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RESEARCH ARTICLE

Persistence of Avian Influenza H5N1 Virus in Surface and Sea Water

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Abstract

The present study aimed to test the persistence of avian influenza H5N1virus (Egyptian isolate) in water with different environmental conditions (temperature and pH) and to test the possible transmission of the virus via aquatic animal *Tilipia zillii*. The persistence of the virus was assessed by using two types of water (Nile water and sea water) adjusted at different pH 6.5, 7.0, 7.5, 8.0 and different temperatures 20 and 34°C. The detection of low pathogenic avian influenza H5N1 virus in water samples was achieved by using real time reverse transcription PCR. Immunofluorescence assay and rt RT-PCR technique were used to detect H5N1 virus in fishes' tissue samples. The results revealed that the low pathogenic avian influenza H5Nl virus persisted for extended periods of time in Nile water than in sea water. In general real time reverse transcription PCR showed that the most optimum pH was 7.0 and the most optimum temperature was 20°C for the persistence of the virus. By using rt RT-PCR and immunofluorescence assay, H5N1 virus was found in the intestine of the fishes that was sampled at 4 and 8 days. Moreover, the viral RNA was detected in gills that were sampled at 4 days only. In conclusion, prevention and control measures can be suggested to minimize, if not eliminate the risk from the consumption of virus-contaminated water.

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INTRODUCTION

Contaminated aquatic habitat with influenza viruses may serve as rich transmission media. Avian influenza viruses (AIVs) have been isolated from the surface water of waterfowl habitats when infected birds were present (Halvorson et al., 1985; Ito et al., 1995), and from the sediment of aquatic habitats following bird migration (Ito et al., 1995; Lang et al., 2008).Transmission of influenza to wild aquatic birds was mainly based on fecal-oral transmission route (Breban et al., 2009).

Laboratory trials using distilled water demonstrated that the infectivity of influenza viruses could remain for weeks to months and the duration of virus persistence showed an inverse relationship to temperature and salinity (Stallknecht et al., 1990a; Stallknecht et al., 1990b). AI H5N1 virus can persist in water for long time. This persistence in water might be enhanced by environmental factors such as temperature, pH and salinity (Animal Health Australia, 2005).The prolonged persistence of influenza viruses might threaten the lives of poultry and mammals, and might be a source for emerging newly reassortant influenza viruses.

Using the poultry and animal manure in some part of the world as a fertilizer in fish farming can increase the spread of AIV (Scholtissek and Naylor, 1988).

Although, many investigators recorded high persistence of AI H5N1 virus in aquatic environment. To clarify the persistence of the virus in water and possible transmission of the virus to fishes, we designed the present work.

Material and Methods:

Virus:

Low pathogenic (LP) AIV, A/chicken/Egypt /Q1995D /2010 (H5N1) virus, was obtained from the Center of Scientific Excellence for influenza viruses, National Research Centre. The pathogenicity of the virus was previously modified by altering the multiple basic amino acids sequence (RRKKR) at the cleavage site of the HA of the highly pathogenic form to create the low pathogenic monobasic sequence (R) using plasmid-based reverse genetics (Webby et al., 2004).

The virus stock was obtained after propagation in Specific Pathogen Free (SPF) 9-to-11-day-old embryonated chicken eggs (WHO, 2002).

Virus Survival in aquatic environment:

Sea and Nile surface waters were separately used. Two types of water were divided into 200 ml aliquots in 500 ml sterile flasks which equipped with aerators. The aliquots were inoculated with LPAIV (A/chicken/Egypt /Q1995D /2010 (H5N1)) at concentration 10^8 EID_{50}/ml and 2 ml of antibiotic-antimitotic mixture were added under a bio-safety level-2 laminar flow cabinet. The pH of the aliquots of the two different water types in the flasks were individually adjusted at 6.5, 7.0, 7.5, and 8.0 by using 1N solution of NaOH or HCl, then exposed to temperatures 20°C and 34°C which represent mean winter and summer temperatures in Egypt. Sampling started at the inoculation day which represents zero time then at weekly basis and tested for the presence of H5N1 virus by real time RT-PCR through 4 weeks as a trial period. This experiment was repeated two times with the same condition.

Viral RNA was extracted from 140 µl of collected water samples by using a QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's protocol. The extracted RNA was aliquoted and kept at -80^{o}C. To detect influenza A, real time reverse transcription PCR (rt RT-PCR) targeting the M gene was performed to all RNA extracted from each water sample according to Kayali et al. (2014).

Virus transmission through *Tilipia zillii* fishes:

For testing the possible transmission of the virus via *Tilipia zillii* fishes, aquarium with a total capacity of 60 liters were filled with 45 liters water and 9 *Tilipia zillii* fishes were added. Then the aquarium was inoculated with LPAI H5N1 at concentration 10^8 EID_{50}/ml. One fish from the aquarium was sampled every 4 days during the experiment period. This experiment was repeated two times with the same condition. The detection of H5N1 virus in fishes' organs was carried out by using rt RT-PCR (Kayaliet al., 2014). Immunofluorescence technique was used to detect H5N1 virus in fishes' tissue samples (Kayali et al., 2014).

Result:

The effect of different pH and temperatures on the persistence of LPAI-H5Nl virus in surface water and sea water:

In general, the real time PCR showed that the concentration of the virus seemed to be lowered in sea water than in Nile water at the same pH values. At pH 6.5 and temperature condition 20^{o}C, the viral RNA was detected till week 3 post inoculation in both surface water and sea water. At pH 7, the viral RNA of H5N1 was stable for 4 weeks in surface water and sea water when the temperature condition was 20^{o}C. The concentration of the virus in surface water was higher than in sea water. At pH 7.5, the viral RNA of H5N1 resisted for 4 weeks in surface water and sea water when the temperature condition was 20^{o}C, while the concentration of the virus was higher in surface water than in sea water. At pH 8, the viral RNA of H5N1 persisted for 3 weeks in surface water and 2 weeks in sea water when the temperature condition was 20^{o}C. In addition, the concentration of the virus was higher in surface water than in sea water at the same conditions (Figure 1).

The viral RNA of H5N1 persisted for 3 weeks in surface water and sea water when pH 6.5 and temperature condition 34^{o}C, while at a fourth week the virus was undetected. The persistence of the virus was better in surface water than in sea water. The persistence of the virus was 4 weeks at pH 7 in surface and sea water when the temperature condition was 34^{o}C. In addition, the concentration of the virus was higher in surface water than in sea water. The resistance of the H5N1 virus was 2 weeks in surface water and in sea water at pH 7.5 when the temperature condition was 34^{o}C. The virus was undetected at the end of third and fourth week in different water types. Also, the concentration of the virus was higher in surface water than in sea water. The stability of the H5N1 virus was 3 weeks in surface water, while it was two weeks in sea water at pH 8 and temperature condition 34^{o}C. Also, the concentration of H5N1 virus was higher in surface water than in sea water (Figure 1).

Detection of LPAI-H5N1 virus in *Tilapia zillii* tissues:

Results were mean of two trails. The obtained results of rt RT-PCR and immunofluorescence assays showed that the virus was found in the intestine of the fishes at 4 and 8 days. Moreover, the virus was detected in the gills of fishes that were sampled at 4 days only. The H5N1 virus was not detected in muscle and liver until the end of the experiments by using real time RT-PCR technique and immunofluorescence assay (Table 1, 2 and Figure 2).

Table 1: Detection of H5N1 virus in fishes' organs by real time RT-PCR.

Sampling day	Log (Viral RNA copy Number)			
	Intestine	Gills	Muscle	Liver
4 day	**3.4**	**2.1**	**0**	**0**
8 day	**1.9**	**0**	**0**	**0**
12 day	0	0	0	0
16 day	0	0	0	0
20 day	0	0	0	0
24 day	0	0	0	0
28 day	0	0	0	0
32 day	0	0	0	0

Table 2: Detection of viral antigen by immunofluorescence assay

Sampling day	Immunofluorescence staining			
	Intestine	Gills	Muscle	Liver
4 day	**+ve**	**+ve**	-ve	-ve
8 day	**+ve**	-ve	-ve	-ve
12 day	-ve	-ve	-ve	-ve
16 day	-ve	-ve	-ve	-ve
20 day	-ve	-ve	-ve	-ve
24 day	-ve	-ve	-ve	-ve
28 day	-ve	-ve	-ve	-ve
32 day	-ve	-ve	-ve	-ve

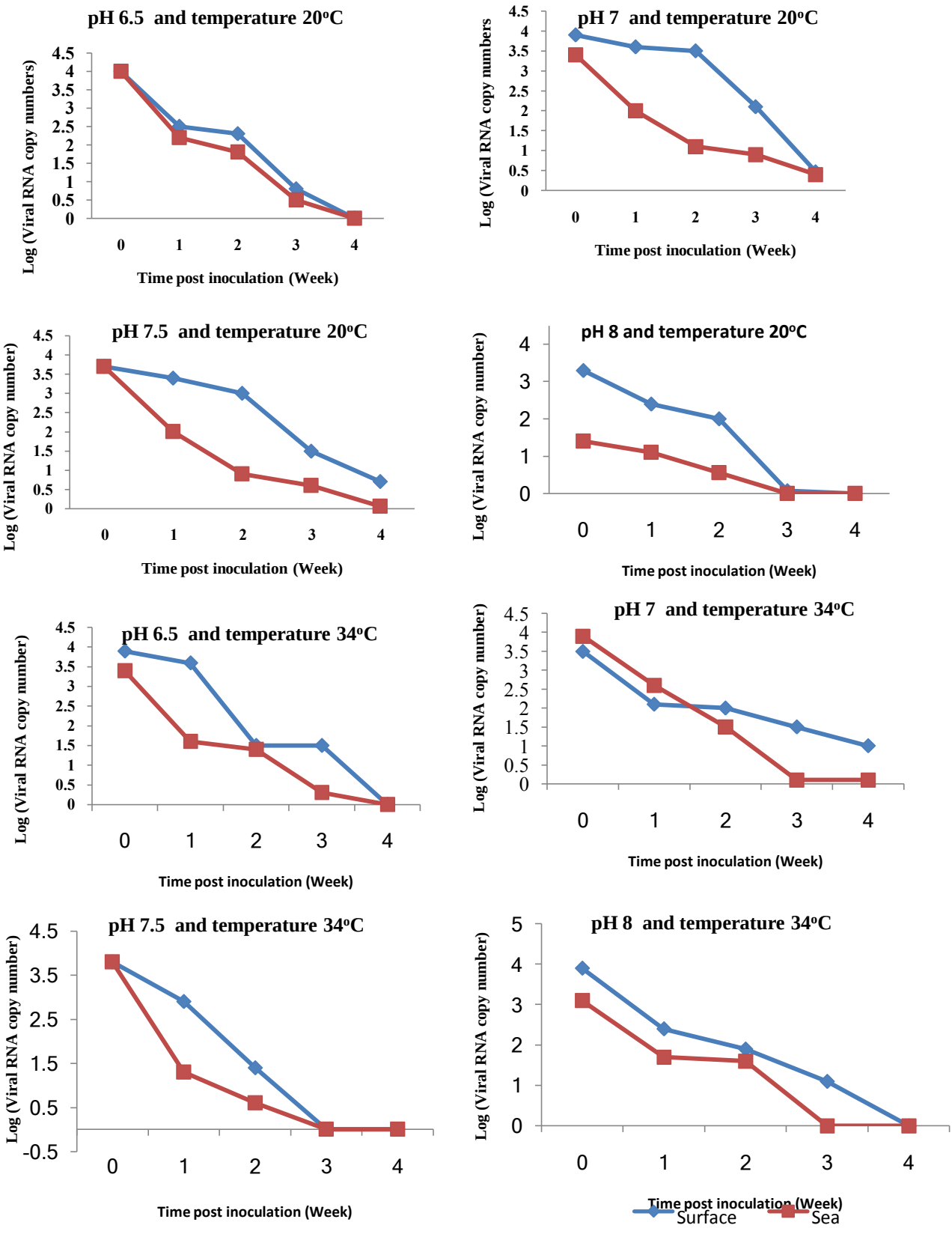

Figure 1: Persistence of H5N1 in different water types at pH 6.5 to 8.0 and temperature 20°C and 34°C.

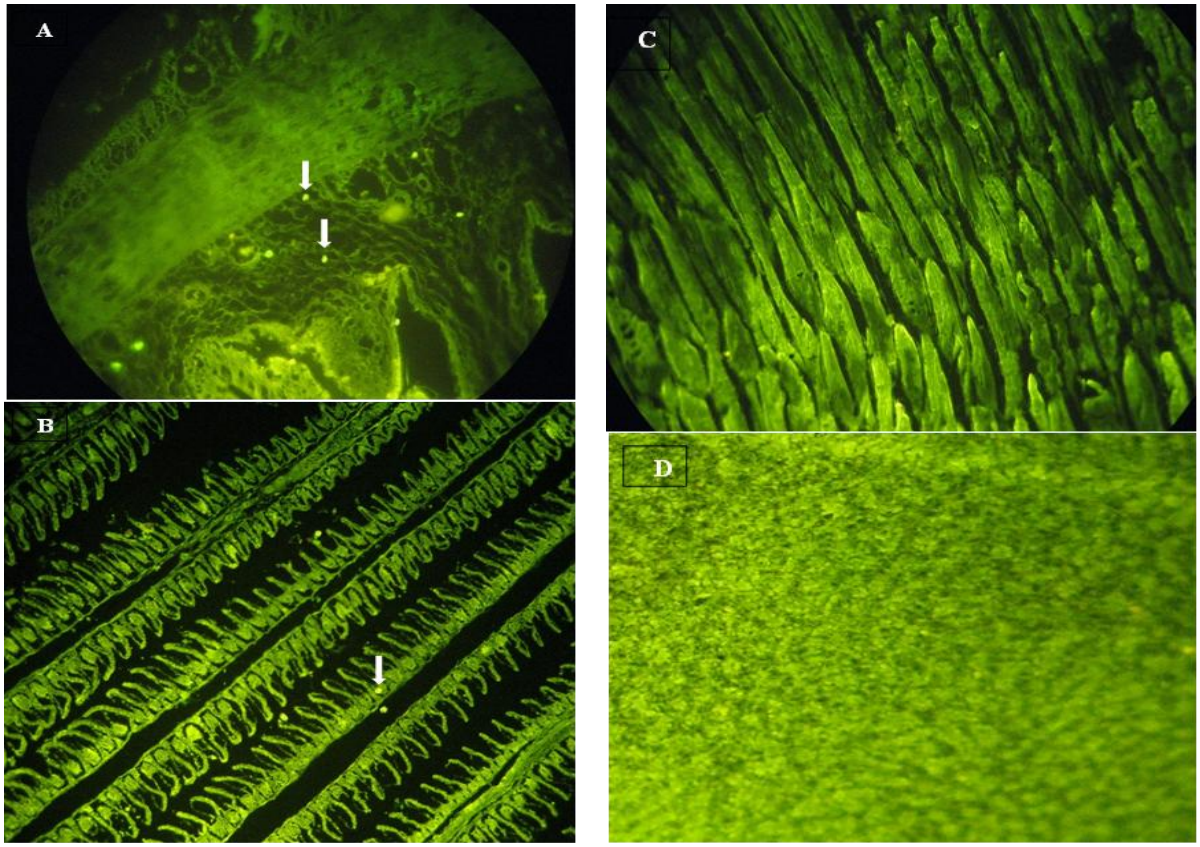

Figure 2: Immunofluorescence staining for fishes' organs. A: Intestines sampled at 4 days, white arrows refer to viruses. B: Gills sampled at 4 days. C: Muscles. D: Livers. Magnification: X 400.

Discussion:

Prolonged persistence and infectivity of the virus in different types of water at different temperatures 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 (de Jong et al., 2005; Buchy et al., 2007; Thiry et al., 2007; Fick et al., 2007). The LPAI-H5Nl virus was found to persist for extended periods of time in water. Such persistence depends on physical and environmental factors such as pH, temperature, salinity and bacterial load (Brown et al., 2007). The results of present study revealed that the virus was found to persist in water for variable periods according to the type of water, degree of temperature and different pH. The current study showed that LPAI-H5Nl virus was persisted for extended periods of time in Nile water than in sea water. This special emphasis on the effect of temperature on the persistence of virus in water was depending on the fact that the temperature is the most important predictor of virus persistence in water (Yates et al., 1985). The present results showed that the LPAI-H5N1 virus had no duration of persistence in acidic conditions (pH=6.5) and alkaline condition (pH=8.0) and warmer temperature (34^{o}C) in sea water. Other study in Egypt showed that H5Nl virus was detected in sea water for more than 8 weeks at 4°C, 4 weeks at 17°C, 1 week at 28°C and was undetectable by the end of the first week at 37°C. In case of waste water, H5Nl virus was detected for more than 8 weeks at 4°C, 5 weeks at 17°C, 1 weeks at 28°C and was undetectable by the end of the first week at 37°C (El-Sayed, 2009). These data supported the fact that the persistence of the virus is inversely proportional with the temperature. These findings were consistent with current study and the previous reports of Yates et al (1985), Stallknecht et al. (1990a), Stallknecht et al. (1990b), Sobsey and Meschke (2003) and Brown et al. (2007) and emphasized on that, temperature is the most important factor affecting the persistence of the virus in water. Brown et al. (2007) concluded that as viral RNA is known to be heat labile. Other researchers concluded that persistence of AI H5N1virus is inversely proportional to temperature (Shahid et al., 2009). This investigation showed that virus persistence was found to be lowered in sea water than that in Nile surface water.Yates et al.

(1985), Sobsey and Meschke (2003) noticed that viruses persisted for longer periods in well water than surface water samples incubated at similar temperature and were less persistent in natural waters and other media compared to the same media that have been sterilized. These studies also revealed that the persistence of the virus in different types of water is inversely proportional to the degree of salinity.

In this investigation, the target aquatic organism was chosen to be *Tilapia zillii* fish because it's wide prevalence in the Nile river and widely used as a food for Egyptian people. *Tilapia zillii* fishes were exposed to water infected with the isolated LPAI-H5Nl for about one month.The obtained results showed that the H5N1 virus found in the intestine of the fishes that was sampled at 4 and 8 days. The viral RNA was detected in gills that were sampled at 4 days only. The H5N1 RNA was not detected in other fishes' organs (muscle and liver) until the end of the experiment by using real time RT-PCR technique. These results might reflect the accumulation of the virus in some fishes' organs. Other study in Egypt, El-Sayed (2009) concluded that the detection of viral RNA by one step RT-PCR and viral antigens by ELISA in such water as well as from the homogenates prepared from the snail tissues might reflect the accumulation and propagation of the virus in the snail tissues and its subsequent release from the snails into the water. Kwon et al. (2005) concluded that many aquatic species have the critical importance role in creating an intermediary link for transmission, processing and spread of influenza viruses to and from vulnerable aquatic and poultry populations. Eissa et al. (2012) detected H5N1 virus in hemolymph of the Red Swamp crayfish (*Procambrusclarkii*) collected from three different provinces across the Nile Delta, Egypt. On the other hand, Eissa et al., found that tissue and mucous samples collected from earthen pond raised tilapia were negative for the virus. And this concluded that, this contamination was probably acquired through fertilization of fish ponds with infected poultry manure, feeding of fish with infected poultry carcasses or presence of infected birds around the aquatic habitats. The erratic 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.

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