

Effect of Disinfectants on the Recovery, Titer and Viral RNA of Highly Pathogenic Avian Influenza Virus (H5N1)

Hussein A. Kaoud^{1*} and Salah Yosseif²

Professor of Veterinary Hygiene and Environmental pollution Department of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, Giza-Egypt, 12211. tel.:

+201224207641; fax: 202-5725240 or 202-5710305.

Veterinary Medical Researcher

Austria. tel.: 0201270972146.

Abstract— *The present investigation was undertaken to evaluate the virucidal activity of five disinfectants; a peroxygen compound (Virkon-S), Glutaldehyde (Aldekol), an organic acid (Longlife 250 S), an innovative Disinfectant-lyte (It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite) and TH4 (a combination of four quaternary ammonium compounds and gluteraldehydes against avian influenza virus (AIV) under laboratory conditions. Disinfectant-Lyte 1/250 (Envirolyte-Egypt) was the most effective disinfectant in killing AIV, showed complete reduction in hemagglutinating (HA) activity) and damage of the nucleic acid (RNA) and viral protein. We report that; disinfectants are inactivating AIV by different methods. The study, however, points out that, we have to know how much damage must be done to the virus before virus infection is prevented.*

Index Terms- Disinfectant, Hemagglutinating activity, Titer; Avian influenza virus, Polymerase chain reaction

I. INTRODUCTION

Influenza A viruses are single-stranded RNA viruses of negative sense with an 8 segmented genome that belongs to the family Orthomyxoviridae [1]. The viral hemagglutinin (HA) and neuraminidase (NA) proteins are envelope glycoprotein's and are the key antigens against which humeral immune responses are directed as well as have special functions (host-cell specificity, release of viral genome, guidance of viral nucleic acid within the host cell cytoplasm). On the viral envelope or within the nucleocapsid there are some particular proteins for the infectivity of the virus and for the replication of the viral genome. It is known that the viral nucleic acid of some viruses can remain infectious when released from the viral capsid.

The viral envelop is highly lipophilic in nature and negatively charged (derive from cytoplasmic membrane) and therefore highly susceptible to a wide range of membrane-active agents, although it is not known how much damage must be done to the virus envelope before virus infection is prevented.

Poultry shed infectious AIV (H5N1) into the environment in both nasal secretions and faeces. Previous studies on HPAI outbreaks have demonstrated that the most important mode of

transmission in domestic poultry is related to the movement of humans, birds, contaminated materials and vehicles [2] - [4] .

It has been demonstrated that the H5N1 virus survives from 4 to 23 days in wet chicken manure [5], many months in cool water [6] , [7] , and 72 hours on plastic, steel and rubber materials [8] .

Disinfectants induced inactivation of AIV has been reported by various researchers all over the world [9], [10] - [12].

“Reference [13] shows the effect of several chemical compounds and compound mixtures (acetic acid, citric acid, calcium hypochlorite, sodium hypochlorite, laundry detergent with peroxygen, commercial iodine/acid disinfectant) to disinfect LPAIV^{ab}”. Documentation of the effectiveness of viral disinfectants against viruses is minimal, and even less information is available on mechanism of action [14]. Since different disinfectants have different modes of action for inactivation of viruses, laboratory studies were performed to examine the effect of most common disinfectants that used in poultry production on the titer, virus recovery and hemagglutinin activity as well as a trial to explain the mechanism of inactivation and their ability to disrupt AIV RNA so that it could not be detected by Real Time -RT-PCR^b.

II. MATERIALS AND METHODS

We tested the efficacy of a new natural disinfectant (Disinfect-lyte, natural superoxide water) to inactivate HPAIV: virus titer, hemagglutinating (HA) activity, viral protein and RNA. We evaluated the virucidal activity (reduction of virus titer, hemagglutinin activity, ability to disrupt or to detect RNA) of five disinfectants; a peroxygen compound (Virkon-S), Glutaldehyde (Aldekol), an organic acid (Longlife 250 S), an innovative Envirolyte-Egypt (It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite) and TH4 (a combination of four quaternary ammonium compounds and gluteraldehydes against avian influenza virus (AIV) under laboratory conditions.

Test virus

Type :H5N1 subtype KC699547 (Egypt/VRLCU-R11/2012)
^c was used in this study. Avian influenza virus was isolated

from infected poultry flocks during recent AI outbreaks in Egypt.

Virus propagation

Tenfold dilutions of H5N1 virus were inoculated into 9-11-day-old ECE 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 as in [15].

Viral RNAs extraction

Viral RNAs were extracted by the use of QIAamp viral RNA Mini Kit (QIAGEN, Germany) Cat.no.52904. The kit combines the selective binding properties of silica-gel-based membrane with the speed of micro spin technology. The kit contain: QIAamp mini spin columns, collection tubes (2 ml), buffer (AVL), buffer AW1, buffer AW2, and buffer AVE and Carrier RNA.

Hemagglutination (HA) test

HA was carried out for standardization of AI antigen used in HI test to 4 Hemagglutination units (HAU); as in [16]. The virus was propagated in the allantoic fluid of 9-11-day-old embrocated specific-pathogen-free (SPF) chicken eggs .The egg infectious dose 50 %; (EID50) was determined by 10-fold dilutions of harvested allantoic fluid. Four days after inoculation the allantoic fluid was harvested and tested for the presence of hemagglutinating activity with the use of 1% chicken red blood cells (RBC) diluted in phosphate-buffered solution (PBS).

Viral protein analysis

For detecting viral proteins a qualitative assay was used in this study as in [14]. Viral proteins analyzed by western blot assay using the monoclonal antibody.

Disinfectants

1- Virkon S (DuPont, UK). It is composed of peroxygen compounds, surfactant, organic acids and an inorganic buffer system. It was used at a concentration of 1%.

2- Aldekol Des 03, (Ewabo, Germany), contains Glutraldehyde 24.8% quaternary ammonium chloride 2.5% and formaldehyde 18.3%. The recommended concentration is (0.5%).

3- Longlife 250 S, (Antec International Limited, UK), contains an active synergistic blend of organic acids, organic biocides and surfactants. It was used the concentration of 0.5%.

4- Disinfectant-lyte (Envirolyte-Egypt) .It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite [(HClO, ClO₂, HClO₃, HClO₄, H₂O₂, O₂, ClO⁻, ClO₂⁻, ClO₃⁻, O⁻, HO₂⁻, OH⁻ - working substances), pH 2.5-3.5, ORP>1150mV, C_{active} ~500 mg I⁻¹, 1\250 = 4 mg I active chlorine)].

5-TH4 (Sogeval, Laval-France), each 1L contains Glutraldehyde (62.50 g) activated by a specific blend of 4 lipophilic biocides (Didecyl dimethyl ammonium chloride 18.75 g, Dioctyl dimethyl ammonium chloride 18.75 g, Octyl

dimethyl ammonium chloride 37. 5 g, Alkyl dimethylbenzyl ammonium chloride 50 g). It was used at a concentration of 0.5%.

Each disinfectant was diluted using single sterile distilled water with the initial v/v or w/v dilution.

II. EXPERIMENTAL DETAILS

For all experiments, infectious virus in allantoic fluid containing KC699547 (H5N1) was diluted 1/10 in BHI broth and 0.5 ml quantities were mixed with equal quantity of diluted disinfectant. Each mixture of virus/disinfectant was incubated for 10 min at room temperature as in [17]. Following incubation, the virus/disinfectant mixtures were either promptly processed for RNA extraction or inoculated into embrocating chicken eggs. An aliquot of 0.5 ml was used for RNA extraction, and the remaining 0.5 ml was inoculated into six eggs.

Inoculated eggs were candled daily for 7 days, and any deaths during the first day were discarded as nonspecific deaths. Prior to analyzing any of the viral ribonucleic acid (RNA) treated with various test disinfectants, preliminary experiments were conducted to ensure that inhibition, due to possible chemical carry-over during extraction, was not resulting in false negatives. None of the test disinfectants used

Table 1: Effect of disinfectants on survival, titer and HA activity of the avian influenza Virus after 10 min exposure [The recovery of AIV (H5N1)]

a Infectivity = (infected embryos)/(total embryos inoculated; n = 6) per treatment.

Disinfectant	Conc.	Embry death	T.of virus	HA activity in the AAF
Virkon-S	1%	4:6 ^a	2 ³	35 % in this study
Aldekol des 03	0.5%	3:6	2 ²	0 % in this study
Longlife 250 S	0.5%	2:6	2 ⁵	50 % in this study
Disinfect-lyte	1:250	0:6	0	0 % in this study
TH4	0.5%	3:6	2 ³	0 % in this study
Control +ve	0	6:6	2 ⁹	100 %
Control - ve	0	0:6	0	0 %

In these experiments resulted in inhibition, based on positive Real Time RT-PCR reactions after spiking the extractions with influenza RNA. Therefore, the observed degradation is due to disinfectant degradation of nucleic acid as opposed to chemical inhibition of the assay itself.

III.RESULTS AND DISCUSSION

Survival, titer and HA activity

The results revealed that Disinfect-lyte (1:250) was very effective in complete destroying of H5N1 virus after 10 min exposure at 25°C. While Aldekol Des 03 (0.5%), reduced virus titer 7 folds (2²). Virkon®-S 1% and TH4 0.5% were reduced virus titer 6 folds (2³), but Longlife 250 S (0.5%)

reduced virus titer only 4 folds (2^5). The allanto-amniotic fluid (AAF) from all of the six embryos inoculated with the treated avian influenza virus showed remarkable decrease in hemagglutinating (HA) activity harvested from inoculated SPF eggs with the exception of Disinfect-lyte (showed complete reduction of hemagglutinating (HA) activity) , as seen in Table and Figure (1).

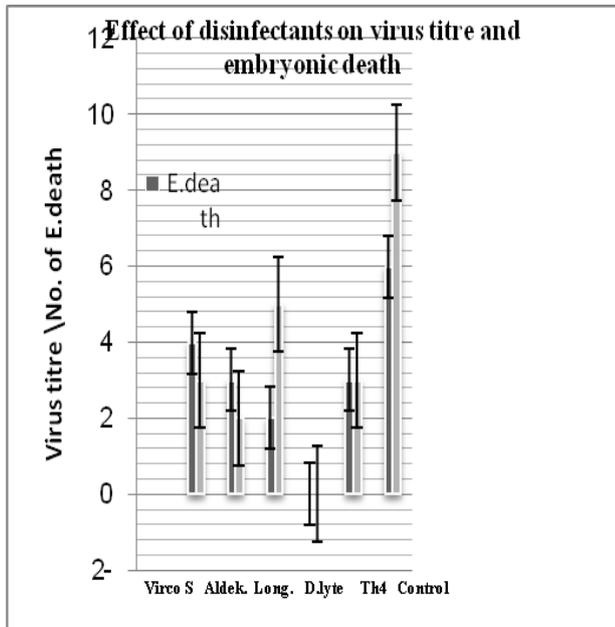


Fig. 1: Effect of disinfectants on the survival and virus titer of AIV (H₅N₁): Disinfect-lyte (1:250) was very effective in completely destroying H5N1 virus after 10 min exposure at 25°C. While Aldekol Des 03 (0.5%), reduced virus titer 7 folds (2^2). Virkon®-S and TH4 0.5% were reduced virus titer 6 folds (2^3), but Longlife 250 S (0.5%) reduced virus titer only 4 folds (2^5). The allanto-amniotic fluid (AAF) from all of the six embryos inoculated with the treated avian influenza virus showed remarkable decrease in hemagglutinating (HA) activity harvested from inoculated SPF eggs with the exception of Disinfect-lyte (showed complete reduction of hemagglutinating (HA) activity).

RNA detected by RRT-PCR

Results of the in vitro evaluation of disinfectants showed that three of the five disinfectants were unable to damage the RNA effectively to prevent detection by RRT-PCR., Table 2. The exceptions were Disinfect-lyte at 1:250 dilutions (resulted in complete degradation of viral RNA following 10 min exposure) and Virkon-S at 1/100 dilution (resulted in 3 log₁₀ reduction following 10 min treatment). Overall, these results indicated that, Disinfect-lyte (1:250) followed by Virkon S (1%) are the most effective disinfectants for inactivation of influenza viruses based on degradation of viral RNA.

These results are as in [18]; sodium hypochlorite and free chlorine were reported as effective for both inactivating AI virus (H5N9, H7N3) and preventing amplification by reverse transcriptase- polymerase chain reaction. Treatment with bleach (10%) with 0% organic challenge resulted in complete degradation of H5N1 viral RNA following 10 min exposure [14].

Table 2: The effect of 10 min exposure of Virkon S Aldekol Des 03, Longlife 250 S, Disinfect-lyte and TH4 on HPAV (H5N1) in relation to their detection by RRT-PCR and virus isolation.

Disinfectant	Concentration	Virus detection
		by RRT-PCR
Virkon-S	1%	±*(3 log ₁₀) in this study
Aldekol Des 03	0.5%	+ (6 log ₁₀) in this study
Longlife 250 S	0.5%	+ (6 log ₁₀) in this study
Disinfect-lyte	1:25	- (0) in this study
TH4	0.5%	+ (6 log ₁₀) in this study
Control	0	+ (7log ₁₀)

*: Virkon-S at 1/100 dilution resulted in 3 log₁₀ reduction

Results observed in this study are similar to results achieved with disinfectants with other viruses. The previous papers have demonstrated the ability of sodium hypochlorite or free chlorine to prevent RT-PCR detection in hepatitis C virus, poliovirus, and rotavirus, respectively [19] - [21]. The results presented here suggest that Virkon-S 1%, Aldekol Des 03 (0.5%), Longlife 250 S (0.5%) and TH4 0.5% are effective for decreasing AIV titre, but they do not adversely affect the viral RNA to the point where it prevents detection by RRT-PCR. Virkon®-S 1% and TH4 0.5% might be affecting viral capsid without damage of viral RNA.

Viral protein

Following a 10 min treatment, viral protein was not visualized with the following treatments: Virkon S 1% Aldekol Des 03, Longlife 250 S and TH4. The study, however, points out that, disinfectants were inactivated AIV by different methods. Disinfectants induced inactivation of AIV has been reported by various researchers all over the world [22], [23], [10] - [13], [17]. Viruses are divided into several subgroups with regard to their resistance to disinfectants, as shown in Table 1, based largely on the presence or absence of an envelope and the size of the virus particle. “Reference [24] shows schematic for dividing viral groups, the least resistant to disinfectants are the enveloped viruses (i.e. influenza)”. Although there is a wealth of information about the viral target to antiviral agents there is little information on the mechanism of action of viral biocide. The structure of avian influenza virus that offer target sites for virucides can be separated into: the envelope, glycoprotein receptors, the capsid and the viral genome. The viral envelope is highly lipophilic in nature and negatively charged (derive from cytoplasmic membrane) and therefore highly susceptible to a wide range of membrane-active agents, although it is not known how much damage must be done to the virus envelope before virus infection is prevented. On the viral envelope or within the nucleocapsid there are some particular proteins for the infectivity of the virus and for the replication of the viral genome. It is known that the viral nucleic acid of some viruses can remain infectious when released from the viral capsid.

Avian influenza viruses continue to threaten globally with pandemic potential. The first step in a potential pandemic is the ability of the virus to enter host cells which is mediated by the viral surface glycoprotein hemagglutinin (HA). Viral entry of influenza is dependent upon the processing of the HA0 polypeptide precursor protein into HA1 and HA2 which is mediated by host cellular proteases [25]–[27] as well as membrane fusion and/or its change [28], [29].

IV. CONCLUSIONS & RECOMMENDATIONS

This experimental study describes the effects of most common disinfectants on infectivity of HPAIV H5N1. It is therefore inferred that H5N1 virus can be inactivated in the poultry farms/hatcheries using disinfectants of the material to be disinfected. However, it may not be practically feasible for the farmers. Use of disinfectants seems more appropriate and practicable capable to inactivate HA and to damage RNA. Consequently there is no need to depopulate the poultry sheds after AIV outbreak for long period of time before arrival of new stock if disinfectants are used appropriately. More research are recommended to explain the mechanism of inactivation and to know how much damage must be done to the virus before virus infection is prevented.

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AUTHOR BIOGRAPHY



Dr. Hussein A. Kaoud is full professor of Professor of Veterinary Hygiene and Environmental pollution and Chairman of Department of Veterinary Hygiene and Environmental Pollution, Faculty of Veterinary Medicine, Cairo University, Giza-Egypt. Dr. Kaoud has written 80 books in Arabian and English language and many Scientific Articles and 10 patents. He has guided many students at Ph.D. and P.G. level and attended many conferences. Dr. Kaoud

is the member of several Egyptian and international societies. He is the editor and reviewer of many international journals. He has received many awards.