Isolation and Characterization of Coronavirus and Rotavirus Associated With Calves, in Central Part of Oromia, Ethiopia

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Abstract

Background: Coronavirus and Rotavirus are most commonly associated etiologies for calves' diarrhea resulting in loss of productivity and economy of farmers. However, various facets of diarrheal disease caused by coronavirus and rotavirus in calves in Ethiopia are inadequately understood. A cross sectional study was conducted with the aim of isolation and molecular characterization of coronavirus and rotavirus from calves in central part of Oromia (Bishoftu, Sebata, Holeta and Addis Ababa), Ethiopia from November 2018 to May 2019. The four study areas were purposively selected and fecal samples were collected by simple random sampling for diagnosis of coronavirus and rotavirus infection by using antigen detection Enzyme linked immunosorbent assay (Ag-ELISA) kit. In addition, this study was carried out to have insight in prevalence and associated risk factors of coronavirus and rotavirus infection in calves.

Result: During the study 83 diarrheic and 162 non-diarrheic fecal samples collected from calves less than 4 weeks of age were screened for coronavirus and rotavirus. Of the 83 diarrheic samples, 1 sample (1.2%) was positive for coronavirus antigen (Ag) and 6 samples (7.2%) were found to be positive for rotavirus antigen (Ag) by Ag-ELISA. All the non-diarrheic samples were negative for both coronavirus and rotavirus Ag. The overall prevalence of coronavirus and rotavirus infection in calves were estimated as 0.4% (1/245) and 2.45% (6/245) respectively. All samples (7) of ELISA test positive of both coronavirus and rotavirus were propagated in Madin Darby bovine kidney cells (MDBK). After 3 subsequent passages, progressive cytopathic effect (CPE) i.e. rounding, detachment as well as destruction of mono-layer cell of five sample (1 sample of coronavirus and 4 sample of rotavirus) (71.4%) were observed. At the molecular stage, reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to determine the presence of coronavirus and rotavirus nucleic acid by using specific primers. The 5 samples that were coronavirus and rotavirus antigen positive by ELISA and develop CPE on cell culture were also positive on RT-PCR technique. Infection prevalence peaked have been obtained at 1^{st} and 2^{nd} weeks of age in male calves.

Conclusion: Diarrheal disease caused by coronavirus and rotavirus has a great health problem in calves that interrupts production benefits with reduced weight gain and increased mortality, and its potential for zoonotic spread. So the present findings show coronavirus and rotavirus infection in calves in Ethiopia that needs to be addressed by practicing early colostrums feeding in newborn calves, using vaccine, or improving livestock management.

Background

Diarrhea in neonatal calves is one of the most challenging clinical syndromes encountered by the practicing large animal veterinarian worldwide. It is caused by multifactorial agents like viruses, bacteria, protozoa (Lorenz *et al.,* 2011). Among these etiological agents of calve diarrhea, coronavirus and rotavirus alone accounts for about 27-36% (Cho and Yoon, 2014). Together with the Bovine rotavirus (BRoV) and bovine coronavirus (BCoV) are the most common viral enteric pathogens. In addition to this

Bovine coronavirus (BCoV) can cause diarrhea and respiratory-tract infections in calves as well as in adult cattle (Nogueira *et al*., 2013).

Coronaviruses (CoVs) (order *Nidovirales*, family *Coronaviridae*, and subfamily *Coronavirinae*) are enveloped viruses with a positive sense, single-stranded RNA genome. With genome sizes ranging from 26 to 32 kilobases (kb) in length, CoVs have the largest genomes for RNA viruses that are responsible for enteric, respiratory, or neurological signs in mammals and birds. CoVs are classified into 3 groups on the basis of antigenic and genetic properties: α-CoVs, β-CoVs, and γ-CoVs. Bovine coronavirus (BCoV) is included in group β-CoVs which also including the closely related HCoV-OC43, which causes respiratory infections in humans, and the human pathogens SARS-CoV and MERS-CoV (Enjuanes *et al*., 2000).

Bovine coronavirus was first identified as the agent of severe diarrhea in neonatal calves (neonatal calf diarrhea). It was also associated with the occurrence of respiratory distress in calves and adults (Lathrop *et al*., 2000; Storz *et al*., 2000). The coronaviral genome encodes four major structural proteins: the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein, all of which are required to produce a structurally complete viral particle (Wang *et al*., 2017). BCoV is transmitted via the fecal-oral or respiratory route (Clark, 1993). It infects epithelial cells in the respiratory tract and the intestines; the nasal turbinates, trachea and lungs and the villi and crypts of the small and large intestine, respectively. Replication leads to shedding of virus in nasal secretions and in feces. Important factors for the pathogenesis are still not fully explored, such as how the virus infects enterocytes shortly after introduction to an animal. Clinical signs range from none to severe, and include fever, respiratory signs and diarrhea with or without blood (Boileau and Kapil, 2010).

Rotavirus is a genus of double stranded RNA virus in the family Reoviridae. The family Reoviridae is composed of eight genera: Orthoreovirus, Orbivirus, Coltivirus, Rotavirus, Aquareovirus, Cypovirus, Phytoreovirus, and Fijivirus. Among these all; Rotavirus species infect humans and animals. Rotaviruses have three important antigenic specificities: group, subgroup, and serotype. Group A rotaviruses are major pathogens in humans and animals. The 70-nm-diameter wheel-shaped particles consist of a double-layered icosahedral capsid enclosing a core particle that contains 11 segments of double-stranded RNA, each segment representing one gene (Kapikian and Shope, 1996).

Bovine coronavirus and rotavirus is the most recognized pathogens causing acute diarrhea in cattle and buffalo calves under one month of age worldwide (Alfieri *et al.,* 2006). It has also been recognized as the major pathogens of acute diarrhea in both humans and animals. So it has the potential of zoonotic and economic impact (Cook *et al.,* 2004). Infection appears and spreads rapidly causing extensive damage to the intestinal lining which results in rapid fluid loss and dehydration (Foster and Smith, 2009). High *genetic diversity* in *coronaviruses* is attributable to the high mutation rates associated with RNA replication, the high recombination frequencies within the coronavirus family and the large coronavirus genomes. Recombination in coronaviruses plays an important role in virus evolution and can result in the emergence of new pathotypes (Woo *et al.,* 2009). Thus far, recombination regions in coronaviruses have been extensively reported for the S gene (Minami *et al*., 2016). Genetic re-assortment is one of the

important mechanisms for generating genetic diversity of rotaviruses and eventually for viral evolution (Zhou *et al.*, 2015). There is no treatment for BCoV and BRoV, but early and confirmatory diagnosis helps to make appropriate prevention and control measures, which could prevent the great economic losses to farmers and the livestock industry (Barua, 2019).

Coronavirus and rotavirus is environmentally distributed worldwide and was highly studied (Straw *et al.,* 2006; Zimmerman, 2006). In a lot of studies BRoV infection rates of 20-60% in samples of diarrhea have been reported (Garaicoechea *et al.,* 2006; Uhde *et al.,* 2008). Prevalence of coronavirus was estimated ranging from 4% to 38.9% in sample of diarrheic calves throughout the world. In developing country like Ethiopia the prevalence of coronavirus and rotavirus were 38.9% and 16.7% (Abraham *et al.,* 1992). Coronavirus and rotavirus in calves is not well studied in Ethiopia. However, only one report by Abraham *et al.* (1992) indicated presence of 38.9% coronavirus and 16.7% rotavirus in calves in central Ethiopia. Such absence of the information could be the reason for lack of the Ethiopian government not to have any strategy for control of coronavirus and rotavirus infection of calves through vaccination. Absence of study conducted on isolation and molecular characterization of coronavirus and rotavirus in calves in Ethiopia may exacerbate the problem. Hence, detecting the circulating strains of coronavirus and rotavirus isolate and molecular evolution of the virus is needed for planning a proper control and preventive measure in the country.

Therefore, the objectives of current study were:

- To detect coronavirus and rotavirus from calves less than one month of age and
- To estimate the prevalence and identify the risk factors coronavirus and rotavirus in calves
- To Isolate and characterize the virus at molecular level in study area.

Methods

Description of the Study Area

The current study was conducted in four selected areas of central part of Oromia, Ethiopia (Bishoftu, Sebata, Holeta and Addis Ababa) from November 2018 to May 2019 (Figure 1). Fecal samples were collected from four selected of dairy farms. There are many small, medium and large-scale dairy farms in four selected area that supply milk and milk products to consumers of the town and surrounding urban areas. These dairy farms contain either local or exotic breeds depending on the scale of production.

Bishoftu town is found in east Shewa Zone, Oromia Regional State, located about 45 km South-east of the capital city, Addis Ababa. The area is located at 9°N latitude and 40°E longitude at altitude of 1850 m.above sea level. According national meterology agence (NMA) (2016), annual rain fall of 866 mm of which 84% is in the long rainy season (June to September) with annual minimum and maximum temperature of 11and 29°C, respectively. The domestic animals reared in Bishoftu town are 30887cattle, 43138 poultry, 9322 equine, 9294 sheep and 4753 goats (Bishoftu City Administration Agricultural Desk, 2014).

Sebeta town is located in the Oromia Special Zone Surrounding Finfinne (Addis Ababa) of Oromia Region. The district is located 25 km south west of Addis Ababa at an altitude of 1800-3385 m above sea level and at latitude and longitude of 8°55-8.917°N and 38°37-38.617°E respectively. It receives an average annual rainfall of 1073 ml and has temperature that ranges from 11.3-28^{0}C. It has a total area of 102,758 km (SHFDO, 2018). According to the information obtained from Sebeta Hawas district Administration Office (2018), both livestock rearing and crop production are the main economic activities of the majority of communities. The major livestock reared in the district include cattle, sheep, goats and poultry.

Holeta Town is located in the central part of the country, 31 km west of Addis Ababa in Oromia Regional state, west Shewa Administrative Region. The area is bounded between latitude 8^o 53’ 75” to 9^o 14’ North and longitude 38^o 21’ 40” to 38^o 36’ 14” East. The Town has an area of 5550 hectares. Holeta Town found at an average 2449 m above sea level. The annual mean maximum and the minimum temperatures are 25.9 and 7.2^{0}C, respectively (HTRADO, 2009).

Addis Ababa, the capital city of Ethiopia, lies at an elevation of 2300 m above sea level and is featured by a grassland biome. It is geographically located at 9°1'48"N latitude and 38°44'24"E longitude. It has a typical highland climate with temperature ranging from 11°C-24 °C. Addis Ababa has a mean annual rainfall of 1300 mm with bimodal distribution (NMA, 2016).

Study Population

The study was conducted in both apparently healthy cow calves and calves having clinical sign of diarrhea namely profuse watery diarrhea, systemic dehydration and depressed during investigation. Cow calves up to 30 days of age groups, all breed and sex reared under intensive management conditions were included in the study. Diarrhea was considered if feces are semi-liquid to liquid, with or without other abnormal characteristics such as presence of blood or mucous. Any calf with feces without these characteristics was considered non-diarrheic or apparently healthy (Ammar *et al.,* 2014).

Study Design

A cross-sectional study was conducted in different dairy farms found in four selected site of (Bishuftu, Sebeta, Holeta and Addis Ababa) central part of Oromia, Ethiopia from November 2018 up to May 2019. Information about the calves was gathered by interviewing farm owners and animal health workers of selected study sites. The information collected were recorded on data collection sheet, and then calves were clinically examined for presence of diarrhea or not and fecal samples were collected for diagnostic testing as follows. At the time of sampling the name of the farm, date of sampling, consistency of feces, age, breed, and tag number was recorded for each calf on proper recording format.

Sampling Technique and Sample Size Determination

Before the commencement of the actual study, preliminary data were sourced from the respective District Agricultural Office and dairy cooperatives to document the lists of dairy farm in to large scale, medium scale, and small scale dairy farm to estimate the size of study population. The Study areas were purposively selected and identified based on transport accessibility, geographical location and on the abundance of dairy farms to get more calves. Clinically diseased and non-diseased calves were sampled for isolation and characterization of rotavirus. The calves from the seven large scale dairy farms namely; Genesis farm, Asterwaqu dairy farm, Mama dairy Farm, Sisay dimma dairy farm, Haddish dairy Farm, Fantu dairy farm, Holeta agricultural research center dairy farm and a representative random sample of calves from 680 medium and small scale dairy farm were selected for the study. The sampling units were both local and crossbred dairy calves aged between birth and 1 month. Farms were categorized in to small, medium and large based on the herd size of (5-20), (21-50), and greater than 50 heads of cattle, respectively. In larger farms, a minimum of 10% of the all calves in the farm were sampled.

Considering individual members of dairy cooperatives in each study location as a cluster, cluster sampling method was used to select calves from medium and small scale dairy farm. In this study, sampling frame for study herds was taken from the dairy cooperatives located in Bishuftu, Sebeta, Addis Ababa and Holeta. A total of 680 medium and small scale dairy producers were registered in the dairy cooperatives of study areas. Accordingly, 170 dairy producer were sampled by using systematic random sampling technique (every 4^{nd} dairy producer) from the documented sampling frame. When a selected dairy farmer did not have calf or no pregnant cows with due calving date in the six month cohort period, it was then replaced by another dairy farmer mostly from the nearby area. Sample size for cluster sampling was determined by adjusting the sample size calculated for simple random sampling. The adjustment is the function of average cluster size and intraculster correlation, and mathematically expressed as follows;

$n' = n[(1+ ((m -1)*\rho)]$

Where; **n'** = sample size for cluster sampling

n = sample size calculated for simple random sampling

m = average cluster size

ρ = intracluster correlation

However, in the present study the average herd (cluster) size (calves per each dairy farm) was 1.6. As clustering was found small, the effect of intracluster correlation would be small and **n'** would approximate **n**. So the sample size calculated for random sampling was taken directly to be the sample size for this study. To estimate the prevalence of bovine rotavirus in calves, sample size was determined by using simple random sampling method (Martin *et al.,* 1987; Thrusfield and Christley, 2018).

p= Expected prevalence

$n = \frac{1.96^2 (p)(1-p)}{d^2}$ d= Desired level of precision (5%)

n= Sample size

Using expected rotavirus prevalence 16.7% in central Ethiopia (Abraham *et al*., 1992), confidence level of 95% and required absolute precision of 5%; a total of 214 sample size was determined for medium and small scale dairy farm of selected study area. However, a total of 245 calves were enrolled during the study period to enhance precision and to compare prevalence across different herd sizes. Of which, 214 calves were from 680 medium and small scale dairy farm and 31 calves from seven large scale dairy farms.

Collection of fecal samples

Fecal samples were collected in sterile tube after cleaning of the anal area with a paper towel and beats by rectal stimulation with the index finger using disposable sterile plastic gloves (Ammar *et al.,* 2014). Approximately 30 grams of fecal material was collected directly from the rectum of calves using disposable latex glove. Collected samples were placed into universal ice box containing ice packs and transported to the virology laboratory at National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta and were stored at -80 °C until processing.

Fecal Sample Processing

Two hundred forty five fecal specimens obtained from calves with diarrheic and non-diarrheic were submitted to the National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta, from November 2018 to May 2019. Fecal samples were prepared as a 10% (wt/vol) suspension of feces in 0.01 M phosphate buffered saline (PBS; pH 7). All samples were centrifuged at 1,500 x g, and the supernatants were tested and then stored in sterile vials at -80°C for further study.

Laboratory Techniques

Detection of bovine coronavirus and bovine rotavirus antigen by ELISA

Multiscreen Ag ELISA Calf digestive (BIO K 314/1, Belgium) is the type a sandwich ELISA capturing mixture of monoclonal antibodies (MAbs) against bovine coronavirus (BCoV) and bovine rotavirus (BRoV) was used to detect BCoV and BRoV Ag in the fecal suspensions. These antibodies capture the corresponding Ag in the fecal samples. The sandwich ELISA procedure was performed according to the manufacturer instruction (Kit reference BIO K 314/1, Belgium).

The ELISA was performed to detect coronavirus and rotavirus Ag in the fecal samples. The 96 well plates provided in the kit contained two different capture antibodies. Rows C, E, D, F, H and G were coated with coronavirus and rotavirus specific capture antibodies and rows A and B coated with non-specific antibodies, which acted as controls (positive and negative control). These control rows allow the

differentiation between specific immunological reaction and non-specific bindings so as to eliminate false positives.

Feces were diluted in the dilution buffer provided in the kit. A volume of 100µl of diluted sample was added to corresponding wells of specific and non-specific antibody coated rows, respectively. Similarly both the positive and negative controls were added to their respective well per plate. The plate was incubated at 25°C for 0.5 hour and washed 3 times with washing solution (diluted in the ratio 1:20 with distilled water) provided in the kit. Ready to use conjugate of coronavirus and rotavirus specific monoclonal antibody labelled peroxidase was used as such and poured in 100µl quantities per well. The plates were covered with a lid and incubated at 25°C for 0.5 hour in a dark room and washed three times with the provided washing buffer. Then 100 µl of the chromogen (tetramehtyl benzidine) solution added to each well on the plate. The plates were then incubated for 10 minutes at 25°C without covering and away from direct light.

Finally the reaction was stopped by adding stop solution (1M phosphoric acid) provided in the kit. The optical density was measured at 450nm after stopping the reaction with 50µl per well of stop solution. The optical density was measured at 450 nm using an ELISA plate reader at 450nm immediately after stopping the reaction with the stop solution. The test was validated using the positive control and data sheet provided by the kit. The net optical density of each sample was calculated by subtracting the reading for each sample well from corresponding negative control. Net optical density (O.D.) = (O.D. of specific binding -O.D. of non-specific binding). The ELISA reader was used to transfer optical density values to excel spread sheet of computer connected to the reader. Even positive and negative reaction results in ELISA was decided based on color changes after adding stop solution as well as calculating the optical density value. Blue color changed to yellow after adding stop solution was recognized as positive and optical density value was > 0.15 Elisa units (EU) for positive and < 0.15 Elisa units (EU) for negative to both bovine coronavirus and rotavirus.

Extraction of bovine coronavirus and rotavirus RNA

Coronavirus and Rotavirus RNA were extracted from the fecal suspension using QIAamp viral RNA mini kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions. About 1g of fecal sample was added to 1ml of phosphate buffer saline (PBS). The mixture was vortexed vigorously for 40 seconds followed by centrifugation at 10,000 rpm for 5 minutes. All the supernatant (about 500µl) was transferred to new tubes.

Briefly, 140 µl of original fecal supernatant was added in to 560 µl buffer AVL-carrier RNA in the microcentrifuge tube, vortexed for 15 seconds to ensured efficient lysis and homogeneous solution, then the incubated at room temperature (15-25°C) for 10 minutes to lysis viral particle. The solution was centrifuged to remove drops from the inside of the lid then 560 µl ethanol (96%) was added in to the sample, mixed by vortexing for 15 seconds and again centrifuged the tube to remove drops from inside the lid. Then 630 µl from the solution was taken and pipetted in to the QIAamp Mini column and centrifuged at 8000 rpm for 1 minutes and the filtered was discarded. This action was repeated twice.

Then 500 µl buffer AW1 was added to QIAamp Mini column and centrifuged at 8000 rpm for 1 min. The filtrate was discarded and the column was placed in a fresh 2ml collection tube .Then 500µl of buffer AW2 were added to the column then centrifuged at 14,000 rpm for 3 min and the filtrate was discarded. Carefully the QIAamp mini column was opened and added 60 µl Buffer AVE equilibrated to room temperature. Then 65µl of Buffer AVE was added to the column, equilibrated at room temperature for 1 minute then centrifuged at 8000 rpm for 1 minute. Finally, the nucleic acid of the virus was obtained. A double elution using 2 x 40 µl Buffer AVE was performed to increased yield. Final extracted viral RNA was stored in -80^{0}C for further processing.

Viral isolation

All ELISA positive fecal samples were taken forward for virus isolation. Approximately 1 gram fecal sample was mixed with 9 ml sterile PBS containing antibiotic. The fecal suspension was then centrifuged at 800 rpm for 15 minutes. The supernatant fluids contain coronavirus and rotavirus positive were filtered through 0.45 µm membrane syringe filter and filtrates were mixed with an equal volume of Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal calf serum (FCS) and 10 µg/ml crystalline trypsin and incubated at 37°C for 60 minutes. After incubation, one ml of the mixture was inoculated into the culture flasks with confluent monolayer of Madin Darby bovine kidney (MDBK) cell lines and kept for 1hour incubation to adsorption virus. After the adsorption at 37°C for 1 hour, the cells were washed three time with plain of DMEM maintenance media and incubated at 37°C in a humidified incubator having 5% CO_2. Monolayers were observed daily for development CPE for five days and viruses were sub-cultured blindly every two days after being subjected to 3 cycle of freezing and thawing. CPE was observed after 48 hours in positive case and it was characterized by a destruction of the monolayer cell, cell rounding and infected cells were disrupted and detached from the flask. Cells showing characteristic CPE were harvested by freezing and thawing thrice and centrifuged at 16,000 rpm for 20 minutes at 4°C for the removal of cell debris. The supernatant containing the virus was collected and stored at -80°C for further passages. If no CPE was observed, the sample was considered as ‘no virus detected’ (NVD) and the culture was frozen at -80°C, then thawed and centrifuged at 3,000 rpm for 10 minutes to collect supernatant for second blind passage (P2). This was repeated for third passage (P3); and if no CPE was observed on the third passage after 48 hours inoculation, then the sample was considered negative for both coronavirus and rotavirus.

Reverse transcription polymerase chain reaction (RT- PCR)

The cDNA synthesis were performed with a RT-PCR Kit (QIAGEN), according to manufacturer's instructions, for the confirmation of bovine coronavirus and rotavirus A and random primers in a 25 uL final reaction volume. The cDNA of each sample was screened separately for the BCV and BRV genome using the primers described in Table 1 based on the previous study *(Park et al., 2006; Tsunemitsu et al*., 1999). PCR reactions were performed according to manufacturer instructions.

Optimized reaction mixture for RT-PCR was dsRNA 2.5 µl, PCR buffer 2.5 µl, dNTPs 2.5 µl, MgC_2 2.5 µl, Forward Primer (10 pmol) 3.0 µl, Reverse Primer 10µM (6µl), DNase/RNase free water 6 µl. 2.5µl of viral dsRNA were denatured at 95°C for 5 minutes and chilled immediately for 5 minutes. Then, reaction was carried out under the following conditions. RT-PCR was carried out with an initial reverse transcription step of 60 minutes at 42^0C, followed by PCR for activation at 94^0C for 15 minutes, 40 cycles of amplification (30 seconds at 94^0C, 45 seconds at 55^0C (annealing), and 45 seconds at 72^0C (extension)), with a final extension of 7 minutes at 72^0C. To analyze the PCR product, agarose gel electrophoresis was performed. For this, 1.5% gel was prepared and 1 µl of 100 base pair (bp) ladder along with the PCR product was run at 110 volts for 45 minutes. The size of the PCR product for gene segment was illuminated in a gel documentation system and a photograph was taken.

Data Management

The collected data were entered in Microsoft Excel. The contingency table was used at 5% significance to assess the differences among the proportions of fecal samples positive to coronavirus and rotavirus variables such as age group and sex of the animals studied by using Chi Square. Quantitative data was coded and entered in a computer spread sheets and the R software was used for the data analysis.

Results

In total, 245 diarrheic and non-diarrheic fecal samples of calves and their relevant herd and farm level information were collected and analyzed to determine the prevalence of bovine coronavirus and rotavirus infection in dairy calves, in central part of Oromia, Ethiopia. Among 245 calves, 83 (33.88%) had diarrhea and 162 (66.12%) had no diarrhea at the time of sampling (Table 2). The overall prevalence of coronavirus and rotavirus infection in calves less than four weeks of age was estimated as 0.4% (1/245) and 2.45% (6/245) by antigen capture sandwich ELISA in selected dairy farm, respectively (Table 2).

Table 2: Frequency distribution of Bovine coronavirus (BCoR) and Bovine rotavirus (BRoV).

Factors	Level		BCoV		BRoV	
		N	-Ve	+Ve	-Ve	+Ve
Location	Bishoftu	60	60	0(0%)	58	2(3.3%)
	Addis Ababa	123	122	1(0.8%)	120	3(2.4%)
	Sebeta	25	25	0(0%)	24	1(4%)
	Holeta	37	37	0(0%)	37	0(0%)
Clinical status	Diarrheic	83	82	1(1.2%)	77	6 (7.2%)
	Non Diarrheic	162	162	0(0%)	162	0 (0%)
Breed	Cross	24	24	0(0%)	23	1(4.2%)
	Local	72	72	0(0%)	70	2(2.8%)
	Exotic	149	148	1(0.7%)	146	3(2%)
Sex	Male	81	80	1(1.2%)	76	5(6.2%)
	Female	164	164	0(0%)	163	1(0.6)
Age	1st week	100	100	0(0%)	96	4(4%)
	2nd week	106	105	1(0.9%)	104	2(1.9%)
	3rd week	18	18	0(0%)	18	0(0%)
	4th week	21	21	0(0%)	21	0(0%)
Floor of the calves area	Concrete	181	180	1(0.6%)	177	4(2.2%)
	Brick	58	58	0(0%)	56	2(3.4%)
	Muddy	6	6	0(0%)	6	0(0%)
First colostrum feeding after birth	Within 30 minutes	132	132	0(0%)	131	1(0.8%)
	Within 2 hours	103	103	1(1%)	98	5(4.9%)
	Within 2-6 hours	10	10	0(0%)	10	0(0%)
Separation of calves from dam	Immediately after birth	29	29	0(0%)	27	2(6.9%)
	<24 hours	71	71	0(0%)	71	0(0%)
	>24 hours	145	144	1(0.7%)	141	4(2.8%)
Total		**245***	**244***	**1(0.4%)***	**239***	**6(2.4%)***

BCoV (Bovine coronavirus), BRoV (Bovine rotovirus), N (Number), -Ve (Negative), +Ve (Positive), (%) represents the percentage of the total number of cases, *, indicate the total of each parameter.

When the results was calculated separately for the two groups of calves (i.e. diarrheic and none diarrheic calves), a prevalence of 1.2% (1/83) of coronavirus and 7.23% (6/83) of rotavirus were observed in diarrheic calves and all none diarrheic samples were negative for both (0/162, Table 2).

Table 3. Evaluation of the association between BRoV, diarrhea, and other variables (age, breed, sex, location, clinical status, means of offering and separation from dam).

	OR	95% CI		*P-value*
(Intercept)	7.323523e-10	NA	2.866901e+112	0.9945
Age	3.459461e-01	3.412473e-02	1.933487e+00	0.2766
Breed	1.745811e+00	2.988061e-01	1.442418e+01	0.5594
Location	6.751928e-01	2.397201e-01	1.824273e+00	0.4238
Clinical Status	6.908461e+08	2.019593e-100	NA	0.9947
Sex	2.896347e-02	3.678763e-04	3.915444e-01	0.0358 *
First colostrum	7.470232e+00	1.001086e+00	9.470379e+01	0.0691
Separate dam	3.066077e-01	4.208658e-02	1.596692e+00	0.1914
Floor area dam	1.401148e+00	1.281755e-01	9.936624e+00	0.7404

NOTE: P value - Level of significance. Significant when P-value ≤0.05. OR (odd ration), 95% CI (95% confidence Interval).

Distribution of antigen positive samples corresponding to ages, breed and sex of calves were shown in Table 2. The results indicate a prevalence for calves in the first and second weeks of ages was 4% and 0.9% for rotavirus and coronavirus, respectively. This shows that new born calves of 1-2 weeks of age were more susceptible to coronavirus and rotavirus infection. But the observed coronavirus and rotavirus prevalence in different calve ages were not significant (P> 0.05) (Table 3).

Current research showed that the males were more susceptible to coronavirus and rotavirus infection as compared to female calves. A higher prevalence of 1.2% (1/81) of coronavirus and 6.2% (5/81) of rotavirus were associated with male calves, while a prevalence of 0% (0/163) and 0.6% (1/164) was recorded in female calves, respectively. The prevalence were significant for only rotavirus between male and female calves. A higher prevalence of coronavirus and rotavirus Ag was observed among calves fed colostrum's from 30 minutes to 2 hours compared to calves given colostrum's within 30 minutes of birth. New born calves of exotic breed cows were more susceptible to coronavirus and rotavirus infection than

local breed. The prevalence was not statically significant (P> 0.05) for breed and between times of colostrum feeding. The current study indicated that the prevalence of coronavirus was higher in Addis Ababa (0.8%) and rotavirus infection were higher Sebeta (4%) as compared with other selected site. The prevalence was not significant (P> 0.05) between the selected locations (Table 3).

Calves floor area was concrete, more susceptible to coronavirus and rotavirus infection than the calves floor area was brick or muddy. The coronavirus and rotavirus prevalence was not significant (P> 0.05) between the calves floor area. The result indicated that 6.9% calves separated immediately after birth from dam were found positive for rotavirus, whereas coronavirus and rotavirus were detected in 0.7% and 2.6% samples of calves separated greater than 24 hours after birth from dam, respectively (Table 2). The prevalence's were not statically significant (P> 0.05) for coronavirus and rotavirus between the times of separations of calves from dam (Table 3). See Table 2 for stratified prevalence of other variables.

In this study, MDBK cell line was used to isolate the virus from all samples of Ag-ELISA test positive samples. Out of 7 samples of both coronavirus and rotavirus cultured on MDBK cell line, CPE was observed in 5 (1 coronavirus and 4 rotavirus) samples, while CPE was not observed on the remaining 2 samples of rotavirus even on third blind passage. In the first passage, infected cells did not show any CPE. But from second passage onwards the infected cells started showing characteristic CPE. At 24 hours post infection (p.i,) the infected cells became round and clumped. At 48 hours p.i, the cells were thin and round shaped. At 72 hours p.i, the cells became small and majority of monolayer detached.

Out of 7 positive samples, 5 (1 sample of coronavirus and 4 samples of rotavirus) samples were screened by RT-PCR for molecular characterization due to the non-availability of sufficient quantity of fecal sample in the remaining samples. The Ag-ELISA negative calves sample and negative on cell culture did not amplify by PCR. After RNA extraction and RT-PCR were done for amplification gene of rotavirus and coronavirus. Out of the 5 (1 sample of coronavirus and 4 samples of rotavirus) fecal samples of coronavirus and rotavirus examined by RT-PCR technique, all samples were identified as positive (100%) for RT-PCR test.

Discussions

Newly born calves are an important source in livestock production in worldwide for meat or breeding i.e. replacement stock (Radostits *et al.,* 2007). These industry faces many series disease problems like calf diarrhea, which usually affect it dramatically. Calf diarrhea is a prime disease affecting newborn calves leading to morbidity and mortality in newborn calves, causing economic losses due to the costs of treatment, diagnostics, weight loss or death in infected animals and poor growth performance. A crucial period for these calves is the first few days following birth. In developing countries like Ethiopia, domestic animals are the major income source for poor families. These families suffered badly due to the neonatal calf mortality curse. Among numerous viral, bacterial and parasitic causative agents, bovine coronavirus and rotavirus is the foremost cause of neonatal calf diarrhea in domestic animals. The cause of neonatal calf mortality is specifically related to bovine coronavirus and rotaviruses (Estes and Kapikian, 2007).

Fecal contamination plays an important role in transmission of coronavirus and rotavirus infection and the infection are widespread globally in cattle populations. For the effective control measures, prompt diagnosis of the disease is important (Dhama *et al.,* 2009).

In the present study, 1 of 245 (0.4%) and 6 of 245 (2.4%), fecal samples screened using Ag-ELISA were positive for coronavirus and rotaviral infection respectively, all none diarrheic samples were negative for both (0/162).

Out of the 83 diarrheic samples 1(1.2%) was found positive for coronavirus by ELISA. Other studies also revealed that prevalence of corona virus in neonatal calf diarrhea is slightly lower than that of rotavirus However, there is paucity of literature stating the corona virus prevalence status in Ethiopia. This study is agreement with the reported of Dash *et al., (2011)* in India (4.76%). Other most reports were dis agree with the current result.

A prevalence of 7.23% (6/83) of rotavirus were observed in diarrheic calves in current study. This result is in agreement with those reported by Prez *et al.* (1998) in Costa Rica (7%), Duman and Aycan (2010) in Turkey (8.5%), Yilmaz (2016) in Turkey (8.92%), and Rajendran and Kang (2014) in India (5.5%). Higher prevalence rate of rotavirus have been reported from many countries including Abraham *et al*. (1992) in Ethiopia (16.7%), Ammar *et al.* (2014) in Algeria (14.63%), Kyle (2007) in Vietnam (15%) , Pisanelli *et al.* (2005) in Southern Italy (16.8%), Jindal *et al.* (2016) in India (27.02%) and Uhde *et al.* (2008) in Switzerland (58.7%). However, the result of this study is higher when compared to that reported by Fiedler *et al.* (1982) in Oldenburg (1.96%). The discrepancy of results could be attributed to the age and the sample size difference. Prevalence of rotaviral infection varies depending on the country and region under study (Basera *et al.,* 2010; Radostitis *et al.,* 2007). All the 6 rotavirus positive samples were from diarrheic calves under the age of 4 weeks. Similar results were recorded by Sharma (2004) in bovine calves.

The current result could suggest that male calves (6.2%) were higher susceptible for rotavirus infection than female calves (0.6%). Other studies like Dash *et al.* (2011) and Sharma (2004) also reported higher susceptibility of male bovine calves (20.37%) and (42.85%) in comparison to female calves (12.76%) and (28.2%), respectively. The possible justification for this could be due to immune system in that Odde (1988) reported higher anti-rotavirus IgG concentrations for female calves compared to male calves. It could be due to the managemental practices as in most of the dairy farms female calves are better looked after than male calves. Previously, Ammar *et al.* (2014) and Dash *et al.* (2011) also reported higher susceptibility of male bovine calves in comparison to female calves against rotavirus infection. In line with this, Clement *et al*. (1995) noticed that males calves were more susceptible to diarrhea as compared to female calves.

Age wise, the susceptibility of newborn calves of 1st week up to 2nd weeks of age to rotavirus infection were more than older calves. The occurrence of rotavirus in the fecal samples of diarrheic calves was found to decrease with increase in the age of the calves. The finding of the present study is in agreement

with the earlier workers reported by Abraham *et al.* (1992), Jindal *et al.* (2000) and Singh *et al.* (2009), higher occurrence of rotavirus infection in diarrheic calves were mainly restricted to the first 2 weeks of life. Maximum prevalence of rotavirus diarrhea was observed in 5-21 days old calves (Radostits *et al.,* 2007). The 2 weeks old calves were the most susceptible to rotavirus infections, which may be due to decreasing of passive immunity and the absence of the natural resistance against infection. The 3-weeks-old calves are characterized by absence of rotavirus, this may be highlighted by an increased natural resistance against infection (Ammar *et al.,* 2014).

The results showed that the prevalence was slightly higher in the Sebeta (4%) than in Bishoftu (3.3%) and Addis Ababa (2.4%) towns. This could be attributed to sample size of the areas and presence of higher number of factories in the near farm that can be a source of contamination for animals. In the present study higher prevalence was recorded in crossbred calves (4.2%) than local calves (2.8%) that similar to previous report of Sharma (2013).

In the present study, viral growth in cell culture was assessed by examining inoculated cells for CPE. Out of 7 samples, only 5 (1 sample of coronavirus and 4 samples of rotavirus) of the Ag-ELISA positive samples established infection in MDBK cells as determined by production of characteristic CPE on the second passage and it continued up to third passages. The CPE observed were characterized by rounding, detachment as well as destruction of mono-layer cell. The CPE produced in this study were in agreement with previous reports (Saravanan *et al.,* 2006).

The RT-PCR technique confirmed the presence of coronavirus and rotaviruses in fecal samples that were previously diagnosed by ELISA and growth in cell culture. The RT-PCR-based genotyping method used was further confirmed to be a useful epidemiological tool and to determine the presence of coronavirus and rotavirus nucleic acid by using specific generic primers for each virus regions in feces samples (Desselberger *et al.,* 2003).

Conclusion And Recommendations

The present investigation was undertaken to investigate the prevalence, isolation and characterization of coronavirus and rotavirus in calves lessen than one month of age at different selected farms in central Oromia. The effect of age, sex, breed and house floor of calves on prevalence of diarrhea was also studied. Using Ag-ELISA, 7 samples (1 coronavirus and 6 rotavirus) were identified as positive, and all of the isolates were obtained from diarrheic calves in the 1^{st} and 2^{nd} weeks of age. The result indicated that there is an association between rotavirus detection and sex of calves in that the prevalence of rotavirus is higher in male calves than female calves. In addition, the prevalence is higher in calves kept in concrete floor, this fed colostrum later (within 2 hours) as well as in local bred calves and in calves separated from their dams immediately after birth. When the 7 Ag-ELISA positive samples were cultured on MDBK cell line, only 5 samples showed cytopathic effect (CPE).

Observational study and questionnaire survey also indicated that only awareness of the advantage of colostrum feeding is not enough, but also times of colostrum administration to neonate calves are crucial for the ultimate development of immune status against pathogens including coronavirus and rotavirus infection. Calving areas should have well-drained grass lots or pastures visible from the barn area and calving areas should be selected or landscaped to allow for adequate drainage. Enteric disease like coronavirus and rotavirus infection is a vital health problem in calves that interrupts production benefits with reduced weight gain and increased mortality, and the virus potential for its zoonotic spread, it is imperative to determine the disease burden and responsible risk factors. This is very useful to execute effective preventive measures such as practicing early colostrum feeding in newborn calves, vaccination in dams and improving livestock management.

Based on the above conclusion the following recommendations were forwarded:

- Awareness creation for researcher and government regarding the effect of coronavirus and rotavirus infection in calves health and growth performance and national economy is very important.
- Further study of coronavirus and rotavirus infection in calves covering larger areas of the country need to be conducted so that representative information of the circulating strains cloud be generated and understood. Availability of such data are critical for control of the disease.

List Of Abbreviations

Ag Antigen

Ag-ELISA Antigen Capturing Eenzyme-Linked Immunosorbent Assay

BCoV Bovine Corona Virus

BRoV Bovine Rotavirus

cDNA Complementary DNA

CPE Cytopathic Effect

DMEM Dulbecco's Modified Eagle Medium

DNA Dioxy Nucleotide Ribonucleic Acid

DNTPs Dioxy nucleotide Triphosphates

dsRNA double stranded RNA

ELISA Enzyme-Linked Immunosorbent Assay

MDBK Madin Darby Bovine Kidney Cell

mRNA Messenger RNA

NAHDIC National Animal Health Diagnostic and Investigation Center

NSP Non Structural Proteins

NVD No Virus Detected

OD Optical Density

p.i Post Infection

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

pH Power of Hydrogen

RNA Ribonucleic Acid

rpm Revolution Per Minute

RT-PCR Reverse Transcriptase Polymerase Chain Reaction

SHFDO Sebeta Hawas Finance and Economic Development Office

UK United Kingdom

VP Structural Proteins

Declarations

Ethics approval to participate Ethical approval for this study was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture Minutes of Animal Research Ethics and Review committee. Verbal consent was also obtained from the farm managers to take samples from their cattle and for further research use of the samples.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions US: Contributed to conception of the research idea, designing and data collection, data analysis, interpretation of data, writing and editing of the manuscript. FD: Contributed to the study concept, interpretation of data, editing or reviewing of the manuscript. MA: Contributed to conception of the research idea. AT: Contributed to the data analysis, interpretation of data, writing and editing of the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

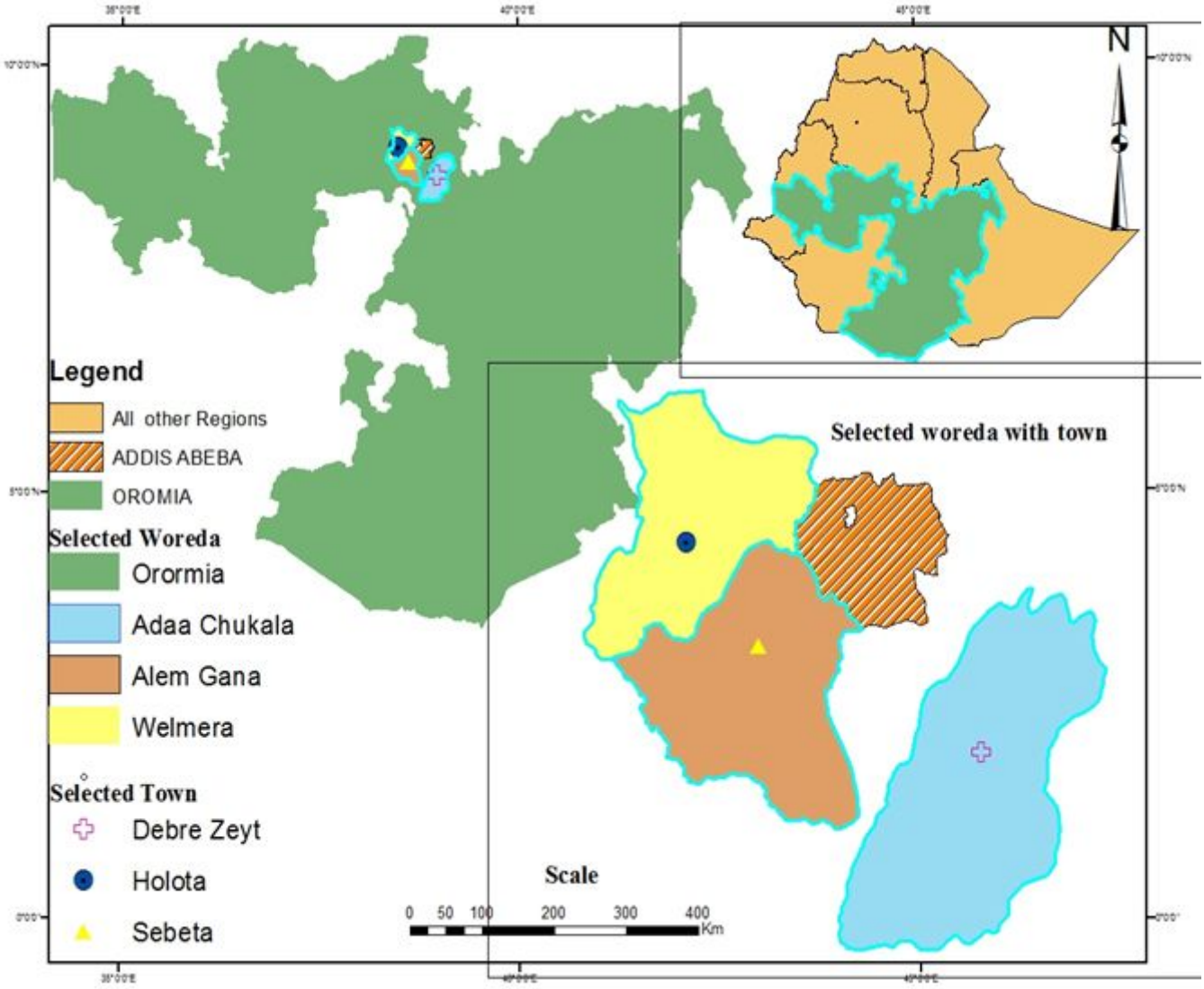

Figure 1

Generated by Umer Seid (corresponding author). To clearly show map of study area and sampling sites. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

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