Comparative efficacy of fluorescent antibody test, immunoperoxidase test and enzyme linked immuno sorbent assay in detection of rotavirus in cell culture

N. N. Barman · A. J. Nath · B. K. Neog ·
G. Das

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Abstract Rotavirus is prevalent worldwide and has been established as a leading cause of mortality due to severe diarrhoea in neonates. Isolation of the virus is a gold standard method for confirmation of rotavirus infection in the host. Propagation of rotavirus in cell culture is a challenge as in many instances the virus does not produce detectable cytopathic effect. This study was undertaken to evaluate the efficacy of fluorescent antibody test (FAT), immunoperoxidase test (IPT) and sandwich ELISA (S-ELISA) to detect rotavirus antigen propagated in MA 104 cell line. The intensity of fluorescence and colour development for I-FAT and I-IPT was categorized and the ELISA OD values were analyzed. The overall mean of detection were 5.16 ± 0.47 , 5.16 ± 0.54 and 5.66 ± 0.33 for I-FAT, I-IPT and S-ELISA, respectively. Significantly less number of samples were positive in the initial one or two passage, which increased up to 100 % from third/fourth passage onwards. The study concluded that I-FAT, I-IPT and S-ELISA were equally effective in detecting propagated rotavirus in cell line, and the former two tests are suitable for in situ demonstration of the virus while the later could be used to assay antigen in cell culture fluid.

Keywords Rotavirus · Cell culture · FAT · IPT · ELISA

Introduction

Rotavirus is a non-enveloped double stranded segmented RNA virus of the family *Reoviridae* with an icosahedral capsid [3] and is prevalent worldwide. In Assam, the epidemiology of rotavirus in pig has been reported [8]. Virus isolation in cell culture is considered as a gold standard for confirmation of rotavirus infection. However, due to non-CPE producing nature of pig and human rotavirus, virus detection and assay in cell cultures is extremely difficult [5]. Various diagnostic methods are being used for detections of rotavirus, yet a comparative evaluation is needed to select a precise and confirmatory technique for an unambiguous diagnosis. The present study compares the efficacy of fluorescent antibody technique (FAT), immunoperoxidase test (IPT) and enzyme linked immuno sorbent assay (ELISA) for detection of rotavirus grown in MA 104 cell line.

Materials and methods

Preparation of samples

Five diarrhoeic samples and one cell culture porcine group A rotavirus (initially isolated from diarrhoeic piglet and maintained at Department of Microbiology, CVSc, Khanapara) isolate were included in the present study. The samples were confirmed to have group A rotavirus by sandwich ELISA (S-ELISA) and ribonucleic acid polyacrylamide gel electrophoresis (RNA PAGE). The samples were filtered through 0.45 μm membrane filter (Millipore) and treated with cocktail antibiotic solution and incubated at 37 °C for 1 h. The inoculums were further treated with trypsin at the rate of 10 $\mu\text{g/ml}$ for 1 h at 37 °C to facilitate adsorption of the virus on to the cells.

N. N. Barman (✉) · B. K. Neog · G. Das
Department of Microbiology, College of Veterinary Science,
Assam Agricultural University, Khanapara, Guwahati, Assam,
India
e-mail: nnbarman@gmail.com

A. J. Nath
Pasteur Institute of India, Coonoor,
Nilgiris 643 103, Tamil Nadu, India

Isolation of rotavirus in MA-104 cell line: preparation of cell monolayer

MA 104 cell line was obtained from NCCS, Pune and maintained in the cell repository of Department of Microbiology, CVSc, Khanapara for routine isolation work. The cell monolayer was detached from the tissue culture flask using 2 ml of freshly prepared trypsin-versene solution and incubating the flask at 37 °C for 10–15 min. The detached cells were re suspended in growth medium (EMEM, JRH, USA) containing 10 % fetal calf serum and antibiotics cocktail (0.02 ml/ml of cell culture suspension). After thorough mixing, the cell suspension were distributed in 100 microlitre volume to 12 well microtitre plates (Corning) containing cover slips for IFT, 96 well tissue culture plates (Nunc) for IPT and test tube/tissue culture flask (Nunc) for S-ELISA, respectively and incubated at 37 °C in a humified atmosphere containing 5 % CO₂ and examined daily under inverted microscope (Axiovert 40, Zeiss, Germany) for growth until a total confluent monolayer.

Infection of monolayers

Test tubes and bottles (25 cm²) with 60 % confluent monolayer were selected for rotavirus infection. Growth media in the tubes and bottles was discarded and washed twice with Ca⁺⁺ Mg⁺⁺ free PBS (pH 7.3, 0.0015 M). The cell monolayers were inoculated with pre-treated 20 % faecal suspension as rotavirus inocula and 3.2×10^4 TCID₅₀ per ml of cell culture virus at the rate of 0.5 ml and 1 ml per tube and bottle respectively, and kept at 37 °C for 1 h to facilitate virus adsorption onto the cells. The inoculated cells were washed with sterile Ca⁺⁺ Mg⁺⁺ free PBS. Serum free maintenance medium containing trypsin (1 µg/ml) was added at a volume of 3 and 10 ml to the tubes and bottles, respectively. Test tubes and bottles containing the monolayers were incubated at 37 °C and were observed at 24 h intervals, under an inverted microscope (American optical) for any alteration of cell morphology up to 4–5 days post infection. Thereafter, the tubes and bottles were kept at –20 °C. For lysis of the rotavirus infected cells, 2–3 cycles of freezing-thawing were done. The inoculum so obtained was clarified by centrifugation at 1,000 g for 15 min. The fluid containing the virus was aliquoted in small screw capped vials and stored at –20 °C until further use. The experiment was carried out till 6th passage level, but without any observable CPE.

Indirect fluorescent antibody test (I-FAT)

Cell culture propagated virus was detected by indirect fluorescent antibody technique (I-FAT) as per the method

described in the European union diagnostic manual [1]. Virus infected cells at different passage level were incubated for 72–96 h and fixed in chilled acetone (–20 °C) for 10 min. After air drying, a selected area of monolayer was marked with PAP pen (Dako, Denmark), washed with wash buffer (0.01 M PBS), treated for 5 min with 0.4 % Triton X-100 prepared in washing buffer followed by blocking with 2 % BSA for 1 h at room temperature. Polyclonal pig anti rotavirus antibody (1:500 dilution, 100 µl volume) was added to all the cover slip containing wells and incubated at 37 °C for 1 h. After thorough washing, rabbit anti pig FITC conjugate (DAKO, Denmark, 1:50 in 1 % BSA) was added and the plates were incubated for 1 h. The final step was washing of the coverslips and then mounting of these on microscopic slides with 20 % glycerine in PBS (0.01 M). The slides were examined under fluorescent microscope (Olympus, Japan) for bright green fluorescence indicative of rotavirus antigen.

Indirect immunoperoxidase test (I-IPT)

Indirect IPT was performed as per the method described in the European union diagnostic manual [1] with slight modification. Cell monolayer attaining approximately 70 % cell growth in 96 well tissue culture plate (Nunc) was infected with rotavirus for 4–5 days. For fixation of cells, acetone/methanol (1:1) solution was added at a volume of 100 µl/well and left for 10 min at room temperature. The cell monolayer was treated with 0.4 % Triton X-100 (in PBS-T) for 5 min. The non specific binding sites were blocked by 2 % BSA (in PBS-T) for 1 h at room temperature. Thereafter, 50 µl of polyclonal pig anti-rotavirus antibody (1:100 in 1 % BSA) was added in the wells. The plate was incubated for 1 h at 37 °C. After that, the plate was washed twice with PBS-T for 5 min. Then, rabbit anti-swine HRPO conjugate (DAKO, Denmark) diluted 1:1000 in 1 % BSA was added at the rate of 50 µl/well. The plate was incubated again at 37 °C for 1 h. After washing with PBS-T three times, the wells were filled with 50 µl of chromogen-substrate solution and stained for 15–30 min at room temperature. After discarding the substrate, the wells were washed with deionized water to stop the colour reaction. The plate was observed under an inverted microscope (Olympus, Japan) for evaluation of the results. Cells showing dark red staining of the cytoplasm were considered to be infected by rotavirus.

For I-FAT and I-IPT the interpretation of results were based on intensity of fluorescence or staining reaction and categorized as less fluorescence/staining in the periphery of cell (+), less fluorescence/staining in the entire cytoplasm (++) and bright fluorescence/staining in the entire cytoplasm (+++) (Fig. 1).

Sandwich enzyme linked immuno sorbent assay (S-ELISA)

The presence of rotavirus in the cell culture fluids was determined by using S-ELISA following the method of Van Nieuwstadt et al. [12] with slight modification. Briefly, 96 well microtitre plates (Maxisorp, Nunc, Denmark) were coated with rabbit anti-rotavirus antibody (1:800 in carbonate-bicarbonate buffer, pH 9.6). The unreactive sites were preblocked with blocking buffer (5 % skimmed milk powder in 0.06 M PBS-T). The test samples were added and serially diluted with an initial dilution of 1:2 in blocking buffer. After incubation, swine anti-rotavirus antibody (1:500) diluted in blocking buffer was added and incubated. This was followed by addition of rabbit anti-swine HRPO conjugate (1:1000, DAKO, Denmark). All these steps were followed by 1 h incubation at 37 °C and washed 5 times with tween PBS and 50 µl volume of each reagents were used. After incubation, freshly prepared substrate (H₂O₂) and chromogen (OPD, Sigma) mixture was added and allowed to react for 15 min. Finally, reaction was stopped by adding 1 M H₂SO₄. Positive control was cell culture propagated group A rotavirus and negative control was meconium. Tracing antibody and conjugate controls were also used in the test. The optical density (OD) of the wells was read at 490 nm wavelength in an ELISA reader (Biorad, USA). The cut-off OD value was obtained by subtracting the mean OD value of negative control from that of the test samples. Difference of OD value ≥ 0.1 was considered as positive.

Results

Detection of field rotavirus grown in cell culture

I-FAT, I-IPT and S-ELISA were employed for detection of the virus in the cell culture at different passage levels

Fig. 1 Rotavirus infected MA 104 cells showing brilliant green fluorescence (a) in I-FAT ($\times 100$) and dark red cytoplasmic staining (b) in I-IPT ($\times 400$) after 72 h of infection

(Table 1). In the first passage 3 (50.00 %) out of 6 isolates were detected by both I-FAT and I-IPT. The number of rotavirus positive isolates increased subsequently, and all isolates (100.00 %) were found positive from the fourth passage onward by I-FAT and third passage onwards by I-IPT, respectively. Using S-ELISA 4 out of 6 samples (66.66 %) were found positive in the 1st passage level but the number of positive samples increased and from 2nd passage onwards, all (100 %) samples yielded positive for rotavirus. Standard group A rotavirus was included as positive control. The intensity of the immunofluorescence and colour development (Fig. 1) in the infected cells was however less in the initial two passages but in the subsequent passage bright green cytoplasmic fluorescence and colour were observed. All the tests could detect the cell culture propagated rotavirus from the first passage onwards. The virus could be detected in significantly higher number of samples from second passage onwards.

Comparative efficacy of I-FAT, I-IPT and S-ELISA

The overall mean as detected by I-FAT, I-IPT and S-ELISA were 5.16 ± 0.47 , 5.16 ± 0.54 and 5.66 ± 0.33 , respectively. Statistical analysis revealed that there was no significant difference between I-FAT, I-IPT and S-ELISA used for detection of propagated virus isolates.

Discussion

The increased intensity of cytoplasmic fluorescence and colour development along with increasing passage level as detected by FAT and IPT test system clearly indicated an increase in viral concentration in the infected cells. These findings are in agreement with observations made by Morera et al. [7], Sestak and Musilov [10]. The intensity of the

Table 1 Detection of cell culture propagated field rotavirus at different passage level using I-FAT, I-IPT and S-ELISA ($n = 6$)

Passage level	No. of samples in which rotavirus detected			Overall mean
	I-FAT	I-IPT	ELISA	
First	3 (50.00)	3 (50.00)	4 (66.66)	$3.33^a \pm 0.33$
Second	5 (83.33)	4 (66.66)	6 (100.00)	$5.00^b \pm 0.57$
Third	5 (83.33)	6 (100.00)	6 (100.00)	$5.66^{bc} \pm 0.33$
Fourth	6 (100.00)	6 (100.00)	6 (100.00)	$6.00^c \pm 0.00$
Fifth	6 (100.00)	6 (100.00)	6 (100.00)	$6.00^c \pm 0.00$
Sixth	6 (100.00)	6 (100.00)	6 (100.00)	$6.00^c \pm 0.00$
Overall mean	5.16 ± 0.47	5.16 ± 0.54	5.66 ± 0.33	

Values in parenthesis indicate percentages, mean value having same superscript does not vary significantly ($P < 0.01$)

cytoplasmic fluorescence was 1.1–2.0 times higher in infected cell than observed in control cells. Sestak and Musilov [10] suggested that the concentration and integrity of virus particles in the sample are the decisive factors for success of an adaptation attempt.

Different antibody based techniques like fluorescent antibody test (FAT), immunoperoxidase test (IPT), enzyme linked immunosorbent assay (ELISA) have been used for detection of cell culture rotaviruses [2, 6, 10, 11]. The usefulness of immunofluorescence is limited by the need for specialized equipment and experienced staff, and also by the difficulty in coping with large numbers of specimens [5]. Despite these limitations, the immunofluorescence test can be used in routine virus diagnosis because it is rapid, accurate and flexible with properly validated reagents [4]. The immunoperoxidase staining can be used as a highly satisfactory alternative to immunofluorescence staining for identification of cell culture propagated rotavirus. IP assay has several advantages over the IF test. It can be easily applied to a large number of samples, is less expensive, and it does not require fluorescent microscope equipment and expertise in reading. In addition, once the enzyme chromogen is developed in the cell monolayer, it provides a permanent record [9, 13]. Both tests have advantages to demonstrate in situ the virus infected cells and can facilitate to count multiplicity of infection (MOI). S-ELISA, on the other hand is simple, sensitive, specific and inexpensive and can be used for screening of large number of samples within a short period and assay the virus concentration. Further, S-ELISA can be considered to be a handy test because the results of the test can be read with the naked eye or a simple colorimeter.

It is concluded that I-FAT, I-IPT and S-ELISA techniques are equally effective in detecting cell culture propagated rotavirus. However, in case of field samples, mere negative result in initial few passages may not be confirmative of absence of virus in the sample.

References

1. Anon. European union diagnostic manual on swine fever. Germany: Hanover; 2002. p. 11–6.
2. Bohl EH, Theil KW, Saif LJ. Isolation and serotyping of porcine rotaviruses and antigenic comparison with other rotaviruses. *J Clin Microbiol.* 1984;19:105–11.
3. Li Z, Baker ML, Jiang W, Estes MK, Prasad BV. Rotavirus architecture at subnanometer resolution. *J Virol.* 2009;83:1754–66.
4. Madeley CR, Peiris JS. Methods in virus diagnosis: immunofluorescence revisited. *J Clin Virol.* 2002;25:121–34.
5. McNulty MS. Rotaviruses. *J Gen Virol.* 1978;40:1–18.
6. Mirazimi A, Magnusson K, Svensson L. A cytoplasmic region of the NSP4 enterotoxin of rotavirus is involved in retention in the endoplasmic reticulum. *J Gen Virol.* 2003;84:875–83.
7. Morera P, Losada A, Fonseca G. Increased of heat shock cognate protein, HSC70, in MA 104 cells following rotavirus infection. *Rev Fac Med Unal.* 2007;55:224–30.
8. Nath AJ, Barman NN, Das SK. Faecal excretion of rota virus in naturally infected pigs: an epidemiological study. *Ind J Anim Sci.* 2007;77:859–61.
9. Schmidt NJ, Dennis J, Lennette EH. Comparison of immunofluorescence and immunoperoxidase staining for identification of rubella virus isolates. *J Clin Microbiol.* 1978;7:576–83.
10. Sestak K, Musilova J. Isolation of rotavirus strains from naturally infected piglets. *Acta Virol.* 1994;38:27–30.
11. Smitalova R, Rodak L, Psikal I, Smid B. Isolation, immunochemical demonstration of field strains of porcine group A rotaviruses and electrophoretic analysis of RNA segments of group A and C rotaviruses. *Vet Med.* 2006;51:288–95.
12. Van Nieuwstadt AP, Cornelissen JBWJ, Zetstra T. Comparison of two methods for detection of transmissible gastroenteritis virus in faeces of pigs with experimentally induced infection. *Am J Vet Res.* 1988;49:1836–43.
13. Zentner BS, Margalith M, Galil A, Halevy B, Sarov I. Detection of rotavirus-specific Ig G antibodies by immunoperoxidase assay and enzyme-linked immunosorbent assay. *J Virol Meth.* 1985; 11:199–206.