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SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE VETERINARIE
CICLO XXVIII

INFECTIOUS BRONCHITIS VIRUS: PHYLOGENY AND EVOLUTION

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RIASSUNTO

Il virus della bronchite infettiva aviare è l'agente patogeno di una malattia altamente contagiosa responsabile di ingenti perdite economiche nel settore avicolo. Il virus è caratterizzato da una grande variabilità genetica e antigenica che ha favorito la comparsa e la diffusione di molteplici e differenti tipi virali. Analisi filogenetiche e calcolo delle distanze nucleotidiche esistenti tra i ceppi sono state utilizzate per classificare la grande varietà di virus appartenenti a questa famiglia di patogeni. Nonostante ciò, non si è ancora raggiunto un accordo sul metodo con il quale i ceppi dovrebbero essere confrontati. Questo ha generato notevole confusione poiché ha favorito lo sviluppo di numerosi sistemi di classificazione, molto spesso contrastanti l'uno con l'altro, e l'utilizzo di nomenclature eterogenee che in molti casi non riflettevano la filogenesi.

Lo scopo di questo lavoro è proporre un sistema di classificazione che sia semplice e ripetibile e basato esclusivamente sulle relazioni filogenetiche esistenti tra i ceppi di bronchite aviaria. Il lavoro si propone inoltre di assegnare una nomenclatura univoca e razionale dei gruppi genici identificati. Mediate l'analisi di tutte le sequenze nucleotidiche della proteina S1 disponibili in banca dati sono stati identificati 32 diversi lineaggi, compresi in 6 genotipi, e un numero di ceppi originati da eventi di ricombinazione tra lineaggi. Poiché la variabilità esistente tra i diversi ceppi di IBV è abbastanza elevata, qui si propone di usare le relazioni filogenetiche piuttosto che il calcolo delle distanze geniche come criterio più adatto per tracciare la storia evolutiva di IBV.

L'adozione di una nomenclatura accettata a livello internazionale è di fondamentale importanza per gli studi futuri sull'epidemiologia ed evoluzione del virus della bronchite infettiva aviaria. Inoltre, così com'è stata sviluppata, la classificazione qui proposta può essere revisionata e aggiornata nel momento in cui saranno disponibili nuove sequenze virali della proteina S1.

SUMMARY

Infectious bronchitis virus (IBV) is the causative agent of a highly contagious disease that results in severe economic losses to the global poultry industry. The virus exists in a wide variety of genetically distinct viral types, and both phylogenetic analysis and measures of pairwise similarity among nucleotide or amino acid sequences have been used to classify IBV strains. However, there is currently no consensus on the method by which IBV sequences should be compared, and heterogeneous genetic group designations that are inconsistent with phylogenetic history have been adopted, leading to the confusing coexistence of multiple genotyping schemes.

Herein, we propose a simple and repeatable phylogeny-based classification system combined with an unambiguous and rationale lineage nomenclature for the assignment of IBV strains. By using complete nucleotide sequences of the S1 gene we determined the phylogenetic structure of IBV, which in turn allowed us to define 6 genotypes that together comprise 32 distinct viral lineages and a number of inter-lineage recombinants. Because of extensive rate variation among IBVs, we suggest that the inference of phylogenetic relationships alone represents a more appropriate criterion for sequence classification than pairwise sequence comparisons.

The adoption of an internationally accepted viral nomenclature is crucial for future studies of IBV epidemiology and evolution, and the classification scheme presented here can be updated and revised novel S1 sequences should become available.

1. LITERATURE REVIEW

1.1. ETIOLOGY

Infectious bronchitis virus (IBV) is a member of the genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, in the order *Nidovirales* (International Committee on Taxonomy of viruses, http://www.ictvonline. org/virustaxonomy.asp).

1.1.1. The family *Coronaviridae*

Coronaviridae contains a wide range of major pathogens infecting a large diversity of organisms, including mammals, birds and fish. In humans, coronaviruses (CoVs) cause about 20% of common colds. However, in recent years members of this family have revealed its high zoonotic potential, posing a more serious threat to human health. The emergence of novel coronaviruses – severe acute respiratory syndrome (SARS) in 2002 and Middle East respiratory syndrome (MERS) in 2012 –, which have caused severe respiratory diseases associated with high rates of mortality in humans, has highlighted the importance of continuous surveillance of these pathogens (Hilgenfeld and Peiris, 2013).

The family *Coronaviridae* is currently comprised of two different subfamilies, *Coronavirinae* and *Torovirinae*. Both are further subdivided into four and two genera, respectively, on the basis of cross-reacting antibodies and more recently of nucleotide sequence relatedness (Gonzalez et al., 2003). The Coronavirinae includes the genera *Alpha*, *Beta*, *Gamma* and *Deltacoronavirus*. The avian coronaviruses are largely classified as gammacoronaviruses. Alpha and betacoronaviruses comprise mammalian coronaviruses, with SARS-CoV and MERS-CoV included in the latter. Viruses isolated from wild birds and pigs, which have a different genomic order and show no close relationship to the gammacoronaviruses, are assigned to the genus *Deltacoronavirus* (Woo et al., 2012). Viral pathogens belonging to the subfamily *Torovirinae* are grouped into the genera *Torovirus* and *Bafinivirus*, which infect mammals and fish, respectively.

The viruses of this family are spherical, 120-160 nm across (Coronavirinae), bacilliform, 170-200 x 75-88 nm (Bafinivirus), or found a mixture of these two forms (Torovirus) and are provided with envelope. The presence of large projections in the virion surface gives the virus the characteristic crown-like appearance in electron micrographs (Lai et al., 2007).

1.1.1.1. Coronavirus replication cycle

Members of the family *Coronaviridae* are the largest positive-stranded RNA viruses identified so far with some of them reaching up to 30 000 nt. They replicate using a nested set of mRNAs that classifies them within the order *Nidovirales* (Sawicki et al., 2007).

All coronaviruses share many biologic and physic features. These include the spherical morphology, the club-shape spike surface projections, the helical nucleocapsid (NC), which is unusual for positive-strand RNA viruses, the structure of the RNA genome, and the organization of the structural proteins.

The isolated genomic RNA is infectious and serves as an mRNA within the infected cell. The first event in infection is the translation of the genome to form the RNA polymerase, which continuously catalyses the synthesis of negative-strand RNA and the sub-genomic mRNAs using the genomic positive-strand as template (Masters, 2006). The newly syntetized negative-strand serves in turn to be copied into more genomes, which are then copied back into positive strand (Fig. 1.1).

The replication of viral RNA takes place in the cytoplasm of the host cells by a unique mechanism in which RNA polymerase allows for the production of a nested set of mRNA molecules with a common leader sequence at the 5’ end and a common 3' end. Therefore, the mRNA coding for the polymerase is the same length as the genomic RNA, whereas the remainder nested set of mRNA molecules, which are capped and polyadenylated, are truncated at the 5' end. Although the truncated mRNA molecules have more than one protein coding sequence, only one protein is translated from each molecule (Bergmann et al., 2006; Sawicki et al., 2007).

As in shown in Figure 1.1, newly synthetized virions acquire their envelopes from the endoplasmic reticulum-derived membrane and are incorporated into vesicles. In proximity of the cell surface, the virions exit the cell following fusion of the vesicles with the plasma membrane.

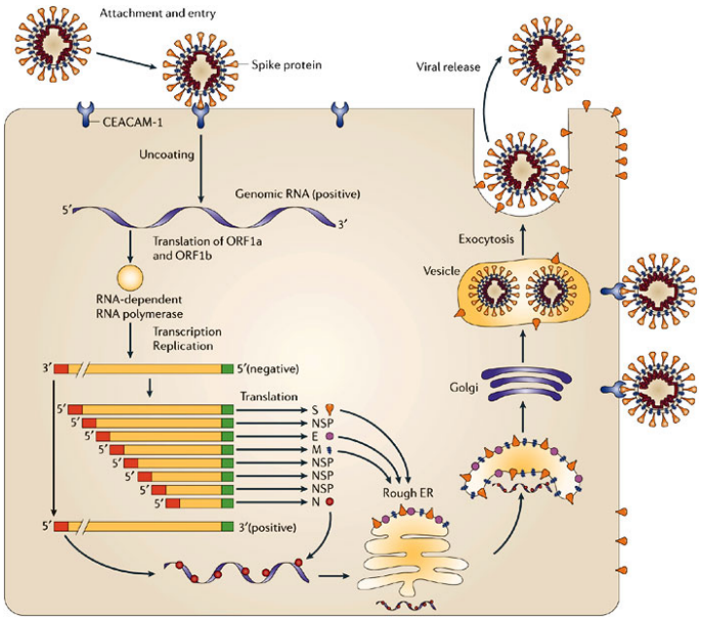

Fig. 1.1. Summary of coronavirus genome replication. The mouse hepatitis virus (MHV) is shown as example of replication. After binding to the host-cell receptor, the virus enters into the cell releasing its genomic RNA into the cytoplasm. Traslation of the ORF1 produces pp1a and pp1ab, which assemble into a replication-transcription complex (RTC). RTC copies the genome either continuously into genome-length template or discontinuously into the various subgenome-length minus-strand templates. The further translation of subgenomic mRNAs gives rise to structural viral proteins. E, envelope protein; ER, endoplasmic reticulum; M, membrane protein; N, nucleocapsid protein; ORF, open reading frame. Reproduced from Bergmann et al., 2006.

1.1.2. The genus *Gammacoronavirus*

Currently the genus *Gammacoronavirus* is classified into two species. In addition to the *Avian coronavirus*, which consists of the infectious bronchitis virus (IBV) and its closely related coronaviruses of turkeys (TCoV) and pheasants, is a CoV identified from a white beluga whale (BWCoV) in 2008 (Mihindukulasuriya et al., 2008). In addition, as a consequence of the recent discovery of a novel CoV from bottlenose dolphin (BdVCoV), a distinct species in gammacoronavirus has been proposed and referred to as Cetacean coronavirus. This would comprise the novel BdV and the BWV (Woo et al., 2014).

1.1.3. The infectious bronchitis virus (IBV)

Infectious bronchitis virus (IBV) is the prototype virus of the genus *Gammacoronavirus* and is the etiological agent of the acute, highly contagious, and economically important viral disease of chicken (Cavanagh and Gelb, 2008). IBV is distributed worldwide and although its natural host is the chicken, the presence of IBV-like and other avian coronaviruses in both domestic and wild animals, including partridge, geese, pigeon, guinea fowl, teal, duck and peafowl has been reported (Cavanagh, 2007, 2005). However, even if the virus may be isolated from a wider host range, it has been postulated that only in the chicken the virus is able to become pathogenic and cause disease (Cavanagh, 2005).

The disease – infectious bronchitis (IB) – was first described by Schalk and Hawn in 1931 in North Dakota, USA (Schalk and Hawn, 1931). It was initially believed that all IBVs comprised a single Massachusetts (Mass) serotype. Jungherr (Jungherr et al., 1956) was the first who revealed that the etiology of IB included more than one serotype, although a retrospective investigation demonstrated that antigenic and genetic variability existed even in the 1940s (Jia et al., 2002). Subsequently, multiple IB serological variants were identified in the United States where in 11 years as many as 11 serotypes or variants of IBV were described (Fabricant, 1998; Hopkins 1969). Since the 1950s IBV outbreaks have occurred globally (Asplin, 1948; Bijlenga 1956; Cumming, 1963; Hipólito, 1957; Sato et al., 1955) and currently more than 50 different IBV genotypes and serotypes, which differ greatly in their pathogenicity for poultry, have been recognized. Some of those have substantial economic impact on the livestock industry, and some others are restricted to specific geographical areas (de Wit et al., 2011a; Jackwood, 2012).

1.1.3.1. Genes and proteins

The IBV genome is composed of @ 27 600 nucleotides and it codes for four major structural genes. These include a spike (S) glycoprotein, small envelope (E) protein, membrane (M) glycoprotein, and nucleocapsid (N) protein (Fig. 1.2) (Spaan et al., 1988; Sutou et al., 1988). Of note, some betacoronaviruses contain a fourth membrane-associated structural protein – the haemagglutinin-esterase (HE) protein – which appears to play a role in binding to or release from the target cell (reviewed by Enjuanes et al., 2000).

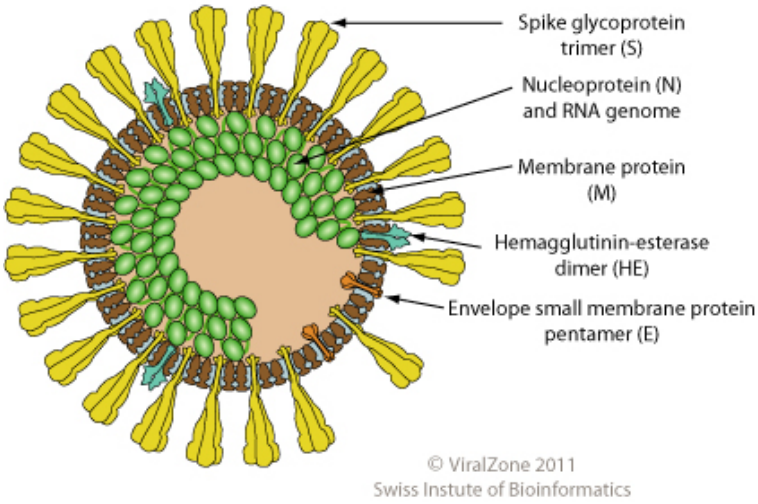

Fig. 1.2. The virion structure of the coronavirus. The viral surface proteins, S and M, are embedded in the viral envelope and the single-stranded positive-sense viral RNA is associated with the nucleocapsid protein. The glycoprotein HE is present only in betacoronaviruses. Reproduced from ViralZone, SIB Swiss Institute of Bioinformatic (http://viralzone.expasy.org/).

At the 5' and 3' ends of the IBV genome are two untranslated regions (UTRs), which play a key role in viral RNA transcription and replication (Boursnell et al., 1987; Ziebuhr et al., 2000). A leader sequence and two overlapping open reading frames (ORFs) are located respectively upstream and downstream the 5'UTR. The ORFs occupy the first two-third of the genome and encode two large polyproteins – 1a and 1ab – that undergo autoproteolytic cleavage into 15 nonstructural proteins (nsps) building up the viral RNA complex (Gorbalenya et al., 2006). Among them, the nsp12, which is the RNA polymerase RNA-dependent (RdRp), is the most conserved and important nsp involved in transcription and viral genome replication (Sawicki et al., 2007; Stephensen et al., 1999). Then, in sequential order, are the four structural genes S, E, M and N (Spaan et al., 1988; Sutou et al., 1988). In addition, two other genes – ORF3 and ORF5 – with a yet unknown function *in vivo*, encode nsps interspersed amongst the structural protein genes (Casais et al., 2005; Hodgson et al., 2006; Lai and Cavanagh, 1997). Genes 3a, 3b and 3c, which is the small E protein, are located between the S and small E protein, while 5a and b are between M and N genes (Fig. 1.3).

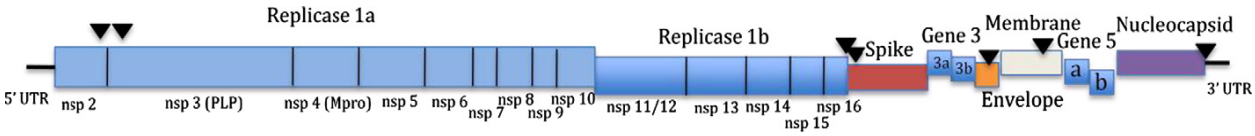

Fig. 1.3. Genome organization of IBV. Black triangles indicate recombination hot spots within the genome. Reproduced from Jackwood et al., 2012.

Within the Coronavirus, the genes have different levels of conservation, which would impact the choice of gene used for various studies. With regards to the relative conservation of the four structural genes, the M is highly conserved between coronaviruses, whereas the E protein is less conserved (Ammayappan and Vakharia, 2009).

The spike glycoprotein

The spike glycoprotein gene is the most variable of all the coronaviruses genes, including IBV (Cavanagh, 2007; Cavanagh and Davis, 1986; Ignjatovic and Galli, 1994; Johnson et al., 2003; Koch et al., 1990; Song et al., 1998a), and is thereby frequently used in phylogenetic and molecular epidemiological studies.

The IBV S protein is a large type I transmembrane protein of @ 1160 amino acids in length (Cavanagh et al., 1986b). This protein is located at the surface of the virion where it forms projections, called spikes glycoproteins, consisting of trimers of S proteins (Delmas and Laude, 1990). During the synthesis, it is post-translationally cleaved into the amino-terminal S1 (~535 amino acids) and the carboxyl-terminal S2 (~627 amino acids) subunits at a multi-basic cleavage site (Cavanagh et al., 1986b).

After initial binding of the receptor, IB enveloped viruses need to deliver their nucleocapsid to the target cell. The spikes play a dual role in this process. At an early stage, the S1 domain recognizes and attaches the host receptor molecules and the S2 is responsible for spanning the viral membrane and anchoring the spikes to the virion (Jackwood et al., 2012). Then, the S protein fuses the virus envelope with the cell membrane through its S2 domain (Bosch et al., 2003). During this process, a series of conformational changes of this domain allow the virion to enter into the targeted host cell (Matsuyama and Taguchi, 2002; Sturman et al., 1990; Zelus et al., 2003).

Because of its function, the spike of many coronaviruses has been recognized as the protein which determines cell and host tropism (Ballestros et al., 1997; Baric et al., 1997; Casais et al., 2003; Fang et al., 2005; Fazakerley et al., 1992; Hingley et al., 1994; Leparc-Goffart et al., 1997; Li et al., 2005; Ontiveros et al., 2003; Phillips et al., 2002; Wesley et al., 1991). In addition, there is strong evidence that the S1 domain is the region mostly involved in immunogenicity complex and binding of virus neutralising antibodies, even though all IBV structural proteins undoubtedly contribute in the generation of an immune response against the virus (Cavanagh, 1983; Cavanagh and Davis, 1986; Koch et al., 1990; Mockett et al., 1984).

Among IBV strains, the S protein represents the most variable gene of the genome. In particular, IB serotypes may differ by 20% to 25% at the genomic scale, and up to 50% of amino acids in the S1 subunit (Cavanagh et al., 2005), while the S2 portion differs to an extent similar to that of other structural proteins (10 to 15%) (Cavanagh, 2007).

The envelope protein

The E protein is a small (~75 to 109 amminoacids) structural protein and represents the minor component of the virion membrane. During infection, only a fraction of the protein is incorporated into the virion envelope, whereas the majority remains localized at the Golgi (Westerbeck and Machamer, 2015). The E protein contains a hydrophobic domain (HD), whose presence suggests that this protein is involved in the release of infectious virus (Ruch and Machamer, 2011; Ye and Hogue, 2007). It has been also proposed a role of the E protein in virus assembly. In particular, the interaction between E and M proteins can be sufficient to direct the production of virus-like particles (VLPs), promoting the virion budding (Lim and Liu, 2001).

The matrix protein

Among the virion membrane proteins, the matrix protein is the most abundant. It is a multifunctional protein that consists of 230 amino acids (Ujike and Taguchi, 2015). The M protein coordinates the process of virus assembly and budding through the interaction with viral nucleoprotein and S glycoprotein at the budding site (de Haan et al., 1999; Escors et al., 2001a,b; Kuo and Masters, 2002; Narayanan et al., 2000; Nguyen and Hogue, 1997; Opstelten et al., 1995; Sturman et al., 1980). Moreover, the interactions between M proteins form a

lattice-like matrix network that is able to exclude some host membrane proteins from the viral envelope during assembly activity (de Haan et al., 2000; Neuman et al., 2008).

The nucleoprotein

The nucleoprotein is a phosphorylated protein consisting of 409 amino acids. It binds to the viral RNA to form a helical complex, called nucleocapsid, which protects the RNA from degradation and ensures its replication and reliable transmission (Davies et al., 1981). Because of its interaction with replicase components of the viral genome and cellular RNA-binding factor, a role in transcription of subgenomic mRNAs has been reported for this protein (Emmott et al., 2013). In addition, the protein seems to be required to guarantee efficient genome replication, even though this process can occur in its absence (Almazán et al., 2004). It has been also suggested that the protein phosphorylation allows the nucleoprotein to take part in the discrimination of viral and non-viral RNA by exposing various functional motifs (Chen et al., 2005; Spencer et al., 2008).

1.2 PATHOGENESIS

The IBV enters the host through either inhalation or direct contact with infected chickens, and it is shed in respiratory discharges and faeces (Cavanagh, 2003). In addition, Gallardo and colleagues reported experimental evidence for IBV venereal transmission from male to female (Gallardo et al., 2011).

The ability of the virus to survive for long period of time in faeces as well as the excretion of virus for up to 20 weeks after infection may aid viral transmission and persistence (Alexander and Gough, 1977). The incubation period is generally 24-48 hours. However, massive infective dose and proximity of the site of virus entry to the upper respiratory tract generally shorten the incubation period (Cavanagh and Gelb, 2008).

Following entry of the virus in a poultry flock, all chicks generally are infected. Young age and poor immunological status of the chickens as well as establishment of secondary bacterial infections affect the course of the infection and in many instances an increased mortality can be observed (Bacon et al., 2004; Vandekerchove et al., 2004). Mortality rate of approximately 25% has been reported in chickens less than 6 weeks of age, while an insignificant variation can be recorded in birds greater than 6 weeks (Cavanagh and Gelb, 2008). Also the causative IBV strain can influence the outcomes of the disease; the mortality increases proportionally to

the virulence of the IBV strain, ranging from reasonable to severe rates.

An important feature in IBV virulence is the changeable affinity of the virus for the diverse host tissues. While the IBV host tropism is restricted to chickens, the cell and tissue tropism in chickens varies greatly between different IBV strains (Cavanagh, 2007). In the host, IBV primarily targets the epithelial surface of the upper respiratory tract and is independent of the strain (Raj and Jones, 1997). The spike glycoprotein acts as the receptor and mediates uptake into the cells through membrane fusion (Belouzard et al., 2012; Heald-Sargent and Gallagher, 2012). The virus then moves into the respiratory system as well as other peripheral non-respiratory tissues, including the gastrointestinal tract, oviduct, and kidneys. Virus replication in enteric tissues is usually not associated with evident clinical signs, whereas those infections involving IBV strains with a predilection for kidneys may result in nephropathogenicity (Cavanagh, 2007).

As mentioned above, abundant fraction of the total variability resides in the S1 protein, which is also the major inducer of neutralizing antibodies; thereby variations of the S1 are partly responsible for differences in virulence and host tissue tropism (Bacon et al., 2004; Casais et al., 2003; Raj and Jones, 1997). Alpha 2,3-linked sialic acids have been identified as receptors determining the binding to the host cells (Abdel Rahman et al., 2009; Winter et al., 2006, 2008a). However, neuroaminic acids are well distributed also in another body districts, which are not susceptible to the IBV infection. Therefore, it remains to be established whether organ specific expression of sialic acids or a yet unidentified host factors contributes to limit the tropism of various IBV strains.

1.3. CLINICAL SIGNS AND PRINCIPAL LESIONS

Avian infectious bronchitis is primarily a respiratory infection of chickens. Nevertheless, depending on the IBV strain, respiratory syndrome, reproductive disorders and nephritis are the three main clinical outcomes which are usually detected in the flocks.

Severity of the infection depends on the age of chicks, virulence of the causative strain and existing level of immunity (Cavanagh, 2007). Moreover, the presence of bacterial pathogens such as *Escherichia coli* or *Mycoplasma* exacerbates the disease, resulting in airsacculitis, pericarditis and perihepatitis.

1.3.1. Respiratory disease

A generalised weakness is observed in chickens with respiratory disease; they appear listless, lethargic and dull with ruffled feathers. Clinical outcomes of IBV respiratory infection include gasping, sneezing, tracheal rales, nasal discharges and conjunctivitis with profuse lacrimation (Cavanagh and Gelb, 2008; Raj and Jones, 1997). These can last less than seven days or several weeks in the case of chronic infection, resulting in mortality between 5% and 25%. The main histological lesions are found in the trachea epithelium, which shows a marked loss of cilia, desquamation, hyperplasia and mononuclear infiltration of the surrounding mucosal layers (Cavanagh, 2003). Post-mortem examination reveals excessive amount of mucus and vascular congestion of the trachea.

1.3.2. Nephropathogenic strains

Nephropathogenic IBV strains are mainly described in broiler-type chickens and cause mild and transient respiratory signs followed by depression, wet droppings, excessive water intake, rapid loss of weight and diarrhoea (Brown et al., 1987; Cowen et al., 1987; Cumming, 1969; Liu and Kong, 2004; Winterfield and Hitchner, 1962).

After infection, nephropathogenic strains firstly replicate in the trachea, where histological lesions identical to those induced by respiratory strains are observed. Then, the virus disseminates in the kidneys replicating in all segments of tubules and ducts, but more often in the epithelial cells of the collecting ducts (Cavanagh et al., 1997). Between five and ten days after infection, the kidneys appear haemorrhagic and degenerative changes in renal tubules and glomeruli as well as focal lymphocytic infiltration can be seen. On necropsy, the kidneys are pale and swollen, and deposits of urates may be present in the ureters of some chicks (Cavanagh and Gelb, 2008; Pohl, 1974; Purcell et al., 1976).

1.3.3. Reproductive disorders

Infection of reproductive tract is associated with lesions of the oviduct resulting in a decline of egg production and quality (McDougall, 1968; Sevoian and Levine, 1957). Severity of the infection varies depending on the IBV causative strain and the age of chicks (Crinion and Hofstad, 1972a). In infected adult individuals the production commonly returns to near normal laying within six-to-eight weeks. In other instances, the performances of the flock remain at a subnormal level, contributing to high economic loss (Cavanagh, 2007; Winterfield

et al., 1984). In female chickens at a young age the infection can cause permanent damage of the developing reproductive tract, inducing the so-called 'false layers syndrome' (Broadfoot et al, 1956; Jones and Jordan, 1970; Jones and Ambali, 1987). However, reproductive disorders generally may not be followed by clinical signs of disease or can result in a mild respiratory syndrome (Cook, 1984; Cook and Huggins, 1986; Muneer et al., 1986, 1988).

At post-mortem examination, the oviduct can show areas of hypoplasia and yolk fluid can be present in the abdominal cavity. The main histopathological changes are in the epithelium of the oviduct (Butler et al., 1972a; Jones and Jordan, 1971); reduction in the height of the epithelial cells and deciliation as well as cellular infiltration in the lamina propria can be observed (Crinion et al, 1971a,b; Crinion and Hofstad, 1972a,b; Sevoian and Levine, 1957).

1.4. DIAGNOSIS

The observation of clinical signs of the chickens is not in itself sufficient to confirm the presence of IBV; that must be achieved by virus isolation, detection of antigens or antibodies, or identification of IBV genome. The test of choice depends on multiple factors, such as the reporting time of the test, the type of sample under investigation, and most importantly, on the purpose of the test. However, all of the diagnostic methods currently available for IBV have limitations and the complementary use of both classical and molecular approaches will help overcome some of them. Apart from the diagnosis, the assessment of the serotype or the genotype of the virus causing disease is recommended to make the surveillance effective (de Wit et al., 2011a; Jackwood and de Wit, 2013).

1.4.1. Virus Isolation

Virus isolation (VI) has been the gold standard for the diagnosis of IBV (Beaudette and Hudson, 1937; Stephensen et al., 1999). The technique allows identifying infectious viral particles after inoculation of the virus in embryonated chick eggs (ECEs), tracheal organ culture (TOC) or cell culture. Among these biological substrates, the latter is the less sensitive (Butler et al., 1972b; Cook et al., 1976; Darbyshire et al., 1975) and requires cell adaptation before virus replication is achieved, and therefore it is not frequently used.

Virus isolation in embryos requires incubation with field material for 5-7 days, followed by a series of embryo passages until embryo malformations or mortality occur (Cavanagh and Naqi, 1997). An alternative and less time-consuming approach is often preferred to the

conventional procedure of isolation. It is first based on the virus replication in the selected substrate and then on the application of immunofluorescent assay (IFA) (Clarke et al., 1972), antigene-capture enzyme-linked immunosorbent assay (ELISA) (Ignjatovic and Ashton, 1996; Naqi et al., 1993) or reverse transcription polymerase chain reaction (RT-PCR) (Adzhar et al., 1997; Andreasen et al., 1991) to confirm the presence of IBV (McMartin, 1993).

Although VI is a sensitive method, several complicating factors such as the presence of another virus interfering with IBV or the inactivation of IBV antigen and the time required for several passages of virus in egg or cell culture limit its use as diagnostic method of choice for IBV infection.

1.4.2. Detection of IBV antigens

The techniques used for detection of IBV-specific antigen all use IBV-specific antibodies, which are in the form of antisera or monoclonal antibodies (Mabs).

1.4.2.1. Agar gel precipitation test (AGPT)

The test is simple, cheap and fast, but is relatively insensitive (de Wit, 2000). The method can be applied to detect IBV after growth in ECEs, using allantoic fluid or the chorioallantoic membrane as source of antigen (Alexander and Gough, 1977; Gelb et al., 1981). In order to prevent false negative results due to imbalance of the antigen, several antisera at different dilutions should be used (Lohr, 1981). However, the test is rarely used.

1.4.2.2. Immunofluorescent assay (IFA) and immunoperoxidase assay (IPA)

The IFA and IPA are two staining-base methods that can be used to detect IBV antigens in infected tissues and cells (reviewed by de Wit, 2000) by using polyclonal anti-IBV serum or specific monoclonal antibodies.

The IFA relies on the ability of a detector molecule, fluorochrome or enzyme, conjugated to an IBV specific antibody. This complex is able to bind to an IBV antigen and can be visualised using ultraviolet microscopy. Although IFA shows variability in sensitivity mainly due to the amount of virus in the starting material, the test is relatively cheap, easy to use and fast (reviewed by de Wit, 2000). As it is mentioned above, the method can be also applied in combination with the VI resulting in a shorter procedure.

The IPA is based on the IBV antigen binding through the complex antibody combined with the peroxidase enzyme. The test is more laborious and time consuming compared to the IFA but it has the advantage to allow evaluation of antigen-bearing cells, as well as general tissue morphology because of the stability of the staining. However, both tests are not commonly used other than for research work (reviewed by de Wit, 2000).

1.4.2.3. ELISA-based method

Enzyme-linked immunosorbent assays (ELISAs) are rapid, cheap and easy techniques that can be used for the detection of infectious bronchitis antigens (Mockett and Darbyshire, 1981) in field samples or in allantoic fluid. However, ELISAs are more suitable for confirming antigen presence in allantoic fluid of inoculated eggs rather than detecting antigens directly in chicken organs because of the low sensitivity of the test (Hesselink et al., 1988; Ignjatovic and Ashton, 1996; Koch et al., 1991; Nagano et al., 1990; Naqi et al., 1993; Yagyu and Ohta, 1987).

1.4.3. Detection of antibodies

IBV infections can be diagnosed by detecting IBV-specific antibodies or by revealing a significant rise in their titre between paired samples. The first sample is taken at onset of disease, while the second one 1 to 4 weeks later (Kreider et al., 1991a,b). At least a four-fold rise in titre is required for a positive diagnosis. A disadvantage of serotyping techniques is the lack of standardization between the different systems and laboratories.

1.4.3.1. ELISA-based method

The ELISA is widely used to identify IBV infections based on high antibody titres. The tests have proved to be highly sensitive, allowing detection of the virus within one week of infection, earlier than by hemagglutination-inhibition (HI) or virus neutralization (VN) tests (de Wit et al., 1998, 1997; Marquardt et al., 1981; Mockett and Darbyshire, 1981). This means that the first sampling must be performed immediately after infection is suspected in order to avoid seroconversion not to be recognized.

The IBV ELISA-based diagnosis can be conducted by using one of the several commercially available ELISA kits or can be *in-house* prepared (Garcia and Bankowski, 1981; Mockett and Darbyshire, 1981; Snyder and Marquardt, 1989). As they lack in type specificity, these tests

are unable to differentiate serotypes; however, they provide a large amount of information very quickly and are invaluable for surveillance purposes and monitoring vaccination responses under field conditions.

1.4.3.2. Virus neutralization

The technique allows identifying virus neutralizing antibodies in ECEs (Davelaar et al., 1984; Gelb et al., 1989), TOC (Darbyshire et al., 1979; Cook, 1984) or cell culture (Hopkins, 1974). VN is less suitable than ELISA for the diagnosis of IBV infection as several of the emerging serotypes do not cross-react with the commonly available antisera, hampering detection of the virus. Nonetheless, VN continues to be considered the gold standard test for the detection of IBV serotype-specific antibodies.

Generally a two-way neutralization test is performed in eggs, first with known antisera and unknown virus and then with known viruses and antisera prepared against the unknown virus. However, lack of method standardization impedes comparison of the results from the different laboratories (de Wit, 2000).

1.4.3.3. Haemagglutination test (HI)

In haemagglutination test for IBV the antibodies are induced against the spike protein and the pre-treatment of IBV with neuraminidase enzyme is necessary because it does not haemagglutinate spontaneously.

The HI test is considered to be type-specific after a single infection or inoculation (de Wit et al., 1997; King and Hopkins, 1983; Monreal et al., 1985), although cross-reactions between serotypes can reduce its specificity (Villarreal, 2010). In the field chickens are usually subjected to more than one immunization with IBV vaccines; therefore, cross-reactive antibodies are likely to be encountered following an anamnestic response induced by a field infection. For example, an HI test using the M41 as an antigen resulted in poor performance in antibody detection following vaccination with H120, although both viruses are of the same serotype (de Wit et al., 1997). This may limit the use of the HI test to monitor vaccine response. Notwithstanding, the test can be useful for routine diagnosis, as it is cheap, quick and easy to perform.

1.4.4. Detection of IBV genome

Nucleic acid based methods are extensively applied for detecting the presence of IBV viral genome and determining the identity of a field strain, and have largely replaced the conventional serotyping techniques. However, lack of consistency and method standardization have been recognised as the main disadvantages to the use of molecular methods.

1.4.4.1. RT-PCR methods

RT-PCR assays are rapid and sufficiently sensitive to detect IBV RNA extracted from tissue or swabs as well as from Flinders Technology Associates (FTA) cards (Moscoso et al., 2005). The test can be performed directly by using the RNA extracted from the infected samples as template (Cavanagh et al., 1999; Jackwood et al., 1997; Li et al., 1993; Worthington et al., 2008) or the infectious allantoic fluid after the virus has been propagated in ECEs (Adzhar et al., 1996, 1997; Andreasen et al., 1991; Kwon et al., 1993; Lin et al., 1991).

The viral RNA is further analysed with RT-PCR by using universal oligonucleotides able to bind to the RNA of many types of IBV (Adzhar et al., 1996; Handberg et al., 1999; Jones et al., 2005; Keeler et al., 1998; Kwon et al., 1993; Lin et al., 1991; Worthington et al., 2008). To this end, RT-PCRs are generally designed on UTR or N genes because of the conserved nature of their target regions among many IBV serotypes (Adzhar et al., 1996; Zwaagstra et al., 1992). Universal primers targeting the S1 gene have been also reported and widely used (Adzhar et al., 1996; Worthington et al., 2008). Otherwise, an approach based on the use of type-specific RT-PCR primers might be preferred (Cavanagh et al., 1999; Keeler et al., 1998), even though this may not be sufficiently specific (Capua et al., 1999).

In addition, the application of a nested PCR-based strategy can be an alternative and useful method to improve the sensitivity especially when only small amount of RNA is in the sample (Cavanagh et al., 1999). In this case, two different RT-PCRs are performed and the amplified product of the first PCR is further used as template for the second amplification. However, false positive results due to sample contaminations of PCR products can occur (de Wit, 2000; Jackwood and de Wit, 2013).

1.4.4.2. Real-Time RT-PCR methods

Real-time RT-PCR assays have been developed to improve the sensitivity and specificity of virus detection. The method has been proven to be more sensitive than virus isolation and conventional specific RT-PCR assays based on N or S1 genes (Meir et al., 2010).
As for RT-PCRs, various protocols have been carried out for IBV detection, with most of them targeting UTR and N genomic regions that are quite conserved among the different IBV strains (Callison et al., 2006; Meir et al., 2010). The application of the RT-PCR assay also allows the IBV viral load to be determined based on viral copy number or fold changes (Callison et al., 2006; Jackwood et al., 2003).

1.4.4.3. Sequence and Phylogenetic Analyses

As live IB vaccines are largely used, the diagnosis of IBV infection may be complicated. A positive Real time RT-PCR- or RT-PCR-based result is not always indicative of the presence of a virulent strain in the field but rather may be due to a re-isolation of a vaccine strain (Cavanagh, 2003; Cavanagh and Gelb, 2008). Thereby, a more reliable diagnosis of IBV requires additional analysis such as sequencing or restriction enzyme fragment length polymorphism (RFLP) or hybridization using IBV specific probes (Binns et al., 1985; Jackwood et al., 1992; Moore et al., 1998) to identify the IBV causative agent. Among the typing methods for IBV, the nucleotide sequencing represents the gold standard. It has aided the rapid genotyping of a large number of viruses by making it possible to easily compare them. The application of phylogeny has allowed the assessment of the phylogenetic relationships between IBVs strains and has further permitted these strains to be assigned into epidemiological and phylogenetic contexts.

Nucleotide heterogeneity is most prevalent in the S1 portion of the S gene and largely contained within three different hypervariable regions (HVRs) (aa 38–67, 91–141 and 274–387) (Cavanagh et al., 1988; Moore et al., 1997). Accordingly, the complete or partial S1 gene is the region convetionally amplified, sequenced, and subjected to bioinformatics analyses to determine the viral genetic types (Abro et al., 2012; Zulperi et al., 2009).

Notably, the lack of method standardization among laboratories, particularly with respect to the portion of the S1 to be used in phylogenetic analysis, limits genotyping to some extent.

1.5. GENETIC AND ANTIGENIC DIVERSITY OF IBV

As described previously, a variety of methods have been developed for the diagnosis and typing of IBV. Systems that examine the antigenic or genetic features of an isolate result in the description of serotypes and genotypes, respectively, whereas methods that are focused on the immune response of chickens against challenge with an IBV strain lead to the designation of protectotypes (Lohr, 1988). Importantly, however, the genotype-, serotype- or protectotype-based approaches do not always group IBVs in the same way making the control of this pathogen extremely challenging.

Due to the nature of IBV, a limited number of amino acid changes in the S1 portion of the S gene may result in relevant modifications in strain antigenicity (Cavanagh et al., 1992a). On the other hand, IBV strains with a very similar S gene sequence but with substantial genetic differences in other parts of the genome can maintain their antigenic features unaltered. In addition, IBV strains can belong to the same genotype and be of the same serotype or they can be of the same genotype and not of the same serotype or, at least, result in weak cross protection (Capua et al., 1998; Cavanagh et al., 1992a,b; Clewley et al., 1981; Kant et al., 1992; Koch et al., 1990; Kusters et al., 1987; Minta et al., 1998; Williams et al., 1992).

However, even with some exceptions (Cavanagh et al., 1992a; Li et al., 2011), the genetic similarity between viruses in the S1 amino acid sequences can be used to predict the level of cross-protection between IBV types (Cavanagh et al., 1997; Ladman et al., 2006; Lee et al., 2003). Generally, a good level of cross-protection has been reported for IBV strains genetically closely related, even if it is also possible that cross-protection might occur between genetically and serologically unrelated viruses (Raj and Jones, 1996).

Another relevant issue to consider in controlling IBV is the lack of standardization on the method by which the IBV strains should be typed, in particular on the different portion and size of the S1 to be used in the assessment of the similarity existing between strains (de Wit et al., 2010a). The parameter is clearly dependant on the region used for its estimation; therefore, different genetic portions lead to different conclusions regarding the relationship between virus strains.

1.5.1. Serotypes

As mentioned before, assessment of the serotype is based on the reaction between an IBV strain with homologous and heterologous antisera produced in chickens. Two strains are considered to be of the same serotype when two-way heterologous neutralization titres differ less than 20-fold from the homologous titres in both directions (Hesselink, 1991). Since every serotype needs its own neutralization test, serotyping became not commonly used because of the increasing emergence of novel IBV variants.

1.5.2. Genotypes

Classifying IBV strains based on molecular characterization of the genome or a part of this leads to the definition of genetic groups, arbitrarily and confusingly defined as genetic types, genotypes, clades or clusters. In the last two decades, genotyping has become the most widely preferred method for IBV typing (Cavanagh et al., 1999; Jackwood et al., 1992; Lee et al., 2000) replacing the serotyping systems (Jackwood and de Wit, 2013). By using RT-PCR techniques followed by sequencing, it is possible to obtain valuable information for epidemiological studies (de Wit, 2000). As discussed before, the analysis of the complete or partial gene coding the S1 portion of the spike protein has been conventionally used to determine viral genetic types.

1.5.3. Protectotypes

Strains that induce protection against each other in chickens belong to the same protectotype (Lohr, 1988). By performing vaccination challenge studies, it is possible to assess the complete immune response of a chicken against an IBV strain and therefore have direct information about the efficacy of a vaccine for IBV (de Wit, 2000). However, the excessive cost and the requirement of high-level facilities are the main disadvantages that make protectotyping extremely difficult to be performed.

1.6. IBV EVOLUTION AND CONTROL MEASURES

Infectious bronchitis viruses are dynamic and are continuously evolving. Because of their nature, these viruses exist in a wide range of antigenically and genetically distinct viral types which frequently develop resistance to vaccines, making the control of this pathogen both

complex and challenging. Typing of IBV strains undoubtedly is essential to choose the right vaccine in the field and to study viral evolution and epidemiology.

1.6.1. IBV evolution

It has been reported that the genetic diversity in coronaviruses is generated by three major key factors (Han et al., 2011): (i) the lack of a proofreading activity of their RNA polymerases, which results in frequent errors during viral RNA synthesis; (ii) the high frequency of homologous RNA recombinations, which is likely due to their unique mechanism of RNA replication (Lai and Cavanagh, 1997); (iii) the large size of their genomes, which provides this viral family an extra flexibility to accommodate and modify genes (Masters, 2006). In addition, the short generation time and large population allow RNA viruses including IBVs to adapt under various environmental conditions (Moya et al., 2000). As a consequence, mutations and recombination events frequently occur in the IBV genomes, resulting in high rates of mutation.

The average mutation rate of coronaviruses is approximately 1.2×10^{-3} nucleotide substitutions per site, per year (sub/site/year) (Hanada et al., 2004; Holmes, 2009). In some fitness landscapes, the existence of a maximum mutation rate, called the error threshold, has been predicted. Beyond this threshold, the quasispecies enter into an error catastrophe, losing its genetic information: consequently, the virus population extinguishes (Duffy et al., 2008; Lauring and Andino, 2010; Tejero et al., 2011). Notably, IBV and some other coronaviruses possess an exoribonuclease (ExoN) domain in nsp14 reported to be involved in proofreading and repair of the viral RdRp (Minskaia et al., 2006; Snijder et al., 2003). Therefore, the increased fidelity of the RdRp results in a higher 'error threshold', which likely permits the virus to maintain a large genome size (Holmes, 2009).

Although mutations occur presumably throughout the entire viral genome, the extensive variation exhibited by the S1 glycoprotein among IBV populations (Kusters et al., 1989, 1987) is undoubtedly the most successfully strategy adopted by these viruses to escape host immunological system and evolve in the environment.

As for many RNA viruses, the recombination is an important mechanism driving IBV genotype diversity. By reducing mutational load they can create new genetic variants that may be very different from parental strains (Worobey and Holmes, 1999). Recombination is the process by which two molecules give rise to a new mutant genome after the exchange of genetic

information. This mechanism of evolution has been reported in many coronaviruses (Brooks et al., 2004; Lee and Jackwood, 2000; Magiorkinis et al., 2004; Thor et al., 2011) and has contributed to the emergence of several alpha- and beta-coronaviruses (Decaro et al., 2009; Woo et al., 2009). Recently the appearance of a novel gamma-coronavirus in turkeys (TCoV) has been attributed to recombination (Jackwood et al., 2010a). Within the IBV genome, the higher incidence of recombination has been observed in the S, followed by nsp2, nsp3 and nsp16 genes (Armesto et al., 2009; Jackwood et al., 2012; Thor et al., 2011).

Factors other than high mutation rates may influence the selection of IBV variants in nature. In the field chickens are typically subjected to more than one immunization with two or more serotypes with the aim to increase the protection. Despite this, sometimes the failure or the insufficient protection of the vaccination may likely promote selection and spread of viruses with an increased viral fitness due to their persistence in vaccinated hosts (Gandon and Day, 2008; Gandon et al., 2001; van Santen and Toro, 2008). For example, it has been suggested that the GA98 variant arose from immune selection caused by DE072 attenuated live vaccine introduced in the country in 1993 (Lee and Jackwood, 2001).

1.6.2. Control measures for IBV

Vaccination is normally an essential tool to increase the resistance of the chickens against challenge with IBV strains (Cook, 2008). Because of the little or no cross-protection between the different virus serotypes, often a combination of two or more antigenic types is included in vaccine formulations in order to achieve a broader protection (Winterfield and Fadly, 1975). Thereby, a well-vaccinated bird is adequately protected against challenge with a virulent homologous IBV and sometimes also partially against IBV of other protectotypes, serotypes or genotypes (Bijlenga et al., 2004). For example, it has been observed that vaccination with Mass followed by 4/91 after a 2-week interval is effective against many different IBV types (Cook et al., 1999; Terregino et al., 2008).

Vaccination programmes and procedures may differ from one country to another depending on local conditions. The most frequently used vaccines in the world are of Mass and H120 types, which are also the only vaccines allowed in some countries. However, vaccines based on other serotypes or local strains are commonly permitted elsewhere.

Both live and inactivated vaccines are used in IB immunization. Live vaccines are especially administered to meat type chickens (broilers) and to young birds. In many countries a first

vaccination of one-day birds is generally practised with low virulence vaccines and a second vaccine dose of the same or different serotype is given at two weeks of age of the chickens. In addition, live vaccines are used for priming of future layers and breeders and also periodically administered during the laying period to achieve local protection of the respiratory tract.

On the other hand, inactivated vaccines are given to layers and breeders of thirteen to eighteen weeks of age, which have been previously vaccinated with live attenuated vaccines. The administration before the onset of egg production is a useful practise to prevent infections of the internal tissues. Inactivated vaccines induce an antibody production that persists for longer periods than that induced by live vaccination (Box et al., 1982, 1980) and provide the chicks with passive maternal antibodies. In contrast to live vaccines, they are not so effective in ensuring good protection of the respiratory tract following challenge with the homologous virulent virus.

However, despite vaccination efforts to control the disease, IB remains one of the most prevalent avian diseases in the world's poultry industry. Control of IBV may be difficult because there is little or no cross-protection between the numerous different serotypes of the virus and the extensive use of vaccines may complicate diagnosis. In particular, field and vaccine strains share a high degree of S1 sequence identity (Gelb et al., 2005) and cannot always be distinguished. Moreover, the persistence of live vaccines may also confuse attempts to identify the causative field strain of IBV resulting in the deployment of the wrong vaccine strain (Cavanagh, 2003; Cavanagh and Gelb, 2008). Other possible reasons of the scarce protection induced by vaccination can be immunosuppression, very short or long intervals between vaccination and challenge or inadequate application of the vaccine (Cavanagh & Gelb, 2008; de Wit et al., 2010b; Jackwood et al., 2009).

1.7. FUNDAMENTALS OF APPLIED EXPERIMENTAL TECHNIQUES

1.7.1. Phylogenetic techniques

Phylogenetic trees have been used to describe the historical relationships of groups of organisms. It is well known that a variety of evolutionary forces act on DNA/RNA sequences. As a result, sequences change in the course of time. One of the first steps in the analysis of aligned nucleotide sequences is the computation of the matrix of genetic distances between all pair of sequences, which provides a measure of the similarity between sequences. The

proportion of different sites between two aligned sequences is called *p*-distance. However, *p*-distance generally underestimates the true genetic distance (d). Indeed, when two sequences are very divergent it is likely that, at a certain position, two or more consecutive mutations have occurred.

As a consequence, the selection of a substitution model, which corrects for multiple hits, is an essential stage in the pipeline of phylogenetic inference. Substitution models of evolution describe the process of genetic variation through fixed mutations and constitute the basis of the evolutionary analysis at the molecular level. The simplest one-parameter Jukes-Cantor model assumes that all nucleotides occur in equal proportions and that the probabilities of each nucleotide substituting for another are equal among all six possible nucleotide pairings (Jukes and Cantor, 1969). However, the Jukes-Cantor model is considered an over-simplification of the process of nucleotide substitution.

The employment of the more complex general time reversible (GTR) model generally provides a statistically better fit to observed patterns of sequence evolution (Yang, 1994). GTR incorporates different rates for every change and different nucleotide frequencies. In addition, a proportion of invariable sites (I) (Shoemaker and Fitch, 1989) and/or rate of variation across sites (Γ) (Yang, 1994) can be incorporated into the model. It is well known that the rate of nucleotide substitution can vary substantially for different positions in a sequence and rate heterogeneity can play a crucial part in the inference of genetic distances. Therefore, an approach that uses a gamma distribution (Γ) is required in order to account for the site-dependent rate variation.

The methods for constructing phylogenetic trees from molecular data can be grouped according to the kind of data – discrete character states or a distance matrix – and the approach they use, clustering algorithm or optimality tree searching.

1.7.1.1. Distance methods

Distance method computes the evolutionary distances for all pairs of taxa and the tree is constructed by inferring the phylogenetic relationships among these determined distance values. The distances are expressed as the fraction of sites that differ between the two sequences. A variety of methods such as the Unweighted Pair-group (UPGMA), the Minimum Evolution (ME) and the Neighbor-Joining (NJ) have been employed to assess the evolutionary distances. Among them, the NJ is the most popular algorithmic method for phylogenetic tree

building. Starting with a star-like tree, the method manipulates a distance matrix, reducing it in size at each step, and then reconstructs the tree from that series of matrices. On the other hand, the NJ does not examine all the possible topologies and does not guarantee finding the tree with the smallest overall distance (Saitou and Nei, 1987).

1.7.2.2. Maximum parsimony

Maximum parsimony (MP) is based on the assumption that the most likely tree is the one that requires the fewest number of changes to explain the data in the alignment. The principle of parsimony is that taxa sharing a common characteristic descend from a common ancestor. Parsimony is the criterion for choosing the best tree by selecting the tree or trees that minimise the number of evolutionary steps, including homoplasmies, required to explain the data (Nei and Kumar, 2000). Thereby, an algorithm operates in determining the minimum number of steps necessary for any given tree to be consistent with the data. That number is the score for the tree, and the tree or trees with the lowest scores are the most parsimonious trees (Nei and Kumar, 2000).

1.7.2.3. Maximum Likelihood methods

The principle of the Maximum Likelihood (ML) is to find the tree topology, branch lengths and parameters of the model of substitution that maximise the probability of observing the sequence data. Compared to other computational methods, the ML is more accurate and robust (i.e. finding the correct tree) and is able to efficiently handle a large number of sequences (Kuhner and Felsenstein, 1994; Huelsenbeck, 1995). By using heuristic hill-climbing algorithms, it is possible to limit the tree space that needs to be explored only to the one adjacent to the temporary tree (Holder and Lewis, 2003). However, it is not guaranteed that the optimal tree is found. The tree with the higher likelihood score can represent a local peak rather than the 'summit' of the tree space. Therefore the most straightforward way to evaluate the success of a tree-search strategy is to generate perturbations in the tree topology by branch-swapping algorithms, such as the nearest-neighbor interchange (NNI), subtree pruning and regrafting (SPR), and tree bisection-regrafting (TBR). These algorithms operate a series of tree rearrangements to test any potential improvements in likelihood scores.

1.7.2.4. Bayesian analysis

Bayesian inference of phylogenies generates a posterior distribution for a parameter, composed of a phylogenetic tree and of a model of evolution, based on the prior for that parameter and the likelihood of the data, generated by a multiple alignment. Both ML and Bayesian methods are considered the preferable methods for tree building, particularly when evolutionary rates differ among lineages. Similarly to the ML, given a model of evolution, Bayesian statistics searches for the best trees which are consistent with both the model and the data (Nei and Kumar, 2000). However, ML seeks the tree that maximises the probability of observing the data given that tree; Bayesian analysis seeks the tree that maximises the probability of the tree given the data and the model for evolution. Of note, within Bayesian inference the posterior probabilities of all the trees usually cannot be calculated analytically. The Markov chain Monte Carlo (MCMC) method, implemented in MrBayes program, can be used for this purpose (Ronquist and Huelsenbeck, 2003).

1.7.2.5. Estimating the reliability of a phylogenetic tree

A tree consists of two elements: nodes and branches. A branch is a line that connects two nodes. Nodes can be either external, which are the tips of the tree or internal, which are the points that represent a common ancestor of two other nodes. Several procedures such as bootstrapping method and Bayesian posterior probabilities have been developed and used to statistically assess the confidence of tree selection.

The bootstrapping method assesses the reliability of a phylogenetic structure by determining the proportion of 'pseudoreplicate' datasets supporting each node (Felsenstein, 1985). These datasets are generated by randomly sampling the original character matrix (the columns of the alignment) to create new matrices of the same size as the original. These proportions (expressed as percentages) can be used as a measure of the reliability of individual branches in the optimal tree. The basis for phylogenetic analysis is the identification of discrete clades of genetically related viral isolates that cluster together on a tree and share a unique common ancestor. Clades (or genetic clusters) are topologically separated by long branches and are considered statistically well supported when bootstrap values exceed 70 (Felsenstein, 1985).

However, despite their popularity, both nonparametric bootstrap frequencies and Bayesian posterior probabilities are computationally expensive and become prohibitive for large data sets. Therefore, new and faster tests to measure tree branch supports of a phylogeny have

been proposed. Anisimova and Gascuel (2006) developed an approximate likelihood ratio test (aLRT), a frequentist test that compares two best nearest-neighbor interchange (NNI) configurations around the branch of interest. In simulations, this test was shown to be accurate, powerful, and robust to moderate model misspecifications. As with any model-based method, more serious model violations may cause the aLRT to become overconfident. This can be generally achieved by using a more conservative approach, which introduces a nonparametric element within an algorithm for evaluating branch supports.

The fast nonparametric version of the aLRT (Shimodaira–Hasegawa [SH]-aLRT), which was developed and implemented in the PHYML phylogenetic inference software (Guindon et al., 2010), helps to reduce the excess of false positives. The method is based on a procedure similar to the SH tree selection (Shimodaira and Hasegawa, 1999) and offers not only speed advantages due to the re-estimation of log likelihoods (RELL), but also excellent levels of accuracy and power (Ansimova et al., 2011).

1.7.2.6. Assessment of temporal structure in a dataset

Gene sequences sampled at different points in time can be used to infer a molecular phylogeny on a natural timescale of months or years. Two sampling times are 'evolutionarily distinct' if genetic sequences sampled at those times differ by a measurable amount of nucleotide or amino acid substitutions within the sampled population (Drummond et al., 2003). In order to infer such a phylogeny it is important to confirm that a 'molecular clock' model, which statistically describes the relationships between observed genetic distances and time, can be applied to the dataset. Therefore, before undertaking computationally more intensive phylogenetic analyses, it is always advisable to explore if sufficient genetic change between sampling times is in the dataset.

By using a simple regression of root-to-tip genetic distance against sampling time, Path-O-Gen program, recently renamed as TempEst, investigates 'clocklikeness' of molecular phylogenies (Rambaut et al., 2016). Specifically, if little 'temporal signal' is in the dataset, this is therefore unsuitable to reconstruct a reliable statistical relationship between genetic divergence and time. This tool can be used to (1) assess whether there is sufficient temporal signal in the data to proceed with phylogenetic molecular clock analysis (implemented in BEAST), and (2) identify sequences whose genetic divergence and sampling date are incongruent (Rambaut et al., 2016).

1.8 OBJECTIVES OF THE STUDY

Advances in molecular biology and bioinformatics analyses have impacted virus classification at all taxonomic levels. The International Committee on Taxonomy of Viruses (ICTV) has no guidelines for the classification of viruses below the species level. However, classification systems have been developed and widely used for a variety of avian pathogens. Due to the virus variability and lack of standardization of tests and nomenclatures, no consensus on IBV classification has so far been reached.

The aim of my research is to construct a simple and robust phylogeny-based classification system combined with an unambiguous and rationale nomenclature of the several genetic groups. This would be of crucial relevance to allow efficient communication on the evolution and emergence of epidemiologically important IBV variants.

To this end I applied recently developed bioinformatics tools (i) to outline the phylogenetic history of this virus and from there derive a rationale lineage nomenclature, (ii) explore whether there is sufficient temporal signal in the IBV data and (iii) evaluate the ability of S1 fragments of different sizes to recapitulate the phylogeny and classification based on full-length S1 sequences.

2. S1 GENE-BASED PHYLOGENY OF INFECTIOUS BRONCHITIS VIRUS: AN ATTEMPT TO HARMONIZE VIRUS CLASSIFICATION

S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification.

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2.1. INTRODUCTION

Infectious bronchitis virus (IBV) is the etiological agent of an acute and highly contagious disease that affects chickens of all ages and poses a major economic burden on the poultry industry. The virus exists in a wide range of antigenically and genetically distinct viral types, making the prevention and the control of this important pathogen both complex and challenging. Although the natural host of IBV is the chicken, the presence of IBV-like and other avian coronaviruses in both domestic and wild animals, including domestic fowl, partridge, geese, pigeon, guinea fowl, teal, duck and peafowl has been reported (Cavanagh, 2007, 2005).

IBV is a single-stranded, positive-sense RNA virus of the family Coronaviridae, genus Gammacoronavirus (Cavanagh and Naqi, 2003; International Committee on Taxonomy of viruses, http://www.ictvonline. org/virustaxonomy.asp). The viral genome comprises two untranslated regions (UTRs) at the 5′ and 3′ ends (Boursnell et al., 1987; Ziebuhr et al., 2000), two overlapping open reading frames (ORFs) encoding the polyproteins 1a and 1ab, and regions encoding the main structural proteins — spike (S), envelope (E), membrane (M) and nucleocapsid (N) (Spaan et al., 1988; Sutou et al., 1988). In addition, two accessory genes, ORF3 and ORF5, expressing proteins 3a and 3b and 5a and 5b, respectively, have been described (Casais et al., 2005; Hodgson et al., 2006; Lai and Cavanagh, 1997). The S protein (~3462 nt), located in the surface of the viral membrane, is the major inducer of neutralizing antibodies (Cavanagh and Naqi, 1997; Winter et al., 2008b) and is responsible for virus binding and entry to host cells (Cavanagh et al., 1986a; Koch et al., 1990; Niesters et al., 1987). It is post-translationally cleaved into the amino-terminal S1 (~535 amino acids) and the carboxyl-terminal S2 (~627 amino acids) subunits at a multi-basic cleavage site (Cavanagh et al., 1986b).

The observation that IB serotypes may differ by 20% to 25% at the genomic scale, and up to 50% of amino acids in the S1 protein (Cavanagh et al., 2005), has warranted considerable attention (Cavanagh and Gelb, 2008). Such variability may lead to important biological differences between strains and novel serotypic variants can emerge as the result of a limited

number of amino acid changes in the spike protein. Nucleotide heterogeneity is most prevalent in the S1 portion of the S gene and largely contained within three different hypervariable regions (HVRs) (aa 38–67, 91–141 and 274–387) (Cavanagh et al., 1988; Moore et al., 1997). Accordingly, the analysis of the complete or partial S1 gene nucleotide sequence has been conventionally used to determine viral genetic types. Currently, more than 50 different antigenic and genetic types of IBV have been recognized, some with substantial economic impact on the livestock industry, and some others restricted to specific geographical areas (de Wit et al., 2011a; Jackwood, 2012).

Effective surveillance is primarily based on the identification of the virus type causing disease (Jackwood and de Wit, 2013). A variety of methods have been developed to differentiate IBV strains. Systems that examine the antigenic or genetic features of an isolate result in the description of serotypes and genotypes, respectively, whereas methods that are focused on the immune response of chickens against challenge with an IBV strain lead to the designation of protectotypes (Lohr, 1988). Importantly, however, the genotype-, serotype- or protectotype-based approaches do not always group IBVs in the same way. In the absence of fast and appropriate biological assays for IBV classification, analyses of S1 sequence data are the most widely used means to assign IBV strains to groups, arbitrarily and confusingly defined as genetic types, genotypes, clades or clusters.

Both phylogenetic analysis and measures of pairwise similarity between nucleotide and amino acid sequences have been used for this purpose. However, there is no agreement on the exact method by which sequences should be compared nor the criteria used to distinguish viral genetic types. This is in part due to the rapid appearance of novel variants and a lack of consistency and uniformity in the nomenclature of the IBV genetic groups. For example, several genotyping studies have been performed on IBV within a specific geographic area without considering a more global context (see below). As a consequence, different clade designations, such ‘Korean New Cluster II’ (Mase et al., 2010), ‘JP-IV’ (Lim et al., 2012) and ‘Chinese New Type’ (Li et al., 2013), have been assigned to describe closely related viruses. Further confusion arises because different regions of the S1 subunit have been used to infer phylogenetic trees, and which region is most informative is debated (Kingham et al., 2000; Lee et al., 2003; Li et al., 2012; Mo et al., 2013; Schikora et al., 2003; Wang and Huang, 2000). Although it is generally true that longer sequences are more informative, several laboratories use a part of S1 that can include one or more HVRs.

The study described here was performed with the aim of constructing a comprehensive, reliable and robust phylogenetic inference on a global scale as the basis for classifying IB viruses for epidemiological purposes. Due to its variability and biological function, the S1 gene is the region commonly sequenced as an ideal target in molecular assays to type IBV strains. Accordingly, we focused on the complete S1 gene. Using all publicly available S1 gene sequence data, our goal was to determine the genetic structure of IBV and to propose a rational and standardized nomenclature of the IBV genetic groups identified here, referred to as lineages. In addition, we evaluated the ability of S1 fragments of different sizes to recapitulate the phylogeny and classification obtained from full-length S1 sequences.

2.2. MATERIALS AND METHODS

All available nucleotide sequences corresponding to the complete coding sequence of the S1 gene (~1620 bp) of IBV (n = 1652) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). Details on these sequences, including their genotype and serotype, were extracted from the GenBank annotations. Sequences shorter than 1440 bp and those of low quality, for example resulting in a nonsense and/or truncated S1 protein, or identical in both sequence and strain name were removed, resulting in a final data set of 1518 sequences. An alignment of the complete S1 gene was performed with a slow and iterative refinement method (FFT-NS-i) implemented in Mafft v.7.0 (http://mafft.cbrc.jp/alignment/software/; Katoh and Standley, 2013) and a maximum likelihood (ML) phylogenetic tree was estimated (see below).

The initial ML tree revealed that some previously recognized IBV groups did not form monophyletic groups (Annex A Fig. A1; see Results) indicative of inter-lineage recombination events that are relatively frequent in IBV (Cavanagh et al., 1992b; Kottier et al., 1995; Lee and Jackwood, 2000). To confirm the occurrence of recombination smaller sequence data sets comprising the suspected recombinant and the putative parental strains were analyzed using the RDP, Geneconv, Maxchi, BootScan, 3Seq and Chimaera methods available in the RDP package v.4 (Martin et al., 2010), applying default settings. The Simplot programv.3.5was also used to define the locations of recombination break-points (Lole et al., 1999). We considered "true recombinants" to be those sequences identified by at least two methods (P b1×10−10) and confirmed by significant phylogenetic incongruence among trees estimated on either side of the putative recombination break-points. All sequences with a history of recombination

determined in this manner were removed from the original 1518 sequence data set used to identify 'pure' IBV lineages, but described as recombinant IBV forms (see Results). In addition, a number of sequences were considered to be unreliable due to a lack of congruence between the strain description in the associated publication and the corresponding nucleotide sequences. This quality control step resulted in a final data set of 1286 full-length S1 sequences, which was used to determine the phylogenetic relationships among IBV strains and to classify them into well established lineages.

Evolutionary distances between lineages and genotypes were inferred using the complete S1 data set, with pairwise (p-distance) comparisons of nucleotide and amino acid sequences performed using the MEGA6 program (Tamura et al., 2013).

To facilitate tree visualization we performed an additional phylogenetic analysis using a smaller subset of full-length S1 sequences (n = 199). This subset comprised, where available, 6 representative sequences of each IBV lineage identified in the final 'cleansed' data set described above. In addition, 26 strains recognized as unique variants because they did not group with any of the identified lineages were included. Detailed information on the selected isolates along with their corresponding nucleotide sequences are provided as Supplementary materials (Table S1, available from http://dx.doi.org/10.1016/j.meegid.2016.02.015). The same data set was also used to assess whether the lineages established using phylogenetic analysis of the complete data set were maintained when only a portion of the S1 gene was analyzed. To that end, two different phylogenetic trees were inferred using the two most common sequenced regions corresponding to the coding sequences of HVRs1 and 2, located between nucleotide positions 112 and 423, and HVR3 between positions 820 and 1161 of the S1 gene, respectively (according to the sequence M21883).

Finally, two additional data sets were created to determine whether there was sufficient temporal structure in the data to undertake a molecular clock dating analysis. The first data set consisted of 372 sequences sampled between 1956 and 2013 and randomly selected from the complete data collection, while the second data set represented a single large lineage (here named as lineage GI-19 but originally designated as QX) of relatively close related viruses sampled between 1993 and 2010. Specifically, all the GI-19 S1 gene full-length sequences collected before the administration of the homologous vaccine in the field (n = 354) were selected. To assess the extent of temporal structure in these data, a regression of root-to-tip genetic distances against date of sampling was performed using the Path-O-Gen

program v.1.4 (http://tree.bio.ed.ac.uk/software/pathogen/) based on an input ML phylogenetic tree (see below).

In all cases phylogenetic trees were inferred using the ML method available in PhyML (Guindon et al., 2010) and implemented in Geneious v.7.1.8 (Kearse et al., 2012), employing a combination of NNI and SPR branch swapping. Prior to phylogenetic analysis, all hypervariable and potentially poorly aligned regions were removed using Gblocks (Castresana, 2000). For this analysis, a less stringent procedure, allowing for gap positions within final blocks, was employed. In addition, the best fit model of nucleotide substitution was inferred using JModeltest v.2.1.4 (Darriba et al., 2012). Accordingly, the General Time Reversible (GTR) model with a discrete gamma distribution (Γ) and allowing for invariant sites (I) was selected in all data analyses based on AICc. Nodal supports in the PhyML analyses were assessed using Shimodaira–Hasegawa (SH)-like branch supports (Anisimova and Gascuel, 2006; Guindon et al., 2010). To further assess the robustness of the phylogenetic tree, additional analyses of the small S1 gene sequence data set were performed using the Bayesian approach within MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001), and the Neighbor-Joining method available in MEGA6 (Tamura et al., 2013). In both these cases we employed the GTR + I + Γ substitution model, with nodal support values obtained by posterior probabilities and 1000 bootstrap replicates, respectively. Topological congruence between trees was compared through visual inspection for (i) ML trees obtained for the complete (n = 1286) and the small data sets (n = 199), (ii) the ML trees estimated for the full-length S1 sequences (~1620 nt) and those corresponding to the HVRs1 and 2 (312 nt) and HVR3 (342 nt) regions, and (iii) the ML, NJ and Bayesian trees all run on the small data set.

2.3. RESULTS

2.3.1 Overall IBV data set

To assess the phylogenetic relationships among the IBV variants and develop a harmonized system to define and name viral lineages, we analyzed all full-length S1 gene IBV sequences available on GenBank. These data comprised 1652 nucleotide sequences obtained from field samples and IBV vaccine strains collected worldwide between 1937 and 2013. After quality control, we inferred a ML phylogenetic tree on a total of 1518 sequences (Fig. A1) with the aim of obtaining a picture of the global genetic variability of this pathogen.

2.3.2. Recombination analysis

The topology of the preliminary ML tree showed evidence for recombination among IBV lineages. In particular, although defined previously, the so-called QX, 793B and Italy02 genetic groups, here referred to as the GI-19, -13 and -21 lineages (see below), no longer appeared as monophyletic groups (Fig. A1). We therefore performed additional analyses to determine whether recombination has occurred within the S1 gene and how this may have impacted the tree topology. This revealed a total of 213 recombinant viruses, which were removed from the data set to enable a more robust phylogenetic inference and identification of major viral lineages. For the purposes of classification, we propose that such recombinant viruses are simply referred to as combinations of the 32 IBV lineages defined below.

Recombination has clearly been of importance in shaping the evolution of some IBV variants. In particular, 143 viruses sampled in China (n= 107) and Korea (n= 36) since the 1990s were found to descend from parental strains belonging to the QX and HN08 (here referred to as lineage 22) genetic groups. This recombination involves, among others, viruses originally described as clustering into Chinese genotype III (Liu et al., 2006b) also known as the ck/CH/LSC/95I-type or tl/CH/ LDT3/03I-type (Han et al., 2011; Mo et al., 2013; Sun et al., 2011), and those previously assigned to the ck/CH/LHLJ/95I-type and BJ-type cluster (Han et al., 2011). In addition, the Korean nephropathogenic strains already known to be recombinants and originally designated as New Cluster I (Lim et al., 2012, 2011), also fell into the group derived from the recombination between QX and HN08. Multiple recombinant break-points were detected within this group, with most located between nucleotides 550 and 652 and 934 and 1125 (according to the sequence AY561711).

In 44 viruses we found evidence of inter-lineage recombination between the 793B and the QX- or HN08 clades, thereby supporting previous observations (Mo et al., 2013). Notably, all sequences possessed break-points located between nucleotide positions 665 and 709 (according to the sequence AY561711). These strains were collected in China from 2004 to 2012 and some were originally grouped by phylogenetic analysis with the 793B or QX genetic groups (Ji et al., 2011). With the exception of few viruses, the remaining recombinant sequences do not share any common break-points or parental strains and were a mosaic of diverse parental lineages. However, taken together, these results reveal that the majority of recombination break-points are located in the intermediate region between theHVRs1 and 2 and the HVR3.

2.3.3. Classification of IB viruses

Our phylogenetic analysis of 1286 IB strains (Fig. 2.1) was used to derive a new and coherent classification scheme for IBV based on the S1 gene. Not only this is the most variable region within the IBV genome, containing abundant phylogenetic information, but it is also the major immunogenic component and the most commonly sequenced region of the IBV genome. Accordingly, 32 IBV lineages, each of which was defined by strongly supported nodes (N0.98 SH-like test support values), were identified using our expansive S1 gene phylogeny. The designation of "lineage" was arbitrarily assigned to monophyletic groups of at least three viruses sampled from at least two different outbreaks. Strains that do not cluster into any lineages according to these subjective criteria are labeled as unique variant (UV) in the phylogenetic tree (n = 26). The lineages further fall into 6 well-supported (i.e. SH-like test support values of 1.0) and more genetically divergent groups, herein termed "genotypes"; 27 lineages cluster into genotype I (GI), which includes the majority of the IBV strains, whereas the remaining 5 genotypes contain one lineage each.

The IBV lineages defined in this manner exhibit uncorrected pairwise distances of 13% and 14% for nucleotide and amino acid sequences, respectively. Similarly, viral genotypes differed at 30% of nucleotides and 31% of amino acids. Importantly, however, because natural virus evolution is unlikely to always produce discrete boundaries, these distance values should only be considered as "rules of thumb" rather than universally valid parameters. Thus, IBV classification should not be undertaken on pairwise distance comparisons alone, but requires input from phylogenetic data.

To avoid confusion, IBV lineages were labeled using the abbreviation of the genotype in which they fall, followed by a consecutive number assigned according to the temporal order of the collection date of the first virus detected per lineage, here referred to as prototype strain. More details on the prototype viruses are provided in Table 2.1. The same temporal scheme was used to assign consecutive roman numbers to the different genotypes. For those viruses collected in the same year and belonging to different lineages within GI we have followed the temporal order of their GenBank sequence submissions. Accordingly, they are labeled GI-1 to GI-27; the oldest IBV in the current study falls into lineage 1, whereas lineage 27 represents the most recently identified cluster within GI. Moreover, to simplify the possible future designation of additional genetic variants, we assigned the number '1' to the all lineages out from genotype I, even if a second IBV lineage is not yet detected in any of these five genotypes.

Accordingly, they are labeled GII-1, GIII-1 GIV-1, GV-1 and GVI-1. Of note is that the lineage GI-24 consists of Indian IB viruses that so far have not been included in a scientific publication or for which a phylogenetic analysis has not been still performed.

The sequence details, where available, were added to the strain name in the format: GenBank accession number, strain name (as reported in the public database), country of origin and collection date.

Fig. 2.1. Phylogenetic tree of complete S1 nucleotide sequences (1456 nt) of 1286 IBVs. The phylogeny shows the evolutionary relationships among all IBV genotypes and lineages proposed here. Each lineage is color-coded and its corresponding designation is reported. Unique variants (UVs) are marked in black. The red box designates the 27 lineages within GI. SH-like branch supports are shown for key nodes. The scale bar represents the number of nucleotide substitutions per site, and the tree is mid-point rooted for clarity only. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

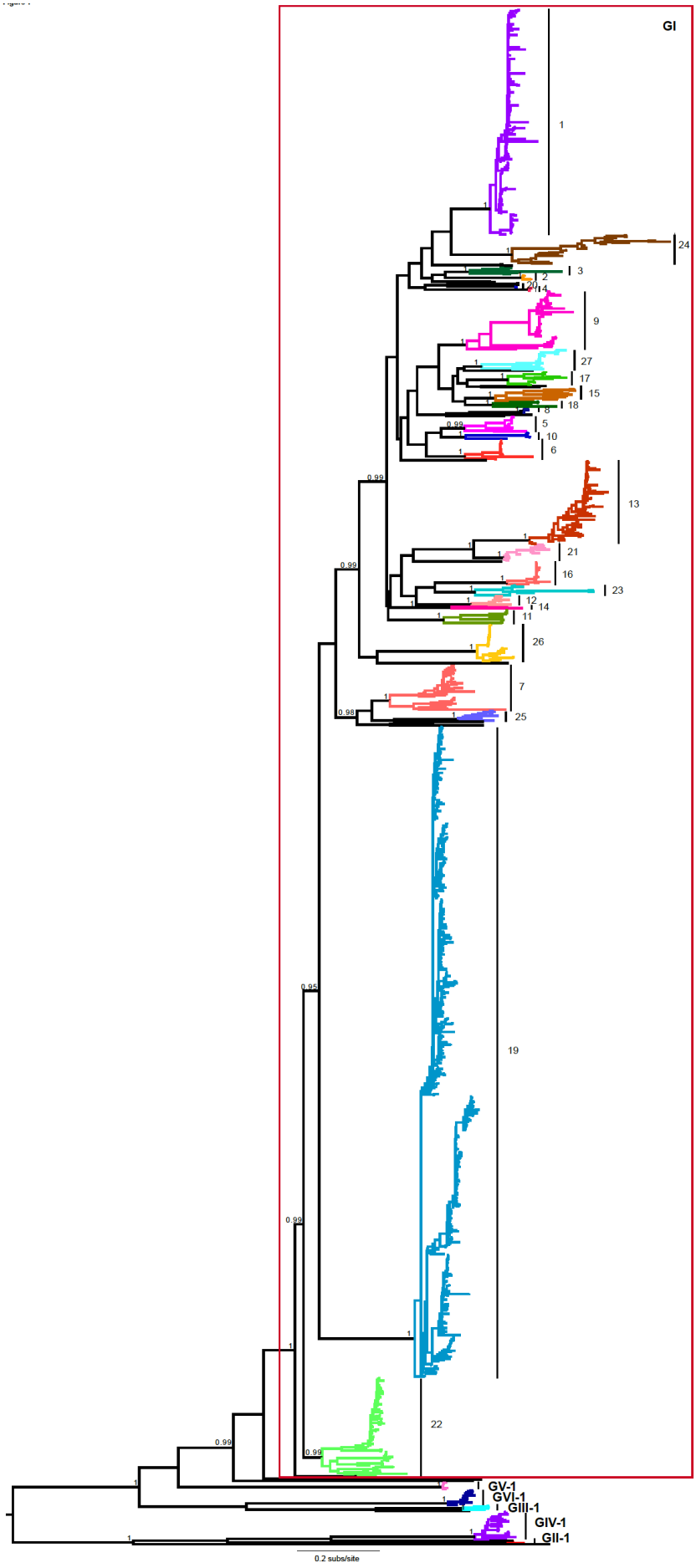
GI
1
24
3
2
20
14
9
27
17
15
18
8
5
10
6
13
21
16
23
12
14
11
26
7
25
19
22
GV-1
GVI-1
GIII-1
GIV-1
GII-1
0.2 subs/site

Table 2.1. Prototype strains and period of circulation of each lineage (data based on the complete S1 nucleotide sequences of the viruses included in the analysis).

Lineage	Period of circulation	Prototype strain			
		Strain name	Country of origin	Collection date	GenBank acc. number
GI-1	1937-2013	Beaudette	USA	1937	M95169
GI-2	1954-2006	Holte	USA	1954	GU393336
GI-3	1960-2006	Gray	USA	1960	L14069
GI-4	1962-1998	Holte	USA	1962	L18988
GI-5	1962-2012	N1/62	Australia	1962	U29522
GI-6	1962-2010	VicS	Australia	1962	U29519
GI-7	1964-2012	TP/64	Taiwan	1964	AY606320
GI-8	1965-1967	L165	USA	1965	JQ964061
GI-9	1973-2011	ARK99	USA	1973	M99482
GI-10	1970s-2000s	B	New Zealand	1970s	AF151954
GI-11	1975-2009	UFMG/G	Brazil	1975	JX182775
GI-12	1978-2006	D3896	The Netherlands	1978	X52084
GI-13	1983-2013	Moroccan-G/83	Morocco	1983	EU914938
GI-14	1984-2006	B1648	Belgium	1984	X87238
GI-15	1986-2008	B4	Korea	1986	FJ807932
GI-16	1986-2011	IZO 28/86	Italy	1986	KJ941019
GI-17	1988-1999	CA/Machado/88	USA	1988	AF419315
GI-18	1993-1999	JP8127	Japan	1993	AY296744
GI-19	1993-2012	58HeN-93II	China	1993	KC577395
GI-20	1996-1999	Qu_mv	Canada	1996	AF349621
GI-21	1997-2005	Spain/97/314	Spain	1997	DQ064806
GI-22	1997-2011	40GDGZ-97I	China	1997	KC577382
GI-23	1998-2012	Variant 2	Israel	1998	AF093796
GI-24	1998-2013	V13	India	1998	KF757447
GI-25	2004-2013	CA/1737/04	USA	2004	EU925393
GI-26	2006-2007	NGA/B401/2006	Nigeria	2006	FN182243
GI-27	2008-2013	GA08	USA	2008	GU301925
GII-1	1979-1984	D1466	The Netherlands	1979	M21971
GIII-1	1988-2008	N1/88	Australia	1988	U29450
GIV-1	1992-2003	DE/072/92	USA	1992	U77298
GV-1	2002-2008	N4/02	Australia	2002	DQ059618
GVI-1	2007-2012	TC07-2	China	2007	GQ265948

2.3.4. Phylogenetic analysis of the small data set

To further assess the reliability of our classification scheme and to better display the IBV phylogeny, we performed an additional ML phylogenetic analysis on a smaller, sub-sampled, data set (n=199), representative of IBV variability in the field (Fig. 2.2). These two trees had very consistent topologies; all lineage-defining branches are distinct from each other and strongly supported (N0.97 SH-like support values). To confirm these findings, we analyzed the smaller data set using different phylogenetic methods. Importantly, equivalent branching patterns were obtained using both NJ and Bayesian methods (Annex A Figs. A2 and A3). Accordingly, we suggest that this smaller data set is used as a reference tool for future epidemiological and evolutionary studies of IBV. The nucleotide sequences of the reference data set are provided as Supplementary materials (Table S1, available from http://dx.doi.org/10.1016/j.meegid.2016.02.015).

Fig. 2.2. Phylogenetic tree of complete S1 nucleotide sequences. The phylogeny contains a total of 199 IBV strains, including 6 representative sequences of each lineage detected and 26 strains recognized as unique variants. Each lineage is color-coded and its corresponding designation is reported. Bars reporting the genotypes in which the lineages fall are shown. GenBank accession number, isolate number or name, country of origin and collection date is given for each strain. The designation "UV" indicates unique variants, here marked in black. A complete list of the 199 sequences used is provided in Table S1. SH-like branch supports are shown for key nodes. The scale bar represents the number of nucleotide substitutions per site, and the tree is mid-point rooted for clarity only.


GI
GII
GIII
GIV
GV
GVI
0.3 subs/site

2.3.5. Description of individual lineages

We used a geography-based system (see below) to describe the 32 IBV lineages reported here. Because of their wide geographic distribution, some lineages are clearly of importance. Among these, lineages GI-1 and -13 (previously named as the Mass and 793B types, respectively) are commonly found, partly reflecting the use of vaccines derived from them in the countries where they have been reported. In contrast, other lineages are confined only to specific countries, many of which are limited to Asia and North America. Africa and South America possess unique lineages as well as some of the European-origin types. Notably, geographically distinct wild-type lineages were identified in Australia and New Zealand, likely reflecting their spatial isolation.

Widely distributed lineages

The GI-1 lineage comprises the first IBV serotype identified and even today is one of the best known and most widely distributed genetic groups, likely due to the extensive use of a homologous vaccine derived from one of its strains. In our data set this group contains 189 viruses collected worldwide (with the exception of Oceania), which were previously assigned to the Massachusetts (also known as Mass or M41), the H120 and the Connecticut (Jungherr et al., 1956) types. The Mass serotype, of which theM41 is the representative strain, is mainly associated with respiratory disease (Cavanagh and Naqi, 1997). The GI-13 lineage is present in many parts of the world and in our study comprises 70 viruses, both vaccine and virulent field strains, previously assigned to the 793B type (also known as 4/91 and CR88) (Gough et al., 1992; Parsons et al., 1992; Picault et al., 1995). Notably, the so-called Israeli variant 1 viruses are members of this lineage (Callison et al., 2001; Gelb et al., 2005). The first known strain of CR88 serotype was isolated in France in 1985 (Picault et al., 1995), whereas the 793B strain emerged in the United Kingdom in 1991 and was originally described as a unique serotype responsible for severe respiratory syndromes (Callison et al., 2001; Cook et al., 1996). A retrospective study revealed a 96% sequence similarity between a strain isolated in Morocco in 1983, which is here referred to as the GI-13 lineage prototype strain, and the 793B variant, suggesting that this North African virus is the progenitor of the lineage (Jones et al., 2004). Recently, this genetic type was identified for the first time in Canada in outbreaks with predominantly respiratory disease and/or egg production problems (Martin et al., 2014). The largest number of IBV strains included in the present investigation comes from the GI-19

lineage that contains 546 viruses collected between 1993 and 2012. The GI-19 variant, the so-called QXIBV strain, was first detected in China in 1996 where it was associated predominantly with severe nephritis, 'false layer' syndrome and potentially proventriculitis (Wang et al., 1998). Since then, several QX-type strains have been identified in China, although most cases have been associated with renal pathology (Liu et al., 2006b). In Europe, numerous reports described QX-like strains following the Chinese index case (Abro et al., 2011; Beato et al., 2005; de Wit et al., 2011b; Gough et al., 2008; Monne et al., 2008; Valastro et al., 2010; Worthington et al., 2008). At the same time, the first QX-like strains were identified in Japan (Ariyoshi et al., 2010; Mase et al., 2004) and Korea (Lee et al., 2008). Thereafter, it was soon reported in such diverse localities as Russia, Africa and the Middle East (Amin et al., 2012; Bochkov et al., 2006; Toffan et al., 2011). Thus, all strains falling in the GI-19 lineage have been previously assigned to the QX clade, also called LX4 (Han et al., 2011; Li et al., 2013; Liu et al., 2009) and A2 (Ji et al., 2011; Li et al., 2010). Confusingly, the same genetic group has also been referred to as Korean-II (K-II) (Lee et al., 2008) and Japanese-III (JP-III) clusters (Ariyoshi et al., 2010; Mase et al., 2004). Of note, a recently submitted sequence (KC577395) shows that the lineage had arisen in China by 1993. The GI-16 lineage contains 19 viruses collected between 1986 and 2011 in China, Taiwan and Italy, previously classified as the Q1 (even known as T3 and J2) or ck/CH/LDL/97I type (Liu et al., 2006b; Yu et al., 2001). The designation of Korean III genotype (K-III) was also used to describe Korean strains clustering with Chinese viruses of LDL-like type. Notably, the classification into K-III was performed using phylogenetic analyses of partial S1 gene sequences (620–642 nt) (Lee et al., 2008), and to our knowledge no complete S1 nucleotide sequences of this genetic group are currently available. The GI-16 lineage has been associated with respiratory syndrome (Ababneh et al., 2012; Yu et al., 2001), severe drops in egg production (de Wit et al., 2012) and nephropathogenic disease (Huang et al., 2004; Toffan et al., 2013). Although it is known to have a more widespread geographic distribution, we only include sequences from three countries. After the first isolation of the Q1 strain in China between 1996 and 1998 (Yu et al., 2001), the lineage was reported in Taiwan in 2002 (Huang et al., 2004), in South America since 2009 (Marandino et al., 2015; Sesti et al., 2014), in some Middle Eastern countries (Ababneh et al., 2012) and in Italy (Toffan et al., 2013) in 2011, and in Colombia in 2012 (Jackwood, 2012). Of note, our phylogenetic analysis reveals that the GI-16 prototype strain is an Italian virus – IZO28/86 – isolated in 1986, approximately 10 years before the first identification of the Q1 strain in China.

Indigenous Asian lineages

In addition to those of European and American origin, we found 6 different lineages to be geographically strictly confined to Asia, of which one constitutes a different genotype (GVI-1). Thus, two distinct genotypes have been present and are probably still circulating in this continent. Most GI-7 lineage viruses were associated with nephropathogenic diseases in infected chickens (Huang et al., 2004). The lineage was detected in Taiwan and China and comprises a total of 43 isolates; the majority were isolated after 1988, with the exception of the TP/64 strain which was isolated in Taiwan in 1964 from layers showing respiratory problems and drop in egg production (Huang et al., 2004). Due to high nucleotide sequence similarity (90%), we propose the existence of a single group comprising strains previously assigned to two different genetic groups referred to as Taiwan-I (TW-I) and Taiwan-II (TW-II) (Liu et al., 2003;Wang and Tsai, 1996). GI-15 consists of 11 respiratory strains collected exclusively in Korea between 1986 and 2008 and previously placed into the genotype named as Korean I (K-I) (Hong et al., 2012; Lee et al., 2010, 2008; Song et al., 1998b). The GI-18 lineage comprises of 3 Japanese and 2 Chinese viruses collected between 1993 and 1999, and contains both respiratory and nephropathogenic strains (Mase et al., 2004; Shieh et al., 2004). The lineage was designated as Japan I (JP-I) (Ariyoshi et al., 2010; Mase et al., 2004; Shieh et al., 2004), as it originally contained only Japanese wild type field strains. However, it is clear that the lineage is no longer confined to Japan. The GI-22 lineage is the only Chinese indigenous genetic type identified here. Since its first detection, it has been of direct relevance to the poultry industry, reflecting its occurrence and widespread distribution in China, as well as its virulence. GI-22 includes 82 field viruses mainly of nephropathogenic nature collected from outbreaks in both broilers and layers flocks during 1997–2011. These local strains were initially assigned to the ck/CH/LSC/99I-type cluster following the inclusion of the Chinese IBV reference strain ck/CH/LSC/99I isolated in 1999 (Han et al., 2011; Liu et al., 2009, 2006b; Mo et al., 2013; Sun et al., 2011), although it is also known as HN08 (Ji et al., 2011; Li et al., 2013). Based on numerous epidemiological surveys conducted in China, the GI-22 lineage along with GI-19, appears to be the dominant viruses in the country (Han et al., 2011; Ji et al., 2011; Li et al., 2013, 2010; Liu et al., 2009, 2006b; Ma et al., 2012; Mo et al., 2013; Sun et al., 2011). The GI-24 lineage contains IB viruses indigenous to India and, to date, no publications describe these strains (i.e. they are only recently reported as accession numbers) such that little epidemiological and clinical information is available. The lineage comprises of 24 viruses collected during the period 1998–2013. Of these, 11 have been assigned to a genotype named

NPR by the submitting authors, while 12 others seem to be of nephropathogenic nature (according to the data reported in GenBank), with no data reported for one strain. The only published data on the circulation of local Indian variant was that of Bayry et al. (2005) who described the emergence in India of a unique nephropathogenic IBV classified as a novel genotype (isolate PDRC/Pune/Ind/ 1/99, AY091551). A BLAST search revealed that the PDRC/Pune/Ind/1/ 99 is 99% similar to the GI-24 prototype strain. However, there is currently insufficient data as to whether this strain can be included in GI-24. As noted above, GVI-1 represents a genetically distinct lineage present in Asia. It comprises 13 isolates collected in China and Korea between 2007 and 2012, which were originally grouped in the 'Korean New Cluster II' (Lim et al., 2012), also designated as Chinese New- Type (Li et al., 2013). The available data on the pathogenicity of these strains revealed them to be of respiratory nature (Li et al., 2010; Lim et al., 2012). Notably, viruses closely related to those included here were also sampled in Japan in 2009 and assigned to a group named JP-IV (Mase et al., 2010) by sequencing of the partial S1 gene (621 nt). To date, no JP-IV-like S1 complete sequences are available so we cannot determine whether the so-called "JP-IV strains" are included in GVI-1 or if they cluster into a separate lineage.

Indigenous North American lineages

A large number of lineages, falling into two well distinct genotypes (GI and GIV), have been reported as indigenous to North America (GI-8, -9, -17, -20, -25, -27 and GIV-1). However, only some of these – GI-9, GI-27 and GIV-1 – have been implicated in widespread disease disseminations and persistent virus infections (Jackwood, 2012). The GI-8 lineage includes one of the first IBV serotypes (SE-17) recognized to be different from the pre-existing IBV antigenic types. However, as this lineage was only detected for a brief period it is likely of limited importance. The variant was isolated in 1967 in Georgia from a chicken flock with acute respiratory distress and was designated as SE-17 (Hopkins, 1969). A retrospective study identified respiratory SE-17 IBVs to be present in USA since 1965 (Mondal et al., 2013). The GI-9 lineage contains vaccine and virulent field strains collected from 1973 to 2011, the majority from the USA (44/49). Herein, we report IBVs previously known to be of Arkansas (Ark) and Ark DPI-like type and strains referred to as California 99 type first detected in North Carolina in 1999 (Martin et al., 2001; Mondal and Cardona, 2004). These viruses are the causative agent of respiratory syndromes, observed in the field as well as under experimental conditions (Fields, 1973; Johnson et al., 1973; Martin et al., 2001; Mondal and Cardona, 2007).

A total of 12 viruses sampled in Pennsylvania, California and Alabama from 1988 to 1999 fall within the GI-17 lineage. This includes strains associated with respiratory distress and renal pathologies, with one also implicated in reproductive pathology (Gelb et al., 2005; Moore et al., 1998; Ziegler et al., 2002). These strains were previously designated as California variants (CAV) (Hein et al., 1989; Moore et al., 1998). Among these, two viruses isolated in the late 1990s in Pennsylvania – PA/Wolgemuth/98 and PA/171/99 – were classified as being two unique genotypes, genetically similar but antigenically distinct from the CA/Machado/88 reference prototype strain (Ziegler et al., 2002). Although we only identified two strains as members of the lineage GI-20, it has been included in our classification because of its epidemiological relevance in Canada. The lineage has been never described outside of Eastern Canada, yet appeared to be the most common lineage circulating in the country between 2000 and 2013 (Martin et al., 2014). The Qu_mv prototype variant (AF349621) was isolated in Quebéc in 1996 from commercial broiler flocks displaying respiratory signs of disease (Ojkic and Binnington, 2002; Smati et al., 2002). Since then, its prevalence in the region has risen, also spreading to Nova Scotia and Ontario. Finally, we group 26 American indigenous viruses collected between 2004 and 2013 and associated with respiratory infection into the GI-25 (n=9) and GI-27 (n=17) lineages, which were previously designated as GA07 and GA08, respectively (Jackwood et al., 2007; Kulkarni and Resurreccion, 2010). GI-24 includes, among others, the prototype CA/1737/04 strain (Jackwood et al., 2007) along with the DMV/5642/06 (Wood et al., 2009) and GA/60,173/07 (Jackwood et al., 2007) variants. The GI-27 lineage contains the most recent IBV defining a lineage, being first identified in 2007. The variant, which became the predominant virus type at that time, was reported to be a novel genotype and designated as GA08 (Jackwood et al., 2010b; Kulkarni and Resurreccion, 2010). Another cluster restricted to the USA is lineage 1 of GIV, which is also the only North American lineage belonging to a different genotype. This group contains both vaccine and field strains (n=24) isolated between 1992 and 2003. Among these is the variant referred to as Delaware variant (DE or DE072), isolated in 1992 from commercial broiler chicks during severe respiratory disease and designated to be of a novel genotype and serotype compared to the others (Gelb et al., 1997; Mondal et al., 2001). In the same lineage are IBV strains previously designated as GA98 and described to be closely related to the DE variant, although of a different serotype (Lee et al., 2001). It has been suggested that the GA98 variant arose from immune selection caused by DE072 attenuated live vaccine introduced in the country in

1993 (Lee and Jackwood, 2001). In addition, viruses recovered in 2000 from layer flocks experiencing reduction in egg production also fell into this lineage (Mondal et al., 2001).

North American and Asian lineages

The GI-2, GI-3 and GI-4 lineages were first described in the USA between the 1950s and the 1960s and later detected in Asia many years later. Notably, however, GI-2 and GI-4were reported in USA only during the 1950s–1960s and never again,whileGI-3was also reported in North America in the late 1990s (Gelb et al., 2005; Gelb et al., 2001) before being identified in Taiwan in 2006. Hence, old lineages may be sporadically re-detected. A total of 5 viruses cluster within the GI-3 lineage, which, among others, includes the serotypes known as Holte and Iowa 97 (Albassam et al., 1986; Hofstad, 1958) and two viruses sampled in China between 2004 and 2006 (Bing et al., 2007). The GI-4 lineage consists of 1 nephropathogenic strain, isolated in USA in 1962 (Winterfield and Hitchner, 1962), whose S1 gene was entirely sequenced in 1994 (accession number L18988; Wang et al., 1994) and two additional viruses collected in China for which no published data are currently available. Of note, the same IB strain nomenclature has been used to identify two viruses genetically distant between each other and belonging to two different lineages. Hence, both the GI-2 and GI-4 lineages include a virus called 'Holte' as prototype strain. The GI-3 lineage contains 7 viruses, comprising both respiratory and nephropathogenic strains. It was originally designated as JMK or the Gray serotype because of the appropriate reference strains (accession numbers L14070 and L14069, respectively). Although these two viruses are antigenically very similar (Cowen and Hitchner, 1975), their pathogenicity is different because the Gray variant can be nephropathogenic while the JMK virus is strictly respirotropic (Kwon and Jackwood, 1995; Thor et al., 2011; Winterfield et al., 1964;Winterfield and Hitchner, 1962).

Indigenous South American lineage

The GI-11 lineage is unique to South America and comprises a total of 13 Brazilian viruses collected between 1975 and 2009. However, novel IBV sequences, which were obtained from field samples from Argentina and Uruguay, have been recently submitted to GenBank (Marandino et al., 2015). By phylogenetic analysis of the complete S1 coding region, the authors included these strains in a genotype referred to as South America I (SAI), which also contains the GI-11 Brazilian viruses. A previous nomenclature based on partial S1 nucleotide

sequences of local Brazilian field variants has been also adopted (Balestrin et al., 2014; Chacón et al., 2011; Fraga et al., 2013; Villarreal et al., 2010), and referred to as the Brazil (Villarreal et al., 2010) or BRI (Chacón et al., 2011) genotypes. The partial Brazilian sequences show a high degree of nucleotide similarity with those of GI-11 (98– 92%), such that it is unclear whether they represent the same genetic type. The GI-11 lineage has been associated with a variety of clinical conditions, ranging from respiratory disease, infertility, drop in egg production and egg quality (Chacón et al., 2011, 2008; Montassier, 2010; Villarreal et al., 2007a) to enteric disorders (Villarreal et al., 2010; Villarreal et al., 2007b). It was recently demonstrated that the Brazilian variant causes predominantly respiratory and kidney diseases under experimental conditions (Chacón et al., 2014: de Wit et al., 2015). Interestingly, our phylogenetic analysis demonstrates that the indigenous GI-11 lineage has been circulating in the country since 1975, supporting the hypothesis of Montassier (2010) that this variant had already been present in the field since at least as early as 1988.

European IBV lineages

Two distinct lineages that fall in two different genotypes – GI-21 and GII-1 – were identified as unique to Europe. Notably, one of these has also been reported in Russia (Bochkov et al., 2006) and recently in Morocco (Fellahi et al., 2015).Within the GI-21 lineage we group 14 viruses sampled between 1997 and 2005 in Italy, the United Kingdom and Spain. The IB viral type of the lineage was originally isolated in Italy in 1999 and designated Italy02 (Bochkov et al., 2007). Thereafter, it was reported to be one of the most predominant genotypes in Spain (Dolz et al., 2009) and the third most frequent in Western Europe over 2002–2006 (Worthington et al., 2008). This variant has mainly been detected in broiler flocks that experienced respiratory signs, as well as adult birds, broiler breeders and layers, associated with drop in egg production (Worthington et al., 2004). It also appeared to induce renal disease in young chickens (Dolz et al., 2012). Although strains in this lineage are related to one of the major and widespread European wild types, a limited number of complete S1 nucleotide sequences are available for analysis. GII-1 lineage is the only group of European viruses that falls in a different genotype to all the other viruses which are classified here as GI. The lineage is comprised of only the Dutch isolates D1466 and V1397, showing a large evolutionary distance compared to the remaining IBV genotypes. The D1466 variant (also called D212) was detected for the first time in The Netherlands in the late 1970s, when it was recognized to have antigenic and molecular properties significantly different from known IBV

strains (Adzhar et al., 1995; Davelaar et al., 1984; Kusters et al., 1989, 1987). Historically, D1466 has never been responsible for major disease in flocks and hence may be of relatively low pathogenicity. However, an increase in virulence of this variant was recently observed. In particular, poor egg production in both layers and broiler breeders was reported between 2005 and 2006 in some countries of Western Europe (Worthington et al., 2008) and more recently in Poland (Domanska-Blicharz et al., 2012).

Indigenous African lineage

The GI-26 lineage represents a unique African cluster of viruses that were identified relatively recently. It contains 32 viruses isolated in Nigeria and Niger between 2006 and 2007, for which no obvious clinical signs were recorded. These local strains were previously grouped into a novel IBV genotype designated as IBADAN, referring to the name of the city (in Nigeria) where the variant was first detected, and were described to be genetically and antigenically clearly distinct from all other known IBV strains (Ducatez et al., 2009).

European and African lineages

Two IBV lineages – 12 and 14 – were found in some European countries as well as in Nigeria. Both fall into GI and were also reported in Russia (Bochkov et al., 2006). Strains previously classified as D207- like, D274-like or UK/6/82-like types fall into the GI-12 lineage. Here, we report 3 Dutch and 3 British strains isolated during 1978–1986 from broilers experiencing respiratory infection and from breeding flocks showing aberrant egg production (Cavanagh et al., 1992a; Cook and Huggins, 1986; Cook, 1984, 1983; Davelaar et al., 1984). In addition, 1 field strain from Russia and 2 from Nigeria (Ducatez et al., 2009), collected in 2002 and 2006, respectively, fall in this lineage. Although the circulation of this variant is well documented (Bochkov et al., 2006; Cavanagh et al., 1999, 1992a; Cook, 1984; Davelaar et al., 1984; Meulemans et al., 2001; Monne et al., 2009; Valastro et al., 2014; Worthington et al., 2008), only a relatively small number of D274-like sequences are available for analysis. A GI-12 like strain was also identified in Egypt in 1989 (Abdel-Moneim et al., 2006), although the status of this virus is ambiguous as only partial S1 sequence (722 nt) is currently available. The GI-14 lineage comprises only two viruses collected in Belgium (B1648) (Meulemans et al., 1987) and Nigeria (NGA/324/2006) (Ducatez et al., 2009), although it merits classification due to its epidemiological relevance and pathogenicity. After its first identification in Belgiumin 1984

(Meulemans et al., 1987), the variant was again reported in the country in 1993 (Meulemans et al., 2001) and later in Italy (Capua et al., 1999), Russia (Bochkov et al., 2006) and Slovenia (Krapez et al., 2011). No other complete S1 gene sequences are available. The viruses related to this variant were previously referred to as the B1848-like type and reported to be mostly nephropathogenic (Meulemans et al., 1987; Capua et al., 1999) and also associated with egg production problems (Capua et al., 1999). The variant was rarely detected in France and Germany between 2002 and 2006 (Worthington et al., 2008), and did not appear to be causing relevant illness in poultry flocks.

Indigenous Middle Eastern lineage

The GI-23 lineage represents the unique wild-type cluster geographically confined to the Middle East. Strains belonging to this lineage have been detected since 1998 in Israel and are still circulating in the area (Ganapathy et al., 2015; Najafi et al., 2015). Some have become dominant in the majority of farms and are involved in respiratory and renal pathologies (El-Mahdy et al., 2012; Meir et al., 2004). However, the complete S1 sequence is only available for a limited number of viruses (n = 9). Some authors have previously assigned these strains as Israeli Variant 2 to distinguish them from those clustering within Israeli Variant 1 (Abdel-Moneim et al., 2002; Callison et al., 2001; Mahmood et al., 2011; Meir et al., 2004). Alternatively, studies performed on the Egyptian isolates divided them into different genotypes on the basis of their HVR3 sequences; they were defined as Egyptian Variant 1, having as reference the strain Egypt/Beni-Suef/01 (Abdel- Moneimet al., 2002) and Egyptian Variant 2,which includes the viruses ck/Eg/BSU-2/2011 and ck/Eg/BSU-3/2011(Abdel-Moneim et al., 2012). To date, no complete nucleotide sequences are available for the three Egyptian strains.

Indigenous Australian and New Zealand lineages

Likely due to their geographical isolation, Australia and New Zealand possess only unique indigenous variants. We found 5 distinct IBV lineages in these localities, 3 falling into GI (GI-5, -6 and -10) and 2 possessing large evolutionary distances between each other and compared to those found elsewhere. Hence, our classification into distinct genotypes designated as GIII-1 and GV-1. The GI-5 and GI-6 lineages contain both vaccine and field strains (13 and 17 viruses, respectively), mostly sampled in Australia. The only Chinese sequences included here

are 4 field viruses (1 in GI-5 and 3 in GI-6) that presumably represent re-isolations of the vaccine strains JAAS and J9,which were from Australia and used in China to control IBV (Liu et al., 2006a). Hence, both these lineages may be geographically confined to Oceania. In addition, one strain sampled in New Zealand falls in GI-6. Among the strains clustering in GI-5 are the Armidale vaccine strain and the nephropathogenic N1/62, also known as T strain. Within the GI-6 lineage is the VicS/62 strain that was introduced as a vaccine into Australia in 1966 (Cumming, 1969). The strains within GI-5 and GI-6 were originally grouped as Australian subgroup I (Ignjatovic et al., 2006), which includes both respiratory and nephropathogenic strains (Sapats et al., 1996). The GI-10 lineage contains 6 New Zealand indigenous viruses; 3 were collected in the 1970s and the remainder in the 2000s (McFarlane and Verma, 2008). This IBV variant was first reported in the country in 1967 (Pohl, 1967), and ten years later 4 different strains designated as A, B, C and D were identified using virus neutralization tests (Lohr, 1977, 1976). Finally, both the lineages falling into GIII and GV contain respiratory and indigenous Australian pathogens (4 and 7 strains, respectively). The GIII-1 lineage was first identified in 1988 (Ignjatovic and McWaters, 1991) and designated as Australian subgroup II (Sapats et al., 1996), whereas the GV-1 lineage was described approximately 14 years later and referred to as Australian subgroup III (Ignjatovic et al., 2006). Both appear to be genetically and antigenically different from the classical strains, here grouped into GI-5 and GI-6 (Ignjatovic et al., 1997; Mardani et al., 2010; Sapats et al., 1996).

2.3.6. Phylogenetic analysis of the HVRs

Since partial S1 gene sequences are often used to classify the IBV strains, we inferred two additional ML phylogenetic trees based on HVRs1 and 2 (312 nt) and HVR3 (342 nt) of the reference subsampled data set (n=199). Strikingly, important topological inconsistencies were observed between the HVRs1 and 2 phylogeny and that inferred using the complete S1 gene. Specifically, although GIII, V and VI exhibit large evolutionary distances compared to the remaining lineages, they cluster within GI in marked contrast to what is seen in the complete S1 tree, while 4 lineages – GI-7, -14, -23, and -27 – do not form monophyletic groups (Fig. 2.3). In addition, while most groups were strongly supported in the SH test (N0.96 SH-like), others were more weakly supported, such as GI-15 which only received 0.60 support, and GI-6 and GI-9, both of which received 0.80 SH-like support. Genetic typing based on HVR3 was similarly inconsistent with that obtained from the whole S1 gene (Fig. 2.4). In particular, 8 lineages (GI-5, -7, -10, -18, -22, -24, -25 and -27) are no longer monophyletic. Overall, these

results indicate that both the genotypes and lineages identified using the HVRs are not representative of those obtained from the phylogenetic analysis of the whole S1 gene, so that only the latter should be used in IBV genetic classification.

Fig. 2.3. Phylogenetic tree of partial S1 nucleotide sequences including HVRs1 and 2. The phylogeny contains a total of 199 IBV strains, including 6 representative sequences of each lineage detected and 26 strains recognized as unique variants. All strains belonging to the same lineage, assessed on the basis of the complete full-length sequences, are labelled with a unique color code as in Figs. 1 and 2. The color-coded boxes reporting the lineage designations are only shown for those lineages correctly identified. GenBank accession number, isolate number or name, country of origin and collection date is given for each strain. The designation "UV" indicates unique variants, here marked in black. SH-like branch supports are shown for key nodes. The scale bar represents the number of nucleotide substitutions per site, and the tree is mid-point rooted for clarity only. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

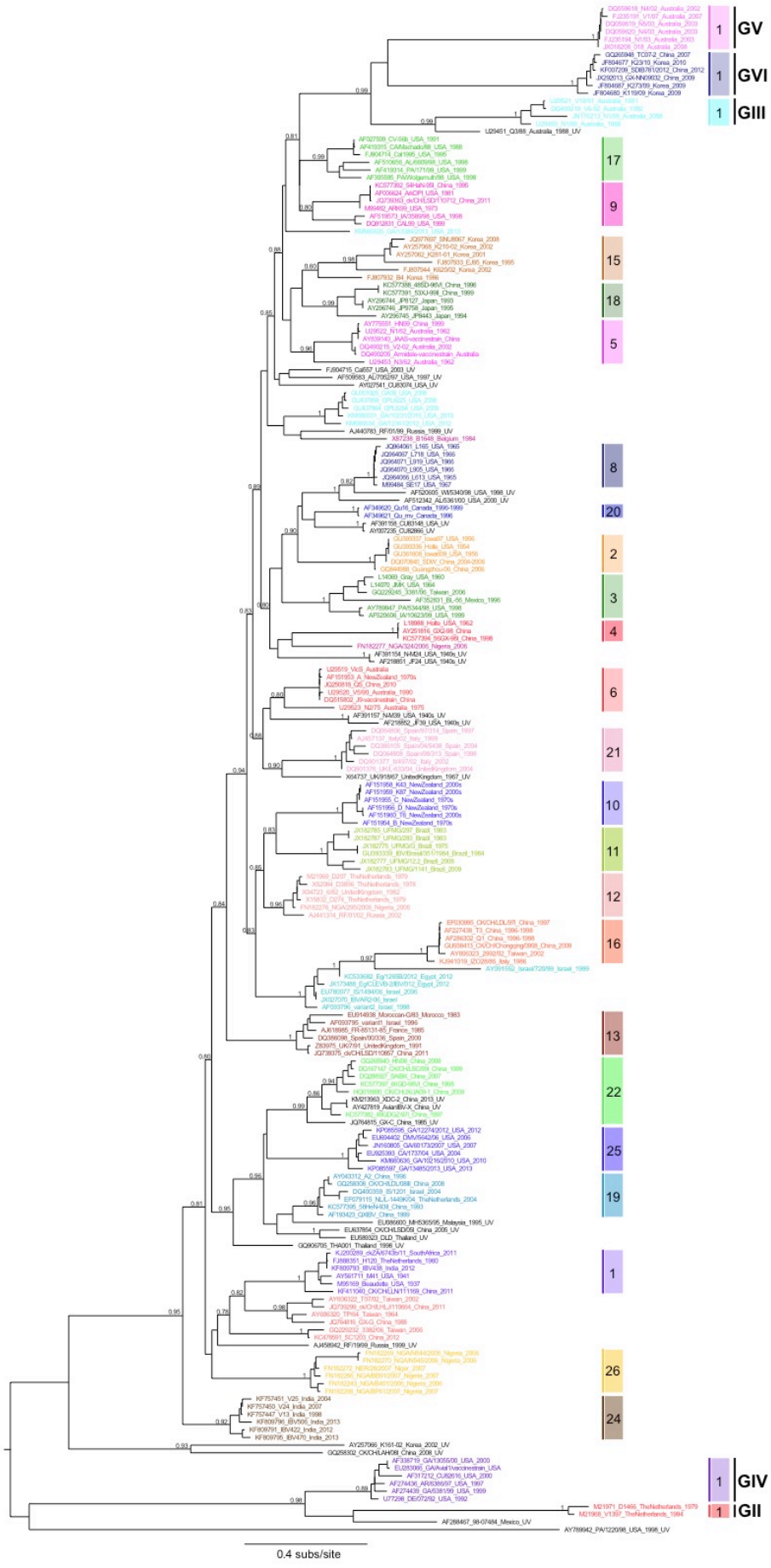
GV
GVI
GIII
GIV
GII
0.4 subs/site

Fig. 2.4. Phylogenetic tree of partial S1 nucleotide sequences including HVR3. The phylogeny contains a total of 199 IBV strains, including 6 representative sequences of each lineage detected and 26 strains recognized as unique variants. All strains belonging to the same lineage, assessed on the basis of the complete full-length sequences, are labeled with a unique color code as in Figs. 1 and 2. The color-coded boxes reporting the lineage designations are shown only for those lineages correctly identified. GenBank accession number, isolate number or name, country of origin and collection date is given for each strain. The designation "UV" indicates unique variants, here marked in black. SH-like branch supports are shown for key nodes. The scale bar represents the number of nucleotide substitutions per site, and the tree is mid-point rooted for clarity only. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)


17
15
6
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23
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16
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14
26
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11
12
1 GVI
1 GIII
1 GIV
1 GII
0.3 subs/site

2.3.7. Assessment of temporal structure

Finally, to determine whether there was sufficient temporal structure for molecular clock dating, we fitted a linear regression of root-to-tip genetic distance from the ML tree against the date (year) of collection for 372 randomly selected sequences from the entire data set. This revealed a weakly negative relationship between genetic distance and time (R-squared=−0.003; correlation coefficient=−0.181 0.181 under the best-fitting root). Such a clear lack of temporal structure means that molecular clock dating schemes based on 'tip dating' alone cannot proceed. An equivalent root-to-tip regression using the GI-19 lineage alone, which includes samples collected from 1993 to 2010 (n = 354) was conducted to determine whether this was also true of more closely related sequences. Similarly, the analysis revealed only weak temporal structure (R-squared = 0.159; correlation coefficient= 0.399).

2.4. DISCUSSION

Advances in molecular biology and bioinformatics analyses have impacted virus classification at all taxonomic levels. The International Committee on Taxonomy of Viruses (ICTV) has no guidelines for the classification of viruses below the species level. However, classification systems have been developed and widely used for a variety of avian pathogens, including Avian influenza (AI) (WHO/OIE/FAO H5N1 Evolution working group) and Newcastle disease (ND) viruses (Aldous et al., 2003; de Almeida et al., 2013), within which distinct "lineages" have been established through phylogenetic analysis and sequence similarities. Herein, we propose a similar framework for IBV. To date, no genetic characterization of IBV has included sequences from all the existing viral variants or adopted a unified system for naming the groups, such that no consensus on IBV classification has been reached. Indeed, the diversity of IBV genetic clustering and naming available at present is highly confusing. Hence, we have attempted to construct a comprehensive phylogenetic history of this virus and from this to derive a rational and harmonious scheme for the classification of IBV that we suggest should be used for future epidemiological studies.

We have focused on the complete nucleotide sequence of the S1 gene as the basis for IBV lineage assignment. Not only it is the most variable region within the IBV genome, containing abundant phylogenetic information, but it encodes the major immunological determinants (Jackwood and de Wit, 2013) and it is used by many laboratories studying IBV. Hence,

phylogenetic analysis of the S1 gene and an S1-based viral classification might provide data of direct epidemiological relevance for controlling IBV spread, particularly as field and vaccine strains share a high degree of S1 sequence identity (Gelb et al., 2005).

Importantly, our classification was exclusively based on the topology of the phylogenetic tree, with strong statistical (SH-like) support values at each node defining monophyletic groups. Hence, IBV strain clustering was evaluated by a robust (maximum likelihood) phylogenetic method that is able to efficiently handle a large number of sequences, and combined with an efficient statistic – the SH-like test – that can rapidly estimate the support for individual groupings on the tree. That very similar tree topologies were estimated using different phylogenetic techniques not only suggests that they are robust, but that faster phylogenetic methods can be used if necessary.

A more challenging issue is recombination, which undoubtedly has major implications for virus classification (Simmonds, 2015). Importantly, however, viral phylogenies based on a single gene (as here) have been previously used to establish viable classification schemes. Notable examples include members of genus Enterovirus (Mirand et al., 2006; Oberste et al., 1999), pestiviruses such as BVDV-1 (Deng et al., 2012; Vilcek et al., 2001) and BVDV-2 (Flores et al., 2002; Jenckel et al., 2014; Weber et al., 2015), circoviruses such as PCV2 (Franzo et al., 2015; Grau-Roma et al., 2008; Segalés et al., 2008), and lentiviruses such as FIV (Marçola et al., 2013; Sodora et al., 1994). In the case of IBV we propose that an effective classification scheme, particularly the designation of lineages and genotypes, should be based on clearly identifiable genetic groups (i.e. with recombinants removed) as these represent a robust phylogenetic backbone. A similar approach has been undertaken for PCV2 (Franzo et al., 2015). Hence, we contend that this is the most coherent and practical way for virus classification in the face of recombination, particularly as it is impractical to integrate multiple incongruent phylogenies and simplistic to think that such complex evolutionary histories will produce more rational classifications. Rather than being defined as unique variants in their own right, recombinants can then be referred to as combinations of these distinct lineages and genotypes, analogous to the definition of ‘circulating recombinant forms’ among HIV subtypes. However, it is evident that more experimental studies are needed to assess how recombination might impact viral fitness. In this respect, the relatively high number of recombinant viruses in our data (n = 213) is in part due to the presence of strains showing an

identical recombinant structure and possessing a strong epidemiological link between each other. Hence, these should not be regarded as result of independent recombination events.

As well as providing the first complete picture of IBV biodiversity, by determining the phylogenetic relationships between all described genetic groups we have provided a well-defined evolutionary history of IBV, which in turn results in a clear definition of viral genotypes and lineages. Accordingly, a total of 6 genotypes (GI-GVI) and 32 lineages were identified, with other potential groups present as unique variants (UVs) and which may become established should future viruses be sequenced. Some well-established lineages such as GI-1 and -13 have a broad geographic distribution, which is presumably associated with the use of vaccines derived from them. Therefore, the majority of the IBV strains included in these lineages might be vaccine and vaccine like strains. The first vaccine to control the disease was developed in the USA in the 1950s using the van Roeckel M-41 strain (van Roeckel et al., 1942) that represents the parent strain of most of the Mass type vaccines used there. By the early 1960s IB had been diagnosed in The Netherlands, leading to the development of a Mass-based vaccine known as the H strain (Bijlenga et al., 2004). The resulting vaccines, H120 and H52, soon became widely used. Today, the Mass and H120 strains of the lineage GI-1 continue to be the most commonly administrated attenuated-live vaccines. In contrast to the GI-1 vaccine strains, the 793B-like vaccines (GI-13), which were developed in Europe in the 1990s and used in many countries, have been never administered in North America. To date, the GI-13 lineage has not been detected in the USA, Oceania and many African and Latin American countries.

The S1 gene phylogeny was also characterized by strong geographic structure, such that IBV strains are often clustered by place of sampling. In particular, with the exception of strains of the GI-1 lineage, IBVs in Europe differ from those found in the USA or Australia, and each geographic group can be distinguished at the phylogenetic scale. That most of the strains in the GI-9 lineage come from the USA might suggest that the pathogenic Ark variant is geographically confined to that country. However, there are unpublished reports recording the circulation of Ark-like strains in South America (Jackwood, 2012; Marandino et al., 2015). The Ark virus is one of the most commonly reported types able to cause widespread disease in the USA, against which an attenuated vaccine was developed. When it first emerged in Arkansas in 1973, it was described as genetically distinct from all the known IBV serotypes recognized at that time and was referred to as Ark99 (Fields, 1973; Johnson et al., 1973).

During the 1980s, an attenuated vaccine derived from an Ark-type virus isolated in the Delmarva Peninsula (Ark DPI strain) (Gelb et al., 1983, 1981) was extensively used in the USA and remains one of the most common vaccines administered to flocks in this country and also in the United Kingdom. In this respect, a previous epidemiological survey reported the identification of GI-9-like strains in Western Europe only in flocks that had received the commercial bivalent IBMM + Ark vaccine (Worthington et al., 2008). However, no European IBV sequences similar to the GI-9 lineage are available in the public database. A similar situation arises with the Chinese IBVs in GI-9 (n = 5). Among these, the Jilin strain (AY839144), which was previously reported to be 100% identical to the Ark DPI strain (Ammayappan et al., 2008), is currently used as vaccine in China (Liu et al., 2006a). This suggests that the Chinese IBVs present in this lineage most likely represent re-isolations of the vaccine strain and not of the Ark field type.

Although the widespread circulation of some specific lineages is probably attributed to the use of vaccination programs based on strains derived from these, this is likely not always the case. In particular, the spread of the nephropathogenic QX-like variant of the GI-19 lineage occurred long before its homologous vaccine was administrated in the field. This Chinese lineage has generated considerable attention due to its ability to become endemic, causing major economic losses in the poultry industry worldwide, with the exception of the Americas and Oceania where it has been never detected. The origin of this lineage and the factors responsible of its distinctive distribution remain unclear (Bochkov et al., 2006; Gough et al., 2008). A role of wild birds has been hypothesized based on evidence that IBV may replicate in Anseriformes (Bochkov et al., 2006; Cavanagh, 2005). Importantly, the present study seems to counter the common assumption that the GI-16 lineage arose in China in 1996. In particular, our analysis provides evidence that the Italian IZO28/86 strain, isolated in 1986, belongs to GI-16 such that it constitutes the lineage prototype strain. This nephropathogenic virus was originally sampled in Italy about 10 years before the first identification of the Q1 strain in China, and its sequence has been only recently submitted to the public database. Additionally, the IZO28/86 sequence is closely related to strain 624/I (JQ901492), suggesting that they belong to the same lineage, which also includes the Q1-like strains. However, they have previously been classified as distinct genotypes. The 624/I virus was first reported as novel variant in Italy in 1993 (Capua et al., 1994) during an outbreak of severe respiratory disease, although only a 350 nt region of S1was sequenced (Capua et al., 1999). Thereafter, it has been sporadically detected in Italy, in Russia (Bochkov et al., 2006) and Slovenia (Krapez et al.,

2011). More recently a longer nucleotide 624/I sequence (1043 nt in length) has been released (JQ901492), which clusters into the same monophyletic group. Based on these observations, it is plausible that both variants belong to the GI-16, unless recombination has occurred in the C-terminal portion of the 624/I S1 sequence.

In the last two decades, a combination of phylogenetic clustering and patterns of sequence similarity in the S1 gene have been conventionally used to group IBV isolates into genetic clades, although a confusing variety of such clustering schemes currently exist. For instance, IBVs have been referred to as novel variants when their S1 nucleotide sequences are at least ≤75% dissimilar from that of any other IBV type (Gelb et al., 2005; Kingham et al., 2000). However, because of rate variation between sequences, reflected here in the lack of temporal structure in the data, distance-based classification methods are susceptible to error. In particular, elevated evolutionary rates leading to individual clusters may result in high genetic distances between sister taxa even though they are closely related. Thus, we suggest that phylogenetic relationships are a more appropriate measure of evolutionary history and hence the basis of a rationale classification than pairwise comparison of sequences. In addition, it is unrealistic to think that nature will create discrete groups of sequences that can consistently be recovered using genetic distances.

Most phylogenetic analyses of IBV have been based on the three more variable regions (HVRs) of the S1 gene. Some investigators have reported that the genetic typing based on HVR1 of the S1 gene is inconsistent with the groupings based on the whole S1 gene (Li et al., 2012; Mo et al., 2013; Schikora et al., 2003), although others disagree (Lee et al., 2003;Wang and Huang, 2000). We clearly show here that the hypervariable fragments (HVRs1 and 2 and HVR3) do not consistently produce clusters that are equivalent to those found through phylogenetic analyses of the S1 phylogeny. Therefore, the risk of misclassification decreases by using a larger portion of the S1 gene, and the sequencing of only one of these regions might result in insufficient phylogenetic resolution. Hence, we strongly recommend a phylogeny that considers the complete S1 gene sequence be employed for future designations of novel IBV lineages or genotypes.

2.5. CONCLUSIONS

Given the rapid evolution of IBV and the use of mass vaccination strategies to control the disease worldwide, additional genetic variants will likely be discovered in the future. As

heterogeneous genetic group designations, which are inconsistent with phylogenetic classification, have largely been used to the present day, it is essential to employ a standard nomenclature of practical use and a well-supported system to identify these novel variants. Herein, we propose a simple and repeatable S1 phylogeny-based classification system combined with an unambiguous lineage nomenclature for the future assignment of IBV strains. Following the suggestions here proposed, at least three complete S1 sequences of viral samples collected at least from two different outbreaks should be available for the identification of a new viral lineage, and genotypes and lineages should be referred to according to the current numerical system. In addition, and in similar manner to the convention in AIV, we encourage the use of a uniform and informative system for naming IBV isolates, which at least should include the name of the strain, country of origin and date of collection (Cavanagh, 2001). Clearly, the adoption of an internationally accepted nomenclature and a common system to coherently designate viruses is central for efficient communication on the evolution and emergence of epidemiologically important IBV variants.

Supplementary Table S1 available from http://dx.doi.org/10.1016/j.meegid.2016.02.015.

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REFERENCES

Ababneh, M., Dalab, A.E., Alsaad, S., Al-Zghoul, M., 2012. Presence of infectious bronchitis virus strain CK/CH/LDL/97I in the Middle East. ISRN Vet Sci. 2012, 201721.

Abdel-Moneim, A.S., Madbouly, H.M., Gelb, J. Jr, Ladman, B.S., 2002. Isolation and identification of Egypt/Beni-Suef/01 a novel genotype of infectious bronchitis virus. Vet. Med. J. Giza 50, 1065-1078.

Abdel-Moneim, A.S., El-Kady M.F., Ladman B.S., Gelb J. Jr, 2006. S1 gene sequence analysis of a nephropathogenic strain of avian infectious bronchitis virus in Egypt. Virol. J. 3, 78.

Abdel-Moneim, A.S., Afifi, M.A., El-Kady, M.F., 2012. Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. Arch. Virol. 157(12), 2453-2457.

Abdel-Rahman, S., El-Kenawy, A.A., Neumann, U., Herrler, G., Winter, C., 2009. Comparative analysis of the sialic acid binding activity and the tropism for the respiratory epithelium of four different strains of avian infectious bronchitis virus. Avian Pathol. 38, 41-45.

Abro, S.H., Renström, L.H., Ullman, K., Isaksson, M., Zohari, S., Jansson, D.S., Belák, S., Baule, C., 2011. Emergence of novel strains of avian infectious bronchitis virus in Sweden. Vet. Microbiol. 155(2-4), 237-246.

Adzhar, A.B., Shaw, K., Britton, P., Cavanagh, D., 1995. Avian infectious bronchitis virus: differences between 793/B and other strains. Vet. Rec. 136, 548.

Adzhar, A.B., Shaw, K., Britton, P., Cavanagh, D., 1996. Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. Avian Pathol. 25, 817-836.

Adzhar, A B., Gough, R.E., Haydon. D., Shaw, K., Britton, P., Cavanagh, D., 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian Pathol. 26, 625-640.

Albassam, M.A., Winterfield, R.W., Thacker, H.L, 1986. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. Avian Dis. 30(3), 468- 476.

Aldous, E.W., Mynn, J.K., Banks, J., Alexander, D.J., 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. 32(3), 239-256.

Alexander, D.J., Gough, R.E., 1977. Isolation of avian infectious bronchitis virus from experimentally infected chickens. Res. Vet. Sci. 23(3), 344-347.

Almazán, F., Galán, C., Enjuanes, L., 2004. The nucleoprotein is required for efficient coronavirus genome replication. J. Virol. 78(22),12683-12688.

Amin, O.G., Valastro, V., Salviato, A., Drago, A., Cattoli, G., Monne, I., 2012. Circulation of QX-like infectious bronchitis virus in the Middle East. Vet. Rec. 171(21), 530.

Ammayappan, A., Upadhyay, C., Gelb, J. Jr, Vakharia, V.N., 2008. Complete genomic sequence analysis of infectious bronchitis virus Ark DPI strain and its evolution by recombination. Virol. J. 5, 157.

Ammayappan, A., Vakharia, V.N., 2009. Complete nucleotide analysis of the structural genome of the infectious bronchitis virus strain md27 reveals its mosaic nature. Viruses 1(3), 1166-1177.

Andreasen, J.R., Jackwood, M.R., Hilt, D.A., 1991. Polymerase chain reaction amplification of the genome of infectious bronchitis virus. Avian Dis. 35, 216-220.

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst. Biol. 55, 539-552.

Anisimova, M., Gil, M., Dufayard, J.F., Dessimoz, C., Gascuel, O., 2011. Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. Syst. Biol. 60(5), 685-699.

Ariyoshi, R., Kawai, T., Honda, T., Tokiyoshi, S., 2010. Classification of IBV S1 genotypes by direct reverse transcriptase-polymerase chain reaction (RT-PCR) and relationship between serotypes and genotypes of strains isolated between 1998 and 2008 in Japan. J. Vet. Med. Sci. 72(6), 687-692.

Armesto, M., Cavanagh, D., Britton, P., 2009. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. PLoS One. 4(10):e7384.

Asplin, F.D., 1948. Identification of infectious bronchitis of chickens in England. Vet. Rec. 60, 485-486.

Bacon, L.D., Hunter, D.B., Zhang, H.M., Brand, K., Etches, R., 2004. Retrospective evidence that the MHC (B haplotype) of chickens influences genetic resistance to attenuated infectious bronchitis vaccine strains in chickens. Avian Pathol. 33, 605-609.

Balestrin, E., Fraga, A.P., Ikuta, N., Canal, C.W., Fonseca, A.S., Lunge, V.R., 2014. Infectious bronchitis virus in different avian physiological systems-a field study in Brazilian poultry flocks. Poult. Sci. 93(8), 1922-1929.

Ballesteros, M.L., Sanchez, C. M., Enjuanes, L., 1997. Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology 227, 378-388.

Bayry, J., Goudar, M.S., Nighot, P.K., Kshirsagar, S.G., Ladman, B.S., Gelb, J. Jr., Ghalsasi, G.R., Kolte, G.N., 2005. Emergence of a nephropathogenic avian infectious bronchitis virus with a novel genotype in India. J. Clin. Microbiol. 43(2), 916-918.

Baric, R.S., Yount, B., Hensley, L., Peel, S.A., Chen, W., 1997. Episodic evolution mediates interspecies transfer of a murine coronavirus. J. Virol. 71, 1946.

Beato, M.S., De Battisti, C., Terregino, C., Drago, A., Capua, I., Ortali, G., 2005. Evidence of circulation of a Chinese strain of infectious bronchitis virus (QXIBV) in Italy. Vet. Rec. 156(22), 720.

Beaudette, F., Hudson, C., 1937. Cultivation of the virus of infectious bronchitis. J. Am. Vet. Med. Assoc. 90, 51-60.

Belouzard, S., Millet, J.K., Licitra, B.N., Whittaker, G.R., 2012. Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses 4, 1011-1033.

Bergmann, C.C., Lane, T.S., Stohlman, S.A., 2006. Coronavirus infection of the central nervous system: host-virus stand-off. Nat. Rev. Microbiol. 4, 121-132.

Bijlenga, G., 1956. Infectious bronchitis in chicks in the Netherlands. Tijdschr. Diergeneesk. 81, 43-54.

Bijlenga, G., Cook, J.K.A., Gelb, J. Jr, de Wit, J.J., 2004. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol. 33(6), 550-557.

Bing, G.X., Liu, X., Pu, J., Liu, Q.F., Wu, QM, Liu, J.H., 2007. Different genotypes of nephropathogenic infectious bronchitis viruses co-circulating in chicken population in China. Virus Genes 35(2), 333-337.

Binns, M.M., Boursnell, M.E.G., Cavanagh, D., Pappin, D.J.C., Brown, T.D.K., 1985. Cloning and sequencing of the gene encoding the spike protein of the coronavirus IBV. J. Gen. Virol. 66, 719-726.

Bochkov, Y.A., Batchenko G.V., Shcherbakova L.O., Borisov A.V., Drygin. V.V., 2006. Molecular epizootiology of avian infectious bronchitis in Russia. Avian Pathol. 35(5), 379-393.

Bochkov, Y.A., Tosi, G., Massi, P., Drygin, V.V., 2007. Phylogenetic analysis of partial S1 and N gene sequences of infectious bronchitis virus isolates from Italy revealed genetic diversity and recombination. Virus Genes 35(1), 65-71.

Bosch, B.J., van der Zee, R., de Haan, C.A., Rottier, P.J., 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J. Virol. 77(16), 8801-8811.

Boursnell, M.E., Brown, T.D., Foulds, I.J., Green, P.F., Tomley, F.M., Binns, M.M., 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J. Gen. Virol. 68, 57-77.

Box, P.G., Beresford, A.V., Roberts, B., 1980. Protection of laying hens against infectious bronchitis with inactivated emulsion vaccines. Vet. Rec. 106, 264-268.

Box, P.G., Roberts, B., Beresford, A.V., 1982. Infectious bronchitis-preventing loss of egg production by emulsion vaccine at point-of-lay. Dev. Biol. Standard. 51, 97-103.

Broadfoot, D.I., Smith, W.M.Jr, 1954. Effects of infectious bronchitis in laying hens on egg production, percent unsettable eggs and hatchability. Poult. Sci. 33, 653-665.

Brooks, J.E., Rainer, A.C., Parr, R.L., Woolcock, P., Hoerr, F., Collisson, E.W., 2004. Comparisons of envelope through 5B sequences of infectious bronchitis coronaviruses indicates recombination occurs in the envelope and membrane genes. Virus Res. 100, 191-198.

Brown, T.P., Glisson, J.R., Rosales, G., Villegas, P., Davis, R.B., 1987. Studies of avian urolithiasis associated with an infectious bronchitis virus. Avian Dis. 31, 629-636.

Butler, E.J., Curtis, M.J., Pearson, A.W., McDougall, J.S., 1972a. Effect of infectious bronchitis virus on the structure and composition of egg albumen. J. Sci. Food Agric. 23, 359-369.

Butler, M., Ellaway, W.J., Hall, T., 1972b. Comparative studies on the infectivity of avian respiratory viruses for eggs, cell cultures and tracheal explants. J. Comp. Pathol. 82, 327-332.

Callison, S.A., Jackwood, M.W., Hilt, D.A., 2001. Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates. Avian Dis. 45(2), 492-499.

Callison, S.A., Hilt, D.A., Boynton, T.O., Sample, B.F., Robison, R., Swayne, D.E., Jackwood, M.W., 2006. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J. Virol. Methods. 138(1-2), 60-65.

Capua, I., Gough, R.E., Mancini, M., Casaccia, C., Weiss, C., 1994. A 'novel' infectious bronchitis strain infecting broiler chickens in Italy. Zentralbl. Veterinarmed B. 41(2), 83-89.

Capua, I., Mawditt, K., Cavanagh, D., Gough, R.E., 1998. Serological and molecular characterisation of recent infectious bronchitis virus strains from Italy, in Proceedings of the International Symposium on Infectious Bronchitis and Pneumovirus infections in Poultry, Rauischholzhausen, Germany, pp. 210-214.

Capua, I., Minta, Z., Karpinska, E., Mawditt, K., Britton, P., Cavanagh, D., Gough, R.E., 1999. Co-circulation of four types of infectious bronchitis virus (793/B, 624/I, B1648 and Massachusetts). Avian Pathol. 28(6), 587-592.

Casais, R., Dove, B., Cavanagh, D., Britton, P., 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77, 9084-9089.

Casais, R., Davies, M., Cavanagh, D., Britton, P., 2005. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. J. Virol. 79, 8065- 8078.

Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17(4), 540-552.

Cavanagh, D., 1983. Coronavirus IBV: structural characterization of the spike protein. J. Gen. Virol. 64, 2577-2583.

Cavanagh, D., Davis, P.J., 1986. Coronavirus IBV: removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. J. Gen. Virol. 67, 1443-1448.

Cavanagh, D., Davis, P.J., Darbyshire, J.H., Peters, R.W., 1986a. Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virusneutralizing or

haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J. Gen. Virol. 67, 1435-1442.

Cavanagh, D., Davis, P.J., Pappin, D.J.C., Binns, M.M., Boursnell, M.E.G., Brown, T. D.K., 1986b. Coronavirus IBV: partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor propolypeptide of IBV strains Beaudette and M41. Virus Res. 4, 133-143.

Cavanagh, D., Davis, P.J., Mockett, A.P., 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11(2), 141-150.

Cavanagh, D., Davis, P.J., Cook, J.K., Li, D., Kant, A., Koch, G., 1992a. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol. 21, 33-43.

Cavanagh, D., Davis, P.J., Cook, J.K., 1992b. Infectious bronchitis virus: evidence for recombination within the Massachusetts serotype. Avian Pathol. 21, 401-408.

Cavanagh, D., Naqi, S., 1997. Infectious bronchitis, in: Calnek, B.W., Barnes, H.J., Beard, C.W., McDougald, L.R., Saif, Y.M. (Eds.), Diseases of poultry, 10th ed. Mosby- Wolfe, London, pp. 511-526.

Cavanagh, D., Elus, M.M., Cook, J.K.A., 1997. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathol. 26(1), 63-74.

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

Cavanagh D., 2001. A nomenclature for avian coronavirus isolates and the question of species status. Avian Pathol. 30, 109-115.

Cavanagh, D., 2003. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol. 32(6), 567-582.

Cavanagh, D, Naqi, S., 2003. Infectious bronchitis, in: Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. (Eds.), Diseases of poultry 11th ed. Iowa State University Press, Ames, IA, pp. 101-119.

Cavanagh, D., 2005. Coronaviruses in poultry and other birds. Avian Pathol. 34(6), 439-448.

Cavanagh, D., Picault, J.P., Gough, R.E., Hess, M., Mawditt, K., Britton, P., 2005. Variation in the spike protein of the 793/B type of infectious bronchitis virus, in the field and during alternate passage in chickens and embryonated eggs. Avian Pathol. 34(1), 20-25.

Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. Vet. Res. 38(2), 281-297.

Cavanagh, D., Gelb J. Jr., 2008. Infectious bronchitis, in: Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne, D.E. (Eds.), Diseases of poultry 12th ed. Blackwell Publishing Professional, Ames, IA, pp. 117-135.

Chacón, J.L., Vejarano, M.P., Ferreira, A.J.P., 2008. S1 gene fragment amplification of Infectious bronchitis virus variant by RT-PCR from Brazil, in: Proceedings of the AVMA/American Association of Avian Pathology Annual Meeting, New Orleans, pp. 76.

Chacón, J.L., Rodrigues, J.N., Assayag, M.S. Jr, Peloso, C., Pedroso, A.C., Ferreira A.J., 2011. Epidemiological survey and molecular characterization of avian infectious bronchitis virus in Brazil between 2003 and 2009. Avian Pathol. 40,153-162.

Chacón, J.L., Assayag, M.S. Jr, Revolledo, L., Astolfi-Ferreira, C.S., Vejarano, M.P., Jones, R.C., Piantino Ferreira, A.J., 2014. Pathogenicity and molecular characteristics of infectious bronchitis virus (IBV) strains isolated from broilers showing diarrhoea and respiratory disease. Br. Poult. Sci. 55, 271-283. doi: 10.1080/00071668.2014.903558.

Chen, H, Gill, A, Dove, B.K., Emmett, S.R., Kemp, C.F., Ritchie, M.A., Dee, M., Hiscox, J.A., 2005. Mass spectroscopic characterization of the coronavirus infectious bronchitis virus nucleoprotein and elucidation of the role of phosphorylation in RNA binding by using surface plasmon resonance. J. Virol. 79(2), 1164-1179.

Clarke, J.K., McFerran, J.B., Gay, F.W., 1972. Use of allantoic cells for the detection of avian infectious bronchitis virus. Arch. Gesamte Virusforsch. 36, 62-70.

Clewley, J.P., Morser, J., Avery, R.J., Lomniczi, B., 1981. Oligonucleotide fingerprinting of ribonucleic acids of infectious bronchitis virus strains. Infect. Immun. 32, 1227-1233.

Cook, J.K.A., Darbyshire, J.H., Peters, R.W., 1976. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch. Virol. 50, 109-118.

Cook, J.K., 1983. Isolation of a new serotype of infectious bronchitis-like virus from chickens in England. Vet Rec. 112, 104-105.

Cook, J.K., 1984. The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. Avian Pathol. 13(4), 733-741.

Cook, J.K., Huggins, M.B., 1986. Newly isolated serotypes of infectious bronchitis virus: their role in disease. Avian Pathol. 15, 129-138.

Cook, J.K., Orbell, S.J., Woods, M.A., Huggins, M.B., 1996. A survey of the presence of a new infectious bronchitis virus designated 4/91 (793B). Vet Rec. 138(8), 178-180.

Cook, J.K.A., Orbell, S.J., Woods, M.A., Huggins, M.B., 1999. Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Pathol. 28, 477-485.

Cook, J.K.A., 2008. Coronaviridae, in: Pattison, M., McMullin, P.F., Bradbury, J.M., Alexander D.J. (Eds.), Poultry Diseases, 6th ed. Philadelphia, Pennsylvania, pp. 340-349.

Cowen, B.S., Hitchner, S.B., 1975. Serotyping of avian infectious bronchitis viruses by the virus-neutralization test. Avian Dis. 19, 583-595.

Cowen, B.S., Wideman, R.F., Rothenbacher, H., Braune M.O., 1987. An outbreak of avian urolithiasis on a large commercial egg farm. Avian Dis. 31, 392-397.

Crinion, R.A.P., Hofstad, M.S., 1972a. Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis. 16, 351-363.

Crinion, R.A.P., Hofstad, M.S., 1972b. Pathogenicity of two embryo-passage levels of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis. 16, 967-973.

Crinion, R.A.P., Ball, R.A., Hofstad, M.S., 1971a. Pathogenesis of oviduct lesions in immature chickens following exposure to infectious bronchitis virus at one day-old. Avian Dis. 15, 32-41.

Crinion, R.A.P., Ball, R.A., Hofstad, M.S., 1971b. Abnormalities in laying chickens following exposure to infectious bronchitis virus at one day-old. Avian Dis. 15, 42-48.

Cumming, R.B., 1963. Infectious avian nephrosis (uraemia) in Australia. Aust. Vet. J. 39, 145-147.

Cumming, R.B., 1969. The control of avian infectious bronchitis/nephrosis in Australia. Aust. Vet. J. 45(4), 200-203. doi: 10.1111/j.1751-0813.1969.tb01930.x.

Darbyshire, J.H., Cook, J.K.A., Peters, R.W., 1975. Comparative growth kinetic studies on avain infectious bronchitis virus in different systems. J. Comp. Pathol. 85, 623-630.

Darbyshire, J.H., Rowell, R.G., Cook, J.K.A., Peters, R.W., 1979. Taxonomic studies on strains of avian infectious bronchitis virus using neutralisation tests in tracheal organ cultures. Arch. Virol. 61, 227-238.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9(8), 772.

Davelaar, F.G., Kouwenhoven, B., Burger, A.G., 1984. Occurrence and significance of infectious bronchitis virus variant strains in egg and broiler production in the Netherlands. Vet. Q. 6(3), 114-120.

Davies, H.A., Dourmashkin, R.R., Macnaughton, M.R., 1981. Ribonucleoprotein of avian infectious bronchitis virus. J. Gen. Virol. 53, 67-74.

de Almeida, R.S., Hammoumi, S., Gil, P., Briand, F.X., Molia, S., Gaidet, N., Cappelle, J., Chevalier, V., Balança, G., Traoré, A., Grillet, C., Maminiaina, O.F., Guendouz, S., Dakouo, M., Samaké, K., Bezeid Oel, M., Diarra, A., Chaka, H., Goutard, F., Thompson, P., Martinez, D., Jestin, V., Albina, E., 2013. New avian paramyxoviruses type I strains identified in Africa provide new outcomes for phylogeny reconstruction and genotype classification. PLoS One. 8(10), e76413. doi: 10.1371/journal.pone.0076413.

Decaro, N., Mari, V., Campolo, M., Lorusso, A., Camero, M., Elia, G., Martella, V., Cordioli, P., Enjuanes, L., Buonavoglia, C., 2009. Recombinant canine coronaviruses related to transmissible gastroenteritis virus of Swine are circulating in dogs. J. Virol. 83, 1532-1537.

de Haan, C.A., Smeets, M., Vernooij, F., Vennema, H., Rottier, P.J., 1999. Mapping of the coronavirus membrane protein domains involved in interaction with the spike protein. J. Virol. 73(9), 7441-7452.

de Haan, C.A., Vennema, H., Rottier, P.J., 2000. Assembly of the coronavirus envelope: homotypic interactions between the M proteins. J. Virol. 74, 4967-4978.

Delmas, B., Laude, H., 1990. Assembly of coronavirus spike protein into trimers and its role in epitope expression. J. Virol. 64(11), 5367-5375.

Deng, Y., Sun, C.Q., Cao, S.J., Lin, T., Yuan, S.S., Zhang, H.B., Zhai, S.L., Huang, L., Shan, T.L., Zheng, H., Wen, X.T., Tong, G.Z., 2012. High prevalence of bovine viral diarrhea virus 1 in Chinese swine herds. Vet. Microbiol. 159(3-4), 490-493.

de Wit, J.J., Mekkes, D.R., Kouwenhoven, B., Verheijden, J.H.M., 1997. Sensitivity and specificity of serological tests for detection of infectious bronchitis virus induced antibodies in broilers. Avian Pathol. 26, 105-118.

de Wit, J.J., Mekkes, D.R., Koch, G., Westenbrink, F., 1998. Detection of specific IgM antibodies to infectious bronchitis virus by an antibody-capture ELISA. Avian Pathol. 27, 155-160.

de Wit, J.J., 2000. Detection of infectious bronchitis virus. Avian Pathol. 29(2),71-93.

de Wit, J.J., Cook, J.K.A., van der Heijden, H.M., 2010a. Infectious bronchitis virus in Asia, Africa, Australia and Latin America - history, current situation and control measures. Braz. J. Poul. Sci. 12, 97-106.

de Wit, J.J., Swart, W.A.J.M., Fabri, T.H.F., 2010b. The efficacy of infectious bronchitis virus vaccinations in the field: association between the a-IBV IgM response, protection and vaccine application parameters. Avian Pathol. 39, 123-132.

de Wit, J.J., Cook, J.K., van der Heijden, H.M., 2011a. Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol. 40(3), 223-235.

de Wit, J.J., Nieuwenhuisen-van Wilgen, J., Hoogkamer, A., van de Sande, H., Zuidam, G.J., Fabri, T.H., 2011b. Induction of cystic oviducts and protection against early challenge with infectious bronchitis virus serotype D388 (genotype QX) by maternally derived antibodies and by early vaccination. Avian Pathol. 40(5), 463-471.

de Wit, J.J., Guerrero, P., Calvo J., Hidalgo, H., 2012. Report of the genotyping, pathotyping and protectotyping of recent strains from Chile, in: Proceedings of the VII International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, pp. 230-241.

de Wit, J.J., Brandao, P., Torres, C.A., Koopman, R., Villarreal, L.Y.B., 2015. Increased level of protection of respiratory tract and kidney by combining different infectious bronchitis virus vaccines against challenge with nephropathogenic Brazilian genotype subcluster 4 strains. Avian Pathol. 44(5), 352-357.

Dolz, R., Bertran, K., Majó, N., 2009. First report of IBV QX-like strains in Spain, in: Proceedings of the VI International symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, pp. 27-33.

Dolz, R., Vergara-Alert, J., Pérez, M., Pujols, J., Majó, N., 2012. New insights on infectious bronchitis virus pathogenesis: characterization of Italy 02 serotype in chicks and adult hens. Vet. Microbiol. 156(3-4), 256-264.

Domanska-Blicharz, K., Lisowska, A., Jatczak, J., Mamczur, J., Minta, Z., 2012. D1466-like genotype of infectious bronchitis virus responsible for a new epidemic in chickens in Poland. Vet. Rec. 171(14), 351. doi: 10.1136/vr.100888.

Drummond, A.J., Pybus, O.G., Rambaut, A., Forsberg, R., Rodrigo, A.G., 2003. Measurably evolving populations. Trends Ecol. Evol. 18, 481-488.

Ducatez, M.F., Martin, A.M., Owoade, A.A., Olatoye, I.O., Alkali, B.R., Maikano, I., Snoeck, C.J., Sausy, A., Cordioli, P., Muller, C.P., 2009. Characterization of a new genotype and serotype of infectious bronchitis virus in Western Africa. J. Gen. Virol. 90, 2679-2685.

Duffy, S, Shackelton, L.A., Holmes, E.C., 2008. Rates of evolutionary change in viruses: patterns and determinants. Nat. Rev. Genet. 9(4), 267-276.

El-Mahdy, S.S., Ekram, S., Ahmed, A., 2012. Efficacy of some living classical and variant infectious bronchitis vaccines against local variant isolated from Egypt. Nat. Sci. 10, 292-299.

Emmott, E., Munday, D., Bickerton, E., Britton, P., Rodgers, M.A., Whitehouse, A., Zhou, E.M., Hiscox, J.A., 2013. The cellular interactome of the coronavirus infectious bronchitis virus nucleocapsid protein and functional implications for virus biology. J. Virol. 87(17), 9486-9500.

Enjuanes, L., Brian, D., Cavanagh, D., Holmes, K., Lai, M.M.C., Laude, H., Masters, P., Rottier, P., Siddell, S.G., Spaan, W.J.M., Taguchi, F., Talbot, P., 2000. Coronaviridae, in: van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S., Maniloff, J., Mayo, M., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.). Virus Taxonomy. Classification and Nomenclature of Viruses, San Diego, Academic Press, pp. 835-849.

Escors, D., Ortego, J., Laude, H., Enjuanes, L., 2001a. The membrane M protein carboxy terminus binds to transmissible gastroenteritis coronavirus core and contributes to core stability. J. Virol. 75(3), 1312-1324.

Escors, D., Camafeita, E., Ortego, J., Laude, H., Enjuanes, L., 2001b. Organization of two transmissible gastroenteritis coronavirus membrane protein topologies within the virion and core. J. Virol. 75(24), 12228-12240.

Fabricant, J., 1998. The early history of infectious bronchitis. Avian Dis. 42, 648-650.

Fang, S.G., Shen, S., Tay, F.P.L., Liu, D.X., 2005. Selection of and recombination between minor variants lead to the adaptation of an avian coronavirus to primate cells. Biochem. Biophys. Res. Commun. 336, 417-423.

Fazakerley, J.K., Parker, S.E., Bloom, F., Buchmeier, M.J., 1992. The V5A13.1 envelope glycoprotein deletion mutant of mouse hepatitis virus type-4 is neuroattenuated by its reduced rate of spread in the central nervous system. Virology 187, 178-188.

Fellahi, S., Ducatez, M., El Harrak, M., Guérin, J.L., Touil, N., Sebbar, G., Bouaiti, el A., Khataby, K., Ennaji, M.M., El-Houadfi, M., 2015. Prevalence and molecular characterization of avian infectious bronchitis virus in poultry flocks in Morocco from 2010 to 2014 and first detection of Italy 02 in Africa. Avian Pathol. 44(4), 287-295.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution 39, 783-791.

Fields, D.B., 1973. Arkansas 99, a new infectious bronchitis serotype. Avian Dis. 17, 659-661.

Flores, E.F., Ridpath, J.F., Weiblen, R., Vogel, F.S., Gil, L.H., 2002. Phylogenetic analysis of Brazilian bovine viral diarrhea virus type 2 (BVDV-2) isolates: evidence for a subgenotype within BVDV-2. Virus Res. 87, 51-60.

Fraga, A.P., Balestrin, E., Ikuta, N., Fonseca, A.S.K., Spilki, F.R., Canal, C.W., Lunge, V.R., 2013. Emergence of a new genotype of avian infectious bronchitis virus in Brazil. Avian Dis. 57(2), 225-232.

Franzo, G., Cortey, M., Olvera, A., Novosel, D., Castro, A.M., Biagini, P., Segalés, J., Drigo, M., 2015. Revisiting the taxonomical classification of Porcine Circovirus type 2 (PCV2): still a real challenge. Virol. J. 12, 131-138.

Ganapathy, K., Ball, C., Forrester, A., 2015. Genotypes of infectious bronchitis viruses circulating in the Middle East between 2009 and 2014. Virus Res. 210, 198-204.

Gandon, S., Mackinnon, M.J., Nee, S., Read, A.F., 2001. Imperfect vaccines and the evolution of pathogen virulence. Nature 414, 751-756.

Gandon, S., Day, T., 2008. Evidences of parasite evolution after vaccination. Vaccine 26, C4-7.

Garcia, Z., Bankowski, R.A., 1981. Comparison of a tissue-culture virus-neutralization test and the enzyme-linked immunosorben t assay for measurement of antibodies to infectious bronchitis. Avian Dis. 25, 121-130.

Gelb, J. Jr, Perkins, B.E., Rosenberger, J.K., Allen, P.H., 1981. Serologic and crossprotection studies with several infectious bronchitis virus isolates from Delmarvareared broiler chickens. Avian Dis. 25(3), 655-666.

Gelb, J. Jr, Leary, J.H., Rosenberger, J.K., 1983. Prevalence of Arkansas-type infectious bronchitis virus in Delmarva Peninsula chickens. Avian Dis. 27(3), 667-678.

Gelb, J. Jr, 1989. Infectious bronchitis, in: Purchase, H.G., Arp, L.H., Domermuth, C.H., Pearson J.E. (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 3rd edn, Kennet Square, PA: American Association of Avian Pathologists. pp. 124-127.

Gelb, J. Jr, Keeler, C.L. Jr, Nix, W.A., Rosenberger, J.K., Cloud S.S., 1997. Antigenic and S1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. Avian Dis. 41(3), 661-669.

Gelb, J. Jr, Ladman, B.S., Tamayo, M., Gonzalez, M., Sivanandan, V., 2001. Novel infectious bronchitis virus S1 genotypes in Mexico 1998-1999. Avian Dis. 45(4), 1060- 1063.

Gelb, J. Jr, Weisman, Y., Ladman, B.S., Meir, R., 2005. S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). Avian Pathol. 34(3), 194-203.

González, J.M., Gomez-Puertas, P., Cavanagh, D., Gorbalenya, A.E., Enjuanes, L.A., 2003. Comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. Arch. Virol. 148(11), 2207-2235.

Gorbalenya, A.E., Enjuanes, L., Ziebuhr, J., Snijder, E.J., 2006. Nidovirales: evolving the largest RNA virus genome. Virus Res. 117(1), 17-37.

Gough, R.E., Randall, C.J., Dagless, M., Alexander, D.J., Cox, W.J., Pearson, D., 1992. A 'new' strain of infectious bronchitis virus infecting domestic fowl in Great Britain. Vet. Rec. 130, 493.

Gough, R.E., Cox, W.J., de B. Welchman, D., Worthington, K.J., Jones, R.C., 2008. Chinese QX strain of infectious bronchitis virus isolated in the UK. Vet. Rec. 162(3), 99-100.

Grau-Roma, L., Crisci, E., Sibila, M., López-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segalés, J., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet. Microbiol. 128, 22-35.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59(3), 307-321.

Han, Z., Sun, C., Yan, B., Zhang, X., Wang, Y., Li, C., Zhang, Q., Ma, Y., Shao, Y., Liu, Q., Kong, X., Liu, S., 2011. A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. Infect. Genet. Evol. 11(1), 190-200.

Hanada, K., Suzuki, Y., Gojobori, T., 2004. A large variation in the rates of synonymous substitution for RNA viruses and its relationship to a diversity of viral infection and transmission modes. Mol. Biol. Evol. 21, 1074-1080.

Handberg, K.J., Nielsen, O.L., Pedersen, M.W., Jørgensen, P.H., 1999. Detection and strain differentiation of infectious bronchitis virus in tracheal tissues from experimentally infected chickens by reverse transcription-polymerase chain reaction. Comparison with an immunohistochemical technique. Avian Pathol. 28(4), 327-335.

Heald-Sargent, T., Gallagher, T., 2012. Ready, set, fuse! The coronavirus spike protein and acquisition of fusion competence. Viruses 4, 557-580.

Hein, R.K., Machado, G.V., Woodward, W.D., 1989. Cooperative investigation for the isolation of respiratory agents on problem cage layer operations, in: Proceedings of the 38th Western Poultry Disease Conference, Tempe, AZ, pp. 79-82.

Hesselink, W.G., Schrier, C., Jagt, E., Hocks, J. 1988. Detection of IBV antigen by enzyme immunoassay: application for diagnostic purposes, in: Proceedings of the I International Symposium on Infectious Bronchitis, Rauischholzhausen, Germany, pp. 54.

Hesselink, W.G., 1991. Serotyping avian infectious bronchitis virus: selection of a unified method, in: Proceedings of the I International Symposium on Infectious Bronchitis, Rauischholzhausen, Germany, pp. 87-97.

Hilgenfeld, R., Peiris, M., 2013. From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. Antiviral Res. 100(1), 286-95.

Hingley, S.T., Gombold, J.L., Lavi, E., Weiss, S.R., 1994. MHV-A59 fusion mutants are attenuated and display altered hepatotropism. Virology 200, 1-10.

Hipólito, O., 1957. Isolamento e identificacão do virus da bronquite infecciosa das galinhas no Brasil. Arquivo Escuela Veterinaria Universidade de Minas Gerais 10, 131–151.

Hodgson, T., Britton, P., Cavanagh, D., 2006. Neither the RNA nor the proteins of open reading frames 3a and 3b of the coronavirus infectious bronchitis virus are essential for replication. J. Virol. 80, 296-305.

Hofstad, M.S., 1958. Antigenic differences among isolates of avian infectious bronchitis virus. Am. J. Vet. Res. 19(72), 740-743.

Holder, M.H., Lewis, P.A., 2003. Phylogeny estimation: traditional and Bayesian approaches. Nat. Rev. Gen. 4, 275-284.

Holmes, E.C., 2009. The Evolution and Emergence of RNA Viruses, first ed. Oxford University Press Inc. New York.

Hong, S.M., Kwon, H.J., Kim, I.H., Mo, M.L., Kim, J.H., 2012. Comparative genomics of Korean infectious bronchitis viruses (IBVs) and an animal model to evaluate pathogenicity of IBVs to the reproductive organs. Viruses 4(11), 2670-2683. doi: 10.3390/v4112670.

Hopkins, S.R., 1969. Serologic and immunologic properties of a recent isolate of infectious bronchitis virus. Avian Dis. 13, 356-362.

Hopkins, S.R., 1974. Serological comparisons of strains of infectious bronchitis virus using plaque-purified isolants. Avian Dis. 18, 231-239.

Huang, Y.P., Lee, H.C., Cheng, M.C., Wang, C.H., 2004. S1 and N gene analysis of avian infectious bronchitis viruses in Taiwan. Avian Dis. 48(3), 581-589.

Huelsenbeck, J.P., 1995. Performance of phylogenetic methods in simulation. Syst. Biol. 44, 17-48.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8), 754-755.

Ignjatovic, J., McWaters, P.G., 1991. Monoclonal antibodies to three structural proteins of avian infectious bronchitis virus: characterization of epitopes and antigenic differentiation of Australian strains. J. Gen. Virol. 72, 2915-2922.

Ignjatovic, J., Galli, L., 1994. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Arch. Virol. 138, 117-34.

Ignjatovic, J., Ashton, F., 1996. Detection and differentiation of avian infectious bronchitis viruses using a monoclonal antibody- based ELISA. Avian Pathol. 25, 721-736.

Ignjatovic, J., Sapats, S.I., Ashton, F., 1997. A long-term study of Australian infectious bronchitis viruses indicates a major antigenic change in recently isolated strains. Avian Pathol. 26(3), 535-552.

Ignjatovic, J., Gould, G., Sapats, S.I., 2006. Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains. Arch. Virol. 151(8), 1567-1585.

International Committee on Taxonomy of Viruses (ICTV), 2012. Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses, in: King, A.M.Q., Adams, M.J., Carstens, E.B. and Lefkowitz, E.J. (Eds.), Elsevier Academic Press, San Diego, CA. http://www.ictvonline.org/virustaxonomy.asp.

Jackwood, M.W., Kwon, H.M., Hilt, D.A. 1992. Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and a DNA probe. Avian Dis. 36, 403-409.

Jackwood, M.W., Yousef, N.M.H., Hilt, D.A., 1997. Further development and use of a molecular serotype identification test for infectious bronchitis virus. Avian Dis. 41(1), 105-110.

Jackwood, M.W., Hilt, D.A., Callison, S.A., 2003. Detection of infectious bronchitis virus by real-time reverse transcriptase-polymerase chain reaction and identification of a quasispecies in the Beaudette strain. Avian Dis. 47, 718-724.

Jackwood, M.W., Hilt, D.A., Williams, S.M., Woolcock, P., Cardona, C., O'Connor, R., 2007. Molecular and serologic characterization, pathogenicity, and protection studies with infectious bronchitis virus field isolates from California. Avian Dis. 51(2), 527-533.

Jackwood, M.W., Hilt, D.A., McCall, A.W., Polizzi, C.N., McKinley, E.T., Williams, S.M., 2009. Infectious bronchitis virus field vaccination coverage and persistence of Arkansas-type viruses in commercial broilers. Avian Dis. 53, 175-183.

Jackwood, M.W., Boynton, T.O., Hilt, D.A., McKinley, E.T., Kissinger, J.C., Paterson, A.H., Robertson, J., Lemke, C., McCall, A.W., Williams, S.M., Jackwood, J.W., Byrd, L.A., 2010a. Emergence of a group 3 coronavirus through recombination. Virology 398, 98-108.

Jackwood, M.W., Hilt, D.A., Sellers, H.S., Williams, S.M., Lasher, H.N., 2010b. Rapid heat-treatment attenuation of infectious bronchitis virus. Avian Pathol. 39(3), 227-233.

Jackwood, M.W., 2012. Review of infectious bronchitis virus around the world. Avian Dis. 56(4), 634-641.

Jackwood, M.W., Hall, D., Handel, A., 2012. Molecular evolution and emergence of avian gammacoronaviruses. Infect. Genet. Evol. 12, 1305-1311.

Jackwood, M.W., de Wit J.J., 2013. Infectious bronchitis, in: Swayne D.E., Glisson J. R., McDougald L.R., Nolan L.K., Suarez D.L., Nair V. John Wiley and Sons eds, Diseases of poultry, 13th ed. Ames, IA, pp. 139-159.

Jenckel, M., Höper, D., Schirrmeier, H., Reimann, I., Goller, K.V., Hoffmann, B., Beer, M., 2014. Mixed triple: allied viruses in unique recent isolates of highly virulent type 2 bovine viral diarrhea virus detected by deep sequencing. J. Virol. 88(12), 6983-6992.

Ji, J., Xie, J., Chen, F., Shu, D., Zuo, K., Xue, C., Qin, J., Li, H., Bi, Y., Ma, J., Xie, Q., 2011. Phylogenetic distribution and predominant genotype of the avian infectious bronchitis virus in China during 2008-2009. Virol. J. 8, 184. doi: 10.1186/1743-422X-8- 184.

Jia, W., Mondal, S.P., Naqi, S.A., 2002. Genetic and antigenic diversity in avian infectious bronchitis virus isolates of the 1940s. Avian Dis. 46, 437-441.

Johnson, R.B., Marquardt, W.W., Newman, J.A., 1973. A new serotype of infectious bronchitis virus responsible for respiratory disease in Arkansas broiler flocks. Avian Dis. 17, 518-523.

Johnson, M.A., Pooley, C., Ignjatovic, J., Tyack, S.G., 2003. A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. Vaccine 21, 2730-2736.

Jones, R.C., Jordan, F.T.W., 1970. The exposure of day-old chicks to infectious bronchitis arid subsequent development of the oviduct. Vet. Rec. 87, 504-505.

Jones, R.C., Jordan, F.T.W., 1971. The site of replication of infectious bronchitis virus in the oviducts of experimentally infected hens. Vet. Rec. 89, 317-318.

Jones, R.C., Ambali, A.G., 1987. Re-excretion of an enterotropic infectious bronchitis virus by hens at point of lay after experimental infection at day-old. Vet. Rec. 120, 617-619.

Jones, R.C., Savage, C.E., Naylor, C.J., Cook, J.K.A., El-Houadfi, M., 2004. A possible North African progenitor of the major European infectious bronchitis virus variant (793B, 4/91, CR88, etc.),

in: Proceedings of the IV International Symposium on Avian Corona- and Pneumovirus Infections, Rauischholzhausen, Germany, pp. 105-111.

Jones, RC, Worthington, K.J., Capua, I., Naylor, C.J., 2005. Efficacy of live infectious bronchitis vaccines against a novel European genotype, Italy 02. Vet. Rec. 156(20), 646-647.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules, in: Mammalian Protein Metabolism, Munro, H.M. (Eds) New York, NY: AcademicPress, 21-132.

Jungherr, E.L., Chomiak, T.W., Luginbuhl, R.E., 1956. Immunological differences in strains of infectious bronchitis virus, in: Proceedings of the 60th Annual Meeting of the United States Livestock Sanitary Association, Chicago, IL, pp. 203-209.

Kant, A., Koch, G., van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A., van der Zeijst, B.A., 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. J. Gen. Virol. 73, 591-596.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30(4), 772-780.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12), 1647-1649.

Keeler, C.L., Reed, K.L., Nix, W.A., Gelb, J.Jr, 1998. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42(2), 275-284.

King, D.J., Hopkins, S.R., 1983. Evaluation of the haemagglutina- tion-inhibition test for measuring the response of chickens to avian infectious bronchitis virus vaccination. Avian Dis. 27, 100-112.

Kingham, B.F., Keeler, C.L. Jr, Nix, W.A., Ladman, B.S., Gelb, J. Jr., 2000. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. Avian Dis. 44(2), 325-335.

Koch, G., Hartog, L., Kant, A., van Roozelaar, D.J., 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. J. Gen. Virol. 71, 1929-1935.

Koch, G., Kant, A., Vos, J.G., de Boer, G.F., 1991. Rapid detection of infectious bronchitis virus by nucleoprotein specific antigen capture assay, in: Proceedings of the II International Symposium on Infectious Bronchitis, Rauischholzhausen , Germany, 168-172.

Kottier, S.A., Cavanagh, D., Britton P., 1995. Experimental evidence of recombination in coronavirus infectious bronchitis virus. Virology 213, 569-580.

Krapez, U., Slavec, B., Rojs, O.Z., 2011. Circulation of infectious bronchitis virus strains from Italy 02 and QX genotypes in Slovenia between 2007 and 2009. Avian Dis. 55, 155-161.

Kreider, D.L., Skeeles, J.K., Parsly, M., Newberry, L.A., Story, J.D., 1991a. Variability in a commercially available enzyme-linked immunosorbent assay system. I. Assay Variability. Avian Dis. 35, 276- 287.

Kreider, D.L., Skeeles, J.K., Parsly, M., Newberry, L.A., Story, J.D., 1991b. Variability in a commercially available enzyme-linked immunosorbent assay system. II. Laboratory Variability. Avian Dis. 35, 288-293.

Kuhner, M.K., Felsenstein, J., 1994. A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. Mol. Biol. Evol. 11(3), 459-468.

Kulkarni, A.B., Resurreccion, R.S., 2010. Genotyping of newly isolated infectious bronchitis virus isolates from northeastern Georgia. Avian Dis. 54(4), 1144-1151.

Kuo, L., Masters, P.S., 2002. Genetic evidence for a structural interaction between the carboxy termini of the membrane and nucleocapsid proteins of mouse hepatitis virus. J. Virol. 76(10), 4987-4999.

Kusters, J.G., Niesters, H.G., Bleumink-Pluym, N.M., Davelaar, F.G., Horzinek, M.C., van der Zeijst, B.A., 1987. Molecular epidemiology of infectious bronchitis virus in The Netherlands. J. Gen. Virol. 68, 343-352.

Kusters, J.G., Niesters, H.G., Lenstra, J.A., Horzinek, M.C., van der Zeijst, B.A., 1989. Phylogeny of antigenic variants of avian coronavirus IBV. Virology 169(1), 217-221.

Kwon, H.M., Jackwood, M.W., Gelb, J.Jr, 1993. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37, 194-202.

Kwon, H.M., Jackwood, M.W., 1995. Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. Virus Genes 9(3), 219-229.

Ladman, B.S., Loupos, A.B., Gelb, J., 2006. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol. 35, 127-133.

Lai, M.M.C., Cavanagh, D., 1997. The molecular biology of coronaviruses. Adv. Virus Res. 48, 1-100.

Lai, M.M.C., Perlman, S., Anderson, L.J., 2007. Coronaviridae, in: Knipe, D.M., Howley, P.M., Griffin, D.E., et al (Eds), Fields Virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia p. 1305.

Lauring, A.S., Andino, R., 2010. Quasispecies theory and the behavior of RNA viruses. PLoS Pathog. 6(7):e1001005. doi: 10.1371/journal.ppat.1001005.

Lee, C.W., Jackwood, M.W., 2000. Evidence of genetic diversity generated by recombination among avian coronavirus IBV. Arch. Virol. 145, 2135-2148.

Lee, C.W., Hilt, D.A., Jackwood, M.W., 2000. Redesign of primer and application of the reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism test to the DE072 strain of infectious bronchitis virus. Avian Dis. 44, 650-654.

Lee, C.W., Jackwood, M.W., 2001. Origin and evolution of Georgia 98 (GA98), a new serotype of avian infectious bronchitis virus. Virus Res. 80(1-2), 33-39.

Lee, C.W., Hilt, D.A., Jackwood, M.W., 2001. Identification and analysis of the Georgia 98 serotype, a new serotype of infectious bronchitis virus. Avian Dis. 45, 164-172.

Lee, C.W., Hilt, D.A., Jackwood, M.W., 2003. Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. J. Vet. Diagn. Invest. 15(4), 344-348.

Lee, E.K., Jeon, W.J., Lee, Y.J., Jeong, O.M., Choi, J.G., Kwon, J.H., Choi, K.S., 2008. Genetic diversity of avian infectious bronchitis virus isolates in Korea between 2003 and 2006. Avian Dis. 52(2), 332-337.

Lee, H.J., Youn, H.N., Kwon, J.S., Lee, Y.J., Kim, J.H., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S., 2010. Characterization of a novel live attenuated infectious bronchitis virus vaccine candidate

derived from a Korean nephropathogenic strain. Vaccine 28(16), 2887-2894. doi: 10.1016/j.vaccine.2010.01.062.

Leparc-Goffart, I., Hingley, S.T., Chua, M.M., Jiang, X., Lavi, E., Weiss, S.R., 1997. Altered pathogenesis of a mutant of the murine coronavirus MHV-A59 is associated with a Q159L amino acid substitution in the spike protein. Virology 239, 1-10.

Li, J., Cook, J.K.A., Brown, T.D.K., Shaw, K., Cavanagh, D., 1993. Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. Avian Pathol. 22, 771-784.

Li, W., Zhang, C., Sui, J., Kuhn, J.H., Moore, M.J., Luo, S., Wong, S.K., Huang, I.C., Xu, K., Vasilieva, N., 2005. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. EMBO J. 24, 1634-1643.

Li, L., Xue, C., Chen, F., Qin, J., Xie, Q., Bi, Y., Cao, Y., 2010. Isolation and genetic analysis revealed no predominant new strains of avian infectious bronchitis virus circulating in South China during 2004-2008. Vet. Microbiol. 143(2-4), 145-154.

Li, M., Wang, X.Y., Wei, P., Chen, Q.Y., Wei, Z.J., Mo, M.L., 2012. Serotype and genotype diversity of infectious bronchitis viruses isolated during 1985–2008 in Guangxi, China. Arch. Virol. 157, 467-474.

Li, M., Mo, M.L., Huang, B.C., Fan, W.S., Wei, Z.J., Wei, T.C., Li, K.R., Wei, P., 2013. Continuous evolution of avian infectious bronchitis virus resulting in different variants co-circulating in southern China. Arch. Virol. 158(8), 1783-1786.

Lim, K.P., Liu, D.X., 2001. The missing link in coronavirus assembly. Retention of the avian coronavirus infectious bronchitis virus envelope protein in the pre-golgi compartments and physical interaction between the envelope and membrane proteins. J. Biol. Chem. 276, 17515-17523.

Lim, T.H., Lee, H.J., Lee, D.H., Lee, Y.N., Park, J.K., Youn, H.N., Kim, M.S., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S., 2011. An emerging recombinant cluster of nephropathogenic strains of avian infectious bronchitis virus in Korea. Infect. Genet. Evol. 11(3), 678-685.

Lim, T.H., Kim, M.S., Jang, J.H., Lee, D.H., Park, J.K., Youn, H.N., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S., 2012. Live attenuated nephropathogenic infectious bronchitis virus vaccine provides broad cross protection against new variant strains. Poult. Sci. 91, 89-94.

Lin, Z., Kato, A., Kudou, Y., Ueda, S., 1991. A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. Arch. Virol. 116, 19-31.

Liu, H.J., Lee, L.H., Shih, W.L., Lin, M.Y., Liao, M.H., 2003. Detection of infectious bronchitis virus by multiplex polymerase chain reaction and sequence analysis. J. Virol. Methods 109, 31-37.

Liu, S., Kong, X., 2004. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non- vaccinated flocks in China. Avian Pathol. 33, 321-327.

Liu, S., Chen, J., Han, Z., Zhang, Q., Shao, Y., Kong, X., Tong, G., 2006a. Infectious bronchitis virus: S1 gene characteristics of vaccines used in China and efficacy of vaccination against heterologous strains from China. Avian Pathol. 35, 394-399.

Liu, S.W., Zhang, Q.X., Chen, J.D., Han, Z.X., Liu, X., Feng, L., Shao, Y.H., Rong, J.G., Kong, X.G., Tong, G.Z., 2006b. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. Arch. Virol. 151(6), 1133-1148.

Liu, S., Zhang, X., Wang, Y., Li, C., Han, Z., Shao, Y., Li, H., Kong, X., 2009. Molecular characterization and pathogenicity of infectious bronchitis coronaviruses: complicated evolution and epidemiology in China caused by cocirculation of multiple types of infectious bronchitis coronaviruses. Intervirology 52(4), 223-234.

Lohr, J.E., 1976. Serological differences between strains of infectious bronchitis virus from New Zealand, Australia and the United States. Avian Dis. 20, 478-482.

Lohr, J.E., 1977. Studies on avian infectious bronchitis virus in New Zealand. I. Serotypes. N. Z. Vet. J. 25(3), 48-51.

Lohr, J.E., 1981. Diagnosis of infectious bronchitis (IB) by examination of tracheal mucus for IB precipitating antigens. Avian Dis. 25, 1058-1064.

Lohr, J.E., 1988. Differentiation of IBV strains, in: Proceedings of the I International Symposium on Infectious Bronchitis, Rauischholzhausen, Germany, pp.199-207.

Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73(1), 152-60.

Ma, H., Shao, Y., Sun, C., Han, Z., Liu, X., Guo, H., Kong, X., Liu, S., 2012. Genetic diversity of avian infectious bronchitis coronavirus in recent years in China. Avian Dis. 56, 15-28.

Magiorkinis, G., Magiorkinis, E., Paraskevis, D., Vandamme, A.M., Van Ranst, M., Moulton, V., Hatzakis, A., 2004. Phylogenetic analysis of the full-length SARSCoV sequences: evidence for phylogenetic discordance in three genomic regions. J. Med. Virol. 74, 369-372.

Mahmood, Z.H., Sleman, R.R., Uthman, A.U., 2011. Isolation and molecular characterization of Sul/01/09 avian infectious bronchitis virus, indicates the emergence of a new genotype in the Middle East. Vet. Microbiol. 150, 21-27.

Marandino, A., Pereda, A., Tomás, G., Hernández, M., Iraola, G., Craig, M.I., Hernández, D., Banda, A., Villegas, P., Panzera, Y., Pérez, R., 2015. Phylodynamic analysis of avian infectious bronchitis virus in South America. J. Gen. Virol. 96, 1340- 1346.

Marçola, T.G., Gomes, C.P., Silva, P.A., Fernandes, G.R., Paludo, G.R., Pereira, R.W., 2013. Identification of a novel subtype of feline immunodeficiency virus in a population of naturally infected felines in the Brazilian Federal District. Virus Genes 46, 546-550.

Mardani, K., Noormohammadi, A.H., Ignjatovic, J., Browning, G.F., 2010. Naturally occurring recombination between distant strains of infectious bronchitis virus. Arch. Virol. 155, 1581-1586.

Marquardt, W.W., Snyder, D.B., Schlotthober, B.A., 1981. Detection and quantification of antibodies to infectious bronchitis virus by enzyme-linked immunosorbent assay. Avian Dis. 25, 713-722.

Martin, M.P., Waknell, P.S., Woolcock, P., 2001. Evaluation of commercially produced infectious bronchitis virus vaccines against an IBV field isolate obtained from broilers in California, in: Proceedings of the 50th Western Poultry Disease Conference, Sacramento, CA, pp. 108-109.

Martin, D.P., Lemey, P., Lott, M., Moulton, V., Posada, D., Lefeuvre, P., 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinfomatics 26, 2462-2463.

Martin, E.A., Brash, M.L., Hoyland, S.K., Coventry, J.M., Sandrock, C., Guerin, M.T., Ojkic, D., 2014. Genotyping of infectious bronchitis viruses identified in Canada between 2000 and 2013. Avian Pathol. 43(3), 264-268.

Mase, M., Tsukamoto, K., Imai, K., Yamaguchi, S., 2004. Phylogenetic analysis of avian infectious bronchitis virus strains isolated in Japan. Arch. Virol. 149(10), 2069- 2978.

Mase, M., Kawanishi, N., Ootani, Y., Murayama, K., Karino, A., Inoue, T., Kawakami, J., 2010. A novel genotype of avian infectious bronchitis virus isolated in Japan in 2009. J. Vet. Med. Sci. 72, 1265-1268.

Masters, P.S., 2006. The molecular biology of coronaviruses. Adv. Virus Res. 66, 193-292.

Matsuyama, S., Taguchi, F., 2002. Receptor-induced conformational changes of murine coronavirus spike protein. J. Virol. 76(23), 11819-11826.

McDougall, J.S., 1968. Infectious bronchitis in laying fowls: its effect upon egg production and sub-sequent egg quality. Vet. Rec. 83, 84-86.

McFarlane, R., Verma, R., 2008. Sequence analysis of the gene coding for the S1 glycoprotein of infectious bronchitis virus (IBV) strains from New Zealand. Virus Genes 37(3), 351-357.

McMartin, D.A., 1993. Infectious bronchitis, in: McFerran, J.B., McNulty, M.S. (Eds.), Virus Infections of Vertebrates. Virus Infections of birds , Vol 4, Amsterdam, Elsevier Science Publishers, pp. 249-275.

Meir, R., Rosenblut, E., Perl, S., Kass, N., Ayali, G., Perk, S., Hemsani, E., 2004. Identification of a novel nephropathogenic infectious bronchitis virus in Israel. Avian Dis. 48, 635-641.

Meir, R., Maharat, O., Farnushi, Y., Simanov, L., 2010. Development of a real-time TaqMan® RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. J. Virol. Methods 163, 190-194.

Meulemans, G., Carlier, M.C., Gonze, M., Petit, P., Vandenbroeck, M., 1987. Incidence, characterisation and prophylaxis of nephropathogenic avian infectious bronchitis viruses. Vet. Rec. 120, 205-206.

Meulemans, G., Boschmans, M., Decaesstecker, M., van den Berg, T.P., Denis, P., Cavanagh, D., 2001. Epidemiology of infectious bronchitis virus in Belgian broilers: A retrospective study, 1986 to 1995. Avian Pathol. 30(4), 411-421.

Mihindukulasuriya, K.A., Wu, G., St Leger, J., Nordhausen, R.W., Wang, D., 2008. Identification of a novel coronavirus from a beluga whale by using a panviral microarray. J. Virol. 82, 5084 - 5088.

Minskaia, E., Hertzig, T., Gorbalenya, A.E., Campanacci, V., Cambillau, C., Canard, B., Ziebuhr, J., 2006. Discovery of an RNA virus 3'-5' exoribonuclease that is critically involved in coronavirus RNA synthesis. Proc. Natl. Acad. Sci. USA 103, 5108-5113.

Minta, Z., Bugajak, P., Karpinska, E., Gough, R, Cavanagh, D., Mawditt, K., Cox, W.J., Bartnicka, B., Wiercinski, J., 1998. Isolation of new strains of IBV from broiler chickens in Poland, in: Proceedings of International Symposium on Infectious Bronchitis and Pneumovirus infections in Poultry, Rauischholzhausen , Germany, 180-188.

Mirand, A., Archimbaud, C., Henquell, C., Michel, Y., Chambon, M., Peigue-Lafeuille, H., Bailly, J.L., 2006. Prospective identification of HEV-B enteroviruses during the 2005 outbreak. J. Med. Virol. 78, 1624-1634.

Mo, M.L., Li, M., Huang, B.C., Fan, W.S., Wei, P., Wei, T.C., Cheng, Q.Y., Wei, Z.J., Lang, Y.H., 2013. Molecular characterization of major structural protein genes of avian coronavirus infectious bronchitis virus isolates in southern china. Viruses 5(12), 3007- 3020.

Mockett, A.P.A., Darbyshire, J.H., 1981. Comparative studies with an enzyme-linked immunosorbent assay (ELISA) for antibodies to avian infectious bronchitis virus. Avian Pathol., 10, 1-10.

Mockett, A.P., Cavanagh, D., Brown, T.D.K., 1984. Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M41. J. Gen. Virol. 65, 2281-2286.

Mondal, .P., Lucio-Mart ne , B., Naqi, S.A., 2001. Isolation and characterization of a novel antigenic subtype of infectious bronchitis virus serotype DE072. Avian Dis. 45(4), 1054-1059.

Mondal, S.P., Cardona, C.J., 2004. Comparison of four regions in the replicase gene of heterologous infectious bronchitis virus strains. Virology 324, 238-248.

Mondal, S.P., Cardona, C.J., 2007. Genotypic and phenotypic characterization of the

California 99 (Cal99) variant of infectious bronchitis virus. Virus Genes 34, 327-341.

Mondal, S., Chang, Y.F., Balasuriya, U., 2013. Sequence analysis of infectious bronchitis virus isolates from the 1960s in the United States. Arch. Virol. 158(2), 497- 503.

Monne, I., Cattoli, G., Jones, R., Worthington, K., Wijmenga, W., 2008. QX genotypes of infectious bronchitis virus circulating in Europe. Vet. Rec. 163, 606-607.

Monne, I., Drago, A., Fasolato, M., Capua, I., Cattoli, G., 2009. Molecular survey of infectious bronchitis virus in Europe in 2008, in: Proceedings of the VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, pp. 34.

Monreal, G., Bauer, H.J., Wiegmann, J., 1985. Comparison of the enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition test and agar gel precipitation test for detection of antibodies to avian infectious bronchitis virus. Avian Pathol. 14, 421- 434.

Montassier, H.J., 2010. Molecular epidemiology and evolution of avian infectious bronchitis virus. Rev. Bras. Cienc. Avic. 12(2), 87-96. Available from: http://dx.doi.org/10.1590/S1516-635X2010000200003.

Moore, K.M., Jackwood, M.W., Hilt, D.A., 1997. Identification of amino acids involved in a serotype and neutralization specific epitope within the s1 subunit of avian infectious bronchitis virus. Arch. Virol. 142(11), 2249-2256.

Moore, K.M., Bennett, J.D., Seal, B.S., Jackwood, M.W., 1998. Sequence comparison of avian infectious bronchitis virus S1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California. Virus Genes 17, 63-83.

Moscoso, H., Raybon, E.O., Thayer, S.G., Hofacre, C.L., 2005. Molecular detection and serotyping of infectious bronchitis virus from FTA® filter paper. Avian Dis. 49(1), 24-29.

Moya, A., Elena, S.F., Bracho, A., Miralles, R., Barrio, E., 2000. The evolution of RNA viruses: a population genetics view. Proc. Natl. Acad. Sci. USA 97(13), 6967-73.

Muneer, M.A., Halvorson, D.A., Sivanandan, V., Newman, J.A., Coon, C.N., 1986. Effects of infectious bronchitis (Arkansas strain) on laying chickens. Avian Dis. 30, 644-647.

Muneer, M.A., Newman, J.A., Halvorson, D.A., Sivanandan, V., Nagaraja, K.V., Coon, C.N., 1988. Efficacy of infectious bronchitis virus vaccines against heterologous challenge. Res. Vet. Sci. 45, 22-27.

Nagano, H., Tsuchimoto, M., Hohdatsu, T., Yamagami, T., Ide, S., Eiguchi, Y., Tanaka, Y., Yamagishi, H., 1990. Enzyme-linked immunosorbent assay for the detection of infectious bronchitis virus (IBV) antigen with monoclonal antibody. Nihon Juigaku Zasshi 52, 657-659.

Najafi, H., Langeroudi, A.G., Hashemzadeh, M., Karimi, V., Madadgar, O., Ghafouri, S.A., Maghsoudlo, H., Farahani, R.K., 2015. Molecular characterization of infectious bronchitis

viruses isolated from broiler chicken farms in Iran, 2014-2015. Arch. Virol. 2015 Oct 14. doi:10.1007/s00705-015-2636-3.

Naqi, S.A., Karaca, K., Bauman, B. 1993. A monoclonal antibody- based antigen capture enzyme-linked immunosorbent assay for identification of infectious bronchitis virus serotypes. Avian Pathol. 22, 555-564.

Narayanan, K., Maeda, A., Maeda, J., Makino, S., 2000. Characterization of the coronavirus M protein and nucleocapsid interaction in infected cells. J. Virol. 74(17), 8127-8134.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics, 1st edn, Edited by Anonymous.

Neuman, B.W., Joseph, J.S., Saikatendu, K.S., Serrano, P., Chatterjee, A., Johnson, M.A., Liao, L., Klaus, J.P., Yates, J.R. 3rd, Wüthrich, K., Stevens, R.C., Buchmeier, M.J., Kuhn, P., 2008. Proteomics analysis unravels the functional repertoire of coronavirus nonstructural protein 3. J. Virol. 82, 5279-5294.

Nguyen, V.P., Hogue, B.G., 1997. Protein interactions during coronavirus assembly. J. Virol. 71, 9278-9284.

Niesters, H.G., Bleumink-Pluym, N.M., Osterhaus, A.D., Horzinek, M.C., van der Zeijst, B.A., 1987. Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. Virology 161, 511-519.

Oberste, M.S., Maher, K., Kilpatrick, D.R., Pallansch, M.A., 1999. Molecular evolution of the human enteroviruses: Correlation of serotype with VP1 sequence and application to picornavirus classification. J. Virol. 73, 1941-1948.

Ojkic, D., Binnington, B., 2002. Phylogenetic analysis of Ontario infectious bronchitis virus isolates, in: Proceedings of 51st Western Poultry Disease Conference, Sacramento, California, 73.

Ontiveros, E., Kim, T.S., Gallagher, T.M., Perlman, S., 2003. Enhanced virulence mediated by the murine coronavirus, mouse hepatitis virus strain JHM, is associated with a glycine at residue 310 of the spike glycoprotein. J. Virol. 77, 10260-10269.

Opstelten, D.J., Raamsman, M.J., Wolfs, K., Horzinek, M.C., Rottier, P.J., 1995. Envelope glycoprotein interactions in coronavirus assembly. J. Cell. Biol. 131(2), 339-49.

Parsons, D., Ellis, M.M., Cavanagh, D., Cook, J.K., 1992. Characterisation of an avian infectious bronchitis virus isolated from IB-vaccinated broiler breeder flocks. Vet. Rec. 131, 408-411.

Phillips, J.J., Chua, M.M., Rall, G.F., Weiss, S.R., 2002. Murine coronavirus spike glycoprotein mediates degree of viral spread, inflammation, and virus-induced immunopathology in the central nervous system. Virology 301, 109-120.

Picault, J.P., Drouin, P., Lamande, J., Allee, C., Toux, J.Y., Le Coq, H., Guittet, M., Bennejean, G., 1995. L'epizootie recente de bronchite infectieuse aviaire en France: importance, evolution et etiologie, in: Proceedings of the 1eres Journée de la Recherche Avicole, Angers, France, pp. 177-179.

Pohl, R.M., 1967. Infectious bronchitis in chickens. N. Z. Vet. J. 15, 151.

Pohl, R., 1974. The histopathogenesis of nephrosis/nephritis syndrome. Avian Pathol. 3, 1-13.

Purcell, D.A., Tham, V.L., Surman, P.G., 1976. The histopathology of infectious bronchitis in fowls infected with a nephrotropic 'T' strain of virus. Aust. Vet. J., 52, 85-91.

Raj, G.D., Jones, R.C., 1996. Protectotypic differentiation of avian infectious bronchitis viruses using an in vitro challenge model. Vet. Microbiol. 53, 239-252.

Raj, G.D., Jones, R.C., 1997. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. Avian Pathol. 26(4), 677-706.

Rambaut, A., Lam, T.T., Carvalho, L.M., Pybus, O.G., 2016. Exploring the temporal structure of heterochronous sequences using TempEst. Virus Evolution 2: vew007 DOI: http://dx.doi.org/10.1093/ve/vew007.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Ruch, T.R., Machamer, C.E., 2011. The hydrophobic domain of infectious bronchitis virus E protein alters the host secretory pathway and is important for release of infectious virus. J. Virol. 85(2), 675-685.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4), 406-425.

Sapats, S.I., Ashton, F., Wright, P.J., Ignjatovic, J., 1996. Sequence analysis of the S1 glycoprotein of infectious bronchitis viruses: identification of a novel genotypic group in Australia. J. Gen. Virol. 77, 413-418.

Sato, T., Sugimori, T., Ishii, S., Matumoto, M., 1955. Infectious bronchitis of chickens in Japan. I. Epidemiological clinical and pathological studies and isolation of the causative virus in embryonated eggs. Jpn. J. Exp. Med. 25, 115-131.

Sawicki, S.G., Sawicki, D.L., Siddell, S.G., 2007. A contemporary view of coronavirus transcription. J. Virol. 81, 20-29.

Schalk, A.E, Hawn, M.C., 1931. An apparently new respiratory disease of chicks. J. Am. Vet. Med. Assoc. 78, 413-422.

Schikora, B.M., Shih, L.M., Hietala, S.K., 2003. Genetic diversity of avian infectious bronchitis virus California variants isolated between 1988 and 2001 based on the S1 subunit of the spike glycoprotein. Arch. Virol. 148, 115-136.

Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grassland, B., Baekbo, P., Allan, G., 2008. PCV2 genotype definition and nomenclature. Vet. Rec. 162, 867-868.

Sesti, L., Sara, L., Alvarado, L., Mató, T., Palya, V., de Wit J.J., 2014. Diagnostic, epidemiology and control of the Q1 infectious bronchitis virus (IBV) variant strain in Peru, Colombia, Argentina and Chile, in: Proceedings of the VIII International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, pp. 56-64.

Sevoian, M., Levine, P.P., 1957. Effects of infectious bronchitis on the reproductive tracts, egg production, and egg quality of laying chickens. Avian Dis. 1, 136-164.

Shieh, H.K., Shien, J.H., Chou, H.Y., Shimizu, Y., Chen, J.N., Chang, P.C., 2004. Complete nucleotide sequences of S1 and N genes of infectious bronchitis virus isolated in Japan and Taiwan. J. Vet. Med. Sci. 66(5), 555-558.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114-1116.

Shoemaker, J.S., Fitch, W.M., 1989. Evidence from nuclear sequences that invariable sites should be considered when sequence divergence is calculated. Mol. Biol. Evol. 6, 270-289.

Simmonds, P., 2015. Methods for virus classification and the challenge of incorporating metagenomic sequence data. J. Gen. Virol. 96, 1193-1206.

Smati, R., Silim, A., Guertin, C., Henrichon, M., Marandi, M., Arella, M., Merzouki, A., 2002. Molecular characterization of three new avian infectious bronchitis virus (IBV) strains isolated in Québec. Virus Genes 25, 85-93.

Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C., Thiel, V., Ziebuhr, J., Poon, L.L., Guan, Y., Rozanov, M., Spaan, W.J., Gorbalenya, A.E., 2003. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol. 331, 991-1004.

Snyder, D.B., Marquardt, W.W., 1989. Enzyme immunoassay for poultry disease monitoring, in: Purchase, H.G., Arp, L.H., Domermuth, C.H., Pearson, J.E. (Eds). A laboratory manual for the isolation and identification of avian pathogens, 3rd ed. Dubuque, Iowa Kendall-Hunt Publishing Co 45.

Sodora, D.L., Shpaer, E.G., Kitchell, B.E., Dow, S.W., Hoover, E.A., Mullins, J.I., 1994. Identification of three feline immunodeficiency virus (FIV) env gene subtypes and comparison of the FIV and human immunodeficiency virus type 1 evolutionary patterns. J. Virol. 68, 2230-2238.

Song, C.S., Lee, Y.J., Lee, C.W., Sung, H.W., Kim, J.H., Mo, I.P., Izumiya, Y., Jang, H.K., Mikami, T., 1998a. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. J. Gen. Virol. 79, 719-723.

Song, C.S., Lee, Y.J., Kim, J.H., Sung, H.W., Lee, C.W., Izumiya, Y., Miyazawa, T., Jang, H.K., Mikami, T., 1998b. Epidemiology classification of infectious bronchitis virus isolated in Korean between 1986 and 1997. Avian Pathol. 27, 409-416.

Spaan, W., Cavanagh, D., Horzinek, M.C., 1988. Coronaviruses: structure and genome expression. J. Gen. Virol. 69, 2939-2952.

Spencer, K.A., Dee, M., Britton, P., Hiscox, J.A., 2008. Role of phosphorylation clusters in the biology of the coronavirus infectious bronchitis virus nucleocapsid protein. Virology 370(2), 373-381.

Stephensen, C.B., Casebolt, D.B., Gangopadhyay, N.N., 1999. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. Virus Res. 60, 181-189.

Sturman, L.S., Holmes, K.V., Behnke, J., 1980. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. J. Virol. 33(1), 449-462.

Sturman, L.S., Ricard, C.S., Holmes, K.V., 1990. Conformational change of the coronavirus peplomer glycoprotein at pH 8.0 and 37 degrees °C correlates with virus aggregation and virus-induced cell fusion. J. Virol. 64(6), 3042-3050.

Sun, C., Han, Z., Ma, H., Zhang, Q., Yan, B., Shao, Y., Xu, J., Kong, X., Liu, S., 2011. Phylogenetic analysis of infectious bronchitis coronaviruses newly isolated in China, and pathogenicity and evaluation of protection induced by Massachusetts serotype H120 vaccine against QX-like strains. Avian Pathol. 40(1), 43-54.

Sutou, S., Sato, S., Okabe, T., Nakai, M., Sasaki, N., 1988. Cloning and sequencing of genes encoding structural proteins of avian infectious bronchitis virus. Virology 165, 589-595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

Tejero, H., Marín, A., Montero, F., 2011. The relationship between the error catastrophe, survival of the flattest, and natural selection. BMC Evol. Biol. 11 doi: 10.1186/1471-2148-11-2.

Terregino, C., Toffan, A., Beato, M.S., De Nardi, R., Vascellari, M., Meini, A., Ortali, G., Mancin, M., Capua, I., 2008. Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial broiler chickens, and evaluation of protection induced by a vaccination programme based on the Ma5 and 4/91 serotypes. Avian Pathol. 37, 487-493.

Thor, S.W., Hilt, D.A., Kissinger, J.C., Paterson, A.H., Jackwood, M.W., 2011. Recombination in avian gamma-coronavirus infectious bronchitis virus. Viruses 3(9), 1777-1799.

Toffan, A., Monne, I., Terregino, C., Cattoli, G., Hodobo, C.T., Gadaga, B., Makaya, P.V., Mdlongwa, E., Swiswa, S., 2011. QX-like infectious bronchitis virus in Africa. Vet. Rec. 169, 589.

Toffan, A., Bonci, M., Bano, L., Valastro, V., Vascellari, M., Capua, I., Terregino, C., 2013. Diagnostic and clinical observation on the infectious bronchitis virus strain Q1 in Italy. Vet. Ital. 49(4), 347-355. doi: 10.12834/VetIt.1303.01.

Ujike, M., Taguchi, F., 2015. Incorporation of Spike and Membrane Glycoproteins into Coronavirus Virions. Viruses 7(4), 1700-1725.

Valastro, V., Monne, I., Fasolato, M., Cecchettin, K., Parker, D., Terregino, C., Cattoli, G., 2010. QX-type infectious bronchitis virus in commercial flocks in the UK. Vet. Rec. 167, 865-866.

Valastro, V., Monne, I., Koopman, R., Currie, R.J.W., Cattoli, G., 2014. An update of infectious bronchitis virus strains circulating in Europe between 2011-2013, in: Proceedings of the XIVth European Poultry Conference, Stavanger, Norway, 444.

Vandekerchove, D., De Herdt, P., Laevens, H., Butaye, P., Meulemans, G., Pasmans, F., 2004. Significance of interactions between Escherichia coli and respiratory pathogens in layer hen flocks suffering from colibacillosis-associated mortality. Avian Pathol. 33, 298-302.

van Roeckel, H., Bullis, K.L., Flint, O.S., Clarke, M.K., 1942. Poultry disease control service Massachusetts Agricultural Experiment Station, MA. Annual Report, Bulletin 388, 99-103.

van Santen, V.L., Toro, H., 2008. Rapid selection in chickens of subpopulations within ArkDPI-derived infectious bronchitis virus vaccines. Avian Pathol. 37, 293-306.

Vilcek, S., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M.T., Paifi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. Virol. 146, 99- 115.

Villarreal, L.Y.B., Brandão, P.E., Chacón, J.L., Assayag, M.S., Maiorka, P., Raffi, P., Saidenberg, A.B.S., Jones, R.C., Ferreira, A.J.P., 2007a. Orchitis in roosters with reduced fertility associated with avian infectious bronchitis virus and avian metapneumovirus infections. Avian Dis. 51, 900-904.

Villarreal, L.Y.B., Brandão, P.E., Chacón, J.L., Saidenberg, A.B.S., Assayag, M.S., Jones, R.C., Ferreira, A.J.P., 2007b. Molecular characterization of infectious bronchitis virus strains isolated from the enteric contents of Brazilian laying hens and broilers. Avian Dis. 51, 974-978.

Villarreal, L. 2010. Diagnosis of infectious bronchitis: an overview of concepts and tools. Rev. Bras. Cienc. Avic. 12, 111-114.

Villarreal, L.Y.B., Sandri, T.L., Souza, S.P., Richtzenhain, L.J., de Wit, J.J., Brandão, P.E., 2010. Molecular epidemiology of avian infectious bronchitis in Brazil from 2007 to 2008 in breeders, broilers, and layers. Avian Dis. 54, 894-898.

Wang, L., Junker, D., Hock, L., Ebiary, E., Collisson, E.W., 1994. Evolutionary implications of genetic variations in the S1 gene of infectious bronchitis virus. Virus Res. 34(3), 327-338.

Wang, C.H., Tsai, C.T., 1996. Genotypic grouping for the isolates of avian infectious bronchitis virus in Taiwan. Arch. Virol. 141, 1677-1688.

Wang, Y.D., Wang, Y.L., Zhang, Z., Fan, G., Jlang, Y., Liu, X., Ding, J., Wang, S., 1998. Isolation and identification of glandular stomach type IBV (QX IBV) in chickens. Chin. J. Anim. Quarantine 15, 1-3.

Wang, C.H., Huang, Y.C., 2000. The relationship between serotype and genotype based on hypervariable region of S1 gene of infectious bronchitis virus. Arch. Virol. 145, 291-300.

Weber, M.N., Streck, A.F., Silveira, S., Mósena, A.C., Silva, M.S., Canal, C.W., 2015. Homologous recombination in pestiviruses: identification of three putative novel events between different subtypes/genogroups. Infect. Genet. Evol. 30, 219-224.

Wesley, R.D., Woods, R.D., Cheung, A.K., 1991. Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus. J. Virol. 65, 3369-3373.

Westerbeck, J.W., Machamer, C.E., 2015. A Coronavirus E protein is Present in two distinct pools with different effects on assembly and the secretory pathway. J. Virol. 89, 9313-9323.

WHO/OIE/FAO H5N1 Evolution Working Group, 2012. Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. Influenza Other Respir. Viruses 6(1), 1-5. doi: 10.1111/j.1750-2659.2011.00298.x.

Williams, A.K., Wang, L., Sneed, L.W., Collison, E.W., 1992. Comparative analyses of the nucleocapsid genes of several strains of infectious bronchitis virus and other corona viruses. Virus Res. 25, 213-222.

Winter, C., Schwegmann-Wessels, C., Cavanagh, D., Neumann, U., Herrler, G., 2006. Sialic acid is a receptor determinant for infection of cells by avian infectious bronchitis virus. J. Gen. Virol. 87, 1209-1216.

Winter, C., Herrler, G., Neumann, U., 2008a. Infection of the tracheal epithelium by infectious bronchitis virus is sialic acid dependent. Microbes Infect. 10, 367-373.

Winter, C., Schwegmann-Wessels, C., Neumann, U., Herrler, G., 2008b. The spike protein of infectious bronchitis virus is retained intracellularly by a tyrosine motif. J. Virol. 82(6), 2765-2771.

Winterfield, R.W., Hitchner, S.B., 1962. Etiology of an infectious nephritis-nephrosis syndrome of chickens. Am. J. Vet. Res. 23, 1273-1279.

Winterfield, R.W., Hitchner, S.B., Appleton, G.S., 1964. Immunological characteristics of a variant of infectious bronchitis virus isolated from chickens. Avian Dis. 8, 40-47.

Winterfield, R.W., Fadly, A.M., 1975. Potential for polyvalent infectious bronchitis vaccines. Am. J. Vet. Res. 36, 524-526.

Winterfield, R.W., Thacker, H.L., Badylak, S.F., 1984. Effects of subtype variations in the Holland strain of infectious bronchitis virus when applied as a vaccine. Poult. Sci. 63, 246-250.

Woo, P.C., Lau, S.K., Huang, Y., Yuen, K.Y., 2009. Coronavirus diversity, phylogeny and interspecies jumping. Exp. Biol. Med. 234, 1117-1127.

Woo, P.C., Lau, S.K., Lam, C.S., Tsang, A.K., Lau, J.H., Bai, R., Teng, J.L., Tsang, C.C., Wang, M., Zheng, B.J., Chan, K.H., Yuen, K.Y., 2012. Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J. Virol. 86(7), 3995-4008.

Woo, P.C., Lau, S.K., Lam, C.S., Tsang, A.K., Hui, S.W., Fan, R.Y., Martelli, P., Yuen, K.Y., 2014. Discovery of a novel bottlenose dolphin coronavirus reveals a distinct species of marine mammal coronavirus in Gammacoronavirus. J. Virol. 88(2), 1318-1331.

Wood, M.K., Ladman, B.S., Preskenis, L.A., Pope, C.R., Bautista, D., Gelb, J. Jr., 2009. Massachusetts live vaccination protects against a novel infectious bronchitis virus S1 genotype DMV/5642/06. Avian Dis. 53(1), 119-123.

Worobey, M., Holmes, E.C., 1999. Evolutionary aspects of recombination in RNA viruses. J. Gen. Virol. 80, 2535-2543.

Worthington, K.J., Savage, C., Naylor, C.J., Wijmenga, W., Jones, R.C., 2004. An RTPCR survey of infectious bronchitis virus genotypes in the UK and selected European countries between 2002 and 2004 and the results from a vaccine trial, in: Proceedings of the IV International Symposium on Avian Corona- and Pneumovirus Infections, Rauischholzhausen, Germany, pp. 125-133.

Worthington, K.J., Currie, R.J., Jones, R.C., 2008. A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. Avian Pathol. 37(3), 247-257.

Yagyu, K., Ohta, S., 1987. Enzyme-linked immunosorbent assay for the detection of infectious bronchitis virus antigens. Nihon Juigaku Zasshi. 49, 757-763.

Yang, Z., 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. J. Mol. Evol. 39, 306-314.

Ye, Y., Hogue, B.G., 2007. Role of the coronavirus E viroporin protein transmembrane domain in virus assembly. J. Virol. 81(7), 3597-3607.

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

Zelus, B.D., Schickli, J.H., Blau, D.M., Weiss, S.R., Holmes, K.V., 2003. Conformational changes in the spike glycoprotein of murine coronavirus are induced at 37 degrees C either by soluble murine CEACAM1 receptors or by pH 8. J. Virol. 77(2), 830-840.

Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E., 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. 81(4), 853-879.

Ziegler, A.F., Ladman, B.S., Dunn, P.A., Schneider, A., Davison, S., Miller, P.G., Lu, H., Weinstock, D., Salem, M., Eckroade, R.J., Gelb, J. Jr., 2002. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. Avian Dis. 46(4), 847-858.

Zulperi, Z.M., Omar, A.R., Arshad, S.S., 2009. Sequence and phylogenetic analysis of S1, S2, M, and N genes of infectious bronchitis virus isolates from Malaysia. Virus Genes 38, 383-391.

Zwaagstra, K.A., van der Zeijst, B.A.M., Kusters, J.G., 1992. Rapid detection and identification of avian infectious bronchitis virus. J. Clin. Microbiol. 30, 79-84.

ANNEX

Figure A1. ML phylogenetic tree of 1518 complete S1 nucleotide sequences. Recombinant sequences and those considered to be unreliable are labelled in red and in green, respectively (and were removed from the main analysis). The coloured boxes designate the GI-19, -13 and -21 lineages that no longer appeared as monophyletic groups. SH-like branch supports are shown for key nodes. The scale bar represents the number of nucleotide substitutions per site, and the tree is mid-point rooted for clarity only.

Figure A2. Neighbor-Joining phylogenetic tree of complete S1 gene nucleotide sequences. A total of 199 IBV strains, including 6 representative sequences of each lineage detected and 26 unique variants were analyzed. Each lineage is colour-coded and its corresponding designation is reported. Bars reporting the genotypes in which the lineages fall are shown. The designation "UV" indicates unique variants, here marked in black. The red box designates the 27 lineages within GI. GenBank accession number, isolate number or name, country of origin and collection date is given for each strain. Bootstrap support values are shown for key nodes. The scale bar represents the number of substitutions per site, and the tree is mid-point rooted for clarity only.

Figure A3. Bayesian (MrBayes) phylogenetic tree of complete S1 nucleotide sequences. A total of 199 IBV strains, including 6 representative sequences of each lineage detected and 26 unique variants were analyzed. Each lineage is colour-coded and its corresponding designation is reported. Bars reporting the genotypes in which the lineages fall are shown. The designation "UV" indicates unique variants, here marked in black. The red box designates the 27 lineages within GI. GenBank accession number, isolate number or name, country of origin and collection date is given for each strain. Posterior probabilities are shown for key nodes. The scale bar represents the number of substitutions per site, and the tree is mid-point rooted for clarity only.

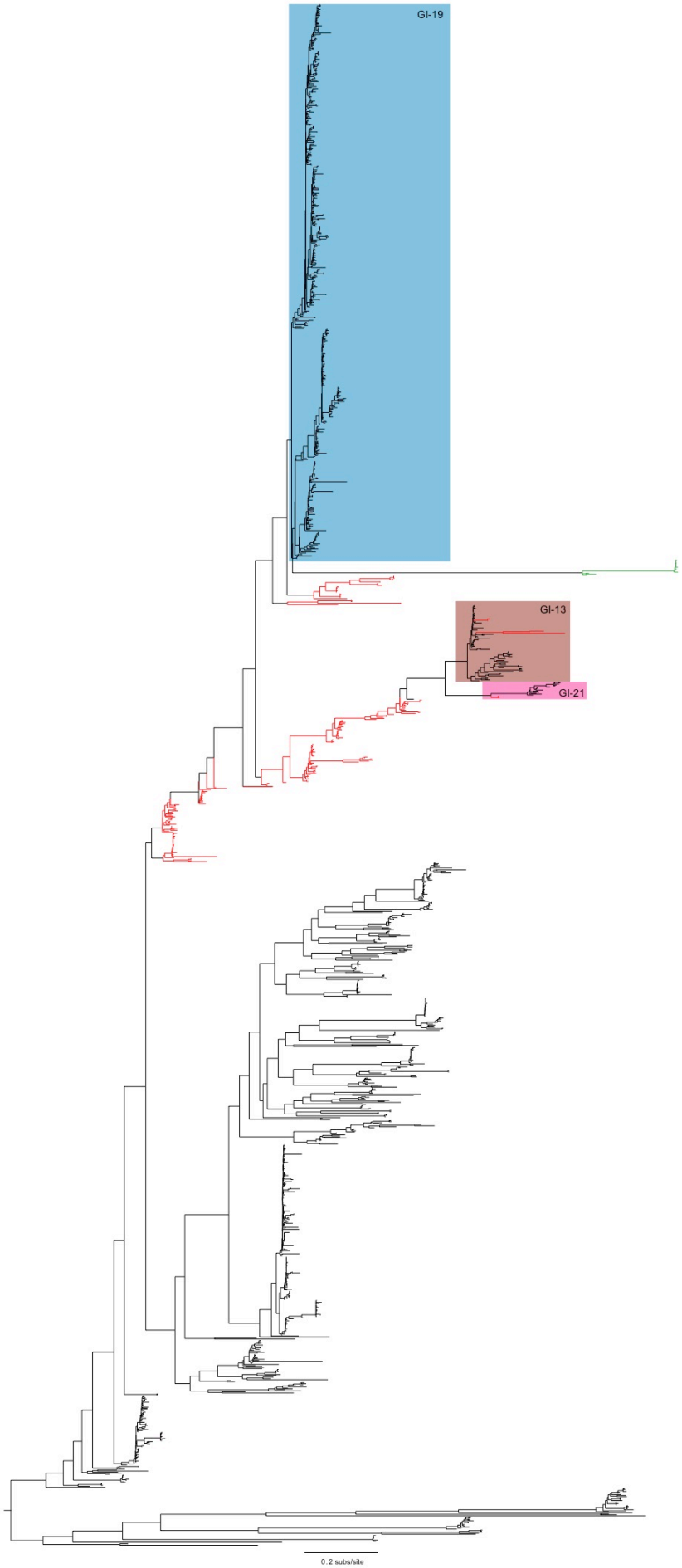

Fig. A1

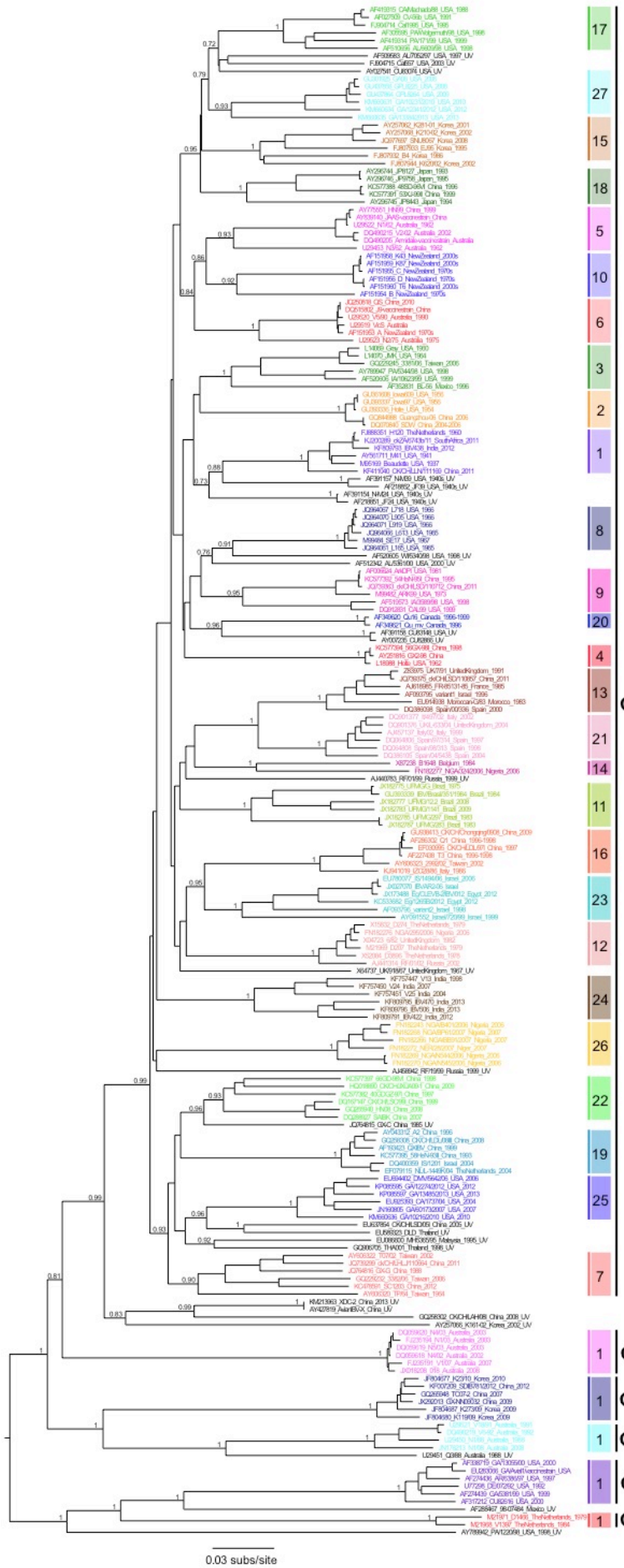
17
27
15
18
5
10
6
3
2
1
8
9
20
4
13
GI
21
14
11
16
23
12
24
26
22
19
25
7
1
GV
1
GVI
1
GIII
1
GIV
1
GII
0.03 subs/site

Fig. A2

Fig. A3

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