

Combination of H120 and 1/96 avian infectious bronchitis virus vaccine strains protect chickens against challenge with IS/1494/06 (variant 2)-like infectious bronchitis virus

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Summary. – Avian infectious bronchitis (IB) is a worldwide chicken disease, caused by avian infectious bronchitis virus (IBV) which infects all commercial poultry lines. The present study was done to evaluate protection caused by two different serotype vaccines (Massachusetts and 793/B) in order to evaluate protection against challenge with IS/1494/06-like virus (variant 2-like virus), which is prevalent in the Middle East. SPF chickens were divided into four groups (n = 20). First and second group as negative control group and non-vaccinated-challenged group received no vaccine. Groups 3 and 4 received H120-H120 and H120-1/96 IBV vaccine strains at the 1st and 14th day, respectively. Twenty one days after last vaccination, non-vaccinated-challenged group and vaccinated group were challenged using variant 2-like IBV. Serum samples were collected before challenge to measure humoral immune response of chickens. Five days after challenge, the tissue samples from the trachea, lungs and kidneys were taken to evaluate ciliary activity, viral load (quantitative real-time RT-PCR), and histopathological evaluation. Clinical sign scores were also recorded after challenge. Overall, the results showed a protective efficacy of the used vaccination program. Best cross protection (69.2%) was obtained in the H120-1/96 vaccinated group. Virus replication of the challenged virus in H120-1/96 group compared with H120-H120 group showed a significant reduction of viral load in trachea (1.5×10^3 compared to 503) and kidneys. Clinical sign scores of the challenged groups showed significant effect of the vaccination program to reduce clinical signs. The trachea pathological scores and histopathological findings in the lungs and kidneys also confirmed better protective efficacy of vaccinated groups. In conclusion, using combination of heterologous IBV vaccine serotypes (Massachusetts and 793/B) would be a better strategy to control variant 2-like viruses, but more evaluation is needed using other circulating isolates to find the best combination of vaccines.

Keywords: avian infectious bronchitis; vaccination; cross protection; 1/96 strain

Introduction

Infectious bronchitis virus (IBV) is a major cause of economic losses in poultry industry and can be involved

in respiratory diseases, nephritis, and poor egg production and quality.

IBV is the coronavirus of the domestic fowl that is mainly observed in chickens. It possesses a positive sense single-stranded RNA genome that ranges from 27 to 31 kb (Cavanagh, 1996). The *Coronaviridae* family is classified into four groups: alpha coronavirus, beta coronavirus, gamma coronavirus, and delta coronavirus. IBV belongs to the gamma coronaviruses (Cavanagh, 2005). IBV genome encodes several viral proteins which play important antigenic and

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Abbreviations: GMT = geometric mean titer; IB = infectious bronchitis; IBV = infectious bronchitis virus; Mass = Massachusetts serotype; SPF = specific pathogen free

immunogenic roles in the virus structure. Spike (S) antigen, which is known as main immunogenic antigen of IBV, has two subunits, S1 and S2. The S1 subunit, which is the most variable part, is responsible for viral variations and recombination. There are hundreds of IBV serotypes and the majority of them differ from each other by 20 to 25% of amino acids of subunit S1 (Cavanagh *et al.*, 1997b; Jackwood *et al.*, 2001), however, some serotypes differ approximately by up to 50% of amino acids (Bande *et al.*, 2015). The S1 subunit of the S protein is the main inducer of protective immunity and it carries majority of the virus neutralizing epitopes, including serotype-specific epitopes, which are usually conformation-dependent in nature (Mockett *et al.*, 1984). New variant strains may differ as much as 55% in their S1 amino acid sequences compared with vaccine strains (Gelb Jr *et al.*, 2005; Kusters *et al.*, 1989). Many new variants have been isolated from commercial flocks (Jackwood, 2012; Sjaak de Wit *et al.*, 2011). Considering the level of homology of S1 gene and the level of cross-protection, it has been shown in several studies that there is a higher chance of cross-protection between strains with a high level of homology than strains with low homology (Cavanagh *et al.*, 1997b). However, the results indicate that this latter relationship is rather weak (De Wit *et al.*, 2011). There is a significant drop in cross-protection in some strains which differ in only a few percents of the genome sequence (De Wit *et al.*, 2012), whereas, there is a high level of cross-protection against other strains with lower homology. The continual emergence of the new IBV serotypes worldwide necessitates prudent evaluation of the level of cross-protection achieved by the use of currently available IBV vaccines (Gelb Jr *et al.*, 1991). As it is expected, cross-protection tends to diminish as the degree of amino acid identity between the S1 proteins of the two IBV strains decreases (Cavanagh *et al.*, 1997a). Revaccination using a different serotype can lead to protection against a broader range of serotypes (Raj and Jones, 1996). Some strains of the IBV are able to induce cross-protection against other genotypes, and these are considered as protectotypes. Protectotypes have been suggested as a valuable approach to develop IBV infection control strategies (Raj and Jones, 1996). In Iran, several genotypes of IBV have been reported (Akbari Azad *et al.*, 2007; Ghahremani *et al.*, 2011). The first isolation of IBV Massachusetts (Mass) serotype in Iranian chicken flocks was reported by Aghakhan *et al.* (1994). Later, several Iranian researchers identified the 793/B serotype (Akbari Azad *et al.*, 2007). The 793/B serotype is regarded as one of the dominant IBV genotypes circulating in Iran (Seyfi Abad Shapouri *et al.*, 2004). Also, the presence of variant 2 viruses (IS/1494/06-like) in Iranian commercial flocks have been demonstrated (Hosseini *et al.*, 2015). The isolation of IS/1494/06 one of the Israeli variant 2 isolates and information on its S1 gene sequence in GenBank (Acc. No. EU780077) has first been reported by Meier and Maher from Israel (Meir *et al.*, 2004).

The IS/1494/06 is still a major IBV variant in Israel, Jordan, Egypt, Turkey and other countries in the Middle East (Hosseini *et al.*, 2015; Hussein *et al.*, 2014; Kahya *et al.*, 2013). IS/1494/06 is known to be nephropathogenic and it also affects the respiratory system (Susan *et al.*, 2010). It should be noted that several Mass and 793/B vaccines have been used in Iran since first isolation of IB viruses. IBV strain 1/96 has been isolated first in Hungary and commercialized by Ceva Santé Animale with trade name "IBird". In this study, we aimed to evaluate cross-protection induced by vaccination using combination of Mass (H120) and 793/B (1/96 strain) against IS/1494 (variant 2) genotype.

Materials and Methods

Experimental design. White specific pathogen-free (SPF) leghorn chickens were divided into four groups containing 20 birds each. Each group in all experiments was housed in a separate negative pressure isolators. They were provided with food and water *ad libitum*. Groups were vaccinated and challenged as shown in Table 1. One and 14 days old chickens were vaccinated with Mass (H120; Ceva Santé Animale) and 793/B vaccines (1/96; IBird, Ceva Santé Animale), respectively using an eye drop. After 35 days, birds in groups 2 (non-vaccinated-challenged group), 3 (H120-H120) and 4 (H120-1/96) were challenged using nasal route (10^4 EID₅₀). Before the challenge, sera samples were taken from the birds to evaluate ELISA titers of IBV vaccination using IDEXX kit (IDEXX Laboratories, Inc., USA). Clinical signs were recorded for five days after challenge in order to evaluate the clinical scores for each group. Five days after challenge, samples were taken from trachea, lungs and kidneys for histopathology, quantitative real-time RT-PCR and ciliary activity of trachea.

Challenge strain history. Tissue samples from trachea and kidneys were collected from chickens showing clinical signs suspected to be related to the IB. Samples were taken from vaccinated flocks from eight Iran provinces. For virus isolation, homogenized samples were inoculated into the allantoic cavity of 9–11 days old SPF embryonated eggs. Primary diagnostics of IBV was done using PCR assay with the target of 5'-UTR part of IBV genome. For genotyping, positive samples were submitted to a nested RT-PCR reaction amplifying S1 gene. Based on genotyping and molecular epidemiology, the IS/1494/06 (variant 2-like) virus was identified as a dominant genotype. From about forty variant 2-like isolates, one was selected at the fifth egg passage level. It was shown to be free from contamination by other avian pathogens such as Newcastle disease virus, avian influenza virus, infectious laryngotracheitis virus, reoviruses, mycoplasma, and other bacteria using molecular detection methods and cultivation. The S1 sequence of the challenge

virus was submitted to the NCBI (Acc. No. KR869776). EID₅₀ was calculated by Reid-Muench method.

Antibody response. The humoral immune response (geometric mean titer: GMT) of birds against IBV before the challenge was measured by ELISA (IDEXX).

Ciliostasis test. Assessment of protection against challenge in each experiment was evaluated using ciliostasis test (Cook *et al.*, 1976). Five days post-challenge, all of the chickens in each group were euthanized. The tracheas were carefully removed and examined for ciliary activity as described previously (Cavanagh *et al.*, 1997b). Briefly, each of 10 tracheal rings (3 rings of upper, 4 rings of middle and 3 rings of lower trachea) were examined by low-power microscopy and ciliary activity was evaluated as follows: 0 = all cilia beating; 1 = 75% beating; 2 = 50% beating; 3 = 25% beating; and 4 = none beating (100% ciliostasis). This gave a maximum possible ciliostasis score of 40 in case of complete ciliostasis (total lack of protection).

Clinical observations. Clinical signs resulting from IBV challenge were registered daily till 5 days post challenge, according to their severity as 0 = normal breathing can be heard, 1 = slight/rare tracheal rales can be heard after provoking by moving the chick, 2 = moderate/frequent tracheal rales can be heard without moving the chick, but not continuously and 3 = marked, continuous tracheal rales can be heard.

Histopathology. Tracheal, lung and kidney samples, taken 5 days after challenge (40 days), were fixed in buffered formalin (10%), processed, sectioned, and stained with hematoxylin and eosin for histopathological evaluation of the lesions produced by the challenge virus. The tracheal lesions were scored from 1 to 4 based on the severity of the observed lesions: 1 = no lesions, 2 = moderate epithelial hyperplasia and subepithelial lymphoid infiltrates, 3 = severe epithelial hyperplasia and subepithelial lymphoid infiltrate described in previous studies (Chen *et al.*, 1996; Nakamura *et al.*, 1991).

RNA extraction, cDNA synthesis and quantitative real-time PCR. Viral RNA was isolated from tissues using Cinna Pure RNA extraction kit (Sinaclone, Iran) according to the sup-

Table 1. Experimental schedule of vaccination using Massachusetts and 793/B-like IBV vaccines and challenge with IS/1494-like IBV 21 days post vaccination

Group No.	Names of groups	Day 1	Day 14	Challenge (day 35)
1	Negative control	-	-	-
2	Non-vaccinated-challenged group	-	-	+
3	H120 -H120	H120 (Ceva)	H120 (Ceva)	+
4	H120-1/96	H120 (Ceva)	IBird-1/96 (Ceva)	+

Table 2. Clinical scores, tracheal pathological scores and ELISA titers of groups receiving different vaccination and challenge scheme (evaluation was conducted 5 day post challenge)

Group No.	Names of groups	Median clinical scores	Median tracheal pathological scores	ELISA titers*
1	Negative control	0	1	0
2	Non-vaccinated-challenged group	1.5	3	0
3	H120-H120	0	2	1064
4	H120-1/96	0	1	1960

*Antibody titers against IBV were measured before challenge.

pliers' instructions. cDNA was synthesized using RevertAid first strand cDNA synthesis kit (Thermo Scientific, Canada). Real-time PCR for IBV detection based on 5' UTR was used in this study. Real-time PCR amplification was done with the amplification kit (Bioneer, South Korea) with forward primer (5'-GCTTTTGAGCCTAGCGTT-3') and reverse primer (5'-GCCATGTTGTCAGTGTCTATTG-3'), and Taqman[®] dual-labeled probe (FAM-CACCACCAGAACCTGTCA CCTC-BHQ1) (Callison *et al.*, 2006). PCR cycling parameters were 95°C for 2 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The reaction was carried out in a QIAGEN Rotor-Gene Q (Corbett Rotor Gene 6000, USA).

Statistical analysis. To evaluate and compare the clinical scores, tracheal pathological scores and ciliostasis between the groups, Mann-Whitney test using SPSS 12.1 software

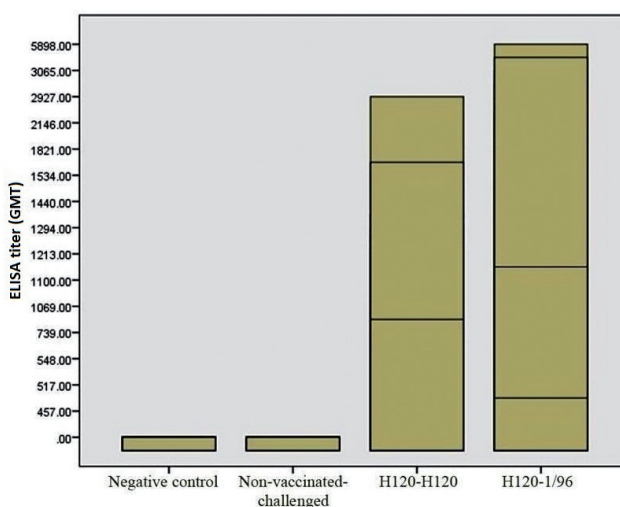


Fig. 1

Comparison of antibody response of vaccinated groups to IBV
Significant differences ($P < 0.05$) were observed between vaccinated groups with higher response seen on H120-1/96 vaccinated group.

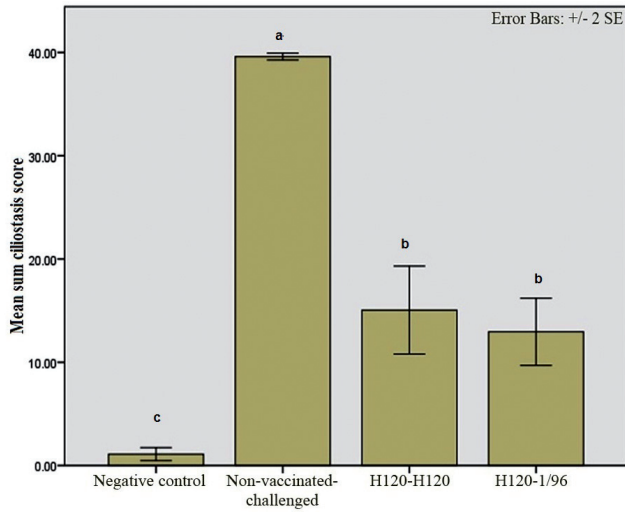


Fig. 2

Mean ciliostasis scores

Significant differences ($P < 0.05$) were observed between vaccinated (H120-H120 and H120-1/96 group) and non-vaccinated-challenged group (a,b,c).

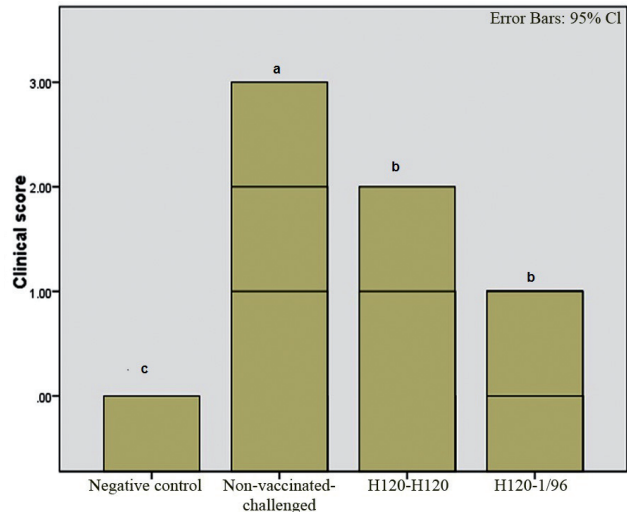


Fig. 3

Clinical signs scores

Significant differences ($P < 0.05$) were observed between vaccinated groups (group 3: H120-H120 and group 4: H120-1/96) and non-vaccinated-challenged group (group 2) (a,b,c).

was used. The 0.02 level was considered as significant. Antibody results were compared using Student's *t*-test. Finally, one-way ANOVA with significance level of 0.05 was used to compare virus loads.

comparing H120-H120 and H120-1/96 groups based on ciliostasis score. Percentage of cross protection obtained by vaccination of H120-H120 and H120-1/96 groups was 60% and 69.2%, respectively (Fig. 2).

Results

IBV antibody detection

To evaluate vaccination program effect on immune system response the titers of antibody against IBV were detected using ELISA method. As expected, no antibody was detected in non-vaccinated-challenged group and negative control group (Table 2). The results showed significant differences ($P < 0.05$) between H120-H120 (GMT: 1064) and H120-1/96 (GMT: 1960) groups using *t*-test analytical method (Fig. 1).

Ciliostasis test

To measure the effect of a virus on the tracheal mucosa we have used the ciliostasis test. The results between groups were compared using Mann-Whitney test based on median ciliary activity score of every bird. Comparing groups in two-tailed comparison showed a significant difference ($P < 0.02$) between non-vaccinated-challenged group and H120-H120 vaccinated and challenged group. The same results were obtained by comparison of non-vaccinated-challenged group and H120-1/96 vaccinated and challenged group ($P < 0.02$). On the other hand, there were no significant differences

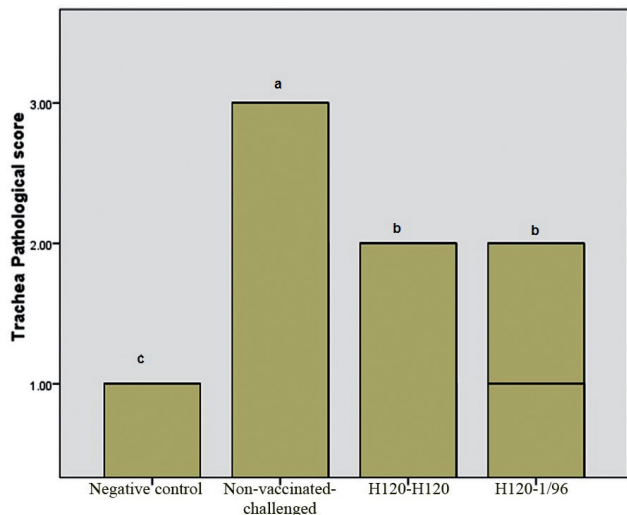


Fig. 4

Tracheal pathological scores

Median tracheal pathological score was significant ($P < 0.05$) between non-vaccinated-challenged to H120-H120, non-vaccinated-challenged to H120-1/96, negative control to non-vaccinated-challenged and negative control to H120-H120 groups. Not significant ($P > 0.05$) differences were detected between H120-H120 and H120-1/96 groups and between negative group and group 4.

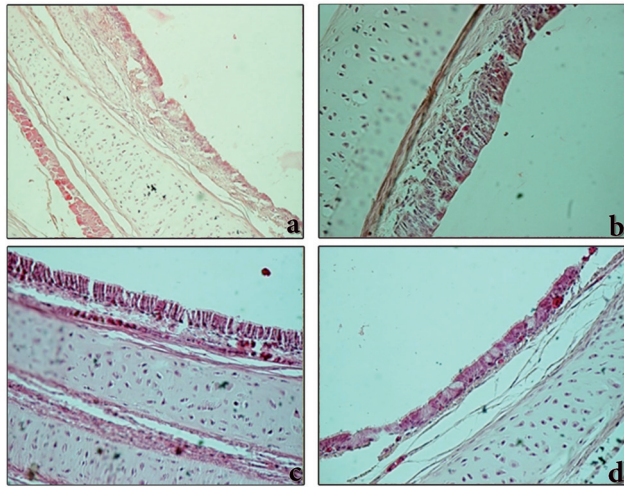


Fig. 5

Histopathology of the trachea

Microscopic changes of the hematoxylin and eosine stained trachea in the SPF chickens infected with IS/1484-like IBV isolate after 5 days post challenge. Negative control group (a); tracheal lesions of non-vaccinated-challenged group receiving challenge virus and non-vaccinated group (b); H120-H120 vaccinated-challenge group showing less lesions (c); non-vaccinated-challenged group, and trachea with almost no changes obtained from H120-1/96 vaccinated-challenged group (d).

Clinical signs score

Clinical observations were recorded and scored from 5 days post challenge. The results of clinical scores are shown in Fig. 3. There were significant differences ($P < 0.05$) between non-vaccinated-challenged group (median score 1.5) vs. H120-H120 (median score 0) group and non-vaccinated-challenged group vs. H120-1/96 (median score 0) group. There was no significant difference between H120-H120 and H120-1/96 groups (Table 2).

Tracheal pathological scores

Based on pathological findings in trachea using scoring method, described previously, all challenged groups were compared two by two with negative control (not vaccinated-not challenged) group. Significant differences were observed comparing non-vaccinated-challenged group (median score 3) and H120-H120 group (median score 2) with negative control group (median score 1) indicating meaningful pathologic lesion scores ($P < 0.05$) (Table 2). No significant results were detected between negative control and H120-1/96 groups (median score 1) indicating proper protection level (Fig. 4). A significant difference was observed by comparing non-vaccinated-challenged group vs. H120-H120 group and non-vaccinated-challenged group vs. H120-1/96 group ($P < 0.05$), but no significant level was observed between H120-H120 and H120-1/96 groups (Fig. 5).

Histopathological evaluation

Histopathological assessment of lungs between challenged groups showed a relative protection in the vaccinated groups.

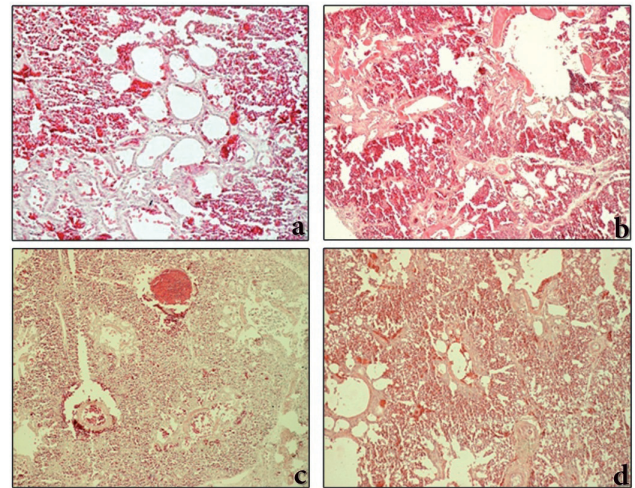


Fig. 6

Histopathology of lungs

Microscopic changes of the hematoxylin and eosine stained lungs in the SPF chickens infected with IS/1484-like IBV isolate after 5 days post challenge. Lungs of control group (a); lungs of non-vaccinated-challenged group with hyperemia, petechia, and infiltration of inflammatory cells (b); H120-H120 group showing mild hyperemia (c); H120-1/96 group with mild hyperemia (d).

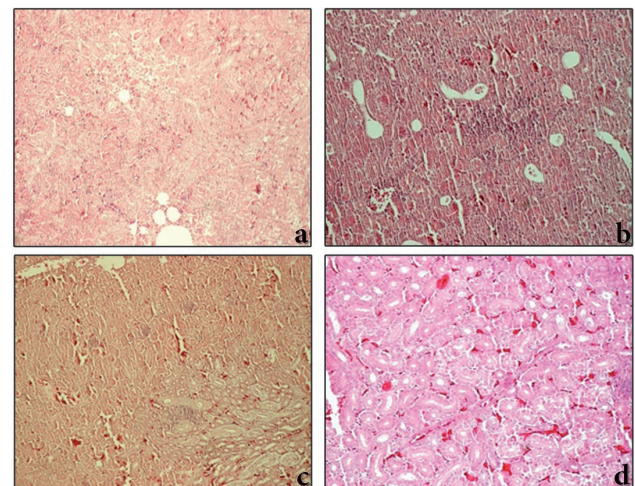


Fig. 7

Histopathology of kidneys

Microscopic changes of the hematoxylin and eosine stained kidneys in the SPF chickens infected with IS/1484-like IBV isolate after 5 days post challenge. Normal histological view of kidneys in control group (a); infiltration of inflammatory cells to interstitial tissue along with hyperemia and petechiae in non-vaccinated-challenged group (b); mild hyperemia and no lesions in H120-H120 group (c), mild hyperemia and no lesions in H120-1/96 group (d).

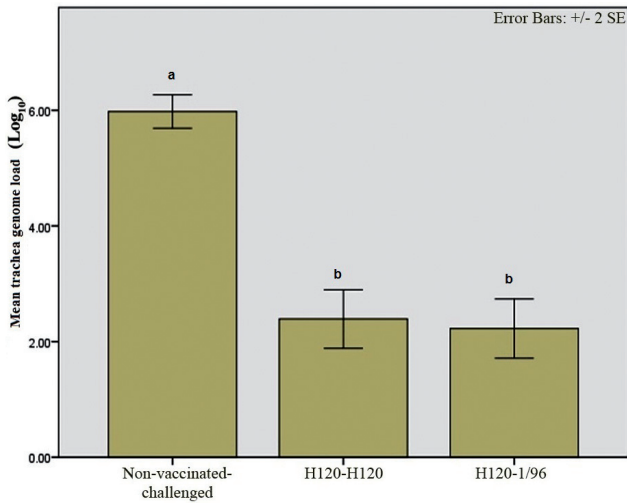


Fig. 8

Real-time PCR of tracheal viral load

Viral load detection in the trachea of the SPF chickens, non-vaccinated, vaccinated with H120-H120 or H120-1/96 and challenged with IS/1484-like IBV isolate, after 5 days post challenge. There was a significant difference ($P < 0.05$) between non-vaccinated-challenged group with other groups, with prominent decrease of viral replication in vaccinated groups (H120-H120 and H120-1/96) (a,b).

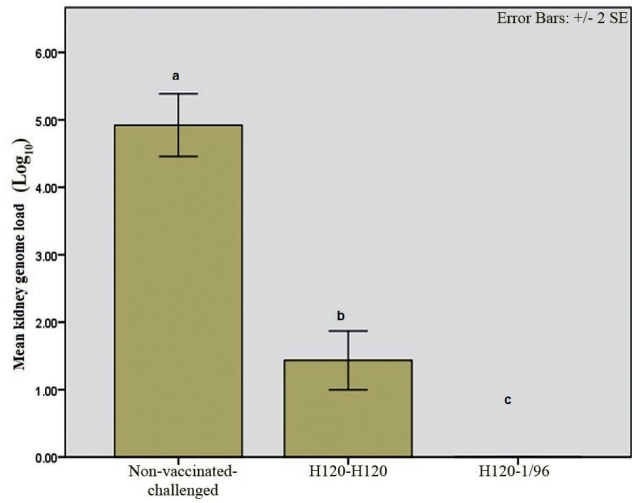


Fig. 10

Real-time PCR of kidney viral load

Viral load detection in the kidneys of the SPF chickens, non-vaccinated, vaccinated with H120-H120 or H120-1/96 and challenged with IS/1484-like IBV isolate, after 5 days post challenge. Significant ($P < 0.05$) differences between the groups were obvious, in which the group receiving H120-1/96 vaccines showed no replication of the challenge virus, although lower and significant ($P < 0.05$) viral replication can be observed in H120-120 vaccinated group compared to non-vaccinated-challenged group (a,b,c).

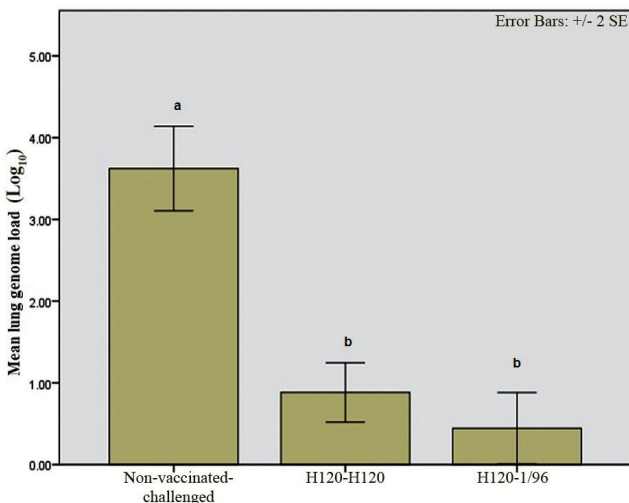


Fig. 9

Real-time PCR of lungs viral load

Viral load detection in the lungs of the SPF chickens, non-vaccinated, vaccinated with H120-H120 or H120-1/96 and challenged with IS/1484-like IBV isolate, after 5 days post challenge. There was a significant difference ($P < 0.05$) between non-vaccinated-challenged group with other groups, with prominent decrease of viral replication in vaccinated groups (H120-H120 and H120-1/96). Non-significant ($P > 0.05$) results were observed between H120-H120 and H120-1/96 vaccinated groups (a,b).

nation in vaccinated groups were mild hyperemia with no lesions in H120-H120 and H120-1/96 groups (Fig. 6). Kidney results evaluation showed almost the same results as in lungs. Hyperemia and petechial, mild infiltration of inflammatory cells, predominantly mononuclear cells, in interstitial tissue was recorded in non-vaccinated-challenged group, whereas mild hyperemia with no lesions in most samples was the only finding in vaccinated groups (Fig. 7).

Quantitative real-time RT-PCR

Mean viral load of virus in trachea, lungs, and kidneys is shown in Fig. 8, 9, and 10. Tracheal investigation on challenged virus in all groups showed significant differences ($P < 0.05$) between non-vaccinated-challenged group compared to H120-H120 and H120-1/96 groups, but no significant difference was detected between H120-H120 and H120-1/96

Table 3. The average of challenge IBV viral loads in trachea, lungs and kidneys

Groups	Trachea	Lungs	Kidneys
1	0	0	0
2	1.62×10^6	1.53×10^4	2.83×10^5
3	1.5×10^3	30.3	190
4	503	102	0

Non-vaccinated-challenged group showed hyperemia, petechia, and mild infiltration of inflammatory (dominantly mononuclear) cells. The only findings of pathological exami-

groups. Regarding lung viral load, exactly the same levels of differences were detected between the groups which showed the same levels of protection obtained by immunization in H120-H120 and H120-1/96 groups. Viral loads in kidney samples of all groups showed significant differences with the best results detected in H120-1/96 group (Table 3).

Discussion

In recent years, the Iranian poultry industry has experienced an increasing incidence of respiratory and nephritis problems with severe economic losses related to IB in flocks. The emergence of new IBV variants has caused several problems to design a proper vaccination program (Liu *et al.*, 2006a). However, the development of vaccines against new IBV variants is not generally an ideal option (Bijlenga *et al.*, 2004; Jackwood *et al.*, 2003). Some IBV vaccine strains from a specific serotype may provide protection against some IBV strains of different serotypes (Cook *et al.*, 1999). Therefore, protection studies on new IBV variants could be a helpful method in control strategy of IBV, as it provides information about the efficacy of available commercial vaccines.

To date, the Mass strains have been primarily used as live vaccines due to their epizootic distributions and cross-protective potency (Bijlenga *et al.*, 2004; Ignjatović and Sapats, 2000). Despite the widespread use of IBV vaccines, outbreaks related to different serotypes of IBV have increased in many countries (Li and Yang, 2001; Liu *et al.*, 2006b). On the other hand, the reported persistence of the Israeli variant isolate (IS/1494/06) related problems in spite of vaccinations of the broiler flocks with H120, supports the argument of IS/1494/06 IBV presence in the Middle East countries (Mahmood *et al.*, 2011; Meir *et al.*, 2004). A pair wise comparison of the S1 gene sequences of IS/1494/06-like viruses showed low level of identity to the Mass (81% percent identity to H120) and 793/B (80% identity to CR88) type viruses (Awad *et al.*, 2014). In this study, we used two different IBV serotypes (H120 and 1/96 (79/B-like)) in order to evaluate their combined effect as protectotypes against IBV IS/1494/06-like viruses.

Vaccination against IBV has been practiced for over half a century. Besides commercial vaccines of Mass serotype, 793/B serotype vaccines have been produced by several companies including 4/91 (Intervet), IB88 (Merial), IBird (Ceva Santé Animale), 233A (Phibro). TABIC IB VAR206 also has been produced as variant 2 vaccine (IS/1494/06 origin) by Phibro. Mass and 793B type vaccines (4/91 and IB88) are main serotypes used in Iran (Cavanagh *et al.*, 2005; Seyfi Abad Shapouri *et al.*, 2004).

In the present study, for the vaccine program we have selected combination of Mass (H120) and 793/B (1/96 strain) serotypes. The usage of both Mass (H120) and 793/B (1/96

in vaccination program makes a broader range of protection against different serotypes of IBV found worldwide (Cavanagh *et al.*, 1999), as observed with different evaluation methods that group receiving H120-1/96 vaccines showed almost the best results among all groups. In our study, the vaccines were administered by eye drop route on 1st and 14th day and since acceptable protection can be frequently seen using suggested protocol (Cook *et al.*, 1999), we have expected the reliable results.

SPF chickens were used in these studies, although, under commercial conditions, it is likely that day old chicks would have maternally derived IBV antibodies. However, earlier studies (Davelaar and Kouwenhoven, 1977) have shown that it is possible to vaccinate successfully even if such antibodies are present. As it is shown in the present study, using vaccine with two serotypes (H120-1/96 group) resulted in a better cross protection (69.2% compared to 60% protection) as previously confirmed (Cook *et al.*, 1999; Davelaar and Kouwenhoven, 1977).

Our results are consistent with a recent study done in Israel with 1494/06 virus challenge which revealed 70% of cilliar protection in H120 (day 0) + 793/B (day 14) vaccinated group. They also reported 83% and 95% protection in chickens vaccinated with 793/B+793/B and Var206+Var206, respectively. It is obvious that homologous challenge-vaccination study (challenge with 1494/06 and vaccination with Var206 vaccine) leads to a higher protection level. Despite the previously mentioned variation (19–21%) in S1 gene of the IS/1494/06-like viruses with H120 and 793/B viruses, the successful protection conferred by our vaccination program may be attributed to the shared characteristics of the S1 protein of the vaccine and challenged IBVs (Cavanagh *et al.*, 1986). A new study has been conducted by Awad (Awad *et al.*, 2015) obtaining almost the same results as we have, in which the group receiving H120 vaccine (1 day old) and 793/B vaccine (14 days old) showed 80% protection of cilia against challenge with IS/1494/06-like virus. They also evaluated vaccination program using H120 and 793/B vaccine (day 1) and 793/B vaccine (day 14) against same challenge virus. The results showed 94% of protection. They concluded that, the combination of these vaccines confers acceptable protection against heterologous challenge. Higher protection of this vaccination program (day 0: H120+793/B and day 14: 793/B) could be due to the higher level of local and cellular immunity at the tracheal site (Lambrechts *et al.*, 1993), in which it has been shown that using both serotype vaccines (H120 and 793/B) in day old chickens induces significant increase in the expression of CD4+, CD8+ and IgA-bearing B cells in the trachea compared with single H120 vaccination (Awad *et al.*, 2014).

In a study by De Wit *et al.* (2011) on commercial broiler breeders, it was reported that Mass (day 0) + 793/B (day 14) vaccines combination and challenge with D388 (QX genotype) after 28 days produced 51% protection in cillioistasis

test 5 days post challenge, while the protection increased to 74% on 7th day after challenge.

Despite the proper protection conferred by Mass and 793/B vaccines against IS/1494/06, there was a study showing challenge with IS/1494/06 Israel 2 variant after vaccination with a Mass-type vaccine followed by QX-like vaccine 14 days later which conferred 50% protection of the vaccinated birds. In vaccinology of IBV, it is important to note that vaccines' efficacy is based on its ability to control cillioistasis caused by the challenge virus. Median clinical sign scores and challenge virus load in different organs also showed the same results considering a better protection of vaccinated groups. These findings are consistent with Cook *et al.* (1976) demonstrating an improved protection that can be achieved after the challenge with many different serotypes using a program of combined serotype vaccine with antigenically different IBV strains incorporated (Adzhar *et al.*, 1995).

It should be noted that in cross-protection experiment, even in closely controlled experimental situations, 10% of vaccinated chicks would not respond with a protective immune response against challenge with the homologous strain (Cook *et al.*, 1996; Nix *et al.*, 2000; Winterfield *et al.*, 1976). These results show that chickens (out-bred, though with restricted sets of parental breeding stocks) are not uniform in their response to IBV vaccination.

The real-time PCR technique is frequently used for IBV infections diagnostics (Cook *et al.*, 1996). To evaluate vaccination ability to control challenge virus, the quantitative real-time PCR was examined for trachea, kidney and lung tissue samples. Titers of the live virus are usually maximal in the nose and trachea within three days and remain so for 2 to 5 days further (Hofstad and Yoder, 1966).

In our findings, although there was no statistically significant difference between H120-H120 and H120-1/96 groups, the results showed that lowest viral load was observed in H120-1/96 vaccinated group and significant virus replication control was recorded between vaccinated and non-vaccinated groups.

In our study, there was no challenge virus detected in the kidneys of H120-1/96 vaccinated group. These results may be due to the ability of vaccines in controlling virus replication in the kidneys comparing to non-vaccinated-challenged group.

It has been reported that low level of IBV replication in the kidney, using combined (H120-1/96) vaccines is due to the low level of virus replication in the trachea and also higher antibody level of the vaccinated birds (Lambrechts *et al.*, 1993), which decreases possibilities of virus replication in the kidneys. Somehow, this finding could be very important in the field conditions as lots of nephrotoxic factors such as high protein and calcium diet, mycotoxins, drugs such as sulfonamides, cold stress, nephropathic diseases, and some other factors can affect flock performance and mortality

which can be impressed by IBV presence in the kidneys. The same results were also recorded for lung samples (3.03E+01 and 1.02E+02 compared to 1.53E+04).

Histopathological evaluation of tracheal samples showed significant differences between non-challenged group (negative control) with H120-H120 and H120-1/96 group, while there were no significant differences between negative control and H120-1/96 vaccinated groups which showed the protective effect against H120 and 1/96 strains combination and limited pathological damages of the trachea.

Trachea lesions in IBV infected birds is often critical in poultry flocks especially in broiler flocks that are dealing with unsuitable conditions such as high ammonia, IBVs dust, CO₂, and several respiratory diseases. Comparison of histopathological lesions between vaccinated and non-vaccinated-challenged groups revealed that vaccination was effective to control the viral related lesions such as severe hyperemia or petechia in the lungs, and lower median pathological score in the trachea.

It is known that under some conditions, which are yet poorly defined, some strains of IBV apparently are able to induce nephrosis (Winterfield and Hitchner, 1962; Zanella, 1988). It is proved that the Mass-like vaccines used alone usually provided poor protection against the nephritis caused by nephrotropic viruses (Cook *et al.*, 2001). As it is observed in present study, vaccination program in H120-H120 could not completely prevent virus replication in kidneys, but as previous reports (Cook *et al.*, 2001) and our findings show combination of H120 and 793/B (1/96) vaccination program could protect chicken from nephritis. Overall, in histopathological evaluation of kidney samples almost no damage was recorded for vaccinated groups (H120-H120 and H120-1/96 groups) showing only very mild hyperemia. In this research, ELISA was used to measure antibody titers against IBV. ELISA detected high levels of antibody against IBV 21 days after last vaccination. Ghadakchi *et al.* (2005) showed that ELISA could be reliable, repeatable and sensitive for monitoring vaccination schedules and the rapid detection of the early rise of antibodies against IBV. These results were consistent with Susan *et al.* (2011).

Our results show that vaccination using two serotypes (H120-1/96) can lead to a better immune response. It also indicates that GMT of H120-1/96 group (GMT: 1960) is significantly higher than H120-H120 group (GMT: 1064). It is believed that cytotoxic T cells play an important anti-viral activity role during early stages of IBV infection while serum IgG is critical at the late stage of IBV infection (Collisson *et al.*, 2000). Therefore, obtaining higher antibody level using combined (H120 and 1/96 strains) vaccination program will surely control IBV infection in the flock.

These results definitely show the important role of 1/96 strain (793/B group) in vaccination program to control IBV infection, especially 1494/06-like IB viruses which are domi-

nant circulating IBVs in commercial flocks in Iran. However, more studies are needed on vaccination policies to control IBV infections in Iranian poultry farms.

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