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NON-ATTENUATION OF HIGHLY PATHOGENIC AVIAN INFLUENZA H_5N_1 BY LABORATORY EXPOSURE TO ULTRAVIOLET RAYS

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Abstract

Avian influenza H5N1 represents one of the most researched viruses in laboratories world-wide in recent times with regards to its epidemiology, ecology, biology and geography. The virus has caused 409 human cases and 256 human fatalities to date. Some laboratory activities and other lab related works predispose certain workers to exposure to this virus. In this work, we assessed the effect of exposure of HPAI infective allantoic fluid to ultraviolet rays for between 15 and 180 minutes. No significant difference was found between the unexposed and exposed viruses. The ability of the virus to haemagglutinate chicken red blood cells, the haemagglutination titre and its pathogenicity in embryonating eggs did not change despite this prolong exposure to UV-light. We call for caution in the handling of HPAI viruses in laboratory inside the microbiological safety cabinet despite sterilization using UV-light.

INTRODUCTION

Avian influenza is a very important zoonotic and trans-boundary animal disease. In recent times, the highly pathogenic avian influenza H5N1 (HPAI H5N1) has been in the centre of attention and activities of most viral research laboratories world widely. This is not unconnected with the recent spread (1996 to date) of the virus in many countries and the trans-boundary cum zoonotic potentials held by the avian influenza

H5N1 virus. The virus has spread in over fifty countries and linked to the death of more than 500 million birds of different species. To date, four hundred and nine (409) number of human infections and two hundred and fifty-six (256) human fatalities has been recorded (1).

While the majority of the countries that has been affected from western countries have got the capacity to easily manage and control the infection using standardized laboratory procedures, achievable policies and management practices, affected countries in developing economies and those country in transition still battle with the ability to cope with effective diagnoses, management and control (2, 3). These latter groups of countries sometimes grapple with inadequate laboratory facilities/expertise and often improvise for equipment and materials.

Ultra-violet (UV) irradiation is a proven germicidal widely used in many research laboratories. A survey of literatures showed that although UV irradiation has been assessed in its ability to inactivate viral, protozoan and bacteria organisms including *Escherichia coli* (4), Sendai virus (5), Polio virus and Adenovirus (6), *Mycobacterium avium* subspp. *paratuberculosis* (7), and protozoans (8), none has been documented with regards to avian influenza viruses.

The manufacturers of most microbiological safety cabinet instruct that the regular assessment of the UV efficiency on work surface be carried out using UV light meter, and several scientists are aware of the need to replace the UV tube should the intensity falls below the adequate requirement (for example 40 microwatts per square centimeter at a wavelength of 253.5 x 10^{-9}m) (The Baker Company, Sanford ME, www.bakerco.com), however, a number of researchers in low income food deficient countries (LIFDC) tend to assume that a relatively new cabinet should have an efficient UV system.

A recent assessment of a set of final year Medical Laboratory Science (virology option) students and other spectrum of research scientists revealed that over 80% believed that the UV rays are able to attenuate/kill any virus after some long period of exposure. Such believe is carried into the work environment and this sometimes unwittingly predispose many researchers to potential hazards associated with undue/unprotected exposure to HPAI H5N1 virus through working in the cabinet, since the aerosolized virus may remain for some time in the cabinet. Technicians, service personnel and laboratory cleaning staff are at similar risk in the course of performing their routine duties.

This work therefore aims at investigating the potential dangers held by the residual avian influenza H5N1 virus following its manipulation in the biological safety cabinets despite extended periods of exposure to ultra-violet radiation.

Materials and Methods

Three candidate H5N1 HPAI viruses were selected from the isolate bank (-70^{o}C) and allow to thaw on wet ice inside a microbiological safety cabinet (SterilGARD® III Advance, The Baker Company, Sanford ME,

www.bakerco.com). The isolates have been characterized using standardized methods as previously reported (9). Briefly described, all un-contaminated allantoic fluids (ALF) arising from inoculation of 20% tissue samples were spot tested by haemagglutination test; the chorio-allantoic membrane (CAM) of positive harvest were further prepared and tested by agar-gel immuno-diffusion (AGID) to detect influenza A group antigen. α-haemagglutination test was conducted to determine the virus subtype. For further confirmation, a cascade-type molecular analysis was performed starting with the M-gene. Every positive result for M-gene was subjected to an RT-PCR for haemagglutinin gene of subtypes H5 and H7. Every positive HA result was confirmed for N1 by RT-PCR. The primers used are listed in Table 1.

The haemagglutination titres of all the viruses were determined afresh using standardized protocol (9). 200µl of each virus sample was inoculated into five 9-day-old embryonating chicken eggs (ECE) and incubated at 37^{o}C. The chicken embryos were monitored for mortality through candling. All dead eggs were chilled at 4^{o}C, aseptically opened and the ALFs tested for haemagglutinating activities and plated on blood agar to exclude bacteria contamination. Fresh ALF was harvested from each of the sample for experimental purposes.

Exposure to UV-light and inoculation

Five pieces each of a new set of 9-day-old ECE were grouped based on assigned timing (0minute, 15 minutes, 30 minutes, 60 minutes, 120 minutes and 180 minutes). All eggs were properly marked and disinfected using 70% ethanol. Freshly harvested ALF were aliquoted and stored at -20^{o}C. Portions of aliquots were placed on wet ice and exposed to UV- irradiation for the time previously assigned. Another portion was left unexposed to UV-light and kept at -20^{o}C.

At the end of each assigned time, 200µl of exposed and unexposed aliquots with the same timing were inoculated through the allantoic route into the marked 9-day-old ECE and sealed with wax. All eggs were incubated at 37^{o}C and monitored for mortality as previously described above. Haemagglutinating ability of the exposed and unexposed ALF arising from the experiment was tested for using the appropriate procedure. Simply described, about 10-20µl of the ALF was mixed with about 20µl of c-RBC on a sterile white porcelain, gently rocked and observed for haemagglutination after about 2-3 minutes.

Portions of all the ALFs were taken for the determination of haemagglutinating titre post- exposure to UV-light. All ALF titres

were determined using standardized procedure (9).

Diagnostic PCR was conducted to determine whether the exposure to ultraviolet rays has had significant effect on the amplicon sizes of the virus samples.

Results

No significant difference exists between the viruses exposed to ultraviolet irradiation at 95% CI (P value =0.3118). The exposure to UV-light does not seem to have any effect on the HPAI H5N1 virus ability to haemagglutinate c-RBC, pathogenicity in eggs and haemagglutination titre (Tables 2-5). However, the unexposed inoculum appears to have increasing titre with longer period of maintenance in the -20^{o}C freezer. The exposure to UV-light does not seem to have any effect on the amplicon sizes of the exposed samples.

TABLE 1: H-GENE AND M-GENE PRIMERS USED IN THE STUDY

H forward	**5′-CCT CCA GAR TAT GCM TAY AAA ATT GTC-3′**
H reverse	**5′-TAC CAA CCG TCT ACC ATK CCY-3′**
M forward	**5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′**
M reverse	**5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′**

TABLE 2: PRE-EXPERIMENTATION VIRUS ISOLATES CHARACTERISTICS.

Passage	Ability to haemagglutinate 10% c-RBC	HA Titre at passage level 1 (Log_2)	Pathogenicity in embryonating eggs (48 hours)	Isolates Designation based on characterization
07/415	100%	5	100%	HPAI (H5N1)
07/456B	100%	4	100%	HPAI (H5N1)
07/439B	100%	5	100%	HPAI (H5N1)

TABLE 3: PATHOGENICITY OF INOCULUM (P2) IN EMBRYONATING CHICKEN EGGS POST EXPOSURE TO UV-LIGHT.

Result of pathogenicity in chicken embryo 48 hours after inoculation					
Virus isolate	15minutes	30minutes	60 minutes	120 minutes	180 minutes
07/456B exposed	100%	100%	100%	100%	100%
07/415 exposed	100%	100%	100%	100%	100%
07/439B exposed	100%	100%	100%	100%	100%
Result of pathogenicity (Unexposed)					
07/456B unexposed	100%	100%	100%	100%	100%
07/415 unexposed	100%	100%	100%	100%	100%
07/439B unexposed	100%	100%	100%	100%	100%

Between 50 and 100% of all embryo die within 24 hours and all embryo die within 48 hours. P2= Passage level 2.

TABLE 4: HAEMAGGLUTINATION TEST OF ALLANTOIC FLUIDS FROM EMBRYONATING CHICKEN EGGS POST-EXPOSURE OF INOCULUM TO UV-LIGHT.

Result of HA after Exposure						
Virus	Unexposed	15minutes	30 minutes	60 minutes	120 minutes	180 minutes
07/456B	3/3 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
07/415	3/3 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)

07/439B	3/3 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)

Results indicate complete haemagglutination of all tested and control samples

TABLE 5: HAEMAGGLUTINATION TITRE OF ALLANTOIC FLUIDS FROM EMBRYONATING CHICKEN EGGS POST-EXPOSURE OF INOCULUM TO UV-LIGHT.

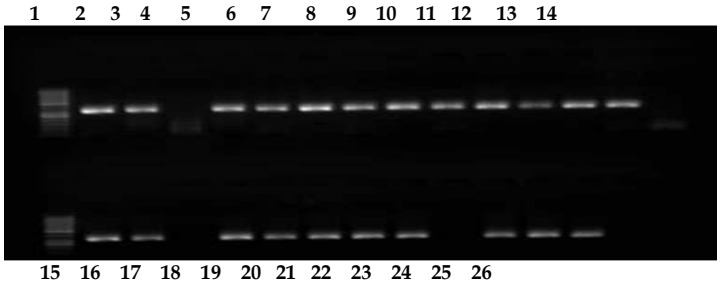
Result of HA titre after UV Exposure P3 (all results in Log_2)					
Virus isolate	15minutes	30 minutes	60 minutes	120 minutes	180 minutes
07/456B	7	6	5	7	6
07/415	4	4	4	8	5
07/439B	6	7	7	7	5
Result of HA titres (Unexposed) P3					
07/456B	4	5	6		
07/415	4	5	6		
07/439B	5	5	8		

P3= Passage level 3

Discussion

Our assessment of the effect of the UV-light rays on virus haemagglutinability, haemagglutination titre, pathogenicity in embryonating chicken eggs and amplicon sizes has shown that the virus seem unaffected by UV-rays. This conflicts with the report of other workers with regards to other viruses affected by UV-light (5, 6). Lowy and co-workers (10) have similarly agreed that gamma irradiation rather that UV irradiation is more effective in penetrating through most biological and non biological agents for purposes of inactivation.

FIGURE 1. AMPLICON SIZE OF THE DIFFERENT SAMPLES ANALYZED FOLLOWING EXPOSURE OF THE INFECTIVE ALLANTOIC FLUIDS TO UV RAYS.



1= 07/456B (60mins, E), 2= 07/456B (P2), 3= 07/149 (Newcastle isolate), 4= 07/439B (60mins, U), 5= 07/439B (15mins, E), 6= 07/456B (15mins, E), 7=07/456B (15mins, U), 8= 07/439B (P2), 9= 07/456B (30mins, U), 10= 07/456B (60mins, U), 11= 07/439B (30mins, E), 12= 07/439B (15mins, U), 13= 07/439B (180mins, E), 14= 07/640 (Newcastle isolate), 15= 07/415 (15mins, E), 16= 07/415 (120mins, E), 17= 07/156 (Newcastle isolate), 18= 07/415 (60mins, E), 19= 07/456B (120mins, E), 20= 07/415 (30mins, E), 21= 07/415 (180mins, E), 22= 07/456B (180mins, E), 23= negative control, 24= 07/456B (30mins, E), 25= 07/439B (120mins, E), 26= 07/415 (60mins, U).
E=Exposed to UV-light; U =Unexposed to UV-Light.

The lack of penetrating power of UV light through the virus may therefore explain why the virus are not inactivated by the UV-rays despite prolong exposure.

Although, we are aware that the degree of thickness of the glass container holding the aliquots may to some extent serve as barrier to penetration of UV light, we ensure the usage of containers with thin

walls (≈1mm thickness) as it will be unethical to expose the naked virus to the environment for such long time in the safety cabinet. However, there is no difference in virus characteristics despite the wide disparity in exposure time (30 minutes up to 180 minutes).

Despite our inability to carry out animal infection assessment study using the exposed virus due to limitations of animal experimentation facilities, it is our strong opinion that the virus may still be able to cause pathogenicity in live chicken comparable to the initial field isolates.
This area of study will need further investigation.

The observed increasing titre recorded in the unexposed inoculum (Table 4) may be as a result of on-going virus activity. Webster and co-workers (9) has indicated that the virus is unstable and may have increasing activity if kept at -20^{o}C for a relatively long time. We therefore affirmed the call for caution in the handling of H5N1 influenza viruses especially on wet ice.

Our opinion survey of virology students and other laboratory staff suggested that 43% have good knowledge, 52% have fair knowledge while 5% have poor knowledge of UV rays. Although 93% agreed that it will attenuate/kill bacteria, only 80% believed that it will attenuate/kill viruses, however two individuals believed that encapsulated bacteria may not be affected and four persons agreed that not all viruses may be affected by UV rays. 74% of the respondents claimed to have knowledge of depreciation in the effectiveness of UV rays over long period of usage time. However, none seem to be sure of whether the UV-rays will inactivate the H5N1 virus. This revealed that virology staff is at high risk of infection with agent like avian influenza H5N1 since most may assume that microbiological safety cabinet is sterile following UV- light exposure.

Further work is encouraged in the areas of laboratory and field assessment of the avian influenza H5N1 virus.

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