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Development of a new enzyme immunoassay for improved detection of rotavirus in fecal specimens of vaccinated infants☆

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ABSTRACT

Background: Group A rotavirus is the most common cause of acute diarrhea in young children worldwide. A simple and rapid enzyme immunoassay (EIA) has been commonly used to detect rotavirus infection and evaluate rotavirus vaccines. Currently licensed commercial EIA kits have low sensitivity. A more sensitive detection of rotavirus can improve rotavirus diagnostics and vaccine efficacy studies.

Objective: A biotin-avidin based sandwich EIA was developed and compared with commercial EIA kits for improved detection of viral shedding in fecal samples from infants who received human rotavirus vaccine Rotarix in Mexico.

Study design: A monoclonal antibody (mAb: 1D4) specific to human rotavirus group antigen VP6 was prepared and used to develop a biotin-avidin based sandwich EIA. This EIA was employed to test 128 fecal samples from vaccinated infants, in comparison with two commercial EIA kits using RT-PCR as a reference.

Results: A new biotin-avidin based sandwich EIA showed specific reaction to group A rotaviruses, but not to other enteric viruses. This new EIA had a detection rate of 36.7% for rotavirus antigen shedding in fecal specimens, which was two times higher (16.4%, 18.0%) than those from two commercial EIA kits.

Conclusion: The new EIA had specificity and higher sensitivity than commercial kits. This new EIA has the potential to detect rotavirus at lower concentration in clinical specimens and thus should be further evaluated as a more sensitive kit for use in diagnostics and vaccine efficacy and effectiveness studies.

1. Background

Group A rotavirus (RVA) is the most common cause of acute viral gastroenteritis in infants and young children and causes approximately 215,000 deaths globally [1,2]. Nearly all children are infected with rotavirus at least once by the age of five years. In the United States, prior to the introduction of rotavirus vaccine, rotavirus gastroenteritis resulted in an estimated 55,000–70,000 hospitalizations in children less than 5 years of age per year [3,4]. Loss of parental time from work, in caring for ill children, has been estimated millions of dollars per year [5]. Rotavirus infection has also been reported to cause mortality for populations at risk, namely the elderly and immunocompromised patients [6–9]. In addition, rotavirus-associated nosocomial infection poses problems in wards of children's hospital and often is costly and difficult to manage.

Two rotavirus vaccines (RotaTeq and Rotarix) have demonstrated high efficacy (> 85%) against severe rotavirus-associated acute gastroenteritis in high- and middle-income countries, but lower efficacy in low income countries [10–12]. Both vaccines have shown herd immunity partially due to transmission of vaccine virus strains from vaccinated children to unvaccinated contacts [13]. However, this transmission could pose the risk of vaccine-derived disease in healthy and immunocompromised children [7,14] The two vaccines have been recommended for use in routine national immunization in more than 80 countries [15]. As more countries are introducing rotavirus vaccines, it is important to effectively monitor the impact of rotavirus vaccination, which will require a simple but more sensitive and specific method to detect rotavirus and assess the risk of vaccine virus shedding and

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transmission. In addition, rapid and accurate diagnosis of rotavirus gastroenteritis is critical for timely and effective patient care and management.

Several methods have been utilized for the detection of rotavirus infection. Detection initially relied on electron microscopy because these fastidious agents were not readily cultivatable in tissue culture [16,17]. Although *in vitro* cultivation of human rotavirus from stool samples has now been achieved, it is not practical and not routinely used for detecting rotavirus because of intensive labor requirement and relatively low isolation rates [17,18]. RT-PCR is a more sensitive method but requires experienced technicians, specialized laboratory equipment and the results may not be relevant to clinical presentation of rotavirus associated acute diarrhea or virus shedding in stool. Consequently, simple and rapid enzyme immunoassays (EIAs) using monoclonal or polyclonal antibodies have been developed and are now commercially available for the detection of rotavirus infection in research laboratories and clinical setting [19–22]. For example, the Premier™Rotaclone® kit, RIDASCREEN® Rotavirus kit and Rotavirus Antigen detection kit use a monoclonal antibody (mAb) specific to the group antigen VP6 of SA11 strain, whereas IDEIA™ Rotavirus EIA kit employs polyclonal antibody to human rotavirus strain. All the current kits have a detection limit of $\sim 10^5$ particles per ml in clinical samples [23–25]. Some commercial EIA kits have low sensitivity or produce false positive results, and thus no longer completely meet the needs for the detection of rotavirus among children who receive rotavirus vaccines and often have much lower rotavirus shedding than those with natural rotavirus infection. Consequently, there is a need to develop a more sensitive and specific EIA for use in diagnostics and surveillance of rotavirus infection.

In this study, a new mAb, specific to VP6 of the human rotavirus strain CDC-5 (G1P [8]) was prepared, characterized and used to develop a biotin-avidin EIA to detect RVA. This in-house EIA along with the two widely used commercial EIA kits were evaluated and compared with RT-PCR test for their sensitivity and specificity to detect rotavirus antigen in fecal samples of infants who received Rotarix vaccine in Mexico.

2. Objective

The aims of this study were to develop a biotin-avidin based EIA and assess whether it had improved detection of viral shedding in fecal samples from infants in comparison with two commercial EIA kits.

3. Study design

3.1. Viruses

The following culture-adapted rotaviruses were used: human strains CDC-5 (G1P[8]), CDC-9 (G1P[8]), Wa (G1P[8]), DS-1 (G2P[4]), P (G3P [8]), ST3 (G4P[6]), MW333 (G8P[4]), WI61 (G9P[8]), L26 (G12P[4]), Rotarix (G1P[8]), bovine strains NCDV (G6P[1]) and WC3 (G6P[5]), porcine OSU (G5P[7]), and simian RRV (G3P[5]). Other gastroenteritis viruses included adenovirus 40 and 41, human astrovirus type 1 (Hast1), enteroviruses ECHO11 and EV71. Rotavirus strains were propagated in MA104 cells and the titers were determined as described [26].

3.2. Preparation of mAb to rotavirus

Hybridoma cells secreted rotavirus-specific mAb were prepared by conventional methods using purified CDC-5 rotavirus as an immunogen, screened by EIA using rabbit serum as a capture antibody [27,28]. Hybridoma cells were adapted to grow in serum-free medium and mAb was purified by the HiTrap protein G HP column according to the manufacturer's instructions. A hybridoma (clone 1D4) was selected and purified 1D4 mAb was conjugated with biotin using EZ-Link NHS-PEO solid phase biotinylation kit (Thermo Scientific), according to the manual.

3.3. Characterization of mAb 1D4

The isotypes of the immunoglobulins produced by the hybridoma were determined by EIA using HRP-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotech), according to the manual. The specificity of the mAb 1D4 was determined against rotavirus and other enteric viruses by dot blot analysis. The mAb from culture supernatant was also examined for protein specificity by immunoprecipitation analysis using protein G immunoprecipitation kit (Thermo Scientific), according to manufacturer's instructions. The elution was run on a 10% sodium dodecyl sulfate polyacrylamide gel and analyzed by immunoblot.

The mAb was further analyzed for epitope mapping. Twenty six peptides spanning amino acids (1–387) with ten amino acid overlapping (Supplemental data) were synthesized at the CDC core facility and dotted onto nitrocellulose membranes and analyzed by dot blot.

3.4. Development of EIA using 1D4 mAb

Microplates were coated with 1D4 mAb overnight at 4 °C, washed with PBS-0.05% Tween 20 solution, and blocked with 3% BSA in PBS-Tween 20 at 37 °C for 1 h. Subsequent, 100 µl of cell culture or clinical sample in PBS was added and incubated for 1 h at 37 °C. Afterwards, 100 µl of the biotin-conjugated 1D4 mAb was added and incubated at 37 °C for 1 h, followed by streptavidin poly-HRP (Thermo Scientific). Once incubated and washed thoroughly, the reactions were developed with TMB substrate and stopped with 1N HCl and OD value was measured.

3.5. Cut-off criteria of new EIA assay

Thirty EIA-negative stool samples were confirmed negative for rotavirus by RT-PCR and used to estimate the cutoff level for new EIA. A sample with an OD450 value above mean +3 standard deviation of negative fecal samples was considered positive.

3.6. Sensitivity and specificity of the EIA

The sensitivity of the EIA was determined by testing serially 2-fold diluted stool specimens or cultured rotavirus strains in diluent buffer. The limit of detection was based on the cutoff value. The specificity of the EIA was evaluated with rotavirus strains and other enteric viruses.

3.7. Evaluation and validation of assay repeatability

The EIA was utilized to test three stool samples with high, intermediate and low EIA OD values. Each sample was tested five times separately in one plate for intra-repeat assay and tested at five different days for inter-repeat assay.

3.8. Detection of rotavirus in clinical samples by EIA and RT-PCR

To evaluate the new EIA for the detection of rotavirus in clinical samples, we tested a fecal collection from infants who received two doses of Rotarix as part of the routine immunization from July through December of 2011 in Mexico [29]. Stool specimens were collected from infants 4–7 days after the first dose of Rotarix vaccination. All specimens were stored at −70 °C and at the time of testing, 10% homogenized extracts were prepared in PBS by centrifugation with a microfuge at maximal speed for 1 min. Supernatants were tested using two commercial EIA kits and the in-house EIA. All stool samples were also tested by RT-PCR and the products were analyzed on a 1% agarose gel [30,31].