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Inactivation of Avian Influenza Viruses by Chemical Disinfectants and the Influence of Faecal Matter

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ABSTRACT

Inactivation of avian influenza viruses (AIVs) using chemical disinfectants is an indispensable biosecurity measure during outbreaks. The efficiency of disinfection process depends on disinfectant type and presence or absence of organic matter. It is also not clear whether AIV pathotype could influence its tolerability to a given disinfectant. Here, we assessed the efficiency of formalin and caustic soda against high (H5N1) and low (H9N2) pathogenic AIVs in the presence and absence of wet faeces. Our results showed that the effectiveness of both disinfectants depends on the target virus with the low pathogenic H9N2 virus being generally more susceptible to disinfection than the high pathogenic H5N1 virus. Wet faeces slightly influenced disinfection action in a virus- and contact time- dependent manner. This study highlights the importance of eliminating faecal matter before initiating a disinfection process and indicates the influence of disinfectant type and AIVs pathotype when selecting the appropriate disinfectant.

1. INTRODUCTION

The circulation of avian influenza viruses (AIVs) in poultry has led to major economic losses in poultry industry and poses risk for humans worldwide, notably in Egypt. In Egypt, AIVs was firstly reported in poultry in 2006 (Saad, Ahmed et al. 2007). Since then and in spite of implementing various control measures, both high and low pathogenic avian influenza viruses (HPAI and LPAI) of many subtypes have become enzootic and continued their circulation in domestic poultry as reported in natural and experimental infections in chicken (Amen, Vemula et al. 2015, Wen, Yang et al. 2021), duck (Hassan, Jobre et al. 2013, Kaoud, Hussein et al. 2014, Campbell, Fleming-Canepa et al. 2021), turkey (Salaheldin, Veits et al. 2017), pigeon (Elgendy, Watanabe et al. 2016) and quail (El-Zoghby, Arafa et al. 2012).

Following AIV outbreaks, the virus can persist for long time in poultry faeces (Nazir, Haumacher et al. 2011), detached feathers (Yamamoto, Nakamura et al. 2010) as well as in dust, mud and soil (Horm, Gutierrez et al. 2012), raising possibilities of virus persistence and dissemination. Considering the lack of efficient vaccination against AIVs and the continuous occurrence of AIVs in poultry, there is an urgent need to put more emphasis on assessing virus inactivation in the environment, prior to host entrance, using chemical disinfectants (Marzouk, Abd El-Hamid et al. 2014). Using various disinfectants against AIVs have been studied (Shahid, Abubakar et al. 2009, Zou, Guo et al. 2013, Marzouk, Abd El-Hamid et al. 2014, Ruenphet, Punyadarsaniya et al. 2019), yet there is a lack in agreement among these studies, making it difficult to draw general conclusions as to the most efficient disinfectant (Weber and Stilianakis 2008). It is also notable that the efficiency of certain disinfectants (e.g. caustic soda (CS)) has not yet been evaluated in details, although being widely marketed.

The presence of organic matter (e.g. poultry faeces) in the disinfectant diluent, application area or on fomites might hamper the germicidal action of the disinfectant, thus interrupt the disinfection process (Quinn 1992). However, little is known about the influence of organic matter (e.g., faeces) on the effectiveness of chemical disinfectant such as formalin and CS.

In the current study, we directly compared the effectiveness of two commonly used chemical disinfectants, formalin and CS, against well-characterized AIVs of high (Ck/H5N1) and low (Ck/H9N2) pathogenicity. We also tested the influence of wet faeces on the virucidal activity of both agents. Our results point toward differences between the virucidal activity of the used disinfectant, and that faecal matter might interfere with their action.

2. MATERIAL AND METHODS

2.1 Source of viruses and their antigenic analyses

In this study, we used two AIVs: (1) HPAI H5N1 virus (A/Chicken/AM-14/2015), (KX230059.1) hereafter referred to as Ck/H5N1. This virus was isolated after a fulminate outbreak in broiler chicken farm in El-Sharkia province, Egypt (Ahmed 2016). (2) LPAI H9N2 virus (A/chicken/Saudi Arabia/CP7/1998) (CY081264.1) hereafter referred to as Ck/H9N2. This virus was firstly isolated from a meat-type chicken flock in Germany (Petersen, Matrosovich et al. 2012). Both viruses are deposited in the influenza research database (Zhang, Aevermann et al. 2017). To confirm the pathotype of both strains, we aligned a stretch of 53 amino acid (aa) residues in our viruses and other 23 HPAI H5N1 and LPAI H9N2 viruses. This aa sequence represents the portion of the virus HA1 subunit that surrounds the proteolytic cleavage site (PCS). This analysis was done using BioEdit sequence alignment software (v. 7.2.0).

2.2 Virus propagation and titration

Viruses were propagated and titrated using Madin Darby canine kidney (MDCK) cells (Veterinary Serum and Vaccine Research Institute (VSVRI), Cairo, Egypt). MDCK cells were grown in 75-cm^2 tissue culture flasks containing Eagle's minimum essential medium (EMEM) supplemented by 50 mM HEBES [*N*-2-hydroxyethylpiperazine-*N*_-2-ethanesulfonic acid] sodium salt (Serva, Heidelberg, Germany), 100 mM sodium bicarbonate, 5% fetal calf serum (Biochrom), 100,000 IU of penicillin G (Grünenthal, Aachen, Germany) and 100 mg of streptomycin sulphate (Sigma, Deisenhofen, Germany) per liter. The MDCK cells were cultivated at 37 ºC under 5% CO2 humified atmosphere. After 48-36 hrs. (80% confluence), the supernatant was poured off and the cells were rinsed twice by phosphate-buffered saline (PBS). For virus propagation, 1% trypsin-EDTA solution was added to trypsinize the confluent MDCK cells and the cells were further seeded into 96-well microtiter plates. Virus propagation was done by allowing virus growth on 150 µl medium (containing approx. 10^6 cell) for five successive passages. The virus stocks (concentration = 10^7 tissue culture infective dose 50, $TCID_{50}$) were harvested after 96 hrs. and stored at -80 until the treatments were performed. All experimental work involving Ck/H5N1 virus was performed at a biosecurity facility level 3 (BSL_3). To exclude the changes that might have occurred in the stock, we measured virus titers at time 0, and was found to be the same as that of stock virus. To compare replication efficiency between Ck/H5N1 and Ck/H9N2 viruses, titers ($TCID_{50}$/ml) of both viruses, that were kept for 5, 10, 15 and 30 min. at 28ºC, were measured after 96-hpi growth time on MDCK cells.

2.3 Faecal sample preparation and virus spiking into the faecal samples.

Wet fresh chicken faecal samples were collected from commercial chicken house and determined for their PH (8.23) and moisture (13.7%). To exclude the presence of toxic materials and pathogenic agents in the faecal sample, 100 gm faeces was diluted in distilled water and the mixture was inoculated into 10 embryonated chicken eggs (ECE) (10 gm/ duplicate egg). The eggs were incubated at 37 ºC and daily monitored for embryo mortalities for 5 days. In addition, the harvested allantoic fluid was assessed for the presence of hemagglutinating pathogen using hemagglutination test (HA) (Killian 2008). No embryo mortalities were observed and hemagglutination test revealed negative results.

2.4. Treatment of AIVs with disinfectants in the presence and absence of wet faeces. We used the suspension test method to assess the anti-viral activity of disinfectants and the influence of wet faeces as described previously (Jeong, Bae et al. 2010, Kurmi, Murugkar et al. 2013). For the effect of disinfectant, 16 set of reaction tube was prepared (eight tube for each of Ck/H5N1 and Ck/H9N2 viruses). In each tube, 100 µl virus stock (10^7 $TCID_{50}$/ml) was mixed thoroughly with 200 µl disinfectant at fixed temperature of 28 ºC and then the effect of disinfectant was stopped by adding 4.5 ml of PBS to the mix after 5, 10, 15 and 30

min. contact time. To test the influence of wet faeces on the disinfection efficiency, another 16 set of reaction tube was prepared (eight tube for each of Ck/H5N1 and Ck/H9N2 viruses). In each tube, 50 gm of wet faeces was added to the virus-disinfectant mix (100µl virus + 200µl disinfectant) at the same temperature and the effect of disinfectant was stooped after the same contact times as described previously. The disinfectants used were: (1) Formalin 10%, purchased from a commercial company as a stock solution (40%) and were further diluted (2) CS 10% (*sodium hydroxide*), prepared by adding 1 gm powder to 10 ml distilled water, purchased from a commercial company (El-gomhoria, Zagazig, Egypt). We used 10% concentration of both disinfectants, as it is commonly used in different poultry facilities in Egypt and following previous reports (Davison, Benson et al. 1999, Lu, Castro et al. 2003). To exclude any effect of the faeces on AIVs survivability, 8 reaction tubes (4 tube for Ck/H5N1 virus and 4 for the Ck/H9N2 virus) were prepared, each contains virus (100µl,10^7 $TCID_{50}$/ml) + 200µl PBS and then 50 gm wet faeces was added to it. The whole mix was kept for 5, 10, 15 and 30 min. contact times at the same temperature and virus titer was measured. Positive control samples include eight tubes (4 tubes for Ck/H5N1 virus and 4 tubes for the Ck/H9N2 virus), each tube contains 100 µl, 10^7 $TCID_{50}$/ml virus added to 200 µl phosphate buffer saline (PBS). The negative control samples include eight tubes (4 tubes for Ck/H5N1 virus and 4 tubes for the Ck/H9N2 virus); each contains 200 µl disinfectants added to 100 µl distilled water. All control tubes were held at the same temperature and times as the treatment materials.

2.5. Quantification of virus titer in MDCK cells using $TCID_{50}$/ml

The virus titers in treatment and control samples were measured using $TCID_{50}$ method. The 96-well microtiter plates containing confluent monolayers of MDCK cells were washed three times with 300 µl PBS solution and the supernatant was poured off after each wash. Each well was prepared to contain 10^6 MDCK cell in 150 µl of serum-free MEM medium supplemented with antibiotics, 25 µl. of MEM containing trypsin (final concentration per well = 1.0µg/ml). Untreated viruses (Virus + PBS) and viruses mixed with various disinfectants for the designated contact times (5, 10, 15 and 30 min.) in the presence or absence of wet faeces were suspended into at least 5-replicate well and was titrated immediately after being collected in 10-fold serial dilutions to the end point using $TCID_{50}$ method. The plates were covered and incubated at 37 ºC under 5% CO_2 for 96 hrs. Virus-induced cytopathic effect (CPE) was observed under light microscope and was confirmed by comparing treated and control MDCK cells (only cell culture medium). Replication of AIVs was validated by performing HA test on 100µl of cell supernatant (Killian 2008). For validation, cytotoxicity test was performed on samples generated for virus titration to control for possible CPE on the MDCK cells. To calculate virus titers, the plates were stained with 1% crystal violet in 10% neutral buffered formalin. Endpoints were recorded as 100% monolayer destruction and the $TCID_{50}$/ml was calculated as previously described (REED and MUENCH 1938). The minimum detectable limit of this assay is 10^2 $TCID_{50}$/ml, independent on the virus strain.

2.6. Virus log_{10} reduction and statistical analyses

Student *T test* (unpaired, two-tailed) was used to assess the difference between the output of the following comparison: 1) between the titer of Ck/H5N1 and Ck/H9N2 viruses ($TCID_{50}$/ml) at each contact time and 2) between the titer of both virus ($TCID_{50}$/ml) at each contact time and their titer when mixed with wet faeces. Two-way ANOVA followed by Bonferroni post-test was used to compare virus titers ($TCID_{50}$/ml) between untreated viruses and their titers when mixed with disinfectants in the presence and absence of wet faeces. For all analyses, the cutoff for significance was 0.05 (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$). The analyses were done using Graph Pad Prism version 5 software (Inc., San Diego, California, USA). We used log_{10} reduction method to provide a numerical comparison between Ck/H5N1 and Ck/H9N2 viruses in their resistance to various disinfectants in the presence or absence of wet feces. Log_{10} reduction was calculated using the equation: log_{10} reduction = log_{10} (A) - log_{10} (B), where A is the mean virus $TCID_{50}$/ml titer before applying the respective treatment and B is the mean virus $TCID_{50}$/ml titer after applying the treatment. Standard error of the mean (SEM) was calculated for a minimum of 5 - replicate value. This analysis was done using *Excel* 2013 function "Log_{10}".

3 RESULTS

3.1 Proteolytic cleavage site as a marker for high pathogenicity of Ck/H5N1, but not Ck/H9N2 virus

As shown in Figure S1, Ck/H5N1 virus along with other H5N1 viruses have multi-basic aa at the PCA site characteristic for HPAI viruses (e.g. *K*, lysine at aa positions 357 and 358 and *R*, Arginine at aa positions 356 and 359). These aa signatures were absent in LPAI viruses including Ck/H9N2 virus (shown as "~" sign in Figure S1). In addition, HPAI H5N1 and LPAI H9N2 viruses differed in certain aa (e.g. Glutamine *Q* at aa position 374 and, Methionine *M* at aa position 376 and Aspartic Acid *D* at aa position 378).

3.2 Replication of Ck/H5N1 and Ck/H9N2 viruses on MDCK cells

After 96-hpi growth time on MDCK cells, Ck/H5N1 virus replicate more efficiently than Ck/H9N2 virus, especially when both viruses were kept at 28 ºC for 10 and 15 min (P value ≤ 0.05) **(Figure 1)**.

3.3 Efficiency of formalin and caustic soda on avian influenza viruses

The effect of formalin and CS on Ck/H5N1 virus is shown in **Figure 2 A** and **Table 1.** After 5 min. contact time, formalin, and CS reduced Ck/H5N1 virus titer non-significantly at similar degree. However, prolonging contact time to 10, 15 and 30 min. resulted in significant reduction in Ck/H5N1 virus titer with formalin exerting more virucidal effect than CS, particularly after 10 and 15 min. On the other hand, both disinfectant reduced Ck/H9N2 virus titer significantly and to a similar degree after all contact times. The virucidal activity of both agents becames more evident especially after 30 min. contact time (Figure 3 A and Table 1).

3.4 Influence of wet feces on the virucidal activity of formalin and CS

In the absence of disinfectants, the presence of wet faeces did not affect Ck/H5N1 and Ck/H9N2 titers (Figure S2). As shown in Figures 2 B and **C**, addition of wet faeces significantly reduced the efficacy of formalin against Ck/H5N1 virus after 5 min. and slightly in a non-significant manner after 10 min and 15 min. contact times, whereas this effect was not observed after 30 min. contact times. On the other side, wet faeces significantly reduced the activity of CS against Ck/H5N1 virus only after 30 min. contact time, while its effect was non-significant after the remaining contact times. As for Ck/H9N2 virus, addition of wet faeces did not alter the anti-viral activity of formalin and significantly reduced the activity of CS only after 10 min. contact time **(Figures 3 B and 3).**

3.5 Potential differences between Ck/H5N1 and Ck/H9N2 viruses in their tolerability to disinfectants.

Table 1 summarizes the differences between titers of Ck/H5N1 and Ck/H9N2 viruses when exposed to formalin and CS in the presence and absence of wet faeces. The results showed that Ck/H5N1 virus was more tolerable to formalin than Ck/H9N2 virus after 5 (average difference = 2.67 virus $\log_{10}$ reduction) and 10 min. (average difference = 1.27 virus $\log_{10}$ reduction) contact times, whereas both viruses tolerated formalin to comparable levels after 15 min. contact times. After 30 min. contact time, Ck/H5N1 was slightly more sensitive to formalin than Ck/H9N2 virus. Regarding CS, Ck/H5N1 virus was more tolerable than Ck/H9N2 virus after 5, 10 and 15 min. contact times with the average differences being 2, 2.94 and 1.32 virus $\log_{10}$ reduction, respectively. The difference between both viruses followed similar pattern when wet feces were added to both disinfectants.

Table 1. Comparison between $\log_{10}$ reduction values in titers of Ck/H5N1 and Ck/H9N2 after applying various disinfectants for the respective contact time in the presence and absence of wet faeces. Standard error of the mean (SEM) was calculated for a minimum of 5-replicate value. This analysis was done using *Excel* 2013 function "Log_{10}".

Disinfection	Formalin		Formalin + Faeces		Caustic Soda (CS)		CS + Faeces	
Viruses	**Ck/H5N1**	**Ck/H9N2**	**Ck/H5N1**	**Ck/H9N2**	**Ck/H5N1**	**Ck/H9N2**	**Ck/H5N1**	**Ck/H9N2**
5 min.	0.33 ± 0.6*	3.00 ± 1.7	0.33 ± 2.5	3.67 ± 1.5	0.33 ± 0.6	2.33 ± 0.6	1.00 ± 1	2.33 ± 0.6
10 min.	3.43 ± 1.4	4.70 ± 0.6	3.43 ± 1.4	4.70 ± 1.2	1.43 ± 1.4	4.37 ± 0.6	2.10 ± 1.6	3.70 ± 1.6
15 min.	5.00 ± 0.0	5.00 ± 1	5.00 ± 1	5.00 ± 0.0	3.68 ± 1.2	5.00 ± 0.0	3.00 ± 0.0	4.67 ± 0.6
30 min.	5.67 ± 0.6	5.23 ± 0.6	5.67 ± 0.6	5.23 ± 0.7	ND†	5.23 ± 0.7	4.13 ± 0.5	4.90 ± 1

* Values in the table represent virus log reduction (TCID_{50}/ml) ± SEM for 5-replicate. † ND : non determined

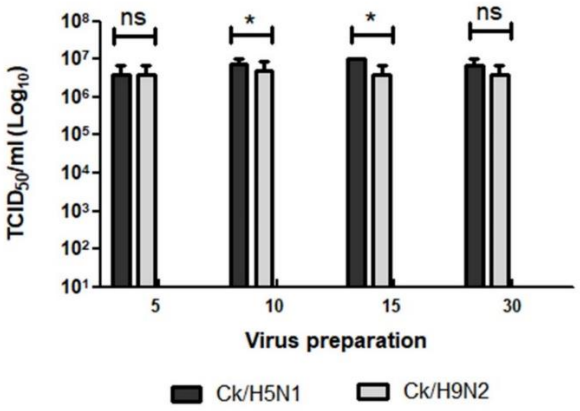

Figure 1. Replication of Ck/H5N1 and Ck/H9N2 viruses in MDCK cells after 96 hrs. growth time. Both virus were kept for 5, 10, 15 and 30 min. X- axis refers to the time where both virus were kept after their growth on MDCK cells for 96 hrs. Virus titer ($TCID_{50}$) are shown on the Y-axis. Data are shown as mean ± SEM of virus titers ($TCID_{50}$) of a minimum of 5-replicate/wells. Asterisks indicate significant difference between mean $TCID_{50}$ of both viruses in each preparation.

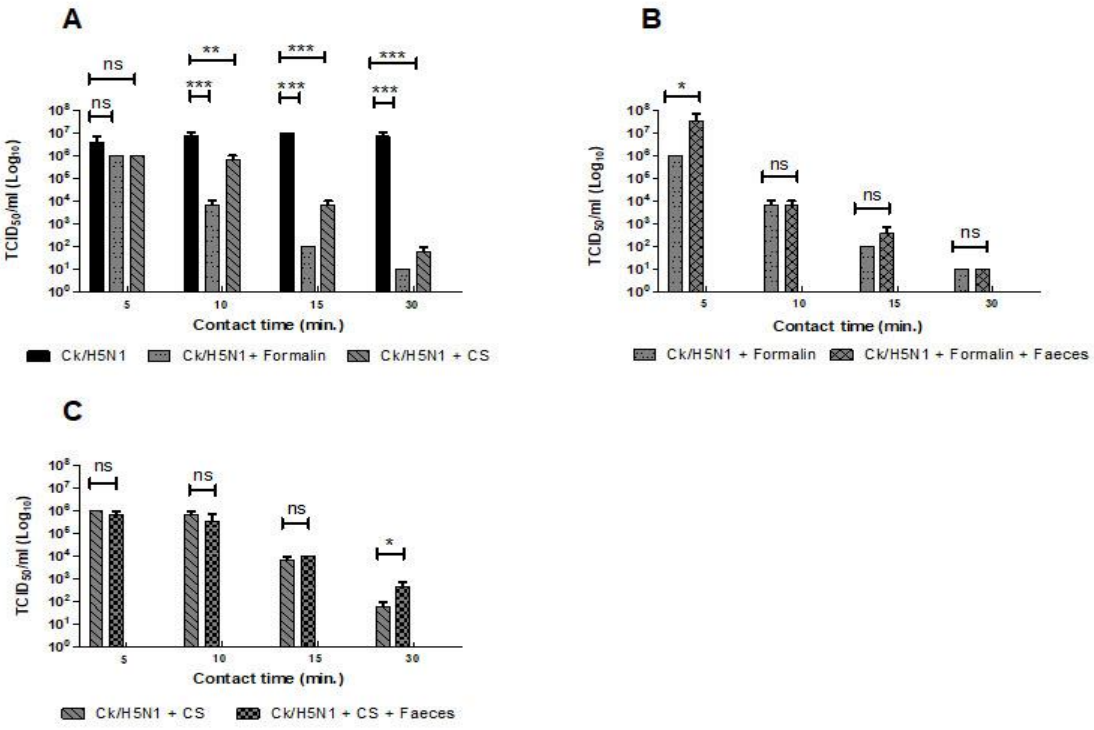

Figure 2. Effect of disinfectants on Ck/H5N1 virus in the absence (A) and presence (B and C) of wet faeces. X-axis refers to the time where both viruses were kept with the disinfectant and faeces. Virus titer ($TCID_{50}$) are shown on the Y-axis. The data are shown as mean ± SEM of $TCID_{50}$ of minimum of 5-replicate wells. To compare replicate means in all conditions, two-way ANOVA followed by Bonferroni post-test was used with the parameters: *, $P \leq 0.05$; **, $P \leq 0.01$;***, $P \leq 0.001$. In **A,** the statistical significance was calculated between Ck/H5N1-disinfectants mix and that of the initial virus titer (black bars).

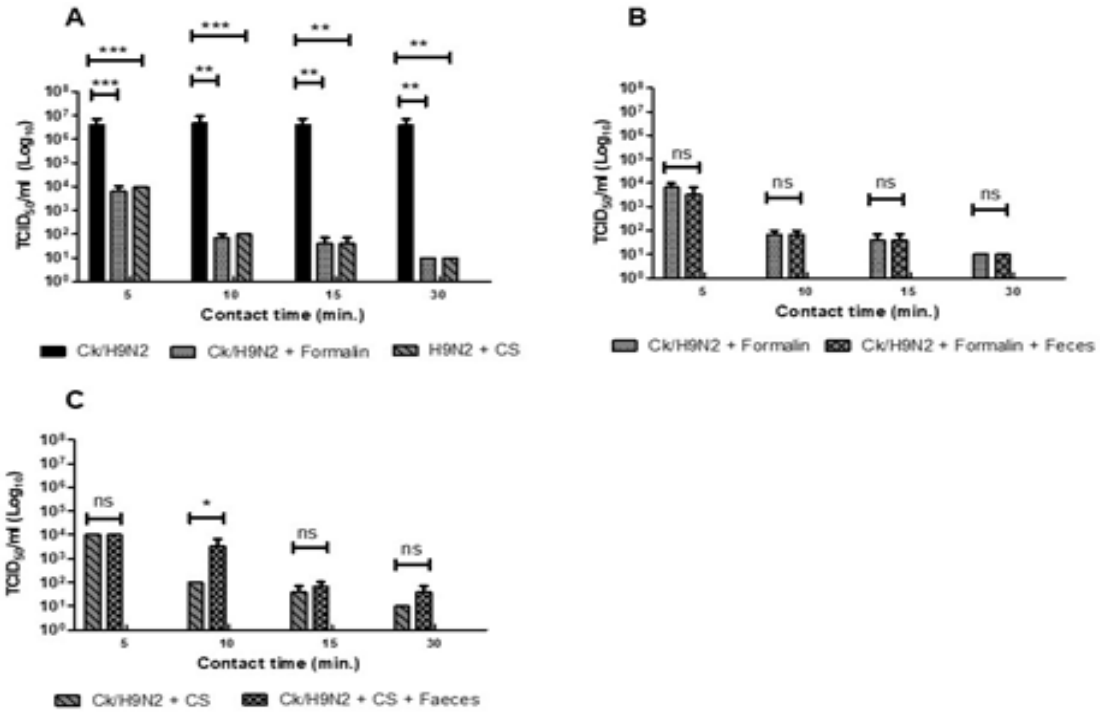

Figure 3. Effect of disinfectants on Ck/H9N2 virus in the absence (A) and presence (B and C) of wet faeces. X-axis refers to the time where both virus were kept with the disinfectant and faeces. Virus titer ($TCID_{50}$) are shown on the Y-axis. The data are shown as mean ± SEM of $TCID_{50}$ of minimum of 5-replicate wells. To compare replicate means in all conditions, two-way ANOVA followed by Bonferroni post-test was used with the parameters: *, $P \leq 0.05$; **, $P \leq 0.01$;***, $P \leq 0.001$. In **A,** the statistical significance was calculated between Ck/H9N2-disinfectants mix and that of the initial virus titer (black bars).

4 DISCUSSION

This study was carried out to assess the virucidal activity of formalin and CS against AIVs in the presence and absence of wet faces, and to determine if there is any differences between HPAI and LPAI viruses in their tolerability to certain disinfectant. The results suggest differences between formalin and CS in their anti-AIV activity and that influence of wet faces on their activity was disinfectant-, virus- and contact time -dependent. These data are highly relevant to the control and prevention of studied AIVs and possibly other related strains.

Ck/H5N1 and Ck/H9N2 viruses differ in their replication competence

The MDCK cells are ideal and permissive for isolation, propagation and titration of human and AIVs (Romanova, Katinger et al. 2003) because they possess both human and avian-type receptors (Tobita, Sugiura et al. 1975, Lugovtsev, Melnyk et al. 2013) that support virus replication (Zhou, Zhu et al. 2010, Ilyushina, Ikizler et al. 2012). As evidenced by its titer on MDCK cells, Ck/H5N1 virus possess higher and faster replication capacity than Ck/H9N2 virus. This agree well with the known increased pathogenicity of HPAI of the H5N1 viruses in inoculated eggs, chickens (Suzuki, Okada et al. 2009) and ducks (Samir, Hamed et al. 2018). The low replication efficiency of the LPAI Ck/H9N2 virus is not attributed to low sensitivity of the used MDCK cells as we added trypsin before virus spiking. In line with the increased replication of Ck/H5N1 virus was the presence of the characteristic polybasic aa (*K* and *R*) signatures surrounding its PCS, which was already absent in Ck/H9N2 virus. This information lay a robust ground for the subsequent analyses.

Anti-AIV activity of formalin and CS

In the current study, we performed the experiments at 28 ºC to provide reliable comparison and to simulate the field situation. Such temperature is not expected to affect virus survivability. Indeed, AIVs loss infectivity

at high (> 56ºC) (Brown, Goekjian et al. 2009, Shahid, Abubakar et al. 2009, Wanaratana, Tantilertcharoen et al. 2010) and low temperatures (< 4 ºC) (Wood, Choi et al. 2010). Formalin and CS are commonly used disinfectants in poultry houses, incubators, transport vehicles and other equipment (Linton 1987). Previous reports have described the efficiency of formalin in inactivating AIVs (Shahid, Abubakar et al. 2009, Wanaratana, Tantilertcharoen et al. 2010), yet these studies used low formalin concentrations and after long contact time, which might be inappropriate intervention during or after epizootics or outbreaks. Thus, formalin at a concentration of 10% was used to allow assessing its activity after short contact time. Addition of formalin (10%) reduced the titers of Ck/H5N1 and Ck/H9N2 viruses after all contact times (Figures 2 A and 3 A) with the highest virucidal activity being evident in long contact time (i.e. 30 min.). This agrees with previous study (Wanaratana, Tantilertcharoen et al. 2010), wherein HPAI H5N1 viruses lost 50% of its infectivity after treatment with formalin irrespective of temperatures or contact times. Independent study showed that formalin eliminated HPAI H5N1 and LPAI H9N2 viruses when used at concentrations of 0.1, 0.04 for contact times of 16, 48 hrs. and one week (Pawar, Murtadak et al. 2015). Similar results have been obtained using low formalin concentration against H7N3 viruses (Muhmmad 2001). Studies investigating the efficacy of CS against AIVs are scarce and to the best of our knowledge, this is the first report deciphering the efficacy of CS (10%) on Egyptian HPAI and LPAI viruses after short contact times. Like formalin, CS (10%) inactivated both viruses at all studied contact times (Figures 2 A and 3 A), indicating the usefulness of CS as a disinfectant against AIVs. Previous reports showed that CS is effective against H5N1 and H1N1 viruses in low concentration (0.1%) after 5 min. contact time (Shahid, Abubakar et al. 2009, Jeong, Bae et al. 2010). Obviously comparing results among studies should be considered with caution as many factors (e.g. temperature, virus strain origin, detection assay and virus pathotype) might influence the results. In our study, the difference between formalin and CS in their anti-AIV activity was only evident in Ck/H5N1 virus, where formalin was more powerful than CS in 3 out of 4 studied contact times. While this indicates difference between the tolerability of both viruses, it suggests that formalin might be appropriate disinfectant when co-infection with both viruses occurs. Whether the difference in the efficacy of formalin and CS against HPAI viruses is due to the chemical nature of disinfectant or its mode of action is not clear and warrants further research.

The influence of "wet faeces" on the anti-AIV activity of formalin and CS

The importance of the influence of faecal matter contamination on disinfection process prompted us to investigate whether wet faeces might reduce the activity of formalin and CS against AIVs. Our data indicated that wet faeces alone did not reduce the studied AIV titers after the indicated contact time (Figure S2), suggesting that the observed reduction in virus titer upon addition of wet faeces to disinfectants is solely due to the inhibitory action of the wet faeces on the disinfectant. Our results showed that the influence of wet faeces on the disinfectant depended on the disinfectant, the virus under study and the contact time. While wet faeces reduced the activity of formalin against Ck/H5N1 virus only after 5 min. contact time, it had no effect on the anti-viral activity of formalin against Ck/H9N2 virus at all studied contact times. This particular result disagrees with previous report (Jang, Lee et al. 2014), wherein 0.1% glutaraldehyde (an aldehyde similar to formalin) was the only one, among other 5-disinfectants, that lost its activity against LPAI H9N2 in the presence of organic matter (fetal bovine serum). In contrast to formalin, wet faeces reduced the activity of CS against Ck/H5N1 only after 30 min. contact time, and reduced CS activity against Ck/H9N2 virus after 10 min. The scarcity of data about CS precluded us to compare our results with others. Since our data indicated that presence of wet faeces partially influenced the disinfectant activity at certain time points, it highlight the importance of the efficient pre-cleaning before using formalin and CS as disinfectants. Further studies are recommended to investigate the link between virus pathotype and efficiency of disinfectant and whether our observation remains true for other concentration of these disinfectants or in longer time.

Tolerability of HPAI Ck/H5N1 and LPAI Ck/H9N2 to disinfectants

Whether AIV pathotype influence their sensitivity to certain disinfectant still unclear. If a farm experienced a mixed HPAI H5N1 and LPAI H9N2 infection, it would be economically beneficial if only the strongest disinfectant that is efficient against both viruses is used. The studies done so far have tested the efficacy

of disinfectants against single virus pathotype (Shahid, Abubakar et al. 2009, Wanaratana, Tantilertcharoen et al. 2010, Wood, Choi et al. 2010) or against two strains belonging to the same virus (e.g. H7N9) (Zou, Guo et al. 2013). The current study added another dimension by directly comparing the antiviral activity of formalin and CS against two well-characterized AIV of high and low pathogenic nature. The data demonstrated that Ck/H5N1 was more tolerable than Ck/H9N2 virus, particularly to CS (Table 1), suggesting that the anti-viral action of disinfectants might be pathotype-dependent. With the observation that formalin is more effective than CS against Ck/H5N1 virus (Figure 3 A), using formalin is sought to be preferable than CS when co-infection with both viruses occurs. Our results agree with Pawar et al (Pawar, Murtadak et al. 2015), but only for some of the studied strains using different formalin concentrations. Previous studies showed that HPAI of H5N1 and H7N1 viruses were more tolerable to ether (Fenters, Yamashiroya et al. 1970) and ammonia (NH_3) (Emmoth, Ottoson et al. 2011) than LPAI AIV strains. There is also evidence that the HPAI H5N8 virus persisted longer than the LPAI H6N2 virus in layer faeces and bedding material obtained from commercial broilers and turkeys when no disinfectants were used (Hauck, Crossley et al. 2017). These data together suggest that the high resistance of HPAI compared to LPAI viruses seems to be a general phenomenon. The question of why HPAI Ck/H5N1 and LPAI Ck/H9N2 viruses responded differently to formalin and CS remains puzzling and this is possibly related to their mode of action, particularly knowing the difference between both viruses in certain aa, and thus protein (Figure S1).

The current study has some shortcomings: due to limited resources at the time of experiments, we investigated only two AIVs strains of differential pathogenicity, which limits some of conclusions. Conduction experiments with additional strains belonging to both pathotype is then warranted. While we used a single concentration of formalin and CS (i.e. 10%), as commonly used, the results might differ if other concentrations were used. Another point is that at the farm or backyard levels, factors such as sun light (UV), desiccation and PH should be taken into consideration as these might accelerate or decelerate AIVs inactivation process (Wanaratana, Tantilertcharoen et al. 2010, Zou, Guo et al. 2013). These should be included in further studies. Noteworthy, including other virus quantification method (e.g. ECE) would also add value to the output data.

This study describes the differential inactivation ability of formalin and CS against AIVs and showed that wet faeces might have some influence on the disinfectant anti-viral effectiveness. It also, for the first time, provides evidence that the two studied viruses, and possibly other viruses, responded differently to these disinfectants. The obtained data should enable formulating recommendation regarding the best disinfectant to be used and highlight the importance of eliminating faecal matter before initiating a disinfection process against AIVs.

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6 Conflict of interest

The authors declare that there was no conflict of interest regarding the publication of this manuscript.

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Figure S1

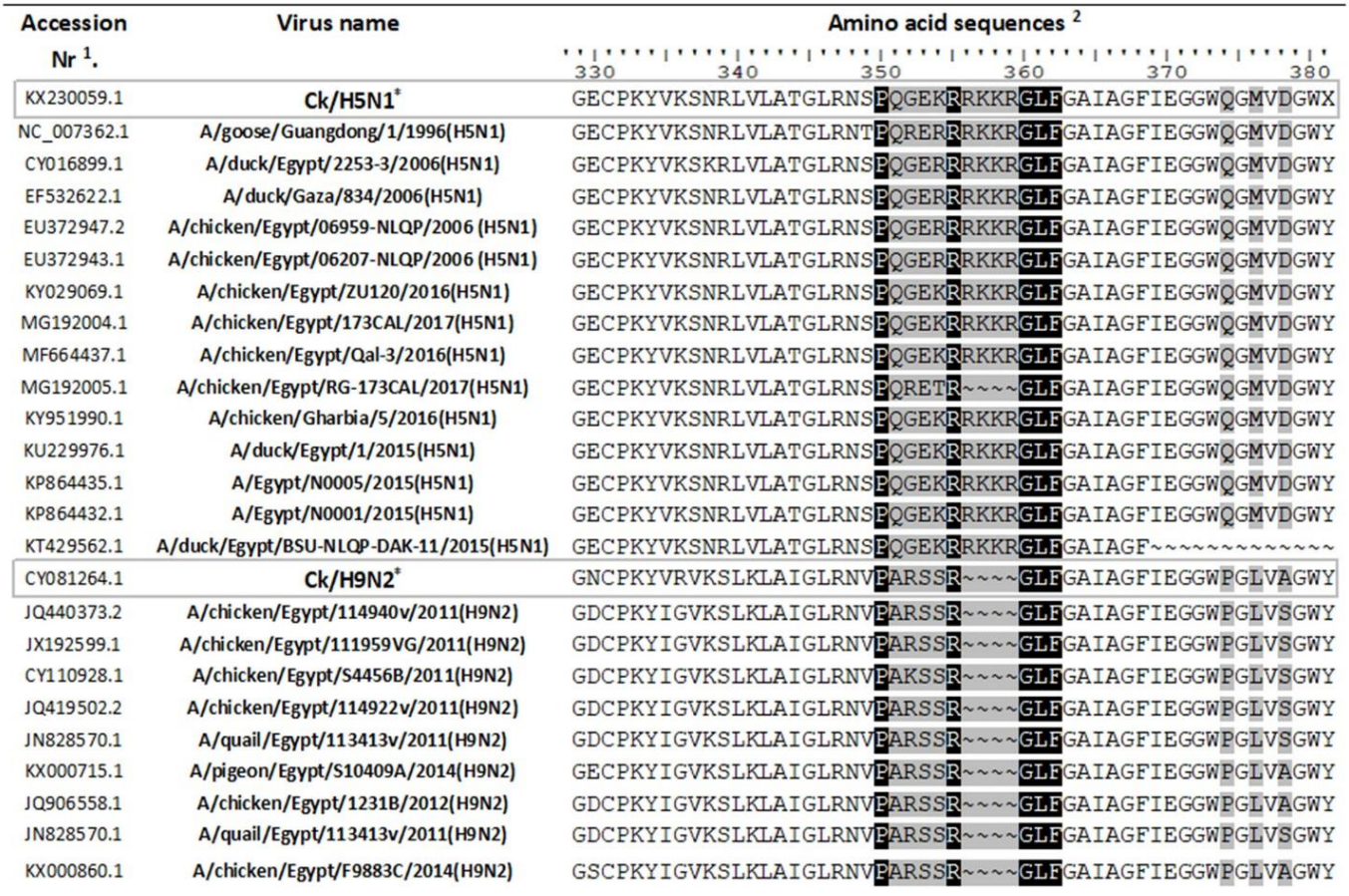

Figure S1. Amino acid alignment between the study viruses (Ck/H5N1 and Ck/H9N2) and other avian influenza viruses prevalent in Egypt. The amino acid (aa) residues that vary between H5N1 and H9N2 viruses are shaded in Grey and the conserved aa are shaded in black. Ck/H5N1 as well as other HPAI H5N1 viruses showed conserved multi-basic amino acids (*K*, lysine at positions 357 and 358 and *R*, Arginine at positions 356 and 359) upstream of the proteolytic cleavage site (between the aa "arginine, *R*" at position 359 and "Glycine, *G*" at position 360). On the other side, these aa residues are absent in Ck/H9N2 and other LPAI H9N2 viruses. [1] the accession numbers are according to the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore). [2] the amino acids presented in the figures represent a part of the translated mRNA nucleotides of the virus HA1 gene. ~: deleted amino acids.* study viruses. *P:* proline, *Q*: Glutamine, *R:* Arginine, *G*: Glycine, *K:* lysine, *L:* Leucine, *F:* Phenylalanine, *E*: Glutamic acid, *T*: Threonine, *S*: Serine, *L*: Leucine, *A*: Alanine, *S*: Serine, *D*: Aspartic Acid, *M*: Methionine.

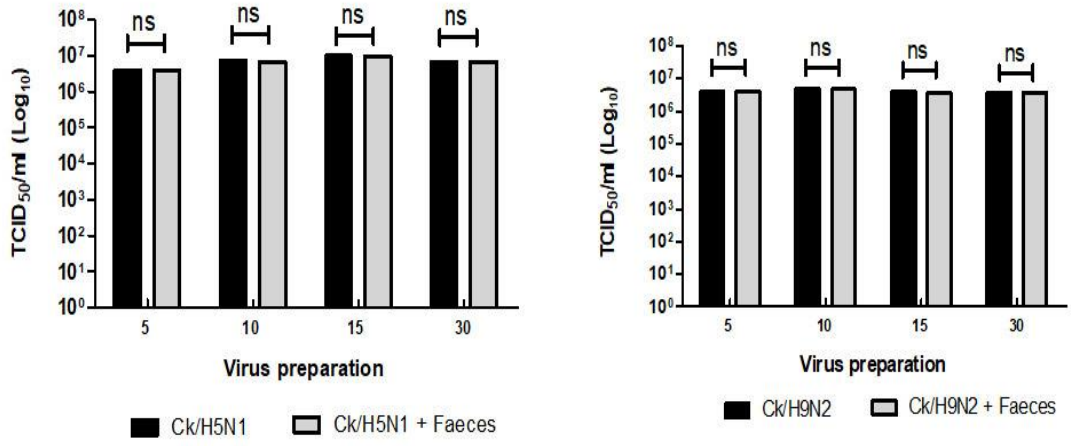

Figure S2. Replication efficiency of Ck/H5N1 and Ck/H9N2 viruses in the presence of wet faeces. X-axis refers to the time where both virus were kept with the disinfectant and faeces. Virus titer ($TCID_{50}$) are shown on the Y-axis. The data are shown as mean ± SEM of $TCID_{50}$ of minimum of 5-replicate wells. Asterisks indicate significant (measured by *T*- test) difference between mean $TCID_{50}$ of viruses alone and when mixed with wet faeces.