Research Article

Efficacy of Disinfectants against Egyptian H5N1 Avian Influenza Virus

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Abstract | Poultry industry in Egypt is facing various management problems along with infectious diseases including avian influenza (AI). Biosecurity measures, controlling poultry movements and inactivated vaccines were devised to combat the spread of highly pathogenic avian influenza (HPAIV) H5N1. HPAIV are highly susceptible to all disinfectants because they are enveloped viruses. Disinfection against avian influenza viruses at the poultry farms would significantly reduce and/or limit the chance for its transmission and outbreaks. Many disinfectants have been evaluated for their inactivation ability, but there is still a need for their evaluation under different conditions and in different ways. In the present study, representative disinfectants from chlorine and non-chlorine oxidizing agents have been evaluated for their virucidal ability against two distinct Egyptian subclades of H5N1 highly pathogenic avian influenza (HPAI); (A/chicken/Egypt/VRLCU67/2011) variant subclade 2.2.1.1 and (A/chicken/Egypt/13VIR3729-4/2013) classic subclade 2.2.1/C that were sodium hypochlorite, calcium hypochlorite (bleaching powder), Virkon® S and Peraclean®. The purpose from using the Egyptian H5N1 viruses in the evaluation was to achieve maximum simulation of Egyptian field reality as the two viruses represent the two main subclades currently co-circulating in Egypt. The disinfectants were tested individually for effectiveness against HPAI H5N1 for 5, 10, 15 and 30 minutes contact time. Numerical method and neutralization test were used to assess the ability of each disinfectant to inactivate the virus. Our results revealed that all the used disinfectants were effective with increasing the contact time more than 15 minutes except with Virkon® S which was effective even at a short contact time, 5 minutes. In conclusion, this study reported that chlorine and non-chlorine oxidizing agents are effective against H5N1 HPAI at the farm level that would be helpful in implementing bio-security measures at farms/hatcheries levels in the wake of avian influenza virus (AIV) outbreak.

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Background

The risk of an avian disease outbreak, especially avian influenza (AI), is always a concern for the poultry industry. AI virus (AIV) is a lipid-enveloped virus of the family *Orthomyxoviridae* and genus Influenza virus A (Swayne and Halvorson, 2003). Based on the genetic differences in the surface glycoproteins, type A influenza viruses can be divided into 18 hemagglutinin (HA; H1-H18) and eleven neuramini-

dase (NA; N1-N11) (Tong et al., 2013). In national poultry production, avian influenza viruses cause two main forms of disease, distinguished by low and high pathogenicity (Swayne and Halvorson, 2003). The low-pathogenicity avian influenza viruses (LPAIV) causes relatively milder symptoms with no serious human health concerns while the high pathogenicity avian influenza viruses (HPAIV) represent much more serious threat to both bird and human health due to their high evolution rate and potential reassortment with other influenza viruses (i.e. H1N1, H9N2) posing a real and potential horrendous threat to human (EPA, 2006). Emergence of HPAIV H5N1 in Hong Kong during 1996-1997 (Swayne and Halvorson, 2003) has posed major concerns to public health and has gained immense attentions in evaluating environmental control measures (Rice et al. 2007).

AIV is relatively easy to disinfect due to the lipid envelope that increases its sensitivity to dehydration, detergents, and surfactants (ARMCANZ, 2000). It has been demonstrated that the HPAI H5N1 virus survives from 4 to 23 days in wet chicken manure (Songserm et al., 2006-a), many months in cool water (Zhang et al., 2006), and 72 hours on plastic, steel and rubber materials (Tiwari et al., 2006). Biosecurity and disinfection are the most essential parts for preventive and post outbreak management of H5N1 AI infections in poultry (Marzouk et al. 2014). Although there are a wide variety of chemical disinfectants available in markets, which considered effective against pathogens, the appropriate disinfectant must be chosen according to the susceptibility of the target virus (Suarez et al., 2003).

This study, therefore, was designed to evaluate the efficacy of various chemicals (commercially available disinfectants) against local Egyptian strains of HPAI H5N1 of different subclades (variant 2.2.1.1 and classical 2.2.1/C) co-circulating in the Egyptian poultry sectors by utilizing an *in vitro* model. The results of this study will be helpful in implementing effective bio-security measures at the farm and hatcheries level.

Materials and Methods

Viruses

Two representative Egyptian HPAI H5N1 from different Egyptian subclades **(**A/chicken/Egypt/VRLCU67/ 2011) variant subclade 2.2.1.1 and (A/chicken/Egypt/13VIR3729-4/2013) classic subclade 2.2.1/C were used in this study to evaluate the virucidal ability of four commercially available chemical disinfectants.

Virus isolation and titration

Specific-pathogen-free (SPF) embryonated chicken egg (ECE) were used at age of 9-11 days for titration of the viral stock (Reed and Muench, 1938) and virus isolation attempted after testing the disinfectants (Swayne and King, 2003; Tiwari et al., 2006). The virus seed stock was determined to have a titer of 10 egg 50% infection dose (EID50)/0.1 ml, and it was used for all tests at this titer.

RNA extraction and Real time RT-PCR assay (rRT-PCR)

Viral RNA extraction and rRT-PCR condition was used for quantitative and sensitive detection of the viral nucleic acid (Abdelwhab et al., 2010).

Chemical disinfectants, neutralizers and interfering agents

Four commonly used chemical (commercial) disinfectants were tested individually for their effectiveness against HPAI for 5, 10, 15 and 30 minutes contact time. The used disinfectants were Virkon® S, calcium hypochlorite (bleaching powder), sodium hypochlorite (Clorox) and Peraclean® as shown in Table 1. Chemical neutralizers were used to remove any residual disinfectants after fixed time of application (Russell et al., 1979; Espigares et al., 2003) as shown in Table 1. A total of 3% yeast extract powder solution was prepared (Meron-India, Cat no. Myep/03/KJ12) as source of organic matter and the tested disinfectants were diluted using 300 ppm hard water solution on the day of use as a source of interfering agent (Bloder, 2009).

Building materials

Cement coupons were manufactured in Arab Contractors Company in Egypt to resemble poultry house floor with dimensions 2x2x 1cm^3 to simulate wall, floor and roof building like the Egyptian field conditions. All coupons were thoroughly dried and sterilized at 121°C for 30 min before use.

Efficacy of tested disinfectant against Egyptian H5N1 HPAIVs

According to the guidelines of The United States Environmental Protection Agency (EPA, 2005), the evaluation test must contain cytotoxicity group, control

Table 1: *Chemical (Commercial) disinfectants and their neutralizers*

Disinfectants	Neutralizers	Disinfectant active ingredients
Virkon® S	Sodium thiosulphate 0.5%	Potassium Peroxymonosulfate (21.41%)
		Sodium Chloride (1.5%)
		Other ingredients (77.09%)
Calcium hypochlorite	Sodium thiosulphate 1%	Calcium hypochlorite (65% available chlorine)
Sodium hypochlorite	Sodium thiosulphate 1%	Sodium hypochlorite (5%)
Peraclean 5% ®	Sodium thiosulphate 1%, Sodium polysorbate (Tween 80) 1% and Sodium bisulphate 1%	Peroxyacetic Acid (4.9%)
		Hydrogen peroxide (26.5%)

group, germicide activity or test group, method for increasing viral titer, method for removal residues of the used disinfectant, initial ID_{50} and reduction of ID_{50} after test expressed as log[10.]

Under complete aseptic condition, four cement coupons sterilized by autoclaving were placed in sterile petri dish. Each coupon was coated with 0.2 ml of infective amino-allantoic fluid (AAF) and 0.2 ml of (organic matter as interfering material) 3% yeast extract, lifted to dry about 1 hour at room temperature (20˚C) (EPA, 2005). Each coupon was covered with 2ml of each tested disinfectant prepared and diluted in hard water previously described. The disinfectant was kept on coupons until the desired contact time then each coupon was scraped with sterile pipette, and the fluid was aspirated from the Petri dish and jetted back onto the coupon three times to dislodge virus from the coupon. The fluids from Petri dish were pooled into a single tube. The pooled fluid then was diluted by making three 10-fold serial dilutions, resulting in dilutions from 10^{-1} to 10^{-3}.

One ml neutralizer (specific for each disinfectant) prepared as previously described was added to the first dilution to inactivate the chemical compounds in question, with subsequent dilutions occurring in PBS. Virus re-isolation attempts were made using each dilution by injecting 9-11 day old SPF ECEs via allantoic route. ECEs were candled daily for 3 days and the dead eggs were chilled for 24 hrs, then opened and the allantoic fluid was aspirated, examined for HA activity and EID50 was calculated according to Reed and Muench (1938). The pooled fluid from the coupons of positive controls was diluted by 10-fold serial dilutions resulting in dilutions from 10^{-1} to 10^{-6}. The cytotoxic control was diluted once resulting in 10^{-1} dilution.

Calculation of neutralizing index (NI)

A numerical method was used to express the ability of disinfectant agent to inactivate virus. An NI of virus inactivation was used to evaluate the efficacy of each agent. This method was a modification of the classical avian serology virus neutralization test (Reed and Muench, 1938). The NI of virus inactivation is calculated using the following equation:

$$NI = T_{Pc} - T_a$$

where T_{PC} is the titer of the positive control plate and T_a is titer of the recovered virus from the disinfectant-treated plates.

Results and Discussion

It is of paramount importance that AI infections in poultry are controlled. International organizations have issued a list of recommendations aiming to control the AI that include implementation of risk reduction interventions such as restriction policies, stamping out, and under certain circumstances appropriate vaccination programmes (OIE, 2005). Secondary spread of AI is mainly caused through human-related activities such as the movement of staff, vehicles, equipment, and other fomites along with restocking of birds in establishments without following adequate biosecurity measures (OIE, 2005). It therefore implies that if disinfection of premises, footwear and 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 halted (OIE, 2005). For this reason, cleaning and disinfecting must be considered an essential part of AI control programs to reduce the bio aerosol contaminants for poultry farms, and that will be a significant step towards disease prevention and elimination (OIE, 2005).

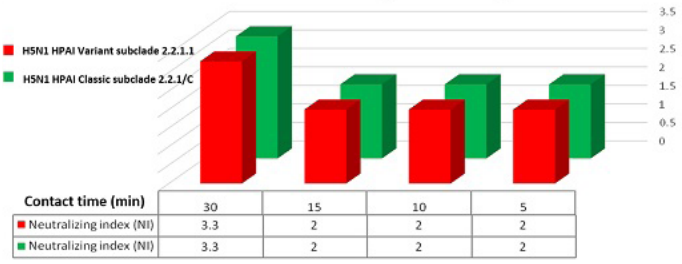
Control of avian influenza (AI) is extremely difficult due to its high level of contagiousness and evolution

rate that pose a threat to avian and human health (Gilsdorf et al., 2006; Songserm et al., 2006b), so the best way to combat with this is to enhance biosecurity. Disinfectants, such as oxidizing agents, phenols, alkalis, alcohols and aldehydes, are all effective against AIV within a relatively short period of contact time (Maillard and Russell, 1997), but the presence of organic materials in the liquids or application area has been found to attenuate their efficacy (Quinn and Markey, 1992; Sattar and Springthorpe, 1999).

Oxidizing disinfectants are a group of disinfectants act by oxidizing the cell membrane of pathogen resulting in cell lysis and death (i.e., Chlorine). Chlorine is considered a universal disinfectant, which can be used as a disinfectant in gaseous form (Cl_2) or in the form of a compound such as sodium hypochlorite, sodium dichloroisocyanurate, calcium hypochlorite and chloramines (White, 1999; Dychdala, 2001). The disinfection efficacy of chlorine increases with decreasing pH while water hardness seems to have no effect on its virucidal efficacy (Dychdala, 2001). Both hypochlorous acid (HOCl) and the hypochlorite ion (-OCl) are strong oxidizing agents that react with a wide variety of biological molecules such as structural proteins (capsid) or surface compounds, lipid envelop (if present) and nucleic acids (DNA or RNA) of viruses (Maris, 1995; Hawkins and Davies, 1999).

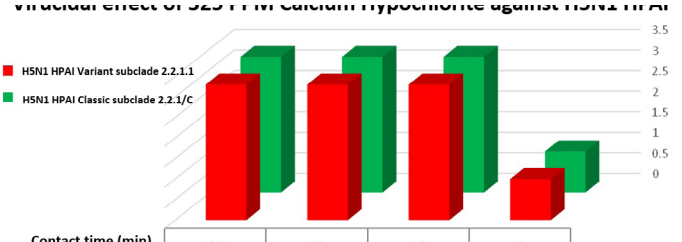
In the present study, four commercial chemical disinfectants were evaluated for their antiviral activity against Egyptian strains of HPAI H5N1 of different subclades (variant 2.2.1.1 and classical 2.2.1/C) co-circulating in the poultry sectors by utilizing an *in vitro* model at different contact time (5, 10, 15 and 30 min.) at the same temperature (Table 1, 2). The results were expressed as neutralization index after contact time 5, 10, 15, and 30 min with each disinfectant. The results had been shown by the mean of four repeat experiments for each disinfectant. For viruses, it is often only practical to measure a 3 to 4 $\log^{10}$ reduction in titer, and no detectable infectious virus in the highest dilution of the virus disinfectant mixture tested. For this reason, inactivation of AIV was considered effective when NI ≥ 2.8, the positive control titer was ≥ 4.0, and there was no recoverable virus from any treated coupon. No recoverable virus equals a titer of <1.2 via the method of Reed and Muench (1938).

Previous studies reported that commercial sodium hypochlorite product at a final dilution 0.125% (w/v) succeed to inactivate 9.8 to 9.4 $\log^{10}$ ELD50 of LPAI H7N2 subtype isolated in Pennsylvania during the 1997–1998 outbreak (Davison et al., 1999). In this study, no difference for both H5N1 AI Egyptian subclades (variant and classical) for the four disinfectants was detected. With 250 ppm Sodium hypochlorite (5%), it seems has no virucidal effect with short contact time (5, 10 and 15 min) that evaluated by the neutralization index (NI=1.8) with detectable virus while with increasing the contact time up to 30 min; the NI increased to be 3.3 and there is no virus recovery (no detectable virus) that confirmed by real time RT-PCR assay as shown in Figure 1 and Table 2.



Contact time (min)	30	15	10	5
Neutralizing index (NI)	3.3	2	2	2
Neutralizing index (NI)	3.3	2	2	2

Figure 1: *Virucidal effect of 250 PPM Sodium Hypochlorite against Egyptian H5N1 HPAI subclades*



Contact time (min)	30	15	10	5
Neutralizing index (NI)	3.3	3.3	3.3	1
Neutralizing index (NI)	3.3	3.3	3.3	1

Figure 2: *Virucidal effect of 325 PPM Calcium Hypochlorite against Egyptian H5N1 HPAI subclades*

Sodium hypochlorite solutions are widely used for disinfection against most of pathogens on hard-surfaces and its use should be discouraged when the organic matter concentrations exceeds 10% (Rutala and Weber 1997). Prince and Prince (2001) reported that for influenza A, a minimum of 200 ppm concentration of sodium hypochlorite is required for inactivation within 10 min contact time. Also, Lombardi et al. (2008) reported that sodium hypochlorite (750 ppm), and calcium hypochlorite (750 ppm), inactivate LPAIV (A/H7N2/Chick/MinhMa/04) effectively on hard and nonporous surfaces.

In contrast, calcium hypochlorite has no virucidal effect with short contact time (5 min) and NI was 1 with

Table 2: *Virucidal effect of chemical disinfectants against Egyptian H5N1 HPAIV with different contact time*

Item		Disinfectant															
		Sodium hypochlorite				Calcium hypochlorite				Virkon®S				Peraclean 5% ®			
Neutralization index (NI)	Contact time (minutes)	5	10	15	30	5	10	15	30	5	10	15	30	5	10	15	30
	Variant subclade 2.1.1.1	1.8	1.8	1.8	3.3	1	3.3	3.3	3.3	3.3	3.3	3.3	3.3	2	2	2	3.3
	Classical subclade 2.1.1/C	1.8	1.8	1.8	3.3	1	3.3	3.3	3.3	3.3	3.3	3.3	3.3	2	2	2	3.3
Detectable virus (Virus recovery) By rRT-PCR assay	Variant subclade 2.1.1.1	Yes			Nil	Yes	Nil			Nil				Yes			Nil
	Classical subclade 2.1.1/C	Yes			Nil	Yes	Nil			Nil				Yes			Nil

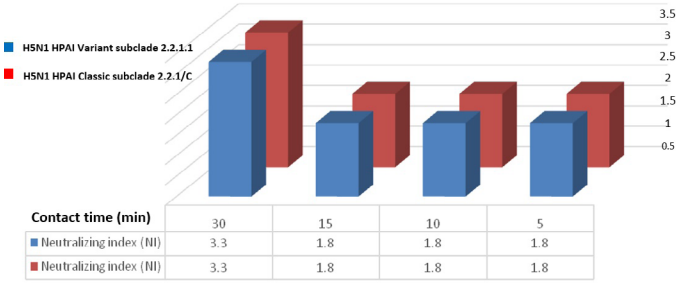


Figure 3: *Virucidal effect of 1% Peraclean® against Egyptian H5N1 HPAI subclades*

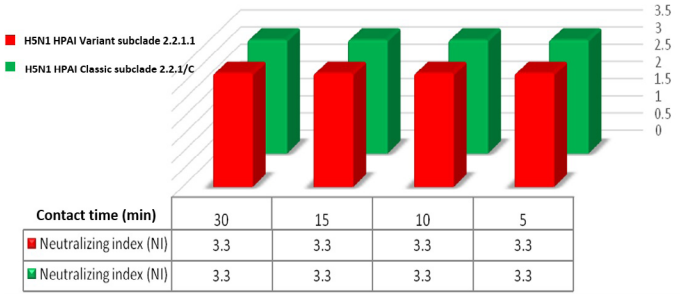


Figure 4: *Virucidal effect of 1% Virkon® S against Egyptian H5N1 HPAI subclades*

detection of virus while with increasing the contact time (10, 15 and 30 min), complete inactivation for the virus (NI=3.3) with no detectable virus as shown in Figure 2 and Table 2. Calcium hypochlorite is an oxidizing agent with a mixture of lime and calcium chloride; it is marketed as chlorine powder or bleach powder for water treatment and as a bleaching agent (Vogt et al., 2010). This compound is relatively stable and has greater available chlorine than sodium hypochlorite (liquid bleach) (Connell, 2014). It is not highly soluble in water and is more preferably used in soft to medium-hard water. Addition of other clutching agent to Clorox and bleach like citric acid or formic acid may increase its effect through addition of formic acid 2% to calcium hypochlorite that increases its efficacy against clostridium perfringens spores (Nasr et al., 2014).

However, Peraclean® 5% (Peroxyacetic Acid Solution) yielded virus recovery (detectable virus) at 5, 10 and 15 min as the NI was 2 while with increasing contact time up to 30 min, complete inactivation for the virus and NI become 3.3 as shown in Figure 3 and Table 2. The combination of peracetic acid and hydrogen peroxide was found to be synergistic and such synergy was maintained with increasing contact time (Alasri et al., 1992). Peracetic or peroxyacetic acid (PAA) is the peroxide of acetic acid (AA) that considers a strong oxidant and disinfectant with high oxidation potential more than chlorine or chlorine dioxide. PAA is commercially available in the form of a mixture containing acetic acid (AA), hydrogen peroxide (HP), and water (Gehr et al., 2002). Although hydrogen peroxide (HP) is contributing to the disinfection power of the PAA mixture; PAA has more potent antimicrobial activity than HP, being rapidly active at low concentrations against a wide spectrum of pathogens (Fraser et al., 1984) while HP requires much high doses than PAA for the same level of disinfection (Wagner et al., 2002). Hydrogen peroxide is a powerful oxidizer that is used primarily as antiseptic, has broad spectrum activity against bacteria, spores, viruses, and fungi when used in appropriate concentration and its byproducts are not toxic and do not pollute the environment (Hancock et al., 2007). Instead of the chlorine formed being given off as a gas, it interacts with the sulphamic acid (acting as a chlorine acceptor) to form an intermediary complex that hydrolyzed (broken down with the formation of water) to release hypochlorous acid. The reaction is

cyclic - the chloride released from the sulphamic acid goes to make more sodium chloride, refueling the cyclic system (Antec International Limited, 1994).

Previous studies reported that the efficacy of 1% Virkon® S was able to inactivate AIV fully after 90 min while 2% concentration achieved virucidal activity in just 30 min (Muhammad et al., 2001). Also, Suarez (2003) reported that 1% Virkon® S was very effective in complete destruction of H5N1 virus in infected poultry premises after the second application at fourth day. In this study, 1% Virkon® S (Peroxyacetic Acid Solution) did not yielded virus recovery (detectable virus) with complete inactivation at different contact time 5, 10, 15 and 30 min as the NI was 3.3 as shown in Figure 4 and Table 2. Our results were coinciding to certain limit with Bieker (2006) who reported that 1% Virkon® S solution might inactivate H1N1 and H7N1 AIV strains after 10 min.

Virkon® S is a balanced, stabilized blend of peroxygen compounds (potassium monopersulphat), surfactant (Sodium dodecyl benzene sulphonate), organic acids (Sulphamic acid and Malic acid), and inorganic buffer (Sodium hex metaphosphate), and inherent part (Sodium chloride). Complex chemical pathway of Virkon salt (sodium chloride) is oxidized by KMPS (triple salt of potassium monopersulphat) that is a multicomponent, optimized, oxidizing system which would destroy all organisms. Efficacy of Virkon® S was performed on a handful viruses, bacteria and fungi and launched it as "the total spectrum disinfectant destroys 100% of all pathogens (Antec International Limited, 1994). The difference between Virkon® S and other chlorine releasing agents is the presence of sulphamic acid and Malic acid/catalyst within Virkon that increase the acidity of Virkon, making it work better than calcium and sod hypochlorites. So, this study confirms that phenolic compounds (Virkon® S) are the most effective disinfectant against H5N1 HPAI with even low concentration and short contact time.

Conclusions

Our results may have an impact on the poultry industry, particularly in view of the current potential for the spread of H5N1 HPAI among poultry and human population. In conclusion, this study describes the efficacy of most commonly used commercial chemical disinfectants on H5N1 HPAIV H5N1 that were effective against avian influenza virus with increasing the contact time more than 15 minutes (sodium hypochlorite, calcium hypochlorite and Peraclean® 5% except in Virkon® S which was effective even at short contact time with complete virus inactivation. It is therefore inferred that H5N1 virus can be inactivated in the poultry farms/ hatcheries by using disinfectants. However, it may not be practically feasible for the farmers. More researches 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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Conflict of Interest

The authors declare that they have no competing interests.

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