

## ORIGINAL ARTICLE

# Bovine Rotavirus and *Cryptosporidium* species Co-infection among Calves in Beni Suf Governorate, Egypt

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### Abstract

Rotavirus A (RVA) and *Cryptosporidium* oocysts infections remain the most recognized worldwide pathogen causing acute diarrhea in calves less than one month of age. The current study was carried out to estimate the prevalence of Bovine Rota virus (BRV) and *Cryptosporidium* spp. infections in calves in Beni Suf governorate, Egypt. Fecal samples of 123 diarrheic calves were subjected for Rapid Rotavirus immunochromatographic assay (ICA) to identify the presence of BRV antigen and modified Ziehl-Neelsen staining technique of *Cryptosporidium* spp. infections. The overall prevalence of Rota virus infection using the ICA was 13(10.57%). *Cryptosporidium* oocysts were detected microscopically in 36(29.27%) calf's fecal samples. The enzootic situations of BRV and *Cryptosporidium* spp. infections have been proved in this study. Rotavirus double-stranded RNA was extracted from the 13 fecal samples and subjected for one-step RT-PCR targeting NSP5 gene, and two-step RT-PCR targeting partial length VP4 gene. The presence of the viral genome of BRV was confirmed by amplification of NSP5 gene and VP4 gene with 155bp and 856bp expected product size, respectively. PCR revealed positivity of 5(4.07%) fecal samples. PCR revealed positivity of 28(22.76%) *Cryptosporidium* spp. infected fecal samples. Mixed infection of BRV and *Cryptosporidium* spp. in this study was 7(5.69 %) cases.

### Keywords

Bovine Rota Virus, *Cryptosporidium* spp., Immunochromatographic Assay

## 1. Introduction

Bovine Rota Virus (BRV) is a major cause of calf diarrhea worldwide (Estes and Greenberg, 2013) with prevalence rate ranges from 7% to 94% worldwide as reported by Falcone et al., (1999). It is the most recognized pathogen causing acute diarrhea in calves less than one month of age worldwide (Alfieri et al., 2006).

BRV belongs to the genus Rotavirus within the family Reoviridae. Rotaviruses are icosahedral and non-enveloped, with 32 capsomers and 11 segments of ds RNA (16–21kbp), well protected by an inner and outer capsid layer (Desselberger et al., 2005; Greenberg and Estes, 2009). The genome encodes six structural proteins and six non-structural proteins (Matthijssens et al., 2012). The segmented nature of this virus favors genetic reassortment and generates new strains that may differ in pathogenicity, virulence and interspecies transmission (Estes and Kapikian, 2007).

*Cryptosporidium parvum* (*C. parvum*) is an enteric protozoan pathogen frequently recovered from diarrheic calves with an important zoonotic significance (Chalmers et al., 2010). Forty-five *Cryptosporidium* spp. have been identified molecularly and more than 100 genotypes exist (Zahedi et al., 2021). *Cryptosporidium* spp. infection is generally observed in calves of one to two weeks, and older calves may be exposed but not develop diarrhea as reported by Holland (1990) and Santín et al., (2004). Diarrhea may be intermittent that peaks at three to five days (Fayer et al., 1998). Profuse yellow to brown diarrhea containing mucus and blood may develop (Kumaresan et al., 2012).

Diarrhea induced by BRV is usually mild to severe. The clinical signs involve depression, anorexia, mild fever and diarrhea. Diarrheic feces vary from liquid to pasty, often yellowish mucoid and occasionally bloody (Youssef and Zaitoun, 2022). The infectious cycle of BRV infections clarifies that the virus targets the enterocytes causing specific lesions in the intestines and mesenteric lymph nodes resulting in morbidity and mortality of calves below three weeks of age (Barua, 2019).

Continuous monitoring of emerging and re-emerging BRV strains is essential for a better understanding of the viral ecology within a region that allows improving the implemented vaccination programs by updating vaccine strains.

The principal aim of this study was the role of BRV and *Cryptosporidium* spp. infections through estimation of their prevalence and clinical significance in Beni Suf, governorate, Egypt and the possibility of their co-infection.

## 2. Material and Methods

The study protocol was approved by the Animal Research Ethics Committee of the Faculty of Veterinary Medicine, Beni-Suef University with a letter for the approval and credibility of the work No. 022-403, in accordance with the international guidelines for animal Research.

## 2.1. Study Area and Animals

A total of 123 diarrheic cattle calves at different localities in Beni Suef governorates were clinically and epidemiologically examined for BRV and *Cryptosporidium* infections during October (2021) to March (2023). Diarrheic calves were especially examined for suckling reflex, septicemia, degree of dehydration and body temperature. Consistency of feces, odor, color, and presence or absence of mucus and blood in feces were checked according to the methods described by [Constable et al., \(2017\)](#).

## 2.2. Fecal Samples

A total of 123 rectal fecal samples were collected from diarrheic calves. These samples were subjected to an immunochromatographic assay (ICA) for rapid detection of BRV infection, as well as microscopic detection of *Cryptosporidium spp.* oocysts using modified Zeihl Neelsen technique.

## 2.3. Rapid Detection Kit used for Detection of BRV Infection

Fecal samples were screened for the presence of BRV antigen by the Rapid Rotavirus Immunochromatographic assay (ICA), (Haicang, Xiamen, China) following manufacturer's instructions.

## 2.4. Microscopy Screening

Detection of *Cryptosporidium spp.* oocysts was performed using modified Ziehl- Neelsen staining technique according to ([Casemore et al., 1985](#))

## 2.5. Molecular Detection and Characterization of BRV Infection in Calves using PCR

Fecal samples (n=13) were diluted with 10% sterile Phosphate Buffered Saline (PBS, pH 7.2) and used for the extraction of viral RNA. Total RNA was extracted using GeneJET Viral RNA Purification Kit (Thermo Scientific, Lithuania catalog no. k0821). One-step RT-PCR targeting NSP5 gene was performed using Verso 1-step RT-PCR kit (ThermoFisher lithuania) to confirm BRV. The primers used for amplifying a NSP5 gene BRV A were mentioned in [Table \(1\)](#), the reaction were prepared according to the manufacturer's directions and the cycling conditions were reverse transcription at 50°C for 30min., primary denaturation at 95°C for 5min. 35 cycle of secondary denaturation at 94°C for 30sec. annealing at 60°C for 30sec. and extension at 72°C for 30sec) and final extension at 72°C for 7min.

## 2.6. DNA Extraction of *Cryptosporidium Spp.*

Fecal samples positive for modified Ziehl- Neelsen staining technique (n=36) were subjected to ten freezing and thawing cycles; by freezing

in liquid nitrogen for five minutes followed by thawing at boiling water bath for five minutes to disrupt the oocyst walls and release the target DNA as described by [Chen et al., \(2002\)](#). DNA extraction was carried out according to the instruction manual of gSync TM DNA extraction kit, Geneaid ([New Taipei City, Taiwan](#)).

## 2.7. DNA Amplification of *Cryptosporidium Spp.*

Amplification of two-step nested PCR for gp60 gene ([Peng et al., 2001](#)), the sequences of the used primer are mentioned in [Table \(1\)](#). This was carried out in a final volume of 25µl containing: 12.5µl 2x Master Mix (applied biotechnology, Egypt), 1µl Forward Primer, 1µl Reverse Primer ([Table, 1](#)), 4µl DNA Template, 6.5µl Nuclease Free Water. The thermo-cycling parameters consisted of an initial denaturation cycle at 95°C for 5min followed by 35 cycles of the following program; 94°C for 45sec. Annealing temperature was 50°C for 45sec for each primer and 72°C for 45sec then followed by final extension step at 72°C for 7min. Amplifications were carried out in 0.2mL tubes using Labnet® Multigene Gradient thermal cycler, Catalog TC9600-G- 230V ([Labnet International, Inc. Edison, NJ, USA](#)). Secondary PCR products were separated by electrophoresis in 1.5% agarose gel for 30 min and visualized by UV transilluminator.

## 3. Results and Discussion

Distribution and prevalence of BRV infected calves among 123 diarrheic calves as estimated by the BRV ICA test revealed 13 (10.57%), [Table \(2\)](#). BRV infection in Assiut Governorate was estimated as 14.60% ([Youssef and Zaitoun, 2022](#)) using ICA and latex agglutination and 29.8% ([Abou El-Ella et al., 2013](#)) using ICA. Variations in prevalence of BRV infection in calves in different studies may be due to geographical variation, difference in hygienic measures, environmental conditions, management system involving the pregnant dams and newborn calves and difference in age groups subjected for investigation and the tests employed for detection of infection on the levels of both sensitivity and specificity.

The obtained results revealed that BRV infection was diagnosed in both males 4(7.14%) and females 2(2.99%), [Table \(3\)](#). Reviewing the literature about the effect of sex on occurrence of BRV infection in different studies, a non-significant difference in rate of BRV infection between male and female calves statistically indicating a non-sex linked disease ([Yahaya and Veronique, 2018; Youssef and Zaitoun, 2022](#)). It was obviously clear that the majority of cases of BRV infection were diagnosed in the first two weeks 6(8.7%), [Table \(3\)](#). Similar results were reported by [Youssef and Zaitoun \(2022\)](#) in Egypt using ICA. The susceptibility of bovine calves decreases with age which may be due to loss of receptors on enterocytes ([Dash et al., 2011; Kumari et al., 2019](#)).

**Table 1.** Primer sets of PCR used for molecular detection of BRV and *Cryptosporidium spp.*

Pathogen	Target gene	Primer	Nucleotide sequence (5'-3')	Amplicon size(bp)	References
BRV	NSP 5	Nsp5-f	GATATTGGACCATCTGATTCTGCT TCAAA	155	<a href="#">Schroeder et al. (2012)</a>
		Nsp5-r	GAAATCCACTTGATCGCACCC AA		
<i>Cryptosporidium Spp.</i>	Glycoprotein 60KDa (Gp60)	Gp60-F	ATAGTCTCCGCTGTATTC	950-1000	<a href="#">Peng et al. (2001)</a>
		Gp60-R	GCAGAGGAACCAGCATC		
		Nest Gp60-F	TCCGCTGTATTCTCAGCC	450	
		Nest Gp60-R	GAGATATATCTTGGTGCG		

**Table 2.** Prevalence of BRV infection, *Cryptosporidium spp.* infection and mixed infection using ICA and microscopic detection.

Examined calves	BRV (ICA)	<i>Cryptosporidium spp.</i> (microscopic detection)	Mixed infection
123	13 (10.57%)	36 (29.27%)	7 (5.69 %)

**Table 3.** Prevalence of BRV infection and Cryptosporidiosis in diarrheic cattle calves in relation to different epidemiological variables.

Parameter	BRV single infection (n=6)	<i>Cryptosporidium</i> spp. single infection (n=29)	Co-infection (n=7)	
Sex	Male (n=56)	4 (7.14%)	16(28.57%)	5(8.93%)
	Female (n=67)	2 (2.99%)	13(19.03%)	2(2.99%)
Age	Birth to 15 d.(n=69)	6 (8.7%)	24(34.78%)	7(10.14%)
	16 to 30 d. (n=41)	0	4(9.76%)	0
	31 to 45 d. (n=13)	0	1(7.69%)	0
Dam parity	1 <sup>st</sup> (n=34)	2(5.88%)	9(26.47%)	3(8.82%)
	2 <sup>nd</sup> (n=28)	2(7.14%)	6(21.43%)	2(7.14%)
	3 <sup>rd</sup> (n=19)	0	10(52.63%)	1(5.26%)
	4 <sup>th</sup> (n=16)	1(6.25%)	2(12.5%)	1(6.25%)
	5 <sup>th</sup> & more (n=26)	1(3.85%)	2(7.69%)	0
Total	123	6 (4.88%)	29 (23.58 %)	7 (5.69%)

Clinical abnormalities observed in BRV ICA positive diarrheic calves in this study, **Table (4)** revealed that the body temperature did not exceed 40°C which is identical to most cases of uncomplicated viral infections. Usually BRV infection is a non-febrile disease, unless complicated by secondary infections (**Chauhan and Singh, 1996**). Diseased calves showed negative suckling reflex which is considered an indication of high susceptibility to enteric infection due to insufficient protective colostrum. Therefore, the crucial plan established for prevention and control of bovine BRV infections include enhancing the immunity of newborn calves through dam immunization and reducing the exposure of calves to infection through strict hygienic measures and adoption of good management system (**Hosein, 2024**).

Severe dehydration was observed, this refers to loss of body fluids through diarrhea. Diarrhea in calves occurs due to interference of absorptive surface function of small intestine (**Nataraju et al., 2009; Vega et al., 2011**).

Clinical examination of BRV infected calves revealed different forms of diarrhea including watery, yellow mucoid and bloody diarrhea. Diarrhea was associated with different degrees of dehydration that may be attributed to loss of body fluids. Watery to pasty yellowish diarrhea followed by dehydration were the prominent clinical findings observed previously by several authors (**Barua, 2019; Abdel-Rady et al., 2022; Youssef and Zaitoun, 2022**). These findings may be attributed to different etiological agents and co-infections involved in each case and secondary infections. Diarrhea and dehydration can be explained on the bases of the pathophysiological changes of the intestinal tracts of BRV infected calves, in which BRV can escape unaffected from acidic pH of stomach and digestive enzymes in gut as a result of the presence of triple protein coat possessed by the virus. BRV invades surface epithelial cells of the small intestinal villi inducing stunts and exfoliation of villi of small intestine inducing reduction in absorption capacity and in secretion of digestive enzymes resulting in profuse viscous fluid containing undigested and unabsorbed nutrients in intestinal lumen (**Hagbom, 2015**).

**Table 4.** Prevalence of BRV infection and Cryptosporidiosis in diarrheic cattle calves in relation to clinical severity.

Parameter	BRV single infection(n=6)	<i>Cryptosporidium</i> spp. single infection (n=29)	Co-infection (n=7)	
Suckling ability (n=123)	Positive (n=38)	2(33.33%)	7(24.14%)	0
	Relative (n=28)	1(16.67%)	9(31.03%)	1(14.29%)
	Non (n= 57)	3 (50%)	13(44.83%)	6(85.71%)
Temperature (n=123)	≤ 37 ° (n=6)	0	0	0
	37.1 to 38 °(n=8)	2(33.33%)	3(10.34%)	0(nil)
	38.1 to 39 °(n=41)	2(33.33%)	13(44.83%)	4(57.14%)
	39.1 to 40 °(n=62)	2(33.33%)	13(44.83%)	3(42.86%)
	40.1°(n=12)>	0	0	0
Dehydration (n=123)	Normal (n=26)	1(16.67%)	1(3.45%)	0(nil)
	Mild (n=13)	0	3(10.34%)	0(nil)
	Moderate (n=32)	1(16.67%)	11(37.93%)	2(28.57%)
	Severe (n=52)	4(66.67%)	14(48.28%)	5(71.43%)
Attitude (n=123)	Standing (n=46)	3 (50%)	8(27.59%)	2(28.57%)
	Recumbent (n=77)	3 (50%)	21(72.41%)	5(71.43%)
Fecal nature (n=123)	Watery (n=53)	5(83.33%)	11(37.93%)	6(85.71%)
	Mucoid (n=64)	1(16.67%)	16(55.17%)	1(14.29%)
	Bloody (n=5)	0	2(6.9%)	0

In this study amplification of RNA of BRV from 13 Positive ICA BRV fecal samples of diarrheic calves was carried out. BRV double-stranded RNA (dsRNA) was extracted from fecal samples and subjected for One-step RT-PCR targeting NSP5 gene. Prevalence of BRV-PCR positive diarrheic calves revealed 5(38.46%), **Table (5)**.

The above mentioned results, showed discrepancy between the prevalence Of BRV infection obtained using the ICA 13(10.57%) and that obtained by PCR 5(38.46%). This might be due to presence of inhibitory substances present in feces. It has been reported that these inhibitory substances interfere with conversion of RNA to cDNA or during PCR as studied by (**Chinsangaram et al., 1993**).

**Table 5.** Results of BRV infection and *Cryptosporidium* spp. using PCR.

Pathogen	Examined samples	Positive
BRV	13	5 (38.46%)
<i>Cryptosporidium</i> spp.	36	28(77.78%)

In the present study, the same total 123 diarrheic fecal samples of calves were subjected to the modified Ziehl Neelsen technique for detection of *Cryptosporidium* spp. infection to quantify the prevalence of *Cryptosporidium* spp. infection and different clinical alterations as well as to investigate the possibility of co-infection with BRV infection. The prevalence of Cryptosporidiosis in calves in this study was estimated as 36(29.27%), **Table (2)**.

The results of this study highlights the enzootic nature of this disease in different localities in Egypt and demonstrates the survival of the causative agent as an important components in the epidemic triangle of this disease which can discussed on the basis of the prolonged survival of this pathogen in the environment as well as the resistance of this pathogen to many disinfectants (**Tzpori and Ward, 2002**).

An increase in prevalence in males 16(28.57%) versus 13(19.03%) in females was detected (Table, 3). Concerning age as risk factor, the results revealed an increase 24(34.78%) in calves from birth to 15 days, versus 4(9.76%) from 16-30 days (Table, 3). The high infection rate and the rapid transmission among calves can be explained (Fayer et al., 1998) who stated that *Cryptosporidium spp.* oocysts are shed from infected calves in huge numbers, ( $10^7$ ) oocysts per gram of feces, as early as 3 days of age, that peaks at 2 weeks of age.

In this study *Cryptosporidium spp.* infected calves showed watery, mucoid or bloody diarrhea. The obtained results ran parallel with those obtained by Kumaresan et al., (2012) who reported similar picture in *Cryptosporidium spp.* infected calves.

Occurrence of diarrhea in *Cryptosporidium* infections can be attributed to exposure of the enterocytes to the pathogen inducing villous atrophy (Heine et al., 1984). Disturbance of the epithelial barrier and secondary infections consequently occur (Gookin et al., 2002). Fermentation of undigested milk in intestine consequently leads to diarrhea of mal absorptive nature (Tzipori and Ward, 2002).

In this study, DNA extraction was carried out on 36 *Cryptosporidium spp.* positive modified Ziehl neelsen stain fecal samples. Amplification of two-step nested PCR for gp60 gene was carried out that resulted in detection of 28(77.78%) (Table, 5).

The severity of clinical signs (Table, 4) especially dehydration observed in some infected calves in this study may be the result of co-infections with other enteric pathogens (Kumaresan et al., 2012). Mixed infection of BRV and *Cryptosporidium spp.* in this study, revealed 7(5.69 %) cases.

In addition, Reynolds et al., (1986) reported that co-infection with multiple enteropathogens is the determining factor of inducing the clinical syndrome. Most European countries reported that mixed infection of BRV and Cryptosporidiosis ranges from (5-50%) (Uhde et al., (2008)). Such dual infection may decrease the resistance of intestinal mucosa leading to severe enteritis with severe clinical outcome.

The prognosis should be regarded as favorable in mono enteropathogen infection compared to situations when dual or mixed infection with *Cryptosporidium spp.* exists. The obtained results clarified the aggravation of the clinical signs in calves after dual infection of BRV, and *Cryptosporidium spp.* compared to BRV infection alone.

#### 4. Conclusion

The present study indicates the high prevalence and the enzootic nature of BRV infection and Cryptosporidiosis in calves in Beni Suef as well as the clinical significance of these diseases in calves. The severity of clinical signs observed in some infected calves in this study was attributed to co-infection of BRV infection and Cryptosporidiosis.

#### 5. Conflict of Interest

The author declare no conflict of interest.

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