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REVIEW ARTICLE

Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review

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The H strain of infectious bronchitis (IB) was one of the earliest live attenuated IB vaccines to be developed and has continued to be used in most parts of the world for almost 50 years. It was developed for use at both the 52nd (H52) and 120th (H120) vaccine levels and, because of it ability to provide heterologous cross-protection against a number of IB viruses of different serotypes, has proved to be one of the most enduring live attenuated IB vaccines. In fact, the H120 vaccine is possibly the most widely used live attenuated IB vaccine globally to this day. The use of H52 has, however, declined with the introduction of safe and highly efficacious inactivated IB vaccines. This review documents the original studies to isolate and attenuate the H strain by serial embryo passage, and describes the early studies to demonstrate its efficacy in laboratory studies and under field conditions. The efficacy of the H vaccine in providing cross-protection against some of the many IB variants now reported worldwide is also discussed, and possible future vaccination strategies for IB considered.

Introduction

Schalk & Hawn (1931) described an unknown respiratory disease in neonatal chicks in the US. Considerable losses were observed and the disease was highly contagious. They named the disease "infectious bronchitis of young chicks". Beach & Schalm (1936) showed that the disease was caused by a specific virus, which is now known as infectious bronchitis virus (IBV), a member of the *Coronavirus* genus within the *Coronaviridae*. For the first time, Beaudette & Hudson (1937) grew the virus on the chorioallantoic membrane of 12-day-old embryonated chicken eggs.

In the past 50 years many new infectious bronchitis (IB) serotypes or variants have been described (Jungherr *et al*., 1956; Anonymous, 1988, 1991, 1998), although until 1960 the Massachusetts serotype was the only one diagnosed in the Netherlands (Hoekstra & Rispens, 1960a). These serotypes may arise either as a result of spontaneous mutation or by recombination (Kusters *et al*., 1990; Cavanagh *et al*., 1992; Wang *et al*., 1993; Jia *et al*., 1995). Cross-protection between different serotypes is variable or poor (Cavanagh, 2003) and therefore the choice of vaccine strain should be carefully considered. Careful and correct diagnosis (De Wit, 2000) is also needed for IBV identification as outbreaks of IB still occur in vaccinated flocks, and the virus strains isolated from such outbreaks are frequently of a different serotype from the vaccine strain applied.

Bijlenga (1956) diagnosed IB for the first time in the Netherlands by means of virus isolation, experimental infection of chickens and serum neutralization (SN) tests. This virus isolate was used to prepare a vaccine by serial passage in embryonated chicken eggs up to the 52nd passage level (Bijlenga, 1960). The vaccine was named IB vaccine strain H52, the H standing for the initial letter of the name (Huyben) of the owner of

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the broiler farm in the province of Brabant, in the southern part of the Netherlands, from which the virus was isolated. The letter H has often been used mistakenly (Rosenberger *et al*., 1976; Winterfield *et al*., 1976a; Winterfield *et al*., 1976b; Owen *et al*., 1991) as an abbreviation of "Holland". This publication, while rectifying this mistake, will concentrate on describing the origin of the H strain vaccine, its isolation and adaptation to embryonated eggs, as well as the original experimental and field vaccinations performed almost 50 years ago.

Because information on the H strain was published in a thesis in German (Bijlenga, 1960), the work is not widely recognized. For this reason this publication will clarify at an international level the continuing confusion (Anonymous, 2003) surrounding the origin and development of this vaccine that is still used worldwide. This review also discusses cross-protection studies using the H vaccine strain and touches on the use of other IB vaccines. Problems related to the occurrence of multiple serotypes and subtypes of IB virus are also considered. Finally, we look into the future and discuss some of the potential attributes of IBV vaccines for the future.

Origin of the IB Vaccine Strain H

On 29 June 1955, 6-week-old broilers were received at the State Serum Institute, Rotterdam, the Netherlands, from Huyben's farm, North Brabant. These broilers showed respiratory problems similar to those observed on the farm since 1954. Paralysis was not observed and the faeces were normal. Mortality in the flock was 30% to 40% with morbidity ranging from 90% to 100%. Haemagglutination inhibition (HI) tests for Newcastle disease (ND), performed on serum samples, were negative, as were virus isolation attempts using brain tissue from two of the broilers. In view of the clinical signs, it was decided to try to isolate the causative agent, presumed to be IB virus.

Isolation and Adaptation of IBV in Embryonated Chicken Eggs to Provide the Vaccine Strain H

Tracheal mucus from two broilers showing respiratory problems was suspended in sterile physiological saline containing antibiotics. After 30 min at room temperature the supernatant was inoculated into the allantoic cavity of five 10-day-old embryonated chicken eggs (0.1 ml per egg). After 6 days of incubation the embryos were still alive and the allantoic fluid (AF) was harvested. No embryonic abnormalities were observed and the AF was inoculated in the same way into another five 10-day-old embryonated eggs (blind passage). At the second egg passage, two embryos died on day 7 of incubation. The AF of these two eggs was harvested and the next passage was performed with this material. All 51 passages were carried out using AF from embryos that died, with the exception of the first blind passage (Table 1). On the third passage, one embryo died 9 days after inoculation. The amnion was thickened and adhered closely to the embryo. The dwarfed embryo weighed only 5 g (at that age a normal embryo weighs approximately 20 g) and was curled into a spherical form with its deformed feet over its head (Bijlenga, 1956). These embryonic changes are typical of an IB infection and the provisional diagnosis was that this virus was the causative agent of the problem at the Huyben farm.

With continuing passage, the virus became adapted to embryonated eggs (Table 1). The dwarfing effect was observed until the 30th passage, but at higher passages this phenomenon disappeared and embryo mortality was observed. After the 51st passage, at which level all the inoculated eggs had died within 48 h of inoculation, it was decided to test the virus as a potential vaccine. A stock of virus was prepared at the 52nd passage level, sufficient to perform experimental vaccination studies under laboratory and field conditions.

Original Laboratory and Field Vaccinations with the IB Vaccine Strain H Performed in 1956

Experimental vaccination under laboratory conditions

In this experiment, 140 1-week-old broiler chicks were vaccinated by mixing the vaccine in the drinking water. Fifty chicks were vaccinated with an early (25th) passage of the vaccine, a second group of 50 chicks with the 52nd passage and the third group of 40 chicks were kept as the non-vaccinated control group. No clinical signs were observed in the group vaccinated with the 52nd passage level, whereas in the group given the 25th passage two chicks died at 24 and 27 days after vaccination, respectively. Macroscopic lesions in the respiratory tract of these chicks included tracheal mucus, tracheitis and airsacculitis.

At 31 days, 6 and 12 weeks, and 6 months after vaccination blood samples were collected from both vaccinated groups in order to perform SN tests using the highly egg-adapted Beaudette strain of IBV at passage level 222 and the constant serum, diluted virus method (Bijlenga, 1956, 1960). Both groups showed very high IB SN titres (i.e. neutralization indices of at least 20,000, while negative titres were 1.3). No marked differences between the groups were observed. Serum antibody titres in the chicks vaccinated with the 52nd passage remained high 6 months after vaccination. The sera from chicks vaccinated with the 25th passage level were not examined at 6 weeks, 12 weeks or 6 months

Table 1. *Mortality following passage of the IB virus H strain 51 times via the allantoic cavity of 10-day-old embryonated eggs*

Passage number	Number of eggs inoculated	Number of dead embryos (days after inoculation)								
		1	2	3	4	5	6	7	8	9
1	5	Blind passage								
2	5							2		
3	5									1
4	4						1		1	
5	4							3		
6	5				3					
7	5				4					
8	5				1					
9	5			1			1			1
10	5		1	1						
11	5		3		1					1
12	5		3	1	1					
13	5		4			1				
14	5		2	1				2		
15	10		4	1		1				
16	5		4	1						
17	5		2							
18	5		3							
19	10		3	3		2				
20	10		5	1	1	1	2			
21	5		1	1						
22	5			1	1	1	2			
23	5		1			1				1
24	5		2	2						
25	15		12	1	2					
26	10		6	2		2				
27	10	1	2	3			4			
28	5		2	2	1					
29	3		3							
30	5			1	1		1			1
31	5		1	1						
32	5			2						
33	5		2		1					
34	5		4							
35	5			3						
36	5		1	2	1					
37	10		1	1	3		1		1	1
38	3			1	1					
39	5				1		2	1		
40	3		3							
41	5		3	2						
42	5			2	3					
43	3		2		1					
44	3		2	1						
45	5			3	2					
46	5		4	1						
47	5		2	2	1					
48	5		4	1						
49	5		1	4						
50	4		4							
51	4		4							

after vaccination as that virus was considered too virulent.

Experimental field vaccinations with the 52nd passage level of IB vaccine strain H

Field trials were performed in 1956 in close collaboration with veterinary services inspectors in the eastern, southern and western part of the Netherlands. All birds were vaccinated via the drinking water with a vaccine virus dose of between 1000 and 8000 median embryo infectious doses per millilitre. Care was taken to use utensils that were clean so that no disinfectant was applied. Also, exposure of the water containers to sunlight (ultraviolet) was avoided to prevent inactivation of the vaccine virus.

Four poultry farms were selected where IB had previously occurred and chicks were vaccinated as follows: 490 at 1 week old, 340 at 3 weeks old and 570 broilers at 5 to 6.5 weeks old. No clinical signs were observed during the first 7 days after vaccination, but at 8 to 14 days after vaccination respiratory problems were seen on three of the poultry

farms where chicks had been vaccinated at between 1 and 3 weeks of age. The mortality varied between 0.2% and 2.4%. The broilers vaccinated at 5 to 6.5 weeks showed no clinical signs. Blood samples were collected at 7 weeks and 4.5 months after vaccination. The majority of sera collected at 7 weeks showed very high SN titres, and 67% of the sera collected at 4.5 months after vaccination still had very high titres (Bijlenga, 1956, 1960).

On another poultry farm in the southern Netherlands, where ND and IB problems had been observed, a combined vaccination experiment using the 52nd passage level of the H strain and the Hitchner B1 strain of ND was carried out via the drinking water. In total, 2400 chicks at 8 days of age were vaccinated against ND and IB. Ten blood samples were collected before vaccination. These sera showed high IB SN titres (probably due to maternal antibodies). At 9 days after vaccination very slight respiratory signs were observed, probably due to the IB vaccine. The mortality observed was less than 1%. At 7 weeks after vaccination very high IB SN titres were observed. No ND HI titres were encountered before vaccination, while at 14 days after the combined vaccination nine out of 10 sera showed ND HI titres.

In order to examine the influence of the 52nd passage of the vaccine strain H on egg production, two poultry farms were selected in the western Netherlands, an area then free of IB. A small number of blood samples of different age groups were collected and the serum samples examined by SN test before vaccination were all negative, thus confirming that IB infection had not occurred in this region. Egg production was recorded for 10 days before vaccination in order to observe any possible subsequent decline in production. Then, 550 19-month-old chickens, 109 at 17 months of age, 105 at 7 months of age, 582 at 6 months and 407 at 5 months of age were vaccinated via the drinking water with a final dose of vaccine of 1000 to 5000 median embryo infectious doses per bird. Hens that had not yet started to lay were thus included in this trial. No clinical signs were observed after vaccination and the vaccination procedure did not have any adverse influence on egg production or on egg shell quality. Six to 8 weeks after vaccination very high SN titres were observed, indicating an immunological response to vaccination.

At this time it was considered that the 52nd passage of the IB vaccine strain H was too virulent for chicks, which was confirmed four years later by Hoekstra & Rispens (1960b). Therefore further egg passages were necessary to attenuate the virus for use as a vaccine in chicks. It was decided to obtain the 120th passage level, which proved to be of greatly reduced virulence (Hoekstra & Rispens, 1960c). This virus is now known as the H120 vaccine, while the lower passaged (52nd passage) vaccine, of higher virulence, is known as the H52 vaccine strain.

Current Application of IB Vaccine Strain H

The H120 vaccine virus has been used successfully as a primary vaccine in broilers worldwide for almost 50 years and also for the initial vaccination of breeders and future layers. Field strains of the Massachusetts serotype are believed to be a continuing cause of disease in many areas of the world (Li & Yang, 2001; Meulemans *et al*., 2001; Farsang *et al*., 2002; Cavanagh & Naqi, 2003) and this, together with the evidence for broad protection with the H strain vaccines (see later), probably accounts for its continuing success. The exception to this is Australia, a country that has developed its own, unique lineages of IB serotypes (Sapats *et al*., 1996) and where the H vaccine strains have not been licensed.

As with any live-attenuated IB vaccine, the H vaccine strains must replicate in the respiratory tract if they are to stimulate protective immunity. It follows, therefore, that some damage to the epithelial layer of the respiratory tract will occur and a fine balance must be achieved between a strain that is so over-attenuated that it is unable to replicate sufficiently to stimulate immunity and one that is insufficiently attenuated so that serious damage may occur. The H120 virus meets these criteria well and is ideally suited for use in young, susceptible chicks, while the H52 vaccine, being less attenuated, is only suitable for use in older chickens that already have some protective immunity as a result of the earlier application of a milder (more attenuated) vaccine or perhaps an IB field exposure. Also, despite the proven safety of the H120 vaccine for young susceptible chicks, it must be remembered that any live IBV vaccine can increase susceptibility to colibacillosis under high *Escherichia coli* pressure (Smith *et al*., 1985; Matthijs *et al*., 2003).

The function of live-attenuated vaccines is to stimulate local, systemic and cell-mediated immunity (CMI) to the virus. Immune responses to IBV have been reviewed (Dhinakar Raj & Jones, 1997b; Cavanagh, 2003). Using either the H strain (Gillette, 1981; Toro & Fernandez, 1994) or the M41 strain (Hawkes *et al*., 1983), the importance of local immunity in the respiratory tract as a first line of defence against IB challenge has been demonstrated. Furthermore, Davelaar & Kouwenhoven (1976, 1980), using both the H120 and H52 vaccine strains, have demonstrated the importance of the Harderian gland in the development of the immunity induced by IB vaccination. These findings were confirmed by Toro *et al*. (1996) who, in a histological study, demonstrated increased plasma cell numbers and enlargement of lymphoid foci following eye-drop administration of

the H120 vaccine. It is therefore essential that this local immunity is stimulated by the careful application of the live attenuated IB vaccines. In addition, the induction of high and persistent levels of systemic immunity is necessary to provide life-long protection against IB challenges. Although the role of CMI in protecting against IB challenges has been less well studied than have other aspects of IB-induced immunity, there is evidence that CMI responses occur following vaccination with the H strain (Timms & Bracewell, 1981). Furthermore, it has been suggested that the CMI response to H120 vaccination may be cross-reactive (Dhinakar Raj & Jones, 1997a). Correct and careful application of the IB vaccine is therefore essential to optimize the stimulation of all types of protective immunity.

Heterologous Cross-protection using IB Vaccine Strain H

Immunity of the chicken to IBV following initial live virus vaccination or natural infection is generally very solid and long-lasting upon challenge with strains of the same (homologous) serotype. Of course the magnitude and duration of the response to vaccination is dependent on many factors, including the age of the chick, levels of maternal immunity, immunogenicity of the vaccine, method of vaccine application, virulence of the field strain challenge, the 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 life-long.

The ability of vaccinated chickens to resist challenge with a strain of a heterologous serotype, however, is much more in doubt. IBV serotypes that are highly unrelated to one another generally do not cross-protect well. This issue is a primary reason why IB is so difficult to control under field conditions, where flocks are likely to become infected with heterologous field strains commonly referred to as variants. The H strain, a Massachusetts serotype IB vaccine, has shown a rare ability to cross-protect against heterologous serotypes, thus making it potentially effective for controlling a broader range of serotypes. In the 1970s, research laboratories evaluated the lower egg-passaged H52 strain as a vaccine against heterologous strains found in the US. Winterfield & Fadly (1975) reported that an H53 vaccine immunized well, providing 70% to 100% protection against challenge with the Connecticut, JMK, Iowa 97, Iowa 609, Florida, Holte, Gray, MD27 and SE17 serotypes. Rosenberger *et al*. (1976) found H52 cross-protected well (58% to 88%) against challenge with US serotypes Connecticut, JMK, Florida and Holte, but to a much lesser degree (11% to 33%) against Arkansas 99, Gray and Maine 209. Protection in these studies was assessed 4 weeks after vaccination by challenging the chicks via the respiratory tract and performing virus recovery attempts from the trachea 4 or 5 days later.

Higher egg-passaged H strain vaccines were also evaluated for their cross-protective ability. Winterfield & Fadly (1975) found that as the egg passage of the vaccine increased, so did attenuation, but cross-protection waned, although not completely. The highly attenuated H121 vaccine protected on average only about 50% as well when compared with the parent H53 strain, only one egg pass removed from the H52 vaccine. The H120 vaccine was found to induce some protection of the respiratory tract against heterologous challenge with strains of the Belgian B1648, French 84084 and French 84221 serotypes (Cook *et al*., 1999), although protection was improved if the chicks were revaccinated with a vaccine (4/91) of a heterologous serotype (Parsons *et al*., 1992). In this work, protection of the respiratory tract was assessed by quantifying the damage caused to the epithelium of the trachea following challenge.

In an attempt to mimic the situation commonly seen in the field, Cook *et al.* (1986) vaccinated 1-day-old chicks with the H120 vaccine and challenged them with a mixed infection comprising a pool of *E. coli* isolates and heterologous IBV strains, not of the Massachusetts serotype. Good cross-protection was found following challenge with several but not all of the heterologous IBV strains examined when given in conjunction with the *E. coli* challenge. Those where good cross-protection was observed included Holte and Iowa 97 (USA), Australian T, D 207, D 3896 (the Netherlands) and several UK isolates.

Research into the use of intermediate egg-passaged levels of H strain vaccines found that they could be used safely in commercial type broilers without serious respiratory side effects and with the retention of cross-protective characteristics. Egg passage 92 H strain used for primary vaccination of 1-day-old broilers initiated cross-protection against heterologous serotype challenge (Winterfield *et al*., 1976a). Booster vaccination with intermediate level H vaccines was found to increase cross-protection levels (Winterfield *et al*., 1976b). Further enhancement of cross-protection against variant IB strains was observed when an intermediate H strain vaccine was given in combination with the Arkansas DPI strain (Gelb *et al*., 1991).

At the present time, although the use of oil adjuvanted, inactivated IB vaccines as booster vaccines for breeder and layer pullets flocks has become common practice in many parts of the world, the H52 strain is still used for this purpose in some areas. However, H52 is not commonly used as vaccine in young broilers or pullets because of potential virulent side effects when used for primary vaccination (Winterfield *et al*., 1976a). On

the other hand, the intermediate passage levels of the H strain are safely used as vaccines when a broader spectrum of cross-protection is needed against variant IBV challenge (Gelb *et al*., 1991).

Prevention of Virus Transmission after H120 Vaccination

In the field, protection at flock level is of greater interest than protection at bird level because it is important to know if a vaccine will prevent transmission of an IB challenge virus from other infected birds to that flock. However, in vaccination and challenge trials, the efficacy of an IB vaccine is usually expressed as the percentage of vaccinated birds protected against challenge. Therefore, in order to obtain more relevant information, a different approach to vaccine efficacy determination has been investigated (De Wit *et al*., 1998). These workers performed a study of flock immunity after H120 vaccination and M41 challenge in order to determine whether vaccination reduced virus transmission; that is, to test whether IBV transmission among vaccinated chickens was significantly reduced compared with that among unvaccinated chickens. In two vaccinated and two unvaccinated groups of specific pathogen free chickens, a standard measure for virus transmission, the reproduction ratio, was determined (the reproduction ratio being defined as the average number of new infections caused by one typical infectious individual during its entire infectious period). It was found that a single eye-drop vaccination with H120 reduced the transmission of the challenge virus among the vaccinated chickens significantly compared with the transmission among the unvaccinated chickens. H120 is the only IBV vaccine that has so far been tested in this way.

Future IB Vaccines

Control of IB is arguably the most challenging of all poultry infectious diseases. New variant serotypes emerge or are introduced into poultry in an unpredictable manner. In many countries, the native indigenous serotypes are only now beginning to be discovered and characterized. Therefore, identification of prevalent serotypes is needed to develop effective vaccines to control the disease.

As discussed earlier, live vaccines provide excellent and often sterilizing immunity against homologous serotype challenge but generally not against variant heterologous serotypes. However, some live vaccine strains such as the H52 strain may also provide cross-protection against heterologous serotypes. Live attenuated vaccines are cost-effective for the poultry industry, especially for broiler production, although two drawbacks of such vaccines are worthy of mention. Live vaccines produce varying degrees of pathology that may make them unsuitable for use in some flocks depending on various factors, such as their age, maternal or active immune status and the presence of concurrent infections. Second, the difficulties associated with, and the importance of, the correct application of live IB vaccines has probably been underestimated. Vaccine instability and reversion to virulence is a serious concern when vaccines are accidentally or purposefully over diluted. Clearly improvements in live vaccine stability and safety are needed in the future.

Inactivated IB vaccines, when used for booster vaccination, are beneficial for the egg-laying performance of breeder and layer hens. However, inactivated IB vaccines are effective only when used as boosters in flocks following priming vaccination with live-attenuated IB vaccines. Immunization with inactivated vaccines reduces, but generally does not eliminate, viral shedding following challenge with a virulent strain of the homologous serotype (Ladman *et al*., 2002). Inactivated IB vaccines do not generally cross-protect well against heterologous challenge, although, if given prior to 18 weeks of age, they may protect pullets after they are placed on layer farms or complexes where variant serotypes are known to exist (Ladman *et al*., 2002). While expensive because of the cost of production and the need for administration by injection, inactivated vaccines are safe because they do not replicate in the bird. Improvement of the efficacy of killed IB vaccines as primary immunogens when applied to the respiratory tract would have the benefit of inducing local respiratory immunity without the drawbacks associated with the use of live vaccines.

New technologies will provide opportunities for vaccine improvement. The use of live attenuated IB vaccines *in ovo* (Wakenell & Sharma, 1986; Wakenell *et al*., 1995) has been investigated but subsequent hatchability was shown to be poor (Chew *et al*., 1997), probably because of the inherent ability of all IBVs, even the attenuated strains, to replicate in and to damage or produce pathology in the chicken embryo. Other immunization technologies include using transgenic-derived IBV protein (Song *et al*., 1998; Zhou *et al*., 2003), DNA vaccination (Seo *et al*., 1997; Kapczynski *et al*., 2003), homologous (IBV) vectored recombinant (Casais *et al*., 2001, 2003; Dove *et al*., 2001; Hodgson *et al*., 2004) and heterologous virus-vectored vaccines (Yu *et al*., 2001; Wang *et al*., 2002; Johnson *et al*., 2003). Vaccine delivery will continue to be an issue and advances in the field of avian immunology may well prove beneficial here. However, much of this technology is in its infancy and, while it is promising, its efficacy, method of delivery and cost are important considerations that will determine the actual utility for the poultry industry. It seems likely that it will be many years

before novel IB vaccines are commercially available and therefore, for the foreseeable future, it will be necessary to continue to optimize the use of the currently available vaccines such as H120 and H52.

Conclusions

Other live attenuated IB vaccines, both of the Massachusetts serotype as well as of other serotypes as these came in to prominence, have been developed over the years. However, the H vaccine strain, particularly the H120 vaccine and the intermediate egg-passaged H vaccines, have stood the test of time and are still used worldwide, some 50 years after their initial development. This indicates the value and efficacy of the H vaccine strain against challenge with both the homologous and some heterologous serotypes. Furthermore, the vaccine has been found to conform to the rigorous safety standards now required of avian vaccines.

Finally, despite the advances in molecular biology and the novel approaches considered for the development of avian and other animal vaccines, the apparently simple method used for attenuation of IBV, namely serial passage through embryonated eggs, has continued to be an effective way of producing safe and inexpensive vaccines for the H strain of IB, as well as for many other variants (Gelb & Cloud, 1983; Jackwood *et al*., 2003).

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