



Development of real-time RT-PCR assay for diagnosis of viral enteritis in neonatal goat kids

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Received: 13 August 2019; Accepted: 3 September 2019

ABSTRACT

Rotavirus gastroenteritis is a worldwide disease affecting primarily infants, young children and young ones of wide variety of mammalian and avian species. Diarrhoea in goat kids is most frequently found associated with Group A rotavirus (GARV) and another enteric pathogen bovine coronavirus (BCoV), a major viral pathogen associated with neonatal diarrhoea. Enteric BCoV replicates in epithelial cells of gut, destroying villi, resulting in severe, often bloody diarrhoea in calves. It requires highly sensitive and specific assays to diagnose the disease at field level. In the present study, a real-time reverse-transcriptase (RT) polymerase chain reaction (PCR) were developed and validated for specific detection and quantification of GARV and BCoV with high sensitivity and specificity. For real-time RT-PCR, primers were designed to target nucleocapsid gene for BCoV; NSP4 gene and VP6 gene were designed for GARV using discontinuous conserved sequences. Real-time RT-PCR assay was standardized by serial dilution of positive GARV and BCoV RNA. The rotavirus real-time RT-PCR assay was found to be specific to rotavirus, but broadly reactive to GARV. The sensitivity of the assay for detecting rotavirus and BCoV in faecal samples and tissue sample was found to be high in such reactions. The real-time RT-PCR assay was effective in detecting GARV and BCoV in all positive samples obtained from sheds, farms and outbreaks. The results of this study demonstrate that the real-time RT-PCR assay for viral enteritis is broadly reactive, specific, and sensitive for detection of GARV and BCoV in faecal sample and tissue samples.

Key words: BCoV, GARV, NSP4 gene, Nucleocapsid gene, Real-time RT-PCR, VP6 gene

Neonatal enteritis is an important disease that causes mortality in young age with significant economic losses. Although the neonatal diarrhea is common, there is still little knowledge about pathology, pathogenesis and immunohistochemical localization of viral agents that cause neonatal enteritis in goat kids. Acute gastroenteritis is caused by a number of viruses, including rotavirus, norovirus, astrovirus, and bovine corona virus (Logan *et al.* 2006; Anderson and Evan, 2010; Maarseveen *et al.* 2010). Transmission is via the fecal–oral route and clinical manifestations range from subclinical infection to varying degrees of fever, diarrhea, and vomiting (Elliott, 2007; Anderson and Evan, 2010). Rotaviruses are dsRNA viruses, and have a segmented genome leading to frequent reassortment (Estes and Kapikian, 2007; Greenberg *et al.* 2009). Serogroups A–C are known to infect humans with

the majority of infections caused by serogroup A (Estes and Kapikian, 2007; Greenberg *et al.* 2009). Bovine coronavirus, of the family *Coronaviridae*, produces a more severe, long-lasting disease compared to rotavirus.

Reduction in goat kid mortality is vital to the economic goat husbandry and is critical for the fast growth of goat industry in the country. The variable mortality rates for small, medium and large categories of goat keeping were 32.8, 32.1 and 15.8% in kids (Kumar, 2006).

Previously, realtime PCR based molecular detections using SYBR green chemistry were developed to detect the group A rotaviruses with higher efficiency in animals (Schwarz *et al.* 2002), and a combined assay for detection of group F adenovirus, rotavirus A, and rotavirus C from stool specimens with the efficiency of 155% compared to electron microscopy (Logan *et al.* 2006). Similarly, TaqMan® probe-based real-time reverse transcriptase (RT) polymerase chain reaction (PCR) assay was developed by Jothikumar *et al.* (2009) to target the non-structural protein region 3 of rotavirus.

Molecular diagnostics offer faster and more accurate reproducible results compared to conventional assays and Real-time PCR offers improved sensitivity and specificity (Yuan *et al.* 2006). We hereby report a real-time PCR based

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diagnostic assay developed for enteric viruses in neonatal goat kids.

MATERIALS AND METHODS

Collection of clinical samples (Fecal samples): A total of 304 diarrhoeal and non-diarrhoeal faecal samples of animals (goat kids and lambs) were collected from Jamunapari, Barbari and Jakrana shed, Experimental shed of Division of Animal Health, ICAR–Central Institute for Research on Goats (CIRG), Makhdoom, Farah, Mathura (Uttar Pradesh) from October, 2016 to March 2019. Samples were collected between the age group of 0–3 months. Out of 304 faecal samples, 254 were diarrhoeal samples, goat kids (232), lambs (22) and 17 tissue samples from different outbreaks and farms. The swab samples were suspended in 2.0 ml sterile double glass distilled water and stored in microtube tubes at 4°C for RNA isolation.

Extraction of RNA: Rotaviral dsRNA extraction with TRIzol reagent was carried out as per the manufacturer's protocol. The RNA pellet was resuspended in 30 µl nuclease free water (NFW), followed by incubation in water bath set at 55–60°C for 10–15 min. The isolated RNA was then used for downstream application including cDNA synthesis or stored at –20°C till further use.

SYBR green real time PCR for GARV detection: The qRT-PCR assay was performed with SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit and CFX96 Real-Time PCR System operated by CFX™ Manager software. The qRT-PCR reaction was run in duplicates, each in a total volume of 20 µl. Primers (Table 1) were designed for the amplification of VP6 gene and NSP4 gene of GARV and Nucleocapsid protein gene for BCoV.

RESULTS AND DISCUSSION

Real-time RT-PCR was standardized and developed for detection of GARV and BCoV in diarrhoeic samples of small ruminants. For GARV we targeted two genes, viz. VP6 and NSP4, and for BCoV, NC gene was targeted. Out of 304 diarrhoeic faecal samples, 94 diarrhoeic neonate fecal samples were tested for GARV VP6 gene in which 5 samples were positive, and out of 74 tested by NSP4 real-time RT-PCR, 4 were positive. These samples were detected with very high sensitivity and specificity for GARV. While NC real-time RT-PCR for detection of BCoV revealed 3

positive out of 94 samples tested.

Detection and quantification of Group A rotavirus targeting VP6 gene: Molecular assay were used for targeting VP6 gene in 94 samples and based on RFU cut-off and melt curve analysis, the positive samples were detected (Fig. 1). Real-time PCR testing of diarrhoeic faecal samples from 94 animals (goat kids-89 and lambs-5), revealed positivity for rotavirus in 5 samples. Thus, the qRT-PCR assay could even detect the rotaviral infection in some of the samples that were negative in conventional RT-PCR, thereby, showing its higher sensitivity compared to the latter.

Detection and quantification of Group A rotavirus targeting NSP4 gene: Molecular assays were used for targeting NSP4 gene in 94 samples and based on RFU cut-off and melt curve analysis, the positive samples were detected (Fig. 2). Real-time PCR testing of diarrhoeic faecal samples from 74 animals (goat kids-69 and lambs-5), revealed positivity for rotavirus in 5 samples. Thus, the qRT-PCR assay could even detect the rotaviral infection in some of the samples that were negative in conventional RT-PCR, thereby, showing its higher sensitivity compared to the latter.

Schwarz *et al.* (2002) have reported the detection of as low as 10 RNA molecules of viral RNA of Group A rotaviruses in clinical samples obtained from various animal species and man by fluorimetry using the SYBR Green I dye in a real-time RT-PCR assay. The testing of diarrhoeic faecal samples from 30 animals (calves-23, goat kids-4 and lambs-3), revealed positivity for rotavirus in 25 samples by qRT-PCR assay. The real-time PCR assay could determine genomic copy numbers present in the diarrhoeic faecal samples Manuja *et al.* (2010), which were earlier detected as positive for rotavirus infection by RT-PCR. In addition to this, rotavirus was detected and quantified in six samples, which were otherwise negative by RT-PCR assay. The results are in agreement with published reports which have described real time RT-PCR to be rapid and more sensitive for the detection and quantitation of rotavirus compared to conventional RT-PCR assay (Kang *et al.* 2004; Logan *et al.* 2006; Gutiérrez-Aguirre *et al.* 2008).

Neonatal diarrhoea induced by animal group A rotavirus also causes significant economic loss in the dairy and meat industry due to increased morbidity and mortality, treatment costs, and reduced growth rates. Diarrhoea is a major cause of death for the young calves under one-month-old and kids and lambs between 0–15 days of age (Maes *et al.* 2003). At the same time, there are evidences of interspecies transmission of rotaviruses from one animal species to others, including humans (Palombo, 2002; Cook *et al.* 2004). This interspecies transmission could occur via genetic reassortment between human and animal rotavirus strains during co-infection of the same cell (Varghese *et al.* 2004). In India, although considerable work has been carried out on human rotaviruses, limited data is available on the epidemiology of animal rotavirus strains and even more restricted observations have been reported regarding the genotype specificities of field isolates.

A diagnosis of viral agents can be made by immune

Table 1. Primers used in the experiment

Name of Primer	Sequence (5'→3')
GARV-VP6-F	GCYAGAGACAARATTGTCGAAG
GARV-VP6-R	YCTARTYGGNARRT TACCRATTCTTCC
GARVNSP4_F-RT	GACAGAGTCGTTAAAG AAATGAGACG
GARVNSP4_R-RT	TCCACTCTCCCATTCTTCTAGCG
BCoVNP_F-RT	CATCCCGCTTCACGTATCTCTTG
BCoVNP_R-RT	ACTGCCTAGGA TACAACACGTCC

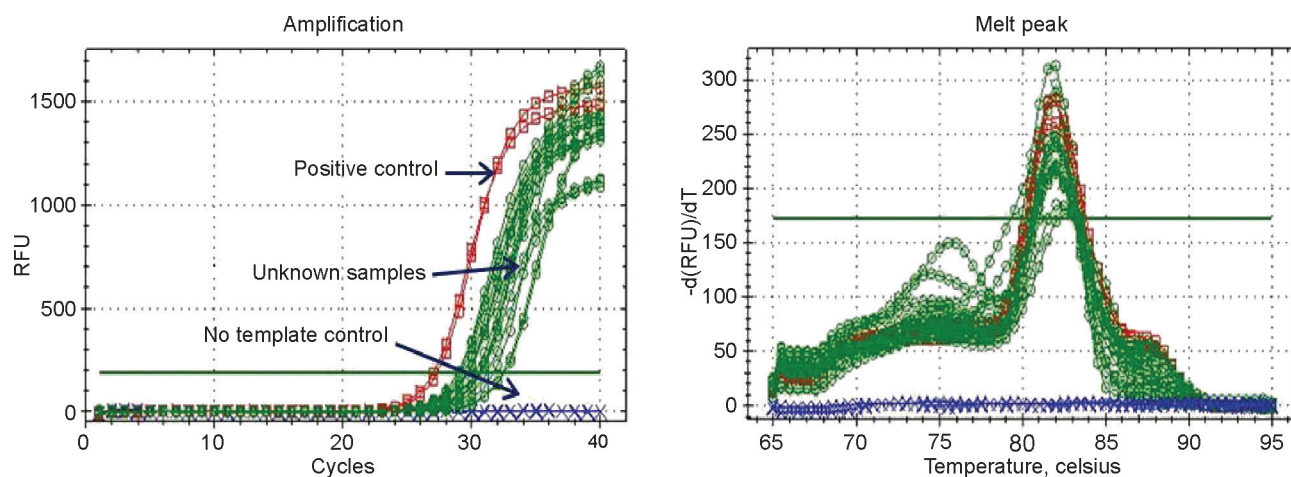


Fig. 1. Cycle quantification and T_m calling of the VP6 gene amplicon of GARV analyzed using SYBR-Green qRT-PCR assay

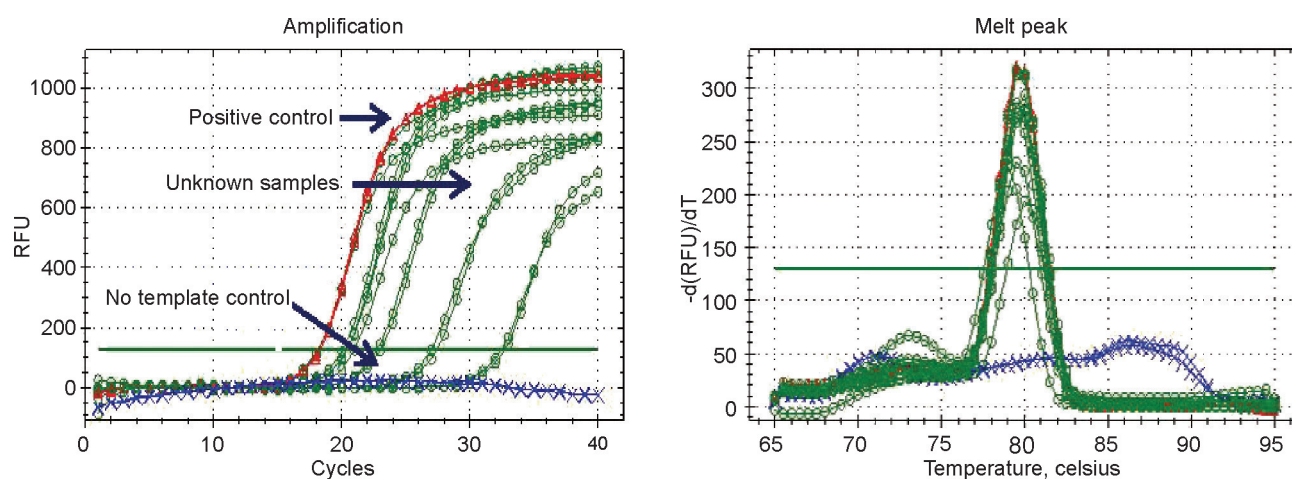


Fig. 2. Cycle quantification and T_m calling of the NSP4 gene amplicon of GARV analyzed using SYBR-Green qRT-PCR assay

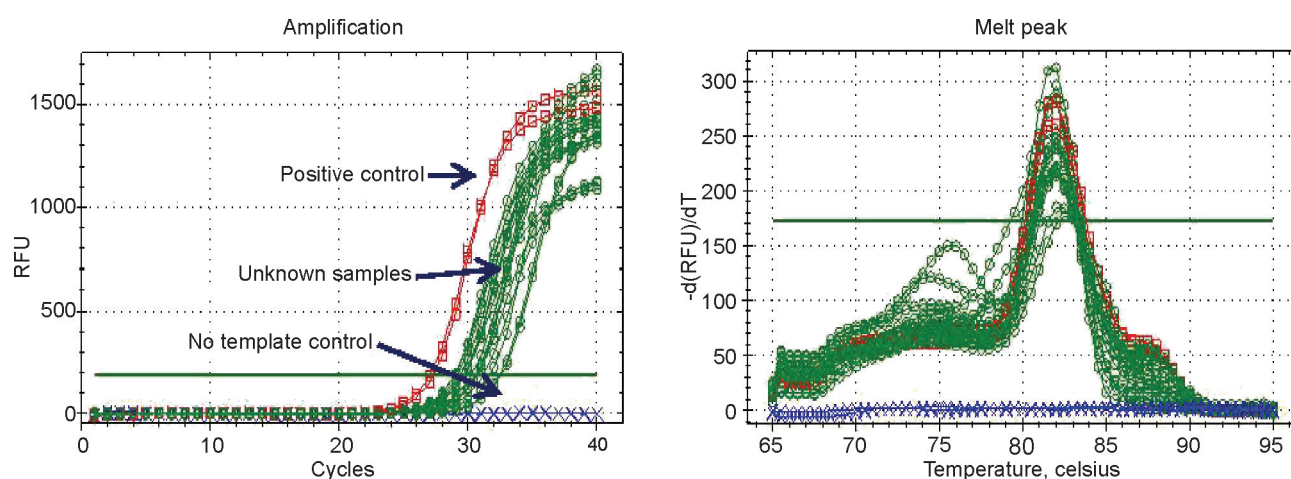


Fig. 3. Cycle quantification and T_m calling of the Nucleocapsid gene amplicon of BCoV analyzed using SYBR-Green qRT-PCR assay.

histochemical examination of tissue fixed in formalin. This study revealed that the most common viral agents in lambs and goat kids neonatal diarrhea was rotavirus. Our current study showed that coronavirus is also an important etiological factor, compared with other viral causes of diarrhea in small ruminants. However, according to a previous study by our group, its incidence has increased and may be an important viral agent in neonatal enteritis in the future. This study showed that viral etiology was very important to occurring of neonatal diarrhea.

Detection and quantification of Bovine Corona Virus (BCoV) targeting Nucleocapsid protein gene: Molecular assays were used for targeting nucleocapsid gene in 94 samples and based on RFU cut-off and melt curve analysis, the positive samples were detected (Fig. 3). Real-time PCR testing of diarrhoeic faecal samples from 94 animals (goat kids-92 and lambs-2), revealed positivity for BCoV in 3 samples. Thus, the qRT-PCR assay could even detect the coronaviral infection in some of the samples that were negative in conventional RT-PCR, thereby, showing its higher sensitivity compared to the latter.

Anamul *et al.* (2015), described study on development and application of a reverse transcription SYBR green real-time PCR assay system for the detection of group A rotaviruses (GARV) in bovines. The examination of clinical samples (n=80) showed consistent diagnostic specificity and sensitivity of the real-time PCR assay in comparison to a routinely used RNA electrophoresis method. The specificity of the assay was confirmed using other enteric viruses with the melting curve analysis of amplified products. The reproducibility of the assay was proved by low values of coefficient of variation in the intra- (1.7%) and inter-assay (2.4%). In comparison to RNA-PAGE screening method which detected 25% (20/80) positive samples for GARV, the newly developed real-time PCR detected 46.25% (37/80) positive samples for GARV, providing higher sensitivity of the assay developed. Reports on development of real time PCR for detection of RVs in animals are limited and in particular from India are not available for the use of the said test for detection of RVs in bovines. The results indicate use of real-time RT-PCR assay as a method of choice for the specific detection of GARV in field samples and particularly in a situation where a number of other clinically resembling infectious agents like picobirnavirus, coronavirus, calicivirus, and astrovirus are known to coincide as mixed infection. In our findings, real time RT-PCR was standardized with SYBR green dye for detection of GARV, GBRV and BCoV.

Feeney *et al.* (2011) reported a study on multiplex TaqMan® assay for detection of viral enteritis. He described the work-up of two internally controlled multiplex, probe-based PCR assays and reports on the clinical validation over a 3-year period, March 2007 to February 2010. Multiplex assays were developed using a combination of TaqMan® and minor groove binder (MGB™) hydrolysis probes. The assays were validated using a panel of 137 specimens, previously positive via a nested gel-based assay. The assays

had improved sensitivity for adenovirus, rotavirus, and norovirus (97.3% vs. 86.1%, 100% vs. 87.8%, and 95.1% vs. 79.5%, respectively) and also more specific for targets adenovirus, rotavirus, and norovirus (99% vs. 95.2%, 100% vs. 93.6%, and 97.9% vs. 92.3%, respectively).

The current study was designed for detection of enteric viruses using molecular assays, to produce accurate, quick with highly sensitive and specific reproducible results. Hence, two genes, viz. VP6 & NSP4 were targeted for GARV and Nucleocapsid for Bovine corona virus. All the assays were set-up using SYBR green Reverse transcriptase real time PCR.

Comparative analysis of GARV VP6 gene, GARV NSP4 gene and BCoV nucleocapsid protein

Neonatal enteritis is an important disease that causes deaths of animals before 3 weeks of age, and results in significant economic losses. Viral agents can predispose the young animals to secondary infections in the gastrointestinal tract, especially in lambs and goat kids younger than 21 days. Although the neonatal diarrhea is common in calves, there is limited knowledge about enteric viruses and their effects including the prevalence and geographic distribution in small ruminants. Hence, in the current study molecular diagnostics were used as a tool to carry out epidemiological investigations with the aim of detecting rotavirus and coronavirus presence goat kids and lambs and its association with viral enteritis.

Neonatal infectious pathologies in goats may be caused by bacteria, viruses, parasites or nutritional deficiencies, often in synergy and usually characterized by high morbidity and mortality. Rotaviruses, Coronaviruses and bacteria, such as *Escherichia coli*, *Salmonella* spp. and *Clostridium* spp., are etiological agents more commonly involved in gastro-enteric diseases in kids (Singh *et al.* 2018a; Singh *et al.* 2018b). In our study, 254 neonatal diarrheic goat kids were studied, of which 47.48% were 0–1 month old and 52.52%

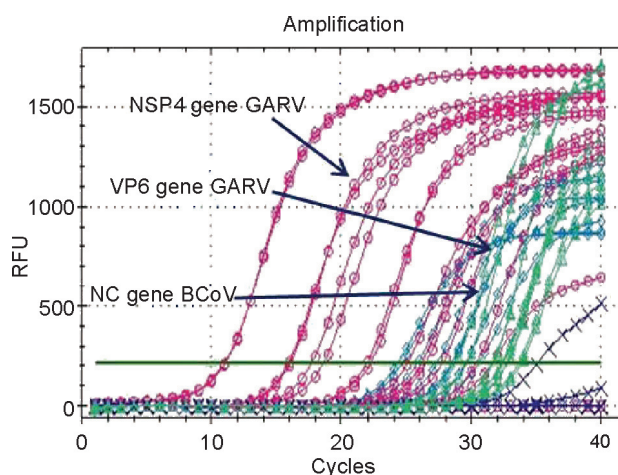


Fig. 4. SYBR green-chemistry based real time PCR assay targeting VP6 gene and NSP4 gene for GARV and nucleocapsid for BCoV showing Cq (cycle quantification or cycle threshold (Ct) of VP6 (Blue), NSP4 (Pink) and Nucleocapsid (NC) (green) in enteric diseases samples of goat kids.

1–3 months of age.

The current study could reveal some important features of the enteric viral infections in small ruminants with respect to various criteria like age, season, gender etc. In conclusion, the real time PCR assays developed for the enteric viruses in the current study were to cater to the higher sensitivity and specificity besides the time involved in conducting the other conventional assays. The GARV were the most common enteric pathogen followed by BCoV and lastly GBRV. The GARV emerged as most important pathogen, that was associated with clinical diarrhoeic cases as evidenced by the conventional RT-PCR assay and the VP6 and NSP4 gene based qRT-PCR. This indicates the fact that GARV needs attention in field conditions leading to diarrhea, slower growth rate and mortality in goat kids, which can only be addressed by vaccination and hygiene measures. Based on the results obtained from the developed real time PCR assays, conclusion could be drawn that there is need to study the epidemiological status of enteric viruses and their pathogenic effects in neonatal kids. Although the GBRV incidence was less than 2%, their importance with respect to clinical diarrhea and its combination with other bacterial pathogens like *Escherichia coli* or *Cryptosporidium* should be explored. The significance of enteric viruses in small ruminants was always not supported by ample data, which the current study could address in a way using the latest tools including qRT-PCR assays for their detection.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, ICAR-Central Institute for Research on Goats, Makhdoom, Mathura and Dean, College of Biotechnology, DUVASU, Mathura, Uttar Pradesh, for providing necessary facilities to carry out the work. The authors are also thankful to CAB in Scheme of ICAR-IASRI and AINP on Neonatal Mortality of Farm Animals (ICAR) for providing financial support to carry out the research work.

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