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Cross-immunity study of two experimentally oil-emulsion inactivated infectious bronchitis vaccines

Momayez[1], R., Bozorgmehrifard[*2], M.H., Toroghi[3], R., Vasfi-Marandi[2], M., Shoshtari[1], A.H.

1. Department of Avian Diseases Research and Diagnostic, Razi Vaccine and Serum Research Institute, Karaj, Iran
2. Poultry Diseases Division, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
3. Department of Research & Biotechnology, Razi Vaccine & Serum Research Institute, Mashhad, Iran.

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ABSTRACT

A study has been carried out for determining of cross-immunity between Massachusetts (Mass) M41 and Iranian IR/773/2001 (793/B) strains of infectious bronchitis virus. Two groups of 20 three weeks old SPF chickens vaccinated subcutaneously with two oil-emulsions inactivated Infectious Bronchitis Vaccines (Mass M41 and Iranian IR/773/2001 strains) that had been manufactured in Razi institute in the laboratory scale. A further group of 20 SPF chickens were kept as non-vaccinated control. Four weeks post vaccination half part of the birds of the each vaccinated groups were challenged by eye drop with 10^3EID50 of M-41 strain of Mass serotype and another part of the birds of the each vaccinated groups were challenged with 10^3EID50 of Iranian IR/773/2001 793/B strain. The birds of the control group were also divided in two parts and challenged with the viruses (M-41 and IR/773/2001) like the birds of the vaccinated groups. Each of the birds of the vaccinated groups manifested a very strong resistance to homologous virus strains and only 10% of them showed ciliostasis in tracheal section examination. Whereas after challenge with heterologous virus strains, the vaccinated birds with IR/773/2001 killed vaccine revealed 50% resistance against M41 and the birds vaccinated with M41 killed vaccine only showed 30% immunity against IR/773/2001 based on the degree of 50% and 70% ciliostasis in tracheal section examination respectively. All of the control birds showed complete ciliostasis without any protection against the challenge of the both viruses. Upon the results, it is suggested that the distribution of 793/B serotype should be carefully studied in different parts of Iran for appropriate vaccination programmes against infectious bronchitis.

Keywords: Infectious bronchitis virus, Cross-immunity, Massachusetts M41, IR/773/2001 (793/B), Inactivated oil emulsion vaccine

INTRODUCTION

Infectious bronchitis (IB) is an acute, highly contagious of upper respiratory and urogenital disease of chicken (Case *et al* 1983, de Wit *et al* 1998 and King & Cavanagh 1991). IB is considered one of the major poultry diseases, being probably endemic in all regions with intensive poultry production and despite the wide use of live and inactivated vaccine; it is still one of the most

* Author for correspondence. E-mail: mhbfard@ut.ac.ir

important poultry diseases in many countries of the world (OIE 2008). Infectious bronchitis virus (IBV) is the causative agent of the disease (King & Cavanagh 1991, Picault *et al* 1986). IBV causes various disease manifestations, as a respiratory disease in chicken that was first reported by Schalk & Hawn (1931) in USA, affecting both quantity and quality of the eggs and also causes renal damage in both young and adult birds (King & Cavanagh. 1991). These viruses are group 3 member of Coronaviridea family, single-stranded positive-sense and containing the largest no segmented RNA genome among the RNA viruses (Cavanagh *et al* 1992, Gelb *et al* 1991, Jia *et al* 1995). As a result of molecular studies, it is now known that the S1 part of the large spike glycoprotein (S) of the IB virus is responsible for determining its serotype (Cavanagh *et al* 1992). Because of its size and molecular composition, IBV has a tendency to form new variant strains that are apparently not protected by commercial vaccines. Therefore, vaccination programs for IBV become difficult to implement because of the face of challenge with a novel virus and it is of great importance to chicken producers to identify the strains of IBV in their flocks so vaccination programs can be modified according to the IBV strains present (Hofstad 1981, Wang *et al* 1996). IBV has been diagnosed in Iran since the early twenties by virus isolation and serological techniques (Aghakhan *et al* 1994). The isolate showed antigenic relationship to the mass serotype. Vasfi Marandi *et al* (*2000)* also identified the IBV in chicken flocks by serological and isolation methods. Momayez *et al* reported the isolation and identification of several IBV isolates from commercial chicken flocks of Iran in 2002. In spite of regular vaccination with live attenuated and inactivated vaccines of mass strains to protect commercial chickens, an epidemiological survey pointed out IB is still responsible for serious financial losses to poultry industry of Iran, causing mortality and adverse effects on quantity and quality of egg production as well as renal failure in broilers and layers. Recently several researchers detected new serotype (793B strain) by RT-PCR technique (Nouri *et al* 2003, Seify Abad Shapouri *et al* 2000, 2002, 2004, Shoushtari *et al* 2006). The 793B serotype may have entered the UK in the winter of 1990/91, when it was sometimes associated with deep pectoral muscle myopathy in layers, in addition to the more usual manifestations of infectious bronchitis (Gough *et al* 1992) and also known as 4/91 (Parsons *et al* 1992). This serotype was isolated in France and known as CR88 (Picault *et al* 1995). Subsequently this serotype has been detected in several European countries, at different frequencies (Cook *et al* 1996, Capua *et al* 1999, Cavanagh *et al* 1999, Meulemans *et al* 2001, Farsang *et al* 2002). Toroghi *et al* identified that the some IBV viruses that had been isolated by Momayez *et al* in 2002 belong to 793/B serotype. The sequence analysis of S1 gene has revealed that Iranian strains of 793/B are very different from the other isolates in the worlds. By RT-PCR using general primer, the Iranian IR/773/2001 isolate amplified 1720 base pairs (bps) of nucleotides of the S1 gene. By using Nested-PCR technique performed on the RT-PCR product of the isolate with the specific primers of Massachusetts (Mass) and 793/B (4/91) types were revealed that the isolate belong to 793/B serotype. The nucleotide sequencing of the whole S1 subunit of the isolate was identified. The sequencing analysis indicated that the virus classified in 793/B serotype. The IR/773/2001 isolate have $\geq$ 97% nucleotide identity and $\geq$ 95.7% deduced amino acid identity with other Iranian isolates. The whole amino acid sequences of 793/B isolate differed by 29 to 30.5% from H120 vaccine strain. Four amino acid substitutions at positions 156, 349, 392 and 393 were found to be specific for Iranian 793/B isolates (Toroghi *et al* 2004). The molecular analysis has shown that 793/B serotype has become a major

population of IBV in regions of Iran (Shoushtari *et al* 2008). Because many differences of S1 gene, this might therefore, suggest that the currently available vaccine with the Iranian793/B isolate could be able to provide better protection against challenge with local strain. Therefore we decided to produce inactivated vaccine with an Iranian isolate of 793/B. This study was conducted to evaluate cross immunity of experimentally inactivated vaccine that produced with Iranian isolate of 793B and M41 strain in SPF chickens to challenge with of the live viruses by the ciliary's activity present in tracheal rings as indicators of immunity.

MATERIALS AND METHODS

Viruses. The Iranian isolate IR/773/2001 (793B serotype) and the Massachusetts M41 strains were used for killed vaccines production as well as challenge examinations in vaccinated chickens. The IR/773/2001 strain was isolated from Iran and classified by RT-PCR, Nested-PCR and sequencing (Momayez *et al* 2002, Toroghi *et al* 2004).

Chick embryos and chickens. White Leghorn Specific pathogen free (SPF) embryonated chicken eggs were obtained from Lohmann Company (Cuxhaven, Germany). Chicken embryonated eggs were used for production of viral seed stocks, virus titration and preparing tracheal rings. White Leghorn chickens SPF was originated from the mentioned above embryonated eggs. The chickens were used for cross-challenge experiments and antisera production. The chickens were housed in restricted place.

Titration of the viruses. The harvested viruses were titrated by inoculating 10-fold serial dilutions (10^{-3}-10^{-9}) with phosphate-buffered saline (PBS) of the virus stocks into the chorioallantoic sac of five 10-day-old SPF chicken embryonated eggs. Allantoic cavity of the embryonated egg was inoculated with 0.1 ml of each dilution of the viruses. The inoculated embryonated eggs were placed into 37 ºC incubator. The eggs were candled daily, with mortality between days 2 to 7 after inoculation considered being virus-specific. The end of 7 days post inoculation, the dead and live embryos were observed for typical IBV lesions, such as stunting, curling, clubbing of the down, or urate deposits in the mesonephros of the kidney. The titers were expressed as the 50% embryo-infective dose (EID50) calculated by the method of Spearman-Karber (Gelb & Jackwood 1998, Villegas, 1998). The titers of the IBV viruses for each dose of vaccines were adjusted to $10^{6.5}$ EID50 before inactivation.

Production of experimental vaccines. Strain of IR/773/2001 a recent Iranian isolate of a new serotype, variously called 793B or 4/91 (Gough *et al* 1992, Parsons *et al* 1992) and M41 of the Massachusetts serotype were used as the IBV seed vaccines. The viruses were inoculated into the allantoic cavity of the SPF chicken embryonated eggs, and the allantoic fluids were harvested 48 h later. The titers of harvested viruses were determined before inactivation. The harvested viruses were inactivated using 0.1% betapropiolactone with continuous stirring at 25º C for 2h. The test for inactivation consisted of 3 blind passages in 10 SPF chicken embryonated eggs. After complete inactivation, the killed viruses were emulsified with ratio 30 to 70 in an aqueous phase of an oil adjuvant emulsion ISA-70 (SEPPIC, Cosmotics/Pharmacy Division, Paris, France). Each dose of the vaccines consisted of 0.5 ml with 0.05 mg Tiomersal. The whole procedures of the vaccines production were carried out in controlled air conditions under a Lamin Air flow unit. The vaccines were stored at 4º C.

Sterility test. The antigens were tested for bacterial and fungal contaminations.

Stability test. The stability of the vaccines was tested for one week at 37º C and different periods of time at 4º C. There should be no physical change in

the vaccine and it should regain its former emulsion state after one quick shake.

Safety test. A double dose of the vaccines was injected subcutaneously into each of ten 21-day-old SPF white leghorn chickens. The chickens were observed for a period of 21 days for abnormal reactions.

Chickens used for experiments. The experimental birds consisted of three groups of twenty one-days-old SPF white leghorn chickens from the poultry diseases vaccines department. Each group of chickens was placed in separated areas and reared to the end of experiments.

Vaccination. At the age of 21, the birds of group one were vaccinated subcutaneously with one dose of the 793B inactivated vaccine. The birds of group two were vaccinated with M41 inactivated vaccine as above. The birds of group three without vaccination were also injected subcutaneously with 0.5 ml tryptose phosphate buffer, raised in restricted area as control group. The design used in this experiment is summarized in Table 1.

Challenge. At approximately 4 weeks post-vaccination, 10 chickens of each group were challenged via eye-drop with 10^3 EID50 IR/773/2001 (793B) strain. The other 10 chickens of each group were challenged as above with 10^3 EID50 Massachusetts (M41) strain.

Serological examination. Chicks were bled prior to challenge at 4 weeks post-vaccination and their sera were tested for determining antibody titers by haemagglutination inhibiting (HI) based on OIE recommendation (Alexander *et al* 1983), using both M41 and 4/91 antigens. Titers are expressed as log2 of the reciprocal value of the highest serum dilution showing complete HI.

Assessment of protection against challenge. In each experiment, at 5 and 7 days post-challenge, half the chicks in each group were humanely killed. The tracheas were carefully removed and examined for ciliary's activity as described previously (Cubillos *et al* 1991, Cavanagh *et al* 1997). Briefly, ten thin tracheal rings (3 from the top and bottom and 4 from the middle of the trachea) are selected from each bird. The rings were examined by low-power microscopy and ciliary activity scored as follows: 0, all cilia beating; 1, 75% beating; 2, 50% beating; 3, 25% beating and 4, none beating (100% ciliostasis).

RESULTS

The results of the vaccines production, the titers of virus yield in harvested allantoic fluid were as high as $10^{8.5}$ EID50/ml for IR/773/2001 and 10^8 EID50/ml for M41 strains. The titers of the IBV viruses in the harvested fluids for each dose of vaccines were adjusted to 6.5 Log10 EID50 before inactivation. After inactivation with betapropiolactone the inactivation of the antigens confirmed by the inactivating test, because the inoculated embryos did not show any specific IBV signs such as mortality and / or dwarfing, stunting, curling and urate deposits in the kidney of the embryos after at least 3 blind passages. The antigens of the vaccine were sterile and the bacterial and fungal media did n't show any contamination. Emulsification of the antigens in aqueous phase of the oil adjuvant (Montanide ISA-70) with ratio 3 to 7 was appropriate by using homogenizer. Vaccines stability test showed that the vaccines were stable without any changes for one week at 37 ºC and at least for 3 months at 4º C. At these temperatures there was no physical change in the vaccines and they regained their emulsion state after one quick shake. The vaccines were safe for chickens and none of the inoculated birds showed noticeable abnormal local or systemic reaction. Feed consumption and water drinking of the vaccinated chickens did not differ from those of the control group. No macropathological reactions were observed at the site of injections. The results of serological responses in SPF chickens, 4 weeks post-vaccination are summarized in Table 1. Both two groups of the vaccinated SPF chickens, showed mean HI titers 6.2 and 6.6 (Log2) for M41 and

IR/773/2001 respectively. The results of challenge in cross immunity test are manifested in Table 2. Ten chickens of the group 1 and group 2 that had been vaccinated with IR/773/2001 and M41 showed a very strong immunity with 90% resistance against homologous viruses, because the vaccinated birds revealed only 10% ciliostasis in tracheal section examination.

Table1. Mean HI titers of the vaccinated and unvaccinated chickens 4 weeks post-vaccination.

Groups	Age of vaccination	Type of vaccine	HI titer $\log_2$ 4 weeks post vaccination
1 (20 chickens)	3-week-old	IR/773/2001/2001	6.6
2 (20 chickens)	3-week-old	Mass (M41)	6.4
3 (20 chickens)	3-week-old	unvaccinated	1.2

Table 2. Evaluation of Cross-immunity in vaccinated chickens by Ciliostasis test.

Groups 3 weeks old SPF chickens	Type of vaccine	Ciliostasis test	
		Challenge IR/773/2001 virus	Challenge M41 virus
Group 1	Inactivated IR/773/2001	10% (score = 0.4)	50% (score = 2)
Group 2	Inactivated M41	70% (score = 2.8)	10% (score = 0.4)
Group 3	unvaccinated	100% (score = 4)	100% (score = 4)

Another ten chickens of the group 1 just showed 50% resistance against heterogonous M41 virus. Whereas another ten chickens of the group 2 showed very weak immunity and revealed 30% resistance with 70% ciliostasis against IR/773/2001 virus. The challenge strains caused 100% typical ciliostasis in control birds. The results of challenge in cross immunity test are manifested in Table 2. Ten chickens of the group 1 and group 2 that had been vaccinated with IR/773/2001 and M41 showed a very strong immunity with 90% resistance against homologous viruses, because the vaccinated birds revealed only 10% ciliostasis in tracheal section examination.

DISCUSSION

Infectious bronchitis (IB) is one of the significant viral diseases in chickens and causes of economic losses in the poultry industry worldwide. IBV also poses a constantly changing threat to the poultry industry because new serotypes and variants of the virus continue to be isolated even from vaccinated flocks. Numerous serotypes or IBV variants with little or even no cross-protection have been identified in the world (Hofstad 1981, Picault 1986, Wang *et al* 1996). In some European countries, strains D274, D1466, and 4/91(793/B) vaccines are used in addition to H120. In Australia, strains of their B and C subtypes are used (Klieve *et al* 1988, Wadey *et al* 1981). Since the winter of 1990/1991, with the occurrence of infection by a new serotype (793/B) of IBV, unusual pathology has been observed in the United Kingdom (Gough *et al* 1992 and Parsons *et al*, 1992) and in France as CR88 (Picault *et al* 1995). This serotype was sometimes associated with deep pectoral muscles myopathy in layers, in addition to the more usual manifestations of infectious bronchitis (Gough *et al* 1992, Parsons *et al* 1992). Subsequently it was discovered that this serotype had been present in France for several years. Indeed, the first known strain of this serotype was isolated there in 1985 (Picault *et al* 1995). Adzhar identified differences between 793/B and other strains in 1995. By using molecular techniques, Adzhar *et al* (1997) also found that the new isolates could be placed into three subgroups and suggested at least three different strains of the 793/B serotype had entered

Britain in or prior to 1990/91. Infectious bronchitis has been reported in commercial poultry flocks of Iran since many years ago which Massachusetts (Mass) was only confirmed serotype (Aghakhan *et al* 1994). Vasfi Marandi *et al* also identified the IBV in chicken flocks by serological and isolation methods in 2000. During recent years, in spite of regular vaccination with live attenuated and inactivated vaccines of Mass strains, an epidemiological survey pointed out IB is still responsible for serious financial losses to poultry industry of Iran. Recently several researchers detected new serotype 793B or 4/91 by RT-PCR technique (Nouri *et al* 2003, Seify Abad Shapouri *et al* 2000, 2004, Shoushtari *et al* 2008). These reports may partially explain the failure of Massachusetts-type vaccines and necessitate revising the vaccination programmes against IB in Iran. Toroghi and co-workers in 2004 identified several isolates of 793/B in Iran that have been isolated by Momayez during the past few years (Momayez *et al* 2002). By sequence analysis of S1 gene they determined that Iranian strains of 793/B are very different from the other isolates in the worlds. Inactivated autogenous vaccines prepared from specific local isolates may be used to immunize commercial layers and breeder chickens. The present results clearly demonstrate the cross-protection that can be achieved against challenge with incorporating two antigenically different IB serotypes. The sequencing analysis indicated that the Iranian isolate IR/773/2001 classified as 793/B or 4/91 serotype with many differences of amino acid sequences from H120 vaccine strain (Toroghi *et al* 2004). Cavanaugh *et al* determined in 2005 that the Iranian and Saudi isolates of 793/B type had approximately 95% identity in the sequenced region with the 4/91 live vaccine strain and with FR/CR88061/88, from which the CR88 live vaccine was derived. Thus the Iranian, Saudi Arabian all differed from the two vaccine strains. Pathological lesions induced by Iranian isolate IR/773/2001 have been determined experimentally in SPF chickens by Mahdavi *et al* in 2007. They indicated that the Iranian isolate is capable to cause lesions in tissue of different organs such as trachea, lungs, intestine and most severely in the kidneys of experimentally infected chicks. These extensive differences of amino acid sequences of 793/B from H120 vaccine strain might explain the reason of increasing recent outbreaks of IB in Iran. The existence of nucleotide differences between Iranian isolate with live 793B vaccine strains showed they were distinct from vaccine strain (Toroghi *et al* 2004). In this study the Iranian IR/773/2001, 793/B isolate and the Massachusetts M41 strain were used as challenge viruses in SPF chickens that had been vaccinated with the provided experimentally oil-inactivated vaccines (IR/773/2001 and M41). The results of tracheas examination for ciliary motility of vaccinated chickens showed evidence of complete protection against the challenge homologous strains of IBV, since all the explants from the vaccinated chickens' demonstrated 90% ciliary activity (OIE recommendation). The opposite was true of the unvaccinated chickens, all of which had tracheal explants without any evidence of ciliary movement, suggesting lack of protection. This clear-cut difference was also observed by Darbyshire in 1979 and Luis in 1982. Gomez and raggi (1974) and Luis *et al* (1982) also reported local resistant in explants of tracheas form chickens immunized by two inoculations of Massachusetts M41 strain, when their explants were examined after challenge with the homologous strain. The results of challenge examination revealed that the IR/773/2001 inactivated vaccine could be able provide 50% immunity against Massachusetts (Mass) M41 serotype while M41 inactivated vaccine could be able provide 30% immunity against IR/773/2001 (793/B serotype) in chickens. Cross-protection produced by some IBV serotypes against antigenically unrelated strains is well known

(Hofstad 1981, Rosenberger *et al* 1976, Winterfield *et al* 1975). Parsons et al in 1992 have reported the Mass-type vaccines to be only partially effective against 793/B serotype.

In the present study the level of antibody response was measured by HI test was found to be protective and compatible with the titer of HI recommended by OIE and also compatible with those of challenge test, therefore this test can be used for the potency control of the oil-emulsion killed IB vaccine. Evidence reported in this paper suggests that the distribution of 793/B serotype should be carefully studied in order to develop and implement appropriate vaccination programmes for given areas.

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