
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Virus Research 194 (2014) 37–48

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

# The avian coronavirus spike protein

I.N. Ambepitiya Wickramasinghe[1], S.J. van Beurden[1], E.A.W.S. Weerts, M.H. Verheije*

*Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL Utrecht, The Netherlands*

ARTICLE INFO

*Article history:*
Available online 17 October 2014

*Keywords:*
Avian coronavirus
*Gammacoronavirus*
Infectious bronchitis virus IBV
Spike
Binding
Tropism

ABSTRACT

Avian coronaviruses of the genus *Gammacoronavirus* are represented by infectious bronchitis virus (IBV), the coronavirus of chicken. IBV causes a highly contagious disease affecting the respiratory tract and, depending on the strain, other tissues including the reproductive and urogenital tract. The control of IBV in the field is hampered by the many different strains circulating worldwide and the limited protection across strains due to serotype diversity. This diversity is believed to be due to the amino acid variation in the S1 domain of the major viral attachment protein spike. In the last years, much effort has been undertaken to address the role of the avian coronavirus spike protein in the various steps of the virus' live cycle. Various models have successfully been developed to elucidate the contribution of the spike in binding of the virus to cells, entry of cell culture cells and organ explants, and the *in vivo* tropism and pathogenesis. This review will give an overview of the literature on avian coronavirus spike proteins with particular focus on our recent studies on binding of recombinant soluble spike protein to chicken tissues. With this, we aim to summarize the current understanding on the avian coronavirus spike's contribution to host and tissue predilections, pathogenesis, as well as its role in therapeutic and protective interventions.



## 1. Introduction

Avian coronaviruses of poultry belong to the genus *Gammacoronavirus* within the order Nidovirales. Avian gammacoronaviruses can cause major health problems with subsequent economic losses in several commercially kept bird species, predominantly chickens (*Gallus gallus*). The genus *Gammacoronavirus* comprises not only viruses of domesticated birds, but also two recently discovered cetacean coronaviruses (Mihindukulasuriya et al., 2008; Woo et al., 2014). In addition, avian coronaviruses belonging to the genera *Gammacoronavirus* and *Deltacoronavirus* have been detected in wild bird species (Chu et al., 2011; Woo et al., 2009, 2012), but details on their pathogenesis and host range are yet unknown. In this review we will focus on the avian gammacoronaviruses of poultry, in particular on the role of the spike protein in the outcome of infection.

The avian infectious bronchitis virus (IBV) causes infectious bronchitis in chickens. It is to date the most important and best-studied *Gammacoronavirus* and is therefore considered the genus' prototype. IBV was the first coronavirus described, and was discovered in the Unites States in the 1930s (Schalk and Hawn, 1931). Currently, it is worldwide present in both industrial and back yard chickens (reviewed by Cook et al., 2012; Jackwood, 2012; Sjaak de Wit et al., 2011). IBV principally infects the epithelium of its hosts' upper airways, which leads to respiratory distress, and predisposes for secondary bacterial airway infections (Dwars et al., 2009; Matthijs et al., 2003). Several IBV strains additionally show a subtype-dependent tropism for other epithelia, including the renal tubuli, the oviduct and parts of the gastrointestinal tract (reviewed in Cook et al., 2012; Ignjatovic and Sapats, 2000; Raj and Jones, 1997). This results in variable morbidity, mortality, pathology and production losses in poultry. The great diversity of IBV strains worldwide makes it difficult to prevent infectious bronchitis in chickens. The presence of IBV-like and other avian coronaviruses in other bird species (including turkey, pheasant, quail, guineafowl, partridge, peafowl, duck, goose and pigeon) (Cavanagh, 2005), complicates the field situation for avian coronaviruses even more.

IBV is an enveloped virus with a positive sense single-stranded RNA genome of 27.6 kb (Masters and Perlman, 2013). The 5′ two-third of the viral genome comprises open reading frame (ORF) 1ab, which encodes for 15 nonstructural replicase proteins (nsp2-16) involved in RNA replication and transcription. The 3′ one-third of the viral genome codes for the structural proteins, which are interspersed by the accessory genes 3a, 3b, 4b/intergenic region, 5a, 5b. These accessory genes are group specific and have, while being dispensable for IBV replication *in vitro* (Casais et al., 2005; Hodgson et al., 2006), yet unknown functions *in vivo*. The structural

* Corresponding author. Tel.: +31 30 253 4296.
*E-mail address:* m.h.verheije@uu.nl (M.H. Verheije).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.virusres.2014.10.009
0168-1702/

proteins of IBV include the spike protein S, the envelop protein E, the membrane protein M and the nucleocapsid protein N (Masters and Perlman, 2013). After genomic replication, the N protein forms together with the RNA genome the ribonucleocapsid, which is encapsidated by the structural proteins E, M and S to generate the virus particle.

The virus' major adhesion molecule is the spike protein S. The characteristics of the S protein are described in detail in Section 4. Much effort has been undertaken to address the role of the spike protein in various steps of the virus' life cycle, and in the outcome of infection *in vivo* with respect to tropism and pathogenesis. Various models have successfully been developed to study different steps of the IBV infection. This review provides an overview of the literature and recent achievements regarding the spike protein of avian coronaviruses, to summarize our current understanding on the spike's contribution to host and tissue predilections, pathogenesis, and its role in therapeutic and protective interventions.

## 2. Infectious bronchitis

The disease described as 'infectious bronchitis' is a collection of symptoms caused by IBV subtypes, which can be discriminated based on genotype, serotype and protectotype (Sjaak de Wit et al., 2011). The classical subtype causing respiratory disease, IBV Massachusetts 41 (M41), was isolated by Van Roekel in the United States in 1941 (reviewed by Fabricant, 1998). Subtypes other than M41 also cause respiratory disease, but with varying severity. Respiratory disease is often clinically characterized by dyspnea, coughing, rales and serous nasal discharge (Cavanagh and Gelb, 2008). It is caused by infection of the ciliated epithelium of the upper respiratory tract (mainly nasal cavity and trachea), resulting in loss of ciliary activity, degeneration, desquamation and loss of these cells. In addition, infected tissues shows hyperemia and inflammation (Fig. 1A), which is mainly characterized by the presence of heterophilic granulocytes and lymphocytes (Fig. 1B). IBV can also spread to the lower respiratory tract and cause aerosacculitis (Bezuidenhout et al., 2011). Usually, the epithelium is restored to normal within 2–3 weeks *via* a state of extensive hyperplasia (Dwars et al., 2009; Nakamura et al., 1991; Purcell and McFerran, 1972).

From the respiratory tract, the virus spreads through the host *via* viremia (Jones and Jordan, 1972) to the epithelial cells of the renal tubuli (Chen and Itakura, 1996; Condron and Marshall, 1986; Purcell et al., 1976) and the ciliated epithelium of the oviduct (Crinion et al., 1971; Jones and Jordan, 1971). Here the virus causes respectively renal failure with urate obstruction due to tubular necrosis with mononuclear inflammation (Chen and Itakura, 1997; Jones, 1974), and oviductal necrosis and malformation leading to abnormal egg production and inability to lay (Chousalkar and Roberts, 2007). The severity of the disease in various organs depends on the IBV subtype and ultimately determines the mortality in chickens.

Minor pathological changes due to IBV infection can occasionally be seen in other organs. The virus has been shown to infect glandular epithelial cells of the proventriculus (Yu et al., 2001), as well as cells resembling histiocytes and lymphoid cells in and enterocytes covering the cecal tonsils (Owen et al., 1991). However, this does not result in significant clinical gastrointestinal disease. IBV can also infect the Harderian gland (Toro et al., 1996, 1997; van Ginkel et al., 2008), an organ involved in the immune response. Finally, it has been reported that testicles can be infected, from which IBV can be venereally transmitted by the semen (Gallardo et al., 2011).

Avian coronaviruses have been detected in various other poultry species. While some of these IBV- or IBV-like viruses display high sequence similarity to IBV or IBV vaccine strains (Liu et al., 2005; Sun et al., 2007), others strains are much more divergent and may represent different virus species (reviewed in Cavanagh, 2005). For example, turkey coronavirus TCoV is very divergent in its spike gene, and causes in contrast to the respiratory disease observed for IBV, gastrointestinal disease in turkeys (*Meleagris*

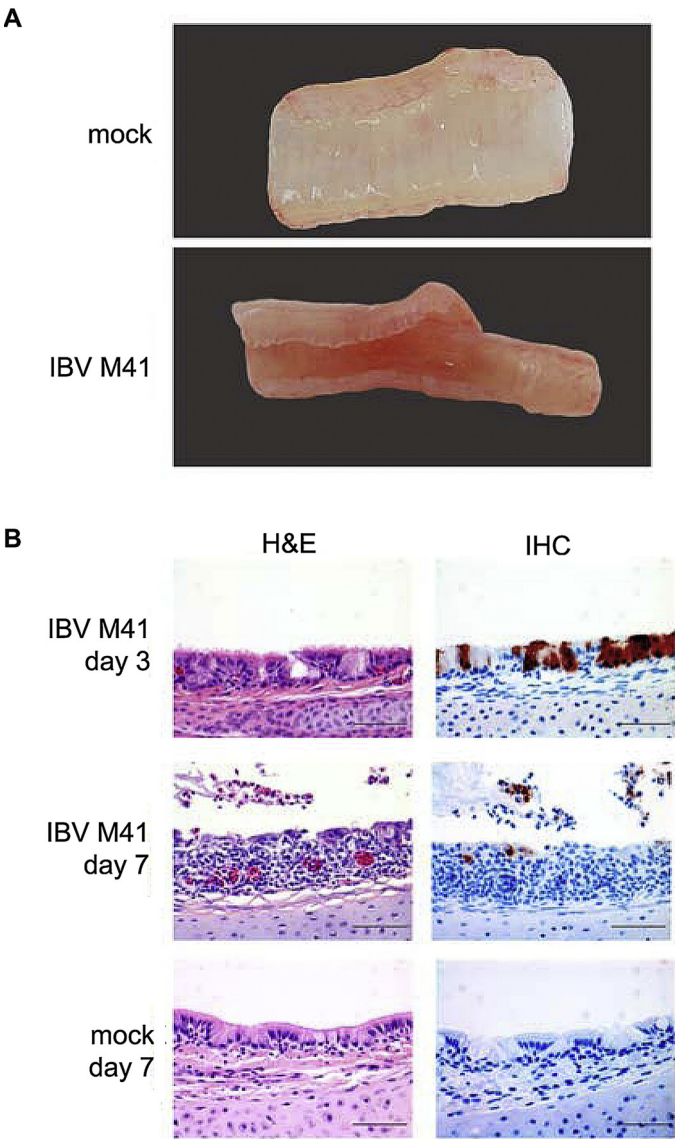



**Fig. 1.** Macroscopic, histological and immunohistochemical analyses of chicken trachea of mock-infected or IBV M41-infected layer chickens. Seven-day-old SPF layer chickens were oronasally infected with PBS (mock) or M41 and sacrificed at various time points after infection. (A) Longitudinally opened trachea of mock (upper) and IBV-M41 infected (lower) chicken at 7 dpi; the M41-infected trachea shows small amounts of mucoid material in the lumen and marked multifocal hyperemia of the mucosa. (B) Hematoxylin and eosin (H&E) (left) and anti-IBV S2 MAb 48.4 immunohistochemistry staining (right) of a section of the trachea of a mock-infected (7 dpi) or M41-infected (3 dpi and 7 dpi) chicken. The trachea of the M41-infected chicken at 3 dpi shows an intact epithelial lining with minimal hyperemia, while both ciliated epithelial cells and non-ciliated mucus-producing epithelial cells show marked intracytoplasmic presence of S2 antigen. At 7 dpi, the trachea has lost normal architecture due to desquamation of the ciliated and non-ciliated epithelium with replacement by a hyperplastic, more squamous non-ciliated epithelium, infiltration by large numbers of lymphocytes, marked hyperemia and in the superficial layer presence of necrotic cells. The lumen contains desquamated epithelial cells, marked numbers of heterophilic granulocytes and abundant mucoid material. Both the epithelial lining and lumen show cells containing the S2 antigen. There are no changes observed in the mock-infected chicken trachea. Scale bars represent 50 μm.

*gallopavo*). Swollen and edematous intestines with frothy content are the result of the observed severe enteritis (Cavanagh, 2001; Guy, 2000; Maurel et al., 2011). Quail coronavirus, which has a high spike sequence identity to TCoV, also causes enteritis with subsequent diarrhea and reduced growth in young quails (*Coturmix coturmix*) (Torres et al., 2013). In guinea fowl (*Numida meleagris*), guinea fowl coronavirus results in acute enteritis and pancreatitis (Liais et al., 2014). Other IBV(-like) coronaviruses cause respiratory and kidney diseases in pheasants (*Phasianus colchicus*) (Cavanagh, 2005; Gough et al., 1996), and respiratory disease and pancreatitis in pigeons (*Columba livia*; Qian et al., 2006). In wild birds, avian gammacoronaviruses are detected, but without clinical signs in the respective hosts (Cavanagh, 2005; Chu et al., 2011).

## 3. Model systems

Laboratory studies on IBV are hampered by the inability of IBV field strains to grow in continuous cell lines. Despite this, several *in vitro* and *ex vivo* models, in addition to *in vivo* models, are available. IBV can be cultured in intra-allantoically inoculated embryonated chicken eggs. The virus infects epithelial cells of the chorioallantoic membrane (CAM) (Fig. 2A) and the chicken embryo. Infectious virus particles accumulate in the allantois fluid, from which they can be harvested. Morphological changes occur in both the CAM (thickening) and the embryo (curling, stunting, growth retardation) (Loomis et al., 1950). In addition, *ex vivo* organ explants have been developed, of which tracheal organ cultures (TOCs) are most commonly used (Darbyshire et al., 1976). As read-out parameter for infection a scoring system for ciliostasis has been developed (Cook et al., 1999).

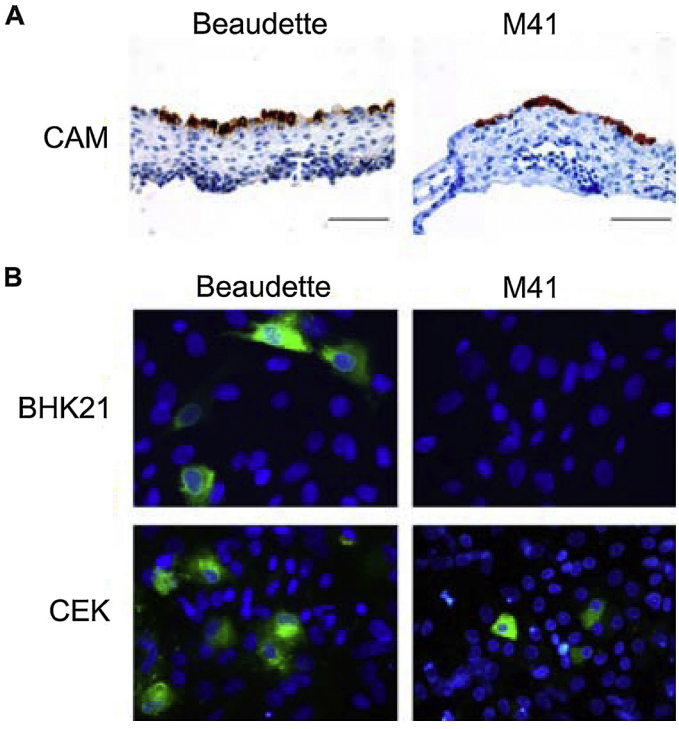



**Fig. 2.** Infection of embryonated eggs, cell culture BHK21 cells, and primary chicken embryonic kidney cells (CEK) with IBV Beaudette and -M41. (A) Ten-day-old embryonated chicken eggs were inoculated with Beaudette and M41 and chorioallantoic membranes (CAM) were stained with anti-IBV S2 MAb 48.4. (B) BHK21 and CEK cells were inoculated with IBV Beaudette and -M41, fixed at 8 hpi and immunofluorescence was performed using the anti-IBV N protein MAb26.1. IBV antigens were present in Beaudette-inoculated BHK21, CEK and CAM, while for M41 infection was only observed in CEK and CAM. Scale bars for CAMs represent 50 μm; pictures of BHK21 and CEK cells were taken at 40× magnification.

Oviduct organ explants (Mork et al., 2014) have been developed to study IBV strains with tropism for reproductive tract. Finally, most IBV field strains grow on isolated primary chicken embryo kidney (CEK) (Gillette, 1973) cells (Fig. 2B), although adaptation may be required.

To study IBV in a laboratory setting, the cell culture adapted IBV strain Beaudette is most commonly used (Beaudette and Hudson, 1937). In contrast to most IBV field strains, Beaudette can infect baby hamster kidney cells (BHK-21) and monkey kidney cells (Vero) (Fig. 2B) (Madu et al., 2007; Otsuki et al., 1979). Beaudette was obtained by serial passaging of the virulent Massachusetts M41 strain in embryonated chicken eggs and cultured cells (Beaudette and Hudson, 1937). As a consequence of the adaptation, Beaudette lost its ability to infect chickens and is not pathogenic for chickens (Geilhausen et al., 1973).

The cell culture adapted IBV strain Beaudette has also been the primary choice as backbone for the development of recombinant viruses (Casais et al., 2001; Fang et al., 2007; Youn et al., 2005). Only recently, a recombinant system based on the attenuated H120 vaccine strain was generated (Zhou et al., 2013). Recombinant viruses are excellent tools to dissect the contribution of each of the viral proteins to the various steps of the infection cycle. Such evaluation is much more cumbersome with wild type IBV strains, where frequently multiple mutations across the genome are present that may all contributing to disease in chickens.

To fundamentally study the role of the spike protein in virus binding, recombinant soluble spike proteins have become practical tools. Protein histochemistry assays in which binding patterns of different IBV spike proteins to various chicken tissues can be compared have been developed by us (Wickramasinghe et al., 2011) and others (Shahwan et al., 2013). These assays also allow the elucidation of specific host attachment factors involved in IBV infection, as detailed in the following sections.

## 4. The spike protein

### 4.1. Characteristics

The spike protein is the largest of the coronavirus structural proteins, and constitutes the characteristic club-like or petal-shaped 16–21 nm protrusions that emerge from the virion surface, giving it a corona-like appearance when visualized by electron microscopy (Masters and Perlman, 2013). The spike protein monomer is a transmembrane glycoprotein with a molecular mass of around 128 kDa before glycosylation (Masters and Perlman, 2013). A cleaved N-terminal signal peptide (Binns et al., 1985) directs the S protein toward the endoplasmatic reticulum (ER), where it obtains extensive terminal N-linked glycosylation (Cavanagh, 1983a,b). Around thirty potential N-linked glycosylation sites have been predicted for the IBV spike protein (Binns et al., 1985), increasing its mass to about 200 kDa. After glycosylation in the ER, the monomers oligomerize to form dimers or trimers (Cavanagh, 1983a,b; Delmas and Laude, 1990; Lewicki and Gallagher, 2002).

The S protein of avian gammacoronaviruses 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,b,c). All reported IBV S protein sequences contain this cleavage recognition site, and minor amino acid variations are believed not to correlate with serotype, pathogenicity and tropism (Jackwood et al., 2001). 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, and the short transmembrane and endodomain.

The coronavirus spike protein is a class I viral fusion peptide, in which the variable S1 domain is involved in host cell receptor binding and the conserved S2 domain mediates fusion of the virion and cellular membranes (Bosch et al., 2003; Masters and Perlman, 2013). All mapped receptor-binding domains (RBD), including that of IBV (Promkuntod et al., 2014), are located at various positions within the S1 domain (Masters and Perlman, 2013). 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 triggered by exposure to acidic pH in endosomes (Chu et al., 2006), resulting in fusion of the viral envelope with the cellular membrane. In contrast to some other coronaviruses, no endosomal proteases have been elucidate to contribute to the infection of IBV (see also Section 6.5). Although the S2 domain is not principally involved in binding to a host cell receptor, the interplay between S1 and S2 might synergistically determine the avidity and specificity of virus attachment (de Haan et al., 2006; Promkuntod et al., 2013).

### 4.2. Sequence diversity

In contrast to virus species belonging to the alpha- and betacoronaviruses, which occur as only one or two different serotypes, there are many different serotypes of the chicken *Gammacoronavirus* IBV (Jackwood, 2012; Sjaak de Wit et al., 2011). As the main antigenic viral protein containing epitopes for neutralization (Cavanagh et al., 1986a,b,c; Kant et al., 1992; Koch et al., 1990; Mockett et al., 1984; Niesters et al., 1987), the high sequence diversity of the S1 domain accounts for this serotypical variation. While amino acid sequence identity for the conserved S2 domain is usually ≥90% between different serotypes, variation in the S1 domain ranges from 2–3% up to 50%, with an average of 20–25% (Britton and Cavanagh, 2007). A phylogenetic tree of S1 proteins of various IBV reference strains is shown in Fig. 3, showing the relationship amongst the selected avian coronavirus species with regard to the spike.

There is a correlation between the amino acid sequence identity and the level of cross-protection: IBV strains within the same serotype usually share more than 95% amino acid identity in S1 (Cavanagh, 2001), whereas IBV strains of other serotypes share less than 85% amino acid identity (Cavanagh, 2005). However, some highly similar strains show only limited cross-protection, whilst a high level of cross-protection may exist for strains with a much lower homology (reviewed in Sjaak de Wit et al., 2011).

Using monoclonal antibodies, five conformation-dependent neutralizing antigenic sites were mapped on S1, as well as another immunodominant region in the N-terminal region of S2 (Koch et al., 1990; Kusters et al., 1989; Lenstra et al., 1989). The five neutralizing antigenic sites on S1 co-locate within three hypervariable regions (HVRs) (Cavanagh et al., 1988, 1992; Moore et al., 1997; Niesters et al., 1987), suggesting the HVRs to be involved in antigenicity and hence serotypical variation. The nucleotide sequence of (part of) the S1 subunit of the spike has traditionally been used for genotyping (OIE, 2013). For diagnostic purposes, genotyping of IBV isolates is usually limited to the hypervariable amino terminus region of S1. As a result of the great genetic variability of IBV variants, a definitive nomenclature and genetic classification system is, however, still lacking.

The high sequence diversity of the IBV S1 domain and the hypervariable amino terminus in particular (Cavanagh et al., 1998; Lee and Jackwood, 2001), is thought to be the result of accumulation of mutations and selection. The average rate of synonymous mutations in the genomes of coronaviruses is about $1.2 \times 10^{-3}$ substitutions/site/year (Hanada et al., 2004). The reason for the high sequence diversity of the IBV spike is unknown, but

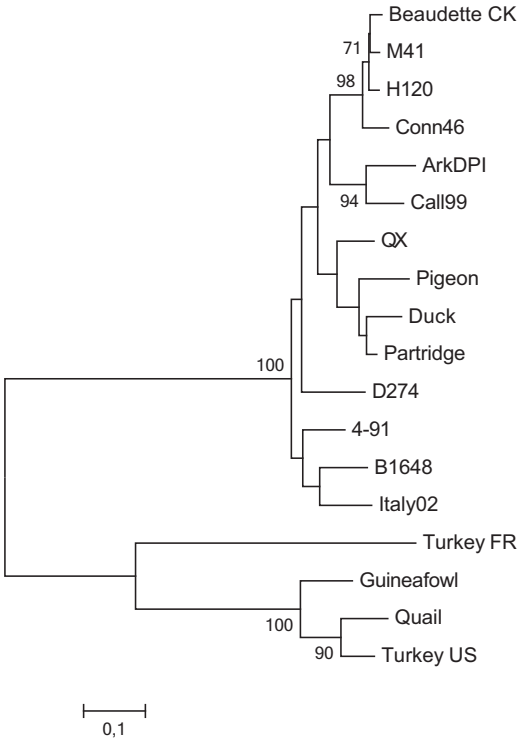


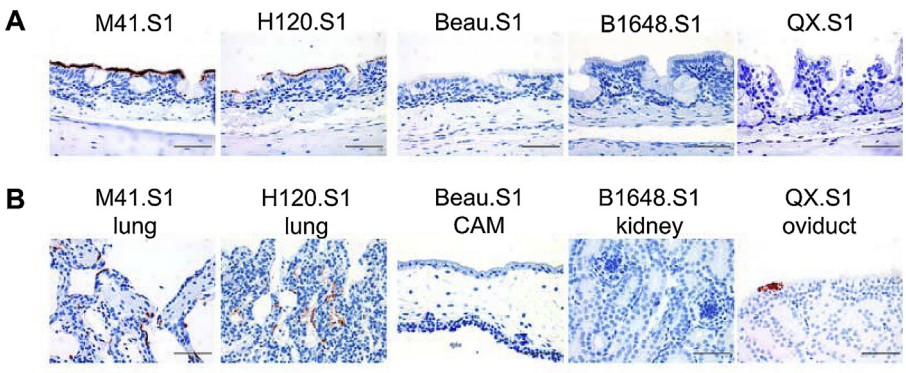

**Fig. 3.** Phylogenetic analysis of the amino acid sequences of the S1 subunit of the spike protein for selected avian gammacoronaviruses. Sequences were aligned using MUSCLE with default settings. The unrooted maximum likelihood tree was constructed using MEGA 6.06 with the best-fit substitution model (LG+G), with complete deletion of gaps. Bootstrap values (500 replicates) are indicated at the nodes when >70.

probably includes selective pressure, high virus titers and high viral subpopulation diversity (Jackwood et al., 2012). Intrahost selection of specific IBV subpopulations may be the result of the chicken immune response, affinity for host receptors and microenvironmental differences between organ systems (Toro et al., 2012a,b). The emergence of new IBV variants is subsequently facilitated by the high basic reproductive number, estimated to be 19.95 (de Wit et al., 1998). The wide-spread use of live-attenuated vaccine strains and the subsequent selective pressure induced by neutralizing antibodies against the spike may force the adaptation of the virus to escape immunity, and hence result in faster evolutionary rates (as discussed in Jackwood et al., 2012).

Next to mutations, genetic recombination may lead to the creation and emergence of new genetic variants that are very different from the parental strains (Masters and Perlman, 2013). The occurrence of recombination has been reported for many coronaviruses, with so-called recombination hot spots representing regions in the genome with higher recombination breakpoint incidence. Such hot spots have been mapped for IBV to nsp2, nsp3, nsp16 and immediately upstream of the spike gene (Thor et al., 2011). The last mentioned hot spot is of special interest, as the S protein contains viral neutralizing epitopes and determines tropism. Hence, recombination of the S gene may result in the emergence of new strains, new serotypes, or even new viruses infecting other hosts. It was hypothesized that a one-time double cross-over event between IBV and a yet unknown other (avian) coronavirus upon infection of a single host led to the emergence of TCoV (Jackwood et al., 2010).

### 4.3. Spikes of other avian coronaviruses

Although IBV has been described primarily as a chicken pathogen, IBV or IBV-like gamma coronaviruses have been found in




C

| | Trachea/Lung | | Kidney | | Oviduct | |
|---|---|---|---|---|---|---|
| | S1binding | Infection | S1binding | Infection | S1binding | Infection |
| M41 | + | + | + | + | + | + |
| H120 | +/- | + | +/- | na | nd | na |
| Beau | - | - | - | - | nd | - |
| B1648 | - | + | - | + | nd | + |
| QX | - | + | - | + | + | + |

**Fig. 4.** Binding of S1 proteins of different IBV strains to trachea and other clinically relevant tissues. Recombinant S1 proteins of IBV M41, H120, Beaudette, B1648 and QX were produced and attachment profiles were compared by performing spike histochemistry as previously described (Wickramasinghe et al., 2011) on trachea (A) and other chicken tissues (B). Binding of the S1 of the virulent Massachusetts M41 strain corresponded with the cells reported to be sensitive for infection with M41. Specifically, M41 S1 had a greater binding avidity than that of the attenuated vaccine strain H120 on trachea and lung (Wickramasinghe et al., 2011). For Beaudette S1 no obvious binding could be observed on chicken trachea (Wickramasinghe et al., 2011), but also not on CAM (Promkuntod et al., 2013). The S1 of the nephropathogenic B1648 did not show any appreciable staining of the trachea and the kidney, while for the S1 of QX, a strain with reported reproductive tract tropism, only mild patchy staining to the oviduct was observed. Scale bars represent 50 μm; (C) schematic representation of the ability of recombinant trimeric spike proteins to bind to a selected set of tissues and the reported ability of the corresponding IBV strains to infect these tissues; na: data not available from literature; nd: not done.

peafowl, partridge, blue-winged teal, pigeon, mallard duck, graylag goose (Cavanagh, 2005), pheasant (Cavanagh et al., 2002), guineafowl (Cavanagh, 2005; Liais et al., 2014) and various wild bird species (Chu et al., 2011). Most of these viruses have not been isolated, but were detected and partly characterized by molecular methods only.

Poultry other than chicken from which avian gammacoronaviruses have been isolated include turkey, quail and guinea fowl (Cavanagh, 2005; Circella et al., 2007; Liais et al., 2014; Torres et al., 2013). TCoV is thought to originate from a recombination of IBV with a gene from unknown origin (Jackwood et al., 2010). IBV and TCoV full genomes are about 86% similar, while the similarity for the S gene is less than 36%. Extensive genetic variation in the S gene suggests that North American and European TCoVs subsequently evolved differently (Fig. 3; Maurel et al., 2011), resulting in the creation of different serotypes (Jackwood et al., 2010). The genome of guinea fowl coronavirus aligns to the TCoV genome, suggesting a common ancestor, but a current separate evolutionary path (Liais et al., 2014). The S1 of quail coronavirus has ~80% amino acid identity with certain TCoV strains, and only ~17% aa identity with IBV. However, as the rest of the genome of quail coronavirus are yet to be determined, it is not known whether quail coronavirus evolved with guineafowl and turkey coronavirus or separately. S1 sequences from coronaviruses detected in some other poultry species, including that of pigeon, partridge and duck, are much less divergent from the S1 of IBV reference strains (Fig. 3). It is, however, unknown whether IBV has a broader host-range than currently thought, or that these various bird species were infected by a number of yet uncharacterized IBV-like viruses.

## 5. The role of the spike protein

As argued above, the spike displays high sequence diversity amongst all circulating IBV strains. It is therefore believed to play a crucial role in the outcome of the infection. Below we will provide an overview of the literature on avian coronavirus spike binding to cells and tissues, and the role of the spike in defining tropism and pathogenicity.

### 5.1. In tissue binding

The effect of sequence diversity in S1 proteins between IBV strains on binding was clearly demonstrated by applying recombinant S1 proteins to chicken tissues using spike histochemistry (Wickramasinghe et al., 2011) (Fig. 4). The binding avidity of recombinant trimeric S1 proteins of IBV Massachusetts strains M41, H120 and Beaudette correlated with the reported pathogenicity of those strains *in vivo* (Wickramasinghe et al., 2011). Strikingly, trimeric soluble S1 proteins of other strains, including the nephropathogenic B1648 and the QX causing reproductive tract disorders, hardly showed any appreciable binding to trachea (Fig. 4A) and other clinically relevant organs, like kidney and oviduct (Fig. 4B). Only for IBV QX limited staining of the oviduct was observed. An overview of the observed binding of various trimeric spike proteins to tissues and the previously reported infectivity of that particular tissue by the corresponding IBV strain is depicted in Fig. 4C.

When expressed as dimers fused to the human Fc tail, M41 S1 showed much less avidity than trimeric M41 S1 for chicken trachea, while B1648 S1 dimers gained, to a limited extend, the ability to bind (Fig. 5). These results are in line with the previously observed binding patterns of dimeric S1 of B1648 and QX to oviduct explants (Mork et al., 2014) and other IBV permissive cells, including tracheal epithelial cells and primary chicken kidney cells (Shahwan et al., 2013). Similarly, dimeric Beaudette S1 bound to chicken cells (Hesse et al., 2012), while trimeric Beaudette S1 was not sufficient for binding to trachea (Fig. 4A; Wickramasinghe et al., 2011) and CAM (Fig. 4B; Promkuntod et al., 2013). Differences in amount of staining between laboratories might be explained by the use of 20-fold higher protein concentrations by Shahwan and coworkers (Shahwan et al., 2013) compared to that used by Wickramasinghe et al. Nevertheless, it is clear that the multimerization state of the spike affects the binding characteristics.

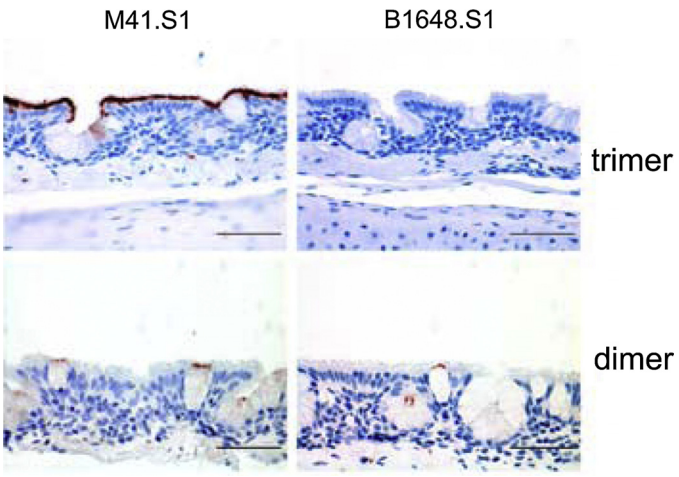



**Fig. 5.** Binding characteristics of recombinant trimeric and dimeric IBV S1 proteins. Recombinant S1 proteins produced in frame with the GCN4 trimerization motif and the Strep-tag ('trimers') or in fusion with the human Fc tail ('dimers') were applied to chicken trachea. Using similar protein amounts for dimeric and trimeric S1 (at a concentration of 0.1 mg/ml), trimeric M41.S1 bound with higher avidity to trachea compared to dimeric M41.S1. In contrast, dimeric B1648.S1 showed limited binding to trachea, while for trimeric B1648.S1 no binding at all could be detected. Scale bars represent 50 μm.

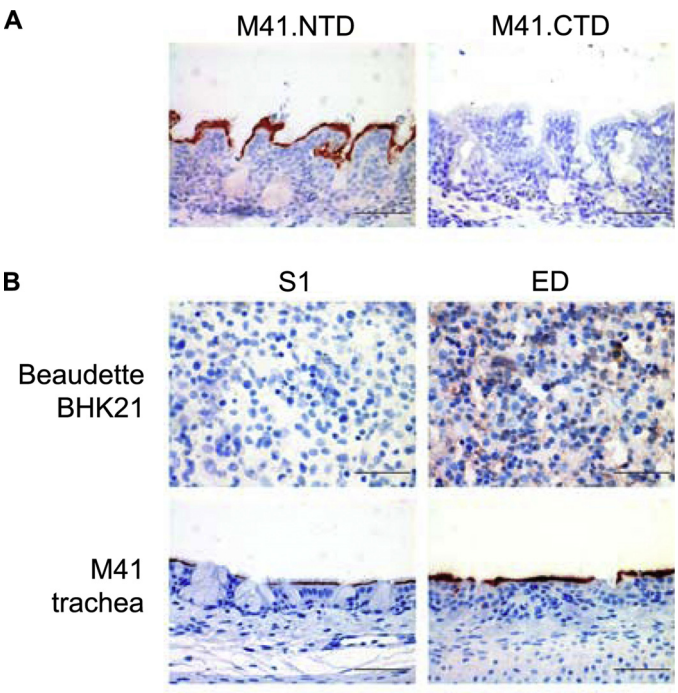



**Fig. 6.** IBV spike domains involved in binding. Spike histochemistry was performed using recombinant soluble spike domains. (A) The N-terminal S1 domain (M41.NTD, comprising aa 19–272 of the spike) and the C-terminal S1 domain (M41.CTD, comprising aa 273–532 of the spike) of M41 were applied to chicken trachea. The NTD was both sufficient and required for attachment to chicken tissues, and thus contained the receptor-binding domain (Promkuntod et al., 2014); (B) recombinant S1 and ectodomains of Beaudette and M41 were produced as soluble recombinant proteins and applied to BHK21 cells and chicken trachea, respectively. Binding of Beaudette spike to cell cultures was only observed for the ectodomain, while the extension of S1 with the S2 ectodomain for M41 increased the binding avidity to the trachea (similarly as observed before for the CAM; Promkuntod et al., 2013). Scale bars represent 50 μm.

With similar spike histochemistry assays, the contribution of the spike domains to binding was elucidated. The N-terminal 253 amino acids of the S1 of the IBV Massachusetts strain M41, but not the C-terminal domain (Fig. 6A) had bound to cells in an α-2,3-sialic acid dependent manner (Promkuntod et al., 2014). Using the differences in binding between M41 and Beaudette S1 proteins amino acids 38, 43, 63, and 69, partly overlapping with HVR1, were identified as critical residues for binding of M41 (Promkuntod et al., 2014). IBV M41 is the only *Gammacoronavirus* for which the RBD has been identified. Likely important binding properties also reside in this domain of other avian gammacoronaviruses. The contribution of domains outside the S1 became clear when studying Beaudette spike binding (Promkuntod et al., 2013). While Beaudette S1 was not sufficient for binding to chicken trachea (Fig. 6A), CAM (Fig. 4B) and BHK21 cells (Fig. 6B), extension of this protein with the S2 part of the spike ectodomain resulted in binding to BHK21 cells (Fig. 6B) and CAM (Promkuntod et al., 2013). Interestingly, extension of the M41 spike, now comprising the complete ectodomain, resulted in an increased affinity and extended binding profile to the chicken trachea (Fig. 6B), and thus S1 and S2 might work together to determine the avidity and specificity of virus attachment.

### 5.2. In cell culture tropism

The IBV spike glycoprotein is a determinant of cell tropism, and the extended host cell tropism of Beaudette (see Section 3 of this review) is merely determined by this protein. By replacing the ectodomain of the spike of Beaudette by that of M41 in the Beaudette viral background using recombinant IBVs (Casais et al., 2003), it was observed that the BeauR-M41(S) acquired the cell tropism of IBV M41. While M41, recombinant Beaudette and BeauR-M41(S) had the ability to produce progeny virus on primary chicken kidney cells, the Vero, BHK-21 and CEF cells only supported infection and replication of Beaudette (Casais et al., 2003). A recombinant Beaudette in which the S1 gene of Beaudette was replaced by that of H120 was recently generated (rBeau-H120(S1e)) and retained its ability to grow on Vero cells (Wei et al., 2014), indicating that the prerequisites for infection of cell culture cells resides in S2 sequence of Beaudette.

There is contradictory data published on the cause of the extended host range of Beaudette. Major putative factors that are studied include additional cleavage of the S protein by host proteases and extended binding to host attachment factors, which are both discussed in Section 6 of this review). In addition, it has been shown that particular mutations in the S protein that drive the cell–cell fusion activity determine the infectivity of Beaudette (Yamada et al., 2009). It might well be that not only attachment to additional host factors, but also fusion and perhaps other downstream events contribute to the cell culture tropism of Beaudette.

### 5.3. In in vivo tropism and pathogenesis

While the S1 domain is important in binding and the S2 domain contributes to determining the *in vitro* tropism of IBV, the role of the S protein *in vivo* is less clear. Exchange of the spike protein of Beaudette by that of virulent M41 and 4/91 IBV strains in the viral Beaudette genome resulted in apathogenic recombinant viruses BeauR-4/91(S) (Armesto et al., 2011) and BeauR-M41(S) (Hodgson et al., 2004); the latter only showing poor replication in trachea and nose compared to M41 (Hodgson et al., 2004). In addition, replacement of only the S1 domain of Beaudette by that of the vaccine strain H120 rendered nonpathogenic virus that could be isolated from tracheal swabs till day 6 (Wei et al., 2014), indicating that the recombinant virus had the ability to infect and replicate in the respiratory tract. To conclude to what extend the spike protein determines *in vivo* tropism clinical studies comparing the organ and cell preferences of the recombinant and parent viruses are needed. The previously mentioned studies nevertheless suggest that the

spike properties do not fully determine pathogenicity of IBV in chickens.

Further elucidation of the contribution of the spike to tissue tropism and pathogenesis *in vivo* came from studies reporting intra-host variation. Viral subpopulations with amino acid differences in S1 distinct from the predominant vaccine populations were selected in the trachea (Gallardo et al., 2010; McKinley et al., 2008; van Santen and Toro, 2008), tears and Harderian gland (van Santen and Toro, 2008), and reproductive tract (Gallardo et al., 2010). These subpopulations were suggested to have different phenotypes across organs (Gallardo et al., 2010; Ndegwa et al., 2014), and their selection might depend on the IBV specific immune responses (Ndegwa et al., 2014; Toro et al., 2012a,b).

The nonstructural replicase proteins encoded by ORF1ab clearly contribute to the fitness and pathogenicity of IBV *in vivo*. A recombinant IBV composed of the structural region of the virulent M41 and the replicase genes of the nonvirulent Beaudette (Armesto et al., 2009) did not show any clinical symptoms after inoculation in chickens, and tracheas of all birds had 100% ciliary activity, in contrast to M41. No virus or viral RNA could be detected in the trachea of birds infected with the recombinant BeauR-Rep-M41-Struct-2. It is of interest to elucidate whether this virus can replicate at the inoculation site, and to determine whether the lack of pathogenicity was due to reduced fitness. Further evidence on the role of the replicase genes in pathogenicity came from a study in which the genome sequences of virulent and avirulent ArkDPI IBV viruses were compared (Ammayappan et al., 2009). Next to the spike, most amino acid substitutions were located in ORF1a. The proteins encoded by this region in other coronaviruses are known to have an immune regulatory role and determine the viral replication rate. Thus, they might be involved in controlling the host response to infection, or the fitness of the virus. The replicase region is, however, not the sole determinant of IBV pathogenicity as replicase gene sequences of IBVs recovered from infected chickens were identical to those of vaccine viruses, with a lack of correlation with the pathotype (Mondal and Cardona, 2004).

## 6. Host determinants

The binding of the coronavirus spike protein to a host factor, and the subsequent fusion of the virus and host cellular membranes, are the first steps in virus' life cycle. The main host factor that is involved in IBV attachment is alpha2,3-linked sialic acid. In addition, specific lectins, heparan sulfate and cellular furin have been shown to play a role in Beaudette infection *in vitro*. Where knowledge on host factors involved in IBV infection is limited, such as with regard to the possible existence of a protein receptor, available data for other coronaviruses is shortly reviewed. The interaction between the spike protein and its specific attachment factors is considered to be mainly responsible for the restricted host species range and tissue tropism of coronaviruses. Hence, the distribution of the receptors is critical for the outcome of infection *in vivo*.

### 6.1. Sialic acids

Alpha2,3-linked sialic acids have been identified as a receptor determinant for IBV in both cells and TOCs (Abd El Rahman et al., 2009; Winter et al., 2006, 2008). Binding of alpha2,3-linked sialic acids to specific lectins corresponded with the susceptibility of these cells to IBV infection, and desialylation by neuraminidase hampered infection. Our lab (Wickramasinghe et al., 2011) and others (Mork et al., 2014; Shahwan et al., 2013) demonstrated that binding of recombinant S1 proteins to chicken tissues *ex vivo* (including trachea, lung, intestine and kidney) also depended on alpha2,3-linked sialic acids (Fig. 7A). In particular, glycan array studies revealed that the S1 protein of the IBV Massachusetts M41 strain specifically bound the di-sialodated glycan Neu5Acα2,3Galβ1,3(Neu5Acα2,3Gal1,4)-GlcNAc (Wickramasinghe et al., 2011). Abd El Rahman et al. (2009) suggested that the observed variation in tissue tropism between different IBV strains might be explained by a difference in preference for specific sialic acids. In this respect, no particular sialic acid could be identified in glycan arrays applying S1 proteins of other IBV strains, including H120, B1648 and QX (data not published). This suggests that either these proteins bind with much lower affinities to the same sialic acid, or that they recognize yet other unidentified host factor, including specific sialic acids which might be not present on this array. While the results of Mork et al. (2014) suggest that binding of recombinant dimeric S1 proteins of IBV strains with different pathogenicity to sialic acids is comparable, our studies using trimeric S1 proteins indicate that there might not only be differences in binding characteristics of different spikes (Fig. 4), but also differences in the attachment profiles of the spike protein to tissues from different animals. For example, binding of the same trimeric M41 S1 protein to oviduct of broiler and layer chickens (Fig. 7B) showed remarkable differences in binding patterns, suggesting differences in attachment factor expression.

Several other coronaviruses use host surface glycans containing sialic acids as attachment factor (Schwegmann-Wessels and Herrler, 2006). It has been shown that sialic acid binding can affect the tropism or the pathogenicity of the virus. For the murine betacoronavirus MHV extended attachment to O-acetylated sialic acids has been shown to affect cell tropism, resulting in increased neurovirulence (Kazi et al., 2005). The alphacoronaviruses porcine respiratory coronavirus and related transmissible gastroenteritis virus both principally use a cellular protein receptor, but the extended ability of the latter to bind to sialic acid is linked to its enterotropism (Krempl et al., 1997; Schultze et al., 1996). Interestingly, IBV does not express a receptor-destroying enzyme (Winter et al., 2006), which is in contrast to avian influenza virus and certain betacoronaviruses (Schwegmann-Wessels and Herrler, 2006). To compensate for the observed lower binding affinity of the IBV spike compared to the HA attachment protein of avian influenza (Wickramasinghe et al., 2011) other host attachment factors might be involved.

### 6.2. Protein receptors

So far, for none of the gammacoronaviruses a functional protein receptor has been identified. For many alpha and betacoronaviruses, specific protein receptors have been identified that restrict the host and tissue infection. Several of the alphacoronaviruses (including porcine, feline, canine and certain human coronaviruses) utilize aminopeptidase N (APN) as receptor protein, while the protein receptor usage of betacoronaviruses is more variable (Masters and Perlman, 2013). Murine coronavirus uses CEACAM1 as receptor (Masters and Perlman, 2013), the human *Alphacoronavirus* NL-63 and the betacoronavirus SARS-CoV both use ACE2 (Masters and Perlman, 2013), while the betacoronavirus MERS-CoV uses DPP4 (Raj et al., 2013). Miguel et al. (2002) suggested a role for APN during IBV entry, but Chu et al. (2007) concluded that feline APN was not a functional receptor for IBV. Whether there actually is a cell surface protein receptor to determine the tropism of IBV remains to be elucidated.

### 6.3. Lectins

Overexpression of the human C-type lectins DC-SIGN or L-SIGN in normally nonpermissive cell lines enhanced infection of IBV in a sialic acid independent manner (Zhang et al., 2012). Similar second or co-receptors, besides the mentioned protein receptors, have been identified for the human coronaviruses SARS-CoV (Jeffers

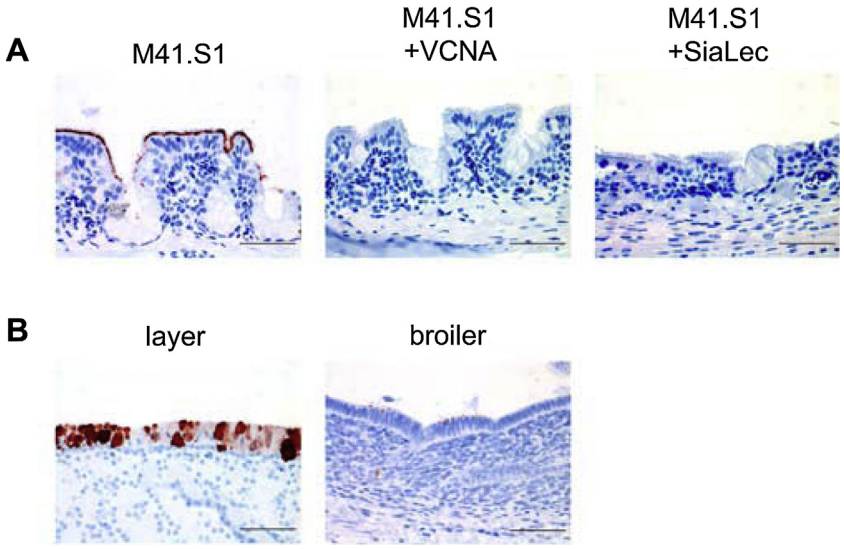



**Fig. 7.** Binding of M41.S1 is sialic acid dependent and differs between the oviduct of broiler and layer. Spike histochemistry using recombinant M41.S1 proteins was performed as described before (Wickramasinghe et al., 2011) on sections of (A) trachea of broiler and (B) oviducts of layer and broiler. Binding of M41.S1 could be inhibited by pretreating the trachea with neuraminidase from *Vibrio cholerae* (VCNA) and by pre-incubating the spike with the Neu5Acα2,3 specific glycan SiaLec (data not published). While M41.S1 has high binding avidity for oviduct of the layer, binding to broiler oviduct almost lacking. Scale bars represent 50 μm.

et al., 2004; Marzi et al., 2004; Yang et al., 2004), 229E (Jeffers et al., 2006) and NL63 (Hofmann et al., 2006), and for the feline coronaviruses FIPV (Regan and Whittaker, 2008) and FeCoV (Regan et al., 2010). However, as DC-SIGN and L-SIGN are mainly expressed on nonepithelial cells, like dendritic cells and endothelial cells of liver and lymph nodes, respectively, the role for chicken homologues of human DC-SIGN/L-SIGN in IBV pathogenesis *in vivo* is hence speculative.

### 6.4. Heparin sulfate

Next to the potential contribution of the S2 domain (Section 5.1), the extended host tropism of the cell culture adapted IBV strain Beaudette might as well be explained by binding to an additional cell surface receptor, namely heparan sulfate (Madu et al., 2007). This is a well-known receptor for many different viruses (Liu and Thorp, 2002), including a murine coronavirus MHV mutant with extended host range (de Haan et al., 2005). Upon passage in cell culture, both IBV Beaudette (Madu et al., 2007) and MHV (de Haan et al., 2005) adapted by the acquisition of heparan sulfate-binding sites, which enabled the use of heparin sulfate as alternative attachment/entry receptor. The putative heparin sulfate-binding site of Beaudette overlaps with a second furin cleavage site in the S2 domain (Madu et al., 2007). Madu et al. showed that the infectivity of Beaudette could be inhibited in the presence of soluble heparin or in cell lines lacking heparan sulfate, and concluded that heparin sulfate had a role as attachment and entry factor for Beaudette. Replacing the proposed heparan sulfate binding sequence (RRKR/S) in S2 by the corresponding sequence of M41 (PRRR/S), however, suggested that the heparan sulfate binding site is not crucial for entry, as infectious viruses could be recovered from Vero cells (Yamada and Liu, 2009). Using our spike histochemistry assay we demonstrated that site was also not required for the attachment of Beaudette spike ectodomain to susceptible chicken tissues (Promkuntod et al., 2013) (Fig. 8).

### 6.5. Host proteases

Furin is a well-known host protease for coronaviruses (Masters and Perlman, 2013). Furin-dependent cleavage of the IBV spike between S1 and S2 is believed to occur during virus assembly (Cavanagh, 1983a,b), and was suggested not to correlate with pathogenicity of the IBV strain (Jackwood et al., 2001). An additional furin-cleavage site in the S2 subunit of the Beaudette was suggested to be involved in the furin-dependent entry and syncytium formation *in vitro* (Yamada and Liu, 2009). This corresponded with the observation that a productiveness of IBV Beaudette infection correlated with cellular furin expression (Tay et al., 2012). Yamada and Liu (2009) hypothesized a novel XXXR/S cleavage site in the IBV spike, corresponding to the position of the additional S2 cleavage site in Beaudette, for cleavage by other yet unidentified host protease. Such a host protease might contribute to the tropism of IBV strain, as it has recently been observed that Beaudette entry, while being furin-dependent on Vero cells, depended on a yet unidentified protease in chicken kidney cells (C. Winter, personal communication).

## 7. Vaccine development and protection

The spike protein induces both humoral and cellular immune responses in chickens infected with IBV (Cavanagh, 2007). The presence of antibodies does not necessarily correlate with protection against IBV (Cavanagh et al., 1986a,b,c; Collisson et al., 2000; Ignjatovic and Galli, 1994). Cytotoxic T lymphocytes (CTL) are also critical in preventing disease (Collisson et al., 2000), and CTL epitopes are present on both the spike protein as well as in the nucleocapsid (Collisson et al., 2000; Liu et al., 2012).

Several different strategies are currently employed to generate vaccines against IBV. The strategies employed to evoke protective immune responses in chickens can be subdivided in the use of live attenuated vaccines, viral vectors and DNA plasmids, and recombinant IBVs. Many of the commercially available vaccines to protect poultry against infectious bronchitis in the field are of the live-attenuated type (Sjaak de Wit et al., 2011), while most of the other approaches are still under experimental investigation. The overview below is given in the context of the spike protein and its role in protection.

### 7.1. Live-attenuated vaccines

Live-attenuated IBV vaccines, obtained by serial passaging of an IBV isolate in embryonated eggs, were among the first IBV vaccines

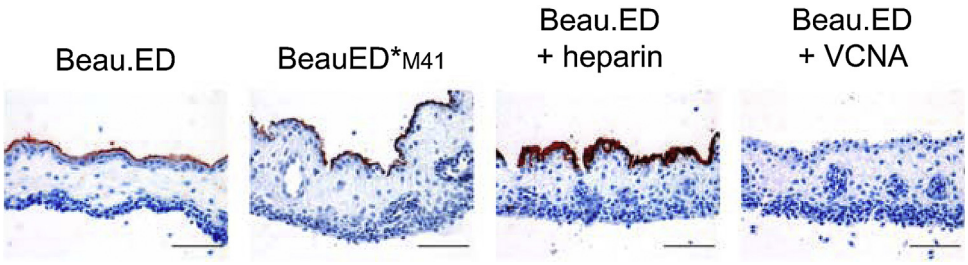



**Fig. 8.** Beaudette spike ectodomain binding to chorioallantoic membrane tissue. Spike histochemistry was performed using recombinant wild type Beaudette spike ectodomains (Beau.ED), or the ectodomain in which the proposed heparan sulfate binding site SHRKHS (aa 686–691) was replaced by the sequence of M41 SPRRRS (Beaud.ED*M41); Beau.ED in the presence of 10 mg/ml heparin, or application of Beau.ED after treatment of tissues with 1 mU neuraminidase of *Vibrio cholerae* (VCNA, as described in Promkuntod et al., 2013). The proposed heparan binding sequence was not required for binding of Beaudette spike, nor could binding be blocked with heparin; binding to CAM was dependent on sialic acids. Scale bars represent 50 μm.

developed and still constitute most of the currently marketed IBV vaccines. Attenuation is based on the adaptation of a virulent field isolate to chicken embryos, with the aim of generating viruses with reduced virulence in chickens, while retaining the ability to induce sufficient immune responses to protect against a challenge virus. In attenuated virus strains, most mutations are observed in the spike and in the region encoding for the replicase proteins (Ammayappan et al., 2009; Mondal and Cardona, 2004). It is, however, yet impossible to predict based on a genetic sequence whether the IBV strain is sufficiently attenuated, while potent enough to induce and effective immune response. In addition, the use of attenuated live-vaccines poses a risk of residual pathogenicity associated with vaccine back-passage in flocks (Abro et al., 2012; McKinley et al., 2011; OIE, 2013). Despite this, live attenuated IBV vaccines are still the golden standard to protect chickens from IBV, as immune responses are high.

### 7.2. Viral vectors and DNA plasmids

Delivery of spike proteins through the use of DNA plasmids or viral vectors to chickens has resulted in variable levels of protective immunity (Cavanagh, 2007). DNA vaccine studies show that the immunization of chickens with genes encoding multiple structural proteins of IBV can increase the level of humoral and cellular responses compared to the use of individual genes (Guo et al., 2010; Yang et al., 2009). In contrast to vector vaccines commonly used in the poultry field to transport and present parts of other avian pathogens (including Newcastle disease virus, fowl pox virus, and herpes virus of turkeys/Marek's disease virus), no such vaccine is marketed for IBV. Delivery of the spike using attenuated strains of other pathogens, including *Mycoplasma gallisepticum* vaccine strain (Shil et al., 2011) and recombinant baculoviruses (Zhang et al., 2014) showed only partial protection against challenge with homologous IBV. Vector vaccines might be improved by addition of immune-related molecules, as these can reduce clinical symptoms and viral RNA levels (Chen et al., 2010; Shi et al., 2011; Wang et al., 2009; Zeshan et al., 2011). Importantly, when cloning into a DNA plasmid or viral vector, the particular sequence of the S1 domain has to be taken into account, as already one amino acid mutation can reduce the ability to confer protection against challenge considerably (Toro et al., 2014a,b). While most studies only express the S1 domain, recently Toro and coworkers showed that the S2 protein can also contribute to the protection against heterologous IBV challenge (Toro et al., 2014a,b). Despite promising developments, viral vector vaccines and DNA vaccines for IBV still perform less well than existing live-attenuated vaccines.

### 7.3. Recombinant viruses

Studies in which recombinant viruses were tested for their ability to protect against challenge *in vivo* have shed more light on the role of the spike and other genes in attenuation and induction of protective immune responses. Vaccination with recombinant IBV Beaudette with spikes of virulent M41 (BeauR-M41(S); Hodgson et al., 2004) and 4/91 (BeauR-4/91(S); Armesto et al., 2011) protected chickens against a challenge with IBV M41 and -4/91, confirming the importance of homologous spike proteins in the induction of protective immune responses. Moreover, the presence of the S1 domain of IBV vaccine strain H120 in a Beaudette background bone was already sufficient to significantly decrease morbidity after challenge with the serologically related virulent M41 (Wei et al., 2014). The recombinant virus induced IBV-specific antibodies to a similar extend as the live attenuated H120 vaccine, and could result in fewer histopathological changes. Recombinant IBVs as vaccines are promising, as in contrast to vector vaccines and DNA plasmids they gain comparable levels of protection as obtained by live-attenuated vaccines. Future research of these recombinant vaccines will likely focus on the quantification of protective immune responses and the ability to induce protection against multiple serotypes.

## 8. Summarizing conclusions

In this review we summarized the current knowledge on avian coronavirus spike proteins. We addressed both the fundamental spike characteristics, and provided an overview of experimental data on the role of the spike in binding and entry in cell culture, tissue tropism *ex vivo*, pathogenesis and protection *in vivo*.

The fundamental characteristics of the spike proteins of avian coronaviruses resemble that of other members of the subfamily Coronavirinae in many ways. However, the serotype diversity caused by the sequence diversity between S1 proteins seems to be unique for IBV. Coronaviruses with divergent spike sequences have also been identified in other poultry species, including turkey. While IBV is primarily a respiratory pathogen in chicken, coronaviruses of turkey cause predominantly gastrointestinal disease. The pathogenesis and tropism of gammacoronaviruses in many other bird species is less clear, and it remains to be determined whether these are truly different viruses or whether IBV has a broader species tropism than previously thought.

The N-terminal domain of the spike protein contains the receptor binding domain. This domain of S1 of the prototype Massachusetts M41 strain is both sufficient and required for attachment to chicken tissues. The binding preference and avidity of trimeric S1 for chicken tissues correlates with the reported tropism and pathogenicity *in vivo*. Binding characteristics of recombinant dimeric S1 proteins differ in this respect, but also require alpha2,3-linked sialic acids on the cell surface to attach. It remains to be established whether host- or organ-specific expression of sialic acids or yet unidentified host factors, including a putative protein receptor

or host protease, contribute to limiting the tropism of various IBV strains.

The spike protein is the main determinant for IBV tropism in cell culture. The extended tropism of IBV Beaudette has been linked to several features of the S2 domain, including a heparan sulfate binding site, a second furin cleavage site, fusion-promoting mutations, as well as its synergistic interaction with S1. Only limited information is available on the actual role of the spike in determining the virus' preference for organs and cells *in vivo*, but despite this, it is clear that the spike is not the sole contributor to the IBV pathogenicity, as viral replicase genes also contribute to the outcome of the disease in chickens.

Finally, both the S1 and S2 domains, but also the nucleocapsid protein are important for the induction of protective immune responses. Selective pressure, likely enhanced by extensive vaccination, as well as intrahost selection results in adaptive evolution of avian coronaviruses by mutation and recombination. Due to the occurrence of many different IBV strains worldwide, and the limited cross protection across serotypes, vaccination to control infectious bronchitis in the poultry industry still remains a big challenge.

## Acknowledgements

We would like to acknowledge N. Promkuntod for his contribution to the manuscripts on which part of this review was based, and G. de Vrieze, A. Berends and M. Woelders for technical assistance. MHV is financially supported by a MEERVOUD grant from the Netherlands Organization for Scientific Research (836.12.012). We would like to acknowledge the COST Action FA1207 on Avian Coronaviruses, in which EU experts are joined to increase our understanding on, and to come to a novel classification and nomenclature system of, avian coronaviruses.

## References

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

Abro, S.H., Renström, L.H., Ullman, K., Isaksson, M., Zohari, S., Jansson, D.S., Belák, S., Baule, C., 2012. Emergence of novel strains of avian infectious bronchitis virus in Sweden. Vet. Microbiol. 155, 237–246.

Ammayappan, A., Upadhyay, C., Gelb Jr., J., Vakharia, V.N., 2009. Identification of sequence changes responsible for the attenuation of avian infectious bronchitis virus strain Arkansas DPI. Arch. Virol. 154, 495–499.

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

Armesto, M., Evans, S., Cavanagh, D., Abu-Median, A.B., Keep, S., Britton, P., 2011. A recombinant avian infectious bronchitis virus expressing a heterologous spike gene belonging to the 4/91 serotype. PLoS ONE 6, e24352.

Beaudette, F.R., Hudson, C.B., 1937. Cultivation of the virus of infectious bronchitis. J. Am. Vet. Med. Assoc. 90, 51–58.

Bezuidenhout, A., Mondal, S.P., Buckles, E.L., 2011. Histopathological and immunohistochemical study of air sac lesions induced by two strains of infectious bronchitis virus. J. Comp. Pathol. 145, 319–326.

Binns, M.M., Boursnell, M.E., Cavanagh, D., Pappin, D.J., Brown, T.D., 1985. Cloning and sequencing of the gene encoding the spike protein of the coronavirus IBV. J. Gen. Virol. 66 (Pt 4), 719–726.

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

Britton, P., Cavanagh, D., 2007. Avian coronavirus diseases and infectious bronchitis vaccine development. In: Thiel, V. (Ed.), Coronaviruses: Molecular and Cellular Biology. Caister Academic Press, Norfolk, UK, pp. 161–181.

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.

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., 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.

Cavanagh, D., 1983a. Coronavirus IBV glycopolypeptides: size of their polypeptide moieties and nature of their oligosaccharides. J. Gen. Virol. 64, 1187–1191.

Cavanagh, D., 1983b. Coronavirus IBV: structural characterization of the spike protein. J. Gen. Virol. 64 (Pt 12), 2577–2583.

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, 281–297.

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., Davis, P.J., Darbyshire, J.H., Peters, R.W., 1986a. 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 (Pt 7), 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., Pappin, D.J., 1986b. Coronavirus IBV glycopolypeptides: locational studies using proteases and saponin, a membrane permeabilizer. Virus Res. 4, 145–156.

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

Cavanagh, D., Gelb Jr., J., 2008. Infectious bronchitis. In: Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne, D.E. (Eds.), Diseases of Poultry. Wiley-Blackwell, Ames, IA, USA, pp. 117–135.

Cavanagh, D., Mawditt, K., Adzhar, A., Gough, R.E., Picault, J.P., Naylor, C.J., Haydon, D., Shaw, K., Britton, P., 1998. Does IBV change slowly despite the capacity of the spike protein to vary greatly? Adv. Exp. Med. Biol. 440, 729–734.

Cavanagh, D., Mawditt, K., Welchman Dde, 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.

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., Itakura, C., 1997. Histopathology and immunohistochemistry of renal lesions due to avian infectious bronchitis virus in chicks uninoculated and previously inoculated with highly virulent infectious bursal disease virus. Avian Pathol. 26, 607–624.

Chen, H.Y., Yang, M.F., Cui, B.A., Cui, P., Sheng, M., Chen, G., Wang, S.J., Geng, J.W., 2010. Construction and immunogenicity of a recombinant fowlpox vaccine coexpressing S1 glycoprotein of infectious bronchitis virus and chicken IL-18. Vaccine 28, 8112–8119.

Chousalkar, K.K., Roberts, J.R., 2007. Ultrastructural observations on effects of infectious bronchitis virus in eggshell-forming regions of the oviduct of the commercial laying hen. Poult. Sci. 86, 1915–1919.

Chu, D.K., Leung, C.Y., Gilbert, M., Joyner, P.H., Ng, E.M., Tse, T.M., Guan, Y., Peiris, J.S., Poon, L.L., 2011. Avian coronavirus in wild aquatic birds. J. Virol. 85, 12815–12820.

Chu, V.C., McElroy, L.J., Aronson, J.M., Oura, T.J., Harbison, C.E., Bauman, B.E., Whittaker, G.R., 2007. Feline aminopeptidase N is not a functional receptor for avian infectious bronchitis virus. Virol. J. 4, 20.

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.

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.

Collisson, E.W., Pei, J., Dzielawa, J., Seo, S.H., 2000. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev. Comp. Immunol. 24, 187–200.

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., Jackwood, M., Jones, R.C., 2012. The long view: 40 years of infectious bronchitis research. Avian Pathol. 41, 239–250.

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.

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

Darbyshire, J.H., Cook, J.K., Peters, R.W., 1976. Organ culture studies on the efficiency of infection of chicken tissues with avian infectious bronchitis virus. Br. J. Exp. Pathol. 57, 443–454.

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.A., Li, Z., te Lintelo, E., Bosch, B.J., Haijema, B.J., Rottier, P.J., 2005. Murine coronavirus with an extended host range uses heparan sulfate as an entry receptor. J. Virol. 79, 14451–14456.

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., 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.
Delmas, B., Laude, H., 1990. Assembly of coronavirus spike protein into trimers and its role in epitope expression. J. Virol. 64, 5367–5375.
Dwars, R.M., Matthijs, M.G., Daemen, A.J., van Eck, J.H., Vervelde, L., Landman, W.J., 2009. Progression of lesions in the respiratory tract of broilers after single infection with *Escherichia coli* compared to superinfection with *E. coli* after infection with infectious bronchitis virus. Vet. Immunol. Immunopathol. 127, 65–76.
Fabricant, J., 1998. The early history of infectious bronchitis. Avian Dis. 42, 648–650.
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. Virology 358, 136–147.
Gallardo, R.A., Hoerr, F.J., Berry, W.D., van Santen, V.L., Toro, H., 2011. Infectious bronchitis virus in testicles and venereal transmission. Avian Dis. 55, 255–258.
Gallardo, R.A., van Santen, V.L., Toro, H., 2010. Host intraspatial selection of infectious bronchitis virus populations. Avian Dis. 54, 807–813.
Geilhausen, H.E., Ligon, F.B., Lukert, P.D., 1973. The pathogenesis of virulent and avirulent avian infectious bronchitis virus. Arch. Gesamte Virusforsch. 40, 285–290.
Gillette, K.G., 1973. Plaque formation by infectious bronchitis virus in chicken embryo kidney cell cultures. Avian Dis. 17, 369–378.
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.
Guo, Z., Wang, H., Yang, T., Wang, X., Lu, D., Li, Y., Zhang, Y., 2010. Priming with a DNA vaccine and boosting with an inactivated vaccine enhance the immune response against infectious bronchitis virus. J. Virol. Methods 167, 84–89.
Guy, J.S., 2000. Turkey coronavirus is more closely related to avian infectious bronchitis virus than to mammalian coronaviruses: a review. Avian Pathol. 29, 207–212.
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.
Hesse, M., Winter, C., Herrler, G., 2012. Analysis of the Sialic Acid Binding Properties of the IBV Spike Protein., pp. 133–135.
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.
Hodgson, T., Casais, R., Dove, B., Britton, P., Cavanagh, D., 2004. Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces protective immunity. J. Virol. 78, 13804–13811.
Hofmann, H., Simmons, G., Rennekamp, A.J., Chaipan, C., Gramberg, T., Heck, E., Geier, M., Wegele, A., Marzi, A., Bates, P., Pohlmann, S., 2006. Highly conserved regions within the spike proteins of human coronaviruses 229E and NL63 determine recognition of their respective cellular receptors. J. Virol. 80, 8639–8652.
Ignjatovic, J., Galli, L., 1994. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Arch. Virol. 138, 117–134.
Ignjatovic, J., Sapats, S., 2000. Avian infectious bronchitis virus. Rev. Sci. Tech. 19, 493–508.
Jackwood, M.W., 2012. Review of infectious bronchitis virus around the world. Avian Dis. 56, 634–641.
Jackwood, M.W., Boynton, T.O., Hilt, D.A., McKinley, E.T., Kissinger, J.C., Paterson, A.H., Robertson, J., Lemke, C., McCall, A.W., Williams, S.M., Jackwood, J.W., Byrd, L.A., 2010. Emergence of a group 3 coronavirus through recombination. Virology 398, 98–108.
Jackwood, M.W., Hall, D., Handel, A., 2012. Molecular evolution and emergence of avian gammacoronaviruses. Infect. Genet. Evol. 12, 1305–1311.
Jackwood, M.W., Hilt, D.A., Callison, S.A., Lee, C.W., Plaza, H., Wade, E., 2001. Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. Avian Dis. 45, 366–372.
Jeffers, S.A., Hemmila, E.M., Holmes, K.V., 2006. Human coronavirus 229E can use CD209L (L-SIGN) to enter cells. Adv. Exp. Med. Biol. 581, 265–269.
Jeffers, S.A., Tusell, S.M., Gillim-Ross, L., Hemmila, E.M., Achenbach, J.E., Babcock, G.J., Thomas Jr., W.D., Thackray, L.B., Young, M.D., Mason, R.J., Ambrosino, D.M., Wentworth, D.E., Demartini, J.C., Holmes, K.V., 2004. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. U. S. A. 101, 15748–15753.
Jones, R.C., 1974. Nephrosis in laying chickens caused by Massachusetts-type infectious bronchitis virus. Vet. Rec. 95, 319.
Jones, R.C., Jordan, F.T., 1971. The site of replication of infectious bronchitis virus in the oviduct of experimentally infected hens. Vet. Rec. 89, 317–318.
Jones, R.C., Jordan, F.T., 1972. Persistence of virus in the tissues and development of the oviduct in the fowl following infection at day old with infectious bronchitis virus. Res. Vet. Sci. 13, 52–60.
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 (Pt 3), 591–596.
Kazi, L., Lissenberg, A., Watson, R., de Groot, R.J., Weiss, S.R., 2005. Expression of hemagglutinin esterase protein from recombinant mouse hepatitis virus enhances neurovirulence. J. Virol. 79, 15064–15073.
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 (Pt 9), 1929–1935.
Krempl, C., Schultze, B., Laude, H., Herrler, G., 1997. Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus. J. Virol. 71, 3285–3287.
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.
Lee, C.W., Jackwood, M.W., 2001. Origin and evolution of Georgia 98 (GA98), a new serotype of avian infectious bronchitis virus. Virus Res. 80, 33–39.
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.
Lewicki, D.N., Gallagher, T.M., 2002. Quaternary structure of coronavirus spikes in complex with carcinoembryonic antigen-related cell adhesion molecule cellular receptors. J. Biol. Chem. 277, 19727–19734.
Liais, E., Croville, G., Mariette, J., Delverdier, M., Lucas, M.N., Klopp, C., Lluch, J., Donnadieu, C., Guy, J.S., Corrand, L., Ducatez, M.F., Guerin, J.L., 2014. Novel avian coronavirus and fulminating disease in guinea fowl, France. Emerg. Infect. Dis. 20, 105–108.
Liu, G., Wang, Q., Liu, N., Xiao, Y., Tong, T., Liu, S., Wu, D., 2012. Infectious bronchitis virus nucleoprotein specific CTL response is generated prior to serum IgG. Vet. Immunol. Immunopathol. 148, 353–358.
Liu, J., Thorp, S.C., 2002. Cell surface heparan sulfate and its roles in assisting viral infections. Med. Res. Rev. 22, 1–25.
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.
Loomis, L.N., Cunningham, C.H., Gray, M.L., Thorp Jr., F., 1950. Pathology of the chicken embryo infected with infectious bronchitis virus. Am. J. Vet. Res. 11, 245–251.
Madu, I.G., Chu, V.C., Lee, H., Regan, A.D., Bauman, B.E., Whittaker, G.R., 2007. Heparan sulfate is a selective attachment factor for the avian coronavirus infectious bronchitis virus Beaudette. Avian Dis. 51, 45–51.
Marzi, A., Gramberg, T., Simmons, G., Moller, P., Rennekamp, A.J., Krumbiegel, M., Geier, M., Eisemann, J., Turza, N., Saunier, B., Steinkasserer, A., Becker, S., Bates, P., Hofmann, H., Pohlmann, S., 2004. DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus. J. Virol. 78, 12090–12095.
Masters, P., Perlman, S., 2013. Coronaviridae. In: Howley, P., Knipe, D.M. (Eds.), Fields Virology. Kluwer, Wolters (Chapter 28).
Matthijs, M.G., van Eck, J.H., Landman, W.J., Stegeman, J.A., 2003. Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. Avian Pathol. 32, 473–481.
Maurel, S., Toquin, D., Briand, F.X., Queguiner, M., Allee, C., Bertin, J., Ravillion, L., Retaux, C., Turblin, V., Morvan, H., Eterradossi, N., 2011. First full-length sequences of the S gene of European isolates reveal further diversity among turkey coronaviruses. Avian Pathol. 40, 179–189.
McKinley, E.T., Hilt, D.A., Jackwood, M.W., 2008. Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. Vaccine 26, 1274–1284.
McKinley, E.T., Jackwood, M.W., Hilt, D.A., Kissinger, J.C., Robertson, J.S., Lemke, C., Paterson, A.H., 2011. Attenuated live vaccine usage affects accurate measures of virus diversity and mutation rates in avian coronavirus infectious bronchitis virus. Virus Res. 158, 225–234.
Miguel, B., Pharr, G.T., Wang, C., 2002. The role of feline aminopeptidase N as a receptor for infectious bronchitis virus. Brief review. Arch. Virol. 147, 2047–2056.
Mihindukulasuriya, K.A., Wu, G., St Leger, J., Nordhausen, R.W., Wang, D., 2008. Identification of a novel coronavirus from a beluga whale by using a panviral microarray. J. Virol. 82, 5084–5088.
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 (Pt 12), 2281–2286.
Mondal, S.P., Cardona, C.J., 2004. Comparison of four regions in the replicase gene of heterologous infectious bronchitis virus strains. Virology 324, 238–248.
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.
Mork, A.K., Hesse, M., Abd El Rahman, S., Rautenschlein, S., Herrler, G., Winter, C., 2014. Differences in the tissue tropism to chicken oviduct epithelial cells between avian coronavirus IBV strains QX and B1648 are not related to the sialic acid binding properties of their spike proteins. Vet. Res. 45, 67–9716.
Nakamura, K., Cook, J.K., Otsuki, K., Huggins, M.B., Frazier, J.A., 1991. Comparative study of respiratory lesions in two chicken lines of different susceptibility infected with infectious bronchitis virus: histology, ultrastructure and immunohistochemistry. Avian Pathol. 20, 241–257.
Ndegwa, E.N., Toro, H., van Santen, V.L., 2014. Comparison of vaccine subpopulation selection, viral loads, vaccine virus persistence in trachea and cloaca, and mucosal antibody responses after vaccination with two different Arkansas Delmarva Poultry Industry – derived infectious bronchitis virus vaccines. Avian Dis. 58, 102–110.

Niesters, H.G., Bleumink-Pluym, N.M., Osterhaus, A.D., Horzinek, M.C., van der Zeijst, B.A., 1987. Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. Virology 161, 511–519.
OIE, 2013. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 2.3.2.).
Otsuki, K., Noro, K., Yamamoto, H., Tsubokura, M., 1979. Studies on avian infectious bronchitis virus (IBV). II. Propagation of IBV in several cultured cells. Arch. Virol. 60, 115–122.
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.
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.
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.
Purcell, D.A., McFerran, J.B., 1972. The histopathology of infectious bronchitis in the domestic fowl. Res. Vet. Sci. 13, 116–122.
Purcell, D.A., Tham, V.L., Surman, P.G., 1976. The histopathology of infectious bronchitis in fowls infected with a nephrotropic "T" strain of virus. Aust. Vet. J. 52, 85–91.
Qian, D.H., Zhu, G.J., Wu, L.Z., Hua, G.X., 2006. Isolation and characterization of a coronavirus from pigeons with pancreatitis. Am. J. Vet. Res. 67, 1575–1579.
Raj, G.D., Jones, R.C., 1997. Infectious bronchitis virus: immunopathogenesis of infection in the chicken. Avian Pathol. 26, 677–706.
Raj, V.S., Mou, H., Smits, S.L., Dekkers, D.H., Muller, M.A., Dijkman, R., Muth, D., Demmers, J.A., Zaki, A., Fouchier, R.A., Thiel, V., Drosten, C., Rottier, P.J., Osterhaus, A.D., Bosch, B.J., Haagmans, B.L., 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature 495, 251–254.
Regan, A.D., Ousterout, D.G., Whittaker, G.R., 2010. Feline lectin activity is critical for the cellular entry of feline infectious peritonitis virus. J. Virol. 84, 7917–7921.
Regan, A.D., Whittaker, G.R., 2008. Utilization of DC-SIGN for entry of feline coronaviruses into host cells. J. Virol. 82, 11992–11996.
Schalk, A.F., Hawn, M.C., 1931. An apparently new respiratory disease in baby chicks. J. Am. Vet. Med. Assoc. 78, 413–422.
Schultze, B., Krempl, C., Ballesteros, M.L., Shaw, L., Schauer, R., Enjuanes, L., Herrler, G., 1996. Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a sialic acid (N-glycolylneuraminic acid) binding activity. J. Virol. 70, 5634–5637.
Schwegmann-Wessels, C., Herrler, G., 2006. Sialic acids as receptor determinants for coronaviruses. Glycoconj. J. 23, 51–58.
Shahwan, K., Hesse, M., Mork, A.K., Herrler, G., Winter, C., 2013. Sialic acid binding properties of soluble coronavirus spike (s1) proteins: differences between infectious bronchitis virus and transmissible gastroenteritis virus. Viruses 5, 1924–1933.
Shi, X.M., Zhao, Y., Gao, H.B., Jing, Z., Wang, M., Cui, H.Y., Tong, G.Z., Wang, Y.F., 2011. Evaluation of recombinant fowlpox virus expressing infectious bronchitis virus S1 gene and chicken interferon-gamma gene for immune protection against heterologous strains. Vaccine 29, 1576–1582.
Shil, P.K., Kanci, A., Browning, G.F., Markham, P.F., 2011. Development and immunogenicity of recombinant GapA(+) *Mycoplasma gallisepticum* vaccine strain ts-11 expressing infectious bronchitis virus-S1 glycoprotein and chicken interleukin-6. Vaccine 29, 3197–3205.
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.
Sun, L., Zhang, G.H., Jiang, J.W., Fu, J.D., Ren, T., Cao, W.S., Xin, C.A., Liao, M., Liu, W.J., 2007. A Massachusetts prototype like coronavirus isolated from wild peafowls is pathogenic to chickens. Virus Res. 130, 121–128.
Tay, F.P., Huang, M., Wang, L., Yamada, Y., Liu, D.X., 2012. Characterization of cellular furin content as a potential factor determining the susceptibility of cultured human and animal cells to coronavirus infectious bronchitis virus infection. Virology 433, 421–430.
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.
Toro, H., Espinoza, C., Ponce, V., Rojas, V., Morales, M.A., Kaleta, E.F., 1997. Infectious bronchitis: effect of viral doses and routes on specific lacrimal and serum antibody responses in chickens. Avian Dis. 41, 379–387.
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., Pennington, D., Gallardo, R.A., van Santen, V.L., van Ginkel, F.W., Zhang, J., Joiner, K.S., 2012a. Infectious bronchitis virus subpopulations in vaccinated chickens after challenge. Avian Dis. 56, 501–508.
Toro, H., van Santen, V.L., Jackwood, M.W., 2012b. Genetic diversity and selection regulates evolution of infectious bronchitis virus. Avian Dis. 56, 449–455.
Toro, H., Zhang, J.F., Gallardo, R.A., van Santen, V.L., van Ginkel, F.W., Joiner, K.S., Breedlove, C., 2014a. S1 of distinct IBV population expressed from recombinant adenovirus confers protection against challenge. Avian Dis. 58, 211–215.
Toro, H., Zhao, W., Breedlove, C., Zhang, Z., Yub, Q., 2014b. Infectious bronchitis virus S2 expressed from recombinant virus confers broad protection against challenge. Avian Dis. 58, 83–89.
Torres, C.A., Villarreal, L.Y., Ayres, G.R., Richtzenhain, L.J., Brandao, P.E., 2013. An avian coronavirus in quail with respiratory and reproductive signs. Avian Dis. 57, 295–299.
van Ginkel, F.W., van Santen, V.L., Gulley, S.L., Toro, H., 2008. Infectious bronchitis virus in the chicken Harderian gland and lachrymal fluid: viral load, infectivity, immune cell responses, and effects of viral immunodeficiency. Avian Dis. 52, 608–617.
van Santen, V.L., Toro, H., 2008. Rapid selection in chickens of subpopulations within ArkDPI-derived infectious bronchitis virus vaccines. Avian Pathol. 37, 293–306.
Wang, Y.F., Sun, Y.K., Tian, Z.C., Shi, X.M., Tong, G.Z., Liu, S.W., Zhi, H.D., Kong, X.G., Wang, M., 2009. Protection of chickens against infectious bronchitis by a recombinant fowlpox virus co-expressing IBV-S1 and chicken IFNgamma. Vaccine 27, 7046–7052.
Wei, Y.Q., Guo, H.C., Dong, H., Wang, H.M., Xu, J., Sun, D.H., Fang, S.G., Cai, X.P., Liu, D.X., Sun, S.Q., 2014. Development and characterization of a recombinant infectious bronchitis virus expressing the ectodomain region of S1 gene of H120 strain. Appl. Microbiol. Biotechnol. 98, 1727–1735.
Wickramasinghe, I.N., de Vries, R.P., Grone, A., de Haan, C.A., Verheije, M.H., 2011. Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. J. Virol. 85, 8903–8912.
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., Lai, K.K., Huang, Y., Lee, P., Luk, G.S., Dyrting, K.C., Chan, K.H., Yuen, K.Y., 2009. Comparative analysis of complete genome sequences of three avian coronaviruses reveals a novel group 3c coronavirus. J. Virol. 83, 908–917.
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 *Alphacoronavirus* and *Betacoronavirus* and avian coronaviruses as the gene source of *Gammacoronavirus* and *Deltacoronavirus*. J. Virol. 86, 3995–4008.
Woo, P.C., Lau, S.K., Lam, C.S., Tsang, A.K., Hui, S.W., Fan, R.Y., Martelli, P., Yuen, K.Y., 2014. Discovery of a novel bottlenose dolphin coronavirus reveals a distinct species of marine mammal coronavirus in *Gammacoronavirus*. J. Virol. 88, 1318–1331.
Yamada, Y., Liu, D.X., 2009. Proteolytic activation of the spike protein at a novel RRRR/S motif is implicated in furin-dependent entry, syncytium formation, and infectivity of coronavirus infectious bronchitis virus in cultured cells. J. Virol. 83, 8744–8758.
Yamada, Y., Liu, X.B., Fang, S.G., Tay, F.P., Liu, D.X., 2009. Acquisition of cell–cell fusion activity by amino acid substitutions in spike protein determines the infectivity of a coronavirus in cultured cells. PLoS ONE 4, e6130.
Yang, T., Wang, H.N., Wang, X., Tang, J.N., Gao, R., Li, J., Guo, Z.C., Li, Y.L., 2009. Multivalent DNA vaccine enhanced protection efficacy against infectious bronchitis virus in chickens. J. Vet. Med. Sci. 71, 1585–1590.
Yang, Z.Y., Huang, Y., Ganesh, L., Leung, K., Kong, W.P., Schwartz, O., Subbarao, K., Nabel, G.J., 2004. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. J. Virol. 78, 5642–5650.
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. Virology 332, 206–215.
Yu, L., Jiang, Y., Low, S., Wang, Z., Nam, S.J., Liu, W., Kwangac, J., 2001. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis. 45, 416–424.
Zeshan, B., Mushtaq, M.H., Wang, X., Li, W., Jiang, P., 2011. Protective immune responses induced by in ovo immunization with recombinant adenoviruses expressing spike (S1) glycoprotein of infectious bronchitis virus fused/co-administered with granulocyte-macrophage colony stimulating factor. Vet. Microbiol. 148, 8–17.
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. Vaccine 32, 664–670.
Zhang, Y., Buckles, E., Whittaker, G.R., 2012. Expression of the C-type lectins DC-SIGN or L-SIGN alters host cell susceptibility for the avian coronavirus, infectious bronchitis virus. Vet. Microbiol. 157, 285–293.
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.