Pakistan J. Zool., pp 1-8, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20240718110524

Clinicomolecular Investigation of Bovine Rota and Coronaviruses and Their Association with Haematobiochemical Parameters in Diarrheic Cattle Calves

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Article Information
Received 18 July 2024
Revised 20 September 2024
Accepted 30 September 2024
Available online 26 November 2024
(early access)

Authors' Contribution
JAK, SSA and AAA conceptualized the research topic of this manuscript. SA conducted the research, analyzed the data statistically and wrote the manuscript. NA, SK and NR helped in the execution of the experimental work.

Key words
Cattle calves, BRoV, BCoV, Rapid kits test, RT PCR, Haematobiochemical parameters

ABSTRACT

Neonatal calf diarrhea (NCD) is the most common cause of disease in newborn calves around the world. Pakistan's young cattle population is severely affected by NCD caused by bovine rotavirus (BRoV) while bovine coronavirus (BCoV) outbreaks. BRoV is a double-stranded RNA virus enteric viral pathogen and BCoV is a positive-sense single-stranded RNA enveloped virus respiratory and enteric pathogen. This project was designed to find out the clinicomolecular investigation of BRoV and BCoV infections and their association with haematobiochemical parameters in diarrheic cattle calves. A total of 200 samples were collected from diarrheic calves to detect BRoV and BCoV antigens using Rapid Detection Kits Test. The samples found positive for said viruses after the initial screening were further subjected to render RT-PCR. The results of the present study demonstrated that the occurrence of BRoV infection using diagnostic screening kits test and RT-PCR was 26% and 21.5%, while the occurrence of BCoV infection was 3.5% and 3.0% respectively. Finally, it was determined that exotic breed calves were more infected than crossbred calves. Calves under one week of age were shown to be more infected than the other old age calves. Milk-fed calves were significantly more likely to be infected with BRoV and BCoV infections than calves on milk replacer. Summer season was more adverse to calves as compare to other seasons. Hematological parameters analysis revealed that mean corpuscular volume (fl) in infected calves dropped while total leukocyte count (thousands/cm3) increased. On analysis of biochemical parameters, it was discovered that sodium (mEq/L), calcium (mmol/L), copper (mol/L), and iron were reduced dramatically but potassium (mEq/L) was not increased significantly. It was concluded that assumed risk factors predisposed the occurrence of BRoV and BCoV infection and haemato-biochemical alterations were observed in calves infected with said viruses. This is the first study report on the occurrence of BCoV infection and the risk factors associated with it in Pakistan.

INTRODUCTION

Livestock is vital subsector of Pakistan. It shares 60.69 % in agriculture sector with the growth rate of 3.06 % in last year. Livestock contribute 11.53% national GDP. Pakistan has a largest population of livestock: 22.42 million cattles, 23.34 million buffaloes, 49.14 million goats, 24.24 million sheep and 0.77 million camel. Livestock are factories that convert grasses into good quality of food like meat and milk.

Neonatal calf diarrhea (NCD) is the most major cause of mostly occurred diseases in new born calves in all over the world is the major cause of morbidity and mortality in newborn calves, mostly less than one month of age. Due to high morbidity and mortality rate in new born calves, NCD is the major cause of a huge productivity and economic losses to dairy industry worldwide (Manzoor, 2018).

It is a complex disease due to the involvement of

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0030-9923/2024/0001-0001 $ 9.00/0

the many different infectious agents isolated from the feces and tissues of the affected calves (Acres, 1975). Among several pathogens associated with NCD, the most common etiologic agents involved in it are *Escherichia coli; Clostridium perfringens* type C, *Salmonella* species, *Cryptosporidium* spp, bovine rotavirus (BRoV) and bovine coronavirus (BCoV). Among these etiological agents, bovine coronaviruses (BCoVs) and bovine rotaviruses (BRoVs) cause infection about 27–36% presenting themselves as the most common and major viral enteric pathogens in newborn calves (Cho and Yoon, 2014; Alfieri *et al*., 2018).

BRoVs are the most common and major viral enteric pathogens (Seid *et al*., 2020) caucusing gastroenteritis, encephalopathy, cerebellitis, meningoencephalitis, and cardiac problems in human beings. Food and water contaminated with bovine rotavirus are the major source of infections in both human and animals (Qin *et al*., 2022).

BCoV is a significant viral pathogen associated with respiratory and enteric problems in newborn calves. Mebus discovered it by accident in 1972, and it was thought to be the most common cause of calf diarrhoea at the University of Nebraska (Vlasova and Saif, 2021). As a result, it was determined that BCoV could cause respiratory and enteric problems in cattle (Zhu *et al*., 2022).

Despite advances in animal husbandry practices, diagnostic techniques, and treatment regimens, calf morbidity and mortality had been remained a major concern (Geletu *et al*., 2021). Pakistan's young livestock population is severely affected by NCD caused by BRoV and BCoV outbreaks. This study was conducted on the occurrence of BRoV and BCoV infection in cattle calves in Pakistan. It also focuses on various hemato-biochemical parameters of the cattle calves infected with BRoV and BCoV.

MATERIALS AND METHODS

Study area and animals

The study was carried out in district Jhelum of Province Punjab, Pakistan (Latitude: 32.940548° N and Longitude: 73.727631° E) on cattle calves less than one month of age exhibiting symptoms of diarrhea and dysentery at different livestock farms and small dairy households in the study area. Calves of Holstein Friesian and crossbred breeds were selected for this study. The duration of this whole study was from July 2020 to June 2021.

Sampling procedure and processing of fecal samples

Four categories were made for samples collection, each category was predefined. Category-1: small households having 1-10 animals, category-2: small livestock farms having 1-50 animals, category-3: medium livestock farms having 51-100 animals and category-4: large livestock farms will be having above 100 animals. The fecal samples were collected from diarrheic calves found in all four categories to perform rapid detection tests and RT-PCR. A total of 200 faecal samples were collected from calves with a history of diarrhoea and dysentery. To screen fecal samples for BRoV and BCoV infections in cattle calves, the S&C biotech bovine rotavirus and bovine coronavirus antigen rapid test with sandwich lateral flow immunochromatographic assay and RT-PCR tests were used.

Molecular characterization and PCR amplification

Following an initial screening with diagnostic kits, faecal samples found positive for BRoV and BCoV infections were subjected to an RT-PCR assay to compare the efficacy of these two diagnostic tools, screening kits and RT-PCR, in detecting the occurrence of BRoV and BCoV infections in cattle calves. This phase included RNA extraction, polymerase chain reaction, and Agarose gel electrophoresis. Total RNA fast extraction stool kit was used to extract RNA from faecal samples (China). The cDNA was synthesized from mRNA or total RNA templates using the Thermo Scientific TM RevertAidTM First Strand cDNA Synthesis Kit, which is commercially available (Sulehria *et al*., 2020). To confirm the presence of BRoV and BCoV in calves' feces, a portion of BRoV and BCoV was amplified by RT-PCR according to the protocol described by (Agnihotri *et al*., 2017).

After the PCR processing, the PCR products were visualized using UV light. After amplification of the VP6 gene fragment of BRoV and N Gene fragment of BCoV, the corresponding electrophoresis was performed to analyse the results of PCR bands. For this purpose, 1.2 % agarose gel was prepared in TAE (Tris-Acetate-EDTA) buffer. For each sample, 8 µLs of the final PCR product and 1 µL of 100 bp DNA ladder were run at 110V for 45 minutes (Mukhtar *et al*., 2016). The gel was stained with ethidium bromide and viewed by trans-illumination to visualize the bands.

Hemato-biochemical analysis

Blood samples were collected from diarrheic calves (who tested positive for BRoV and BCoV infections after preliminary screening tests) for haemato-biochemical analysis. A total of 10 ml of blood was collected from each calf, 5 ml for CBC and 5 ml for serum electrolytes analysis. The CBC was performed using a VET haematology analyzer. The serum was kept at -20°C until further testing. A semi-automated clinical chemistry analyzer machine was used to estimate biochemical parameters in

serum samples. All the tests were performed following the manufacturer's directions (Sulehria *et al*., 2020).

Risk factor analysis and husbandry practices

A questionnaire was constructed to study the assumed risk factors like age, sex, breed, body condition, body weight, location, vomiting, diarrhea, sample source, body size, cohabitation with other animals, living environment, food type, deworming history, contact with the feces of other species, vaccination history, presence of other co-pathogens, any other human infected with BRoV and BCoV. Animal husbandry practices like calf housing either caged or confined or living with other animals, calf housing hygienic conditions, early colostrum feeding and navel cord management also were observed.

Statistical analysis

SPSS version 20 was used for statistical analysis. The t-test was used to analyse data on hemato-biochemical parameters, and Chi Square was used to analyse data on risk factors. The degree of association of risk factors with the occurrence of BRoV and BCoV infections in cattle calves was determined using an odds ratio, and a *p*-value of (<0.05) was considered significant.

RESULTS AND DISCUSSION

BRoV and BCoV infection in calves

After initial screening of fecal samples through rapid diagnostic kits test, it was found that out of 200 fecal samples, 52 samples were found positive for BRoV and 7 were found positive for BCoV, respectively. After performing the RT-PCR, out of 52 samples, 43 was found positive for BRoV and among 7, 6 were confirmed for BCoV, respectively (Table I). Previous findings from different laboratories (Uddin *et al*., 2022; Ammar *et al*., 2014; Wei *et al*., 2021; Lotfollahzadeh *et al*., 2020; Brunauer *et al*., 2021; Barkley *et al*., 2021; Singh *et al*., 2020) supported the current study's findings. The variations in results could be attributed to the virus's inability to remain intact in the faeces due to activities of the endogenous RNase enzyme, and the lack of partially degraded RNA may affect the sensitivity of RT-PCR or intermittent virus shedding in faecal materials (Vermeulen *et al*., 2011).

When data was statistically analysed, then it was seen that the percentage occurrence in case of BRoV (OR=1.283, *p*=0.290) when checked through diagnostic screening kits, it was 1.283 times more than checked through RT-PCR but the results of percentage occurrence when checked through kits and PCR were non-significant (*p*>0.05). Similarly, in case of BCoV (OR=1.173, *p*=0.778), the occurrence percentage when checked through screening kits was 1.173 times more than when checked through RT-PCR but the results of percentage occurrence when checked through kits and PCR were non-significant (*p*>0.05) as shown in Table I.

Category wise BRoV and BCoV infection

Among results found, category-1, among 50 diarrheic samples collected, no case was found positive for BRoV through kits and RT-PCR. Among 50 samples collected from category-2, 7 cases were found positive for BRoV through kits and 5 cases through RT-PCR. The highest % age occurrence of 54% and 44% through kits and RT-PCR was found in category-3 followed by category-4 in which 38% occurrence was found through kits and 32% through RT-PCR (Table II). Similarly in case of BCoV, category-1 and category-2, among 100 diarrheic samples collected, no case was found positive for BCoV through kits and RT-PCR. Among 50 samples collected from category-3, 5 cases were found positive through kits and 4 cases through RT-PCR. Similarly, when screening of samples of category-4 was done, only two cases were found positive for BCoV both through kits and RT-PCR as shown in (Table II).

When categories were ranked on the basis of %age occurrence, the category-3 was on rank-1 followed by category-4 in case of both BRoV and BCoV infections as shown in Table III.

Risk factors associated with BRoV and BCoV infection

Various risk factors associated with the occurrence of BRoV and BCoV infections were studied and analyzed. When data on calves' breeds was statistically analyzed, it was discovered that the breeds had the highest potential for

Table I. Occurrence of BRoV and BCoV in diarrheic cattle calves as determined by diagnostic screening kits test and PCR.

Species	Total samples	Kits positive samples	PCR positive samples	Occurrence (kit positive)	Occurrence (PCR positive)	Odd ratio (OR)	95%CI for OR	Chi square	P-value
BRoV	200	52	43	26%	21.5%	1.283	0.808-2.037	1.118	0.290
BCoV	200	7	6	3.5%	3.0%	1.173	0.387-3.553	0.080	0.778

Table II. Category wise occurrence of BRoV and BCoV in infected calves.

Area	Tech-nique	Calves screened (no)	Rota posi-tive (no)	Percentage occurrence
BRoV-infected calves				
Categpry-1	Kits	50	0	0%
	PCR	0	0	0%
Categpry-2	Kits	50	7	14%
	PCR	7	5	10%
Categpry-3	Kits	50	27	54%
	PCR	27	22	44%
Categpry-4	Kits	50	19	38%
	PCR	19	16	32%
BRoV infected calves				
Categpry-1	Kits	50	0	0%
	PCR	0	0	0%
Categpry-2	Kits	50	0	0%
	PCR	0	0	0%
Categpry-3	Kits	50	5	10%
	PCR	5	4	8%
Categpry-4	Kits	50	2	4%
	PCR	2	2	4%

disease dynamics, implying that the breeds were highly significantly ($p<0.000$) affected by BRoV and BCoV infections. The odds ratio (OR=10.628; 95% CI=4.291-26.323) indicated that Holstein Friesian calves had the greatest potential for disease dynamics, followed by crossbred calves. The OR revealed that exotic breed calves were 10.628 times more affected than crossbred calves.

Age was also a significant assumed risk factor; calves were divided into three groups based on their age, namely 1 to 10 days, 11 to 20 days, and 21 to 30 days. When the data was divided into three groups, calves aged one to ten days were found to be significantly ($p<0.001$) more affected by BRoV and BCoV infections than the other two age groups. Another risk factor associated with the occurrence of BRoV and BCoV infection in calves was sex and was significantly ($p<0.034$) associated. The findings of Seid *et al*. (2020) and Bertoni *et al*. (2021) regarding breed, age, and sex supported the current study's findings. Male calves were two (OR=2.011) times more affected than female calves. The seasonal association between winter and monsoon had no significant ($p>0.586$) effect on the occurrence rate in calves, whereas spring and summer had a highly significant ($p<0.001$) effect on calves infected with BRoV and BCoV infections. The highest occurrence of BRoV and BCoV infections was observed during the summer season, while zero occurrences were observed during the winter season. Weather and climate change were the most important risk factors that contributed to the spread of disease caused by BRoV and BCoV infections (Boileau and Kapil, 2010). The current study's findings were very similar to those of Trotz-Williams *et al*. (2007) and Tamrat *et al*. (2020).

When data from normal, emaciated, and fatty calves was compared, the emaciated calves were significantly ($p<0.02$) more affected with BRoV and BCoV infections than the normal and fatty calves. This finding was also consistent with the findings of a study conducted in North West Ethiopia by Tamrat *et al*. (2020) and Kayasaki *et al*. (2021). The findings (OR=8.793; 95% CI=1.003-77.097) also revealed that emaciated calves were 8.793 times more likely to be affected by BRoV and BCoV infections than fatty and normal calves. Milk-fed calves were significantly ($p<0.005$) more likely to be infected with BRoV and BCoV infections than calves on milk replacer. According to the findings (OR= 3.188; CI= 1.399-7.262), milking calves were 3.188 times more likely to be infected with BRoV and BCoV infections than calves on milk replacer. These findings were similar to those of Kayasaki *et al*. (2021).

In this study, open housing had a significantly greater ($p<0.000$) effect on calves with BRoV and BCoV infection than confined housing. The previous findings of others (Waltner-Toews *et al*., 1986; Quigley *et al*., 1995; Bazeley, 2003; Bertoni *et al*., 2021) corroborated the current study's findings. According to the findings (OR=8.313; CI=2.467-26.006), open housing affected calves with BRoV and BCoV infection 8.313 times more than confined housing. Further investigation into the cohabitation of calves living with other animals revealed that those calves who had a history of living with other animals were significantly ($p<0.000$) affected with BRoV and BCoV infections.

Hematological analysis of infected calves

Data regarding hematological parameters were analyzed by t-test. On analyzing the data in infected cattle calves, it was found that mean corpuscular volume (fl) decreased significantly ($p<0.001$) while total leukocyte count (thousands/cm^3) increased significantly ($p<0.006$). TEC (million/cm^3), WBC (m/mm^3), RBC (m/mm^3), and hematocrit (%) were all significantly ($p<0.000$) increased in BRoV and BCoV infected calves. Mean corpuscular haemoglobin (pg) and monocytes (%) were also significantly ($p<0.05$) lower in infected calves. Other parameters such as haemoglobin (g/dL), mean corpuscular haemoglobin concentration (g/dl), lymphocytes (%), packed cell volume (%), and basophils (%) were not significantly ($p>0.05$) associated with the occurrence of BRoV and BCoV infections (Table IV). The findings of previous studies of supported the current study's findings

(Barua *et al*., 2018; Song *et al*., 2020; Naylor, 1987; Al-Robaiee and Al-Farwachi, 2012; Brar *et al*., 2015).

Table III. Category wise ranking order of occurrence of BRoV and BCoV.

S. No	Category	Ranking	Percentage occurrence (Kits)	Percentage occurrence (PCR)
BRoV infected calves				
1	Category-3	Rank 1st	54%	44%
2	Category-4	Rank 2nd	38%	32%
3	Category-2	Rank 3rd	14%	10%
4	Category-1	Rank 4th	0%	0%
BCoV infected calves				
1	Category-3	Rank 1st	10%	8%
2	Category-4	Rank 2nd	4%	4%
3	Category-2	Rank 3rd	0%	0%
4	Category-1	Rank 4th	0%	0%

Biochemical analysis of infected claves blood

Biochemical analysis revealed that potassium (mEq/L) was not significantly (p>0.090) increased in BRoV and BCoV infected calves, whereas sodium (mEq/L) was significantly (p<0.000) decreased. Calcium (mmol/L), copper (mol/L), and iron (mol/L) levels were also significantly (p<0.000) lower in diarrheic calves infected with BRoV and BCoV infections (Table IV). The current study's findings were similar to those reported by others (Barua *et al*., 2018; Tajik *et al*., 2012; Klinkon and Jezek, 2012; Singh *et al*., 2006; Kaur *et al*., 2006; Klinkon and Jezek, 2012; Sobiech *et al*., 2013; Brar *et al*., 2015).

CONCLUSION

It was concluded that BRoV and BCoV infections were present in cattle calves in Punjab, Pakistan. Assumed risk factors like breed, age, sex, season, housing type, food type, hygiene, environmental conditions, body conditions, and contact with animals were all found to be less or more significantly associated with the occurrence of BRoV and BCoV infections. Rapid diagnostic kits tests were proved to the best diagnostic tool for the immediate diagnosis of BRoV and BCoV. Furthermore, it was concluded that haematobiochemical alterations can aid in the diagnosis of BRoV and BCoV infections in calves.

Table IV. Hematological and biochemical parameters of diarrheic cattle calves infected with BRoV and BCoV.

Parameters	Mean±SD	SEM	t statistic	P-value	95% C.I
Hematological parameters					
PCV (%)	43.136 ± 1.602	0.71661	0.888	0.425	41.15-45.13
Hb (g/dL)	10.396 ± 1.922	0.85990	-1.284	0.269	8.01-12.78
MCV (fl)	38.00 ± 3.391	1.51658	-7.913	0.001**	33.79-42.21
MCH (pg)	12.932 ± 0.539	0.24130	-4.426	0.011*	12.26-13.60
MCHC (g/dl)	33.358 ± 2.684	1.20059	-1.368	0.243	30.02-36.69
Monocytes (%)	2.434 ± 0.452	0.20247	-2.795	0.049*	1.87-3.00
Lymphocytes (%)	53.648 ± 5.978	2.67375	-2.376	0.076	46.22-61.07
Basophils (%)	0.556 ± 0.096	0.04331	-10.251	0.001**	0.44-0.68
TLC (thousand/cm^3)	13.124 ± 1.441	0.64483	5.310	0.006**	11.33-14.91
TEC (million/cm^3)	8.716 ± 0.280	0.12540	4.912	0.008**	8.37-9.06
WBC (m/mm^3)	21.374 ± 2.764	1.23628	10.818	0.000**	17.94-24.81
RBC (m/mm^3)	12.086 ± 1.152	0.51530	6.959	0.002**	10.66-13.52
Hct (%)	56.626 ± 1.546	0.69162	27.654	0.000**	54.71-58.55
Biochemical parameters					
Na (mEq/L)	127.892 ± 0.957	0.42804	-37.632	0.000**	126.70-129.08
K (mEq/L)	6.242 ± 0.293	0.13113	2.227	0.090*	5.88-6.61
Ca (mmol/L)	1.324 ± 0.238	0.10647	-11.985	0.000**	1.03-1.62
Cu (µmol/L)	7.554 ± 0.426	0.19072	-31.176	0.000**	7.02-8.08
Fe (µmol/L)	8.382 ± 0.350	0.15682	-77.813	0.000**	7.95-8.82

Data are indicated as (Mean±SD, SEM, t statistic, P-value, 95% confidence interval) and ** shows that the values are highly significant (p<0.01), while * shows values are significant (p<0.05). BCoV stands for bovine coronavirus.

DECLARATIONS

Funding

The study received no funding.

IRB approval

This study was ap-proved by Advanced Studies and Research Board at University of Veteri-nary and Animal Sciences (UVAS), Lahore, Pakistan 0n 09-10-2019. (Approval no: DAS/:8250 Dated: 28/10/2019).

Ethical approval

The study design was presented to the Ethics Committee, University of Veterinary and Animal Sciences, Lahore, for ethical approval. This study was approved by Advanced Studies and Research Board at University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan 0n 09-10-2019. (Approval no: DAS/:8250 Dated: 28/10/2019).

Statement of conflict of interest

All authors have no conflicts of interest.

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