Journal of Virological Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

HA1-specific indirect ELISA for serological detection of canine influenza virus H3N2 infection in dogs

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Article history:
Received 5 June 2014
Received in revised form
13 December 2014
Accepted 31 January 2015
Available online xxx

Keywords:
Canine influenza
H3N2
Indirect ELISA
HA1

ABSTRACT

An indirect ELISA using recombinant HA1 protein of canine influenza virus (CIV) as a coating antigen was developed and characterized for its application to serosurveillance in dogs. The CIV H3N2-specific indirect ELISA was developed using recombinant HA1 protein (baculovirus-expression system) as a coating antigen. A total of 65 CIV H3N2-positive or negative canine sera were tested by the indirect ELISA for receiver operating characteristic (ROC) analysis and results compared to those generated by the hemagglutination inhibition (HI) test. Canine sera collected 10 days following intranasal inoculation with canine H3N2, seasonal H3N2 (A/Brisbane/10/2007) or pandemic H1N1 influenza virus (A/California/04/2009) were used for the cross-reaction test. An adjusted optical density (OD) of 0.17 was determined to be the optimal cut-off value for seropositivity. The indirect ELISA showed 95.7% sensitivity and 94.7% specificity when compared to the HI test. A cross-reaction test was also performed using canine sera reactive with CIV H3N2, seasonal H3N2 (human) and pandemic H1N1 (human) influenza viruses. Based on the data generated in this study, the canine H3N2-associated ELISA using baculovirus expressed HA1 antigen will be useful for herd-based serological survey of the canine H3N2 virus infection in dogs.

1. Introduction

Following the discovery of interspecies transmission of avian influenza virus (H3N2) to dogs, intraspecies transmission of canine influenza virus (CIV H3N2) was demonstrated by experimental and surveillance studies (Lee et al., 2009; Song et al., 2008, 2009). Previously, serological detection of canine H3N2 infection was based on the hemagglutination inhibition (HI) test or a commercialized competitive ELISA which was established to detect universal avian influenza A virus-specific antibodies using the NP protein (Song et al., 2009). However, use of these methods for CIV H3N2 serology proved to be problematic. The HI test is labor-intensive due to its requirements for serum and RBC preparation protocols that minimize non-specific reactions, and the commercialized competitive ELISA, with its own systemic characterization, cannot clarify that the positive result is due to infection with canine H3N2 instead of other avian influenza viruses. However, the previous study showed that a hemagglutinin (HA)-specific ELISA successfully detected avian influenza A (H5N1) virus-specific antibodies, and this result was confirmed by subtype-specific HI and microneutralizing assays (Rowe et al., 1999). Therefore, in this study, an indirect ELISA using recombinant HA1 protein of CIV H3N2 as a coating antigen was developed and characterized to determine its application for serosurveillance against the virus in dogs.

2. Materials and methods

2.1. Preparation and purification of recombinant HA1 protein

First, the canine H3N2 HA1 coding sequence was amplified by PCR using two oligonucleotides: the 5′-oligonucleotide (5′-CAG

Abbreviations: CIV, canine influenza virus; HI, hemagglutination inhibition; HA, hemagglutinin; ROC, receiver operating characteristic; OD, optical density; PBST, phosphate buffered saline containing 0.05% Tween 20; TMB, 3,3′,5,5′-tetramethylbezidine; AUC, area under the curve.

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http://dx.doi.org/10.1016/j.jviromet.2015.01.007
0166-0934/

GAT CCC AAT CTT CCA GGA AAT GAA AAT AA) contained a BamHI site and the coding sequence for the N-terminal amino acid residues of canine H3N2 HA1, the 3′-oligonucleotide (5′-CAG CGG CCG CTC AAT GGT GAT GGT GAT GAT GGG TTT GCC TCT CAG GGA C) contained a NotI site, stop codon, C-terminal 6×His tag, and the encoding sequence for the C-terminal amino acid residues of canine H3N2 HA1 (A/canine/Korea/GCVP01/2007(H3N2). The PCR product was purified with a QIAquick PCR purification kit (Qiagen Korea, Seoul, South Korea), restricted with BamHI and NotI, repurified with a QIAquick PCR purification kit (Qiagen Korea, Seoul, South Korea), and subcloned in BamHI-NotI restricted pAcGP67A, which resulted in pAcGP67A-cH3N2 HA1.

The pAcGP67A-cH3N2 HA1 DNA (2 μg) was co-transfected with 0.5 μg of BaculoGold AcNPV DNA into 2×10^6 sf9 cells following the recommendations of the supplier. On post-transfection days 5 and 8, the medium was centrifuged and the supernatant tested in a limiting dilution assay with sf9 cells, which were incubated with 10-fold dilutions of supernatant in 6-well plates or T-75 flasks. Recombinant virus in the supernatants was then amplified by infecting sf9 cells.

Next, 2×10^6 High5 cells in Sf-900 II SFM medium were infected with recombinant baculovirus in spinner flasks at moi of 0.01–10. Supernatant was harvested after 3 days and centrifuged at 3000 × *g* for 10 min to remove cell debris, then loaded on Ni-NTA resin equilibrated with 20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, and 5 mM imidazole. Protein was washed with the resin in 5 mM imidazole and eluted with 250 mM imidazole. The eluted protein, canine H3N2 HA1, was dialyzed with 20 mM Tris–HCl (pH 8.0) and 10% glycerol.

2.2. Indirect ELISA

After antigen preparation, the coating, blocking, and dilutions of indirect ELISA were optimized to detect HA-specific canine IgG. The prepared antigen was coated on the plate, HI test-positive and HI test-negative canine sera reactive with CIV H3N2 was used as primary antibody, and sheep anti-dog IgG and anti-sheep IgG-HRP were used for detection. The optimal conditions for the antigen concentration and the serum dilution were obtained using a checkerboard titration method.

ELISA plates (Greiner Bio-One, Frickenhausen, Germany) were coated with canine H3N2-derived HA1 (50 ng/well) in cold carbonate buffer and incubated overnight at 4 °C. Antigen-free carbonate buffer wells were coated for endpoint assay controls. Plates were blocked with 0.2% skim milk in phosphate buffered saline containing 0.05% Tween 20 (PBST) at 37 °C for 1 h). After washing the plates, 100-fold diluted canine serum samples (serum diluent: 0.2% skim milk in PBST including 10 μg/ml of hi5 culture supernatant) were applied in duplicate to the antigen coated and non-coated wells and incubated for 90 min at 37 °C. Sheep anti-dog IgG (1:1000) was added to each well and incubated under the previously stated same conditions. After washing, anti-sheep IgG-HRP (1:5,000) was added and incubated for 60 min. For color development, 100 μL of peroxidase substrate, 3,3′,5,5′-tetra-methylbezidine (TMB) in acidic buffer (KPL, Gaithersburg, United States) was added to each well and incubated for 5 min at RT. To stop the reaction, an equal volume of the manufacture's recommended solution (1 N HCl) was added, and plates were read at a wavelength of 450 nm within 30 min. Endpoint titers of canine H3N2-HA-specific IgG Abs were expressed as the optical density (OD) values of the uncoated wells subtracted from the HA coated well values (adjusted OD).

The established indirect ELISA was compared with HI test and competitive ELISA as described below. The HI test was performed with 8 HA units of canine influenza virus (H3N2) (A/canine/Korea/GCVP01/2007) following the OIE manual. To validate the obtained cut-off value, an antiserum against canine H3N2 (kindly provided by Green Cross Veterinary Products Co., Ltd.) was serially diluted to produce HI titers of 40, 80, 160, and 320. The prepared sera were tested in duplicate using indirect ELISA.

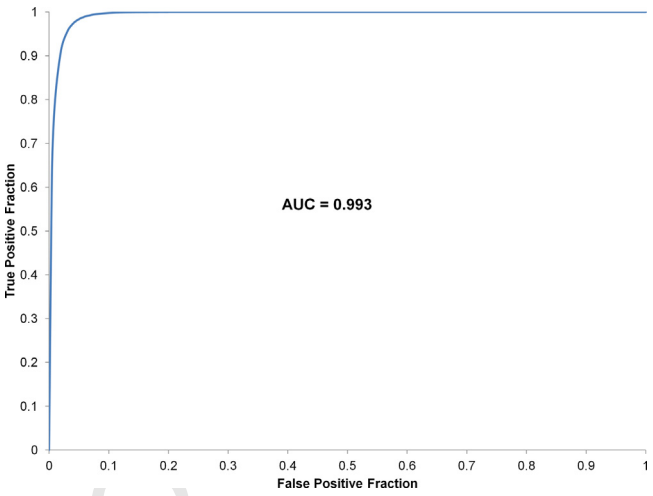

Fig. 1. ROC curve analysis comparing the indirect ELISA with the HI test.

2.3. Sera

To test the indirect ELISA specificity and sensitivity and to determine cutoff values, a total of 65 canine sera were tested. The sera were provided by the Animal and Plant Quarantine Agency, Anyang, Korea and Green Cross Veterinary Products Co., Ltd., Yong-In, Korea. Negative sera (obtained from 44 experimental dogs) showed a HI titer less than 10, the positive sera were obtained from 16 canine sera that tested positive from a serological survey (160 ~ >1280 HI titers against canine influenza H3N2 virus) and five reference sera obtained from the experimental challenge (160–640 HI titers against canine influenza H3N2 virus). To test for cross-reactivity, archived canine sera ($n = 10$) which were collected 10 days following intranasal inoculation with canine H3N2, seasonal H3N2 (A/Brisbane/10/2007) or pandemic H1N1 influenza viruses (A/California/04/2009) were used in the cross-reaction test. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB-AEC-12054).

2.4. Statistical analysis

The ROC analysis was performed with a web-based ROC analysis tool (Eng, 2007), and the cut-off value was determined when the optimal values of sensitivity and specificity were obtained.

3. Results

3.1. Comparison of indirect ELISA and HI assay

Using the HI test as a gold standard, the ROC curve is depicted in Fig. 1. The area under the curve (AUC) value, which is indicative of the accuracy of the test, was 0.993(an AUC value of 1 = 100% sensitivity and specificity). When the cut-off value was set at 0.17, sensitivity and specificity were 95.7% and 94.7%, respectively. When the cut-off value was set at 0.36, sensitivity and specificity were 93.5% and 94.7%, respectively. Since it showed the best sensitivity and specificity, the cut-off value was set at 0.17 of adjusted OD for the indirect ELISA performed in this study.

In addition, the indirect ELISA was compared with the HI test using sera with a low range of HI titers. When canine sera diluted

Please cite this article in press as: Shim, D.H., et al., HA1-specific indirect ELISA for serological detection of canine influenza virus H3N2 infection in dogs. J. Virol. Methods (2015), http://dx.doi.org/10.1016/j.jviromet.2015.01.007