



Inactivation of Avian Influenza Virus using Commercial Chemical Disinfectants in Small Scale Poultry Production

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Key words

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Carrier test
Evaluation

ABSTRACT:

Two commercial chemical disinfectants which are commonly used currently in the Egyptian markets were tested individually for effectiveness against highly pathogenic avian influenza virus (HPAIV) A/chicken/Egypt/13VIR3729/4/2013 (H5N1), which currently hit the Egyptian poultry farms at 2013. The tested agents were sodium hypochlorite 5% available chlorine (NaOCl) and PERACLEAN 5%[®] (Peroxyacetic Acid 4.9% and hydrogen peroxide 26.5%). The test was performed in accordance to the guidelines of American environmental protection agency (EPA), using a carrier test with surfaces (coupons) designed specially to mimic the poultry house floor and made from concrete cement, (under dirty condition resembled phase two, step two of European Committee for Standardization (CEN)). At room temperature which mimic the field condition in the Egyptian poultry farms, both sodium hypochlorite with concentration (250ppm), and PERACLEAN 5%[®] with concentration (1%), were not able to inactivate the virus after 5 minutes contact time, while inactivation was achieved within 30 minutes contact time, which proved one of the golden rules when applying a disinfectant, that was allowing the increase of contact time between the disinfectant and influenza virus.

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1. INTRODUCTION

Since 2006 and after it had been introduced to Egypt, highly pathogenic avian influenza virus (HPAIV) strain H5N1 had caused many outbreaks, and formed the main threat to the commercial and small scale poultry production industry in Egypt (Aly et al., 2008).

Despite the taken control strategies including vaccination, surveillance, and the depopulation of more than 40 million birds, the disease was not totally eliminated and over one billion dollars losses were estimated in the commercial poultry and backyard sectors due to the virus (Meleigy, 2007). By 2008 the virus had become endemic (Beato et al., 2013)

Because of the current situation of endemicity of the virus in Egypt, improving the biosecurity of poultry production had become essential to reduce the incidence of the disease (Negro et al., 2013).

HPAI viruses are spread to domestic poultry primarily through direct or indirect contact with infected birds. Transmission also occurs through movement of infected poultry and contaminated organic material and fomites (Capua and Marangon,

2006). Thus, the application of good biosecurity practices along the marketing chain has become very important in the prevention, control and eradication of HPAI (FAO, 2006)

According to the Egyptian integrated national plan for avian and human influenza (FAO, 2010). Biosecurity is a key strategy to control avian influenza virus.

(Noll and Youngner, 1959) divided viruses into three categories denoted A, B and C based on their resistance to chemical agents, this classification depends on the presence or absence of virus envelope and on the virus size itself, which appear to be the most important characteristics that affect the resistance to chemical agents. AIVs belonged to category A, which includes all the enveloped intermediate to large sized viruses.

Although there are many chemical disinfectants available in markets, which considered effective against avian pathogens, the appropriate disinfectant must be chosen according to the susceptibility of the target virus (Suarez et al., 2003).

Disinfectant agents such as chlorine and per oxygen compounds are capable in inactivating the AIV (Swayne et al., 1998).

Sodium hypochlorite is a strong oxidizer, and it has been used as a disinfectant since World War I when

it was used to prevent infection in wounds. Sodium hypochlorite reacts in water, producing hypochlorous acid and hypochlorite anions. The resulting hypochlorous acid has strong antimicrobial properties, even in low concentrations. The antimicrobial mechanism of chlorine is unknown, but it most likely causes breakdown of enzymatic reactions within the cell and protein denaturation (Silim, 1998). The disinfecting efficiency of chlorine increases with decreasing pH. Water hardness seems to have no effect on the ability of chlorine to inactivate viruses, but chlorites show reduced efficacy in the presence of organic matter, because hypochlorous acid reacts with the organic matter leaving less available to react with microorganisms (Dychdala, 2001). In general, the use of hypochlorite solutions should be discouraged when the organic matter concentrations exceeds

10%. For influenza A, a minimum of 200 ppm concentration of sodium hypochlorite is required for inactivation within 10 min (Prince and Prince, 2001). Sodium hypochlorite, however, can be corrosive to some materials (AUSVETPLAN, 2000).

Peracetic acid or peroxyacetic acid (PAA) is the peroxide of acetic acid (AA). PAA is a strong oxidant and disinfectant. Its oxidation potential is larger than that of chlorine or chlorine dioxide. PAA is commercially available in the form of a mixture containing AA, hydrogen peroxide (HP), PAA, and water (Gehr et al., 2002).

Although HP is also a disinfectant contributing to the disinfection power of the PAA mixture, PAA is a more potent antimicrobial agent than HP, being rapidly active at low concentrations against a wide spectrum of microorganisms (Fraser et al., 1984). It was found that HP required much larger doses than PAA for the same level of disinfection (Wagner et al., 2002).

Although limited work has been done to explore the mode of action of PAA as an antimicrobial agent, it is speculated that it functions as other peroxides and oxidizing agents (Block, 1991). Its disinfectant activity is based on the release of active oxygen (Liberti and Notarnicola, 1999).

The aim of this work was to evaluate the common household disinfectant easily found in markets as sodium hypochlorite and PERACLEAN5%[®] as they commonly used in bleaching clothes and could be used for small number of rear poultry producers

2. MATERIALS AND METHODS

Virus: Attempts to isolate the virus were carried out specially to examine the effect of disinfectants against a field strain which achieves maximum simulation of Egyptian field reality. HPAI strain H5N1 (A/chicken/Egypt/13VIR3729-4/2013) was isolated from a commercial broiler flock in Beni-Suif during 2013. Molecular identification, sequencing of the HA gene and phylogenetic analysis were carried out in OIE/FAO Reference Laboratory in Italy (IZSVe, Legnaro-Padova). Phylogenetic analysis revealed that the virus follows clade 2.2.1/c, a classical virus.

Chicken embryos: Specific-pathogen-free (SPF) eggs were used for the titration of the viral stock and virus isolation attempts after testing the disinfectants.

Building materials: Cement coupons were manufactured in Arab Contractors Company in Egypt to resemble poultry house floor with dimensions 2 x 2x 1cm³ as shown in Fig (1).



Fig (1): Cement coupons Yeast extract:

3% yeast extract powder solution was prepared by adding 3g yeast extract to 100ml bi-distilled water. (Meron- India- patch number MYEP/03/KJ12)

Chemical disinfectants:

Two chemical disinfectants were tested individually for effectiveness against HPAI for a 5 and 30 minutes contact time. The used disinfectants were 250 ppm sodium hypochlorite 5% available chlorine and 1% PERACLEAN 5%[®].

The tested disinfectants were diluted using 300 ppm

ppm hard water solution on the day of use. The hard water solution was prepared according to (Bloder, 2009) as following:

1. 986 ml bi-distilled water
2. 6ml solution A (19.84 g anhydrous MgCl₂) + (46.24 anhydrous CaCl₂)/ L
3. 8ml solution B (35.02 g NaHCO₃/L)

Sodium hypochlorite solution was prepared by diluting 5 ml of sodium hypochlorite solution, with or equal to 4% available chlorine (chlorox[®]) into 100 ml of prepared hard water solution, forming 250 ppm final concentration.

PERACLEAN 5%[®] was prepared by diluting 1 ml of PERACLEAN 5%[®] into 100 ml of prepared hard water solution.

PBS was prepared by adding 8.5 g of sodium chloride, 1.18 g of dibasicsodium phosphate, and 0.22 g of monobasic sodium phosphate to 1liter of bi-distilled water. Cold, sterile PBS was used to dilute antibiotic solution (Penicillin G 2x10⁶IU, Streptomycin, 200 mg, Mycostatin 0.5x10⁶IU, Gentamycin250 mg) for 1 liter PBS. This PBS antibiotic mixture was used for all necessary dilutions.

Preparation of chemical neutralizers: was used to remove any residual disinfectants.

Efficacy of tested chemical compounds to inactivate Egyptian strain of HPAIV-H5N1:

According to the guidelines of the United States Environmental Protection Agency (EPA, 2005), disinfectant must be validated for each individual organism for which disinfection efficacy evaluation will be made, the evaluation test must contain cytotoxicity

group, control group, germicide activity or test group, method for increasing viral titer, method for removal residues of the used disinfectant, initial ID50 and reduction of ID50 after test expressed as log 10.

Under working laminar air flow, three cement coupons sterilized by autoclaving were placed in sterile Petri dish. Each coupon was coated with 0.2ml of infective amnio-allantoic fluid (AAF) and 0.2 ml of 3% yeast extract, lifted to dry about 1 hour at room temperature (20^oc).

Every coupon was covered with 2ml of the tested disinfectant prepared as previously described. The disinfectant was kept on coupons till 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

1 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 9-11day old SPF chicken eggs via allantoic route.

Eggs were candled daily for 3 days and the dead eggs were chilled for 24 h, then opened and the allantoic fluid was aspirated, examined for HA activity and EID50 was calculated via the method of Reed and Muench (1938).

The pooled fluid from the coupons of positive controls was diluted using six 10-fold serial dilutions, resulting in dilutions from 10⁻¹ to 10⁻⁶. The cytotoxic control was diluted once resulting in a 10⁻¹ dilution.

Table .1. List of Neutralizers:

Neutralizer	Disinfectant	
1 Sodium thiosulphate 1%	Sodium hypochlorite	Russell et al., (1979)
2 Sodium thiosulphate 1% - Sodium polysorbate (Tween 80) 1% – Sodium bisulphate 1%	PERACLEAN5% [®]	Espigares et al., (2003)

Calculation of neutralizing index (NI):

A numerical method was used to express the ability of a 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 serological virus neutralization test (Swayne and King 2003).

The NI of virus inactivation is calculated using the following equation

$$NI = P_c - T_a$$

Where T_a is titer of the recovered virus from the disinfectant-treated plates and P_c is the titer of the positive control plate.

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 more than or equals 2.8, the positive control titer was more than or equals 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 (Lombardi et al., 2008).

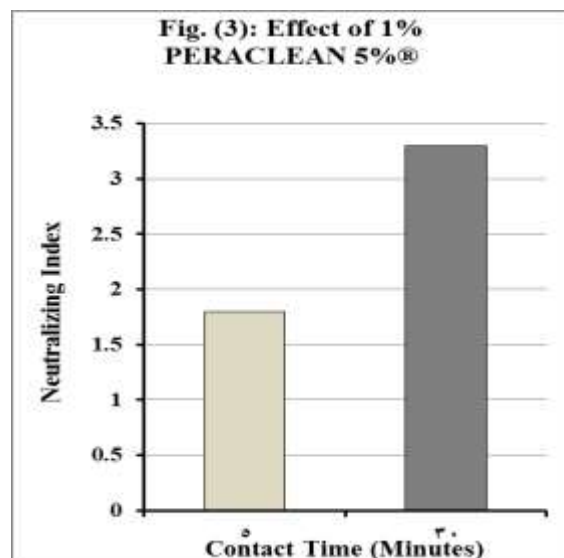
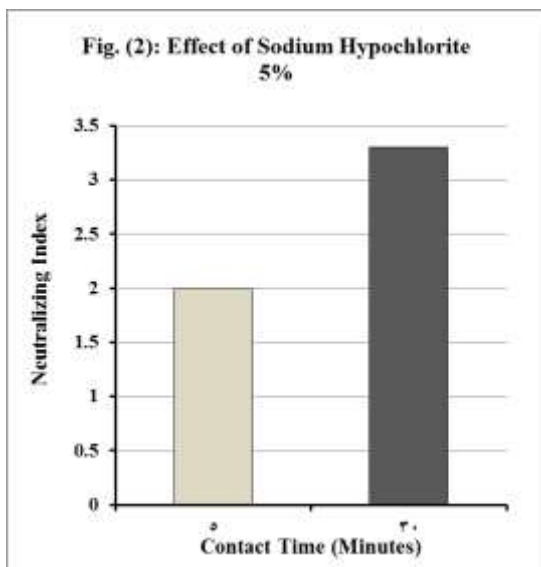
3. RESULTS AND DISCUSSION.

Table (2):250 ppm sodium hypochlorite (5% available chlorine)

Time (Minutes)	Positive Control (PC) /($\log 10$)	Average Titer (T_a) /($\log 10$)	Neutralizing index (NI)	Recoverable viruses
5	4.5	2.5	2	Yes
30	4.5	1.2	3.3	Nil
Time (Minutes)	Positive Control (PC) /($\log 10$)	Average Titer (T_a) /($\log 10$)	Neutralizing index (NI)	Recoverable viruses
5	4.5	2.5	2	Yes
30	4.5	1.2	3.3	Nil

Table (3): 1% Peraclean 5%:

Time (Minutes)	Positive Control (PC)/($\log 10$)	Average Titer (T_a)/($\log 10$)	Neutralizing index (NI)	Recoverable viruses
5	4.5	2.7	1.8	Yes
30	4.5	1.2	3.3	Nil



EPA guidelines considers a disinfectant agent to be effective if the product can achieve a complete inactivation of the virus at all dilutions, while at least 4 logs of virus particles per milliliter must be recovered from the non-veridical treated control carrier. When the EPA criteria for effectiveness is applied both 250 ppm sodium hypochlorite 5% available chlorine and 1% PERACLEAN 5%[®] were not able to inactivate the virus after 5min, while after 30 min inactivation level was achieved.

In the current study the used test for the evaluation of the disinfectants virucidal ability was a carrier test which was modified to mimic the field condition as much as possible, cement coupons which were designed similar to the Egyptian poultry houses floor were used, disinfectants were diluted using hard water and organic (protein) load was added to the coupons.

NI values were used to determine whether a disinfection agent was effective. The NI value, however, is dependent mainly on the positive control titer for a given test. A low positive control titer does not indicate that a disinfection agent was ineffective.

A summary of the NI indices for the two tested disinfectants is shown in Table (2) and (3), Fig (2), (3).

Our result had nearly matched with the results obtained from Lombardi et al., (2008) who used sodium hypochlorite to inactivate LPAI, the used concentration was 750 PPM (5% available chlorine) and inactivation was reached after 10 min at non porous surfaces, but the virus was not totally inactivated when he used wood surfaces (pours surface), while our results showed that after 30 min, HPAI was in activated with the use of 250 PPM concentration, and the used surface was a porous one.

Davison et al., (1999) inactivated LPAI H7N2 using sodium hypochlorite product at a final dilution 0.125% (w/v), using in use dilution test after 10 min, (Suarez et al., 2003) used sodium hypochlorite at a dilution 1: 10 in the inactivation of two different LPAI, found that it was also able to inactivate the virus. Bieker, 2006 inactivated two different LPAI AIV strains using 1% sodium hypochlorite after 1 min but without using organic load, on using organic load Following a 10 min treatment, 1% concentration failed to give 4 log₁₀ TCID₅₀/ml reductions in viral titer.

From our results we notice that, although the used strain was HPAI, the used concentration was lower than the concentrations commonly used,

the virus was effectively inactivated, which may be attributed probably because of the increase of contact time used in this study.

There are little researches available discussing the veridical effect of peracetic acid against AIV, however Songserm et al.,(2005) evaluated the veridical ability of Peracetic acid against the HPAI H5N1 Thai strain with a titer of 10^{6.3} ELD₅₀/ml, and found it completely inactivated after 10 min exposure time.

Our results indicated that the Egyptian HPAIV inactivated following the exposure to 1% PERACLEAN 5%[®] after 30 min, the difference in the inactivation results can be attributed to the difference in the type of the used HPAIV, and the used Peracetic acid.

The results of this work highlight the sensitivity of HPAIV H5N1 to the disinfectants, which may improve biosecurity measures on the farms and reduce the economic losses caused by HPAIV H5N1.

In order to achieve a successful biosecurity programs which mainly depend on disinfection processes, the used disinfectants should be tested first against the locally and concurrent AIV strains circulating in Egypt. The contact time should be regarded in order to achieve a successful inactivation of the virus and a successful biosecurity program even on small scale backyard systems.

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