Virological and molecular studies for detection of bovine Rotavirus in neonatal calves in some farms in Egypt

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ABSTRACT

The present work was designed to investigate bovine rotavirus antigen in fecal samples of forty diarrheic new borne calves of cattle (32) and buffaloes (8) below three months in some farms in Egypt. The detection was done by ELISA and indirect fluorescent antibody technique (FAT) as well as molecular method as real-time quantitative reverse transcriptase PCR (RT-qPCR) from samples collected during neonatal diarrhea. Six out of forty tested samples were found positive for rotavirus (15%) infection using ELISA test. Successful isolation of the rotavirus on MDBK cell line from two samples from the positive ELISA samples. The beginning of characteristic cytopathic effects (CPE) were observed from the 3rd passages as clumping, rounding of infected cells with syncytia and leaving empty vacuole space in MDBK cell line. The detected rotavirus with tissue culturing was identified by FAT with apple green fluorescence in the cytoplasm of infected cells. Viral RNA was extracted and the virus was confirmed by RT-qPCR assay utilized primers and probe that were designed to the target gene for rotavirus. The presence of rotavirus in fecal samples obtained from neonatal calves suggested its etiological roles and it is the main causative virus involved in neonatal calf diarrhea.

Key wards: Rotavirus – RT-qPCR – ELISA – Isolation-FAT.

INTRODUCTION

Rotaviruses infections are major cause of acute diarrhea in animals and humans which caused by rotaviruses belonging to RNA viruses from the family Reoviridae contains 11 segmented of no enveloped double-stranded RNA (ds RNA) genome (Estes, 1996), packed inside three protein layers of core, inner capsid, and outer capsid. The segmented nature of the viral genome allows reassortment in the mixed infection in natural conditions in animals leading to emergence of new serotypes of the virus (Niture et al., 2009).

Rotaviral diarrheas are common in calves and the results of its infections range from subclinical infection to death. The affected young calves may die as a result of severe dehydration or secondary bacterial infections (Holland, 1990). Rotavirus (RoV) is the leading causes of epidemic gastroenteritis and almost half of all deaths worldwide are estimated to occur in Africa (Mwenda et al., 2010 and Patel et al., 2011). RoV diarrhoea is the major cause of death of millions of children in developing countries besides being economically significant malady in neonates of many domestic animals (Dhama et al., 2009; WHO, 2009; Martella et al., 2010 and Suresh et al., 2013) causing severe diarrhea among infants and young children, with an estimated 611,000 deaths from rotavirus infection per year worldwide (Maclntyre and De Villiers, 2010).

The viral genome encodes six structural proteins (VP1 to VP4, VP6 and VP7) and six non-structural proteins (NSP1 to NSP6) (Greenberg and Estes 2009). These segments are classified serologically into seven different groups or species (A-G) (Estes and Kapikian 2007). Viral protein 6 (VP6) of the second layer of the capsid is called the group antigen. It is used for

rotavirus species identification and classification into 7 groups designated A–G (Smitalova et al., 2009). Group A rotavirus constitute the principal pathogen cause in human and animals (Versalovic et al., 2011 and Manuja et al 2010). Group A rotavirus is classified as G and P genotypes or serotypes according to the genetic or antigenic characteristics presented by the proteins VP7 and VP4, both located in the virus outer capsid. At least 25 different G genotypes and 33 P genotypes have been described based on molecular differences (Abe et al., 2011). The most predominant G serotypes in diarrheic calves in Egypt are G6 and G10 (Hussein et al., 1993 and 1999)

Diagnosis of rotaviral diarrhea requires detection of viral antigens or viral nucleic acids in feces. These diagnostic techniques including enzyme-linked imunosorbent assay (ELISA), polyacrylamide gel electrophoresis, nucleic acid hybridization and immune electron microscopy (Benfield et al., 1984 and Hammami et al., 1990).

PCR is an assay with higher sensitivity to diagnose rotavirus infection, especially when low levels of virus are shed in the feces because of its high sensitivity, however, this test should be performed by well-trained diagnostic personnel to avoid contamination with any trace amount of nucleic acid, which may lead to false-positive diagnosis (Chinsangaram et al., 1993). In vitro cultivation of rotaviruses is not easy as they require the presence of proteolytic enzymes both in the sample and maintenance medium. These enzymes are essential for outer capsid VP4 digestion. Usually trypsin is used to digest VP4 to VP5 and VP8 thus enabling virus penetration into a cell (Estes and Kapikian, 2007).

Molecular characterization was performed by multiplex semi-nested RT-PCR reactions, which indicated the association s of genotypes circulating in herds in Brazil (Silva et al., 2012). The presence of Rota virus in neonatal calves may constitute public health risks. The objective of this study was to identify and quantify the presence of the virus in fecal samples by different methods including isolation of the virus from fecal samples, ELISA, FAT and molecular diagnosis to help in production of vaccine against the local strain of virus to help the control of the disease.

MATERIALS AND METHODS

Specimens and sample preparation:

Fecal samples were collected from 40 calves of farm cattle (32) and buffaloes (8), from less than three months of age, showing symptoms of diarrhea from cattle farm and prepared according to Suresh et al., (2013). All fecal samples were transported immediately forwarded to the laboratory on ice packs and then each fecal sample was suspended in10% (W/V) phosphate buffered saline (PBS, pH 7.2-7.4). The suspensions were clarified by centrifugation at 3,000 rpm for 10 min; supernatants were collected and stored at – 20°C until analyzed (Tate et al., 2013).

Enzyme-linked immunosorbent assay (ELISA):

Clinical samples were screened for the presence of rotavirus using commercially available ELISA kit (Bio-X Diagnostics, S.P.R.L.U). All procedures were performed as manufacturer's recommended protocol.

Hyperimmune sera:

Bovine rotavirus antibody was obtained from Animal Health Research Institute (AHRI), Virology Department.

Virus isolation:

Trail of isolation of rotaviruses from field fecal samples was carried out on MDBK cells line obtained from (Vac. Sera, El-Agouza, Giza) grown in Eagle's minimum essential medium (MEM) supplemented with 10% bovine calf serum without antibodies to rotavirus. Each 25

cm^3 tissue culture flask was inoculated with a volume of 0.2 mL of trypsin treated sample and incubated for 1-2 hours at 37 oC. The inoculum was then removed and the flask was washed three times. The spacemen suspension was pre-incubated for 45 min at 37°C in Eagle's medium MEM with 10 µg/ml trypsin (Trypsin 1:250, Sigma, USA) as prescribed by (Ali et al., 2008). Cytopathic effect (CPE) was monitored for 7 days post inoculation.

Identification by FAT:

Indirect fluorescent antibody technique (IDFAT) was applied for confirmation of the detected virus in MDBK cell on cover slip as described previously by (Hansa et al., 2013). The infected cell showing characteristic CPE stained for bovine Rotavirus antigen detection using specific anti- bovine rotavirus antibody and anti-bovine antibody conjugated with fluorescine iso-thiocyanate.

RT- PCR

Extraction of viral RNA:

Viral RNA was extracted from the infected tissue cultures using EZ1 Virus Mini kit with EZ1 Advanced automatic Extractor (Qiagen – Germany). The manufacturer's protocol was followed.

Primer/probe design for real time RT-qPCR:

The design of real time PCR kits using Genetic PCR Solutions TM Kit (GPS) – Target Species dtec-qPCR-kit (Spain) specific targeted reagents designed for pathogen detection by using qPCR for detection of rotavirus including positive control. The kit includes a mixture of highly specific oligonucleotide forward/reverse primers and dual-labeled probes for rotavirus at optimal concentration (ready-to-use the primers/probe mix).

Negative controls (PCR-grade H_2O without template) and positive controls were incorporated with each set of test tissue culture samples and subjected to PCR assays to avoid the number of false-positives resulting using a safety precausions.

Real time RT-PCR for (BRoVA):

Real Time RT-PCR was done using GPS. The thermal profile used was reverse transcription process at 50^0C for 10 min and then initial denaturation at 95^0C for 5min. followed by 45 cycle of denaturation at 95^0C for 30sec and 60^0C for 1min for annealing, extension and data collection according to the manufacturer's guidelines. The thermal cycling protocols used for the respective viruses are shown in Table (1). The mixture was added to a well of a 96-well micro-plate and loaded into the Step OnePlus Real time PCR System (Version 2.1, Applied Biosystems, Foster City, CA, USA). Fluorescence data were collected at the end of annealing step.

Table (1): Thermal cycling protocol for detection of rotavirus by RT-qPCR.

Operation	Temp.	Time	Cycle
RT-PCR	50°C	10min.	1
Enzyme activation	95°C	5min.	1
Denaturation	95°C	30sec.	45
Hybridization, elongation, data collection	60°C	1min	

Fluorogenic signal should be collected during the last step by using the FAM channel.

RESULTS

Isolation of Rotavirus:

The characteristic cytopathic effects (CPE) were observed from the third passage as clumping and rounding of infected cells, detachment of monolayer, syncytia and leaving empty vacuole space in MDBK cell line (Figure, 2), while negative control show no CPE (Figure, 1).

ELISA:

Six out of forty (15%) samples from diarrheic calves screened by ELISA were found positive for rotavirus (Table, 2).

Indirect fluorescent antibody technique (IDFAT):

Infected cell cultures on cover slip were detected for rotavirus antigen by indirect FAT revealed specific granular, diffuse intracytoplasmic apple green fluorescence of infected cells (Figure, 3). Negative control didn't show fluorescence in FAT.

A sample with threshold cycle (C_T) value of ≤35 was defined as positive result. The PCR cycle at which an increase in the fluorescence signal is detected initially (C_T) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product (Figure, 4).

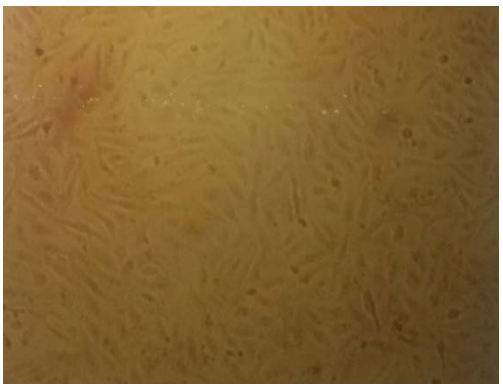

Figure (1): None infected MDBK cell line (negative control).

Figure (2): Infected MDBK cells with Rotavirus 4th day PI showing clumping, rounding and syncytia formation leaving empty vacuole space at 3rd passage.

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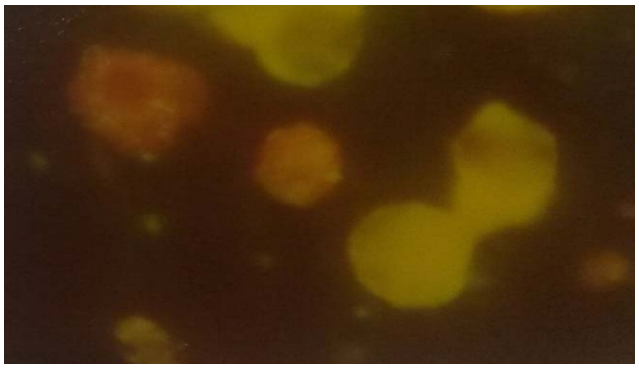

Figure (3) indirect fluorescent antibody assay showing specific granular intracytoplasmic apple green fluorescence in rotavirus infected MDBK cells at 72 hr post infection. Real time PCR for Bovine Rotavirus (BRVA)

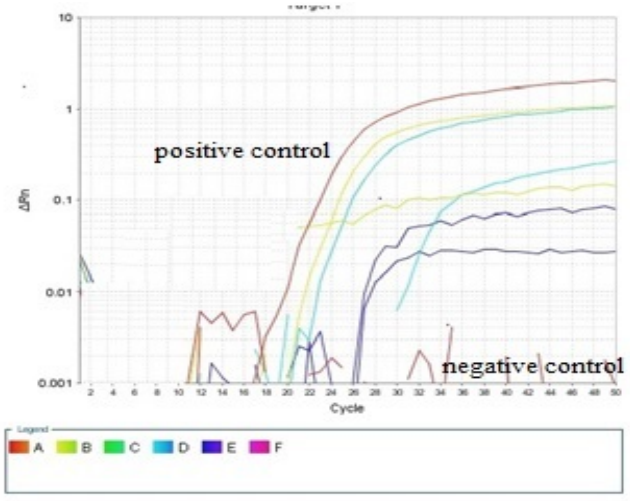

Figure (4): Amplification curve of Bovine Rota virus detection from infected MDBK cell lines by real time PCR showing threshold cycle (C_T) value of 17 for positive control and C_T of 20, 22, 26 and 27 for positive sample results.

Table (2): Comparison of different methods for detection of Rota virus from fecal samples.

Test	Isolation (Fecal)	ELISA (Fecal)	RT-PCR (Infected cells)
No. of samples	40	40	10
Infected T.C.	2	6	4
% positive	5%	15%	30%

DISCUSSION

Neonatal diarrhea in calves is one of the most important disease conditions which cause substantial economic losses to farmers due to increased mortality, costs of treatment and diagnosis and poor growth performance. Rotavirus is known to be the most important agent causing neonatal calf diarrhea (Ambily and Mini, 2014).

As rotavirus is an important agent associated with gastroenteritis in domestic animals. The 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 adopting effective control measures, rapid diagnosis of the disease is important (Dhama et al., 2009).

The present study was designed to investigate the BRoV infection by ELISA and isolation of rotavirus in cell culture as standard diagnostic methods and confirmation by FAT and RT-PCR methods during neonatal calf diarrhea.

However, ELISA being simple, fast and sensitive assay that can be performed routinely and can act as instrumental for the diagnosis of rotavirus and field epidemiological studies as well as the sensitivity and specificity of ELISA was 100% (Suresh *et al.,* 2013).

In the present study, specific diagnosis of infection with rotavirus is made by identification of the virus in the fecal samples by ELISA which is sensitive, specific and detect all serotype of rotavirus (Table, 2).

Although, virus isolation (VI) has been the gold standard for viral detection, it was the least sensitive technique for the detection of rotavirus (Benfield et al., 1984) and also the expense and time involved keep VI less popular. Previous studies indicated that isolation of BRV on MDBK cell line in presence of trypsin increases the viral growth by 100 fold when incorporated in maintenance medium (Albert, 1990).

Viral growth in cell culture was assessed by examining inoculated cells for CPE and indirect fluorescent antibody test. The CPE produced in this study (Figure, 2) were in agreement with previous report of (Saravanan et al., 2006 and Suresh et al 2013), who recorded that 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 typically after third passages. In first passage, infected cells did not show any cytopathic effect (CPE). From the second passage onwards the infected cells started to form clusters of the virus or intra-cytoplasmic inclusion bodies that indicated with FAT as cytoplasmic granulation (Figure, 3). The finding of intracytoplasmic inclusion body (IB) findings was in agreement with the earlier reports (Suresh et al 2013). He considers virus isolation followed by indirect FAT as standard test, and specificity of these assays was 92.39% of ELISA.

The real time PCR requires less time and labor, reduces the risk of cross contamination, and so has been described as a high sensitive quantitative detection method for viral nucleic acids (Li et al., 2010).

In our study molecular methods such as RT-qPCR have been used to detect Rota virus genome in few hours. The target sequence has been selected by GPS team for detection of rotavirus by Target Species dtec-qPCR-kit, designed for primers/probes by using the best software available of phylogenetic criteria for an optimal design for real time PCR with positive and negative controls (Figure, 4).

It has been found that RT-PCR is not only a highly sensitive method in detecting small concentrations of rotavirus in fecal samples but it can also be used for strain identification and further differentiation (WHO 2009 and Suresh et al., 2013). Also rotavirus real-time RT-PCR assay utilized primers and probe that were designed to target the non-structural protein region 3 (NSP3) of rotavirus (Jothikumar et al., 2009).

The presence of coronavirus and rotavirus in fecal samples obtained from neonatal calves suggested their etiological roles and they are the most common viruses involved in neonatal calf diarrhea. Several studies on the seasonality of rotavirus at various locations with different climatic conditions in South Africa identified two recurrent features of the disease. First, rotavirus infection occurred year-round in all locations studied; and secondly, in each region, rotavirus cases increased during the cooler and drier months (Steele and Glass, 2011).

RoVs were detected in each of the four seasons. Also, 80% of all RoV detections were made in the cooler months of August to October in 2010 and April to July in 2011. Only 20% of RoV detections occurred in summer months of December and February (Chigor and Okoh, 2012).

Although qRT-PCR increases the sensitivity of rotavirus detection in fecal specimens, some of these cases may be in children with low-level viral shedding from a resolved or asymptomatic wild-type rotavirus infection and not true disease. Rotavirus might have been the cause of symptoms in some children whose specimens tested negative for rotavirus by ELISA but showed low levels of qRT-PCR–detected virus (Tate et al., 2013).

Data obtained in this study suggest that, for routine purposes, ELISA remains the test of choice due to it is simple, convenient procedure and detect accepted limit. However, when in doubt, isolation in cell culture should be used to verify findings; RT-PCR assay provides highly sensitive, specific and rapid detection of the virus. These rabid techniques may facilitate diagnosis of rotaviral diarrheas circulating in the country to improve its prevention and control and greatly assist in molecular studies of the virus.

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