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Detection of Bovine Rotavirus from Diarrheic Bovine calves in Gujarat region, India

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ABSTRACT

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The aim of the current study was to identify the prevalence of bovine rotavirus infection in bovine calves aged from 1 day to 2 months old in Gujarat region. Studied samples were divided into 4 age groups, age group I (1-10 days) age group II 11-20 days), age group III (21-30 days) and age group IV (31-60 days). All the samples were screened by latex agglutination test (LAT), ELISA and RT-PCR technique. Among the 117 fecal sample screened, 23.93%, 15.38% and 22.22% found positive for *Rotavirus* by LAT, ELISA and RT-PCR respectively. Age wise, rotavirus infection was highly prevalent in age group-I (1-10 days) i.e. 38.46%. Sex wise, there was no clear-cut establishment of correlation for *Rotavirus*. The present study showed that rotaviruses are involved in the neonatal calves' diarrhea in Gujarat area

Introduction

Bovine rotavirus is a primary and most common pathogen responsible for the NCD. Rotavirus diarrhea in calves, piglets, foals and lambs is often referred as "white scour" or "milk scour". Bovine rotavirus usually causes diarrhea in calves at 1 to 2 weeks of age

(Dhama *et al.*, 2009). It has been reported that diarrhea in calves from 5-10 days of age is commonly due to rotavirus and infected calves excrete rotavirus in their feces upto the age of 6 to 8 weeks. Rotavirus was first isolated from calves with diarrhea and since then has been isolated from cattle, sheep, horses, dogs, cats, chickens and turkeys (Mebus *et al.*, 1969).

The disease is usually seen in young animals and the susceptibility to disease decreases as the age progress, most likely due to changes in animal physiology and/or acquired immunity due to previous exposures (Estes and Kapikian, 2007).

The virus belongs to the genus Rotavirus of subfamily Sedoreovirinae in the family Reoviridae. Rotavirus is dsRNA, non-enveloped, 65-75 nm in diameter, icosahedron symmetry having 11 double-stranded RNA segments (16~21 kb) and is very stable over a wide pH range with heat stability (MacLachlan *et al.*, 2016). The fully infectious rotavirus particle consists of three protein layers (i.e. core, inner capsid and outer capsid) and is also termed triple-layered particle (TLP). By electron microscopy, TLPs resemble wheels (latin. rota), and this appearance has led to the name of Rotavirus for the genus (Flewett *et al.*, 1974).

The dsRNA segments of Rotavirus code for six structural proteins (SPS) viz. VP7VP4-VP6-VP1-VP2-VP3 and six non-structural proteins (NSPs) viz. NSP1–NSP2–NSP3–NSP4–NSP5/6. The VP6 protein comprises the middle layer of the capsid and is the protein, encoded by gene segment 6, to which common immunodiagnostics are directed. Rotavirus has 11 segments of the dsRNA as the genome and that could be detected by RNA-Polyacrylamide Gel Electrophoresis (RNA-PAGE). The eleven segments of the dsRNA are separated from each other based on their electrophoretic mobility and it is very sensitive method for determination of the presence of Rotavirus in fecal samples. The detection of 11 segments of dsRNA of RVA in RNA-PAGE exhibited the 4-2-3-2 pattern of migration. RVA are excreted in large numbers in the feces and can be detected by the various methods viz. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Enzyme Linked Immunosorbent Assay

(ELISA), PAGE, Latex agglutination test (LAT), Electron microscopy, Passive hemagglutination assay (PHA), Immunoblotting and Immunofluorescence test.

Materials and Methods

The present study was undertaken to ascertain detection of bovine rotavirus from fecal samples of diarrheal calves of cattle and buffalo by Enzyme Linked Immunosorbent Assay (ELISA) and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) techniques.

Collection of specimens

All the 117 fecal samples were collected from calves of cattle and buffalo of 1 day to 2 months of age. Samples were collected from farms of various region viz. Anand (n=31), Surat (n= 68) and Junagadh(n=18). The fecal samples were collected as per the method of Yilmaz (2016). The samples were transferred to sterile disposable plastic specimen vial. The specimen vial was labelled properly according to the species, age, sex and name of owner etc. The sample was stored in deep freeze (-40⁰C) for detection of viral antigen until use.

Screening by Latex Agglutination Test (LAT)

All the fecal samples were screened for rotavirus antigen by LAT(Figure 1). LAT was performed to detect rotavirus antigen in fecal supernatants as per instruction by the kit manufacturer (HiRotavirus Latex Test Kit, LK08,HimediaPvt. Ltd.).

Sample Preparation for ELISA

The fecal sample was diluted in dilution buffer (provided by kit manufacturer) volume per volume (v/v). This was a qualitative dilution only, which must allow the pipetting the fecal

suspensions. The gruds were discarded by natural sedimentation for about 10 minutes without centrifugation.

Screening by ELISA

All the samples again screened rotavirus antigen by ELISA. ELISA was performed to detect bovine rotavirus in the fecal samples by the kit manufacture (Bio X Diagnostic Multiscreen Ag ELISA kit (BIO K 315/1, Belgique). This 96 well plate ELISA kit also used for diagnosis of Coronavirus and *E. coli* F5 attachment factor from fecal sample of calves along with Rotavirus(Figure 2). The ELISA plate is sensitised with a mixture of antibodies that are specific for the above 3 pathogens. These antibodies capture the corresponding pathogens in the faecal samples. The net optical density of each sample was calculated by following formula:

$$Val(ue) = \frac{\text{Delta OD Sample} \times 100}{\text{Delta OD Positive}}$$

The optical density of ≥ 6.0 at 450nm wavelength were considered as positive for Rotavirus by antigen detection sandwich ELISA

Extraction of Viral RNA

Viral RNA extraction was done from fecal suspensions (10% v/v) by using a QIAamp Viral RNA Mini Kit, QIAGEN (Cat. No. 52904) following the manufactures protocol.

Quality and Quantity Check of RNA

For quantification of viral RNA, absorbance was read in Nanodrop Spectrophotometer at 260nm.

An optical density (OD) 1 corresponds to 40µg/ml for dsRNA. A ratio of 260:280 provides an estimate of purity of the nucleic acid. Pure preparations of RNA have 260:280 ratio of 2.0. The viral RNA from fecal samples

was quantified and the ratio of purity was evaluated following the method of Sambrook and Russel (2012).

Molecular Detection of Rotavirus by RT-PCR

The RT-PCR assay for specific VP6 gene was standardized using the viral genomic RNA extracted by QIAamp RNA Mini Kit, QIAGEN (Cat. No. 52904)(Table 1).

The genomic RNA was quantitated by Nanodrop and used as a template for cDNA synthesis by using components of PrimeScript™ RT-PCR kit, TaKaRa (Cat. No. RR014A). The reaction conditions for RT-PCR using PrimeScript™ RT-PCR kit, TaKaRa were standardized to get the desired specific product. The reaction mixture of polymerase chain reaction (PCR) as per Table 2 and thermo cycling conditions used for PCR to amplify full-length VP6 gene as per Table.3.

Visualization of PCR products by agarose gel electrophoresis

To confirm the targeted PCR amplification, 5µl of the PCR products from each tube was mixed with 1µl of 6X gel loading buffer and electrophoresed along with DNA molecular weight marker (HiMediaPvt. Ltd) on 2.0 % agarose (Low EEO, SeaKem) gels containing 0.5µg/ml ethidium bromide (Sigma-Aldrich, USA) at 80V in 0.5X Tris Borate EDTA (TBE) buffer. The amplified product was visualized as single compact band of expected size under UV light and documented by gel documentation system (Genetix Biotech Pvt. Ltd, Delhi).

Results and Discussion

A total 117 fecal samples were collected from diarrheic calves from farms of various region

viz. Anand, Surat and Junagadh, and screened for the presence of Rotavirus using latex agglutination test (LAT), ELISA and RT-PCR.

Out of 117 diarrheic samples screened for Rotavirus, 28 (23.93%), 18 (15.38%) and 26 (22.22%) samples were found positive for Rotavirus by LAT, ELISA and RT-PCR method, respectively as mentioned in Table 4. The results of RT-PCR were taken in to consideration to show the prevalence of Rotavirus. The RT-PCR technique is widely used for the detection of the RNA viruses. It helps in the detection of viral nucleic acid during initial stages of infection without waiting for higher virus titre and development of immune response in the affected host species. The amplification of fragment of VP6 gene of Bovine rotavirus in RT-PCR indicated that calves were suffering from rotaviral infection (Figure 3). In the present study, overall prevalence of Rotavirus recorded was almost similar to Suresh *et al.*, (2012), Abdulazeez *et al.*, (2017), and Pardo-Mora (2018) they observed 24.10%, 25.60% and 19.70%, respectively. In contrast to present study, Mondal *et al.*, (2013), Yilmaz (2016) and Gill *et al.*, (2017) observed low prevalence of Rotavirus i.e. 11.23%, 7.57% and 10%, respectively.

The difference in Rotavirus prevalence in diarrheic calves which established in the present study and by above mentioned investigators is in agreement due to scientific fact that rotaviruses are worldwide distributed and able to infect humans and domesticated animals. Various factors like hygienic measures, proper access to maternal colostrum, nutrition season and climatic factors such as rainfall, temperature, relative humidity etc. might be responsible for rotaviral diarrhea as reported by Abdulazeez *et al.*, (2017). Negative results may occur because some cases of diarrhea might not be

associated with infectious agents, and probably due to nutritional or other management factors, or because other non-investigated pathogens might be involved as reported by Ammar *et al.*, (2014).

Out of 117 fecal samples, 28 (23.93%) samples were found positive for Rotavirus by latex agglutination test as indicated in the form of clump/agglutination of latex particles in the test sample and positive control, and absence of agglutination in negative control (Figure 4). AlYousif *et al.*, (2001) and Singh (2011) reported higher 52% and 32.07% prevalence of rotavirus infection whereas Sukura *et al.*, (1990) reported lower (12%) prevalence of Rotavirus by LAT.

In the present investigation, 18 (15.38%) samples were found positive by ELISA (Figure 5). The result is in agreement with Al-Robaiee and Al-Farwachi (2013), Ammar *et al.*, (2014) and Singh *et al.*, (2015), as they reported 15.50%, 14.63 and 12% prevalence of Rotavirus.

In contrast to present study, Wei *et al.*, (2013), Khamees *et al.*, (2015) and Abdulazeez *et al.*, (2017) reported 42.05%, 43.50% and 36% prevalence of Rotavirus.

Region wise Prevalence of Rotavirus

Rotavirus was highly prevalent in Junagadh (38.88%) as compared to Anand (22.58%) and Surat (17.64%) region. This high and low prevalence in various regions might be due to the difference in climatic conditions, sample size, management practices, personal hygiene, the age at which sample was collected and farm size (Table 5).

Age and Sex wise Prevalence of Rotavirus

In the present study, the association between the different age groups and sex with the

occurrence of diarrhea due to Rotavirus was studied (Table 6).

The rotavirus infection (38.46%) highly

prevalent in 1-10 days of age group of calves followed by 30.76%, 19.23% and 11.53% for age group of 11-20 days, 21-30 days and 31-60 days.

Table.1 Oligonucleotide primer (VP6) sequence used in RT-PCR of *BRV*

Primers	Sequences (5'-3')	Expected product size (bp)	Reference
VP6 F	GACGGVGCRACTACATGGT (19)	379	Yilmaz, 2016
VP6 R	GTCCAATTCATNCCTGGTGG (20)		

Table.2 Reaction of polymerase chain reaction (PCR)

Reagents	Volume (µl)
10 X PCR Buffer II	5
dNTP Mixture (10 Mm each)	2
VP6 F (20 µM)	10
VP6 R (20 µM)	10
TaKaRa Ex Taq HS (5 U/ µl)	0.5
cDNA	5
Sterile water	17.5
Total	50

Table.3 Thermo cycling conditions used for PCR to amplify full-length VP6 gene

Sr. No.	Step	Temperature	Time	40 Cycles
1	Denaturation	94°C	30 seconds	
2	Annealing	55°C	30 seconds	
3	Extension	72°C	90 seconds	

Table.4 Detail information of *Rotavirus* positive samples by various methods

Sr. No.	Sample code	Bovine Calf		LAT	ELISA	RT-PCR
		Age (Days)	Sex			
1	A-8	15	Female	++++	++++	Positive
2	A-9	3	Female	-	++	Negative
3	A-10	27	Female	++	-	Positive
4	A-11	45	Male	++++	++++	Positive
5	A-17	20	Male	+	-	Positive
6	A-18	7	Female	++	-	Positive
7	A-30	30	Male	+	++	Positive
8	A-31	7	Male	++	++	Positive

9	S-34	45	Female	+++	-	Positive
10	S-35	30	Female	+	-	Positive
11	S-43	8	Male	+++	++	Positive
12	S-49	11	Male	++++	++	Positive
13	S-50	7	Male	++	-	Negative
14	S-55	6	Male	++++	+++	Positive
15	S-56	11	Male	++++	+++	Positive
16	S-75	2	Male	+++	+++	Positive
17	S-76	25	Male	++	-	Negative
18	S-84	1	Female	+++	+++	Positive
19	S-86	1	Female	+++	+++	Positive
20	S-88	2	Female	+++	+++	Positive
21	S-90	14	Male	++	-	Positive
22	S-92	35	Female	+++	+++	Positive
23	J-101	7	Male	+	-	Positive
24	J-102	15	Female	+	-	Positive
25	J-103	5	Male	+++	+++	Positive
26	J-104	25	Female	++	-	Positive
27	J-105	14	Male	+++	+++	Positive
28	J-108	20	Male	+++	+++	Positive
29	J-115	30	Female	+++	+++	Positive
Total				28/117 (23.93%)	18/117 (15.38%)	26/117 (22.22%)

Table.5 Rotavirus prevalence in various regions

Regions	Total no. of sample	No. of positive samples	Prevalence %
Junagadh	18	07	38.88
Anand	31	07	22.58
Surat	68	12	17.64
Total	117	26	22.22

Table.6 Rotavirus prevalence by RT-PCR in various age groups and sex

Age groups(Days)	No. of positive samples	Sex wise Prevalence of Rotavirus	
		No. of male calves Infected	No. of female calves infected
I (1-10)	10 (38.40%)	6 (60%)	4 (40%)
II (11-20)	8 (30.76%)	6 (75%)	2 (25%)
III (21-30)	5 (19.23%)	1 (20%)	4 (80%)
IV (31-60)	3 (11.53%)	1 (33.33%)	2 (66.66)
Total	26	14 (53.85%)	12 (46.15%)

Fig.1 Latex agglutination test kit

Fig.2 Components of Sandwich ELISA kit

Fig.3 Agarose gel showing amplified product for VP6- gene of *Rotavirus* isolates (approximately 379bp)
L: DNA Molecular weight ladder – 1000bp,
1-4: Isolates (Samples)
P: Positive control
N: Negative control

Fig. 4 Screening for *Rotavirus* using Latex Agglutination Test kit (1-4: Samples, 5: Positive control, 6: Negative control)

Fig.5 Screening for *Rotavirus* using Sandwich ELISA (C1-H9: Samples, A1: Positive control, B1: Negative control)

The prevalence of Rotavirus is higher in 1-10 days age group, this finding is supported by Ammar *et al.*, (2014) and Khamees (2015) they also reported (60.80% and 19.23%) and higher prevalence of Rotavirus infection in 1-10 days age group. A higher infection rate in the first week of life suggests widespread

rotavirus in this group of younger calves reported by Selim *et al.*, (1991) and Majeed *et al.*, (2011). As numerous factors interplay in precipitating clinical diarrhoea, it is difficult to make absolute conclusions based on the limited information. However, rotavirus infection has been shown to be more

important than other agents in diarrhoea in young calves of around 1 week of age (4-14 days) (Selim *et al.*, 1991). In the present investigation, 22 (18.80%) diarrheic calves were infected with both *E. coli* and Rotavirus.

The resultant of effectiveness of sex on infection rate in present study which slightly higher in male (53.84%) than female (46.15%) but not much difference similar finding to those of Dash *et al.*, (2011) and Hassan *et al.*, (2014), they recorded infection rate (20.37%) in male and (12.76%) in female and 37.5% in males and 40% in females, respectively. In contrast to the present study, Ammar *et al.*, (2014) recorded higher frequency of infection of rotavirus in female (60.86%) as compared to the male (39.13%). The anatomical, functional and hormonal similarities of body systems of male and female calves in early ages lead to non-specific resistance against infection, but degree of contamination with virus, dose of viruses, exposing to stress factors, consumption of colostrum or not and other many environmental and management factors, all effect on infection rate and severity in both sexes of calves in same or different periodic age as reported by Hassan *et al.*, (2014). Calves within their first month of life are highly susceptible to viral diarrhea probably due to suckling milk, which neutralizes the acidic pH of their digestive tract and in turn allows several pathogens to survive (Dhama *et al.*, 2009; Cho and Yoon, 2014). Several improvements in vaccination, medication and management have been implemented to reduce the incidence of NCD. However, NCD is still persistent because it is complex and multifactorial and can be triggered by several different infectious and non-infectious causes (Cho and Yoon, 2014). Direct losses are due to dehydration, reduced growth rate, and high morbidity and mortality.

Indirect losses are due to the imposition of trade restrictions and increased costs of

management and veterinary care as well as animal suffering.

Comparison of LAT, ELISA and RT-PCR for Rotavirus detection

In the present study, the difference in the results of nine samples was recorded for LAT and ELISA i.e. LAT showed nine samples positive for Rotavirus while the same samples were negative by ELISA. The LAT results (23.93%) were almost similar to that of the RT-PCR (22.22%). These results indicated that LAT was more sensitive than ELISA and thus, former technique should be used for screening of Rotavirus detection. However, two fecal samples were weak positive by LAT and negative by ELISA and RT-PCR. This variation in result of LAT and RT-PCR might be due to cross agglutination with another organism present in the fecal sample.

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