Original Article

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

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Abstract

Rotavirus A (RVA) and *Cryptosporidium* oocysts infections remain the most recognized pathogen worldwide causing acute diarrhea in calves less than one month of age. The current study was carried out to estimate the prevalence of Bovine Rotavirus (BRV) and *Cryptosporidium spp.* Infections in calves in Beni-Suef governorate, Egypt. Fecal samples of 123 diarrheic calves were subjected to 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 Rotavirus 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 to one-step RT-PCR targeting the 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 the 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 Rotavirus, *Cryptosporidium spp.*, Immunochromatographic Assay

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1. Introduction

Bovine Rota Virus (BRV) is a major cause of calf diarrhea worldwide (MK and HB, 2013) with prevalence rates ranging 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 (16–21 kbp), well protected by an inner and outer capsid layer (Greenberg and Estes, 2009; Desselberger, 2014). The genome encodes six structural proteins and six non-structural proteins (Matthijnssens 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., 2011). 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, which allows for the improvement of 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-Suef, governorate, Egypt, and the possibility of their co-infection.

2. Materials and methods

The study protocol was approved by the Animal Research Ethics Committee of the Faculty of Veterinary Medicine, Beni-Suef University (approval 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 from 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 the 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 the manufacturer's instructions.

2.4. Microscopy Screening

Detection of *Cryptosporidium spp.* oocysts was performed using the 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 the GeneJET Viral RNA Purification Kit (Thermo Scientific, Lithuania catalog no. k0821). One-step RT-PCR targeting the NSP5 gene was performed using Verso 1-step RT-PCR kit (ThermoFisher Lithuania) to confirm BRV. The primers used for amplifying an NSP5 gene BRV A were mentioned in Table 1 the reaction was prepared according to the manufacturer's directions and the cycling conditions were reverse transcription at 50°C for 30 min., primary denaturation at 95°C for 5 min, 35 cycles of secondary denaturation (at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec), and final extension at 72°C for 7 min.

2.6. DNA Extraction of *Cryptosporidium spp.*

Fecal samples positive for the 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 the 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 25 μl containing: 12.5 μl 2x Master Mix (applied biotechnology, Egypt), 1 μl Forward Primer, 1 μl Reverse Primer (Table 1), 4 μl DNA template, 6.5 μl Nuclease Free Water. The thermo-cycling parameters consisted of an initial denaturation cycle at 95°C for 5 min followed by 35 cycles of the following program; 94°C for 45 sec, annealing temperature was 50°C for 45 sec for each primer, and 72°C for 45 sec then followed by final extension step at 72°C for 7 min. 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 As-

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	GAT ATT GGA CCA TCT GAT TCT GCT TCA AA	155	Schroeder et al. (2012)
		Nsp5-r	GAA ATC CAC TTG ATC GCA CCC AA		
	Glycoprotein 60KDa (Gp60)	Gp60-F	ATA GTC TCC GCT GTA TTC	950-1000	
		Gp60-R	GCA GAG GAA CCA GCA TC		
Cryptosporidium spp.		Nest Gp60-F	TCC GCT GTA TTC TCA GCC	450	Peng et al. (2001)
		Nest Gp60-R	GAG ATA TAT CTT GGT GCG		

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

Examined calves	BRV (ICA)	*Cryptosporidium spp.* (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)	*Cryptosporidium 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%)
	Birth to 15 d.(n=69)	6 (8.7%)	24(34.78%)	7(10.14%)
Age	16 to 30 d. (n=41)	0	4(9.76%)	0
	31 to 45 d. (n=13)	0	1(7.69%)	0
	1st (n=34)	2(5.88%)	9(26.47%)	3(8.82%)
Dam parity	2nd (n=28)	2(7.14%)	6(21.43%)	2(7.14%)
	3rd (n=19)	0	10(52.63%)	1(5.26%)
	4th (n=16)	1(6.25%)	2(12.5%)	1(6.25%)
	5th & more (n=26)	1(3.85%)	2(7.69%)	0
Total	123	6 (4.88%)	29 (23.58 %)	7 (5.69%)

siut 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 the prevalence of BRV infection in calves in different studies may be due to geographical variation, differences in hygienic measures, environmental conditions, management systems involving the pregnant dams and newborn calves, and differences 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 the occurrence of BRV infection in different studies, a non-significant difference in the rate of BRV infection between male and female calves statistically indicating a non-sex linked disease (Monney et al., 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).

Clinical abnormalities observed in BRV ICA positive diarrheic calves in this study revealed that the body temperature did not exceed 40°C which is identical to most cases of uncomplicated viral infections (Table 4). 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 the prevention and control of bovine BRV infections includes enhancing the immunity of newborn calves through dam immunization and reducing the exposure of calves to infection through strict hygienic measures and the adoption of good management systems (Hosein, 2024).

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

Parameter		BRV single infection(n=6)	*Cryptosporidium 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)	$\leq$ 37°C (n=6)	0	0	0
	37.1 to 38°C (n=8)	2(33.33%)	3(10.34%)	0(nil)
	38.1 to 39°C (n=41)	2(33.33%)	13(44.83%)	4(57.14%)
	39.1 to 40°C (n=62)	2(33.33%)	13(44.83%)	3(42.86%)
	40.1°C (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

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

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

Severe dehydration was observed, this refers to loss of body fluids through diarrhea. Diarrhea in calves occurs due to interference with the absorptive surface function of the 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 based on the pathophysiological changes of the intestinal tracts of BRV-infected calves, in which BRV can escape unaffected from the acidic pH of the stomach and digestive enzymes in the 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 the small intestine inducing reduction in absorption capacity and in secretion of digestive enzymes resulting in profuse viscous fluid containing undigested and unabsorbed nutrients in the intestinal lumen (Hagbom, 2015).

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 to One-step RT-PCR targeting the NSP5 gene. Prevalence of BRV-PCR positive diarrheic calves revealed 5(38.46%) (Table 5).

The above-mentioned results showed a 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 the presence of inhibitory substances present in feces. It has been reported that these inhibitory substances interfere with the conversion of RNA to cDNA or during PCR as studied by (Chinsangaram et al., 1993).

In the present study, the same total of 123 diarrheic fecal samples of calves were subjected to the modified Ziehl-Neelsen technique for the 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 highlight the enzootic nature of this disease in different localities in Egypt and demonstrate the survival of the causative agent as an important component in the epidemic triangle of this disease which can discussed based on the prolonged survival of this pathogen in the environment as well as the resistance of

this pathogen to many disinfectants (Tzipori 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 a risk factor, the results revealed an increase of 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 a similar picture in *Cryptosporidium spp.* infected calves.

The occurrence of diarrhea in *Cryptosporidium* infections can be attributed to the 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 the intestine consequently leads to diarrhea of malabsorptive 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 the gp60 gene was carried out that resulted in the 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 outcomes.

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.

3.1. 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.

Article Information

Ethical Approval. The study protocol was approved by the Animal Research Ethics Committee of the Faculty of Veterinary Medicine, Beni-Suef University (approval no. 022-403).

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Conflict of Interest. The authors declare no conflict of interest.

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