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Research Article

Survival of LPAI H5N1 in Water Containing *Tilapia zillii*

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ABSTRACT
The present study aimed to test the survival of LPAI H5N1 in water in the presence of *Tilapia zillii* fishes, the experiment was conducted using aquarium filled with 45 liters of Nile water and nine fishes of *Tilapia zillii*. The presence of the virus was tested in water samples by real-time RT-PCR. The viability and the titer of the virus in water samples extracted from aquarium were detected by Plaque infectivity assay and Heamagglutination assay, respectively. The results showed that, the viral RNA persisted 28 days in aquarium water. The CT values showed that the concentration of the virus seemed to be lowered by the time. The virus was survived in water containing fishes *Tilapia zillii* for 8 days by using plaque assay technique. In water containing *Tilapia zillii* fishes, the titer of LPAI-H5N1 virus was 512 and 64 HA at zero time and 4 days, respectively. In conclusion, prolonged persistence and infectivity of the virus in water might provide an evidence for the possibility of re-infection of poultry and human who might use or deal with this contaminated water for different purposes.

Keywords: Survival, LPAI-H5N1, Nile water, *Tilapia zillii.*

INTRODUCTION

The avian influenza virus belongs to the Orthomyxoviridae family of segmented negative-sense RNA viruses that are divided into six different genera, including influenza types A, B, C, Isavirus, Thogotovirus, and Quaranjavirus[1]. There are 18 different hemagglutinin (HA) antigens (H1 to H18) and eleven different neuraminidase (NA) antigens (N1 to N11) subtypes of influenza A, labeled according to H number (for the type of HA) and N number (for the type of NA)[2-4]. Water habitats contaminated with influenza viruses may serve as a rich transmission medium. Avian influenza viruses have been isolated from surface water of waterfowl habitats when infected ducks were present,[5,6] and from aquatic habitats sediment following bird migration[6,7]. Several aquatic species have the critical importance role in creating an intermediary link for spreading and transmission of influenza viruses to and from vulnerable aquatic and poultry populations. The throwing dumping of dead bird carcasses into water bodies as well as faulty usage of inefficiently treated poultry manure in organic fertilization of fish ponds would result in catastrophic eruption and evolution of new influenza viral hybrids with an ultimate disaster of state wide pandemic[8]. Drinking water sources that may be susceptible to avian influenza virus deposition include surface water bodies (e.g.; rivers, lakes, ponds and reservoirs), rainwater collection systems and groundwater aquifers. From these sources, open water bodies where infected waterfowl gather are the most likely potential route of entry of virus into the drinking water supply. The viral inactivation rates can be quite slow, especially in colder temperatures though it is not known how long the H5N1 strain can persist in the environment[9]. Studies of the survival of AI H5N1 in water containing fishes are rare worldwide and in Egypt are absent. So, the aim of this study is to test the survival of LPAI H5N1 in water in the presence of *Tilapia zillii*.

MATERIAL AND METHODS

Virus

Low pathogenic avian influenza virus (LPAIV), A/chicken/Egypt /Q1995D /2010 (H5N1) [HA Genbank accession no. KC436135], was obtained from Center of Scientific Excellence for influenza viruses, National Research Centre. The pathogenicity of obtained virus was previously modified by altering the multiple basic amino acids coding sequence (RRKKR) at the cleavage site of the HA of the highly pathogenic H5N1 virus to create the non-pathogenic form monobasic sequence (R) using plasmid based reverse genetics[10].

Virus propagation

Low pathogenic avian influenza virus (LPAIV), A/chicken/Egypt /Q1995D /2010 (H5N1) was used to conduct the experiments. The virus stock was obtained after propagation in Specific Pathogen Free (SPF) 9-to-11-day-old embryonated chicken eggs[11].

Experimental procedure

Aquarium with a total capacity of 60 liter (35 x 35 x 50cm) was filled with 45 liters of Nile water and 9 fishes of *Tilapia zillii*. *Tilapia zillii* fishes were placed in aquarium that supplied by aerator to make the fishes adapted in Nile

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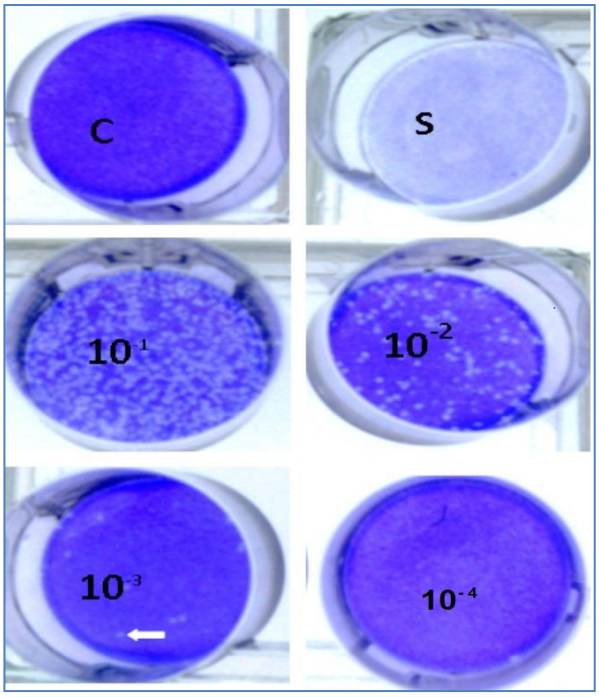

Figure 1: Plaque infectivity assay to detect the viability of H5N1 in aquarium water samples at zero time. C: control negative, S: samples were collected at zero time, dilutions 10^{-1}—10^{-4}: dilution of zero time samples.

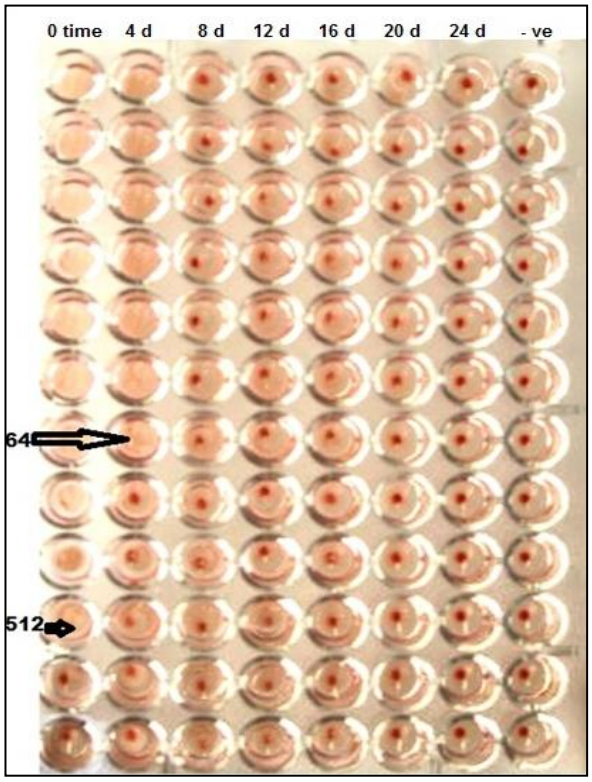

Figure 2: Hemaglutination assay for detection of H5N1 in aquarium water samples. The LPAI-H5N1 virus was detected in aquarium water samples at zero time with titer 512 and at 4 days with titer 64 HA. –ve: negative control, 0 time: samples were collected at zero time, 4 d: samples were collected at the fourth day.

Table 1: Detection of viral persistence in aquarium water by real time RT-PCR.

Sampling day	Ct values (*)
0	24.67
4	25.17
8	27.33
12	27.45
16	28.42
20	28.40
24	28.78
28	32.39
32	39.44

(*): All CT values are mean of two trails.

water with optimum pH and temperature condition. Five milliliter of avian influenza H5N1 virus (A/chicken/Egypt /Q1995D /2010 (H5N1)) (virus titer = 10^8 EID50/ml) was inoculated in aquarium water then water samples were sampled at zero time and every 4 days for 5 weeks as a trial period. This experiment was repeated two times with the same condition. The presence of the virus was tested in water samples by real-time RT-PCR. Real time reverse transcription PCR (rt RT-PCR) targeting the M gene was performed on all RNA extracted from each water samples according to Kayali et al.[12]. The viability of the virus in water was detected by Plaque infectivity assay, For counting of viral particles in water sample, plaque infectivity assay was carried out according to Tobita et al. (1975)[13], and the titer of virus in water samples was detected by Heamagglutination assay according to WHO procedure[11].

RESULTS

Persistence of LPAI H5N1 RNA in aquarium water

Viral RNA was extracted from water sampled from aquarium and subjected to real time RT-PCR. The CT values results are mean of two trails. The results showed that, the viral RNA persisted 28 days in aquarium water. By the time, the concentration of the virus seemed to be lowered (Table 1).

Plaque infectivity assay to detect the viability of LPAI H5N1 in water

Water containing *Tilapia zillii* fishes was sampled and inoculated on MDCK cells for plaque assay of LPAI-H5N1 virus. The obtained results showed that the infectious virus was detected at zero time, 4 and 8 days with titers 1.7×10^5, 9.9×10^3 and 1.98×10^3 pfu/ml, respectively (Figure 1).

Hemagglutination (HA) assay to detect the titer of the virus in aquarium water

Hemagglutination tests were performed to detect the presence of virus in water containing *Tilapia zillii*. Hemagglutination tests were performed in 96-wells micro-titer plates with 0.5% chicken red blood cells. The LPAI-H5N1 virus was detected in water containing *Tilapia zillii* fishes only at zero time and 4 days with titers 512 and 64 HA, respectively (Figure 2).

DISCUSSION

Information about the presence of aquatic animals that might mediate virus entry, survival and persistence for

long periods is unavailable[14,15]. One of the disadvantages of the conventional methods for studying the persistence and the infectivity of the AIV H5Nl in water is that such methods neglect the possibility of the presence of aquatic animals. The present study deals with the persistence and survival of LPAI- H5N1 in water containing *Tilapia zillii* fishes. The results of the current study showed that the viral RNA of LPAI- H5N1 persisted 28 days in aquarium water containing *Tilapia zillii*. In other study, when molluscs, fish or tadpoles were introduced in aquariums containing rain water, the H5N1 viral RNA persistence increased up to 20 days**[16].** The authors suggested a probable impact of these aquatic animals on the biological cycle of H5N1 virus[16]. found that the viral RNA persisted in water until the last day of the experiment (day 8) even in the presence of mussels. They observed a decrease of the viral load measured in water (3 logs in 8 days) but also in mussels (1 log in 8 day). Infectious particles were detected in water during 4 days, and during 6 days in mussels. The results of Horm *et al.*[16] were in concordance with the present results. The obtained results showed that the infectious virus was detected at zero time, 4 and 8 days with titers 1.7×10^5, 9.9×10^3 and 1.98×10^3 pfu/ml, respectively. In other study, Low Pathogenic Avian Influenza viruses were reported to survive in some lake sediments between 2 and 4 days at temperatures ranging from 20 to 30ºC[17]. These results did not agree with that of[17], and this might be due to the difference in substrates. In other study, it has been reported that several physical factors as organic solvents and detergents inactivated avian influenza viruses in the environment due to their lipid envelopes[18]. Webster *et al.*[19] found that mixed infected feces obtained from ducks naturally infected with A/duck/Memphis/546/74 (H3N6) influenza virus in non-chlorinated river water and stored the suspension at 4 and 22°C. With a starting viral concentration of $10^{8.10}$ EID_{50}/ml, virus infectivity did not drop after 7 days at 4 °C and gradually decreased thereafter, but residual infectivity was still detectable after 32 days. At 22 °C the infectivity of virus in the water decreased more rapidly. Significant levels of virus were detected after 4 days but no virus was detectable at 7 days and afterwards. These results were nearly similar to the present result. In other study, infectious HPAI H5N1 virus could be recovered from water during a maximum of 4 days post-contamination at 25ºC. Interestingly, Viral infectious particles, in similar conditions, could not be isolated from any of the natural surface water specimens tested (ponds and lake)[12]. The authors concluded that main microbiological parameters and physicochemical which differed between rain and lake /pond water specimens were: a total absence of chemical oxygen demand with globally lower concentrations of nitrite and nitrate, a globally less abundant bacteriological flora in rain water and a higher concentration of sodium[12]. Infected ducks can shed a large number of virus particles not only in their feces but also in saliva and nasal discharge which can therefore easily lead to water contamination[18]. The same authors concluded that influenza viruses are protected by organic materials such as nasal secretions or feces, which may increase their resistance to physical and chemical inactivation. Previous publications supported the idea that AIV could survive for longer in lake sediments than in lake water[17]. Stallknecht *et al.*[20] used AIV isolates derived from four waterfowl species in Louisiana, USA. The experiment was conducted to study the effects of water temperature on the persistence of AIV under sterile laboratory conditions at 4°C, 17 °C and 28 °C for 60 days. Linear regression models for these viruses predicted that an initial concentration of 1×10^6 tissue culture infectious dose 50 ($TCID_{50}$)/ml of water could remain infective for up to 207 days at 17 °C and up to 102 days at 28 °C. It was estimated that at 4 °C the contaminated water with a concentration of 1×10^6 $TCID_{50}$/ml could remain infective for 1,333 days. These results disagree with the current result this might be due to the authors used sterile distilled water but in the current study we used the surface Nile water.

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