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Efficacy of disinfectants to inactivate H1N1 influenza A virus isolated from pigs[1]

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ABSTRACT.- Lara A.C., Fernando F.S., Takeuti K.L., Bortolozzo F.P. & de Barcellos D.E.S.N. 2022. **Efficacy of disinfectants to inactivate H1N1 influenza A virus isolated from pigs**. *Pesquisa Veterinária Brasileira 42:e06987, 2022*. Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Bairro Agronomia, Porto Alegre, RS 91540-000, Brazil. E-mail: davidbarcellos@terra.com.br

The aim of this study was to access the efficacy of four disinfectants to inactivate influenza A [H1N1] 0 hour and 72 hours after disinfectant dilution. A pandemic H1N1 influenza virus isolated from a pig with respiratory disease was used to obtain inoculums containing 6.4log$_{10}$ EID$_{50}$/mL; 5.4log$_{10}$ EID$_{50}$/mL; 4.4log$_{10}$ EID$_{50}$/mL and 3.4log$_{10}$ EID$_{50}$/mL. Suspension test was composed of 400µL of viral inoculum, 100µL of organic load and 500µL of each individually diluted disinfectant and incubated for ten minutes of contact time. After a neutralizing step, each mixture was filtered on a 0.22µm membrane and 0.2mL was inoculated in six 9-day-old embryo chicken egg through allantoic route. The allantoic fluid from eggs was harvest for RT-PCR and hemagglutination test. The experiment was repeated 72 hours after disinfectant dilution. On the first assessment with fresh disinfectant, influenza virus was inactivated by oxidizing compost disinfectant and phenolic disinfectant in all virus concentrations, the quaternary ammonium compound (QAC) and glutaraldehyde association inactivated the virus up to a concentration of 5.4log$_{10}$ EID$_{50}$/mL. QAC disinfectant did not eliminate virus viability. Seventy-two hours after disinfectants were diluted, oxidizing compost disinfectant and QAC and glutaraldehyde association disinfectant demonstrated the same result as the evaluation with fresh disinfectant solution. Phenolic disinfectant inactivated viral inoculum up to a concentration of 5.4log$_{10}$ EID$_{50}$/mL. QAC had no effect on inactivating 3.4log$_{10}$ EID$_{50}$/mL of influenza virus. In conclusion, three of the four disinfectants tested were effective to inactivate pandemic H1N1 influenza virus in the presence of organic load. Test result performed 72hours after disinfectant dilution suggest a decrease in the effectiveness of one disinfectant.

INDEX TERMS: Disinfection, disinfectants, influenza A virus, organic load, pigs, H1N1.

RESUMO.- [**Eficácia de desinfetantes para inativar o vírus da influenza A H1N1 isolado de suínos.**] O objetivo deste trabalho foi avaliar a eficácia de quatro desinfetantes em inativar o vírus da influenza A [H1N1] 0-hora e 72-horas após a diluição dos produtos. Um vírus H1N1 pandêmico isolado previamente de um suíno com doença respiratória foi utilizado e foram obtidas quatro concentrações de inóculo contendo 6,4log$_{10}$ EID$_{50}$/mL; 5,4log$_{10}$ EID$_{50}$/mL; 4,4log$_{10}$ EID$_{50}$/mL and 3,4log$_{10}$ EID$_{50}$/mL. Para compor o teste em suspensão foram adicionados 400µL de inóculo viral, 100µL de matéria orgânica e 500µL de cada desinfetante diluído individualmente e a mesma foi incubada por 10 minutos. Após a etapa neutralizante, a suspensão foi filtrada em membrana 0,22µm e 0,2mL foi inoculado em seis ovos de galinha embrionados de nove dias de incubação, via rota alantóide. O fluido alantóide foi colhido após 72 horas para testes de hemaglutinação e RT-PCR. O mesmo protocolo experimental foi repetido usando as soluções desinfetantes 72 horas após a diluição. O vírus da influenza foi inativado pelo composto oxidante e também pelo desinfetante fenólico em todas as concentrações virais

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testadas 0-hora após diluição. O desinfetante com associação de amônia quaternária e glutaraldeído inativou o vírus na concentração de até 5,4log$_{10}$ EID$_{50}$/mL. O desinfetante à base de amônia quaternária não inativou o vírus. Os resultados 72-horas após a diluição não diferiram quando comparado com 0-hora, exceto o desinfetante fenólico, o qual inativou o vírus da influenza somente até a concentração 5,4log$_{10}$ EID$_{50}$/mL. Concluindo, três dos quatro desinfetantes testados foram efetivos ao inativar o vírus da influenza [H1N1] pandêmico na presença de matéria orgânica. Os resultados do teste com produtos diluídos após 72 horas sugerem redução da efetividade em, pelo menos, um desinfetante.

TERMOS DE INDEXAÇÃO: Desinfecção, desinfetantes, matéria orgânica, suínos, virus influenza A, H1N1.

INTRODUCTION

Influenza A virus (IAV) is endemic in swine herds (Torremorell et al. 2012), causing health and economic losses and is an important pathogen involved in porcine respiratory disease complex (Rech et al. 2018). IAV is a zoonotic pathogen and its transmission among species is relevant to public health (Nelson & Vincent 2015, Anderson et al. 2021). Some measures must be considered to control IAV in pigs, as increasing biosecurity, adopting practices to prevent the introduction of new strains in the herd by pigs or workers, implementing all-in all-out system and following disinfection protocols. Influenza virus can persist in some surfaces as wipes, plastic and stainless steel (Bean et al. 1982, Perry et al. 2016), and in the presence of organic load and mucus, IAV can increase the viability period (Hauck et al. 2017, Hirose et al. 2017). Moreover, Neira et al. (2016) detected significant levels of IAV on barn surfaces and air during an outbreak of influenza in pigs. Even though the most common route of influenza transmission among pigs is direct contact with shedding individuals, indirect transmission by fomites has also been demonstrated (Allerson et al. 2013). Thus, disinfection procedures can reduce the contamination of surface facilities, equipment and fomites, reducing the risk of the pathogen spread.

Chemical disinfection is widely used in pig farms and there are several classes of disinfectants available including aldehydes, oxidizing agents, phenols and ammonia compounds (Dvorak 2008). The action mechanism varies regarding each class of disinfectant, but the main mechanisms consist on viral protein or lipids denaturation, nucleic acid disruption and/or damage and changes on membranes (Prince & Prince 2001, Dvorak 2008). However, some other factors can influence the effectiveness of the disinfectant such as dose, time of contact, surface composition and characteristics, organic load, temperature and pH (De Benedictis et al. 2007).

In vitro tests can provide information regarding effectiveness of chemical disinfectants against specific pathogens and guide the appropriate choice for a disinfection program. Some studies have reported the susceptibility of H1N1 influenza virus isolated from humans after the pandemic event in 2009 (Jeong et al. 2010, Subhash et al. 2014, Perry et al. 2016), but usually the disinfectants tested are not used in livestock due to the cost and applicability. In addition, cleaning conditions, levels of organic load and type of surface can be a challenge to disinfection procedures in livestock. The aim of this study was to evaluate the efficacy of four disinfectants, commonly used on swine farms, to inactivate a pandemic H1N1 influenza virus using fresh disinfectant solution and 72 hours after dilution.

MATERIALS AND METHODS

Influenza virus, isolation and titration. A brazilian field pandemic H1N1 influenza virus[4] was previously isolated from a lung sample collected from a pig with clinical respiratory signs. The inoculum was diluted in PBS, 0.01M, pH 7.4, clarified by centrifugation at 12.000rpm for 20 min at 4°C, filtered through a 0.22µm membrane (Milliplex™, Millipore corp., Bedford, USA) and inoculated in specific pathogen-free embryonated chicken eggs (ECE). Inoculated eggs were candled daily for seven consecutive days and any deaths during the first day were discarded as non-specific deaths. The isolated virus was titrated in ECE, and the 50% embryo-infective doses (EID$_{50}$) were determined (Reed & Muench 1938). Three ten-fold dilution were performed in order to obtain four concentrations of inoculums containing 6.4log$_{10}$ EID$_{50}$/mL; 5.4log$_{10}$ EID$_{50}$/mL; 4.4log$_{10}$ EID$_{50}$/mL and 3.4log$_{10}$ EID$_{50}$/mL. All inoculums were tested by RT-PCR to assess the initial cycle threshold (Ct) and stored at -70°C until use.

***In vitro* evaluation of disinfectants.** Disinfectant concentration (%) followed the manufacturer's guidelines (Table 1), using a standard hard water prepared according to AOAC 960.09 E and F (AOAC 1990). In order to simulate a surface cleaning failure, organic load was included in the test. Pig feces were previously autoclaved at 121°C for 20 minutes and the sterility test was performed on blood agar, Sabouraud (Thermo Scientific, Waltham, US) and brain heart infusion (BHI; Merck KGaA, Darmstadt, Germany). BHI and blood agar were incubated for 72 hours at 37°C and Sabouraud agar at 25°C for five days. After approval of sterility, feces were diluted at 1/3 (v/v) in PBS pH 7.4 and homogenized. An aliquot of 0.2mL

Table 1. Disinfectants and concentrations tested against four concentrations of influenza A virus (IAV)

Study group	Disinfectant concentration	IAV inoculum (log$_{10}$EID$_{50}$/mL)	Organic load	Contact time
Oxidizing agent[a]	0.5%	6.4 5.4 4.4 3.4	Yes	10 minutes
Phenol[b]	0.4%	6.4 5.4 4.4 3.4	Yes	10 minutes
Quaternary ammonium (QAC) and glutaraldehyde association[c]	0.1%	6.4 5.4 4.4 3.4	Yes	10 minutes
Quaternary ammonium compound[d] (QAC)	0.1%	6.4 5.4 4.4 3.4	Yes	10 minutes

[a] Virkon™S, Lanxess, Germany, [b] Biophene, Neogen, US, [c] TH4, Theseo, Brazil, [d] Germon 80, Sanphar, Brazil.

[4] SISGEN A60FC25.

was inoculated in ten 9-day-old ECE and eggs were candled daily to determine the viability of embryos for ten days.

On a safety cabinet, 400µL of viral inoculum were added to 100µL of organic load solution and 500µL of each individually diluted disinfectant, vortexed for 30 s and incubated for ten minutes. At the end of the contact time, 1000µL of neutralizer solution (1.5g sodium thiosulfate [$Na_2S_2O_2$, Thermo Fisher Scientific, Waltham, US], 0.07g lecithin [Thermo Fisher Scientific, Waltham, US], and 0.1mL Tween 80 [Thermo Fischer Scientific, Waltham, US] in 100mL phosphate buffered saline [PBS, pH 7.2]) was added to the solution. To ensure that neutralizer solution was not toxic to the embryos, ten 9-day-old ECE were previously inoculated with the mixture of each disinfectant and neutralizer solution and eggs were candled daily for 10 days. The final suspension from each mixture of disinfectant and inoculum was filtered through a 0.22µm membrane (Milliplex™, Millipore corp., Bedford, USA) and 0.2mL inoculated in six 9-day-old ECE through allantoic route. Inoculated ECE were candled daily for 72 hours and observed to assess embryos viability, any deaths during the first 24 hours were discarded as non-specific deaths. Allantoic fluid from reminiscent eggs were harvest for RT-PCR and hemagglutination test.

The experiment was conducted simultaneously with negative and positive controls. A negative control consisted of ten 9-day-old ECE inoculated with 0.2mL of a mixture containing water (400µL), organic load solution (100µL) and neutralizer solution (1000µL). Another negative control was composed of ten 9-day-old ECE that were inoculated with 200µL of a mixture containing each disinfectant (400µL), organic load solution (100µL) and neutralizer solution (1000µL) to assess the toxicity of the disinfectant on embryos. Finally, the positive control consisted of ten 9-day-old ECE with each of four viral inoculum from $6.4\log_{10}EID_{50}$/mL to $3.4\log_{10}EID_{50}$/mL. The test was performed in duplicates resulting in 44 samples. The experiment was repeated with the same protocol described above, 72 hours after dilution of the disinfectants have been performed. Therefore, a total of 88 samples were tested.

Hemagglutination assays. Hemagglutination assays were carried out using 0.5% chicken erythrocytes as previously described (Swenson et al. 2018).

Real-time reverse transcription-polymerase chain reactions (RT-PCR). RNA was extracted from allantoic fluid using the Cador Pathogen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One Step-RT-qPCR analyses were run on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City/CA, US). The reaction was performed with the LSI VetMAX® Swine Influenza A kit (A/H1N1/2009) (Thermo Fisher Scientific, Waltham/MA, US) for a final volume of 20µl/reaction.

Data analysis. The difference between the positive control and the test was recorded and Ct values were used to interpretate whether disinfectants reduced virus viability and infectivity. The results considered the Ct means of duplicate sets of control and test at time 0-hour and 72-hours post disinfectant dilution.

RESULTS

Influenza A [H1N1] virus titration and detection by RT-PCR are demonstrated on Table 2. The organic load, the neutralizer and the four disinfectants had no effect on embryos viability, demonstrating no toxicity.

In the first evaluation using fresh disinfectant solution (Hour 0), oxidizing compost disinfectant and phenolic disinfectant inactivated influenza at all tested virus concentrations (Table 3). The association of quaternary ammonium (QAC) and glutaraldehyde inactivated the virus up to a concentration of $5.4\log_{10}EID_{50}$/mL, therefore the highest virus concentration ($6.4\log_{10}EID_{50}$/mL) tested was still viable. The quaternary ammonium disinfectant did not eliminate virus viability after a 10-minute contact (Table 3). Seventy-two hours after disinfectants were diluted, oxidizing compost disinfectant demonstrated the same result as the assessment with fresh disinfectant solution, inactivating influenza at all tested virus concentration. The phenolic disinfectant and the association of QAC and glutaraldehyde inactivated the virus up to $5.4\log_{10}EID_{50}$/mL. Lastly, QAC had no effect on influenza virus inactivation (Table 3).

DISCUSSION AND CONCLUSION

The experiment evaluated four commercial disinfectants and their effectiveness on inactivating four concentrations of a pandemic H1N1 influenza virus. The tested disinfectants are widely used in swine production in order to decrease contamination on farms after cleaning facilities, equipment and vehicles. Our results demonstrated that oxidizing compost disinfectant and phenolic disinfectant were the most effective, even at high levels of influenza virus ($6.4\log_{10}EID_{50}$/mL). The efficacy of these active ingredients has previously been reported using the high pathogenic avian influenza virus H7N2 (Suarez et al. 2003), H7N1 (Sonthipet et al. 2018) and H5N1 (Marzouk et al. 2014) when tested at the manufacturer's recommended dose. The QAC disinfectant did not inactivate the influenza virus under our in vitro test conditions, considering the initial virus concentration of $3.4\log_{10}EID_{50}$/mL. QAC has previously been reported to be effective for enveloped virus (Jeffrey 1995, Suarez et al. 2003) due to its hydrophobic activity (Gerba 2015). It is important to note that there are different commercially available QAC-based disinfectants, regarding generation and active ingredients, which can lead to different results. Our results demonstrated that IAV is susceptible to QAC and glutaraldehyde association disinfectant, but the concentration may influence its efficacy. Marzouk et al. (2014) reported the inactivation of two strains of avian influenza virus ($7.15\log_{10}EID_{50}$/mL and $8.13\log_{10}EID_{50}$/mL) using 1% QAC and glutaraldehyde association with a contact time of 15 minutes.

Influenza viruses are described as relatively susceptible to chemical disinfection, particularly due to the presence of the viral envelope, which is composed of lipid bilayers (De Benedictis et al. 2007, Ivanova et al. 2015). Therefore, IAV is inactivated by most disinfectants when used properly. Organic

Table 2. Influenza virus titration (IAV) on embryonated chicken eggs (ECE) and cycle threshold (Ct) values from RT-PCR of viral inoculum

Viral titration ($\log_{10}EID_{50}$)	IAV RT-PCR (Ct)	
	0 h	72 h
6.2	21.7	22.6
5.2	24.3	25.2
4.2	28.8	28.1
3.2	31.0	31.6

matter has been described as an enhancer to increase viral persistence and tenacity (Hauck et al. 2017). Since organic load also decreases the efficacy of chemical disinfectants (Marzouk et al. 2014, Chandler-Bostock & Mellits 2015), we considered the worst-case scenario and included autoclaved feces to simulate a real situation of an unsuccessful cleaning procedure, similar to other studies (Guan et al. 2014, Sonthipet et al. 2018).

The test was repeated 72 hours after disinfectants were diluted to assess effectiveness over time. All tested products demonstrated similar performance, except for phenolic disinfectant that decreased effectiveness when compared to the fresh diluted test (Hour 0). The effectiveness of oxidizing agent to inactivate the avian influenza virus was obtained after a 10-day-dilution and the virus was not eliminated, demonstrating the importance of the freshness of the disinfectant solution (Suarez et al. 2003). It is important to understand the stability of disinfectants, since some biosecurity procedures in a pig farm include disinfection arch or footbaths where the disinfectants are not necessarily diluted at the time of use, and could result in disinfection failure.

In vitro test to assess virus susceptibility to disinfectants may have some protocol variation, in addition to the class of disinfectants and virus strain. Susceptibility tests can be performed with carriers, using coupons with different surfaces or suspension model and can also be evaluated under different conditions, as temperature, pH, contact time between disinfectants and viruses, different concentrations of organic load and disinfectant neutralizing step. In our study, we standardized four different concentrations of the IAV and the disinfectants were diluted according to the manufacturer's recommendations. Viral recovery was performed using ECE inoculation in order to assess virus viability and infectivity. Allantoic fluid was harvested from the ECE 72 hours post inoculation, RT-PCR and hemagglutination assays were run simultaneously and the agreement between assays was 100%, since all replicates had the same results using both methods. The limitation of our experiment is that our test was carried out with one virus strain. Hauck et al. (2017) reported different persistence time in manure when they tested two avian influenza viruses with different levels of pathogenicity. Moreover, viruses can tolerate extensive variations in the glycerophospholipids composition of their envelopes (Ivanova et al. 2015) and it is unknown how this characteristic may influence the virus persistence and susceptibility.

Information on the effectiveness of disinfectants in inactivating pandemic H1N1 influenza virus isolated from pig is limited, therefore, we evaluated four disinfectants widely used on pig farms under the same controlled conditions. In conclusion, three of the four disinfectants tested were effective to inactivate pandemic H1N1 influenza virus in the presence of organic load. Test result performed 72 hours after disinfectant dilution suggest a decrease in the effectiveness of one disinfectant and further investigation is needed to understand the limit period of action of each active ingredient.

Conflict of interest statement.- The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Table 3. Results of influenza A virus (IAV) hemagglutination and RT-PCR at 0 hour and 72 hours after disinfectant dilution

Study group	Virus titer (log_{10} EID_{50})	Hemagglutination	IAV RT-PCR	Hemagglutination	IAV RT-PCR
		0 h		72 h	
Oxidizing agent	6.4	-	-	-	-
	5.4	-	-	-	-
	4.4	-	-	-	-
	3.4	-	-	-	-
	Neg. control	-	-	-	-
Phenol	6.4	-	-	+	+
	5.4	-	-	-	-
	4.4	-	-	-	-
	3.4	-	-	-	-
	Neg. control	-	-	-	-
Quaternary ammonium (QAC) and glutaraldehyde association	6.4	+	+	+	+
	5.4	-	-	-	-
	4.4	-	-	-	-
	3.4	-	-	-	-
	Neg. control	-	-	-	-
Quaternary ammonium compound (QAC)	6.4	+	+	+	+
	5.4	+	+	+	+
	4.4	+	+	+	+
	3.4	+	+	+	+
	Neg. control	-	-	-	-
Positive control	6.4	+	+	+	+
	5.4	+	+	+	+
	4.4	+	+	+	+
	3.4	+	+	+	+

"+" positive, "-" negative.

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