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Evaluation of different inactivation methods for high and low pathogenic avian influenza viruses in egg-fluids for antigen preparation

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In view of the emerging avian influenza (AI) viruses, it is important to study the susceptibility of AI viruses to inactivating agents for preparation of antigens and inactivated vaccines. The available information on susceptibility of both the high and low pathogenic AI viruses to different inactivating agents is inadequate and ambiguous. It has been shown that different subtypes of influenza viruses require different physical and chemical conditions for inactivation of infectivity. The present study was undertaken to evaluate the use of beta-propiolactone (BPL), formalin and ether for inactivation and its impact on antigenicity of AI viruses.

A total of nine high and low pathogenic AI viruses belonging to four influenza A subtypes were included in the study. The H5N1 viruses were from the clades 2.2, 2.3.2.1 and 2.3.4. The H9N2 virus included in the study was of the G1 genotype, while the H11N1 and H4N6 viruses were from the Eurasian lineage. The viruses were treated with BPL, formalin and with ether. The confirmation of virus inactivation was performed by two serial passages of inactivated viruses in embryonated chicken eggs.

The infectivity of all tested AI viruses was eliminated using 0.1% BPL; 0.04% and 0.1% formalin. Ether eliminated infectivity of all tested low pathogenic AI viruses; however, ether with 0.2% or 0.5% Tween-20 was required for inactivation of the highly pathogenic AI H5N1 viruses. Treatment with BPL, ether and formalin retained virus hemagglutination (HA) titers. Interestingly ether treatment resulted in significant rise in HA titers ($P < 0.05$) of all tested AI viruses. This data demonstrated the utility of BPL, formalin and ether for the inactivation of infectivity of AI viruses used in the study for the preparation of inactivated virus antigens for research and diagnosis of AI.

1. Introduction

Influenza type A viruses belong to the family Orthomyxoviridae and they are single-stranded, negative-sense RNA viruses, with segmented genome. Influenza viruses are enveloped and around 80–100 nm in diameter. On the basis of the antigenicity of the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) molecules, influenza A viruses are classified into 18 HA subtypes (H1–H18) and eleven NA subtypes (N1–N11) (Knipe and Howley, 2007; Tong et al., 2012, 2013). Wild birds, water-fowl, gulls, shore birds and bats are the natural host and reservoir for type A influenza viruses (Kawaoka et al., 1988; Tong et al., 2013).

Avian influenza (AI) viruses are classified into two forms based on the severity of the illness caused in the poultry. Low pathogenic avian influenza (LPAI) viruses cause no observable or mild clinical symptoms whereas highly pathogenic avian influenza (HPAI) viruses cause severe respiratory illness and death among infected chickens (Alexander, 2000). The outbreaks of HPAI viruses have been reported from Asia, Europe, Africa, America and Australia in the poultry (Oie update on avian influenza, 2014). India also reported outbreaks of HPAI H5N1 virus and the presence of LPAI viruses in poultry (Chakrabarti et al., 2009; Nagarajan et al., 2009; Pawar et al., 2012a,b. Human Q3 infections with AI viruses such as H5N1, H9N2, H7N1, H7N3, and H7N9 have been reported from Asia, Africa, and Europe (CDC information on avian influenza, 2014). This shows that AI viruses are imminent threats to public health as zoonotic pathogens.

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The handling of HPAI viruses needs biosafety level-3+ (BSL-3+) facility which limits work on HPAI viruses (Jonges et al., 2010). Therefore, the virus inactivation is a necessary step for the preparation of the virus antigens and inactivated vaccines. The inactivated antigens could be used as reagents for various serological assays and diagnosis of AI.

A rational choice of inactivating agent and inactivation conditions is critical for preparation of inactivated vaccines (Budowsky et al., 1993). Beta-propiolactone (BPL), formalin, and ether have been conventionally used for preparation of inactivated vaccines (Chu, 1948; Francis, 1953; Goldstein and Tauraso, 1970; Swayne and Kapczynski, 2008). BPL is an alkylating agent which modifies the structure of nucleic acids inducing nicks in DNA, cross-linking between DNA and proteins as well as between DNA strands in the double helix (Perrin and Morgeaux, 1995). The mechanism with which viral inactivation is achieved by aldehydes is through the alkylation of amino and sulphydrilic groups of proteins and purine bases (De Benedictis et al., 2007). Formalin, which is an aldehyde derivative, cross-links the viral proteins inhibiting the viral replication (Swayne and Kapczynski, 2008). Ether treatment of influenza virus causes disruption of the viral envelope to smaller fragments (Choppin and Stoeckenius, 1964). Generally, less than 0.1% formalin and BPL is used for inactivated vaccine preparations (WHO, 2003).

The available information on susceptibility of AI viruses to different inactivating agents is inadequate and ambiguous (De Benedictis et al., 2007). It has been shown that different subtypes of influenza viruses require different physical and chemical conditions for inactivation of infectivity (Zou et al., 2013). The present study was undertaken to evaluate the use of BPL, formalin and ether for inactivation of high and low pathogenic AI viruses used in the present study and also to investigate its impact on the antigenicity of AI viruses.

2. Materials and methods

2.1. Viruses used

A total of nine representative high and low pathogenic AI viruses comprising four influenza A virus subtypes were used in the study. The tested viruses were isolated from different avian species (chickens, ducks, crows and wild aquatic bird) either during routine surveillance or during the AI outbreaks in different states of India. The viruses generated by reverse genetics (RG) method were also included. The purpose of using H5N1 viruses generated by RG method was that these viruses are used as seed strains for vaccine production and it is necessary to understand the inactivation properties of these viruses. The viruses used were: four isolates of HPAI H5N1 viruses (A/chicken/Manipur/NIV9743/2007 (clade 2.2) [accession number: FJ719834], A/crow/Jamshedpur/NIVAN1117307/2011 (clade 2.3.2.1), A/chicken/Navapur/India/33487/2006 (clade 2.2) [accession number: DQ887061], A/crow/WB/NIVAN1117301/2011) (clade 2.3.2.1); two reverse genetically modified H5N1 viruses (A/Anhui/2005/PR8-IBCDC-RG-6 (clade 2.3.4), A/India/NIV/2006/PR8-IBCDC-RG-7) (clade 2.2) and three LPAI viruses [A/chicken/Pune/India/099321/2009 (H9N2)-G1 lineage, A/duck/WB/India/101006 (H4N6)-Eurasian lineage [accession number: JX310061], A/Aquatic bird/India/NIV-17095/2007 (H11N1)-Eurasian lineage [accession number: CY055175]. These viruses were propagated in 10-day-old embryonated chicken eggs and the virus titers were determined by the hemagglutination (HA) assay using 0.5% turkey red blood cells (TRBCs) (Pawar et al., 2012a,b; WHO, 2002).

2.2. Inactivation of viruses

2.2.1. Virus inactivation using BPL

BPL (Ferak, Berlin) was diluted in phosphate buffered saline (PBS) and mixed with virus suspension (10 ml) to attain the final concentrations of 0.05% and 0.1%. The preparation was mixed using vortex to form a homogenous mixture and was transferred to a fresh container (15 ml sterile tubes, Tarsons, India) and kept at 4 °C for 16 h in the refrigerator. The preparation was subsequently incubated at 37 °C for 2 h for hydrolysis of BPL (Goldstein and Tauraso, 1970; Jonges et al., 2010). After completion of treatment the virus aliquots were stored at −80 °C until further use.

2.2.2. Virus inactivation using formalin

One percent formalin was prepared in PBS using commercially available formaldehyde solution (Fisher Scientific, India) and mixed with virus suspension (10 ml) to obtain the final concentrations of 0.02%, 0.04% and 0.1%. The preparation was stirred, transferred to a fresh container and incubated at three different time points (16 h at 37 °C, 48 h at 37 °C, 1 week at 4 °C) (Furuya et al., 2010; Jonges et al., 2010; Takada et al., 2003). The virus suspension was stored at −80 °C until further use.

2.2.3. Virus inactivation using ether

Equal quantity of the virus suspension (5 ml) and anesthetic grade ether (5 ml) [Qualigens] were mixed in a 15 ml flat-bottom glass tube with a screw-cap. Adhesive tape was wrapped around the cap to reduce the loss of ether (Andrewes and Horstmann, 1949). The preparation in the tube was stirred for 30 min at room temperature using magnetic stirrer and magnetic bar. The preparation was then centrifuged at 3000 rpm (604 × *g*) for 15 min. The separated aqueous phase was collected in another tube, the residual ether was allowed to evaporate by keeping the preparation open for 1 h in class II B biosafety cabinet, aliquoted and stored at −80 °C until further use (Takada et al., 2003).

Those HPAI (H5N1) viruses which could not be inactivated by ether, were treated with a mixture of ether and Tween-20 (Hi-Media) (Fenters et al., 1970). Tween-20 (1%) was prepared in PBS and added to the virus and ether mixture to obtain the final concentrations 0.05%, 0.1%, 0.2% and 0.5% of Tween-20. The further procedure was similar as above.

2.2.4. Untreated virus controls

The untreated virus controls were subjected to similar experimental conditions as the treatment groups without the addition of inactivating agent. Such controls were included in each experiment.

2.3. Hemagglutination (HA) and hemagglutination inhibition (HI) assays

Titration of untreated and treated viruses was performed in 96-well-V-bottom micro-plates (Tarsons, India). Twofold serial dilutions of viruses were prepared in PBS and incubated with 50 μl 0.5% turkey RBCs (Pawar et al., 2012a,b). The HA titer was expressed as the reciprocal of the highest dilution giving complete hemagglutination. Allantoic fluids from the eggs inoculated with untreated and treated viruses were harvested and tested by HA assay (WHO, 2012). HI assay was performed to determine the reactivity of the inactivated viruses with antibodies. The HI assay was performed using influenza A antisera raised in fowl (*Gallus gallus*) against the viruses used in the study. The antisera were treated with the receptor destroying enzyme (RDE) (Denka Seiken, Japan) at 37 °C for 16 h and then incubated for 30 min at 56 °C to inactivate the (RDE) activity. The assay was performed as described in the WHO Manual on Animal influenza diagnosis and surveillance (WHO, 2002).

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