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Use of embryonated chicken egg as a model to study the susceptibility of avian influenza H9N2 viruses to oseltamivir carboxylate

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A B S T R A C T

Avian influenza (AI) H9N2 viruses are endemic in many bird species, and human infections of H9N2 viruses have been reported. Oseltamivir phosphate (Tamiflu®) is the available antiviral drug for the treatment and prophylaxis of influenza. There are no reports of use of embryonated chicken egg as a model to study susceptibility of AI viruses to oseltamivir carboxylate (OC), the active metabolite. The present study was undertaken to explore the use of embryonated chicken eggs as a model for testing OC against the AI H9N2 viruses. A total of three AI H9N2 viruses, isolated in poultry in India, were used. Various virus dilutions were tested against 14 μg/ml of OC. Three methods, namely (1) the *in vitro* virus–drug treatment, (2) drug delivery and virus challenge by allantoic route, and (3) drug delivery by albumen route and virus challenge by allantoic route were explored. The viruses were also tested using the fluorescence-based neuraminidase inhibitor (NAI) assay. There was significant inhibition ($p < 0.05$) of the H9N2 viruses in presence of OC. The infectious virus titers as well as hemagglutination titers were significantly lower in presence of OC as compared to controls. The *in vitro* treatment of virus and drug; and drug and virus delivery at the same time by allantoic route showed significantly higher inhibition ($p < 0.05$) of virus growth than that by the albumen route. In the NAI assay, the half maximal inhibitory concentration (IC_{50}) values of the H9N2 viruses were within the standard range for known susceptible reference virus. In conclusion, the H9N2 viruses used in the study were susceptible to OC. Embryonated chicken egg could be used as a model to study susceptibility of AI viruses to antiviral drugs.

1. Introduction

Influenza viruses are single stranded, negative-sense RNA viruses, which belong to the family Orthomyxoviridae. The world experienced the most recent influenza pandemic in the year 2009 caused by the H1N1 virus. Avian influenza (AI) viruses such as H5N1, H7N9, and H9N2 are emerging and are potential threats to human and animal health. In addition to this, there are reports of resistance of influenza viruses to the antiviral drugs such as oseltamivir (Monto et al., 2006). Therefore, it is necessary to screen existing as well as emerging influenza viruses against the available antiviral agents.

Oseltamivir (oseltamivir phosphate) (Tamiflu®) is an orally administered antiviral drug, recommended by the World Health Organization (WHO) for use in the clinical management of pandemic and seasonal influenza virus infections of varying severity. Oseltamivir carboxylate (OC) is the active metabolite of oseltamivir, and is a transition-state analog of sialic acid that is a potent selective inhibitor of influenza A and B virus neuraminidases (Hayden, 2005).

There have been increasing speculations about the pandemic potential of AI H9N2 viruses (Paul, 2008). There are reports of prevalence of H9N2 viruses in avian species from several parts of the world, and also from poultry in India (Pawar et al., 2012a; Nagarajan et al., 2009). Human infections of H9N2 virus have been reported from China, Hong Kong, and Egypt (Peiris et al., 1999; Butt et al., 2005; World Health Organization, 2015), and seroprevalence of antibodies against H9N2 viruses has also been reported (Xiong et al., 2014; Zhou et al., 2014; Huang et al., 2013; Pawar et al., 2012b; Hadipour, 2011; Guo et al., 1999).

The existing methods for studying the susceptibility of influenza viruses to oseltamivir include use of neuraminidase inhibition assay and analysis of neuraminidase (NA) gene sequence for the markers of drug resistance to the drug (Hurt et al., 2004; McKimm-Breschkin, 2000). The use of tissue culture, mice, and ferrets for antiviral studies on influenza viruses has been reported

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(Govorkova et al., 2001; Govorkova et al., 2007). Moreover, no correlation between the susceptibility of H5N1 viruses by NAI assay and the protection offered by oseltamivir in mice (*in vivo*) was found (Govorkova et al., 2009).

Embryonated chicken eggs have been conventionally used for isolation and propagation of influenza viruses (World Health Organization, 2002). The antiviral activities of amantidine, rimantidine, and zanamivir against influenza viruses have been studied using the egg model (Haertl et al., 2004; Sauerbrei et al., 2006). In the reported studies, the drug delivery was *via* the albumen route and virus challenge was by the chorioallantoic membrane. The use of the egg model by exploring the allantoic route for virus inoculation and the albumen route for drug delivery has been reported (Wang et al., 2008). However, there are no studies comparing various routes for drug delivery of OC in eggs for AI viruses. The present work was undertaken to study the susceptibility of three isolates of AI H9N2 viruses from India to OC, and to explore three methods for virus and drug treatment and delivery using embryonated chicken egg as a model.

2. Materials and methods

2.1. Viruses used

AI H9N2 viruses isolated from India, namely, A/chicken/India/WB-NIV1057183/2010 (H9N2) [H9N2-WB-1057183] (GenBank: **JX310069**), A/chicken/India/WB-NIV1057209/2010 (H9N2) [H9N2-WB-1057209], and A/chicken/India/JH-NIV 124248/2012 (H9N2) [H9N2-JH-124248], were used in the study.

2.2. Virus propagation and detection

The virus stock was prepared in embryonated chicken eggs by inoculating the virus by the allantoic route. The eggs used in all the experiments were 10-days-old at the time of inoculation and 13-days-old at the time of completion of experiment. The eggs were incubated for 72 h at 37 °C, and were observed daily. After completion of the incubation, the embryos were chilled overnight at 4 °C. The allantoic fluid was harvested, and hemagglutination (HA) assay was performed using 0.5% turkey red blood cells (World Health Organization, 2002). The virus stock was stored at −80 °C till further use. The clinical end point was detection of virus by HA assay as a measure of virus growth in eggs. The study was conducted in accordance with the institutional guidelines.

2.3. Preparation of antiviral drug stock solution and toxicity testing

The antiviral drug OC was kindly provided by Hoffmann-La Roche, Basel, Switzerland. A suspension of the drug was made in phosphate buffered saline (PBS) (pH 7.2). Peak plasma concentration of OC after a 75 mg dose in humans has been reported to be 0.35 μg/ml (Hayden, 2005). Toxicity of OC was tested in eggs by inoculating 14 μg/ml, 28 μg/ml, 56 μg/ml, and 112 μg/ml of OC. The inoculated eggs were observed for 72 h. The embryos were decapitated, and fixed in formalin for a minimum period of 48 hs for histopathological analysis. Whole sections of the embryos were processed for paraffin embedding, sectioned at 4 μm and stained with hematoxylin and eosin; and were examined for histopathological changes.

2.4. 50% egg infectious dose in presence and absence of OC

The 50% egg infectious dose (EID_{50}) titer of H9N2 virus was determined in the presence and absence of 14 μg/ml of OC. The virus was serially diluted tenfold (undiluted to 10^{-9}) and treated by the *in vitro* drug treatment method (Song et al., 2007; Rajik et al., 2009). Each dilution was inoculated in ten eggs. Eggs showing HA titer ≥ 2 HA units were considered positive, while those showing no titer were negative. The EID_{50} was calculated using the Reed and Muench method (Reed and Muench, 1938). The mean log HA titers for individual dilutions were also compared. The experiment was performed three times. 100 EID_{50} virus was then used for the further experiments using three different methods.

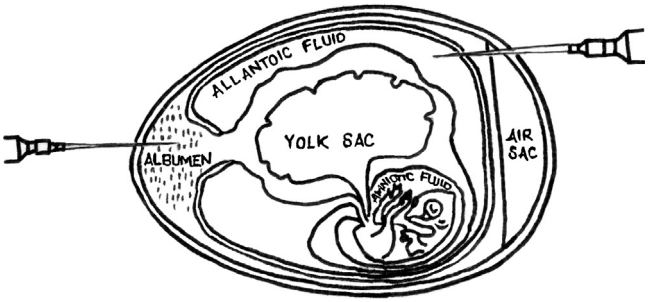

Fig. 1. Schematic diagram of 10-days-old embryonated chicken egg showing the allantoic and albumen routes used for virus and drug inoculation.

2.5. In ovo *antiviral assays*

Various drug concentrations of OC, 1.75 μg/ml, 3.5 μg/ml, 7 μg/ml, 14 μg/ml, 28 μg/ml, 56 μg/ml, and 112 μg/ml were tested against 100 EID_{50} virus, using the *in vitro* drug treatment method (described below), to determine the concentration of drug required for complete inhibition of virus growth. Each drug concentration was inoculated in ten eggs. Drug concentrations 1.75 μg/ml, 3.5 μg/ml, and 7 μg/ml did not show any significant drop in the virus HA titers after treatment as compared to the untreated controls ($p > 0.05$). OC concentrations of 14 μg/ml and above showed complete inhibition of the virus. Therefore, 14 μg/ml of OC was used for the *in ovo* antiviral assays.

The following three methods were then used for the *in ovo* antiviral assays:

(a) ***In vitro* treatment of virus and drug:** Equal volumes of the drug and virus were mixed and incubated for 1 h at 37 °C. This mixture (0.2 ml virus + 0.2 ml drug) was inoculated into the allantoic cavity.
(b) **Virus and drug delivery by the allantoic route:** In the allantoic cavity, 0.2 ml virus suspension was inoculated. In addition, 0.2 ml drug was administered at two time points [namely 0 h (ALL 0 h) and 2 h (ALL 2 h)] by the allantoic route.
(c) **Drug delivery by the albumen route:** In the allantoic cavity, 0.2 ml virus was inoculated and 0.2 ml drug was administered, through albumen at two time points; 0 h (ALB 0 h) and 2 h (ALB 2 h) (Fig. 1) (Wang et al., 2008).

Each treatment group, including the virus control group, consisted of ten eggs. There was no drug administration in the virus controls, PBS was used instead of OC. Total controls, which had no virus or drug inoculated, were also included in each experiment. The inoculated eggs were incubated at 37 °C for 72 h. The eggs were observed after an interval of 24 h till 72 h. At the end of 72 h, the eggs were chilled at 4 °C overnight. The allantoic fluids were harvested, and tested by the HA assay.