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Isolation, identification and chemical inactivation of infectious laryngotracheitis virus for use as a vaccine candidate

Mohammad Majid Ebrahimi[1], Shahla Shahsavandi[1], Ali Reza Yousefi[1], Nikdokht Ebrahimi[2]

[1]Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), P.O. Box 31975-148, Karaj, Iran; [2]School of Pharmacy, Tehran University of Medical Sciences, P.O. Code 1417614411, Tehran, Iran

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Summary. – Infectious laryngotracheitis (ILT) is a poultry respiratory disease associated with considerable mortality in chicken and decreasing egg production. Vaccination along with biosecurity measures are considered as the main strategy for ILT control. This study was aimed to evaluate the potency of an inactive ILT vaccine candidate derived from a local ILTV isolate. The isolated virus was characterized and treated with various chemicals and their concentrations. The virus infectivity was entirely abolished by treatment of 3 mM binary ethylene imine following 16 h incubation. The immune response of inactivated ILTV suspension with adjuvans was evaluated in both SPF chickens (experiment-I) and Hyline pullets (experiment-II). Efficacy of the inactivated and live ILT vaccines combination was compared. The results of experiment-I showed that the inactivated antigen induced specific antibody titers against ILTV. In experiment-II, despite the increase in serum antibody level administration of the inactivated antigen alone did not offer sufficient protection. The full protection was found in chickens that received the combination regimen. We conclude that simultaneous administration of the inactivated and live ILT vaccines was efficient for induction of immunity against ILTV.

Keywords: infectious laryngotracheitis virus; vaccine; inactivation; immune response

Introduction

Infectious laryngotracheitis (ILT), as an acute viral disease of avian respiratory tract, is associated with considerable economic loss in commercial farms. The disease is caused by *Gallid herpesvirus 1,* the genus *Iltovirus,* the subfamily *Herpesvirinae* of the family *Herpesviridae* (Garcia *et al*., 2013; MacLachlan and Dubovi, 2017), forming three categories according to course of infection: acute, subacute and mild or chronic (Samour, 2016; Swayne, 2016). The ILT is characterized by sneezing, rales, nasal exudate, and conjunctivitis, swollen inferior orbital and nasal sinuses and ocular discharge. In chronic form of the disease, it may cause dyspnoea, gasping and death as a result of asphyxiation of discharges. However, ILT-associated morbidity is depending on the virulence of ILT virus (ILTV) strain as well as flock immunity status (Davison *et al*., 2009; Jordan, 1993; Noormohammadi and Devlin, 2018). In addition, drastic loses in egg production are a common eventuate in the ILTV-infected laying flocks. Although all the chickens are susceptible to the ILTV, 4 to 18-month-old birds are more prone to the disease and may show 0.1% to 70% mortality. Immunosuppressive agents such as aflatoxins can increase the ILT disease rates in poultry (Ebrahimi and Shahsavandi, 2008; Garcia *et al*., 2013). The infected birds may carry the disease and become a source of infection for susceptible birds, even after recovering. At stressful conditions, the latent ILTV has a great opportunity to be activated in the recovering birds, leading to ILT outbreak in sensitive chickens. Me-

E-mail: mm.ebrahimi@rvsri.ac.ir, mm.ebrahimi@gmail.com; phone: +98-2634570038.

Abbreviations: AGID = agar gel immunodiffusion; BEI = binary ethyleneimine; BPL = beta-propiolactone; CAM = chorioallantoic membrane; ILT = infectious laryngotracheitis; ILTV = ILT virus; IM = intramuscular; ML = modified-live; SC = subcutaneous; SPF = specific pathogen free; TK = thymidine kinase

chanical spread, such as contaminated crates and littering are also involved in the ILT outbreak (Garcia *et al.*, 2013; Jordan, 1993).

Vaccination against ILT and biosecurity management are the main strategies in prevention of the disease. Because of economic losses attributed to ILT, vaccination of susceptible chickens by low-virulence ILTV strain or attenuated modified-live (ML) viruses in the endemic areas is recommended (Garcia *et al.*, 2013). Despite the successes which have been obtained in prevention of ILT using ML vaccine viruses, the vaccinated flocks are still at risk of ILT outbreak, as the virus has a potential of reversion to virulence. The ILTV could also spread from vaccinated to unvaccinated contact birds. Meanwhile, insufficient attenuation of ML vaccine viruses and interaction with undefined infectious, nutritional, or management factors are also involved in ILT outbreak (Ebrahimi *et al.*, 2001; Guy *et al.*, 1991; Kotiw *et al.*, 1995; Ou and Giambrone, 2012).

Inactivated viral vaccines, however, have no risk of reversion to virulence in administrated birds. Various factors affected the quality of an inactivated vaccine and consequently changed the immunization and protection against the pathogen. For instance, the materials and methods used for inactivation of ILTV during the vaccine manufacturing process have great impact on vaccine quality outcome (Bahnemann, 1997). Formalin, beta-propiolactone (BPL) and binary ethyleneimine (BEI) are the most common inactivators used for inactivation of antigens and commercial vaccines (Anderson *et al.*, 2008; Bahnemann, 1997; Bomford, 1997; Delrue *et al.*, 2012; Palya, 1991; Reddy and Srinivasan, 1991). In addition, the optimum concentration of inactivator and the duration of inactivation process have critical roles in manufacturing of the inactivated viral vaccine.

In this study, ILTV isolated from suspected commercial pullets was identified, and inactivated using various inactivators. Efficacy of the inactivated virus was evaluated by serological tests after intramuscular (IM) and subcutaneous (SC) inoculations at an experimental condition. The most effective vaccine regimen was then examined in a field trial.

Materials and Methods

Virus isolation. An outbreak of a respiratory disease was detected in the layer flock composed of 50,000 chickens in five widely separate farms. The affected chickens showed clinical signs including spasm, coughing, oral and nasal discharge, hemorrhagic conjunctivitis, swelling of eyelids and infra orbital sinuses. The mortality rate was about 3% at the end of disease's period.

Under minimal contamination conditions, the laryngotracheal tissues of birds susceptible to ILT infection were dissected. A portion of the trachea was homogenized in 1 ml of phosphate buffer saline (PBS) and stored at -70°C until further analyses. The homogenized samples were then diluted and mixed in 1:10 (v/v) ratio by nutrient broth (Merck, Germany) containing penicillin, streptomycin (1 mg/ml) and gentamicin (1000 IU/ml). The suspension was centrifuged (15 min, 1,800 x g at 4°C) and a 0.2 ml of supernatant fluid was inoculated to the dropped chorioallantoic membrane (CAM) of five 10- to 11-day-old embryonated specific pathogen free (SPF; RVSRI, Iran) eggs. The eggs were incubated for 7 days at 37°C and the CAMs were then collected and examined for pock formation.

Virus identification – Agar gel immunodiffusion (AGID). The AGID test was conducted to specify ILTV isolates (Swayne, 2016). Briefly, melted 1.5% Nobel agar (containing 8% sodium chloride and 0.01% merthiolate) was poured to the Petri dish and one central well surrounded by six outer wells were cut out. The surrounding wells were filled with the isolated samples, while the central well contained a hyperimmune serum of ILTV infected birds. Dishes were incubated at room temperature for 24–48 h and examined for precipitation lines.

Molecular identification – DNA extraction. A 100 µl aliquots of each homogenized sample was incubated with 100 µl of lysis buffer (1% sodium dodecyl sulfate in 10 mM Tris and 1 mM EDTA) and 200 µg/ml of proteinase K (Roche, Germany) at 56°C for 4 h. Virus DNA was extracted using GeneAll® ExgeneTM genomic DNA micro kit (South Korea) according to the manufacturer's instructions.

PCR. A 675 bp fragment of ILT thymidine kinase (TK) gene sequence was amplified using the designed primer set: 5'-ATGC CAAATTGGAGAGGTTG-3' and 5'-CGTCTGGTCGATTGAAGGAT-3' and *Taq* DNA polymerase master mix red (Ampliqon, Denmark). The cycling conditions were as follows: an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 40 s, and extension at 72°C for 30 s. The final extension was performed at 72°C for 10 min. The DNAs extracted from ILT vaccine (RVSRI, Iran) and an uninfected CAM were used as positive and negative controls, respectively. Ten microliters of each final PCR product sample was separated in a 1% agarose gel with a 100 bp DNA molecular weight marker (Fermentas, Germany).

Virus propagation. A 0.2 ml of virus sample was inoculated to the CAM of 10 to 11-day-old embryonated SPF chicken eggs and incubated at 37°C for a week. The infected CAMs were harvested, homogenized and after a freeze-thawing process, the samples were centrifuged (1,800 x *g* at 4°C) for 15 min. The titer of ILTV in collected samples was evaluated.

Virus titration. Ten-fold serial dilutions of the ILT virus suspension from 10^{-1} to 10^{-6} were carried out. For each dilution (0.1 ml per each egg), CAMs from 6 SPF eggs were inoculated and incubated at 37°C up to a week. After incubation, the CAMs were

removed and observed for pock presence. The infectivity titer was calculated with the Spearman-Karber formula.

Virus inactivation and evaluation. Different concentrations of BPL, formalin, and BEI were applied for inactivation of the ILTV at various incubation times (Table 1). Inactivation process was evaluated by inoculation of 0.1 ml of each treated virus suspension into CAM of three 10- to 11-day-old SPF embryonated eggs and examined for subsequent infectivity and pock formation during three serial passages. In order to evaluate antigen potency, forty 60-day-old SPF chickens were divided into four equal groups. Three samples of 0.2 ml of each provided ILTV antigen were IM inoculated to the three groups of birds and the 4th group without vaccination was considered as negative control. To determine the best serological response to the inactivated virus, blood samples were collected three weeks after immunization and ELISA antibody titer and the serum neutralization (SN) index was determined.

Serum neutralization (SN). A 10-fold diluted virus (10^{-1} to 10^{-6}) was added to undiluted chicken sera. The mixtures were then incubated at room temperature for 1 h. CAMs of 11-day-old SPF embryonated chicken eggs (n = 6 egg per dilution) were inoculated with 200 µl of serum-virus mixtures and then sealed and incubated at 37°C during the next seven days. The end point was recorded as the highest dilution of the virus without pocks on the CAMs.

ELISA measurement of ILT antibody titer. Sera antibody against ILTV was measured by specific ELISA kit (Synbiotics, USA) according to manufacturer's instructions. Briefly, diluted sera samples were left to bind to the wells. Unbound material was washed and an alkaline phosphatase labelled donkey anti-chicken IgG conjugate reagent was added. Unbound conjugate was washed away and phenolphthalein monophosphate substrate was added to the wells. The optical density (OD) was directly related to the amount of antibody present in the sample. The anti-logarithm of log10 titer was calculated (Synbiotics software) and recorded as the quantity of ILTV antibodies in each sample.

Vaccine preparation. Based on the results obtained in virus inactivation and potency tests as well as the ILTV antibody levels, the antigen inactivated by 3 mM BEI during 16 h was chosen for inactivated ILTV vaccine preparation. The inactivated ILTV suspension containing ≥$10^{5.5}$ EID_{50}/ml was mixed and homogenized with ISA 70 (SEPPIC Co., France) adjuvant and 0.5 ml of the prepared antigen was consider as one dose. The efficacy of the inactivated ILTV vaccine candidate was then evaluated in experimental and field trial studies.

Efficacy evaluation. Experiment-I. In order to evaluate serological responses to the inactivated ILTV antigen, a total of eight-weeks old SPF chickens were randomly divided to four groups (each of 20 birds). Treated groups included two groups were chickens received the inactivated ILTV plus ISA 70 by SC (ISA-SC) and IM (ISA-IM) administration. A group receiving commercial live-attenuated ILTV vaccine (RVSRI, Iran) via eye-drop was considered as positive control group, while non-vaccinated group was used as negative control group. Blood samples were collected from 10 birds per group four weeks post-vaccination (12 weeks of age). The serological immune responses were evaluated by comparing the serum antibody level against ILTV by ELISA and SN tests. The international and institutional guidelines for the care and use of animals were performed in the study. Experiment-II. Based on the results obtained in the experiment-I, ISA-SC was chosen. Four 8-week-old groups of Hyline pullets housed in experimental facilities were vaccinated with 1) attenuated commercial ILTV vaccine (RVSRI, Iran) as the positive control (n = 300 chickens/group), 2) the inactivated ILTV vaccine (n = 300), 3) combination of commercial live and the provided inactivated ILTV vaccines (live+inactivated; n = 300), and 4) without vaccination as the negative control (n = 100). In order to measure antibody titers against ILTV, blood samples were collected at 0, 1, 2, 3 and 4 months post-vaccination (0 = before administration). At 2 and 4 months post-vaccination, ten birds in each group were challenged via intra-tracheal inoculation of 10^3 EID_{50}/bird of a virulent ILTV. The birds were observed for general and specific clinical signs of the diseases during the next 3 weeks post-challenge.

Statistical analysis. Data were subjected to analyses of variance using generalized linear model (GLM) procedure of SAS 9.2 (SAS, 2004). Before analyses, normal distribution of continuous data was tested by UNIVARIATE procedure and Shapiro-Wilk test. To compare proportion of birds protected in challenge tests, data were analyzed by GENMOD procedure using a binary distribution and a logit odds ratio link. The mathematical model for data which were measured once was: $Y_{ij}=\mu + T_i + e_{ij}$, in which, Y_{ij} is observations, T_i is treatment effect, and e_{ij} is a residual random error. For analyses, data were measured over time by ELISA for ILTV antibodies detection, the effect of time and two-way interaction effect of treatment×time were included in the model. Results were reported as mean and standard error (SE). Tukey's test was used for multiple comparisons of the mean and statistical differences and tendencies were declared at $P < 0.05$ and $0.05 < P < 0.10$, respectively.

Results

Isolation and identification of ILTV

Hemorrhage in tracheas and larynxes with mucus exudates were observed in postmortem examination of the suspected birds (Fig. 1a). Formation of typical pocks on CAM of embryonated SPF eggs inoculated with the homogenized laryngotracheal samples indicated the presence of ILTV (Fig. 1b). Amplification of a 675 bp sequence from ILTV TK gene in PCR (Fig. 2) and also appearance of the clear precipitation lines in AGID test confirmed successful isolation of ILTV. The TK ILTV sequence is accessible in the GenBank under Acc. No. JF838220.

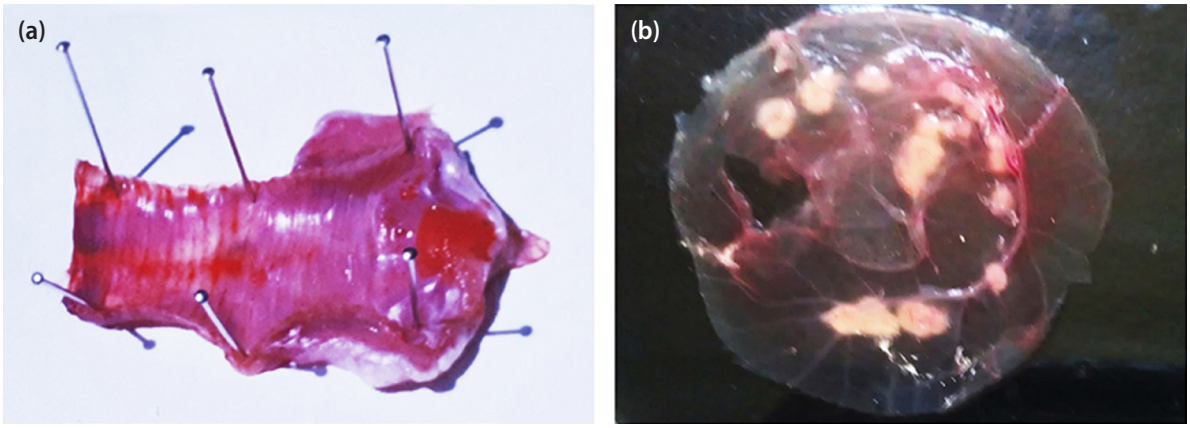

Fig. 1

ILTV pathology

Hemorrhagic laryngotrachea of chickens infected with infectious laryngotracheitis virus with mucus exudate **(a)**; formation of white pock as a typical pathological lesion of the virus on chorioallantoic membrane of embryonated SPF egg **(b)**.

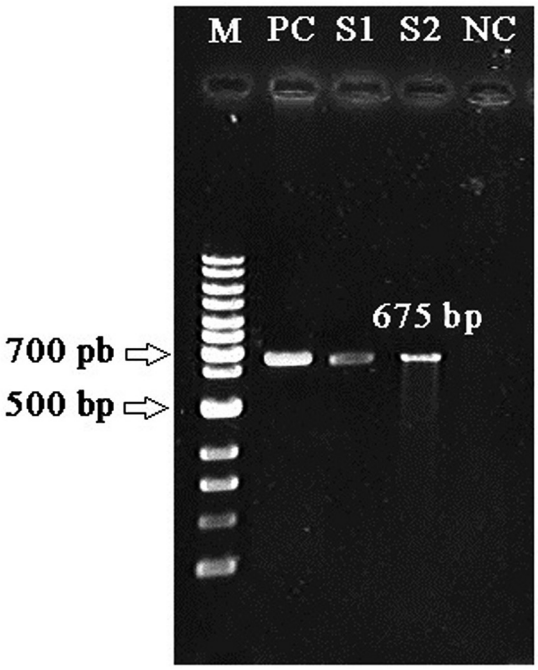

Fig. 2

Amplification of ILTV thymidine kinase gene

ILTV thymidine kinase gene fragment with molecular weight of 675 bp; M, 100 bp molecular weight marker; PC: positive control (purified ILTV DNA of ILTV vaccine); S1 and S2: field isolates; NC: negative control.

Virus inactivation

The effect of inactivators concentration and inactivation time on infectivity of ILTV and specific antibody titers are represented in Table 1. Inactivation of ILTV was confirmed after three passages in CAM of chicken embryonated eggs. No pock formation was observed when the virus was completely inactivated. The highest sera antibody titer against ILTV not being associated with infectivity was noted when the antigen was inactivated by 3 mM BEI ($P < 0.05$). In addition, there was no significant difference between ELISA antibody titer of the prepared antigen inactivated by BEI during 16 or 24 h. The results suggested that treatment of ILTV by 3 mM BEI during 16 h resulted in better inactivation and induction of higher antibody levels.

Experiment-I

Neutralizing index and mean antibody titers against ILTV in the treated SPF chickens are represented in Table 2. Administration of the adjuvant ISA 70 combined with inactivated virus either by IM or SC administration resulted in higher ($P < 0.05$) neutralizing index and ELISA antibody titer compared to the positive control birds receiving live-attenuated ILTV vaccine via eye-drop. However, route of administration did not affect ($P > 0.05$) neutralizing index or antibody titer in response to the inactivated ILTV.

Experiment-II

Neutralizing index and mean antibody titers against ILTV in the treated chickens under field trial conditions are represented in Fig. 3 and 4. As shown in Fig. 3, the highest neutralization index was observed in chickens inoculated by combination of the inactivated virus and the live ILTV vaccine compared to the other corresponding groups ($P < 0.05$). Similar results were recorded for antibody titers against ILTV (Fig. 4), where both ISA-inactivated virus and

Table 1. Effect of three inactivators at different concentrations and inactivation times on infectivity and ELISA antibody titer of infectious laryngotracheitis virus

Inactivator											
Formalin				BPL				BEI			
Con (%)	Time (h)	Infectivity	Antibody titer	Con (%)	Time (min)	Infectivity	Antibody titer	Con (mM)	Time (h)	Infectivity	Antibody titer
0.05	16	+	ND	0.05	90	+	ND	1	16	+	ND
0.05	24	+	ND	0.05	120	+	ND	1	24	+	ND
0.1	16	+	ND	0.1	90	+	ND	3	16	-	1919±205
0.1	24	-	1404±145[a]	0.1	120	-	1540±176	3	24	-	1727±140
0.2	16	-	1069±117[ab]	0.2	90	-	1267±152	5	16	-	1714±188
0.2	24	-	965±108[b]	0.2	120	-	1195±121	5	24	-	1505±176

Note: BPL, beta-propiolactone; BEI, binary ethyleneimine; Con, concentration; ND, not done. [a,b]Within each column, values with different superscripts are significantly different (P <0.05).

live ILTV vaccine induced significant amount of antibodies. However, the highest level of antibodies was noted in the group receiving inactivated virus and live vaccine (P <0.05). The ratio of survived birds in the first and second challenge tests was higher in the inactivated virus and live vaccine combination group compared to either the inactivated virus or the control groups (P <0.05; Table 3). Receiving only inactivated ILTV was associated with 30–40% protection which was significantly lower than in combination treated group (P <0.05). All the birds in the negative control group showed symptoms of the disease following challenges.

Discussion

Vaccination against ILTV in the area with history of ILTV prevalence is the main strategy on controlling the disease. Currently, the ILTV vaccines available for use in the poultry industry are chicken embryo origin and tissue-culture origin modified live vaccines. Like other herpes viruses, ILTV becomes latent and may reactivate at the stressful conditions leading to horizontal transmission to naïve chickens (Garcia *et al*., 2013; Noormohammadi and Devlin, 2018). Use of the live ILTV vaccine requires strict safety standards due to concerns about reversion to virulence and the possibility of virus passaging on the farms. The inactivated ILTV vaccine could help to mitigate production loss in laying farms. In the present study, a local ILTV isolate was inactivated with formalin, BPL, and BEI at different concentrations and different incubation times. The best inactivated antigen was formulated with ISA 70 adjuvant and administered via SC and IM routes to chickens in order to determine its immunogenicity and potency. The capability of the adjuvanted-inactivated ILTV to enhance antibody immune responses was also investigated in a field trial conditions.

Table 2. Efficacy of the inactivated infectious laryngotracheitis virus antigen adjuvanted with ISA 70 in the different routes of administration in SPF chickens

Experimental group*	Neutralizing index (log_{10})	Antibody titer
ISA-SC	1.83	2849±217[a]
ISA-IM	1.83	2685±209[a]
Positive control	1.50	2142±178[b]
Negative control	0	146±28[c]

*Treatments were included ISA-IM, ISA 70 adjuvant and intramuscular administration; ISA-SC, ISA 70 adjuvant and subcutaneous administration; Positive control, administration of live-attenuated ILT vaccine by eye drop; Negative control, no vaccine administration. [a-b]Within antibody titer column, values with different superscripts are significantly different (P <0.05).

Table 3. Survival percentage of commercial pullets infected by infectious laryngotracheitis virus at two time-point by challenge tests

Treatment group	Vaccination route	Post-vaccination month	
		2	4
Inactivated antigen	subcutaneous (SC)	40% (4/10)[b]	30% (3/10)[b]
Live attenuated vaccine	eye drop (ED)	90% (9/10)[a]	80% (8/10)[a]
Lnactivated + attenuated	SC+ED	100% (10/10)[a]	100% (10/10)[a]
Negative control	no vaccination	0% (0/10)[c]	0% (0/10)[c]

[a-c]Within each column, values with different superscripts are significantly different (P <0.05).

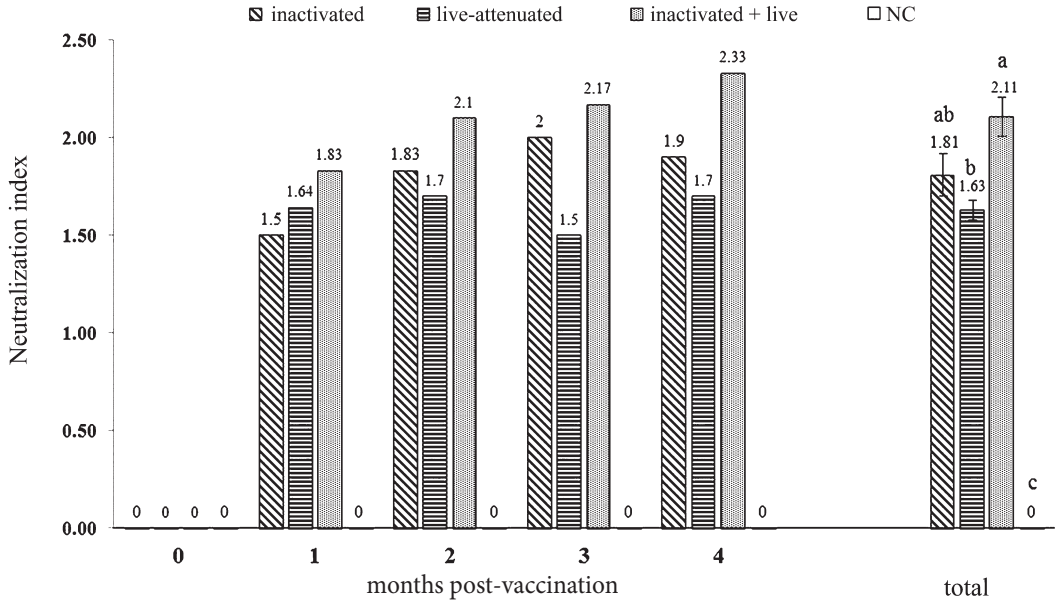

Fig. 3

Neutralization index of ILTV

Comparison of neutralization index in 8-week-old commercial chickens inoculated with the inactivated ILTV antigen and live-attenuated vaccine. NC: negative control (no vaccine administration). a-c: values with different superscripts are significantly different (P <0.05).

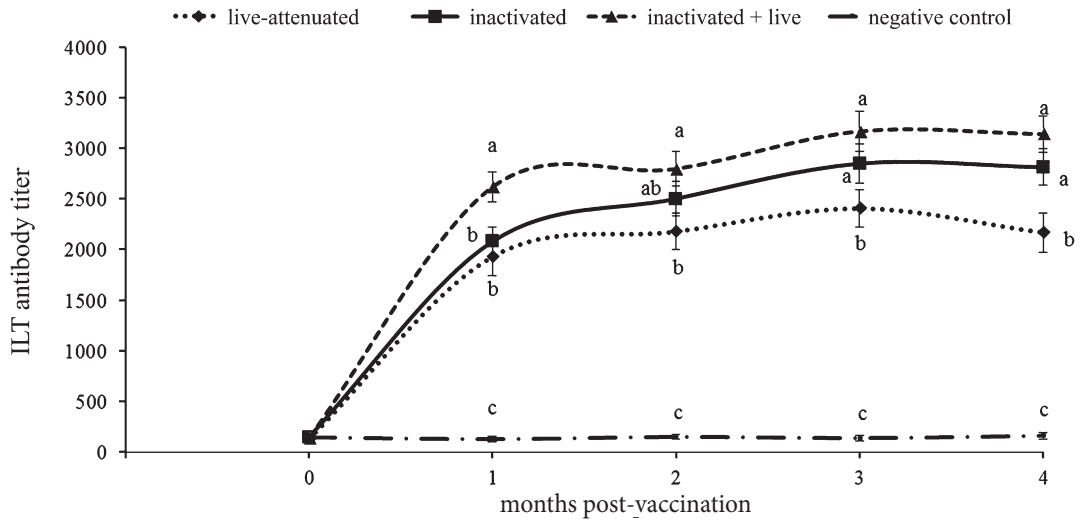

Fig. 4

Antibody titers after ILTV vaccination

ELISA antibody titers against ILTV in 8-week-old commercial chickens vaccinated by an inactivated, live-attenuated, or combination of live-attenuated and inactivated ILTV vaccines up to 4 months post-vaccination. Negative control, no vaccine administration; a-c: within each post-vaccination time; values with different superscripts are significantly different (P <0.05).

The complete inactivation of ILTV with BEI was achieved at a concentration of 3 mM within 16 h incubation at 37°C after three passages in the CAM of SPF embryonated eggs. For many years BEI is widely used for the inactivation of viruses in the vaccine production procedure. This substance is a member of aziridin group

with two protonizable amino groups and two methylene bridges (Delrue *et al.*, 2012), mainly affecting the viral DNA or RNA. BEI was used to inactivate many viruses, such as avian and human influenza viruses (Herrera-Rodriguez *et al.*, 2019), Newcastle disease virus (Jagt *et al.*, 2010), and West Nile virus (Chowdhury *et al.*, 2015). It has been shown that by 1.6 mM concentration of BEI foot-and-mouth disease virus was inactivated within 8–10 h of incubation (Bahnemann, 1975). Also treatment of rabies virus with low concentration of BEI (1 mM) inactivated the virus within 2 h (Mondal *et al.*, 2005).

The surface glycoproteins B and D of ILTV play an essential role in eliciting protective immune responses and the conformational integrity of these proteins should not be changed during the vaccine development procedure. Here, despite the complete inactivation, the antigenicity of ILTV can still be preserved. No decrease in ELISA antibody titer and SN index was found when the SPF chickens received the inactivated ILTV antigen indicating that BEI does not affect the immunogenic properties of the surface proteins. BEI is an alkylating agent and reacts with the nucleophilic guanine of viral nucleic acids. It has been found that the nucleophilic substitution leads to the opening of the BEI ring and alkylation of N7-guanine and adenine. Due to the faster rate of ring-opening reaction in RNA than DNA (Delrue *et al.*, 2012), BEI is not interacting with proteins especially when used at low concentrations. In contrast, formalin can denature the virus envelope proteins which serve as a target for immune responses. Such a change in conformational integrity of a protein and also RNA-protein cross linking affects its function; therefore, the viral antigen inactivated with formalin may not be ideal vaccine antigen. The other observed problem is the persistence of incompletely inactivated virus particles in the formalin inactivated vaccine, which can cause poor induction of neutralizing antibody responses (Metz *et al.*, 2004).

As a result of the inactivation trial, we evaluated the efficacy of adjuvanted BEI-inactivated ILTV antigen in SPF chickens and Hyline pullets. The significant higher antibody response and SN index were found in SPF chickens when subcutaneously administrated with ISA-ILTV antigen compared to the live ILTV vaccine. A similar trend for increased antibody titer has been reported in sensitive chickens vaccinated with inactivated ILTV vaccine (Bahnemann, 1997; Fahey *et al.*, 1983; Palomino-Tapia *et al.*, 2019). By comparing two vaccine administration routes, higher ELISA titer was observed in the groups of chickens which received the vaccine by SC administration, although the difference was not significant. In the field trial, while induction of a high level of serum antibody response was observed in both the ISA-inactivated virus and live ILTV vaccine groups, only the inactivated virus in combination with live ILTV group showed full protection. A single administration of inactivated ILTV induced significant humoral immune responses but could not provide sufficient protection against the virus challenge indicating no significant correlation between protection against ILTV and general antibodies (Devlin *et al.*, 2010; Gharaibeh *et al.*, 2008; Sabir *et al.*, 2019). In agreement with our results, it has been shown that administration of ILTV recombinant or inactivated vaccines in chickens could not induce complete protection against the disease, while vaccination with ILTV modified live vaccines in combination with recombinant or inactivated vaccines had a better protection (Johnson *et al.*, 2010; Oldoni *et al.*, 2009; Palomino-Tapia *et al.*, 2019; Rodriguez-Avila *et al.*, 2008; Vagnozzi *et al.*, 2012). Due to the responsibility of innate and cell-mediated responses for protection against ILTV rather than humoral immunity (Coppo *et al.*, 2013), a combination of inactivated ILTV and live ILTV vaccines was also included in the experiment to determine a more efficacious vaccination regimen. This regimen was able to induce stronger humoral responses and protect chickens better than the live attenuated vaccine.

One potential approach to improve the less immunogenic inactivated vaccines is to include adjuvants. The oil adjuvanted-vaccines are generally acting as direct immune system activating potentiators, maintain antigen at the injection site, increase antigen uptake by antigen presenting cells, and induced antigen-specific antibody responses early after immunization (Aucouturier *et al.*, 2001; Jansen *et al.*, 2005). Such availability of an antigen is necessary for the maintenance of the circulating antibodies for a prolonged time. On the other hand, the mucosal delivery of virus greatly influences the replication of ILTV and lymphocyte infiltration in avian trachea and conjunctiva (Beltrán *et al.*, 2017). In our study, neither of the vaccines could provide full protection against the challenged ILTV. It seems that the effectiveness of vaccination against ILTV depend on the correlation between the adjuvanted-ILTV antigen response in promoting B cell activation and the live attenuated virus in eliciting cell-mediated immunity.

Conclusion

The implementation of a vaccination program against ILTV has reduced the incidence of the infection in poultry. We managed to produce an effective inactivated ILTV antigen based on a local isolate and demonstrated its effect on the immune response following vaccination in chickens. A combination of ILTV inactivated ILTV and live ILTV vaccines is suggested in terms of inducing the highest serum antibody titers and sufficient protection

against the infection; however, this vaccination regimen warrants further optimization.

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