Pakistan J. Zool., vol. 48(5), pp. 1443-1447, 2016.

Comparative Antiviral Efficacy of Zanamivir and Amantadine Against Tunisian Isolate of Avian Influenza Virus (H9N2)

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ABSTRACT

New emerging avian influenza A viruses pose a continued threat, not only to avian species but also to the humans. Avian influenza viruses increasingly crossing species barriers possibly with increased zoonotic potential. Due to rapid spread of influenza viruses, zoo animals and birds are at great risk. There are many species in zoos that are part of worldwide programme to save endangered species. Vaccination may protect birds kept in zoo against avian influenza as preventive and eradication programme along with culling and biosecurity. However, to eradicate influenza infection from valuable bird species in zoos, novel strategies are needed, including antiviral treatments. Antiviral treatments of infected birds kept in zoos are appropriate to prevent death of those birds that are part of genetics pool. The present study evaluated the anti-influenza efficacy of the potent neuraminidase inhibitor zanamivir and ion channel blockers amantadine in avian species using chickens. Zanamivir showed high antiviral efficacy than amantadine in the chicken model. Sequence analysis revealed mutation in matrix (M2) gene of virus for reduction in antiviral efficacy of amantadine. Anti-influenza drug administration combined with active surveillance and vaccination strategies could be useful for control of AIV in precious captive birds.

Article Information
Received 20 April 2015
Revised 29 December 2015
Accepted 17 March 2016
Available online 1 August 2016

Authors' Contributions
SU performed virus genome sequencing. MAAS performed virus isolation in eggs. AUK prepared virus inoculum. QUN performed haemaglutination inhibition assay and isolated the virus. MIK and MY maintained and monitored the experimental birds. MY performed necropsy of turkey and scoring of clinical lesion. SU and MAAS analyzed the data. SU and MY wrote the article.

Key words
Zanamivir, Amantadine, Efficacy, Avian influenza virus (H9N2).

INTRODUCTION

Avian influenza (AI) is a respiratory disease. Its causative agent influenza A viruses are prevalent worldwide and classified as either highly pathogenic AI (HPAIV), causing severe systemic disease with high mortality, or low pathogenic AI (LPAIV) inducing relatively mild clinical signs in birds (Alexander, 2007;Subtain *et al.*,2011; Umar *et al.*, 2016). Highly pathogenic avian influenza (HPAI) is one of the most devastating viral diseases in bird species; it exacts high mortality in poultry and, increasingly, is a potential source of widespread and grave infections of mammals, including humans. Since 1997, H5N1 HPAI has caused over 500 human infections with approximately 60% mortality (WHO, 2010). Furthermore, avian influenza virus (AIV) is known to have a propensity for interspecies transmission and potential for pandemicity (Baigent and McCauley, 2003; Morens and Taubenberger, 2010). For example, during the HPAI H5N1 virus outbreak in Thailand In December 2003, two tigers (*Panthera tigris*) and two leopards leopards (*Panthera pardus*) at a zoo in Thailand died unexpectedly, highlighting the interspecies transmission ability of AIV (Keawcharoen *et al.*, 2004; Quirk, 2004). In addition, there is evidence that the transmission of low pathogenic avian influenza (LPAI) virus from avian to mammalian hosts continues to occur, perhaps preluding the emergence of a new pandemic virus (Butt *et al.*, 2005; Cui *et al.*, 2014). LPAI H9N2 virus can pose a significant zoonotic threat like H5N1 (Ahad *et al.*, 2013; Umar *et al.*, 2015a). Monitoring AI viral infections in domestic and wild birds is therefore important to control animal diseases and prevent humanpandemics (Zhang *et al.*, 2009; Tombari *et al.*, 2013). Through the acquisition of gene segments from other viruses H9N2 has under gone evolution to a more diverse genotype in terrestrial poultry birds since the early 1990. Genome study of recently isolated H9N2 viruses has shown extensive genetic re-assortment of these viruses with highly pathogenic avian influenza (HPAI) viruses (Tombari *et al.*, 2011; Iqbal *et al.*, 2009).To control influenza infection, a variety of vaccines and antiviral drugs have been developed for administration to humans and animals (Boltz *et al.*, 2010; Salomon and Webster, 2009; Sambhara and Poland, 2010). In avian species, AIV

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0030-9923/2016/0005-1443 $ 8.00/0

vaccination is generally not allowed in many countries. However, based on a desire to protect genetically unique birds, Europe and Singapore granted permission for an emergency AIV vaccination, allowing zoos to vaccinate valuable stock with an inactivated vaccine (Philippa *et al*., 2007; Elahi *et al*., 2015). Although vaccination of exotic and zoo birds for the prevention of AIV infection has been suggested (Bertelsen *et al*., 2007; Furger *et al*., 2008; Koch *et al*., 2009; Lecu *et al*., 2009; Philippa *et al*., 2007), a significant species variation in serologic response was reported in previous vaccine studies using zoo birds (Bertelsen *et al*., 2007). Furthermore, the lag time between identification of a newly emerging strain and vaccine development/ distribution, and concerns regarding vaccine efficacy and safety are problematic (Boltz *et al*., 2010). The use of neuraminidase inhibitors in humans was very effective during the initial phases of the 2009 H1N1 pandemic when vaccines were not available (Boltz *et al*., 2010). However, in animals, vaccines are only considered with a comprehensive program including biosecurity, culling, diagnostics, and surveillance to control and eradicate AIV (Kapczynski and Swayne, 2009). Therefore, novel strategies such as antiviral treatment are needed for the protection of valuable zoo birds from AI infection. In a previous study, *in ovo* studies demonstrated that the neuraminidase inhibitor Zanamivir is nontoxic for chicken embryos and prevents entirely the replication of a HPAI of the subtype H7N1 (Kaleta *et al*., 2007; Shaukat *et al*., 2011). However, in avian species, the antiviral efficacy of neuraminidase inhibitors and protein clockers has not yet been evaluated for clinical applications. The present study evaluated the anti-influenza activity of the potent neuraminidaseinhibitors (zanamivir) and viral matrix protein (M2) inhibitor (amantadine) in chicken. This report is the first study conducted on the efficacy of antiviral drugs against circulating LPAI (H9N2) virus in chickens in Tunisia.

MATERIALS AND METHODS

Virus inoculum stocks

Experimental study protocol was approved by the Animal care and research committee of the Pir Mehr Ali Shah Arid Agriculture University Rawalpindi and experimentation were carried out according to the guidelines of committee. Avian influenza A virus, (A/ chicken/Tunisia/12/2010 (H9N2) was a field isolate obtained National Veterinary School Tunisia. Viral stocks were prepared and titrated in 9 to 10-day-old chicken embryonated eggs. Median embryo infectious dose (EID_{50}) was calculated using previously reported methods (Reed and Muench, 1938). The viral stocks were diluted in medium containing antimicrobials to yield a final titre of 10^6 EID_{50}/0.5 ml.

Animals

Forty 3 weeks-old broiler chickens (*Gallus gallus*) were purchased from local hatchery and used in the experiment. All birds were declared serologically naïve and free from influenza viruses before the start of the experiment using haemagglutination inhibition and virus isolation (Iqbal *et al*., 2013; Umar *et al*., 2015b).

Drug administration

Zanamivir (Relenza® GalxosmithKline) and amantadine (Symmetrel® Endo Pharmaceuticals) were separately mixed 1:1 with phosphate buffered saline (PBS) and administered orally. Zanamivir and amantadine treatment (0.5 mg/kg of body weight/twice a day (1mg/kg of body weight/day) for 5 days began 4 hr before virus inoculation. Control inoculated chickens received sterile PBS on the same schedule (Lee *et al*., 2011).

Experimental design

Each group of experimental birds was kept in cages in separate rooms. General animal care, water and standard poultry feed ration were provided throughout the experiment by animal house staff according to the requirement of birds. The birds were divided randomly into 4 groups; zanamivir treated group, amantadine treated group, PBS treated mock infected control group and PBS treated non infected control group. Each group (n =10) were housed in separate animal isolators. The birds of the drug-treated groups (zanamivir & amantadine) and the PBS treated group were infected intranasally with a titer of 10^6EID50/bird (50% egg infective dose /bird). The chickens were sacrificed at day 5 post-infection for virus isolation and titration. For organ samples, trachea and cecal tonsil homogenates were supplemented to 10% (w/v) with 1% streptomycin (300 mg/ml) and the suspensions were centrifuged. Each supernatant was serially diluted 10-fold and aliquots of each dilution were inoculated into 10-to-11-day-old embryonated chicken eggs. After 3 days of incubation, allantoic fluid was collected and tested for haemagglutination activity. The virus titer of each specimen was calculated by the Reed-Muench method and is expressed as the mean±SD.

Statistical analysis

Statistical analysis and graphical presentation was performed using GraphPad Prism 6 software (GraphPad Software Inc. La Jolla, CA, USA) and values were expressed as the mean ± standard deviation of the mean (SDM). One way analysis of variance (ANOVA) was used to analyse tissue virus titre. The number of birds shedding virus were tested for statistical significance

using Fisher's exact test. Statistical significance was set at $P< 0.05$ unless otherwise stated.

Table I.- Antiviral effects of zanamivir and amantadine against avian influenza virus in broiler chickens.

Group	Virus isolation		Virus titre ($log_{10}EID_{50}/g$)[c]	
	Trachea	Cecal tonsils	Trachea	Cecal tonsils
Zanamivir treated[a]	3/10*	0/10**	2.4±0.5	0.0±0.0
Amantadine treated[b]	8/10	6/10	4.7±0.8	4.2±0.4
PBS treated mock infected control group	10/10	10/10	5.9±1.2	5.1±0.5
PBS treated non infected control	0/10	0/10	0.0±0.0	0.0±0.0

[a,b]1 mg/kg/day, p.o. b.i.d. x5 days beginning at 4 hr pre–virus exposure.
Number of chickens shedding virus/total number of chickens; virus isolation was done at 5 days post-infection.
*P <0.05, **P <0.001 by Fisher's exact test compared to PBS-treated control negative group.

RESULTS AND DISCUSSION

Virus replication was detected in all ten trachea and cecal tonsil samples of the mock infected control group of chickens (Table I). However, compared to the non infected control group, zanamivir significantly reduced viral replication from both trachea (three of ten samples positive for viral replication; P <0.05), and cecal tonsil (none of ten samples positive for viral replication; P <0.001) at day 5 post-infection. On the other hand, when compared to control group, amantadine showed non-significant reduction in virus replication (P>0.05) in both trachea (eight of ten samples positive for viral replication; P >0.05) and cecal tonsils (six of ten samples positive for viral replication; P>0.05). Zanamivir showed high antiviral efficacy than amantadine in the chicken model. Reduced antiviral efficacy of amantadine suggests high possibility of mutation in matrix gene of studied virus. Amantadine drugs inhibit the growth of virus by blocking the ion channel formation of M2 protein during the early stage of infection. Substitution of amino acids within M2 results in loss of antiviral capability of amantadine. Amino acid substitutions at positions 26, 27, 30, 31, and 34 within the transmembrane domain of M2 have been reported a key factor in loss of sensitivity to M2 blockers. Previously, it was shown that H5, H7 and H9 influenza A viruses had the $V^{27}A$ and $S^{31}N$ amino acid substitutions in the M2 protein (Ilyushina *et al*., 2005). Later on, sequence analysis on matrix (M2) gene of studied avian influenza virus (H9N2) revealed substitution at $S^{31}N$ (data not published). We did not find an $R^{292}K$ substitution, which is associated with resistance to the sialidase inhibitors zanamivir, in the NA proteins of virus studied.

Among avian models, chicken is widely used for evaluating AIV vaccines (Hsu *et al*., 2010), but fewer studies have involved anti-influenza viral drug evaluation. In our previously developed avian models, H9N2- infected 3 weeks-old chickens displayed a high level of virus shedding from trachea and cecal tonsil cells on day 5 post-infection (data not shown). Considering these previous results, we presently measured virus shedding from the respiratory and digestive tracts on day 5 post-infection in the chicken model. The results indicate the potential of chicken models for evaluation of new anti-AIV drugs for birds. In the poultry industry, massive zanamivir administration might not be suitable because of the high cost. However, in zoos, where avian species is in danger of becoming extinct in the wild and genetically unique birds are housed, conservation demands the prevention and eradication of AIV, since massive culling is not an option. Furthermore, in zoos and in the home, pet birds are in close contact with humans, particularly during feeding and handling. This contact may lead to the avian-to-human transmission of AIV (Stirling *et al*., 2008). In this light, the use of zanamivir with zoo birds could be a prudent disease prevention policy in AI outbreaks. In previous studies, only vaccines have been considered as an option for the eradication of AIV in zoos (Bertelsen *et al.*, 2007; Kapczynski and Swayne, 2009; Koch *et al.*, 2009; Lecu *et al*., 2009). However, in metaphylactic vaccination, there would be no effective vaccine during the lag time for the development of vaccine to novel AIV strains (Boltz *et al*., 2010). Furthermore, even developed inactivated vaccines may be poorly immunogenic in some bird species (Bertelsen *et al*., 2007), and several weeks may be required to induce protective levels of neutralizing antibody. Therefore, zanamivir could be effective to reduce AIV infection in valuable birds during the lag time for vaccine development and in the early phase after metaphylactic vaccination. The role of zanamivir in preventing AIV infection during the period of production of sufficient neutralizing antibody after vaccination warrants study. Prophylactic administration of zanamivir during epizootic outbreaks could be effective for preventing AIV outbreaks in zoos. However, zanamivir is expensive and may also produce unwanted side effects in long-term treatment. Therefore, the clinical application of zanamivir to zoo birds and pet birds requires appropriate administration guidelines. In zoologic pharmacology, the decisions concerning dosage

and dosing regimen are often made with limited species-specific information, with extrapolation to non- approved species (Hunter and Isaza, 2008). The present study also evaluated zanamivir only in the orders Galliformes. Therefore, effective methods of extrapolating a dosage to zoo birds and pet birds should be considered. Further, in susceptible species, early recognition of illness is required to treat infected birds subsequently. On the other hand, the natural reservoirs for AIV are orders *Anseriformes* and *Charadriiformes*, which normally undergo a subclinical course of infection (Hunter and Isaza, 2008; Umar *et al*., 2015a). In these species, routine virus monitoring with active surveillance is required to determine appropriate prevention and treatment measures. In the present study, we examined the antiviral efficacy of zanamivir and amantadine against LPAI viruses using chicken models and provided a possibility of zanamivir administration in avian species. Further study is required to evaluate the efficacy of zanamivir against HPAI using avian models for optimizing the zanamivir application guideline for HPAI control. We recommend use of zanamivir is to treat cases of avian influenza in precious captive birds. Anti-influenza drug administration combined with active surveillance and vaccination strategies could be useful for control of AIV infection in precious avian species.

CONCLUSION

It can be concluded that Zanamivir is better antiviral agent than amantadine against H9N2 viruses circulating in poultry of Tunisia and surrounding countries. Anti-influenza drug administration combined with active surveillance and vaccination strategies could be useful for control of AIV in precious captive birds

Statement of conflict of interest

Authors have declared no conflict of interest.

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