The validation of Haemagglutinin Inhibition test for the detection of antibodies against canine influenza virus in a guinea pig serum model

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Abstract

Haemagglutination inhibition (HI) assay is a useful method for detecting antibodies against haemagglutinating viruses such as the influenza virus. An interaction between the influenza surface protein, haemagglutinin (HA) and the receptors on the surface of erythrocytes can result in agglutination when sufficient viral particles are present. Influenza positive serum can inhibit the haemagglutinin activity whereas negative serum will have no effect. In this study, known positive canine influenza (H3N2) serum from guinea pigs was evaluated by four different factors of HI assay. The four different factors in HI assay included a) elimination of non-specific inhibitors (20% kaolin or receptor destroying enzyme (RDE)), b) type of red blood cells (RBCs), c) percentage of RBCs, and d) haemagglutination unit (HAU) of virus. Our results showed that the receptor destroying enzyme (RDE) treated serum with 1% turkey RBCs and incubation with 4HAU/25µl of virus provided highest HI value (no statistic significant among groups). In conclusion, the HI protocol for testing canine influenza virus specific antibodies in guinea pig serum model has been validated and can be used for influenza research in the future.

Introduction

Influenza virus causes respiratory disease in several animal species as well as humans. The equine-origin canine influenza virus subtype H3N8 (CIV-H3N8) was first reported in racing greyhounds in the US in 2004. The CIV infected dogs showed clinical signs of upper respiratory tract infection such as cough, nasal discharge, fever and subsequently self-recovery (Crawford *et al.*, 2005; Zhang *et al.*, 2007). After the first outbreak, the CIV-H3N8 spread out to other states in the US and the United Kingdom (Daly *et al.*, 2008; Newton *et al.*, 2007; Payungporn *et al.*, 2008). In 2008, avian-origin CIV-H3N2 emerged in dogs in Korea and spread rapidly worldwide including Southeast Asia and North America (Bunpapong *et al.*, 2014; Li *et al.*, 2010; Lin *et al.*, 2012; Martella *et al.*, 2010; Voorhees *et al.*, 2017; Weese *et al.*, 2019).

Experimental studies of inter-species transmission of influenza have been useful to predict the pandemic potential that could happen in humans. The first case of inter-species transmission of CIV was reported in Korea (Song *et al.*, 2011). CIV can be transmitted from dogs to other host species such as guinea pigs, ferrets and cats both under experimental settings and by natural infection (Kim *et al.*, 2013; Lyoo *et al.*, 2015). The genetic characterization of the viruses has revealed the identical genetic composition of CIV from dogs and these animals suggesting potential intermediate hosts of CIV-H3N2 (Lyoo *et al.*, 2015; Song *et al.*, 2011).

There have been several mammal models used for influenza research. For example, the ferret model is a common and useful model for influenza study. The ferret model can be used for direct and indirect contact and aerosol study (Belser *et al.*, 2011; Herlocher *et al.*, 2001). However, the ferret model presents several disadvantages such as its expense, limited animal supply and difficulty in handling (Lowen *et al.*, 2006). The mouse model can be used as a mammal model since the model is inexpensive and easy to handle. However the mouse model is not suitable for transmission study (Schulman and Kilbourne, 1963). Another alternative mammal model, the guinea pig model can be used for influenza research (Azoulay-Dupuis *et al.*, 1984). The guinea pig model is suitable for infection and transmission study of both large droplet and airborne particles (Mubareka *et al.*, 2009). For example, some studies have used the guinea pig model for experimental challenges of IAV-H5N1 and CIV-H3N2 (Bushnell *et al.*, 2010; Van Hoeven *et al.*, 2009).

In general, the Haemagglutination inhibition (HI) test is commonly used for the detection of antibodies against influenza virus due to its rapidity, simplicity and cost effectiveness. However, the sensitivity of the HI test can vary due to the compatibility of the receptors on RBCs and viral proteins (Pedersen, 2014). Unfortunately, for guinea pigs, the standard protocol for an HI test specific to guinea pig serum is still unavailable. The aim of this study was to validate an HI test for the detection of antibodies against canine influenza virus in guinea pig serum. In this study, known positive canine influenza (H3N2) serum from guinea pigs was evaluated by four different factors of HI assay including a) the elimination of non-specific inhibitors (20% kaolin or receptor destroying enzyme (RDE)), b) type of red blood cells (RBCs), c) percentage of RBCs, and d) the hemagglutination unit (HAU) of the virus. The HI protocol for testing canine influenza virus in guinea pig serum model has been validated.

Materials and Methods

Virus: The canine influenza virus subtype H3N2 (CIV-H3N2) used in this study was isolated from a dog in Thailand in 2014. This virus, A/canine/Thailand/CU-DC5299/12, had been previously characterized by whole genome sequencing and the whole sequences were submitted to the GenBank (Accession #KC599545-52). Based on phylogenetic analysis, Thai CIV-H3N2 was closely related to the CIV-H3N2 from China and Korea of the avian Eurasian lineage (Bunpapong *et al.*, 2014). In this study, the virus was propagated in embryonic chicken eggs to a concentration of 10^6 EID$_{50}$ per milliliter at the 4th passage level.

Validation of HI test: To acquired known positive influenza A serum of guinea pigs, blood samples were collected from CIV-H3N2 infected SPF guinea pigs at 14 days post-inoculation. The guinea pigs were inoculated intranasally with 300 µl of CIV-H3N2 (A/canine/Thailand/CU-DC5299/12). Blood samples were collected at 14 dpi and centrifuged at 1000Xg for 10 minutes to separate serum immediately after blood collection. The separated serum was kept at -80 ^{0}C until further use. This study was conducted under the ethical approval of the Chulalongkorn University Animal Care and Use Committee (CU-VET, IACUC# 1431100).

For HI protocol standardization, the serum sample was subjected to HI test for specific influenza antibodies (CIV-H3N2). Four different factors were evaluated including a) elimination of non-specific inhibitors (20% kaolin or receptor destroying enzymes (RDE)), b) type of red blood cells (RBCs) and c) percentage of RBCs and d) hemagglutination unit (HAU) of the virus. In total, 16 experimental groups (A-P) were included in the study.

For sera treatment, the positive serum samples of guinea pigs were divided into 16 groups including group A – P, one serum per group (Table 1). The serum samples were treated by either receptor destroying enzymes (RDE) or 20% Kaolin with different types (chicken or turkey RBCs). In detail, for groups A-H, the serum samples were treated with 20% Kaolin (20 g of Kaolin mixed with 100 ml of 1x phosphate buffer solution (PBS) at room temperature for 30 minutes and centrifuged at 1000xg for 10 minutes. Treated samples were absorbed with 100 µl of 50% turkey RBCs (group A-D) or 50% chicken RBCs (group E-H). Then, all samples were incubated at room temperature for an hour. For groups I-P, positive sera samples were treated with receptor destroying enzyme (RDE) (RDE mixed with 20 ml of 0.9% normal saline solution) at 37°C for 20 hours then inactivated by heat at 56 °C for an hour and absorbed with 100 µl of 50% turkey RBCs (group I-L) or 50% chicken RBCs (group M-P). Then, all samples were incubated at room temperature for an hour. After treatment, treated serum samples were

two-fold diluted in 96-well micro-titer plates by phosphate-buffered saline (PBS). Each sample was incubated with 4 or 8 haemagglutination units (HAU) per 25 or 50 µl of each virus for 45 mins at room temperature. Then 0.5% or 1% of chicken RBCs or turkey RBCs were added and incubated for 1 hour. The

HI titer was determined by the reciprocal of the last dilution which presented non-agglutination. Positive samples were identified with samples showing a titer ≥ 40 (Bunpapong *et al.*, 2014).

Table 1 Experimental groups (A-P) for HI protocol standardization based on sera treatment (a) 20% Kaolin and RDE (b) types of RBCs and HI step (c) HAU and (d) % RBCs

HI protocol standardization				Experimental group
Sera treatment		HI step		
Serum treatment	Types of RBCs	HAU	% RBCS	
20% Kaolin	Turkey RBCs	4HAU/25µl	1% RBC	A
			0.5% RBC	B
		8HAU/50µl	1% RBC	C
			0.5% RBC	D
	Chicken RBCs	4HAU/25µl	1% RBC	E
			0.5% RBC	F
		8HAU/50µl	1% RBC	G
			0.5% RBC	H
RDE	Turkey RBCs	4HAU/25µl	1% RBC	I
			0.5% RBC	J
		8HAU/50µl	1% RBC	K
			0.5% RBC	L
	Chicken RBCs	4HAU/25µl	1% RBC	M
			0.5% RBC	N
		8HAU/50µl	1% RBC	O
			0.5% RBC	P

Statistical analysis: The descriptive statistics including geometric mean and standard deviation of HI titer are described for each group. Analysis of statistical differences of HI titers among groups was done by two sample t-test with equal variance using Software for Statistics and Data Science (Stata) version 13.0. Graphs were plotted with Prism 8.

Results

In this study, known positive guinea pig sera samples were treated with four different factors including a) elimination of non-specific inhibitors (20% kaolin or receptor destroying enzyme (RDE)), b) type of red blood cells (RBCs), c) percentage of RBCs, and d) haemagglutination unit (HAU) of virus and designated into 16 experimental groups.

For the non-specific inhibitors factor, the serum treated with RDE (group I-P) possessed geometric mean and SD of HI titer at 160 ± 189.96. While the geometric mean HI titer of the serum treated with 20% Kaolin (A-H) was 61.69 ± 42.68. Although, both RDE and Kaolin can be used for romoving non-specific inhibitors, the geometric mean HI titer of serum treated with RDE is higher than that of 20% Kaolin but there is no statistically significant difference between two treatments (p=0.0574) (Figure 1).

For RBCs types factor, the serum treated with 20% Kaolin and absorbed with turkey RBCs (group A-D) possessed geometric mean HI titer 80.00 ± 50.33, while the serum treated with 20% Kaolin and absorbed with chicken RBCs (group E-H) had geometric mean HI titer 47.57 ±30.00. There was no statistically significant difference between the two treatments (p=0.2773). For

the serum treated with RDE and absorbed with turkey RBCs (group I-L) present geometric mean HI titer 190.27 ± 256.12 and with chicken RBCs (group M-P) present geometric mean HI titer 134.54 ± 114.89. Even though, the serum absorbed with turkey RBCs showed the higher mean HI titer than that absorbed with chicken RBCs, the statistic analysis showed no statistic significance between types of RBCs absorption (p=0.5452) (Figure 2).

For the concentration of virus, haemagglutination units (HAU) of virus, the samples in the HI incubation step using 4HAU/25µl provided HI titer (160, 80, 80 ,40, 640, 160, 320, 160; geometric mean and standard deviation = 146.72 ± 195.30) which higher than those using 8HAU/25 µl (80, 40, 80, 20, 160, 80, 160, 40; geometric mean and standard deviation = 67.27 ± 52.85). The haemagglutination units (HAU) of virus, the samples with HI step using 4HAU/25µl provided HI titer equal or higher than those using 8HAU/25 µl either turkey RBCs and chicken RBCs. For the percentage of RBCs, the samples using 1% and 0.5% turkery RBCs present geometric mean 190.27 and 80.00 respectively whereas the samples using 1% and 0.5% chicken RBCs present the geometric mean 134.54 and 47.57 respectively. The samples in the HI step using 1% either turkey RBCs or chicken RBCs had HI titer higher than those using 0.5% either turkey RBCs or chicken RBCs. Due to the limited number of groups, there was no statistical analysis among treatments (Figure 3).

Overall, the serum treated with receptor destroying enzyme (RDE) absorbed with turkey RBCs with HI step of the concentration 4HAU/25µl and 1% of turkey RBCs (group I) yielded the highest HI titer compared with other groups. The serum treated with receptor

destroying enzyme (RDE) absorbed with chicken RBCs and 4HAU/25µl and 1% of chicken RBCs (group M) showed the second highest HI titer (Figure 4). According to the cut-off of HI titer ≥ 40, one sample treated with 20% Kaolin and absorbed with chicken RBCs with a concentration 8HAU/50µl and 0.5% of chicken RBCs showed negative results (group H).
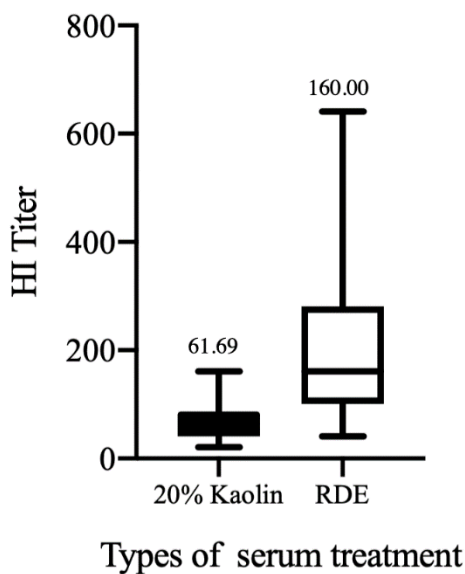

Figure 1   HI titer of serum treated with the non-specific inhibitors factor (20% Kaolin and RDE). Number and bar represent geometric mean and standard deviation of HI titer of each group.
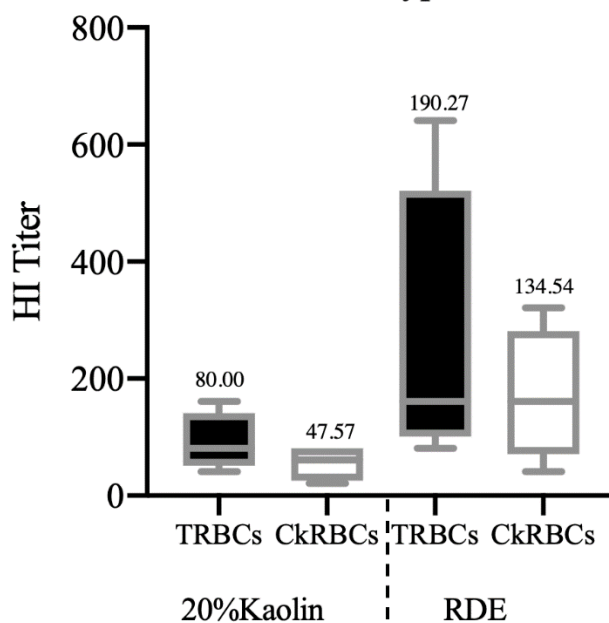

Figure 2   HI titer of serum treated with different type of red blood cells. Number and bar represent geometric mean and standard deviation of HI titer of each group.

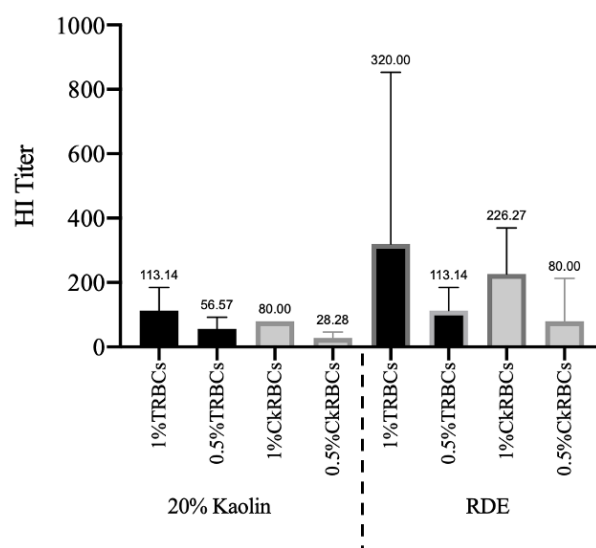## HI Titer with different concentrations and types of red blood cells

Figure 3 HI titer of serum treated with different concentrations of virus and percentage of red blood cells. Number and bar represent geometric mean and standard deviation of HI titer of each group.
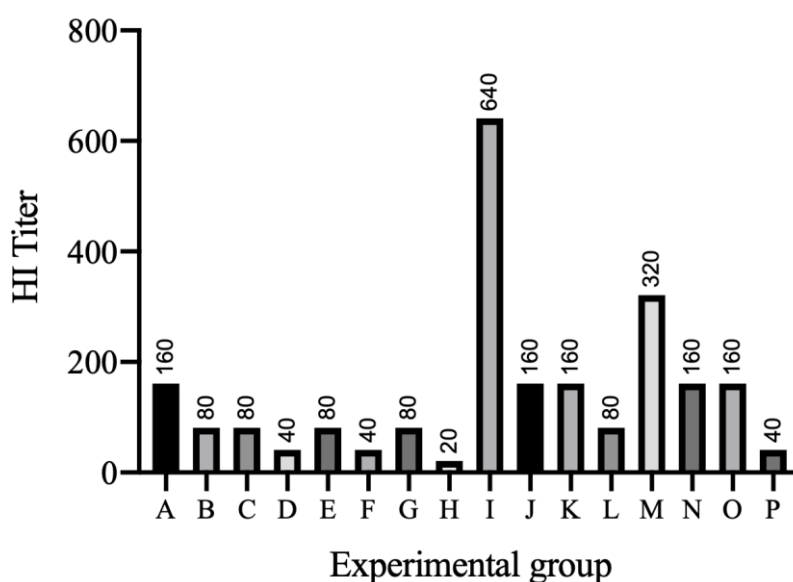
HI Titer in each experimental group

Figure 4 HI titers of each treatment group by 4 different factors (16 experimental groups; A-P)..

Discussion

There are HI standard protocols for the detection of specific antibodies against influenza A viruses for multi-species. For example, an HI protocol for avian and swine species (OIE, 2021a, b). However, there is no specific HI standard protocol for guinea pig serum. In this study, we developed the standard protocol for HI test in guinea pig serum prior to the pathogenicity and animal challenge studies. The appropriate HI standard protocol in guinea pig serum is the serum treated with receptor destroying enzyme (RDE) absorbed with turkey RBCs and incubating with the virus concentration 4 HAU/25µl and 1% of turkey RBCs.

For the HI protocol, serum treatment by the elimination of non-specific inhibitors can be performed by adding either 20% kaolin or receptor destroying enzyme (RDE). It is known that the sensitivity and specificity of the HI test can be reduced due to nonspecific inhibitors in serum (Ryan-Poirier and Kawaoka, 1991; Ryan-Poirier and Kawaoka, 1993). Thus, there are several serum treatment methods used for HI test including serum treatment with 20% kaolin or RDE (Boliar *et al.*, 2006). The presence of non-specific

inhibitors (NSIs) in human and animal sera can affect the HI result, thus the use of either 20% kaolin or RDE is recommended for the elimination of non-specific inhibitors (Kim *et al.*, 2012b). The advantages of 20% Kaolin and RDE are their commercial availability and ease of preparation and use. As for the disadvantages, Kaolin is suitable for removing the gamma globulin fraction, while the RDE inactivates all non-specific inhibitors (Tauraso *et al.*, 1971). Previous studies have shown that RDE is able to inactive NSIs more efficiently and more consistently than Kaolin when used for serum treatment in other species (Favaro *et al.*, 2017; Kim *et al.*, 2012b). Our result in this study showed that serum treated with RDE yielded higher HI titer than that treated with 20% Kaolin which is similar with other studies (Boliar *et al.*, 2006; Kim *et al.*, 2012a).

For type of red blood cell, the HI test could be influenced by the types or species of RBCs. It is noted that the agglutination between RBCs and virus depends on Sialic acid α 2.3 and α 2.6 Gal linkages present on the RBC surface. For example, previous studies have shown that human, chicken, pig and guinea pig RBCs express both α 2.3 and α 2.6 Gal linkages and can be used in HI test for influenza research. Notably, swine RBCs contain SA α 2.6 Gal linkage more than α 2.3 Gal linkage, while chicken RBCs have a higher proportion of SA α 2.3 Gal linkage than those of guinea pigs and humans (Stephenson *et al.*, 2004). In general, turkey RBC is commonly used for HI test due to its small, nucleated cells and rapid sedimentation (Stephenson *et al.*, 2004). In this study, serum treated with turkey RBCs yielded higher HI titer than that used chicken RBCs. The result of this study is similar to other previous studies in which turkey RBCs were appropriate for avian, swine and canine LPAI viruses (Anderson *et al.*, 2012; Pawar *et al.*, 2012; Thontiravong *et al.*, 2016). Another previous study reported that using either turkey RBCs or chicken RBCs provided highly sensitive and specific HI test for canine H3N8 challenged study (Anderson *et al.*, 2012).

For the concentration of the virus, haemagglutination unit (HAU) of virus, the samples with HI step using 4HAU/25µl provided HI titer equal or higher than those using 8HAU/50 µl both with turkey RBCs and chicken RBCs. In a previous study, HI test with serum treated with RDE and 0.5% of chicken RBCs with 4HAU/25µl was done in the human H3N2 influenza virus challenge study in the guinea pig model (Bushnell *et al.*, 2010). Notably, the recommendation of optimum HI test for avian and swine serum samples is available and provided by OIE (OIE, 2021b). However, there is no specific guidline for HI test in guinea pig serum. Thus, the evaluation of the HI test by varing percentages of RBC and concentration or haemagglutination unit (HAU) of virus was performed.

This study provides an additional standard protocol of HI test for guinea pig serum samples. Thus, the use of a standard protocol for HI test for the detection of influenza antibodies in guinea pig serum has made available by using the serum treated with receptor destroying enzyme (RDE) absorbed with turkey RBCs and incubating with the virus concentration 4 HAU/25µl and 1% of turkey RBCs. In conclusion, the HI protocol for testing canine influenza

virus in the guinea pig serum model has been validated and can be used for influenza research in the future.

Acknowledgements

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