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Isolation and Identification of Rotavirus Infection in Diarrheic Calves at El Gharbia Governorate

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Abstract: Neonatal diarrhea is one of the most important diseases of calves worldwide, causing economic loss in both dairy and beef cattle herds. The current study was designated for isolation and identification of Rotavirus infections in calves as one of the most important and main viral infection causing diarrhea in newborn calves in El-Gharbia province in the Nile Delta of Egypt by testing 50 fecal samples of diarrheic calves below three months of age from September 2015 till March 2016. The samples were inoculated in MDBK cells revealed successful isolation of rotavirus in five samples with characteristic cytopathic effects showed rounding of cells, shrinkage of cell wall together with an increase of granularity and progressed to form a bunch of grapes within 72-96 hrs. by using reverse transcription polymerase chain reaction (RT-PCR). Out of the 50 fecal samples examined only 5 samples were identified as positive (10%). The proportion of positivity in fecal samples suggested that rotavirus infections are common in Egypt.

Key words: Rotavirus · Calves · Diarrhea · RT-PCR

INTRODUCTION

Diarrhea has been recognized as a serious condition of animals because of high morbidity and mortality and has been ranked as one of the main six causes of all deaths from infectious diseases [1-3]. Amongst infectious agents, Rotavirus, Coronavirus, Cryptosporidium and *Escherichia coli* which all are responsible for 75-95% of infection in neonatal calves worldwide; and especially rotavirus accounts for about 27- 36% [3, 4]. One of the most important and main viral factors that cause diarrhea in calves is rotaviruses [5-7]. In Egypt rotavirus infection represents a serious economic problem threating animal production [8]. Rotavirus is non-enveloped, double stranded RNA virus, with a diameter of 65-70 nm and belongs to the genus Rotavirus of the subfamily Sedoreovirinae in the family Reoviridae and characterized by segmented genomes comprising of 11 segments of double stranded RNA which is surrounded by an inner and outer capsid layers[9]. There are several methods for diagnosis of rotavirus like electron microscope (EM), RNA-polyacrylamide gel electrophoresis(RNA-PAGE) [10] and ELISA [10, 11], cell culture FAT [12] and reverse transcription polymerase chain reaction (RT-PCR) [10, 13, 14]. Although isolation of rotavirus using cell culture is a laborious process and also less sensitive, it gives the ultimate proof of virus association with the disease. Isolation of bovine rotavirus (BRV) in the lab can be performed using specific primary cell cultures (calf kidney cells) and cell lines (MA 104-Simian origin, MDBK and PK-15). The incorporation of trypsin in the culture medium of the rotavirus in small quantities was found to enhance the cytopathic effect (CPE) and also by the pretreatment of fecal samples with trypsin [15].

The goal of this study was to investigate the prevalence of rotavirus infections in diarrheic calves in El Gharbia province.

MATERIALS AND METHODS

Sample Collection and Preparation: For this study 50 fecal samples were collected from calves from different dairy farms in Qutour city.Calves aging from one day to 3 month, suffering from diarrhea. After collection, fecal

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samples were put in isopor boxes with cooling blocks and immediately transported to the lab and kept at -80°C. Fecal suspensions were prepared in 1.5 ml Eppendorf tubes by mixing feces and DEPC water (ultra-pure water previously treated with 0, 1% diethyl-pyrocarbonate) w/v 50%. Suspensions were thereafter centrifuged at 12000x for 5 minutes at 4°C. 250µl of the supernatants were transferred to new 1, 5 ml Eppendorf tube for virus isolation.

RNA Extraction: RNA was extracted from isolated samples in MDBK cells using easy-RED ™ total RNA Extraction kit according to manufacturer manual. The obtained RNA was stored at -20c until used in the downstream applications.

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 rotavirus was performed according to Saravanan *et al*. [16].

Reverse Transcription Polymerase Chain Reaction (RT-PCR): RT-PCR technique was applied using primers developed by Asano *et al*.[17] that specifically detect bovine Rota virus (Table 1).

RT-PCR was performed using Thermo Scientific Verso 1-Step RT-PCR Reddy Mix Kit with total volume of 25 µl containing 2X 1-Step RT-PCR Reddy Mix (12.5µl), Verso Enzyme Mix(0.5 µl), RT Enhancer (1.25µl), Forward Primer 10µM (0.5µl), Reverse Primer 10µM (0.5µl), 1.25 uM, Template RNA(2.5µl), DNase/RNase free water (to 25 µl). The thermal profile was applied as mentioned in (Table 2). 8 µl of the generated PCR products were migrated on 1.5 % ethidium bromide stained agarose gel under constant volt of 80 V for 40 min. the gel was then visualized using UV- Transilluminator then photographed by the associated camera.

Table 1: Primer details

Primer	Primer sequences	Size of amplicons
ROT1(sense)	CTCTGGCAAARCTGGTGTCA	492Pb
ROT2(antisense)	CATTCGACGCTGATGACATY	

Cycling Conditions of (RT-PCR)

Table 2: Cycling conditions

Step	Temp. in °C	Time	Cycles
cDNA synthesis	50	15 min	1
Verso inactivation	95	2 min	1
Denaturation	94	30s	40
Annealing	50	30s	
Extension	75	45s	
Final extension	72	5 min	1
Hold	4	Infinite time	1

RESULTS

Isolation of Bovine Rotavirus in MDBK Cell Line: In the first passage, infected cells did not show any cytopathic effect (CPE). But from second passage onwards the infected cells started showing characteristic CPE. On H&E staining, uninfected MDBK cells showed normal staining characteristics, while the infected cells showed rounding of cells, shrinkage of cell wall together with an increase of granularity and progressed to form a bunch of grapes within 72-96 hrs. (Fig. 2). Out of 50 samples only 5 samples were able to infect MDBK cells.

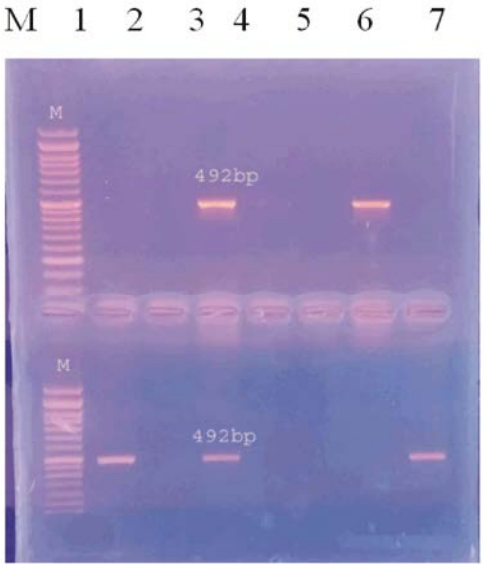

Fig. 1: Agarose gel electrophoresis of PCR product of rotavirus isolate showing specefic target band size at 492 bp.
M: molecular base marker (50 bp ladder)

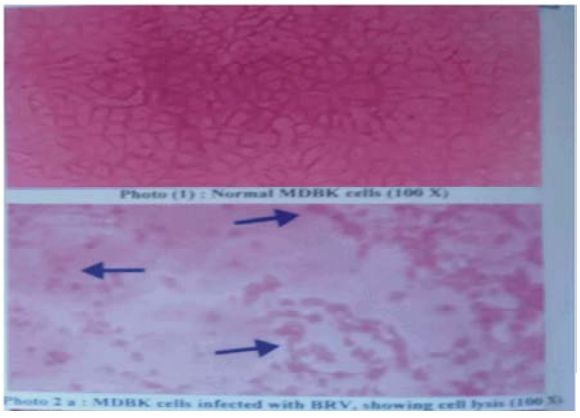

Fig. 2: Photo (1): Normal MDBK cells (100x)
Photo 2: MDBK cells infected with rotavirus showing rounding, shrinkage and cytoplasmic granularity (100x)

Results of PCR: Out of the 50 fecal samples examined by RT-PCR technique, 5 samples were identified as positive (10%) and give specific target band size(492 bp). In this study, the presence of rotavirus antigen was detected in calves ageing between 1-15 days.

DISCUSSION

Newly born calves represent worldwide an important source in animal production either for meat or breeding [18]. Such industry faces many disease syndromes which usually affect it dramatically. One of these diseases is diarrhea which may lead to 75% mortality of calves less than three weeks of age [19].

Rotavirus is the major cause of acute gastroenteritis in newborn calves. Rotavirus infection is non-viremic, have very short incubation period and the calf suffer from profuse diarrhea and severe dehydration [3]. Rotavirus causes significant economic losses for cattle farms due to death or weight loss in infected animals, in addition to the high cost of veterinary services and other reasons. Fecal contamination plays a major role in transmission of rotavirus infection and also around the world have demonstrated that the infection is widespread in populations of cattle. Many methods are used to determine the presence of rotavirus antigen. ELISA, Polyacrylamid Gel Electrophoresis (PAGE), Latex Agglutination (LA), Electron Microscopy (EM), Immunofluorescen (IF) and Immunoperoxidase (IP) are most widely used methods[20- 22]. Recently, RT-PCR, using the VP4 or VP7 gene primers, is much widely used for detection of animal rotaviruses [23-25]. Also, semi-nested or multiplex RT-PCR is being developed. Besides the sequencing of the other capsid genes has also helped in differentiating various rotavirus isolates [21]. 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 [15, 26].

In the present study, viral growth in cell culture was assessed by examining inoculated cells for CPE. Out of fifty samples, only five inoculated MDBK cells producing characteristic CPE at the level of second passage till the six passages. The CPE produced in this study were in agreement with previous reports [16, 27]. 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 current study, these changes were observed only after 72 hrp.i.. Infected cells stained with H&E staining method showed characteristic syncytia and eosinophilc intracytoplasmic inclusion body. And these findings were in agreement with the earlier reports [28].

In this study the RT-PCR for isolated samples using specific primer for amplification of target sequence of VP4 revealed that out of 50 tested samples only 5 samples were positive giving specific size 492 bp. (10%) and the presence of rotavirus antigen was determined in calves ageing between (1-15) days, two samples were positive from (1-7 days) while the high percent three samples were from (8-15 days) and this result agree with Robaiee and Farwachi [29], who had detected rotavirus antigen in neonatal calves, aged between 8-15 days by antigen ELISA from different part of Mosul city, Iraq. In Egypt, many studies showed that the presence of rotavirus as the cause of neonatal calf diarrhea. Shalaby *et al*. [30] was the first to report the presence of rotavirus, then followed by other studies like Iman *et al*.[31] and Perk *et al*. [32] and Shimaa *et al*. [33].

The PCR technique, which is a rapid and sensitive genomic detection tool, can be used to detect the genomic RNA extracted from feces, after reverse transcribing it with reverse transcriptase enzyme to obtain the cDNA, followed by PCR amplification, using specific gene primers. Many researchers have exploited this technique for detection of the BRV in clinical samples using VP4 or VP7 primers [34-36]. PCR have been shown to have a higher sensitivity than both antigen detection methods and EM and some of the commercial ELISA kits have shown a low sensitivity and specificity. PCR are therefore an increasingly common way of diagnosis by Blanchard [37].The reverse transcriptase-polymerase chain reaction (RT-PCR) offers many advantages besides high sensitivity and specificity in detection of rotavirus in fecal samples by Kang *et al*. [38]. It helps in the detection of viral nucleic acid during initial stages of infection without waiting for higher virus titer and development of immune response in the affected host species. Detection of rotavirus infection in reservoir animals and symptom less carriers is another advantage of RT-PCR.

CONCLUSION

The virological data that were collected in this study indicate the presence of rotavirus infection in diarrheic calves in Qutour city of Egypt. An evaluation of the data leads us to the conclusion that a vaccination program needs to be implemented to bring rotavirus infections under control.

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