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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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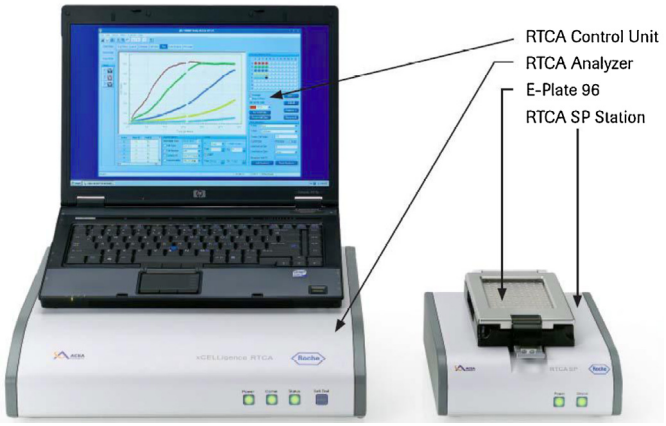

Fig. 1. xCELLigence system (RTCA). xCELLigence system (RTCA) is composed of a control unit, analyzer, E-plate 96, and Sp station.

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