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REVIEW ARTICLE

Infectious bronchitis virus variants: a review of the history, current situation and control measures

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The history, current situation and control measures for infectious bronchitis virus (IBV) variants are reviewed. A large number of IBV variants exist worldwide; some being unique to a particular area, others having a more general distribution. The possible reasons why some strains spread readily over major parts of the world, whereas other strains stay more localized are discussed. The advantages and disadvantages of strain classification by protectotyping, serotyping and genotyping are discussed in relation to *in vivo* protection. The different vaccination strategies are also considered.

Introduction

Infectious bronchitis virus (IBV) is ubiquitous in most parts of the world where poultry are reared and is able to spread very rapidly in non-protected birds. It is shed via both the respiratory tract and the faeces, and can persist in the birds and the intestinal tract for several weeks or months. Outdoors, survival of IBV for 56 days in litter has been reported (Cavanagh & Gelb, 2008). Although strict biosecurity and working with a one-age system are essential control measures, vaccination is normally required to increase the resistance of the chickens against challenge with IBV strains. This is made more difficult to achieve because IBV exists in the form of many different antigenic or genotypic types, commonly referred to as variants. Being a coronavirus and therefore a single-stranded RNA virus, IBV has an enormous capacity to change both by spontaneous mutation and by genetic recombination (Cavanagh & Gelb, 2008). When these events occur with IBV, both are most likely to result in the emergence of new variants if they occur in the hypervariable regions of the spike gene. Whilst many new variants are unable to replicate or survive for only a short time, a few emerge that become of economic importance either worldwide or in restricted geographic areas (see below).

The present paper presents an overview of the history and current situation of IBV worldwide, including the measures necessary to control the many infectious bronchitis (IB) variants found throughout the world.

History of infectious bronchitis variants

For many years it was widely believed that the first variants of IBV occurred in the early 1950s when Jungherr *et al*. (1956) in the USA showed that the Connecticut (Conn) isolate of 1951 neither cross-neutralized nor cross-protected with the original Massachusetts (Mass) isolate from the early 1940s. However, a retrospective study (Jia *et al.*, 2002), using monoclonal antibodies and molecular analysis of part of the S1 subunit of the spike glycoprotein (S) gene, identified non-Mass IBVs among isolates made in the USA as early as the 1940s. Although some of these are found elsewhere, most countries are now known to have their own indigenous variants as well, and this paper will consider the current situation worldwide.

The USA

In the USA a number of different IB variants had been identified by the early 1970s, generally on the basis of serological analysis (Hitchner *et al.*, 1966; Hopkins, 1974; Cowen & Hitchner, 1975; Johnson & Marquardt, 1976). Hofstad (1961, 1981) showed a poor level of cross-protection between several of them. New variants continued to be identified and poor cross-protection was shown (Gelb *et al.*, 1981). In a survey using a reverse transcriptase polymerase chain reaction (RT-PCR) for typing, Jackwood *et al.* (2005) identified 82 different IB variants among 1523 submissions to their laboratory over an 11-year period. As the number of variants identified in the USA increased, a few were shown to be both widely distributed and of major economic importance. Possibly the most significant of these was the variant known as Arkansas (Ark) (Fields, 1973); its importance quickly being recognized by the development of live-attenuated vaccines from this strain (Gelb & Cloud, 1983). Vaccines developed from Mass, Conn and Ark IBVs are widely used in the USA, either singly or in

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combination (e.g. a combined Mass–Conn commercial product) and these vaccines have also proved beneficial in providing adequate cross-protection against other IB variants including California 99 (Alvarado *et al.*, 2003), thereby indicating the possibility of cross-protection between IBVs. The Ark serotype continued to be of economic importance in the main poultry-producing areas, including Georgia and Delmarva (Gelb *et al.*, 1983). By the mid-1990s, the availability of molecular diagnostic methods led to the identification of subtypes or "quasi-species" within the Ark serotype (Nix *et al.*, 2000; Jackwood *et al.*, 2005). However, suggestions that the currently available Ark vaccines were providing incomplete protection against more recent isolates of this serotype were discounted by Sander *et al.* (1997), who demonstrated that poor protection was more likely to be due to poor vaccine application than to antigenic drift in the virus in the field. This finding, supported by the recent work of Jackwood *et al.* (2009), confirms the importance of careful application of IB vaccines.

Another IB variant of importance in the USA, Delaware (DE) 072—first reported in the northeast of the country in 1992 (Gelb *et al.*, 1997)—was found to show little genomic relatedness in the S1 region of the S gene to other US variants, but interestingly to be closely related to the Dutch variant, D1466 (Lee & Jackwood, 2001b) (see below). DE072 increased in incidence throughout the next decade, also causing major disease problems in vaccinated flocks in Georgia (Lee *et al.*, 2001). It has apparently undergone both genetic drift and recombination (Lee & Jackwood, 2000, 2001a), such that DE072 vaccines provide only poor protection against field strains, and molecular analysis of field isolates has led to the recognition of a new IB variant, designated Georgia 98 (GA98) (Lee *et al.*, 2001). A homologous vaccine was developed from the Georgia 98 (GA98) strain that provided adequate protection after two applications against a homologous challenge but also against a DE072 challenge (Jackwood *et al.*, 2003). Recently, a new IBV variant designated GA08, for which existing vaccines were not protective, was detected in the same region (Jackwood *et al.*, 2010). Genetic analyses, serotyping and cross-protection trials with strains from California have now shown that different parts of the USA can have their own IBV variants and have confirmed that new ones continue to emerge (Jackwood *et al.*, 2007).

Latin America

IBV had appeared in Latin America by the 1950s and the first reported isolate was of the Mass serotype in Brazil (Hipólito, 1957), although isolation of a variant (Ark) was not reported in that country until some 10 years later (Branden & Da Silva, 1986). In a study carried out in the mid-1990s, IBV isolates of at least five different antigenic types were found in commercial chickens of all types throughout Brazil, but mainly in the major poultry-producing area of the south (Di Fabio *et al.*, 2000). Several different genotypes have now been identified in Brazil by analysis of either the S1 gene (Montassier *et al.*, 2006, 2008; Villarreal *et al.*, 2007a, 2007b) or the nucleoprotein (N) gene (Abreu *et al.*, 2006), but protection studies have not been performed. More recently it has been shown (Villarreal *et al.*, 2010) that both a unique cluster of IBV strains (subdivided into three subclusters) as well as the 4/91 genotype currently co-exist in that country. It is important to remember that in Brazil, as in many other parts of the world, the only live attenuated IB vaccines licensed for use are of the Mass serotype and that protection studies (Cook *et al.*, 1999; Di Fabio *et al.*, 2000) showed that the currently available Mass vaccine provided inadequate protection against some of the Brazilian variants.

Hidalgo *et al.* (1976) reported the first isolation of IB (Mass serotype) in Chile in 1975 and variants were reported for the first time there some 10 years later (Hidalgo *et al.*, 1986). By the mid-1980s, IB was reported as a serious problem in commercial chicken flocks and novel variants as well as the Mass and Conn serotypes were isolated from broiler and layer flocks, and again Mass vaccines were found to protect poorly against challenge with these variants (Cubillos *et al.*, 1991). Elsewhere in Latin America a variant IBV was isolated from commercial chickens in Honduras in 1997 and poor protection against Mass vaccines was demonstrated (Cook *et al.*, 1999). Following the isolation of the Ark serotype in Mexico in the early 1990s (Quiroz *et al.*, 1993), the use of molecular methods subsequently identified IBV variants unique to that country in commercial chickens (Escorcia *et al.*, 2000; Callison *et al.*, 2001; Gelb *et al.*, 2001). They were shown by neutralization tests to be different from Mass or Conn serotypes, but *in vivo* protection studies were not performed. Rather surprisingly, IBV variants do not appear to have been reported in Argentina until very recently when Rimondi *et al.* (2009), using only molecular techniques, detected three unique genotype clusters (in addition to Mass and Conn); one of these was closely related to isolates from Brazil. Similar techniques had been used a few years earlier to identify for the first time a genetically unique IB variant in Colombia (Alvarado *et al.*, 2005).

Europe

Until the late 1970s, it was believed that only IBVs of the Mass serotype were important causes of disease in Europe. Then Dawson & Gough (1971) reported the detection of IBV variants in the UK and workers at the Doorn Institute in The Netherlands isolated IBVs belonging to at least four different IBV serotypes associated with disease outbreaks in Mass-vaccinated commercial flocks (Davelaar *et al.*, 1984). These IBVs belonged to novel serotypes—D207 (also known as D274), D212 (better known as D1466), D3896 and D3128—and experimental data showed that existing IB vaccines protected poorly against them. Vaccines were developed using some of these variants, with several still in use today. The development in the UK of techniques that made use of tracheal organ cultures for both the isolation and serotyping of IBVs (Cook, 1984; Cook & Huggins, 1986) resulted in increased interest in the detection of new IB variants. Many new ones were isolated, not only in the UK (Gough *et al.*, 1996) but also in other European countries including France (Picault *et al.*, 1986), Belgium (Meulemans *et al.*, 1987), Italy (Capua *et al.*, 1994; Zanella *et al.*, 2000, 2003), Poland (Minta *et al.*, 1998) and Spain (Dolz *et al.*, 2006, 2008).

Many, perhaps the majority, of these variants were detected for only a brief period and were probably of limited importance. However, occasionally a variant that caused major disease outbreaks did occur. One example of this is the IB variant B1648, mainly associated with renal problems in vaccinated flocks in Belgium and neighbouring countries for a relatively short period in the 1990s (Lambrechts *et al.*, 1993; Pensaert & Lambrechts, 1994). However, of major importance internationally was the variant called variously 4/91, 793B and CR88 (here named 4/91) (Gough *et al.*, 1992; Parsons *et al.*, 1992; Le Gros, 1998) that emerged in the 1990s, associated with major welfare and economic problems in apparently well-vaccinated flocks. IB 4/91 quickly spread to many parts of the world and necessitated the development of live-attenuated IB vaccines to control it. This virus 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.

More recently, the use of RT-PCR technology has led to an increase in the detection of novel IB variants in Europe (Worthington *et al.*, 2008). However, it is important to remember that detecting an IB variant by molecular methods does not necessarily mean that the virus concerned is causing major disease problems. This can be illustrated by the IB variant Italian 02 (It-02). This virus appears to be easily detected by RT-PCR (Jones *et al.*, 2005; Dolz *et al.*, 2006) but is difficult to isolate, and its association with widespread disease outbreaks remains to be established. On the other hand, the variant QX IBV is easy to detect by RT-PCR, is readily isolated and is associated with major disease outbreaks in many areas. This virus, first reported associated with proventriculitis in China in the late 1990s (Yu *et al.*, 2001), spread throughout Russia (Bochkov *et al.*, 2006), and then appeared in much of Europe (Beato *et al.*, 2005; Landman *et al.*, 2005; Benyeda *et al.*, 2009). It is associated with nephritis and respiratory distress in broilers and with the so-called "false layer syndrome" and drops in egg production in breeders and layers. It is therefore causing major economic problems in IB-vaccinated flocks. Although no homologous vaccine was available until very recently, some success is reported in controlling its effects using currently available IB vaccines (De Wit *et al.*, 2006, 2009; Worthington & Jones, 2006; Terregino *et al.*, 2008; De Wit & Van de Sande, 2009).

Africa

IBV associated with swollen head syndrome and causing severe problems throughout southern Africa was isolated in the early 1980s (Morley & Thomson, 1984), confirmed as a variant and shown to be poorly protected against by Mass vaccines (Cook *et al.*, 1999). The only other known incidence of IBV variants in sub-Saharan Africa is the recent report by Ducatez *et al.* (2009), who detected a novel IBV in Nigeria and Niger that was antigenically and genetically distinct from other known IBVs. However, no association with disease was demonstrated and there is no information on the ability of currently available vaccines to protect against it.

IBV variants have been recognized in Egypt since the 1950s (Sheble *et al.*, 1986; Eid, 1998) with the isolation of a variant shown by neutralization tests to be closely related to the Dutch variant D3128. Subsequently, variants related to Mass, other European IBVs and one related to an Israeli variant have been identified by genome analysis in that country (Abdel-Moneim *et al.*, 2006). Bourogaa *et al.* (2009) used both molecular methods and cross-neutralization tests to identify IBV isolates from Tunisia as variants that are closely related to ones found in Europe. 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). The suggestion was made that parts of Africa, where variants have been little studied, might be a reservoir for such viruses, although the increasing number of variants being reported in other countries, such as Brazil and China, indicate that several such reservoirs might exist. More recently, El-Bouqdaoui *et al.* (2005) used RT-PCR and restriction fragment length polymorphism techniques to study outbreaks of nephritis associated with IB in Morocco, where Mass and 4/91 vaccines have been used since 1960 and 2000, respectively. Three novel genotypes were identified, against which Mass vaccines provided poor protection.

Middle East

Variant IBVs have been recognized in Israel since at least the mid-1990s (Meir *et al.*, 1998, 2004; Callison *et al.*, 2001) on the basis of both virus neutralization tests and molecular techniques, and protection studies have shown that Mass vaccines provide inadequate protection against some of these novel variants. In Jordan, the use of RT-PCR enabled European IBV variants D274 and 4/91 to be detected (Roussan *et al.*, 2008), but since the primers used were designed to detect only these specific variants, it is possible that others are present in that country. Similar methods have been used to identify 4/91 in Iran (Seyfi Abad Shapouri *et al.*, 2004).

India and Pakistan

Antibodies to several "American" (Muneer *et al.*, 1987a) and "European" (Ahmed *et al.*, 2007) IBV variants have been demonstrated in Pakistan, but virological studies have still to be performed. An IBV isolated in India in the early 2000s from cases of nephritis was reported to have a unique S1 sequence, indicating it to be different from other known IBVs (Bayry *et al.*, 2005).

Asia

In Asia, many studies have been performed in different countries in recent years. In Malaysia, where IBV was first isolated in 1967, variants have been present since at least 1979 (reported by Lohr, 1988). More recently, molecular epidemiological studies of IBVs isolated in Malaysia and Singapore showed that whilst some were of the Mass serotype (probably identical to the H120 vaccine), others were similar to IBVs reported from China and Taiwan (Yu *et al.*, 2001b). These studies led the authors to suggest that IBV variants have existed in Asia for some time. This finding was substantiated by

Zulperi *et al.* (2009) who used sequence and phylogenetic analysis to study two variants isolated in Malaysia, 10 years apart. One was similar to several Chinese variants whilst the other was characterized as unique to Malaysia, but no protection studies were performed. IB has been a problem in Thailand since the 1950s, despite the use of many different IB vaccines, and a recent molecular study by Pohuang *et al*. (2009) has identified two groups of IBV variants in Thailand by phylogenetic analysis of the SI gene: Group I appeared to be unique to that country, whilst Group II showed a close relationship to Chinese IBVs, including variant A2 (see below).

IBV variants have been associated with disease outbreaks in Korea since at least the mid-1980s (Song *et al.*, 1998). Initially Mass vaccines were successful in controlling disease, but since 1990 IB outbreaks have been experienced in well-vaccinated flocks with an increased incidence of renal problems. Song *et al.* (1998) classified 40 IBV isolates into Mass plus four local genotypes, one of which was not only the predominant type but, in pathogenicity studies, caused 50% mortality in specific pathogen free (SPF) chicks inoculated at 1 day old. More recent studies (Lee *et al.*, 2004) have extended this work and reported further genetic diversity amongst Korean IBV variants isolated from diseased flocks; some of which are indigenous to Korea, whilst others share genetic relationships with IBV variants from other countries in the region (Lee *et al.*, 2008). It is suggested that Korean IBVs are continually evolving (Jang *et al.*, 2007).

Doi *et al*. (1982) studied the antigenic relationship of eight Japanese IBV isolates obtained between 1960 and 1974 and concluded that there were many distinct serotypes of IBV in Japan. Mase *et al*. (2004) carried out a detailed analysis of Japanese IBV variants by looking at the N-terminus of the S1 glycoprotein and identified three major genetic groups not found in other countries. One group, present in Japan since at least the 1960s, may be found only in Japan, whilst the other two, which were more recent, are related to Chinese and Taiwanese variants (see below). These groups were distinct from those found in Europe or the USA, although the 4/91 serotype has been isolated in Japan (Mase *et al.*, 2008; Shimazaki *et al.*, 2008). The origin of these variants is not clear, but Shieh *et al.* (2004) reported the close relationship of isolates from Taiwan and Japan to IBVs found in Australia and the USA, and suggested that the Asian variants are recombinants; their S gene being derived from Australian variants and the N genes from US strains. More recently, analysis of the hypervariable region of the S1 subunit of Japanese field isolates has demonstrated the presence of a novel IBV variant (Mase *et al.*, 2010).

IBV variants have been recognized in Taiwan since at least the mid-1960s and two distinct lineages have been identified, as well as Mass and IBVs related to those reported in neighbouring countries (Wang & Tsai, 1996; Wang & Huang, 2000; Huang *et al.*, 2004). However, recent data have suggested that the currently dominant IB variant in Taiwan may have arisen as a result of recombination in the 5′ end of the N gene between a local IBV and a foreign IBV (Kuo *et al.*, 2010). The failure of Mass vaccines to provide adequate protection has led to the development of vaccines from indigenous strains (Huang & Wang, 2006).

For many years little was known of the situation regarding IBV variants in China, but the fact that Mass-type vaccines were used successfully suggests that variants may not have been a problem before the 1980s. However, by the mid-1990s this was clearly no longer the case and in the past decade many published reports have revealed the diversity of IBVs causing disease in that country. By means of *in vivo* studies and antigenic typing using monoclonal antibodies and cross-neutralization tests, Wu *et al.* (1998) identified highly pathogenic variant IBVs in China associated with both respiratory disease and nephritis and showed that H120 vaccine provided poor protection against challenge with these isolates. Possibly the most significant IBV variant worldwide to have emerged from China is the QX variant reported by YuDong *et al*. (1998) in association with proventriculitis (see above). Another IBV genotype Q1 has also been associated with proventriculitis (Yu *et al.*, 2001a). However, it remains unclear whether the problems with proventriculitis that were associated with the detection of QX or Q1 strains were really caused by the IBV infection or by another factor because local replication of the IBV strain in the proventriculis was not shown. In a recent study of 26 IBVs isolated between 1985 and 2008 from a variety of disease conditions in the Guangxi region of China, Wei *et al*. (2009) identified four clusters on the basis of RT-PCR analysis of the N gene. They were grouped into seven serotypes by neutralization tests, but there was poor correlation between the results of genotyping and serotyping. In an analysis of the genome of 26 IB variants isolated from the kidneys, proventriculus and oviduct in different areas of China between 1995 and 2004, Liu *et al.* (2006) identified Mass type IBVs plus five genotypes apparently found only in China and co-circulating there. One of these (genotype A2) was subsequently shown to be the dominant indigenous type in China at that time (Liu *et al.*, 2009b), although more recently, based on analysis of S1 gene sequences, Zou *et al*. (2010) have reported that a different genotype, designated LX4, is now dominant. Others showed close relationships with either Korean or Taiwanese IBV variants, and one was closely related to an Australian isolate (Liu *et al.*, 2006). Cuiping *et al.* (2007) identified the 4/91 variant in China, along with the Australian T strain, as well as one variant indigenous to China. Thus the diversity of IB variants in China is now well established, some being restricted to and co-circulating in that country, whilst others show similarities with IBV variants identified in other countries in the region.

Russia

Molecular characterization using a part of the S gene of 91 IBV strains isolated between 1998 and 2002 from chickens in Russia showed the complexity of the IBV situation in that country (Bochkov *et al.*, 2006). The major group of isolates (38 viruses) was of the Mass genotype, circulating in Russia since the early 1970s; a second group of 22 strains were of the known European genotypes D274, 4/91, B1648, 624/I and It-02. Two further isolates from very distant geographic locations in Russia (the Far East and European parts) clustered together with Chinese strains of the QX IBV genotype. The remaining 27 Russian isolates were divided into 11 novel genotypes.

Australia and New Zealand

In Australia, where IBV has always evolved independently from the rest of the world due to its geographical isolation (Ignjatovic *et al.*, 2006), many different IBV variants have been isolated and characterized since the early 1960s (Cumming, 1963) and *in vivo* protection studies have been performed with these variants (Klieve & Cumming, 1988). Using both monoclonal antibodies directed against the major IBV proteins and sequencing studies, several distinct lineages have now been recognized (Ignjatovic *et al.*, 1997, 2006), all different from those found elsewhere. Ignjatovic *et al.* (2002) in a study comparing the pathogenicity of 25 Australian IBV strains isolated between the 1960s and 1990s found that, of 12 IBVs isolated between 1961 and 1976, nine were nephropathogenic, whilst only three of 13 strains isolated between 1981 and 1994 were associated with nephritis. They suggested that this indicated a change in the prevalent IBV strains from highly nephropathogenic (1960s to 1970s) to respiratory (1980s to early 1990s). Their work also indicated the emergence in the late 1980s of respiratory strains with altered tissue tropism in Australia. It does not appear to be clear as yet whether similar changes in pathogenicity have occurred in other parts of the world.

Interestingly, in New Zealand, IB problems were uncommon before the 1970s, when IBV variants were first reported (Lohr, 1988). It was initially believed, on the basis of cross-neutralization tests, that IBV variants had evolved independently of those reported in Australia or the USA and at least four different variants were identified (Lohr, 1976, 1977). However, sequencing of the S1 gene has recently revealed genetic relationships between these early IBV isolates and ones made since 2000, and phylogenetic analysis has also shown that they are more closely related to Australian than to European or North American ones (McFarlane & Verma, 2008).

Spreading of the variants

The above account highlights the large number of IBV variants that exist worldwide; some being unique to a particular area, others having a more general distribution. The reason why some strains spread readily over major parts of the world whereas others remain more local is unknown. Variants of major importance in major parts of the world, such as 4/91 or QX that have spread over Asia, Europe and Africa in a short period, have not been reported in, for example, the USA or Australia. On the other hand, the major strain of the USA, the Arkansas strain has hardly been reported outside the USA. A pathogenic strain like D1466 that has been in some countries in Western Europe for three decades now has hardly been reported outside Western Europe, although it is very difficult to achieve a sufficient level of protection (Cook *et al.*, 1999) against this strain, which would make it easier for the strain to spread to other areas. It seems likely that geographical isolation and control measures employed in countries may play a part in preventing entry of IBV variants. The recent discoveries of IBV and IBV-like strains in species of birds other than the chicken (Cavanagh, 2005), such as geese, ducks, and pigeons, might also play a role in the spread of IBV strains over the world. Specific IBV strains that were able to infect another bird species, especially if it is a migratory bird, would spread more easily over long distances than a strain that could not replicate in that bird species. An unknown IBV-like virus that might be common in a migratory bird could infect the poultry industry in different parts of the world, leaving us with the mystery of how this new virus spread so fast within the poultry industry. Although the role of the wild birds in the world of IBV is largely unknown and speculative, it is certainly an area that deserves more attention and research.

Current situation

As discussed, it is becoming clearer that many countries have to deal with many types of IBV. Currently, genotyping is by far the most used system and has largely replaced serotyping and protectotyping. Does this create a problem? The preferred 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 (De Wit, 2000). Typing by functional tests results in protectotypes and antigenic types (serotypes and epitope types). Tests that look at the genome result in genotypes.

Protectotypes. With protectotyping, the complete immune response of a chicken against an IBV strain is measured. 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. However, protectotyping is laborious and expensive and requires both SPF chickens and high-level facilities for performing vaccination-challenge studies.

Serotypes. Serotyping is based on the reaction between an IBV strain and chicken-induced IBV serotype-specific antibodies. Two strains (A and B) are considered to be of the same serotype when two-way heterologous neutralization titres (antiserum A with virus B, and antiserum B with virus A) differ less than 20-fold from the homologous titres (antiserum A with virus A, antiserum B with virus B) in both directions (Hesselink, 1991). Serotyping becomes less practical as more IBV types are detected in a certain area since every serotype needs its own neutralization test, and for new strains that appear to be different, an antiserum has to be raised in SPF birds. As mentioned before, more and more countries have to deal with an increasing number of variants, which decreases the practicability of serotyping.

Genotypes. Grouping of strains based on genetic characterization of (a part of) the genome results in genotypes. Methods include sequencing, detection of genotype-specific parts of the genome by RT-PCR, or determination of the position of enzyme cleavage sites (De Wit, 2000). Genomic information is objective and provides essential information for epidemiological

studies (Figure 1). Most used for genotyping is the part of the genome that codes for the S1 subunit of the spike glycoprotein, which is the major inducer of protective immunity and carries most of the virus-neutralizing epitopes, including serotype-specific epitopes, which are usually conformation dependent (Mockett *et al.*, 1984; Cavanagh & Davis, 1986; Koch *et al.*, 1990; Cavanagh *et al.*, 1992).

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 the 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 other larger percentages of mutations at other parts of S1 might not result in a relevant change in antigenicity of the virus. On the other hand, IB viruses 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 & Galli, 1995). These features of the IB virus result in a disadvantage of genotyping for use in the field, as direct translation of information about usually only a part of the genome (generally a part of the S gene) of an IBV strain to biological function or antigenicity of the virus is not possible or is not without risk. Despite these limitations, there are reports that the S1 gene sequence comparison (part of 700 nucleotides) is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization (Ladman *et al.*, 2006). Whether this is a general rule is unknown, as only a small number of strains and vaccines have been compared, and also different parts of the S1 gene were used for the comparison of the homology. In general, however, a lower homology in sequence of the S1 subunit of two strains (e.g. a vaccine and a field strain) means a greater chance that relevant mutations have occurred, which might result in a lower cross-protection.

An analysis of several papers reporting the level of homology of the S1 gene or a part of it and level of cross-protection (Cavanagh *et al.*, 1997; Cook *et al.*, 2001; Meir *et al.*, 2004; Gelb *et al.*, 2005; Abdel-Moneim *et al.*, 2006; Ladman *et al.*, 2006; Liu *et al.*, 2009a) shows that,

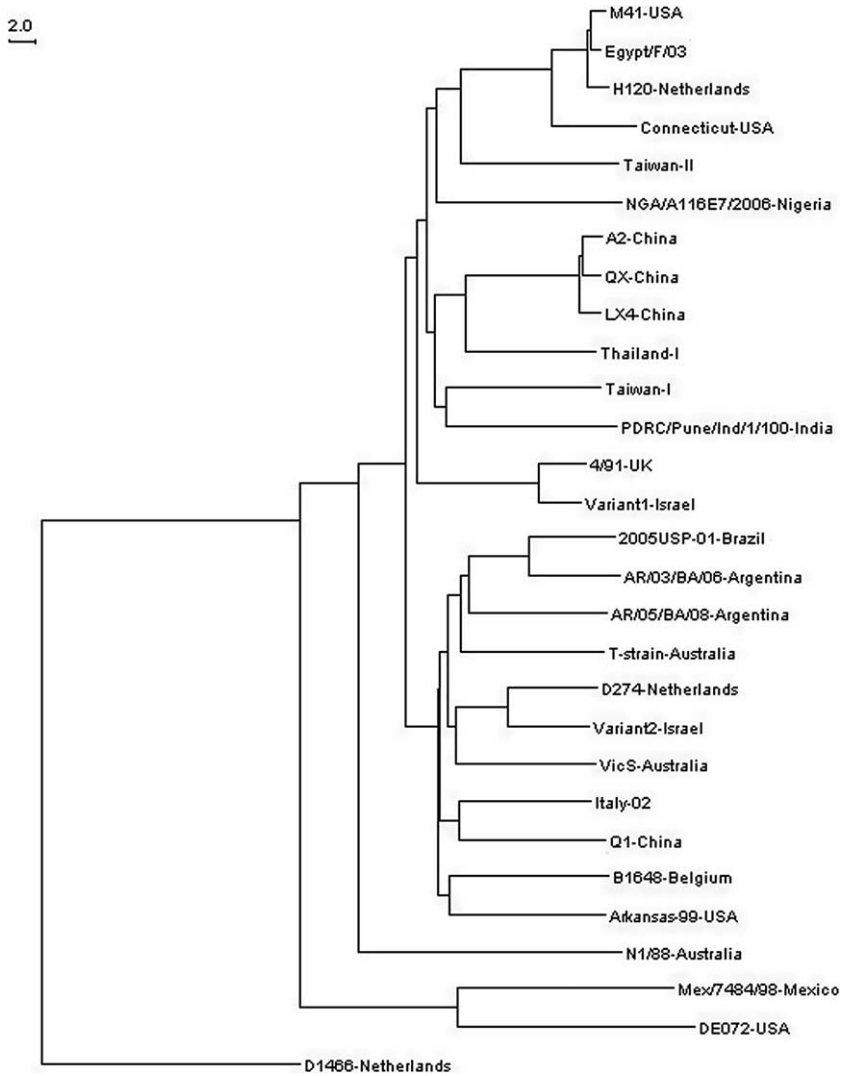

Figure 1. *Phylogenetic tree for a selection of IBV variants from different parts of the world showing the wide diversity of IBV. The tree is based on comparison of the partial S gene (between nucleotides 20,447 and 20,924—numbering compared with the genome of Ark DPI; Ammayappan* et al.*, 2008), coding for a part of the S1 glycoprotein including the highly variable regions 1 and 2. The phylogenetic tree analysis was conducted by neighbour-joining method using bootstrap analysis (100 replications).*

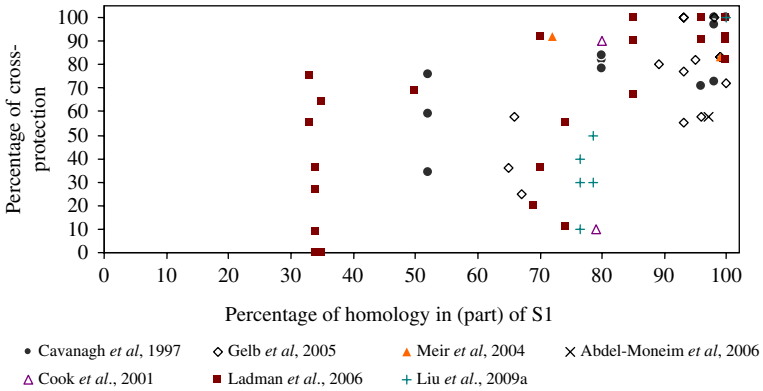

Figure 2. *Correlation between the level of homology in the S1 region of the S gene of IBV strains and the level of cross-protection between these strains as reported in seven publications.*

in general, there is a higher chance of a good level of cross-protection between strains with a high level of homology than between strains with a low homology (Figure 2). However, these data also show that the relationship is not very strong. Some strains that differ by only a few percent from other strains in the sequenced part of the genome showed a significant drop in cross-protection (Meir *et al.*, 2004; Abdel-Moneim *et al.*, 2006), whereas there was a high level of cross-protection against other strains with a much lower homology (Meir *et al.*, 2004). Figure 2 also shows the wide variation in level of cross-protection that is detected for strains with the same level of homology in comparison with the strains that are used as vaccine. Another limitation that needs to be considered is the different size and location of the S1 region of the S gene that different laboratories and research groups use for their analyses of the level of homology (De Wit *et al.*, 2010a). Several laboratories sequence a part of S1 that includes the highly variable regions 1 and 2; others do not. Analysis of different parts of S1 can result in different levels of homology leading to different conclusions regarding the relationship between virus strains. An example is shown in Table 1 and Figures 3, 4, and 5. For this analysis the sequences of four IBV strains of different genotypes and serotypes, as published by Adzhar *et al.* (1997), were used. The homology of the four strains—7/91 (genotype 4/91), H120 (Mass serotype), D274 and D1466—was compared for nucleotides 1 to 1600 of the S1 region of the S gene. The extent of nucleotide identity between the strains was expressed on nucleotide level as follows: score 100% when all four strains had the same nucleobase (in the copy DNA); score 75% when three out of four strains had the same nucleobase; score 50% when two strains had the same nucleobase and the two other strains had another nucleobase in common; score 25% when two strains had the same nucleobase and the two other strains each had another different nucleobase; and score 0% when all strains had a different nucleobase at that position of the genome. In this comparison, a missing nucleotide (by deletion) was considered to be a different nucleobase. Table 1 shows the overview of the comparison. When windows of 10 nucleotides were used for the comparison of the four strains, the average homology of the four strains could have been 42.5% but also 100% based on the location of the 10 nucleotides that have been used for the comparison. When windows of 100 nucleotides were used for the comparison of the four strains, the average homology of the four strains could have been 68.0% but also 86.8% based on the location of the 100 nucleotides that were used for the comparison. When windows of 400 nucleotides were used for the comparison, the average homology of the four strains varied from 73.2 to 81.6% based of the location of the 400 nucleotides that were used for the comparison.

When the IBV strain tested is the result of a recombination event between different IB genotypes, examination of different parts of S1 can mean the detection of a different genotype (Wang *et al.*, 1993; Jia *et al.*, 1995; Dolz *et al.*, 2008). The detected homology with other strains is then very dependent on the part of the genome that is being used for the comparisons of the homology. Using larger parts of S1 for the comparison of strains results in a decreased risk

Table 1. *Average level of genetic homology for four IBV strains using different nucleotide window sizes across the entire S1 gene of different genotypes/serotypes (Adzhar* et al.*, 1997).*

Number of nucleotides per window used to calculate the homology	Number of windows	Lowest homology[a]	Highest homology	Difference in homology between highest and lowest windows (%)
Per 1	1600	0	100	100
Per 10[b]	160	42.5	100	57.5
Per 50	32	64.5	91.0	26.5
Per 100	16	68.0	86.8	18.2
Per 200	8	69.6	83.9	14.3
Per 400	4	73.2	81.6	8.4
Per 800[c]	2	76.8	80.2	3.4
All 1600	1	78.5[d]		

[a]The extent of nucleotide identity between the strains was expressed on the nucleotide level as follows: score 100% when all four strains had the same nucleobase (in the copy DNA); score 75% when three out of four strains had the same nucleobase; score 50% when two strains had the same nucleobase and the two other strains had another nucleobase in common; score 25% when two strains had the same nucleobase and the two other strains each had another different nucleobase; and score 0% when all strains had a different nucleobase at that position of the genome. In this comparison, a missing nucleotide (by deletion) was considered to be a different nucleobase. [b]Homology in nucleotides 1 to 10, 11 to 20, 21 to 30, and so on to 1591 to 1600. [c]Homology in nucleotides 1 to 800 and 801 to 1600. [d]Average homology.

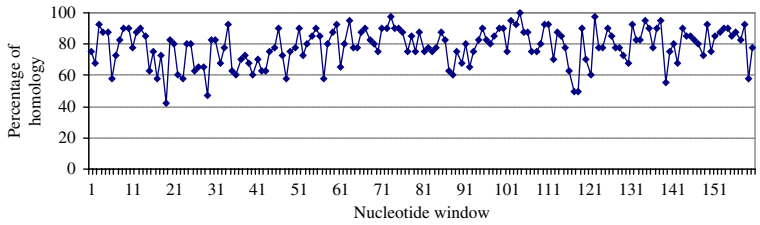

Figure 3. *Average level of homology between four strains of IBV in 160 different windows of 10 nucleotides of nucleotides 1 to 1600 of the S1 region on the S gene of different genotypes/serotypes (Adzhar* et al.*, 1997).*

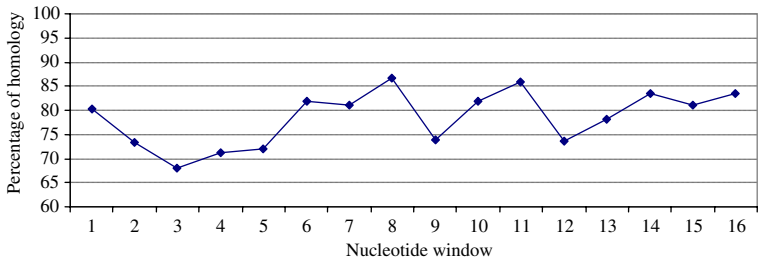

Figure 4. *Average level of homology between four strains of IBV in 16 different windows of 100 nucleotides of nucleotides 1 to 1600 of the S1 region on the S gene of different genotypes/serotypes (Adzhar* et al.*, 1997).*

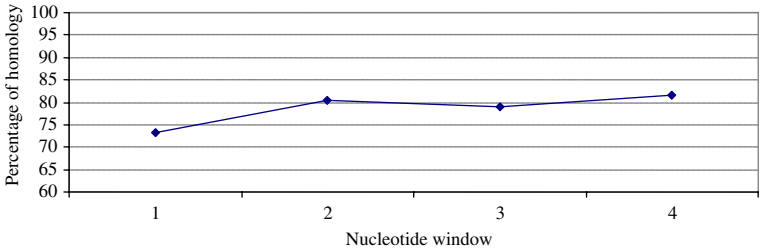

Figure 5. *Average level of homology between four strains of IBV in four different windows of 400 nucleotides of nucleotides 1 to 1600 of the S1 region on the S gene of different genotypes/serotypes (Adzhar* et al.*, 1997).*

of finding high levels of homology between strains where in fact this is not really the case. In the field situation there is another limitation on the predictive value of a vaccine against the field challenge strain based on the homology in the S1 gene. In many countries, IBV vaccines are used that are a combination of two strains, or the vaccination programme contains two vaccinations with two (or more) different IBV strains. The level of cross-protection then depends on the efficacy of the combination of strains, which makes it impossible to determine a level of homology between the field strain and the "vaccine strain".

This leads to the conclusion that genotyping is an excellent tool for epidemiological studies (Figure 1), and is a convenient, practical tool for typing that can be used best as a means of screening to select potentially important strains. In situations where there is suspicion in the field that the genotype of recent isolates does not provide accurate information about the true antigenic nature of these IBV isolates, then conventional testing (serotyping) and especially *in vivo* protection studies are required.

Control measures

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). It is shed via both the respiratory tract and the faeces and can persist in the birds, intestinal tract and faeces for several weeks or months. 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 strains (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 against challenge and also for priming of future layers and breeders that will be boosted with the inactivated vaccines. 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.

Most used in the world are vaccines of the Mass serotype. In several parts of the world, Mass vaccines are the only vaccines allowed, but elsewhere vaccines of one or more other serotypes are permitted. 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 protectotypes, serotypes or genotypes that can vary from high to low (Figure 2 and reviewed for the H-strain by 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 vaccinated under optimal conditions may have immunity lasting many months and for broilers, this may be lifelong (Bijlenga *et al.*, 2004).

It has been shown that vaccination with two antigenically distinct live-attenuated vaccines such as Mass and 4/91 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.

Results of challenge studies and field work have shown that vaccination with a bivalent vaccine containing the Mass and Ark strains (Gelb *et al.*, 1989, 1991, 2005) provided, on average, a higher level of cross-protection against certain heterologous field strains than other combinations of vaccines such as Mass together with Conn or with JMK. However, the Mass and Ark vaccine did not provide significant protection against challenge with another strain (Ladman *et al.*, 2002). It is unknown whether the separate application of these two IBV strains would have resulted in a higher (or lower) level of cross-protection against the same heterologous challenges.

A well-vaccinated chicken is protected against challenge with a virulent homologous IBV strain. This means that this well-vaccinated chicken is also protected against an early revaccination with a homologous vaccine (Davelaar & Kouwenhoven, 1980). Despite this, revaccination of young birds, especially broilers, using a vaccine of the same serotype as the first vaccine has proven to be beneficial under field conditions. This is an indication that the quality of the first vaccination might need careful attention. 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.*, 2010b). The vaccine 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, resulting in an increased risk of infections with *Escherichia coli* or other bacteria (Goren, 1978; Hopkins & Yoder, 1984; Smith *et al.*, 1985; Cook *et al.*, 1986; Matthijs *et al.*, 2003) and even increase of virulence of the virus (Hopkins & Yoder, 1986).

In order to achieve an increased level of protection during the laying period of commercial layers and parent stock, the use of inactivated IBV vaccines after a priming with live attenuated IBV vaccines has been shown to be effective against homologous Mass challenges (Gough *et al.*, 1977; Box *et al.*, 1980; Timms & Bracewell, 1983; Box & Ellis, 1985; Box *et al.*, 1988). The efficacy of increasing the level of protection against heterologous challenges in the laying period has rarely been reported (De Wit *et al.*, 2009), although birds that had been vaccinated twice with a live Mass-type vaccine and boosted with a killed oil-emulsion vaccine containing a Mass strain showed no protection against challenge with a strain of the Ark type (Muneer *et al.*, 1987b).

Finally, it must be emphasized that whilst high-quality live-attenuated and inactivated IB vaccines are available for use worldwide, two points require careful consideration by those responsible for the welfare of commercial chicken flocks. Firstly, the importance of careful application of the vaccines cannot be overstated. Secondly, there may be a need to amend vaccine programmes, using vaccines licensed for use in a particular area, in order to optimize protection against the IB variants currently circulating in that area.

Conclusion

The problems of how to type the large number of IBV variants that are now found worldwide, and how to relate the findings to the best vaccination strategy for protection, are clearly complex. Whilst genotyping has advantages of ease and speed, the different sizes and locations of the part of the S1 region of the S gene that are used by different groups for the comparison of strains makes interpretation of results very difficult, and the present review emphasizes the need for a standardized method of performing genotyping. Another major point that needs to be remembered is that only the vaccinated chicken decides whether genetic or antigenic differences as shown by genotyping or serotyping are relevant for the level of cross-protection in that bird.

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