Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Veterinarie

Ciclo XXX

Settore Concorsuale: 07/H3

Settore Scientifico Disciplinare: VET05

Full-length genomes molecular investigation of Infectious Bronchitis Virus strains

Presentata da: Valeria Listorti

Coordinatore Dottorato **Supervisore**

Prof. Arcangelo Gentile **Prof. ssa Elena Catelli**

Esame finale anno 2018

Table of content

Introduction

Infectious Bronchitis is a ubiquitous, highly contagious viral disease of chickens. It is caused by the Infectious Bronchitis virus (IBV). While the virus has an initial respiratory tropism, it can also affect the female reproductive tract, causing loss of production and poor egg quality. Some strains have predilection for the kidney of young chickens, resulting in nephritis associated with significant mortality. Control is attempted using live attenuated and inactivated vaccines. IBV is able to genetically mutate and recombine, resulting in antigenic shift and drift. This means that vaccination programmes are constantly being challenged. Thus, even though the disease was first reported more than 60 years ago, control is never complete even today, because new strains emerge continuously (Cook *et al*., 2012).

The aim of this thesis was to analyze the full-length genome sequences of different strains of IBV. In the first part of the project, we obtained and analyzed the sequence of a Q1 strain isolated in Italy in 2013 in order to have more information on the genetic characteristics and the origin of this strain. The Q1 genotype was for the first time isolated in China in 1996 and then have spread in Far East, Middle East, Europe and South America. In Italy, it was isolated for the first time in 2011 and is still circulating. The results of this study led to the hypothesis that the Q1 genotype was genetically related to the 624I genotype, circulating in Italy from the 1960s. So, the full-length analysis of a 624I IBV strain isolated in Italy in 1996 was carried out and the genetic correlation between Q1 and 624I genotypes was assessed in order to understand the evolutionary scenario. IBV QX genotype was detected for the first time in China in 1996 and then spread worldwide becoming one of the most commonly encountered genotypes. IBV QX genotype causes sufficient disease in Europe for several commercial companies to have started developing live attenuated vaccines. Effective knowledge of the prevalence and circulation of different IBV genotypes in any area is not straightforward. One complication can be the presence of the live vaccines previously applied for disease control and the inability to distinguish between the field viruses and vaccines. The possibility to analyze field strain (progenitor) used to make any particular vaccine is vital in tracking IBV vaccines. This is because comparison of the full sequences of progenitor and vaccine strains reveals the changes which occurred in the attenuation process, and those changes are generally unique to that particular vaccine. In the second part of the study, the full genome of an IBV QX vaccine (L1148 strain) was consensus sequenced alongside its progenitor field strain (1148-A strain) to determine vaccine markers, thereby enabling detection on farms and to investigate the changes occurred during the attenuation process.

1. Infectious Bronchitis Literature Review

1.1 Aetiology

1.1.1 Taxonomy

Infectious bronchitis (IB) is caused by a virus traditionally named IBV which has been recently included in the species *Avian coronavirus* (AvCoV) belonging to the genus *Gammacoronavirus* in the family *Coronaviridae* (order *Nidovirales*).

The *Coronaviridae* family includes two subfamilies, *Coronavirinae* and *Torovirinae* and within the subfamily *Coronavirinae* there are 4 genera: *Alpha-*, *Beta-*, *Gamma-* and *Deltacoronavirus*. The genus *Gammacoronavirus* includes two species: *Avian coronavirus* and *Beluga Whale coronavirus SW1* (https://talk.ictvonline.org/taxonomy/). *Alphacoronavirus* (α-CoV) and *Betacoronavirus* (β-CoV) infect many mammalian species ranging from bats to humans, whereas *Gammacoronavirus* (γ-CoV) and *Deltacoronavirus* (δ-CoV) are largely established in birds and to a lesser degree in mammals. It has been hypothesized a model of CoV evolution, in which a Coronavirus ancestor has evolved separately in bats in *Alphacoronavirus* and *Betacoronavirus* and in birds in *Gammacoronavirus* and *Deltacoronavirus* (Fig.1) (Woo *et al.*, 2012).

The classification of the family *Coronaviridae* has been reviewed in 2009 by the International Committee on Taxonomy of Viruses. The strains showing a 90% pair-wise amino acid sequence identity in seven conserved replicase domains, are now classified in the same species. Therefore, all the different species of avian *Gammacoronaviruses* previously existing, including IBV, IBV-like isolated from different avian species and the *Turkey coronavirus* (TCoV) have been grouped together in the AvCoV species.

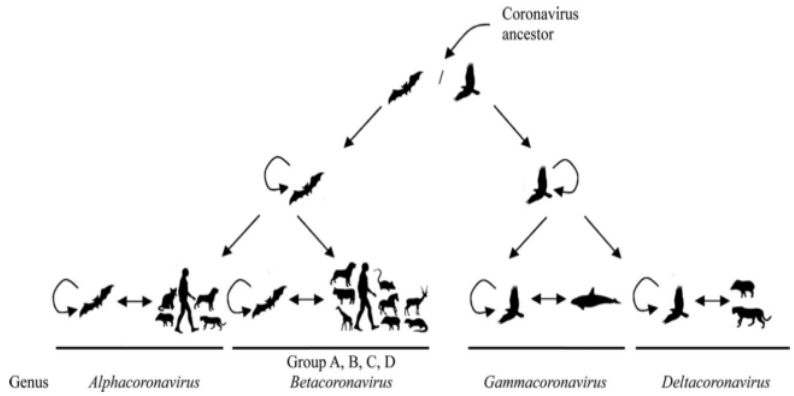

Fig.1 A model of CoV evolution (Woo *et al.*, 2012).

1.1.2 Avian coronavirus nomenclature

Many different antigenic forms, serotypes or variants of AvCoV exist worldwide and this variability results in an inconsistent nomenclature. For this reason, a new nomenclature "CoVGenus/AvCov/host/country/specimen id/year" has been recently proposed to refer the AvCoV strains (Ducatez *et al*., 2016).

1.1.3 Morphology

IBV is an enveloped virus with a round to pleomorphic shape. The virus particles, of approximately 120nm in diameter, are covered by club-shaped surface projections (spikes), formed by the Spike (S) proteins, of about 20nm in length which gives to the virus a crown-like appearance and hence the name "*corona*" (latin for crown), (Fig. 2). IBV, like other coronaviruses has a helical nucleocapsid that enclose the genetic material and is surrounded by membrane proteins (M) and, in a smaller number by envelope proteins (E), all of which are embedded in a lipid membrane (Fig. 3), (Becker *et al*., 1967; Jackwood and de Wit 2013).

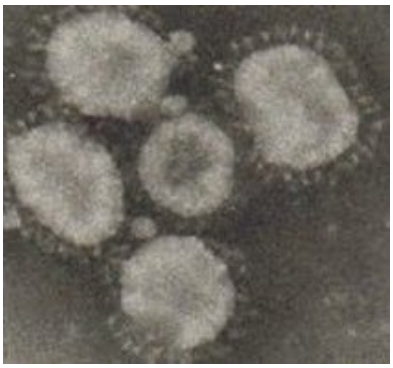

Fig. 2 Electron micrograph of Infectious Bronchitis virus particles (Cook et al., 1983)

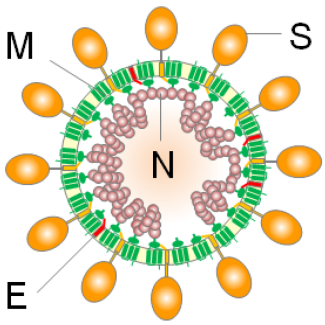

Fig. 3 Coronavirus virion structure (Ujike and Taguchi 2015)

1.1.4 Genome organization and replication

IBV has a single strand (ss) RNA positive sense (ps) genome of approximately 27.5 to 28 kb. The genome of IBV is typical of other coronaviruses and comprises the following genes: gene 1, encoding for the polyproteins 1a-1ab, spike gene (S), accessory genes 3a and 3b, envelope gene (E), membrane gene (M), 5a and 5b accessory genes and the nucleocapsid gene (N) (Jackwood and de Wit, 2013). Additionally, either for IBV and the closely related Turkey CoV, there is a region located between the membrane (M) gene and the group-specific gene 5 referred to the intergenic region (IR), also known as Open Reading Frame (ORF) 4b or 10, and

recently for some strains also ORFs 4c and 6b have been described (Cao *et al.*, 2008; Hewson *et al.*, 2011, Bentley *et al.*, 2013, Albonik, 2015). In the figure 4 the most common IBV genome organization: 5'UTR-1a/1ab-S-3a-3b-E-M-4b-5a-5b-N-3'UTR is reported (Cavanagh, 2005).

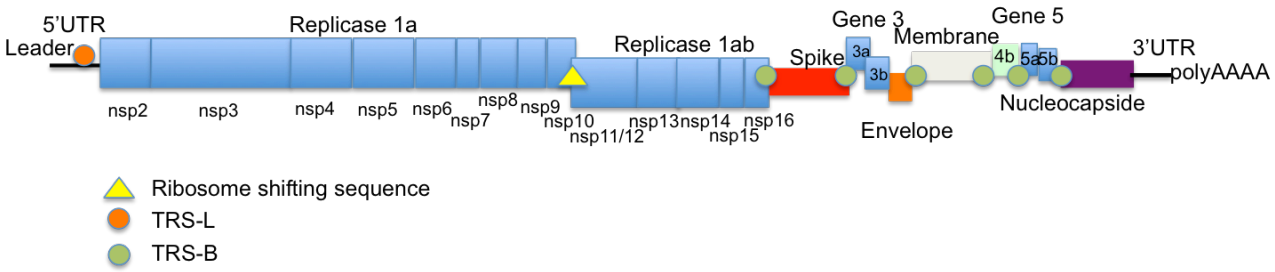

Fig. 4 IBV genome organization

The 5' end of the genome is capped, although the exact structure of the capped 5'end has not been determined. The 3' end is polyadenilated (poly-(A)). At the 5'end there is an untraslated region (5' UTR) of approximately 500 nucleotides (nts). At the end of the genome there is an untraslated region (3'-UTR) of approximately 300 nts, before the poly-(A), (Cavanagh and Britton 2008).

Gene 1 occupies the first two-thirds of the entire genome and consists of two large ORFs 1a, and 1ab. ORF 1a and 1ab overlap slightly and are translated by a -1 frameshift mechanism into two long polyproteins: pp1a and pp1ab (Namy *et al.*, 2006). Both proteins are proteolytically cleaved by two virus-encoded proteases (Ziebhur *et al.*, 2000) in 15 non-structural proteins (nsp). Nsp 2 to 11 are encoded in ORF 1a (most coronaviruses have 16 nsp but IBV lack the nsp 1) and nsp 12 to 16 are encoded in ORF 1ab. The nsps make up the replication-transcription complex (RTC).

The remaining one third of the genome encodes for structural and group-specific accessory proteins.

Transcription Regulatory Sequences (TRS), consisting in short, repeated, AT-rich sequences, are found upstream the 5' end of each gene (TRS-B) and at the 3' end of the leader sequence (TRS-L).

The genome replication strategy of coronaviruses was originally proposed in 1995 (Sawicki and Sawicki, 1995). The ORF 1 is directly translated to form the polyproteins 1a and 1ab that are then cleaved to form the replication-transcription complex (RTC). The RNA dependent RNA polimerase (RdRp) copies the genome continuously from the 3' end to produce a complementary negative sense (ns) copy of the genome, that serves in turn to be copied into more genomes (genome replication). Furthermore, the RdRp generate a series of subgenomic ns genomes for each gene or group specific genes by a process of discontinuous transcription that is typical of the coronaviruses replication (Fig 5). The RdRp starts the synthesis of the subgenomic ns genomes from the 3' end of the genomic RNA. Elongation of the nascent subgenomic ns genome continues until the first functional TRS-B motif is encountered. After the copy of the TRS-B, the RdRp will either disregard the TRS-B motif and continue to elongate the nascent strand, or stop the elongation of the nascent ns genome and relocate and complete its synthesis. This relocation will be guided by complementary between the 3'end of the nascent ns strand (TRS-B) and the TRS-L. Then the translocated ns strand will be extended by coping the 5'end of the genome (Fig. 5). Because of this discontinuous event, the subgenomic ns templates and the genomic ns template, all have the same 3' end and would be equally recognized and copied into subgenomic or genomic mRNAs respectively, by the viral transcriptase. Furthermore, both the genomic and the subgenomic mRNAs could be used to produce the new proteins, because they have the same 5'end which would give each the same ribosome recognition signal (Sawicki *et al.*, 2007; Sawicki, 2009).

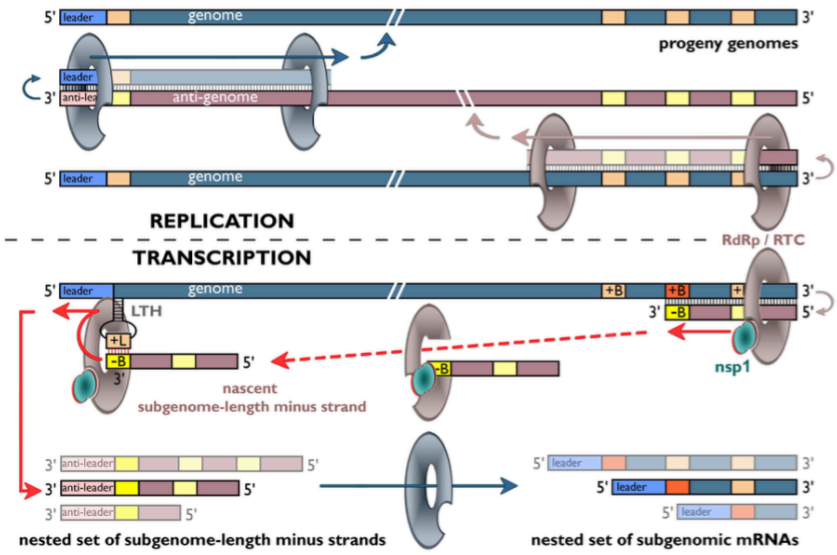

Fig.5 Schematic view of Coronavirus replication and transcription (Posthuma et al., 2017)

1.1.5 Proteins characteristics and roles

1.1.5.1 Non structural proteins

The nsps have at least eight enzymatic activity and are involved in the replication-transcription process (Sawicki *et al*., 2007; Snijdr *et al*., 2003).

The nsp 11 and 12 form the RNA dependent RNA polimerase. In the nsp 3 resides a proteolitically active domain PL^{pro} for the cleavage of the polyproteins 1a-1ab in sites between nsp 2 and 3, and nsp 3 and 4 (Lim and Liu 1998; Lim *et al*., 2000; Ziebuhr *et al*., 2001). The Nsp 5 contains the Main protease M^{pro} responsible for cleaving nsp 4 through 16 (Jackwood *et al*., 2012).

The CoV nsps assemble themselves together with the N protein into the RTC, forming double-membrane vescicles (DMV) in which viral RNA synthesis occurs (Gosert *et al*., 2002). The nsp 4, for its multiple highly hydrophobic stretches of amino acids, is predicted to span intracellular membranes (Bonilla *et al*., 1994). Together, nsps 3, 4, and 6 are predicted to anchor viral RTC on double-membrane vesicles in the cytoplasm (Graham *et al*., 2008). Also nsp 2 has been shown to be recruited to RTCs and no exchange of nsp 2 between the cytoplasm or other DMVs occurs (Hagemeijer *et al*., 2010).

Nsp 7 and nsp 8 form a super-complex that may act as a cofactor to the RdRp (Zhai *et al*., 2005). The nsp 9 is a ss RNA binding protein and interacts with the super-complex (Sutton *et al*., 2004; Egloff *et al*., 2004). Nsp 8 and nsp 9, also bind the RNA, and this possibly suggest their ability to interfere with host-cell translation (Ponnusamy *et al*., 2006). Nsp 10 is involved in RNA synthesis (Donaldson *et al*., 2006). Nsp 14 has 3'-5' exonuclease activity (Minskaia *et al*., 2006). Nsp 15 is an endoribonuclease and nsp 16 is an RNA methyltransferase (Eckerle *et al*., 2006).

1.1.5.2 Spike protein

The Spike protein is the largest structural protein of coronaviruses and forms the characteristic club-like or petal-shaped protrusions that emerge from the virion surface (reviewed in Wickramasinghe *et al*., 2014). The S protein of IBV is cleaved by a furin-like host cell protease at the highly basic pentapeptide motif RRFRR, generating the subunits S1 and S2 of about 500 and 600 amino acids in size, respectively (Cavanagh *et al*., 1986a). The N

terminal S1 subunit is part of the large ectodomain and forms the bulb of the oligomeric S protein. The C-terminal S2 subunit comprises the other part of the ectodomain forming a narrow stalk, the short transmembrane (TM) and the endodomain (Cyto) of the protein (Fig.6), (rewieved in Wickramasinghe *et al*., 2014).

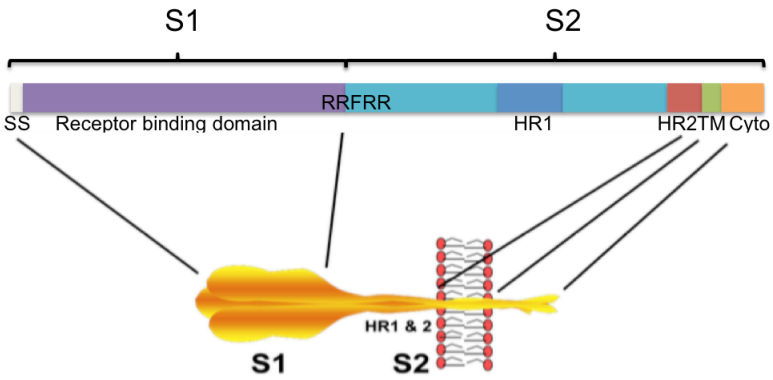

Fig. 6 Schematic representation of the S gene coding regions (Armesto et al. 2012, slightly modified)

The coronaviruses spike protein is a class I viral fusion peptide, in which the variable S1 domain is involved in host cell receptor binding. The conserved S2 domain mediates the anchor and the fusion of the virion to the cellular membranes and the releasing of the viral ribonucleoprotein in the cytosol of the infected cell (Bosch *et al*., 2003). All mapped receptor-binding domains (RBD) are located at various positions within the S1 domain (Promkuntod *et al*., 2014). The S2 membrane fusion unit of the ectodomain contains two heptad repeat regions (HR1 and HR2), which interact to form the coiled-coil structure of the stalk (de Groot *et al*., 1987), and a putative fusion peptide. After endocytosis, conformational changes in the S protein are trigged by exposure to acid pH in endosomes (Chu *et al*., 2006), resulting in the fusion of viral envelope with the cellular membrane. Although the S2 domain is not principally involved in binding to host cell receptors, the interplay between S1 and S2 domains might synergically influence the avidity and specificity of virus attachment (de Haan *et al*., 2006; Promkuntod *et al*., 2013). The S protein is the main antigenic viral protein containing epitopes for virus neutralization. Using monoclonal antibodies, five conformation-dependent neutralizing antigenic sites have been mapped on S1 (Cavanagh *et al*., 1984, Cavanagh *et al*., 1986b; Kant *et al*., 1992; Koch *et al*., 1990; Mockett *et al*., 1984; Niesters *et al*., 1987), and another immunodominant region in the N-terminal region of the S2 (Koch *et al*. 1990; Kusters

et al., 1989; Lenstra *et al*., 1989). The five neutralizing antigenic sites found in S1 co-locate within three hypervariable regions (HVRs) (Cavanagh *et al*., 1988, 1992; Moore *et al*., 1997; Niesters *et al*., 1987), suggesting HVRs to be involved in antigenicity variation. Within the IBV strains, variation in the S1 domain in amino acid sequence, ranges from 2-3% to 50% with an average of 20-25%, while identity, for the conserved S2 domain, is usually >90% (Britton and Cavanagh 2007). It has been demonstrated that the S protein is a determinant of cell tropism (Casais *et al*., 2003). Alpha2,3-linked sialic acid have been identified as a receptor determinant in cells for primary attachment of IBV (Winter *et al*., 2006, 2008).

1.1.5.3 Membrane protein

The M protein is a type III membrane protein, it is the most abundant structural glycoprotein in the virus particles and plays an essential role in virion assembly and budding (Stern and Sefton., 1982; Sturman *et al*., 1980). It spans the membrane bilaterally three times, leaving a short N-terminal ectdomain and a long C-terminus inside the virion (Armstrong *et al*., 1984). The M protein is essential for coronavirus envelope formation. It has been demonstrated that Virus Like Particles (VLPs), with similarity in size and shape to the authentic virions, can be formed from cells that express both E and M proteins (Vannema *et al*., 1996). It has been proposed that the envelope formation is mediated by lateral interaction between M molecules that form a two-dimensional lattice in intracellular membranes (Opstelten *et al*., 1995). The M protein interacts also with the viral nucleocapsid and with the S protein (McBride *et al*., 2007), for virus particles assembling (Sturman *et al*., 1980; Narayanan *et al*., 2000).

1.1.5.4 Envelope protein

The membrane topology of E protein is not completely resolved but most data suggest that it is a transmembrane protein. The E protein has a N-terminal ectodomain and a C-terminal endodomain and has ion channel activity. While the M protein is relatively abundant, the E protein is only present in small quantities in the virion (Fehr and Perlman, 2015). It is unknown how E protein assists M protein in assembly of the virion, and several possibilities have been suggested. Some works have indicated a role for the E protein in inducing membrane curvature (Raamsman *et al*., 2000; Corse *et al*., 2000; Fischer *et al*., 1998), although other authors have suggested that E protein prevents the aggregation of M proteins (Boscarino *et al*., 2008). The E protein may also have a separate role in promoting viral release by altering the host secretory pathway (Ye *et al*., 2007). Furthermore, the E protein

forms cation-selective ion channels in the lipid envelope, enhancing membrane permeability (Wilson *et al*., 2004).

1.1.5.5 Nucleocapsid protein

The N protein is the only protein present in the nucleocapsid. It is composed by two separate domains, an N-terminal domain and a C-terminal domain, both capable of binding RNA *in vitro.* It has been suggested that optimal RNA binding requires contribution from both domains (Chang *et al*., 2006; Hurst *et al*., 2009). N protein also binds nsp 3, (Hurst *et al*., 2009; 2013) a key component of the RTC, and the M protein (Sturman *et al*., 1980). These proteins interactions likely help to tether the viral genome to the RTC, and subsequently the packaging of the genome into viral particles.

1.1.5.6 Accessory proteins

It has been demonstrated that the accessory proteins 3a, 3b, 5a and 5b are not essential for virus replication. They have accessory functions, possibly associated with the virus-host interaction (Casais *et al*., 2005; de Haan *et al*., 2002; Hodgson *et al*., 2006; Yount *et al*., 2005; Cavanagh, 2007). For example, it has been demonstrated that accessory protein 5b is required for the inhibition of IFN production by blocking host translation, better known as host shutoff, (Kint *et al*., 2016).

1.1.6 Cell infection and virus replication

The attachment of the virion to the host cells is initiated by the interaction between the S protein and its receptor. Following receptor binding, the virus must next gain access to the host cell cytosol by fusion of the viral and cellular membranes and release of the viral genome into the cytoplasm. The next step, in the coronaviruses lifecycle, is the translation from the virion genomic RNA of the replicase gene, which origins the nsps. They assemble into the RTC to create an environment suitable for RNA synthesis. The nsps are also responsible for genomic RNA replication and transcription of the sub-genomic RNAs. Following replication and subgenomic RNA synthesis, the viral structural proteins S, E, and M are translated and inserted into the endoplasmic reticulum (ER). These proteins move along the secretory pathway into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). Viral genomes, encapsidated by N protein, bud into the membranes of the ERGIC containing viral

structural proteins, forming mature virions. Following assembly, virions are transported to the cell surface in vesicles and released by exocytosis (Fig. 7) (Fehr and Perlman, 2015).

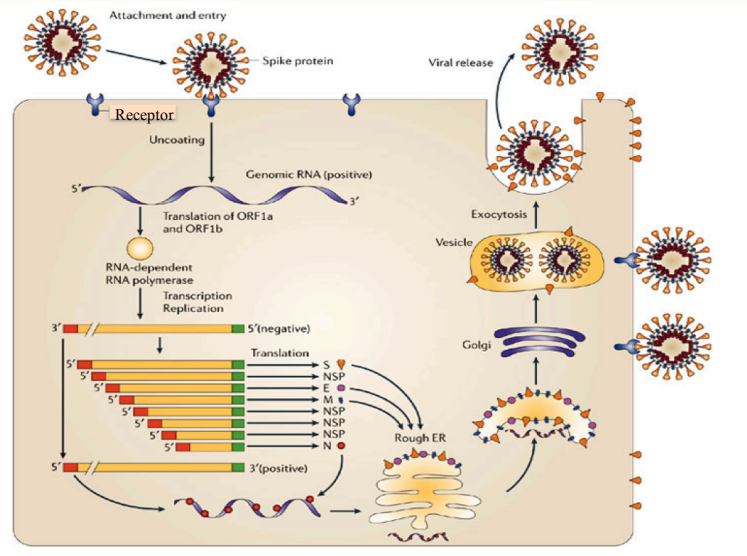

Fig 7 Coronavirus replication in host cells (Bergmann et al., 2006 slightly modified)

1.1.7 Susceptibility to Chemical and physical agents

Coronaviruses are heat liable, being inactivated after 15 minutes at 56°C, but samples containing proteins should be treated at 60°C for at least 30 minutes to inactivate the virus (Rabenau *et al*., 2005). Long term storage of IBV is recommended at -80°C. Survival of the virus up to 12 days in spring, and 56 days at temperatures below freezing, has been reported. Cold chain should be maintained for samples sent to the laboratory for diagnosis (Jackwood and de Wit, 2013). Infectious allantoic fluid lyophilized, sealed under vacuum, and stored in a refrigerator might remain viable for at least 30 years (Cavanagh and Jelb, 2008). The reduction in titre following extremes of pH, is variable depending on virus strain. A pH 3 treatment, at room temperature for 4 hours, results in reduction of titre of 1-2 $\log_{10}$ for most isolates, but up to 5 $\log_{10}$ for others (Cavangh and Jelb, 2008). IBV in cell cultures is more stable in medium at pH 6.0 and 6.5 than pH 7.0 to 8 (Alexander and Collins, 1975). IBV, being an enveloped virus, is sensitive to either 50% chloroform, and 0.01% sodium dexycholate at 4°C for 18 hours. Most common disinfectants used in poultry houses inactivate IBV. The area to be disinfected should be free of organic material. Treatment with 0.05% or 0.1% beta-propriolactone (BPL) or 0.1% formalin eliminates IBV infectivity. Only BPL treatment had no adverse effect on IBV hemoagglutination (HA) activity, making it a good choice for creating HA antigen and killed vaccine (Jackwood and de Wit, 2013).

1.1.8 IBV typing

The preferred IBV typing system depends on the goal (e.g. selection of vaccination programmes, or epidemiological studies), available techniques, experience, field situation and costs. Classification systems are divided into two major groups: functional tests, which regard the biological function of a virus; and non-functional tests, which examine the viral genome. Typing by functional tests results in protectotypes and serotypes. Tests which look at the genome result in genotypes (de Wit 2000; de Wit *et al*., 2011a).

1.1.8.1 Genotypes: Grouping strains based on genetic characterization provide essential information for epidemiological studies. Most used for genotyping is the part of the genome that encodes for the S1 subunit of the spike glycoprotein, which is the major inducer of the virus-neutralizing epitopes, including serotype-specific epitopes which are usually conformation dependent (Mockett *et al*., 1984; Cavanagh and Davis, 1986; Koch *et al*., 1990; Cavanagh *et al*., 1992). A new classification of IBV based on the phylogenetic analysis of complete S1 nucleotide sequences has been recently proposed (Valastro *et al*., 2016). Six genotypes have been defined that together comprise 32 distinct viral lineages. The IBV lineages, on the new classification scheme, exhibit a pairwise distances of 13% and 14% for nucleotide and amino acid respectively. Similarly, viral genotypes differ at least of 30% in the nucleotide, or 31% in amino acid sequences. Twenty-seven lineages cluster into genotype 1 (GI), which includes the majority of the IBV strains, whereas the remaining 5 genotypes contain only one lineage each.

1.1.8.2 Serotypes: Two IBV strains (e.g. A and B) are considered to be of the same serotype when two-way heterologous neutralization titers (antiserum A with virus B, and antiserum B with virus A) differ less than 20-fold from the homologous titers (antiserum A with virus A, and antiserum B with virus B) in both directions (Hesselink, 1991). Serotyping became less practical as more IBV types are detected in a certain area, since every serotype need its own neutralization test, and for new strains that appear to be different, an antiserum has to be raised in specific pathogens free (SPF) birds. More and more countries have to deal with an increasing number of variants, which decreases the practicability of serotyping (de Wit *et al*., 2011a).

1.1.8.3 Protectotypes: For the field, grouping of IBV strains into protectotypes is the most important system from a practical point of view, because it provides direct information

about the efficacy of a vaccine. Strains that induce protection against each other in chickens belong to the same protectotype (Lohr, 1988; Cook *et al*., 1999). However, protectotyping is laborious and expensive and required both SPF chickens and high-level facilities for performing vaccination challenge studies.

1.1.8.4 Relationship between genotype, serotype and protectotype

A complicating factor with regard to genotyping of IBV is that a change of only a small percentage of amino acids in the S1 protein can result in a change of serotype (Cavanagh *et al*., 1992) due to a change in virus neutralizing epitopes, whereas mutations in other parts of S1 might not result in a relevant change in antigenicity of the virus. On the other hand, IBVs of different serotypes and genotypes not only have different epitopes but also share common epitopes that are of importance in cross-immunity (Cavanagh *et al*., 1992; 1997) and cell mediated immune responses (Boots *et al*., 1992; Ignjatovic and Galli, 1995). Despite these limitations, S1 gene sequence comparison is reported as a better predictor of immunity in chickens than serotyping by virus neutralization (Ladman *et al*., 2006). In general, a lower homology in the sequences of the S1 subunit of two strains means greater chance that relevant mutations have occurred, which might result in a lower cross-protection (de Wit *et al*., 2011a). Genotyping is an excellent, convenient and practical tool for epidemiological studies, and it can be used best as a means of screening to select potentially important strains. In field situations, where there is suspicion that the genotyping of a recent IBV isolate does not provide accurate information about the true antigenic nature of the virus, then conventional testing (serotyping) and especially *in vivo* protection studies are required (de Wit *et al*., 2011a).

1.1.9 IBV mutation and recombination

Genetic diversity in coronaviruses is due to adaptive evolution driven by high mutation rates and genetic recombination (Holmes *et al*., 2009). Mutations includes substitutions, which are the result of high error rate and limited proofreading capability of the viral RdRp, as well as insertions and deletions, caused by recombination events or by RdRp stuttering or slippage (Jackwood and de Wit, 2013). Although IBV (and other coronaviruses) has a 3'-5' exoribonuclease (ExoN) domain in nsp 14 that is involved in proofreading and repair (Minskaia *et al*., 2006), the average rate of synonymous mutations is approximately 1.2 X 10^{-3}

substitutions/site/year, that is still high (Hanada *et al*., 2004, Holmes, 2009).

Recombination has been associated with the expansion of viral host range (Brown *et al*., 1997; Gibbs and Weiller., 1999), increases in virulence (Khatchikian *et al*., 1989), evasions of host immunity (Malim *et al*., 2001) and evolution of resistance to antivirals (Nora *et al*., 2007).
The process of recombination, that takes place in RNA viruses, corresponds to the formation of chimeric molecules from parental genomes of mixed origin. In the family *Coronaviridae* it has been well documented and is thought to be a contributing factor in the emergence and evolution of different IBV genotypes as well as different species of coronaviruses (Thor *et al*., 2011). In these viruses, recombination is thought to be due to a unique template switching "copy-choice" mechanism during RNA replication (Lai *et al*., 1992). The RdRp switches from one RNA molecule (the donor template) to another (the acceptor template) during synthesis, while remaining bound to the nascent nucleic acid chain, thereby generating an RNA molecule with mixed ancestry (Aaziz and Tepfer, 1999; Breyer *et al*., 2001; Von Hippen *et al*., 1994), (Fig.8). It is common opinion that the emergence of new strains and serotypes of IBV is largely due to the accumulation of mutations in the S gene over time rather than to recombination events (Jackwood, 2012).

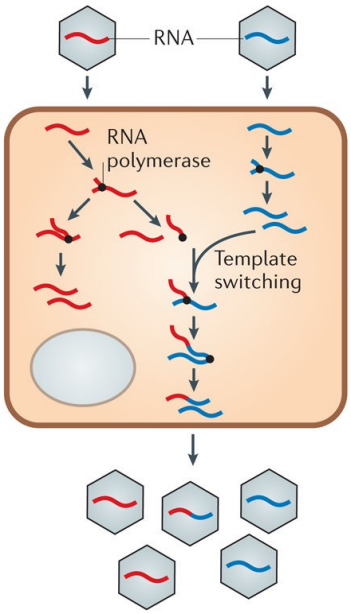

Fig.8 Schematic view of recombinant virus generation by Copy-Choice recombination mechanism (Simon-Loriere and Holmes, 2011).

1.2 Epidemiology

1.2.1 IBV world distribution

Infectious bronchitis virus is worldwide distributed and many serotypes and genotypes have been detected in all continents, except Antarctica. While the vast majority of variants remained confined in time and space, some of them have managed to invade broader regions or even to emerge as a worldwide threat (de Wit *et al*., 2011a). IBV was reported for the first time in USA in 1931 (Schalk and Hawn, 1931) and the first IBV variants were detected in the early 1950s (Jungherr et al., 1956). Since then, in USA numerous IBV strains have been identified, of which the Massachusetts or 'Mass' serotype become dominant (Jackwood *et al*., 2005). Recently it has been reported that the most commonly isolated types of IBV in the USA are Arkansas and Ark-like strains. Other IBV types reported in the USA are the California-type viruses, nephropathogenic strains identified in Pennsylvania, strains GA07 and GA08 detected in Georgia and South Carolina, SE17, Connecticut and Delaware strains (Jackwood *et al*., 2005; Jackwood, 2012).

IBV had appeared in Latin America by the 1950s and the first reported isolate in Brazil was of the Mass serotype (Hipolito, 1957). About 10 years later, the Ark type emerged, causing huge economic losses in Brazilian poultry industry (Branden and De Silva, 1986). Recently it has been shown that a Brazilian cluster of viruses subdivided in three subclusters (Brazil 01, 02, and 03) as the Mass and 793B genotype currently co-exist in that country (Villareal *et al*., 2010). Most recent works conducted in Perù, Colombia, Argentina, Chile and Uruguay revealed the circulation of the Q1, South America I (SAI), and Mass genotype in those countries (Sesti *et al*., 2014a,b; Marandino *et al*., 2015). In Colombia, in addition, three other variant strains have been reported (Sesti *et al*., 2016). The Ark type has been recently identified in Mexico (Jackwood, 2012). In Cuba three isolates have been characterized, and respectively shown to be similar to Mass type, Belgian and Californian strains (Acevedo *et al.*, 2012).

In Europe, the Mass serotype was detected for the first time in the 1940s (Cavanagh and Davis, 1993). Variant IBVs were first reported in early 1970s (Dawson and Gough, 1971). These IBVs belonged to four novel serotypes: D207 (also known as D274), D212 (better known as D1466), D3896 and D3128. Vaccines were developed using some of these variants, of which several are still in use today. The variant D1648 was able to cause major disease outbreaks for a relatively short period of time in the 1990s, mainly associated with renal

problems in flocks in Belgium and in the neighboring countries. The variant called 793B (also known as 4/91 or CR88) emerged in the 1990s (Gough *et al*., 1992) and had the major importance internationally. This variant caused major welfare and economic problems in apparently well vaccinated flocks. IBV 793B quickly spread to many parts of the world and necessitated the development of homologous live-attenuated IB vaccines to control it. This genotype continues to be a major concern in poultry of all ages in many parts of the world, but interestingly has not been reported in the USA (de Wit *et al*., 2011a). In Italy, a recent study showed that the 793B strains circulating are only of vaccine origin (Franzo *et al*., 2014)

The genotype Italy-02 was detected in Germany for the first time in 2000, then in Italy in 2002, and then spread in many European countries (Jones *et al*., 2005). After 2004, its prevalence declined in all countries except Spain (Worthington *et al*., 2008), but also in this Country has recently been replaced by other genotypes as the QX and 793B (Moreno *et al*., 2017). IBV 624I has been reported for the first time in Italy in 1993 (Capua et al., 1994, Capua et al., 1999), but a retrospective study demonstrated its circulation from 1965 (Taddei *et al*., 2012). This genotype had largely circulated in the Country until 2004; then it seemed to have disappeared up to 2010 when again viruses belonging to 624I genotype were detected; in the two following years the number of detections increased (Massi, 2013). After 2013, 624I genotype has not been reported anymore (Massi, 2015).

Because of its pathogenicity, the most important IBV type which become widespread in Europe in recent years is the QX genotype (Beato *et al*., 2005), isolated for the first time in China in 1996 (Wang *et al*., 1998). The Q1 genotype, after its first detection in China in 1996 (Yu *et al*., 2001), was detected in Europe for the first time in 2011, where spread in most of the EU countries (Toffan *et al*., 2011).

Early isolations from Russia and neighboring countries were closely related to the Mass type, whereas isolation from the late 1990s up to 2002 were recognized to be related to European types 793B, D274, B1648, 624/I and Italy-02 (Bochkov *et al*., 2006). A more recent extensive epidemiological study performed in Russia, Ukraine and Kazakhstan revealed the prevalence also of QX and Italy-02 genotypes, Ark variants and local strains (Ovchinnikova *et al*., 2011)

The prevalence of IBV strains in the Middle East varied from Country to Country. In Israel, Mass was the only genotype detected for many years until 793B genotype was identified in 1996 (Meir *et al*., 1998). Few years later a new variant, Israel/Variant-2/98 (Var2), was

reported (Callison *et al*., 2001) along with another variant named IS720; it spread in Israel and also in other countries like Iraq and Egypt, where also 793B and D274 genotypes were reported (Jackwood 2012). In Iraq, a recent study revealed the presence of the Israel/Variant-2/98, 793B, QX genotypes and the DY 12-2-like strain, that is a Chinese recombinant strain reported for the first time in Middle East (Seger *et al*., 2016). In Iran, Var2, 793B, QX, IS720, Mass and IR-1 type have been detected (Najafi *et al*., 2016).

The Var2 has been recently detected and characterized in Poland, revealing that this genotype that have circulated only in Middle East, for more than 20 years, spread in a European Country becoming one of the predominant genotype circulating in that Country (Lisowska *et al*., 2017).

It has been speculated that the IBVs have long been in existence in Asia. In China IBV was first reported in the mid 1980s and showed great diversity, although several Mass and 793B isolates were reported (Bande *et al*., 2017). Recently, nine different genetic groups have been recognized in China: LX4, LDT3, LHLJ, BJ, LDL, N1/62 and LSC, as well as Mass and 793B genotypes (Han *et al*., 2011). Possibly the most significant IBV variant worldwide emerged from China, is the QX genotype, reported for the first time by YuDong *et al*. (1998) in birds affected by proventriculitis and kidney lesions and later classified in the LX4 group (Han *et al*., 2011). Another IBV genotype, the Q1, also associated with proventriculitis, have emerged from China (Yu *et al*., 2001). It was later included in the LDL group (Han *et al*., 2011).

In the early 1990s a nephropathogenic strain designated KM91 was identified and became widespread in Korea (Lee *et al*., 2004). Ten years later, strains similar to that circulating in China (including the QX genotype) were identified in Korea (Liu *et al*., 2006). Characterization of IBV isolates between 2003 and 2006 identified three genetic groups, designated Korea (K)-I, K-II (LX4-type), and K-III (LDL-type) (Lee *et al*., 2008). More recently, strains that appear to be recombinants of KM91 and QX were detected (Lim *et al*., 2011).

Since 1995 in Japan, four different genetic groups have been identified: Japan (JP)-I, JP-II, JP-III (LX4), and JP-IV (Ariyoshi *et al*., 2010; Mase *et al*., 2004). The JP- III group falls into the China LX4 group (QX IBV), whereas JP-I, JP- II, and JP-IV appear to be unique variants. The 793B type, as well as Mass and, interestingly, Gray genotypes have also been reported (Ariyoshi *et al*., 2010; Mase *et al*., 2010).

In Thailand, a recent molecular study based on phylogenetic analysis of the S1 gene, has

identified two groups of IBV variants: Group I appeared to be unique to that Country, whilst Group II showed a close relationship to Chinese IBVs (Pohuang *et al*., 2009). IBV variants have been recognized in Taiwan since the mid-1960s. Local strains divided into two distinct lineages (TWI and TWII) have been identified, as well as Mass and IBVs related to the Q1 genotype (Wang & Tsai, 1996; Wang & Huang, 2000; Huang *et al*., 2004). However, recent data have suggested that the currently dominant IBV variant in Taiwan may have arisen as a result of recombination in the 5' end of the N gene between Taiwanese, Chinese and US IBVs (Kuo *et al*., 2010)

In Africa IBV was reported for the first time in the middle 80s in South Africa (Morley & Thomson 1984). In the early 1980s the unusual enterotropic variant, known as IB ''G'', was isolated in Morocco (El-Houadfi & Jones, 1985; El-Houadfi *et al*., 1986). Interestingly, S1 sequence data have shown that IBV G and 4/91 are very closely related, possibly with a common origin (Jones *et al*., 2004). Recent studies report in this Country the presence of the genotypes Mass, Italy 02 and 793B and the emergence of a new Moroccan genotype (Fellahi *et al*., 2015 a,b)

An epidemiological survey conducted in Nigeria and Niger between 2006 and 2007 identify a unique African cluster of viruses for which no clinical signs were recorded. These local strains were grouped into a novel IBV genotype designated as IBADAN, referring to the name of the city (in Nigeria) where the viruses were first detected, and were described to be genetically and antigenically clearly distinct from all other known IBV strains (Ducatez *et al*., 2009). In Tunisia, many strains have been isolated forming a new genotype, being genetically distinct from the other genotypes and are serologically related to the 793B and D274 genotypes, also the Italy-02 genotype has been reported (Borugaa *et al*., 2009; 2012). In Libya strains clustering with Egyptian and Israeli genotypes have been recently characterized (Awad *et al*., 2014). In Algeria, strains genetically related to the Mass genotype have been detected (Sid *et al*., 2015). In the "Horn of Africa", in Ethiopia recently IBV 793B has been reported (Hutton *et al*., 2016). In Sudan, strains belonging to the 793B and Mass genotype have been reported (Ballal *et al*., 2005). More recently an epidemiological survey revealed the presence in this Country of strains similar to Korean and Russian strains and QX genotype, and recombinant strains that could have likewise risen from H120, 4/91 and an Italian QX strain (Naguib *et al*., 2016).

In Australia IBV has always evolved independently from the rest of the world. IBV isolates

have been separated into subgroup 1 (classical strains), and subgroups 2 and 3 (novel strains). Subgroup 1 viruses include, among others, the nephropathogenic Australia/N1/62 strain, the first isolate of IBV in Australia, and the vaccine strain Australia/VicS/62. The subgroup 2 viruses were identified in 1988 and include Australia/ subgroup 2/N1/88, Australia/subgroup 2/Q3/88, and Australia/ subgroup 2/V18/91 (Ignjatovic *et al*., 1997). The subgroup 3 strains were first identified around 2002 (Ignjatovic *et al*., 2006) and appear to be chimeras resulting from recombination between subgroup 1 and 2 viruses (Mardani *et al*., 2010). Both subgroup 2 and 3 viruses are respiratory pathogens, and to date, none have been found to be nephropathogenic, like the classical strains in subgroup 1.

12.2 IBV in Italy

In Italy, a recent epidemiological study conducted mainly in the Emilia Romagna region, but also in other Italian regions, revealed that, currently, the main genotype circulating in the Country is the QX (28,6%) followed by the 793B (28%), M41 (9,3%), Q1 (7,8%) and D274 (1,4%), (Massi 2017). It has been hypotized that the 793B strains circulating are of vaccine origin (Franzo *et al*., 2014), so it is reasonable to assert that the vast majority of the field strains circulating belonging to the QX genotype. Another genotype circulating in Italy is the recombinant Guandong/Xindadi (4,1%) resulted from the recombination between QX and 793B genotypes, that probably co-infect frequently the birds (Moreno *et al*., 2017).

1.2.3 Natural and experimental hosts of Avian coronaviruses

It is no longer accepted that the chicken is the only host for IBV, although it is possible that IBV only causes disease in the chicken. IBV has been detected in many species of gallinaceous and non-gallinaceous and some of them could be considered a *reservoir* for IBV (Liu *et al*., 2005). IBV strains with high genetic identity in S1 gene with Mass-type vaccines, has been recovered from peafowl (*Pavo cristatus*) (Liu *et al*., 2005). Ito and colleagues, in 1991 (Ito *et al*., 1991) have isolated from guinea fowls (*Numida meleagridis*) a coronavirus antigenically related to IBV. The affected birds had been suffering high mortality, low feed consumption and enteritis. Whether the guinea fowl virus was a ‘‘genuine’’ guinea fowl coronavirus (i.e. a separate species) or an IBV that had spread from nearby chickens to the guinea fowl is not known (Cavanagh, 2005).

In China, a *Gammacoronavirus* has been isolated from a teal (*Anas crecca*) kept domestically near chickens. The isolate had a spike protein with 90% identity with some known field IBV strains, including a nephropathogenic one. When this isolate was inoculated into chickens, it caused disease, including kidney involvement. This, plus the very high relationship found between the genes of the teal isolate and of IBV, including the S gene, makes probable that the teal isolate was actually an IBV strain that had spread to the teal from nearby chickens (Liu *et al*., 2005).

During a recent epidemiological investigation in northern Italy, IBV strains with very high percentages of identity with IBV vaccine strains, applied in the neighboring chicken flocks, have been detected in quails (*Coturnix Coturnix*) and pheasants (*Phasianus Colchicus*) (Torres *et al*., 2016)

Virus similar to IBV (IBV-like), have been isolated from different avian species.

The *Turkey coronavirus* (TCoV) is associated with highly contagious gastroenteritis in young poults (Barnes *et al*., 2000) and was first identified in 1951 (Peterson and Hymass, 1951). Experimental inoculation of TCoV into chickens resulted in replication in the alimentary tract, although asymptomatically (Ismail *et al*., 2003). The sequences of the genes of the Turkey coronaviruses in the US, and those subsequently detected in the UK (Cavanagh *et al*., 2001), had 85 to 90% identity with those of IBV, which is high given that strains of IBV commonly differ from each other by this amount. The exception was in the spike protein; a research has revealed that there is only approximately 34% identity between the spike protein of the TCoV isolates and those of IBVs, a lower identity than the lowest yet seen between IBV strains. The emergence of coronaviruses in turkeys in the USA was proposed to have resulted from recombination events involving IBVs and an as-yet-unidentified coronavirus donating a spike (S) gene (Lin *et al*., 2004). This is suspected to have resulted from a host shift from chickens to turkeys and the virus has altered the tissue tropism from upper respiratory to intestinal. Recombination has been proposed in preference to evolution or selection of a subpopulation of viruses owing to this low S protein amino acid identity and because under experimental conditions exposure/passage of TCoVs in chickens did not result in selection for genetic changes that were sufficient to maintain infection and replication in chickens (Jackwood *et al.*, 2010). A recent analysis on the complete genome sequence of a European TCoV demonstrated that European turkey and guinea fowl coronaviruses share a common genetic backbone and suggests that these recombined in two separate events with different, yet related, unknown

Avian coronaviruses, acquiring their S-3a genes. The study also showed that the North American viruses do not share a common backbone with European turkey and guinea fowl viruses; however, they do share similar S-3a genes with guinea fowl virus (Brown *et al*., 2016).

Pheasants were infected by a coronavirus (PhCoV), sometimes associated with respiratory disease and sometimes associated with kidney disease (Spackman & Cameron, 1983; Lister *et al*., 1985; Gough *et al*., 1996; Pennycott, 2000). Inoculation of chickens with several PhCoVs resulted in production of antibodies, indicative of replication, but without disease (Gough *et al*., 1996). A molecular investigation of coronaviruses isolated from pheasants showed a high genetic similarity of these strain with IBV (Cavanagh *et al*., 2002). The degree of genetic relatedness to IBV is the same as that between IBV and TCoV except with regard to the S protein, in which PhCoV and IBV are more closely related to each other than to TCoV.

Recently a *Gammacoronavirus* has been isolated from quails showing an enteric syndrome. The amino acid (aa) sequence identity in the S1 gene with IBVs was of 16-18%, suggesting that *Quail coronavirus* (QCoV) is not an IBV variant. In contrast, the S1 of QCoV displayed 79-81% aa identity with TCoV strains suggesting that the QCoV is a TCoV-like virus (Circella *et al*., 2007).

Gammacoronaviruses, genetically different from IBV and other IBV-like viruses, have been detected in fecal samples or cloacal swabs of graylag geese (*Anser anser*), pigeons (*Columba livia*) and mallard ducks (*Anas platyrhynchos*), (Jonassen *et al*., 2005).

The relatedness at the genetic level and the capacity of IBV, TCoV and PhCoV to infect chickens, raised the question as to whether these three viruses should be considered as three distinct species or as one species (Cavanagh, 2005). For these reasons, to overcome a classification problem, the new nomenclature of the coronaviruses includes all the IBV-like viruses and the *Gammacoronaviruses* isolated in avian species within IBV in the *Avian Coronaviruses* species.

The presence of IBV and other *Gammacoronaviruses* in the same host could determine recombination between them.

1.2.4 Transmission

IBV is highly contagious and spreads rapidly among chickens in a flock. Transmission may occur by either inhalation, thought oculo-nasal route, or ingestion of infectious virus

particles by direct contact between infected and susceptible birds; by indirect contact trough aerosol droplets of faeces; and by exposure to virus-contaminated fomites, such as clothing, shoes, tools etc. Aerosol generation from the respiratory tract is a significant mode of transmission because of high virus concentration in the respiratory tract during the acute stage of the infection. Most likely, transmission by aerosol is especially effective over short distances, such as within a flock or premises, because the enveloped virion is inactivated relatively quickly in the environment (Jackwood and De Wit 2013). The virus is also excreted by faeces and urine. A long-term recovery (2-7 months) of IBV has been reported from infected or vaccinated flocks (Naqi *et al*., 2003). Vertical transmission does not seem to be relevant for IBV, although Cook and colleagues could re-isolate the challenge virus after infection in laying SPF hens and cockerels from the vitelline membrane of the eggs, for 1-7 weeks, and from semen of cockerels for two weeks; virus could be isolated even from a small number of hatched chicks. However, the implication for the field of this last finding remains unclear, because these chicks developed no clinical signs, did not seroconvert and were not protected against challenge (Cook *et al*., 1971).

1.3 Pathogenesis

The incubation period of IBV is dose dependent and can be as short as 18 hours for intratracheal inoculation and up to 36 hours for ocular application (Jackwood and de Wit, 2013). Generally, the disease has a short incubation period: susceptible birds placed with recently infected chickens usually develop clinical signs within 24-48 hours.

IBV initially infects the upper respiratory tract where infection is restricted to the ciliated and mucus secreting cells. This is the main site of IBV replication, following which a viraemia occurs and the virus gets widely disseminated to other tissues (Mc Martin, 1993). IBV is epitheliotropic and is able to replicate in different tissues depending on the tropism of the strain. In the trachea, the virus causes the stasis of cilia, both *in vivo* and *in vitro* and damages of the respiratory epithelium often predisposing chickens to secondary infections such as *E.coli*, which can be the main cause of most debilitating disease, including mortality (Peighambari *et al*., 2000; Matthijs *et al*., 2005). Titers of live virus are maximal in the nose and trachea within three days and they remain so for two to five days further (Ambali and Jones, 1990). After this period, virus titres in the respiratory tract drop rapidly and in the

second week p.i. can be below the level of detection. It has been suggested that the tracheal damage in terms of ciliary activity is variable since it depends on the virulence of IBV strains for the trachea (Otsuki *et al*., 1990; Dhinakar Raj and Jones, 1997). Tissue recovery is normally seen after 10 to 14 days (Cook *et al*., 2012).

IBV is able to replicate also in the epithelial cells of the lungs (Janse, 1994) and airsacs (Nauwync and Pensaert, 1988). High virus titer has been seen in these tissues between 4 to 11 days p.i. Some strains of IBV are intrinsically nephropathogenic i.e. they cause nephritis when inoculated experimentally into specific pathogen free chickens, causing mortality (Pensaert and Lambrechts, 1994; Cook *et al*., 1996). IBV infects mainly the lower nephron down to the collecting duct epithelial cells and replicate in all segments of tubules and ducts, but more frequently in the epithelial cells of the collecting ducts, collecting tubules, distal convoluted tubules and Henle's loops (Chen and Itakura, 1996; Chen *et al*., 1996). Structural alterations in the tubular epithelial cells (Condron and Marshall, 1986) causes impaired fluid and electrolyte transport leading to acute renal failure. It has been hypothesized that the kidney is the site of persistence of IBV (Dhinakar Raj and Jones, 1996), and is an immunoprivileged site where virus could survive away from the immune response (Cook *et al*., 2012). Modest to high titer of IBV in the kidney do not necessarily correlate with overt kidney disease. For example, in an experimental study, the Moroccan G strain replicated to similar titer in kidney as in trachea, though no gross kidney changes were observed (Ambali and Jones, 1990).

Some IBV strains are able to replicate and cause lesions in the reproductive tract. It has been demonstrated that the ciliated and granular cells of the surface epithelia and secretory epithelial cells of the tubular glands of the infundibulum and magnum are the target cells for the virus (Chousalkar *et al*., 2007). Furthermore, there are IBV strains able to cause cystic lesions of the oviduct when layers hens are infected in the first days of life (Crinion and Hofstad, 1972; Landman *et al*., 2005; Benyeda *et al*., 2009). These viruses cause permanent damage of the oviduct that could be evident only several weeks after infection, when birds come into lay leading to reduced egg production and quality, and increasing the incidence of "false layers" in the flock (De Wit, 2006). Within a few weeks, the susceptibility of the young chicks to develop damage of the oviduct that leads to the false layers syndrome has been shown to drop dramatically (de Wit *et al*., 2011b). The pathogenesis of this syndrome has not been elucidated. In hens, experimentally infected with the QX genotype, the absence of obvious histological changes and measurable replication of IBV in the oviduct point to the

presumption that the dilatated lumen and the accumulation of serous fluid in the oviduct is not the direct effect of the virus. It is likely that infection during the first week of life may affect the last step of the post-embryo development of the oviduct, which enables the Mullerian duct (developing oviduct) to open into the lumen of the cloaca. An inflammation and the subsequent stricture of the wall of the cloaca or of the entry of the oviduct might let to the obstruction of the posterior part of the oviduct resulting in fluid accumulation and dilatation. This hypothesis explains the occurrence of false layer syndrome only following early IBV infection (Benyeda *et al*., 2010).

It has been suggested that IBV is implicated in the formation of epydidimal stones, in rooster epidydimis, that has detrimental effects on sperm production and fertility (Boltz *et al*., 2004).

IBV have been isolated from cloacal swabs, faeces and caecal tonsils (Alexander and Gough, 1977; Alexander *et al,* 1978; Cook, 1984; Lucio and Fabricant, 1990). *In vitro* explants of gut tissues have been shown to support the growth of IBV (Bhattacharjee and Jones, 1997; Darbyshire *et al.,* 1976). IBV has been isolated also from oesophagus (Lucio and Fabricant, 1990; Dhinakar Raj and Jones, 1996). However, because of the method used for the virus detection, it is not clear whether the virus actually multiplies in the oesophagus or whether virus is swallowed after being expelled from the trachea. IBV has also been isolated from proventriculus, duodenum and jejunum (Ambali and Jones, 1990; Lucio and Fabricant, 1990). Darbyshire and colleagues have shown that proventriculus was inferior only to respiratory tissues and oviduct in supporting virus multiplication in *vitro* (Darbyshire *et al*., 1976).

IBV replication has been described in cells resembling histiocytes and lymphoid cells of the caecal tonsils (Owen *et al.,* 1991) and demonstrated by IF in apical epithelial cells of the villi in ileum and rectum (Ambali and Jones, 1990; Dhinakar Raj and Jones, 1996). Despite IBV has a wide tropism for gut tissues, gross and histological changes have been described only in proventriculus, following Q1 and QX genotypes infection (Wang *et al.*, 1998; Yu *et al*. 2001, Toffan *et al*., 2013).

Gross pectoral muscle lesions have been observed in chickens infected with the 793B genotype. The genesis of these lesions has not been elucidated, it has been tempted to speculate that it could have been a result of immune complex deposition in the capillary walls of the muscles (Gough *et al*., 1992; Dhinakar Raj and Jones 1996).

There is evidence of replication of the virus also in lymphoid tissues including harderian glands and bursa of Fabricius, it might be reasonable to suppose that infection could result in immunosuppression (Dhinakar Raj and Jones, 1996).

1.5 Clinical signs

The clinical outcome of an infection in chickens, and occurrence of mortality depend on many variables such as the virus strain, sex, age and immune status (vaccination, immune suppression, and maternally derived antibodies) of chickens, co-infections, environmental factors such as climate, dust, ammonia, and cold stress (Jackwood and de Wit, 2013). Usually all birds in the flock became infected. Mortality may be as high as 25% or more in chickens less than 6 weeks of age and usually is negligible in chickens greater than 6 weeks of age (Cavanagh and Naqi, 2008).

IB respiratory signs in chicks are: gasping, coughing, sneezing, tracheal rales, and nasal discharge; wet eyes and swollen sinuses may be observed (Fig.9).

Fig. 9 IBV Infected chick showing gasping and respiratory distress (https://partnersah.vet.cornell.edu/avian-atlas/#/)

The chicks appear depressed and may be seen huddled under a heat source. Feed consumption and weight gain are significantly reduced. In chickens greater than 6 weeks of age and in adult birds, the signs are similar to those in chicks, but nasal discharge does not occur as frequently, and the disease may go unnoticed unless the flock is examined carefully by handling the birds or listening to them at night when the birds are normally quiet (Hofstad,

1984). Broiler chickens infected with a nephropathic strain may appear to recover from respiratory phase and then show depression, ruffled feathers, wet dropping and increased water intake (Jackwood and de Wit, 2013).

In laying flocks, decline in egg production and quality are seen in addition to respiratory signs. The severity of the production declines might vary with the period of lay (Eck, 1983) and with the causative virus strain. Six to eight weeks may elapse before production returns to the pre-infection level, but in most cases, this is never attained. Soft-shelled, misshapen and rough-shelled eggs are produced (Fig.10) and the internal quality of the eggs may be inferior. The albumen may be thin and watery without definite demarcation between the thick and thin albumen of the normal fresh egg (Cavanagh and Naqi, 2008). In addition to production declines, for the breeders, the number of eggs unacceptable for setting is increased and hatchability is reduced (Crinion and Hofstad, 1972).

Fig.10 Soft shelled and misshaped eggs from IB affected hens; ("Atlante di anatomia patologica della gallina ovaiola", Elanco)

Flocks with false layers fail to reach the normal rate of lay, whereas the flock looks healthy, and behaves normally. The peak of production can be as low as 35% of expected production values (Cavanagh, 2000). The large cysts can distend the hen's abdomen and the hens stands like penguins (de Wit, 2006) (Fig. 11).

Fig. 11 Hen affected by false layer sindrome, standing like penguin ("Atlante di anatomia patologica della gallina ovaiola", Elanco)

1.4 Macroscopic and microscopic lesions

The post mortem examination of the respiratory tract in affected chickens shows, macroscopically, serous, catarrhal, or caseous exudate in the trachea, nasal passages and sinuses. The mucosa of the trachea is edematous and congested (Fig.12). A caseous plug may be found in the lower trachea or bronchi of chicks that are found died (Cavanagh and Naqi 2008, Benyeda *et al*., 2010).

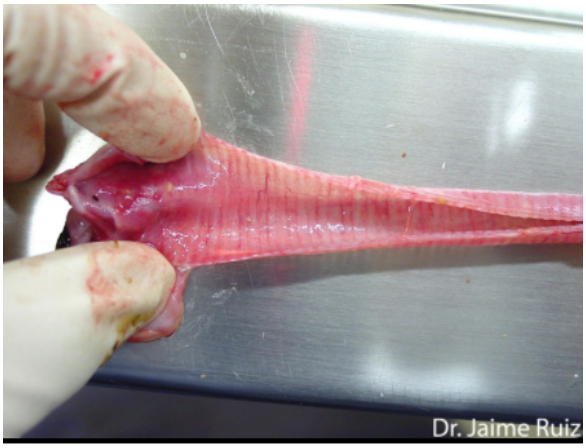

Fig. 12 Post mortem examination of IBV infected chicken showing oedema and congestion of the proximal trachea (https://partnersah.vet.cornell.edu/avian-atlas/#/)

Within 18 hours of infection, histological exam shows loss of cilia, rounding and sloughing of epithelial cells, and minor infiltration of heterophils and lymphocytes. Regeneration of the epithelium starts within 48 hours. Hyperplasia is followed by massive infiltration of the *lamina propria* by lymphoid cells and the formation of a large number of germinal centers, which may be present after seven days.

Air sacs may appear macroscopically cloudy or contain a yellow caseous exudate. Microscopically there is oedema, epithelial cells desquamation, and some fibrinous exudation within 24 hours, lymphoid nodules, increased heterophils, fibroblast proliferation, and later regeneration by cuboidal epithelial cells (Riddell, 1987). Small areas of pneumonia may be observed around the large bronchi (Jackwood and de Wit 2013).

In the Harderian gland the presence of IBV can be characterized microscopically by a sharpe increase in the number of plasma cells, hyperemia, and exstensive lymphoid follicle formation (Toro *et al*., 1996).

Nephropatic infections produce macroscopically swollen and pale kidneys with the tubules and ureters often distended with urates (Fig.13) (Cumming *et al*., 1969; Gillette *et al*., 1973).

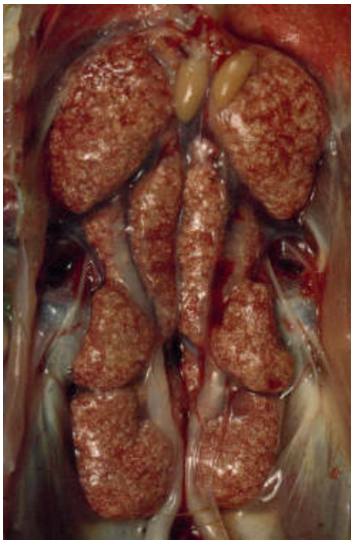

Fig.13 IBV infected chicken, pale kidneys distended with urates (Avian Pathology Service-University of Bologna, Photo Archive)

The microscopic kidney lesions of IB are principally those of an interstitial nephritis. The virus causes granular degeneration, vacuolation and desquamation of the tubular epithelium, and massive infiltration of heterophils in the interstitium in acute stage of the disease.

The lesions in tubules are most prominent in the medulla. Focal area of necrosis may be seen as well as indications of attempted regeneration of the tubular epithelium.

During recovery, the inflammatory cell population changes to lymphocytes and plasma cells. In some cases, degenerative changes may persist and result in severe atrophy of one or all of the divisions of the nephrons. In urolithiasis, the ureters associated with atrophied kidneys are distended with urates and often contain large calculi composed mainly of urates (Riddell, 1987).

When the reproductive system is affected, large accumulation of yolk fluid may be seen in the abdominal cavity, often associated with bacterial infection in laying hens. The infection of the reproductive tract of hens determines microscopically patchy loss of cilia from the anterior segment as well as the posterior segment of the infundibulum, that could be seen even 30 days after infection, and plasma cells and lymphocytes infiltration in the *lamina propria*. Lymphoid nodules have been recorded in the *lamina propria* and muscularis region of the infundibulum (Chousalkar and Roberts, 2007).

In the magnum IBV causes microscopically loss of cilia and patchy loss of mucopolysaccharides of the epithelial cells, loss of cilia and lymphocyte and plasma cell infiltration in both the tubular shell gland and shell gland pouch, and a persistent lymphocytic and plasma cell infiltration in the subepithelial space. The lymphocytes surround also blood vessels in the muscularis area (Chousalkar *et al*., 2007)

Few strains of IBV such as those of QX genotype, when infects hens in the first days of age, are able to cause the false layer syndrome macroscopically characterized by permanent damage of the oviduct, causing dilatation of the oviduct with serum-like fluid accumulation, accompanied by very few microscopic changes. In the dilated oviducts, flattening of the developing folds and thinning of the walls could be seen, with focal mononuclear cell infiltration beneath the epithelial layer or the serosal lining (Fig.14).

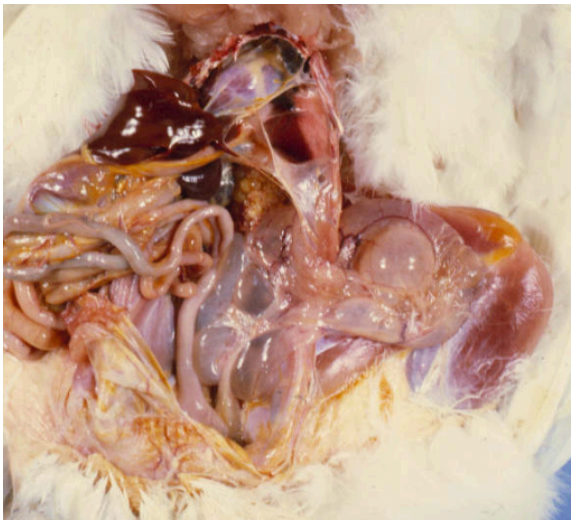

Fig.14 Post mortem examination of hens affected by false layer syndrome showing oviduct serum-like fluid accumulation in the oviduct (Avian Pathology Service-University of Bologna, Photo Archive)

In the bursa of Fabricius it has been described macroscopically the presence of yellowish brown material filling the serrated lumen of the bursa and microscopically the necrosis of lymphocytes in the follicles with the presence of numerous heterophils in the bursal secretion. The surface epithelium of the plicae lining the lumen of the bursa shows mild hyperplasia and forms a series of cystic cavities. The follicles could be repopulated with lymphocytes but the lymphocytolysis and cystic hyperplasia of the epithelium could persist 21 days p.i. It has been observed also proliferation of fibrous tissue between the follicles accompanied atrophy of these structures and resulted in shrinkage of the bursa (Mac Donald and Mc Martin, 1976).

It has been observed that the myopathy affecting both deep and superficial pectoral muscles associated to IBV (Gough *et al.*, 1992) is characterized by marked swelling and pallor of deep pectoral muscles together with the presence of occasional fascial hemorrhages and a layer of gelatinous oedema over its surface.

Lesions of the proventriculus are characterized by thickening of the wall, proventricular congestion at the point of emergence of the glandular ducts (*papilla*), (Fig.15).

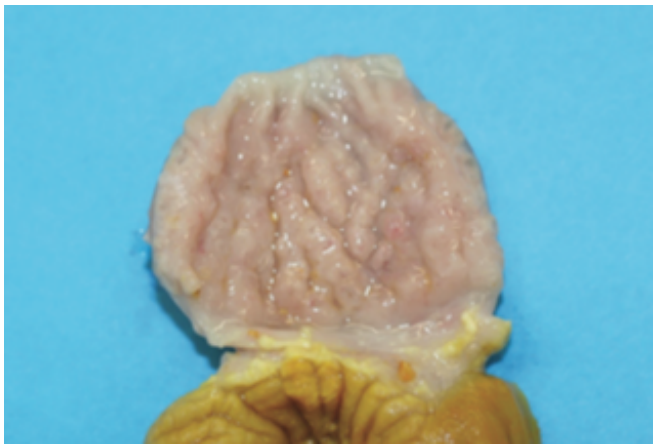

Fig. 15 Post mortem examination of Q1 IBV infected chicken, showing thickening of proventricular wall with prominence of papilla (Toffan et al., 2013)

Histologically, the proventriculus is affected by mild to severe proventriculitis, characterized by multifocal erosion and necrosis of the tunica mucosa and glandular epithelium, associated with infiltration of lymphocytes, plasma cells and heterophils, as well as fibroplasia in the lamina propria (Toffan *et al.*, 2013).

Except for the proventriculus, although IBV has a wide tropism for gut tissues no gross or

histological changes have been reported.

1.6 Immunity

The immune system works through a complex interplay of several cell types and proteins that protect the host from invading pathogens. The first line of defence for IBV is the mucous membranes of the respiratory tract, and of the gut. The tracheal mucosa is lined by ciliated epithelium that contains mucus producing goblet cells.

The innate immune system represents the second line of defence consisting of proteins and different cell types.

Finally, T and B cells, cells of the adaptive immune system are activated by antigen presenting cells (APC) to exert their effector functions. Chickens have a well-developed mucosal immune system, with several organised mucosa-associated lymphoid tissues in the respiratory tract, eye/head region (that are very important in the response to IBV infection) and intestinal tract. In the respiratory tract, lymphoid tissues are located in Harderian Gland (HG), conjunctiva (conjunctiva associated lymphoid tissue; CALT), and nose (nasal-associated lymphoid tissue; NALT). Lymphoid follicles and scattered lymphoid cells found in the *lamina propria* consist of APC, CD4+ and CD8+ lymphoid cells. In the lung, there is the bronchus associated lymphoid tissue (BALT) (Vervelde and de Wit, 2014).

1.6.1 Innate immunity

The innate and the adaptive immune responses to IBV are highly integrated. The innate immune system is involved in directing the adaptive response and can also act as expresser of the adaptive immunity (Juul-Madsen *et al*., 2011). IBV induces a diversity of local innate effectors. The type I Interferon (IFN), produced in the early phase of infection, has an antiviral activity against IBV (Wang *et al*., 2006; Pei *et al*., 2001) and could induce an up-regulation of the expression of the Toll like receptors (TLR) 2 and 3. It has been demonstrated that also Interleukin-1, macrophage colony-stimulating factor I receptor precursor (CSF-1-R), chemokine ah294, and common cytokine receptor chain, all significantly increased their transcription during the early phase of infection (Wang *et al*., 2006). IBV induces the activation of Natural killer cells (NK) producing IFN-γ, able to kill the virus-infected cells at 1

day p.i. in the lung and in the blood (Vervelde *et al*., 2013). Mannose-binding lectin (MBL), an innate pathogen pattern-recognition molecule, is involved in the regulation of the adaptive immune response to IBV (Juul-Madsen *et al*., 2011).

1.6.2 Active Immunity

1.6.2.1 Cell-mediated immunity

The Cytotoxic T Lymphocytes (CTL) response of chicks to IBV infection plays a critical role in the elimination of virus during acute infection (Collison *et al*., 2000). It has also been demonstrated that adoptive transfer of CTLs with alpha/beta T cell receptor (TCR2) could protect chicks from acute infection of IBV in the respiratory tract (Seo *et al*., 2000). IBV specific CTL epitopes were mapped within the carboxy-terminal 120 amino acids of the nucleic capsid protein (Timms and Bracewell, 1983). Furthermore, during a primary IBV-specific immune response, induced by ocular inoculation of attenuated IBV, only the HG and the CALT exhibit a response with a noticeable change in CD3+ and CD44+ T cells, IFN-γ, granzyme A and perforin mRNA expression while little change is noticed in the spleen. This observation is consistent with the observation that HG is the initial site of replication for IBV after ocular inoculation (Gujar *et al*., 2013).

1.6.2.2 Humoral immunity

The protective effect of antibodies is not only achieved by neutralising the free virus. Virus specific antibodies lead to a plethora of antiviral activities. Coating of virus with antibodies can result in virolysis by complement activation and enhance phagocytosis (Vervelde and de Wit, 2014).

Usually the first antibodies can be detected in serum and lacrimal fluid between 1 and 2 weeks p.i.: IgA, IgG, and IgM can be detected in serum; while in lacrimal fluid and tracheal washing, IgA and IgG are the commonly detected antibody classes (reviewed in Jackwood and de Wit, 2013). An important part of the IgA in the lacrimal fluid originated from the HG (Baba *et al*., 1988; Develaar *et al*., 1982), while the IgG concentration is largely the result of passive transport of IgG from the serum (Develaar *et al*., 1982; Toro *et al*., 1993). IgM is only present for a few weeks after vaccination or infection; therefore, its detection is indicative of a recent vaccination or infection. Serotype specific virus neutralizing antibodies are induced by the amino-terminal S1 subunit of the S glycoprotein (Cavanagh *et al*., 1992). The protective role of

antibodies has been shown by the significantly longer period of virus persistence p.i. in chickens that were immunocompromised by Infectious Bursal Disease virus infection (Rosemberg and Gelb, 1978). A similar effect was reported after in-ovo bursectomy in white leghorn chickens (Cook *et al*., 1991). The IBV ELISA and Virus Neutralization (VN) antibodies levels in tears are not accurate indicators of IBV immunity (Gelb *et al*., 1998), moreover no correlation was found between the serum ELISA antibody titres and the degree of kidney protection against a nephropathogenic strain (Pensaert and Lambrechts, 1994). In another study, no antibodies were detectable after vaccination in post-hatch cyclophosphamide bursectomized chickens that still resisted to challenge (Chubb, 1974). In laying birds, a clear correlation was found between the level of antibodies against the challenge virus and the level of protection against egg drop (Finney *et al.*, 1990).

1.6.3 Maternally derived immunity

High levels of maternally derived antibodies (MDA) significantly reduced the extent of clinical signs or damage to trachea, kidneys and oviduct due to IBV infection in chicks during the first days of life (reviewed in Jackwood and de Wit, 2013). An experimental study conducted by de Wit and colleagues in 2009 showed that a higher level of neutralizing antibodies in the breeders, for the strain used in that study, is correlated with a higher level of protection against early challenge in the progeny, giving to unvaccinated and challenged birds ciliostasis protection ranging between 50% to 100%, at 6 days of age (de Wit *et al*., 2009). A recent study on the protection of birds against a QX strain (D388) suggests that virus neutralizing MDA are important for the early protection of birds against the false layers syndrome, especially associated with the use of a heterologous vaccine at one day of age (De Wit *et al*., 2011b).

1.7 Diagnosis

Diagnosis of IB is based on clinical signs, lesions, detection of IBV RNA, virus isolation, IBV antigen detection and seroconversion (Gelb and Jackwood, 2008).

1.7.1 Factors influencing IBV detection and isolation

One of the most important factor for a successful detection of IBV is the time of sampling. All the IBV strains can be isolated from the respiratory tract during the first 3 to 5 days p.i., after this period the virus titre drop rapidly (de Wit, 2000). When chickens are sampled in the chronic stage of an IBV infection, the value of sampling intestinal tract and associated lymphoid tissues (e.g. cecal tonsils) is higher than sampling tracheas (reviewed in de Wit, 2000). The level of immunity of the chicken at the moment of infection has a major influence on the amount of IBV that can be detected. When collecting samples from a large flock, both healthy birds and those with clinical signs should be sampled. Typically, clinical signs begin 3-5 days post infection, when the virus is no longer at the peak titre in the trachea. In mild cases of the disease, clinical signs may go unnoticed until secondary pathogens become involved, at which time IBV is no longer present (Jackwood and de Wit, 2013).

Samples for IBV isolation must be chilled at 0° to 4° as soon as possible to preserve the viability of the virus. If virus isolation is to be attempted within 1 day, no other storage precaution is necessary. For longer storage, the samples should be frozen at below -20°C as soon as possible. Swabs should be placed in cold sterile media with antibiotics to suppress bacterial and fungal growth (Gelb, 1989; de Wit, 2000). In case of identification by molecular techniques, swabs from respiratory tract or cloaca may also be submitted without being placed in liquid transport media (Cavanagh *et al*., 1999).

1.7.2 Virus Isolation and serotyping

Virus isolation (VI) can be laborious, time consuming and costly. The isolation has to be confirmed using a second technique, such as retro-trascription polimerase chian reaction (RT-PCR), immunofluorescence assay (IFA) or antigen ELISA, for genome or antigen detection. IBV can be isolated using different biological systems: embryonated chicken specific pathogen free (SPF) eggs, chicken embryo tracheal organ cultures (TOC) and cell cultures (McMartin, 1993).

IBV grows well in embryonated chicken eggs. The eggs are inoculated at 9-11 days of incubation by corion-allantoic route (Gelb and Jackwood 2008). The maximum virus titre in allantoic fluids can be reached 1 to 2 days post-inoculation (Hitchner and White, 1955), although this peak can be delayed for non-egg-adapted field strains. For isolation of non-egg-

adapted field strains several sequential passages can be given to increase the amount of virus before performing the antigen detection method. The extent of changes to the infected embryos that are induced by IBV vary greatly. IBV produces embryo stunting, dwarfing, curling, clubbing of the down, or urea deposit in the mesonephros. Especially for field strains, the visible changes in the embryos in the first passage can be minimal. Usually, embryo mortality and dwarfing increase as the number of serial passages increases.

In general, adaptation of IBV strains is necessary for fluent replication and induction of cytopathic effect (CPE) in cell cultures (Gillette, 1973). Chick embryo kidney (CEK) cells and chicken kidney cells show the highest sensitivity for adapted IBV strains (Otsuki *et al.*, 1979).

TOCs, usually prepared from 20-day- old (SPF) embryos, have proved very successful for the isolation, titration and serotyping of IBV (Cook, 1984) because no adaptation of field strains is required for growth and induction of ciliostasis. Ciliostasis, easily observed by low-power microscopy, usually occurs within 3 to 4 days after inoculation, but this period can differ between strains, inoculation dose (Colwell & Lukert, 1969) and, possibly, genetic factors of the host (Otsuki *et al.*, 1990). The presence of IBV has to be confirmed by an IBV-specific test, since ciliostasis can also be induced by many other agents.

Serotyping of IBV isolates and strains can be done using haemoagglutination inhibition test (HI) (Alexander *et al*., 1983; King and Hopkins, 1984) and virus neutralization (VN) tests in chicken embryos (Dawson and Gough, 1971), TOCs (Darbyshire *et al*., 1979) and cell cultures (Hopkins, 1974).

Monoclonal antibodies (MAbs), usually employed in ELISA, have proven useful in grouping and differentiating strains of IBV (Ignjatovic and McWater, 1991; Koch *et al*., 1986). The limitation of MAb analysis for IB serotype definition are the lack of availability of MAbs and the need to produce new MAbs, with appropriate specificity, to keep pace with the ever-growing number of emerging IB-variant serotypes (Karaca *et al*., 1992).

1.7.3 Detection of IBV genome

A wide range of genetically distinct IBV genotypes exists and novel variants continue to emerge. With the advent of molecular biology technique, many different protocols of RT-PCR, RT-Nested PCR and Real Time RT-PCR have been developed, for detection and typing of IBV.

Protocols direct on conservative regions of the viral genome, such as the 3'UTR (Adzhar *et al*., 1996), the 5' UTR (Callison *et al*., 2006) and the RdRp (Poon *et al*., 2005) are used for IBV detection. Protocols targeting part of the S1 gene (Cavanagh *et al*., 1999; Jones *et al*., 2005; Valastro *et al*., 2010) are typically used for genotyping. However, due to the availability of different protocols for IBV genotyping, in the online databases are present sequences of different parts of the S1 gene. In order to standardize all the available data, it has been recently proposed to use only the sequencing of the complete S1 gene for the genotypization (Valastro *et al*., 2016). An issue that make difficult the diagnostic of IBV is the frequent presence in the same host of different IBV strains, including vaccine strains, and the inability to unequivocally distinguish between them (Franzo *et al.*, 2014). Although universal oligonucleotides are adequate for the detection of most known IBV strains, emerging variants or some strains in a mixed sample may be missed. In many laboratories, after the IBV detection using universal primers, genotype specific protocols are used for the identification of different genotypes co-infecting the same host. The traditional molecular diagnostic tools applied to genotype identification are relatively laborious and frequently require additional analyses procedure to ensure unambiguous identification of IBV strains, and to type all IB variants potentially co-infecting the same host. As consequence, new molecular methods for genotype characterization are needed and have been investigated as the use of microsphere-based assay (Valastro *et al*., 2014).

1.7.4 Antigen detection methods

All the technique used for identification of IBV-specific antigen employ IBV-specific antibodies. These antibodies are either in form of antisera or MAbs. Agar Gel precipitation test (AGPT), IFA, Immunoperoxidase assay (IPA) and antigen ELISA could be used for detecting IBV antigen directly in tracheal exudate (Lohr, 1981) or to confirm the presence of IBV in inoculated embrionated chicken eggs (Alexander and Gough, 1977, reviewed in de Wit, 2011).

1.7.5 Serology

A number of serological tests have been described, including VN, agar gel immunodiffusion (AGID), HI and ELISA. Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, for routine serological testing VN tests are too expensive and impractical, while AGID tests lack sensitivity. ELISA and HI tests are most

suitable for routine serology. ELISAs are useful for general monitoring of IBV exposure and can detect antibody responses to all serotypes. Strains and isolates of IBV will agglutinate chicken red blood cells after neuraminidase treatment. HI when used on serial sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. Because chicken sera from older birds contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB cannot be used with a high degree of confidence. Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine or field challenge responses (OIE, 2017).

1.8 Control

1.8.1 Biosecurity and traditional vaccines

IBV is ubiquitous in most parts of the world where poultry are reared and is able to spread very rapidly in non-protected birds (De Wit *et al*., 1998). Although strict biosecurity and working with a one-age system are essential control measures, vaccination is normally an essential tool to increase the resistance of the chickens against challenge with IBV (Cook, 2008).

For vaccination of chickens against IBV, both live attenuated and inactivated (usually oil-adjuvanted) vaccines are used. Live vaccines are especially used in young birds to achieve early protection and also for priming of future layers and breeders that will be boosted with the inactivated vaccines. Live attenuated vaccines are important for the stimulation of the local protection, but the protection last not very long in the field. In areas with an increased level of field challenge, live attenuated vaccines are also used periodically during the laying period with the intention of keeping the local protection of the respiratory tract at a high level.

The vaccines of Mass serotype are the most used in the world. In several parts of the world, Mass vaccines are the only allowed vaccines, but elsewhere, vaccines of other serotypes are permitted. In Europe, vaccine strains of the 793B and QX genotype are commonly used. Depending on the epidemiological situation of the area, also other genotypes are used.

Vaccines of a certain serotype (or genotype) are normally able to protect the well-vaccinated

chicken against a homologous challenge. Often there is a partial protection against strains of other serotypes or genotypes, that can vary from low to high (Bijlenga *et al*., 2004). The magnitude and duration of the response to vaccination is dependent on many factors, including age of the chick, levels of maternal immunity, immunogenicity of the vaccine, method of vaccine application, virulence of the field strain challenge, interval between vaccination and challenge and immunocompetency of the host. Chickens live vaccinated under optimal conditions may have immunity lasting many months and for broilers, this may be life-long (Bijlenga *et al*., 2004).

It has been shown that vaccination with two antigenically distinct live-attenuated vaccines such as Mass and 793B can result in a broad cross-protection against many different IBV types (Cook *et al*., 1999; Terregino *et al*., 2008). The cross-protection was broader when these vaccines were applied with a 2-week interval than when the vaccines were combined on the same day. Whatever live vaccine is used, the application is a very critical step. IBV virus is a sensitive virus that can be inactivated easily (Cavanagh & Gelb, 2008), which may result in inadequate efficacy of the vaccination under field conditions (Jackwood *et al*., 2009; De Wit *et al*., 2010). Live attenuated vaccines may be applied by eye drop or nasal drop, spray or drinking water routes and it is essential that a high percentage of the birds receive a required dose of the vaccine in the right tissue. Inadequate ‘‘take’’ of the vaccine may result in no or a decreased level of protection, delayed protection, or prolonged presence/circulation of the vaccine virus in the flock, that could result in reversion to virulence of the virus (Hopkins & Yoder, 1986). Commonly, broilers are vaccinated in the hatchery at one day of age by coarse spray using a live attenuated M41 vaccine strain. A booster vaccination at 10-15 days with a live attenuated vaccine of a different genotype, depending on the epidemiological situation of the area, is used to give broad protection to the vaccinated birds. In Italy, usually, for the booster vaccination a 793B or a QX vaccine strain is used. It is also possible to combine at one day of age different vaccine strains in the hatchery (Pascucci, 2015). Inactivated oil-emulsion vaccines are administered to breeders and layers prior to the onset of egg production. Pullets may be vaccinated between 10 and 18 weeks of age depending on the immunization program. The effects of inactivated vaccines depend heavily on proper priming with live vaccines. Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Inactivated vaccines induce high level of serum antibodies and provide protection to internal tissues, kidney, and reproductive tract (de Wit *et al*., 2011a; Landman *et al*., 2002). In contrast to live vaccines, inactivated vaccines are not nearly as

effective at preventing infection of the respiratory tract following challenge with homologous virulent virus (Cook *et al*., 1986). To achieve an early post hatch protection against IBV, Avian metapneumovirus (AMPV) and a Newcastle disease virus (NDV), it is possible to apply simultaneously different live attenuated vaccines in the hatchery. Different studies demonstrated that the simultaneously application of an IBV, an AMPV and a NDV live attenuated vaccine in dual or triple combination did not interfere in the production of IBV humoral antibody responses, neither in the level of protection against IB challenge (Cook *et al*., 2001; Tarpey *et al*., 2007; Awad *et al*., 2015).

1.8.2 New generation Vaccines

Molecular vaccines, including subunit vaccines, DNA vaccines, virus-like particles (VLP) and recombinant vector vaccines have all been developed and tested for their efficacy against IBV (Cook *et al*., 2012). Already in 1994, Ignjatovic and Galli produced S1, N, and M proteins by immunoaffinity purification of IBV strain N1/62 and used them for immunization of chickens. Multiple immunizations were necessary for induction of an antibody response. Neither the N nor the M antigen induced protection to a virulent challenge after 4 immunizations.

A recombinant CVI1988/Rispens Marek's disease virus (MDV) vaccine (rMDV-S1) expressing the S1 gene of a QX IBV inserted into the genome of virus has been developed. SPF chickens that were vaccinated at day old with rMDV-S1 were found to have a low-level of antibody response until 4 weeks post-vaccination, but after IBV challenge, performed at 4 weeks of age, the antibody level increased more rapidly when compared to the control group. The chickens were protected when challenged with the QX-like IBV (Zhang *et al*., 2012). Falchieri et colleagues in 2013 developed a recombinant vaccine expressing either S1 or N genes, or both, of IBV in AMPV vector. Day-old chicks were vaccinated by eye-drop. The vaccine induced partial protection against virulent IBV QX challenge 3 weeks later, as assessed by tracheal ciliar activity test. Nonetheless no sero-conversion nor major tracheal rAMPV/IBV replication (assessed by real time RT-PCR) in vaccinated chicks was observed (Falchieri *et al*., 2013). Toro and colleagues in 2014 developed a recombinant vaccine using a Newcastle disease virus (LaSota) expressing IBV S2 gene. IBV heterotypic protection was assessed using a prime–boost approach along with a commercial attenuated Mass type vaccine. Their results demonstrated that priming the birds with IBV S2, which overexpose the chicken immune system to S2, followed by boost with whole virus protects chickens against IBV (Toro *et al*., 2014a). Different research groups have used Adenovirus as vector for the expression of IBV

proteins. A recombinant Fowl Adenovirus (FAV) that expresses the S1 from IBV Vic S strain has been developed and tested in birds at day six, 90–100% protection was induced against either homologous or heterologous challenge, demonstrating that the recombinant FAV/S1 IBV vaccine could be a potential alternative vaccination strategy against IBV (Johnson *et al*., 2003).

Toro and colleagues developed a recombinant Adenovirus vaccine expressing S1 protein of Ark genotype IBV; one of the vaccine developed caused, after a single vaccination dose, an effective protection of chickens from clinical signs and significantly reduced viral load after IBV Ark virulent challenge; moreover, the number of both IgA and IgG IBV specific antibody secreting lymphocytes in the spleen increased after challenge (Toro *et al*., 2014b). Liu and colleagues, in 2013, produced and assembled a IBV VLPs vaccine by inserting and M and S proteins in baculovirus grown in Sf9 cells. The generated VLPs induced, 28 days post vaccination humoral immune responses in a level comparable to that of inactivated IBV vaccine and elicited significantly higher cellular immune responses than the inactivated vaccine (Liu *et al*., 2013). In the 2014 a modified baculovirus expressing the S1 glycoprotein of a M41 strain was produced. This vaccine was able to generate strong humoral and cell-mediated immune responses, and showed good induction of cytotoxic T lymphocyte response, giving to the vaccinated birds a good protection following challenge with a virulent IBV M41, almost comparable to an inactivated IBV vaccine (Zhang *et al*., 2014). In the same year, Lv et colleagues (2014) developed a chimeric virus-like particle (VLP). The VLP was composed of matrix and neuraminidase proteins of avian influenza (AI) (H5N1) and of protein S1 of IBV. The chimeric VLPs induced in SPF chickens a significantly higher neutralization antibody level when compared to an inactivated H120 virus**.** A recombinant S-ectodomain protein subunit vaccine has recently been developed and tested in experimental trial. This vaccine showed a reduced viral loads 5 days post-challenge in both tears and tracheas statistically significant different from chickens immunized with a recombinant S1 protein. Consistent with viral loads, significantly reduced tracheal mucosal thickness and tracheal lesion scores revealed that recombinant S-ectodomain protein provided improved protection of tracheal integrity compared to S1 protein. These results indicate that the S2 domain has an important role in inducing protective immunity. Thus, including the S2 domain in IBV recombinant vaccine might be promising for better viral vectored and/or subunit vaccine strategies (Eldemery *et al*., 2017). Different research groups have developed a reverse genetic system for manipulation of IBV genome in order to develop of rationally designed live-attenuated IBV

vaccines (Casais *et al.*, 2001; Fang *et al.*, 2007; Youn *et al.*, 2005; Zhou *et al.*, 2013; van Beurden *et al.*, 2017).

To date only traditional live attenuated and inactivated vaccines are commercially available for the control of the disease.

1.9 References

- Aaziz R., Tepfer M. (1999). *Recombination in RNA viruses and in virus-resistant transgenic plants*. J Gen Virol 80: 1339-1346.
- Acevedo A. M., Diaz de Arce H., Brandao P.E., Colas M., Oliveira S., Perez L.J. (2012). *First evidence of the emergence of novel putative infectious bronchitis virus genotypes in Cuba*. Res Vet Sci 93: 1046-1049.
- Adzhar A., 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.
- Abolnik C. (2015). *Genomic and single nucleotide polymorphism analysis of infectious bronchitis coronavirus*. Infect Genet Evol 32: 416-424.
- Alexander D.J., Collins M.S (1975). *Effect of pH on the growth and cytopathogenicity of avian infectious virus.* Avian Dis 30: 468-476.
- Alexander D.J., Gough R.E. (1977). *Isolation of avian infectious bronchitis virus from experimentally infected chickens.* Res Vet Sci 23: 344-347.
- Alexander D.J., Allan W.H., Biggs P.M., Bracewell C.D., Darbyshire J.H., Dawson P.S., Harris A.H., Jordan F.T., Macpherson I., Mcferran J.B., Randall C.J., Stuart J.C., Swarbrick O. & Wilding G.P. (1983). *A standard technique for haemagglutination inhibition tests for antibodies to avian infectious bronchitis virus*. Vet Rec 113: 64.
- Ambali A.G., Jones R.C. (1990). *Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus*. Avian Dis 34: 809-817.
- 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: 687-692.
- Armesto M., Bentley K., Keep S., Bickerton E., Britton P. (2012) *Chapter 2: Coronavirus reverse genetics.* In Reverse genetics of RNA viruses: Application and Prespectives. A. Bridgen (ed.), Wiley-Blackwell, Chichester, UK, pp.27-65.
- Armstrong J., Niemann H., Smeekens S., Rottier P., Warren G. (1984). *Sequence and topology of a model intracellular membrane protein, E1 glycoprotein, from a coronavirus*. Nature 308: 751-752.
- Awad F., Forrester A., Baylis M., Lemiere S., Jones R.C., Ganapathy K. (2015). *Immune*

responses and interactions following simultaneous application of live Newcastle disease, infectious bronchitis and avian metapneumovirus vaccines in specific-pathogen-free chicks. Res Vet Sci 98: 127-133.

- Baba T., Masumoto K., Nishida S., Kajikawa T., Mitsui M. (1988). Harderian gland dependency of immunoglobulin A production in the lacrimal fluid of chicken. Immunol 65: 67-71.
- Ballal A., Karrar A.E., El Hussein A.M. (2005). *Isolation and characterization of infectious bronchitis virus strain 4/91 from commercial layer chickens in the Sudan*. J Anim Vet Adv 4: 910–912.
- Bande F., Arshad S.S., Omar A.R., Hair-Bejo M., Mahmuda A., Nair V. (2017). *Global distribution and strain diversity of avian infectious bronchitis virus: a review.* Animal Health Research Reviews 18: 70-83.
- Barnes H. J., Guy J. S., Vaillancourt J. P. (2000). *Poult enteritis complex*. Rev Sci Tech 19: 565-588.
- Bhattacharjee P.S., Jones R.C. (1997). *Susceptibility of organ cultures from chicken tissues for strains of infectious bronchitis virus isolated from the intestine*. Avian Pathol 26: 553-563.
- 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 (QX IBV) in Italy*. Vet Rec 156: 720.
- Becker W.B., McIntosh K., Dees J.H., Chanock R.M. (1967). *Morphogenesis of Avian Infectious Bronchitis Virus and a Related Human Virus (Strain 229E)*. J Virol 1: 1019-1027.
- Benyeda Z., Mato T., Suveges T., Szabo E., Kardi V., Abonyi-Toth Z., Rusvai M., Palya V. (2009). *Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions*. Avian Pathol 38: 449-456.
- Benyeda Z., Szeredi L., Mató T., Süveges T., Balka G., Abonyi-Tóth Z., Rusvai M., Palya V. (2010). *Comparative histopathology and immunohistochemistry of QX-like, Massachusetts and 793/B serotypes of infectious bronchitis virus infection in chickens.* J Comp Pathol 143: 276-283.
- Bentley K., Keep S.M., Armesto M., Britton P. (2013). *Identification of a noncanonically transcribed subgenomic mRNA of infectious bronchitis virus and other gammacoronaviruses*. J Virol 87: 2128-36.

- Bijlenga G., Cook J.K.A., Gelb J., 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: 550-557.
- 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: 379-393.
- Boltz D.A., Nakai M., Bahra J.M. (2004). *Avian infectious bronchitis virus: a possible cause of reduced fertility in the rooster*. Avian Dis 48: 909-915.
- Boots A.M.H., Benaissa-Trouw B.J., Hesselink W., Rijke E., Schrier C., Hensen E.J. (1992). *Induction of anti-viral immune responses by immunization with recombinant-DNA encoded avian coronavirus nucleocapsid protein*. Vaccine 10: 119-124.
- Bourogâa H., Miled K., Gribâa L., El Behi I., Ghram A. (2009). *Characterization of new variants of avian infectious bronchitis virus in Tunisia*. Avian Dis 53: 426-433.
- Bourogâa H., Hellal I., Hassen J., Fathallah I., Ghram A. (2012). *S1 gene sequence analysis of new variant isolates of avian infectious bronchitis virus in Tunisia*. Veterinary Medicine: Research and Reports 3: 41-48.
- Boscarino J.A., Logan H.L., Lacny J.J., Gallagher T.M. (2008). *Envelope protein palmitoylations are crucial for murine coronavirus assembly*. J Virol 82: 2989-2999.
- Bosch B.J., van der Zee R., de Hann C.A.M., Rottier P.J.M. (2003). *The Coronavirus Spike Protein Is a Calss I Virus Fusion Protein: Structural and Functional Characterization of the Fusion Core Complex.* J Virol 77: 8801-8811.
- Boursnell M.E.G., Brown T.D.K., 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.
- Breyer W.A., Matthews B.W. (2001). *A structural basis for processivity*. Protein Sci 10: 1699-1711.
- Britton, P., Cavanagh, D., (2007). *Avian coronavirus diseases and infectious bronchitis vaccine development.* In: Coronaviruses: Molecular and Cellular Biology. Thiel V. (ed.), Caister Academic Press, Norfolk, UK, pp. 161-181.
- Brown D.W. (1997). *Threat to humans from virus infections of non-human primates*. Rev Med Virol 7:239-246.
- Brown P.A., Touzain F., Briand FX., Gouilh A.M., Courtillon C., Allée C., Lemaitre E., De Boisséson C., Blanchard Y., Eteradossi N. (2016). *First complete genome sequence of*

European turkey coronavirus suggests complex recombination history related with US turkey and guinea fowl coronaviruses. J Gen Vir 97: 110-120.

- 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: 492-499.
- Callison S.A., Hilt D.A., Boynton D.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 Method 138: 60-65.
- Cao J., Wu C.C., Lin T.L. (2008). *Complete nucleotide sequence of polyprotein gene 1 and genome organization of turkey coronavirus*. Virus Res 136: 43-49.
- Capua I., Gough R.E., Mancini M., Casaccia C., Weiss C. (1994). *A 'novel' infectious bronchitis strain infecting broiler chickens in Italy.* Zentralblatt für Veterinärmedizin. Reihe B. J Vet Med 41: 83-89.

  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.
- Cavanagh D. (2000). *Coronaviruses and Toroviruses*. In A. Z. Zuckerman, J. E. Banatvala, and J. R. Pattison (eds.). Principles and Practice of Clinical Virology, 4th ed. John Wiley and Sons: Chichester, United Kingdom, pp. 345-356.
- Cavanagh D. (2001). *A nomenclature for avian coronavirus isolates and the question of species status*. Avian Pathol 30: 109-115.
- Cavanagh D. (2005). *Coronaviruses in poultry and other birds*. Avian Pathol 34: 439-448.
- Cavanagh D. (2007). *Coronavirus avian infectious bronchitis virus*. Vet Res 38: 282-297.
- Cavanagh D., Britton P. (2008) *Chapter 3: Nidovirus Genome Organization and Expression Mechanisms*. In Nidoviruses, Perlman S., Gallagher T., Snijder E.J. (eds.), Washington D.C., pp. 29-46.
- 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. (1993). *Sequence analysis of strains of avian infectious bronchitis coronavirus isolated during the 1960s in the U.K.* Arch Virol 130: 471-476.
- Cavanagh D., Naqi S., (2008). *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 edition. Iowa State University Press, Ames, pp. 101-120.
- Cavanagh D., Jelb J.Jr. (2008). *Infectious Bronchitis*. In Diseases of Poultry 12th ed, Saif Y.M. (ed.), Blackwell Publishing, Ames, Iowa, pp. 117-135.
- Cavanagh D., Darbyshire J.H., Davis P., Peters R.W. (1984). *Induction of humoral neutralising and haemagglutination-inhibiting antibody by the spike protein of avian infectious bronchitis virus.* Avian Pathol 13: 573-583.
- Cavanagh D., Davis P.J., Pappin D.J., Binns M.M., Boursnell M.E., Brown T.D. (1986a). *Coronavirus IBV: partial amino terminal sequencing of spike polypep- tide 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., Darbyshire J.H., Peters R.W. (1986b). *Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection.* J Gen Virol 67: 1435-1442.
- 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: 141-150.
- Cavanagh D., Davis P.J., Cook J.K., Li D., Kant A., Koch G. (1992). *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., Ellis 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: 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., Mawditt K.D., Welchman de B., Britton P., Gough R.E. (2002). *Coronaviruses from pheasants (Phasianus colchicus) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys.* Avian Pathol 31:

81-93.

- Casais R., Thiel V., Siddell S.G., Cavanagh D., Britton P. (2001). *Reverse genetics system for the avian coronavirus infectious bronchitis virus*. J Virol 75: 12359-12369.
- Chang C.K., Sue S.C., Yu T.H., Hsieh C.M., Tsai C.K., Chiang Y.C., Lee S.J., Hsiao H.H., Wu W.J., Chang W.L., Lin C.H., Huang T.H. (2006). *Modular organization of SARS coronavirus nucleocapsid protein.* J Biomed Sci 13: 59-72.
- Chen B.Y., Itakura C. (1996). *Cytopathology of chick renal epithelial cells experimentally infected with avian infectious bronchitis virus*. Avian Pathol 25: 675-690.
- Chen B.Y., Hosi S., Nunoya T., Itakura C. (1996). *Histopathology and immunohistochemistry of renal lesions due to infectious bronchitis virus in chicks.* Avian Pathol 25: 269-283.

  Chousalkar K.K., Roberts J.R. (2007). *Ultrastructural study of infectious bronchitis virus infection in infundibulum and magnum of commercial laying hens*. Vet Microb 122: 223-236.
- Chousalkar K.K., Roberts J.R., Reece R. (2007). *Histopathology of two serotypes of infectious bronchitis virus in laying hens vaccinated in the rearing phase*. Poult Sci 86: 59-62.
- Chu V.C., McElroy L.J., Chu V., Bauman B.E., Whittaker G.R. (2006). *The avian coronavirus infectious bronchitis virus undergoes direct low-pH-dependent fusion activation during entry into host cells*. J Virol 80: 3180-3188.
- Chubb R.C. (1974). *The effect of the suppression of circulating antibody on resistanc to the Australian avian infectious bronchitis virus in poultry*. Dev Comp Immunol 24: 187-200.
- Circella E., Camarda A., Martella V., Bruni G., Lavazza A., Buonavoglia C. (2007). *Coronavirus associated with an enteric syndrome on a quail farm*. Avian Pathol 36: 251-258.
- Collison E.W., Pei J., Dzielawa J., Seo S.H. (2000). *Cytotoxic T lymphocites are critical in the control of infectious bronchitis virus in poultry.* Dev Comp Immunol 24: 187-200.
- Colwell W.M., Lukert P.D. (1969). *Effects of avian infectious bronchitis virus (IBV) on tracheal organ cultures.* Avian Dis 13: 888-894.
- Condron R.J., Marshall A.T. (1986) *Pathogenesis of infectious bronchitis nephritis 1. Morphometric analysis of kidney proximal tubular epithelium in chickens*. J Comp Pathol 96: 47-61.

- Cook J.K. (1968). *Duration of experimental infectious bronchitis in chickens*. Res Vet Sci 9: 506-514.
- Cook J.K. (1971). *Recovery of Infectious Bronchitis virus from eggs and chicks produced by experimentally inoculated hens*. J Comp Pathol 81: 203-211.
- Cook J.K.A. (1983). *Isolation of a new serotype of infectious bronchitis-like virus from chickens in England.* Vet Rec 112: 104-105.
- Cook J.K.A. (1984). *The classification of new serotypes of IBV isolated from poultry flocks in Britain between 1981 and 1983*. Avian Pathol 13: 733-741.
- Cook J.K.A. (2008). *Coronaviridae*. In M. Pattison, P.F. McMullin, J.M. Bradbury & D.J. Alexander (Eds.). Poultry Diseases 6th edn., Philadelphia, Pennsylvania, USA: Saunders Elsevier, pp.340-349.
- 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.A., Smith H.W., Huggins M.B. (1986). *Infectious bronchitis immunity: its study in chickens experimentally infected with mixtures of infectious bronchitis virus and Escherichia coli.* J Gen Virol 67: 1427-1434.
- Cook J.K., Davison T.F., Huggins M.B., McLaughlan P. (1991). *Effect of in ovo bursectomy on the course of an infectious bronchitis virus infection in line C White Leghorn chickens*. Arch Virol 118: 225-234.
- 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: 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., Huggins M.B., Orbell S.J., Mawditt K., Cavanagh D. (2001). *Infectious bronchitis virus vaccine interferes with the replication of avian pneumovirus vaccine in domestic fowl.* Avian Pathol 30: 233-242.
- Cook J.K., Jackwood M., Jones R.C. (2012). *The long view: 40 years of infectious bronchitis research*. Avian Pathol 41: 239-250.
- Corse E., Machamer C.E. (2000). *Infectious bronchitis virus E protein is targeted to the Golgi complex and directs release of virus-like particles*. J Virol 74: 4319-4326.

- Chousalkar J.R. Roberts J.R. (2007). *Ultrastructural study of infectious bronchitis virus infection in infundibulum and magnum of commercial laying hens.* Vet Microb 122: 223-236.
- Crinion R.A.P., Hofstad M.S. (1972). *Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages.* Avian Dis 16: 351-363.
- Darbyshire J.H., Cook J.K.A., Peters R.W. (1976). *Organ culture studies on efficiency of infection of chicken tissues with avian infectious bronchitis virus*. British J Exp Pathol 57: 443-454.
- Dawson P.S., Gough R.E. (1971). *Antigenic variation in strains of avian infectious bronchitis virus*. Archiv f˙ur die gesamte Virusforschung 34: 32-39.
- de Groot R.J., Lenstra J.A., Luytjes W., Niesters H.G., Horzinek M.C., van der Zeijst B.A., Spaan W.J. (1987). *Sequence and structure of the coronavirus peplomer protein*. Adv Exp Med Biol 218: 31-38.
- de Haan C., Masters P., Shen X., Weiss S., Rottier P. (2002). *The group- specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host*. Virol 296: 177-189.
- de Haan C.A., Te Lintelo E., Li Z., Raaben M., Wurdinger T., Bosch B.J., Rottier P.J. (2006). *Cooperative involvement of the S1 and S2 subunits of the murine coronavirus spike protein in receptor binding and extended host range*. J Virol 80: 10909-10918.
- de Wit J.J. (2000). *Detection of infectious bronchitis virus*. Avian Pathol 29: 71-93.
- de Wit J.J. (2006). *Un nuovo capitolo sull'argomento false ovaiole*. Atti della giornata di studio Intervet, Italia, Bologna, 7 giugno 2006.
- De Wit J.J., de Jong M.C., Pijpers A., Verheijden J.H. (1998). *Transmission of infectious bronchitis virus within vaccinated and unvaccinated groups of chickens*. Avian Pathol 27: 464-471.
- de Wit J.J., Swart W.A.J.M., Fabri T.H.F. (2010). *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.A, van der Heijden H.M.J.F. (2011a). *Infectious bronchitis virus variants: a review of the history, current situation and control measures*. Avian Pathol 40: 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: 463-471.

- Develaar F.G., Noordzij A., Vanderdonk J.A. (1982). *A study on the synthesis and secretion of immunoglobulins by the harderian gland of the fowl after eyedrop vaccination against infectious bronchitis at 1-day-old.* Avian Pathol 11: 63-79.
- Dhinakar Raj G., Jones R.C. (1996) *Immunopathogenesis of infection in SPF chicks and commercial broilers of a variant infectious bronchitis virus of economic importance.* Avian Pathol 25: 481-501.
- Dhinakar Raj G., Jones R.C. (1997). *Infectious bronchitis virus: Immunopathogenesis of infection in the chicken.* Avian Pathol 26:677-706.
- Donaldson E., Sims A., Deming D., Baric R. (2006). *Mutational analysis of MHV-A59 replicase protein nsp10*. In Perlman, S. and Holmes, K. (eds), The Nidoviruses: toward control of SARS and other Nidovirus diseases. Advances in experimental medicine and biology, Spinger 581: 61-66.
- Droual R., Woolcock P.R. (1994). *Swollen head syndrome associated with E. coli and infectious bronchitis virus in the Central Valley of California.* Avian Pathol 23: 733-742.
- 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.
- Ducatez M., Europenian Union COST Action FA1207 (2016). *Recommendations for a standardized avian coronavirus (AvCoV) nomenclature: outcome from discussions within the framework of the European Union COST Action FA1207: "towards control of avian coronaviruses: strategies for vaccination, diagnosis and surveillance".* Avian Pathol 45: 602-603.
- Eckerle L., Brockway S., Sperry S., Lu X., Denison M. (2006). *Effects of mutagenesis of murine hepatitis virus nsp1 and nsp14 on replication in culture*. In Perlman, S. and Holmes, K. (eds), The Nidoviruses: toward control of SARS and other Nidovirus diseases. Advances in experimental medicine and biology, Springer 581: 55-60.
- Egloff M., Ferron F., Campanacci V., Longhi S., Rancurel C., Dutartre H., Snijder E., Gorbalenya A., Cambillau C. and Canard B. (2004). *The severe acute respiratory syndrome-coronavirus replicative protein nsp9 is a single-stranded RNA-binding subunit unique in the RNA virus world*. Proc Natl Acad Sci USA 101: 3792-3796.

- El-Houadfi M., Jones R.C. (1985). *Isolation of avian infectious bronchitis viruses in Morocco including an enterotropic variant*. Vet Rec 116: 445.
- El-Houadfi M., Jones R.C., Cook J.K., Ambali A.G. (1986). *The isolation and characterisation of six avian infectious bronchitis viruses isolated in Morocco*. Avian Pathol 15: 93-105.
- Eldemery F., Joiner K.S., Toro H., van Santen V.L. (2017). *Protection against infectious bronchitis virus by spike ectodomain subunit vaccine*. Vacc 35: 5864-5871
- Falchieri M., Lupini C., Cecchinato M., Catelli E., Kontolaimou M., Naylor C.J. (2013). *Avian metapneumoviruses expressing infectious bronchitis virus genes are stable and induce protection*. Vac 31: 2565–2571.
- Fang S., Chen B., Tay F.P., Ng B.S., Liu D.X. (2007). *An arginine-to-proline mutation in a domain with undefined functions within the helicase protein (Nsp13) is lethal to the coronavirus infectious bronchitis virus in cultured cells*. Virol 358: 136-147.
- Fehr A.R., Perlman S. (2015). *Coronaviruses: An overview of their replication and Pathogenesis.* Methods Mol Biol 1282: 1-23.
- 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. (2015a). *Prevalence and molecular characterization of avian infec- tious bronchitis virus in poultry flocks in Morocco from 2010 to 2014 and first detection of Italy 02 in Africa.* Avian Pathol 44: 287-295.
- Fellahi S., El Harrak M., Ducatez M., Loutfi C., Koraichi S.I., Kuhn J.H., Khayi S., El Houadfi M., Ennaji M.M. (2015b). *Phylogenetic analysis of avian infectious bronchitis virus S1 glycoprotein regions reveals emergence of a new genotype in Moroccan broiler chicken flocks.* Virol J 12: 116
- Finney P.M., Box P.G., Holmes H.C. (1990). *Studies with a bivalent infectious bronchitis killed virus vaccine.* Avian Pathol 19: 435-450.
- Franzo G., Naylor C.J., Lupini C., Drigo M., Catelli E., Listorti V., Pesente P., Giovanardi D., Morandini E., Cecchinato M. (2014). *Continued use of IBV 793B vaccine needs reassessment after its withdrawal led to the genotype's disappearance*. Vaccine 32 6765-6767.
- Gillette K. G. (1973). *Plaque formation by infectious bronchitis virus in chicken embryo kidney cell cultures*. Avian Dis 17: 369-378.
- Gelb J. Jr. (1989). *Infectious bronchitis*. In H.G. Purchase, L.H. Arp, C.H. Domermuth & J.E. Pearson (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.J., Jackwood M.W. (2008). *Infectious Bronchitis*. In: A laboratory manual for the isolation, identification, and characterization of avian phatogens, 5th ed. L. Dofour-Zavala, D.E. Swayne, J.R. Glisson, J.E. Pearson, W.M. Reed, M.W. Jackwood and P. Woolcock, eds. American Association of Avian Pathologists, Kennet Square, PA, pp.146-149.
- Gurjar R.S, Gulley S.L., van Ginkel F.W. (2013). *Cell-mediated immune responses in the head-associated lymphoid tissues induced to a live attenuated avian coronavirus vaccine.* Dev Comp Immunol 41: 715-722.
- Hesselink W.G (1991). *Serotyping avian infectious bronchitis virus: selection of a unified method*. In E.F. Kaleta & U. Heffels-Redmann (Eds.). Proceedings of the II International Symposium on Infectious BronchitisRauischholzhausen, Germany, pp. 87-97.
- Hewson K.A., Ignjatovic J., Browning G.F., Devlin J.M., Noormohammadi A.H. (2011). *Infectious bronchitis viruses with naturally occurring genomic rearrangement and gene deletion*. Arch Virol 156: 245-252.
- 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. (1984). *Avian infectious bronchitis*. In M. S. Hofstad, H. J. Barnes, B. W. Calnek, W. M. Reid, and H. W. Yoder, Jr. (eds.). Diseases of Poultry, 8th ed. Iowa State Uni- versity Press: Ames, IA, pp. 429-443.
- Hopkins S.R., Yoder H.W.Jr. (1986). *Reversion to virulence of chicken-passaged infectious bronchitis vaccine virus*. Avian Dis 30: 221-223.
- Fischer F., Stegen C.F., Masters P.S., Samsonoff W.A. (1998). *Analysis of constructed E gene mutants of mouse hepatitis virus confirms a pivotal role for E protein in coronavirus assembly*. J Virol 72: 7885-7894.
- Gelb J., Nix W.A. Jr., Gellman S.D. (1998). *Infectious bronchitis virus antibodies in tears and their relationship to immunity*. Avian Dis 42: 364-374.
- Gibbs M.J., Weiller G.F. (1999). *Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus*. Proc Natl Acad Sci USA 96: 8022-8027.
- Gelb J.Jr, Jackwood M.W. (2008). *Infectious Bronchitis*. In: A Laboratory Manual for the

Isolation, Identification, and Characterization of Avian Pathogens. 5° ed., L.Dufour-Zavala, D.E. Swayne, J.R. Glisson, J.E. Pearson, W.M. Reed, M.W. Jackwood e P. Woolcock. Ed. American Association of Avian Pathologists, Kennet Square, PA, pp. 146-149.

- Gosert R., Kanjanahaluethai A., Egger D., Bienz K. Baker S. (2002). *RNA replication of mouse hepatitis virus takes place at double-membrane vesicles*. J. Virol. 76: 3697-3708.
- Graham R.L., Sparks J.S., Eckerle L.D., Sims A.C., Denison M.R. (2008). SARS coronavirus replicase proteins in pathogenesis. Virus Res 133: 88-100.
- 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: 493-494.
- Gough R.E., Cox W.J., Winkler C.E., Sharp M.W., Spackman D. (1996). *Isolation and identification of infectious bronchitis virus from pheasants.* Vet Rec 138: 208-209.
- Hagemeijer M., Verheije M.H, Ulasli M., Shaltie I., de Vries L., Reggiori F., Rottier P., de Haan C. (2010). *Dynamics of coronavirus replication- transcription complexes*. J Virol. 84: 2134-2149.
- 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.
- 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: 581-589.
- Hipolito, O. (1957). *Isolamento e identificacao do virus da bronquite infecciosa das galinhas no Brasil.* Arquivo Escuela Veterinaria Universidade de Minas Gerais, 10: 131-151.
- Hitchner S.B. and White P.G. (1955). *Growth curve studies of chick embryo-propagated infectious bronchitis virus*. Poultry Sci 34: 590–594.
- Holmes E.C. (2009). *The Evolution and Emergence of RNA Viruses, 1st ed.;* Oxford University Press Inc.: New York, NY, USA.
- Hurst K.R., Koetzner C.A., Masters P.S. (2009). *Identification of in vivo-interacting domains of the murine coronavirus nucleocapsid protein*. J Virol 83: 7221-7234.
- Hurst K.R., Koetzner C.A., Masters P.S. (2013). *Characterization of a critical interaction between the coronavirus nucleocapsid protein and nonstructural protein 3 of the viral replicase-transcriptase complex*. J Virol 87: 9159-9172.

- Hutton S, Bettridge J, Christley R, Habte T and Ganapathy K (2016). *Detection of infectious bronchitis virus 793B, avian metapneumovirus, Mycoplasma gallisepticum and Mycoplasma synoviae in poultry in Ethiopia.* Trop Anim Health Prod 49: 317–322.
- 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.
- Ignatovic 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., Galli L. (1995). *Immune responses to structural proteins of avian infectious bronchitis virus*. Avian Pathol 24: 313-332.
- 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: 535-552.
- Ignjatovic J., Gould G., Sapats S. (2006). *Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains*. Arch Virol 151: 1567-1585.
- Ismail M.M., Tang A.Y., Saif Y.M. (2003). *Pathogenicity of turkey coronavirus in turkeys and chickens*. Avian Dis 47: 515-522.
- Ito N.M.K., Miyaji C.I., Capellaro C.E.M.P.D.M. (1991). Studies on broiler's IBV and IB-like virus from guinea fowl. In E.F. Kaleta & U. Heffels-Redmann (Eds.), Proceedings of the II International Symposium on Infectious Bronchitis pp. 302 -307.
- Janse E.M., Van Roozelaar D., Koch G. (1994) *Leukocyte subpopulations in kidney and trachea of chickens infected with infectious bronchitis virus*. Avian Pathol 23: 513-523.
- Jackwood M.W. (2012). *Review of Infectious bronchitis virus around the world*. Avian Dis 56: 634-641.
- Jackwood M.W. and De Wit S. (2013). *Infectious bronchitis*. In: Diseases of poultry, 13th edition. Swayne D.E., Glisson J.R., McDougald L.R., Nolan L.K., Suarez D.L., Nair V. (eds.). Wiley-Blackweel, pp. 139-159.
- Jackwood M.W., Hilt D.A., Lee C.W, Kwon H.M., Callison S.A., Moore K.M., Moscoso H., Sellers H., Thayer S. (2005). *Data from 11 Years of Molecular Typing Infectious Bronchitis Virus Field Isolates*. Avian Dis 49: 614-618.
- 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., Hall D., Handel A. (2012). *Molecular evolution and emerge of avian gammacoronaviruses.* Infect Genet Evol 12: 1305-1311.
- Jonassen C.M., Kofstad T., Larsen I-L., Lovland A., Handeland K., Follestad A., Lillehaug, A. (2005). *Molecular identification and characterization of novel coronaviruses infecting greylag geese (Anser anser), feral pigeons (Columba livia) and mallards (Anas platyrhynchos)*. J Gen Virol 86: 1597-1607.
- 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 variant (793B, 4/91, CR88)*. In E.F. Kaleta & U. Heffels-Redmann (Eds.). Proceedings of the IV International Sympo- sium on Avian Corona- and Pneumovirus Infections, Rauischholzhausen, Germany, pp. 105-111.
- Jones R.C., 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: 646-647.
- 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.
- Juul-Madsen H.R., Norup L.R.; Jørgensen P.H.; Handberg K.; Wattrang E.; Dalgaard T.S. *Crosstalk between innate and adaptive immune responses to infectious bronchitis virus after vaccination and challenge of chickens varying in serum mannose-binding lectin concentrations*. Vaccine 29: 9499-9507
- Juungherr E.I., Chomiak T.W., Luginbuhl R.E. (1956). *Immunologic differences in strains of infectious bronchitis virus.* Proceeding of 60th Annual Meeting US Livestock Sanitary Association, 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.
- Karaca K., Naqi S., Gelb J. (1992). *Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes*. Avian Dis 36: 903-915.
- Khatchikian D., Orlich M., Rott R. (1989). *Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus*. Nature 340: 156-157.

- Kint J., Langereis M.A, Maier H. J., Britton P., van Kuppeveld F. J., Joseph Koumans J., Wiegertjes G.F., Forlenza M. (2016). *Infectious Bronchitis Coronavirus Limits Interferon Production by Inducing a Host Shutoff That Requires Accessory Protein 5b*. J Virol 90: 7519-7528.
- Koch G., Hartog L., Kant A., van Roozelaar D., de Boer G.F. (1986). *Antigenic differentiation of avian bronchitis virus variant strains employing monoclonal antibodies*. Israel J Vet Med 42: 89-97.
- 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.
- Kuo S.M., Wang C.H., Hou M.H., Huang Y.P., Kao H.W., Su H.L. (2010). *Evolution of infectious bronchitis virus in Taiwan: characterization of RNA recombination in the nucleocapsid gen*e. Vet Microb 144: 293-302.
- 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: 217-221.
- 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. (1992). *RNA Recombination in animal and plant viruses.* Microbiol Rev 56: 61-79.
- Landman W.J.M., Pope C.R., Ziegler A.F., Swieczkowki T., Callahan C.J., Davison S., Gelb J.Jr. (2002). *Protection of chickens after live and inactivated virus vaccination against challenge with nephropathogenic infectious bronchitis virus PA/Wolgemuth/98*. Avian Dis 46: 938-944.
- Landman W.J.M., Dwars R.M., De Wit J.J. (2005). *High incidence of false layers in (re)production hens supposedly attributed to a juvenile infectious bronchitis infection*. In Proceedings of the XIVth International Congress of the World Veterinary Poultry Association, Turkey, Istanbul, p.269.
- 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: 332-337.
- Lee S. K., Sung H.W., Kwon H.M. (2004). *S1 glycoprotein gene analysis of infectious bronchitis viruses isolated in Korea*. Arch Virol 149: 481-494.
- Lenstra J.A., Kusters J.G., Koch G., van der Zeijst B.A. (1989). *Antigenicity of the*

peplomer protein of infectious bronchitis virus. Mol Immunol 26: 7-15.

- Lim K. P.., Liu D.X. (1998). *Characterization of the two overlapping papain-like proteinase domains encoded in gene 1 of the coronavirus infectious bronchitis virus and determination of the C-terminal cleavage site of an 87-kDa protein*. Virology 245: 303-312.
- Lim K.P., Ng L.F., Liu D.X. (2000). *Identification of a novel cleavage activity of the first papain-like proteinase domain encoded by open reading frame 1a of the coronavirus Avian infectious bronchitis virus and characterization of the cleavage products*. J Virol 74: 1674-1785.
- 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: 678-685.
- Lin T.L., Loa C.C., Wu C.C. (2004). *Complete sequences of 3' end coding region for structural protein genes of turkey coronavirus*. Vir Res 106: 61-70.
- Lister S.A., Beer J.V., Gough R.E., Holmes R.G., Jones J.M.W., Orton R.G. (1985). *Outbreaks of nephritis in pheasants (Phasianus colchicus) with a possible coronavirus aetiology*. Vet Rec 117: 612-613.
- Liu S., Chen J., Chen J., Kong X., Shao Y., Han Z., Feng L., Cai X., Gu S., Liu M. (2005). *Isolation of avian infectious bronchitis coronavirus from domestic peafowl (Pavo cristatus) and teal (Anas)*. J Gen Virol 86: 719-725.
- 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. (2006). *Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004*. Arch Virol 151: 1133-1148.
- Liu G., Lv L., Yin L., Li X., Luo D., Liu K., Xue C., Cao Y. (2013). *Assembly and immunogenicity of coronavirus-like particles carrying infectious bronchitis virus M and S proteins*. Vac 31: 5524-5530.
- Lisowska A., Sajewicz-Krukowska J., Fusaro A., Pikula A., Domanska-Blicharz K. (2017). *First characterization of a Middle-East GI-23 lineage (Var2-like) of infectious bronchitis virus in Europe*. Vir Res 242: 43-48.
- Lucio, B., Fabricant J. (1990). *Tissue tropism of 3 cloacal isolates and Massachusetts strains of infectious bronchitis virus*. Avian Dis 34: 865-870.
- 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 E.F. Kaleta & U. Heffels-Redmann (Eds). Proceedings of the First International Symposium on Infectious Bronchitis, Rauischholzhausen, Germany, pp.199-207.
- Lv L., Li X., Liu G., Li R., Liu Q., Shen H., Wang W., Xue C., Cao Y. (2014). *Production and immunogenicity of chimeric virus-like particles containing the spike glycoprotein of infectious bronchitis virus.* J Vet Sci 15: 209-216.
- Macdonald J. W., McMartin D.A. (1976). *Observations on the effects of the H52 and H120 vaccine strains of the infectious bronchitis virus in the domestic fowl*. Avian Pathol 5: 157-173.
- Malim M.H., Emerman M. (2001). *HIV-1 sequence variation: drift, shift, and attenuation*. Cell 104: 469-472.
- Marandino A., Pereda A., Tomás G., Hernández M., Iraola G., Craig M.I., Hernández D., Banda A., Villegas P., Panzera Y., Perez R. (2015). *Phylodynamic analysis of avian infectious bronchitis virus in South America*. J Gen Virol 96: 1340–1346.
- Marandino, A., Tomas G., Panzera Y., Greif G., Parodi-Talice A., Hernandez M., Techera C., Hernandez D., Perez R. (2017): *Whole-genome characterization of Uruguayan strains of avian infectious bronchitis virus reveals extensive recombination between the two major South American lineages*. Infect Genet Evol 54: 245-250.
- 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.
- Mase M., Tsukamoto K., Imai K., Yamaguchi S. (2004). *Phylogenetic analysis of avian infectious bronchitis virus strains isolated in Japan.* Arch Virol 149: 2069-2078.
- Mase M., Kawanishi N., Ootani Y., Murayama K., Karino A., Inoue T., and J. Kawakami J. (2010). *A novel genotype of avian infectious bronchitis virus isolated in Japan in 2009.* J Vet Med Sci 72: 1265-1268.
- Massi P. (2013). *Situazione epidemiologica della Bronchite Infettiva in Italia*. Rivista di medicina veterinaria, pp. 13-20.
- Massi P. (2017). Aggiornamenti sulla Bronchite Infettiva Aviare. Atti del della Societa' Italiana di Patologia Aviare 2017- LVI convegno e II° simposio scientifico, Forlì, Italy 6-7 Aprile 2017- Parma, Italy, 22 Settembre 2017 pp. 43-53.
- Massi P., Barbieri I., Fiorentini L., Casadio M., Parigi M., Tosi G. (2015). *Analisi molecolare di ceppi del Virus della Bronchite Infettiva aviare negli anni 2013 e 2014,*

considerazioni sui genotipi circolanti in Italia e in altri paesi europei ed extra-europei. Atti del LIV convegno annuale della Societa' Italiana di Patologia Aviare, Forlì, Italy 16-17 Aprile 2015, pp. 220-229.

- Matthijs M.G., van Eck J.H., de Wit J.J., Bouma A., Stegeman J.A. (2005). *Effect of IBV-H120 vaccination in broilers on colibacillosis susceptibility after infection with a virulent Massachusetts-type IBV strain*. Avian Dis 49: 540-545.
- McBride C., Li J., Machamer C. (2007). The cytoplasmic tail of the severe acute respiratory syndrome coronavirus spike protein contains a novel endoplasmic reticulum retrieval signal that binds COPI and promotes interaction with membrane protein. J Virol 81: 2418-2428.
- McMartin D.A. (1993). *Infectious bronchitis*. In J. B. McFerran. & M. S. McNulty (Eds) Virus Infections of Vertebrates. Virus Infections of Birds, Vol. 4, pp. 249-275. Amsterdam: Elsevier Science Publis
- Meir R., Malkinson M., Weisman Y. (1998). *Characterization of IBV isolates in Israel using RT-PCR and RFLP*. Proceedings of the International Symposium on Infectious Bronchitis and Pneumovirus Infections in Poultry. Rauischholzhausen, Germany, pp. 229-234.
- Mockett A.P., Cavanagh D., Brown T.D. (1984). *Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M41*. J Gen Virol 65: 2281-2286.
- 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: 2249-2256.
- Monne I. (2016). *Stability and diversity: The Yin and Yang of gammacoronaviruses genome.* Proceedings of the 9th International Symposium on Avian Corona-and Pneumoviruses and Complicating Pathogens, Leusden/Utrecht, The Netherlands, 21-24 June 2016, p.151.
- Moreno A., Franzo G., Massi P., Tosi G., Blanco A., Antilles N., Biarnes M., Majo N., Nofrarìas M., Dolz R., Lelli D., Sozzi E., Lavazza A., Cecchinato M. (2017). *A novel variant of the infectious bronchitis virus resulting from recombination events in Italy and Spain*. Avian Pathol 46: 28-35.
- Morley A.J., Thomson D.K. (1984). *Swollen-head syndrome in broiler chickens*. Avian Dis 28: 238–243.

- Namy O., Moran S.J., Stuart D.I., Gilbert R.J., Brierley I. (2006). *A mechanical explanation of RNA pseudoknot function in programmed ribosomal frameshifting.* Nature 441: 244-277.
- Najafi H., Langeroudi A.G., Hashemzadeh M., Karimi M., Madagar O., Ghafouri S.S., Maghsoudlo H., Farahani R.K. (2016). *Molecular characterization of infectious bronchitis viruses isolated from broiler chicken farms in Iran, 2014-2015*. Arch Vir 161: 53-62
- Nauwynck H., Pensaert M. (1988) *Studies on the pathogenesis of infections with a nephropathogenic variant of infectious bronchitis virus in chickens*. In Proceedings of the 1st International Symposium on Infectious Bronchitis, Rauischholzhausen, Germany, pp. 113-119.
- Narayanan K., Maeda A., Maeda J., Makino S. (2000). *Characterization of the Coronavirus M protein and Nucleocapsid interaction in infected cells*. J Gen Virol 74: 8127-8134.
- Naqi S., Gay K., Patalla P., Mondal S., Liu R. (2003). *Establishment of Persistent Avian Infectious Bronchitis Virus Infection in Antibody-Free and Antibody-Positive Chicken*. Avian Dis 47: 594-600.
- Naguib M.M., Höper D., Arafa A.S., Setta A.M., Abed M., Monne I., Beer M., Harder T.C. (2016). *Full genome sequence analysis of a newly emerged QX-like infectious bronchitis virus from Sudan reveals distinct spots of recombination*. Infect Genet Evol 46: 42–49.
- 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*. Virol 161: 511-519.
- Nora T., Charpentier C., Tenaillon O., Hoede C., Clavel F., Hance A.J. (2007). *Contribution of recombination to the evolution of human immunodeficiency viruses expressing resistance to antiretroviral treatment*. J Virol 81: 7620-7628.
- Office International des Epizooties (2017). *Avian Infectious Bronchitis*. Manual of standards for diagnostic tests and vaccines for terrestrial animals, chapter 2.3.2.
- Ovchinnikova E. V., Bochkov Y.A., Shcherbakova L.O., Nikonova Z.B., Zinyakov N.G., Elatkin N.P., Mudrak N.S., Borisov A.V., and V. V. Drygin V.V. (2011). *Molecular characterization of infectious bronchitis virus isolates from Russia and neighbouring countries: identification of intertypic recombination in the S1 gene*. Avian Pathol 40: 507-514.
- Opstelten D. J., Raamsman M.J., Wolfs K., Horzinek M.C., Rottier P.J.M. (1995). *Envelope*

glycoprotein interactions in coronavirus assembly. J Cell Biol 131: 339-349.

- Otsuki K., Noro K., Yamamoto H., Tsubokura M. (1979). *Studies on avian bronchitis virus (IBV). Propagation of IBV in several cultured cells*. Arch Virol 60: 115-122.
- Otsuki K., Huggins M.B., Cook J.K.A. (1990). *Comparison of the susceptibility to avian infectious bronchitis virus infection of two inbred lines of white leghorn chickens.* Avian Pathol 19: 467-475.
- Owen R.L., Cowen B.S., Hattel A.L., Naqi S.A., Wilson R.A. (1991). *Detection of viral antigen following exposure of one-day-old chickens to the Holland 52 strain of infectious bronchitis virus*. Avian Pathol 20: 663-673.
- Pascucci S. (2013). *Le basi dell'immunità e l'induzione della protezione*. Rivista di medicina veterinaria, pp. 31-36.
- Pei J., Sekellick M.J., Marcus P.I., Choi I., Collisson E.W. (2001). *Chicken Interferon Type I Inhibits Infectious Bronchitis Virus Replication and Associated Respiratory Illness*. J Interferon Cytokine Res 21: 1071-1077.
- Peighambari S.M., Julian R.J., Gyles CL. (2000). *Experimental Escherichia coli respiratory infection in broilers*. Avian Dis 44: 759-769.
- Pennycott T.W. (2000). *Causes of mortality and culling in adult pheasants*. Vet Rec 146: 273-278.
- Pensaert M., Lambrechts C. (1994). *Vaccination of chickens against a Belgian nephropathogenic strain of infectious bronchitis virus B1648 using attenuated homologous and heterologous strains.* Avian Pathol 23: 631-641.
- Peterson E.H., Hymass T.A. (1951). *Antibiotic in treatment of unfamiliar turkey disease*. Poult Sci 30: 466-468.
- Pohuang T., Chansiripornchai N., Tawatsin A., Sasipreeyajan J. (2009). *Detection and molecular characterization of infectious bronchitis virus isolated from recent outbreaks in broiler flocks in Thailand*. J Vet Sci 10: 219-223.
- Poon L.L., Chu D.K., Chan K.H., Wong O.K., Ellis T.M., Leung Y.H., Lau S.K., Woo P.C., Suen K.Y., Yuen K.Y., Guan Y., Peiris J.S. (2005). *Identification of a novel coronavirus in bats*. J Virol 79: 2001-2009.
- Ponnusamy R., Mesters J., Ziebuhr J., Moll R. and Hilgenfeld R. (2006). *Non structural proteins 8 and 9 of human coronavirus 229E*. In Perlman S. and Holmes K. (eds), The Nidoviruses: toward control of SARS and other Nidovirus diseases. Advances in experimental medicine and biology, Spinger, 581: 49-54.

- Promkuntod N., Wickramasinghe I.N., de Vrieze G., Grone A., Verheije M.H. (2013). *Contributions of the S2 spike ectodomain to attachment and host range of infectious bronchitis virus*. Virus Res 177: 127-137.
- Promkuntod N., van Eijndhoven R.E., de Vrieze G., Grone A., Verheije M.H. (2014). *Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus*. Virology 448: 26-32.
- Raamsman M.J., Locker J.K., de Hooge A., de Vries A.A., Griffiths G., Vennema H., Rottier P.J. (2000). *Characterization of the coronavirus mouse hepatitis virus strain A59 small membrane protein E.* J Virol 74: 2333-2342.
- Rabenau H.F., Cinatl J., Morgenstern B., Baur G., Preiser W., Doerr H.W. (2005). Stability and inactivation of SARS coronavirus. Med Microbiol Immunol 194: 1-6.
- Riddell C. (1987). Avian Histopathology. American Association of Avian Pathololology: Kennett Square, PA.
- Rosemberg J.K., Gelb J. (1978). *Response to several avian respiratory viruses as affected by infection in the chicken*. Avian Dis 22: 95-105.
- Sawicki S. G., and Sawicki D.L. (1995). Coronaviruses use discontinuous extension for synthesis of subgenome-length negative strands. Adv Exp Med Biol 380: 499-506.
- Sawicki S. G., Sawicki D.L., Siddel S.G. (2007). A contemporary view of Coronavirus transcription. J Virol 81: 20-29.
- Sawicki S.G. (2009). Coronavirus Genome Replication. In: Viral genome replication. Cameron C.E., Gotte M., Raney K. (eds). Spinger Science+Business, New York, pp. 25-39.
- Schalk A.F., Hawn M.C. (1931). An apparently new respiratory disease of chicks. J Am Vet Med Assoc 78: 413-422.
- Seger W., GhalyanchiLangeroudi A., Karimi V., Madadgar O., Marand M.V., Hashemzadeh M. (2016). *Genotyping of infectious bronchitis viruses from broiler farms in Iraq during 2014-2015*. Arch Vir 161: 1229-37.
- Seo S.H., Pei J., Briles W.E., Dzielawa J., Collison E.W. (2000). *Adoptive transfer of infectious bronchitis virus primed alphabeta T cells bearing CD8 antigen protects chicks from acute infection*. Virology 269: 183-189.
- Sesti L., Sanguinetti H.R., Zenobi C.R., Terrera M.V., Jauregui M., Chacón J., Sara L., Paulet P. (2014a). *A vaccine combination trial for the control of the Q1 Infectious Bronchitis virus (IBV) variant strain in South America*. Proceedings of the 8th International

Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, 17-20 June 2014, pp. 311-316.

- Sesti L., Sara L., Alvarado L., Mató T., Palya V., de Wit J.J. (2014b). Diagnostic, epidemiology and control of the q1 infectious bronchitis virus (IBV) variant strain in Peru, Colombia, Argentina and Chile. Proceedings of the 8th International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, 17-20 June 2014, pp. 56-64.
- Sesti, L., Ardilas C., Alvarado L., de Wit S., Dijkman R. (2016). *Multi-strain infection by infectious bronchitis variant viruses in broiler and breeder flocks in Latin America*. Proceedings of the 9th International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Utrecht, The Netherlands, 21-24 June 2016, pp. 64-76.
- Sid H., Benachour K., Rautenschlein S. (2015*). Co-infection with multiple respiratory pathogens contributes to increased mortality rates in Algerian poultry flocks*. Avian Dis 59: 440–446.
- Simon-Loriere E., Holmes EC. (2011). *Why do RNA viruses recombine?* Nat Rev Microbiol 9: 617-626.
- 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.
- Spackman D., Cameron I.R.D. (1983). *Isolation of infectious bronchitis virus from pheasants*. Vet Rec 113: 354 -355.
- Stern D. F., Sefton B.M. (1982). *Coronavirus proteins: structure and function of the oligosaccharides of the avian infectious bronchitis virus glycoproteins*. J Virol 44: 804-812.
- Sturman L. S., Holmes K.V., Behnke J. (1980). *Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid*. J Virol 33: 449-462.
- Sutton G., Fry E., Carter L., Sainsbury S., Walter T., Nettleship J., Berrow N., Owens R., Gilbert R., Davidson A., Siddell S., Poon L., Diprose J., Alderton D., Walsh M., Grimes J. and Stuart D. (2004). *The nsp9 replicase protein of SARS-coronavirus, structure and functional insights*. Structure 12: 341-353.
- Taddei R., Tosi G., Boniotti M.B., Casadio M., Fiorentini L., Fabbi M., Massi P. (2012) *Caratterizzazione molecolare di ceppi del virus della bronchite infettiva aviare isolati in*

Italia tra il 1963 ed il 1989. Proceedings of 51 Convegno annuale Societa' Italiana Patologia Aviare (SIPA): 11-12 Ottobre 2012, Salsomaggiore Terme (PR), Italy, pp.332-341.

- Tarpey I., Huggins M.B., Orbell S.J. (2007). *The efficacy of an avian metapneumovirus vaccine applied simultaneously with infectious bronchitis and Newcastle disease virus vaccines to specific-pathogen-free chickens*. Avian Dis 51: 594-596.
- 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: 1777-1799.
- Timms L. M., Bracewell C.D. (1983). *Cell mediated and humoral immune response of chickens to inactivated oil- emulsion infectious bronchitis vaccine*. Res Vet Sci 34: 224-230.
- Toffan A., Terregino C., Mazzacan E., Castaldello I., Capua I., Bonci, M., (2011). *Detection of Chinese Q1 strain of infectious bronchitis virus in Europe.* Vet Rec 169: 212-213.
- 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: 347-355.
- Toro H., Lavaud P., Vallejos P., Ferreira A. (1993). *Transfer of IgG from serum to lachrymal fluid in chickens*. Avian Dis 37: 60-66.
- Torres C.A., Listorti V., Lupini C., Franzo G., Drigo M., Catelli E., Brandão P.E., Cecchinato M. (2017). *Gamma and Deltacoronaviruses in quail and pheasants from Northern Italy*. Poult Sci 96: 717-722.
- Toro H., Godoy V., Larenas J., Reyes E., Kaleta E.F. (1996). *Avian infectious bronchitis viral persistence in the Harderian gland and histological changes after eyedrop vaccination*. Avian Dis 40:114-120.
- Toro H., Zhao W., Breedlove C., Zhang Z., Yu Q., Van Santen V. (2014 a). *Infectious bronchitis virus S2 expressed from recombinant virus confers broad protection against challenge*. Avian Dis 58: 83-89.

- Toro H., Zhang J.F., Gallardo R.A., van Santen V.L., van Ginkel F.W., Joiner K.S., Breedlove C. (2014 b). *S1 of distinct IBV population expressed from recombinant adenovirus confers protection against challenge*. Avian Dis 58: 211-215.
- Ujike M., Taguchi F. (2015). *Incorporation of Spike and Membrane Glycoproteins into Coronavirus Virions.* Viruses 7: 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., Schivo A., Mozzo V., Dalla Pozza M.C., Koopman R., Currie R.W. Cattoli G. (2014). *Simultaneous detection of multiple IBV genotypes by a microsphere-based assay.* Proceedings of the 8th International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, 17-20 June 2014, pp. 147-149.
- Valastro V., Holmes E.C, Britton P., Fusaro A., Jackwood M.W., Cattoli G., Monne I. (2016). *S1 gene-based phylogeny of infectious bronchitis virus: an attempt to harmonize virus classification*. Infect Genet Evol 39: 349-364.
- van Beurden S.J., Alinda J. Berends A.J., Krämer-Kühl A., Spekreijse D., Chénard G., Philipp H.C., Mundt E., Rottier P.J.M., Verheije H.M. (2017). *A reverse genetics system for avian coronavirus infectious bronchitis virus based on targeted RNA recombination.* Virol J 14: 109.
- Vennema H., Goedeke G.J., Rossen J.W.A., Voorhout W.F., Horzinek M.C., Opstelten D.J.E., Rottier P.J.M. (1996). *Nucleocapsid-independent assembly of coronavirus-like particles* by co-expression of viral envelope protein genes. EMBO J 15: 2020-2028.
- Vervelde L., de Wit J.J, (2014). *Mucosal immune responses to infectious bronchitis virus: an overview*. Proceedings of the 8th International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany, 17-20 June 2014, pp. 222-227.
- Vervelde L., Matthijs M.G., van Haarlem D.A., de Wit J.J., Jansen C.A. (2013). *Rapid NK-cell activation in chicken after infection with infectious bronchitis virus M41*. Vet Immunol Immunopathol 151: 337-341.
- Villarreal L. Y., Sandri T.L., Souza S.P., Richtzenhain L.J., de Wit J.J., Brandao 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.

- Von Hippel P.H., Fairfield F.R., Dolejsi M.K. (1994). *On the processivity of polymerases*. Ann NY Acad Sci 726: 118-131.
- Wang C.H., Huang Y.C. (2000). *Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus*. Arch Virol 145: 291-300.
- Wang C.H., Tsai C.T. (1996). *Genetic grouping for the isolates of avian infectious bronchitis virus in Taiwan*. Arch Virol 141: 1677-1688.
- Wang Y.D., Wang Y.L., Zhang Fan G.C., Jiang Y.H., Liu X.E., Ding J., Wang S.S. (1998). *Isolation and identification of glandular stomach type IBV (QXIBV) in chickens*. Chinese J Anim Quarantine 15: 1-3.
- Wang X., Rosa A.J., Oliverira H.N., Rosa G.J., Guo X., Tavnicek M., Girshick T. (2006). *Transcriptome of local innate and adaptive immunity during early phase of infectious bronchitis virus infection.* Viral Immunol 19*: 768-774.*
- Wickramasinghe I.N., van Beurden S.J., Weerts E.A., Verheije M.H. (2014). The Avian Coronavirus spike protein. Virus Res 194: 37-48.
- Wilson L., McKinlay C., Gage P., Ewart G. (2004). SARS coronavirus *E protein forms cation-selective ion channels*. Virology 330: 322-331.
- Winter C., Herrler G., Neumann U. (2008). *Infection of the tracheal epithelium by infectious bronchitis virus is sialic acid dependent*. Microbes Infect 10: 367-373.
- 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.
- Woo P. C., Lau S.K., Lam C.S., Lau C.C., 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 Alphacoron- avirus and Betacoronavirus and Avian Coronaviruses as the Gene Source of Gammacoronavirus and Deltacoronavirus*. J Virol 86: 3995-4008.
- 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: 247-257.
- Ye Y., Hogue B.G. (2007). *Role of the coronavirus E viroporin protein transmembrane domain in virus assembly*. J Virol 81: 3597-3607.

- Youn S., Leibowitz J.L., Collisson E.W. (2005). *In vitro assembled, recombinant infectious bronchitis viruses demonstrate that the 5a open reading frame is not essential for replication*. Virol 332: 206-215.
- Yount B., Roberts R.S., Sims A.C., Deming D., Frieman M.B., Sparks J., Denison M.R., Davis N., Baric R.S. (2005). *Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice*. J Virol 79: 14909 - 14922.
- Yu L., Jiang Y., Low S., 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.
- Zhai Y., Sun F., Li X., Pang H., Xu X., Bartlam M., Rao Z. (2005). *Insights into SARS-CoV transcription and replication from the structure of the nsp7- nsp8 hexadecamer*. Nat. Struct. Mol Biol 12: 980 -986.
- Zhang X., Wu Y., Huang Y., Liu X. (2012). Protection conferred by a recombinant Marek's disease virus that expresses the spike protein from infectious bronchitis virus in specific pathogen-free chicken. Virol J 9: 85.
- Zhang J., Chen X.W., Tong T.Z., Ye Y., Liao M., Fan H.Y. (2014). *BacMam virus-based surface display of the infectious bronchitis virus (IBV) S1 glycoprotein confers strong protection against virulent IBV challenge in chickens.* Vac 32: 664-670.
- Zhou Y.S., Zhang Y., Wang H.N., Fan W.Q., Yang X., Zhang A.Y., Zeng F.Y., Zhang Z.K., Cao H.P., Zeng C. (2013). *Establishment of reverse genetics system for infectious bronchitis virus attenuated vaccine strain H120.* Vet Microbiol 162: 53-61.
- Ziebuhr J., Snijder E.J., Gorbalenya A.E. (2000). *Virus-encoded proteinases and proteolytic processing in the Nidovirales*. J Gen Virol 81: 853-879.
- Ziebuhr J., Thiel V., Gorgalenya A.E., (2001). *The autocatalytic release of a putative RNA virus transcription factor from its polyprotein precursor involves two paralogous papain-like proteases that cleave the same peptide bond*. J Biol Chem 276: 33220-32

Chapter 2: Published Paper I

NOTICE: this is post-print (final draft post-refereeing) author's version of a work that was accepted for publication in Virus Research. Changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version is published in: Franzo G., Listorti V., Naylor C.J., Lupini C., Laconi A., Felice V., Drigo M., Catelli E., Cecchinato M. "Molecular investigation of a full-length genome of a Q1-like IBV strain isolated in Italy in 2013." Virus Resarch 2015 Dec 2;210:77-80. doi: 10.1016/j.virusres.2015.07.008. Epub 2015 Jul 17

Short Communication

Molecular investigation of a full-length genome of a Q1-like IBV strain isolated in Italy in 2013

Giovanni Franzo[a,*,1], Valeria Listorti[b,1], Clive J. Naylor[c], Caterina Lupini[b], Andrea Laconi[b], Viviana Felice[b], Michele Drigo[a], Elena Catelli[b], Mattia Cecchinato[a]

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

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

[c] Department of Infection Biology, University of Liverpool, Leahurst Campus, Neston, Cheshire CH64 7TE, United Kingdom

Abstract

Since 1996 a new Infectious Bronchitis virus (IBV) genotype, referred to as Q1, circulated in China and was reported for the first time in Italy in 2011, associated with an increase of mortality, kidney lesions and proventriculitis. During northern Italian outbreak of respiratory disease in a broiler flock in 2013, an IBV strain was detected by RT-PCR and characterized as Q1-like based on partial S1 sequence. The virus was isolated and named γCoV/Ck/Italy/I2022/13. All coding regions of the isolate were sequenced and compared with 130 complete genome sequences of IBV and TCoV, downloaded from ViPR. This showed the highest identity with a Chinese strain CK/CH/LDL/97I (p-distance=0.044). To identify potential recombination events a complete genome SimPlot analysis was carried out which revealed the presence of possible multiple recombination events, but the minor parent strains remained unknown. A phylogenetic analysis of the complete S1 gene was performed using all complete S1 sequences available on ViPR and showed the isolate clustered with an Q1-like strain isolated in Italy in 2011 (p-distance=0.004) and a group of Chinese Q1-like strains

isolated from the mid 90's (p-distance equal or higher than 0.001). It could be hypothesized that the isolate descended from the Italian 2011 Q1-like strain or was the result of a separate introduction from China through commercial trade or migratory birds; but the data currently available does not distinguish between these possibilities.

Main text

Infectious bronchitis virus (IBV), belonging to family *Coronaviridae*, genus *Coronavirus*, is a positive-sense, single-stranded RNA virus of about 27.6 kb (5-UTR-1a/1ab-S-3a-3b-E-M-5a-5b-N-3 UTR) causing major economic losses in the poultry industry (Jackwood and de Wit, 2013). Currently, control strategies are mainly based on widespread use of vaccination. Nevertheless, vaccine-induced immunity is generally poorly protective between strains due to limited cross protection afforded between strains (Cook et al., 2012; Sjaak de Wit et al., 2011b) and this is currently considered to result from antigenic diversity due to S1 protein variability. The viral genome can mutate rapidly through substitutions, insertions, deletions and recombinations and this results in the emergence of a large number of IBV variants, characterized by little or negligible cross protection (Jackwood et al., 2012; Thor et al., 2011). In recent times, different genotypes of apparent Asian origin, have spread to other countries and continents, sometimes with economic consequences (De Wit et al., 2011a). Different strains closely related to the proposed Q1 genotype, isolated for the first time in the mid 90's (Yu et al., 2001a; Yu et al., 2001b), have more recently been described in Italy (Franzo et al., 2014; Toffan et al., 2011; Toffan et al., 2013) in association with respiratory disease. However, little sequence information is available about this genotype despite its presence in Asia, Middle East, Europe and South America (Ababneh et al., 2012; Alvarado, 2012; de Wit et al., 2012; Huang et al., 2004; Jackwood, 2012a; Jackwood, 2012b; Rimondi et al., 2009; Sesti et al., 2014a; Sesti et al., 2014b). This is the first report of a full genome sequence of a Q1 like isolate together with its comparison to currently available full length IBV genomes on world databases. Additionally, a comparison with a broader S1 protein database, typically used for classification purposes, was performed.

To this end, 10 swabs were collected (May 2013) from 35 days old chickens showing respiratory signs, raised in a commercial broiler farm located in Northern Italy. Virus was isolated in chicken embryo tracheal organ cultures (TOC) (Cook et al., 1976). Ciliostasis observed 3 days after inoculation was taken as the indicator of the presence of the virus. This was confirmed when RNA was extracted from the TOC medium and an IBV specific RT-Nested PCR was performed (Cavanagh et al., 1999). Virus recovered from the third passage was

named γCoV/Ck/Italy/I2022/13 (proposed classification Ducatez, 2014) and used for the following steps. A two-step RT-PCR protocol was developed to amplify the full genome of IBV through several overlapping amplicons. Different sets of primers were designed using Primer3 on the basis of the sequence already published. Reverse transcription was performed with the commercial kit Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific, Carlsbad, CA) while PCR was performed using the Phusion Hot Start II High-Fidelity DNA Polymerase kit (ThermoFisher Scientific, Carlsbad, CA). Both RT and PCR phases were thoroughly optimized with respect to primers (available on request), thermal profiles and reagents, so as to achieve an acceptable final yield in absence of non-specific products. The final protocol included the following steps.: 2µL of RNA were added to a pre-mix comprising dNTP mix (final concentration 0,5mM), 15pmol of primer and water up to a volume of 14,5 µL. After a denaturation step at 65°C for 5 minutes the pre-mix was added with 5X RT Buffer, 20U of Thermo Scientific RiboLock RNase Inhibitor, 20U Maxima H Minus ReverseTranscriptase. Water was added to reach final volume of 20µL. Reverse transcription was performed at 50°C for 90 minutes and terminated through a step at 85°C for 5 minutes. Several PCRs were optimized to cover all coding regions of IBV genome using overlapping amplicons of approximatively 2kbp. Four µL of cDNA were added to a standard mix including 5X Phusion HF buffer, 200µM of each dNTP, 0,5 µM of forward and reverse primers, and 0,02U/µL of Phusion Hot Start II DNA polymerase. Nanopure water was added to a final volume of 50µL Sequencing was performed at Macrogen (Macrogen Europe). After the initial activation at 98°C for 30s, 40 cycles were performed at 98°C for 10s, 60°C for 20s and 72°C for 150s. Each amplicon was sequenced using 4 primers including those used for PCR plus two additional internal primers. The list of primer used for reverse transcription, PCR and sequencing is available as Supplementary material 1.

All chromatograms were visually inspected using FinchTV (http://www.geospiza.com/Products/finchtv.shtml) and sequences were trimmed to remove primer contamination and bases with a phred score lower that 20 using Geneious 8.0.5 (http://www.geneious.com/). Sequences were aligned to a reference IBV-QX genotype sequence (Acc.Num. JQ088078) and consensus sequences was generated using the same software. Complete genome sequences including IBV and TCoV (130) were downloaded from ViPR (Pickett et al., 2012) and aligned using MAFFT version 7(Katoh and Standley, 2013). The substitution model was selected on the basis of Bayesian information criterion (BIC) calculated using Jmodeltest 2.1.6 (Darriba et al., 2012). A complete genome phylogenetic tree was reconstructed using the MaximumLikelihood (ML) method implemented in PhyML 3.0

(Guindon et al., 2010). A combination of Nearest neighbor interchange (NNI) and sub-tree pruning and regrafting (SPR) were selected as the tree rearrangement strategy. To evaluate robustness of the monophyly of the taxa subsets, a fast non-parametric version of the aLRT (Shimodaira–Hasegawa [SH]-aLRT), developed and implemented in the PhyML 3.0 (Anisimova et al., 2011), was used.

A NeighborNet network was reconstructed using SplitsTree4 v4.12.3 (Huson and Bryant, 2010). Phylogenetic network was used to display the incompatibilities and ambiguous phylogenetic signals within datasets and provided a preliminary overview on the extent of recombination phenomenon. Presence of recombination within dataset was also tested using the Phi test implemented in the same software. Possible recombinant nature of the isolated strain was also evaluated using SimPlot. Briefly, a sliding window of 300nt was moved along the alignment 20 nucleotide at a time and the pairwise percentage of identity between γCoV/Ck/Italy/I2022/13 and each of the 130 complete genome sequences was calculated for each step. When the percentage of identity was lower than 95% a BLAST search was performed selecting the corresponding region as query in order to obtain the closely related sequences. The region coding for S1 protein is traditionally used for genotyping and classification purpose. All available complete or nearby complete S1 sequences (length at least 1500bp) were downloaded from ViPR (http://www.viprbrc.org/brc/home.spg?decorator=vipr). This dataset was reduced in order to reduce the computational load. To this end, CD-HIT (Li and Godzik, 2006) was used to cluster sequences with identity over 95% and one sequence within each cluster was selected. After clustering, 153 sequences remained and substitution model and phylogenetic tree were reconstructed as previously described. Based on these results, a subset of sequences part of the subtree including the strain γCoV/Ck/Italy/I2022/13 were selected. This dataset was expanded to its original number of taxa and a new phylogenetic tree was obtained.

This study reports, for the first time, the whole genome sequence (coding regions) of a Q1 like strain (γCoV/Ck/Italy/I2022/13) isolated in Italy from chickens with respiratory signs.

A 27403 nt consensus sequence was obtained (minimum coverage 2X). Gene length is reported in Table 1. The phylogenetic tree based on complete genome sequence demonstrated that strain γCoV/Ck/Italy/I2022/13 clusters with Chinese sequences JX195177 and JX195178 (p-distance=0.044) (Supplementary figure 1). The high IBV recombination frequency (Thor et al., 2011) was confirmed also in present study (Phi test p-value=0.0). Phylogenetic network, confirming JX195177 and JX195178 as the most closely related to our isolate, was characterized by the presence of several reticulations (Figure 1),

strongly suggestive of extensive recombination within the database and of the presence of contrasting phylogenetic signal within the sequence. Pairwise comparison with 130 available complete genomes confirmed the close relationship between γCoV/Ck/Italy/I2022/13 and JX195177/JX195178 along the whole genome with the exception of region 841-2812, 3389-9926, 22355-23143 and 25356-26027(data not shown), corresponding to the 1ab, Spike, 5a and N genes. The absence of double peaks in the chromatograms as well as the location of the recombination breakpoints (i.e. within PCR amplicons) discount for mixed infections, *in vitro* and *in silico* recombination, supporting the genuine nature of the reported event. The sharp change in percentage of identity in these positions strongly suggests that γCoV/Ck/Italy/I2022/13 had undergone multiple recombination events. A BLAST search using all publicly available sequences, performed on these regions, did not find closely related sequences (no identity >95%). Unfortunately, the limited number of sequences in regions different from the S gene impedes the study of the recombination patterns and the identification of the strains involved.

Currently, the vast majority of sequencing efforts are focused on S1 gene (or part of it) and consequently few sequences are available for other genome regions, thus currently precluding identification of the minor parents. Hopefully, the advent of next generation sequencing technologies might contribute to an increase in availability of full length genome sequences, hence leading to a deeper understanding of the evolution of other, currently neglected, genes.

Phylogenetic trees of the full S1 gene confirmed γCoV/Ck/Italy/I2022/13 to belong to the same cluster as JX195177/JX195178. This clade comprised only Chinese isolates (Figure 2) often classified as belonging to the Q1 genotype. However, a closely related Italian strain JQ290229 (p-distance=0.004) was previously sampled in 2011 (Toffan et al., 2011). The reconstruction of the actual origin of strain γCoV/Ck/Italy/I2022/13 is challenging. It could be the descendant of strain JQ290229 or occur as the result of separate introduction events from China. Even if clear link are still lacking, several hypothesis can be advocated, including undeclared commercial trade or migratory birds (Cavanagh, 2005). While a closer relationship with Chinese strains makes the second hypothesis more likely, some sort of convergent evolution affecting Italian and Chinese strains can't be excluded. Further investigation based on the comparison of broader regions of the genome and taking advantage of the analysis of recombination patterns could provide more information on this issue. It could also highlight introduction into Italy of one of the most challenging diseases of

commercial poultry. Interestingly, other two Italian sequences (JQ901492 and KJ941019) were part of the γCoV/Ck/Italy/I2022/13 related cluster (Figure 2) and displayed a p-distance of 0.058 and 0.062. Isolate JQ901492, belonging to the genotype 624I, has been previously hypothesized as an ancestor of the Q1 genotype (Massi, 2013) which was imported to China in the past and introduced again in Italy after a period of independent evolution. The high genetic diversity of 624I and the first Q1, sampled only few years apart, hardly fit with this hypothesis. Unfortunately, even if 624I had been reported and sequenced in Italy since 60's (Taddei et al., 2012), JQ901492 is the oldest public available 624I sequence. Such scarcity of data makes it impossible to confidently support or refute this hypothesis, highlighting the importance of the need for further sequence data availability (Brister et al., 2010; Scotch et al., 2011).

Acknowledgements

The work here described was supported by University of Padua grant (ex 60%, 2014).

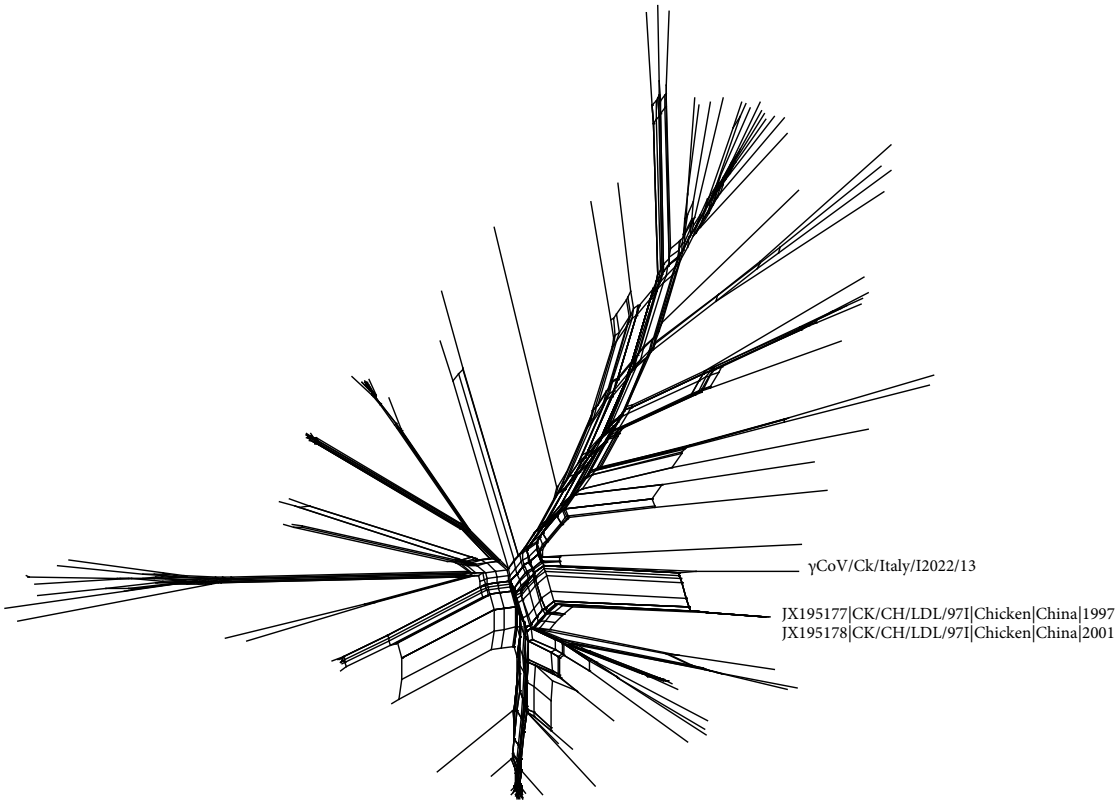

Fig.1 Phylogenetic network based on NeighborNet method including 130 complete IBV and TCoV genomes. For easiness of representation, only strain reported in the present study and closely related strains (i.e., JX195177 and JX195178) are labelled.

Table 1 Gene and protein lengths of strain γCoV/Ck/Italy/I2022/13.

Gene	***1ab***	***Spike***	***3a***	***3b***	***3c***	***M***	***5a***	***5b***	***N***
Length nt	19893	3501	174	195	309	681	198	249	1230
Length aa	6631	1167	58	65	103	227	66	83	410

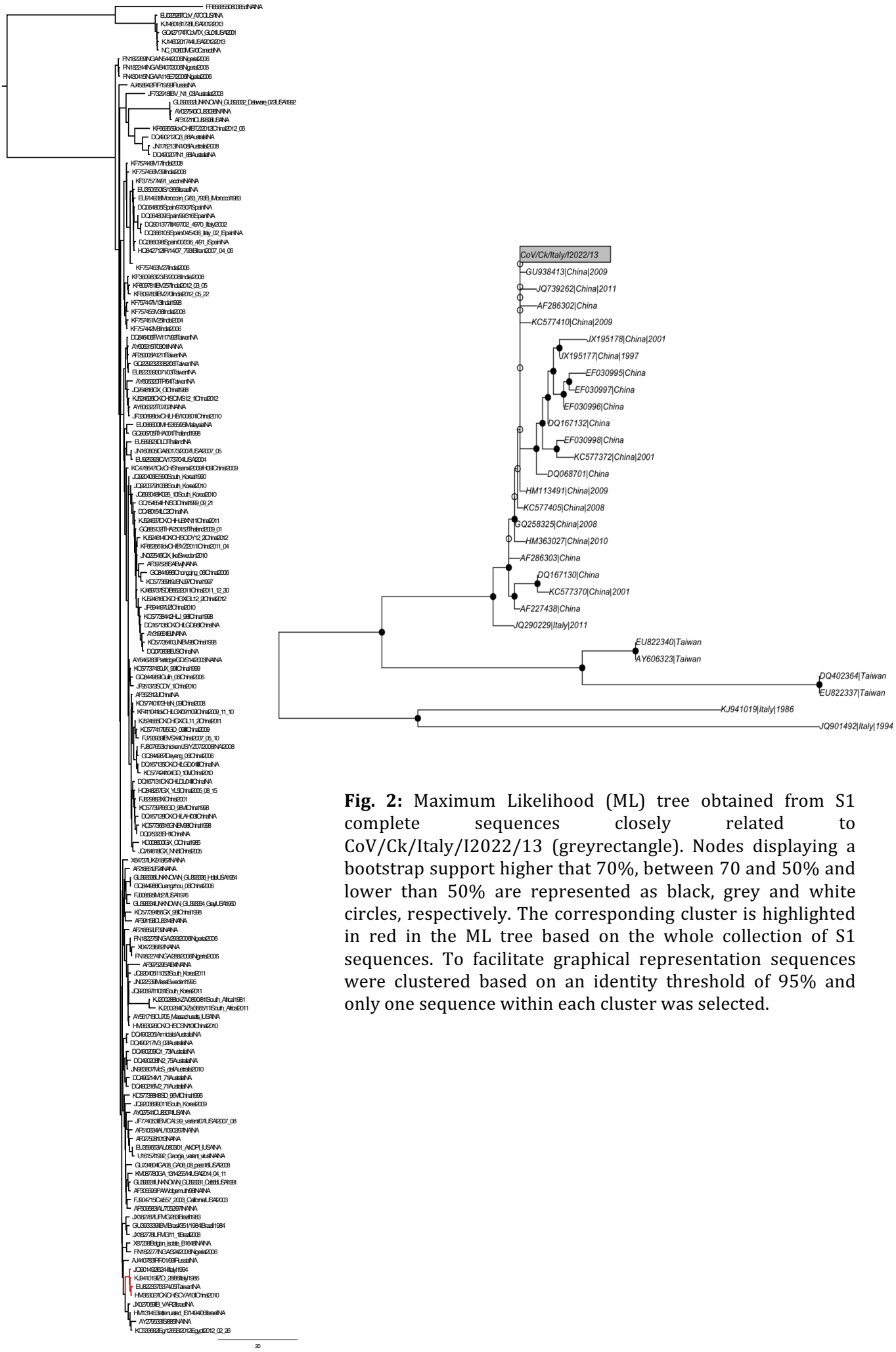

Fig. 2: Maximum Likelihood (ML) tree obtained from S1 complete sequences closely related to CoV/Ck/Italy/I2022/13 (greyrectangle). Nodes displaying a bootstrap support higher that 70%, between 70 and 50% and lower than 50% are represented as black, grey and white circles, respectively. The corresponding cluster is highlighted in red in the ML tree based on the whole collection of S1 sequences. To facilitate graphical representation sequences were clustered based on an identity threshold of 95% and only one sequence within each cluster was selected.

Supplementary material 1. Primers used for reverse transcription, PCR and sequencing. Primers are named according to their approximate position in the viral genome reported in kb and rounded to two decimal digits. Forward and reverse primers are coded with "+" and "-" symbols, respectively.

RT	Sequence 5'-3'	PCR	Sequence 5'-3'	Seq	Sequence 5'-3'
2,06-	tttagtaaaaagaccacc	0,06+	gcgctagatttccaacttaacaaaacg	0,67+	cctaaggattacgctgatgcttttgc
4,26-	catacttttgcgcatc	2,03-	gacttgcgaaacaagatgccaaatgcc	1,26+	ttcgcaggaacttgtcttgcaagc
6,18-	agaaaacctacaccag	1,92+	tggaggcttgcatatggaaaagtgcg	2,52+	tagaggaatgtcacagcttggtgc
8,18-	gtaaagaatgtactaacc	4,2-	ggtataaagaggatttctttatcctcaagatcatg	3,1+	ctctcgatgttgtgaatttaccatctgg
10,1-	atagtatcaaagactacagg	4,1+	cggaggatggtgttaaataccgc	4,8+	tgattgtgatgttgtgaatttaccatctgg
12,1-	ctccataagaatcctg	6,08-	caaataatattagaaagaccaaataaagccaattcc	5,32+	ctattagtcttagggcaatatggg
14,13-	taaaacttggttgttcc	5,93+	gattcttttgatgtgttacgctattgtgcag	6,53+	gttaaacctacagcatatgcttacc
16,1-	ttcacataaagcatcaac	8,05-	cctggtttagtatactcacatacactacc	7,17+	aatgctcctccggtagtatggaag
18,04-	gtcatactcaaactgc	7,97+	cctaatggtgttaggcttatagttcc	8,6+	gtatgatggcaacgagtttgttgg
19,73-	caaaatgcattactcgc	10,1-	gtatcaaagactacaggatcataccattg	9,26+	cctgtcactatgcgttctaatggtac
22,51-	catatcttctttttgacc	10,02+	cagttattattggagtttgtgctgaag	10,6+	ggtaaatccacctaaaactgtgtggg
24,08-	tttgaatcattaaacagac	12,08-	gaatcctgatccggagttggacttggc	11,13+	gaagttagatagcatggcagaacg
26,23-	accaactttaggtggc	12,01+	gtggcagcaggtaatcaacctttagg	12,64+	gacttaaagtcagaagtaacagctg
27,89-	ttgctctaactctatac	14,12-	ataaaacttggttgttccaataactacagg	13,18+	gatctcctcaagtatgattatactgagg
		14,05+	gtgtctatcctttctactatgactaataggc	14,69+	caaggtcttgtagcagatatttctgg
		16,09-	cacataaagcatcaacagctgcatgag	15,25+	aagtgttgctatgaccatgtcatgc
		15,99+	ggcaagcagaagcgtactacagtac	16,62+	catgaaagtggctcagcctacaac
		18,02-	ctgcttgacattgggtactattggattc	17,18+	caacatgttttataacacgtgatgaggc
		17,94+	cgttgtctatgatataggcaaccctaaagg	18,18+	taacctacctggttgtaatggtgg
		19,67-	gtattgacagagttgtgtatactttgcc	18,71+	gaagagaaatattcgcacactgcc
		19,46+	gtaacagtgtcaattgattaccatagc	19,97+	gatagccaataatggcaatgatgacg
		22,01-	gaagctgagaaccagtgtcattacg	20,38+	gtgtggtaagttactggtaagag
		22,26-	tccatacgcgtttgtatgtactcatctg	21,66-	gctcttagtaacataaactaacagtccac
		21,95+	ccagcagtttgtagtttctggtgg	22,48+	ccatttcttagtaatgttagcactgg
		24,07-	gaatcattaaacagacttttttaggtctg	22,99+	ctctagcattacaacaaattcaagatgttg
		23,99+	cattatgcctctaatgagtaagtgtgg	24,55+	aagcatttgtacaggctgctgatgc
		26,22-	aactttaggtggctttggtcctcc	25,27+	gacgtaatatctatcgtatggtgcag
		26,02+	gaaaagcgcgaatttatctgagagaagg	26,49+	gatagccaagatggtatagtgtggg
		27,83-	catagccaattaaacttaacttaaactaaaatttagctc		

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.
- Alvarado, I.R., 2012. *Panorama de Bronquitis Infecciosa en Latino América*. Proceedings of the X International Poultry Seminar - ASPA.
- Anisimova, M., Gil, M., Dufayard, J.-., Dessimoz, C., Gascuel, O., 2011. *Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes.* Syst. Biol. 60, 685-699.
- Brister, J.R., Bao, Y., Kuiken, C., Lefkowitz, E.J., Mercier, P.L., Leplae, R., Madupu, R., Scheuermann, R.H., Schobel, S., Seto, D., 2010. *Towards viral genome annotation standards, report from the 2010 NCBI annotation workshop.* Viruses 2, 2258-2268.
- Cavanagh, D., Mawditt, K., Britton, P., Naylor, C., 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., 2005. *Coronaviruses in poultry and other birds*. Avian Pathol. 34, 439-448.
- Cook, J.K., 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., Jackwood, M., Jones, R.C., 2012. *The long view: 40 years of infectious bronchitis research*. Avian Pathol. 41, 239-250.
- Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. *JModelTest 2: More models, new heuristics and parallel computing*. Nature Methods 9, 772.
- de Wit, J., Guerrero, P., Calvo, J., Hidalgo, H., 2012. *Report of the genotyping, pathotyping, and protectotyping of resent from Chile*. Proceedings of the VII International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, 61-67.
- Ducatez, M., 2014. *Towards suggestions on a unified nomenclature and classification of IBV strains*. 8th SYPMOSIUM On ACOV & AMPV / 2nd MEETING COST ACTION 1207, 416.
- Franzo, G., Naylor, C.J., Lupini, C., Drigo, M., Catelli, E., Listorti, V., Pesente, P., Giovanardi, D., Morandini, E., Cecchinato, M., 2014. *Continued use of IBV 793B vaccine needs reassessment after its withdrawal led to the genotype's disappearance*. Vaccine 32, 6765-6767.
- Guindon, S., Dufayard, J.-., 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, 307-321.
- Huang, Y., Lee, H., Cheng, M., Wang, C., 2004. *S1 and N gene analysis of avian infectious bronchitis viruses in Taiwan*. Avian Dis. 48, 581-589.
- Huson, D.H., Bryant, D., 2010. User Manual for SplitsTree4 V4.11.3.
- Jackwood, M., 2012a. *Molecular diagnostics and characterization of infectious bronchitis virus*. Proceedings of XXXI Seminario Avícola Internacional de AMEVEA.
- Jackwood, M.W., 2012b. *Review of infectious bronchitis virus around the world*. Avian Dis. 56, 634-641.
- Jackwood, M.W., de Wit, J.J., 2013. *Infectious Bronchitis*. In: Anonymous Diseases of Poultry. Wiley-Blackwell, pp. 139-159.
- Jackwood, M.W., Hall, D., Handel, A., 2012. *Molecular evolution and emergence of avian gammacoronaviruses*. Infect. Genet. Evol. 12, 1305-1311.
- Katoh, K., Standley, D.M., 2013. *MAFFT multiple sequence alignment software version 7: improvements in performance and usability*. Mol. Biol. Evol. 30, 772-780.

- Li, W., Godzik, A., 2006. *Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences.* Bioinformatics 22, 1658-1659.
- Massi, P., 2013. *Situazione epidemiologica della bronchite infettiva in Italia*. Rivista di Medicina Veterinaria, Speciale 2013, 13-20.
- Pickett, B.E., Greer, D.S., Zhang, Y., Stewart, L., Zhou, L., Sun, G., Gu, Z., Kumar, S., Zaremba, S., Larsen, C.N., Jen, W., Klem, E.B., Scheuermann, R.H., 2012. *Virus pathogen database and analysis resource (ViPR): a comprehensive bioinformatics database and analysis resource for the coronavirus research community*. Viruses 4, 3209-3226.
- Rimondi, A., Craig, M.I., Vagnozzi, A., König, G., Delamer, M., Pereda, A., 2009. *Molecular characterization of avian infectious bronchitis virus strains from outbreaks in Argentina (2001–2008)*. Avian Pathol. 38, 149-153.
- Scotch, M., Sarkar, I.N., Mei, C., Leaman, R., Cheung, K., Ortiz, P., Singraur, A., Gonzalez, G., 2011. *Enhancing phylogeography by improving geographical information from GenBank*. J. Biomed. Inform. 44, S44-S47.
- Sesti, L., Sanguinetti, H.R., Zenobi, C.R., Terrera, M.V., Jauregui, M., Chacón, J., Sara, L., Paulet, P., 2014a. *A vaccine combination trial for the control of the Q1 Infectious Bronchitis*
  virus (IBV) variant strain in South America. VIII International Symposium on Avian Corona- and Pneumoviruses and complicating pathogens, 311-316.
- Sesti, L., Sara, L., Alvarado, L., Mató, T., Palya, V., de Wit, J.J., 2014b. *Diagnostic, epidemiology and control of the q1 infectious bronchitis virus (IBV) variant strain in Peru, Colombia, Argentina and Chile*. 8th SYPMOSIUM on ACOV & AMPV / 2nd MEETING COST ACTION 1207.
- Sjaak de Wit, J.J., Cook, J.K., van der Heijden, H.M., 2011. *Infectious bronchitis virus variants: a review of the history, current situation and control measures*. Avian Pathol. 40, 223-235.
- Taddei, R., Tosi, G., Boniotti, M.B., Casadio, M., Fiorentini, L., Fabbi, M., Massi, P., 2012. *Caratterizzazione molecolare di ceppi del virus della Bronchite Infettiva Aviare isolati in Italia tra il 1963 ed il 1989*. Atti del LI Convegno Annuale della Società Italiana di Patologia Aviare, 316-325.
- 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, 1777-1799.
- Toffan, A., Bonci, M., Bano, L., 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, 347-355.
- Toffan, A., Terregino, C., Mazzacan, E., Castaldello, I., Capua, I., Bonci, M., 2011. *Detection of Chinese Q1 strain of infectious bronchitis virus in Europe*. Vet. Rec. 169, 212-213.
- Yu, L., Jiang, Y., Low, S., Wang, Z., Nam, S.J., Liu, W., Kwang, J., 2001a. *Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens*. Avian Dis., 416-424.
- Yu, L., Wang, Z., Jiang, Y., Low, S., Kwang, J., 2001b. *Molecular epidemiology of infectious bronchitis virus isolates from China and Southeast Asia*. Avian Dis. 45, 201-209.

Chapter 2: New findings on the genetic correlation between Q1 and 624I genotypes of Infectious Bronchitis Virus

Abstract

A new Infectious Bronchitis virus (IBV) genotype, referred to as Q1, was detected for the first time in China in 1996, and then has spread worldwide. The first report of Q1 genotype in Italy occurred in 2011 and a deep molecular investigation of a Q1 IBV strain isolated in Italy in 2013 has raised hypotheses regarding the origin of this genotype. The phylogenetic analysis of the S1 gene sequence of the Q1 Italian strain revealed, beyond an obvious correlation with Q1 sequences available in web databases, a close relationship with sequences of the 624I strains circulating in Italy in the early '90s. The close genetic correlation observed led to the hypothesis that 624I was an ancestor of the Q1 genotype. Despite the fact that most heterogeneity of IBVs occurs in the S1 gene, the sequence analysis of this single gene was not sufficient neither to confirm nor deny this hypothesis. In the present study an Italian 624I strain isolated in 1996 and identified as gammaCoV/AvCov/Ck/Italy/IP14425/96 was fully sequenced. This represents the first full genome sequence of a virus belonging to the 624I genotype. The presence of potential recombination events was evaluated not only for gammaCoV/AvCov/Ck/Italy/ IP14425/96 but also for all the complete Q1 full genome sequences available. Recombinant nature of some Q1 viruses was demonstrated and the parental strains identified. IBV full genome sequences phylogenetic analysis confirms the genetic correlation between gammaCoV/AvCov/Ck/Italy/IP14425/96 and Q1 strains, suggesting that 624I was introduced in China somewhere in the past and there it has evolved into the Q1 genotype, before its global dissemination. Taken as a whole the results presented in this study confirm the hypothesis of 624I genotype as Q1 genotype ancestor.

Introduction

Infectious bronchitis (IB) is an avian disease distributed worldwide that represents one of the most persistent health problems of the commercial poultry industry (Cook et al., 2012, de Wit et al., 2011a). The aetiological agent of the disease is a virus belonging to the genus Gammacoronavirus, characterized by a positive sense single stranded RNA genome (27.6kb) called Infectious Bronchitis Virus (IBV) (Jackwood and de Wit, 2013). IBV genome can evolve rapidly by mutation and recombination events, resulting in the continue emergence of new IBV variants, characterized by little or negligible cross protection. The majority of the variants causes a transitory problem and then disappears or remains confined into a specific geographical region, but few variants are able to persist for extend period of time and spread to new areas. Recently, genotypes of Asian origin have spread worldwide causing important economic losses (de Wit et al., 2011a). One of these genotypes is the Q1 that was detected for the first time in China in 1996 (Yu et al., 2001) and then reported in Asia, Middle East, Europe and South America (Ababneh et al., 2012, Huang et al., 2004, Jackwood, 2012, Marandino et al., 2015, Rimondi et al., 2009). In Italy the Q1 genotype was reported for the first time in 2011, causing an outbreak of disease associated with respiratory signs, increased mortality, kidney lesions and proventriculitis (Toffan et al., 2013). Since then, the genotype has been continuously detected in Italy (Massi et al., 2015). Phylogenetic analysis performed using full or partial S1 sequences showed a high similarity (>99%) between Italian and Chinese Q1 isolates (Franzo et al., 2015, Massi et al., 2015, Toffan et al., 2013). Interestingly a high homology (94,1%) between strains belonging to the Q1 genotype and strains belonging to the 624I genotype was observed (Franzo et al., 2015, Massi et al., 2015), such that the recently proposed new IBV nomenclature based on the S1 sequence placed them in the same lineage (GI-16) (Valastro et al., 2016). IBV 624I has been reported for the first time in Italy in 1993 (Capua et al., 1994, Capua et al., 1999), in outbreaks of diseases associated with kidney lesions and drop in egg production in breeders and layers (Capua et al., 1996). This genotype had largely circulated in the country until 2004; then it seemed to have disappeared up to 2010 when again viruses belonging to 624I genotype were detected in few broiler farms affected by respiratory disease located in different areas of Italy; in the two following years the number of detections increased (Massi, 2013). After 2013, 624I genotype has not been reported anymore (Massi et al., 2015). A recent retrospective study carried out on 123 IBV strains isolated in Italy between 1963 and 1989 revealed that 624I genotype had not only circulated long before its first reporting in 1993, but that in fact it was one of the major IBV genotypes

circulating in the Country at that time (Taddei et al., 2012). Evidences of the presence of this genotype have also been found in Slovenia, where several 624I strains were isolated between 1991 and 1999 (Krapez et al., 2010), in Poland and South Africa (Capua et al., 1999) and eventually in Russia where 624I genotype was reported in 2002 (Bochkov et al., 2006).

The high homology observed between Q1 and 624I genotypes arose questions regarding their origin and evolution. In particular 624I has been hypothesized as an ancestor of the Q1 genotype (Franzo et al., 2015, Massi, 2013), but unfortunately the unavailability of any 624I full genome sequence didn't allow to draw any final conclusions.

With the present study we aimed to clarify the genetic correlation between 624I genotype and Q1 genotype. A 624I IBV genotype strain was fully sequenced and phylogenetic analysis performed both using a dataset based on available IBV full length genome sequences and, due to the larger number of published sequences, a dataset based on full S1 gene. Furthermore, recombination analysis was carried out along the whole genome sequences of 624I or Q1 strains.

Materials and Methods

Virus

624I IBV strain was isolated in 1996 in Specific Pathogens Free (SPF) chicken eggs and the 3th passage was propagated in SPF chicken embryo tracheal organ coltures (TOC). The virus will be referred hereafter as gammaCoV/AvCov/Ck/Italy/IP14425/96.

RNA extraction, RT-PCR and sequencing

The RNA was extracted using Qiamp viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacture's protocol. Viral RNA was firstly retro-transcribed using Super Script III enzyme (Invitrogen, Carlsbad, USA) and then amplified using Ranger enzyme (Bioline, London, UK) according to the manufacturer's instructions**.** Retro-transcription, amplification and sequencing were carried out using primers previously designed for IBV full genome sequencing (Franzo et al., 2015, Listorti et al., 2017). Where primers did not work due to sequence differences, new primers were designed based on the newly determined sequences flanking those genome regions (Table 1). Sequencing was performed by Source BioScience (Nottingham, UK).

RT-PCR of the 3' END of genome

3' end of the genome was determined using a 3'RACE protocol previously described (Laconi et al., 2016). Briefly, RT was performed with a primer containing 20 Ts followed by an adaptor sequence at its 5' terminus. This was amplified by PCR using 2 primers, one within the end of the genome and one matching the adaptor (Table 1). These PCR products were sequenced towards the polyA tail.

Sequences analysis and comparison

Chromatograms were analysed using the program Chromas (http://technelysium.com.au/wp/chromas/) and sequences aligned using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Open Reading Frame (ORF) prediction was carried out using ORFfinder program (https://www.ncbi.nlm.nih.gov/orffinder/).

Complete genome sequences analysis

A data set containing 313 complete genome sequences of IBV was downloaded from ViPr, an open bioinformatics database and analysis resource for virology research. This data set was reduced in order to minimize the computational load. To this end, cd-hit-est test of the CD-HIT Suite (Li and Godzik, 2006) was used to cluster sequences that shared over 98% identity and one sequence within each cluster was selected. After clustering, 187 sequences remained. Sequences were aligned using ClustaW method and phylogenetic analysis was carried out with MEGA7 software (Kumar et al., 2016) using Maximum Likelihood method with Tamura-Nei substitution model and 1,000 bootstrap replicates to assess the robustness of the branches.

S1 gene sequences analysis

A data set containing all available complete or nearly complete S1 sequences (at least 1000bp) was downloaded from ViPr. In order to minimize the computation load the data set was reduced as described in the previous paragraph. After clustering, 320 sequences remained. The sequences were aligned using ClustalW method, and a phylogenetic tree was reconstructed using the Maximum Likelihood method with Kimura 2parameter substitution model and 1,000 bootstrap replicates to assign confidence level to the branches in MEGA 7 software. Based on these results a subset of sequences clustering with gammaCoV/AvCov/Ck/Italy/IP14425/96 was selected and expanded to its original number

of taxa. Sequences belonging to this dataset were aligned with ClustalW method, and a phylogenetic tree was reconstructed using the parameters previously described.

Recombination event analysis

Recombinant nature of strain gammaCoV/AvCov/Ck/Italy/ IP14425/96 was evaluated using RDP4 software (http://web.cbio.uct.ac.za/~darren/rdp.html)(Martin et al., 2017). Occurrence of possible recombination events was also evaluated for the available Q1 full genome sequences: gammaCoV/Ck/Italy/I2022/13 (KP780179), an Italian isolate from 2013, CK/CH/LDL/97I (JX195177), a Chinese isolate from 1997 and UY/09/CA/01 (MF421319) a Uruguayan isolate from 2009. The Kimura 2 parameter substitution model with a window size of 200 nucleotides and a step size of 20 nucleotides was used to calculate the pairwise percentage of identity between gammaCoV/AvCov/Ck/Italy/IP14425/96, the Q1 strains and 13 complete genome sequences of relevant strains, selected on the previous phylogenetic analysis. Phylogenetic analysis was performed for those genome portions where a sharp change in percentage of identity strongly suggested recombination events using a dataset including all the sequences available for the given regions.

Results

Genome organization of strain gammaCoV/AvCov/Ck/Italy/ IP14425/96

A consensus sequence of 27.573bp was obtained (minimum coverage 2X), with the 5' UTR incomplete by approximately 100nt. The ORF analysis predicted 13 ORFs and revealed the following genome organization: 5'UTR-1a-1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b-3'UTR (Table 2). The same genome organisation was observed for viruses gammaCoV/Ck/Italy/I2022/13, CK/CH/LDL/97I and UY/09/CA/01, all belonging to the Q1 genotype (Table 2).

Accession number

Sequence of the IBV strain gammaCoV/AvCov/Ck/Italy/IP14425/96 was submitted to the GenBank database and the following accession number was assigned: MG021194.

Phylogenetic analysis of full genomes of IBV strains

The phylogenetic analysis of the 187 representative full IBV genome sequences demonstrated that gammaCoV/AvCov/Ck/Italy/IP14425/96 clustered together with Q1 strains gammaCoV/Ck/Italy/I2022/13, CK/CH/LDL/97I and UY/09/CA/01, occupying a basal

position in the specific cluster (Figure 1). In the same clade was also present the Uruguayan strain UY/11/CA/18 (MF421320), previously ascribed to the SAI genotype (Lineage G-11) (Figure 1) (Marandino et al., 2015).

GammaCoV/AvCov/Ck/Italy/IP14425/96 624I strain showed the highest sequence identity with the Italian Q1 strain gammaCoV/Ck/Italy/I2022/13 (*p*-distance 0.054), while the percentage of identity slightly decrease when the virus was compared to the Chinese (*p*-distance 0.058) and the Uruguayan (*p*-distance 0.062) Q1 strains.

Phylogenetic analysis of full S1 sequences

Phylogenetic analysis using a dataset characterized by 320 representative full S1 sequences showed that gammaCoV/AvCov/Ck/Italy/ IP14425/96 clustered together with strains previously identified as 624/I genotype isolated in Italy in the late '80s and early '90s and strains belonging to Q1 genotype, isolated in Italy, in China, in Taiwan and South America (Figure 2A). The highest sequence identity was observed with strain 624I/94/JQ901492.1 (*p*-distance = 0.036) while the identity was lower when compared to Q1 strains (Table 1 – supplementary material).

The subtree obtained with the expanded dataset shows 3 clades, of which the one comprising all 624/I strains occupies a basal position in respect to the others. In the remaining two clades, Q1 Italian, Chinese and some of the Taiwanese strains, cluster together, while the remaining Q1 Taiwan strains and all South American Q1 strains form a distinctive phylogenetic group (Figure 2B).

Recombination analysis

Recombination analysis was performed to assess the possible recombinant nature of the 624I strain gammaCoV/AvCov/Ck/Italy/ IP14425/96 and the Q1 strains gammaCoV/Ck/Italy/I2022/13, CK/CH/LDL/97I and UY/09/CA/01.

Possible recombination events were identified in the 1a gene sequence of the strain CK/CH/LDL/97I with a H120 vaccine strain (FJ888351) (Figure 3A) and of the strain UY/09/CA/01 with UY/11/CA/18 strain (Figure 3D). Nor gammaCoV/Ck/Italy/I2022/13 neither gammaCoV/AvCov/Ck/Italy/IP14425/96 Q1 strains showed a similar recombination event in the 1a gene (Figures 3B and 3C).

The analysis revealed a possible recombination event between Q1 strains UY/09/CA/01 and gammaCoV/Ck/Italy/I2022/13 in the 3' portion of the genome, including the whole S gene (Figure 3D).

A phylogenetic analysis was performed considering the merely 1a gene, revealing that CK/CH/LDL/97I clustered with H120 and Mass strains, UY/09/CA/01 clustered with UY/11/CA/18 (SAI genotype - GI-11 lineage) and strains of North American origin (California genotype – lineage GI-17; Georgia genotype – lineage GI-8; Arkansas genotype – lineage GI-9), while gammaCoV/AvCov/Ck/Italy/IP14425/96 and gammaCoV/Ck/Italy/I2022/13 form a distinctive clade together with a QX-like Italian strain (ITA/90254/2005 - FN430414) (Figure 4).

The phylogenetic analysis performed using a dataset based on the last 3' 7kb of 187 IBV genomes showed that UY/09/CA/01 and gammaCoV/Ck/Italy/I2022/13 clustered together, while UY/11/CA/18 clustered with two other viruses belonging to the SAI genotype (lineage G-11) (Figure 5).

Discussion

In the present study the genome of the IBV strain gammaCoV/AvCov/Ck/Italy/ IP14425/96 isolated in Italy in 1996 was fully sequenced. To our knowledge this is the first report of a full genome sequence of a virus belonging to the 624I genotype. The isolate shows a genome organisation slightly different when compared to the genome organization of most IBVs previously reported (5'UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3'UTR) (Cavanagh, 2005), since ORF analysis showed the presence of accessory genes 4b, 4c and 6b already reported for TCoV and other IBVs (Abolnik, 2015, Hewson et al., 2011).

Full genomes of Q1 IBVs analysed in the present paper showed the same genome organization observed in 624I strain. It is not clear whether the scarcity of reports of presence of the accessory genes 4b, 4c and 6b in IBVs is due to their absence in some genomes; or whether it depends on algorithms and software used by other authors for the ORFs detection. As a matter of fact a recent ORF analysis of the genome of the Q1 strain gammaCoV/Ck/Italy/I2022/13, (Marandino et al., 2017) didn't support the presence of ORF 6b in the analysed virus, an outcome clearly in contrast with the results presented here.

A recent study confirmed the expression of the 4b protein after M41 IBV infection *in vitro* (Bentley et al., 2013). IBV accessory genes 3a, 3b, 5a and 5b are known to be not necessary for viral replication, but several studies demonstrated their involvement in the pathogenicity of the virus (Kint et al., 2015a, Kint et al., 2015b, Kint et al., 2016, van Beurden et al., 2017). A similar function might be hypothesised also for genes 4b, 4c and 6b, especially in the light that the 4b homologous gene in the MERS-CoV has been reported as an antagonist of type I interferon response (Yang et al., 2013) and that the 6b homolog in SARS-CoV was shown to be

able to induce apoptosis (Ye et al., 2008). More studies need to be done to improve the knowledge on these 3 accessory genes, in particular whether they are peculiar of certain genotypes and whether their expression influences the pathogenicity or the tropism of the virus.

Phylogenetic analyses performed using two different datasets, one built with IBV complete genome sequences and one built with IBV complete or nearly complete S1 gene sequences, showed that 624I and Q1 genotypes clustered together. The basal location of the 624I strain in both the phylogenetic trees strongly suggests that this genotype has played an important role in the emergence of the Q1 genotype and supports the hypothesis that elects the 624I genotype as Q1 genotype ancestor formulated by Massi (2013).

Our findings, coupled with the epidemiological data available on 624I and Q1 genotypes, strongly suggest that 624I genotype was introduced in China somewhere in the past and there has evolved into Q1 genotype before its global dissemination. This model implies a long-distance intercontinental dispersion of 624I genotype, whose mechanism is still not fully understood; however 624I has proven its ability to circulate for extend periods in a geographical area (Taddei et al., 2012) and to spread outside of it (Capua et al., 1999, Krapez et al., 2010). The intercontinental dispersion of 624I genotype is further supported by the detection of this genotype in Russia (Bochkov et al., 2006); due to the intermediate position between Europe and the far East it is not unlikely that the circulation of 624I in this country might have played an important role on its introduction in China.

The relatively high genetic diversity between the 624I strain gammaCoV/AvCov/Ck/Italy/ IP14425/96 and the Q1 Chinese strain CK/CH/LDL/97I, two viruses isolated only one year apart, seems not to support this evolutionary model. However, the phylogenetic analysis carried out using the expanded S1 sequences database showed that the sequences of Chinese Q1 strains were closely related with the oldest 624I sequence available in web databases (KJ941019.1), obtained from a virus isolated in Italy in the middle ‘80s. These results suggests that the introduction of 624I in China might have occurred long before the period of isolation of gammaCoV/AvCov/Ck/Italy/ IP14425/96 and CK/CH/LDL/97I strains. To further assess the robustness of this evolutionary model the release of the full genome sequences, or at least the S1 sequences, of 624I strains isolated in Italy from the late ‘80s backwards will be crucial.

The relatively low identity between the 624I gammaCoV/AvCov/Ck/Italy/ IP14425/96 strain and Q1 CK/CH/LDL/97I strain observed might also rely on the recombinant nature of the latter. Recombination analyses revealed in fact that strain CK/CH/LDL/97I potentially underwent recombination with a H120 vaccine strain, which has been previously

demonstrated to be involved in recombination events leading to phenomena of reversion to virulence and the emerge of new genotypes in China (Zhang et al., 2010).

However, the absence of such recombination in the genome of Q1 Italian strain gammaCoV/Ck/Italy/I2022/13 suggests that not all Q1 strains emerged as a result of a recombination event with a H120 strain. Recombination has proved to be a fundamental biological event behind the emerge of new IBV genotypes (Jackwood, 2012, Quinteros et al., 2016), but it's important to keep in mind that recombination might have high fitness costs, because of the decline of optimal intra- and inter-protein interactions (Simon-Loriere and Holmes, 2011), therefore it's more likely that non-recombinants Q1 strains disseminated worldwide. The absence of the recombination event in the 1a gene of Q1 Italian strain gammaCoV/Ck/Italy/I2022/13 might also lead to the hypothesis that the Chinese and Italian Q1 strains are the results of an independent evolution from 624I genotype. However, the huge differences in field conditions between the two countries, causing a different genetic pressure on IBV viruses, together with the results of the phylogenetic analyses based on the S1 gene sequences dataset, in which Q1 Italian strains didn't form a distinctive phylogenetic clade, but clustered together with Chinese and Taiwanese strains, make this hypothesis highly unlikely. A further evolutionary scenario might be that Q1 genotype evolved in Italy from 624I genotype and then spread worldwide. However, this hypothesis is not supported by the epidemiological data; in fact Q1 genotype was detected for the first time in China, in 1996 (Yu et al., 2001), long before its first detection in Italy (Toffan et al., 2013).

To obtain a better understanding about the worldwide dissemination of Q1 genotype, the full genome sequence of Q1 virus UY/09/CA/01 was taken into account. This is a Q1 strain isolated in Uruguay in 2009, which had been proven to have undergone recombination with a major genotype circulating in that area, SAI genotype – lineage GI-11 (Marandino et al., 2015, Marandino et al., 2017). Full genome sequence comparison showed that UY/09/CA/01 strain had higher identity with the Q1 CK/CH/LDL/97I Chinese strain (*p*-distance 0.051) rather than with the Q1 gammaCoV/Ck/Italy/I2022/13 Italian strain (*p*-distance 0.053). Moreover the recombination analysis revealed that UY/09/CA/01 sequence presented a recombination event in the 1a gene, as it has occurred in CK/CH/LDL/97I strain. However, the major parental sequences for the recombination event in the 1a gene of UY/09/CA/01 strain were identified to belong to UY/11/CA/18 strain and to Arkansas genotype viruses (lineage GI-9). This finding was further confirmed by the phylogenetic analysis of the merely 1a gene sequence, which showed that strain UY/09/CA/01 clustered together with strain UY/11/CA/18 and North American strains. Interestingly the recombination analysis

identified gammaCoV/Ck/Italy/I2022/13 as potential major parental strain in the last third of UY/09/CA/01 genome, as further suggested by the phylogenetic analysis on that portion of the genome. These data seem to support the previously suggested hypothesis that Q1 genotype has not been introduced in South America directly from China, but via an indirect route from Italy (Marandino et al., 2015). However, the scarcity of South American Q1 sequences available in this portion of the genome, makes this hypothesis not conclusive.

In accordance with previous studies, we observed that recombination events can occur not only within the Spike gene, known to generally lead to the emerge of new genotypes (Lim et al., 2011, Moreno et al., 2017), but also in other regions of the IBV genome (Quinteros et al., 2016, Zhang et al., 2010) with potential effects on the pathogenicity of the virus (Armesto et al., 2009).

Taken as a whole, the data presented in this study clearly support the hypothesis that identifies 624I genotype as the ancestor of Q1, one of the recently emerged Asian IBV genotypes. An evolutionary model, which implies the introduction of 624I in China and a subsequent evolution into the Q1 genotype in that country before its dissemination worldwide, is proposed. Unfortunately the scarcity of availability of 624I and Q1 full genome sequences makes difficult to accept beyond any doubts the evolutionary and dissemination models proposed, remarking the urgency of increase sequence data availability.

Table 1: Primers used for reverse transcription, PCR and sequencing.

RT	Sequence 5'-3'	Sequencing	Sequences 5'-3'
2.06neg	tttagtaaaaagaccacc	0.67+	cctaaggattacgctgatgcttttgc
4.26neg	catacttttgcgcatc	1.26+	ttcgcaggaacttgtcttgcaagc
6.18neg	agaaaacctacaccag	2.52+	tagaggaatgtcacagcttggtgc
8.18neg	gtaaagaatgtactaaac	3.04+	tacaccaatgtcacagcttggtgc
10.10neg	cacagttgtgtgcactaactcaaag	3.10+	ctctcgatgttgtgaatttaccatctgg
12.10neg	ctccataagaatcctg	IB4.6+†	gtacggatgaagtaatagaagcttc
14.13neg	taaaacttggttgttcc	4.80+	tgattgtgatgttgtgaatttaccatctgg
16.10neg	ttcacataaagcatcaac	IB5.15neg†	catcagtatcaggtgttaacttataag
QX18.10neg	catagaagaagaatggcatagctttc	5.32+	ctattagtcttagggcaatatggg
19.73neg	caaaatgcattactcgc	IB5.70+†	gtgtggtttatttacacaagtaatccag
22.51neg	catatcttctttttgacc	6.53+	gttaaacctacagcatatgcttacc
24.08neg	tttgaatcattaaacagac	IB6.7neg†	ctaatcgttctgaaagtgcctgatcaag
26.24neg	ccaagatacatttccag	IB7.15+†	ctttataacaagatctggtgctaaac
27.89neg	Ttgctctaactctatac	7.17+	aatgctcctccggtagtatggaag
Dta-Adaptneg	gcatctcgaggcttgtggctttttttttttttttttttta	IB8.2neg†	caacccaaactagcattattgtaaacac
Dtc-Adaptneg	gcatctcgaggcttgtggctttttttttttttttttttc	8.60+	gtatgatggcaacgagtttgttgg
Dtg-Adaptneg	gcatctcgaggcttgtggctttttttttttttttttttg	IB8.61neg†	taccaacaaactcgttgccatc
PCR	Sequence 5'-3'	9.26+	cctgtcactatgcgttctaatggtac
0.06+	gcgctagatttccaacttaacaaaacg	10.60+	ggtaaatccacctaaaactgtgtggg
2.03neg	gacttgcgaaacaagatgccaaatgcc	11.13+	gaagttagatagcatggagagacg
1.92+	tggaggcttgcatatggaaaagtgcg	12.64+	gacttaaagtcagaagtaacagctg
4.20neg	ggtataaagaggatttctttatcctcaagatcatg	13.18+	gatctcctcaagtatgattatactgagg
4,10+	cggaggatggtgttaaataccgc	QX13.90+	gaggtgacgtctaaatattttgaatg
6.08neg	caaataatattagaaagaccaaataaagccaattcc	14.69+	caaggtcttgtagcagatatttctgg
5.90+†	gactatggtaaagactcatttgacg	15.25+	aagtgttgctatgaccatgtcatgc
8.05neg	cctggtttagtatactcacatacactacc	IB15.60+†	ggtgcagcttggtgattttacctttg
7.97+	cctaatggtgttaggcttatagttcc	IB16.40neg†	ctataaccttgaaacactgacgtg
10.10neg	gtactaaagactacaggatcataccattg	QX16.80neg	gaatcagctgtaacacagaatataac
10.02+	cagttattattggagtttgtgctgaag	16.62+	catgaaagtggctcagcctacaac
12.08neg	gaatcctgatccggagttggacttggc	17.18+	caacatgttttataacacgtgatgaggc
12.01+	gtggcagcaggtaatcaacctttagg	18.02neg	ctgcttgacattgggtactactggattc
14.12neg	ataaaacttggttgttccaataactacagg	18.18+	taacctacctggttgtaatggtgg
14.05+	gtgtctatcctttctactatgactaataggc	18.71+	gaagagaaatattcgcacactgcc
16.09neg	cacataaagcatcaacagctgcatgag	19.67neg†	gtattgacagagttgtgtatactttggc
QX15.06+	gatgattgcactcgcatagtacctc	19.46+	gtaacagtgtcaattgattaccatagc
QX18.10neg	catagaagaagaatggcatagctttc	19.97+	gatagccaataatggcaatgatgacg
17.62+†	tactcaggcttatgcttgttggaagc	IB20.40+†	cattgtttatagtgtctcttttgtttgcac
19.67neg	gtattgacagagttgtgtatactttgcc	IB21.70neg†	gtcacaaattgcccttatgtaagttatgg
19.46+	gtaacagtgtcaattgattaccatagc	IB21.95+†	ccagcagtttgtagtttctggtgg
22.26neg	tccatacgcgtttgtatgtactcatctg	IB23.60neg†	cacgtgcagtgatgtagtaactacc
IB21.95+	ccagcagtttgtagtttctggtgg	QX24.20neg	ctacttacactgtttcaattgttttctc
IB24.07neg	gaatcattaaacagacttttaggtctg	IB24.70+†	gtactcttggtactgaacaagcag
23.99+	cattatgcctctaatgagtaagtgtgg	IB25.60neg†	ctcttgaaaagagagcatgaaacaaagagg
26.24neg†	ccaagatacatttccag	QX27.20+	cctacatgtctatcgccaggg
26.02+	gaaaagcgcgaatttatctgagagaagg		
27.83neg	catagccaattaaacttaacttaaactaaaatttagctc		
26.49+	gatagccaagatggtatagtgtggg		
Adapt neg	gcatctcgagggttgtggc		

Primer names generally indicate approximate binding positions in the 624I genome.
Coding and anticoding sense primers are labeled + and neg respectively.
† *Indicates primers designed for the 624I IBV strain sequencing based on the newly determined sequences.*

Table 2 Genomic organization of 624I and Q1 strains. Strains belonging to the two genotypes show the same genome organization. Accessory genes 4b, 4c and 6b have been identified in the genome of all the 4 viruses.

Gene	Genome position			
	gammaCov/AvCoV/Ck/Italy/IP14425/96	gammaCoV/Ck/Italy/I2022/13	CK/CH/LDL/97I	UY/09/CA/01
1a	433-12288	432-12290	433-12291	432-12254
1ab	12363-20321	12365-20323	12366-20324	12329-20287
S	20272-23772	20274-23774	20275-23775	20238-23711
3a	23772-23945	23774-23947	23775-23948	23738-23911
3b	23945-24136	23947-24141	23938-24142	23911-24105
E	24117-24404	24122-24430	24123-24431	24086-24394
M	24394-25074	24423-25103	24424-25104	24387-25067
4b	25075-25359	25104-25388	25105-25377	25068-25352
4c	25280-25450	25309-25470	25310-25387	25273-25434
5a	25434-25631	25454-25651	25466-25663	25418-25614
5b	25628-25876	25648-25896	25660-25908	25612-25860
N	25819-27045	25839-27068	25851-27080	25803-27032
6b	27054-27278	27058-27222	27089-27403	27041-27265

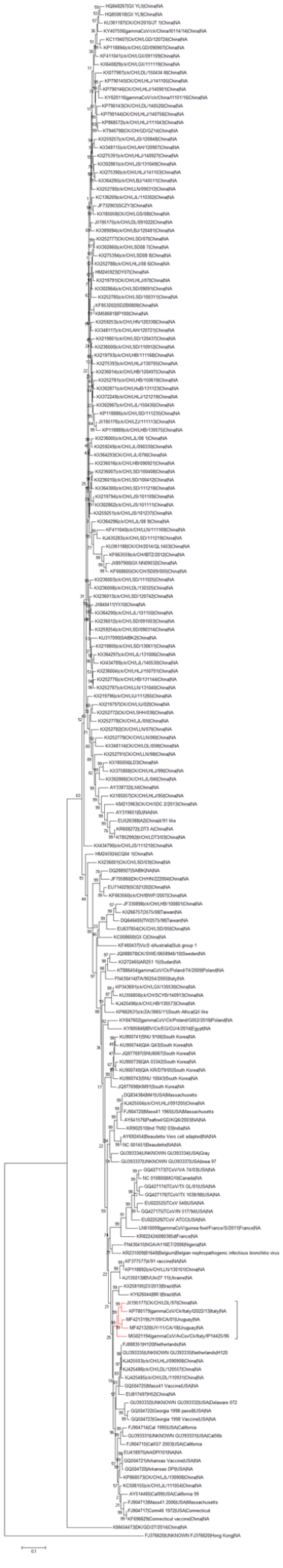

Figure 1 Phylogenetic tree based on Maximum Likelihood method with Tamura-Nei substitution model using 187 representative complete IBV and TCoV genomes. For easiness of representation, strains considered in the present study are marked in red.

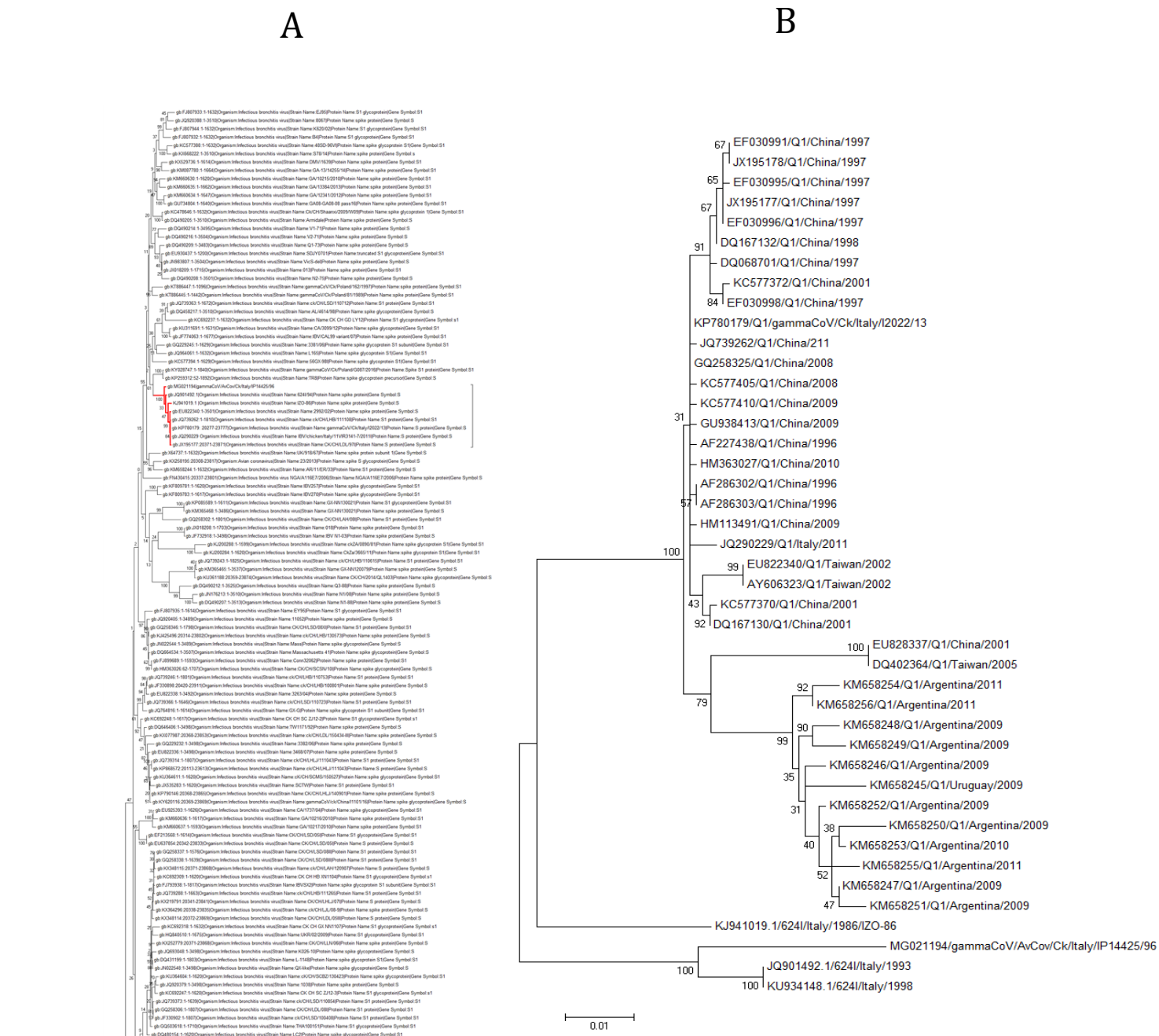

Figure 2 **A** Phylogenetic tree based on Maximum Likelihood method with Kimura-2 model using 320 S1 gene complete sequences. For easiness of representation, strain reported in the present study and closely related strains are marked in red. **B** Expanded phylogenetic tree based on Maximum Likelihood method with Kimura-2 model using S1 complete sequences closely related to gammaCoV/AvCov/Ck/Italy/ IP14425/96.

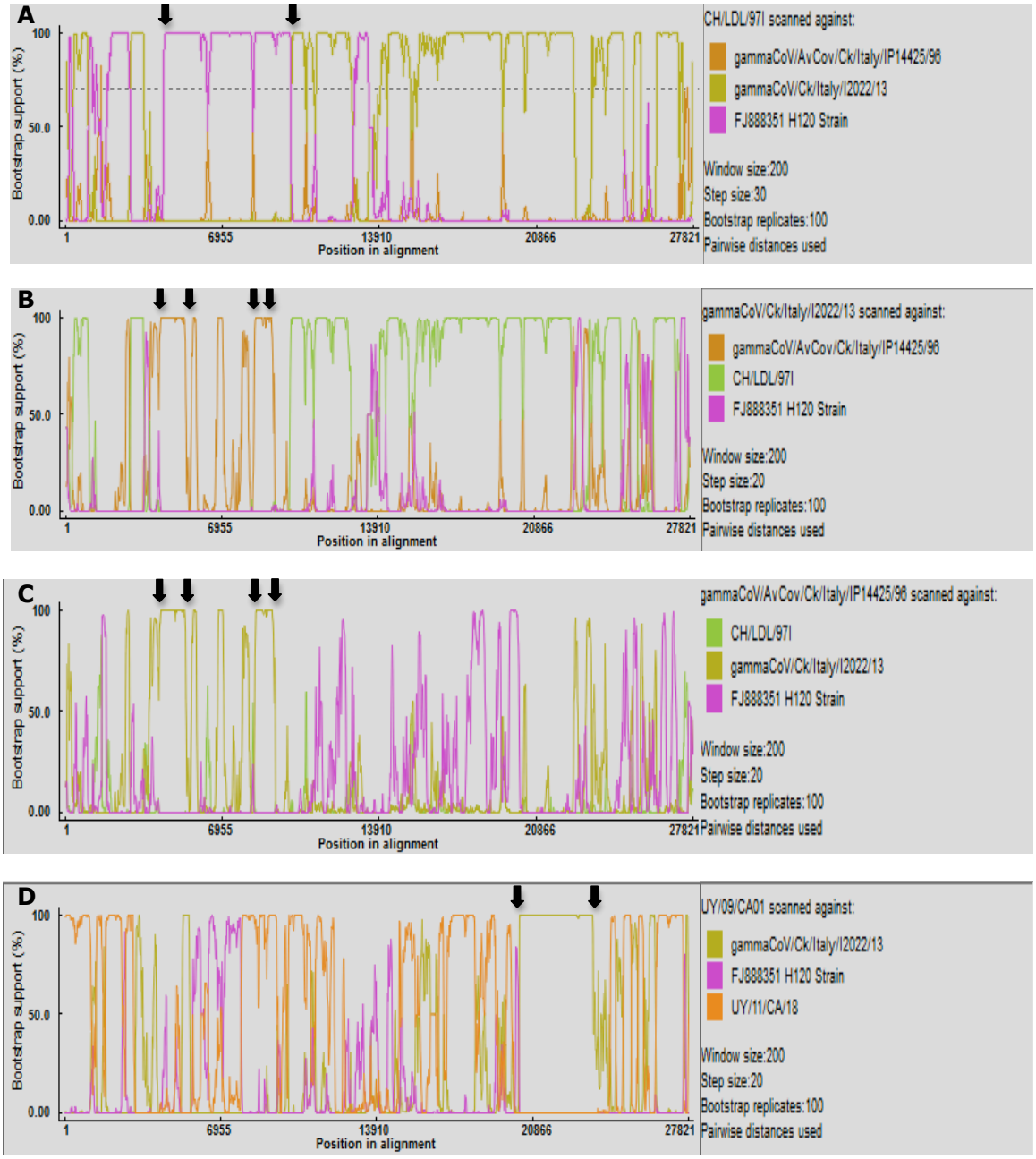

Figure 3 RDP screenshots displaying the possible recombination events associated with CK/CH/LDL/97I (A), gammaCoV/Ck/Italy/I2022/13 (B), gammaCoV/AvCov/Ck/Italy/IP14425/96 (C) and UY/09/CA/01 (D). Each panel displays the pairwise identities among the possible recombinant and its putative parents. Crossover sites indicated sharp changes in pairwise identity are indicated by arrows. A Comparisons among the putative recombinant CK/CH/LDL/97I shows sharp changes in the pairwise identity within the 1a gene, H120 vaccine strain FJ888351 has been identified as putative parental strains for that genomic region. B Comparisons among the putative recombinant gammaCoV/Ck/Italy/I2022/13 shows sharp changes in the pairwise identity within the 1a gene, strain gammaCoV/AvCov/Ck/Italy/IP14425/96 has been identified as major parental strain for that region. C Comparisons among the putative recombinant gammaCoV/AvCov/Ck/Italy/IP14425/96 shows sharp changes in the pairwise identity within the 1a gene, strain gammaCoV/Ck/Italy/I2022/13 has been identified as major parental strain for that genomic region. D Comparisons among the putative recombinant UY/09/CA/01 shows sharp changes in the pairwise identity in the last 7kb of the genome, strain gammaCoV/Ck/Italy/I2022/13 has been identified as major parental strain for that genomic region.

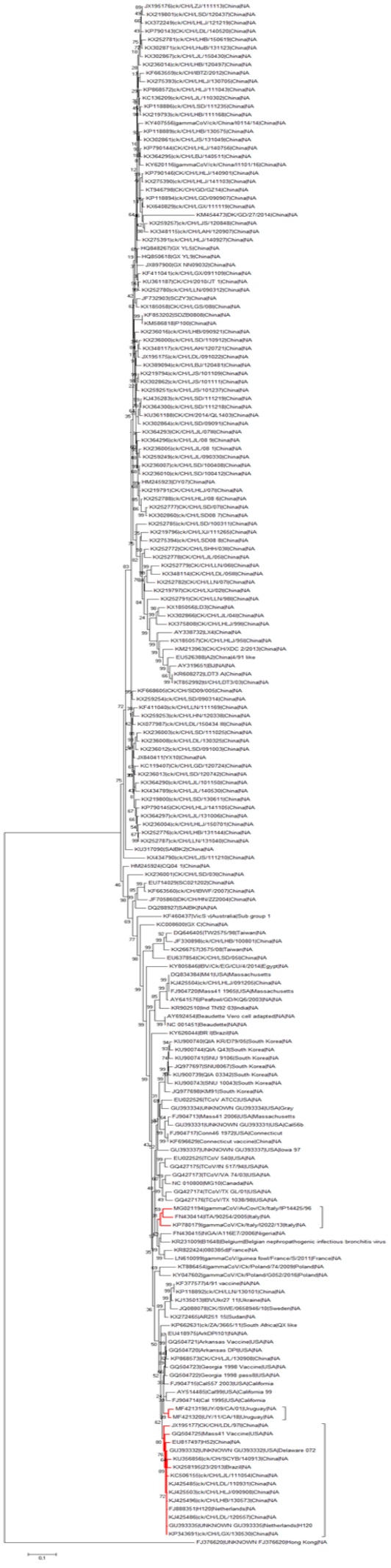

Figure 4 Phylogenetic tree based on Maximum Likelihood method with Tamura-Nei substitution model using 187 representative IBV and TCoV 1a gene. For easiness of representation, strains considered in the present study are marked in red

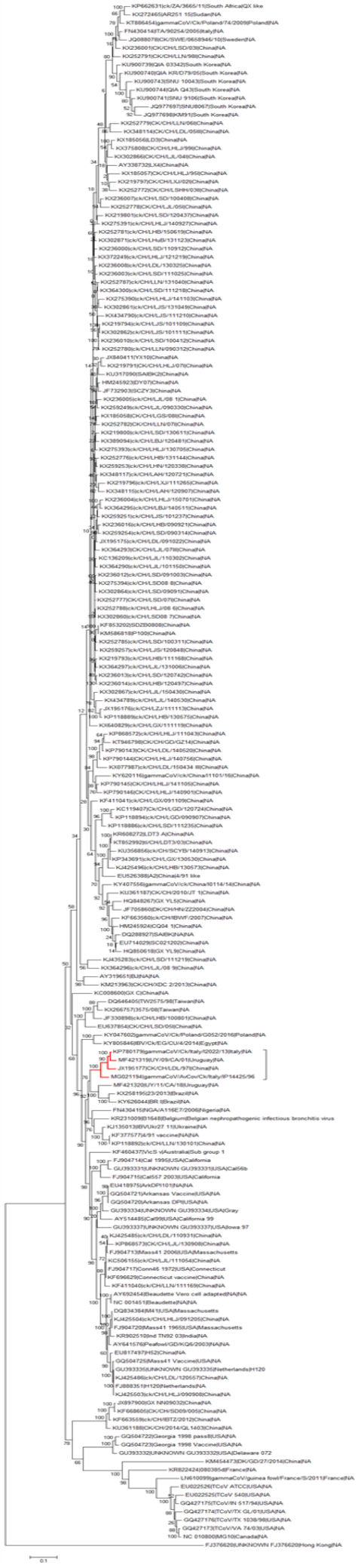

Figure 5 Phylogenetic tree based on Maximum Likelihood method with Tamura-Nei substitution model using 187 representative IBV and TCoV sequences of the last 7kb of the genome. For easiness of representation, strains considered in the present study are marked in red

References

- Ababneh, M., A. E. Dalab, S. Alsaad and M. Al-Zghoul, 2012: Presence of Infectious Bronchitis Virus Strain CK/CH/LDL/97I in the Middle East. *ISRN Vet Sci,* 2012, 201721.
- Abolnik, C., 2015: Genomic and single nucleotide polymorphism analysis of infectious bronchitis coronavirus. *Infect Genet Evol,* 32, 416-424.
- Armesto, M., D. Cavanagh and P. Britton, 2009: The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. *PLoS One,* 4, e7384.
- Bentley, K., S. M. Keep, M. Armesto and P. Britton, 2013: Identification of a noncanonically transcribed subgenomic mRNA of infectious bronchitis virus and other gammacoronaviruses. *J Virol,* 87, 2128-2136.
- Bochkov, Y. A., G. V. Batchenko, L. O. Shcherbakova, A. V. Borisov and V. V. Drygin, 2006: Molecular epizootiology of avian infectious bronchitis in Russia. *Avian Pathol,* 35, 379-393.
- Capua, I., R. E. Gough, M. Mancini, C. Casaccia and C. Weiss, 1994: A 'Novel' Infectious Bronchitis Strain Infecting Broiler Chickens in Italy. *Journal of Veterinary Medicine Series B*, 83-89.
- Capua, I., G. Grasso, S. Ferdinandi, C. Weiss and C. Casaccia, 1996: Studies on outbreak of nephritis and of drop in egg production associated with infectious bronchitis strain 624/I. *Zootecnia International* 49-51.
- Capua, I., Z. Minta, E. Karpinska, K. Mawditt, P. Britton, D. Cavanagh and R. E. Gough, 1999: Co-circulation of four types of infectious bronchitis virus (793/B, 624/I, B1648 and Massachusetts). *Avian Pathology,* 28, 587-592.
- Cavanagh, D., 2005: Coronaviruses in poultry and other birds. *Avian Pathol,* 34, 439-448.
- Cook, J. K., M. Jackwood and R. C. Jones, 2012: The long view: 40 years of infectious bronchitis research. *Avian Pathol,* 41, 239-250.
- de Wit, J. J. S., J. K. Cook and H. M. van der Heijden, 2011: Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian Pathol,* 40, 223-235.
- Franzo, G., V. Listorti, C. J. Naylor, C. Lupini, A. Laconi, V. Felice, M. Drigo, E. Catelli and M. Cecchinato, 2015: Molecular investigation of a full-length genome of a Q1-like IBV strain isolated in Italy in 2013. *Virus Res,* 210, 77-80.
- Hewson, K. A., J. Ignjatovic, G. F. Browning, J. M. Devlin and A. H. Noormohammadi, 2011: Infectious bronchitis viruses with naturally occurring genomic rearrangement and gene deletion. *Arch Virol,* 156, 245-252.
- Huang, Y. P., H. C. Lee, M. C. Cheng and C. H. Wang, 2004: S1 and N gene analysis of avian infectious bronchitis viruses in Taiwan. *Avian Dis,* 48, 581-589.
- Jackwood, M. and J. J. de Wit, 2013: Infectious bronchitis. *Diseases of Poultry. ,* 13th edition, 139-159.
- Jackwood, M. W., 2012: Review of infectious bronchitis virus around the world. *Avian Dis,* 56, 634-641.
- Kint, J., A. Dickhout, J. Kutter, H. J. Maier, P. Britton, J. Koumans, G. P. Pijlman, J. J. Fros, G. F. Wiegertjes and M. Forlenza, 2015a: Infectious Bronchitis Coronavirus Inhibits STAT1 Signaling and Requires Accessory Proteins for Resistance to Type I Interferon Activity. *J Virol,* 89, 12047-12057.
- Kint, J., M. Fernandez-Gutierrez, H. J. Maier, P. Britton, M. A. Langereis, J. Koumans, G. F. Wiegertjes and M. Forlenza, 2015b: Activation of the chicken type I interferon response by infectious bronchitis coronavirus. *J Virol,* 89, 1156-1167.

- Kint, J., M. A. Langereis, H. J. Maier, P. Britton, F. J. van Kuppeveld, J. Koumans, G. F. Wiegertjes and M. Forlenza, 2016: Infectious Bronchitis Coronavirus Limits Interferon Production by Inducing a Host Shutoff That Requires Accessory Protein 5b. *J Virol,* 90, 7519-7528.
- Krapez, U., B. Slavec, D. Barlic-Maganja and O. Z. Rojs, 2010: Molecular analysis of infectious bronchitis viruses isolated in Slovenia between 1990 and 2005: a retrospective study. *Virus Genes,* 41, 414-416.
- Kumar, S., G. Stecher and K. Tamura, 2016: MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol,* 33, 1870-1874.
- Laconi, A., J. Clubbe, M. Falchieri, C. Lupini, M. Cecchinato, E. Catelli, V. Listorti and C. J. Naylor, 2016: A comparison of AMPV subtypes A and B full genomes, gene transcripts and proteins led to reverse-genetics systems rescuing both subtypes. *J Gen Virol,* 97, 1324-1332.
- Li, W. and A. Godzik, 2006: Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics,* 22, 1658-1659.
- Lim, T. H., H. J. Lee, D. H. Lee, Y. N. Lee, J. K. Park, H. N. Youn, M. S. Kim, J. B. Lee, S. Y. Park, I. S. Choi and C. S. Song, 2011: An emerging recombinant cluster of nephropathogenic strains of avian infectious bronchitis virus in Korea. *Infect Genet Evol,* 11, 678-685.
- Listorti, V., A. Laconi, E. Catelli, M. Cecchinato, C. Lupini and C. J. Naylor, 2017: Identification of IBV QX vaccine markers : Should vaccine acceptance by authorities require similar identifications for all live IBV vaccines? *Vaccine,* 35, 5531-5534.
- Marandino, A., A. Pereda, G. Tomas, M. Hernandez, G. Iraola, M. I. Craig, D. Hernandez, A. Banda, P. Villegas, Y. Panzera and R. Perez, 2015: Phylodynamic analysis of avian infectious bronchitis virus in South America. *J Gen Virol,* 96, 1340-1346.
- Marandino, A., G. Tomas, Y. Panzera, G. Greif, A. Parodi-Talice, M. Hernandez, C. Techera, D. Hernandez and R. Perez, 2017: Whole-genome characterization of Uruguayan strains of avian infectious bronchitis virus reveals extensive recombination between the two major South American lineages. *Infect Genet Evol,* 54, 245-250.
- Martin, D. P., B. Murrell, A. Khoosal and B. Muhire, 2017: Detecting and Analyzing Genetic Recombination Using RDP4. *Methods Mol Biol,* 1525, 433-460.
- Massi, P., 2013: Situazione epidemiologica della Bronchite Infettiva in Italia. *Rivista di medicina veterinaria*, 13-20.
- Massi, P., I. Barbieri, L. Fiorentini, M. Casadio, M. Parigi and G. Tosi, 2015: Analisi molecolare di ceppi del Virus della Bronchite Infettiva aviare negli anni 2013 e 2014 . considerazioni sui genotipi circolanti in Italia e in altri paesi europei ed extra-europei. *Atti del LIV convegno annuale della Societa' Italiana di Patologia Aviare,Forlì, 16-17 Aprile 2015.*, 220-229.
- Quinteros, J. A., S. W. Lee, P. F. Markham, A. H. Noormohammadi, C. A. Hartley, A. R. Legione, M. J. Coppo, P. K. Vaz and G. F. Browning, 2016: Full genome analysis of Australian infectious bronchitis viruses suggests frequent recombination events between vaccine strains and multiple phylogenetically distant avian coronaviruses of unknown origin. *Vet Microbiol,* 197, 27-38.
- Rimondi, A., M. I. Craig, A. Vagnozzi, G. Konig, M. Delamer and A. Pereda, 2009: Molecular characterization of avian infectious bronchitis virus strains from outbreaks in Argentina (2001-2008). *Avian Pathol,* 38, 149-153.
- Simon-Loriere, E. and E. C. Holmes, 2011: Why do RNA viruses recombine? *Nat Rev Microbiol,* 9, 617-626.
- Taddei, R., G. Tosi, M. B. Boniotti, M. Casadio, L. Fiorentini, M. Fabbi and P. Massi, 2012: Caratterizzazione molecolare di ceppi del virus della bronchite infettiva aviare isolati

in Italia tra il 1963 ed il 1989. *Proceedings of 51 Convegno annuale Societa' Italiana Patologia Aviare (SIPA) : 11-12 Ottobre 2012 , Salsomaggiore Terme (PR)* 332-341.
- Toffan, A., M. Bonci, L. Bano, L. Bano, V. Valastro, M. Vascellari, I. Capua and C. Terregino, 2013: Diagnostic and clinical observation on the infectious bronchitis virus strain Q1 in Italy. *Vet Ital,* 49, 347-355.
- Valastro, V., E. C. Holmes, P. Britton, A. Fusaro, M. W. Jackwood, G. Cattoli and I. Monne, 2016: S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infect Genet Evol,* 39, 349-364.
- van Beurden, S. J., A. J. Berends, A. Kramer-Kuhl, D. Spekreijse, G. Chenard, H. C. Philipp, E. Mundt, P. J. M. Rottier and M. H. Verheije, 2017: A reverse genetics system for avian coronavirus infectious bronchitis virus based on targeted RNA recombination. *Virol J,* 14, 109.
- Yang, Y., L. Zhang, H. Geng, Y. Deng, B. Huang, Y. Guo, Z. Zhao and W. Tan, 2013: The structural and accessory proteins M, ORF 4a, ORF 4b, and ORF 5 of Middle East respiratory syndrome coronavirus (MERS-CoV) are potent interferon antagonists. *Protein Cell,* 4, 951-961.
- Ye, Z., C. K. Wong, P. Li and Y. Xie, 2008: A SARS-CoV protein, ORF-6, induces caspase-3 mediated, ER stress and JNK-dependent apoptosis. *Biochim Biophys Acta,* 1780, 1383-1387.
- Yu, L., Y. Jiang, S. Low, Z. Wang, S. H. Nam, W. Liu and J. Kwang, 2001: Characterization of Three Infectious Bronchitis Virus Isolates from China Associated withProventriculus in Vaccinated Chickens. *Avian Dis,* 45, 416-424.
- Zhang, Y., H. N. Wang, T. Wang, W. Q. Fan, A. Y. Zhang, K. Wei, G. B. Tian and X. Yang, 2010: Complete genome sequence and recombination analysis of infectious bronchitis virus attenuated vaccine strain H120. *Virus Genes,* 41, 377-388.
-

Chapter 4: Published Paper II

NOTICE: this is post-print (final draft post-refereeing) author's version of a work that was accepted for publication in Vaccine. Changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version is published in: Listorti V., Laconi A., Catelli E., Cecchinato M., Lupini C., Naylor C.J., "Identification of IBV QX vaccine markers : Should vaccine acceptance by authorities require similar identifications for all live IBV vaccines?" Vaccine 2017 Oct 9;35(42):5531-5534. doi: 10.1016/j.vaccine.2017.06.021. Epub 2017 Sep 13.

Short Communication

Identification of IBV QX vaccine markers: Should vaccine acceptance by authorities require similar identifications for all live IBV vaccines?

Valeria Listorti [a], Andrea Laconi [a], Elena Catelli [b], Mattia Cecchinato [c], Caterina Lupini [b], Clive J. Naylor [a,⇑]

[a] Department of Infection Biology, University of Liverpool, Leahurst Campus, Neston, Cheshire CH64 7TE, United Kingdom

[b] Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra, 50, 40064 Ozzano dell'Emilia, BO, Italy

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

Abstract

IBV genotype QX causes sufficient disease in Europe for several commercial companies to have started developing live attenuated vaccines. Here, one of those vaccines (L1148) was fully consensus sequenced alongside its progenitor field strain (1148-A) to determine vaccine markers, thereby enabling detection on farms. Twenty-eight single nucleotide substitutions were associated with the 1148-A attenuation, of which any combination can identify vaccine L1148 in the field. Sixteen substitutions resulted in amino acid coding changes of which half were in spike. One change in the 1b gene altered the normally highly conserved final 5 nucleotides of the transcription regulatory sequence of the S gene, common to all IBV QX genes. No mutations can currently be associated with the attenuation process. Field vaccination strategies would greatly benefit by such comparative sequence data being mandatorily submitted to regulators prior to vaccine release following a successful registration process.

1.Introduction

Infectious Bronchitis virus (IBV) primarily infects domestic fowl and causes respiratory and other diseases [1] resulting in serious losses in unprotected birds. It is mainly controlled using live vaccines made from field viruses isolated during disease outbreaks. Most IBV vaccines are prepared by multiple passage of such IBV field viruses in eggs to yield attenuated strains which no longer cause disease but can still stimulate a protective immune response ([2,3]). There are many IBV genotypes and generally optimal protection relies on vaccination with the same genotype.

Some genotypes have been continuously detectable since suitable detection techniques were developed while others have appeared, briefly caused a problem, then disappeared ([4,5]). In general such transient genotypes do not cause sufficient problem to warrant a commercial live vaccine development. However other genotypes have caused a problem for a longer period such as IBV 793B, and led several companies to prepare appropriate vaccines. IBV QX (newly classified as GI-19 lineage [6]) increasingly appears to fall into this category. It appeared in China and then Europe some 15 years ago [7] and has already caused sufficient problems [8] for several major European located poultry biological companies to develop live vaccines.

Effective knowledge of the prevalence and circulation of different IBV genotypes in any area is not straightforward. One complication can be the presence of the very live vaccines previously applied for disease control. A recent example concerned IBV 793B in an area of Italy where withdrawal of IBV 793B live vaccine led to the genotype's disappearance; thereby suggesting that, virulent IBV 793B, previously thought ubiquitous, was largely absent [9]. Most situations are not clear-cut and conclusions become hampered by the inability to distinguish between a genuine disease- causing-field-virus and the attenuated commercial vaccine applied to protect birds against it. Fortunately molecular diagnostic developments make such differentiation relatively straightforward, but only if the field strain used to make any particular vaccine (progenitor) is available to diagnosticians. Specifically, comparison of the full sequences of the vaccine and the progenitor reveals the changes which occurred in the largely random attenuation process, and those changes are invariably unique to that particular vaccine. While for IBV little work has been undertaken or published, for AMPV, it has been shown that two

vaccines produced by passaging the identical progenitor field strain, did not contain any of the same attenuation-associated mutations (manuscript in preparation). Furthermore where AMPV vaccines persist in the field and mutate further, those mutations are never found to be back- mutations to the original progenitor virus [10,11].

In the current work we fully consensus sequenced the complete genomes of a commercial IBV QX vaccine which had been prepared by 80 chicken-embryo-passages of its virulent progenitor 1148-A [12]. We continue to show how the sequence comparison can be used to assist in the assessment of IBV QX prevalence on farms.

2. Materials and methods

2.1. Viral RNA extraction, RT-PCRs and sequencing

Samples of a production batch of vaccine strain L1148 and an aliquot of its egg isolated progenitor strain 1148-A, were com- pared. The RNA of both viruses was extracted using Qiamp viral RNA minikit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Viral RNA was retro-transcribed using Super Script III enzyme (Invitrogen, Carlsbad, USA) then amplified using Ranger enzyme (Bioline, London, UK) according to each manufacturer's instructions. Retro-transcription, amplification and sequencing used primers previously designed for IBV full genome sequencing [13]. Where primers did not work due to QX sequence differences, new primers were designed based on the newly determined sequences flanking those genome regions (Table 1). Sequencing was performed by Source BioScience (Nottingham, UK). All regions were sequenced twice. Where vaccine and progenitor sequences differed, the locations were sequenced again, starting with a new retro-transcription of the region.

2.2. RT-PCR of the 3′ END of genome

The 3′ end of the genome was determined using a 3′RACE protocol previously described [14]. Briefly, RT was performed with a primer containing 20 Ts followed by an adaptor sequence at its 5′ terminus, then amplified using 2 PCR primers, one within the genome end and the other matching the adaptor (Table 1). PCR products were sequenced towards the polyA tail using a genome end primer.

2.3. Sequences analysis and comparison

Chromatograms were analysed using the program Chromas (http://technelysium.com.au/wp/chromas/) and sequences aligned using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Open Reading Frame (ORF) prediction was carried out using ORF- finder program (https://www.ncbi.nlm.nih.gov/orffinder/).

2.4. Accession numbers

Sequences of IBV strains L1148 (QX vaccine strain) and 1148-A (QX progenitor strain) were submitted to the GenBank database and were assigned the following accession numbers: KY933090 (L1148) and KY933089 (1148-A).

3. Results and discussion

A consensus sequence of both viruses was obtained, except for approximately the first 100nt of the 5'UTRs. The ORF analysis predicted 13 ORFs for each, resulting in the genome organization: 5'UTR-1a-1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b-3'UTR. Transcription regulatory sequences excluding that for the polymerase gene (TRS-Bs) were identified and are reported in Table 2. For the S gene, a substitution was detected within the normally highly conserved final 5 nucleotide region of the 8 nucleotide TRS-B, as shown in Table 2.

Comparison of the two sequences showed 28 nucleic acid substitutions between the vaccine strain and its progenitor. Eleven nucleic acid substitutions were located in the 1a-1b genes, 8 in the S gene, two changes each in the 5'UTR, 5b gene and 3'UTR, and one change each in the E, M and 5a genes (Table 3). Of the 28 nucleic acids differences, 16 resulted in amino acid changes, and of the remainder, 8 were silent mutations and 4 were in un- translated regions. Of 15 coding changes, 7 were located in the S gene, five in the 1a-1b gene and one each in the E, M and 5b genes (Table 3).

Nucleotide substitutions likely to affect protein structure and function occurred in S1 5b and Nsp 13. In S1, amino acid charge alterations occurred in the S1 gene corresponding to nucleotide positions 20,460 (Met->His) and 21,438 (Glu->Gln), and in the 5b gene at position 25,664 (Asp->Tyr). In Nsp13, an aromatic Pro was substituted by smaller and aliphatic Leu corresponding to nucleotide position 15,693. The availability of vaccine and progenitor sequences, and comparison of those sequences to determine vaccine markers is vital in tracking IBV vaccines. For QX L1148 vaccine, field detections containing only one of the 28 nucleotide substitutions arising from the attenuation process would be highly likely to have

arisen from that vaccine having been applied within transmissible distance. Indeed, a commonly used S1 diagnostic RT-PCR [15] covers the mutation at 21,073. Sequencing of genome sections covering several of the substitutions would make that conclusion irrefutable. For simplicity, sequencing of a region containing a high density of the changes would be ideal. The region of S1 containing four mutations could be readily RT-PCR amplified and economically sequenced using primer sequences provided here. However as for any IBV genotype, recombination may have occurred hence only for those genome sections actually sequenced could vaccine QX L1148 origin be attributed.

Similar to our previous diagnostic Avian metapneumovirus vaccines studies [16], our research facility was fortunate to have isolated, and hence have access to, the IBV QX progenitor strain. Unfortunately vaccine progenitors or their sequences are not generally made available to the research community by the companies producing the vaccines. This leads to an inability to distinguish vaccine and field virus, hence prevents intelligent tailoring of IBV vaccine administration in a disease situation. In our opinion, this is unacceptable. This situation also means that vaccines can become wrongly implicated in causing a disease problem, say if a disease outbreak becomes wrongly attributed to a suspected reversion to virulence event. Hence all parties aspiring to open and informed vaccination of poultry have an interest in improving this situation. In our view, vaccine regulatory bodies should require that both the vaccine and vaccine progenitor sequences are deter- mined and made available at the time of vaccine registration or release in their jurisdiction. While the consensus sequences presented in our study relied on the possession of oligonucleotides designed over a considerable period, vaccine producers could readily use next generation sequencing to this end.

A further potentially useful aspect of having identified mutations occurring during the attenuation process is that they could seed further studies to determine which were responsible of the virulence loss, and beyond that, by what mechanism they arose. For example, the S gene TRS mutation may have either up or down regulated S gene transcription [17], 5b protein changes may have altered visibility of the virus to the host immune response due to its reported involvement in immune shut off [18] and alterations in the replicase affecting pathogenicity [19] may have all contributed to the vaccine phenotype. The changes in S may also be involved in attenuation but there remains some uncertainty as to this protein's role in virulence [20]. Currently no role can be attributed to any of these mutations but further studies might inform further and assist in rational vaccine design by making use of

the IBV reverse genetics systems now available

Table 1. Primers used for reverse transcription, PCR and sequencing. Primer names generally indicate approximate binding positions in the QX genome. Coding and anticoding sense primers are labeled + and neg respectively.

RT	Sequence 5'-3'	Sequencing	Sequence 5'-3'
2.06neg	tttagtaaaaagaccacc	0.67+	cctaaggattacgctgatgcttttgc
4.26neg	catacttttgcgcatc	1.26+	ttcgcaggaacttgtcttgcaagc
6.18neg	agaaaacctacaccag	2.52+	tagaggaatgtcacagcttggtgc
8.18neg	gtaaagaatgtactaacc	3.04+	tacaccaatgtcacagcttggtgc
QX10.20neg	cacagttgtgtgcactaactcaaag	3.1+	ctctcgatgttgtgaatttaccatctgg
12.1neg	ctccataagaatcctg	4.8+	tgattgtgatgttgtgaatttaccatctgg
14.13neg	taaaacttggttgttcc	5.32+	ctattagtcttagggcaatatggg
QX16,15neg	ggtcaacatactaacctcatctacc	6.53+	gttaaacctacagcatatgcttacc
QX18.10neg	catagaagaagaatggcatagctttc	7.17+	aatgctcctccggtagtatggaag
19.73neg	caaaatgcattactcgc	QX 8.50 +	gtgctatacatgtacacaccgctg
22.51neg	catatcttctttttgacc	9.26+	cctgtcactatgcgttctaatggtac
QX24.20neg	ctacttcacctgtttcaattgttttctc	QX 9.40neg	ctaatttatcgggttgaactttctgagc
26.23neg	accaactttaggtggc	10.6+	ggtaaatccacctaaaactgtgtggg
27.89neg	ttgctctaactctatac	QX10.90neg	cagccaagtctctatcaaagattgac
Dta-Adaptneg	gcatctcgaggcttgtggctttttttttttttttttttta	QX11.50+	gaagcaggcaatcaaatttatgttgac
Dtc-Adaptneg	gcatctcgaggcttgtggctttttttttttttttttttc	11.13+	gaagttagatagcatggagagacg
Dtg-Adaptneg	gcatctcgaggcttgtggctttttttttttttttttttg	12.64+	gacttaaagtcagaagtaacagctg
		13.18+	gatctcctcaagtatgattatactgagg
PCR	**Sequence 5'-3'**	14.69+	caaggtcttgtagcagatatttctgg
0.06+	gcgctagatttccaacttaacaaaacg	15.25+	aagtgttgctatgaccatgtcatgc
2.03neg	gacttgcgaaacaagatgccaaatgcc	16.09neg	cacataaagcatcaacagctgcatgag
1.92+	tggaggcttgcatatggaaaagtgcg	15.99+	ggcaagcagaagcgtactacagta
4.2neg	ggtataaagaggatttctttatcctcaagatcatg	16.62+	catgaaagtggctcagcctacaac
4.1+	cggaggatggtgttaaataccgc	QX16.80neg	gaatcagctgtaacacagaatataac
6.08neg	caaataatattagaaagaccaaataaagccaattcc	17.18+	caacatgttttataacacgtgatgaggc
5.93+	gattcttttgatgtgttacgctattgtgcag	18.18+	taacctacctggttgtaatggtgg
8.05neg	cctggtttagtatactcacatacactacc	18.71+	gaagagaaatattcgcacactgcc
QX7.80+	cttccatagcattgtttgcttctag	19.86+	tgcacatgtttctgttgctttcagattg
10.02+	cagttattattggagtttgtgctgaag	QX19.50+	agtagccaatgatggcaatgacgatg
12.08-	gaatcctgatccggagttggacttggc	QX19.90+	gcttcagtcggcttggacatgtgg
12.01+	gtggcagcaggtaatcaacctttagg	19.97+	gatagccaataatggcaatgatgacg
14.12-	ataaaacttggttgttccaataactacagg	QX20.60+	ctgcacatgagtgcactgttggtg
QX13.90+	gaggtgacgtctaaatattttgaatg	QX21.70neg	caaaattcgtgcttaattcacctg
QX16.15-	ggtcaacatactaacctcatctacc	22.48+	ccatttcttagtaatgttagcactgg
QX15.06+	gatgattgcactcgcatagtacctc	22.99+	ctctagcattacaacaaattcaagatgttg
QX18.10neg	catagaagaagaatggcatagctttc	24.60+	cttgttgtctttttttcgtatacatgggtag
17.94+	cgttgtctatgatataggcaaccctaaagg	25.27+	gacgtaatatctatcgtatggtgcag
19.67neg	gtattgacagagttgtgtatactttgcc	QX25.87+	ctcaattaagggttttagataggttaattc
QX18.10neg	catagaagaagaatggcatagctttc	QX26.88neg	gacatgtaggtaatcaaactacatgcc
19.46+	gtaacagtgtcaattgattaccatagc	QX27.20+	cctacatgtctatcgccaggg
22.26neg	tccatacgcgtttgtatgtactcatctg		
QX21.90+	ggttaatccttgtgaagatgttaatcaac		
QX24.20neg	ctacttcacctgtttcaattgttttctc		
23.99+	cattatgcctctaatgagtaagtgtgg		
26.22neg	aactttaggtggctttggtcctcc		
26.02+	gaaaagcgcgaatttatctgagagaagg		
27.83neg	catagccaattaaacttaacttaaactaaaatttagctc		
26.49+	gatagccaagatggtatagtgtggg		
Adapt neg	gcatctcgagggttgtggc		

Table 2. TRS sequences of progenitor strain IBV QX 1148-A and QX vaccine L1148 showing showing the S gene A to C substitution.

Gene	TRS start position	TRS sequence in progenitor	TRS sequence in vaccine
S	20208	AGG AACAA	AGG A**C**CAA
3	23740	CTG AACAA	CTG AACAA
M	24326	CTT AACAA	CTT AACAA
4b	24800	CTG AACAA	CTG AACAA
5	25390	ACC AACAA	ACC AACAA
N	25740	CTT AACAA	CTT AACAA
6b	26901	CAA AACAA	CAA AACAA

Table 3. Nucleoside substitutions arising during the attenuation of QX 1148-A to yield vaccine L-1148. Shading denotes the 16 amino acid changes occurring.

Genome region	Genome position	Nucleotide		Amino acid	
		progenitor	vaccine	progenitor	vaccine
5'UTR	78	C	T	n.c.	n.c.
5'UTR	257	C	T	n.c	n.c.
Nsp 2	548	C	T	N	N
Nsp 2	1970	C	T	F	F
Nsp 3	3322	C	T	T	I
Nsp 3	4597	T	G	I	S
Nsp 3	6209	C	T	C	C
Nsp 4	8688	G	A	N	N
Nsp 8	10929	C	A	L	I
Nsp 12	14440	C	T	V	V
Nsp 13	15693	C	T	P	L
Nsp 14	17239	C	T	N	N
Nsp 16	20212	A	C	E	D
S1	20460	G	C	M	H
S1	20521	C	A	D	K
S1	21073	C	T	T	I
S1	21435	G	T	G	F
S1	21436	G	T	G	F
S1	21438	G	C	E	Q
S2	22902	T	A	S	T
S2	22921	C	T	S	L
E	24281	G	T	A	S
M	25048	A	C	E	A
5a	25616	C	T	Y	Y
5b	25664	G	T	D	Y
5b	25831	C	T	N	N
3'UTR	27074	C	T	n.c.	n.c.
3'UTR	27539	T	C	n.c.	n.c.

References

[1] Jackwood M, de Wit JJ. Infectious bronchitis. Diseases of poultry, 13th ed.; 2013. p. 139–59.

[2] Bijlenga G, Cook JK, Gelb Jr J, de Wit JJ. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol 2004; 33:550–7.

[3] Gelb JJ, Cloud SS. Effect of serial embryo passage of an Arkansas-type avian infectious bronchitis virus isolate on clinical response, virus recovery, and immunity. Avian Dis 1983; 27:679–987.

[4] Jackwood MW. Review of infectious bronchitis virus around the world. Avian Dis 2012; 56:634–41.

[5] Sjaak de Wit JJ, Cook JK, van der Heijden HM. Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol 2011; 40:223–35.

[6] Valastro V, Holmes EC, Britton P, Fusaro A, Jackwood MW, Cattoli G, Monne I. S1 gene-based phylogeny of infectious bronchitis virus: an attempt to harmonize virus classification. Infect Genet Evol 2016; 39:349–64.

[7] YuDong W, YongLin W, ZiChun Z, GenChe F, YiHai J, XiangE L. Isolation and identification of glandular stomach type IBV (QX IBV) in chickens. Chinese J Anim Quarantine 1998; 15:1–3.

[8] Stooker L, Mer Van C, Andrews S, Wijmenga W, Vancraeynest D. PanEuropean survey on the distribution of different strains of Infectious Bronchitis virus in 2011. Book of abstracts, XVIII congress world veterinary poultry association French branch (WVPA), Nantes, France, 19th–23rd August 2013; 2013. p. 383.

[9] Franzo G, Naylor CJ, Lupini C, Drigo M, Catelli E, Listorti V, Pesente P, Giovanardi D, Morandini E, Cecchinato M. Continued use of IBV 793B vaccine needs reassessment after its withdrawal led to the genotype's disappearance. Vaccine 2014; 32:6765–7.

[10] Catelli E, Cecchinato M, Savage CE, Jones RC, Naylor CJ. Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. Vaccine 2006; 24:6476–82.

[11] Lupini C, Cecchinato M, Ricchizzi E, Naylor CJ, Catelli E. A Turkey rhinotracheitis outbreak caused by the environmental spread of a vaccine- derived avian metapneumovirus. Avian Pathol 2011; 40:525–30.

[12] Geerligs HJ, Boelm GJ, Meinders CA, Stuurman BG, Symons J, Tarres-Call J, et al. Efficacy and safety of an attenuated live QX-like infectious bronchitis virus strain as a vaccine for chickens. Avian Pathol 2011; 40:93–102.

[13] Franzo G, Listorti V, Naylor CJ, Lupini C, Laconi A, Felice V, et al. Molecular investigation of a full-length genome of a Q1-like IBV strain isolated in Italy in 2013. Virus Res 2015; 210:77–80.

[14] Laconi A, Clubbe J, Falchieri M, Lupini C, Cecchinato M, Catelli E, et al. A comparison of AMPV subtypes A and B full genomes, gene transcripts and proteins led to reverse-genetics systems rescuing both subtypes. J Gen Virol 2016; 97:1324–32.

[15] Jones RC, Worthington KJ, Capua I, Naylor CJ. Efficacy of live infectious bronchitis vaccines against a novel European genotype, Italy 02. Veter Record 2005; 156:646–7.

[16] Cecchinato M, Catelli E, Lupini C, Ricchizzi E, Prosperi S, Naylor CJ. Reversion to virulence of a subtype B avian metapneumovirus vaccine: is it time for regulators to require availability of vaccine progenitors? Vaccine 2014; 32:4660–4.

[17] Bentley K, Keep SM, Armesto M, Britton P. Identification of a noncanonically transcribed subgenomic mRNA of infectious bronchitis virus and other gammacoronaviruses. J Virol 2013; 87:2128–36.

[18] Kint J, Langereis MA, Maier HJ, Britton P, van Kuppeveld FJ, Koumans J, et al. Infectious bronchitis coronavirus limits interferon production by inducing a host shutoff that requires accessory protein 5b. J Virol 2016; 90:7519–28.

[19] Armesto M, Cavanagh D, Britton P. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. PLoS One 2009; 4: e7384.

[20] Stevenson-Legget P, Britton P, Bickerton E. Generation of a recombinant infectious bronchitis virus suggests that the S protein is a determinant of pathogenicity. Book of abstracts, 9th international symposium on avian corona-and pneumovirus, Utrech, The Netherlands, 21st–24th June, 2016; 2016. p. 122.

Conclusions

The sequencing of the complete genome of IBV is of vital importance to obtain information about the epidemiology of this virus and for diagnostic purposes. IBV is able to mutate rapidly and to recombine and this causes the continuous emergence of new strains worldwide. The study of the genomic characteristics of the strains circulating is fundamental for the developing of new controlling strategies.

In recent times, different genotypes of apparent Asian origin, such as the Q1 and the QX genotype have spread to other countries and continents, sometimes with economic consequences. On this thesis the attention was focused on the characterization of strains belonging to these two genotypes.

In the first part of the project we reported, for the first time, the whole genome sequence of a Q1 strain (gammaCoV/Ck/Italy/I2022/13) isolated in Italy from chickens with respiratory signs. This genotype, after the first isolation in China in the mid 90' has spread in many countries. In Italy, it was reported for the first time in 2011. The phylogenetic analysis of the complete genome of gammaCoV/Ck/Italy/I2022/13 Q1 strain demonstrated that it clusterized with a Chinese Q1 strain. Surprisingly, the analysis of the S1 gene revealed also a high homology with an old genotype circulating in Italy from the 60', the 624I genotype. In order to clarify the genetic correlation between 624I genotype and Q1 genotype we sequenced and analysed the complete genome of a 624I strain isolated in Italy in 1996 (gammaCov/Ck/Italy/IP14425/96). The basal location of the 624I strain in both phylogenetic trees, constructed on the complete genome sequence and on the S1 gene, strongly suggests that this genotype has played an important role in the emerge and in the evolution of Q1 genotype and supports the hypothesis that elects 624I genotype as Q1 ancestor. The findings reported in this study strongly suggest that 624I genotype was introduced in China somewhere in the past, there evolved into Q1 genotype, before its reintroduction in Italy.

In the second part of the project we sequenced and compared a commercial vaccine strain of QX genotype and its progenitor strain. The QX genotype was isolated in China about 15 years ago and then spread worldwide becoming one of the major genotype circulating, this epidemiological situation has prompted the production of homologous vaccines. In this study the sequencing of the QX vaccine strain L1148, widely used in the field, and its progenitor strain 1148-A led to the identification of 28 markers in the vaccine sequence. The sequencing of the region of the S1 gene containing four vaccine markers could be an efficacious and economic method for the irrefutable identification of this vaccine in the field. A further

potentially useful aspect of having identified mutations occurring during the attenuation process is that they could seed further studies to determine which were responsible of the virulence loss, and beyond that, by what mechanism they arose.

In conclusion, it is important to remark the importance of performing complete genome sequencing of viruses and the urgency of an increase sequence data availability that, in the future, with the increasing use of sequencing techniques, might be more achievable and economic.