



A novel assay for detecting canine parvovirus using a quartz crystal microbalance biosensor



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ABSTRACT

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Rapid and accurate diagnosis is crucial to reduce both the shedding and clinical signs of canine parvovirus (CPV). The quartz crystal microbalance (QCM) is a new tool for measuring frequency changes associated with antigen–antibody interactions. In this study, the QCM biosensor and ProLinker™ B were used to rapidly diagnose CPV infection. ProLinker™ B enables antibodies to be attached to a gold-coated quartz surface in a regular pattern and in the correct orientation for antigen binding. Receiver operating characteristics (ROC) curves were used to set a cut-off value using reference CPVs (two groups: one CPV-positive and one CPV-negative). The ROC curves overlapped and the point of intersection was used as the cut-off value. A QCM biosensor with a cut-off value of -205 Hz showed 95.4% (104/109) sensitivity and 98.0% (149/152) specificity when used to test 261 field fecal samples compared to PCR. In conclusion, the QCM biosensor described herein is eminently suitable for the rapid diagnosis of CPV infection with high sensitivity and specificity. Therefore, it is a promising analytical tool that will be useful for clinical diagnosis, which requires rapid and reliable analyses.

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1. Introduction

Canine parvovirus (CPV), a member of the genus *Parvovirus* within the family *Parvoviridae*, first emerged as a canine pathogen in the late 1970s (Appel et al., 1978; Kelly, 1978). A few years after the first outbreak, CPV-2 was completely replaced by two antigenic variants, termed CPV-2a and CPV-2b (Parrish et al., 1988). In 2000, a novel mutant of CPV, harboring an amino acid substitution at position 426 (CPV-2c), emerged in Italy and rapidly spread to several countries (Buonavoglia et al., 2001; Decaro and Buonavoglia, 2012). Infection by canine CPV results in a highly contagious enteric disease with a high rate of morbidity and mortality (Larson and Schultz, 2007; Parrish, 1990, 1999).

CPV is a small, non-enveloped single-stranded DNA (ssDNA) virus with a genome of approximately 5.2 kb that encodes two structural (VP1 and VP2) and two non-structural (NS1 and NS2) proteins (Cotmore and Tattersall, 1987). VP2 is the major capsid protein, and plays an important role as an antigenic determinant (Chang et al., 1992; Horiuchi et al., 1994).

Currently, a diagnosis of CPV is based on clinical signs, which include vomiting and diarrhea; however, a definitive diagnosis is difficult because these symptoms are common to other enteric diseases (Elia et al., 2007; Hirasawa et al., 1994). Conventional detection methods such as electron microscopy (Teramoto et al., 1984), virus isolation (Mochizuki et al., 1993), latex agglutination (Veijalainen et al., 1986), hemagglutination (Mochizuki et al., 1993; Teramoto et al., 1984), and enzyme-linked immunosorbent assay (Drane et al., 1994; Teramoto et al., 1984) have been developed for the detection of CPV, and all are effective and accurate. Molecular detection techniques, including PCR (Mochizuki et al., 1993), real-time PCR (Decaro et al., 2005), nested PCR (Hirasawa et al., 1994), and loop-mediated isothermal amplification (Cho et al., 2006; Mukhopadhyay et al., 2012) have also been used to diagnose CPV, although the sensitivity and specificity of these techniques are variable; however, a major drawback is that these methods are both laborious and time-consuming. The key to preventing the spread of CPV is early and rapid diagnosis. Infected dogs can then be isolated and given supportive treatment to reduce both morbidity and mortality. Therefore, a rapid, on-site diagnostic method is required for the early detection of CPV.

The quartz crystal microbalance (QCM) is a nanogram-sensitive technique that utilizes acoustic waves generated by oscillating a piezoelectric, single crystal quartz plate to measure mass (Dixon,

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2008). The piezoelectric effect is due to the electrical charge that accumulates in certain solid materials, such as crystals, in response to an applied mechanical stress. The QCM biosensor measures mass per unit area by measuring the change in the frequency of a quartz crystal resonator, and is a convenient method for detecting antigen-antibody interactions. The QCM biosensor is a “label-free technology” because binding is detected directly without the need for labeled reagents. Many studies have used the QCM biosensor to detect pathogenic microorganisms such as *Vibrio cholerae*, *Bacillus anthracis*, malaria, *Salmonella Typhimurium*, *Leishmania infantum*, and canine influenza virus (Cabral-Miranda et al., 2014; Carter et al., 1995; Cooper et al., 2001; Hao et al., 2009; Ittarat et al., 2013; Kim et al., 2014; Salam et al., 2013).

The aim of the present study was to use the QCM biosensor in conjunction with ProLinker™ B (used to immobilize CPV-specific antibodies onto a gold-coated quartz surface) to develop a suitable method for the rapid and accurate diagnosis of CPV. The change in mass at the quartz crystal surface induced by antigen-antibody interactions generates a measurable change in frequency. The QCM biosensor described herein constitutes a rapid and sensitive method for diagnosing CPV infection in dogs.

2. Materials and methods

2.1. Reference and field samples

The 27 CPV-positive references (virus isolation) were obtained from the Korea veterinary culture collection (KVCC) (<http://kvcc.kahis.go.kr>) (Supplemental Table 1). There were also 40 CPV-negative references, which comprised of 36 CPV-negative fecal samples, two bacterial (*Escherichia coli* and *salmonella*) and two viral (canine coronavirus and canine rotavirus) isolates. All of the CPV-positive and CPV-negative reference samples were confirmed as positive and negative by PCR, respectively. The field samples comprised 261 fecal samples taken from dogs with suspected CPV which were being treated at animal hospitals in Seoul and Gyeonggi province in South Korea from 2012 to 2013. DNA was extracted using a Qiagen DNA extraction kit (Hilden, Germany), according to manufacturer's instructions. All samples were identified as CPV-positive or -negative by PCR using the following CPV-specific primers: forward, 5'-CAAATAGAGCATTGGGCTTACC-3' and reverse, 5'-CAATCTCCTTCTGGATATCTTC-3'. The PCR reaction mixture comprised 11 μ L of DNase-free water, 5 μ L of 5 \times PCR buffer, 1 μ L of each primer, 1 μ L of dNTP mix, 1 μ L of PCR enzyme mix, and 5 μ L of extracted DNA as a template (total volume 25 μ L). The PCR conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension at 72 °C for 10 min.

Supplementary Table 1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2015.03.015>.

2.2. Commercial immunochromatography Ag kit

A commercial immunochromatography Ag kit was purchased from the Bionote Inc (Hwaseong, South Korea). The test device that comes in the kit shows the letters “T” (test line) and “C” (control line) on its surface. The control line should always be visible if the test has been performed properly. A purple “test line” is visible if sufficient CPV antigen is detected. Briefly, fecal samples were placed in sample collection tubes containing assay diluent and mixed vigorously. The tube was then left to stand for 1 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 read after 10 min.

2.3. Measurement of the TCID₅₀

An endpoint dilution assay was used to measure virus titer. Serial dilutions of KVCC-VR0900161 (Supplemental Table 1) were prepared and inoculated onto replicate Crandall Ress feline kidney (CRFK) cell cultures in 96-well plastic plates. The number of infected cell cultures at each virus dilution was then determined by looking for a cytopathic effect (CPE). After an incubation period for 4 days, wells in plates that displayed a CPE were scored as positive. The TCID₅₀ was calculated using the Reed & Muench endpoint method.

2.4. Preparation of the quartz

The quartz was soaked in a solution containing MeOH and 35% HCl (1:1) for 30 min. A freshly prepared piranha solution (a 3:1 mixture of concentrated H₂SO₄ and 30% H₂O₂) was then added for 10 min. The quartz was then thoroughly washed with distilled water and dried under a stream of nitrogen (N₂) gas. The pre-cleaned quartz was then gold-coated by thermal evaporation in a Magnetron Sputtering System (Clotech, Seoul, South Korea) before immersion in a 3 mM ProLinker™ B solution (Proteogen, Chuncheon, South Korea), which contains exposed –SH groups, for 1 h. The quartz was then rinsed sequentially with CHCl₃, EtOH, and distilled water before drying under a stream of N₂ gas.

2.5. Immobilization of monoclonal antibodies onto the gold-coated quartz

A monoclonal anti-CPV IgG antibody was purchased from Median Diagnostics Inc (Chuncheon, South Korea). The monoclonal anti-CPV IgG antibody (50 μ g/mL) was mixed with PBS (1 mL containing 30% glycine) and 50 μ L of the mixture added to the gold-coated quartz and incubated overnight at 4 °C. The quartz was then rinsed twice with PBS and deionized water, and dried under a stream of N₂ gas. The antibody-coated quartz was stored at 4 °C until use.

2.6. Measurement of frequency changes

A X-delic XQ10 instrument (UBTgen, Seoul, South Korea) was used to measure frequency changes in the gold-plated quartz. Briefly, the monoclonal CPV IgG antibody-coated quartz was placed horizontally and loaded with 100 μ L of PBS. When the frequency reached equilibrium, 100 μ L of fecal sample was loaded onto the quartz surface. Real-time changes in 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

All of the CPV-positive ($n=27$) and -negative reference samples ($n=40$) were classified according to their measured frequency changes (Supplemental Table 2) and receiver operating characteristics (ROC) curves constructed (Fig. 1). Reference samples were divided into two groups, namely, CPV-positive and CPV-negative. Although both groups showed clear separation, there was a point of overlap and this point of intersection was used as the cut-off value. The cut-off value was the threshold value that determined whether a sample was identified correctly as positive or negative. According to the ROC, the cut-off value was set at a frequency change of –205 Hz. The diagnostic sensitivity and specificity were calculated as follows: sensitivity = true positive / (true positive + false negative); specificity = true negative / (true negative + false positive). As

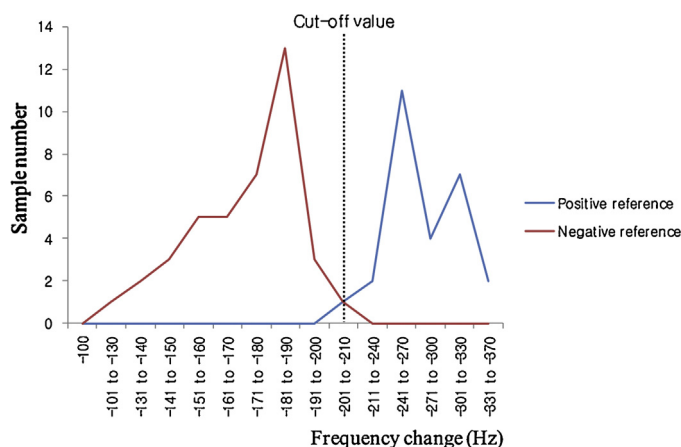


Fig. 1. Determining the cut-off value using canine parvovirus (CPV)-positive and -negative reference samples. All the CPV-positive ($n=27$; blue) and -negative reference samples ($n=40$; red) were classified according to their measured frequency changes and receiver operating characteristics (ROC) curves. The point of intersection between the ROC curves was used as the cut-off value (dotted line).

shown in Supplemental Table 2, a cut-off of -205 Hz yielded a sensitivity of 100% and a specificity of 97.5%.

Supplementary Table 2 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2015.03.015>.

3.2. Identification of the detection limits

A CPV-positive reference sample registered with the KVCC (KVCC-VR0900161) was used to set the detection limit. The $TCID_{50}/mL$ for this sample, as measured by a commercial immunochromatography Ag kit (CIAK) and PCR, was 10^3 and 10^2 , respectively (Table 1). Using the test sample in the QCM biosensor revealed that frequency changes increased gradually as the concentration of the test sample increased (Fig. 2). Frequency changes exceeding the cut-off value (-205 Hz) were generated at a minimum $TCID_{50}/mL$ value of 10^2 .

3.3. Useful life-span of CPV-specific antibody-coated quartz

If the QCM biosensor is to be of practical use in the field, the antibodies immobilized on the surface must retain long-term activity. Therefore, we stored the CPV IgG antibody coated quartz at $4^\circ C$

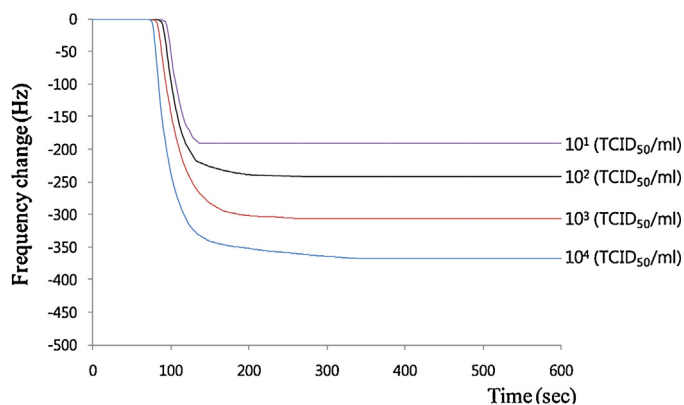


Fig. 2. The frequency change at different canine parvovirus (CPV) concentrations. A CPV standard (KVCC-VR0900161) was tested at concentrations of 10^1 – 10^4 $TCID_{50}/mL$. The frequency change (Hz) measured at each concentration was as follows: 10^1 , -191.5 ; 10^2 , -245.3 ; 10^3 , -299.8 ; and 10^4 , -361 Hz. A frequency change exceeding the cut-off value (-205 Hz) was observed at concentrations above 10^2 $TCID_{50}/mL$.

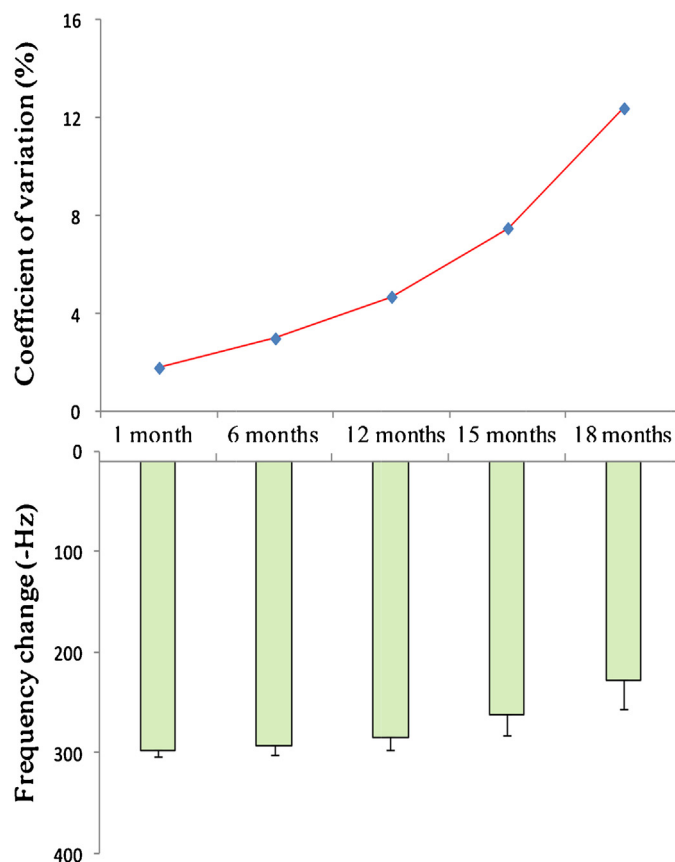


Fig. 3. Measuring the “shelf-life” of the biosensor. Frequency changes and coefficient of variation were measured after storing the biosensor at $4^\circ C$ for 1, 6, 12, 15, and 18 months. The sensor was then reacted with CPV at a concentration of 10^3 $TCID_{50}/mL$ (KVCC-VR0900161). The frequency change and the coefficient of variation were made by graph in a time-dependent manner. Upper panel, coefficient of variation; lower panel, frequency change.

for 1, 6, 12, 15 and 18 months. The diagnostic accuracy at each time point was then tested using KVCC-VR0900161 (Supplemental Table 1) at 10^3 $TCID_{50}/mL$. As shown in Supplemental Table 3, the coefficient of variation (CV) at 1, 6, 12, 15 and 18 months increased gradually (1.8%, 3.0%, 4.7%, 7.5% and 12.4%) in a time-dependent manner. The frequency change and CV at each time point are shown in Fig. 3. A CV $< 5\%$ was maintained for 12 months and thus quartz-immobilized CPV IgG antibody can be stored at $4^\circ C$ for up to 1 year, making them eminently suitable for field use.

Supplementary Table 3 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2015.03.015>.

3.4. Field sample application

All suspected CPV-positive fecal samples were confirmed as CPV-positive or -negative by PCR before application to the QCM biosensor. Of the 261 field samples tested, 41.8% (109/261) were CPV-positive and 58.2% (152/261) were CPV-negative; of the 109 CPV-positive samples confirmed by PCR, CPV-2a, 2b and 2c comprised to 95.4% (104/109), 4.6% (5/109) and 0% (0/109), respectively. The QCM biosensor identified 104/109 as positive (95.4% sensitivity) and 149/152 as negative (98.0% specificity). However, the sensitivity and specificity of the CIAK were 80.7% (88/109) and 89.5% (136/152), respectively.

Table 1
Detection limits of different assays at different CPV concentrations.

Assay	Concentration of CPV (TCID ₅₀ /mL)			
	10 ⁴	10 ³	10 ²	10 ¹
ClIAK	Pos	Pos	Neg	Neg
PCR	Pos	Pos	Pos	Neg
QCM (FC ± SD)	(−361 ± 7.15)	(−299.8 ± 5.87)	(−245.3 ± 6.31)	(−191.5 ± 3.59)

ClIAK, commercial immunochromatography antigen kit; CPV, canine parvovirus; FC, frequency change (Hz); SD, standard deviation; Pos, positive; Neg, negative.

4. Discussion

The gold-coated quartz surface is a relatively new class of device to which biological recognition elements can be attached. When measuring the frequency change that caused the antigen–antibody interaction, it is important that the frequency is highly stable and that background noise is kept to a minimum; this will ensure maximum sensitivity. Several factors may cause frequency drift, including the electrode surface and the viscosity of the liquid (Buttry and Ward, 1992; Tsionsky et al., 1995). Previous studies used silver and gold electrodes as QCM biosensors (Kim et al., 2014; Su et al., 2000); however, gold electrodes produce more consistent results because the surface is less rough than that of silver (Su et al., 2000). The roughness of the electrode surface is critical if frequency drift is to be minimized. Frequency drift is also associated with temperature (Rocklein and George, 2003; Martin et al., 1991; Wang et al., 2005). If the temperature fluctuates, both real mass changes over time and apparent mass changes must be measured concurrently; thus temperature-dependent apparent mass changes must be characterized and minimized to allow the QCM biosensor to provide an accurate readout of the frequency change (Rocklein and George, 2003). Over the last decade, the use of the QCM has been extended to the liquid phase (Bruckenstein et al., 1994). The resonance frequency of the crystal responds to the properties of a contacting liquid. Inconsistencies in liquid viscosity are easily overcome by using PBS or water; thus the electrode surface, the temperature, and the viscosity of the liquid must all be taken into account if frequency drift is to be minimized.

If a QCM biosensor is to have both high sensitivity and specificity, important issues such as antibody orientation on the sensor surface must be resolved. There are two main approaches to preparing the surface on an antibody-based sensor: random antibody immobilization and site-directed antibody immobilization. The simplest technique is to allow random adsorption of the antibody onto the quartz surface. Although simple, this method has major drawbacks, including low stability and lower activity due to random orientation (Buijs et al., 1996; Makaraviciute and Ramanaviciene, 2013). Direct antibody immobilization via a self-assembled monolayer has multiple advantages, namely, that the technique is simple and the covalent bonding means that the surface is both stable and reusable; however, a major drawback is the relatively low sensitivity due to random antibody orientation and the low availability of antibody binding sites (Kausaite-Minkstimiene et al., 2010; Tsai and Pai, 2009). Therefore, site-directed antibody immobilization methods have been developed to address these short-comings (Della Ventura et al., 2011; Hussack et al., 2009; Kwon et al., 2004; Um et al., 2011; Yang et al., 2013; Yoon et al., 2011). A previous study showed that ProLinker™ B, a calixcrown-5 derivative, is able to orient antibodies in a site-directed manner, leading to a more consistent position on the surface and a significant increase in sensitivity for the target antigen (Lee et al., 2003).

As mentioned above, gold electrodes are the preferred carrier for use in QCM biosensors because they generate a stable frequency (Steebhorn and Sklādál, 1997); however, the major drawback is the high unit price of gold and thus gold-coated electrodes must be reusable to minimize the cost of each experiment. The

antigen–antibody interaction is usually irreversible; however, the interaction can be disrupted by chaotropic reagents, organic solvents, or even ultrasonic radiation (Marazuela and Moreno-Bondi, 2002). ProLinker™ B can easily be detached from quartz surfaces using piranha solution (Lee et al., 2003). The gold-coated quartz crystal described herein is reusable, which significantly reduces the cost of the QCM biosensors used to detect CPV.

In conclusion, the QCM biosensor described herein represents a promising analytical tool for use in clinical diagnosis, where rapid and reliable analyses are needed. Therefore, the QCM biosensor is a potential sensitive and specific assay for the rapid and early diagnosis of CPV infection.

Conflicts of interest

The authors have no conflicts of interest to declare.

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