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Detection of H3N2 canine influenza virus using a Quartz Crystal Microbalance

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ABSTRACT

Label-free technology-based Quartz Crystal Microbalance (QCM) is an emerging tool in biological research. In this study, QCM was applied successfully for the rapid diagnosis of H3N2 canine influenza virus (CIV) infection. ProLinker™ B, a calixcrown derivative, enables antibodies to be attached to a gold-coated quartz surface and positioned in a regular pattern with the correct orientation. The ProLinker-coated quartz-based assay detected H3N2 CIV at lower concentrations (2^2 HA unit) than a commercial immunochromatography Ag kit (2^3 HA unit). Three independent experiments in which H3N2 CIV-positive reference samples were applied to an anti-CIV nucleoprotein (NP) monoclonal antibody immobilized on a quartz surface yielded standard deviations (SD) of ≤5%, indicating high reproducibility. In addition, the QCM assay with a cut-off value (−140 Hz) showed 97.1% (34/35) sensitivity and 94.7% (36/38) specificity in testing 73 field saliva samples, respectively. Thus, the QCM assay described herein will be a valuable tool for the rapid diagnosis of H3N2 CIV infection with high sensitivity and specificity, and should overcome several of the disadvantages and limitations inherent in the commercial immunochromatography Ag kit.

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1. Introduction

H3N2 canine influenza virus (CIV) is an enveloped virus belonging to the genus *Influenza A virus* (IAV) within the family *Orthomyxoviridae*, which has an eight-segmented, negative-sense, single-stranded RNA genome (Webster et al., 1992). Canines infected with H3N2 CIV develop fever, nasal discharge, anorexia, lethargy, and mild-to-fatal respiratory symptoms.

H3N2 CIV caused an outbreak of contagious canine respiratory disease in South Korea in 2007 (Song et al., 2008), and the results of a study examining the systemic transmission of H3N2 CIV between dogs were published the following year (Song et al., 2009). Furthermore, interspecies transmission of CIV to cats was reported in both South Korea and China (Jeoung et al., 2013a; Kim et al., 2013; Lei et al., 2012). In China, the H3N2 CIV subtype, which originated from avian species, was isolated from pet dogs in Guangdong and Jiangsu Provinces (Li et al., 2010; Lin et al., 2012). Recently, novel subtypes (e.g., H1N1 and H9N2) were identified as the causative agents of a flu-like disease in dogs (Su et al., 2014; Sun et al., 2013).

Virus isolation from cell culture is the standard method for diagnosing influenza virus infection (Stamboulian et al., 1999). However, molecular biological methods such as one-step reverse transcription polymerase chain reaction (RT-PCR) (Jeoung et al., 2013b), real-time RT-PCR (Van et al., 2001), nucleic acid sequence-based amplification (NASBA) (Van et al., 2006), loop-mediated isothermal amplification (LAMP) (Imai et al., 2006; Masahiro et al., 2006), transcription-mediated assay (TMA) (Hill, 2001), microarray analysis (Dawson et al., 2007; Wang et al., 2006), and a commercial immunochromatography Ag kit (Mitamura et al., 2013; Sasaki et al., 2012) have now been developed for detecting influenza A viruses.

Quartz Crystal Microbalance (QCM) was first developed as a mass sensor for use in the gas phase under vacuum (King, 1964). However, it has since become a powerful biological research technique for monitoring the adsorption of particles onto solid surfaces (Carter et al., 1995; Hao et al., 2009; Ittarat et al., 2013; Wen-Chi and Pi-Ju, 2009). QCM is a convenient method of detecting antigen:antibody interactions by measuring frequency changes at a quartz crystal surface. When an antigen adsorbs to the surface, the quartz crystals vibrate at a reduced frequency; this change in frequency can be detected and measured. QCM is a "label-free technology" because binding is detected directly without the need to label reagents. In addition, QCM is rapid and reliable, and requires

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minimal sample handling or laboratory skill. Owing to its simplicity and cost effectiveness, QCM has been investigated extensively for its ability to detect pathogenic microorganisms such as *Vibrio cholerae*, *Bacillus anthracis*, malaria and *Salmonella Typhimurium* (Carter et al., 1995; Cooper et al., 2001; Hao et al., 2009; Ittarat et al., 2013; Salam et al., 2013).

In this study, QCM assay was developed based on ProLinker^{TM} B, which was used to immobilize H3N2 CIV-specific antibodies onto a gold-coated quartz surface. The antibody:antigen interaction was measured in terms of a frequency shift, which occurs due to a change in mass at the quartz crystal surface. This report describes the development of a rapid and sensitive immunoassay and its successful application for the detection of H3N2 CIV antigens in canine saliva samples.

2. Materials and methods

2.1. Reference and field samples

Reference H3N2 CIV samples were used to set a cut-off value for discriminating positive samples from negative in specimens in the field. A total of 30 H3N2 CIV samples, all registered in the Korea veterinary culture collection (KVCC) (http://kvcc.kahis.go.kr), were used as positive reference samples (Supplemental Table 1), and 31 saliva samples collected from canines in South Korea and diagnosed as H3N2 CIV-negative by RT-PCR were used as negative reference samples. H3N2 CIV-positive reference samples were thawed and inoculated into specific pathogen-free embryonated chicken eggs. At 3 days post-inoculation, the allantoic fluids were collected and viral RNA was extracted using the Qiagen viral RNA kit (Hilden, Germany), according to manufacturer's instructions.

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.07.022.

To test the QCM assay's application to field samples, 73 saliva samples from dogs with suspected H3N2 CIV were obtained from animal hospitals in Seoul and Gyeonggi province in Korea from 2012 to 2013. All samples were identified as H3N2 CIV-positive or -negative by RT-PCR using the following primers targeting CIV: forward, 5′-GAAGCTCGGCATTGTAAGAC-3′, and reverse, 5′-ATTCAGGCCTCAGGAAGA GT-3′. RT-PCR reactions were performed using a Qiagen onestep RT-PCR kit in a mixture (25 μL) containing 11 μL of RNase-free water, 5 μL of 5× RT-PCR buffer, 1 μL of each primer, 1 μL of dNTP mix, 1 μL of RT-PCR enzyme mix, and 5 μL of extracted RNA. The RT-PCR conditions were as follows: 45 °C for 30 min for RT, 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 50 °C for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The size of the amplicons was 697 bp, as determined by agarose gel electrophoresis.

2.2. Commercial immunochromatography Ag kit

A commercial immunochromatography Ag kit was purchased from the Bionote (Hwaseong, South Korea) and used as follows. A commercial immunochromatography Ag kit has a letter of "C" as control line and "T" as test line on the surface of the device. Both the control line and test line are not visible before applying a sample. The control line is used for procedural control and should always appear if the test procedure is performed properly. Whereas, a test line will be visible if there is H3N2 CIV antigen in the saliva. Briefly, canine saliva samples were placed in sample collection tubes containing assay diluent and mixed vigorously. The tube was then left to stand for 3 min at room temperature to allow any particles to settle. Finally, four drops of sample solution were loaded into the sample hole using a disposable dropper and the results were interpreted after 10 min.

2.3. Hemagglutination (HA) assay

Chicken blood cells were pipetted into a conical tube and centrifuged. The supernatant was then removed without disturbing the blood cells, and phosphate buffered saline (PBS) was added to the tube. The saline and cells were mixed by inverting the tube several times. The tube was spun at 800 rpm for 5 min. This procedure was repeated two more times. After the final wash, the supernatant was removed and PBS was added to yield a 1% solution of red blood cells. The HA assay was performed in a round bottomed 96-well plate. Briefly, 50 μL of PBS was added to each well of the plate. Next, 50 μL of virus sample was added to the first well. The contents were mixed and then 50 μL was transferred to the next well. This process was repeated across the plate, with 50 μL of solution being discarded from the final well. Finally, 50 μL of the 1% red blood cell solution was added to each well and mixed gently. The plate was left at room temperature for 30 min. The virus dilution at which agglutination first occurred was determined visually.

2.4. Preparation of quartz

Quartz was soaked in a solution containing MeOH and 35% HCl (1:1) for 30 min. Freshly prepared piranha solution (a 3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2) was then added for 10 min. The quartz was then washed thoroughly with distilled water and dried under a stream of nitrogen (N_2) gas. The pre-cleaned quartz was then gold-coated by thermal evaporation in a Magnetron Sputtering System (Cliotech, Seoul, South Korea) before being immersed in a ProLinker^{TM} B solution (3 mM) (Proteogen, Chuncheon, South Korea), which contains exposed —SH groups, for 1 h. The quartz was rinsed sequentially with $CHCl_3$, EtOH, and distilled water before drying under a stream of N_2 gas.

2.5. Monoclonal antibody immobilization on gold-coated quartz

Nucleoprotein (NP) specific monoclonal antibodies for H3N2 canine influenza virus were purchased from Median Diagnostics (Chuncheon, South Korea). The monoclonal anti-NP antibodies (50 μg/mL) were dissolved in PBS (1 mL containing 30% glycine), and 50 μL of the mixture was immobilized onto the gold-coated quartz by incubation overnight at 4 °C. The quartz was then rinsed twice with PBS and deionized water, and dried under a stream of N_2 gas. The antibody-coated quartz was stored in 4 °C until use.

2.6. Measurement of frequency shifts

Frequency shifts were measured using the Xdelic XQ-10 (UBTgen, Seoul, South Korea) according to the manufacturer's instructions. Briefly, the antibody-coated quartz was placed horizontally so that it made contact with a equilibrium buffer solution (PBS). The quartz surface was then loaded with 100 μL of PBS. When the frequency reached equilibrium states, 100 μL of each saliva sample was loaded onto the quartz crystal and real-time changes of resonant frequency were recorded until the signal again reached equilibrium. Each experiment was performed at room temperature and repeated three times.

3. Results

3.1. Determining the cut-off value

As shown in Table 1, all reference samples were classified according to the generated frequency shift. All H3N2 CIV-positive

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