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Real-time cell analysis – A new method for dynamic, quantitative measurement of infectious viruses and antiserum neutralizing activity

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A B S T R A C T

A newly developed electronic cell sensor array – the xCELLigence real-time cell analysis (RTCA) system is tested currently for dynamic monitoring of cell attachment, proliferation, damage, and death. In this study, human enterovirus (HEV71) infection of human rhabdomyosarcoma (RD) was used as an in vitro model to validate the application of this novel system as a straightforward and efficient assay for quantitative measurement of infectious viruses based on virus-induced cytopathic effect (CPE). Several experimental tests were performed including the determination of optimal seeding density of the RD cells in 96-well E-plates, RTCA real-time monitoring of the virus induced CPE and virus titer calculation, and viral neutralization test to determine HEV71 antibody titer. Traditional 50% tissue culture infective dose ($TCID_{50}$) assay was also conducted for methodology comparison and validation, which indicated a consistent result between the two assays. These findings indicate that the xCELLigence RTCA system can be a valuable addition to current viral assays for quantitative measurement of infectious viruses and quantitation of neutralization antibody titer in real-time, warranting for future research and exploration of applications to many other animal and human viruses.

1. Introduction

Since the discovery and identification of viruses in the late 19th century (Levy, 1994), numerous viruses have been reported to cause a variety of diseases among humans, animals, and plants (Levy, 1994). Since viruses are obligate intracellular pathogens, the isolation, propagation, and study of viral pathogens and new isolates are largely dependent on the availability of a live host system, such as in vitro cell cultures. Currently, viruses are commonly identified by using molecular technology like PCR and RT-PCR. However, confirmative diagnosis and quantitation of infectious viruses are still dependent on traditional infectivity methods using permissive cell lines established in vitro; including viral plaque assay and median tissue culture infective dose ($TCID_{50}$) assays.

Viral PFU assay is known to be an enumerative quantitation method used to measure infectious viruses by counting the number of plaques produced in the monolayer of permissive cells within a semisolid medium that restricts progeny viruses from being released to neighbor cells. In contrast, $TCID_{50}$ assay is a quantitation method that uses microscopic observation of viral induced cytopathic effect (CPE) to determine the endpoint dilution of a virus causing 50% cell death or CPE of inoculated cells. Between these two virus infectivity assays, the end-point dilution based $TCID_{50}$ assay is used more commonly. Particularly, in clinical research for the determination of the lethal dose of pathogenic viruses and viruses like retroviruses which infect and replicate in host cells without killing them, resulting in no plaques in liquid and semisolid medium. This method is based primarily on the endpoint dilution to determine the amount of viruses required to kill 50% of test cells or to produce CPE in 50% of tested cell cultures. This method of viral quantitation requires manual observation and recording of all affected wells using an inverted phase contrast microscope to calculate the $TCID_{50}$ titer based on the end-point dilution.

The xCELLigence system RTCA, developed by Roche Applied Science, uses microelectronic biosensor technology to do dynamic, real-time, label-free, and non-invasive analysis of cellular events, including cell number change, cell adhesion, cell viability, cell morphology, and cell motility (Solly et al., 2004; Atienza et al., 2006; Fang et al., 2011; Irelan et al., 2011; Ke et al., 2011; Moodley et al., 2011). With the increased attention to cell-mediated cytotoxicity, virus-mediated cytotoxicity, and profiling tumor cell response to

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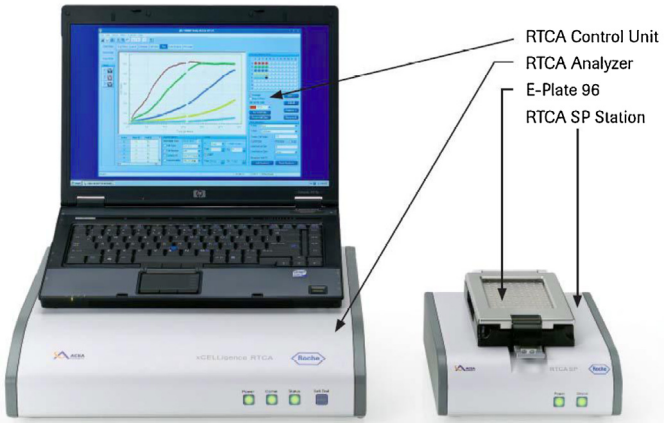

Fig. 1. xCELLigence system (RTCA). xCELLigence system (RTCA) is composed of a control unit, analyzer, E-plate 96, and Sp station.

treatment, this RTCA system presents a unique, impedance-based system for cell-based assays; allowing for label-free and real-time monitoring of cellular processes such as cell growth, proliferation, cytotoxicity, adhesion, morphological dynamics, and modulation of barrier function. This system has been used recently in monitoring virus infection and virus-induced cytopathology (Yama et al., 2009; Fang et al., 2011; Yian et al., 2012). To determine the feasibility and efficiency of this new technology in virology, the RTCA system was also tested for possible quantitation of infectious virus titers based on cell viability and morphology changes. In this study, human enterovirus type 71 (HEV71) infection of RD cells was used as an in vitro model in comparison to traditional $TCID_{50}$ viral assay to validate the RTCA method.

2. Materials and methods

2.1. xCELLigence system (RTCA)

The xCELLigence RTCA SP system is composed of four components: a computer, electronic sensor analyzer, workstation, and 96-well microtiter detection device (Fig. 1). The microelectrode sensor arrays are coated in 96-well microtiter plates and the microtiter plate detection device is connected to the workstation from the inside of the cell culture incubator. A cable connects the workstation to the sensor analyzer for impedance measurement. The impedance data from the selected wells is exported to the computer and analyzed using RTCA software. A parameter termed cell index is used to quantify cell status based on detected cell-electrode impedance. Cell attachment and proliferation from selected wells of the plate were monitored and recorded every 15 min using the RTCA SP.

2.2. Cell and virus

The human rhabdomyosarcoma (RD) cell line was obtained from the National Polio Laboratory in China and employed for human enterovirus type 71 (HEV71) replication and infectivity assay. RD cells were propagated and maintained at 37 °C with 5% CO_2 with Eagle's Minimum Essential Medium (Eagle's MEM, GIBCO) supplemented with 10% fetal bovine serum (FBS). RD cells were harvested at their exponential growth phase using 0.25% trypsin–EDTA digestive solution and used for viral infectivity tests.

A HEV71 stock was obtained from the National Polio Laboratory in China and used as a reference virus to test and establish the RTCA based viral infectivity measurement. The HEV71 reference strain was prepared from one patient infected with HFMD during the HEV71 outbreak in Anhui province in 2008. This particular HEV71 isolate is known to induce visible CPE in the RD cells following a short period of viral infection (Chua et al., 2008). Therefore, the HEV71–RD cell system is designed as an in vitro model to assess whether the RTCA SP system can be used as a viral assay to measure viral infectivity and infectious dose; through comparison with virus titer determined by currently used ($TCID_{50}$) assay. The calculation of viral $TCID_{50}$ value was conducted using the method described by Reed–Muench method (1938).

2.3. Antiviral HEV71 sera

A viral neutralization assay was also performed in this study using positive anti-HEV71 sera obtained from three individuals who were confirmed with HEV71 infection in 2008 (unpublished data from this laboratory). The neutralization test was performed to determine if the RTCA SP-based viral assay system can be used to effectively measure antibody mediated viral neutralization activity.

2.4. Optimized cell density for RTCA

To establish a background reading for the RTCA system, 100 μL of growth media containing 10% FBS was added to each well of the 96-well E-plates and tested. Then, 100 μL/well of 2-fold serial dilutions of RD cell suspensions with cell density ranging from 7.5×10^3 to 6.0×10^4 cells/well were seeded into the E-plate. An additional 100 μL/well of the growth medium was added to the E-plate for a total volume of 200 μL/well before plating the plates at 37 °C incubators with 5% CO_2. Cell growth status from each E-plate were monitored every 15 min and cell index values were calculated and expressed accordingly based on observing dynamic cell growth curves during post-growth time.

Meanwhile, the same preparation of RD cell suspension was also seeded into a regular 96-well plate (Nunclon) at 200 μl/well of cell suspension containing 4 selected cell densities (from 7.5×10^3 to 6.0×10^4 cells/well). Cell attachment and growth were examined using phase contrast inverted microscopy as a parallel comparison. Eight replicate wells were used for each seeded cell density and three experimental tests were independently conducted in order to determine an optimal cell seeding density in cell attachment and viability for a total of 6 days, the time required to complete the HEV71 infectivity test.

2.5. Viral infectivity assay

RD cells at their exponential growth phase were harvested using trypsin–versene solution and individual cell suspensions were prepared at a cell density of 1.5×10^4 cells/mL. RD cells were then seeded into an E-96-well plate at 100 μL/well and incubated at 37 °C to allowed cell attachment and formation of a cell monolayer.

A HEV71 viral stock was diluted 10-fold with Eagle's MEM and used for a viral infectivity assay. According to pre-experimentally determined dynamic cell proliferation, 100 μL/well of diluted HEV71 was added to the E-plate at 8 replicate wells per viral dilution. In addition, the same volume of growth medium was added to the cell cultures as mock-infected control wells. After 90 min of viral adsorption at 37 °C, the test cells were supplemented with 100 μL/well of Eagle's MEM growth media containing 5% FBS, and then incubated 37 °C. Infectious viral titers were determined based on cell index values obtained through the RTCA system.

As a parallel comparison, the same 10-fold dilutions of HEV71 samples were tested and tittered by infecting RD cells grown in normal 96-well cell culture plates at 100 μL/well and 8 wells per dilution. Infected cultures were incubated at 37 °C and viral-induced cytopathic effect (CPE) was monitored daily using inverted phase contract microscopy (Nikon 80i). After day 8, the viral

infectivity test was terminated and infectious viral titers were calculated as $TCID_{50}$ units based on viral induced CPE.

2.6. Viral neutralization assay

To validate and explore the possible use of RTCA as a real-time monitoring tool for viral infectivity, a viral neutralization test was performed using anti-HEV71 serum samples collected in 2008. In this test, the anti-HEV71 sera were inactivated with 56 °C water bath for 30 min. Then, 2-fold serial dilutions of the sera ranging from 1:2 to 1:32 were performed with Eagle's MEM containing no FBS. To determine the viral neutralization titer, each diluted serum solution was mixed with an equal volume of a pre-diluted viral stock containing 100 $TCID_{50}$/mL of HEV71. The resulting mixture was incubated at 37 °C along with both positive (viral solution alone) and negative (dilution medium alone) controls under the same experimental conditions. At the end of a 90 min incubation, each serum–virus mixture was inoculated into both the 96-well E-plates and regular 96-well cell culture plates seeded with RD cell at 1.5×10^4 cells/well, 19 h prior to viral inoculation at 100 μl/well and 4 replicated wells per mixture. The infected cultures were incubated at 37 °C with 5% CO_2 and continually monitored using RTCA software and cell index values were documented. Viral neutralization titer of these three serum samples were defined as the reciprocal of the highest dilution of these sera which caused the complete blockage of viral infectivity of 100 $TCID_{50}$ HEV71 in all the test wells. The same mixtures of serum–virus dilutions were also tested for viral neutralization activity using traditional $TCID_{50}$ viral infectivity assays, and serum-mediated viral neutralization titers were determined based on microscopic observation of infected cell cultures for viral induced CPE. A comparative analysis was conducted between the two methods for viral neutralization effect from the three sera samples.

3. Results

3.1. Optimal density for cell proliferation and viability

To facilitate the use of the RTCA SP system as a biological tool to measure viral infectivity, it requires firstly establishing optimized seeding densities for test cells. Cell impedance was measured from each individual well of a 96-well E-plate and was automatically converted to cell index values by the RTCA Software. The cell index value represents a quantitative measure of the growth status of the test cells. Under the same test conditions, increased cell attachment in an E-plate well was detected by the electrodes leading to a relatively large value of cell index. Furthermore, even when the same number of cells was seeded in the wells of an E-plate, a detected change in the cell index value can be an indication of a different rate of cell growth.

To determine an optimal seeding density of test cells to be used for the RTCA system, RD cells were seeded into the E-plates in four different densities ranging from 7.5×10^3 to 6.0×10^4 cells/well. Respective impedance was determined and shown in Fig. 2A. The cell index values from the wells with 6.0×10^4 and 3.0×10^4 cells/well settings showed a decrease at post growth time 85 h and 119 h, respectively. However, at the seeding density of 1.5×10^4 cells/well, the cell index value continued to be increased and reached its highest point at 151 h post growth. In contrast, the cell index value from the well seeded with 7.5×10^3 cells/well

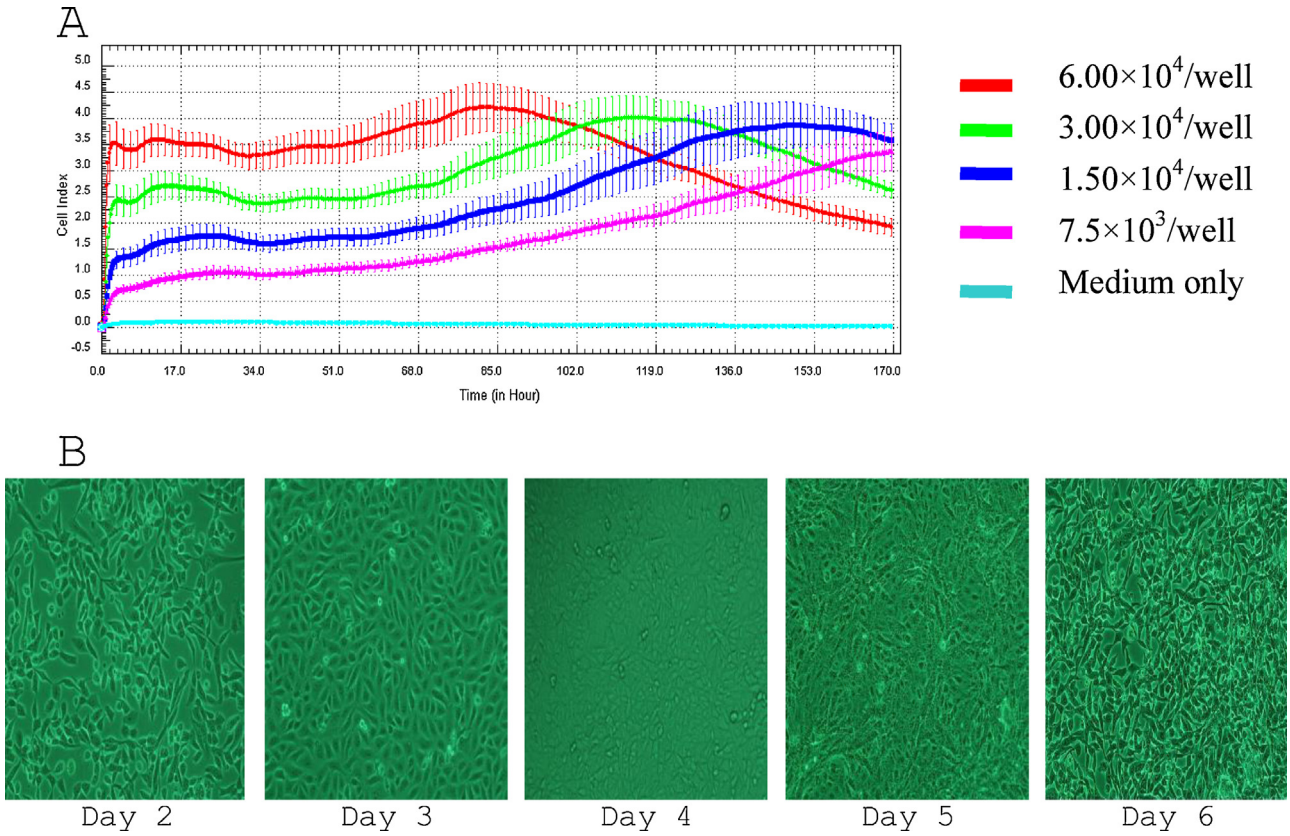

Fig. 2. (A) The density of cells titration based on RTCA. Four densities of RD cells were seeded in the E-plate of 96 wells with 8 replicated wells and continuously monitored by measuring the CI to identify a suitable time point for viral infectivity. The adhesion and proliferation of RD cells were dynamically monitored every 15 min using the RTCA SP Instrument. The colored curves indicate the average CI of different cell densities seeded per well in an E-plate. (B) Cell status observation via microscopy. RD cells (1.5×10^4/well) were seeded into the 96-well plate (Nunclon) as a parallel comparison being observed from the second day to the sixth day after cell seeding by microscope (10×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

continued to be increased up to 170 h, but at a relatively low level. Thus, the optimal seeding density of RD cells for this study was determined to be 1.5×10^4 cells/well.

In comparison with the same seeding density of RD cells (1.5×10^4 cells/well) in normal, 96-well plates, cell growth status was monitored daily using inverted microscopy (Fig. 2B). RD cells showed good attachment and growth for up to 6 days, which is comparable with the experimental findings obtained from the RTCA SP system by measuring cell index values.

3.2. HEV71 infectivity

When the test cells were monitored using the RTCA system, a clear correlation was noted between the amount of infectious

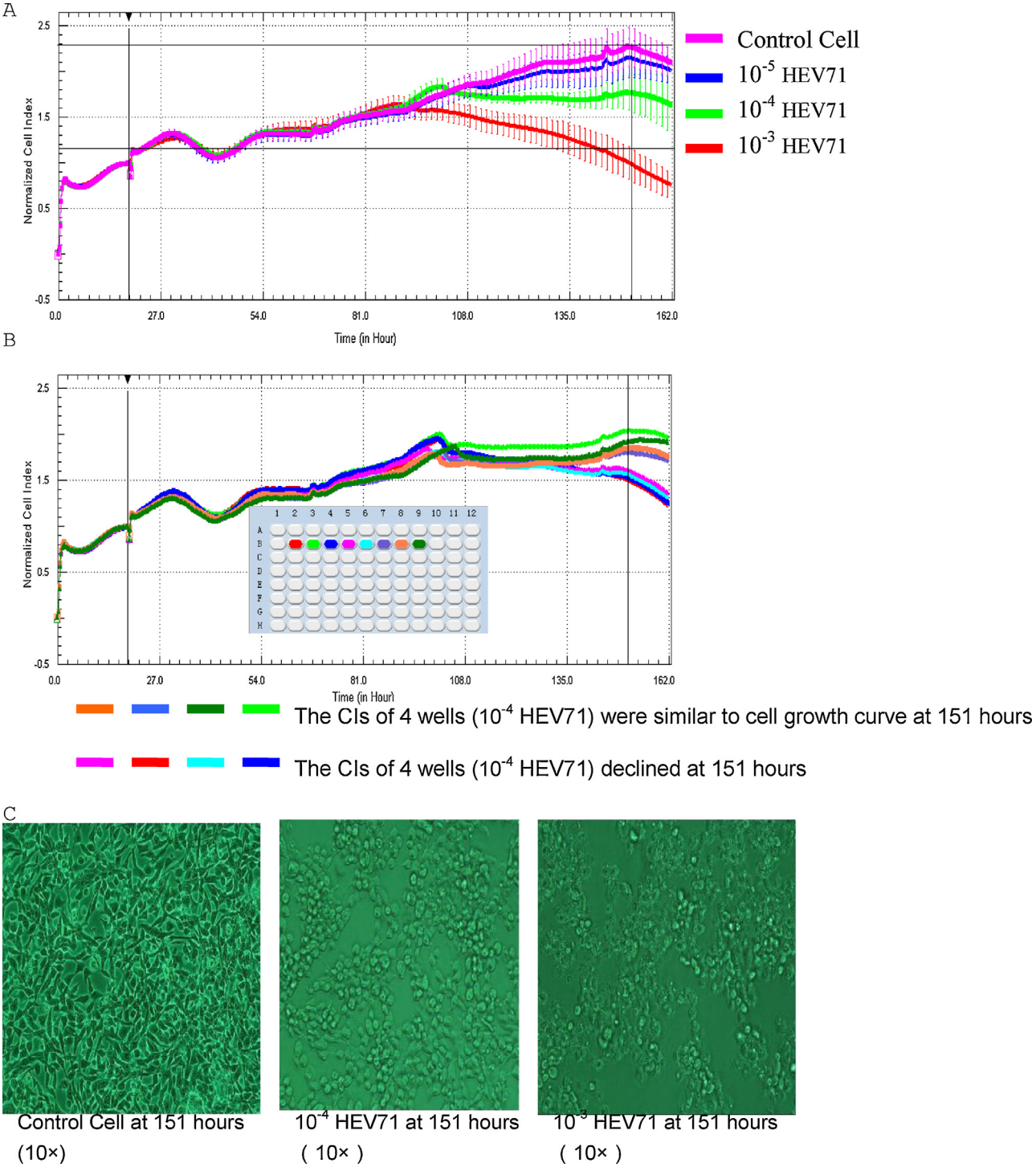

Fig. 3. HEV71 induced cytopathogenicity. Dynamic monitoring of RD cells during HEV71 infection (A) the HEV71 mediated effect on adhesion and proliferation of the cells was monitored by measuring cell impedance every 15 min using the RTCA SP Instrument. The black vertical line indicates the time HEV71 was added (19 h) and (B) we regarded 151 h as the endpoint of our results. Each dilution was done in 8 replicate wells. The CI from each well infected with a 10^{-4} dilution were observed, the CI of 4 wells continued to decline and the CI of the 4 other wells were similar to the cell growth curve. (C) Phase contrast microscopy (10×) illustrates cytopathic effect in the HEV71-infected RD cells at 151 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

virus inoculated and detected changes of CELL INDEX values. As shown in Fig. 3A, while the cell index value from the noninfected wells remained increased up to 151 h, affected wells showed a viral dose-dependent faster decline of cell index values. This difference signifies the occurrence of cell apoptosis. The magenta curve shows the average cell index of the growth of unaffected cells with 151 h considered as the endpoint for test analysis. Compared to the control wells, the average cell index of RD cells infected with the 10^{-5} dilution of HEV71 showed a similar pattern to the control cells, despite a notably slightly lower level (Fig. 3A, purple curve). In contrast, the average cell index of cells infected with the 10^{-3} dilution began to decline at approximately 89 h post virus infection (Fig. 3A, red curve), and dropped dramatically afterward. This pattern of decline in cell index values was detected in all 8 wells infected with the 10^{-3} virus dilution (data not shown). The average cell index of cells infected with the dilution of 10^{-4} HEV71 began to decline at approximately 100 h following virus inoculation (Fig. 3A, green curve). We determined that the dilution of 10^{-4} virus appeared to be the dilution closest to the endpoint of infectious HEV71, and thus, the average cell index curves were characterized from each of the eight, individual wells infected with the 10^{-4} dilution. As shown in Fig. 3B, while a similar pattern of cell index curves were observed from 4 wells, the other 4 wells showed a continued decline of the average cell index values after post infection time of 151 h. These observations allowed us to determine the $TICD_{50}$ titer of the HEV71 stock which was calculated as: inoculum volume (mL) $\times 10^4$.

Titration of the HEV71 stock was also conducted using a regular 96-well plate for traditional $TCID_{50}$ assays as a parallel comparison and the experimental results were shown in Fig. 3C. The control cells appeared healthy with good cell attachment at post growth time of 151 h. However, cells infected with the 10^{-4} viral dilution showed viral-induced CPE in approximately half of the 8 wells at 151 h post infection time, while all the 8 wells infected at 10^{-3} virus

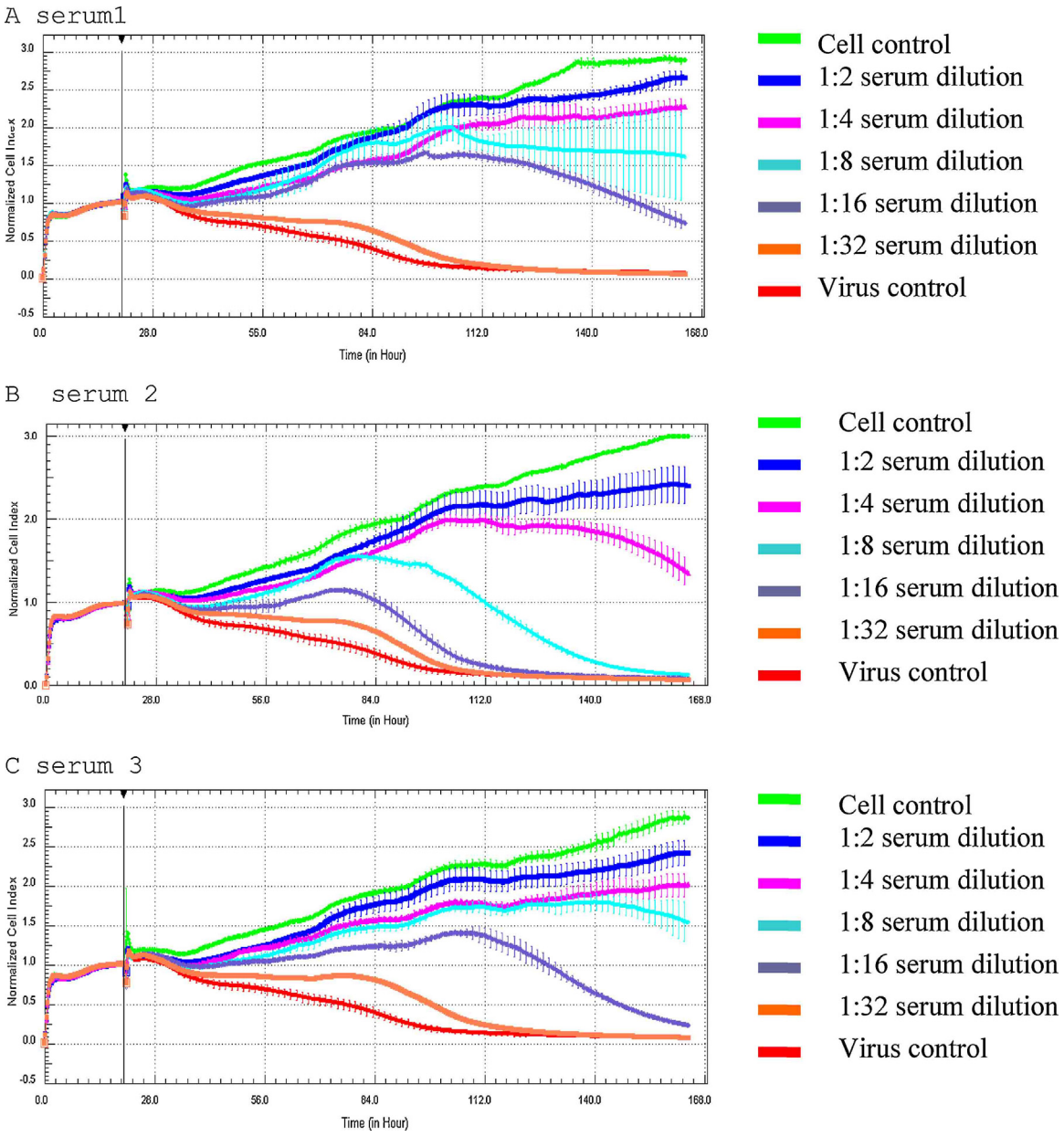

Fig. 4. Dynamic monitoring of neutralizing activity of diluted antiserum samples. The virus-mediated effect on adhesion and proliferation of the cells was monitored by measuring cell impedance every 15 min using the RTCA SP Instrument. The final dilution of HEV71 neutralization antibody used in this test was 1:2–1:32. The black vertical line indicates when the virus was added (19 h). Results are the average of 4 replicate wells.

dilution showed viral induced CPE. Therefore, the HEV71 virus stock has a $TCID_{50}$ titer falling to the dilution of 10^{-4}.

3.3. Neutralizing antibody test

In this study, the possible use of RTCA SP to determine neutralization titer of unknown anti-sera was also explored. Particularly, this study defined the neutralization antibody titer as the reciprocal of the lowest concentration of test sera showing complete blockage of the viral infectivity of 100 $TCID_{50}$ HEV71 in all the test wells of RD cells. Compared to the controls, a similar pattern of the average cell index of cell growth was observed for sera #1 and #3 at dilutions of 1:4 and for serum #2 at 1:2 dilution (Fig. 4A–C). Apparent decline of the cell index values were observed when the sera was further diluted to 1:4 (serum 2) and 1:8 (sera 1 and 3). According to the CI curves, the neutralization antibody titer in serum 1, serum 2, and serum 3, were determined to be 4, 2, and 4, respectively. The average cell index curve from the RD cells exposed to the dilutions of these sera alone showed no difference to the cell controls, indicating no cytotoxic effect of these three serum samples (data not shown).

Additionally, Fig. 4 showed the time of onset of the average decrease of cell index values for serum 1 at 98 h, serum 2 at 75 h, and serum 3 at 106 h post viral infection. According to the delayed time for cell death, serum 3 exhibited the highest viral neutralization activity while serum 2 was the lowest.

4. Discussion

In vitro cultivation of animal cells has become an extremely important and essential biological tool for the study of animal and human viruses today. Currently, many cell lines have been established from the tissues and organs of various animal species (Barron et al., 1970; Wolf and Mann, 1980; Fryer and Lannan, 1994; Lu et al., 1999, 2001; Rougee et al., 2007; Lakra et al., 2011). These cell lines are widely used for virus isolation, characterization, and studies (Menna et al., 1975; Lifson et al., 1986; Smith et al., 1986; Payment and Trudel, 1994; Shimizu et al., 1994; Lu et al., 1997, 2003; Leland and Ginocchio, 2007; Ilarraza et al., 2011; Rice, 2011; Sainz et al., 2012), and other related studies including antiviral drug screening (Yasuhara-Bell et al., 2010; Wozniak et al., 2011; Tong et al., 2012) and preparation of attenuated anti-viral vaccines (Barrett et al., 2009; Liu et al., 2009; George et al., 2010; Monath et al., 2011; Ilyushina et al., 2012; Perdue et al., 2011; Lohr et al., 2012). Since virus infection of in vitro cells cultures can lead to visible cellular damage and death (CPE), this virus infection characteristic has been utilized for the detection of viral pathogens and the quantitation of infectious virus titers through limited endpoint dilution tests. Among the methods for viral titration, $TCID_{50}$ assay is considered rapid and readily reproducible since semisolid overlay medium or specific antibody conjugate are not required. Despite the limitation of tedious manual examination of each individual well for virus infection (CPE), $TCID_{50}$ is widely used today for the titration of many animal viruses including clinical virus quantitation where the lethal dose of virus must be determined since being established in 1938 (Reed and Muench, 1938).

In the present study, the possible use of the new xCELLigence RTCA system as a tool to quantify infectious virus was evaluated by measuring the change of cell-electrode impedance of HEV71 infected RD cells. This application of the RTCA system in virology is based on the understanding that cell-electrode impedance is primarily influenced by three factors; cell numbers, cell morphology, and cell adhesion to the electrode surface. Productive infection of pathogenic viruses such as HEV71 is known to induce cell damage and death (CPE) resulting in decreased number of test cells that can visualized under inverted microscope. These cellular death events and cell population changes could also be readily detected using the RTCA system which reflects the decreased level of cell-electrode impedance. Such changes form the basis of using the RTCA system to detect and measure the infectious titer of a test virus.

Unlike traditional tests which measure viral infectivity based on microscopic observations of viral induced CPE, the RTCA system mediated viral infectivity measurement is based on the cell growth curve as determined by changes in cell index. In particular, each cell index change indicates changes of cell status in each well and these two parameters can be used to reflect the magnitude of viral induced CPE: one parameter was the onset time point when cell index values begin to decrease, and the other was the cell index curve of affected cell cultures. By comparing these parameters to the cell index values and curves of uninfected control cells, no viral infectivity was determined when the cell index curve from an individual well/sample is similar to that observed in the control cell cultures. In contrast, when test cells shows an earlier decline of the cell index curve as compared to control cells, this suggested the occurrence of active viral infection of the cultures. Furthermore, a large decrease of cell index or dramatic decline of the cell index curve indicated the presence of a massive virus infection in the cell cultures. Monitoring and analysis of the cell index curves could be done on individual wells with respect to time and this forms the basis to calculate the titers of test viruses and/or neutralization antibody titer by measuring the number of wells showing the cell index declined. Infectious titer of a virus stock can be calculated similarly according to the Reed–Muench method based on detecting the change of cell index values using a computer instead of detecting viral induced CPE via inverted microscopy.

To establish the xCELLigence RTCA system as tool for effective measurement of virus infection and quantitation of infectious viruses, three independent experimental tests were conducted using HEV71–RD cells as a model, including the use of the RTCA system to quantify the infectious titer of a HEV71 stock through measuring electronic impendence; and titration of the same virus dilution using traditional $TCID_{50}$ assay so the infectious titer of the virus stock can be compared and validated between these two methods. Results obtained from this study have clearly shown that the infectious titer of the HEV71 stock detected from these two methods is in complete agreement. Despite the limitation of present testing with one virus strain (HEV71), findings from this study have clearly demonstrated the potential and application of the RTCA system to many other human viruses for quantitation as long as they are able to induce a productive infection in their permissive cell cultures. A major advantage of the RTCA system in virus titration testing is the ability of the system to monitor and measure virus-induced cell damage and death in a very dynamic fashion which is easy and convenient. Specifically, since each well can be precisely monitored and visualized through a computer, this eliminates the need to manually check each individual wells using an inverted phase contrast microscope. It should be noted that the high cost of purchasing and operation of the RTCA system may be a significant limitation. However, the exploration and extension of its use to virology may be a cost-effective application in situations where this system is currently available for other cell-based assays.

Before conducting the cytopathic assay, an initial test for quantitative analysis of cell proliferation was carried out using the RTCA system to determine the optimal density for cell seeding. This test demonstrated that 1.5×10^4 cells/well was the optimal seeding density for E-plates with RD cells to be used for HEV71 infections. At this density, seeded RD cells showed no obvious decrease of cell index for approximately 151 h, which meets the 6-day requirement for HEV71 to induce a detectable cytopathic change in RD cells. It should be pointed out that since the productive infection time for viruses can vary significantly in different host cell cultures, a

pre-test for cell seeding density for a particular virus-cell infection system is required when using the RTCA system.

In this study, the neutralization antibody titer and the time of onset of CPE were recorded in a real-time manner. In addition, even though the neutralization titer can be the same for the three different antiserum samples, the onset time of neutralization activities on virus inactivation have different impedance curves (i.e. serum samples 1 & 3 in Fig. 4). Therefore, traditional methods are not just laborious and difficult for high throughput detection, it is also incapable of detecting the onset time of virus-induced and antiserum-mediated cellular change.

In summary, the RTCA SP system was assessed to be a useful tool for quantitation of infectious virus and testing the neutralizing activity of serum. The RTCA system provides the opportunity to screen serum samples in a high throughput environment, by reducing overall workload and the laborious task of manual examination of test cells for the presence/absence of virus-induced cytopathic effects and neutralizing activity.

Competing interests

The authors declare that they have no competing interests.

Authors contributions

ZT performed experiments and contributed to data analysis, and prepared the manuscript preparation. XZK and JYW participated and assisted in experimental tests, XZ conceived of the study, experimental design, data analysis, and manuscript revision. All authors read and approved the final manuscript.

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