

Prevalence of bovine rota viral antigen infection and associated biochemical changes in buffalo-calves at Kaliobeia governorate

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Abstract

Neonatal diarrhea is an important worldwide problem that results in major economic losses in many dairy and beef cattle herds that result from treatment costs and calf deaths.

Determination of prevalence of Bovine Rota Virus (BoRV) was carried out on 100 buffalo-calves aged from 1- 6 month belonging to several dairy buffalo farms at Kaliobeia governorate, animals were suffered from anorexia with high body temperature, diarrhea, with gradual weight loss and varying degree of dehydration. Bovine Rota viral antigen was detected in 38% (38/100) in faecal samples by antigen captured ELISA. Neutralizing antibodies (Ab) of (BoRV) was 30% (30/100) by serum neutralization test in serum samples. Molecular identification of (BoRV) in 5 representative positive ELISA samples by using RT-PCR, were positive for amplification of bands at the predicted molecular size (1356 bp of VP6 genes).

Biochemically, the examination of serum samples revealed a highly significant decrease of total protein, albumin, globulin and sodium. On the other hand, a significant increase was recorded in serum potassium, urea nitrogen, creatinine and liver enzymatic activities of AST, ALT and ALP.

The recommendation from this study revealed vaccination of pregnant dairy buffalo to achieve passive immunity in the off springs, as a part of preventive program. Early vaccination of calves born from non-vaccinated buffaloes is also required.

Key words: BoRV, PCR, ELISA, AST, ALT, ALP.

Introduction

Neonatal diarrhea caused by Rota virus is a worldwide problem and is seen as one of the biggest challenges for both the beef and dairy industry (Lorenz *et al.*, 2011).

Rotaviruses belong to the family *Reoviridae*, subfamily *Sedoreoviridae* and genus *Rotavirus* where the genus *Rotavirus* includes five different species *Rotavirus A-E* (Mattijnsens *et al.*, 2011).

Neonatal diarrhea causes a lot of economic damage to the farmers through need for increased management, veterinary treatment, reduced growth and deaths of the calves.

The disease varies from calf to calf, some of them suffering from acute dehydration and

death while others suffer from sub-acute forms with malnutrition that lasts for several days (Gay *et al.*, 2012).

The occurrence of diarrhea during the first six months caused reduction on growth and it causes a prolonging of the time from birth to when the heifers have their first calving, that had a big impact on the economy by influencing the mortality. A higher incidence rate of diarrhea caused a higher mortality amongst the calves. Decreased growth was also seen as a big factor influencing the economy (Razzaque *et al.*, 2009).

The genome of rotavirus consists of 11 segments of dsRNA averaging 18,550 bp with segments at a varying size between 663 and

3302 bp. All segments share a short common pattern of nucleic acid at the 5' and 3' terminals, 10 and 8 nucleotides respectively. Within these there are another common pattern of 30-40 nucleotides that are segment specific.

The 11 segments of dsRNA encode 13 different proteins ranging from VP1-VP7 and NSP1-NSP6 where segment 9 encodes for 2 different types of VP7. Of the proteins six have been found to be structural (**Blanchard, 2012**).

Rotavirus that infects calves causes an often severe and sometimes life-threatening diarrhea. The virus replicates in mature enterocytes on the villi of the small intestines. The replication eventually causes lysis of the cells. The mature enterocytes are then replaced by immature enterocytes from the crypts of the villi. The balance between absorption and secretion of fluid is then changed resulting in an accumulation of fluid in the small intestine. Loss of mature enterocytes also contributes to a systemic insufficiency of bicarbonates, sodium, potassium, chloride and water causing acidosis. The loss of enterocytes reduces the ability to digest milk and the undigested milk is further fermented by microorganisms, which also contributes to cause acidosis. Low lactase, due to the loss of enterocytes, in the intestinal lumen further contributes to fluid accumulation by a failed osmotic regulation (**Mohd Nor et al., 2012**).

The incubation period of the virus is 12-24 hours and the diarrheic calves are most often feverless. The disease may result in severe dehydration and mortality rates differ, but reports have estimated it to be in average 5-20 %. The diarrhea is self-limiting and animals most often recover if not to severe dehydration have occurred. Animals that recover properly usually return to normal bodyweight within 10-28 days post infection (**Dhama et al., 2009**).

There are a variety of diagnostic methods available for the detection of rotavirus including PCR, ELISA. For rotavirus sample material that may be used for detection are feces and biopsies of small intestine. Biopsy being an alternative if the animal has died and necropsy is performed.

A polymerase chain reaction (PCR) protocol

has been developed for identification of bovine group (A) rotavirus infection in feces (**Chinsangaram 1993, Parra and Espinola, 2006**).

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. RT-PCR was efficiently able to detect artificial mixes field infections. RT-PCR provided a very sensitive method for typing BRV field isolates (**Hussein et al., 1996**). PCR are therefore an increasingly common way of diagnosis (**Blanchard, 2012**).

The aim of this study is detection of Bovine Rota Viral antigen in fecal samples in newly borne calves by ELISA test, determination of antibodies against Bovine Rota Virus in newly borne calves by serum neutralization test, molecular detection of Bovine Rota Virus by PCR, and determination of total protein, albumin, globulin's, ALT, sodium, potassium, creatinine and urea in serum samples.

Materials and Methods

Animals: A total of 100 diseased neonatal calves aged 1-6 months which showing diarrhoea from buffalo farms at Kaliobeia governorate.

Rotavirus: Rotavirus group A was kindly obtained from the department of virology, Animal Health Research Institute, Dokki, Giza. The virus had a titer of $10^6 \log_{10}$ TCID₅₀/ ml in MDBK cells. It was used as positive control in ELISA and RT-PCR.

Blood samples: blood samples were collected and kept at -20°C until analysed. Serum samples were used for detection of antibodies against BoRV newly born calves by serum neutralization test.

Fecal samples: A total of 100 fecal samples were collected from diarrheic non vaccinated bovine calves (**table 1**). These samples were collected in sterilized plastic container, transported on ice and stored at -20°C till further processing. The samples were used in trials for detection of rotaviruses using sandwich ELISA and RT-PCR.

Table (1). Total number of collected fecal samples of different ages from different localities in Kalubeya governorate.

Localities	Age of calves	No. of collected fecal samples
Kaloub	Up to 1 week	50
Toukh	1-2 weeks	20
Benha	2-4 weeks	10
Shibeen El Kanater	6 weeks	20
	Total	100

Immune captured enzyme linked immune sorbent assay (ELISA):

All fecal samples were analyzed using commercial ELISA kits (Bio-x Diagnostic, Belgium), for detection of Bovine Rota Viral antigen in fecal samples. Positive results were expressed by calculation of NET OD of sample = OD of the sample – OD of –ve control Divide the OD of the sample on the OD of positive control x100. Positive sample = > 5.97 .

MDBK cell culture (madin darby bovine kidney cell culture VACSERA, Agoza, Egypt) 96 - well tissue culture plate .MDBK cells were added to all the wells of the micro plates (100 µl per well 3 × 10⁵ cells per ml) and observed daily until confluence' Then, 50 µl per well of pooled serum samples sequential 10-fold dilutions were added eight wells per dilution. The plate was freezed and thawed three times and the contained tissue and fluid were centrifuged at 5000 cultured for 3 days at 37°C in a humid atmosphere containing 5% CO₂ incubator. The virus was then titrated and used for detection of antibodies against bovine Rota virus in serum of newly borne calves by serum neutralization test.

Serum neutralization test Was performed by the use of serial 2-fold dilutions of heat inactivated serum, a tissue culture infective dose (TCID₅₀) of BoRV was used to quantitate serum neutralizing antibodies in newly borne calves serum samples according to **Edwards et al., (1986)**.

Detection of Bovine Rota Viral antigen by polymerase chain reaction PCR. QIA amp® Viral RNA mini Kit (QIAGEN, USA):

QiAamp viral RNA mini Kit (Cat no 52904) contain QIAamp mini spin columns, collection tubes (2ml), Buffer (AVL), Buffer AW1, Buffer AW2, Buffer AVE and Carrier RNA. The kits used for extraction of Rota genomic RNA from fecal suspension.

Rotavirus primer sequence (QIAGEN, USA):

A set of primers was designed according to the published database to be used for amplification of full length VP6. The sequence of oligonucleotides are: VP6 F 5'-GGCTTTTAAACGAAGTCTTCAACATGG–3' (forward primer).

VP6 R 5'- GGTCACATCCTCTCACTACGC - 3' (reverse primer)

The primers amplify a product of about 1356 bp in length according to **Brian (2007)**.

Determination of biochemical parameters:

10 blood sample were taken from both clinically healthy and diseased neonatal calves aged 1- 6 month for colorimetric determination of aspartate amino transferase (AST), alanine amino transferase (ALT) (**Reitman and Frankle 1957, Ghanem et al., 2012**), alkaline phosphatase (ALP) according to **Szasz, 1976**, total protein and albumin as described by (**Dumas et al., 1971**). Determination of blood urea nitrogen (BUN) and blood serum creatinine was carried out according to (**Richerich, 1968 and Giorio, 1974**), respectively.

Also serum sodium and serum potassium were estimated (Trinder, 1951 and Miller, 1984).

Statistical analysis Data of biochemical analysis were expressed as means $\pm$ standard error

($M \pm SE$) and was conducted by one way analysis of variance (SPSS 1993).

Results

Table (2). Detection of Bovine Rota Viral antigen fecal samples by using immune captured ELISA.

No. of fecal samples Age		No. of +ve Samples
50	Up to 1 week	23
20	1-2 weeks	9
10	2-4 weeks	3
20	6 weeks	3
100		38

Table (3). Antibody titre against Bovine Rota Virus in tested calves sera by serum neutralization test.

No. of tested serum samples	No. of +ve samples	Antibody titre of BoRV		
		2	4	8
100	30	No.	No.	No.
		20	7	3

Detection of Rotavirus in feces of calves using RT-PCR:

We take 5 representative samples for confirmation the positive Rota Virus samples. Primer set were designed according to the published database to be used for amplification of the full length of VP6 gene of rotavirus.

The specific primer for VP6 was designed to amplify 1356 bp fragment of VP6 gene of rotavirus. The total RNA was extracted from the ultra-centrifuged pellets of fecal samples. Then the one-step RT-PCR was applied to amplify the VP6 gene of rotavirus.

Analysis of the RT-PCR products in ethidium bromide-stained agarose gel reveals positive for amplification of bands at the predicted molecular size (1356 bp of VP6 gene) as represented in **figure (1)**.

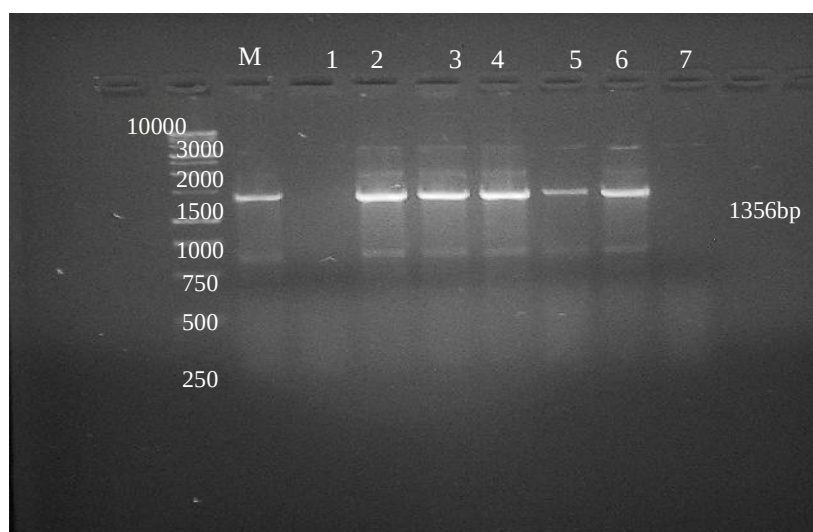


Figure (1): Electrophoresis of the obtained PCR specific bands for VP6 gene of Rotavirus.

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

Lane 1: positive control.

Lane 2: negative control

Lane 3, 4, 5, 6, and 7: Represent the amplified product of correctly VP6 gene size 1356 bp of samples.

Table (4). Means and standard errors of serum biochemical constituents of clinically healthy and diarrheic buffalo- calves.

Parameters	Clinically healthy calves	Diarrheic calves
Total protein (gm/dl)	7.7±0.66	4.8±0.20 **
Albumin (gm/dl)	4.1 ± 0.15	2.8 ± 0.10**
Globulin (gm/dl)	3.6 ± 0.26	2.00 ± 0.11*
Sodium (mEq/l)	136.12 ± 5.2	108.3 ± 8.11 **
Potassium (mEq/l)	6.81 ± 0.11	7.01 ± 0.22 *
AST (IU/L)	60.12 ± 2.2	85.2 ± 3.6 **
ALT (IU/L)	40.00 ± 4.20	70.0 ± 3.8 **
ALP (IU/L)	89.5 ± 4.3	105 ± 6.3 **
Urea nitrogen (mg%)	20.3 ± 2.77	32.0 ± 2.98 **
Creatinine (mg%)	0.06	2.00± 0.15 **

*Significant (p<0.05)

** highly significant (p<0.01)

Discussion

Diarrhea is not a specific disease with a specific cause but it is actually a clinical sign of disease complex with many possible causes, there have been a number of infectious viral or bacterial agents occur singly or in combination are incriminated in causing calf diarrhea. (Mahmoud and Zeidan 2004). Neonatal calf diarrhea remains one of the most common and economically devastating syndrom on cattle industry, especially occurs in the first week of life (Azizzadeh *et al.*, 2012)

Calve diarrhea associated with loss of water and electrolytes that leads to an isotonic or hypotonic dehydration in diarrhoeic calves. Infectious diarrhoea in calves is most commonly associated with rotavirus, coronavirus, enterotoxigenic *Escherichia coli*, *Cryptosporidium parvum*, or some combination of these pathogens (Foster *et al.*, 2009).

Rotaviruses are classified as a member of family Reoviridae. The virus genome is consists of 11 segments of double stranded RNA surrounded by a double capsid (Estes and Cohen, 1989). The virus particles are icosahedral, 55-75 nm in diameter and non-enveloped (Estes *et al.*, 1979 and Holmes, 1983). The viral genome is surrounded by three layer of structural proteins which is VP1, VP2 and VP3 (Ericson *et al.*, 1982). Group A rotavirus is the most important etiological agent of acute diarrhea in young calves and children (Bridger and Woode, 1975).

In this study, as in Table (2) 100 serum samples were for the detection of Rota virus in fecal samples by sandwich ELISA, the results has proved the presence of rotavirus in calves diarrhea with the percentage (38%) 38 from 100 total examined samples ,this results agree with (Perk *et al.*, 2000) who reported that incidence of BoRV affecting neonates in Egypt was (37.3%). Also with (Fassi *et al.*, 1988) who reported that presence of rotavirus diarrheas in calves is (29.7%).

For the detection of Rotavirus antigen correlated with different ages, the study showed that

a higher Rotavirus antigen percent was detected in 1 week old animals, this results agree with (Erdogan *et al.*, 2003), who reported that calves 1-7 days of age seem to be most often affected with Rotavirus. But disagree with Dhama (2009), who reported that the disease is usually seen in young calves of 2–8 weeks of age and the susceptibility decreases as the age progresses. Also the results disagree with (Thrusfield 2005), who reported that Rotavirus was the most common in the third week of life (46-66%).

Out of total 100 serum samples 30 (3%) were positive for antibodies against BoRV by serum neutralization test as shown in Table (3). the results revealed that the most prominent causative viral agent of Neonatal calf diarrhea was rotavirus. This results come in agree with the results of other studies (Haschek 2006).

The molecular characterization of rotavirus is most important in the current research. The PCR has proved its efficacy in detecting rotavirus in the fecal samples. Several other researches have been developed and used PCR in detecting and characterization of rotavirus (Gouvea *et al.*, 1994). The assay was based on the extraction of ds RNA and amplification of rotavirus VP6 genes 1356 bp of the RT - Product using specific primers complementary to full length of VP6 gene which were designed according to database in gene bank (El Sabagh, 2006). The 5 representative positive ELISA samples (3,4,5,6 and 7)give bands which were detected in predicted size for VP6 1356 bp in figure (1).

The molecular tools and techniques are being widely used in animal disease diagnosis including PCR and allied techniques as RT-PCR, real-time PCR, nucleotide sequencing and phylogenetic analysis

Antigen and antibody ELISA are readily available and easy to use, and viral antigen can be detected in serum as well as whole blood and even skin biopsies. Reverse-transcription PCR (RT-PCR) has become a routine diagnostic method and is highly sensitive as well as specific, and has the advantage that it allows pool-

ing of samples, and viral RNA can be detected independently of specific antibodies, (**Hilbe, 2007**).

The biochemical analysis as shown in **table (4)** revealed a highly significant decrease in serum total proteins, albumin and globulins values in diarrheic buffalo – calves when compared with clinically healthy ones. The reduction of total proteins, albumin and globulins may be attributed to the general unthriftiness which may affect worsely the hepatic paranchyma resulting in the failure of the liver for protein synthesis (**Tawfik et al., 2004 and Azizzadeh et al., 2012**). Regarding serum sodium level there was a highly significant decrease in diarrheic calves. This decrease may be due to decreased absorption of this element from the inflamed or irritated intestinal tract or may be as a result of the rapid and continuous excretion of this element along with excessive secretion of intestinal juices in cases of enteropathies (**Nasser et al., 2000 and Ramkumar, 2012**). The finding of hyperkaleamia in diarrheic buffalo- calves was attributed to the dehydration and peripheral circulatory failure that results in an aerobic oxidation, liberation of lactic acid and occurrence of acidosis that lead to the drawing back of potassium from the cell to the plasma causing hyperkalaemia (**Foster and Smith 2009 and Hamdy et al., 2005**). Blood urea nitrogen and creatinine in serum of diarrheic buffalo – calves revealed a highly significant increase in comparison with the clinically healthy ones. Such finding attributed to excessive production of urea and creatinine by increase protein catabolism processes in severe toxic, febrile condition, reduce renal function and the beginning of nephropathological changes (**Tawfik et al., 2004**). A significant elevation in serum AST, ALT and ALP activities in diseased calves. These elevation referred to the degenerative changes and necrotic processes which accompanied the formation of intestinal and hepatic lesions due to viral infection and its toxin (**Radostits et al., 2002**).

Conclusion

The recommendation figured from this study are vaccination of pregnant dairy buffalo to achieve passive immunity in the off springs, as a part of preventive program. Early vaccination of calves born from non-vaccinated buffaloes is also required.

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معدل الإصابة والتغيرات البيوكيميائية المصاحبة لأنتجين فيروس الروتا في عجول الجاموس الحلاب في محافظة القليوبية

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معهد بحوث صحة الحيوان بالدقى

الملخص العربى

يعتبر الإسهال من أهم المشكلات على مستوى العالم والذي له آثار إقتصادية كبيرة سواء في عجول التسمين أو الحلاب وذلك بسبب تكلفة العلاج المرتفعة وزيادة معدل الوفيات.

تم دراسة معدل الإصابة بأنتجين فيروس الروتا على ١٠٠ من عجول الجاموس الحلاب عمر ١-٦ أشهر في محافظة القليوبية والتي تعاني من أعراض الإسهال ، ارتفاع درجات الحرارة ، فقدان الشهية ، مع انخفاض في وزن الجسم. وجد أن نسبة أنتجين فيروس الروتا كانت ٣٨% (١٠٠/٣٨) في عينات البراز باستخدام اختبار الإليزا. أما الأجسام المناعية ضد فيروس الروتا فكانت ٣٠% (١٠٠/٣٠) باستخدام اختبار السيرم المتعادل.

التعرف الجزيئى على فيروس الروتا أعطى نتائج إيجابية باستخدام اختبار البلمره المتسلسل. الإختبارات البيوكيميائية لسيرم العجول تشير إلى انخفاض ملحوظ في البروتين الكلى ، الألبومين ، الجلوبيولين ، الصوديوم وكذلك انخفاض ملحوظ في نسبة البوتاسيوم ، اليوريا نيتروجين ، الكرياتينين وانزيمات الكبد ALT, AST.

وتوصى هذه الدراسة بتحسين الأمهات العشار ضد فيروس الروتا وذلك لزيادة الأجسام المناعية السالبة ضد الفيروس بالإضافة إلى تحصين العجول حديثة الولادة ضد فيروس الروتا.