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Development of an enzyme-linked immunospot assay for determination of rotavirus infectivity

Tingdong Li[a], Haijun Lin[b], Linqi Yu[a], Miaoge Xue[b], Shengxiang Ge[a,*], Qinjian Zhao[a], Jun Zhang[a], Ningshao Xia[a,b]

[a] *State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, National Institute of Diagnostics and Vaccine Development in Infectious Disease, School of Public Health, Xiamen University, Xiamen, China*
[b] *School of Life Science, Xiamen University, Xiamen, China*

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Conventional rotavirus infectivity assays are time consuming, labor intensive, and with low sample throughput. To overcome these problems, a 96-well microplate enzyme-linked immunospot assay (Elispot) was developed for the measurement of rotavirus infectious titers. The infected MA104 cells were stained with a horseradish peroxidase-conjugated anti-VP6 monoclonal antibody followed by detection with an ELISPOT analyzer. A linear relationship was found between spot number and input of rotavirus dose in SA11 and 10 rotavirus isolates of different genotypes. The propagation of rotavirus SA11 in MA104 cells was monitored, and the neutralizing activity of serum samples and monoclonal antibodies was determined. The 50% neutralizing titer (NT_{50}) of serum and 50% inhibitory concentration (IC_{50}) of monoclonal antibodies were correlated well with the results determined by ELISA-based neutralization assay. In conclusion, a rapid and semi-automated procedure to determine rotavirus infectivity was developed, which will be useful to study the infectivity and the neutralizing epitopes of rotavirus.

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1. Introduction

Rotaviruses, member of *Reoviridae*, are non-enveloped icosahedral viruses with a diameter of about 70 nm. The rotavirus genome consists of 11 segments of double-strand RNA encoding six structural proteins (VP1 to VP4, VP6 and VP7) and five nonstructural proteins (NSP1 to NSP5) (Estes and Cohen, 1989). Rotaviruses are classified into seven groups designated A–G based on the antigenicity of VP6 (Ramig et al., 2005) and group A rotavirus is the most common cause of severe diarrhea in infants and young children globally. Each year, approximately 2 million hospitalizations and 453,000 deaths in children <5 years of age were due to rotavirus infection around the world (Parashar et al., 2003; Tate et al., 2012). Group A rotavirus can be further classified into more than 19 G serotypes and 27 P serotypes according to the genetic and antigenic diversity of the two outer capsid proteins, VP7 and VP4, respectively (Santos and Hoshino, 2005; Matthijnssens et al., 2008).

In general, vaccination is the most effective mean of protection against rotavirus infection. Three live attenuated vaccines have been approved by FDA (Parashar and Glass, 2009), but one of which, Rotashield^{TM}, was withdrawn from the market due to the increased risk of intussusception (Murphy et al., 2001). The other two vaccines, RotaTeq^{TM} and Rotarix^{TM}, both have high efficacy against severe rotavirus gastroenteritis in developed countries (Linhares et al., 2008; Plosker, 2010; Wang et al., 2011). However, they were both less effective in developing countries (Armah et al., 2010; Madhi et al., 2011; Snelling et al., 2011; Feikin et al., 2012) where the vaccines would be most critical to saving lives. Researchers continued to investigate more effective vaccines and other inhibitors, such as siRNA to prevent rotavirus infection (Lopez et al., 2012).

Detection of rotaviruses depends on electron microscopy (Brandt et al., 1981), enzyme-linked immunosorbant assay (Beards et al., 1984), latex agglutination (Kohno et al., 2000), lateral-flow immunoassay (Al-Yousif et al., 2002) and nucleic acid amplification (RT-PCR) (Kang et al., 2004; Ranheim et al., 2006). However, cell culture method remains the gold standard for virus diagnosis because it is the only method for detection of infectious viral particles. In addition, quantitation of virus infectivity is important for determination of the potency of live virus vaccines and the screening of therapeutic molecules with the potential to inhibit rotavirus replication.

Different assays were developed for the determination of rotavirus titers, and the advantages and disadvantages of these assays were summarized in Table 1. Traditionally, the infectivity

* Corresponding author.
E-mail address: sxge@xmu.edu.cn (S. Ge).

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Table 1
The comparison of the methods for quantitative determination of rotavirus.

Method	Detection	Advantages	Disadvantages	References
$TCID_{50}$ assay Plaque assay	Cytopathic effect Plaques	Classical Classical, broad linear range	Time consuming, labor intensive, subjective and highly variable	Wyatt et al. (1982), Chang et al. (1999)
Fluorescent focus assay	Rotavirus proteins	Rapid, not dependent on cytopathic effect	Manual enumeration of infected cells, subjective due to observer bias, highly variable	Coulson and Masendycz (1990), Yang et al. (1998)
qRT-PCR	Genome RNA	Objective, rapid and simple	Not correlate with the infectious titer	Kang et al. (2004), Ranheim et al. (2006)
ELISA	Rotavirus proteins	Objective, simple, cheap	Needs freezing and thawing, highly variable	Knowlton et al. (1991)
Elispot	Rotavirus proteins	Objective, rapid, simple, automated and high-throughput	The ELISPOT ANALYZER is a bit expensive	This work

of rotaviruses is determined by $TCID_{50}$ assay (Chang et al., 1999) or plaque assay (Wyatt et al., 1982). These assays are typically highly variable, labor intensive and time consuming, requiring 5 days or more for the appearance of cytopathology. Antibody based detection assays were developed to monitor viral antigen production within infected cells prior to release of progeny viruses by fluorescent- or peroxidase-conjugated reagents (Yang et al., 1998; Asensi et al., 2006). However, manual enumeration of labeled cells can be still time consuming and lead to variable results due to bias in the selection of the fields of view. An ELISA assay was developed to determine rotavirus antigen production, which is less laborious and more objective, but it could not be performed in cell (Knowlton et al., 1991). Recently, infrared imagers have been used to automate the counting procedure for immunofluorescence-based rotavirus infectivity assay (Iskarpatyoti et al., 2012; Lopez et al., 2012), which fully automates data collection and can be adapted for high-throughput screening. Both the imager and secondary antibodies were expensive for high throughput screening.

The Enzyme-linked immunospot (ELISPOT) assay is one of the most sensitive and robust immunological methods for enumerating antigen specific lymphocytes (Lalvani et al., 2001; Karlsson et al., 2003), cytokine-producing cells (Streeck et al., 2009). Recently, it has been used widely in virus titration (Kang and Shin, 2012; Wang et al., 2012) and microneutralization assays for some viruses such as respiratory syncytial virus (Zielinska et al., 2005), human cytomegalovirus (Abai et al., 2007; Tang et al., 2011) and dengue virus (Liu et al., 2011) with the advantages of being objective, rapid and with high throughput.

In this study, based on a horseradish-peroxidase conjugated monoclonal antibody (mAb) against rotavirus VP6 and the Elispot reader, a rapid and automated rotavirus infectivity assay was developed. The spot number detected in Elispot assay were highly correlated with the input of rotavirus dose and it works well in detecting of rotavirus neutralizing antibodies in both sera samples and monoclonal antibodies.

2. Material and methods

2.1. Cells and viruses

MA-104 cells (ATCC® CRL-2378.1, ATCC, Manassas, VA, USA) were maintained as monolayer cultures at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA). Simian rotavirus strain SA11 (ATCC® VR-1565™, Manassas, VA, USA) was propagated in MA104 cells (ATCC® CRL2378.1) (ATCC, Manassas, VA, USA). Ten human rotavirus (P1-P10) isolated from diarrhea patients were provided kindly by Xiamen Center for Disease Control and Prevention (Xiamen, Fujian, China) and propagated in MA104 cells. Rotavirus infectious titers were determined by $TCID_{50}$ assay (Table 2). All virus stocks were stored in 0.1 ml aliquots at −80 °C until use.

2.2. Monoclonal antibodies and sera

Monoclonal antibodies (mAbs) were prepared with standard Hybridoma technology (Zhang, 2012). Balb/c mice were immunized with three injections (0, 2, 4 weeks) of simian rotavirus SA11. Mouse sera were collected 14 days after the each immunization. The anti-VP6 antibody titer in mouse serum was monitored by enzyme-linked immunosorbant assay (ELISA) with VP6 coated microplates. Neutralizing antibody titer in mouse serum was monitored by ELISA-based neutralization assay reported in previous studies (Knowlton et al., 1991). Anti-VP6 mAbs were screened by ELISA with VP6 coated microplates. Neutralizing mAbs were screened by ELISA-based neutralization assay. The mAbs were produced in mouse ascites fluids and were affinity-purified with a Protein A column. The concentrations of purified IgGs were determined using OD at 280 nm. The mAbs were diluted into 1.0 mg/ml in PBS and stored in PBS at −20 °C. An anti-VP6 mAb, 7H11 and 4C2, was conjugated with horseradish peroxidase (HRP) by sodium periodate oxidation method (Nakane and Kawaoi, 1974). Human sera were provided by Guangxi Center for Disease Prevention and Control, which were collected in 2004 in Lingyun county, Guangxi province, China.

2.3. Cytopathic effect assay ($TCID_{50}$) assay for determination of rotavirus infectious titer

MA104 cells were seeded into 96-well microplates at 20,000 cells per well and incubated at 37 °C until confluent cell monolayers

Table 2
Infectious titers and genotype of rotavirus.

Virus strain	$TCID_{50}$/ml	G type	P type
SA11	1.58×10^6	G3	P[2]
P1	2.37×10^6	G1	P[8]
P2	4.22×10^5	G3	P[8]
P3	6.31×10^5	G1	P[8]
P4	3.16×10^6	G1	P[8]
P5	6.81×10^5	G1	P[8]
P6	2.08×10^6	G1	P[8]
P7	1.47×10^6	G9	P[8]
P8	4.81×10^5	G1	P[8]
P9	3.16×10^5	G1	P[8]
P10	4.22×10^6	G1	P[8]

The ten strains of rotavirus designated P1 to P10 were isolated from rotavirus positive stool samples, which were kindly represented by Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.

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