-administered, that could lower the vaccine titre or viability, leading to low coverage, rolling reactions and reversion to virulence. Nevertheless, it is still used, mainly for booster administration of less attenuated vaccines in the field [83]. Gel vaccination, initially studied for coccidiosis [132,133], has also been applied to IBV providing a chemically stable environment, without altering the vaccine kinetics [124,134]. A possible beneficial impact of gel vaccination on the chick body temperature with respect to the spray technique has also been hypothesized, although consistent experimental confirmation is still lacking. Vaccination timing is another field of intense debate and research. IB vaccination at hatchery is almost universally adopted, because of the higher coverage obtained while chicks are in the boxes. However, the ability of the birds to mount a proper immune response after an early priming is still questioned: experimental evidences suggest that the latter the birds are vaccinated, the highest antibody levels are reached [84,135], and the presence of maternally derived antibodies (MDA) could also complicate early vaccination by interfering with vaccine replication [136]. In field conditions, IB infection often occurs around 30 days of age [126,135]: this late appearance could be explained by the vaccine titre decrease and fading competition among replicating vaccines and field strains [68]. It is also worth to note that, when resorting to combined vaccination, an interval of at least two weeks between subsequent administrations appears beneficial likely because of a more mature immune system of the chick and a recovery of the respiratory epithelium [50,84].

On the other hand, many registered vaccines for broilers are declared to guarantee protection from day-old vaccination up to the end of the cycle, according to the supplementary protection certificates. An early combined vaccination at 1 day of age was proven to confer an efficient immune response [119],

and epidemiological studies showed a lower likelihood of developing IB in flocks vaccinated with a combination of two strains at hatchery, probably because of a better standardization of vaccine administration conditions and procedures at the hatchery rather than at the farm [137]. Moreover, it should be considered that a delay in vaccination could expose the birds to an earlier entrance of the virus. Early vaccination appears therefore as a good compromise between bird biology and production constraints, ensuring fair conditions of vaccine administration, maximum coverage and economical sustainability of the procedures.

It should not be under-emphasized that inconsistencies between experimental and field results could be due to different conditions and better control of environmental, managerial and biological variables in experimental settings [50], which prevent a full inference of the field conditions based on the obtained results. A certain delay was highlighted in the field response to a change of the vaccination strategy, demonstrating the existence of complex interactions among cross-protection between field and vaccine strains, infectious pressure and outbreak occurrence [126], stressing the need to wait at least two complete cycles in broiler production before assessing the actual effect of a particular vaccination strategy, especially in high infectious pressure situations.

As extensively described, IB control complexity is determined by several factors, from field conditions to the biological properties of the virus, which need unceasing research efforts and studies. New vaccines and new control strategies have to overcome old problems, such as costs and management issues, cross-protection limitations and variant emergence, whose knowledge and evaluation must be considered as essential requisites for strategy planning.

At the moment, IB eradication is a distant goal and all efforts in the field should aim at optimizing the implementation of measures of well-known efficacy. A steady and rigorous monitoring based on objective criteria and knowledge of the local epidemiological scenario should be also strictly implemented.

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

Legnardi, M., Franzo, G., Koutoulis, K.C., Wiśniewski, M., Catelli, E., Tucciarone, C.M., Cecchinato, M.

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

Matteo Legnardi ,*,[1] Giovanni Franzo,* Konstantinos C. Koutoulis,† Marek Wiśniewski,‡ Elena Catelli,§ Claudia Maria Tucciarone ,* and Mattia Cecchinato*

*Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università, 16, 35020, Legnaro (PD), Italy; †Department of Poultry Diseases, Faculty of Veterinary Science, University of Thessaly, Trikalon 224, 43100, Karditsa, Greece; ‡Ceva Animal Health Polska Sp. z o.o., ul. Okrzei, 1A, 03–715 Warszawa, Poland; and §Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra, 50, 40064 Ozzano dell'Emilia (BO), Italy

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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[1]Corresponding author: matteo.legnardi@gmail.com

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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Evolution of infectious bronchitis virus in the field after homologous vaccination introduction

Giovanni Franzo*†, Matteo Legnardi†, Claudia Maria Tucciarone, Michele Drigo, Marco Martini and Mattia Cecchinato

Abstract

Despite the fact that vaccine resistance has been typically considered a rare phenomenon, some episodes of vaccine failure have been reported with increasing frequency in intensively-raised livestock. Infectious bronchitis virus (IBV) is a widespread avian coronavirus, whose control relies mainly on extensive vaccine administration. Unfortunately, the continuous emergence of new vaccine-immunity escaping variants prompts the development of new vaccines. In the present work, a molecular epidemiology study was performed to evaluate the potential role of homologous vaccination in driving IBV evolution. This was undertaken by assessing IBV viral RNA sequences from the ORF encoding the S1 portion of viral surface glycoprotein (S) before and after the introduction of a new live vaccine on broiler farms in northern-Italy. The results of several biostatistics analyses consistently demonstrate the presence of a higher pressure in the post-vaccination period. Natural selection was detected essentially on sites located on the protein surface, within or nearby domains involved in viral attachment or related functions. This evidence strongly supports the action of vaccine-induced immunity in conditioning viral evolution, potentially leading to the emergence of new vaccine-escape variants. The great plasticity of rapidly-evolving RNA-viruses in response to human intervention, which extends beyond the poultry industry, is demonstrated, claiming further attention due to their relevance for animal and especially human health.

Introduction

Avian infectious bronchitis is a well-recognized and widespread disease, which entails remarkable economic losses to the poultry industry by inducing respiratory and reproductive signs, decreased productive performances and increased mortality, particularly when nephropathogenic strains or secondary infections are involved [1].

The etiological agent, avian infectious bronchitis virus (IBV), is a member of the species *avian Coronavirus*, order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Gammacoronavirus*, subgenus *Igacovirus* [2]. The viral genome is about 27 kb long and encodes non-structural (such as the RNA-dependent RNA polymerase (RdRp) and other accessory and regulatory proteins) and structural proteins (i.e., the spike, envelope, membrane, and nucleocapsid) [3]. Among the others, the spike protein (S), and the sub-unit S1 in particular, is widely studied because of its role in cell tropism, receptor attachment, neutralizing antibodies and cell-mediated immune response induction [1, 4, 5]. Additionally, the high genetic variability of the S1 gene has prompted its use for the classification of IBV strains into genotypes and lineages, which can display significantly different geographical distribution and, in some instances, biological behavior and immunological features [6].

In fact, as other positive-sense single-stranded RNA viruses, IBV is able to evolve rapidly and to recombine [1, 7, 8], leading to the emergence of a remarkable genetic and phenotypic variability over time. This heterogeneity poses noteworthy challenges to the

*Correspondence: giovanni.franzo@unipd.it
†Giovanni Franzo and Matteo Legnardi contributed equally to this article
Department of Animal Medicine, Production and Health (MAPS), University of Padua, Viale dell'Università 16, 35020 Legnaro, PD, Italy

understanding of IBV epidemiology and its control. Currently, vaccination represents the most applied and effective strategy to limit the disease impact. Nevertheless, the antigenic variability entails the existence of several serotypes and protectotypes, which translate in a poor cross-protection among genotypes [9], requiring the use of different vaccine combinations in order to broaden the protection spectrum or the development of new vaccines against recently emerged or introduced genotypes [10, 11]. Unfortunately, even closely related vaccines can fall into episodes of incomplete protection or vaccine immune-escape because of amino acid substitution in specific antigenic sites [9].

Despite the quite clear scenario, the understanding of underlying forces prompting the viral phenotypic variability is much more nuanced.

A high mutation rate does not automatically lead to a comparably elevated heterogeneity in biological features: the persistence and spread of new phenotypic variants implies that they must be favorably selected by the environment [12]. Although different kinds of mutations can alter the viral behavior and biology, the occurrence of non-synonymous mutations in relevant protein regions is probably the most recognized and studied evolutionary mechanism.

In this sense, the host population immunity represents one of the most obvious forces that can promote viral diversification, especially in antigenic regions. Besides natural immunity, vaccine administration could significantly contribute to this process. The role of vaccination in driving viral evolution has been reported for different diseases affecting both animals and human beings. When immunity is not sterilizing, wild strains are able to circulate in a new and more challenging environment, potentially adapting to it [13, 14]. Numerous examples can be mentioned for viruses that, to different degrees, circumvented vaccination strategies by immuno-escape (Hepatitis B virus, avian Metapneumovirus, Porcine circovirus type 2) [15–17], virulence increase (Marek disease virus) [18], or both (Infectious bursal disease virus) [18].

Nevertheless, the confirmation of wide vaccination application as a driver of IBV evolution is challenging. Particularly, the lack of systematic studies and the application of different vaccination protocols in different farms create a multitude of confounding effects hindering the identification of the actual underlying biological phenomena.

The present study investigates the impact of the homologous vaccination application on the evolution of IBV QX genotype (GI-19 lineage) S1 subunit in field conditions, circumventing the above-mentioned limitations and benefiting of the peculiar Italian epidemiological scenario and related control strategies applied in response to QX introduction in this country.

The proposed model was selected and considered extremely favorable for several reasons:

1. The QX genotype, and IBV in general, displays a high mutation rate (i.e. approximatively 10^{-3}–10^{-5} substitutions/site/year) and therefore a relevant mutation number is expected to accumulate even over a relatively short time period [19].
2. The immunological features of IBV have been extensively studied. Particularly, the spike protein has been recognized as the most relevant target of the host immune response [4, 20]. Therefore, a S1-focused study design, limiting the technical problems and maximizing the biological information can be defined.
3. GI-19 is one of the most relevant lineages in the world, and the most significant one in Italy. Our previous studies have provided a detailed characterization of its molecular epidemiology in Italy and integrated this information with the remarkable, and ever increasing, number of sequences released worldwide [19, 21, 22]. This bulk of data allowed to demonstrate that after the introduction in Italy, QX strains had a substantially independent evolution [19].
4. The Italian poultry industry is dominated by few integrated companies, mainly located in northern Italy. Consequently, the applied control strategies are highly homogeneous among farms. Particularly, a homologous QX vaccine has recently been introduced by some of these companies and applied to all affiliated broiler farms, replacing a Mass+793B based vaccination. Therefore, a clear comparison between the pre- and post-homologous vaccination application was possible.

Materials and methods

Sample collection and sequencing

Four hundred and ten samples (pools of ten tracheal swabs), previously tested IBV positive using the Virus-IBV-kit (Gensig, Southampton, UK), were collected between 2012 and 2017 from broiler farms located in northern Italy and delivered to the laboratory of infectious diseases of the Dept. Animal medicine, Production and Health (Padua University) for sequencing and genotyping. An IBV hypervariable region was amplified using the XCE1 and XCE2 primers as described by Cavanagh et al. [23] and Sanger sequenced in both directions using the same primers. All obtained sequences were first genotyped by comparison with the reference set provided by Valastro et al. [6]. Particularly, sequences were aligned

using the MAFFT method [24] and the obtained alignment was trimmed in order to include the hypervariable region only. A phylogenetic analysis was performed using the IQ-TREE software [25], selecting as a substitution model (GTR + G [4]) the one with the lowest Akaike Information Criterion (AIC) calculated using Jmodeltest [26].

Strains clustering in the GI-19 lineage were selected and the sequencing of the full S1 gene was attempted.

To this purpose an additional external primer pair was designed using Primer3: QXS1F (5′-TGGGTGACAGTG GAAAACTG-3′) and QXS1R (5′-TGTGTTTGTATG TACTCATC-3′). The full S1 gene was thus amplified by two overlapping RT-PCR using the primer pair QXS1F-XCE2 and XCE1- QXS1R. The reaction was performed using the SuperScript ™ III One-Step RT-PCR System with Platinum™TaqDNA Polymerase kit (Thermo fisher) at the following conditions: 1X reaction mix, 0.5 µM of each primer, 1 µL of SuperScript™III RT/Platinum™Taq Mix and 5 µL of RNA template. Molecular biology grade water was added up to a final volume of 25 µL.

The cycling parameters were set at 50 °C for 30 min, 95 °C for 5 min, 45 cycles of 95 °C for 20 s and 50 °C for 20 s and 68 °C for 50 s. The presence and specificity of the PCR products was visualized on a SYBR Safe stained agarose gel. Positive samples were Sanger-sequenced in both directions using the same primer pairs.

The obtained chromatogram quality was evaluated with FinchTV (Geospiza) and consensus was obtained with CromasPro (CromasPro Version 1.5).

Sequence analysis

All the obtained complete S1 sequences were aligned at the amino acid level and then the nucleotide sequences were back-translated using the MAFFT algorithm implemented in TranslatorX [27]. Genotyping was confirmed using the previously described approach. To evaluate the distribution of Italian GI-19 strains in the international scenario, an extensive dataset of S1 IBV sequences was downloaded from GenBank and included in the phylogenetic tree. Additionally, the presence of recombination events in the GI-19 full S1 dataset was assessed using the RDP4 software [28]. A recombination event was accepted if detected by more than two methods with a significance level lower than p value < 0.001 with Bonferroni correction. For each method, settings were adjusted according to the software manual. The absence of undetected recombination events was evaluated using the SBP method implemented in HyPhy [29].

The total dataset was divided in two subsets according to the vaccination protocol.

More in detail, sequences collected before GI-19 homologous vaccination introduction (i.e. November 2014) were included in the "pre-vaccination" dataset, while the remaining were in the "post-vaccination" dataset. Because of the high turnover featuring broiler farms, the new vaccination scheme was applied to all considered farms by the beginning of January 2015. No samples obtained in the intermediate period were included in the study.

Several methods, based on the ratio between synonymous (dS) and non-synonymous (dN) substitution rate estimation, were applied independently on both datasets. The presence of pervasive purifying and diversifying selection was tested using SLAC, FEL and FUBAR methods [30, 31]. Sites were considered under pervasive diversifying selection only when detected by at least two of the implemented methods, similarly to what Franzo et al. performed [17]. Episodic diversifying selection was tested with MEME [32].

The presence of episodic diversifying and directional selection was also tested on the whole dataset with FEEDS and MEDS methods, marking the post-vaccination sequences as foreground branches [33]. All analyses were performed using the multiprocessor version of HyPhy [29]. Phylogenetic trees required for the analysis were reconstructed using IQTree [25].

The significance level was set to p-value < 0.1 (i.e. method's default) for the SLAC, FEL, FUBAR, FEEDS and MEDS methods, while the posterior probability threshold for the FUBAR method was set to 0.9.

S1 subunit homology modeling

The nucleotide sequence of an Italian GI-19 strain S1 subunit was translated at amino acid level and the SWISS-MODEL web server was used to identify the best template for which quaternary structure had been experimentally determined [34]. The same program was used to estimate the protein structure through a homology-modeling approach. The obtained model was visualized and edited with Chimera [35].

Results

Samples and sequencing

One hundred and fifty-five samples were preliminary classified in the GI-19 lineage based on the hypervariable region analysis and were therefore included in the study. The complete S1 gene could be sequenced for 95 strains and the genotype classification was confirmed for all of them. Because prolonged circulation of live attenuated vaccines has been demonstrated in the field, the detection and sequencing of vaccine strains was considered likely. Therefore, the obtained sequences were compared with the S1 of the GI-19 based vaccines currently registered in Italy.

Four sequences, all obtained from samples collected after vaccination introduction, were removed from the dataset because of their identity with vaccine ones (strain D388).

All considered field strains formed a monophyletic group including only Italian strains (Additional file 1).

Finally, one sequence (strain 25088) was demonstrated to be a recombinant between QX and 793B strains and was also removed before further analyses. The final dataset included 90 sequences: of those, 39 and 51 were collected in the pre-vaccination and post vaccination period, respectively.

Genetic distance distribution demonstrated a substantially comparable profile between the two datasets, although the "post-vaccination" period showed a slightly higher genetic distance (Additional file 2). Therefore, the hypothesis that a higher antigenic variability may simply reflect a broader genetic heterogeneity can be disregarded. The obtained sequences have been submitted to GenBank (Acc.Numbers MK491671–MK491761).

Selective pressure analysis: pervasive selections

Although FUBAR detected as positively selected sites codons 52, 54, 58, 65 and 132 in both datasets and 9, 64, 389 and 539 in the post-vaccination period only, pervasive diversifying selection was not detected in any site according to the decided criteria, regardless of the considered dataset.

To further evaluate the presence of a differential selective pressure strength before and after the vaccination introduction, the standardized differences in dN−dS before and after vaccination introduction, estimated using FUBAR, FEL and SLAC, were calculated for each position in the alignment. Scores higher than 0, suggestive of a more prominent diversifying selection following the vaccination introduction, appeared predominant with all considered methods (Figures 1 and 2). Additionally, this value was used to calculate a cumulative score by summing it codon by codon. This score allowed to highlight the diversification tendency of S1 protein regions in the two considered time periods. Also in this case, the vast majority of S1 protein regions were characterized by higher dN−dS values in the post-vaccination introduction period, particularly in the area between aa 300 and 400 (Figures 2 and 3).

Selective pressure analysis: episodic selections

Episodic diversifying selection could not be proven in the pre-vaccination dataset using MEME. However, positions 9, 29, 54, 65, 96 and 483 were detected in the post-vaccination dataset.

Partially comparable results were obtained using FEEDS. Codons 9, 65, 225 and 483 showed signs of

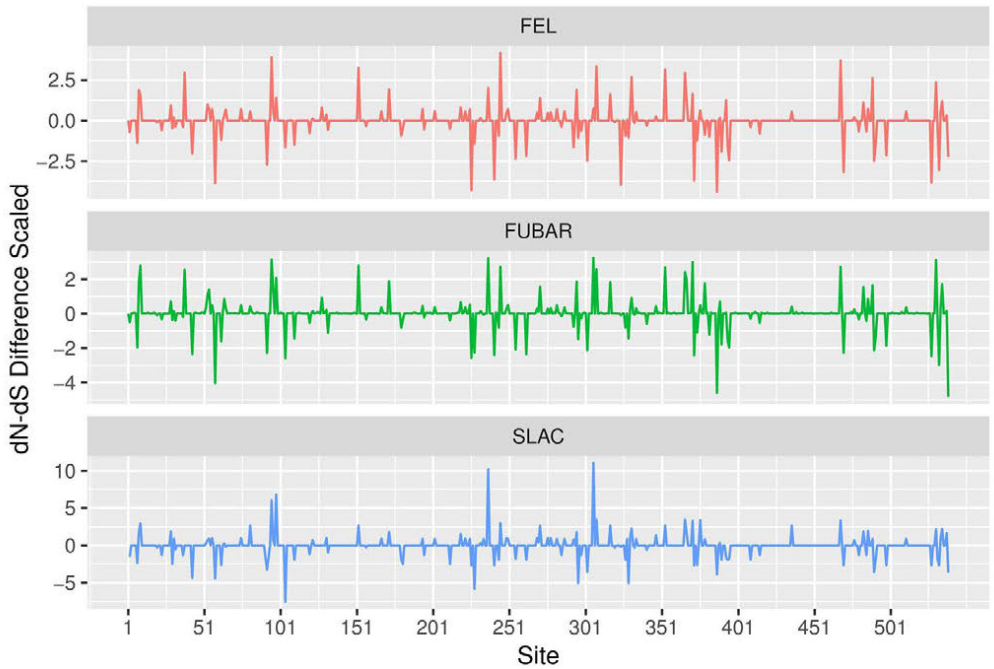

Figure 1 Plot reporting the scaled dN-dS difference between the post-vaccination and pre-vaccination periods for each codon positions calculated with different methods (FEL, FUBAR and SLAC). Positive scores are suggestive of a more intense selective pressure in the post vaccination period.

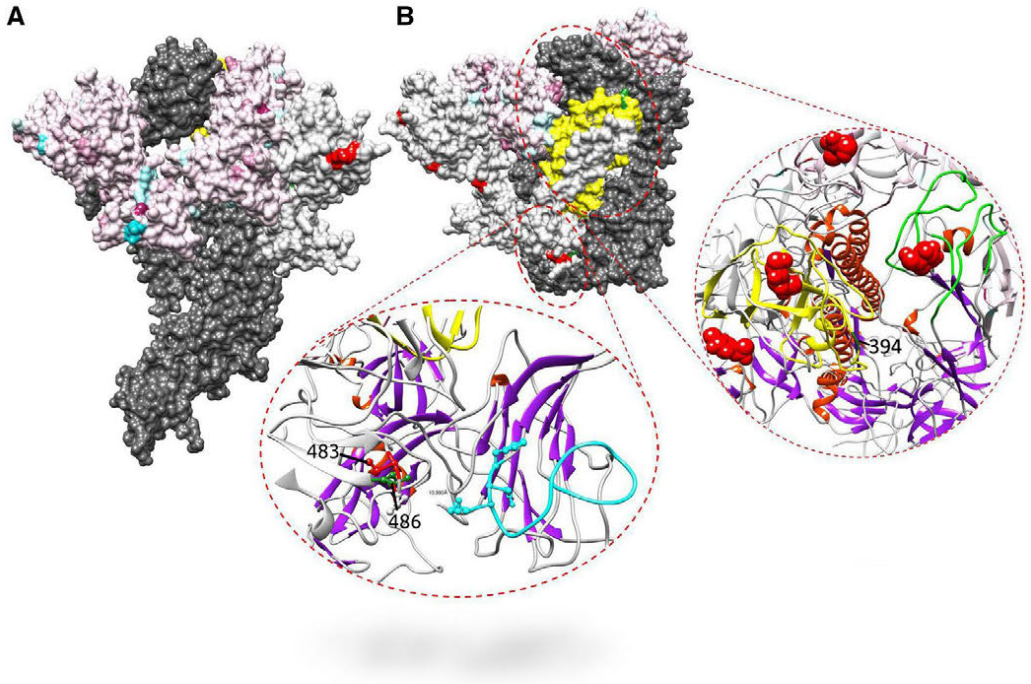

Figure 2 Lateral (A) and upper (B) view of the quaternary structure of the IBV spike protein. The full spike protein is reported for one monomer only (dark gray). The S1 region is shown for the two remaining monomers, which have been edited to highlight different selective pressure features. In the color-coded monomer, the scaled dN-dS difference between pre- and post-vaccination, calculated using FUBAR, is reported on the protein surface ranging from purple (higher pressure in the post-vaccination period) to light blue (higher pressure in the pre-vaccination period). In the white-colored monomer, sites under episodic diversifying or directional selection in the post-vaccination period are reported in red and green, respectively. The region demonstrating the higher cumulative dN-dS score (AA 300–400) is highlighted in yellow. The ribbon visualization of relevant protein domains is reported as inserts. Lower insert reports S1 NTD RBM and the partial ceiling over it (coded in light blue). Sites of the nearby monomer under episodic diversifying and directional selection in the post-vaccination period are reported in red and green, respectively. Right insert displays the S1 C-terminal domain (CTD) region. Two monomers are represented: one depicting the region with the highest cumulative score in dN-dS difference between vaccination periods (in yellow) and the other highlighting the corresponding extended putative RBM loops (in green). A more detailed representation of the overall protein structure is reported as Additional file 4.

diversifying selection acting on the foreground tree branches (i.e. post-vaccination period). Finally, episodic directional selection was demonstrated on 3 amino acids using MEDS, after the vaccination introduction. Particularly, a tendency to mutate toward Asparagine (N), Threonine (T) and Valine (V) was detected in positions 29, 394 and 486, respectively (Figure 2 and Additional file 3). The frequency of those amino acids rose from 0% before homologous vaccination introduction to respectively 4%, 12% and 6% after the new vaccination program implementation.

Homology modeling

Homology modeling based on an IBV spike reference (pdb template:6CV0) [36] allowed the mapping of selective pressure sites and strength on the quaternary viral protein structure. Although minor differences between the predicted and actual QX Spike structure cannot be excluded, the good model-quality evaluation statistics provided by SWISS-MODEL support the accuracy of the obtained quaternary model.

Most of the surface amino-acids are featured by a stronger diversifying selection in the "post-vaccination" dataset (Figure 2). Particularly, the region with the higher cumulative score in dN-dS difference between pre- and post-vaccination periods corresponds to the S1 C-terminal domain (CTD) where the putative receptor-binding motif (RBM) loops are located.

The same region, exposed on the spike surface, also hosted the aa 394, detected under directional selection. Similarly, aa 486, also exposed on the spike subunit, is in close relationship (approximatively 10 Å) with the partial ceiling nearby another putative RBM in the S1 N-terminal domain (NTD) of the adjacent subunit (Figure 2B). The last aa detected under directional selection (aa 29) was located within the viral protein structure, instead.

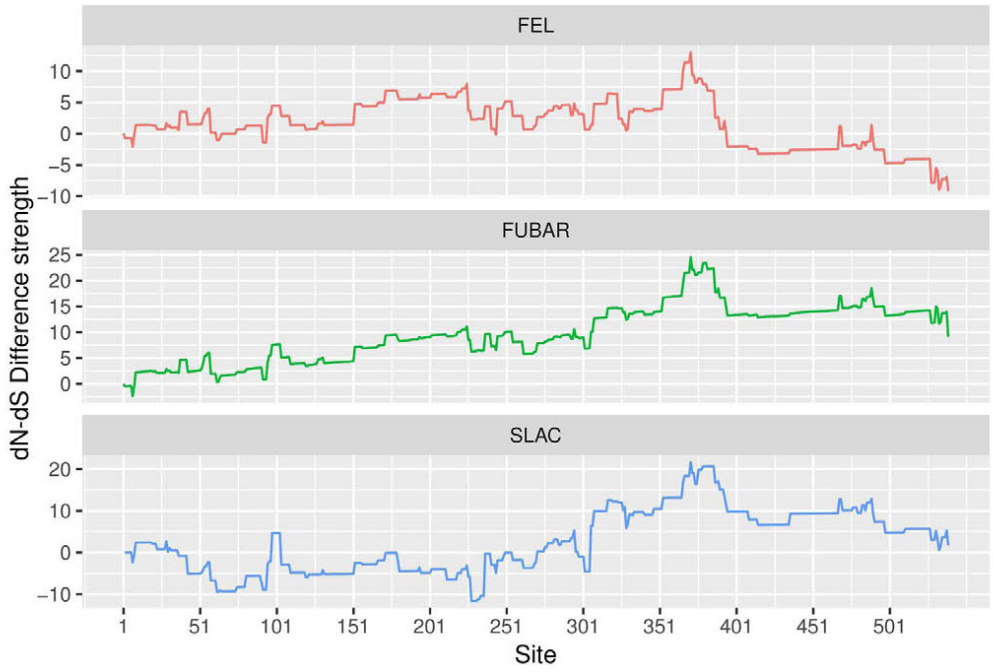

Figure 3 Plot reporting the cumulative score based on the dN-dS difference between the pre- and post-vaccination periods for each codon position calculated with different methods (FEL, FUBAR and SLAC).

All sites detected under episodic diversifying selection in the post-vaccination period are located on the spike protein surface (Figure 2).

Discussion

Human intervention has dramatically changed the environment where pathogens evolve [18, 37]. The most evident examples are represented by the prompt emergence and spread of antimicrobial and antiviral resistance [38]. Rapidly-evolving RNA viruses seem particularly favored in the arms race against human treatments because of their ability to quickly explore the fitness landscape and new evolution pathways [39].

However, several studies have demonstrated the presence of noteworthy exceptions in veterinary medicine, most notably in intensively raised livestock like poultry and pigs [16–18]. The present work confirms this trend also for IBV, since an overall higher selective pressure strength was proven after homologous vaccination was introduced. Of note, differently from experimental infections, the molecular epidemiology-based statistical approach applied in the present study allows modelling viral evolution in its natural environment. The bias and confounding effects due to experimental conditions, animal genetic line and health status, infectious dose, selected viral strain etc. are thus avoided. Moreover, the random inclusion of several farms located in the same area before and after homologous vaccination introduction controls for the individual flock peculiarities and contingent situations, provide an overall picture of the investigated phenomenon. Several sites were detected to be under a significant episodic diversifying or directional selection in the post vaccination period only. Significantly, all the corresponding amino acids were exposed on the viral S1 surface (Figure 2), thus allowing the speculation of the role of host derived immune selection in affecting evolution.

On the contrary, pervasive diversifying selection could not be detected at the set criteria in any dataset. Based on this evidence and considering that episodic diversifying selection could be detected only in the post-vaccination dataset, an evolution occurring through a selective burst induced by homologous vaccination introduction can thus be hypothesized. Nevertheless, a lower statistical power of methods dealing only with pervasive selection could be expected [35]. When the difference between post- and pre-vaccination period in dN-dS was calculated, an overall balance in favor of a higher diversifying selection was evidenced after the homologous vaccination introduction. Therefore, although not statistically significant, a trend featured by a higher selective pressure was confirmed also by these methods (Figures 1 and 3). The highest cumulative score in dN-dS difference between vaccination periods was located in the S1

C-terminal domain (CTD), where the two extended putative receptor-binding motif (RBM) loops are located (Figure 2) [36]. This finding is particularly suggestive since antibodies raised against the RBM domain could display a higher neutralizing activity and, therefore, exert a stronger pressure toward viral diversification. Accordingly, the corresponding region (including the aa 394, detected under directional selection in the present study) was demonstrated to be involved in the selection of neutralization-resistant D274 genotype variants [40].

Similar evidence can be claimed for other sites identified in the present study, which are located within, or in close proximity to, regions like aa 24–62 and 87–93, reported as epitopes in previous experimental studies. Particularly, region 87–93 was proven to be an antigenic region specifically in the QX (GI-19) genotype [41].

Interestingly, different amino acids, i.e. aa 483 (under episodic diversifying selection) and 486 (under episodic directional selection), are located in close proximity with a loop structure constituting a partial ceiling over the S1 NTD RBM of the nearby S1 subunit (Figure 2).

Since coronavirus S1-NTDs are under the host immune pressure, the evolution of a ceiling has been suggested to provide a better protection from host immune surveillance to the sugar-binding site [39]. Antibodies binding to a nearby region could therefore decrease/inhibit the functionality of domains of pivotal relevance for viral life cycle by sterical interference. However, a potential direct role of aa 484–486 in host immune response cannot be excluded.

The congruence between the findings obtained in the present study and other experimental evidence supports the immune-induced nature of the detected pressures. Experimental confirmation could complement and confirm the results herein described, evaluating the progressive evolution of GI-19 strains in a more standardized "vaccine environment".

The actual reasons behind the strength of selective pressures imposed by vaccination, especially in veterinary medicine, are still not fully understood.

IBV vaccine immunity is not sterilizing and a certain viral persistence in vaccinated animals is possible [42]. The scenario is further worsened by the typically partial coverage achieved by routine vaccination protocols in field conditions that, although usually effective in preventing clinical outbreaks and reducing the infectious pressure, facilitates the circulation of field viruses in a partially immunized population [21, 43]. A previous study based on a phylodynamic approach, performed in the same geographic area and timeframe, demonstrated the benefits of the homologous vaccination introduction in reducing viral population size and outbreak frequency [21]. Nevertheless, the IBV QX genotype has continued to circulate in Italy. These weaknesses can allow the presence of a viral population size high enough for natural selection to act and favor the emergence of more fit variants in a new immune-environment. Due to the early vaccination and short life span of the animals, the vaccine-induced immunity is expected to be the dominant force interacting and shaping viral evolution, rather than natural infection. Additionally, the immunity induced by a single strain (i.e. vaccine virus) is likely to be more homogeneous compared to the one elicited against heterogeneous field strains. Although the *treatment-mosaic*-like situation is considered one of the key factors limiting the emergence of vaccine-escape mutants, the reduced genetic variability of the poultry population imposed by commercial constraints could imply a decreased immune response spectrum, facilitating the selection of specific mutations.

Besides homologous vaccination, IBV is often controlled providing a combination of vaccines based on different genotypes (i.e. heterologous vaccination). Since a more diversified and heterogeneous immune response spectrum has been reported to arise, the emergence of specific-immunity escaping variants could be hindered.

Therefore, the benefits of heterologous versus homologous vaccination in terms of vaccine-escape induction should be carefully evaluated in the framework of the best vaccination guideline definition.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13567-019-0713-4.

Additional file 1. Maximum likelihood phylogenetic tree based on an extended collection of IBV S1 sequences. The QX (GI-19) strain are highlighted ocher, while the Italian sequences have been coded in red. It is possible to zoom into appreciate further details and tip labels.

Additional file 2. Density curves representing pairwise p-distance calculated for the pre- and post-vaccination datasets.

Additional file 3. Time scaled phylogenetic trees reconstructed using the QX sequences obtained in the present study. The ancestral history of the amino acids detected under episodic directional selection has been reconstructed over time through a discrete trait analysis using the Bayesian approach implemented in BEAST1.8. Each tree has been color coded to depict the evolution of one of the considered amino acids.

Additional file 4. Animation displaying the quaternary structure of the IBV spike protein and the action of selective pressures.

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Authors' contributions
GF and MC conceived and planned the experiments. ML and GF performed the experimental procedures, data analysis and contributed equally to the manuscript writing. CMT participated in data acquisition and interpretation. ML, MM, MD, GF and MC discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All obtained sequences have been made available in Genbank (Acc. Numbers MK491671–MK491761).

Competing interests
The authors declare that they have no competing interests.

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Phylodynamic analysis and evaluation of the balance between anthropic and environmental factors affecting IBV spreading among Italian poultry farms

Giovanni Franzo[1]✉, Claudia Maria Tucciarone[1], Ana Moreno[2], Matteo Legnardi[1], Paola Massi[3], Giovanni Tosi[3], Tiziana Trogu[2], Raffaella Ceruti[4], Patrizia Pesente[5], Giovanni Ortali[5], Luigi Gavazzi[4] & Mattia Cecchinato[1]

Infectious bronchitis virus (IBV) control is mainly based on wide vaccine administration. Although effective, its efficacy is not absolute, the viral circulation is not prevented and some side effects cannot be denied. Despite this, the determinants of IBV epidemiology and the factors affecting its circulation are still largely unknown and poorly investigated. In the present study, 361 IBV QX (the most relevant field genotype in Italy) sequences were obtained between 2012 and 2016 from the two main Italian integrated poultry companies. Several biostatistical and bioinformatics approaches were used to reconstruct the history of the QX genotype in Italy and to assess the effect of different environmental, climatic and social factors on its spreading patterns. Moreover, two structured coalescent models were considered in order to investigate if an actual compartmentalization occurs between the two integrated poultry companies and the role of a third "ghost" deme, representative of minor industrial poultry companies and the rural sector. The obtained results suggest that the integration of the poultry companies is an effective barrier against IBV spreading, since the strains sampled from the two companies formed two essentially-independent clades. Remarkably, the only exceptions were represented by farms located in the high densely populated poultry area of Northern Italy. The inclusion of a third deme in the model revealed the likely role of other poultry companies and rural farms (particularly concentrated in Northern Italy) as sources of strain introduction into one of the major poultry companies, whose farms are mainly located in the high densely populated poultry area of Northern Italy. Accordingly, when the effect of different environmental and urban parameters on IBV geographic spreading was investigated, no factor seems to contribute to IBV dispersal velocity, being poultry population density the only exception. Finally, the different viral population pattern observed in the two companies over the same time period supports the pivotal role of management and control strategies on IBV epidemiology. Overall, the present study results stress the crucial relevance of human action rather than environmental factors, highlighting the direct benefits that could derive from improved management and organization of the poultry sector on a larger scale.

Infectious bronchitis virus (IBV) is currently classified in the species *Avian coronavirus*, genus *Gammacoronavirus*, family *Coronaviridae* (https://talk.ictvonline.org/). The viral genome is about 27Kb long and encodes different non-structural, accessory and structural proteins. Among those, the structural spike protein is by far the most extensively studied, because of its role in viral attachment, cell tropism and immunity[1]. Additionally, the current

[1]Dipartimento di Medicina Animale, Produzioni e Salute (MAPS), Università di Padova, Legnaro, (PD), Italia. [2]Dipartimento di Virologia, Sezione di Brescia, Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna, Brescia, (BS), Italia. [3]Sezione di Forlì, Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna, Forlì Cesena, (FC), Italia. [4]Gesco sca, Cazzago San Martino, (BS), Italia. [5]Laboratorio Tre Valli, San Michele Extra, (VR), Italia. ✉e-mail: giovanni.franzo@unipd.it

IBV classification in genotypes and lineages is based on the phylogenetic analysis of the corresponding genome region[2].

IBV is one of the most relevant infectious diseases of poultry, causing remarkable economic losses due to respiratory and reproductive signs and increased mortality, especially when secondary infections occur or when nephropathogenic strains are involved[3].

Currently, the most effective control measure is vaccine application, which proved to effectively reduce clinical signs emergence, infectious pressure and viral population size, at least when properly performed[4,5]. However, this approach cannot be considered a panacea, and some drawbacks cannot be denied. In fact, it must be kept in mind that IBV vaccination is not able to avoid animal infection and a prolonged circulation of field strains has been demonstrated even in vaccinated flocks[6]. Even when infectious bronchitis is properly controlled (i.e. asymptomatic infections), some economic losses have been reported in subclinically infected flocks[7]. Moreover, the co-circulation of field and live attenuated vaccines could enhance the likelihood of recombination and the vaccine induced immunity could promote and/or guide viral evolution[8,9].

Biosecurity measures and the proper understanding of the factors influencing viral spread are thus of primary importance. The risk factors of virus introduction in a farm have been investigated in several studies, and include transport of live poultry, species and productive category, dominant winds[10,11], sharing of personnel, fomites and means of transportation[12–16]. Other routes of transmission are summarized under the umbrella of 'contiguous spread'. However, most of these studies are based on single outbreaks, during emergency scenarios (e.g. avian influenza outbreaks), and on the evaluation of factors associated to farm infection using a traditional statistical approach[17,18]. Therefore, a more comprehensive analysis of the ecological aspects behind the spread of IBV is largely lacking. Recently, advances in bioinformatics tools have allowed to link viral phylogeny and evolution to epidemiological factors, modeling the process of viral spreading over time, investigating its determinants and integrating landscape ecology with molecular epidemiology[19]. Therefore, a more accurate evaluation of IBV spreading determinants, informed on data collected over several years, could contribute in the understanding of this disease epidemiology.

One of the anthropic factors that affects and complicates the understanding of IBV epidemiology is the interaction between viral behavior and poultry production management. Modern poultry farming is typically featured by an integrated system, where all the production phases are organized and managed by a single entity/organization. The independence among the different integrated companies (i.e. separate hatcheries, means of transportation, feed delivery, slaughterhouses, etc.), even in the same country, should guarantee a certain protection from the introduction of new strains, at least from farms belonging to other companies.

Although theoretically plausible, this assumption has not been rigorously verified for IBV and the extent of potential breaches has never been quantified.

In the present study, the spreading process of the most relevant field genotype in Italy, IBV GI-19 lineage (previously known as QX), has been investigated using a continuous phylogeographic approach. The effect of different environmental and social variables, like altitude, road density, poultry population density, etc. on the spreading patterns has been also investigated using dedicated statistical tests. Finally, the migration of viral strains among integrated poultry companies was assessed and quantified.

Results

Dataset. A total of 361 QX sequences were included in the final dataset. Of those, 135 belonged to "Company A" and 226 to "Company B". The sampled farm location is reported in Fig. 1. Overall, farms were mainly located in the "Pianura Padana" region (central area of Northern Italy) and, to a lesser extent, in North-Eastern, North-Western, Central and Southern Italy. Although the two companies tend to operate in different Italian regions, a clear overlapping was present in the high densely populated poultry area of Northern Italy (Fig. 1).

QX-population genetics parameter estimation. All considered field sequences formed a monophyletic group including only Italian strains (Supplementary Fig. 1). TempEst investigation revealed that the positive correlation between genetic divergence and sampling time (i.e. $R = 0.335$) was suitable for phylogenetic molecular clock analysis[20].

The tMRCA of the overall QX population in Italy (i.e. QX genotype introduction) was estimated in 2003.52 [95HPD: 1999.73-2006.76] using the structured coalescent approach. Almost identical results were obtained including a third "*ghost*" *deme* (i.e. an estimated *deme* for which no sequences were available, representative of other unsampled companies and farms) in the analysis or using the "traditional" coalescent approach. When strains collected from integrated poultry companies were considered independently, the tMRCA was predicted in 2003.19 [95HPD: 1994.11-2010.3] for *Company A* and in 2010.6 [2007.26-2011.99] for *Company B*. The viral population dynamics evidenced a substantially constant Ne*t (Effective population size * generation time, or relative genetic diversity) with the remarkable exception of the period between mid-2014 and mid-2015, when a sudden fluctuation was observed. However, a quite different scenario was demonstrated between the two integrated poultry companies. In fact, *Company A* was featured by a substantially constant population size, with a minor decrease affecting particularly the period 2013-2015. However, the Ne*t 95HPD were relatively broad and at odds with the significance of the observed variations. On the contrary, a much more changeable pattern was observed in *Company B* (Fig. 2).

Migration among companies. The structured coalescent model fitted with the two company, evidenced the presence of 2 separate clades (Fig. 3A) for the 2 companies, with only 11 exceptions, represented by strains sampled in *Company B* but clustering in the *Company A* clade, thus suggesting the migration of strains from *Company A* to *Company B*. Accordingly, the migration rate from *Company B* to *Company A* was $2.5*10^{-2}$ [95HPD: $6.02*10^{-2}$-$8.40*10^{-2}$], while the one from *Company A* to *Company B* was $6.66*10^{-2}$ [95HPD: $2.2*10^{-2}$-0.12]. The

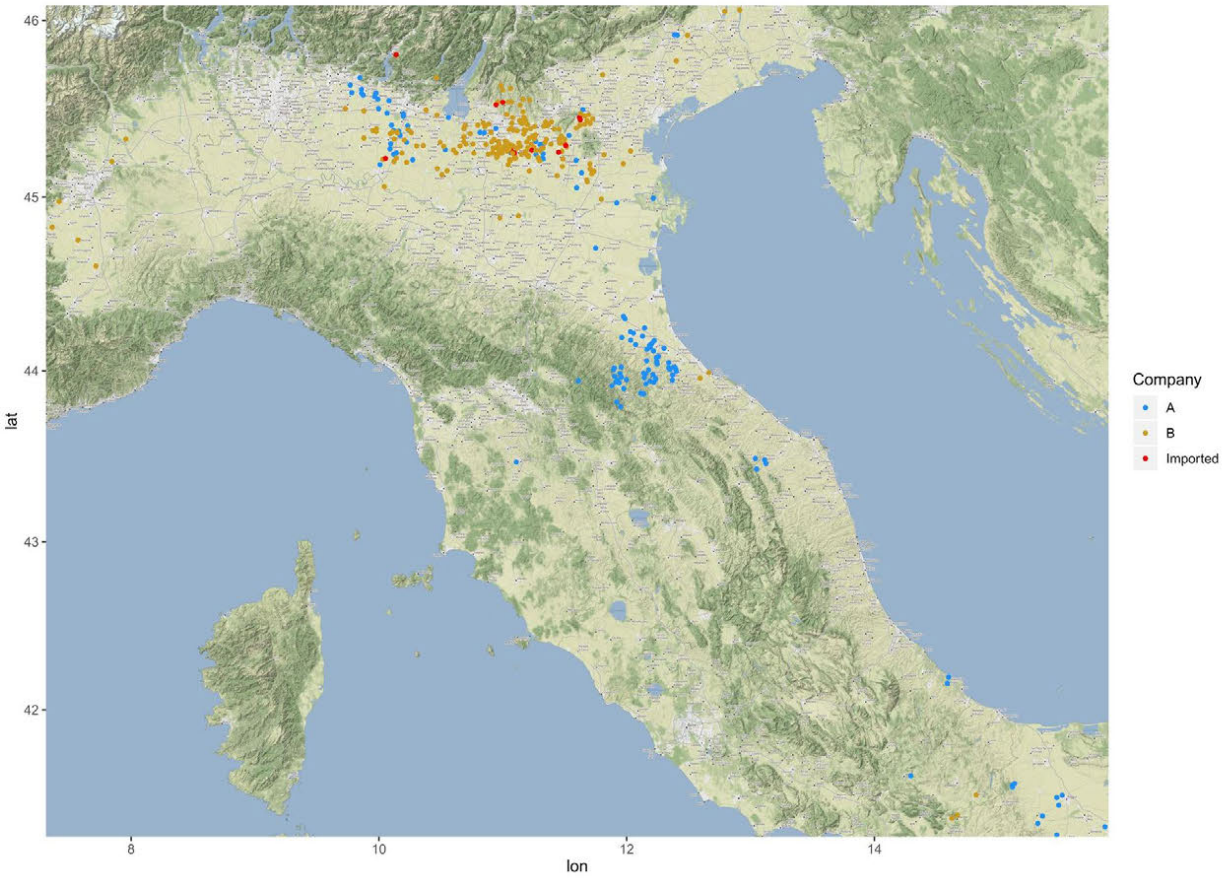

Figure 1. Location of farms from which samples have been obtained. Different companies have been color coded. Samples collected in *Company B* but clustering with *Company A* clade have been colored in red (herein named "Imported"). Farm location has been jittered using an internal routine of *ggplot* library to guarantee anonymity. The map was generated in R (version 3.4.4), using the library *ggmap*[51].

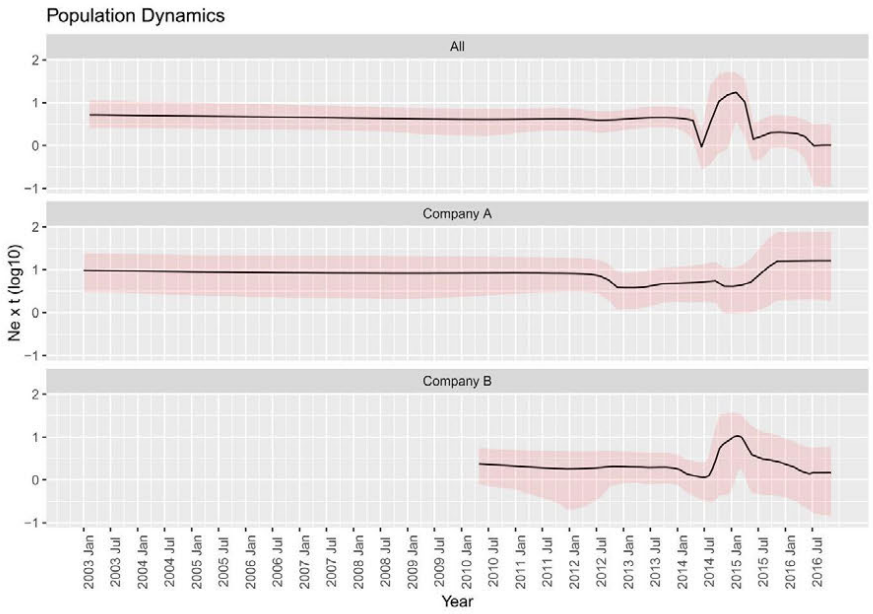

Figure 2. Mean relative genetic diversity (Ne x t) of the Italian GI-19 population over time. The results of the All Italian strains and of those sampled from (Company A and B) have been reported in different graphs. The upper and lower 95HPD values are reported as shaded areas.

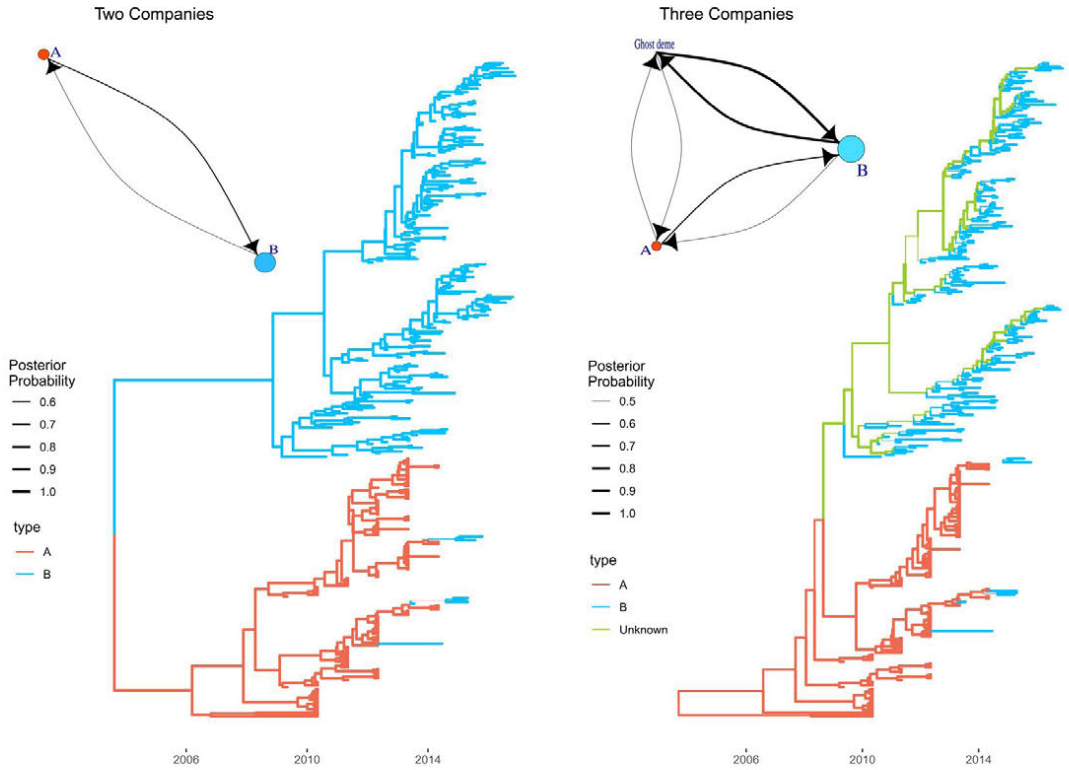

Figure 3. Structured coalescent-based phylogenetic tree of the samples included in the present study. Branch colors, as from legend, mark the inferred company where the ancestral strain was circulating, while branch width represents the posterior confidence of the inference. The trees reconstructed assuming just the *Company A* and *B demes* (left figure) and the one including also the *ghost deme* (right figure) are reported. In the top left insert it is reported the network depicting the migration rate between *Company A* and *Company B*. Arrows and circles size are proportional to the inferred migration rate and population size, respectively. Similarly, the top right insert reports the network of the migration rate between *Company A, Company B* and *ghost demes*.

demes population size estimation suggested the viral population size of *Company B* being 1.86 times greater than the *Company A* (Fig. 3A).

When the third ghost *deme* was included in the analysis, a partially different scenario was observed (Fig. 3B).

Company A formed an independent clade (with the exception of the 11 strains sampled from *Company B*), evidencing transmission events occurring essentially within the same company.

On the other hand, several of the internal branches leading to strains collected from *Company B* were predicted to belong to the "ghost" *deme*, although not always with a high posterior probability (Fig. 3B). The viral spreading among farms of *Company B* appears to be at least partially meditated by other unsampled farms/companies (Fig. 3B). Even if the population size of the *ghost deme* was predicted to be 6.87 and 19.23 times smaller than the *Company A* and *Company B* ones, respectively, its role in the viral transmission was not negligible, being the estimated transmission rates:

from *Company B* to *Company A*: $1.56*10^{-2}$ [95HPD: $2.20*10^{-7}$-$9.16*10^{-2}$]; from *ghost* to *Company A*: $1.27*10^{-2}$ [95HPD: $5.59*10^{-8}$-$7.02*10^{-2}$]; from A to B: $5.68*10^{-2}$ [95HPD: $6.74*10^{-4}$-0.13]; from *ghost* to *Company B*: 1.58 [95HPD: 0.57- 2.73]; from *Company A* to *ghost*: $8.36*10^{-2}$ [95HPD: $1.64*10^{-6}$-0.43]; from *Company B* to *ghost*: 1.44 [95HPD: $4.04*10^{-2}$-3.51] (Fig. 3B).

Phylogeographic analysis. All the samples phylogenetically belonging to *Company A* but collected from *Company B* originated from farms located in the high densely populated area of Northern Italy (Fig. 1). The continuous phylogeographic analysis reconstructed a spreading pattern originating from a single introduction in Emilia Romagna region (*Company A*), followed by a progressive expansion and persistence at high level in the Pianura Padana region. More rarely, spreading episodes toward other Italian regions were observed (Fig. 4). After QX introduction, the infection wave front increased slowly approximatively until 2009, when a rapid expansion led to the final distribution range by the middle of 2012 (Fig. 5). Accordingly, the dispersal velocity progressively increased in the first years after QX genotype introduction, peaking in the period 2009–2011 and then remaining essentially constant, despite some fluctuations (Fig. 5). The presence of a high dispersal velocity after 2012, when no further increase in wave front was observed, suggests that IBV continued to circulate at high rate after its first establishment in a region.

The analysis of the effect of different environmental factors on QX genotype dispersal velocity led essentially to negative results (i.e. absence of significant correlation). The only exception was represented by the poultry density

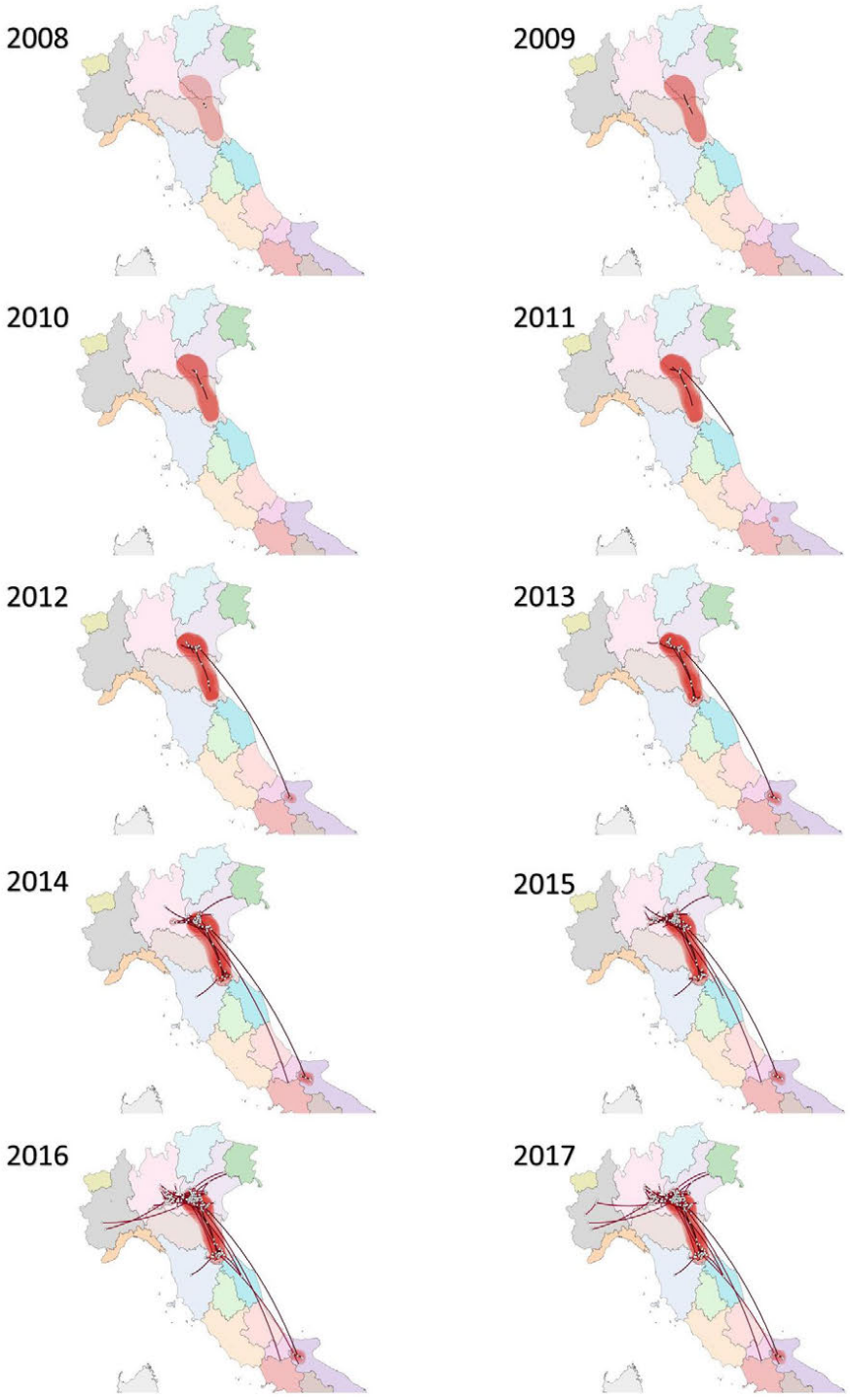

Figure 4. Results of phylogeographic analysis. The full posterior distribution of trees obtained in the continuous phylogeographic analysis is reported and the uncertainty (95HPD) on these estimates is reflected by contouring them with red polygons. Viral dispersal time has been represented by color coding the respective arrows from black (older) to red (newer).

SL model, which was positively and significantly correlated to viral dispersal velocity: D = 0.0225, percentage of D with p-value < 0.005 = 74%.

Discussion

Despite the economic relevance, the epidemiology of IBV and the factors affecting its behavior have been only partially investigated. Even if a huge amount of knowledge and literature has accumulated over time, most of the reports are anecdotal or based on the analysis of single clinical outbreaks[17,21,22]. Although relevant pieces of information could be obtained, the risk of being biased by personal believes or the particular condition under

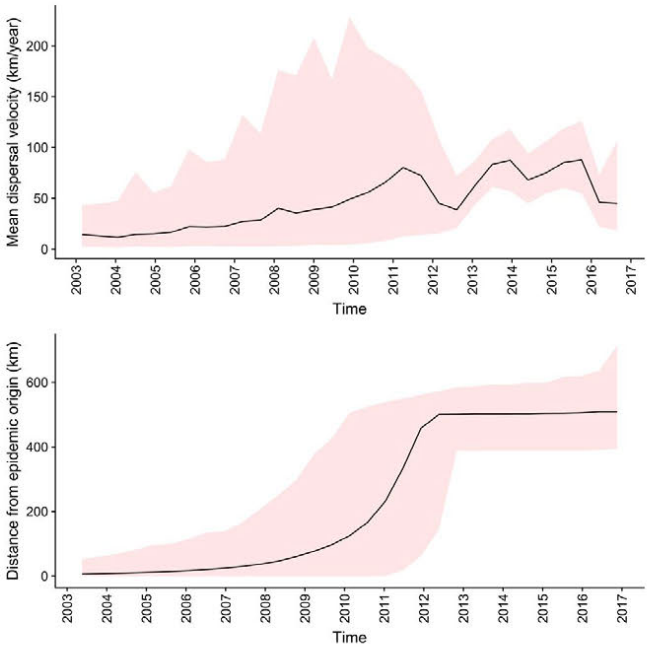

Figure 5. Estimated dispersion statistics of the QX epidemics. Upper figure: the mean dispersal velocity is reported over time. The red area corresponds to the 95% credible regions of the estimation. Lower figure: The geographic distance of the outbreaks wavefront from the estimated initial introduction is reported over time. The red area corresponds to the 95% credible regions of the estimated wavefront position.

investigation is high. A certain caution is thus required when inferring and extending the same conclusion on a broader/general scale. Moreover, most of the available studies are focused on Avian influenza and, to a lesser extent, Newcastle disease and Infectious laryngotracheitis[17,23,24].

The aim of the present study was to construct an objective and statistically sound framework to understand IBV field strains behavior, the effect of control measures and the factors conditioning their epidemiology. The field of phylodynamics, and all related extensions, provides an invaluable tool for the study of viruses and particularly of rapidly evolving ones, whose evolution can be measured in "real time", over the course of an epidemic[25].

IBV QX genotype is the most relevant field strain in Italy[26,27], and despite a relatively long circulation and the efforts devoted to its control, it still remains one of the main menaces for poultry industry profitability. Therefore, the understanding of the forces shaping its epidemiology would be of remarkable relevance in order to prevent the induced damages, rather than try to control them. Remarkably, the Italian IBV strains appear to originate from one introduction events only, as previously reported[27]. Therefore, it was possible to reconstruct IBV Italian strain evolution and epidemiological pattern without the biasing effect of strains recently introduced from other counties.

The implemented approach allowed to reconstruct the migration history of the QX genotype over time. The estimated introduction, in Emilia Romagna region, shortly predates the first detection, posing in favor of the effectiveness of the Italian monitoring and early detection systems. All the analyses, independently of the underlying statistical model, support that *Company A* was the first introduction site (Fig. 3). Thereafter, the virus circulation was limited to farms belonging to this company for years, until approximatively 2010, when *Company B* became involved. Contextually, a progressive increase in diffusion speed was noticed (Fig. 5), not unexpectedly considering the rising number of involved farms (especially at the border between Veneto and Lombardy regions, where most farms are located) and thus the increase in spreading potential and opportunity. The high farm density of this area has been described as a risk factor for different infectious diseases[22], and IBV seems to be no exception. Interestingly, the viral population size remained relatively constant in this time period, evidencing that, even if QX strains were able to effectively spread from farm to farm, their replication was adequately controlled, likely by effective vaccination strategies. Actually, a certain slowdown in dispersal velocity was noticed in 2011-12, potentially because of a progressive decrease in naive populations availability. A dramatic change was observed in 2014, when a new spreading wave (Fig. 4) and an increase in diffusion rate (Fig. 5) and population size (Fig. 2) were detected. A more detailed analysis demonstrated that this variation affected Company B only (Fig. 2). A previous study has ascribed this episode to a change in the vaccination scheme adopted by this company, which moved from a heterologous Mass+793B based vaccination to a Mass only vaccination leading to an increased viral circulation and clinical outbreaks number[4]. Moreover, experimental studies demonstrated a significant reduction in R_0 in vaccinated groups compared to unvaccinated ones[28]. It can therefore be speculated that the increase in infectious pressure within-farm and the higher flock susceptibility to infection could have enhanced the risk of IBV spreading to other farms and regions. In support of this hypothesis, the geographical spreading affected mainly Northern Italian farms (where *Company B* is located). Moreover, when a new double vaccination was implemented, the decrease in viral population size was mirrored by a reduction of dispersal velocity.

Continuous phylogeography showed that the areas interested by a more intensive viral circulation were those featured by a higher poultry density, and this evidence was confirmed by a statistically significant correlation between poultry density and dispersal velocity. The association between spatial proximity and farm infection is probably the most consistently reported risk factor for poultry infectious diseases[17,23,29]. Although an airborne transmission has been proposed for IBV, its occurrence has rarely been demonstrated experimentally[30]. However, the spatial proximity likely increases the likelihood of a greater number of horizontal contacts between farms, including the movement of people, vehicles and fomites between farms, as well as sharing similar risk factors (e.g. environmental conditions, climate, presence of wild animals, etc.)[16,23]. Based on these premises, the presence of segregated poultry companies should represent an effective obstacle to viral shedding and the obtained results partially confirm these evidence. The strains from different poultry companies formed two independent clusters, which suggests the effectiveness of independent production flow/chain in protecting farms from exogenous introductions. Additionally, the application of adequate biosecurity measures, enforced also by the Italian legislation, likely contributed in limiting new strain introduction.

The exceptions to this general rule were farms located in the high densely populated poultry area of Northern Italy, where an overlap between the two companies occurs. The unidirectionality of the viral flux from *Company A* to *Company B* implies that other factors, besides spatial proximity, must be in place. A detailed survey could shed some insights into relevant factors like different biosecurity measures, structural factors, vaccination strategy etc.. The mediation of other "actors" cannot also be excluded. In fact, the analysis of just two companies, however predominant they are on the Italian poultry sector, cannot be considered an accurate depiction of the Italian situation. Remarkably, the inclusion of a third *deme* (representative of other unsampled companies and farms) in the analysis model highlighted that several transmission events could be mediated by smaller entities operating in the same region. Actually, the high migration rate estimated between *Company B* and this *ghost deme* poses in favor of its pivotal role in maintaining an active IBV circulation.

Even if the idea of modeling *demes* for which no sequences are available could seem counterintuitive, previous studies showed that the structured coalescent can provide meaningful estimates even in absence of samples from one population[31] and this approach has already been applied and proven effective for other diseases, including Ebola[32]. Since also Company A was evaluated in the same analysis run, the absence of relevant links between this company and the ghost *deme* further supports the analysis reliability, posing in favor of an actual interaction between *Company B* and the *ghost deme* rather than a mere low specificity of the method. A less effective control of IBV infection could be speculated for small companies, whose management capability and resources are limited compared to big-integrated companies. In fact, all Italian farms have to follow national legislation[33] dictating the minimum biosecurity measures to be applied. However, integrated poultry farms, part of major companies, enforce additional managerial practices to increase biosecurity levels. Personnel and veterinarian formation, internal audits and periodic controls guarantee a higher level of application of the required standards, compared to most of small non-integrated farms.

The higher spatial overlap and the likely sharing of some infrastructures (e.g. streets, accessory personnel, services and infrastructures) could nevertheless have a negative indirect effect on the major companies, especially in Northern Italy where *Company B* is located. However, differences between *Company A* and *Company B* in the application of biosecurity measures and production flow management could also explain the different IBV epidemiology, as demonstrated by the dissimilar patterns in viral population fluctuations in the two companies (Fig. 2). A further risk factor that would deserve further investigation is the presence of the rural sector, which is highly concentrated in the densely populated poultry area of Northern Italy. This sector is characterized by a complex mix of growers, dealers and backyards flocks, often applying poor biosecurity measures and linked together by a poorly traceable contact network[34]. Although interactions with industrial poultry farming is hardly discouraged, illegal/indirect interactions have been documented and multiple epidemiological connections could result in a bidirectional transmission between the two sectors, as demonstrated in the Italian low pathogenicity Avian influenza (AI) outbreaks occurred in 2007–2009[34]. After these episodes, a stricter legislation has been developed, imposing limits to animal movements and more active surveillance in the rural sector. Nevertheless, no measures were taken for the monitoring and control of IBV in these enterprises, and therefore their role as sources of encroachment in intensive farming cannot be excluded.

Other environmental factors do not seem to play a relevant role in affecting viral dispersal. While climatic conditions like temperature, humidity and wind could actually affect viral viability and spreading, their effect could be circumvented by a transmission mediated by "fast-moving" vectors like trucks, personnel and, potentially, wild species[35,36]. More surprising could be the non-significant role of road density. However, it must be stressed that the available raster reported the overall density of roads, which could significantly differ from those preferentially used for live animal or their byproduct transportation, hindering the detection of an otherwise plausible risk factor. Therefore, the mapping of the live animal transportation pathways could provide remarkable benefits in IBV (and other infectious diseases) epidemiology understanding and control.

The present study demonstrates that IBV spreading potential is mainly affected by farm and poultry density overall, which can be reasonably claimed as a major risk factor. Other environmental/climatic variables do not seem to affect IBV epidemiology, stressing the pivotal role of human action and thus highlighting the direct benefits that could derive from an improved management and organization of the poultry sector on a larger scale. Actually, the integration of poultry production seems to provide a relevant constrain to IBV circulation, even though some differences were noted between the two considered companies. In fact, despite differences in management and applied control strategies likely playing a role, the presence in the same area of other minor poultry companies seems to represent a major issue, probably due to the less effective infection control ascribable to the sometimes lower organization capability and resources of small enterprises. The present study results emphasize the need of an active sharing of sequences and related molecular epidemiology data originating from all the actors in poultry production, allowing a proper depiction of the viral exchange dynamics, based on actual data rather

than estimations. The obtained information would represent a fundamental substrate for the implementation of effective and shared efforts for the infection control on a broad regional scale.

Materials and Methods

IBV strain sampling, diagnosis and sequencing. Samples were collected for routine diagnostic purpose in the period 2012-2016 from poultry flocks belonging to the two main poultry companies (here named *Company A* and *Company B*) operating in Italy, which account together for about 90% of Italian poultry production. Samples were obtained mainly from outbreaks of respiratory disease, following a standard protocol that enforced the collection of a pool of 10 tracheal swabs from randomly selected birds. For each sampling, collection date and farm localization were recorded. All considered samples had been performed in the context of routine diagnostic activity and no experimental treatments or additional assays were implemented during the study. Therefore, no ethical approval was required to use specimens collected for diagnostic purpose. Additionally, several samples from *Company A* were already sequenced using the same protocol and published in Franzo *et al.*[27]. When detailed information on sampling farm and time could be traced back, these samples were included in the study. The permission to use the collected samples for research purpose was obtained from each company.

Swab pools were resuspended in 2 ml of PBS and vortexed. Thereafter, RNA was extracted from 200 µl of the obtained eluate using the High Pure Viral RNA Kit (Roche Diagnostics, Monza, Italy) kit. Diagnosis was performed by amplification and Sanger sequencing of the hypervariable region of the S1 region using the primer pair described by Cavanagh *et al.*[37]. Obtained chromatograms quality was evaluated using FinchTV (http://www.geospiza.com) and consensus sequences were generated using CromasPro (CromasProVersion 1.5).

Sequence dataset preparation. All obtained sequences plus the reference dataset provided by Valastro *et al.* (2016) were aligned using MAFFT[38] and a phylogenetic tree was reconstructed using IQ-TREE[39] selecting as the best substitution model the one with the lowest Akaike's information criterion, calculated using Jmodeltest[40]. The strains clustering with the GI-19 lineage (previously known as QX genotype) were selected and further evaluated for the presence of recombination in the considered region using RDP4[41] and GARD[42]: to limit the computational burden the sequences were clustered using a 99% identity threshold using CD-HIT[43] and a single representative sequence for each cluster was selected. These sequences plus the Valastro *et al.* (2016) references were re-aligned and recombination analysis was performed. Recombinant sequences, including the ones belonging to the same cluster, were removed from the dataset. Finally, the dataset was re-expanded to the original size and sequences identical or closely related (p-distance <0.01) to the QX-based vaccines administered in Italy were also excluded. To evaluate the distribution of Italian GI-19 strains in the international scenario, an extensive dataset of S1 IBV sequences was downloaded from GenBank and a phylogenetic tree was reconstructed as previously described. To reduce computational complexity and increase interpretation easiness (without losing information), only one sequence representative of all identical ones was selected using CD-HIT and included in the analysis.

The presence of an adequate phylogenetic signal was assessed by a likelihood mapping analysis performed with IQ-TREE. TempEst was used to preliminarily evaluate the temporal signal of the Italian QX phylogeny and therefore the applicability of molecular clock-based methods[20].

Strain migration among integrated poultry companies. IBV QX strain migration among companies was evaluated using the structured coalescent-based approach implemented in the MultiTypeTree extension of BEAST2[44]. According to this model, the considered population is divided in a series of *demes*, which can be imagined as different islands, featured by their own populations size and interconnected by a certain migration rate among them.

In the particular Italian QX scenario, the serially sampled (i.e. with known collection date) strains were used to infer the migration rate and history between the two integrated poultry companies (i.e. considered as different *demes*) over time. Additionally, the Bayesian approach implemented in BEAST allowed to contextually estimate other population parameters, including the time to most recent common ancestor (tMRCA), evolutionary rate and population size.

Accounting for the presence of other farms and companies operating in the Italian poultry sector, which could take part in or mediate the viral transmission among the investigated major companies, a third *"ghost" deme* (a *deme* for which no sequences were available) was added to the model[31]. The priori of the *ghost deme* size was set to one tenth of the other *demes*, according to the estimated poultry population distribution. However, broad priori distribution (i.e. relatively uninformative priori) was chosen to avoid constrains or biases in the parameter posterior estimation.

For all analyses, the best substitution model ($TN93 + G_4$) was selected based on the Bayesian information criterion, calculated using Jmodeltest[40], while the relaxed lognormal molecular clock model was selected based on marginal likelihood calculation and comparison using the Path Sampling and Stepping Stone method[45].

The final estimations were obtained performing a 200 million generation Markov chain Monte Carlo run, sampling parameters and trees every twenty thousand generations. Results were visually inspected using Tracer 1.5 and accepted only if mixing and convergence were adequate and the Estimated Sample Size was greater than 200 for all parameters.

Parameter estimation was summarized in terms of mean and 95% Highest Posterior Density (HPD) after the exclusion of a burn-in equal to 20% of the run length. Maximum clade credibility (MCC) trees were constructed and annotated using Treeannotator (BEAST package).

Results consistency was also evaluated performing a "traditional" serial coalescent analysis in BEAST 1.8.4[46]. The same substitution and clock model of the structured coalescent analysis were selected, while a nonparametric skyline population model was chosen to reconstruct the viral population dynamic over time[47]. Independent

analysis for each integrated company were also performed using the same approach but generating two new datasets including only the sequences collected from a specific company. However, sequences introduced from one company to the other were excluded from the company-specific analysis since they did not share a common evolution history.

Continuous phylogeography and determinants of IBV spreading. The history of QX dispersal was reconstructed over time using the continuous phyogeographic approach described by Lemey et al.,[48] using BEAST 1.8.4. Substitution and clock models were selected as previously described. Similarly, the Gamma Relaxed Random Walk was preferred over the other phylogeographic continuous diffusion models based on the marginal likelihood calculation and comparison using the Path Sampling and Stepping Stone method[45,48]. The final estimations were obtained performing a 200 million generation Markov chain Monte Carlo run, sampling parameters and trees every twenty thousand generations. Results were visually inspected using Tracer 1.5 and accepted only if mixing and convergence were adequate and the Estimated Sample Size was greater than 200 for all parameters. The reconstruction of QX movements over time within Italian borders was obtained using SpreaD3, summarizing and visualizing the full posterior distribution of trees obtained in continuous phylogeographic analyses[49].

Pattern and determinants of viral spreading were evaluated as described by (Dellicour *et al.*)[19], using the *seraphim* R library[50]. The history of lineage dispersal was recovered from the posterior trees generated using BEAST and annotated with ancestral longitude and latitude reconstruction. Particularly, the distance, duration and velocity of spatial dispersal were recoded as vectors and used to generate different summary statistics of viral spreading, including dispersal velocity and maximal wave front distances (measured from the location of the tree root).

Several environmental/social variables were considered to determine if they were associated with the dispersal rate of IBV lineages. The environmental rasters describing the variables of are shown in Supplementary Fig. 2.

More in detail, the values in the raster (i.e. altitude, population density, poultry density, temperature, etc.) were used to associate a weight to the abovementioned vector. Two models of spatial movements were considered: (1) "straight line (SL) path" model, assuming a straight movement between the starting and ending locations of each branch (i.e. the branch weight is computed as the sum of raster cells through which the straight line passes); (2) "least cost (LC) path" model, using a least cost algorithm (i.e. the branch weight is computed as the sum of the values of cells transition values between adjacent cells along the least-cost path). In this model, the analyzed environmental variable can be considered both as a conductance (i.e. enhancing viral dispersal through the cells with higher values) or resistance factor (i.e. allowing an easier dispersal through cells with lower values). Both instances were evaluated for each considered factor.

The obtained "environmental" weights were used to calculate a regression with the branch duration and the corresponding coefficient of determination (R^2_{env}) was obtained. A null coefficient of determination (R^2_{null}) was also calculated assuming the null raster (i.e. when only the spatial distance of each movement is assumed to affect branch duration). The statistic $D = R^2_{env} - R^2_{null}$ was selected as final outcome, and describes how much the regression is strengthened when the spatial variation in the environmental variable is included. To account for the phylogenetic uncertainness, the D statistic was calculated for each tree of the posterior distribution. However, for computational constraints, the number of posterior trees was down-sampled to 1000 after discharging a 20% burn-in. Only the environmental variables with more than 90% of D statistics > 0 were considered for further analysis. Particularly, the significance of D statistic of those variables was assessed against a D null distribution obtained by randomizing 1000 times the phylogenetic nodes location under the constraint that branch length remained equal. A p-value was generated for each initial tree, therefore a percentage of the trees with p-value $<$ 0.05 could be calculated, which can be interpreted as a posterior probability of observing a significant correlation between lineage movements and considered environmental variable. According to Dellicour *et al.*, (2016), a percentage of p-value < 0.05 greater than 50% was considered a strong evidence that the environmental variable is associated to viral movement speed[19].

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Author contributions

G.F., A.M. and M.C. planned the study, G.F., C.M.T., M.L., T.T., R.C., P.P. performed laboratory work and generated the sequences obtained in the present study, C.M.T. and M.L. curated the sequences dataset, G.F. analyzed the data, M.C., A.M., P.M., G.T., G.O., L.G. supervised the respective research groups, G.F. wrote the manuscript, C.M.T., M.L., M.C. revised and improved the manuscript. All authors reviewed and agreed on the current version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to G.F.

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Effect of genome composition and codon bias on infectious bronchitis virus evolution and adaptation to target tissues

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Effect of genome composition and codon bias on infectious bronchitis virus evolution and adaptation to target tissues

Giovanni Franzo*, Claudia Maria Tucciarone, Matteo Legnardi and Mattia Cecchinato

Abstract

Background: Infectious bronchitis virus (IBV) is one of the most relevant viruses affecting the poultry industry, and several studies have investigated the factors involved in its biological cycle and evolution. However, very few of those studies focused on the effect of genome composition and the codon bias of different IBV proteins, despite the remarkable increase in available complete genomes. In the present study, all IBV complete genomes were downloaded ($n = 383$), and several statistics representative of genome composition and codon bias were calculated for each protein-coding sequence, including but not limited to, the nucleotide odds ratio, relative synonymous codon usage and effective number of codons. Additionally, viral codon usage was compared to host codon usage based on a collection of highly expressed genes in IBV target and nontarget tissues.

Results: The results obtained demonstrated a significant difference among structural, non-structural and accessory proteins, especially regarding dinucleotide composition, which appears under strong selective forces. In particular, some dinucleotide pairs, such as CpG, a probable target of the host innate immune response, are underrepresented in genes coding for pp1a, pp1ab, S and N. Although genome composition and dinucleotide bias appear to affect codon usage, additional selective forces may act directly on codon bias. Variability in relative synonymous codon usage and effective number of codons was found for different proteins, with structural proteins and polyproteins being more adapted to the codon bias of host target tissues. In contrast, accessory proteins had a more biased codon usage (i.e., lower number of preferred codons), which might contribute to the regulation of their expression level and timing throughout the cell cycle.

Conclusions: The present study confirms the existence of selective forces acting directly on the genome and not only indirectly through phenotype selection. This evidence might help understanding IBV biology and in developing attenuated strains without affecting the protein phenotype and therefore immunogenicity.

Keywords: Infectious bronchitis virus, Codon Bias, Genome composition, Evolution

* Correspondence: giovanni.franzo@unipd.it; giovanni.franzo1@gmail.com
Microbiology and Infectious Diseases, Department of Animal Medicine, Production and Health (MAPS), University of Padua, Viale dell'Università 16 - 35020 Legnaro, Padua, Italy

Background

Infectious bronchitis virus (IBV), a member of the family *Coronaviridae,* genus *Coronavirus,* classified within the species *Avian coronavirus* (https://talk.ictvonline.org/), is one of the most relevant viral poultry pathogens and responsible for remarkable economic losses worldwide due to both direct and indirect costs [1]. IBV mainly causes upper respiratory tract disease, which can lead to high mortality when secondary infections occur. High mortality is also associated with some strains able to cause nephritis. Additionally, the genital tract of layer and breeder birds can be affected, causing reproductive disorders and altered egg production [2].

IBV is characterized by a single-stranded positive-sense genome of approximately 27 kb that codes for at least 10 open reading frames (ORFs) [1]. The 5′ two-thirds of the genome encodes two polyproteins, pp1a and pp1ab, which are then proteolytically cleaved in 15 nonstructural proteins. Production of pp1ab requires the translating ribosome to change the reading frame at the frameshift signal that bridges ORF1a and ORF1ab [3].

The rest of the genome encodes structural proteins, including Spike (S), Envelope (E), Matrix (M) and Nucleocapsid (N) [1]. Accessory proteins (3a, 3b, 5a and 5b) not fundamental for virus replication [4] have been identified and proven to be involved in virus–host interactions and immune response modulation during infection [5]. Coronaviruses are well-known to interact at various levels with cell signalling and innate and adaptative responses to maximize their replicative success and limit recognition by the host defence system [6, 7]. Although most of the current knowledge is based on experimental evidence, the increasing sequencing capability, coupled with improved modelling approaches, has contributed in several ways to the study of these viruses. Indeed, sequence analysis has allowed us to reconstruct the epidemiology of IBV strains, identify their differences, estimate the causes and strength of selective pressures shaping their evolution and evaluate the consequences, just to mention a few [8–10]. However, with limited exceptions, genome analysis has been considered an indirect and easier way to investigate IBV protein features. Nevertheless, it must be stressed that the viral RNA genome cannot be reduced to the genotype concept (i.e., a mere "string of text" coding for a certain phenotype), as the RNA molecule has its own phenotypic features and is thus under the action of direct selective pressures. For example, genome base composition can alter physical properties, such as stability at different temperatures, pH, and metal concentration [11–13], as well as functional aspects, such as those ascribable to the presence of secondary structures. Several studies have demonstrated the presence of a relevant genomic signature in dinucleotide frequencies in different organisms. In eukaryotic genomes, TpA is broadly under-represented, likely because of the higher susceptibility to degradation by ribonucleases, lower thermal stability and occurrence of the TA dinucleotide in two stop codons as well as in many regulatory regions [14, 15]. In addition, the CpG dinucleotide is similarly underrepresented because cytosine in CG dinucleotides is easily methylated, and this form tends to spontaneously deaminate to thymine [16].

Interestingly, even the microbiota of different environments features distinct patterns, supporting the direct or indirect effect of environmental conditions on organism genome composition [17].

Codon bias is another phenomenon potentially affecting organism fitness in the absence of a direct effect on protein primary structure. Because of the degeneracy of the genetic code, the 20 amino acids are encoded by 61 codons. As there are more codons than amino acids, the genetic code is necessarily redundant, and most amino acids are encoded by two to six different codons [16]. However, different synonymous codons are used with different frequencies among organisms or even among tissues of the same organism [18, 19].

Two non-conflicting hypotheses have been proposed to justify codon bias occurrence: 1) the mutational hypothesis suggests that uneven codon usage is due to the underlying genome composition and therefore to forces favouring certain types of mutations [20]; 2) the selectionist hypothesis postulates the occurrence of selective forces directly acting on codon bias. In fact, a positive correlation has been observed between gene expression and codon bias, with highly expressed genes enriched in the most frequent optimal codons. In addition to translation efficiency, codon usage has been related to gene expression level, translation fidelity, appropriate protein folding and overall organism fitness [16, 21, 22].

Currently, the most accepted model, the mutation-selection-drift balance model of codon bias, proposes selective forces favouring preferred codons, whereas mutation pressure and genetic drift allow for the persistence of minor ones [23, 24].

Although the intensity of selective forces acting on codon bias are often considered weak [16], viruses can represent a remarkable exception. As intracellular obligate parasites, they must accomplish two fundamental tasks: escaping from the host immune system and being able to efficiently exploit the cell synthetic machinery. Accordingly, the virus-host association in terms of codon bias and genome composition has been reported by different authors [25–28], and in some instances, progressive viral adaptation after a host jump has been proven [28, 29].

These viral features can clearly affect IBV biology, fitness and virulence, although the issue has rarely been

investigated [30], despite the availability of a remarkable number of complete genomes and host tissue-specific gene expression levels.

Results

Genome base composition

Overall, IBV coding regions showed a lower percentage of C and G nucleotide, although with a certain variability among proteins. When the distribution was evaluated for different codon positions, the CG content decreased from the first to the third codon position. A summary of genome composition features is provided in Additional file 1 and Additional file 2.

Dinucleotide pairs *Rho* statistic calculation analysis revealed that several residues could be considered as over- or underrepresented (Additional file 3) according to the cut-offs proposed by Karlin et al., (1998) [31]. However, the limited sequence length and the likely confounding effect of codon bias and amino acid sequence suggest caution in the results interpretation. The Z-score calculated by random permutation of synonymous codons represents thus a more robust estimation. This statistic confirmed the presence of different dinucleotide pairs significantly over or under-represented compared to what is expected by chance. Particularly, CpG and TpC were highly under-represented in pp1a and pp1ab and to a lesser extent in the two main structural proteins, S and N. Accessory, M and E proteins were within the expected ranges. Similar patterns were observed for ApT and GpA in the pp1a, pp1ab and S. On the contrary, pp1a, pp1ab and S revealed over-represented ApC, ApG, CpA, GpT and TpG dinucleotide pairs. CpT and GpC were overrepresented in polyprotein region only (Fig. 1). Overall, accessory, E and M proteins had a dinucleotide content essentially explainable by C and G frequency only.

The 2 principal components of PCA performed on Z-score explained almost 80% of the overall variability, and were therefore used to summarize the dinucleotide features of IBV genes.

Two different patterns were clearly observed. pp1a, pp1ab and S protein formed separate clusters on the

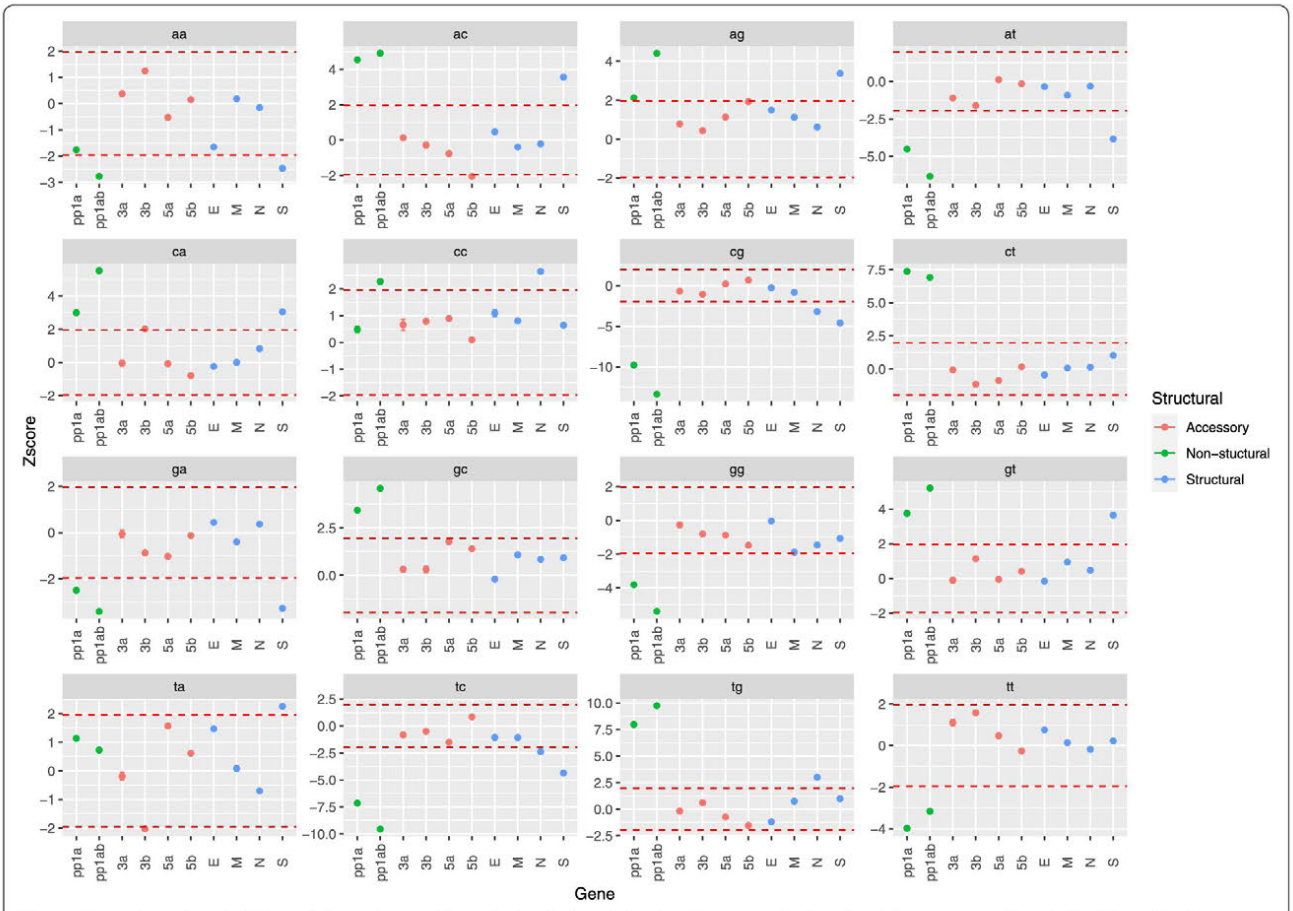

Fig. 1 Mean (point) and 95% confidence interval (errorbar) calculated for the Z-score each dinucleotide-gene pair. The dashed lines (i.e. Z-score ± 1.96) highlight the cut-off for significantly under- and overrepresented dinucleotides. Structural, non-structural and accessory proteins have been color-coded. The figure was generated using ggplot2 [32]

negative side of PC1, while the rest of the proteins constituted a more homogeneous group, being the accessory proteins located on the positive extreme of PC1. M and N proteins, featured by less positive values, were differentiated based on PC2 values. Similarly, 5a and 5b were differentiated from 3b through PC2 scores, although a sparser distribution and relevant overlapping were present, involving especially protein 3a (Fig. 2a). Principal components loading analysis confirmed the high weight of several nucleotide pairs in differentiating the two main gene groups along the PC1 (e.g.. CpG, TpC, ApT, etc. were positively correlated to PC1), while TpA and CpC were especially correlated with PC2 scores (Fig. 2a).

Relative synonymous codon usage

Relevant differences in RSCU were observed among codons, similarly to what observed for dinucleotide frequency. Although a certain variability was observed among proteins, some common patterns could be observed. Particularly, codon containing the CpG dinucleotide were within the expected ranges or, more frequently, under-represented (Fig. 3). Codon CGT and CGC were the only exceptions, being the former slightly overrepresented in 1a, 1ab, N and S genes and the latter in 5b one (Fig. 3). Based on PCA eigenvalues evaluation, the first two principal components (PC1 and PC2) were maintained since explaining more than 30% of the overall variability. The observed pattern was featured by a higher similarity in codon bias usage among structural and non-structural proteins compared to accessory ones (Fig. 2b). Particularly, a closer relationship was observed between pp1a, pp1ab and S protein, and between M and N ones. The E protein was the only exception, forming a separated cluster largely overlapping with the codon usage pattern of 3a and 3b proteins, which had a highly heterogeneous distribution. Although comparably heterogeneous, 5a and 5b formed essentially independent groups. PCA loading analysis highlighted the primary contribution of CpG enriched/depleted codon in determining PC1 values (being 6 out of 8 positively correlated to PC1) (Additional file 4). Similarly, 6 out of 8 of CpG enriched codons contributed positively to the PC2. In both instances, the CpG demonstrated higher loadings

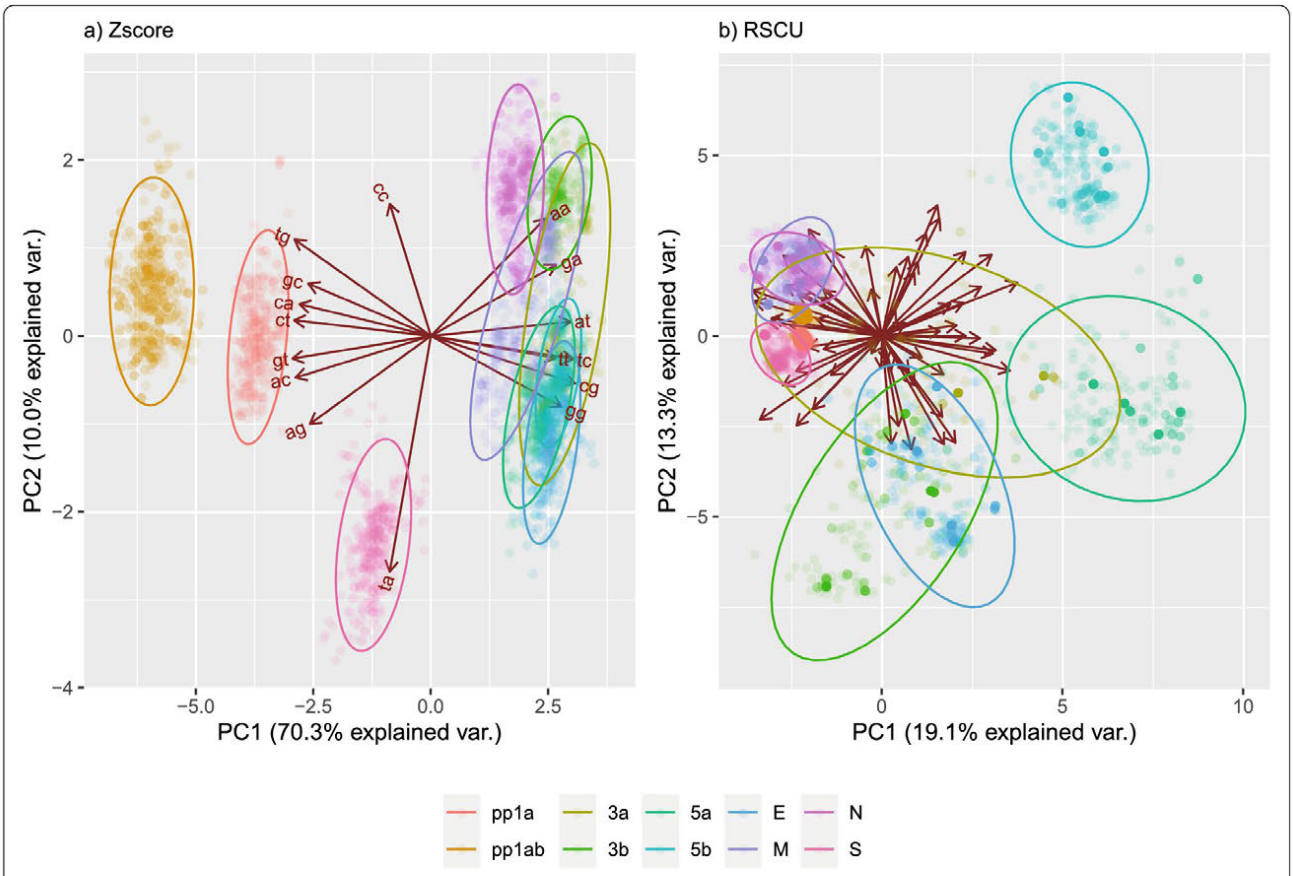

Fig. 2 Scatter plot based on the first two components of the PCA performed on Z-score (**a**) and RSCU (**b**) calculated for all IBV proteins (color-coded). The PCA loadings are represented as arrows. However, for graphical reasons the labels have been removed from (**b**) and the loading values are provided in Additional file 4. The 95% confidence ellipses around clusters are also reported. The figure was generated using ggplot2 [32]

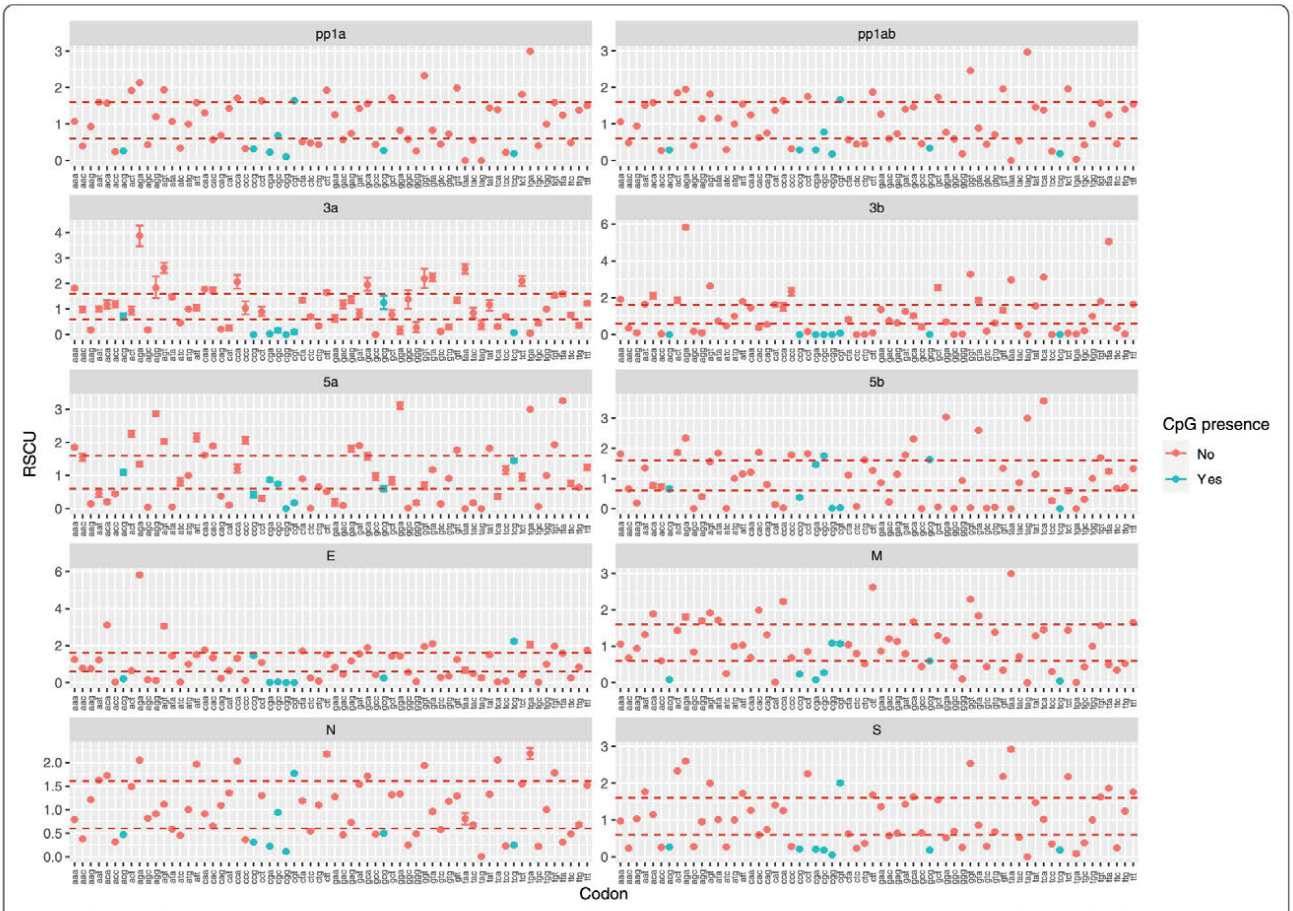

Fig. 3 Mean (point) and 95% confidence interval (errorbar) of RSCU statistic calculated for each gene–codon pair. When the CpG pair was present in the codon, it has been highlighted in blue. The dashed lines (i.e. RSCU = 0.6 and 1.6) highlight the cut-off for significantly under- and overrepresented codons, respectively. The figure was generated using ggplot2 [32]

on average compared to the other codons. However, the limited number of CpG rich codons prevented any robust statistical inference. Therefore, structural and non-structural proteins were located in PCA regions representing codons with low CpG content, i.e. negative values on PC1 (pp1a, pp1ab, M, S and N) or PC2 (E).

Nc and Nc plot

Effective number of codon calculation revealed a relevant difference among IBV proteins. Accessory proteins showed the more biased codon usage, with lower Nc values compared to structural and non-structural ones (Additional files 1 and 5). When nucleotide composition was accounted for, higher Nc' values were obtained. However, the above-mentioned difference remained or was even magnified (Fig. 4).

The Nc values were constantly lower than the ones expected based on CG3 content only. While this remained true for accessory, E and M protein coding regions even after accounting for genome composition, the Nc' of the N gene lied on the expected value and was higher for the polyproteins and S genes, which overall showed values comparable with the host ones (Fig. 4).

Neutrality plot, general average hydropathicity (gravy) and aromaticity (aroma) indices

A significant association ($p < 0.05$) between GC12 and CG3 content was demonstrated for pp1a (b = 0.10), 3a (b = 0.10), 5b (b = 0.17), E (b = 0.16), M (b = 0.15), S (b = 0.11) and N (b = 0.09) genes. Therefore, mutation drift accounted for approximately 10% of the codon bias of 1a, 3a, S and N genes, while a more intense effect (approximatively 15–20%) was estimated for 5b, E and M ones. Overall, the impact of mutation bias can be considered low. Similarly, regression analysis demonstrated that Gravy and Aroma indices were significantly associated ($p < 0.05$) with the PC1 and/or PC2 of Z-score and/or RSCU (Additional file 6), a trend confirming the occurrence of additional selective pressure acting on codon and dinucleotide composition rather than the effect of genome composition or mutation bias only.

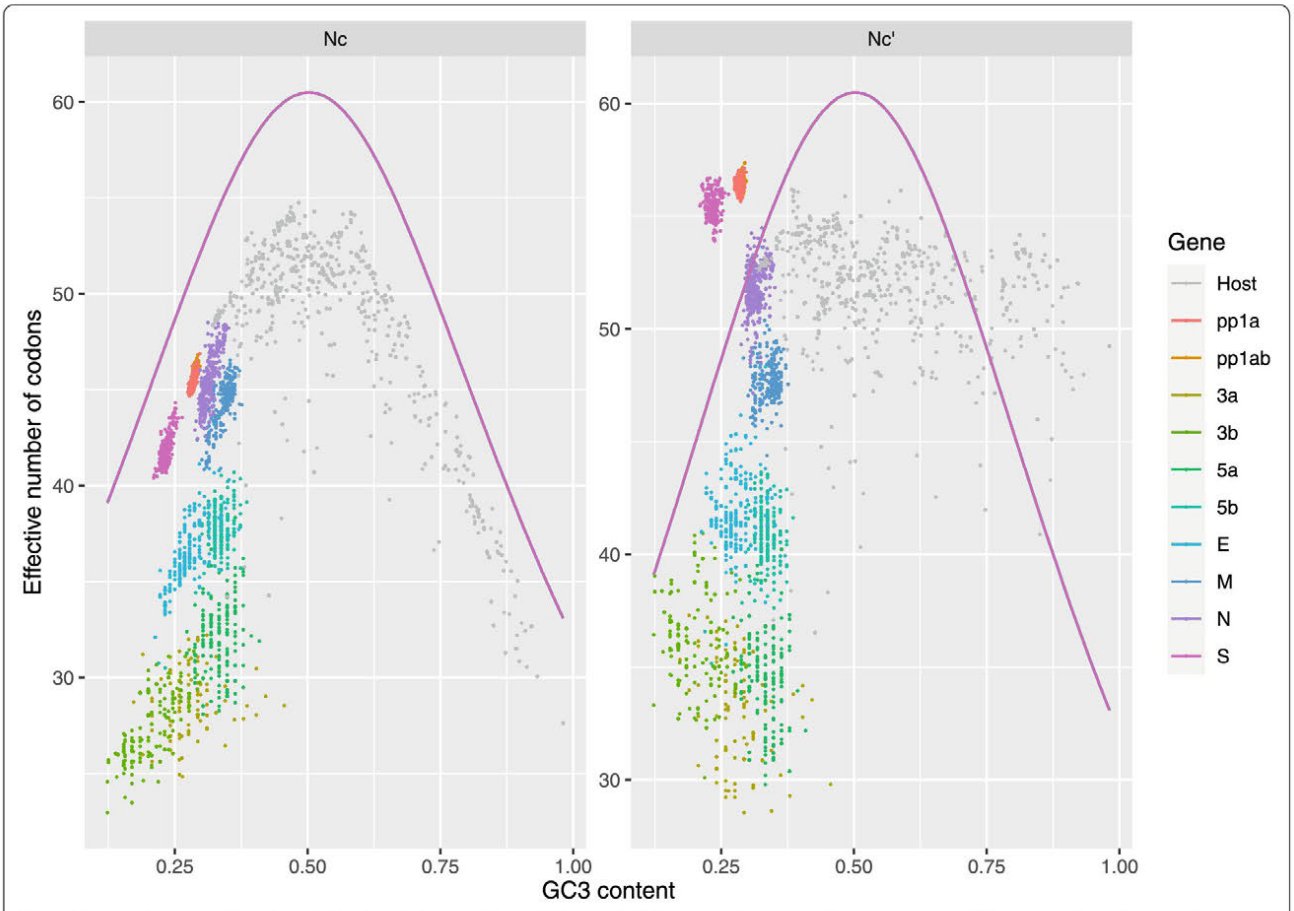

Fig. 4 Scatterplot reporting the relationship between Nc and Ncp and GC3 content of IBV coding sequences. IBV proteins have been color –coded while the host genes have been reported in grey. The line representing the expected Nc values, which would result from GC composition being the only factor influencing the codon usage bias, has been superimposed. The figure was generated using ggplot2 [32]

CAI analysis

The CAI of IBV proteins was calculated based on the relative adaptiveness of each codon based on the most expressed genes of considered tissues. Irrespectively of the considered organ, the CAI was on average lower for accessory proteins compared to non-structural and especially structural ones (Fig. 5a). However, when single genes were evaluated, a more complex pattern was observed. Most genes had a value of approximate 0.7, N showed the higher value while accessory protein 3a and 3b had the lowest CAI value. E gene was the structural protein coding gene with the lowest CAI value (Fig. 5b and Additional file 1). Despite these differences, a constantly lower CAI was observed in non-target tissues compared to target ones.

Discussion

The present study highlights a relevant heterogeneity in genome composition and codon bias among IBV genes. Different dinucleotide pairs were shown to be significantly underrepresented, as demonstrated by several dinucleotide odds ratio values lower than the 0.78 and 1.23 cut-offs proposed by Karlin et al. (1998) [31] (Additional file 3). However, these thresholds can be considered accurate for long sequences only [31]. Additionally, dinucleotide frequency might be affected by codon bias and by amino acid composition imposed by protein functional constraints. After accounting for the codon bias and amino acid constraints of the studied sequences using a permutation approach, several dinucleotide pairs still significantly deviated from what was expected by chance alone (Fig. 1). Similar to what has been described for influenza A virus (IAV) [34], noteworthy variability was observed among IBV genes. In particular, the CpG pair was highly underrepresented in the genes encoding polyprotein, spike and nucleocapsid. This pair is well known to be underrepresented in eukaryotic genomes, as cytosine in CG dinucleotides are easily methylated and tend to spontaneously deaminate to thymine [15, 16]. However, methylation does not seem to occur in viruses, especially in RNA viruses that use their own synthetic apparatus for genome replication and transcription [35]. Other causes should thus be

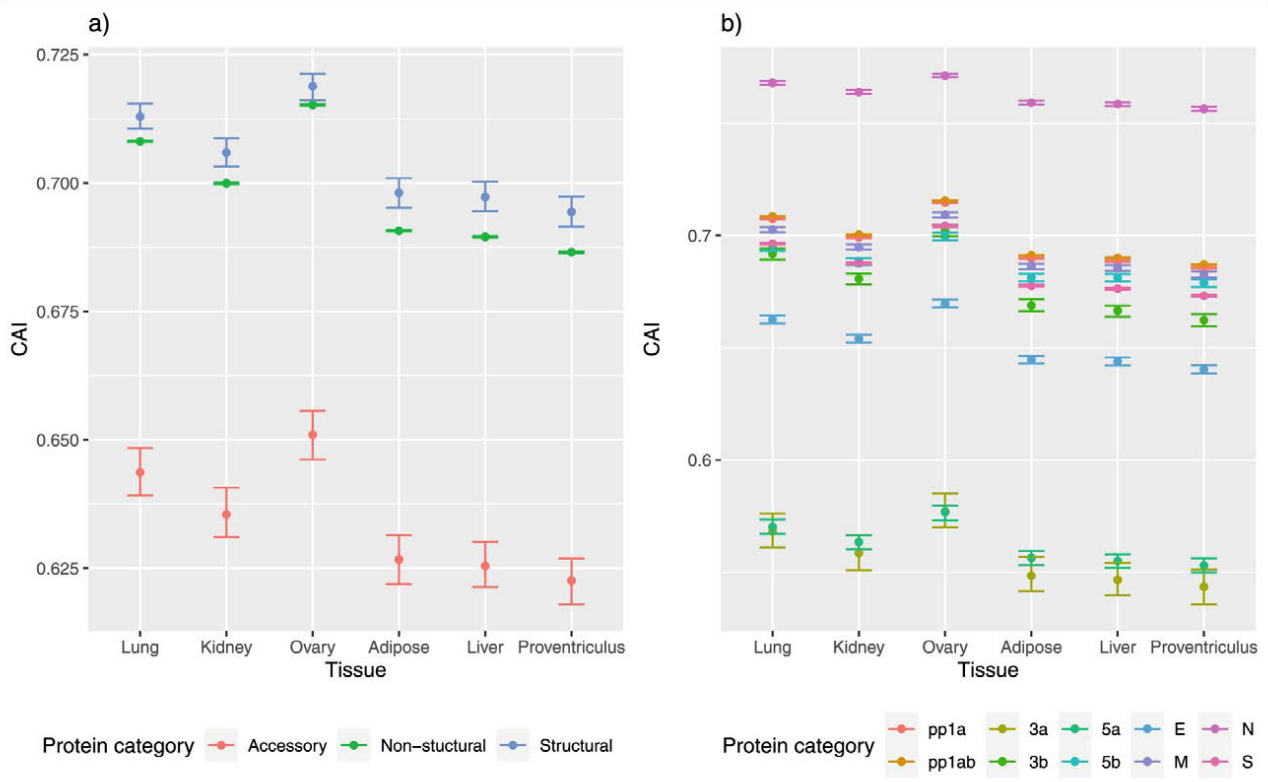

Fig. 5 Mean (point) and 95% confidence interval (errorbar) of the CAI index calculated for genes corresponding to different protein category (**a**) and proteins (**b**) (color-coded) with respect to different host tissues. The figure was generated using ggplot2 [33]

evaluated. Unmethylated DNA is a well-known target of the pattern recognition receptor (PRR) Toll-like receptor 9 (TLR-9) in mammals and is thus involved in innate immune response activation. Interestingly, TLR-9 is absent from the avian genome, and no orthologue gene has been identified [36, 37]. Nevertheless, chicken TLR-21 has a comparable function [38], despite some differences in activation when stimulated by pathogens [39, 40]. Therefore, the tendency of DNA viruses to reduce their CpG content can be easily explained. Much more under debate is whether similar forces act on RNA viruses. Other TLRs, such as TLR3, TLR7, and TLR8 (which is a pseudogene in chickens), PRRs such as RIG-I (absent in chickens) and MDA5 have been demonstrated to target RNA viruses [41, 42], but none has been proven to recognize CpG regions. Nevertheless, more recent evidence suggests that ssRNA oligonucleotides expressing unmethylated CpG can elicit monocytes and stimulate PBMCs through a mechanism independent of TLR3, – 7, – 8 or – 9 [43].

Atkinson and colleagues demonstrated that experimentally increasing the CpG and, to a lesser extent, the TpA content leads to echovirus 7 attenuation, lower replication rates and low competitive fitness relative to wild-type [44]. More recently, Takata et al. (2017) proved that the zinc-finger antiviral protein (ZAP) selectively binds to sequences containing CpG dinucleotide and that HIV strains with a modified CpG content are defective in normal cells but able to replicate in ZAP-defective cells [45].

Therefore, host immune pressure can be considered the most likely selective force shaping IBV genome towards a reduction in CpG motifs, as proposed for other viruses [46, 47]. In contrast to influenza, TpA was not underrepresented in any of the viral proteins, similar to what was previously reported for other members of *Coronaviridae* [48]. This evidence was unexpected, as TpA upregulation had detrimental effects on viral fitness according to Atkinson et al. (2014) [44]. Thus, other host response mechanisms might be involved and potentially circumvented in various ways by viruses belonging to different families.

Interestingly, the polyproteins and spike protein exhibited the most biased dinucleotide usage and were clearly differentiated from the others in PCA (Fig. 2). A lower variability, suggestive of stronger constraints, was also evidenced, especially when compared to accessory proteins. Two phenomena might contribute to the observed scenario. The first involves a higher transcription level and mRNA abundance of genomic regions coding for abundant viral proteins (S) or functional ones (pp1a and pp1ab). Additionally, a large number of genomic RNAs (constituted for two-thirds by the polyprotein coding region) are produced and present in the cytoplasm before

encapsidation. RNA abundance might represent a factor imposing the minimization of immune-stimulatory domains such as CpG ones. Regardless, transcription of these genes appears to be comparable to that of other IBV proteins [49]. An alternative hypothesis involves the absolute number of CpG molecules. Significantly, these proteins are the longest ones, and a significant negative correlation was found between the CpG Z-score and IBV CDS length ($b = -0.002$; $p < 0.001$). Therefore, the higher absolute CpG content in mRNA molecules might reduce the relative amount, minimizing viral recognition. Interestingly, the relationship between the CpG absolute count and CDS length can be adequately described by two ratios: one representative of pp1a, pp1ab and S and another, higher one, of the remaining CDS (Additional file 7). Therefore, additional forces are to a certain extent, likely contributing to the observed dinucleotide composition.

The evaluation of codon bias based on PCA allowed us to classify IBV proteins, albeit less clearly. In particular, pp1a, pp1ab and S had a significantly overlapping distribution, closely mimicked by the N and M proteins. On the other hand, E and accessory proteins demonstrated a different and much sparser distribution (Fig. 2). It could be speculated that these differences are linked to differential gene expression and that proteins expressed at similar levels, especially interacting ones, tend to have correlated levels of codon bias [16, 50]. CAI evaluation supported the proposed hypothesis. Overall, IBV codon bias appears to be more adapted to that of target tissues than non-target tissues, including other epithelial and *parenchymatous* organs (Fig. 5). Additionally, higher CAI was observed for the nucleocapsid protein, which is an abundant structural protein, followed by other structural and non-structural proteins vital for viral replication and whose concerted interaction is necessary for viral encapsidation and infectivity [51, 52]. These results, although based on a broader database and evaluated in a tissue-specific fashion, are largely in agreement with the evidence obtained by Brandao et al. (2013) [53], strengthening their robustness. In general, we observed evidence for selective forces optimizing codon profiles, especially for genes coding for highly expressed proteins and fundamental for viral viability. Accessory proteins were also featured by a higher heterogeneity in RSCU distribution (Fig. 2b), which appears to involve lower constraints in their codon usage bias. Nevertheless, the shorter length of their coding region might also have magnified the effect of single, random mutations, thus increasing the "background noise" and affecting the observed variability.

The effective number of codon estimations confirmed the opposite tendency of these groups of proteins. The overall Nc was consistently lower for accessory proteins, indicating to a more biased use of synonymous codons (Fig. 4 and Additional file 5). The genome composition, although relevant, cannot fully explain this pattern. In fact, even after accounting for this confounding factor, most of the genes still deviate significantly from the expected values based on GC3 content only, confirming the action of additional pressures other than mutation bias, in agreement with neutrality plot results. Features allowing viral strains to mimic the genome composition and codon bias of the host or tissues where they replicate can be expected to be under strong selective pressures. Moreover, the huge sample size of viral populations within the same host or cell can favour natural selection over genetic drift, even in the presence of modest selective coefficients.

The Gravy and Aroma significant correlation with PC representative of dinucleotide and Codon composition suggests that also selective pressures acting on proteins could indirectly affect these viral features. Interestingly, the previously reported differences among structural, non-structural and accessory proteins do not seem to hold for Gravy and Aroma indices (Additional file 6). Therefore, differential selective patterns can be suggested to act on viral genome and proteins, although both can indirectly affect the nucleotide and codon composition.

In particular, the polyprotein, S and N coding genes showed a lower bias compared to what was expected by chance alone and mimicked the effective number of codons used by the host (Fig. 4). In contrast, accessory proteins exhibited a much more restricted codon usage (and therefore lower adaptation) than chicken proteins. It could therefore be proposed that structural and non-structural proteins exhibit a broad codon spectrum (i.e., lower codon bias), more similar to the host, to overcome potential replication restriction due to the limiting effect of rare tRNAs, which can induce long waiting times and stall elongation [15].

However, the results of a recent study evaluating IBV gene expression by ribosome profiling appear to reject this hypothesis: despite being highly transcribed, the translation efficacy of the polyprotein, S and N genes was reported to be lower than that of other proteins, including accessory proteins [49]. A limited role of codon bias in gene expression regulation thus cannot be excluded [54]. Our study demonstrates that codon bias is highly affected by overall nucleotide composition, particularly by dinucleotide frequency. Therefore, it is likely that the selection apparently acting on codon bias is largely ascribable to the underlying selection aiming to minimize CpG content and limit viral recognition [34]. Regardless, the remaining differences in terms of codon bias, ENC and CAI among proteins with similar dinucleotide patterns and the different adaptations to target and non-target tissues can hardly be justified by

dinucleotide composition alone. Consequently, an actual effect of codon bias on viral fitness can likely be claimed, despite apparently conflicting with experimental evidence [49].

Although extremely accurate, the study of Dinan et al. (2019) [49] analysed IBV gene expression in chicken kidney primary cell culture, which likely does not represent the actual cell biology in vivo. Additionally, only a "snapshot" of IBV and cell gene expression was obtained, corresponding to a particular moment of the viral cell cycle. Nevertheless, cell transcription activity and pathways can change remarkably at different cycle stages, and differential tRNA abundance can influence viral RNA protein synthesis [55–57] Continuously expressed proteins such as structural ones and those critical for viral replication might benefit from a lower codon bias, be more adapted to the codon spectrum used by target tissue cells and less susceptible to the variation in tRNAs throughout the cell cycle. The high codon bias of some proteins, accessory ones in particular, might contribute to the regulation of expression of these proteins, favouring their presence in particular cell phases. Additional and more focused experimental studies should be performed to evaluate this theoretically plausible hypothesis.

Conclusions

Overall, the present study demonstrates that different forces shape the IBV genome and coding sequences in addition to those acting at the protein level [9]. Constraints in dinucleotide frequency reducing viral recognition by innate immune response likely play both a direct role, conditioning genome composition, and an indirect role, affecting codon bias. However, several lines of evidence support the presence of residual selection acting directly on codon usage, which appears to be linked to host tissue adaptation and potentiality in the regulation of individual protein expression.

This evidence might help understanding IBV biology and the development of attenuated strains without affecting the protein phenotype and therefore immunogenicity. Dedicated experimental studies, based on reverse genetics also, could be of remarkable benefit in confirming the association between viral fitness indexes and codon bias or dinucleotide composition.

Methods

Dataset

The whole collection of IBV complete or almost complete (including all coding regions) genomes (n = 383) was downloaded from Genbank (accessed 28/03/2020). In-house developed Python scripts were used for gene and feature extraction, using the Biopython library functions [58]. Different datasets were created for each coding sequence (CDS) and sequences with unknown nucleotide, frameshift mutations or premature stop codons were excluded from further analysis.

Viral genome composition analysis

For each sequence, the following statistics were obtained: content of each nucleotide, total GC content (GC) and in codon positions 1 (GC1), 2 (GC2) and 3 (GC3).

The dinucleotide odds ratio (*Rho)* was computed for each dinucleotide pair using the R library *seqinr* [59]. The *Rho* represents the frequency of dinucleotide (xy) divided by the product of frequencies of nucleotide (x) and nucleotide (y) and should thus be equal to 1.00 when dinucleotide (xy) is formed by chance. Since dinucleotide frequency can be biased by the protein primary structure (i.e. amino acid sequence) and codon usage bias of these genes, a Z-score was calculated normalizing the observed *Rho* by its expectation and variance estimated performing a random sequence generation, which allowed to consistently evaluate the degree of over- or underrepresentation and its statistical significance. Particularly, the selected models generate random sequence by shuffling of synonymous codons, without affecting the codon usage bias and the protein structure. For each sequence, a total of 1000 simulated sequences were generated for dinucleotide pair.

Relative synonymous codon usage (RSCU) and effective number of codons (Nc)

The RSCU was calculated using the *seqinr* package in R. This statistic, indicative of codon bias, is calculated based on the count of a particular codon, relative to the number of times that the codon would be observed assuming a uniform synonymous codon usage. Consequently, in absence of any codon bias a value close to 1 is expected, while synonymous codons with values lower than 0.6 or greater than 1.6 are classified as under or over-represented, respectively [28, 60].

The Nc values were calculated using the http://agnigarh.tezu.ernet.in/~ssankar/cub.php website [61]. This summary statistic describes the total number of different codons used in a sequence and can thus range between 21 (only one codon used for each amino-acid) and 60 (all synonymous codons are uniformly used) [62]. A second parameter, the Nc' statistic, also ranging between 21 and 60, was calculated to account for the genome composition effect on codon bias [15, 63]. Obtained Nc and Nc' values were plotted against the GC3 content of the relative sequence and compared with the expected Nc distribution under the assumption that it is determined only by GC3 content.

Neutrality plot, general average hydropathicity (gravy) and aromaticity (aroma) indices

A linear regression was calculated between the GC content in the first two codon positions (GC12) of each sequence and the respective GC3 content.

This analysis evaluated the influence of mutational pressure and natural selection on codon usage patterns. The presence of a statistical association and a regression coefficient close to 1 are indicative of mutational bias being the predominant force shaping codon bias patterns.

On the contrary, a regression slope approximating 0 suggests the presence of selective pressure acting on and shaping the codon bias evolution. In this sense, the regression coefficient can be interpreted as a quantitative measure of the mutation-selection equilibrium [64–66].

Gravy and Aroma indices were calculated using the Peptides [67] package in R. Briefly, the Gravy value is the sum of hydropathy values of all amino acids in a sequence divided by the number of residues, while the Aroma value is the frequency of aromatic amino acids in a given amino acid sequence.

Principal component analysis (PCA)

A principal component analysis was performed independently on the dinucleotide Z-score and RSCU of all genes, after centering and scaling, using the *prcomp* function of the *stats* library in R [68]. Loadings and eigenvalues associated to each principal component (PC) were evaluated using the same library.

Codon adaptation index (CAI) calculation

CAI is a summary value (ranging from 0 to 1) that describes the codon usage of a gene relative to the codon usage of a reference set of genes, defining as translationally optimal codons those frequently present in highly expressed genes. It is therefore commonly used to predict the gene expression level based on its coding sequence. In this particular scenario, the CAI value was used to identify the degree of different viral proteins adaptation to the tissue-specific translational machinery. To this purpose, all chicken CDSs were downloaded from Genbank (GCF_000002315.6). Gene expression profiles of different tissues were downloaded from Chickspress [69]. Particularly, lung, kidney and reproductive tract were selected as IBV "target" tissues, while liver and proventriculus were included as "non-target" and "marginal target" tissues, respectively. These were selected as controls representative of tissue with similar features (i.e. parenchymatous or epithelial tissues) known to be irrelevant IBV replication sites. Adipose tissue was also included as representative of a non-target tissue with remarkably different biological features. A collection of tissue-specific highly expressed genes was obtained selecting those whose expression level was in the higher 25 percentile of the considered tissue. The relative CDSs were selected, manually cured (i.e. those with unknown bases or incomplete sequences were removed) and used to calculate the tissue-specific relative adaptiveness of each codon, which was in turn used to calculate the CAI of different proteins for each IBV strain using the *seqinr* [59] package in R. All images of the present manuscript were drawn using the *ggplot2* library [33].

Abbreviations

IBV: Infectious bronchitis virus; ORF: Open reading frame; PCA: Principal Component Analysis; RSCU: Relative synonymous codon usage; Nc: Effective number of codon; CAI: Codon Adaptation Index; CDS: Coding DNA sequence; TLR: Toll-like receptor; RIG-I: Retinoic acid-inducible gene I; MDA5: Melanoma differentiation-associated protein 5

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-021-07559-5.

Additional file 1. Summary of different genome composition and codon bias statistics calculated for each IBV protein. The *P*-value refers to the presence of a significant difference in the mean value of the considered statistic among proteins.

Additional file 2. Density plot representing the distribution of nucleotide composition for different IBV coding regions.

Additional file 3. Mean and and 95% confidence intervals of Rho statistic calculated for each gene–dinucleotide pair. Structural, non – structural and accessory proteins have been color–coded. Dahed lines represent the cut–offs defined by Karling et al., 1998.

Additional file 4. Loadings associated to the RSCU of each codon. When the CpG pair was present in the codon, it has been highlighted in blue.

Additional file 5. Scatterplot reporting the relationship between Nc and Nc' and GC3 content of IBV coding regions. Structural, non – structural and accessory proteins have been color–coded. The line representing the expected Nc values, which would result from GC composition being the only factor influencing the codon usage bias, has been superimposed.

Additional file 6. Table reporting the regression coefficient between Gravy or Aroma indexes and the first 2 principal components (PC1 and PC2) of the principal component analyses (PCAs) based on Z-score and RSCU. Regression coefficients have been calculated for different genes, independently. * indicates statistical significance ($p < 0.05$).

Additional file 7. Relationship between gene length and total CG count. Two regression lines representative of pp1a, pp1ab and S coding regions (in blue) and another for the remaining proteins (in black) have been superimposed.

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Not applicable.

Authors' contributions

GF conceptualized and designed the study, performed the analysis and wrote the manuscript draft. GF, CMT, and ML organized the data. GF and MC supervised the study. GF, CMT, ML and MC colloaborated to results interpretation. All the authors revised and approved the final manuscript version.

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Availability of data and materials

All used IBV sequences are freely available in GenBank (https://www.ncbi.nlm.nih.gov/sites/myncbi/giovanni.franzo.1/collections/59873661/public/),

while chicken CDS are available under the genome assembly accession number GCF_000002315.6.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

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ORIGINAL ARTICLE

Effect of assay choice, viral concentration and operator interpretation on infectious bronchitis virus detection and characterization

Claudia Maria Tucciarone[a], Giovanni Franzo[a], Matteo Legnardi[a], Andrea Fortin[b], Viviana Valastro[b], Elena Lazzaro[a], Calogero Terregino[b] and Mattia Cecchinato[a]

[a]Department of Animal Medicine, Production and Health (MAPS), Università degli Studi di Padova, Viale dell'Università, Legnaro PD, Italy; [b]Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, Legnaro PD, Italy

ABSTRACT

Despite the efforts to achieve a consistent classification scheme based on the complete S1 gene, the genetic characterization of infectious bronchitis virus (IBV) is often performed on partial S1 regions due to economic and time constraints in the diagnostic routine. Sanger sequencing remains the most common and cost-effective option even if the analysis of samples where multiple field and vaccine strain populations coexist can lead to partial or misleading results. The present study aimed to evaluate the different diagnostic outcomes of three commonly used RT–PCR methods targeting two regions of the S1 gene. A possible bias in IBV detection and characterization was investigated in relation to the adopted method, the strain concentration as well as their ratio in mixed samples. Thirty samples were prepared by artificially mixing two vaccine strains, combined at different ratios and selected among four different IBV lineages, i.e. GI-1 (Mass), GI-13 (793/B), GI-19 (QX), GI-23 (Israeli Variant 2). Sequence analysis was conducted both manually and with bioinformatic methods. The result agreement among methods, replicates and analysis approaches was statistically evaluated. Consistent results emerged among the three assays, with a few discrepancies likely caused by primer affinity and target amount. This study confirms the complexity of IBV strain identification and highlights the importance of evaluating and updating the available diagnostic assays for a reliable detection of all circulating IBV strains.

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Introduction

Infectious bronchitis virus (IBV), classified within the avian coronavirus species, is the cause of infectious bronchitis (IB), which is still one of the most burdensome and frequent diseases in poultry, affecting both bird health and management costs (Jackwood *et al.*, 2012). The clinical manifestations are generally characterized by respiratory signs, and a reduction in growth and egg-laying, and they are commonly controlled by biosecurity measures and vaccination (Jackwood & De Wit, 2020). To achieve the widest cross-protection levels, different vaccine strains have historically been associated and administered together (Cook *et al.*, 1999; Terregino *et al.*, 2008; Awad *et al.*, 2016), at least in Europe, where Mass-based (GI-1) vaccines are commonly combined with either a 793/B-based (GI-13) vaccine or a vaccine based on locally circulating strains (Franzo *et al.*, 2016) to provide homologous protection.

Nonetheless, this well-known strategy based on the association of different strains and/or multiple administrations (Jordan, 2017) presents some drawbacks other than the direct costs. Typically, IBV vaccines are attenuated and can replicate and persist for a long time in birds (Tucciarone *et al.*, 2018). Since the induced immunity is not sterilizing, coinfection with field strains can occur, increasing the likelihood of recombination events (Jackwood, 2012). Moreover, it has been proven that some vaccines can persist at the population level as long as they are administered to the flocks (Franzo *et al.*, 2014). Therefore, the simultaneous presence of several strains confuses both the epidemiological picture (Legnardi *et al.*, 2019) and the diagnostic process.

The coinfection issue is particularly challenging considering that routine diagnostic assays are typically unable to identify more than one strain at the same time. The identification of multiple strains within one sample can be obtained only by screening with several genotype-specific molecular assays or by using deep sequencing techniques, the costs of which often make them unviable options for everyday use. For IBV characterization, routine diagnosis mainly relies on RT–PCR and Sanger sequencing, making the best of costs, rapidity and efficiency of the assays. However, no standardized protocol has been defined, and each laboratory adopts different assays in the attempt to keep up with local epidemiology, IBV

CONTACT Giovanni Franzo giovanni.franzo@unipd.it
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evolution and technological progress (De Wit, 2000). In fact, due to the extreme genetic variability of IBV (Valastro *et al.*, 2016), the selection of a single assay able to identify every strain appears impossible and requires the careful evaluation and update of current methods, accounting for the virus propensity to evolve rapidly, the local epidemiological situation and administered vaccines. While a relevant amount of information can still be achieved by RT–PCR followed by sequencing, this technique is not free from limitations, primarily the inability to identify minor viral subpopulations and coinfections, and can also be affected by possible flaws in the RT–PCR process, such as poor sensitivity and specificity.

In an attempt to evaluate the influence of the diagnostic method and operator interpretation on the diagnostic outcome, this study was designed to compare (both manually, and with a bioinformatic approach) the results of three common RT–PCR assays followed by Sanger sequencing, on artificially coinfected samples.

Materials and methods

Sample preparation, amplification and sequencing

Four common vaccines based on GI-1 (Mass), GI-13 (793/B), GI-19 (QX), and GI-23 (Israeli Variant 2) lineages were selected and RNA was extracted using High Pure Viral RNA Kit (Roche, Basel, Switzerland) following the manufacturer's instructions.

Samples were tested with a real-time RT–PCR targeting the UTR region (Callison *et al.*, 2006), and diluted in order to achieve a comparable initial concentration (C_T value ~18) for each vaccine. These were considered as starting samples (SD, starting dilution) for the artificial coinfection reproduction. Each SD was diluted again at 1:100 (−2) and 1:10,000 (−4) and mixed with another vaccine dilution, to produce 30 samples (Supplementary Table 1).

Samples were then amplified in triplicate with the following RT–PCR assays:

- Method A, a one-step RT–PCR performed with primers XCE1+ and XCE2− (Cavanagh *et al.*, 1999);
- Method B, a two-step nested RT–PCR performed with primers SX1+ and SX2−, SX3+ and SX4− (Worthington *et al.*, 2008);
- Method C, a one-step RT–PCR performed with primers IBV260+ and IBV548− (Valastro *et al.*, 2010).

Assay A was performed using the SuperScript™ III One-Step RT–PCR System with Platinum™ Taq DNA Polymerase kit (Invitrogen™, Waltham, MA, USA), assay C with the QIAGEN OneStep RT–PCR Kit (QIAGEN, Hilden, Germany), whereas the retrotranscription phase of assay B was performed separately with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA) and the following PCRs were performed with the Platinum™ Taq DNA Polymerase Kit (Invitrogen, USA).

Products were visualized by capillary gel electrophoresis. Amplicons were Sanger sequenced in both directions with the same primers that were also used in the amplification. Chromatograms were both manually inspected and automatically analysed with bioinformatic means.

Operator-driven analysis and lineage classification

For the manual analysis, chromatograms were visually inspected for a preliminary quality check, and trimmed to remove the primer sequence using FinchTV software (Geospiza, Inc., Seattle, WA, US). Sequences were copied in FASTA format before and after the consensus assembly with ChromasPro 2.1.8 (Technelysium Pty Ltd, Brisbane, QLD, Australia). Consensus, forward and reverse sequences were aligned to the vaccine reference sequences, and a phylogenetic tree was reconstructed within assay to allow a comparison of the characterization outcome.

Read quality evaluation and automatic lineage classification

The operator-independent chromatogram analysis was performed in R using the sangeranalyseR library. Briefly, chromatograms were automatically trimmed in the regions with a Phred quality score lower than 20. Since coinfection can lead to double peaks, the occurrence of these events was annotated. A ratio of 0.2 of the height of the secondary peak to the primary peak was selected to differentiate random noise from actual sub-population presence.

A database was created including the following features for each chromatogram: raw read length, trimmed read length, mean raw read quality, mean trimmed read quality, number of secondary peaks in the raw and trimmed sequences. Additionally, composite measures were created describing the percentage of information lost after trimming compared to the expected read length. Similarly, the percentage of nucleotide positions with double peak evidence was calculated.

The effect of different methods, vaccine and dilution combination on mean trimmed quality, secondary peaks and percentage of information lost were statistically evaluated using a mixed linear model, accounting for the same samples being tested in multiple runs for different methods. All models

were fitted using the nlme (Pinheiro *et al.*, 2019) library in R 3.4.4, and the statistical significance of each model improvement over simpler ones was assessed by the likelihood-ratio test.

When forward and reverse chromatograms of adequate quality were available, the respective FASTA files were generated as well as consensus sequences. For each method, the obtained sequences were aligned with the respective references and a neighbor-joining phylogenetic tree based on the p-distance was reconstructed using MEGA X (Kumar *et al.*, 2018) to classify them. The agreement between runs, methods, manual and automatic classification was assessed by calculating the Fleiss' kappa (K) using the irr library in R. The statistical significance level was set to P-value < 0.05 for all mentioned tests.

Results

All 30 combinations tested positive by every RT–PCR method and run, and therefore a total of 270 amplicons was obtained and then Sanger sequenced.

Operator-driven analysis and lineage classification

Overall, 212 out of 270 (78.5%) forward, 209 reverse (77.4%) and 197 (72.9%) consensus sequences of adequate quality were obtained. In detail, 21, 26, 22 consensus sequences were assembled in the three runs for method A; 24, 24, 24 for method B; and 18, 18, 20 for method C (Table 1). Method B globally yielded the highest number of sequences and appeared the most reproducible method. Forward and reverse sequences were always concordant, although, in some cases, only the forward or reverse sequence was obtained, hence preventing the consensus assembly. Generally, samples with a low and similar amount of vaccines were sequenced with difficulty, especially with methods A and C, whereas method B seemed affected by a more prominent within-run variability (Table 2). A substantial agreement ($K = 0.645$; P-value < 0.001) was observed among methods and among runs, within the method, while the agreement among forward and reverse reads was almost perfect ($K = 0.860$).

Read quality evaluation and automatic lineage classification

Overall, 192 out of 270 (71.1%) forward sequences, 190 (70.4%) reverse sequences, and 190 (70.4%) consensus sequences of adequate quality were obtained. In detail, 19, 23, 20 consensus sequences in the three runs for method A; 23, 24, 24 for method B; and 19, 16, 22 for method C were assembled (Table 1).

A moderate agreement ($K = 0.410$; P-value < 0.001) was observed among methods, although it was significantly depressed by the disagreement among N.S. reads. On the other hand, the agreement among runs within method was substantial ($K = 0.68$; P-value < 0.001), whereas the agreement among forward and reverse reads was almost perfect ($K = 0.99$; P-value < 0.001) (Table 2).

Finally, the overall agreement between operator-driven and automatic classification was almost perfect ($K = 0.892$; P-value < 0.001) (Table 2).

Additionally, the following features were selected to evaluate the effect of different experimental conditions on the read quality:

(1) average Phred score after trimming;
(2) % of information lost due to poor quality region trimming;
(3) % of secondary peaks in the read after trimming.

Independently of the considered outcome, the effect of RT–PCR method, lineage and dilution combinations was proven to be statistically significant, as well as their interaction (Table 3).

While the significant effect of so many independent variables makes a point by point dissertation challenging, some main trends can be identified. Independently of the method, the most challenging reads were obtained when both lineages were present at low viral titre (Figures 1 and 2). On the contrary, the combination of high and low viral titres for different lineages guaranteed the overall best performances. Besides, different methods revealed different performances depending on the tested lineage combination. The presence of double peaks appears more influenced by the ratio of the two lineages rather than the absolute titre. However, an effect of the particular combination of method and lineage was also clear in this case (Figure 3).

Discussion

The present study systematically evaluated the effect of co-infection, at variable ratios, with different IBV lineages on commonly applied diagnostic assays, followed by Sanger sequencing, thus simulating a regular scenario of the everyday diagnostic process. For this reason, four vaccines were selected to represent the most frequently encountered genotypes in the routine diagnostic activity, at least in Europe. The use of vaccines rather than isolated field strains was necessary to ensure the presence of a single population in the specimen.

An undeniable impact of all tested variables on the chromatogram quality was observed (Figure 1), thus leading to an extremely complex pattern and poor outcome predictability. Each method performed differently based on the combination of lineages involved and their ratio, as demonstrated by the statistical

Table 1. Summary of sample classification into lineages based on forward and reverse reads and consensus sequences obtained from the 270 amplicons.

	Method	Lineage	Consensus per run			Forward per run			Reverse per run		
			1	2	3	1	2	3	1	2	3
Operator-driven analysis	A	GI-1 (Mass)	4	5	4	5	6	4	4	6	4
		GI-13 (793/B)	8	11	9	8	11	9	9	11	9
		GI-19 (QX)	4	3	3	4	3	3	4	3	3
		GI-23 (Var2)	5	7	6	6	7	7	5	7	6
		Total	21	26	22	23	27	23	22	27	22
		N.S.	9	4	8	7	3	7	8	3	8
	B	GI-1 (Mass)	8	8	10	8	8	10	10	8	10
		GI-13 (793/B)	12	13	10	12	13	10	12	13	11
		GI-19 (QX)	–	–	–	–	–	–	–	–	–
		GI-23 (Var2)	4	3	4	4	3	5	5	3	5
		Total	24	24	24	24	24	25	27	24	26
		N.S.	6	6	5	6	6	5	3	6	4
	C	GI-1 (Mass)	4	3	4	4	4	5	6	3	5
		GI-13 (793/B)	6	9	9	7	9	11	6	9	9
		GI-19 (QX)	6	3	4	8	3	5	6	4	5
		GI-23 (Var2)	2	3	3	3	4	3	2	3	3
		Total	18	18	29	22	20	24	20	19	22
		N.S.	12	12	10	8	10	6	10	11	8
	All	Total	197/270 (72.9%)			212/270 (78.5%)			209/270 (77.4%)		
Automatic genotype classification	A	GI-1 (Mass)	4	4	4	4	4	4	4	4	4
		GI-13 (793/B)	8	11	9	8	11	9	8	11	9
		GI-19 (QX)	3	3	3	3	3	3	3	3	3
		GI-23 (Var2)	4	5	4	4	5	4	4	5	4
		Total	19	23	20	19	23	20	19	23	20
		N.S.	11	7	10	11	7	10	11	7	10
	B	GI-1 (Mass)	9	8	10	9	8	10	9	8	10
		GI-13 (793/B)	11	13	10	12	13	10	11	13	10
		GI-19 (QX)	–	–	–	–	–	–	–	–	–
		GI-23 (Var2)	3	3	4	3	3	5	3	3	4
		Total	23	24	24	24	24	25	23	24	24
		N.S.	6	6	5	6	6	5	7	6	6
	C	GI-1 (Mass)	5	2	5	5	2	5	5	2	5
		GI-13 (793/B)	6	8	10	6	8	10	6	8	10
		GI-19 (QX)	6	3	4	6	3	4	6	3	4
		GI-23 (Var2)	2	3	3	2	3	3	2	3	3
		Total	19	16	22	19	16	22	19	16	22
		N.S.	11	14	8	11	14	8	11	14	8
	All	Total	190/270 (70.4%)			192/270 (71.1%)			190/270 (70.4%)		

Note: The count has been divided according to the diagnostic method, run replicate and analytical approach (operator-driven/automatic classification). N.S. = not sequencable. Method A: (Cavanagh *et al.*, 1999); B (Worthington *et al.*, 2008); C (Valastro *et al.*, 2010).

significance of interactions for all considered outcomes (i.e. read quality, information loss and double peak presence) (Figures 1–3). Despite this, some general patterns could still be highlighted.

The presence of an equal template amount was definitely the most challenging scenario, leading to an average lower read quality and the presence of a higher percentage of double peaks, with the partial exception of method B (Figure 3). A comparable template amount can, in fact, lead to a similar ratio of amplicons and comparable fluorescence during Sanger sequencing. The percentage of lost information was also affected (Figure 2), since a more intensive trimming was requested in case of a similar template amount. However, this was particularly severe at the lowest dilutions, suggesting a limit of the RT–PCR process rather than sequencing. The lineage effect is likely due to the differential primer affinity, and the overall outcome was further magnified by the interaction between these factors (Table 3, Figures 1–3).

In addition to a decreasing sequence quality, sequence exclusion due to mixed templates also

Table 2. Summary of *K* values of agreement among methods, runs and sequencing direction within analytic approach (operator-driven and automatic genotype classification) and between the two analytic approaches (*P*-value < 0.001).

	Operator-driven analysis			Automatic genotype classification			
Agreement K	Among methods	Among runs	Among forward and reverse	Among methods	Among runs	Among forward and reverse	Between operator-driven and automatic genotype classification
Overall	0.645	0.654	0.860	0.410	0.680	0.990	0.892
GI-13 (793/B)	0.773	0.798	0.958	0.659	0.784	0.991	0.969
GI-1 (Mass)	0.743	0.751	0.863	0.492	0.782	1.000	0.909
GI-19 (QX)	0.777	0.777	0.892	0.307	0.802	1.000	0.937
GI-23 (Var2)	0.736	0.722	0.898	0.574	0.750	0.982	0.865
N.S.	0.266	0.290	0.687	0.052	0.415	0.982	0.788

Note: Var 2: Israeli Variant 2; N.S.: not sequencable.

Table 3. Summary of the statistical significance of independent variables (i.e. diagnostic method, vaccine and dilution combination) for the considered outcome of interest.

	Variable	Chisq	Df	*P*-value
Average quality	Method	25.5173	2	<2.2e-16
	Dilution Combination	1.7882	4	0.002877
	Vaccine Combination	20.2766	5	0.001109
	Method:Dilution Combination	34.5755	8	0.0003194
	Method:Vaccine Combination	65.6222	10	3.081E-07
	Dilution Combination: Vaccine Combination	39.2442	20	0.006216
	Method:Dilution Combination: Vaccine Combination	119.6775	40	7.1E-07
Percentage of lost information	Method	25.7565	2	0.0002553
	Dilution Combination	0.1348	4	0.997827
	Vaccine Combination	19.6651	5	0.001444
	Method:Dilution Combination	18.6311	8	0.016962
	Method:Vaccine Combination	67.8230	10	<0.001
	Dilution Combination: Vaccine Combination	29.5777	20	0.076991
	Method:Dilution Combination: Vaccine Combination	72.4347	40	0.001276
Percentage of secondary peaks	Method	30.8671	2	0.0001983
	Dilution Combination	1.4068	4	0.8430049
	Vaccine Combination	21.8664	5	0.0005551
	Method:Dilution Combination	38.7083	8	5.567E-05
	Method:Vaccine Combination	65.4242	10	3.362E-07
	Dilution Combination: Vaccine Combination	42.2161	20	0.0025917
	Method:Dilution Combination: Vaccine Combination	102.8153	40	0.0001957

Note: Both main effects and interactions (coded by ":") are reported.

occurred, just as it can happen during the diagnostic activity when a confident characterization cannot be achieved. In this framework, the subjective evaluation of the operator could significantly discern if the reads can be retained or excluded, or affect the length of the trimmed regions, potentially leading to a different percentage of reads that were not sequencable (N.S. reads) or lineage classification. Even if understandable from a technical perspective, this last circumstance can cause misleading results, complicating the interpretation of the epidemiological scenario or leading to a decrease in clients' trust. For this reason, the common operator-dependent approach was compared to an automatic approach, and the agreement between them was assessed, as well as the agreement among methods, runs and sequencing direction (i.e. forward or reverse).

The agreement among methods in lineage classification was substantial in most instances, although lower for the automatic approach. However, the main source of disagreement was represented by the N.S. reads, where a much higher variability was observed. This evidence was particularly clear for the automatic approach, where the presence of fixed cut-offs for exclusion led to the discordant exclusion of "borderline" reads. Conversely, a well-trained operator was able to preserve a higher number of reads and therefore of consensus sequences.

The inability to achieve a sequence can have serious implications under field conditions, including false-negative results if the diagnostic protocol requires sequencing confirmation, costs for sample retesting, or at least the lack of a proper classification of the detected strains (i.e. field/vaccine strain, lineage, historical or emerging/recently introduced strain), severely hampering our control capabilities.

Although much less marked, the imperfect agreement among methods in the final lineage outcome can also lead to misleading results. The primer affinity for different templates clearly plays a role in this sense. For example, the genotype detected with method A corresponded mainly to the most abundant strain in the sample, with the sole exception of one sample composed by low concentration of GI-19 (QX) and GI-23 (Israeli Variant 2) vaccines, which was identified as GI-19 (QX) in the first run and as GI-23 (Israeli Variant 2) in the second (data not shown). This inconsistency could come from the randomness in primer annealing when the target templates have a similar concentration and from the rapid excess of amplification of one strain over the other. With method B, the most frequently detected strain was GI-13 (793/B), reflecting a primer affinity that was probably intensified by the three-phased process (cDNA synthesis and double amplification). Remarkably, the GI-19 (QX) vaccine was never identified by this method. A more focused analysis highlighted that the primers of the first PCR round presented the highest number of mismatches with GI-19 (QX) lineage (up to five mismatches in the forward primers, up to six in the reverse primers) (data not shown), thus supporting the primer-affinity hypothesis. On the contrary, method C was particularly efficient in detecting GI-19 (QX), since this assay was expressly validated for this purpose (Valastro *et al.*, 2010). Method C appeared heavily affected by the presence of multiple strains in the samples though, resulting in low-quality to unreadable sequences.

Even so, a generally high agreement was observed among different runs, suggesting that the final output is the result of a deterministic process, influenced by template concentration and primer affinity, rather than a stochastic one. Also in this case, the most common discrepancies were related to discarded reads. Unfortunately, the complexity of the observed patterns makes this process hardly predictable. Additionally, it must be stressed that the observed results were obtained with reference vaccine strains, leaving out the evaluation of how the within-lineage heterogeneity

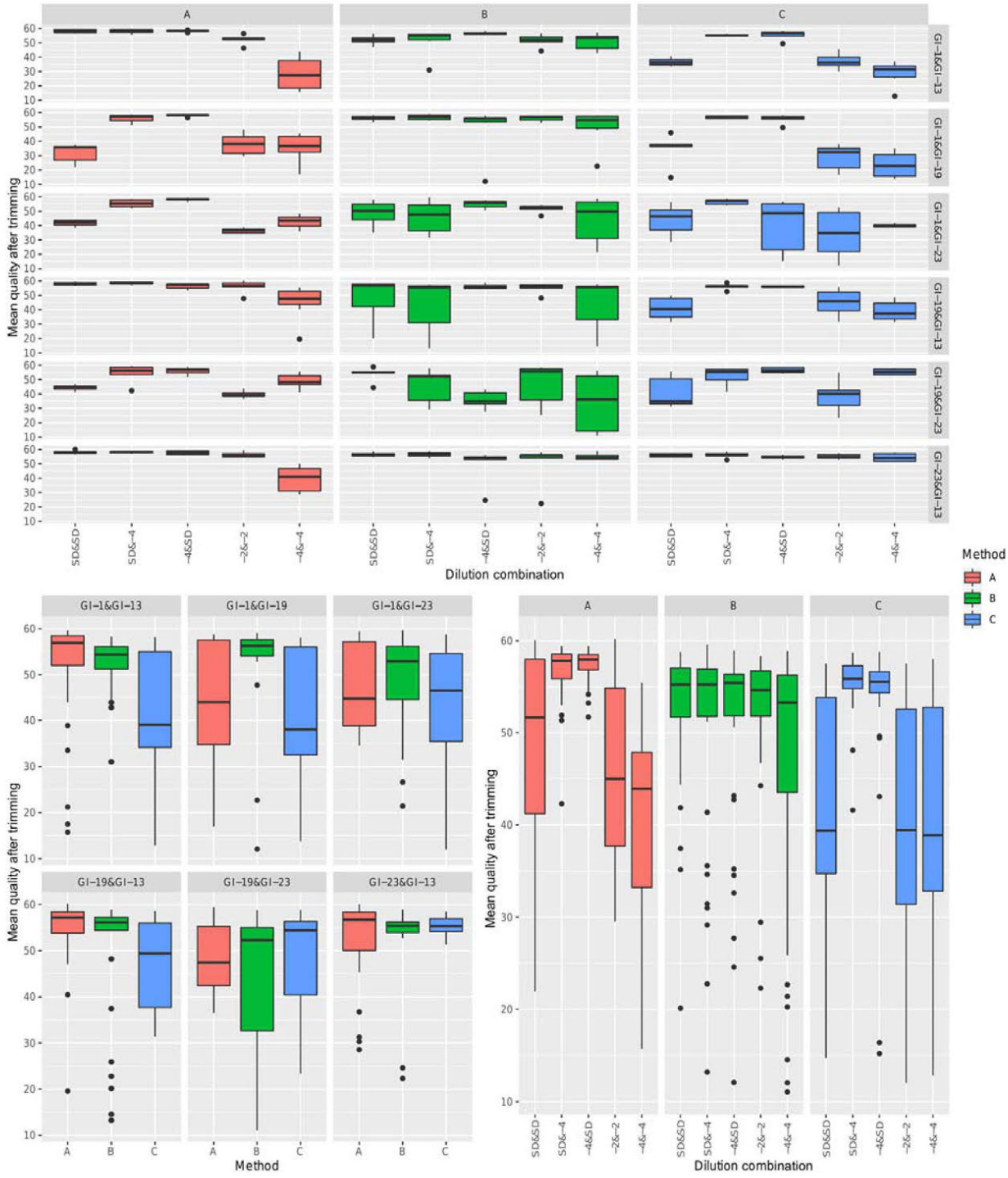

Figure 1. Boxplot reporting the distribution of average read quality after automatic trimming. Different diagnostic methods have been colour-coded. In the upper figure, score values have been faceted based on both vaccine and dilution combination. The overall effect of vaccine combination or dilution has been reported in the lower left and right panels, respectively.

could affect primer affinity and the relative sequencing results. Finally, an almost perfect agreement was proven between forward and reverse reads, testifying that the strain classification is determined by RT–PCR reaction dynamics rather than sequencing dynamics.

Overall, the present study discloses how a standardized laboratory process could still influence the results when working on tricky samples. Although Sanger sequencing can give some hints of mixed populations, such as by the presence of a strong background signal or double peaks, the method itself is not suited for detecting more than one strain. Therefore, while an expert laboratory worker can inform field veterinarians of such evidence, further insights into the actual coinfection composition are prevented. Neither the automatic nor the operator-driven approach was able to effectively deal with the presence of coinfections in the achievement of a confident characterization, which was significantly determined by the selected diagnostic method. Human operators are

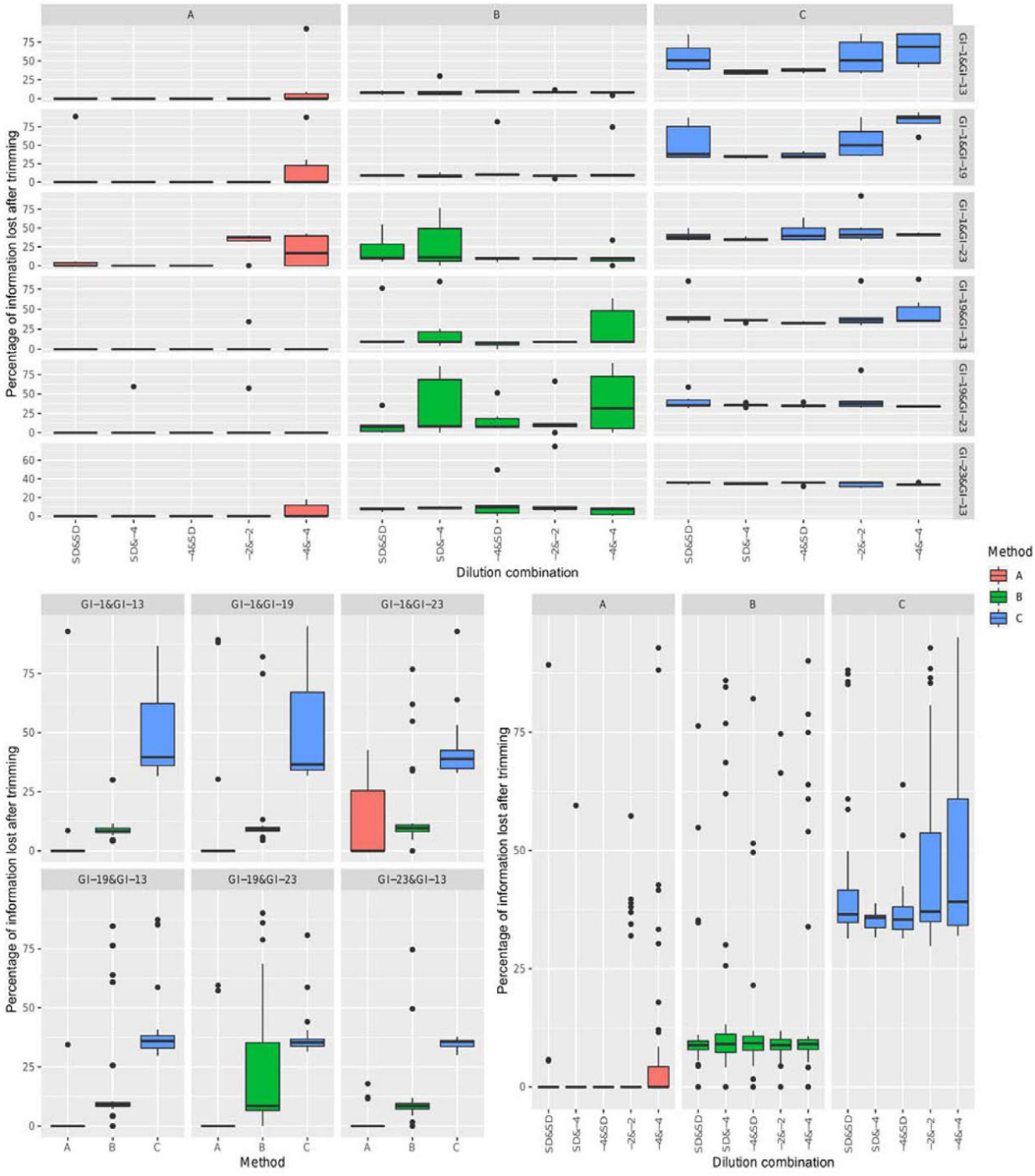

Figure 2. Boxplot reporting the distribution of percentage of information lost due to trimming. Different diagnostic methods have been colour-coded. In the upper figure, values have been faceted based on both vaccine and dilution combination. The overall effect of vaccine combination or dilution has been reported in the lower left and right panels, respectively.

generally able to more efficiently retain and interpret poor quality sequences, which are typically excluded by automatic approaches. While this could represent an economical and practical advantage for the veterinarians in everyday diagnostic activity, the effect of such subjective, operator-based, sequence evaluation should be carefully evaluated and accounted for when more precise epidemiological studies are the final target.

It cannot be underemphasized that, although some "general" rules were identified in the specific cases, the complexity of the interactions among the considered variables, including specific lineage pairs and their concentration, prevents any inference or prevision on the method performances in unknown field samples. Additionally, the experimental conditions poorly represent the field conditions, where samples are frequently coinfected but rarely contain equally concentrated strains, further complicating the diagnostic scenario.

Even if the evaluation of the different outcome and possible determinants of this variability aimed to assist in the choice of the diagnostic assay, a decisive answer is impossible to obtain and different assays exhibited

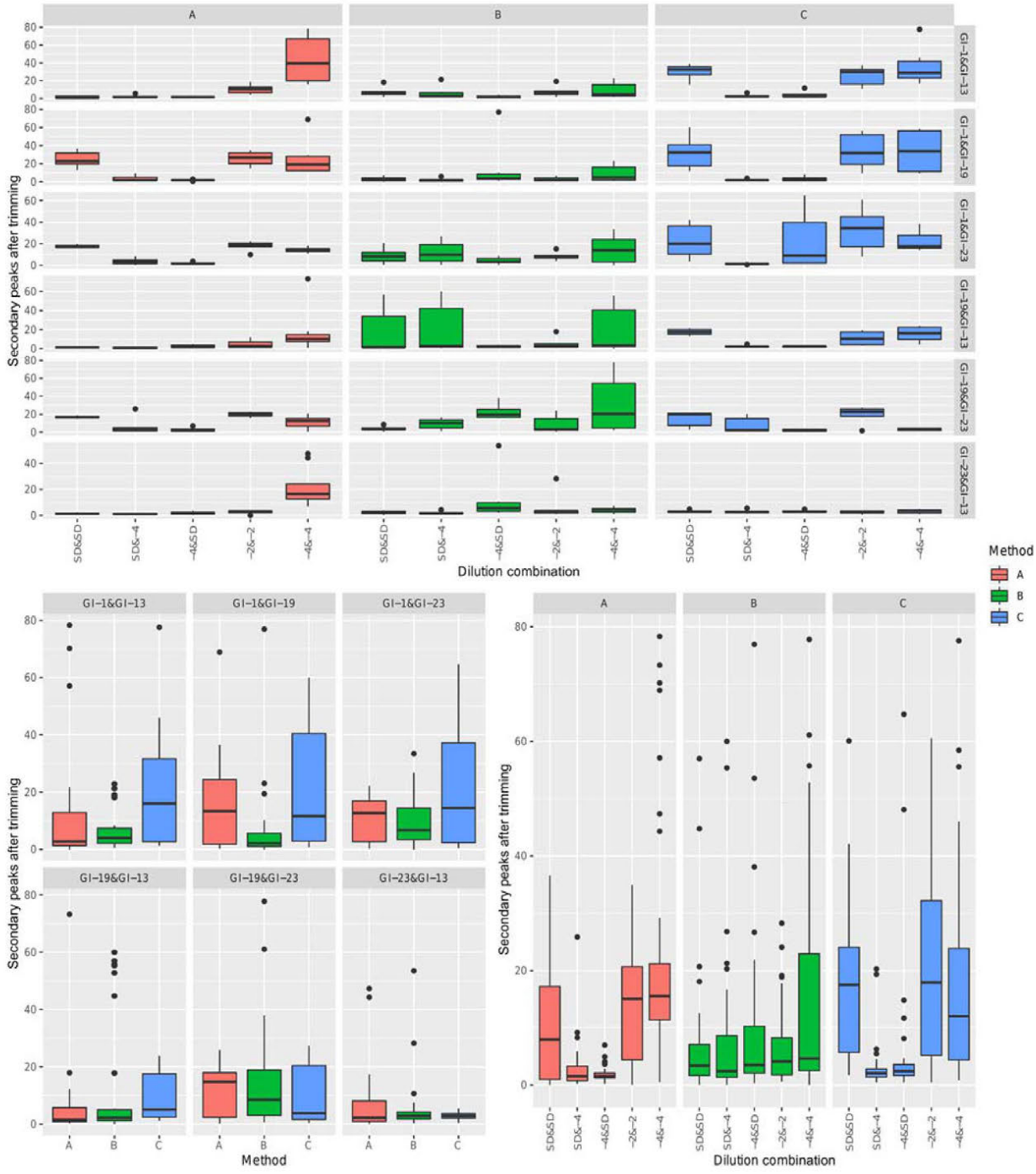

Figure 3. Boxplot reporting the distribution of the percentage of secondary peaks in the read after trimming. Different diagnostic methods have been colour-coded. In the upper figure, values have been faceted based on both vaccine and dilution combination. The overall effect of vaccine combination or dilution has been reported in the lower left and right panels, respectively.

pros and cons. The variability of the target region, the primer design and assay update including the emerging strains are only a few of the elements that can influence the performances of the diagnostic process.

The choice of the diagnostic assay should be based on the specific diagnostic purpose and consider all available elements indicating the presence of multiple strains, such as recent vaccination, clinical symptoms and epidemiological data. Information about the vaccination protocol could facilitate the interpretation of the results or suggest the use of a strain-specific assay to specifically detect, in light of a particular clinical suspicion, the most likely field strain rather than a co-infecting vaccine one. Otherwise, in complete absence of indications, the least biased assay should be preferred, aiming to identify the prevalent strain, still acknowledging the possibility of other undetected strains, and being aware of potentially misleading results, the occurrence of which is largely unpredictable *a priori*.

Based on these considerations, a diagnostic algorithm combining several assays with different designs and features, should be developed to process difficult samples and assist in the achievement of a truthful

characterization of the strain composition of the samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

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

Legnardi, M., Franzo, G., Tucciarone, C.M., Catelli, E., Menandro, M.L., Cecchinato, M.

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

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Legnardi Matteo[1*], Franzo Giovanni[1], Tucciarone Claudia Maria[1], Catelli Elena[2], Menandro Maria Luisa[1], Cecchinato Mattia[1]

[1] Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università, 16, 35020, Legnaro (PD), Italy

[2] Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra, 50, 40064 Ozzano nell'Emilia (BO), Italy

* Corresponding author at: Dipartimento MAPS, Università di Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italia; Tel: +39 0498272968; E-mail: matteo.legnardi@gmail.com

Running title: Effect of vaccination strategies on IBV epidemiology, diagnosis and control

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 one 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 in Italy have been caused primarily by QX field strains.

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.

Keywords

IBV, infectious bronchitis virus, molecular epidemiology, poultry, QX, vaccination.

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 one 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-I Mass	GI-12 D274	GI-13 793B	GI-16 Q1	GI-19 QX	GII-I 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 I. Yearly number of detections of each lineage and symptoms possibly ascribable to IB.

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 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.

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

Table II. Yearly number of QX detections divided in vaccine and field strains. 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.

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 due 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 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

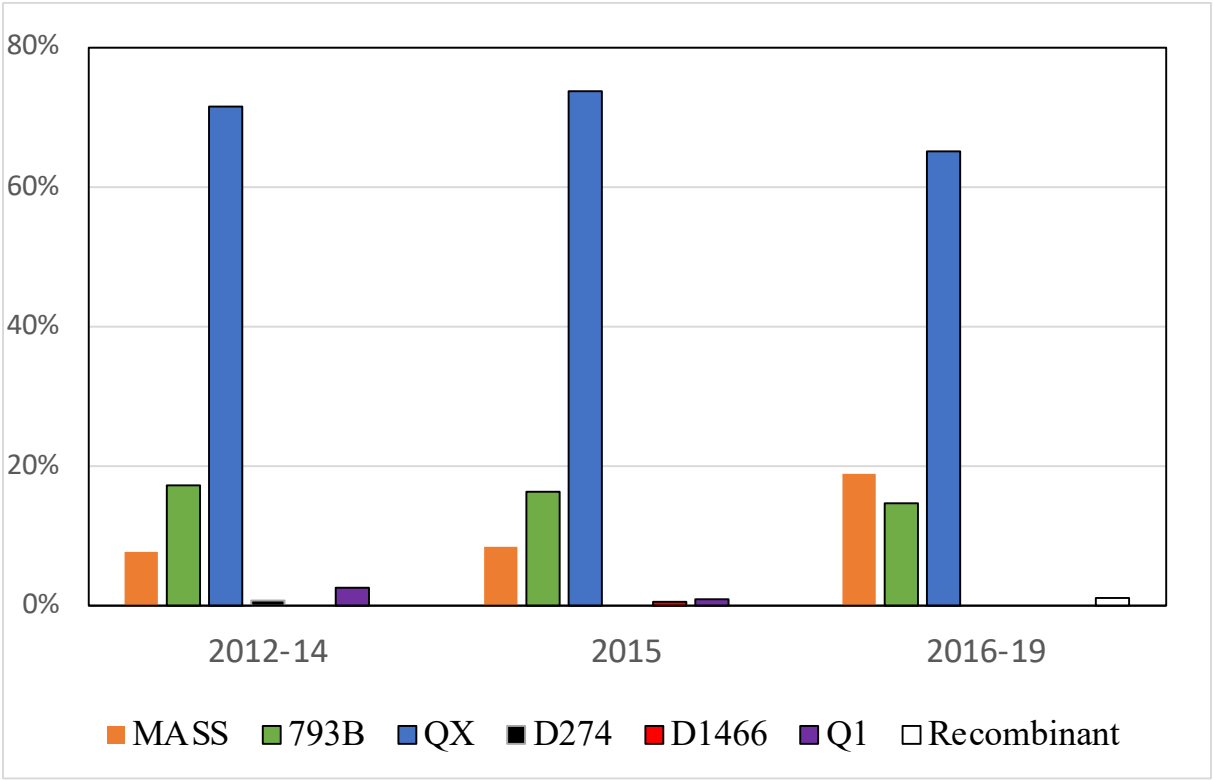

Figure 1. Relative frequency of detection of different lineages throughout the three considered periods.

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 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).

Conclusion

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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Infectious bronchitis hatchery vaccination: comparison between traditional spray administration and a newly developed gel delivery system in field conditions

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Infectious Bronchitis Hatchery Vaccination: Comparison between Traditional Spray Administration and a Newly Developed Gel Delivery System in Field Conditions

Matteo Legnardi [1], Henrik Baranyay [2], Csanád Simon [2], János Molnár [3], Tiede Bijlsma [4], Mattia Cecchinato [1], András Gáspárdy [5], András Bersényi [5], Claudia Maria Tucciarone [1], Giovanni Franzo [1] and László Kőrösi [6,*]

[1] Department of Animal Medicine, Production and Health (MAPS), University of Padua, 35020 Legnaro (PD), Italy; matteo.legnardi@phd.unipd.it (M.L.); mattia.cecchinato@unipd.it (M.C.); claudiamaria.tucciarone@unipd.it (C.M.T.); giovanni.franzo@unipd.it (G.F.)
[2] Phlatus ZRT, Kisperkáta telep 1., 2431 Perkáta, Hungary; henrik@phlatus.hu (H.B.); simon@phlatus.hu (C.S.)
[3] Laboratory of Plastic and Rubber Technology, Department of Physical Chemistry and Materials Science, Budapest University of Technology and Economics, Műegyetem rkp. 3.H.ép, H-1111 Budapest, Hungary; molnar.janos@mail.bme.hu
[4] BioChek B.V. Fokkerstraat 14, 2811 ER Reeuwijk, The Netherlands; tiedebijlsma@biochek.com
[5] Department of Animal Breeding and Genetics, University of Veterinary Medicine Budapest, István utca 2., H-1078 Budapest, Hungary; gaspardy.andras@univet.hu (A.G.); bersenyi.andras@univet.hu (A.B.)
[6] AgriAL Bt, Béri Balogh Ádám u 42, 2100 Gödöllő, Hungary
* Correspondence: korosidr@gmail.com

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Abstract: The control of infectious bronchitis (IB) is essential in intensive broiler production and is pursued through strict biosecurity and mass vaccination. Despite effective and routinely adopted, hatchery spray vaccination has been hypothesized to affect chicks' body temperature and wellbeing. Recently, gel administration has been proposed as an alternative and proved feasible in experimental settings. In this study, IBV spray and gel vaccination methods were compared in field conditions. One hundred birds from the same hatch were enrolled in the study and vaccinated, half by spray and half by gel, with 793B and Mass vaccines. After vaccination, rectal temperature was measured and vaccine intake assessed. The two groups were housed for 35 days in separate pens and swabs and blood samples were collected at multiple time points for genotype-specific molecular analyses and serology, respectively. The temperature was significantly lower in spray-vaccinated chicks 10 min and an hour after administration. A similar trend in 793B titres was observed in both groups, while the Mass vaccine was detected later but persisted longer in gel-vaccinated chicks. No differences were observed in mean antibody titres. Compared to spray, gel administration appears equally effective and less impactful on body temperature, thus supporting its application for IBV vaccination.

Keywords: Infectious bronchitis virus; gel vaccination; spray vaccination; broiler; hatchery

1. Introduction

Avian coronavirus, the causative agent of infectious bronchitis (IB), is one of the most relevant and impactful pathogens to affect the poultry industry worldwide [1]. IBV belongs to the genus *Gammacoronavirus*, family *Coronaviridae*, order *Nidovirales*, and is an enveloped virus with a single-stranded positive-sense RNA genome [2]. IBV is responsible for aspecific respiratory signs in chickens of every age, but which tend to be more severe at early ages. Depending on the pathogenicity of the strain involved, IBV may also damage the kidneys and oviduct, the latter resulting in drops in egg production and quality [3]. Mortality is usually low, but it may increase significantly in case of secondary infections by pathogens such as *E. coli* or *Mycoplasma* spp. Interactions with other respiratory or immunosuppressive viral diseases have also been documented [4].

The effective control of this highly contagious disease is essential for every type of production and is mainly achieved through rigorous biosecurity and mass vaccination [5].

Unfortunately, the efficacy of IBV vaccination is hindered by its remarkable evolutionary rate, ascribable to the frequent occurrence of both mutation and recombination events. This causes the emergence of a plethora of genetic IB variants, often found in cocirculation and coinfection and whose cross-protection is usually limited [6]. According to a phylogeny-based classification of the S1 gene sequence, a total of eight IBV genotypes are currently recognized, further divided into 36 lineages [7–11]. Based on which strains are circulating, several vaccination strategies can be adopted, either reliant on the administration of a homologous vaccine to the field strain or on a combination of heterologous vaccines able to provide a broader immunity, following the so-called "protectotype" concept [6,12].

In addition to the used vaccine strains, the conferred protection also depends on other factors related to vaccination procedures, including vaccine type, administration route and schedule [13]. Specifically, the broiler sector relies on the use of live attenuated vaccines, usually mass administered by drinking water or, more and more frequently, by spray [13]. The matter of vaccination timing is particularly debated. Multiple studies seem to suggest that the humoral immunity induced by early vaccination may be suboptimal, possibly because of interference with maternally derived antibodies (MDA), thus leaving the chicks unprotected against field viruses and favoring immuno-escape and recombination [14,15]. Despite this evidence, a clear trend towards hatchery administration has been observed recently in intensive poultry farming [16–18]. Moreover, even if an interval between subsequent administrations is traditionally recommended for a better immune response, likely to allow for tracheal epithelium recovery [19], the combined application of multiple vaccines at hatchery level has become the norm in many countries [16,17].

Hatchery vaccination by spray offers many advantages, such as lower labour costs and improved standardization of administration procedures and conditions compared to farm vaccination [6,17]. Several studies support its efficacy, with many vaccines being marketed as protective for the entire broiler cycle when administered at day-old [6]. On the other hand, it has been hypothesized that spray administration at the hatchery may result in a sudden drop in body temperature during a critical phase of chickens' life, when the thermoregulatory system is not yet fully developed and they still act as poikilotherms [20], possibly affecting their growth and wellbeing [21].

Gel administration has recently drawn some interest as an alternative route for IBV vaccine delivery at the hatchery. Superabsorbent hydrogels are materials with a three-dimensional framework that retains stability even after absorbing large amounts of water. In the poultry sector, they have been used for a long time to supply live coccidiosis vaccines [22] and more recently probiotics and prebiotics [23]. Some studies have reported that IBV vaccination by gel allows for a proper immunization in experimental conditions [23–26]. Compared to spray vaccination, it may also have the additional advantage of a lesser impact on chicks' body temperature [26].

The aim of this study was to assess the applicability of IBV gel vaccination at the hatchery in typical field conditions, by comparing gel administration with a newly developed patented system to spray vaccination, considered as the reference method.

2. Materials and Methods

2.1. Animals

The study was conducted on two groups of 50 chicks, one vaccinated by spray and the other by gel. The chicks were randomly selected from the same daily hatch of 114,300 Ross308 broilers, of which 109,300 were vaccinated by spray and 5000 by gel. Both groups were vaccinated at the hatchery with two live attenuated IBV vaccines, Cevac® Ibird (strain 1/96, batch 2512H4D1KL) and Cevac® Bron 120 L (strain H120, batch 1111H4D1KI) (Ceva Santé Animale, Libourne, France). Strain 1/96 belongs to the 793B lineage (GI-13), while strain H120 falls into the Mass lineage (GI-1). The combination of Mass and 793B vaccines was chosen as it is one of the most commonly adopted dual vaccination protocols [6,18], granting immunity against a broad range of variants [27,28].

2.2. Vaccination

The chicks were vaccinated in boxes of 90 birds each. Spray vaccination was administered using a SmartCount™ machine (Royal Pas Reform, Zeddam, The Netherlands), setting the droplet size at 200–250 µm (with ~10% of the volume of sprayed vaccine consisting of drops smaller than the average at 2.5 bar pressure). The spray dose per box was 15 mL (0.17 mL/chick), in which 90 doses of the two vaccines were mixed.

The newly developed SmartDrops (Phlatus ZRT, Perkáta, Hungary) application system, consisting of a drug-delivery superabsorbent hydrogel product and an application device, was used for gel administration. The implemented blue-colored hydrogel was based on superabsorbent polymers and aliphatic polycarboxylic acids, in accordance with European Union EC 1831/2003 standards. The application device allowed for an accurate delivery of the hydrogel by automatically controlling the shape, size, and size distribution of the droplets. Forty-five grams of gel, containing a mixture of 90 doses of the two vaccines, were administered to each box (0.5 g/chick).

2.3. Vaccine Intake

Vaccine intake was assessed by visually inspecting the chicks of both groups right after administration. Additionally, vaccine ingestion was evaluated in the gel-vaccinated group by individually checking whether the chicks' tongue was blue-tinged after 15 min.

2.4. Body Temperature

The cloacal temperature of 25 randomly selected birds from each group was measured at take-off, and then 10 min and 1 h after vaccination. During that time, the two groups were kept in separate boxes in the chick holding room at 24 °C.

2.5. Bird Housing

After vaccination, the birds were individually identified with wing bands and the two groups were housed in separate pens. Every aspect of the management—Housing, feed specification, lighting, etc.—Was conducted following the Ross broiler manual recommendation [29].

2.6. Quantitative Real Time RT-PCR

Choanal swabs were collected from ten randomly selected chicks from each group at the first time-point at 4 days of age (DOA). The same animals were followed longitudinally by subsequent sampling at 8, 12, 16, 21, and 35 DOA. Spare samples were also taken from other individually identified birds in case of deaths among the initially selected chicks during the trial.

Each swab was individually eluted in 2 mL of PBS, and viral RNA was extracted with High Pure RNA Isolation Kit (Roche, Basel, Switzerland). Two previously validated real time quantitative RT-PCR (qRT-PCR) assays [30], one specific for Mass strains and the other for 1/96-like strains, were then used to assess vaccine coverage, titres and kinetics. The standard curves for the two assays were prepared by testing serial dilutions of the respective titrated vaccine, thus allowing an accurate quantification of vaccine titres. The implemented primers and probes are detailed in Table 1.

Table 1. Primers and probes implemented in the Mass and 1/96-like specific real time RT-PCR assays, previously designed by Tucciarone et al. [30].

Primer/Probe	Sequence
1/96-like Forward primer	5′-CCTGGTTCAGGTTGGCATTT-3′
1/96-like Reverse primer	5′-ATGCACTGCCTGCATTGTTG-3′
1/96-like Probe	5′-FAM-TCTACTGCATAAGCACCCCCATG-BHQ1-3′
Mass Forward primer	5′-AGCAGACGCAGGTTTGGCTA-3′
Mass Reverse primer	5′-TGGTTGACATCTTCGCAAGG-3′
Mass Probe	5′-FAM-CATCTGGTTCCATAGACATCTTTGTCG-BHQ1-3′

The assays were performed on LightCycler® 96 Instrument (Roche Life Science, Penzberg, Germany) using SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen, Waltham, MA, USA). Titrated dilutions of Cevac® IBird (1/96 strain) and Cevac® Bron 120 L (H120 strain) vaccines were used as positive controls.

2.7. Serology

For serological monitoring, blood samples were taken from 16 animals of each group prior to vaccination to evaluate the presence of MDA, then at 21 and 35 DOA. The collected sera were tested at BioChek B.V. laboratories (Reeuwijk, The Netherlands) using CK119 IBV ELISA kit (Lot n.: FS7886).

2.8. Statistical Analyses

Differences among groups in terms of body temperature and antibody titres were analyzed with the two-sample *t*-test setting. Differences in viral titres were analyzed with the Kruskal–Wallis test, followed by the Mann–Whitney U test with Bonferroni adjustment as a post hoc test. For all analyses, the significance level was set at $p < 0.05$.

3. Results

3.1. Vaccine Intake

After administration, the spray-vaccinated birds appeared wet and stained with the blue-dyed solution (Figure 1a), while gel droplets were visible on the group vaccinated by gel (Figure 1b). After 15 min, all gel-vaccinated chicks showed a blue-tinged tongue (Figure 1c).

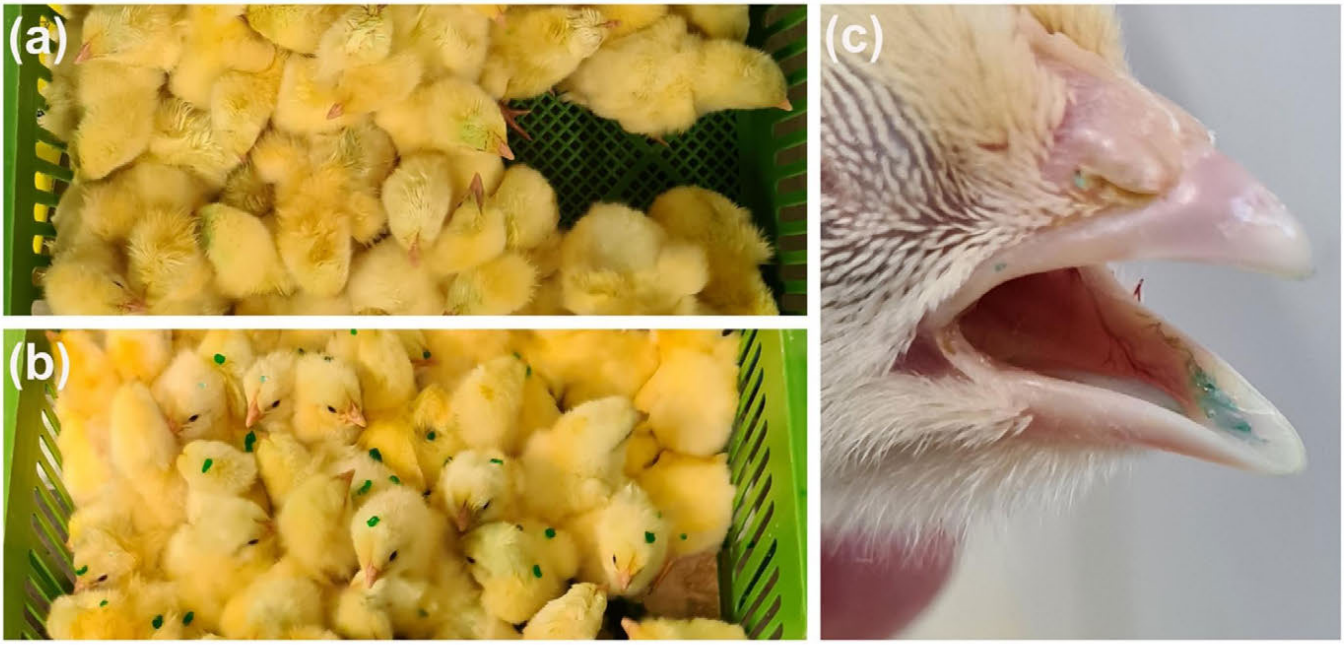

Figure 1. Post-administration evaluation of vaccine intake. Boxes of spray (**a**) and gel (**b**) vaccinated chicks immediately after vaccination, and tongue of a gel-vaccinated chick after 15 min from the administration (**c**).

3.2. Chick Temperature Following Vaccination

The body temperatures measured in the two groups are detailed in Table 2. A drop of more than 1 °C was observed in the spray-vaccinated group 10 min after administration, while the temperature of the gel-vaccinated birds remained stable. A slight decrease in temperature was observed in both groups after one hour in the chick holding room. The differences between the two groups were statistically significant at both sampling points.

Table 2. Average body temperatures (°C) and standard deviations measured in the spray and gel-vaccinated groups.

	Take-Off	Spray-Vaccinated Chicks (N = 25)		Gel-Vaccinated Chicks (N = 25)	
		After 10 min	After 1 h	After 10 min	After 1 h
Average	40.9	39.6	39.2	40.8	40.2
SD	0.19	0.70	0.39	0.27	0.24

3.3. Vaccine Coverage

Detailed individual results from each group and sampling point are provided in Figure 2. All animals tested positive for H120 and 1/96 at least at one sampling point. For 1/96, some birds of both groups were already positive at day 4, and positive results were still observed at day 35. Individual 1/96 detections were more frequent in the spray-vaccinated group, especially in the first three time points. Conversely, a higher number of H120 detections were observed in the gel-vaccinated group, where the detection period (from 8 to 35 DOA) was slightly delayed compared to spray vaccination (from 4 to 21 DOA). In every group, the number of positive individuals peaked around day 16 and then started to decrease. Intermittent detections were also observed in both groups and for both vaccines.

Bird ID	Spray 1/96						Spray H120					
	4	8	12	16	21	35	4	8	12	16	21	35
1	-	-	+	+	+	-	+	-	-	+	+	-
2	-	+	-	+	+	+	-	+	+	+	-	-
3	+	-	+	+	+	+	-	-	-	+	-	-
4	+	+	+	+	-	-	+	-	+	+	-	-
5	-	-	+	+	+	-	-	-	-	+	-	-
6	-	+	+	+	+	-	-	-	-	+	-	-
7	+	-	+	+	-	-	-	-	-	+	-	-
8	+	+	-	+	+	+	-	-	-	+	+	-
9	-	-	+	+	+	-	-	+	+	+	-	-
10	+	-	+	+	+	-	-	-	-	+	-	-
Bird ID	**Gel 1/96**						**Gel H120**					
	4	8	12	16	21	35	4	8	12	16	21	35
1	+	+	+	+	-	-	-	+	-	+	+	+
2	-	-	-	-	+	-	-	+	+	+	+	-
3	-	-	-	+	+	+	-	+	-	+	+	-
4	-	-	+	-	+	-	-	+	-	+	-	-
5	-	-	-	+	+	+	-	+	-	+	+	-
6	-	-	-	+	+	+	-	-	-	+	+	-
7	-	-	+	+	+	+	-	+	-	+	+	-
8	+	-	-	+	+	+	-	+	+	+	-	-
9	-	-	+	+	+	-	-	+	-	+	-	-
10	-	-	+	+	+	-	-	+	-	+	+	-

Figure 2. Individual qRT-PCR results. Individual samples from ten chicks per group and sampling point were evaluated with two qRT-PCR assays.

All animals of both groups were found positive for H120 by day 16. For 1/96, all animals were positive by day 12 in the spray-vaccinated group, and by day 21 in the gel-vaccinated one (Figure 3).

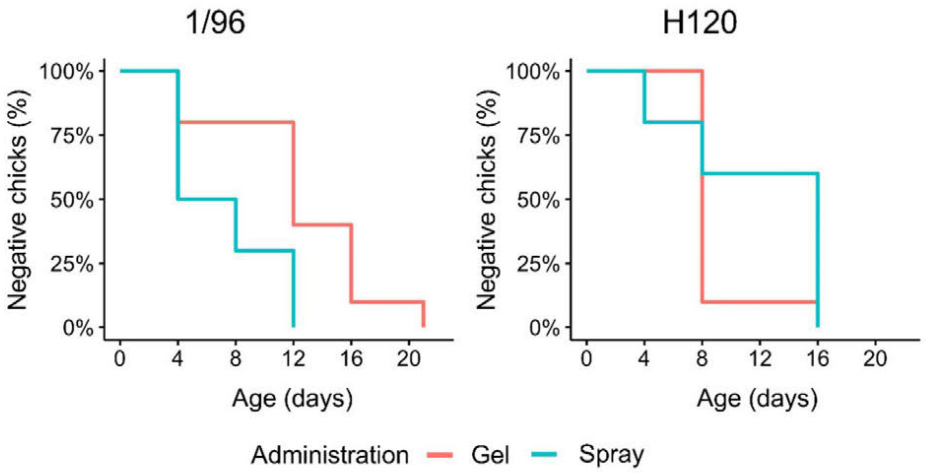

Figure 3. Percentage of negative chicks for vaccine strains 1/96 and H120. Kaplan–Meyer plots show the percentage of birds still negative for the two vaccines at the relative sampling point.

3.4. Vaccine Kinetics

The replication of live attenuated IBV vaccines, administered by spray or gel, is detailed in Figure 4. 1/96 vaccine titres peaked at 12 DOA in both groups, showing a similar trend regardless of the route of administration. As for H120 vaccination, the observed titres peaked at day 8 in the gel-vaccinated group and at day 16 in spray-vaccinated animals. In both groups, H120 titres showed a temporary decline at 12 DOA, when 1/96 titres were at the highest point (Figure 4). No statistically significant differences between the two administration methods were detected at any of the sampling points, with the exception of H120 vaccination at 8 DOA.

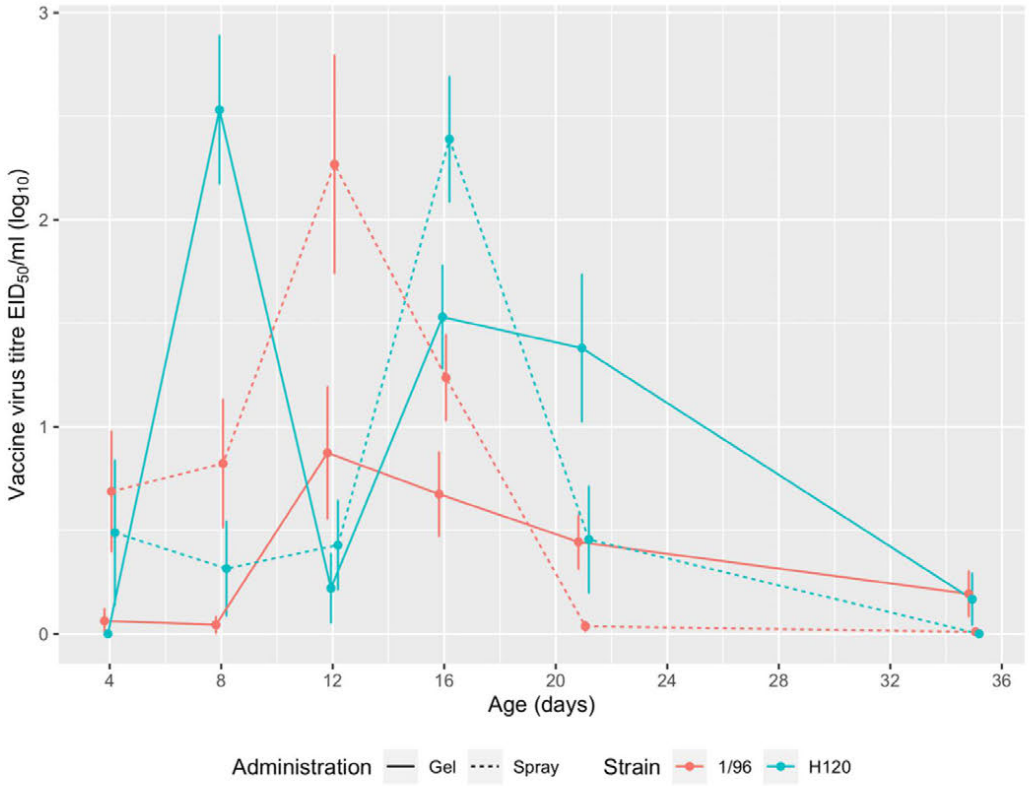

Figure 4. Replication of live attenuated IBV vaccines. For each group and sampling point, ten swabs were analyzed. Mean vaccine titres of positive results are expressed as $\log_{10}$ of embryo infectious doses 50 (EID_{50}) per mL.

3.5. Serology

Anti-IBV antibody titres detected in the two groups are shown in Figure 5. Regardless of the route of administration, the titres observed in 21-day-old-chicks were significantly lower than the MDA titres observed at the hatchery, while a slight rise was observed at 35 DOA. The differences between the two groups were not statistically significant at any sampling point.

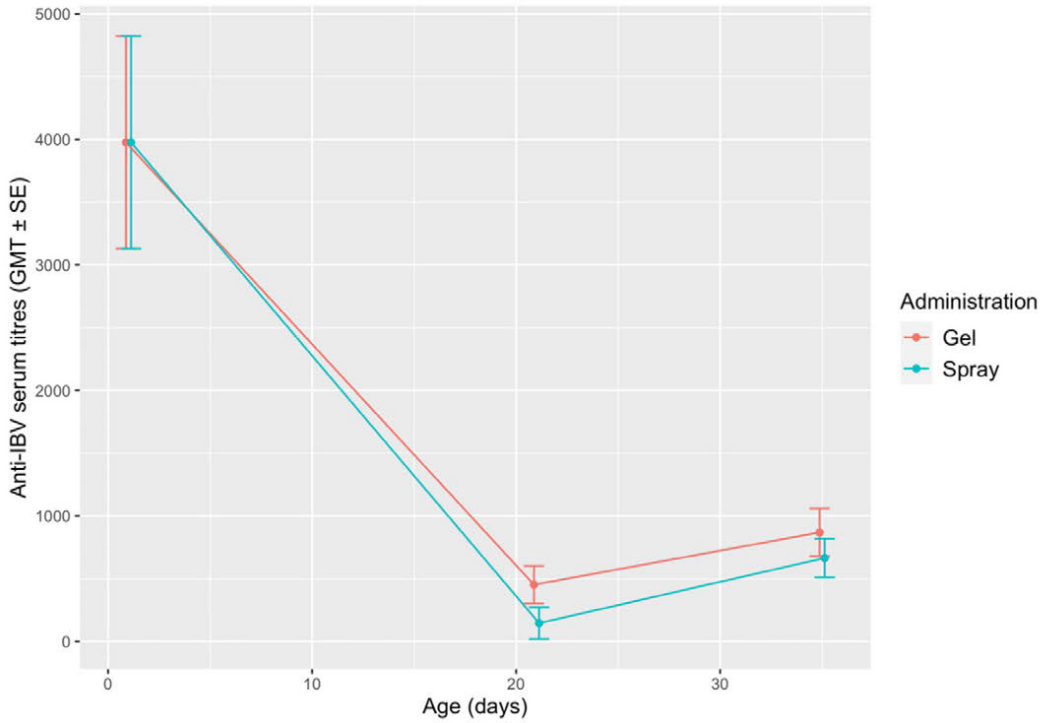

Figure 5. Mean anti-IBV antibody titres in the gel and spray-vaccinated groups. The results are shown as geometric mean titres (GMT) ± standard error (SE).

4. Discussion

Based on the herein presented comparison, the administration of multiple IBV live vaccines at the hatchery yielded similar results when performed by spray, which is the routinely adopted method, and by gel. In typical field conditions, the two routes of administration were comparable in terms of coverage and elicited immune response, as shown by vaccine replication (Figure 4) and antibody titres (Figure 5). For 1/96 strain, a 100% coverage was reached earlier (12 DOA) in the gel-vaccinated birds than in the spray group (20 DOA). For both vaccine strains, intermittent detections were observed, in agreement with previous observations by Tucciarone et al. [26]. This finding could be tentatively ascribed to the persistent circulation of the live vaccine virus among chicks of the same group, which may elicit subsequent reinfections.

No statistically significant differences were found in serology. MDA titres were high before vaccine administration in both groups, which belonged to the same hatch. A marked decline was observed at 21 DOA, followed by a slight increase at 35 DOA. This trend is consistent with the results of previous works [15,27,31]. The low antibody levels observed at 21 and 35 DOA may be a consequence of MDA interference, particularly since vaccines were administered at the hatchery [15]. However, this should not be interpreted as a vaccination failure, as it is well-established that humoral antibody titres correlate poorly to the protection conferred by IBV vaccines [6,27].

On the other hand, protection seems to be associated to cellular and local immunity at the tracheal level [27]. Despite not allowing a direct evaluation of the elicited immune response, the assessment of vaccine viral titres at this level, that are thought to compete

with field strains for tracheal receptors [12,30], is commonly used as a proxy for vaccination quality and efficacy [6,30].

Slight differences were observed between the two groups in terms of vaccine kinetics. Vaccine strain H120 was detected from 4 to 21 DOA in the spray-vaccinated group, and from 8 to 35 DOA in the gel-vaccinated one, with comparable titres between the two groups. Strain 1/96 was found at every sampling point (from 4 to 35 DOA) in both groups, with slightly higher titres in the spray-vaccinated birds until 16 DOA and in the gel-vaccinated ones at later sampling points. However, the observed differences may be deemed inconclusive to decide whether the two supply methods differ in terms of vaccine take, and therefore in potential efficacy.

The comparison of the vaccine kinetics observed in the gel vaccinated group with those observed in an experimental setting [30] reveals a substantially overlapping pattern. Although absolute titres were slightly different, this could be ascribed to the differences in study conditions and/or implemented Mass vaccine (based on strain B48 instead of H120).

No notable signs were observed in the two groups for the duration of the trial, and the growth performances were in line with Broiler Ross standards (data not shown). Even if a rigorous assessment of vaccination efficacy and safety was beyond the scope of this work, this may be seen as proof supporting the safety of gel vaccination, at least comparatively to spray. While the two administration methods provided similar results in terms of coverage, gel vaccination did not affect the chicks' body temperature, contrary to spray vaccination which caused a 1 °C drop in body temperature within an hour after administration. A decrease equal to roughly 2 °C was previously observed in the hatchery where the study was conducted, when 20 mL of spray were administered to each box (data not shown). Cold stress at the hatchery is reported to affect muscle growth and development [32] and could even predispose to necrotic enteritis [33]. However, it is worth noting that the observed drop in temperature, even if statistically significant, may not be enough to cause any problem [34], and further studies are needed to prove the biological significance of this side effect of spray vaccination.

Besides the actually implemented Mass and 793B vaccines, whose combination represents one of the most commonly adopted IBV vaccination protocols [27,28], gel administration is likely suitable for every other live IBV vaccine with similar features. Likewise, further research efforts may be devoted to assessing whether gel delivery systems are suitable for other vaccines that are commonly administered at the hatchery against different diseases. Gel administration also allows to effectively combine different substances, such as coccidiosis vaccines and probiotics, granting their stability [23]. Being able to administer IBV vaccines with other active principles would be of great practical value, allowing for a holistic approach to chicks' health and early immunization while minimizing stressful procedures during a critical phase of the cycle.

Complementing the first studies conducted in experimental conditions, the present results provide further evidence supporting the feasibility of IBV hatchery vaccination by gel in field conditions. Considering its possible benefits over traditionally implemented methods, it would be worth testing this technique in standardized in vivo challenge experiments involving different vaccines and field strains, to better characterize its features and provide definite evidence on its efficacy.

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General conclusions

The outcomes of the herein reported studies demonstrate that IBV evolution, diagnosis and control are inextricably linked in many ways. First and foremost, IBV genetic variability is the main factor driving the choice of vaccination protocols. Strategies relying on homologous vaccines clearly depend on knowing which variant is the most relevant in the context of interest, but, even when resorting to broad-spectrum protocols based on the protectotype concept, it is essential to know whether the administered combination of heterologous vaccines is effective against the dominant field threats.

Genetic heterogeneity also has many repercussions on diagnostic approaches. Especially when using molecular assays, diagnostic choices need to be tailored on the current epidemiological situation, i.e., using a combination of different assays, implementing methods specific to the relevant field lineages, sampling suitable matrices based on the tropism of the suspected strain, and designing new assays to cope with emerging variants. In addition, knowing the epidemiological scenario also allows for a more precise interpretation of the obtained results.

While the implications of IBV evolution for diagnosis and control are the most immediate to understand, the interactions between these three aspects should not be interpreted as unidirectional, but rather as mutual (Figure 2).

The reported studies show how not only vaccination, but also biosecurity, may profoundly alter IBV evolution. Despite their efficacy in reducing disease occurrence and viral circulation, IBV vaccines are not sterilizing, allowing for a persistent circulation of field strains. The significant immune pressure exerted by vaccines, especially by homologous ones, could result in both directional and diversifying selection, thus favoring the emergence of vaccine-escaping variants.

Biosecurity and management procedures, on the other hand, do not have similar unwanted effects, at least when properly planned and executed. Nonetheless, their role in shaping IBV evolution should not be overlooked. It was observed that a strong integration of poultry farming may lead to a compartmentalization of IBV evolution and subsequent selection of distinct subpopulations. This is likely attributable to several factors contributing to the separation between poultry companies, including farm location, production chain segregation and differences in vaccination protocols.

While diagnostic procedures do not directly affect IBV genetic heterogeneity, they represent the only gateway to its study. However, diagnostic tests will always capture only part of the actual epidemiological scenario. Besides the obvious importance of choosing specific tests based on which strains are circulating and which should be detected, novel evidence provided in this thesis demonstrates how, even when implementing generic molecular assays, some strains may be preferentially amplified over others based on the used primers. Other practical determinants that may affect test results include the timing of sampling activities, the type of sample and its shipping and storage conditions. For these reasons, attentive planning and interpretation of diagnostic tests are of the utmost importance in order to avoid a skewed perception of the epidemiological reality.

Laboratory methods are also essential to determine vaccine efficacy. Their usefulness is not limited to *in vivo* challenge studies, which are the only way to directly evaluate the protection conferred by vaccines against a given field strain. In fact, both molecular and serological assays may be used to assess the quality of vaccination and the achieved coverage. The detection and quantification of vaccine strains are commonly accepted as a proxy of live vaccination efficacy, as is the detection of high, uniform and lasting antibody titres. However, for these results to be considered significant, it is essential to compare them to a baseline in terms of vaccine kinetics and elicited immune response.

Conversely, the implemented control measures should always be considered when deciding which assays to use and how significant are the obtained results. When resorting to live vaccines, their replication and persistence could mask the presence of field strains, and even of other vaccines. Possessing information on the vaccination protocol could also suggest the appropriate time of sampling: for instance, when early vaccination is adopted, field strains are more frequently detected towards the end of the cycle, while the preferable time window to assess vaccine replication is usually between 7 and 10 days post-vaccination.

Other than allowing for a more precise depiction of IBV epidemiology, such a holistic approach may also help identifying where to act to improve our knowledge of IBV, and subsequently our control capabilities. Since novel vaccine types do not currently represent a viable option, the efforts towards a more effective IBV control should be focused on the optimization of currently available vaccination procedures. Besides choosing an appropriate protocol, great attention should be given to vaccine administration. Suboptimal and unstandardized administration may result in a limited coverage, allowing for a greater (and frequently undetected) circulation of field strains in a partially immune environment, which in turn may favor the occurrence

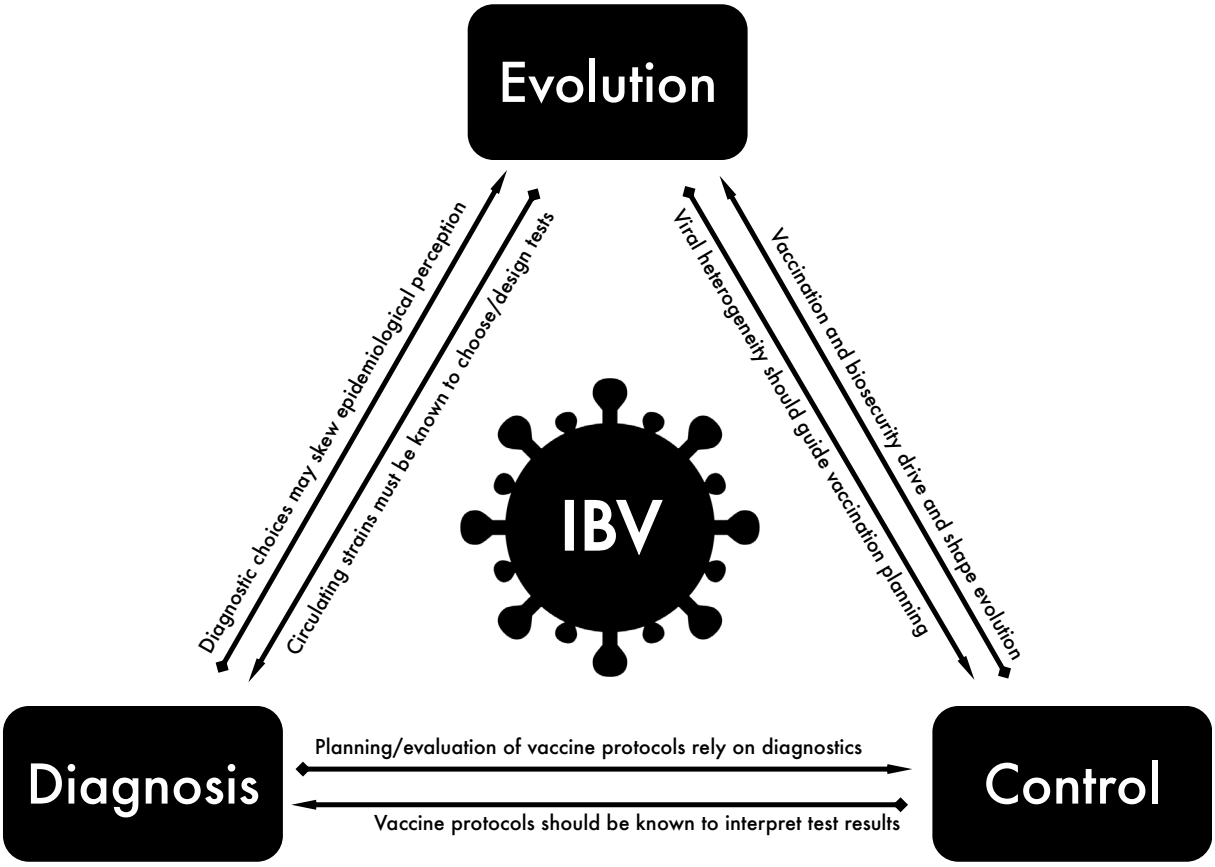

Figure 2. Mutual interactions between IBV evolution, diagnostic choices and control measures.

of immune-escape events. An attempt should also be made to reduce the amount of stress caused by vaccination procedures, which would positively impact the birds' overall health. The herein reported field testing of gel vaccination at the hatchery provided promising results in this regard, as this administration method seems to have a lesser impact on chicks' body temperature compared to spray.

Many developments may also be envisioned in diagnostics. Today, IBV diagnosis mainly relies on RT-PCR assays targeting a portion of the S1 gene, followed by strain characterization by Sanger sequencing. Although the current classification should be based on the full S1 gene, considering a shorter region is usually enough for a classification up to the lineage level. On the other hand, this practice hinders the sharing of genetic data between different laboratories adopting different tests, thus crippling the execution of large-scale phylogenetic studies. Aside from this issue, Sanger sequencing is also limited by a lower resolution compared to NGS methods. This may be particularly significant in the case of IBV, whose remarkable variability results in the coexistence of different subpopulations even in a single host or vaccine batch, which cannot be properly characterized through Sanger sequencing. NGS would also allow

gathering more information on genomic regions outside of the S1 gene, which are almost always overlooked but are known to play a role in IBV evolution and pathogenesis.

However useful these improved protocols and techniques may be, their mass adoption ultimately depends on overcoming some major practical and economical constraints. A much more achievable goal would be to make optimal and rational use of currently available resources. In the end, despite being the most obvious of all possible recommendations, ensuring a steady, attentive and coordinated monitoring at the largest scale possible would still represent the biggest advancement towards a greater knowledge of IBV epidemiology.

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