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RESEARCH ARTICLE

DETECTION OF BOVINE ROTAVIRUS IN NEONATAL CALF DIARRHOEA BY ELISA, FAT AND TRANSMISSION ELECTRON MICROSCOPY

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ABSTRACT

The present study was designed to investigate and compare the sensitivity of enzyme linked immunosorbent assay (ELISA), fluorescent antibody test (FAT) and transmission electron microscopy (TEM) for the detection of bovine rotavirus during neonatal calf diarrhea. A total of 112 faecal samples of diarrhoeic calves below three months were collected from organized dairy farms of Namakkal (Tamil Nadu) and Bareilly (Uttar Pradesh), India. Out of these, 27 faecal samples (24.10%) were found positive for rotavirus by ELISA and TEM. The percent positivity ranged from 18.86 % to 55.55% in different organized farms. The rotavirus samples when subjected to isolation in MDBK cells resulted in successful isolation of bovine group A rotavirus in twenty samples. The characteristic cytopathic effects (CPE) were observed from second passage onwards and comprised of clumping and rounding of infected cells, detachment of monolayer, intracytoplasmic eosinophilic inclusions, syncytia and leaving empty vacuole space in MDBK cell line. Rotavirus antigen in cell culture adapted virus was detected by direct fluorescent antibody test (dFAT). The sensitivity and specificity of ELISA and TEM were 100% and 92.32% respectively, considering virus isolation followed by dFAT as standard test. Study indicates that ELISA is most suitable and sufficient test for routine diagnosis of bovine rotaviral as compared to laborious TEM analysis for diarrheic samples; however, isolation of the virus in cell culture remains indispensable for the identification of doubtful specimens. To our knowledge, this is the first report from India employing TEM, ELISA and cell culture based FAT for detection and comparison of these tests for bovine rotavirus group A from diarrhoeic calves.

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INTRODUCTION

Rotavirus diarrhoea is major cause of death of millions of children in developing countries besides being economically significant malady in neonates of many domestic animals (Bishop *et al*., 1973; Gumusova *et al*., 2007; Uhde *et al*., 2008; Dhama *et al*., 2009; WHO, 2009; Martella *et al*., 2010; Suresh *et al.,* 2012). In bovines, rotavirus is the most common agent responsible for neonatal calf diarrhoea (Singh and Singh, 1971; Acres *et al*., 1975; Woode, 1978; Khan and Khan 1991; Chauhan and Singh 1996; Svensson *et al.,* 2003; Dhama *et al*., 2009; Martella *et al.,* 2010). Rotavirus is non-enveloped, double stranded RNA virus, with a diameter of 65-70 nm and belongs to family *Reoviridae*. The geneome of rotavirus comprises of 11 segments of double stranded RNA (16-21 kbp) which is surrounded by an inner and outer capsid layers (Desselberger *et al*., 2005; MacLachlan and Dubovi, 2010). Formation of reassortants is in part responsible for the wide variety of rotavirus strains found in nature; even reassortants of animal-human strains have been identified. Since rotavirus diarrhoea is a major health problem of young animals, several methods like agar gel immunodiffusion and counter-immunoelectrophoresis, complement fixation, radio-immunoassay, haemagglutination and haemagglutination inhibition (Mohammed *et al.,* 1978), dot immunobinding (Chauhan and Singh, 1992a), RNA-polyacrylamide gel electrophoresis (RNA-PAGE) (MacLachlan and Dubovi, 2010; Manuja *et al*., 2010), electron microscopy (EM) (Benfield *et al*., 1984; MacLachlan and Dubovi 2010), ELISA (Benfield *et al*., 1984; Manuja *et al*., 2010), cell culture FAT (Hammami, *et al*., 1989; Khattar and Pandey, 1990; Chauhan and Singh, 1992b), reverse transcription polymerase chain reaction (RT-PCR) (Gouvea *et al*., 1990; MacLachlan and Dubovi 2010; Manuja *et al*., 2010; Suresh *et al*., 2011), integrated cell culture and reverse transcription quantitative PCR (ICC-RT-qPCR) assay (Li *et al*., 2010) have been employed for the detection of rotavirus (Dhama *et al*., 2009). However, each of these methods has its own limitations. Examination of faecal samples with EM although provides rapid results is generally not carried nowadays for routine diagnostis and as the samples must contain approximately10^6 virions/ml (Athanassious *et al.,* 1994; MacLachlan and Dubovi, 2010). Transmission electron microscopy (TEM) appears to be an indispensable diagnostic tool with negatively stained faecal samples. Reports indicated that TEM is more sensitive and valuable technique than FAT in isolation and detection of coronavius from diarrhoeic samples, and comparison of diagnostic tests has also been described likewise for rotavirus diarrhea (Dar *et al*., 1998; Benfield *et al*., 1984; Khattar and Pandey, 1990; Athanassious *et al*., 1994 ; Martin and Follet, 1997). However, ELISA remains to be most preferred test for detecting viral antigen and have been used during the efficacy trials of rotavirus vaccine (Joensuu *et al*., 1997; Perez-Schael *et al*., 1997). While ELISA is 10–100 times more sensitive than EM, there is variability of the sensitivity and specificity of type of enzyme immunoassay assay (EIA) used (Lipson *et al*., 1990, 2001).

Isolation of the rotavirus in cell culture is generally less sensitive and is laborious process but gives the ultimate proof of virus association with the disease. Isolation of bovine rotavirus (BRV) is performed in rotavirus specific primary cell cultures (calf kidney cells) and cell lines (MA 104-Simian origin, MDBK, and PK-15). Enhancement of

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cytopathic effect (CPE) has been shown to be increased by incorporation of trypsin in the medium in minute quantities and by the pretreatment of faecal samples with trypsin (Almeida *et al.,* 1978; Chauhan and Singh 992c; Steele *et al*., 2003). The objective of this study was to compare three diagnostics tests viz., ELISA, TEM and cell culture based direct (d) FAT for diagnosis of bovine rotavirus.

MATERIALS AND METHODS

Collection of faecal samples

During the period from September 2008 to March 2009, faecal samples were collected from 112 calves, below three months of age, evincing symptoms of diarrhoea, from Military Dairy Farm, Bareilly (Uttar Pradesh), Organized Dairy Farm of Namakkal (Tamil Nadu), India and Post Mortem Room, Indian Veterinary Research Institute, (IVRI), Izatnagar. All the samples were collected from calves of different breeds namely non-descript, Frieswal, Holstein Friesian and Jersey directly from the rectum. All faecal samples were suspended in 10% (W/V) phosphate buffered saline (PBS, pH 7.4), clarified by centrifugation at 8000 x g for 10 min at 4°C and supernatants were collected and stored at – 20°C till further use.

Detection of bovine rotavirus by ELISA

All the 112 clinical samples were screened for the presence of rotavirus using commercially available ELISA kit (Bio-X Diagnostics, Belgium) as per manufacturer's recommended protocol.

Detection of bovine rotavirus by transmission electron microscopy (TEM)

Faecal samples were processed for TEM as described by Theil and McCloskey (1995) with slight modifications. Briefly, A 10% fecal suspension in PBS (pH 7.4) was sonicated for 20 seconds and then cleared by centrifugation at 15,000 x g for 60 minutes. About 200µl supernatant was then stained with phosphotungstic acid (pH 6.4), nebulized on a 200-mesh collodion-coated grid and viewed under an electron microscope at a magnification of 40,000 to 80,000x.

Isolation of bovine rotavirus in MDBK cell line

The Madin Darby bovine kidney (MDBK) cell line obtained from CADRAD-Virology, IVRI, was used in this study. Isolation of bovine group A rotavirus was performed as per the method of Saravanan *et al.* (2006). Briefly, the rotavirus positive supernatant fluids were filtered through 0.45 µm membrane syringe filter and filtrates were mixed with an equal volume of Eagle's Minimum Essential Medium (EMEM) containing 2% fetal calf serum (FCS) and 10 µg/ml crystalline trypsin (Amersco, USA), and incubated at 37°C for 60 min. After incubation, one ml of the mixture was inoculated into the culture flasks with confluent monolayer of MDBK cell lines and kept for 1 hr incubation. After adsorption at 37°C for 1 hr, the cells were washed thrice with plain EMEM and then over layered with maintenance medium containing 1 µg /ml of crystalline trypsin and incubated at 37°C. Cytopathic effects (CPE) were observed for 5 days post infection (p.i) and cells showing characteristic CPE were harvested by freezing and thawing thrice and centrifuged at 12000 x g for 20 min at 4°C for the removal of cell debris. The supernatant containing the virus was collected and stored at -20°C for further passages. Rotavirus infected MDBK cells were stained with hematoxylin and eosin (H and E) to observe characteristic CPE (Chauhan and Singh, 1992c; Saravanan *et al.,* 2006).

Identification of isolated bovine rotavirus by fluorescent antibody test (FAT)

The fifth passage virus was used as the inoculum for chamber slide cultures to be used for detection of virus by dFAT as described previously with some modifications (Hansa *et al.,* 2012). After the characteristic CPE was observed, the chamber slides were washed with sterile PBS solution (pH 7.4) and cells were fixed with absolute cold methanol for 15 min. the cells were then washed with PBS and incubated with 1:20 dilution FITC anti-rotavirus monoclonal antibody (Bio-X Diagnostics, Belgium) at 25ºC for 1hr. Finally, cells were washed with PBS and mounted with aqueous glycerol mountant and examined under UV microscope at 40x.

Statistical analysis

Sensitivity and specificity were calculated at 95% confidence level using Vassar Stats: statistical computation website (http://faculty. vassar.edu/lowry/VassarStats.html).

RESULTS

Detection of bovine rotavirus by ELISA

Out of 112 faecal samples from diarrhoeic calves screened by ELISA, 27 samples were positive for bovine group A rotavirus. The percent positivity for BRV was 18.86%, 24.0% and 55.55% in Military dairy farm (Bareilly, Uttar Pradesh), Dairy farm, (Namakkal, Tamil Nadu) and PM room (IVRI, Bareilly, Uttar Pradesh) with an overall average of 24.10% (Table 1). The sensitivity and specificity of ELISA to detect to BRV was 100% and 92.39% respectively (Table 2).

Detection of bovine rotavirus by TEM

Transmission electron microscopy (TEM) studies revealed the presence of double-shelled rotavirus like particles, approximately with the diameter of 55-70 nm (Fig. 1). Out of 112 faecal samples, rotavirus was observed in 27 samples. The percent positivity for rotavirus was 18.86%, 24.0% and 55.55% in Military Dairy Farm (Bareilly, Uttar Pradesh), Dairy Farm, (Namakkal, Tamil Nadu) and PM Room (IVRI, Bareilly, Uttar Pradesh) with an overall average of 24.10% (Table 1, 2).

Isolation of bovine rotavirus in MDBK cell line

In first passage, infected cells did not show any cytopathic effect (CPE). But from second passage onwards the infected cells started

Table 1. Detection of group A rotavirus in faecal samples from different organized farms by virus isolation based dFAT, ELISA and TEM

Name of the farm/PM room	No. of samples	ELISA positive	TEM positive	Virus Isolation followed by DFAT
Military dairy farm, Bareilly	53	10 (18.86%)*	10 (18.86%)	9 (16.98%)
Dairy farm, Namakkal	50	12 (24.0%)	12 (24.0%)	10 (20%)
PM room, IVRI	9	5 (55.55%)	5 (55.55%)	1 (11.11%)
Total	112	27 (24.10%)	27 (24.10%)	20 (17.85)

*Figures in the parenthesis are the percentage of animals positive for the particular test

Table 2. Sensitivity and specificity of ELISA and TEM compared with virus isolation followed by dFAT for detection of bovine rotavirus

Tests	Result	Virus Isolation followed by DFAT		Total	Sensitivity[a] (%)	Specificity[a] (%)
		Positive	Negative			
ELISA/ TEM	Positive	20	7	27	100	92.39
	Negative	0	85	85		
	Total	20	92	112		

[a] The values are calculated at 95% confidence interval

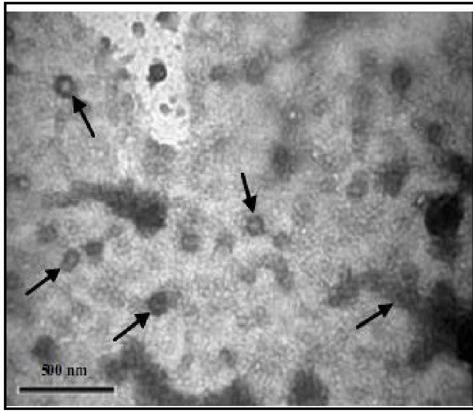

Fig. 1. A transmission electron micrograph showing negatively stained rotavirus. Rotavirus particles are approximately 60-70 nm in diameter. (A 500 nm size bar is included for comparison),x80,000.

showing characteristic CPE. At 24 hrs post infection (p.i,) the infected cells became round and clumped. At 48 hrs p.i, the cells were thin, round shaped and have tendency to form syncytia. At 72 hrs p.i, the cells became small and majority of monolayer detached and left empty vacuoles. At 96 hrs p.i, only few cells were found attached to the glass surface and the rest were detached and were found floating in the medium and the monolayer showed typical moth eaten appearance. At 120 hrs p.i, the monolayer was completely washed out (Fig. 2).

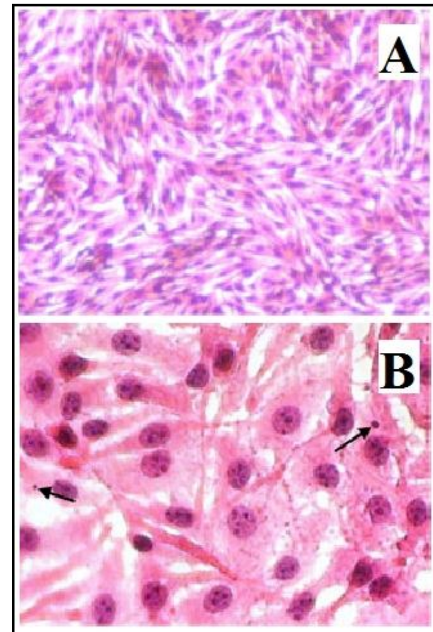

Fig. 3. MDBK cells infected with BRV and stained by routine H & E staining procedure (A): Uninfected control, x100, (B) Cells showing syncytia, eosinophilic intracytoplasmic inclusion body at 48 hrs p.i, ×200.

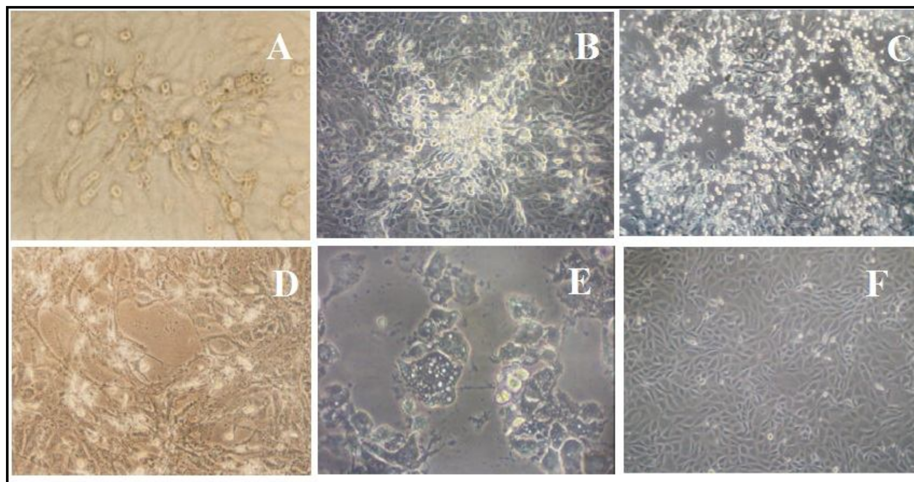

Fig. 2. Isolation of the bovine rotavirus in the MDBK cell line. (A) Unstained infected cells showing clumping and rounding, 48 hr p. i., ×400. (B) Cells showing syncytia formation and detachment, 72 hr p.i., unstained, ×100. (C) Detached cells floating in the media, 96 hr p.i, x100. (D) Moth eaten appearance of infected MDBK cells, 120 hr, p. i x100. (E) cells showing vacuolation, 120 hr p.i. (F) Uninfected MDBK cell control.

On H&E staining, uninfected MDBK cells showed normal staining characteristics while the infected cells showed syncytia, eosinophilic intracytoplasmic inclusion body and cells were surrounded by a halo space (Fig. 3). Out of 27 samples only 20 samples were able to infect MDBK cells. The percent adaptation for rotavirus was 16.98%, 20% and 11.11% in samples of Military Dairy Farm (Bareilly, Uttar Pradesh), Dairy Farm, (Namakkal, Tamil Nadu) and PM Room (IVRI, Bareilly, Uttar Pradesh) with an overall average of 17.85% (Table 1).

Identification of isolated bovine rotavirus by FAT

The infected chamber slide cultures were stained for rotavirus antigen revealed specific granular, diffuse apple green fluorescence in the cytoplasm of infected cells. In few places, inclusion body, syncytia of infected cells were also seen. All the 20 samples adapted in MDBK cells were positive for bovine group A rotavirus antigen. Negative control didn't show fluorescence in FAT (Fig. 4).

DISCUSSION

Rotaviruses are the single most important cause of severe diarrheal illness in infants and young children in both developed and developing countries as well as in intensively reared farm animals worldwide. It has been estimated that rotaviruses in humans account for 18% (i.e., >2 million) of the 10.6 million deaths, with the greatest toll being in the developing countries (Estes and Kapikian 2007). In animals' disease is usually seen only in young animals, 1–8 weeks of age and the severity of disease ranges from subclinical, through enteritis of varying severity, to death. For the effective control measures prompt diagnosis of the disease is important (Dhama *et al*., 2009). The present study was designed to investigate and to compare the sensitivity and specificity of ELISA and TEM diagnostic methods using isolation of the virus in cell culture and confirmation by dFAT as standard method. TEM and ELISA are more sensitive and valuable techniques than virus isolation for detecting rotaviruses but the later remains standard for rotavirus confirmation (Dar *et al*., 1998). Previously, electron microscopy has been used as standard for comparison of

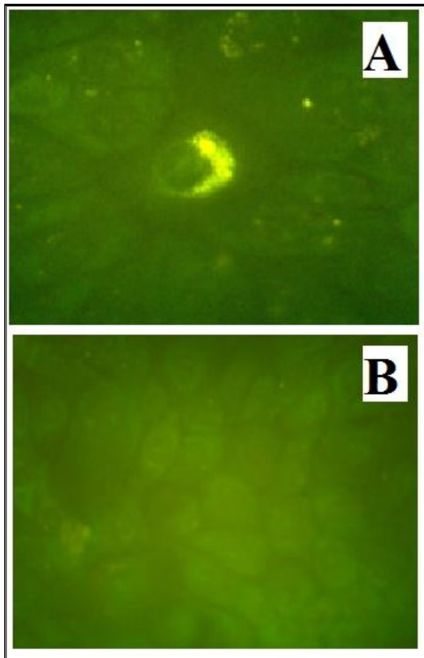

Fig. 4. Direct fluorescent antibody assay showing specific granular apple green fluorescence, syncytia and inclusion body in the cytoplasm of rotavirus infected MDBK cells at 48 hr post infection(A) and uninfected control (B) ×400

commercial latex agglutination tests and enzyme immunoassay for the detection of rotavirus in animal faeces (Goyal *et al*., 1987). Research reports have indicated that the comparative sensitivity and specificity of ELISA, RNA-PAGE and RT-PCR were 100%, 66.67% and 71.43% and 97%, 100% and 100%, respectively, considering virus isolation as standard test (Benfield *et al*., 1984; Athanassious *et al*., 1994; Martin and Follet, 1997; Dhama *et al*., 2009; Manuja *et al*., 2010; Martella *et al*., 2010; Suresh *et al.,* 2011). However, ELISA being simple, fast and sensitive assay can be performed routinely and can act as instrumental for the diagnosis of group A rotavirus and field epidemiological studies. In the present study, out of 112 faecal samples of diarrhoeic calves tested, 27 (24.10%) samples were found positive by ELISA and but from only 20/27 (74%) samples virus infected MDBK cell as detected and confirmed by dFAT. To our knowledge, this is the first report from India employing TEM, ELISA and cell culture based DFAT for detection and comparison of these diagnostics for bovine rotavirus group A from diarrhoeic calves.

Previous studies indicated that isolation of BRV in the MDBK cell line in presence of trypsin increases the viral growth by 100 fold when incorporated in maintenance medium (Almeida *et al*., 1987; Albert, 1990). In the present study, viral growth in cell culture was assessed by examining inoculated cells for CPE and direct fluorescent antibody test (dFAT). Out of twenty seven samples, twenty viral samples infected MDBK cells as determined by production of characteristic CPE at second passage level and it continued up to eight passages. The CPE produced in this study were in agreement with previous reports (McNulty *et al.,*1977; Nagesha *et al.,* 1985; Saravanan *et al.,* 2006). The virus replicates and multiplies in endoplasmic reticulum and the clusters of viruses are seen as intra-cytoplasmic inclusion bodies on detachment and vacuolation of MDBK cells. In the present study, these changes were observed only after 72 hr p.i.. Infected cells stained with H&E staining method showed characteristic syncytia and eosinophilc intracytoplasmic inclusion body. Confirmation of the virus by FAT indicated specific granular, diffuse apple green fluorescence in the cytoplasm of infected cells. In few places, inclusion body, syncytia of infected cells was also observed and theses findings were in agreement with the earlier reports (Chauhan and Singh 1992c; Winiarczyk and Gradzki, 1999).

When the two techniques were compared, sensitivity of ELISA and TEM was found to be 100%, considering virus isolation followed by dFAT as standard test, and specificity of these assays with virus isolation followed by dFAT was 92.39% for both ELISA and TEM. The results of present study are in accord with other reports showing respective sensitivities and specificities of 100%, 94.7% for detection of porcine rotavirus (Winiarczyk and Gradzki, 1999) and 100%, 97 % for detection of bovine rotavirus by ELISA (Manuja *et al.,* 2010). The results of present study showed TEM is also highly sensitive technique for detection of bovine rotavirus than virus isolation in cell line and final confirmation dFAT in fecal samples. It is possible that there may be strain variation among the samples and few variants were not able to get adapted to the particular cell line. Also, the collection, transportation and processing of the fragile RNA virus samples increases the possibilities of viral death, although every step was given due consideration during the present study. Molecular methods of BRV diagnosis are being used as more sensitive and alternate methods. It has been found that RT-PCR is not only a highly sensitive method in detecting small concentrations of rotavirus in fecal samples but can also be used for strain identification and further differentiation (WHO 2009; Suresh *et al.,* 2011).

Taken together, the results of the present investigation demonstrated that TEM, ELISA, and cell culture based dFAT can be effectively employed in a diagnosis of rotavirus diarrhoea. However, for routine purposes, ELISA remains a choice due to its simple, convenient procedure and detection limit. However, when in doubt, isolation in cell culture should be used to verify findings. Although not suitable for the large scale screening of specimens, isolation and detection in cell culture remains indispensable for the identification of selected or doubtful specimens. Though TEM was found to be equally sensitive for viral identification it needs an experienced investigator. Further investigations need to be carried out to know the real epidemiological status and magnitude of infection of this important virus in animal population, studying its economical impacts and finding out genomic variations among the bovine rotaviruses circulating in the country.

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