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DETECTION AND ISOLATION OF ROTA (GROUP A) AND CORONA (GROUP 2) VIRUSES FROM NOMADIC CAMELS WITH SPECIAL REFERENCE TO PATHOLOGICAL ALTERATIONS

(With One Table and 6 Figures)

By

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استكشاف وعزل فيروس الروتا (مجموعة أ) وفيروس الكورونا (مجموعة 2) من الجمال الرحالة مع الأشارة للتغيرات الباثولوجية المصاحبة

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لدراسة تواجد فيروس الروتا (مجموعة أ) وفيروس الكورونا (مجموعة 2) في قطعان جمال جماعات البدو الرحالة بصحارى محافظة الشرقية (2006 -2007)، تم جمع عدد (229) عينة براز من جمال شملت (130) عينة من جمال مصابة بالإسهال وعدد (64) عينة من جمال مصابة بالإسهال ومختلطة مع فصائل من حيوانات أخرى مصابة بالإسهال وعدد (35) عينة من جمال سليمة ظاهريا. تم إكتشاف وجود فيروسى الروتا والكورونا بإستخدام إختبارى الفلورسنت المناعى والإليزا. كما تم عزل فيروس الروتا على خلايا كلية القرد الأفريقى الأخضر ومشاهدة التأثير الباثولوجى الخلوى وحدوث ظاهرة إدمصاص كرات الدم الحمراء حول الخلايا المحقونة والتى تميز فيروسات الروتا كما تأكد وجود فيروس الروتا بإستخدام إختبار الإليزا فى الخلايا المحقونة ، على آن محاولات عزل فيروس الكورونا على نفس الخلايا لم تكن ناجحة. إستكشاف فيروسى الروتا أو الكورونا أو كلا من الفيروسين معا فى إجمالى العينات بإستخدام الإليزا كانت: 44.01% (101 \229)، 23.14% (53\229) و10.91% (25\229) إلا أن نسبة وجود فيروس الروتا أو الكورونا أو هما معا كانت: 53.07% (69\130)، 34.61% (45\130) و 17.69% (23\130) فى الجمال المصابة بالإسهال و كانت بنسبة 34.37% (22\64)، 12.50% (8\64) و 3.12% (2\64) فى الجمال المصابة بالإسهال والمخالطة لحيوانات أخرى مصابة بالأسهال إلا أن النسبة كانت 28.57% (10\35) لفيروس الروتا فى الجمال السليمه ظاهريا. أشارت التغيرات الباثولوجية الميكروسكوبية المصاحبة للإصابة بفيروس الروتا فى الجمال إلى حدوث ضمور في بعض الخملات المعوية وتسطح الخلايا الطلائية المعوية مع وجود ارتشاحات لخلايا وحيدة الأنوية (الليمفوسايت) وعديدة الأنوية بينما كانت الأمعاء الدقيقة والغليظة المصاحبة للإصابة بفيروس الكورونا يظهر بها بؤر عديدة من تقرحات الطبقة المخاطية ودرجات مختلفة من الاستسقاء

(الاوديما) الممتد من الطبقة المخاطية إلى طبقة التحت مخاطية مع تراكم الخلايا الليمفوسايت. تشير نتائج الدراسة إلى أهمية دور كلا من فيروسى الروتا أو الكورونا فى الإصابة المعوية بين قطعان الجمال المصرية.

SUMMARY

To study the presence of rota (group A) and corona (group 2) viruses in camels, a total of 229 fecal samples were collected from diarrheic (130) as well as contact diarrheic(with other animal species) (64) and healthy camels (35) from different nomadic flocks from desert areas in Sharkia Governorate, Egypt during 2006- 2007. Group A rota virus infection was confirmed by direct fluorescent anti-body technique (FAT), virus isolation and detection of rota virus antigen by immune-captured enzyme linked immune sorbent assay (Ic-ELISA) in feces and harvested inoculated cell, as well as demonstration of cytopathic effect (CPE) and haemadsorbtion criteria of rota virus on Vero cell. Corona virus antigen was detected by FAT, Ic-ELISA but all attempts for corona virus isolation were entirely failed. Specific rota virus or corona virus antigens or both; were detected by Ic-ELISA; in 44.01% (101/229), 23.14% (53/229) and 10.91% (25/229) of the total examined fecal specimens, respectively, of which 53.07% (69/ 130), 34.61% (45/130) and 17.69% (23/130) were from diarrheic, 34.37% (22/64), 12.50% (8/64) and 3.12% (2/64) from contact animals for rota, corona or both virus antigens, respectively and 28.57% (10/35), for rota virus only from healthy camels. The results show that the rota virus is widespread, mainly among camels from 6 to 12-months old, while corona virus alone or mixed with rota virus was 1 to 2 years old. The histopathological changes in small intestine associated with rota virus infection were observed in villus atrophy and flattening of the epithelial cells. The small and large intestines infected by corona virus had multifocal mucosal ulceration and various degrees of mucosal to submucosal edema with moderate accumulation of lymphocytes. These findings emphasis the significant role of rota virus and corona virus or both in the enteric infection of camels in Egypt.

Key words: *Camel, corona virus, rota virus, pathology.*

INTRODUCTION

Rota and corona viruses encountered as an important potential pathogens of different mammals including camels causing serious enteritis, pneumonia, dehydration and some death particularly neonated cases (Berrada *et al.,* 1999, Wűnschmann *et al.,* 2002, Cebra *et al.,* 2003 and Tibary *et al.,* 2006) (Orr 1984, Alfieri *et al.,* 2006; Oliveira Filho *et al.,* 2007). Rota viruses are taxonomically grouped in the Reoviridae family characterized by a double-stranded RNA genome that consists of 11 gene segments enclosed in a double-shelled protein capsid (Derbyshire and Woode, 1978 and Kalica *et al.,* 1978). Rota viruses are divided into 6 distinct groups designated A through F, with evidence accumulating to support the possible inclusion of one more group. Group A rota viruses (GARV) are most often associated with diarrhea among farm animals and humans (Bridger, 1987). Corona virus has been reported in sheep, goats and llamas (Evermann and Benfield, 2001 and Cebra *et al.,* 2003), though it is more commonly identified as a problem in cattle (Tzipori, 1981 and Decaro *et al.,* 2008) causing infections of mucosal surfaces, of the respiratory and digestive systems (Evermann and Benfield, 2001 and Lai *et al.,* 2006). Corona viruses are large, enveloped single-stranded with positive polarity RNA viruses in the family Coronaviridae order Nidovirales (Vries *et al.,* 1997), have three distinct antigenic groups based on their serologic characterization (Claude *et al.,* 2005). Corona viruses isolated from mammals and humans are categorized in Groups 1 and 2 (Lai *et al.,* 2006). Bovine corona virus (BCoV) and alpaca corona virus (ApCoV) are both members of group 2 (Davis *et al.,* 2000 and Claude *et al.,* 2005). Detection of rota or corona viruses in fecal samples was confirmed with FAT, ELISA, RT-PCR, or nested PCR (Park *et al.,* 2006). However, there are inadequate data on the incidence, as well as the isolation and histopathology of rota virus and corona virus infections, in camels located in Egypt. The objective of the present work was planned for detection, isolation and estimation the rate of rota and corona viruses infection in nomadic camels at Sharkia Governorate.

MATERIALS and METHODS

1. Sample collection and preparation:

During 2006 – 2007, a total of 229 fecal samples were randomly collected from diarrheic as well as contact diarrheic (with other animal species) and healthy camels from different nomadic Bedouin camel

flocks from desert areas in Sharkia Governorate, Egypt. Fecal samples were collected from the rectum following digital stimulation. A suspension of fecal material was prepared in a 1:10 proportion (p/v) in phosphate-buffered saline (PBS 0.01 M pH 7.2), centrifuged at 7000 rpm/m under refrigeration for 30 minutes. Supernatant was filtered through a 0.45 µm (Millex® USA) system. One portion included various regions of small and large intestines of each diarrheic slaughtered animal were fixed in neutral buffered formalin, for routine histopathological examination.

2. Direct fluorescent anti-body technique (FAT):

The lumenal surface of intestinal segments was cellularly scraped, transferred to an eppendorf centrifuge tube, and 1 ml PBS was added. The sample was vortexed and clarified by centrifugation at 5000 rpm/m for 5 min. The supernatant was decant and sediment cells were resuspended in 1 ml PBS with vortex, then pipetted (50 µl) over a depressed microscope slide, dried at 37 ^{0}C then fixed at aquas acetone ethanol (1:1) over night at 4 ^{0}C, then washed triple with PBS. Hyper immune serum fluorescein-labelled [fluorescein isothiocyanated] for bovine rota or bovine corona viruses (VHRD, Pullman, USA) was added (50 µl /slide). All examined slides were incubated at 37 ^{0}C for one h., followed by triple washing with PBS containing 0.1% bovine albumin (BD-BBL, Ireland), then mounted in glycerol saline and examined under fluorescent microscope (Burleson *et al.*, 1997).

3. Immune captured enzyme linked immune sorbent assay (Ic-ELISA):

All collected fecal samples were submitted to commercial Ic-ELISA [Bio-x Diagnostic, Belgium], as well as all inoculated cells (positive or negative CPE) to confirm and identify the obtained viral isolate. The kits can only detect the presence of group A bovine rota virus (BRoV) and group 2 BCoV antigens.

4. Cell culture, viral isolation and titeration:

Of the positive in Ic-ELISA, some of the tested samples (5 +ve rota & 5 +ve corona) were chosen and attempts for viruses isolation in cell culture was carried out. Monolayers of Vero cells adapted to growth in Minimum Essential Medium Eagle (MEM - Cultilab®) with 10% of bovine fetal serum (Cultilab®) were used. One ml of the diluted (1:10) filtered fecal suspension was inoculated, in triplicate, in monolayers Vero cell with 48 hours of growth in plastic bottles (25 cm^2). Previous

growth medium was discarded and monolayers were washed twice in MEM before the inoculation. After 0ne hour of adsorption under low stirring at 37ºC, the inoculum was discarded. Eight ml of MEM were added. Bottles were incubated at 37ºC and cytopathic effect (CPE) was observed until 96 hours after inoculation. Monolayers were then frozen for the next passages, which were performed with 1 ml of the respective previous passage. As the negative control, inoculum with PBS was used; isolates were considered positive when the cytopathic effect was observed till the 3rd passage to exclude non specific CPE. Random positive harvested inoculated plastic bottles (CPE, haemadsorbtion and Ic-ELISA +ve) were polled for estimation the infectivity titration of the viral isolate to obtained $TCID_{50}$/ml according to Reed and Muench, (1938).

5. Haemadsorbtion assay:

The test was performed according to Vogel and Shelokov (1957). After 2 or 3 days of incubation; whether or not suspected CPE has occurred; the tissue culture media are removed and cells were washed twice with PBS and then incubated for one h. with a 0.4% suspension of guinea pig erythrocytes (RBCs). The bottles are incubated for 1h. at 4ºC, 22º C or at 37ºC, rinsed briefly with PBS to resuspend the settled RBCs, and then examined under low or high magnification. Positive haemadsorption is detected by firm adhesion of the RBCs which must not float across the monolayer when the bottle is gently agitated. Individual infected cells as well as the ratio of infection can be demonstrated.

6. Histopathological examination:

Representative tissue specimens were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin (Bancroft and Stevens, 1990).

RESULTS

Examination of intestinal sediment cells of totally 30 samples by FAT revealed 3, 7 and 3 positive samples for rota, corona and both virus antigens, respectively (Fig. 1).

Specific rota virus or corona virus antigens or both; were detected (Table 1) by Ic-ELISA, in 44.01% (101/229), 23.14% (53/229) and 10.91% (25/229) of the total examined fecal specimens, respectively, of which 53.07% (69/ 130), 34.61% (45/130) and 17.69% (23/130) were

from diarrheic, 34.37% (22/64), 12.50% (8/64) and 3.12% (2/64) from contact and 28.57% (10/35), from healthy camels for rota, corona or both virus antigens, respectively. The results show that the rota virus; 54.83% (34/62); is widespread, mainly among camel from 6 to 12-months-old, while corona virus alone or mixed with rota virus was 38.23%, 14.70% (13/34, 5/34), respectively at 1 to 2 years old.

Among fecal samples studied for the presence of rota or corona virus by Ic-ELISA, 10 positive samples were tested to isolate the viruses in Vero cells, 5 samples (5/5 = 100%) for rota virus presented rounding, granulation, processing and syncytial cytopathic effect until the fifth passage with haemadsorbtion for added RBCs (Fig. 2). The CPE in Bottles indicated that the isolates were confirmed as positive for rota virus by Ic-ELISA. When titration of the rota virus isolate was performed, a titer equal to $10^{5.4}$ $TCID_{50}$/ml was obtained. All trails for corona virus isolation have been failed.

The histopathological examination of rota virus infected intestines revealed that the virus specially attacks the epithelium of small intestine. The epithelial cells are lost from the tips of villi. These desquamated cells are replaced by cuboidal, then flattened squamous epithelial cells resulting in villus atrophy. Some villi remained denuded and their stroma became internally infiltrated with mononuclear inflammatory cells and moderate neutrophilic infiltration in the lamina propria (Fig.3). The small and large intestines infected by corona virus had multifocal mucosal ulceration and various degrees of mucosal to submucosal edema with moderate accumulation of lymphocytes and plasma cells (Fig.4). Severe congestion of blood capillaries and wide dilatation of the lymphatics in some villi were found (Fig.5). Mild hypercellularity was observed in the mucosal lamina propria of most sections of colon and cecum. In mixed infections of rota and corona viruses of intestine destruction and necrosis of the intestinal villi and mononuclear inflammatory cell infiltration were found (Fig. 6).

Table 1: Detection of rota (group A) and corona (group 2) viruses antigens in camel fecal samples by ELISA:

Age	No. of Samples tested	Signs	No. Samples	ROTA				CORONA				ROTA & CORONA			
				+V	% of positive samples	Total +V samples	%of positive samples	+V	% of positive samples	Total +V samples	%of positive samples	+V	% of positive samples	Total +V samples	%of positive samples
> 3 months	74	H[1]	11	2	18.18%	32/74	43.24%	0	0.00%	20/74	27.02%	0	0.00%	11/74	14.86 %
		C[2]	18	7	38.88%			3	16.66%			1	5.55%		
		D[3]	45	23	51.11%			17	37.77%			10	22.22%		
6-12 months	62	H	9	4	44.44%	34/62	54.83%	0	0.00%	13/62	20.96%	0	0.00%	8/62	12.90 %
		C	12	3	25.00%			1	8.33%			0	0.00%		
		D	41	27	65.85%			12	29.26%			8	19.51%		
1-2 year	34	H	6	2	33.33%	12/34	35.29%	0	0.00%	13/34	38.23%	0	0.00%	5/34	14.70 %
		C	11	4	36.36%			2	18.18%			1	9.09%		
		D	17	6	35.29%			11	64.70%			4	23.52%		
<2 year	59	H	9	2	22.22%	23/59	38.98%	0	0.00%	7/59	11.86%	0	0.00%	1/59	1.69%
		C	23	8	34.78%			2	8.69%			0	0.00%		
		D	27	13	48.14%			5	18.51%			1	3.70%		
No. of each clinically samples tested		H	35	10	(10/35) 28.57%			0	0.00%			0	0.00%		
		C	64	22	(22/64) 34.37%			8	(8/64%) 12.50%			2	(2/64) 3.12%		
		D	130	69	(69/130) 53.07%			45	(45/130) 34.61%			23	(23/130) 17.69%		
Total examined samples			229	101	(101/229) 44.10%			53	(53/229) 23.14			25	(25/229) 10.91%		

1: Healthy; 2: Contact diarrheic and 3: diarrheic.

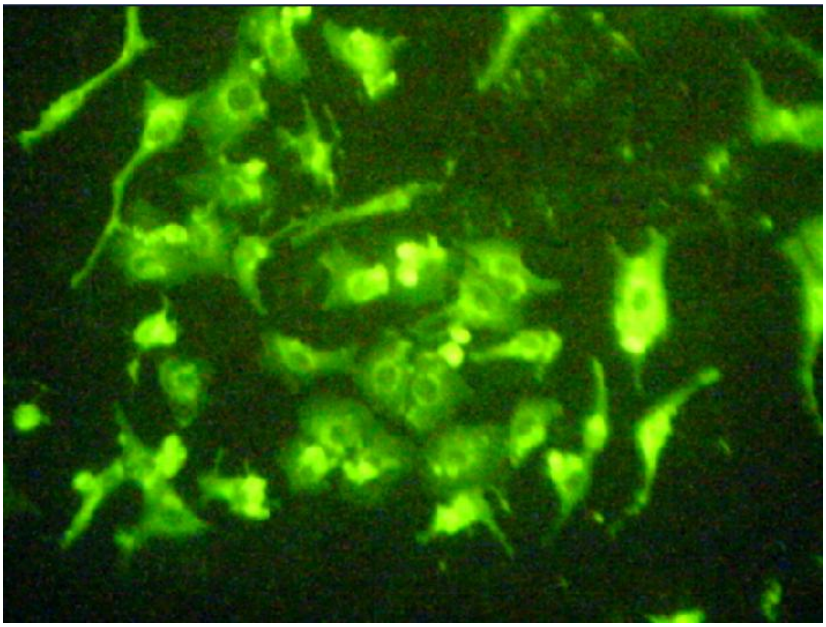

Fig. 1: FAT, Intracytoplasmic perinuclear fluorescence in the positive enterocyte.

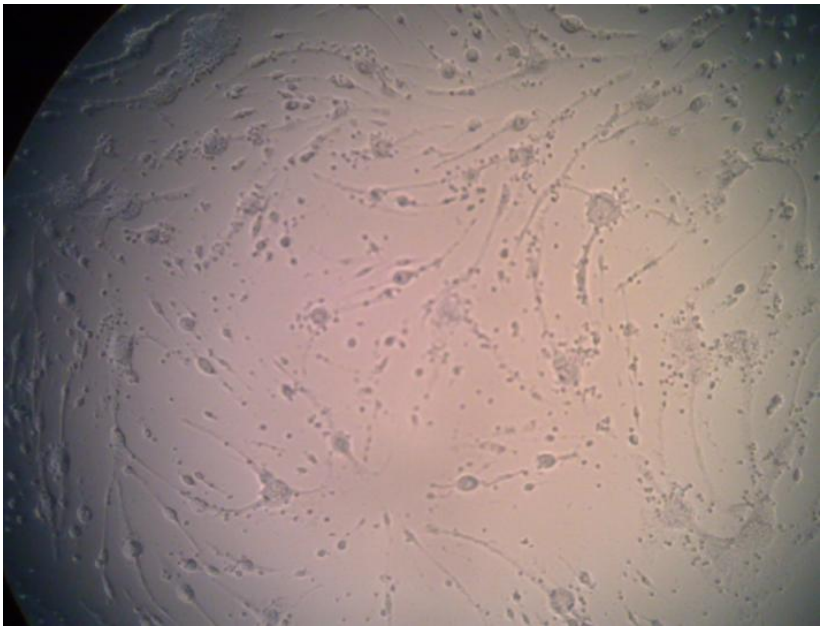

Fig. 2: Camel rota virus infected Vero cells reveals positive haemadsorption.

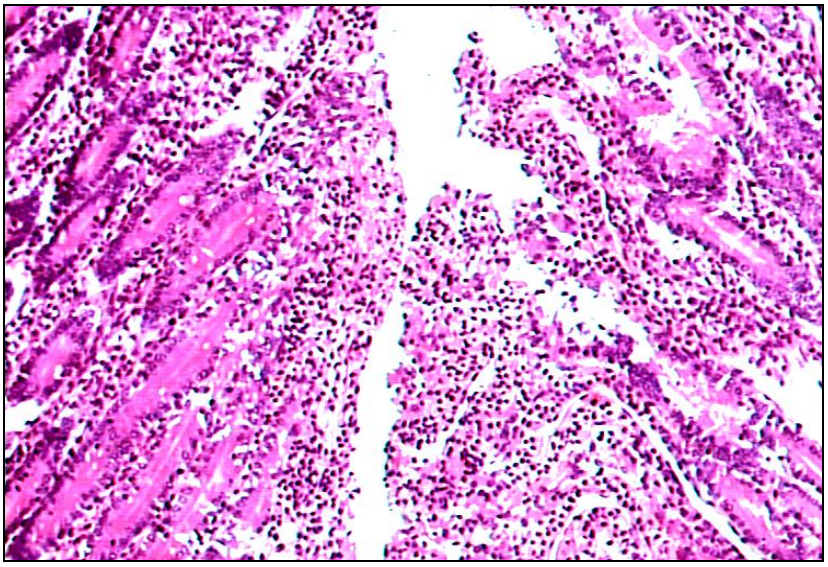

Fig. 3: Duodenum of camel calf infected with *Rota virus* showing intense infiltration of mononuclear inflammatory cells and moderate neutrophilic infiltration in the lamina propria (H&E; X100).

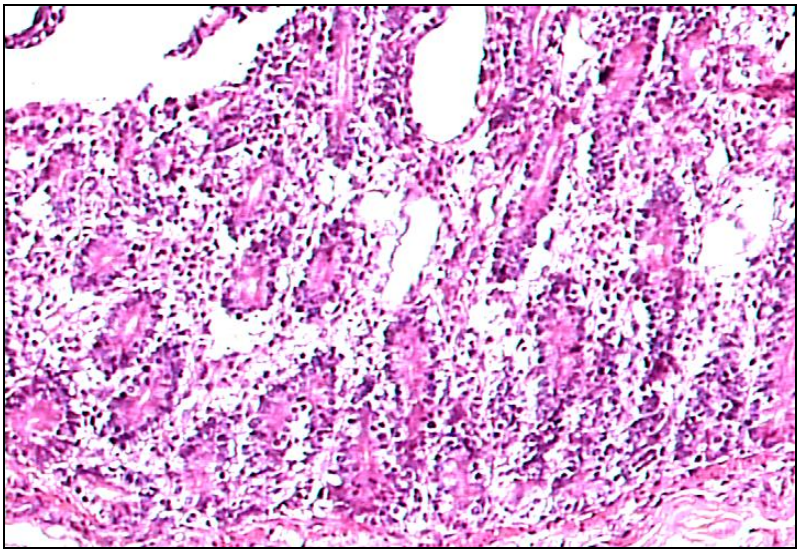

Fig. 4: Jejunum of camel calf infected with *Corona virus* showing mononuclear inflammatory cells and moderate edema in the lamina propria (H&E; X100).

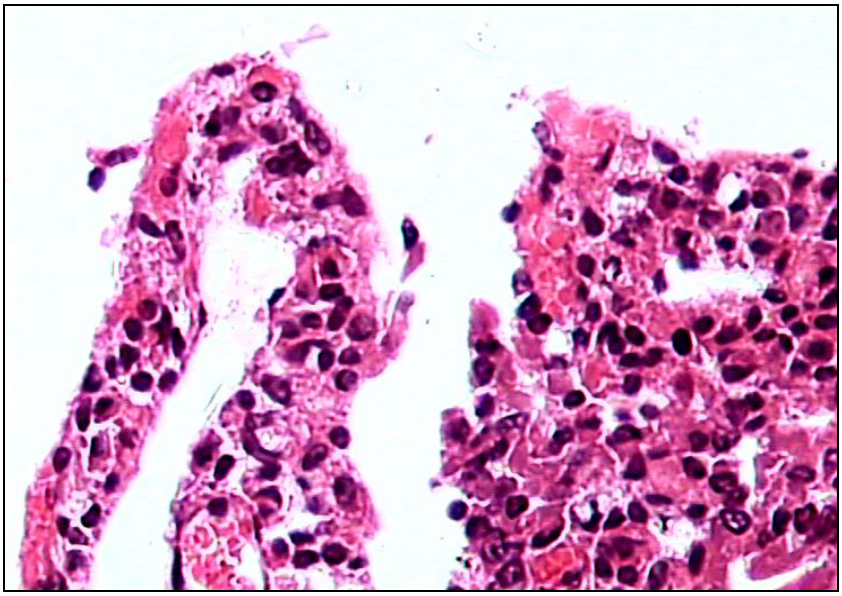

Fig. 5: Ileum of camel calf infected with *Corona virus* showing severe congestion of the blood capillaries and wide dilatation of the lymphatic in some villi (H&E; X400).

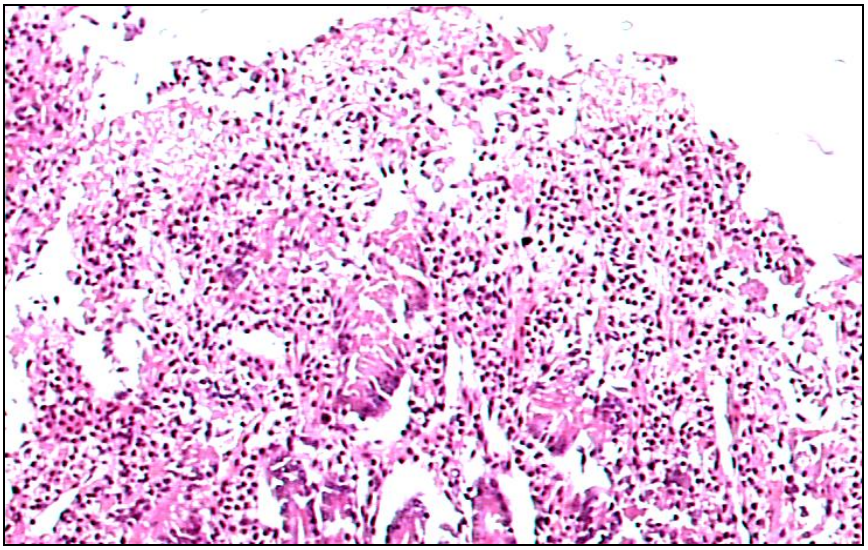

Fig. 6: Ileum of camel calf infected with *Rota virus and Corona virus* showing destruction and necrosis of the intestinal villi with mononuclear inflammatory cell infiltration (H&E; X100).

DISCUSSION

In this study, FAT has been used to stain intestinal cells from infected camels, where positive rota and corona virus antigens were detected, although, the staining results (positive/negative) tend to lack detail on intensity/distribution of staining. Although the immunofluorescence (IF) has been used to stain cells or cell debris excreted in stools from viral infected calves; the problem of non-specific fluorescence has prevented its widespread use on stools, but this can be overcome if the virus antigen is first separated by immune precipitation prior to staining (WHO, 1980). However, this method is laborious and time-consuming when done on a large scale. A simpler method is needed to make detection of rota or corona viruses in camel feaces more acceptable. The ELISA is reproducible, rapid and quantitative method for the detection of corona virus, rota virus and reovirus antigens particularly suited for the large-scale testing of fecal samples, the prevalence of rota and corona virus antigens was higher by ELISA than by EM. (Yolken *et al.,* 1977 and Dea and Tijssen, 1988).

In this work, an indirect antigen capture ELISA employing monoclonal antibodies (MAbs) to BRoV and BCoV as the capture antibodies was used to detect BRoV and BCoV in the camel fecal suspensions because of their relative simplicity and speed. The results obtained revealed that rota virus or corona virus antigens or both; were detected, in 44.01%, 23.14% and 10.91% of the total examined fecal specimens respectively. These results like to some extent the frequency of bovine GARV (11 to 19.4%), BCoV (14 to 39%) (Alfieri *et al.,* 2006; Takiuchi *et al.,* 2006 and Oliveira Filho *et al.,* 2007) and the bovine GARV and BCoV co-infection in (15.9%) recorded in cattle herds (Fernandes *et al.,* 2009).

Our positive Ic-ELISA revealed 53.07%, 34.61% and 17.69% were from diarrheic, 34.37%, 12.55% and 3.12% from contact for rota, corona or both virus antigens respectively and 28.57%, from healthy camels for rota virus only. A high frequency of viruses associated were with that diarrheic camels, as previously described for prevalence of rota virus (26.98%) and Corona virus (3.17%) infection in diarrheic calves in Iran (Mayameei *et al.,* 2009). In addition, rota virus infection were the most common causes of diarrhea in camel calves from 6 to 12-months-old (Wernery, 1999 and Wernery and Kaaden, 2002), while corona virus alone or mixed with rota virus was at 1 to 2 years old. Anyhow; bovine corona virus was the principal virus particle present in samples of winter

dysenteric adult cattle (Saif *et al.*, 1988), whereas chronically infected adult animals are often a source of the corona virus infection (Clark, 1993).

Interestingly, in the current study; healthy camels had a score for rota virus antigen detection. The exact reason for this finding is unknown. However Since the initial description of the clinical signs of rota virus-induced diarrhea in calves, much has been learned about the epidemiology of rota virus infection (Mebus *et al.,* 1969). Rota viruses can be found in fecal samples from normal healthy animal or in animals exhibiting clinical disease of varied severity (Dea, *et al.,* 1985). The source of the rota or corona viral infection is uncertain in the diarrheic or healthy camels present case. The fact that other contact animals species in the herd had a history of diarrhea supports the concept that there were either acquired or exchange of rota or corona viral infection among camels and the other animal species. Additionally, the possible interspecies corona virus infection may have implication for the cohusbandry of individuals of the family Camelidae with individuals of other families such as Bovidae, Cervidae, and Equidae (Wűnschmann *et al.,* 2002). The findings of the present study are supported by a recent report in which a corona virus antigenically related to BCoV was identified in a wild ruminants (Tsunemitsu *et al.,* 1995) elk calves (Majhdi *et al.,* 1997), foal (Davis *et al.,* 2000 and Elizabeth *et al.,* 2000) llamas and alpacas (Cebra *et al.,* 2003), While the group A rota virus was identified to be a common intestinal infection in animals and has been demonstrated as a cause of diarrhea in mice, calves, piglets, foals, young rabbits, deer, pronghorn antelope, chickens, turkeys, goats, kittens, a chimpanzee, and a gorilla. There is also evidence that it can infect other animals, as shown by virus isolation studies in monkeys and dogs (WHO, 1980 and Ijaz *et al.,* 1989).

Although most field strains of BCoV grow poorly in cell culture and fail to produce CPE until after blind passage, primary calf kidney (PCK) and Vero cells have permitted primary isolation of virus (Linda *et al.,* 1988) while; BRV were isolated from fecal specimens in Vero cells (Sato *et al.,* 1997). In the present study, isolation of camel corona virus in Vero cells were completely failed where no CPE or Ic- ELISA viral detection; until after 5 blind passages; were obtained, whereas camel group A rota virus was isolated in Vero cells and identified by Ic-ELISA.where CPE were manifested on days 3-5 on the 2^{nd}-5^{th} passages as rounding, elongation, and granulation of cells. This characteristic CPE goes hand in hand with the growth characteristics in MDBK cells of a

calf rotavirus isolated in Northern Ireland (McNulty *et al.*, 1977) and those recorded in MA104 cells during detection and isolation of group A rota virus from camel calves in Sudan which were identified by ELISA and EM (Ali *et al.*, 2004). This finding may attributed to the presence of proteolytic enzymes. The ability of pancreatic proteolytic enzymes to enhance rota virus infectivity in both permissive and semipermissive cell lines was recorded, the degree of enhancement observed was dependent upon the initial multiplicity of infection and the host cell line employed, Vero cells showed that trypsin treatment allowed multiple rounds of rota virus replication to occur in these cells (Graham and Estes, 1980).

In present work, camel rota virus infected Vero cells were positive for haemadsorption assay. This finding strongly pointed to the relation of this isolated virus to BRoV as among the rota viruses for which the presence of a haemagglutinin has been demonstrated are rota calf diarrhoea virus (Spence *et al.*, 1976 & 1978 and Inaba *et al.*, 1977).

In titration of the camel rota virus isolate, a greatest titer observed was equal to $10^{5.4}$ $TCID_{50}$/ml, thus confirm the hypothesis of high viral concentration, what may contribute for more knowledge of CRV antigenic properties and consequently; seroneutralization assay in microplates may be used in the future to detect and titerate the camel rota virus antibodies in camel flocks.

The histopathological changes associated with rota virus infection were observed in villous atrophy and flattening of the epithelial cells. These changes were consistent with the lesion previously described in natural or experimental rota virus infections in bovine calve, piglets and mice (Mebus *et al.*, 1971, Carpio *et al.*, 1981, and Jos *et al.*, 2003). In many domestic animals, the features of the infection, the intestinal involvement and the pathogenesis are similar to bovines, even though considerable serotype differences exist among animal rota viruses of different geno-groups (Wani *et al.*, 2004 and Fukai *et al.*, 2006). After entry through oral route, rota viruses have been found to replicate in the mature villous epithelial cells (enterocytes). For their efficient colonization and infectivity, viruses need their outer capsid to be cleaved and removed, which is facilitated by intestinal proteases like chymotrypsin (Ramig, 2004 and Desselberger *et al.*, 2005). The small and large intestine infected by corona virus had multifocal mucosal ulceration and various degrees of mucosal to submucosal edema with moderate accumulation of lymphocytes. This is in agreement with previously record in bovine calves (Mebus *et al.*, 1973, Mebus *et al.*, 1975 and Sharpee *et al.*, 1976). On the other hand, Wűnschmann *et al.*

(2002) recorded that pathological changes were probably marked in the colon and cecum than the parts of small intestine in camel calves and mixed nonhemolytic coliforms and nonhemolytic *Streptococcus* sp. were cultured from colon. In addition, the corona virus infection may have predisposed the animal to the putative fatal clostridial infection.

In conclusion; the results of the present study refer to the significant role of rota virus and corona virus or both in the enteric infection of camels in Egypt. In addition, the study refers the antigenic similarities between bovine and camel rota and corona viruses and suggest that camel may be a source for rota or corona viruses that infect domestic ruminants or vice versa. However, Molecular studies should warranted to clear-up the detailed pathogenesis and strains variance.

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