The detection of influenza A and B viruses in clinical specimens using a quartz crystal microbalance

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Abstract

Current methods for the accurate diagnosis of influenza based on culture of the virus or PCR are highly sensitive and specific but require specialised laboratory facilities and highly trained personnel and, in the case of viral culture, can take up to 14 days to obtain a definitive result. In this study, a quartz crystal microbalance-based immunosensor (QCM) has been developed and its potential evaluated for the rapid and sensitive detection of both influenza A and B viruses in laboratory-cultured preparations and clinical samples. The effective limit for detection by QCM for stock preparations of both A/PR/8/34 and B/Lee/40 viruses was 1 x 10^4 pfu/mL, associated with observed frequency shifts of 30 (± 5) and 37 (± 6.5) Hz respectively. Conjugation of 13 nm gold nanoparticles to the detecting antibody improved the mass sensitivity of the immunosensor, resulting in a 10-fold increase in sensitivity and a detection limit of 1 x 10^3 pfu/mL for both preparations, with resulting frequency shifts of 102 (±11) and 115 (±5) Hz respectively. Detection of virus in nasal washes with this technique was achieved by overnight passage in MDCK cultures prior to analysis. A comparison of results obtained from 67 clinical samples using existing RT-PCR, shell vial, cell culture and ELISA methods showed that QCM techniques were comparable in sensitivity and specificity to cell culture methods.

Keywords

Influenza

Quartz Crystal Microbalance

Detection

Immunosensor

1. Introduction

The contribution of diagnostics to the management of patients with viral infections has increased considerably in the last decade, led by improved technologies that allow rapid, accurate and sensitive diagnosis of viral pathogens for an increasing range of infections. Rapid diagnosis allows the prompt initiation of effective antiviral therapy, especially in immunocompromised patients. Viral culture, usually in combination with immunofluorescence, has been regarded as the *gold standard* for the laboratory diagnosis of respiratory viruses (Doing et al., 1998; Johnston and Siegel, 1991). However, these tests are not rapid and their clinical value is often limited. In the case of influenza viruses, the isolation and identification by culture requires 2-14 days for the diagnosis of an illness whose duration is typically 5-7 days (Covalciuc et al., 1999). Rapid antigen detection tests (≤ 1hr) are less sensitive and sometimes less specific than culture or molecular methods but, nevertheless, can serve as a guide for appropriate treatment with antiviral agents (Storch, 2003). PCR and real-time-PCR techniques are highly sensitive, relatively specific and more rapid than cell culture (Atmar et al., 1996; Kehl et al., 2001; Liolios et al., 2001; Templeton et al., 2004; van Elden et al., 2002) and are widely used for the diagnosis of respiratory viruses but are more complex and require highly trained personnel. False-positives can result due to the high sensitivity of the PCR amplification system (Storch, 2003; Su et al., 2003). Accordingly, the need for rapid detection methods with high sensitivity and specificity that are easy to perform and interpret remains a research priority (Su et al., 2003).

Considerable effort has been directed towards the development of simple biosensors for the detection of viruses in point-of-care tests (Critchley and Dimmock, 2004; Eun et al., 2002; Hardy and Dimmock, 2003; Su et al., 2003; Wu et al., 2005; Zhou et al, 2002). Biosensors which detect interactions between viral antigens and specific antibodies (immunosensors) can be classified according to the type of transducer used in the device (Eun et al., 2002; Mecea, 2005). Piezoelectric sensors, such as the quartz crystal microbalance (QCM), detect mass changes due to molecular interactions on the surface of the transducer (Gajendragad et al., 2001; Vaughan et al., 1999). The application of an external electrical potential to a piezoelectric material, such as quartz, produces internal mechanical stresses that induce an oscillating electric field which, in turn, initiates an acoustic wave throughout the crystal. These waves travel in a direction perpendicular to the plate surfaces (Ebato et al., 1994; Janshoff and Steinem 2001; Mecea, 2005).

Sauerbrey (Sauerbrey, 1959) first described the relationship between observed frequency decrease (Δf) and deposited mass (m) on the crystal surface in air or a vacuum (Mecea, 2005; Sauerbrey, 1959; Vaughan et al., 1999). The frequency changes observed when a liquid is passed over the QCM crystal surface are also dependent on both the density and viscosity of the solution (Kanazawa and Gordon, 1985). QCM devices are relatively simple and convenient to use and can detect rapid, real-time responses to binding events on the crystal surface, such as antigen-antibody interactions (Lee and Chang, 2005; Park et al., 2003; Skládal et al., 2004; Uttenthaler et al., 1998), and have been applied to several areas in biotechnology including clinical diagnosis (Janshoff and Steinem, 2001; Nath and Chilkoti, 2002)

and environmental monitoring (Kurosawa et al., 2006). More recently, a new approach has been described to improve the sensitivity of QCM biosensors by the use of antibody-functionalized nanoparticles as a mass amplification probe for a QCM sensor, resulting in significant enhancement of sensitivity (Chu et al., 2006). In the current study, a QCM-based immunosensor was developed and evaluated for possible application in the rapid and sensitive detection of influenza viruses in clinical specimens, and compared with other commonly used diagnostic techniques.

2. Materials and Methods

2.1 Clinical specimens.

Nasal wash samples were collected from 67 hospital patients with acute signs and symptoms during the Australian winter of 2005 and provided by two Australian national reference laboratories: (a) Victorian Infectious Diseases Reference Laboratory (VIDRL), Melbourne, Australia and (b) WHO Collaborating Center for Reference and Research on Influenza, Melbourne, Australia.

2.2 Cells and viruses.

Cultures prepared from cells of the Madin-Darby canine kidney (MDCK) line (obtained from CSL Ltd, Parkville, Australia) were used in all studies and were grown in Eagle's Minimal Essential Medium (MEM) containing 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid), 0.14% (w/v) sodium bicarbonate, 100 U/mL penicillin G, 100 μg/L streptomycin and 1 μg/L amphotericin B, supplemented with fetal calf serum to 5% (v/v) pH 7.2 in a 5% (v/v) CO_2 incubator at 37°C. Human influenza A/PR/8/34 (H1N1) and B/Lee/40 viruses were

included in the study for reference purposes and were grown in 10-11 day-old embryonated hens' eggs.

2.3 Virus purification.

Allantoic fluid containing virus was clarified by centrifugation at 3000 × *g* for 5 min. The supernatant fluid was then centrifuged at 160 030 × *g* for 60 min at 4°C using an SW41 Ti rotor in a Beckman Optima™ L-80 XP Ultracentrifuge (Beckman Coulter Inc., Fullerton, USA) which was used for all subsequent ultracentrifuge separations. The virus pellet was resuspended in 500 μL of TNE (0.05M Tris-HCl pH 7.4 containing 0.15 M NaCl, 1mM EDTA) buffer. Concentrated virus was centrifuged to a 60% (w/v) sucrose cushion through a 30% (w/v) sucrose interface at 160 030 × *g* for 90 min. Virus was collected by aspiration and was then diluted 1:5 in TNE buffer and pelleted by centrifugation at 160 030 × *g* for 60 min. It was then resuspended in TNE buffer and centrifuged through a 15-60% (w/v) sucrose gradient at 160 030 × *g* for 12 h at 4°C. The virus band was diluted 1:5 in TNE buffer and centrifuged at 160 030 × *g* for 60 min at 4°C. The pellet was resuspended in TNE buffer and stored at –70°C.

2.4 Pre-treatment of clinical specimens

A volume of 50 μL of each nasal wash sample from patients was incubated in duplicate tubes containing confluent MDCK cultures in 1 mL of serum-free MEM growth medium containing 1 μg/mL trypsin and incubated overnight at 34°C in a 5% (v/v) CO_2 incubator. The samples were centrifuged at 5000 × g for 20 min prior to collecting the supernatant fluid for QCM analysis.

2.5 Plaque assays.

These were performed in 6-well confluent MDCK monolayer cultures, as previously described (Tannock et al., 1984).

2.6 Shell vial assays.

Twenty four-hour MDCK cell cultures were prepared on the surfaces of circular 12 mm coverslips in shell vials and washed twice with PBS prior to adding 50 μL of a fresh clinical sample to each cover slip. The shell vials were then centrifuged at 700 × *g* for 60 min and, after adding 1 mL of MEM containing 1 μg/mL trypsin (bovine pancreas, ICN Biomedicals, Seven Hills NSW, Australia), were incubated for 48 h at 34°C in a 5% (v/v) CO_2 incubator. The cultures were then washed twice with PBS and fixed by adding 1 mL of chilled 80% (v/v) acetone in PBS for 15 min at 4°C. Finally, the cultures were stained by indirect immunofluorescence (IFA) with monoclonal antibodies (MAbs) specific for nucleoprotein of influenza A and B viruses (Bartholoma and Forbes, 1989) followed by FITC-labeled goat anti-mouse antibody (Chemicon International Inc., Temecula, USA).

2.7 Directigen® FLU-A enzyme immunoassay.

The Directigen® influenza A *in vitro* enzyme immunoassay (Becton, Dickinson and Co., Sparks, USA) was used for the rapid detection of influenza A antigen from nasal washings, according to the manufacturer's instructions.

2.8 Antigen ELISA.

An antigen ELISA was developed using the method described by Bucher et al., (Bucher et al., 1991). The wells of a 96 well Immulon 2HB microtiter plate (Thermo

Scientific, Milford, USA) were coated by adding MAbs specific for the M1 protein of influenza A or B viruses (4-8 μg/100 μL in 0.1M sodium carbonate buffer pH 9.6) to each well and leaving overnight at 4°C. Unattached antibody was removed by washing and dilutions in PBS containing 50 μL of purified A/PR/8/34 or B/Lee/40 (initial concentration 100 μg/mL) were added to individual wells. Then 50 μL of a 1:1000 dilution of chicken antisera prepared against a purified preparation of the M1 protein of either A/PR/8/34 or B/Lee/40 were used as primary antibodies. After washing, 50 μL of a 1:2000 dilution of goat anti-mouse-HRP conjugate (Chemicon International Inc., Temecula USA) was used as the secondary detection antibody. Following color development using 3,3',5,5' tetramethyl-benzidine (TMB, Becton, Dickinson and Co., Sparks, USA) as the substrate, absorbance values were determined using an ELISA plate reader (Dynatech MR 7000, Dynatech Laboratories, Chantilly, USA) set at 450 nm.

2.9 RT-PCR.

Viral RNA was extracted from allantoic fluid or nasal wash samples using a QIAamp® Viral RNA Mini Kit (Qiagen, Hamburg GmBH). RT-PCR was performed using the Titan™ One Tube RT-PCR system (Roche Diagnostics, Mannheim GmBH) according to the manufacturer's instructions. Egg-grown influenza A/PR/8/34 (H1N1) and B/Lee/40 viruses and all nasal samples were tested by RT-PCR using the method described by Poddar (Poddar, 2002).

2.10 QCM sensor system.

A Maxtek RQCM sensor coupled to a flow injection system was used with 25 mm 9 MHz quartz crystals mounted in a Maxtek CHC-100 crystal holder (Maxtek Inc.,

Cypress, USA). An oscillator/frequency counter collected the output signal of the oscillator and outputs measured frequency changes (Δf), resistance changes and changes in mass. The flow injection system consisted of a fluid circuit with a flow-through cell (FC-550 Flow Cell, Maxtek Inc., Cypress, USA), Rheodyne injection switching valve (Model 5020, Rheodyne LLC, Rohnert Park, USA) and a Razel syringe pump (Model A-99, Razel Scientific Instruments Inc., Stamford USA). A schematic diagram of the apparatus used in this work is shown in Fig. 1. All experiments were carried out at room temperature.

2.11 Pre-treatment of QCM crystals.

The piezoelectric quartz crystal was immersed in 1.2 N NaOH for 30 min to remove impurities from the crystal surface and then washed with distilled water, air dried and placed for 5 min in 1.2 N HCl. It was then washed with distilled water and ethanol and dried in a stream of nitrogen (Park et al, 2000).

2.12 Immobilization of antibodies on the quartz crystal.

One hundred microlitres of Protein A dissolved in PBS (2 mg/mL) were added to the gold electrode of the quartz crystals and incubated at room temperature in a humid environment for 2 h. Analysis of the binding characteristics of the anti-M1 MAbs in ELISA experiments indicated that optimum binding was achieved at 8 μg/mL of the MAb specific for the M1 protein of A/PR/8/34 and 4 μg/mL for that of B/Lee/40. Protein A-coated crystals were incubated with 100 μL of each antibody solution at these concentrations for 1 h at 37^{o}C in a humidified chamber, rinsed with PBS and distilled water, and dried in a stream of nitrogen.

2.13 Preparation of gold nanoparticles.

Gold nanoparticles were prepared using a modification of the method of Grabar et al., (Grabar et al, 1995). A total of 190 mL of 5×10^{-3} M of gold (III) chloride trihydrate ($HAuCl_4.3H_2O$) in distilled water was brought to the boil with vigorous stirring in a 500 mL round-bottomed flask. Following the addition of 10 mL 0.5% (w/v) of trisodium citrate, the solution was boiled for an additional 10 min and allowed to cool while stirring for a further 15 min. The colloidal solution was stored in dark bottles at 4°C. All glassware used was thoroughly cleaned in aqua regia ($HCl:HNO_3$, 3:1), rinsed in distilled water and oven-dried prior to use.

2.14 Preparation of the antibody-nanoparticle conjugates.

Antibody-nanoparticle conjugates were prepared by the addition of 30 μg of MAb specific for the M1 protein of either A/PR/8/34 or B/Lee/40 to 1 mL of a gold nanoparticle suspension containing 1.4×10^{12} nanoparticles in 5 mM sodium carbonate buffer pH 9.0, followed by incubation at room temperature with gentle mixing for 1 h. Then 200 μL of 5% (w/v) BSA in PBS were added and mixed for 30 min at room temperature to block free binding sites on the nanoparticles and to stabilize the nanoparticle suspension. The antibody-nanoparticle conjugate was centrifuged at 10 000 × *g* for 10 min. The supernatant fluid containing unbound antibody was discarded and the red-coloured antibody-nanoparticle conjugate pellet gently resuspended in 1 mL of TBS-BSA (20mM Tris pH 8.0 containing 150 mM NaCl, 0.1% (w/v) BSA). Finally, after a second centrifugation step at 10 000 × *g* for 10 min, the supernatant fluid was removed and the antibody-colloidal gold conjugate pellet resuspended in 100 μL of TBS-BSA and stored at 4°C (Chu et al., 2006).

3. Results

3.1 Determination of the sensitivity of the QCM for the detection of A/PR/8/34 and B/Lee/40.

The resonance frequency shift of a QCM sensor is influenced by many factors, such as changes in mass, viscosity, dielectric constant of the solution and the ionic status of the crystal interface with the buffer solution (Kim et al., 2004; Mecea, 2005; Park et al., 2000). Accordingly, a number of parameters, including efficiency of the blocking buffer, the pH of the reaction buffer, the flow rate, the concentration of antibody and the orientation of the antibody in the presence of Protein A were optimized to determine the limits of detection for A/PR/8/34 and B/Lee/40 (data not shown).

Following immobilization of the MAb to the crystal electrode, dilutions of either A/PR/8/34 or B/Lee/40 (1×10^3 - 1×10^8 pfu/mL) in PBS were injected continuously onto the immunosensor at a constant flow rate of 3 mL/h using a syringe pump. The flow path of the sensor was rinsed with PBS prior to loading of each virus dilution analysed. Typical recordings of frequency changes observed with the binding of either virus are shown in Fig. 2 (A and B), which shows a frequency shift in the range 15-493 Hz for A/PR/8/34 and 16-410 Hz for B/Lee/40 viruses respectively, and was dependent on the titer of the virus added. The data show frequency changes (ΔHz) after introducing the virus samples to the sensor surface for 60 min post-injection. These data suggest that the effective minimum detection limit for both A/PR/8/34 and B/Lee/40 was 1×10^4 pfu/mL, which was associated with frequency shifts of 30 ($\pm$5) and 37 ($\pm$6.5) Hz, respectively (n=3).

3.2 Use of nanoparticles to increase the sensitivity of the biosensor.

Gold nanoparticles conjugated with antibodies were used in an attempt to increase the sensitivity of the QCM immunosensor for the detection of influenza viruses. The diameter of the nanoparticles used was determined to be 13 nm by transmission electron microscopy (TEM). Conjugation of the anti-influenza MAbs to nanoparticles was confirmed by visible absorbance measurements, which showed that unconjugated nanoparticles had an absorbance maximum (λ_{max}) at 520 nm that increased to 527 nm following conjugation. This wavelength shift of 7 nm indicates that protein was bound to the surface of the nanoparticles resulting in an increased primary gold plasmon resonance peak (Nath and Chilkoti, 2002).

3.3 Frequency changes observed with the antibody-nanoparticle conjugates.

Following attachment of antibody to the quartz crystal, purified A/PR/8/34 and B/Lee/40 preparations (titers 1×10^3 -1×10^8 pfu/mL in PBS) were injected at a flow rate of 3 mL/h for 30 min. Then PBS was passed through for 15 min to remove unbound antigen, followed by injection of the nanoparticle-MAb conjugates specific for influenza A or B viruses. Frequency changes following the binding of the MAb-nanoparticle conjugates to the cognate antigen were recorded for 60 min. Typical sensor profiles for purified A/PR/8/34 and B/Lee/40 are shown in Fig. 2 (C and D). From this data, the lowest detectable infectious titer for purified preparations of both A/PR/8/34 and B/Lee/40 was 1×10^3 pfu/mL (10 μg/mL) corresponding to frequency shifts of 102 ($\pm$11) and 115 ($\pm$5) Hz, respectively (n=3). Negative control background measurements in the absence of virus were 65 ($\pm$10) Hz for each nanoparticle-antibody conjugate preparation used.

Comparisons of the frequency shift responses to different viral dilutions in the range 1×10^3 - 1×10^8 pfu/mL, determined in both the presence and absence of nanoparticles are summarized in Table 1. These results support those shown in Fig. 2 (C and D) that the conjugation of nanoparticles with virus-specific MAbs substantially increases the sensitivity of the assay. Use of the nanoparticle-antibody conjugates resulted in 5.8-fold and 6.7-fold increases in frequency shift responses in the detection of 1×10^3 pfu/mL of A/PR/8/34 and B/Lee/40 respectively, compared with measurements taken without the use of nanoparticles. At higher titers, these differences in sensitivity were less; at 1×10^7 pfu/mL, increases in frequency shift of 0.2-fold were observed for A/PR/8/34 and 0.15-fold for B/Lee/40 (Table 1; Fig. 2).

3.4 Relationship between infectious titer and observed frequency change.

Fig. 3 shows the relationship between the infectious titer of A/PR/8/34 and B/Lee/40 and the frequency changes recorded in semi-logarithmic plots. This analysis indicates an approximate linear relationship between virus concentration and observed frequency change for each virus. The frequency changes were observed with both viruses in the titer range of 1×10^3 - 1×10^8 pfu/mL lie within the calibration range, thus allowing an estimation of A/PR/8/34 and B/Lee/40 virus titers in unknown samples.

3.5 Detection of influenza viruses in nasal washings.

Dilutions of purified A/PR/8/34 (1×10^3 - 1×10^8 pfu/mL) were used to spike a 20% (v/v) nasal wash specimen in PBS from a normal asymptomatic volunteer. The diluted sample was mixed and centrifuged at 5000 × *g* for 20 min and the supernatant collected for analysis. Significant noise in the frequency response was observed in

all samples, including the negative control that consisted of the sample spiked with the same volume of PBS without virus (data not shown). Therefore, direct detection of the virus from diluted nasal washes by this technique does not appear practicable. The signal noise resulting from testing of the clinical samples could not be eliminated by simple pre-treatment of clinical samples either by microfiltration through 0.2 μm and 0.45 μm Anodisc filters (Whatman plc Kent, UK) or by overnight digestion with up to 15 mg/mL (600 000 units) chicken egg white lysozyme (Sigma Aldrich, St Louis, MO, USA) followed by centrifugation at 5000 × *g* for 20 min (data not shown).

However, when the same samples were inoculated into MDCK cell cultures and incubated overnight prior to QCM analysis, significant and stable signal frequency shifts for the culture maintenance fluids of positive samples were observed in the range 10^3–10^9 copies of M1 cDNA/mL (Fig 4). For stocks of purified A/PR/8/34, 1 pfu/mL was equivalent to $10^{4.54}$ copies/mL and $10^{4.46}$ copies/mL for B/Lee/40 (44). These signals appeared in the absence of a visible CPE in infected cultures and the procedure was used in all subsequent evaluations of the QCM technique on clinical samples.

3.6 Detection of influenza viruses by currently available methods and QCM.

The results from these tests on the 67 clinical samples are summarized in Table 2. Based on data from Fig. 2, the readout for determining positive samples was 40 Hz for the QCM analyses that did not involve the use of conjugated nanoparticles and 120 Hz for the QCM analyses when nanoparticles were used. Influenza A and B viruses were detected by RT-PCR, shell vial assay, cell culture and ELISA in 52, 40,

45 and 34 of the 67 clinical specimens, respectively. The Directigen Flu A test detected influenza A virus in only 13 specimens. The QCM and nanoparticle-enhanced QCM (NP-QCM) tests detected influenza A and B viruses in 40 and 43 specimens, respectively. The influenza viruses present in the clinical samples were A/New Caledonia/20/99–like (H1N1), A/Wyoming/3/2003-like (H3N2); influenza B viruses were B/Brisbane/32/2002–like. However, 12, 7 and 18 RT-PCR-positive specimens were negative by the shell vial, cell culture and ELISA methods for influenza A and B viruses, respectively. Twelve and nine of the 52 samples positive by the RT-PCR technique were negative by QCM and NP-QCM methods.

As shown in Table 3, the respective sensitivities of the tests for the detection of influenza A and B viruses by the shell vial, standard cell culture, ELISA, Directigen Flu A test, QCM and NP-QCM tests were 76 and 80, 81 and 100, 68 and 60, 35 (for influenza A only) and 76 and 80 and 81 and 87% for influenza B viruses (Table 3). The specificity, positive and negative predictive values for the detection of each virus are shown in Table 3. The highest number of false-negative results (24) was obtained with the Directigen Flu A test.

4. Discussion

This study was conducted in light of a continuing need for rapid sensitive methods for the identification and antigenic characterization of influenza viruses for epidemiological purposes and for the selection of vaccine strains. A QCM-based immunosensor was developed and evaluated for the rapid detection of both influenza A and B viruses in 67 nasal wash samples obtained during the 2005 Australian winter. Their sensitivity was compared with PCR, the shell vial and standard cell

culture methods, an antigen ELISA and the commercially available Directigen Flu A kit (for influenza A viruses only).

One of the most important factors that affects the sensitivity of the QCM sensor is the optimal concentration of antibody immobilized on the crystal surface which leads to improved reaction kinetics and the avoidance of unfavourable effects such as minimal non-specific binding (Jenkins et al., 2004; Luppa et al., 2001). In this study, the optimum monoclonal antibody concentration immobilized on the QCM for maximum sensitivity was 8 μg/mL for A/PR/8/34 and 4 μg/mL for B/Lee/40 viruses. These results are consistent with the optimized concentrations that were used in the ELISA assays (data not shown). Once the optimal antibody concentration was determined, the antigen concentration was varied to determine the optimal antigen:antibody ratio to maximize the sensitivity of the sensor.

The results presented in Fig. 2 show that the infectious titer of the virus in the sample was directly proportional to the mass accumulation on the immobilized monolayer which was, in turn, proportional to the rate of frequency shift recorded. Results obtained with different concentrations of purified viruses in Fig. 2 (A and B) showed that the lowest detectable infectious titer of both purified A/PR/8/34 and B/Lee/40 viruses was 10^4 pfu/mL (25 μg/mL). Using a similar approach with Protein A-immobilized anti-virus antibodies, König and Grätzel (König and Grätzel, 1994) obtained detection limits of $5x10^4$ pfu/mL for of human herpes viruses. However, Zuo et al. (Zuo et al., 2002) were able to detect as little as 0.6-4 μg/mL of the SARS coronavirus (SARS-CoV) in sputa in a gas phase using a QCM sensor coated with Protein A and polyclonal antibody.

In this study, anti-influenza A and B monoclonal antibodies were bound to 13 nm gold nanoparticles and these conjugates were used to improve the mass sensitivity of the sensor for influenza detection. As shown in Fig. 2 (C and D) and summarized in Table 1, the lowest detectable viral infective titer of purified A/PR/8/34 and B/Lee/40 was 10^3 pfu/mL (10 μg/mL), a 10-fold increase in sensitivity for the QCM sensor using nanoparticle-bound anti-influenza A and B monoclonal antibodies. Given that viral titres in nasal wash samples of symptomatic patients are usually 10^3 pfu/mL or greater (35, 39), this step clearly increases the potential of such biosensors for the detection of influenza virus in clinical samples.

However, the data presented in Fig. 2 (C and D) and summarized in Table 1 indicate that the use of nanoparticles did not result in a proportional increase in the sensitivity of the QCM sensor for the detection of influenza viruses with in samples with high infectious titers. Maximum enhancement of the sensitivity was observed only in the range 10^3-10^4 pfu/mL, suggesting that the adsorption of excess antibody–colloidal gold conjugate on the electrode surface leads to steric hindrance effects (Chu et al., 2006).

In order to quantitate viruses detected by the QCM sensor, calibration curves were established for both the QCM and NP-QCM techniques (Fig. 3). These curves describe the relationship between the frequency shift and the titer of the influenza virus suspension injected on to the sensor. When these data are plotted on a logarithmic-linear scale, an apparent linear relationship between amount of antigen and frequency shift was observed. A similar relationship was observed when nanoparticle-conjugated antibodies were used instead of antibody alone. Based on

this apparent linear semi-logarithmic relationship between frequency shift and virus concentration, and with the use of nanoparticle-antibody conjugates, it should be possible to use this calibration curve to determine unknown viral titers for samples stored under optimal conditions within the range 10^3–10^7 pfu/mL. (Fig. 3).

The basic parameters developed in this study for the QCM-based immunosensor were subsequently applied to the detection of influenza A and B viruses in clinical specimens. In this study, the capacity of the QCM and the NP-QCM methods to detect influenza viruses in the nasal washes of patients with influenza-like symptoms were compared with other currently used methods, including RT-PCR, standard cell culture, shell vial, ELISA and the Directigen Flu A (for influenza A virus only) methods.

QCM offers a number of potential advantages over existing techniques, which include obviating the need for labeling techniques to measure the binding reaction between virus and antibody, the use of short measurement times, operational simplicity, low cost, the opportunity to re-use the crystal sensors and the potential for online data collection. The QCM technique developed in this study was shown to be as sensitive as the shell vial method. The NP-QCM technique was more sensitive than the shell vial method and equally as sensitive as cell culture (Table 2), but required an additional step involving the use of a secondary nanoparticle-antibody conjugate. Direct detection of the virus from the nasal wash samples by QCM was not possible because of significant frequency fluctuations that occurred, possibly due to the viscosity of the samples. The heterogeneous nature of clinical samples appears to be an inherent limitation for diagnostic tests utilising piezoelectric immunosensors (Wu et al., 2005). Attempts to reduce the viscosity of the nasal wash samples of

infected patients by centrifugation or microfiltration prior to analysis on our immunosensor proved unsuccessful (data not shown). However, Zuo and colleagues (Zuo et al., 2004) overcame this limitation by the atomisation of sputum samples containing SARS-CoV by ultrasonication prior to adsorption and detection on the immunosensor. In this initial study, an additional overnight culture step was used to overcome the viscosity effects of sputum samples resulting in an increased processing time of 2 days.

The sensitivities of tests for detection of influenza A and B by the shell vial, standard cell culture, ELISA, Directigen Flu A test, QCM and NP-QCM tests were 76 and 80, 81 and 100, 68 and 60, 35 (influenza A), 76 and 80 and 81 and 87%, respectively (Table 3). These results differ from those of Quach *et al*., (Quach et al, 2002) who reported sensitivities between 64.2 and 84.7% and specificities of 90 to 100% compared with RT-PCR. However, in the present study, frozen samples were used and virus in newly collected samples could be expected to be more readily detected since proteolysis or denaturation of influenza antigens has been previously observed in stored nasal washes (Quach et al, 2002).

When the data for influenza A and B were combined, the sensitivities of each method were, in descending order, RT-PCR (100%), NP-QCM (83%), QCM (81%), standard cell culture (81%), shell vial (77%) and ELISA (69%) (Table 3). The times required to complete each test were: Directigen Flu A 20 min, RT-PCR 1 day, ELISA 1 day, shell vial 2 days, QCM and NP-QCM 2 days and the standard cell culture method up to 14 days (including the time for repassage of initially negative samples). Egg-grown preparations of A/PR/8/34 and B/Lee/40 could be detected by the QCM and NP-QCM methods within 30 min and 1 h, respectively.

Despite requiring a similar time interval (2 days) to complete the test that involves blind passage of clinical specimens, the NP-QCM method described is more economical, and simpler, more sensitive and objective in interpretation than the widely used Shell Vial Assay, often regarded as the 'gold standard' which requires the use of IFA, and cell culture. With further refinements, especially pre-treatment of samples to reduce viscosity effects, the direct analysis of patient specimens would greatly increase the utility of this immunosensing technique and save considerable time in a clinical setting.

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Figure Legends

Fig. 1. Schematic of the apparatus used for continuous flow detection of influenza using a quartz crystal microbalance.

Fig. 2. Time-dependent frequency changes at different virus concentrations. (A) Frequency changes of the immunosensor using A/PR/8/34 virus. (B) Frequency changes of the immunosensor using B/Lee/40 virus. (C) Nanoparticle-conjugated anti-influenza A monoclonal antibody (A/PR/8/34). (D) Nanoparticle-conjugated anti-influenza B monoclonal antibody (B/Lee/40). 10^8 pfu/mL, 10^7 pfu/mL, 10^6 pfu/mL 10^5 pfu/mL, 10^4 pfu/mL, 10^3 pfu mL, Negative

Fig. 3. Relationship between influenza virus concentration and frequency shifts in the presence and absence of nanoparticles. (A) A/PR/8/34. (B) B/Lee/40.

- Without nanoparticles
- With nanoparticles

Fig. 4. Time-dependent frequency changes of clinical samples in cell culture medium (A) Frequency changes of clinical samples (without nanoparticles). (B) Frequency changes of clinical samples using nanoparticle-conjugated influenza A monoclonal antibody. 10^9 copies/mL, 10^6 copies/mL, 10^5 copies/mL, 10^4 copies/mL, 10^3 copies/mL, negative

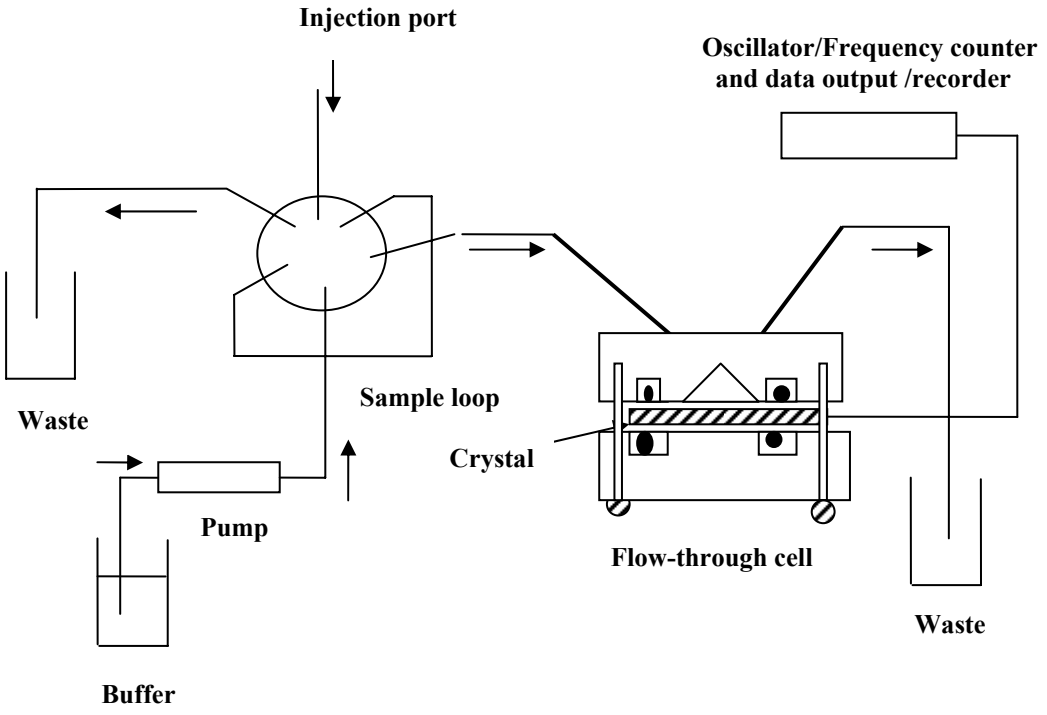

Injection port
Oscillator/Frequency counter
and data output /recorder
Waste
Sample loop
Crystal
Pump
Flow-through cell
Waste
Buffer

Figure 1

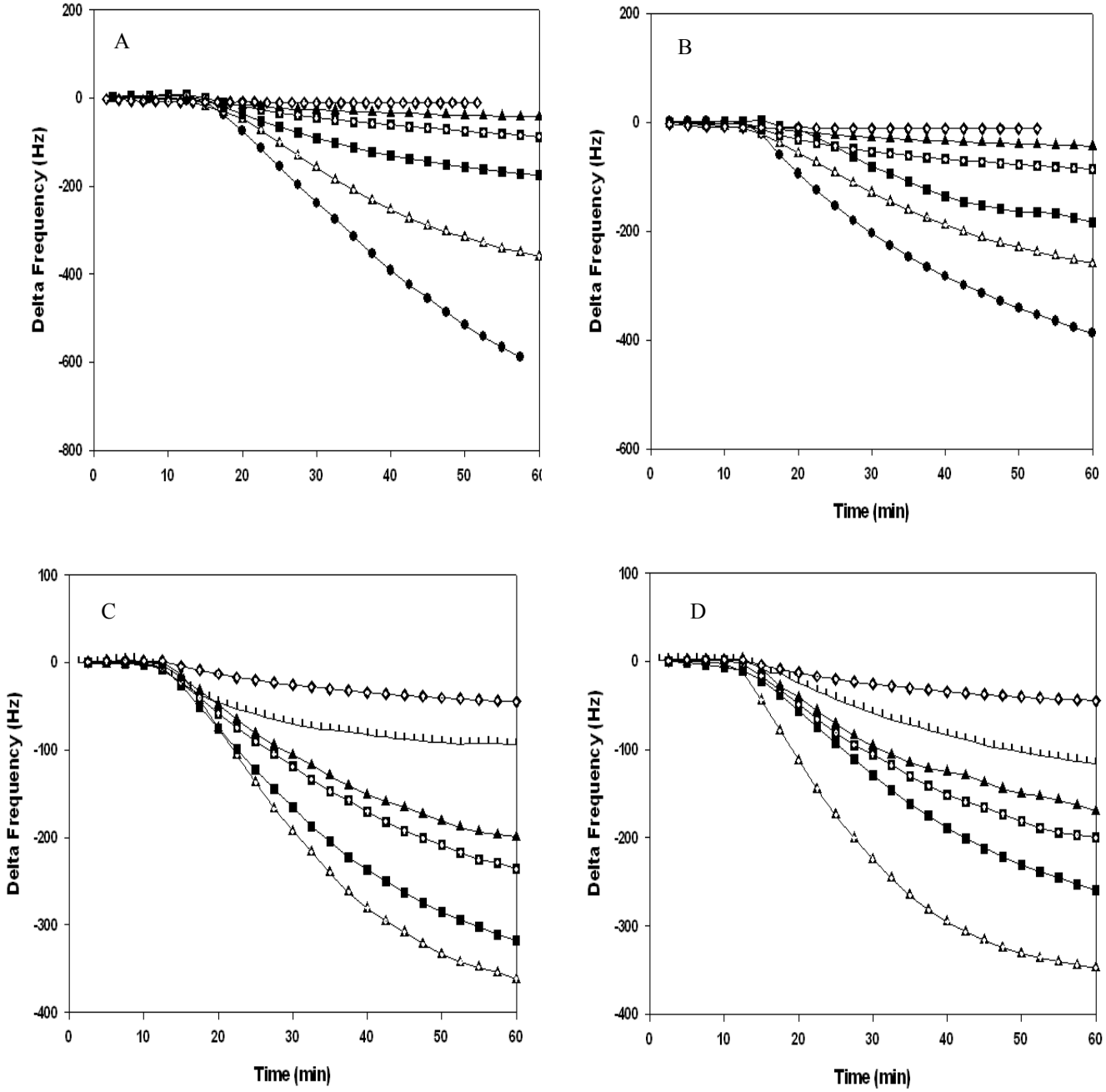
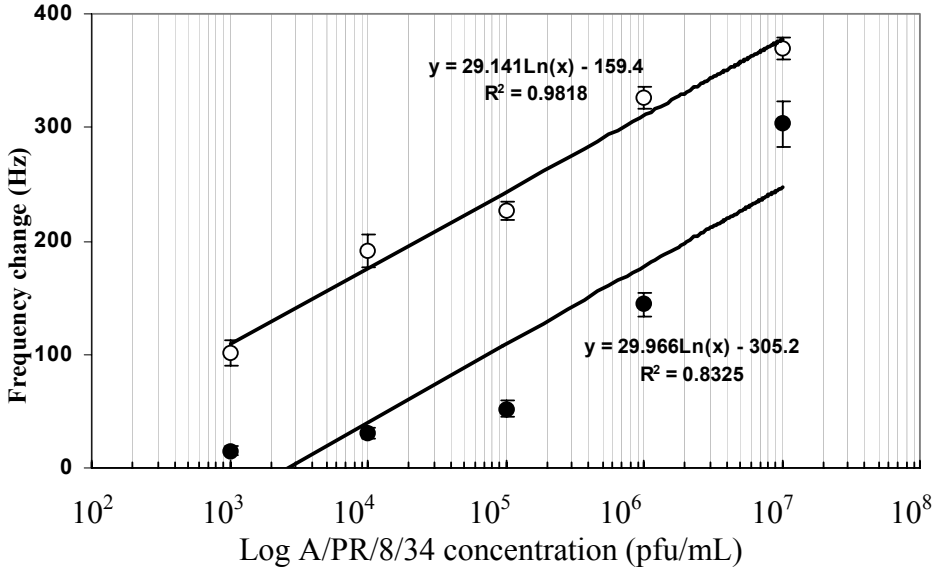


Figure 2.


y = 29.141Ln(x) - 159.4
R² = 0.9818
y = 29.966Ln(x) - 305.2
R² = 0.8325
Frequency change (Hz)
Log A/PR/8/34 concentration (pfu/mL)

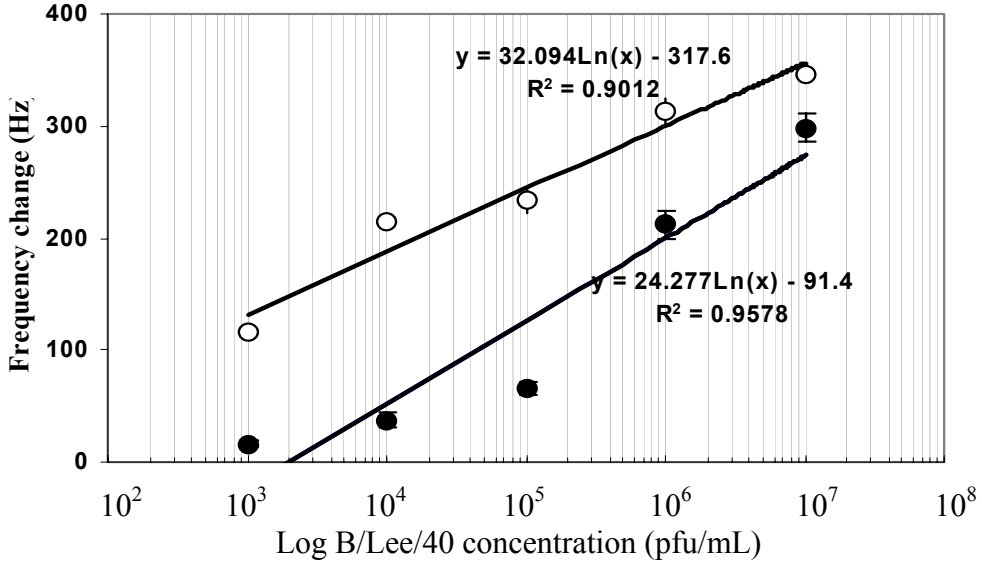
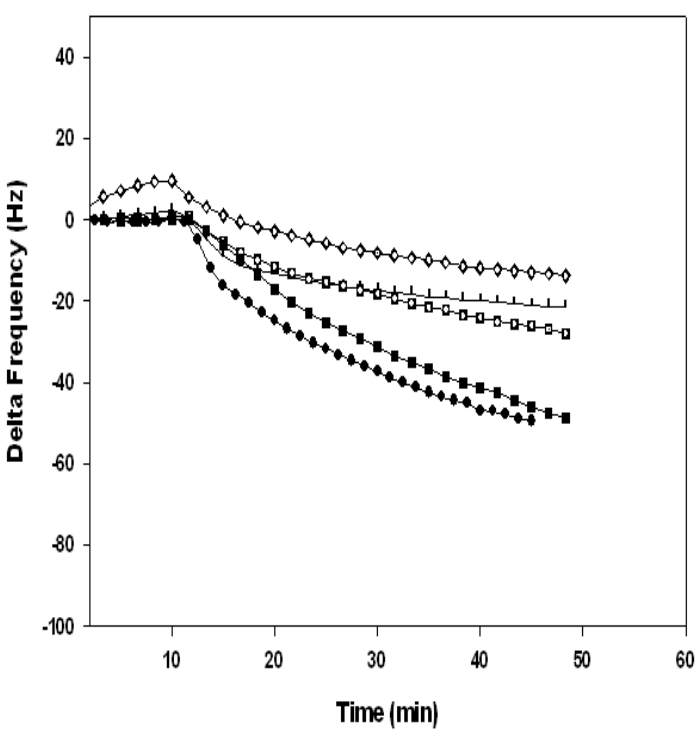

y = 32.094Ln(x) - 317.6
R² = 0.9012
y = 24.277Ln(x) - 91.4
R² = 0.9578
Frequency change (Hz)
Log B/Lee/40 concentration (pfu/mL)

Figure 3


Delta Frequency (Hz)
40
20
0
-20
-40
-60
-80
-100
10
20
30
40
50
60
Time (min)

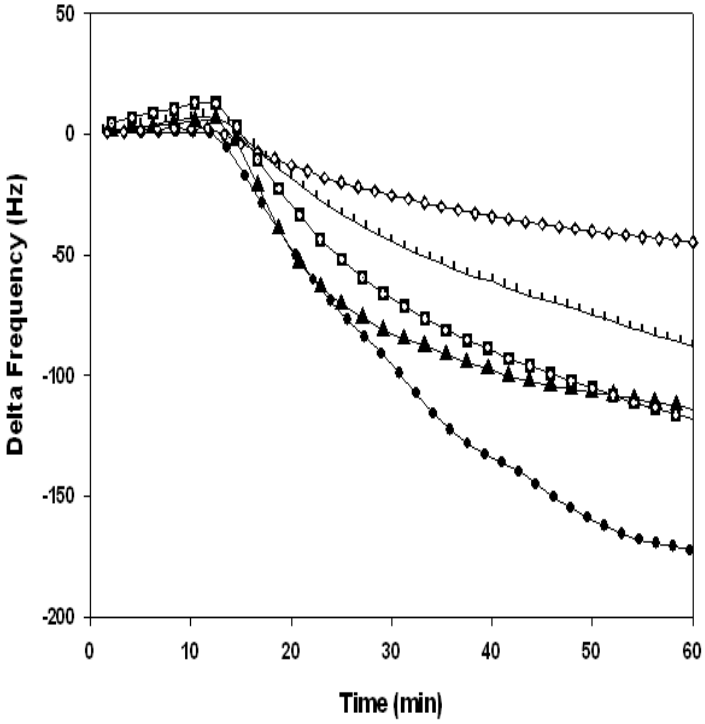

Delta Frequency (Hz)
50
0
-50
-100
-150
-200
0
10
20
30
40
50
60
Time (min)

Figure 4

Virus		Negative control	10^{3a}	10^4	10^5	10^6	10^7
Influenza A Frequency shift (Hz)	*without* nanoparticles	15[b]±2	15±4.5	30± 5	52±7.6	144±10.4	303±20.2
	with nanoparticles	65±10	102± 11	191± 14	227± 8	326± 9	370± 9.5
Percentage increase for influenza A		333	580	536.6	336	126	22
Influenza B Frequency shift (Hz)	*without* nanoparticles	15[b]±2.5	15±4.5	37±6.5	66±5	212±12.5	298±12.5
	with nanoparticles	65±7.5	115±5	214± 5.2	233±10.4	313± 12.5	345 ± 5
Percentage increase for influenza B		333	666	478	253	47	15

Table 1. Detection of A/PR/8/34 and B/Lee/40 viruses with and without nanoparticles.

[a] pfu/mL

[b] Results are expressed as mean values; ± standard deviation; n=3

Virus type	No (%) of specimens found positive by[a]:						
	RT-PCR	Shell vial	Cell culture	ELISA	Directigen Flu A	QCM	QCM/ nanoparticle
Influenza A[b]	37 (55)	28 (42)	30 (45)	25 (37)	13 (19)	28(42)	30 (45)
Influenza B[c]	15 (22)	12(18)	15 (22)	9 (13)	NA	12(18)	13 (19)
Totals	52 (77)	40 (60)	45 (67)	34 (50)	13(19)	40 (60)	43 (64)

Table 2. Detection of influenza A and B viruses in the nasal wash samples from 67 patients

[a] Times for the tests were RT-PCR: 1 day, shell vial: 2 days, cell culture: 14 days, ELISA: 1 day; Directigen Flu A: 20 min, QCM: 2 days.

[b]During the Australian winter of 2005, all influenza A viruses detected were A/New Caledonia/20/99 – like (H1N1) (2/37) and A/Wyoming/3/2003 - like (H3N2) (35/37).

[c]Influenza B viruses were B/Brisbane/32/2002 – like.

Test		No of specimens				Sensitivity[e]	Specificity[f]	PPV[g]	NPV[h]
		TP[a]	TN[b]	FP[c]	FN[d]				
Shell vial	Influenza A	28	27	3	9	76	90	90	75
	Influenza B	12	52	0	3	80	100	100	95
Cell culture	Influenza A	30	30	0	7	81	100	100	81
	Influenza B	15	52	0	0	100	100	100	100
ELISA	Influenza A	25	30	0	12	68	100	100	71
	Influenza B	9	52	0	6	60	100	100	90
Directigen Flu A	Influenza A	13	30	0	24	35	100	100	56
	Influenza B	NA	NA	NA	NA	NA	NA	NA	NA
QCM	Influenza A	28	30	0	9	76	100	100	77
	Influenza B	12	52	0	3	80	100	100	95
QCM (nanoparticles)	Influenza A	30	30	0	7	81	100	100	95
	Influenza B	13	52	0	2	87	100	100	96

Table 3. Results obtained for shell vial, standard cell culture, ELISA, Directigen Flu A and QCM methods in comparison with RT-PCR

A total of 67 nasal samples were compared with RT-PCR which was used as the reference method (*gold standard*).

[a] TP, true-positives; [b] TN, true-negatives; [c] FP, false-positives; [d] FN, false-negatives.

[e] Sensitivity = number of TP specimens/ (number of TP + number of FN specimens) x 100;

[f] Specificity = number of TN specimens/ (number of TN specimens + number of FP specimens) x 100;

[g] PPV (positive predictive value) = TP/ (TP+ FP) x100 ; [h] NPV (negative predictive value) = TN / (TN+ TN) x 100.

Samples positive by shell vial, standard cell culture, ELISA, Directigen Flu A kit and QCM, but negative by RT-PCR, were considered FP. Samples that were identified by RT-PCR assays were considered as TP. Samples that were negative by shell vial, standard cell culture, ELISA, BD Directigen Flu A kit and QCM but positive by PCR were regarded as FN. A sample that was negative by RT-PCR was a TN.