

Effect of some physical factors on Egyptian subtype H5N1 and its survivability

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Abstract: Dry and wet litters were spiked with the virus to determine the survivability of the virus in litter at 4, 37, 40, 56 and 70°C.

Infected litter packets were exposed to 4°C, 28°C and 56°C, respectively, for different time intervals (½, 12, 24, 48 hr) to determine the infectivity and haemagglutination activity of the virus. The results were revealed that:

(1) The virus in water was inactivated after 24 hr at 28 and 37 °C and at 40 °C after 60 min. At 56°C the virus lost its infectivity within 5-15 min. At 70°C the virus was killed in a short time and after only 5 minutes of incubation and could not be recovered.

(2) The results from the pH inactivation test showed that none of the pH levels (5 and 9) were able to inactivate the tested H5N1 virus following the exposure of 5 min, 10 min and 1 hr.

(3) Litter exposed to 37 °C for ½, 12, 24, 48 hr, all embryos were dead within 48 hr of incubation and all the harvested allantoic fluid showed haemagglutination (HA test) were $2^{10}, 2^6, 2^5, 2^4$, respectively.

(4) At 4 °C, the virus was detected up to 30 th day in wet litter only but the virus lost about 50 % infectivity. At 37 °C, virus lost 33.32–33.32%, 66.62–50% infectivity within 12 hr and 24 hr exposure in dry litter, respectively.

Keywords: HPAI H5N1, Physical environment, Infectivity, Haemagglutination, survivability.

1. Introduction

The AIVs subtype H5N1, belonged to the family Orthomyxoviridae, genus Influenza A, are negative single stranded, enveloped RNA viruses. The H5N1 virus also poses a significant threat to human health as this virus has been shown to be able to infect humans [1,2]. In order to limit the animal and human health impact, it is of crucial importance that H5N1 virus infection in poultry is controlled and eradicated [3,4].

Highly pathogenic avian influenza (HPAI) is a highly infectious viral disease that affects a wide range of bird species. Clinical signs of the disease depend on the strain and subtype of virus and the species of bird infected. Avian influenza (AI) is classified according to disease severity, with two recognized forms: HPAI, also known as fowl plague, and low-pathogenic avian influenza (LPAI).

Flu viruses are well-protected from inactivation by organic material and infectious virus can be recovered from manure for up to 105 days, especially in high moisture and low temperature conditions.

AI viruses can survive in cool and moist conditions, particularly when organic material is present [5]. Under field conditions virus has been shown to survive in river water and faeces [6, 7, 8, 9]. Also, it can survive in the environment for 6 days at 37 °C [10].

For the highly pathogenic form, studies have shown that fecal material from infected birds may contain up to 16×10^6 virions/gm of faeces and one gram contains enough viruses to infect one million birds [11]. Proper sanitation and biosecurity cannot be over emphasized; they are the first line of defense against avian influenza virus (AIV). Thus, all methods for preventing and controlling the spread of AI V are related to controlling the contamination of houses, equipment, wastes and personnel.

One important consideration in this aspect from the epidemiology point of view could be the close association of the persistence of virus in the litter and the water bodies present in the many parts of the poultry farms.

Therefore, the objective of the present study was to determine the survivability of Egyptian H5N1 under various temperatures and different levels of pH and contaminated poultry litter.

2. Materials and Methods

Virus

H5N1 isolated in Egypt 2012: Phylogenetic analysis of the HA1 of the two isolated HPAI H5N1 strains {KC699547 [Influenza A virus (A/duck/Egypt/VRLCU-R11/2012(H5N1)]. The virus was isolated from infected duck flock during recent AI outbreaks in Egypt.

Virus propagation

H5N1 virus was propagated in 11 day old chicken embryonated eggs (CEE) where, tenfold virus dilutions were inoculated into 11-day-old chicken embryonated eggs in six-replications and then incubated in a 37 °C humidified incubator and candled twice a day for 7 days. The virus titers determined from the allantoic fluid (AF) as ELD₅₀/ml and evaluated [12]

Haemagglutination (HA) test

HA was carried out for standardization of AI antigen used in HI test to 4 Haemagglutination units (HAU); as recommended by [12].

Temperature inactivation

The H5N1 virus was subjected to the conditions of various temperatures. The tested protocol was modified from [13]. Briefly, the H5N1 viruses were added into 15 ml-sterile screw-capped, polypropylene centrifuge tubes and placed in a water bath at various temperatures, including 28, 37, 40, 56 and 70 °C, for 5, 15, 30, 45 and 60 min. Following the incubation, 0.1 ml of all treated samples was inoculated into 11-day-old CEE in six-replications and followed the previously described protocol [14].

pH inactivation

The H5N1 viruses were subjected to the conditions of various pH. The tested protocol was modified from [15]. Briefly, infectious AF containing H5N1 viruses with adjusted pH to 2, 5, 9, 10 and 13, using sodium hydroxide (NaOH) or hydrochloric acid (HCl) was incubated for 1, 12 and 24 hr at room temperature. Following the incubation period, 0.1ml of all treated samples was inoculated into 11-day-old CEE in six-replications and followed the previously described protocol.

The duration of survivability was determined in terms of the percent infectivity to the ECEs in first passage and recovery of the virus in subsequent passages as described earlier [16].

Effects of temperature on survival and virus titre of AIV (H5N1) in litter

The preserved virus was cultivated in 9 to 11-day-old embryonated chicken eggs. Harvested amnioallantoic (AAF) fluid was titrated on the basis of haemagglutination (HA) potential. Peptone water was prepared, autoclaved and

incubated at 37°C for 24 h to check sterility. AAF was diluted in peptone water to have 4 HA unit titer. It was divided into aliquots in sterilized glass vials with 4 ml each. Chicken litter was packed in quantities of 300 g in autoclaved bags and incubated at 121 °C for 60 min. To determine the moisture content of the chicken litter, 100 g were weighed before and after drying. 300 grams of sterile chicken litter were placed under sterile conditions into sterile plastic boxes with lids. The chicken litter was contaminated with 4 ml of allantoic fluid of H5N1 diluted in 11 ml of sterile DMEM, which resulted in a titer of 10^{8.35} EID₅₀/300 g chicken litters. The packets were exposed to 4°C, 28°C and 56°C, respectively, for different time intervals (½, 12, 24, 48 hr).

1) Effect of wet and dry conditions on survival of AIV (H5N1) under variable temperatures in wet and dry litter

Fresh chicken litter was collected and packed in quantities of 1 g in autoclaved bags and incubated at 121 °C for 60 min. The packed were divided into two parts; one part was used as such as the wet litter and another part was dried aseptically in the oven at 50 °C to bring the moisture level below 20 % and used as the dry litter.

Spiking of the Virus in litter

The spiking of litter (dry and wet) was done in order to ensure a final virus concentration of around 10⁶ EID₅₀/ml, which is often shed by the infected chickens in the faeces. Both dry and wet litter were spiked with the original virus (EID₅₀ 10^{10.33}/ml, diluted 1:100) in the ratio of 1 part diluted virus: 1 part litter and triturated with mortar pestle to ensure proper mixing [17].

Survivability of virus in litter was examined at 4, 37, 40, 56 and 70°C. Spiked litter samples (1 g) were aliquoted into the 4ml screw capped vials. Five sets of 30 vials were made for both dry and wet litter; each set was stored at the five different temperatures mentioned above. Positive control and negative control for both dry and wet litter was kept at -80 °C and processed along with the treated samples.

3. RESULTS AND DISCUSSION

The virus was inactivated after 24 hr at 28 and 37 °C and at 40 °C after 60 min. At 56°C the virus lost its infectivity within 5-15min [18].

At 70°C the virus was killed in a short time and after only 5 minutes of incubation and could not be recovered [7].

On the other hand, we recorded that the virus completely inactivated within 30 min after direct sunlight exposure at an environmental temperature of 32 to 35°C.

pH inactivation

The effect of pH condition on the infectivity of H5N1 viruses is summarized in Table 2. The pH of 5, 9 and 10 had no effect on the infectivity of the H5N1 viruses regardless of the tested contact time.

The results from the pH inactivation test in this study showed that none of the pH levels (5 and 9) were able to inactivate the tested H5N1 virus following the exposure of 5 min, 10 min and 1 hr. While, a pH 2 and 13 were able to inactivate the tested H5N1 virus following the exposure of 5 min, 10 min and 1 hr [19,16,20].

Table 1. Temperature inactivation of the virus at 28, 37, 40, 56 and 70 ° C, for 5, 15, 30, 45, 60 m 24hr.in and

Exposure time(min) Temp	Infectivity (%)				
	28C°	37 C°	40C°	56C°	70 C°
5	6/6	6/6	4/6	1/6*	0/6*
15	6/6	6/6	2/6*	1/6*	-
30	6/6	3/6*	1/6*	0/6*	-
45	4/6	2/6*	0/6*	-	-
60	3/6*	1/6*	0/6*	-	-
24 hr	0/6*	0/6*	-	-	-

Infectivity to embryonated chicken eggs (infected embryos) / (total embryos: n=6) per treatment. *P* < 0.05*

Table 2. Effect of pH levels on the infectivity of the avian influenza H5N1 viruses.

pH	Contac time,5 min	Contact time,10 min	Contact time,1 hr
2	2/6*	1/6*	0/6*
5	6/6	5/6	5/6
9	6/6	6/6	6/6
10	6/6	6/6	6/6
13	2/6*	1/6*	0/6*

* Infectivity to embryonated chicken eggs (infected embryos)/(total embryos: n=6) per treatment. *P* < 0.05*

Table 3. Effect of temperature on the survival and virus titre of AI (H₅N₁) in litter.

Exposure time(hrs) Temp	Embryonic death (%)			HA activity in the AAF			HI test		
	28C°	37 C°	56 C°	28C°	37 C°	56C°	28C°	37C°	56 C°
½	0/6	0/6	0/6	2 ⁷	2 ¹⁰	2 ⁶	+ve	+ve	+ve
12	0/6	0/6	0/6	2 ⁶	2 ⁶	2 ⁶	+ve	+ve	+ve
24	0/6	0/6	6/6	2 ⁵	2 ⁵	0	+ve	+ve	-ve
48	0/6	0/6	6/6	2 ⁵	2 ⁴	0	+ve	+ve	-ve

(n=6) per treatment Infectivity to embryonated chicken eggs (infected embryos)/(total embryos: n=6) per treatment.

Effect of temperature on the survival and virus titre of AI (H₅N₁) in litter

Results of temperature effect are recorded in Tables and Figures ...

Exposure of litter to 28 °C for ½, 12, 24, 48 hr led to death 100 % for all inoculated embryonated chicken eggs. All embryos were dead within 48 hr of incubation and all the

harvested allantoic fluid showed haemagglutination by HA test. It was noticed that the HA readings were 2⁷, 2⁶, 2⁵, 2⁵, respectively.

Litter exposed to 37 °C for ½, 12, 24, 48 hr, all embryos were dead within 48 hr of incubation and all the harvested allantoic fluid showed haemagglutination (HA test) were 2¹⁰, 2⁶, 2⁵, 2⁴, respectively.

Contaminated litter exposed to 56 °C for ½, 12, 24, 48 hr and inoculated in specific pathogen free embryonated chicked after ½ and 12 hr, showed embryonic death and the harvested allantoic fluid showed 2⁶ by HA test for both intervals. On the other hand, exposure to 56 °C for 24 and 48 hr, the embryos stilled viable with no deaths and the allantoic fluid were did not show haemagglutination activity for any time of exposure.

At 4°C infectivity of HPAI (H5N2) was detected after 35 days and for 2 days after incubation at 25°C, but detected for 7 days at 20°C. [21,22].

The persistence of AIV H5N1 was inversely proportional to temperature [23]. Virus could survive more than 100 days at 4°C but was inactivated after 24 hr at 28°C and after 30 min at 56°C. At 25°C the virus lost its infectivity within 24 hr. and at 40°C the virus in the manure was killed in a short time and after only 15 minutes of incubation, the virus could not be recovered [18].

On the other hand, the virus completely inactivated within 30 min after direct sunlight exposure at an environmental temperature of 32 to 35°C. and after exposure for 3 min at 70°C [7].

Table 4. Percent infectivity of H5N1 avian influenza virus in dry and wet litter at 4, 37, 40, 56 and 70°C.

Temp. Time	0		4hr		12hr		24hr		48hr		7days		15 days		30 days	
	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D
4°C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/6	0/6*
37°C	6/6	6/6	4/6	4/6	2/6*	3/6*	2/6*	3/6*	0/6*	1/6*	0/6*	0/6*	-	-	-	-
40°C	6/6	6/6	6/6	6/6	3/6*	3/6*	0/6*	1/6*	0/6*	1/6*	0/6*	0/6*	-	-	-	-
56°C	6/6	6/6	5/6	4/6	1/6*	1/6*	0/6*	0/6*	-	-	-	-	-	-	-	-
70°C	6/6	6/6	1/6*	1/6*	0/6*	0/6*	-	-	-	-	-	-	-	-	-	-

* Infectivity to embryonated chicken eggs (infected embryos)/(total embryos: n=6) per treatment. *P* < 0.05*

At 4 °C, the virus was detected up to 30 th day in wet litter only but the virus lost about 50 % (3/6) infectivity (Table 3). However the virus was recovered up to 15 days in dry litter (the virus lost about 50 % infectivity) [16].

At 37 °C, virus lost 33.32–33.32% (4/6), 66.62(2/6) - 50% (3/6) infectivity within 12 hr and 24 hr exposure in dry litter, respectively. The virus was recovered only up to 48 hr in dry litter (1/6).

At 40 °C, the virus lost 50 % (3/6) infectivity in both wet and dry litter within 12 h of incubation (Table 3) and after 48 hr (1/6) in dry litter ,but it could not be detected after 7 days in and dry litter [17].

At 56 °C, the virus was detected up to 12 hr in wet and dry litter in percentage of 16.33% (1/6).. But, the virus completely could not be detected after 24hr in both wet and dry litter.

At 70 °C, the virus was detected up to 4hr in litter. However the virus completely could not be detected after 12 hr in both wet and dry litter.

At high temperatures the virus is destroyed in minutes, the higher the temperature the shorter the survival time of the virus ,at 70°, 75° and 80°C, AIV was destroyed in 30, 5 and 1 minutes, respectively [24].

In this study the virus survived up to 4 weeks at 4 °C in both wet and dry litter. Extremely low temperatures in the environment results in the prolonged survivability of the

virus and enhances the risk of recurrent bouts of outbreaks in the same geographical regions.

The mechanism of the effect of temperature may kill or disable the viruses by two possible ways. High temperature (> 56 °C) may alter the envelope or damage the proteins only. Denatured proteins can result in fatal mutations (haemagglutination and infectivity were observed in our study). The presented data are agree with Kurmi et al. [17] with 6-6.5 logs of H5N1 virus, in wet and dry chicken feces no virus was detected at 24 hours at 42 C, 30 hrs at 37 C, 5 days at 24 C, and 7 (wet) or 8 (dry) weeks at 4 C.

High temperature (> 56 °C) may damage the proteins and nucleic acid. Enveloped viruses can be rendered harmless when their envelope is destroyed, because the virus no longer has the recognition sites necessary to identify and attach to host cells [25, 19, 3, 16, 26, 17] and also can halt the process of protein synthesis, by damaging RNA.

Extremely low temperatures in the environment results in the prolonged survivability of the virus and enhances the risk of recurrent bouts of outbreaks in the same geographical regions.

Proper sanitation and biosecurity cannot be over emphasized; they are the first line of defense against AI. Thus, all methods for preventing and controlling the spread of AI are related to controlling the contamination of equipment and personnel.

Conclusion

From these results we concluded that: (1) AI viruses can survive in cool, moist and dry conditions, particularly when organic material is present. (2) H5N1 virus in litter could be inactivated by increasing temperature above 56°C for at least 24 hr. In a chicken house, the temperature could be successfully raised to above 56°C by ordinary litter heaps. Raising the temperature in an empty house could help in disinfection, especially in endemic areas. This is useful for the prevention and control of the spread of the virus. (3) The survivability of the virus depends on the medium in which the virus is present. The persistence of virus in the water greatly differs from that in litter and faeces where, the virus survives longer in the water. (4) pH =2 and 13 were able to inactivate the H5N1 virus following the exposure of few minutes. (5) One important consideration in this aspect from the epidemiology point of view could be the close association of the persistence of virus in the litter and the water bodies present in the many parts of the poultry farms. However, data from many experiments indicate that heating to 37 C reduces survival to days, and heating to 56-60 C reduces survival to minutes to a few hours.

Conflict of interest

The author has declared no conflict of interest.

References

[1] WHO, 2008. Cumulative Number of Confirmed Human Cases of Avian Influenza A(H5N1) Reported to WHO: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_04_30/en/

[2] Kaoud, H.A. 2007. Eco-Epidemiologic Impacts of HPAI on Avian and Human Health in Egypt. *International Journal of Poultry Science*, 7 (1):72-76.

[3] Songserm, T., Jam-on, R., Sae-Heng, N., Meemak, N. 2006b. Survival and stability of HPAI H5N1 in different environments and susceptibility to disinfectants. In: Schudel A, Lombard M, editors. *Development of biological*. Switzerland: Karger. p. 254.

[4] Kaoud, H.A. 2007. HPAI Epidemic in Egypt: Evaluation, Risk Factors and Dynamic of Spreading. *International Journal of Poultry Science*, 6 (12): 983-988.

[5] Swayne, D.E. 2008. *Epidemiology of Avian Influenza in Agricultural and Other Man-Made Systems*, in David E. Swayne, ed., *Avian Influenza*. Ames, IA: Blackwell Publishing.

[6] Webster R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J. and K. Copal Murti. 1978. Intestinal influenza: Replication and characterization of influenza viruses in ducks. *J. Wildl. Diseases*, 14(2): 268-278.

[7] Songserm, T., Jam-on, R., Sae-Heng, N., Meemak, N., 2005. Survival and stability of HPAI H5N1 in different environments and susceptibility to disinfectants. In: Schudel, A., Lombard, M. (Eds.), *Developments in Biologicals*, Proceedings of the OIE/FAO International Conference on Avian Influenza (Abstract 73). Paris, France, April 7-8, 2005 254 pp.

[8] Brown, J., Stallknecht, D., Valeika, S. and Swayne, D. 2007. Susceptibility of wood ducks to H5N1 highly pathogenic avian influenza virus. *J. Wildl. Diseases*, 43:660-667.

[9] Nagarajan S, Tosh C, Smith DK, Peiris JSM, Murugkar HV, Sridevi R, et al. 2012. Avian Influenza (H5N1) Virus of Clade 2.3.2 in Domestic Poultry in India. *PLoS ONE* 7(2): e31844.

[10] World Health Organization. WHO interim guidelines on clinical management of humans infected by influenza A (H5N1). Vol. 2004. Geneva: The Organization; 2004.

[11] WHO, The Writing Committee of the World Health Organization. Consultation on Human Influenza A/H5 Avian influenza A (H5N1) infection in humans. *New Eng J Med*. 2005;353:1374-1385. doi: 10.1056/NEJMra052211.

[12] OIE, 2009. Version adopted by the World Assembly of Delegates of the OIE in May 2009 OIE *Terrestrial Manual* 2009. Chapter 2.3.4. Avian influenza

[13] Swayne, D.E., Beck, J.R., 2004. Heat inactivation of avian influenza and Newcastle disease viruses in egg products. *Avian Pathol*. 33, 512-518.

[14] OIE. 2005. Office International des Epizooties and Food agricultural organization "OIE/FAO" (2005): Recommendations of the World Health Organization for Animal Health/Food and Agriculture Organization International scientific conference on Avian

[15] Stallknecht, D.E., Kearney, M.T., Shane, S.M., Zwank, P.J., 1990. Effect of pH, temperature, and salinity on persistence of avian influenza viruses in water. *Avian Dis*. 34; 412-418.

[16] Lu, H., Castro, A.E., Pennick, K., Liu, J., Yang, Q., Dunn, P., Weinstock, D., Henzler, D., 2003. Survival of avian influenza virus H7N2 in SPF chickens and their environments. *Avian Dis*. 47;1015-1021.

[17] Kurmi, B., H. V. Murugkar, S. Nagarajan, C. Tosh, S. C., Dubey, and Kumar, J. 2013. Survivability of Highly Pathogenic Avian Influenza H5N1 Virus in Poultry Faeces at Different Temperatures. *Indian J Virol*. 24(2); 272-277.

[18] Chumpolbanhorn, K., Suemanotham, N., Siripara, N., Puyati, B. and Chaichoun, K. 2006. The effect of temperature and UV light on infectivity of avian influenza virus (H5N1, Thai field strain) in chicken fecal manure. *Southeast Asian Trop Med Public Health*, 37;102-105.

[19] Muhammad, K., Das, P., Yaqoob, T., Riaz, A., Manzoor, 2001. R. Effect of physicochemical factors on survival of avian influenza virus (H7N3 type) *Int J Agric Biol*. 4;416-418.

[20] Wanaratana, S.; Tantilertcharoen, R.; Sasipreeyajan, J. and Pakpinyo, S. 2010. The inactivation of avian influenza virus subtype H5N1 isolated from chickens in Thailand by chemical and physical treatments. *Vet. Microbiol.*, 140 (1-2); 43-48.

[21] Beard, C.W., Brugh, M., Johnson, D.C. 1984. Laboratory studies with Pennsylvania avian influenza viruses (H5N2). *Proceedings of the US Animal Health Association*, 4th Worth, TX, USA, 88;462-473.

[22] Stallknecht et al., 1990b)

[23] Shahid, M.A., Abubakar, M., Hameed, S. and Hassan, S., 2009. Avian influenza virus (H5N1); effects of physicochemical factors on its survival. *Virol. J.*, 6; 38.

[24] Department of Primary Industries and Energy "DPIE" .1996. *Disease strategy on avian influenza*. 2nd ed. Canberra: Australian Veterinary Emergency Plan.

[25] York, J.L. 1997. Enzymes: classification, kinetics, and control. In: Devlin TM, editor. *Textbook of biochemistry*

with clinical correlations. 4. New York: Wiley-Liss; pp. 127–178.

[26] Brown, J.D., Goekjian, G., Poulson, R., Valeika, S., Stallknecht, D.E. 2009. Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. *Vet Microbiol.*,136;20–26.



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