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Effect of Disinfectants on Highly Pathogenic Avian Influenza Virus (H5N1) in Lab and Poultry Farms

Hussein A.Kaoud
Professor of Veterinary Hygiene and Environmental pollution
Department of Veterinary Hygiene and Environmental Pollution, Faculty of Veterinary Medicine, Cairo University, Giza-Egypt, 12211. tel.: +201224207641; fax: 202-5725240 or 202-5710305.

Abstract*— This study was carried out in 6 layer houses at Giza province, during2010-2011 outbreaks of high pathogenic avian influenza (HPAI) diagnosed in Egypt. The present investigation was undertaken to evaluate the virucidal activity of different disinfectants against avian influenza virus (AIV) under laboratory and field conditions. Anigen Rapid AIV Ag Test Kit was used for detection of AIV from environment and embroynated chicken's eggs (ECE). Five disinfectants were evaluated for their effectiveness against AIV contaminated premises (in vitro and vivo). They were an organic acid (Longlife 250 S), a peroxygen compound (Virkon-S)-, Glutraldehyde (Aldekol) and (TH4) - (a combination of four quaternary ammonium compounds and gluteraldhydes) and innovative Envirolyte-Egypt (It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite. Envirolyte-Egypt (1/250) and Virkon S 1% were the most effective disinfectants in killing AIV. Despite the good results obtained with Aldekol 0.5%, Longlife 250 S 0.5% and TH4 0.5% in laboratory test after 10 min, but the effect of both disinfectants on AIV infected premises was failed.

Index Terms— **Highly pathogenic, Avian influenza, Virucidal activity, Disinfectants; Field study.**

I. INTRODUCTION

An outbreak of Avian Influenza (H5N1), has been reported in Egypt in 2006 [1]. 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. The influenza viruses are relatively unstable in the environment. It is easily destroyed by heat (56º C for 3 hours or 60º C or more for 30 minutes); extreme changes of pH, no isotonic conditions and dryness. AIV is very sensitive to most detergents and disinfectants [2]. However, 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. In water, the virus can survive for up to four days at 22º C and more than 30 days at 0º C. Also, it can survive in the environment for 6 days at 37 º C [3]. For the highly pathogenic form, studies have shown that fecal material from infected birds may contain up to 16 x 10^6 virions/g of feces and one gram contains enough viruses to infect one million birds [4].

Studies for the determination of the efficacy of chemical substances [5-9] demonstrated a high sensitivity of influenza viruses, but the test conditions chosen were not very suitable for evaluating the efficacy of disinfectants against IVA in animal husbandry. Documentation of the effectiveness of viral disinfectants is minimal, and even less information is available on the mechanism of action and their efficacy in the presence of organic challenge. In addition to the lack of efficacy data, the data that are available in the literature are difficult to interpret and compare against other data due to lack of standardized testing protocols for the inactivation of viruses. The objective of the present study was to evaluate the disinfectant efficacy of Aldekol des 03, Virkon S, Longlife 250 S, TH4 and Envirolyte-Egypt for the control of AIV either by laboratory and field tests.

II. MATERIALS AND METHODS

A. Experimental Details

Samples

Swab samples were collected from 6 premises of commercial layers houses during 2010 and 2011 at Giza province. These houses were suspected to be contaminated with AIV as a result of disease symptoms in these farms. Swab samples were collected from dead and sick birds (dry swabs used for collecting samples from dead birds and swabs moistened with viral transport medium for live birds). Lungs, intestines, tracheas and livers samples were collected

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and transported with viral transport medium [2]. The virus confirmed after submission to the reference laboratory (National Laboratory for Veterinary Quality Control of Poultry Production, Ministry of Agriculture, Egypt) for characterization.

Disinfectants

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

2- 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%.

3-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%.

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

5- Envirolyte-Egypt (It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite (HClO, ClO_2, $HClO_3$, $HClO_4$, H_2O_2, O_2, ClO^-, ClO_2^-, ClO_3^-, O^-, HO_2^-, OH^- - working substances, pH from 2.0 to 8.5, 1\500 = 2 mg /L active chlorine, 1\1000 - 1mg /L active chlorine.).

The tested protocol was followed as in [10]. Briefly, all disinfectants were diluted with distilled water following the manufacturer's recommendation. The exposure time was 10 min and 1hr in laboratory evaluation and floor house application of the disinfectants while it was extended to 4 and 8 days in poultry house.

Antigen detection tests

Antigen Rapid AIV Ag Test Kit used for detection of AIV in avian droppings, with a high degree of accuracy. A product was manufactured by Antigen Biotechnology Institute Animal Genetics, Inc. of Korea. The principle is Immunochromatographic assay (the kit uses a monoclonal antibody against the nucleoprotein and able to detect any influenza A virus within 20 minutes).

Virus propagation

Tenfold dilutions of H5N1 virus were inoculated into 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 ELD_{50}/ml and evaluated according to the method described by [11], [12].

Virus identification and characterization

The infectious AF was harvested from each ECE and subjected to HA and HI test according to OIE standard methods [2]. HA and HI tests were done in a conventional micro plate procedure. The HA titer equal to or greater than 1:4 was determined as infectivity. The infectious AF showing HA titer was considered to be randomly collected for the HI test. Concern HI test, Antigen rapid AIV Ag Test Kit used for detection of AIV and infectious AF containing 4 HA units of virus were performed in this test. The HI end point was read at the highest reciprocal of the dilution exhibiting complete inhibition of HA activity.

B. Experimental Work

Laboratory evaluation of the disinfectants

This was done in the Dept. of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. The tested disinfectants were evaluated according to the protocols as in [10] – [14]. Briefly, all disinfectants were diluted with distilled water following the manufacturer's recommendation. About a 0.5 ml of AIV containing approximately 1.0 x 106 ELD50 was mixed with 0.5 ml of diluted disinfectants and incubated for 10 min and 1 hr at room temperature. In addition, 0.5 ml of AIV was mixed with0.5 ml of PBS, and 0.5 ml of disinfectant was mixed with 0.5 ml of distilled water and served as the positive and negative control, respectively. After 10 min and 1 hr incubation period, 0.1 ml of the virus-disinfectant mixture, the positive and negative controls were inoculated into11-day-old ECE in three replications and candled twice a day for 7 days. Inoculated ECE dying prior to 24 h were discarded. The allantoic fluid was harvested from each egg on day 7 post inoculation or upon death and the hem agglutination test was performed to determine the presence of the virus propagation.

Field Trials

Evaluation of disinfectants on the poultry house floor

A poultry farm infected with high pathogenic avian influenza (HPAI) was chosen for carrying out the field trial. Experimental test units were 1-ft^2 floor plots. A half ml of AIVs containing approximately 1.0 x 10^6 ELD_{50} was placed in the center of the plot. Each disinfectant was applied to 10 plots as a coarse spray at a low application rate

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of 125 ml/plot. The rate was chosen due to its ability to create a good surface coverage. Five untreated plots, receiving no disinfectant, served as negative control and also another five virus treated plots, served as positive control.

Evaluation of disinfectants in the poultry house

Six AIV infected houses naturally infected due to outbreak were used in this trial, after depopulation and removal of the droppings and litter; the houses were dry-cleaned and wet cleaned by using Polycar 1% (washing detergent). AIV infected houses were divided into 2 groups. First group had one house which disinfected by Aldekol Des 03 (0.5%). Second group had 5 houses, the first house disinfected by Virkon S 1%, the second house disinfected by Longlife 250 S 0.5% , third house was disinfected by TH4 0.5% and the fourth and fifth houses were disinfected by Envirolyte-Egypt (1/500) and (1/250),respectively.

Swabs were taken from different parts of the poultry houses (cage walkways, fan areas, walls, feeders and egg belts) to detect the presence of the virus. When these samples test positive, cleaning and disinfection repeated to ensure complete killing of the virus [15]. Swabs were inserted in isotonic phosphate buffered saline of pH 7.0-7.4 containing antibiotic (penicillin 10,000 units/ml). The supernatant fluids (0.2 ml) obtained through the centrifugation at 1000 rpm were inoculated into the allantoic sac of 11-day-old embryonated chicken eggs. The eggs were incubated at 37°C for 4-7 days and candled daily for presence of dead embryos. The allantoic fluid was harvested from each egg and tested by HA and HI as in [16]. Detection of HA activity indicates a high probability for the presence of an influenza A virus or of an avian paramyxovirus. Samples that give a negative reaction should be passage into at least one further batch of eggs**.**

III. RESULTS AND DISCUSSION

Avian influenza is one of the devastating viral diseases of poultry with a tendency of rapid spread and inducing high morbidity (100%) and mortality (up to 80%). The casual agent of the disease is excreted in droppings of the diseased bird which results in the contamination of litter, feed, feeders, water, drinkers, air, eggs/egg trays, sheds and surroundings. The movement of the contaminated materials and persons from the infected farm disseminate virus to the other farms and susceptible birds in the vicinity [17], 18]. The infectivity of the AIV is eliminated by the natural physical factors and chemical agents [19]. Previous investigations on AIV disinfection performed with different substances in suspension tests with and without organic load or on carriers like line or batiste gave important information on the effects of disinfectants against AIV [5], [19], [20], [6], [21], [7], [8]. However, most of the disinfectants tested in these studies are not very common nowadays, and the methods used were not very suitable for testing the ability of a disinfectant for veterinary field conditions. Especially in animal husbandry, the requirements on a disinfectant are very high, as a lot of factors like high organic soiling even after proper cleaning, different materials with often porous surfaces, low temperatures and short contact times can negatively influence its efficacy.

Laboratory evaluation of the disinfectants

The results from the present study indicated that H5N1 isolated virus could be moderately inactivated by exposure to the disinfectants including, Aldekol Des 03 0.5%, Virkon S 1%, Longlife 250 S 0.5%, TH4 0.5% and Envirolyte-Egypt (1/500) for 10 min. The Envirolyte-Egypt at concentration of (1/250) was superior for the complete inactivation of high pathogenic avian influenza virus than the other disinfectants (see Table 1).

Table 1 Laboratory evaluation of the disinfectant efficacy of Aldekol des 03, Virkon S , Longlife 250 S , TH4 and Envirolyte-Egypt on HPAV (H5N1).

Disinfectant		Time of exposure				
	Concent.	10 min	1 hr	+ Control‡	-Control‡	Reference
Aldekol des 03	0.5%	2#:6[a]	0:6	5:6	0:6	in this study
Virkon S	1%	2:6	0:6	5:6	0:6	in this study
Longlife 250 S	0.5%	2:6	1:6	6:6	0:6	in this study
TH4	0.5%	3:6	2:6	5:6	0:6	in this study
Envirolyte-Egypt	1/500	3:6	2:6	6:6	0:6	in this study
Envirolyte-Egypt	1/250	0:6	0:6	6:6	0:6	in this study

#: Virus propagation.

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

‡Control for 1hr exposure time.

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In general, on the basis of their resistance to chemical agents, viruses can be divided into three categories (A, B and C) according to the presence/absence of lipids on the virus particle and size of virus. Avian influenza viruses belong to category A, which is the easiest to inactivate and can be inactivated by all of the major classes of disinfectants, if used properly including pH, concentration, temperature ,organic matter and time of exposure [22].

Field Trials

Evaluation of disinfectants on the poultry house floor

The results of evaluation of disinfectants on the poultry house floor showed that the disinfectant Envirolyte-Egypt could completely inactivate H5N1 virus when used at concentration of (1/250) for only 10 min. While, other disinfectants; Aldekol Des 03, Virkon S, Long life 250 S and TH4 could efficiently inactivate H5N1 virus when used at the manufacturer's recommended concentration for 1 hr (see Table 2). Similar results were demonstrated by Songserm *et al.* [23] where the Thai strain of HPAI H5N1 at a titer of $10^{6.3}$ ELD_{50}/ml was completely inactivated after exposure to glutaraldehyde, phenol, parasitic acid, ammonium chloride or acid hyper chloride for 10 min. "Reference [24] shows that Virkon S and Aldekol were effective for AIV at double of their recommended concentration in 45 minutes ".

Table 2 Evaluation of the efficacy of Aldekol des 03, Virkon S , Long life 250 S , TH4 and Envirolyte-Egypt on floor house contaminated with HPAV (H5N1).

Disinfectant	Concent.	Time of exposure				
		10 min	1 hr	+ Control‡	- Control‡	Reference
Aldekol des 03	0.5%	2#:6	0:6	5:6	1:6	in this study
Virkon S	1%	2:6	0:6	5:6	1:6	in this study
Longlife 250 S	0.5%	2:6	0:6	6:6	2:6	in this study
TH4	0.5%	3:6	0:6	5:6	0:6	in this study
Envirolyte-Egypt	1/500	3:6	2:6	6:6	1:6	in this study
Envirolyte-Egypt	1/250	0:6	0:6	5:6	0:6	in this study

Virus propagation.
‡Control for 1hr exposure time.

Virucidal effect of tested disinfectants on avian influenza virus infected poultry premises

Disinfectants used frequently in the poultry industry are inactivated to varying degrees by organic material present in poultry houses at depopulation. It seems likely therefore that the standard Chick-Martin test [25] using 5% dried yeast or 3% dried human faces, might not give a satisfactory estimation of the ability of disinfectants to remain active under the conditions in which they would be used [26] .The results in Table 3, showed that Envirolyte-Egypt (1/250) was very effective in complete killing of H5N1 virus in infected poultry premises within 10 min exposure. While Virkon S (1%) was very effective in complete killing of H5N1 virus in infected poultry premises after the second application at 4 th day. These results coincided with the results recorded by the "reference" [10] concerning the Virkon S, 1%. The efficacy of Virkon-S (0.5%) dilution against H7N3 subtype was able to inactivate AIV fully after 90 min while 1% and 2% concentration achieved virucidal activity in just 30 min [24].

Table 3 Virucidal effect of tested disinfectants; Aldekol des 03, Virkon S , Longlife 250 S , TH4 and Envirolyte-Egypt on avian influenza virus infected poultry premises

Disinfectants	Aldekol		Virkon S		Longlife		TH4		Env.		Env.	
Conc.	0.5%		1%		0 .5%		0.5%		1/500		1/250	
Houses	no.1		no.2		no.3		no.4		no.5		no.6	
Time	10 m	2nd ap*	10 m	2nd ap	10 m	2nd ap	10 m	2nd ap	10 m	2nd ap	10 m	2nd ap
	D	N	D	N	D	D	D	N	D	N	N	N

N: No virus detected D: Virus detected
Time: 10minutes; *: second application of the disinfectant after 4days from the first one (samples were taken 10 m post-exposure) (Suarez et al., 2003; Lamichhane, 2006; OIE, 2004; Reed &Munench, 1938)

It was also noticed that Long life (0.5%) in houses failed to control AIV even after the second application at 4 th day. Aldekol 0.5%, TH_4 0.5% and Envirolyte-Egypt (1/500) after the second application in houses gave complete sanitation of the houses from AIV. Disinfectants induced inactivation of AIV has been reported by various researchers all over the world [27] – [29]. "Reference [30] used several chemical compounds and compound

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mixtures (acetic acid, citric acid, calcium hypochlorite, sodium hypochlorite, laundry detergent with peroxygen, commercial iodine/acid disinfectant) to disinfect LPAIV".

IV. CONCLUSIONS & RECOMMENDATIONS

Envirolyte-Egypt (1/250) followed by Virkon S 1% were the most effective disinfectants in killing AIV after 10 minutes in laboratory test and application in poultry house. Virkon S (1%) was effective in complete killing of H5N1 virus in infected poultry premises after the second application at 4 th day. Despite the good results obtained with Aldekol 0.5%, Longlife 250 S 0.5% and TH4 0.5% in laboratory test after 10 min, but their disinfectant efficacy on AIV infected premises was failed. Envirolyte-Egypt (Disinfectant-lyte) is natural phisicho-chemical electrically activated water. It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite (HClO, ClO_2, $HClO_3$, $HClO_4$, H_2O_2, O_2, ClO^-, ClO_2^-, ClO_3^-, O^-, HO_2^-, OH^- - working substances}. In animal husbandry, the requirements on a disinfectant are very high, as a lot of factors like high organic soiling even after proper cleaning, different materials with often porous surfaces, low temperatures and short contact times can negatively influence its efficacy.

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AUTHOR BIOGRAPHY

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 society's. He is the editor and reviewer of many international journals. He is received many awards.