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Isolation and Antigenic and Molecular Characterization of G10 of Group A Rotavirus in Camel

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We are here reporting the successful Isolation and characterization of G10 serotype of group A rotaviruses from fecal samples collected from camel farms suffering from diarrhea in Alexandria and Esmalia governorates. After preparation of fecal samples and inoculation on MA 104 cell line for five passages, eight isolates were successfully isolated with a clear and reproducible CPE on the inoculated cells. The isolates were identified antigenically using VP6 monoclonal antibodies (MAbs) based antigens capture ELISA that able to detect any group A rotavirus. The viral RNA was extracted from the tissue culture harvest of the propagated viruses and RT-PCR using primers specific for VP6 and VP7 of group A rotaviruses was employed and confirmed the molecular characterization of the isolates viruses with the correct and expected bands. The RT-PCR specific band of VP7 gene of two selected isolates was eluted from the agarose gel and sequenced using VP7 specific primers sequence. The obtained sequence was analyzed using computer software (BLAST) which revealed that both isolates had Maximum identity to the G10 serotype of group A bovine rotaviruses ranging from 90-93%. This is the first report on the circulation of G10 serotype of group A rotaviruses in camel.

Key words:

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INTRODUCTION

Rotavirus (RV) is recognized as the single most important cause of sever acute dehydrating diarrhea in the young human and many animals species including calves **(Kapikian and Chanock, 1996)**. High mortality and morbidity among young calves due to rotavirus disease is a serious cause of economic loss to animal farms and diary industry **(Deleeuw et al., 1980, House et al., 1978, Saif and Fernandez , 1996, Woode and Bridger, 1975).** The calf rotavirus infection has a worldwide distribution and associated with 40 – 48% of the neonatal calves, **(Morin et al., 1976 and Snodgrass and Wells , 1976).** The morbidity and mortality rates can reach up to 30% and 90% respectively due to camel calf diarrhea **(Schwartz and Dioil, 1992)**. Only few reports on viral causes of camel calf diarrhea were published **(Mahin et al., 1983)**. In general there is a lack of details study on the role of rotavirus in camel calf diarrhea.

Rotavirus is composed of triple-layered protein capsid which encloses a genome of eleven segments of double – stranded ds RNA **(Estes et al., 1996).**The genome primarily encodes six structure and six non structure protein **(Estes and Cohen , 1989)**. VP4 and VP7, encoded respectively by gene segments 4 and 7, 8 or 9 depend on the strain. Specify two distinct serotype specificities termed P (Protease sensitive) and G (Glycoprotein) serotypes **(Estes and Cohen , 1989)**. The intermediate capsid protein VP6 possesses the group and subgroup specific epitopes. Rotaviruses are classified into 7 groups (A-G) based on the antigenic properties of VP6 protein. Group A rotaviruses constitute the major pathogens in human and animals **(Kapikian and Chanock, 1996)**. On the basees of the VP7 and VP4 proteins and their coding nucleic acid, group A rotaviruses are classified into different serotypes and genotypes. 15 G serotypes and 16 G genotypes have been identified in diarrheic calves **(Estes and Kapikian, 2007 andGulati et al., 2007)** and there is 14 P serotypes and 27 P genotypes **(Khamrin et al., 2007)** . The most predominant G serotypes in diarrheic calves in Egypt are G6 and G10 **(Hussein et al., 1993b and Hussein et al., 1999c)** There is no available data on the circulating camel rotaviruses in

Egypt. In this study, trial for isolation, antigenic and genetic characterization of rotaviruses in fecal samples of diarrheic camel calves was achieved.

MATERIALS AND METHODS

1.Fecal samples

Eighty five fecal samples from 2 weeks till 4 months day old camel calves were collected from four different governorates in Egypt (Fayoum, Alexandria, Ismailia and Giza) during period 2004-2005. Fecal samples were tested for group A rotavirus by Mabs–based ELISA **(Hussein et al., 1995)**. Positive fecal samples for RV were used for isolation trial.

2.Electron microscopy

Positive fecal samples in ELISA were examined with electron microscopy **(Alain et al., 1987)** for shown the characteristic feature of rotavirus particles.

3.Tissue culture

Eight positive fecal samples and tissue culture supernatant in Mabs based- ELISA were propagated after treatment with trypsin on rhesus monkey kidney (MA104)cells in the presence of 0.5ug of trypsin per ml as described by **(Saif et al., 1988)**.

4.Extraction of Rotavirus ds RNA

The dsRNA was extracted from fecal samples using RNA extraction kit [GIBCO] according to recommended procedures that involved dissociating cells by Trizol then chloroform, isopropanol and ethanol 75% with different centrifugation then suspended the extracted RNA in nuclease free water **(Chomeznski and Sacchi, 1987)** the RNA suspension was kept at -85 ◦C till used for reverse transcriptase RT-PCR .

5.Oligonucleotide primers

Primers was designed according to publish database **(El- Sabagh, 2006)** to be used for amplification of full length VP6 gene, the sequence of forward primer is (5'-GGCTTTTAAACGAAGTCTTCA ACATGG-3') and the VP6 reverse primer (5'-GGTCACATCCTCTCACTACGC -3') were used to amplify of 1356 bp fragment of rotavirus VP6 gene. The VP7 forward primer is (5'-GCGGTTAGCTCCTTTTAATGT ATGG-3') and the reverse primer is

(5'-GGTCACATCATATACAAC-CTCTAATCTAACAT-3') were used to amplify 1030bp fragment of VP7 gene.

6.RT-PCR

RT-PCR was performed by modification according to **(Chang et al., 1996)** the 30ul of RNA were mixed with 5ul of dimethylsulfoxide and the mixture incubated at 95◦C for 5 minutes followed by rapid cooling on ice the denaturated ds RNA of VP6 and VP7 genes were amplified using one step RT-PCR kit. The first – strand cDNA synthesis was accomplish by incubating the mixture for 30 minutes at 47◦C then at 94◦C for 2 minutes and then 35 amplification cycles of 95◦C for 45 second (denaturation), 55◦C for 45 second (annealing), 72◦C for 1.5 minutes (extension) and conducted followed by a (final extension cycle) of 5 minutes at 72◦C . The PCR products 10ul were loaded on to agarose gel was prepared by dissolving of 1.25 gram agarose gel in 100ml (1X) TAE buffer with 0.5ug/ml ethidium bromide. Electrophoresis was conducted for 1hr at 120 v and the gels were photographed under UV light according to **(Sambrook et al., 1989)** the bands of expected correct size were cut from gel and gel slices containing DNA bands were placed in montage DNA gel extraction device. Then the eluted DNA was sent to Agricultural Genetic Engineering Research Institute (AGERI) with forward primer to be sequenced. The sequencing was analyzed by Blast computer utility of the National Center for Biotechnology and Information (NCBI) web of internet.

RESULTS

In a total of 85 fecal samples obtained from the diarrheic calves, eight were positive for Mabs- based ELISA as in table (1), then the eight positive samples were concentrated by ultracentrifugation and examined under electron microscopy as in figure (1).

Trail for isolation of CRV from positive fecal samples of diarrheic camel calves in MA104 cell culture till 5th passage.

The propagated eight samples were identified using Mabs- based ELISA in harvested tissue culture supernatants in (table 2).

Table (1): Different Absorbances of Field Fecal Samples of diarrheic calves in ELISA Reader

Cod. No. of samples	Governorate	Absorbances at wave length 450
3	Fayoum	0.219
6	Alexandria	0.127
10		1.084
19		0.453
25		1.137
33	Esmailia	0.188
34		0.227
67	Giza	0.449
Normal control		0.125

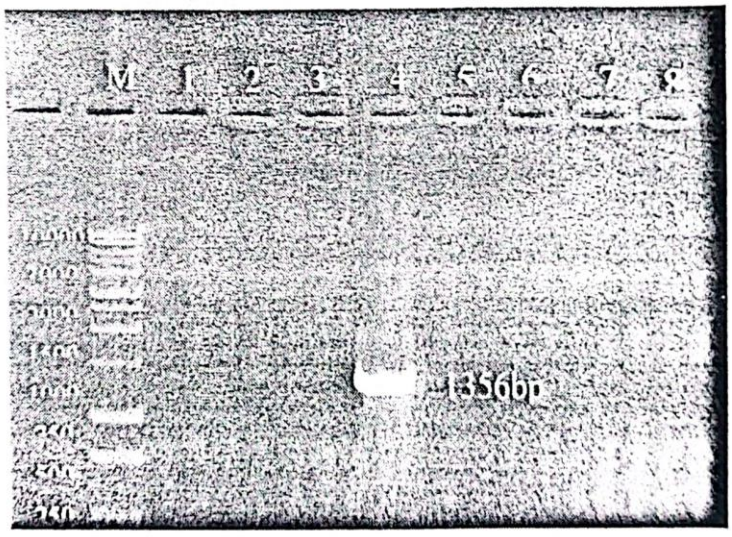
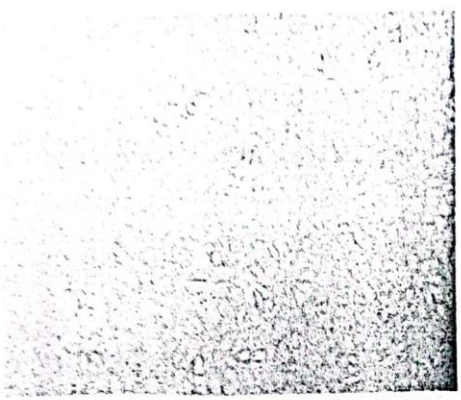


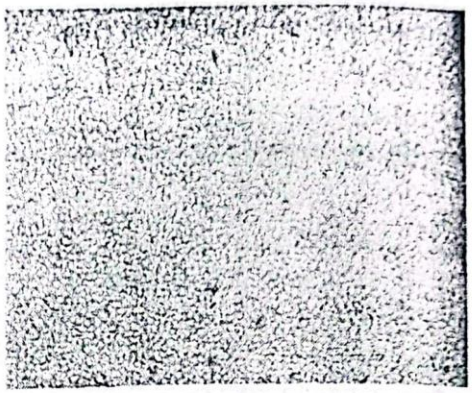
Figure (2): Ethidium bromide stained agarose gel electrophoresis of the RT-PCR products of VP6 gene of CRV in fecal samples along with 1Kbp DNA ladder.

M: Represents 1kbp DNA ladder.

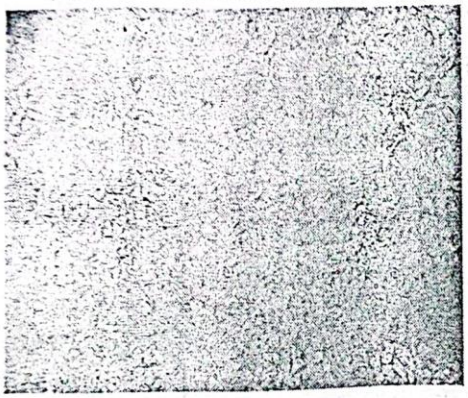
Lane 4: Represents the amplified product of CRV in sample no. 19
Lanes 1, 2, 3, 5, 6, 7 and 8: Represent the negative samples for amplification.



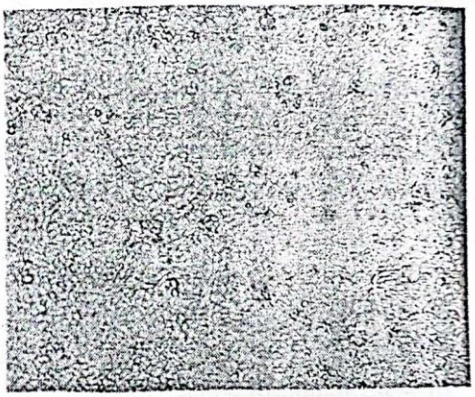
(A) non-inoculated cell control with high power



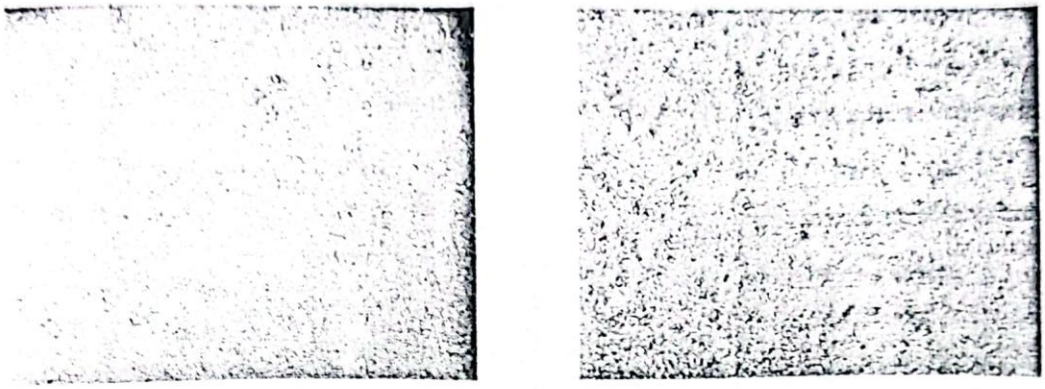
B) non-inoculated cell with low power



(C) Rounding and clumping of inoculated cells high power



(D) cell rounding and clumping cells with low power



(E) Detachment of inoculated cells sheet with high power (F) detachment of inoculated cells with low power.

Figure (3): Showing the sequential appearance of the characteristic CPE of rotavirus in comparison with control cells.

Table(2): The optical densities of the harvested tissue culture supernatant of the eight propagated isolates in comparison with normal control.

Cod. No. of samples	Absorbances of test samples measured by ELISA reader at wave length 450
3	0.102
6	0.112
10	0.145
19	0.111
25	0.162
33	0.115
34	0.138
67	0.203
Normal control	0.080

Table (3): The cytopathic behavior of the inoculated samples on MA104 cell line after five passages.

No. Of samples	Degree of CPE Days post inoculutation						
	1st	2nd	3rd	4th	5th	6th	7th
3	+	++	+++	++++			
6	–	–	+	++	++	+++	+++
10	+	++	+++	++++			
19	+	++	+++	++++			
25	+	++	+++	++++			
33		+	++	+++	++++		
34	–	–	++	++	++	+++	+++
67	+	++	+++	++++			

After 3rd and 5th passages , the RT-culture supernatant 7 out of 8 samples PCR using VP6 primers was carried produced the specific band 1356bp as out on the extracted RNA from tissuin figure 4 and 5.

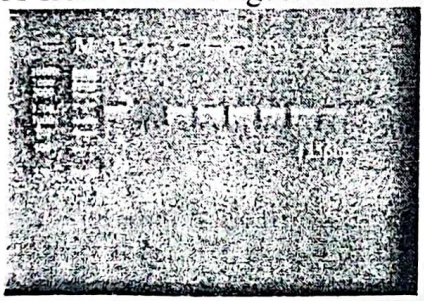


Fig.(4) :RT-PCR products of VP6 (1356bp) of CRV isolates after 3rd passage in ethidium bromide stained agarose gel electrophoresis, along with 1Kbp DNA ladder (M) that contains 13 size bands ranged between 250 and 10.000 bp

M: Marker represents bands of molecular sizes of (10,000-3000-2000-1500, 1000, 750, 500. 250 bp)

Lane 1, 3, 4, 5, 6, and 7: Represent the amplified product of correctly VP7gene size 1356 bp of isolates No. 3, 10, 19, 25, 33 and 34.

Lane 2, 8: Represent the negative for (amplification isolates numbers: 6 and 67) .

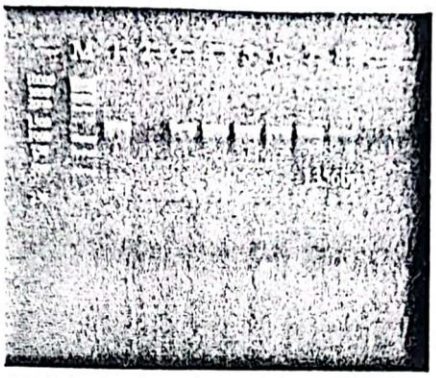


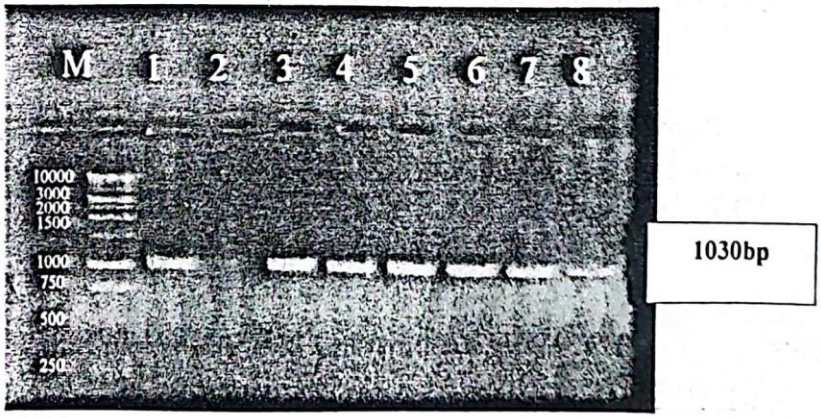
Fig.(5): RT- PCR products of VP6 (1356bp) of CRV propagated in tissue cell cultureMA104 of 5th passage in ethiduim bromide stained agarose gel electrophoresis , along with 1Kbp DNA ladder (M)

M: Represents 1kbp DNA ladder.

Lanes 1, 3, 4, 5, 6, 7and 8: Represents the amplified product of VP6 genes For the positive propagated isolates No. 3, 10, 19, 25, 33, 34 and 67.

Lane 2: Represents the negative isolates No. 6.

The RT-PCR using VP7 specific primers was carried out on the extracted RNA from tissue culture supernatant of the 5th passage. Figure 6 showing 7 out of 8 propagated samples produce the specific band of VP7 at 1030bp



(Fig.6): RT-PCR products of VP7 (1030 bp)of 5th passage of propagated isolates in MA104 cells of CRV in ethidium bromide stained agarose gel electrophoresis, along with 1kbp DNA ladder

Analysis of the nucleotide sequence of VP7 gene fragments of CRV:

The purified DNA representing the outer capsid VP7 gene of CRV was 1030bp in size only sequence of 580 bp as obtained. The obtained nucleotide sequence shown in the following box.

TTGANTCCCATCCTACTCTTGATTNTCTTATCTATGGATTCATATTAATG
AGTATAACTAGAATGATGGACTACATAATTTATAAATTTTTNGCTTATAG
TCACGATCACTTCAATTGTTGTTAACGCACAAAATTACGGTATCAATTTA
CCAGTAACTGGATCGATGGATATGTCATATGTGAACGTTACTAAAGATGA
GCCATTTCTAACATCAACATTATGTTTATACTATCCAACAGAAGCCAGAA
CAGAAATAAATGATAACGAGTGGACAAGTACGTTGTCGCAGTTGTTCCTG
ACAAAGGGATGGCCAACTGGATCCGTATACTTTAAGGAATACGATGATAT
AGCTACCTTTTCAGTAGATCCACAATTATATTGCNGACTATAACATAGTT
TTGATGAGATACAATTCGGATTTAGAACTTGATATGTCGGAATTGGCAAA
TCTAATATTAAATGAATGGCTGTGCAATCCAATGGACATTACATTATATT
ATTATCAACAAACGGACGAAGCAAACAAATGGANAGCAATGGGGCAATCA
TGTACAATAAAAGTATGTCCACTAAATCC

The obtained sequence was analyzed using computer software (BLAST) via the internet (www.ncbi.nlh.gov.) which revealed that the nucleotide sequence was highly related to strains of G10 Bovine Rotavirus in percentage range between 88% to 92% so, the CRV were related to G10 BRV lineage as shown in the following (Table4).

Table-7: Homology% of the two highly positive samples sequenced fragment to the published group A rotavirus sequences in the gene bank (NCBI):

Camel Rotavirus (10)

Accession	Description	Max. identity
L07658.1	Rotavirus sp. outer capsid protein (VP7) mRNA, complete cds	90%
X52650.1	Bovine rotavirus protein 7 (VP7), segment 8, genomic RNA	91%
X57852.1	Bovine rotavirus (strain B223) VP7 mRNA	91%
M64679.1	Bovine rotavirus B11 glycoprotein (VP7) gene, complete cds	91%
D01056.1	Bovine rotavirus mRNA for VP7, complete cds, strain:KK3	90%
D01055.1	Bovine rotavirus mRNA for VP7, complete cds, strain:A44	90%
AY644385.1	Bovine rotavirus strain CIT10A/02 outer capsid protein gene, partial cds	89%
AF386918.1	Bovine rotavirus strain B75 outer capsid protein VP7 gene, complete cds	89%
AF507093.1	Bovine rotavirus G10 isolate	93%

Accession	Description	Max. identity
	Mordva/11/99 glycoprotein (VP7) gene, partial cds	
AF507098.1	Bovine rotavirus G10 isolate Sobinka/10/99/C glycoprotein (VP7) gene, partial cds	92%
AF507095.1	Bovine rotavirus G10 isolate Pavlovo/04/01 glycoprotein (VP7) gene, partial cds	92%
AF507111.1	Bovine rotavirus G10 isolate Kavernino/01/01 glycoprotein (VP7) gene, partial cds	93%

Rotavirus (25) Camel

Accession	Description	Max. identity
X52650.1	Bovine rotavirus protein 7 (VP7), segment 8, genomic RNA	91%
M64679.1	Bovine rotavirus B11 glycoprotein (VP7) gene, complete cds	91%
X57852.1	Bovine rotavirus (strain B223) VP7 mRNA	91%
L07658.1	Rotavirus sp. outer capsid protein (VP7) mRNA, complete cds	91%
D01056.1	Bovine rotavirus mRNA for VP7, complete cds, strain:KK3	90%
U14996.1	Bovine rotavirus 2292B major capsid glycoprotein (VP7) mRNA, complete cds	89%

Accession	Description	Max. identity
X53403.1	Bovine rotavirus strain 61A VP7 gene RNA	89%
AY644385.1	Bovine rotavirus strain CIT10A/02 outer capsid protein gene, partial cds	89%
D01055.1	Bovine rotavirus mRNA for VP7, complete cds, strain:A44	89%
EU221265.1	Human rotavirus A isolate CRI 1444 capsid glycoprotein (VP7) gene, partial cds	88%
AF507095.1	Bovine rotavirus G10 isolate Pavlovo/04/01 glycoprotein (VP7) gene, partial cds	93%
AF507111.1	Bovine rotavirus G10 isolate Kavernino/01/01 glycoprotein (VP7) gene, partial cds	93%
AF507098.1	Bovine rotavirus G10 isolate Sobinka/10/99/C glycoprotein (VP7)	93%
AF507104.1	Bovine rotavirus G10 isolate Sobinka/04/01/A/G10 glycoprotein (VP7) gene, partial cds	91%

Discussion

In this study, we have detected of group A rotavirus in fecal samples of diarrheic camel calves using Mabs- based ELISA and electron microscopy **(House et al., 1978, Yolken et al., 1978 , Herrman et al., 1985 and Hussein et al., 1995)**. The availability of local strain of CRV was limited so, important to continue in trail to isolate CRV using MA104 cell culture **(Babuik et al., 1977)**. The cytopathic effect (CPE) on the inoculated cells were varied between the samples among the applied five passages **(Saif and Theil, 1985)**.

The application of Mabs- based ELISA to identify rotavirus in cell culture was of great help **(Hussein et al., 1995).**

The molecular characterization of CRV is most important in current study . the PCR has proved its efficacy in detecting rotavirus in the samples of both fecal and tissue culture **(Estes and Kapikian, 2007, Gentsch et al., 1993 , Isegawa et al., 1993** and **Gouvea et al ., 1994)** and the developed RT-PCR genotyping assay based detecting of G6 and G10 serotypes of group A bovine**(Hussein et al., 1996)** the RT-PCR based assay for genotyping of CRV was used in our study to genomic characterization of local 7 isolated stains based on extraction of ds RNA and amplification of CRV (VP6) gene 1356bp and VP7 gene 1030bp of the RT product using specific primers complementary to full length of each VP6 and VP7 genes**(Hussein et al., 1995).** The obtained bands from RT-PCR were detected in the accurate size 1356 and 1030 bp for both VP6 and VP7 gene . Also, the original samples give one band which was detected in predicted size for VP6 1356 bp in figure (2). After 3rd passages of the inoculated samples in cell culture, RT-PCR products revealed six positive isolates bands at size 1356 bp for VP6 gene at (figure 4) .After the 5th Passage of isolates in tissue cell culture, RT-PCR amplified products were seven positive isolates with bands at predict size 1356 bp for VP6 gene and six positive isolates with 1030 bp of the VP7 gene(figure 5, 6) .Two highly positive isolates coded no. (10, 25). Extracted dsRNA which was amplified by RT-PCR for VP7 gene. The eluted (purified) DNA after electrophoresis of the products was sent to be sequenced with the forward primer to be sequenced. The obtained sequence results of only 580 bp fragment of the amplified 1030bp PCR product revealed the specificity of the product to rotaviruses .Nucleotide sequence analysis has indicated the high identity of the sequenced fragment of both isolates **(Estes and Kapikian, 2007and Morin et al., 1976)** to the G10 serotype of group A rotavirus. This is the first report on the existence of G10 serotype of group A rotavirus in camel. Characterization of such serotype in camel population will add more interest on gathering the diversity information of rotaviruses.

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