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A TRIAL FOR DETECTION OF RABBIT ROTA VIRUS BY CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

During winter 2005 an out break of diarrhea was recorded in a rabbit stock farm in Faculty of Agriculture, Benha University. A total of forty intestinal fecal samples of dead diarrheic rabbits were submitted for detection of rabbit rotavirus by indirect capture ELISA, involving capture of rabbit rotavirus by means of bovine rota polyclonal antibodies and detected with another monoclonal antibodies against the major group A specific rotavirus antigen (VP6). The detection of rotavirus indicated that; 17 out of 40 samples (42.5%) were positive. Almost all ages of examined rabbits revealed rotavirus infection, where 5 out of 13 (38%), 7 out of 15 (47%) and 5 out of 12 (42%) of rabbits less than 1 month old, 1-2 months old and greater than 6 months old were positive, respectively. This is the first report of rabbit rotaviruses in Egypt, so further investigations are recommended.

INTRODUCTION

Rotaviruses are a major cause of acute viral gastro-enteritis and diarrhea in animals including cattle ***(Kapikian and Chanock, 1990 Saif et al., 1994 and Estes 2001)*** and rabbits ***(Bryden et al., 1976, Petric et al., 1978, Sato et al., 1982a,b, Castrucci et al., 1985 Schoeb et al., 1986, Thouless et al., 1986, Yu et al., 1994, Rizzi et al., 1995 and Banyai et al., 2005).***

Rotaviruses have a complex architecture, non-enveloped icosahedral virions belong to the reoviridae family and characterized by a genome consisting of 11 segments of double- stranded RNA, enclosed in a triple-layered protein capsid ***(Estes 1996).***

The innermost layer is composed of VP_1, VP_2, VP_3 and the genome; the middle layer is composed of trimerized VP 6 which makes up over 51% of the virions, and the outer layer is composed of the glycoprotein VP7 and spikes of VP4 dimers ***(Prasad et al., 1990 and Ester 1996)***.

Three Major antigenic specificities are assigned to rotaviruses: group, subgroup, and serotypes. Both group and subgroup specificities are mediated mainly by VP6, which is the major inner capsid protein, seven groups-designated as A, B, C, D, E, F and G-have been distinguished in human, other mammals, and birds ***(Estes 1996)***. Two subgroups I and II, can be identified within group A, as determined by monoclonal antibodies ***(Greenbergy et al., 1983)***. The classification of rotaviruses in seotypes is based on both glycoprotein VP7 (G types) and protease- sensitive protein VP4 (P types) antigenic specificities ***(Estes and Cohen 1989, Blacklow and Greenberg 1991)***. To date 14 G-types (among group A) have been described, with G1, G6 G8 and G10 among bovine isolates ***(Bellinzoni et al., 1989, Estes and Cohen 1989, Snodgrass et al., 1990, Blackhall et al 1992, Parwani et al., 1992, Parwani et al., 1993 and Woode et al., 1983)***. Group A lapine rotavirus isolates have been characterized as types G3 ***(Petric et al., 1978, Sato et al., 1982a,b, Castrucci et al., 1985, Conner et al., 1988, Thouless 1988 Ciarlet et al., 1997, De-Leener et al., 2004 and Banyai et al., 2005)***.

Group A rotaviruses share a common group antigen ***(Thouless et al., 1977)*** of VP6 ***(Woode et al., 1976, and Choi et al., 1997)***. A variety of techniques have been used to detect rotavirus in stool specimens, including electron microscopy ***(Morisse 1982 and Nieddu et al. 2000)***, serum-neutralization test (SNT) ***(Castrucci et al. 1985)***, polymerase chain reaction ***(Cere et al. 2000)*** and ELISA ***(Morisse 1982, Digiacoma and Thouless 1986, Thouless et al., 1986 and Cere et al., 2000)***. Hence

SNT revealed antigenic relationship between group A rabbit and bovine rotaviruses (BRV) *(Castrucci et al., 1984, 1985, 1988)*, antis

era against bovine rotavirus in ELIAS for detection of human rotavirus antigen were used (***Sarkkinen 1981)***.

Up till now there is no available reports on rabbit rotavirus in Egypt. This allowed us to study the incidence of rotavirus among dead diarrheic rabbits. Therefore, the objective of this study was to apply capture ELISA to detect rabbit rotavirus by using polyclonal bovine rotavirus as captured antibodies and common rota VP6 monoclonal antibodies.

MATERIAL AND METHODS

I- Samples:

During winter 2005 a sèvere diarrhea with high morbidity and mortality were recorded in a rabbit stock farm in Faculty of Agriculture, Benha University, the animals were submitted to clinical and P.M examination. A forty dead rabbits of different ages were identified as diarrheic [diarrhea is defined as characterized by fluid stools accompanied by fecal staining of the perineum (*Conner et al., 1988*)] considered to be naturally infected, presumably by rotavirus; for each animal one gram of intestinal contents was collected and suspended in 5 ml of phosphate buffered saline. The sample was filtrated through 0.45 um memberan and stored at -70°C till used.

II- Antisera:

a) Polyclonal antibody to Rotavirus: Reference polyclonal antibodies against BRV were kindly supplied by Department of Virology, Faculty of Veterinary Medicine, Cairo University. The polyclonal antibody was prepared at Dr. El-Azhary laboratory, Department of Virology, Faculty of Veterinary Medicine, Montreal University,

Canada; the lyophilized polyclonal antibodies were re-suspended in distilled water and used in a concentration of 1:100.

b) Monoclonal antibody (Mabs) to Rotavirus: VP6 s

c) pecific Mabs RQ34 (an IgG2a) and RQ 64 (an IgG2b) were mixed in a ratio (1:1) and used in a concentration of 1:20 These Mabs were produced and characterized in Dr. El-Azhary laboratory, Department of Virology, Faculty of Veterinary Medicine, Montreal University, Canada. The Mabs were kindly supplied by, Department of Virology, Faculty of Veterinary Medicine, Cairo University.

III- Buffers:

a) Phosphate buffered saline (PBS) PH 2.7-2.4:

- Adjust the pH to 7.2-7.4 using 1N HCL and complete the volume to 1 liter, sterilized by autoclaving.

b) Blocking buffer: 5% non-fat dry milk (NFDM) in PBS was used.

c) Washing buffer: 0.05% Tween 20 (SERVA) in PBS (50ul tween 20 in 100 ml (PBS) was used.

d) Diluting buffer: 0.5% non-fat dry milk (NFDM) in PBS was used.

e) Substrate buffer:

1. Phosphate citrate buffer pH 5:

- **Buffer stock A:** 10.5 grams of citric acid (Sigma) were dissolved in 500ml-distilled water (0.1 M).
- **Buffer stock B:** 17.8 grams of disodium hydrogen phosphate (2H2O) (Sigma) were dissolved in 500ml-distilled ware (0.2 M).
- **Phosphate-citrate working solution:** 49 ml of buffer A were mixed with 51 of buffer B and the pH is then adjusted to 5 and stored at 4°C until used.

2. OPD- Substrate working solution (freshly prepared): 17 mg of orthophenylene dimaine (OPD) (KOCK-LIGHT LABORATORIES) were dissolved in 50ml Phosphate citrate buffer, to which 25ul H_2O_2 30% (ASH) were added.

- The solution is light sensitive, so must be kept in dark bottles.

f) Stopping buffer: 5% sodium dodecyle sulphate(SDS (SYNBIOTICS) in distilled water was used.

IV- Horse radish peroxidase conjugate goat antimous IgG (whole molecule) (Sigma): A working solution was prepared at a concentration of 1/5000 in dilution buffer.

V- Identification of BRV using sandwich ELISA:

The indirect Mab based antigen capture ELISA was performed according to ***Hussein et al., (1995):***

1. ELISA plate was coated with 100 ul containing 3.4 ug of Rotavirus specific polyclonal antibodies in distilled water 1: 20. The plate was then incubated for 1 hour at 37°C.
2. The plate was blocked using 200 ul/well blocking buffer and incubated over night at 4°C.
3. The blocking buffer was decanted by inverting and flipping the plate several times.
4. 100 ul of suspended intestinal content were added in the blocked plate (100 ul of P.B.S were used as negative control well). The plate was then incubated in a humid chamber at 37°C for 1 hour.
5. The plate was inverted and flipped several times then 3X washing using 300 ul/well of the washing buffer were applied.
6. 100 ul of monoclonal antibodies in diluting buffer (1: 20) were added then the plate was incubated at 37°C in a humid chamber for 1 hour.

7. The plate was washed as in step 5.
8. 100 ul of anitmous conjugated peroxidase diluted 1/5000 in dilution buffer were added to each well. The plate was then incubated in a humid chamber at 37°C for 1 hours.
9. The plate was washed as in step 5.
10. 100 ul of OPD substrate working solution were added to each well of the ELISA plate. The plate was incubated in a dark place at room temperature for 30 minutes to allow color development.
11. Further color development was stopped by adding 100 ul stopping solution to each well.
12. Absorbencies were read at 450 nm wavelength filters in an automated ELISA reader.
13. The optical densities of the tested samples as well as control wells were recorded.

RESULTS

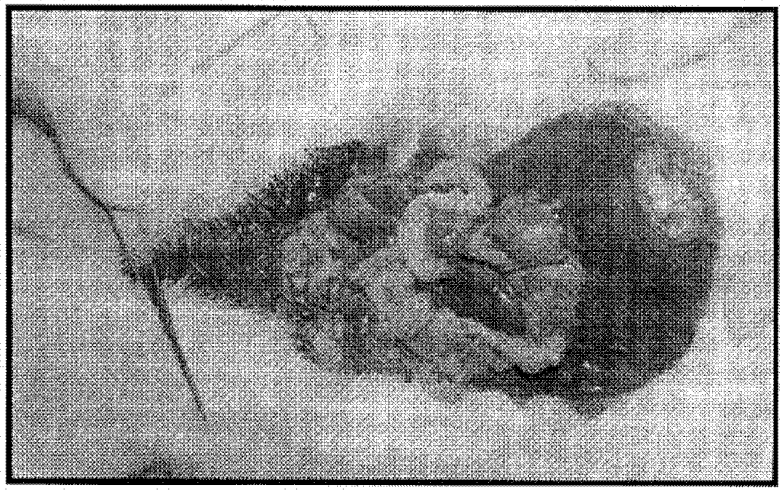

Fig. (1): Rabbit naturally infected with rotavirus showing severe inflammation of small intestine with distended caecum.

Table (1): Detection of group A rabbit rotavirus antigen by capture ELISA.

Age	No. of examined samples	+ ve result	+ve result %
< 1 month	13	5	38%
1-2 months	15	7	47%
> 6 months	12	5	42%
Total	**40**	**17**	**42.5%**

The ELISA results for detection group A rabbit rotavirus antigen are shown in table (1). It is evident from the results that; polyclonal bovine rotavirus antisera can be used effectively as a diagnostic reagent in diagnosis of group A rabbit rotavirus, as 17 out of 40 (42.5%) of examined samples were positive.

The results showed clearly that; rabbits of different ages were susceptible.

DISCUSSION

Our results firmly established group A rotavirus infection in rabbits by showing that: An outbreak of serious digestive problems with high morbidity and mortality in rabbit of different ages were recorded, clinical signs revealed soft or fluid stools and faecal staining of the perineum, affected rabbits rapidly became dehydrated and most of them died within two days of the onset of the diarrhea. The post mortem lesions (photo 1) illustrated severe inflammation of the intestine and distended caecum and colon with fluid and gases, these observations mentioned by many authors ***(Bryden et al 1976, Peeters et al 1982, 1984, 1988, Vetesi et al 1982, Castrucci et al 1985, Schoeb et al 1986 Thouless et al 1986, 1988, Yu et al 1994 Cere et al 2000, and Banyai et al 2005).*** These drastic

diarrhea is probably due to the capacity of rotavirus to increase the fluid and electrolytes secretion of the enterocyte; after its release from virus infected cells *(Estes et al 2001 and Morris and Estes 2001)*.

Positive detection of rabbit rotavirus (table 1) indicated that, bovine polyclonal antibodies can be used effectively as a diagnostic reagent in ELISA for detection of rabbit rotaviruses, these result come in agreement with ***Sarkkinen 1981*** who detected human rotavirus antigen in stool samples by ELISA with antisera against bovine rotavirus. Moreover, antigenic relationships between rotavirus strain of bovine and rabbit origin were recorded *(Sato et al 1982b, and Castrucci et al 1984, 1985)*. Detail of ELISA results showed that: 17 out of 40 samples (42.5%) were positive, the same ratio were recorded by (*Peeters et al 1984 Xu and Cui 1992, Nieddu et al 2000)* Almost all ages were infected where 5 out of 13 (38%) were positive for rabbits less than one month old, the same ratio were recorded by *(Bryden et al 1976, Peeters et al, 1982, 1984, 1988, Vetesi et al 1982, Castrucci et al 1985 and Schoeb et al 1986)*. The most susceptible age among rabbits was 1-2 months old; where 7 out of 15 (47%) were positive, the same results were documented by (*Moriss 1982, Thouless et al 1988, and XU and Cui 1992)*. Rabbits over 6 months old showed 5 out of 12 (42%) were positive, the same results were stated by *(Cere et al 2000)*. Any how, study of antibody titer to rabbit rotavirus over time within individual rabbits litters and in colony of rabbits of different ages showed that, transplacentally derived maternal antibodies declined to low levels by about one month of age, so most rabbits at 1 to 2 months of age had a low antibody titer while rabbits over 2 months old had to same what high antibody level *(Digiocamo and Thouless 1984, 1986)*.

In the view of these results we concluded that, rabbit rotaviruses were detected in Egypt for the first time by capture ELISA using polyclonal bovine rotavirus and VP6 monoclonal antibodies, so further investigations are recommended.

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محاولة لاستكشاف فيروس الروتا فى الأرانب باستخدام الأختبار المناعى الإنزيمى المرتبط والماسك

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أثناء عام ٢٠٠٥ حدث إسهال وبائى بين قطيع مزرعة الأرانب بكلية الزراعة- جامعة بنها، أربعـــون عينة برازية أخذت من محتوى أمعاء الأرانب النافقة وأخضعت للكشف عن وجود فيروس الـــروتا بهـــا وذلك بالأختبار المناعى الإنزيمى المرتبط والماسك والمثبت لفيروس الروتا من العينات باســـتخدام أجسام مناعية متعددة ضد فيروس الروتا الذى يصيب الماشية ثم الكشف عن هذا الفيروس الممســـوك والمثبـــت باستخدام أجسام مناعية وحيدة ضد البروتين السادس (VP6) والمولد للمناعة الخاصة بمجموعة الروتا الرئيسية (A) .

أشـــارت النتائج إلى أن مجموعة العينات الإيجابية لوجود فيروس الروتا فى الأرانب كانت ١٧ عيـــنة إيجابـــية من الأربعين عينة وبنسبة ٤٢,٥% كذلك تم استكشاف فيروس الروتا فى مختلف أعمـــار الأرانـــب ولكـــن بنسب مختلفة، حيث كان وجود الفيروس إيجابيا ١٣/٥ وبنسبة ٣٨% فى الأرانـــب الـــتى عمـــرها أقـــل من شهر فى حين كان ١٥/٧ وبنسبة ٤٧% و ١٢/٥ وبنسبة ٤٢% للأرانـــب ذات الأعمار التى تتراوح من شهر إلى شهرين والأرانب التى عمرها أكبر من ٦ شهور على الترتيب.

هـــذا وتعـــد هـــذه الدراسة الأولى من نوعها فى مصر للكشف عن وجود فيروس الروتا فى الأرانب الأمر الذى يتطلب مزيد من الدراسة.