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Original Article

Isolation and G-typing of Rotaviruses from diarrheal Calves in Tehran and Alborz provinces, Iran

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ABSTRACT

Rotaviruses group A are the major cause of diarrhea in calves under one month and every year causes enormous economic losses. Serological and molecular techniques can be used for rapid detection of rotaviruses but virus isolation requires specific methods of cell culture and suitable cell lines. In this study, 41 samples were collected from diarrheal calves up to the age of one month, from industrial and semi-industrial farms in Tehran and Alborz provinces. The samples were positive by RT-PCR on VP6 gene. After preparation and inoculation onto MA104 cells in roller tube culture and constant cell culture system, the cytopathic effect (CPE) was observed. Eligible cultured with CPE, were confirmed by two-step RT-PCR using VP6 gene primers. Semi-nested PCR using VP7 gene primers was also performed for G-genotyping in which 11 and 2 samples were detected as G6 and G10, respectively. This is the first report on isolation and identification of rotaviruses, one of the causative agents of viral diarrhea in Iran. The results of this research suggest that, these two types, can be used as the dominant strains for manufacturing a suitable vaccine against the rotaviruses in Iran.

Keywords: Rotavirus, Isolation, Iran

INTRODUCTION

Rotavirus is the most common cause of acute diarrhea among infants and children in both humans and animals throughout the world. Although rotavirus infection is common among adults (Dhama *et al* 2009, Murphy *et al* 1999, Radostitis *et al* 2007, Trojnar *et al* 2013). Rotaviruses often cause disease in calves aged 1 to 2 weeks. Some calves are infected again or continuously excreted the virus through the feces but the infection in older is often subclinical (Knipe & Howley 2013, Castrucci *et al* 1983, Dhama *et al* 2009). The virus genome contains 11 pieces of double-stranded RNA (dsRNA). Viral replication occurs in the cytoplasm of infected cells. Virus cultured in vitro is facilitated by the treatment with proteolytic enzymes such as trypsin. Cleavage of the outer capsid VP4 to VP5 and VP8 by polypeptide enzymes facilitates virus entry into cells (Knipe & Howley 2013, Altenburg *et al* 1980, Estes & Cohen, 1989, Martin *et al* 2004). The use of MA104 cell with trypsinization of the fecal specimens were introduced for isolation the bovine rotavirus by several references.

The cell lesions were observed as rounding, enlarged cells and bead-like structure in cell culture. In the first and second passages, the appearance of CPE, was visible 48-60 hours after inoculation. With continued passage the CPE were visible 24 hours after inoculation and destruction was complete 2 days after inoculation (Rodriguez *et al* 2004, Castrucci *et al* 1983, Villarreal *et al* 2006). In the absence of added trypsin to fecal samples, the viral infection in cell culture vastly reduced or even disappears and it is not possible to isolate the virus (Babiuk *et al* 1977, Lopez *et al* 1986, Sunil-chandra & Mahalingam 1996). Rotavirus detection methods are direct method (cell culture isolation and detection by electron microscopy), viral antigen detection (ELISA, latex agglutination, and fluorescence immunoassay method) and detection of viral nucleic acids on gel electrophoresis or electrophoretype, hybridization-point detection method and PCR (Hammond *et al* 1984, Hughes *et al* 1984, Buesa *et al* 1996). The RT-PCR has been reported as more efficient method capable of typing a broader range of samples positive for rotavirus. This method multiplies the viral nucleic acid extracted from feces using specific primers for detection and typing rotaviruses. Also the PCR is more sensitive and much faster than the serotyping by ELISA. PCR can be used on fecal samples that have not been kept in good condition, (Buesa *et al* 1996, Gentsch *et al* 1992). Rotaviruses have seven groups from A to G. These groups are distinguished by middle capsid protein (VP6). VP6 gene are used for verification these groups. Group A rotaviruses can be classified into several types based on the antigenic and genomic characteristics of VP7 proteins. G-typing can be done using VP7 specific primers (Matthijnssens *et al* 2012, Medeiros *et al* 2014). There are several reports on the prevalence of rotavirus in calves in Iran, but isolation of the virus and serotyping have not been reported yet. (Keyvanfar *et al* 2001, Kargar 2002, Rabbani *et al* 2007). This is the first report on isolation and typing of bovine rotaviruses in Iran. The results are useful for further virological and epidemiological studies and also prerequisite for manufacturing a potent vaccine against the virus in the country.

MATERIALS AND METHODS

Sampling. A total of 41 feces samples from calves up to the age of one month from the industrial and semi-industrial dairy herds in Shahriar, Eslamshahr, Robatkarim, Savojbolagh, Varamin, Rey, Pakdasht and Damavand in the Tehran and Alborz provinces were received and kept in the -70 °C until use.

Sample preparation and inoculation on the cell culture. Initially, 10% feces suspension was prepared by phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 15-20 minute. Then, the supernatant was removed and filtered through a 0.45µm membrane. Trypsin (5µg/ml, Difco) was added and incubated for 30 minute at in 37 °C. The MA104 grown in cell culture tubes and cell culture flasks (Nunc) were washed with PBS solution three times to remove serum. Then the samples were inoculated on cell cultures and incubated in 37 °C for 1-1.5 hours to absorb the virus. The culture medium, DMEM (GIBCO-High glucose) containing trypsin (0.5µg/ml) and 10% tryptose phosphate broth (HIMEDIA,) was added to the cells. Inoculated cells were incubated in 37oC and were observed for 14 days to see the cytopatrhic effect (CPE). After three times freeze-thawing, the blind passages were prepared. This action was continued up to five blind passages. Case of CPE in any passage was inoculated after three times freeze-thawing, in order to increase and enhance viral titer, for 2-3 another passages to the new constant cell culture. If cellular lesions was observed, the presence of viral genome was confirmed by RT-PCR (Lopez *et al* 1986, Babiuk *et al* 1977, Rodriguez *et al* 2004).

Viral RNA extraction. In cell cultures possess cell lesions, the extraction of RNA by RNA extraction solution (Cinagen) was performed according to manufacturer's instructions.

Reverse Transcription (RT). RT reaction optimized based on using Revert Aid TM First Strand cDNA synthesis kit (Fermentas). The RNA and random

hexamer primer were heated at 95 °C for 5 minute and the thermal procedure was performed as follows: 25 °C (5 minute), 42 °C (60 minute) and 70 °C (5 minute)

PCR Reaction

Primers. A series of primers were used for amplification of VP6 and VP7 gene fragments as outlined below:

VP6 oligonucleotide primers

VP6-F 5' GAC GGVGCR ACT ACA TG GT 3' (Iturriza Gomara *et al* 2002)

VP6-R 5' GTC CAA TTC ATN CCT GGT G 3'

Product: 382bp

G-typing oligonucleotide primers

1. First round: product 881bp (Asmah *et al* 2001)

VP7-F 5' ATG TAT GGT ATT GAA TAT ACC AC 3' (nt 51-71)

VP7-R 5' AAC TTG CCA CCA TTT TTT CC 3' (nt 914-932)

2. Second round typing primers (Asmah *et al* 2001, Gouvea *et al* 1994)

VP7-F 5' ATG TAT GGT ATT GAA TAT ACC AC 3' (nt 51-71)

Genotype G5:

FT5 5' CAT GTA CTC GTT GTT ACG TC 3' (nt 779-760)

product size: 729bp

Genotype G6:

DT6 5' CTA GTT CCT GTG TAG AAT C 3' (nt 499-481)

product size: 449bp

Genotype G8:

HT8 5' CGG TTC CGG ATT AGA CAC 3' (nt 273-256)

product size: 223bp

Genotype G10:

ET10 5' TTC AGC CGT TGC GAC TTC 3' (nt 714-697)

product Size: 664bp

Genotype G11:

BT11 5' GTC ATC AGC AAT CTG AGT TGC 3' (nt 336-316)

product size: 286bpVP6 gene PCR

VP6 gene PCR. The cDNA applied in PCR reaction using lyophilized tube (Bioneer; Korea). The amplification for VP6 gene RT-PCR was done at the following temperature cycle: 94 °C for 5 minute (one cycle), 94 °C for 1 minute, 55 °C for 1 minute, 72 °C for 30 second (30 cycles) and final elongation 72 °C for 3 minute.

PCR amplification for G-typing

VP7 gene initial PCR. For genotyping of VP6 positive rotavirus samples, PCR reactions were performed on the cDNA using specific primers for VP7 gene. VP7 gene was amplified with the following temperature cycle: 95 °C for 4 minute (1 cycle), 95 °C for 50 seconds (35 cycles), 51 °C for 50 seconds (35 cycles), 72 °C for 50 seconds (35 cycles) and 72 °C for 5 minute (1 cycle).

Semi-nested PCR for G-genotyping. Finally, for G-genotyping of rotavirus-positive samples, the semi-nested PCR reactions were performed on initial PCR products of VP7 gene. Thus, the PCR with specific primers for genotypes G5, G6, G8, G10, and G11 were performed. Semi-nested PCR amplification program for VP7 gene G genotypes was done as follows: 94 °C for 3 minute (one cycle), 94 °C for 1 minute (30 cycles), 55 °C for 50 seconds (30 cycles), 72 °C for 40 seconds (30 cycles), 72 °C for 5 minute. Distilled water was used instead of DNA sample in all PCR reactions as negative control.

Electerophoresis. The Ladder DNA 100 bp (GeneRulerTM 100 bp DNA Ladder-Fermentas) was used as marker. After preparing the 1.6% gel (Ultra pure agarose-invitrogen) using TBE solution and electrophoresis, the specific bands were observed in the gel documentation system.

RESULTS

Cell culture. Of the 41 samples which were inoculated into cell cultures, the cytopathic effect (CPE) was observed in 13 samples (4 samples from Shahriar, 5 samples from Robatkarim, 3 samples from Eslamshahr and 1 sample from Pakdasht). The CPE was observed in the initial passages (2 samples), first blind passages (3 samples), second blind passages (5 samples) and third blind passages (3 samples). The cytopathic effects usually began in the third or fourth day after inoculation and were reached approximately

to 80-90% cell damage after 2-3 days. In maximum cell damages, the tubes and flasks were transferred to -70 oC. The three times frozen-thawed samples passed 3-4 times on new cell cultures. In all of re-inoculations, the CPE was observed at earlier time. So in the final passages, the CPE was observed 1-2 days after inoculation. In each CPE, the round cells, dark cells, enlargement and three hexagonal cells were observed. Finally, for confirmation of diagnosis, the samples were tested by molecular methods for validation the virus.

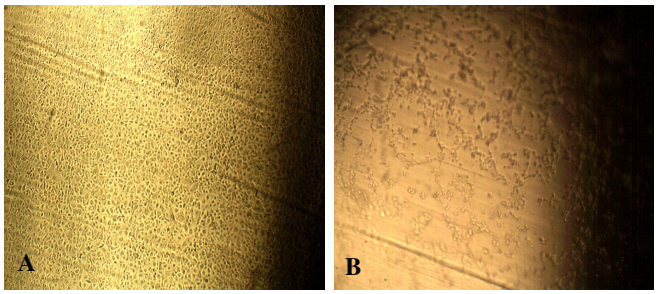

Figure 1. MA104 cells in roller tube culture A. Normal cell B. CPE in cells at 5 days after inoculation in second blind passage.

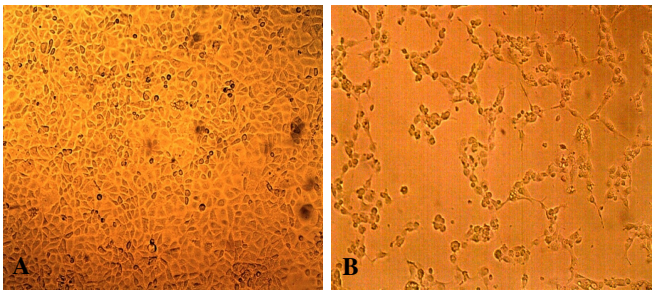

Figure 2. MA104 cells in constant cell culture A. Normal cell B. CPE in cells at 5 days after inoculation in the third blind passage (damaged cells, are large, round, dark, three-sided and granular).

VP6 gene PCR. From 41 samples were taken from cell cultures, the RNA extraction with phenol-chloroform (Cinagen RNX) and two step RT-PCR of VP6 gene were performed. In 13 samples that were eligible for CPE, the VP6 gene was positive in PCR. The size of amplified bands was 382 bp. Negative control showed no band.

G-genotyping of rotavirus-positive samples. The PCR was performed for VP7 gene on all cDNA which were positive for VP6 gene. 13 samples were positive for VP7 gene (881bp). Finally, for G genotyping, semi-nested PCR were performed. No band was observed for negative controls.

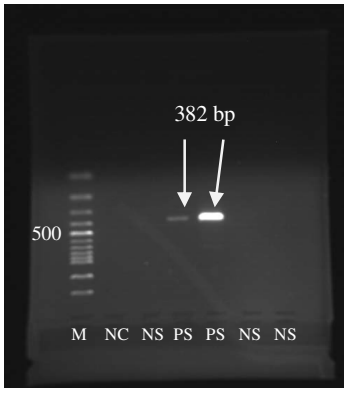

Figure 3. PCR product performed on cells containing CPE for rotavirus VP6 gene. [M: The Ladder DNA, 100 bp (GeneRulerTM 100 bp DNA Ladder-Fermentas) as a marker, NC: Negative control (distilled water), NS: Negative sample, PS: Positive sample].

Semi-nested PCR for G-genotyping. From 13 samples isolated on the cell cultures and positive in VP6 and VP7 genes PCR, in semi-nested PCR using specific primers for genotypes G5, G6, G8, G10, and G11, 11 samples (3 samples from Shahriar, 4 samples from Robatkarim, 3 samples from Eslamshahr and 1 sample from Pakdasht) were G6 and 2 samples (1 sample from Shahriar and 1 sample from Robatkarim) were G10. The size of the amplified fragment of the VP7 gene for G6 was 449bp, and for G10, was 664bp.

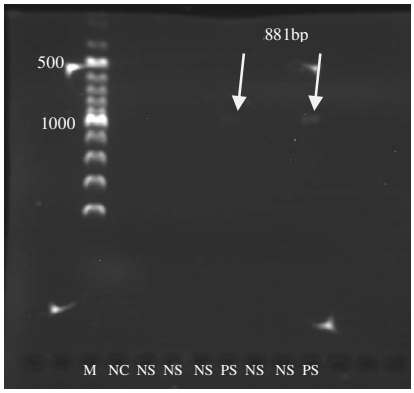

Figure 4. VP7 gene PCR products performed on cells containing CPE. [M: The Ladder DNA, 100 bp (GeneRulerTM 100 bp DNA Ladder-Fermentas) as a marker, NC: Negative control (distilled water), NS: Negative sample (cells with no CPE), PS: Positive sample].

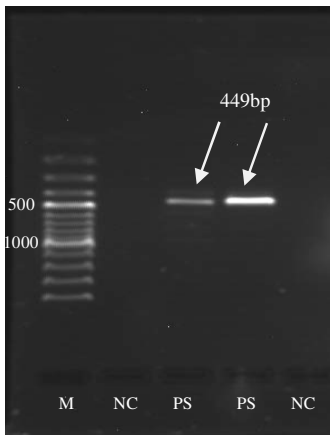

Figure 5. Semi-nested PCR products performed on cells containing eligible CPE for rotavirus VP7 gene. The G6 genotype is identified. [M: The Ladder DNA, 100 bp (GeneRulerTM 100 bp DNA Ladder-Fermentas) as a marker, NC: Negative control (distilled water), NS: Negative sample (cells with no CPE), PS: Positive sample].

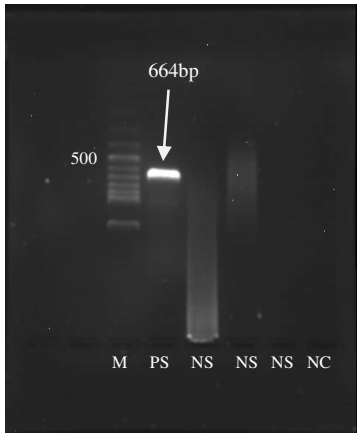

Figure 6. Semi-nested PCR products performed on cells containing CPE for rotavirus VP7 gene. The G10 genotype is identified. [M: The Ladder DNA, 100 bp (GeneRulerTM 100 bp DNA Ladder-Fermentas) as a marker, NC: Negative control (distilled water), NS: Negative sample, PS: Positive sample].

DISCUSSION

Group A rotaviruses are the most common primary cause of acute gastroenteritis in infants of many mammals and young birds (broiler chicken, turkey, pigeon) (Knipe & Howley 2013, Trojnar *et al* 2013). Rotavirus infection in calves caused high morbidity and mortality resulting in heavy economic losses. (Dhama *et al* 2009, Murphy *et al* 1999, Radostitis *et al* 2007). Based on antigenic characteristic or genomic sequence of VP4 and VP7, group A rotaviruses are classified to P and G type respectively. So far, 15 G genotypes and 26 P genotypes have been identified. Eight G genotypes (G1, G3, G5, G6, G7, G8, G10 and G11) and six P genotypes (P [1], P [5], P [11], P [14], P [17] and P [21]) have been identified in bovine rotavirus diarrhea and among these genotypes, G6, G8 and G10 and P (1), P (5) and P (11) are significantly prevalent (Matthijnssens *et al* 2012, Medeiros *et al* 2014, Altenburg *et al* 1980, Estes & Cohen 1989, Martin *et al* 2004). In a study conducted in Brazil, 50 rotavirus positive feces samples were collected from dairy and beef cattle during 1996-1999. Eighty eight percent of samples were typed in which G6 was the most common genotype (68%) while G10, G8 and G5 were reported as 17%, 6% and 2%, respectively (Alfieri *et al* 2004). In another study in Japan, 28 of 167 calves infected with rotavirus were isolated and identified by semi-nested PCR with specific primers as G6P [5] (89/3%), G5P [11] (7/1%) and G10 [P11] (3/6%) (Okada & Matsumoto 2002). In most studies, the three genotypes G6, G8 and G10 are observed and genotype G5, with low percentage except for epidemic condition has been reported. Also, genotype G11 has been found in cases of pig farms (Ishizaki *et al* 1996). The prevalence of the virus has been reported in previous studies in Iran. Keyvanfar et al. (2001) investigated the prevalence of the virus in dairy calves around Tehran and determined the group A rotavirus using ELISA. The prevalence of rotavirus infections was 28.8 %. Using monoclonal antibodies against serotypes G6 and G10, 41.7% of samples infected with serotype G6, 33.3% infected with serotype G10 and 8.3% were mixed infection with both serotypes and 16.7% were unknown (Keyvanfar *et al* 2001). Kargar et al. (2002) studied on 63 feces samples from calves with diarrhea in farms around Tehran. They showed viral particles like rotavirus, under electron microscopy in 20 cases (31.74%). Rabbani et al. (2007) in a study that measured the presence of rotavirus antibodies in serum of 184 calves under one month, performed by ELISA showed that all serum samples from calves with diarrhea and 99% of

serum samples of healthy calves had the antibodies against rotavirus antigen. Mayameii et al. (2007) used semi-nested PCR method for rotavirus G typing for G6 and G8, and G10 in 142 samples were collected from Khouzestan and Tehran provinces and reported G6 and G10, respectively, were common (20.51% and 64.1 in Khouzestan and 37.9% and 20.96% in Tehran) and G8 has not been detected. These results are consistent with the findings of researchers in other countries. The present results suggest that the major types of bovine rotavirus in industrial and semi-industrial dairy farms in Tehran and Alborz are G6 and G10 types. These studies suggest that the vaccine strains to prevent rotavirus infection should include the G6 and G10 types. The typing of bovine rotavirus circulating in these areas and other parts of Iran should be performed for better understanding of the epidemiology of bovine rotavirus prevalence to achieve the best prevention and control pathways.

Our study showed that PCR-RFLP is useful epidemiological tool identifying of *p. multocida* serovars with using three different restriction endonuclease enzymes and concluded that PCR-RFLP was a rapid test and could be done for *p. multocida* species but until now no PCR-RFLP based techniques have been used for detection somatic serotyping of *p. multocida*. Hence one of the purpouse in present study was undertaken to develop a simple DNA based typing scheme.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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