Effect of Physico-chemical Factors on the Survival of Avian Influenza Virus (H9N2)

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Summary: The wide spread outbreaks of avian influenza (AI) virus in the different regions of the world has highlighted the problems in controlling its persistence and spread. Among the stakeholders, poultry industry, poultry consumers and associated human resources are particularly important, which eventually effect economic losses. There are serious threats to human health because of the risk of formation of new mutants. In this study, some physical and chemical agents have been investigated and optimized to inactivate AI virus (H9N2) type isolated from the chickens. We artificially infected the chicken eggs and determined inactivation under the influence of different physical factors (temperatures, pH and UV radiations) and chemical agents (formalin, phenol, iodine and fin virus) at different values and exposure time. Among the physical agents, the virus became inactivated at pH 5 and 9 after 30 min., at 56°C after 45 min and at 280 nm after 90 min. The chemical agents formalin, phenol, iodine and fin virus inactivate the virus in 0.5 % solution after 15, 30, 30 and 15 minutes respectively. These optimized parameters will be helpful in controlling the spread of AI virus (H9N2) in different parts of the world according to their available resources.

Introduction

Avian influenza (AI) is an infectious disease of birds caused by type A strains of the influenza virus. The disease was first identified in Italy more than 100 years ago and occurs worldwide. Sixteen subtypes of influenza virus are known. All birds are thought to be susceptible to the infection with AI, though some species are more susceptible to infection than others. Infection causes a wide spectrum of symptoms in birds, ranging from mild illness to a highly contagious and rapidly fatal disease that results in severe epidemics. Avian influenza viruses of all subtypes can cause low pathogenicity in avian influenza susceptible birds. Pathogenic symptoms occurring in the respiratory, reproductive or enteric tracts have been observed in different subtypes such as H1 [1], H3 [2], H5 [3], H6 [4], H7 [5] and H9 [6,7] viruses.

Some sub types of avian influenza viruses can affect the human health and pose a significant threat. These are H5 and H9, endemic in poultry in some regions of the world [8, 9]. In future, a dual risk is ascertained, one is the risk to human health from the avian host and the other is genetic re-assortment between a mammalian and an avian influenza virus, which could become the basis for a generation of a new pandemic virus for humans.

Further spread of AI is done through human-related activities following re-stocking of birds with inadequately sanitized conditions. Therefore, it follows that if disinfection of premises, footwear, clothing, vehicles, crates, farm equipment and other materials is not carried out properly, infection will persist in the avian population and the concurrent damage to the poultry industry and the public health threat will not be checked. For this reason, cleaning and disinfecting must be considered an essential part of AI control programs. Approval to release a microbial pest control agent depends on the experimental results obtained after a titer testing protocol, as detailed in subdivision M of the pesticide assessment guideline.

In order to limit the animal and human health impacts, it is critically important that AI infections in poultry are controlled and eradicated. The present work has been done with the objective to investigate different physical and chemical methods of inactivating AIV, and to indicate specific

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situations in which the use of a given method or product is recommended or discouraged using the locally isolated strain (H9 N2), thus providing concise and practical information on how to develop specific sanitation programs aimed at the inactivation of AI virus.

The present study has been conducted with the objective of inactivating AIV local isolated strain (H9N2) and indicating specific situations in which the use of specific physical and chemical agents are either recommended or discouraged. Hence, the ultimate goal of this study is to provide the reader with concise and practical information on how to develop specific sanitation programs aimed at the inactivation of AIVs.

Results and Discussion

Physical Methods

pH

Orthomyxoviridae is considered to be sensitive to acid pH values, although its retention of infectivity is dependent on the degree of acidity, as well as on the virus strain [10]. We have studied the effect of various pH conditions provided to the virus environment for different intervals of time. The virus survives at pH 7 in the maximum time period given (i.e. 45 minutes), but the survival of the virus is affected at pH 5 and 9 within a 30 minutes period (Table-1). The mechanism of pH infectivity of Influenza virus has been reported [10-14]. The reports reveal that virus incubation at pH 5.0 induces viral fusion with the cell host membrane [11]. A study (14) has described a kinetic model for the activation and inactivation of type A human influenza virus, depending on the pH of the medium and using two different tested strains (H1N1 and H2N2). This model has shown that the pKa was 5.6–5.7 and the pKi was 4.8–4.9, respectively, for the two viruses. Previous studies have demonstrated that treatments of 2, 5 and 15 min at pH 4.9 give different results for the activation/inactivation of two different human influenza A strains [10]. There is some variation between different studies regarding the effect of pH on the survival of different strains. This may be due to the genetic make up among the different strains and their adaptability in the different environmental conditions. A study [15] has shown that 4HA units of AIV H7N3 lost their infectivity after 48 h at the acid values of pH 1 and 3, as well as at the basic pH values of 10 and 14. Other factors related to the medium could interfere with the effect of pH on virus infectivity. In another study [16], it has been concluded that the effect of pH varies according to the salinity of the medium. They have reported the existence of three different AIVs (H6N2, H4N6 and H10N7) in water according to the waterfowl habitat conditions. The maximum infectivity for these viruses in salt-less water was detected at the pH range of 7.4– 7.8, whereas in saline water, the optimum pH tolerance shifts to acidic range.

Temperature

The combined effect of time and temperature on the stability of avian influenza virus (H9N2) isolates was investigated. 4HA (the virus) units were subjected to 56^{0}C for 15, 30 and 45 minutes. The virus retained 80 % of its activity until 30 minutes, while it became inactive after a time period of 45 minutes (Table-1). AIVs can persist in distilled water for more than 100 days at 28^{0}C and 200 days at 17^{0}C [16]. The time of virus survival at 4^{0}C was estimated to be more than 1300 days [16]. Others have demonstrated that an AIV H7N3 subtype at a concentration of 4 HA units in peptone water (pH 7.0) decreased its HA activity. However, it preserved its infectivity when incubated at 4, 30 and 37^{0}C for 35 days, but lost the activity after a prolonged exposure at 56^{0}C for 60 min [15]. Studies on the resistance of four low pathogenic AI strains of H7N2 showed the retention of their infectivity for 30 min at

Table-1: Effect of physical factors on haemagglutination activity of AI virus (H9N2).

Interaction time (min.)	Temperature (56^{0}C)		pH 5		pH 7		pH 9		UV light (280 nm)	
	HA activity n=5	% HA activity	HA activity n=5	% HA activity	HA activity n=5	% HA activity	HA activity n=5	% HA activity	HA activity n=5	% HA activity
15 (30*)	+++++	100	---++	40	+++++	100	--+++	60	+++++	100
30 (60*)	++++-	80	-----	0	+++++	100	-----	0	++++-	80
45 (90*)	-----	0	-----	0	+++++	100	-----	0	-----	0
Negative control	+++++	100	+++++	100	+++++	100	+++++	100	+++++	100

*Interaction time for UV light.
N = number of eggs.

56^{0}C. They were completely inactivated after 60 min at the same temperature or after 10 min at 60^{0}C [17,18]. Comparative evaluation of the studies on different strains shows similarity and does not show variation among the different types.

UV Light

UV light treatment has potential and feasible applications towards control of the spread of micro-organisms and use as a sterilization tool in the microbiological laboratories. UV light exposure to the virus at 280 nm for 30, 60 and 90 minutes resulted in the inactivation of virus after 90 minutes exposure, whereas at 60 minutes, only 20 % of the activity was lost (Table-1). Muhammad *et al* [15] showed that UV light is unable to inactivate the AI virus (H7N3) after the exposure for 45 minutes. Apparently, it seems a contradiction; however, in his study, the maximum time duration was 45 minutes, which is half of our exposure time (i.e. 90 minutes). Furthermore, the UV radiation is effective only when the surface is well cleaned and the source of light is positioned very close to the surface that needs to be disinfected [19]. The type of AI virus may also be the reason of variations in the results.

Chemical Factors

Viruses can be divided into three categories (A, B, C) on the basis of their resistance to chemical agents [20]. This classification is based on the presence/ absence of lipids on the virus and on the virus size, which appear to be the characteristics that mostly influence the resistance to chemical agents. AIVs belong to category A, in which all enveloped viruses of intermediate to large size are present. The presence of lipids is associated with a high susceptibility to all disinfectants [21,22].

The effect of different chemical agents on the survival of avian influenza virus also varies. The virus was treated with 0.5 %, 1 %, and 1.5 % formalin, fin virus, iodine and phenol for 15, 30, and 45 minutes. The results are presented in Table-2.

Formalin

It was observed that virus lost its viability in 0.5 % formalin in 15 minutes. It has been reported [23] that formalin at low concentrations, such as 0.04 % and 0.1 %, is able to inactivate three strains of AIVs (H5N2, H5N9 and H9N2) after 16 hours at 37°C. The efficacy of formalin has also been tested at three different dilutions (0.06 %, 0.12 % and 0.24 %) against the H7N3 subtype for 6, 12, 18 and 24 hours, and it was revealed that a period of 12 hours was the minimum time period to inactivate the virus [15]. In the present study, we used higher concentrations in order to check whether the increase in concentration reduces the time period of virus inactivation which turned out to be the case.

Iodine

The virus (4HA units) was treated with 0.5 %, 1 % and 1.5 % iodine solution. The results showed that the virus was inactivated within 30 minutes at all tested concentrations of iodine. In some studies [24,25] the virucidal effect of iodine containing disinfectants was observed against Newcastle disease and influenza viruses. They found out that various dilutions of iodine products showed excellent virucidal activity against Newcastle disease and influenza viruses. Furthermore, 3 % solution of iodophore showed bactericidal and virucidal activity within 30 minutes of its application. In comparison, the particular strain of virus used in our study requires less than half the concentration to be inactivated.

Table-2: Effect of chemical factors on haemagglutination activity of AI virus (H9N2).

Conc.	Inter-action time (min)	Formalin HA activity n=5	Formalin % HA activity	Iodine HA activity n=5	Iodine % HA activity	Phenol HA activity n=5	Phenol % HA activity	Fin virus HA activity n=5	Fin virus % HA activity
	15	-----	0	---++	40	----+	20	-----	0
0.5%	30	-----	0	-----	0	-----	0	-----	0
	45	-----	0	-----	0	-----	0	-----	0
	15	-----	0	-----	0	-----	0	-----	0
1.0%	30	-----	0	-----	0	-----	0	-----	0
	45	-----	0	-----	0	-----	0	-----	0
	15	-----	0	-----	0	-----	0	-----	0
1.5%	30	-----	0	-----	0	-----	0	-----	0
	45	-----	0	-----	0	-----	0	-----	0
Negative control		+++++	100	+++++	100	+++++	100	+++++	100

Phenol

4HA units of the virus were treated with 0.5 %, 1 % and 1.5 % phenol crystals solution. The results showed that AI virus was inactivated within 30 minutes when treated with concentrations of phenol as mentioned above. A study [26] reported that poly phenolic complex had a deleterious effect on the protein synthesis of AIV. Furthermore, the phenolic compound had antiviral properties and its inhibitory effect was dose dependent. In a study [15], it has been reported that phenol crystal has the ability to kill a Pakistan H7N3 isolate at different concentrations (0.1 %, 0.2 % and 0.4 %). Each concentration was tested after 6, 12, 18 and 24 h. Reports reveal that a 0.1 % phenol crystal solution can not kill AIV after 24h, while a 0.2 % solution killed AIV after 18h, and a 0.4 % solution required only 12 h to do so. A study [27] using two distinct disinfectants (Tektrol and One stroke environment) reported that 0.1 % dilution inactivated the AIV (H5N9A/ turkey/ Wisconsin/ 68, H7N3 A/ turkey/ Oregon/71) after an exposure of 60 minutes. In our study, 0.5 % solution of phenol has killed the AIV (H9N5) within 15 minutes. These variations may be explained in terms of the concentration, as well as the strain of virus being used.

Finvirus

The virus (4HA units) was treated with 0.5 %, 1 % and1.5 % fin virus (HBTA cresylic 20 g, detergents / emollients 20 g, organic acids 30 gm and ecipient q.s.p. 100 g). The results showed that it was inactivated in all of the concentrations of finvirus when it was kept for 15, 30 and 45 minutes. It has been reported [28] that 0.5 % fin virus inactivated avian influenza virus in a minimum duration of 5 minutes

The choice of an agent (physical or chemical) to be utilized for disinfection depends on the agent's ability to permeate through the bacteria or the virus. Furthermore, the destruction of viral infectivity depends on the strain of virus, length of exposure time, quality of virus, the nature of medium in which it is suspended and the interaction between treatments.

Experimental

Source of Avian Influenza Virus (AIV)

The avian influenza virus H9N2 type was obtained from Olympia Laboratory, Sheikhupura and stored at -20^{0}C.

Cleaning and Selection of Fertile Eggs

A total of 310, eight days old chicken embryonated eggs were purchased from local hatchery. All eggs were cleaned with 5 % formalin and kept in an automatic incubator at 37°C temperature with 60-80 % relative humidity. The incubated eggs were candled on the 11th day, in order to separate the live and dead embryos. All the infertile eggs were discarded. Live embryonated eggs were selected for propagation of avian influenza virus H9N2 type. The boundary of the air sac and the position of head were marked on live embryonated eggs.

Cultivation of AIV

The AIV H9N2 type was cultivated in 11 days old embryonated eggs. For this purpose 10 embryonated eggs were inoculated via the allontoic sac route [29]. The inoculum was brought to room temperature. Antibacterial (Gentamicin 100 μg/ml) and antifungal (Nystatin 40 I.U/ml) drugs were added to the inoculum. A small hole was drilled through the egg shell with a sharp pointed nail at one centimeter above the circumference opposite to the head of embryos. Next, 0.1 ml inoculum was injected through the opening with 01ml sterile disposable syringe by inserting the needle about ¾ of an inch (25 mm) into the allantoic sac. Then, the opening of each egg was sealed with sterilized molten wax. All inoculated and uninoculated control eggs were incubated at 37°C with 60-80 % relative humidity.

Candling of Inoculated eggs

The inoculated eggs were candled every 24 hours post inoculation and those showing mortality were kept at 4°C overnight. After 72 hour incubation, all the dead and live embryos were chilled and processed for ascertaining the existence of virus [30].

Harvesting of Allantoic Fluid

The shell over the air-sac was first disinfected with 70 % alcohol and then removed carefully with the help of sterile forceps, discarding its pieces in a beaker containing disinfectant. The chorioallantoic membrane (CAM) and amniotic membrane were turned with forceps to release allantoic fluid. The fluid was aspirated with 5 ml disposable syringe, and was then pooled in sterile plastic bottles and stored at –20°C. Harvested avian

influenza virus H9N2 type was checked for its haemagglutination (HA) activity by the HA test [30].

Collection of Blood & Washing of RBC

5 ml blood was collected from the wing vein of the broiler using a disposable syringe containing an anticoagulant (5 mg ethylenediamine tetra acetic acid). The contents were mixed by gentle rotation to avoid rupture of the suspended erythrocytes [31]. Next, blood samples were centrifuged at 1500 rpm for 5 minute for RBCs collection. The plasma was separated without disturbing the sediment with the help of a sterile pasture pipette. Physiological saline solution (0.9 %) was added to the sediment and the cells were resuspended by gently shaking the tube. This tube was again centrifuged at 1500 rpm for 5 minute, giving the cells a total of three washings in this manner. A 0.5 % suspension of washed erythrocytes was prepared in physiological saline.

Titration of Harvested Virus

The presence of virus was checked by the haemagglutination (HA) test [30]. The test was performed to estimate 4HA activity of test virus. According to this method a microtitre plate was used for determination of haemagglution activity of AIV. A volume of 50ul of physiological saline (pH 7.2) was suspended in all wells of microtitre plate with a multichannel micropipette. 50ul of virus was added to well 1 of each row, and was mixed with physiological saline using micropipette. 2 fold dilutions of virus were prepared for all wells up to well 11. A 50ul of 0.5 % RBCs suspension was then added to each well of the microtitre plate, and was then shaken gently to allow the mixing of contents of wells. Next, the plate was incubated at room temperature. The results were recorded after 15-20 minutes. It must be noted over here that uniform distribution of RBCs in the micro wells indicates positive results, while a small sharply outlined button of RBCs at the bottom of the well indicates negative results.

Calculation of 4HA units

The HA titer was the reciprocal of the highest dilution exhibiting haemagglutination. The end point titer and 4HA units of avian influenza virus H9N2 type were calculated as follows (Table-3):

HA unit of AAF: 512

4HA units of AAF: 512/4 =128

Physicochemical Factors

After determining the HA activity of the virus in amnioallantoic fluid, it's HA unit was subjected to physical and chemical factors and then inoculated into embryonated eggs.

Effect of Physical Factors

Heating Period

HA activity of virus was checked at 56^{0}C for 15, 30 and 45 minutes. Peptone water (0.5 %) containing HA units of virus were kept at 56^{0}C in a water bath. Then, 1 ml of virus was collected from the tube at 15, 30, and 45 minute intervals and inoculated in to three sets of eggs. These inoculated eggs were then kept in an incubator for 72 hours.

pH

The effect of pH on the HA activity of virus was determined at pH 5, 7 and 9. Peptone water (0.5

Table-3: Haemagglutination titers of AI virus (H9N2).

	1 (1:2)	2 (1:4)	3 (1:8)	4 (1:16)	5 (1:32)	6 (1:64)	7 (1:128)	8 (1:256)	9 (1:512)	10 (1:1024)	11 (1:2048)	12 (con.)
A	+	+	+	+	+	+	+	+	+	-	-	-
B	+	+	+	+	+	+	+	+	+	-	-	-
C	+	+	+	+	+	+	+	+	+	-	-	-
D	+	+	+	+	+	+	+	+	+	-	-	-
E	+	+	+	+	+	+	+	+	+	-	-	-
F	+	+	+	+	+	+	+	+	+	-	-	-
G	+	+	+	+	+	+	+	+	+	-	-	-
H	+	+	+	+	+	+	+	+	+	-	-	-

Con. control

Positive(+) Haemagglutination activity.

Negative(-) Haemagglutination activity.

* The HA titer of AIV was the reciprocal of highest dilution indicating uniform distribution of RBC in the micro wells of the plate.

%) having HA units of virus was distributed in 3 glass bottles. Then, the pH of virus suspension was adjusted as 5, 7 and 9 respectively by adding hydrochloric acid (HCl) or sodium hydroxide (NaOH). From each vial, 1 ml of virus was collected at 15, 30 and 45 minutes intervals and inoculated in to three sets of eggs. These eggs were kept in an incubator at 37^{0}C for 72 hours.

Ultraviolet Light

Peptone water (0.5 %) containing 4HA unit was dispersed in a Petri-dish and placed in a chamber of UV light of 280 nm wave length. The distance between the UV light chamber and the petridish was one foot. From the Petri dish, 1ml of sample was collected after 30, 60 and 90 minute intervals, and was then inoculated into three sets of eggs. These eggs were kept in an incubator for 72 hours at 37 ^{0}C

Chemical Factors

Among chemical factors formalin, phenol, iodine and fin virus of 0.5 %, 1 % and 1.5 % concentration were used. Peptone water (0.5 %) containing 4HA units of virus were distributed in 3 glass vials for each chemical. Then, 640 μl, 1280 μl, 1920 μl volumes of each chemical were added to each bottle and final concentrations of 0.5 %, 1 % and 1.5 % were prepared. The virus and disinfectant mixtures were incubated at 37^{0}C for 15, 30 and 45 minutes. After each interaction time, three sets of 11 days old embryonated chicken eggs were inoculated with 0.1 ml of inoculum from each prepared concentration of chemicals through the allantoic route. These inoculated eggs were kept in an incubator for 72 hours at 37^{0}C.

Spot Agglutination Test

The presence of avian influenza virus after various physical and chemical treatments is confirmed by collecting amnioallantoic fluid (AAF) 72 hours post inoculation through the spot agglutination test [32]. According to this method, one drop of allantoic fluid from the treated eggs and equal quantity of 0.5 percent washed erythrocytes of chicken were added and mixed on to a clean glass agglutinated slide. The clumping or agglutination of erythrocytes occurred within 03 minutes. This was taken as a positive heamaglutination reaction, thereby indicating the presence of virus.

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