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Short Communication

Optimizing the process of inactivating influenza virus subtype H9N2 by formalin in the production of killed avian influenza vaccine

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ABSTRACT

Avian influenza is one of the most important diseases in avian industry, which also threats human population. Thus, vaccination is necessary for controlling this viral disease. In this study, killed vaccine of avian influenza subtype H9N2 and formalin solution (for virus inactivation) were used. It is necessary to study the effect of different factors such as formalin concentration, as well as incubation temperature and duration on inactivation process. For this purpose, after preparation and measurement of antibody titers of vaccinal strains of avian influenza, 27 experimental samples of H9N2 avian influenza virus were prepared at different formalin concentrations (0.1%, 0.05%, and 0.025%), at different incubation temperatures (4 °C, 25 °C, and 37 °C), and in different incubation durations (12, 18, and 24 h). In addition, three control samples were prepared at three different test temperatures without adding formalin. All the samples were evaluated by inactivation test, hemagglutination assay, and measurement of free formaldehyde. All the experiments were repeated within three consecutive periods. Considering the findings and destructive effects of long incubation durations at 37°C on antigens, the temperature of 25 °C was more suitable. Furthermore, the free formaldehyde amounts at different concentrations at 25 °C were slightly different in comparison with 37 °C. Therefore, formalin concentration of 0.1% at 25 °C completely inactivated the virus within 24 h and was proposed as the optimal condition.

Keywords: Inactivation, Formalin, Influenza virus H9N2, Avian influenza vaccine

Optimisation de la procédure d'inactivation par la formalin edu sous-type H9N2 du virus de l'influenza pour la production de vaccins inactivés

Résumé: L'influenza aviaire est l'une des maladies les plus importantes touchant l'industrie avicole, qui menace également la santé des populations humaines. La vaccination est donc nécessaire pour le contrôle de cette maladie. Afin d'optimiser la procédure d'inactivation du sous-type H9N2 du virus de l'influenza aviaire, les effets de facteurs variés, comme la concentration de formaline ainsi que les durées et températures optimales d'incubation ont été étudiés. A cet effet, après la préparation et l'évaluation des titres d'anticorps induits par les souches vaccinales de l'influenza aviaire, 27 échantillons expérimentaux du sous-type H9N2 du virus de l'influenza aviaire ont été préparés à différentes concentrations de formaline (0,1%, 0,05%, et 0,025%) ainsi qu'à différentes températures (4 °C, 25 °C et 37 °C) et durées (12 h, 18 h, et 24 h) d'incubation.

De plus, 3 échantillons préparés à trois températures différentes et sans formaline ont été utilisés comme témoins. Tous les échantillons ont été ensuite soumis à des tests d'inactivation et d'hémagglutination ainsi qu'à une évaluation du taux de formaldéhyde libre. Toutes ces analyses ont été répétées trois fois. Nos De plus, 3 échantillons préparés à trois températures différentes et sans formaline ont été utilisés comme témoins. Tous les échantillons ont été ensuite soumis à des tests d'inactivation et d'hémagglutination ainsi qu'à une évaluation du taux de formaldéhyde libre. Toutes ces analyses ont été répétées trois fois. Nos résultats ont révélé l'effet délétère d'une incubation prolongée à 37 °C sur les antigènes d'intérêts alors qu'une incubation à 25 °C s'est avérée être plus appropriée. D'autant plus que la formation de formaldéhyde libre différait sensiblement lors des incubations à 25 °C et 37 °C. En résumé, une incubation de 24 h en présence de formaline 0,1% à 25 °C été capable d'inactiver complétement le virus et représente donc la condition optimale proposée dans cette étude.

Mots clés: Inactivation, Formaline, Influenza virus H9N2, Vaccin influenza aviaire

INTRODUCTION

Regulations and safety concerns mandate viral clearance in biopharmaceuticals and vaccines, which involves the removal and/or inactivation of viruses. Viruses require different inactivation methods depending on their type (Vijayasankaran et al., 2009). Avian influenza is a major viral disease in the industrial poultry of many countries, such as Mexico, Pakistan, China, Italy, and Iran (De Benedictis et al., 2007). According to the literature, inactivated monovalent and trivalent avian influenza vaccines could provoke the immune system to protect the host against viral infections and death, thereby leading to the avoidance of reduced egg production in avian industry and prevention of virus transfer from the host to human (Butterfield and Campbell, 1979; Stone, 1987; Swayne et al., 2001; Swayne et al., 2006). Avian influenza viruses (AIVs) belong to the *Orthomyxoviridae* family and influenza virus A genus. AIVs are single-stranded, negative-sense, enveloped RNA viruses. Envelope of these viruses is sensitive to ether, chloroform, and various chemical disinfectants and Haemagglutinin (HA) and neuraminidase (NA) proteins are known as the main sources of the antigenic features of viruses (Portela and Digard, 2002; Shojaei et al., 2015). Low-pathogenic AIV subtype H9N2 was identified following the infection of chickens in Italy, pheasants in Ireland, ostriches in South Africa, turkeys in the United States, and chickens in Korea and China, as well as other reported cases in Pakistan and chicken influenza outbreak in Iran (1998) (Brown, 2010). In Pakistan and Mexico, vaccination of chickens with inactivated oil-emulsion vaccines under subtypes H5N2 and H7N3 has been used against avian influenza outbreak (Alexander, 2000). Production of inactivated vaccines, also known as "killed vaccines", involves the use of microorganisms, which must be killed or inactivated since they are considered unsafe in the living form. Inactivation of these microorganisms is possible through using certain physical or chemical factors in such vaccines. Some of the most important physical factors in the inactivation process include heat, pasteurization, ultraviolent radiation, and Gamma ray. As for chemical factors, use of formaldehyde, β-Propiolactone, Sodium periodates, solvents or detergents, psoralens, and aziridines is essential to the completion of the inactivation process (Hilleringmann et al., 2014). In Iran, one of the vaccines produced in Razi Vaccine and Serum Research Institute (northwest branch) is the avian influenza vaccine using the killed AIV subtype H9N2. It is noteworthy that the inactivation stage is considered crucial to the production of this vaccine. In this institute, 37% formalin (w/w) is an inherent element in the inactivation process, which has long been used for the proper conduction of this stage. Formaldehyde is an organic compound in the form of colorless gas with an unpleasant smell, which is generally available as a

watery solution with weight percentage of 30-50 in the commercial market; this solution is known as formalin. In global markets, the most common form of this solution is 37% formalin (w/w) (Salthammer et al., 2010). In the production of inactivated vaccines, some of the most prominent influential factors during virus inactivation by formalin include temperature, time and concentration, all of which significantly affect the proper completion of the inactivation process. This study aimed to achieve the optimal conditions for complete inactivation and determine the required concentration of formalin to achieve minimum value of free formaldehyde in the final product. Furthermore, we assessed the antigenicity and immunogenicity of the obtained vaccine with the least possible changes.

MATERIALS AND METHODS

Preparation of virus. The seed used in this study was AIV subtype H9N2 (A/Chicken/Iran/99/H9N2), which was inoculated and proliferated in the allantoic cavity of 11-day embryonated eggs after the preparation stages. Following the inoculation, the eggs were incubated at the temperature of 37.5 °C with 60% humidity for 72 h. Candling was performed once every 24 h and wasted eggs in the first 24-h candling were eliminated from the experiment. After 72 h, the remaining eggs were kept in a refrigerator at the temperature of 4°C for 24 h. After refrigeration, the eggs were opened to collect the amnio-allantoic fluid separately, which was the same active antigen used in the experiments (Abdi et al., 2016).

Virus titration. Collected amnio-allantoic fluid (active antigen) was evaluated in terms of haemagglutination in a 96-well V-bottom microwell plate using 1% chicken red blood cells and prepared with final titration of HA=10 $\log_2$ (OIE, 2014). In addition, infective titers of the obtained antigen were determined using the method proposed by Reed and Muench and reported as EID_{50}=9.8 $\log_{10}$ (Reed and Muench, 1938).

Formaldehyde solution (37% w/w). For this study, formaldehyde solution was purchased from Merck company, Germany (Batch No: K42183502, Lot No: 10.04002.2500) in a 2.5-liter container. The solution was diluted using 1% (v/v) phosphate-buffered saline to be applied in the experiments.

Sample preparation. In this study, formaldehyde solution 37% (w/w) was used in three concentrations of 0.1%, 0.05%, and 0.025% (v/v). Incubation periods were determined at 12, 18 and 24 h at temperatures of 4, 25 and 37 °C. Samples were classified into 27 groups, nine of which were administered with 0.1% formalin concentration at the mentioned incubation temperatures and periods. Other sample groups were administered with formalin concentrations of 0.05% and 0.025%. For the proper classification of samples, sample groups with 0.1%, 0.05%, and 0.025% formalin concentration were represented as A, B and C, respectively. Incubation temperature and duration of each sample group was briefed in the following manner: C-25-24 (0.025% concentration, 25 °C temperature, and 24-h incubation). In this study, we assessed three control groups without formalin at temperatures of 4, 25, and 37°C. Finally, sample groups were preserved in a total of 30 vials. To prepare the samples with 0.1% concentration, 90 cc of the active antigen and 10 cc of 1% formalin were added to nine sterile vials in aseptic conditions. In the next stage, three vials were transferred to a refrigerator with the temperature of 4 °C, three vials were placed in an incubator at the temperature of 25 °C, and three vials were transferred to an incubator with the temperature of 37 °C. Related experiments were carried out after 12, 18, and 24 h, and the other vials were prepared similarly.

Inactivation test. From each of the experimental vials, 0.2 cc was injected to the allantoic cavity of 10 embryonated eggs (9-11 days) using insulin syringes, and all the eggs were labeled with the properties of each sample group. After inoculation, the eggs were incubated for 72 h at the temperature of 37.5 °C. Moreover, the eggs were candled once every 24 h and the wasted eggs were removed. After 72 h, the remaining eggs were kept in a refrigerator at the

temperature of 4°C for 24 h. Following that, the eggs were opened and evaluated in terms of macro-haemagglutination using 10% chicken red blood cells (RBCs). This process was repeated for three passages. In case any of the eggs (n=10) in each passage proved positive for macro-haemagglutination and showed agglutination with 10% RBC, the test would be repeated. Obtaining the same results was indicative of incomplete or improper inactivation process.

Haemagglutination (HA) assay. In accordance with virus titration, HA assay was performed for each vial in order to evaluate the antigenic features of the samples under different conditions (OIE., 2014).

Free formaldehyde test. Free formaldehyde test is considered a fundamental physicochemical analysis to determine the value of free formaldehyde in the final product based on the optical absorption at wavelength of 410 angstrom. In brief, this test consists of four main stages: 1) preparation of standards, 2) dilution of samples, 3) addition of the complex component, and 4) reading of the results at wavelength of 410 angstrom (Appendix, 2011).

RESULTS AND DISCUSSION

In the present study, in order to optimize the inactivation process in the production of avian influenza vaccine, we used formaldehyde in three concentrations of 0.1%, 0.05%, and 0.025% at temperatures of 4, 25 and 37°C with incubation periods of 12, 18, and 24 h. All the samples were evaluated based on three main tests, including inactivation, HA assay, and free formaldehyde test. In the sample group with incubation temperature of 4 °C, all embryos died due to the absence of formalin effects at this temperature. As such, inactivation test results were disregarded in this group. Inactivation test results of other experimental groups are presented in tables 1 and 2. In this study, complete inactivation was considered a prerequisite to the selection of the optimal sample group; in other words, results of the inactivation test had to be negative in all the three passages. According to our findings, groups A-25-24, A-37-18, and A-37-24 contained the optimal samples, and one of these groups could be selected after performing the HA assay and free formaldehyde test. Results of the HA assay are shown in Table 3. In the current research, results of t-test were indicative of no statistically significant differences between the sample groups administered with 0.1% formalin concentration at different incubation temperatures and durations (P<0.05). In this regard, Ghadimipour et al. (2013) evaluated the inactivation process in different conditions and reported no effect on the virus HA titer.

Table 1. Results of inactivation test for avian influenza virus subtype H9N2 using different concentrations of formalin at 25 °C

Concentration	0.1%			0.05%			0.025%		
	F.P	S.P	T.P	F.P	S.P	T.P	F.P	S.P	T.P
12 h	-	-	+	-	-	+	-	+	+
18 h	-	-	+	-	-	+	-	+	+
24 h	-	-	-	-	-	+	-	+	+

F.P: first passage, S.P: second passage, T.P: third passage - = absence of agglutination, + =agglutination and incomplete inactivation

Table 2 . Results of inactivation test for avian influenza virus subtype H9N2 using different concentrations of formalin at 37 °C

Concentration	0.1%			0.05%			%0.025		
	F.P	S.P	T.P	F.P	S.P	T.P	F.P	S.P	T.P
12 h	-	-	+	-	-	+	-	+	+
18 h	-	-	-	-	-	+	-	-	+
24 h	-	-	-	-	-	+	-	-	+

F.P: first passage, S.P: second passage, T.P: third passage - = absence of agglutination, + =agglutination and incomplete inactivation

Results of free formaldehyde test are presented in tables 4 and 5. Values of free formaldehyde in different sample groups were based on g/l. Results of t-test were indicative of no statistically significant differences between the samples administered with 0.1% formalin concentration at different incubation temperatures and durations in terms of free formaldehyde (P<0.05). In

the present study, in addition to sample group A-25-24, two groups of A-37-18 and A-37-24 showed negative results in all the three passages. In other words, complete inactivation of AIV subtype H9N2 was carried out at the temperature of 37 °C using 0.1% formalin concentration with the incubation period of 18 and 24 h. This finding is consistent with the results of previous studies in this regard. For instance, in a research by De Benedictis et al. (2007), inactivation of low- and high-pathogenicity AIV was performed through incubation for 16 h at the temperature of 37 °C and 0.12% formalin dilution.

Table 3 . Results of hemagglutination assay in different samples

Concentration	0.1%			0.05%			0.025%		
	4°C	25°C	37°C	4°C	25°C	37°C	4°C	25°C	37°C
12 h	9	9	10	9	9	10	9	10	9
18 h	9	10	9	9	10	9	10	9	10
24 h	9	9	9	10	9	9	9	9	9

According to the findings of Chatchai et al. (2007), formalin inactivation at the temperature of 37 °C has greater effects compared to inactivation at 25 °C.

Table 4 . Calculation of final free formaldehyde concentration in different samples

Conc. of Standards	Abso .of Standards	Abso .of Samples	Conc. of Samples based on abso.	Conc. Of samples based on dilution	Final Conc.	Groups
0.000314	0.078	0.122	0.00053263	0.026631498	0.266314983	A-37-24
0.000627	0.14	0.059	0.000264613	0.013230665	0.132306645	B-37-24
0.001254	0.285	0.012	6.46643E-05	0.003233217	0.032332171	C-37-24
0.002509	0.59	0.119	0.000519867	0.025993363	0.259933634	A-37-18
		0.057	0.000256105	0.012805241	0.128052412	B-37-18
		0.014	7.31728E-05	0.00365864	0.036586403	C-37-18
		0.118	0.000515613	0.025780652	0.257806517	A-37-12
		0.06	0.000268868	0.013443376	0.134433762	B-37-12
		0.011	6.04101E-05	0.003020505	0.030205054	C-37-12
		0.137	0.000596443	0.029822173	0.298221731	A-25-24
		0.051	0.000230579	0.011528971	0.115289713	B-25-24
		0.031	0.000145495	0.007274738	0.072747384	C-25-24
		0.139	0.000604952	0.030247596	0.302475964	A-25-18
		0.053	0.000239088	0.011954395	0.119543946	B-25-18
		0.033	0.000154003	0.007700162	0.077001617	C-25-18
		0.135	0.000587935	0.02939675	0.293967498	A-25-12
		0.049	0.000222071	0.011103548	0.11103548	B-25-12
		0.031	0.000145495	0.007274738	0.072747384	C-25-12

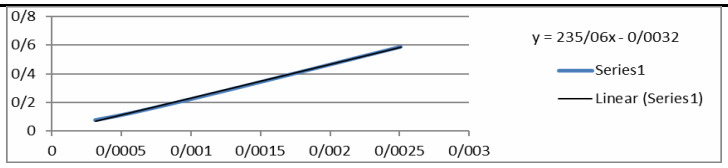

Table 5 . Summarized results of free formaldehyde test in different samples

Concentration	0.1%		0.05%		0.025%	
	25°C	37°C	25°C	37°C	25°C	37°C
12 h	0.29	0.25	0.11	0.13	0.07	0.03
18 h	0.3	0.25	0.11	0.12	0.07	0.03
24 h	0.29	0.26	0.11	0.13	0.07	0.03

Furthermore, the mentioned study confirmed that complete virus inactivation could be achieved at the incubation temperature of 37 °C for 18 h, while the same conditions were compared with the temperature of 25 °C, and agglutination was observed in the third passage. Another research by Tian et al. (2005) was conducted on AIV subtype H5N1, which suggested that complete inactivation, could be carried out at the incubation temperature of 37 °C for 24 h using 0.02% formalin concentration. Inconsistently, findings of the present study indicated that the inactivation of AIV subtype H9N2 could not be completed using 0.05% formalin concentration (twice higher than the concentration used in the study by Tian et al. (2005) at the incubation temperature of 37 °C for 24 h. With respect to low- and high-pathogenicity AIV subtype H9N2, findings of Angin et al. (2012) indicated that complete inactivation could be achieved using 0.1% and 0.04% formalin concentrations at the incubation temperature of 37 °C for 16 h, which is in line with our findings in the case of 0.1% formalin concentration, while it is inconsistent with regard to 0.05% formalin concentration. Since lower temperatures are considered suitable for biological contents, 25 °C is more advantageous compared to 37 °C for the proper conduction of the inactivation process. Therefore, in the current study, sample group A-25-24 with inactivation at the incubation temperature of 25 °C for 24 h using 0.1% formalin concentration was considered to have the optimal condition. With regard to the sample group with 0.1% formalin concentration at the temperature of 4 °C, the inactivation test results were negative up to the second passage for one week, while agglutination was observed in the third passage, which was suggestive of incomplete inactivation. Previous studies have proposed that effects of formalin inactivation may decline at low temperatures. In their research, Jang et al. (2014) used a commercial composition consisting of 20% formaldehyde and 12% oligomer pentaerythritose to inactivate AIV subtype H7N1 at low temperatures, which was reported to be effective in this regard. Moreover, another study by Lombardi et al. (2008) focused on the inactivation of AIV subtype H7N2 using a formalin composition with propylene glycol (50% mass/mass) at low temperatures, which was reported to be effective in the inactivation process. Considering the results of the current and aforementioned studies (Lombardi et al., 2008; Jang et al., 2014), further investigations on the proper concentration of antifreeze materials in avian influenza viruses' inactivation process at low temperatures is recommended. Some of the influential factors in the optimization of the inactivation process are the type and concentration of antifreeze and incubation period at the temperature of 4 °C, which must be taken into account in other studies in this regard.

In the present study, complete inactivation was achieved in sample group A-25-24, while it could not be achieved in sample group A-25-18 (interval of groups: 6 h). Complete inactivation could be possible in incubation periods ranging between 18-24 h. Therefore, it is recommended that to obtain the optimal incubation time, periods of 19, 20, 21, 22, and 23 h be further examined.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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