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Development of a multiplexed Luminex assay for simultaneous detection of enteric viruses in cattle

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Abstract

Viral and bacterial gastroenteritis and diarrhea have long been a problem in livestock with devastating effects on animal health and production causing a heavy financial burden on producers. Therefore, the bead-based multiplex detection assay was created for simultaneous detection of three livestock viral diarrheic agents *viz*. bovine rotavirus (BRV), bovine coronavirus (BCoV) and bluetongue virus (BTV). The primers and probes for triplex MAGPIX assay for simultaneous detection of three enteric viruses were designed and the assay was optimized for hybridization temperature, primer-probe and bead concentrations. The newly developed MAGPIX assay was used to determine the prevalence of these diarrhea-associated viruses by testing 200 fecal samples collected from Haryana state of India during 2018-2019. The limit of detection of the developed triplex assay was 1×10^5, 1×10^4, and 1×10^5 RNA copies for BRV, BCoV, and BTV, respectively, being lower than the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). However, it was higher than the conventional RT-PCR, showing it to be more sensitive. The newly developed MAGPIX assay was a rapid, cost-effective and high throughput diagnostic tool for identification of three major entero-pathogenic diarrhea associated viruses, either alone or in tandem, with the aim to prevent and control viral diarrhea in animals.

Introduction

In the dairy sector, viral enteritis diarrhea is a major and potentially fatal health issue. Diarrhea in newborn calves is caused by a wide variety of viruses, bacteria and protozoan parasites, including but not limited to bovine rotavirus (BRV), bovine coronavirus (BCoV), *Escherichia coli* and *Salmonella* strains, *Cryptosporidium parvum* etc. The three most notable enteric viral infections are bluetongue virus (BTV), BCoV and BRV, all of which cause significant morbidity and economic losses in livestock.[1]

Group A BRV is a non-enveloped, double-stranded RNA virus that has 11 segments and is classified in the genus *Rotavirus* of the family *Reoviridae*.[2] There is a large economic loss for the cattle sector due to calf diarrhea and BRV is the major cause of this. Among BRV strains, genotypes G4–G6–G8–G10–G12 and P1–P5–P11 are predominant. While group A rotaviruses are responsible for 95.00% of BRV infections worldwide, group B and C rotaviruses have also been discovered in instances of BRV infections acquired in the field. It is common to find BRV and BCoV in calves that are scouring.[3]

The BCoV, also known as betacoronavirus 1, is an enveloped virus having a positive-sense and single-stranded RNA genome belonging to the genus *Betacoronavirus*. Among the most significant livestock viruses, BCoV causes diarrhea in both newborn calves and adult cows (winter dysentery). Both beef cattle pneumonia and respiratory illnesses in young calves have been linked to this virus.[4] In addition to causing gastroenteritis and pneumonia, several BCoV strains are known to induce co-infection.[5]

The World Organization for Animal Health has classified the arboviral non-contagious illness known as bluetongue (BT) in domestic and wild ruminants as a reportable disease.[6,7] After infection with BTV, most cattle show no outward signs of illness; therefore, the disease is often overlooked and allowed to spread rapidly across the herd. It is difficult to eradicate BTV because asymptomatic carrier cattle can act as a virus reservoir and source of infection in the farm and virus spread to the herd is

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primarily through the bites of biological vectors of *Culicoides* spp.[8] Though, direct transmission has also been observed. Although BTV may cause serious complications, the first symptoms are often non-specific and mimic those of other viral diseases.

Luminex® technology or xMAP® technology is a bead-based multiplex assay for multi-analyte profiling present in the sample. This multiplex technology enables a high degree of multiplexing and can be configured to perform a wide variety of assays quickly, cost-effectively and accurately with high degree of multiplexing within a single sample volume. This can simultaneously detect and quantify multiple RNA or protein targets in a single assay. The Luminex-based assays were based on microsphere-based multiplexing in which microspheres having internal proportions of red and infra-red fluorescent dyes were bound with specific probes for detection of virus. With Luminex® xMAP technology, it is possible to identify a large number of distinct targets in a single reaction. Here, x represents an unknown analyte, and MAP denotes multi-analyte profiling. The xMAP multiplex technology has turned into a diagnostic tool in clinical and veterinary contexts despite the fact that Luminex technology was first created to detect numerous analysts such as cytokines and B cells.[9,10] This innovation has been put into commercial usage, with great success, as a part of the xTAG® gastrointestinal pathogen panel.[11] The Luminex xMAP approach is superior to other molecular diagnostic techniques like polymerase chain reaction (PCR) and quantitative PCR (qPCR) in terms of speed, sensitivity, specificity and reliability. The xMAP assay has been developed to detect rotavirus, norovirus, sapovirus, astrovirus and adenovirus in clinical and environmental samples.[12]Earlier most of the bead-based assays were immunoassays to identify antigens, proteins, serological measure of various antibody production and differentiation of vaccinated animals from infected. However, these xMAP assays have now been used to detect both proteins and nucleic acids.[13,14] Consequently, with consideration of above perspectives, the current study was undertaken to develop a triplex nucleic acid based MAGPIX assay for simultaneous detection of three major entero-pathogenic viruses responsible for diarrhea and enteritis in animals *viz*. BRV, BCoV and BTV.

Materials and Methods

Collection of sample and viral RNA extraction. From March 2018 to February 2019, a total number of 200 fecal samples of buffalo and cow calves (< 1 month - 1 year) from organized and unorganized dairy farms of Haryana, India, were collected. Samples were collected in sterile stool collection containers and stored at – 20.00 ˚C until processed. Large fecal debris was separated out by diluting the solution with phosphate-buffered saline (10.00% w/v), vortexed and centrifuged at 10,000 rpm for 10 min before being frozen at – 20.00 ˚C for later use. Viral RNA was extracted using a modified version of the Trizol technique.[15] The extracted RNA was quantified by Nano-drop (Thermo Fisher Scientific, Waltham, USA) and the purity of RNA was tested by optical density of 260 per 280.

Design of primers and probe for Luminex/MAGPIX assay. Conserved sequences of BRV, BCoV and BTV available in the GenBank® were used for designing primers and probe for species specific Luminex/MAGPIX assay. The primers were designed targeting the *N* gene of BCoV, *VP6* gene of BRV and *Seg-10* of BTV. The forward primers and the 5' biotin modification of reverse primers depending on strand complimentary to the probe sequence at 100 nM concentration with High Performance Liquid Chromatography (HPLC) purification, obtained from Integrated DNA Technology (IDT, Coralville, USA) were used. The probes had 5' amino C12 spacer modification and HPLC purification.[16]

Commercially synthesized gene as a positive control. The gene constructs specific for BRV, BCoV and BTV were commercially synthesized from Bio Basic Inc. (Markham, Canada). These gene constructs were transformed in the PUC57 synthetic construct cloning vector in the correct (5'-3') orientation. The *E. Coli* transformants having plasmid were grown on Luria Bertani (LB) agar (Himedia, Maharashtra, India), further expanded in LB broth (Himedia) and used for plasmid isolation using standard procedure. Throughout the study, these plasmids were used as positive controls for the respective viruses.

Optimization of multiplex PCR/reverse transcription-PCR (RT-PCR). The multiplex PCR/RT-PCR assay was optimized for triplex combinations. The triplex consisted of BRV (*VP6* gene), BcoV (*N* gene) and BTV *(Seg-10* gene). The triplex RT-PCR was optimized for cDNA template concentration (10.00 - 20.00 ng μL^{-1}) and primer concentrations (0.50 - 1.00 uM). Initial denaturation at 94.00 ˚C for 3 min was followed by 35 cycles at 94.00 ˚C for 30 sec, 50.00 ˚C for 30 sec and 72.00 ˚C for 1 min, with a final extension at 72.00 ˚C for 10 min; this was the cycling protocol for triplex RT-PCR.

Bead coupling and count. Specific capture probes were bound to three different spectrally unique fluorescent beads for the multiplex reaction. The BoCV probe was coupled on bead No. 28, BTV probe on bead No. 34 and BRV probe on bead No. 37. The bead coupling protocol and bead count by hemocytometer was conducted as per xMAP cookbook. Briefly, 5.00×10^6 of the stock microspheres were pelleted by micro-centrifugation at ≥ 8,000 *g* for 1- 2 min. Supernatant was discarded and beads were re-suspended in 50.00 µL of 0.10 M 2-(N-morpholino)-ethanesulfonic acid monohydrate (pH: 4.50; Sigma-Aldrich, St. Louis, USA), by vortexing and sonication for approximately 20 sec. The designed probes for all the

three viruses were mixed with these beads along with the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich), compounded and incubated for 1 hr. After coupling, beads were washed with 1.00 mL of 0.02% Tween 20 (Sigma-Aldrich) and 1.00 mL of 0.10% sodium dodecyl sulfate (Sigma-Aldrich). Finally, the beads were re-suspended in 100 µL of Tris-EDTA buffer (Sigma-Aldrich) in pH of 8.00 through vortexing. The bead concentration used was 1.25×10^7 mL^{-1}. The bead volume was optimized at 1.25×10^6 beads per virus specific probe (10.00 pmol), being three times lesser than the recommended concentration of beads.

Probe hybridization. The bead labeled probe was hybridized with biotinylated PCR products in 1.50X tetramethyl ammonium chloride (TMAC; Sigma-Aldrich) hybridization solution. Biotinylated PCR product (5.00 µL), 33.00 µL of working hybridization solution, 1.00 µL of each labeled bead from the designated triplex set and 11.00 µL of nuclease-free water (Qiagen, Hilden, Germany) were used to achieve the optimal reaction volume of 50.00 µL. Multiplex set performance was measured at 50.00, 53.00 and 55.00 ˚C during hybridization at various incubation periods (10, 15 and 25 min). The detection dye streptavidin-phycoerythrin (SAPE; Thermo Fisher Scientific) was also optimized for multiplex set at 1.00 µL in 1,000 L (1.00 mg mL^{-1}; Sigma-Aldrich) in 1.00X TMAC reporter solution.

Interpretation of results. The wash protocol for hybridization was used in which the products were transferred to the 96-well plate and washed twice with 100 µL of 1.00X TMAC buffer on the plate magnet. The beads were re-suspended in TMAC with concentration of 75.00 µL reporter buffer containing an optimal amount of 25.00 µL SAPE per reaction. The results were observed on the Luminex xPONENT Software (version 4.3; BioRad, Hercules, USA) and expressed in term of mean fluorescence intensity (MFI). The MFI is usually reported as two standard deviations of the background MFI or negative control.

Assessment of sensitivity and specificity of assay. The assay reactions were first optimized in the monoplex format using ten-fold serial dilutions for each virus cDNA/plasmid DNA followed by the multiplex/triplex format. The analytical sensitivity or limit of detection (LOD) was expressed in terms of copy number. The copy number was calculated using the quantified DNA concentration according to the following formula for copy number:

$$Copy\ number = A \times No / Length \times 10^9 \times 650.$$

where, *A* is the DNA concentration in ng μL^{-1}, *No* is Avogadro's number (6.022×10^{23}) and length is amplicon size (base pair).

The LOD was calculated for both the monolpex and multiplex formats. The specificity of the bead-based multiplex assay was assessed using the DNA from the synthesized plasmid/cDNA prepared from viral RNAs for all three viruses. A negative control consisting of labeled beads with nuclease free water and SAPE was also included. The MFI of the bead coupled probes and specific target biotin labeled PCR product was recorded. Furthermore, an *in silico* comparison for cross reactivity of the primers and probe sequences with closely related virus species to the targeted three viruses was also done.

Repeatability of the assay. The intra- and inter-assay reproducibility was calculated using the methods outlined in the Luminex cookbook. According to the Luminex xMAP recipe book (https://www.luminexcorp.com/xmap-technology), we ran three repetitions of each sample to assess intra-assay repeatability and five replicates of each sample to determine inter-assay repeatability. Intra-assay repeatability should be within a coefficient of variation (CV) range of 10.00%; while, inter-assay repeatability should fall within a CV range of 20.00%.

Validation of the assay. During the 2018-2019 study period, a total number of 200 fecal specimens were collected from both organized and unorganized dairy farms in Haryana, India, and subjected to the testing and analysis using the developed multiplex MAGPIX assay for the prevalence of diarrhea-associated viruses. To determine whether approach was more sensitive for detecting individual viruses in feces, the novel triplex test was compared with traditional PCR and real-time PCR.

Results

Sequence comparison and assay design. The nucleotide sequences of enteric viruses from various reference strains of distinct enteric viruses were compared using publicly available and in house generated nucleotide sequences. A conserved region of genome was located, enabling target footprints for primers and probes for a virus-specific MAGPIX assay for BCoV, BRV and BTV. *In silico* analysis of these primers and probes sequences with other sequences revealed no resemblance in footprints, indicating that they are BRV, BCoV and BTV specific.

Singlex/multiplex PCR. The PCR/RT-PCR assay was optimized for both singlex and triplex combinations. The PCR reaction mixture was optimized with 10.00 - 20.00 µL of DNA/cDNA template (plasmid DNA or cDNA prepared from viral RNA) and 0.80 µM of each forward and reverse biotin-labelled primers, in 25.00 µL reaction solution using TopTaq master mix. The optimized triplex RT-PCR reaction mixture consisted of cDNA template that was 10.00 - 20.00 µL of template of each virus cDNA, 0.80 µM of each forward primer, 0.80 µM of each five biotin-labelled reverse primers and 25.00 µL of 2.00 X Toptaq master mix to the final volume of 50.00 µL with addition of 15.00 µL of nuclease free water. The triplex consisted of BRV (*VP6* gene), BCoV (*N* gene) and BTV (*Seg-10* gene).

Hybridization and Luminex analysis. Three different types of diarrheal viruses may now be detected with the single detection equipment thanks to the Luminex xTAG multiplex detection technology. The following describes the best hybridization system and reaction conditions: The total reaction volume (50.00 µL) was prepared using 5.00 µL of the biotinylated PCR product with 33.00 µL of the hybridization solution with 1.50X TMAC and 1.00 µL of each labelled beads from the triplex set and 11.00 µL of nuclease free water was optimized as a working mixture of the assay. The conditions for hybridization were optimized as denaturation at 96.00˚C for 1.50 min and annealing at 50.00 ˚C for 15 min for triplex assay. The thermocycler was used to incubate the reporter mix at 55.00 ˚C for 5 min and then, the MAGPIX multiplex reader equipment was used to analyze each well at the holding temperature of 55.00 ˚C. The results were observed on the Luminex xPONENT Software (Fig. 1). A low MFI was calculated on lower dilution for different viruses observed at lower dilution that then became constant on further dilution for all three viruses in the triplex format.

Analytical specificity of the assay. The specificity analysis for BRV, BCoV and BTV was carried out using probe-coupled beads. The SAPE and labelled beads in nuclease-free water used as the negative control. No probe cross reactivity was observed and no dye signal corresponding to the negative control was seen. The specificity of the Luminex xTAG assay technique was examined and the results confirmed that the primer pairs were highly specific for each virus. The effectiveness of the new assay was further evaluated using it on a variety of target combinations. In this regard, five sets of mixed samples and a negative control were included. This showed high specificity of the developed assay for detection of the target with an error bar at 5.00%.

Analytical sensitivity of the assay. The sensitivity analysis for BRV, BCoV and BTV was carried out using probe-coupled beads. On the y axis, dye signal MFI in arbitrary units was plotted against the number of fluorescent beads that were detected for each individual virus. The z axis represents the capture probes used to identify each peak (Fig. 1). As shown in Table 1, the LOD was determined by testing triplicates of the positive control in both monoplex and triplex assay formats using serial 10-fold dilutions. The sensitivity of both traditional multiple RT-PCR and real-time PCR was compared to that of established method. Although being lower than real-time PCR, the minimum detection rate was nevertheless greater than that achieved by traditional multiplex PCR.

Repeatability of the assay. Table 2 displays the %CV results for the multiplex sets for both inter- and intra-assay repeatability. The %CV for each target virus in triplex varied from 7.90 to 11.00% within a run, and from 4.47 to 7.40% between runs. As compared to the RT-qPCR test, the xMAP assay obtained a larger %CV when the lower LOD was approached.

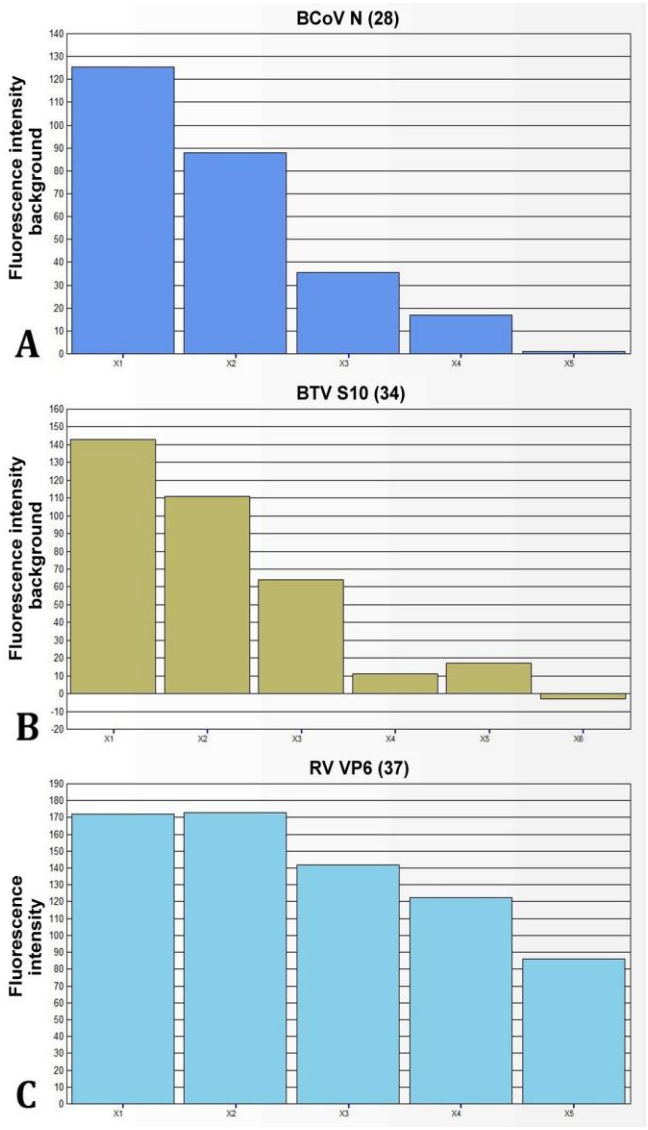

Fig. 1. Luminex assays showing 10-fold serial dilutions for **A)** Bovine coronavirus (BCoV), **B)** Bluetongue virus (BTV) and **C)** Rotavirus (RV). Low mean fluorescence intensity (MFI) was detected on lower dilution for the different viruses. The positive samples show MFI above background level (though in different concentrations).

Table 1. The limit of detection (LOD) in monoplex and triplex formats.

Sample	Virus	LOD (Copy Number)			
		Conventional RT-PCR	Monoplex MAGPIX assay	Triplex MAGPIX assay	Real-time RT-PCR
1	Bovine rotavirus	1.10×10^5	1.62×10^5	2.43×10^5	5.00
2	Bovine coronavirus	1.61×10^4	2.91×10^4	4.87×10^4	4.00
3	Bluetongue virus	2.26×10^5	3.74×10^5	2.13×10^5	5.00

Table 2. Coefficient of variation (%) for inter-assay and intra-assay repeatability for each target virus in triplex format and quantitative polymerase chain reaction (RT-qPCR).

Sample	Analyte	Luminex Assay		RT-qPCR Assay	
		Inter-assay	Intra-assay	Inter-assay	Intra-assay
1	Bovine rotavirus	7.40	11.00	0.74	0.41
2	Bovine coronavirus	4.60	9.10	0.68	0.90
3	Bluetongue virus	4.47	7.90	0.16	1.00

Evaluation of clinical specimens. For evaluation of the Luminex assay performance, 200 diarrheic samples were tested. The confirmed positive samples by conventional RT-PCR and RT-qPCR for each virus (11 RV, 9 BCoV and 9 BTV) were also found positive by Luminex assay (Fig. 2). The results suggested no difference in identification of samples by the developed Luminex beads based multiplex assay and real-time RT-PCR method.

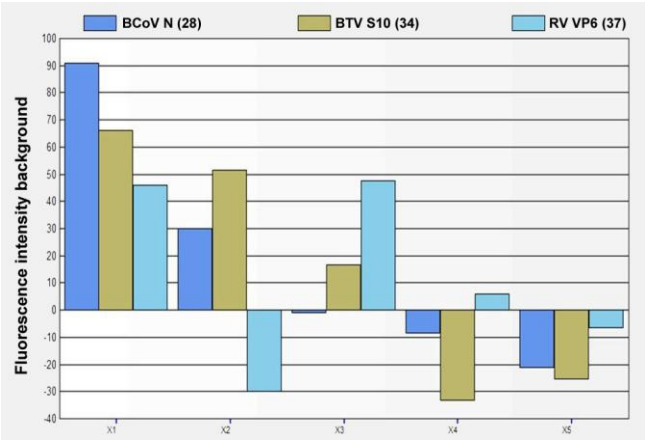

Fig. 2. Validation of the artificial mixed infection samples. X1: Positive control; X2: Bovine coronavirus (BCoV) and bluetongue virus (BTV) positive samples; X3: BTV and rotavirus (RV) positive samples; X4: RV positive sample; X5: Negative control. Low mean fluorescence intensity (MFI) was detected on lower dilution for different viruses. The positive samples show MFI above background level (though in different concentrations). The negative samples show MFI below background level.

Discussion

Most cases of diarrhea are caused by viruses, and as a result, animal producers throughout the globe suffer substantial financial losses due to viral gastroenteritis. Rapid onset and dissemination of infection leads to significant damage to the intestinal lining and subsequent fast fluid loss and dehydration.[17] Multiplex infections which include two or more viruses may occur frequently and significantly impede clinical diagnosis. Consequently, pathogen surveillance is required for precise causal agent differentiation in specimens and to avoid the trans-boundary spread of viral diarrheal illness. Diarrhea in farm animals may be difficult to diagnose; thus, rapid and accurate diagnostic tests are essential. Today, PCR-based approaches have been shown to be both quick and accurate in identifying viruses linked to diarrhea.[18] The authors' laboratories have developed and frequently used multiplex PCR and RT-PCR methods for detecting up to four viral infections. Multiple infections, especially when they are complicated, need more precise diagnosis procedures. Increased test throughput capacity and potential cost savings are driving the rising use of multiplexing technologies.

To quickly, specifically and simultaneously identify RNA from three enteric viral infections in a single biological sample, we further developed a Luminex xTAG high-throughput detection technique, taking use of the features of high flux, wide range detection with small sample volumes. Furthermore, this works well for multiplex pathogen identification in samples, making it a good choice for large-scale screening of field samples. Using xMAP technology multiplex PCR in conjunction with a bead solution, we were able to construct standard curves for the aforementioned three diarrheal infections and then, quantify the concentration of each pathogen by measuring the MFI value in each sample.

In this study, three different types of beads were coupled with gene specific probes. The BoCV probe was coupled on bead No. 28, BTV probe on bead No. 34 and BRV probe on bead No. 37. It was found that 90 sec at 96.00 ˚C followed by 15 min at 50.00 ˚C was the optimum hybridization temperature and time. The hybridization was carried out at 95.00 ˚C for three min, followed by 50.00 ˚C for 30 min and the working microsphere mixture included seven different types of beads each of which was attached to a separate gene-specific capture probe.[11]

In order to get the best possible outcomes, multiplex assays must take into account a number of parameters, the most important of which are the assay dynamic range, cross-reactivity and biological matrix. It is also important to know how each analyte functions in the body, how each test reagent binds to a sample, and what the sample itself looks like. Avoiding interference from the matrix effect during hybridization calls for diluting the sample and performing an extra washing step beforehand.[19] Hybridization protocols of the analytes at various concentrations were adjusted in the present investigation to improve binding specificity and sensitivity. The LOD was found to be consistent with a previous investigation on the detection of seven enteric viruses in terms of copy number per response. In contrast, a detection threshold of 100 copies μL^{-1} was achieved using the multiplex PCR-based Luminex x-TAG test designed for the diagnosis of rabbit hemorrhagic illness.[20] The LOD in terms of copy

number of MAGPIX assay (monoplex and triplex formats) was comparable with conventional RT-PCR which was 10^5 for BRV and BTV and 10^4 for BCoV; however, it was lower than real-time RT-PCR assays.

The analytical specificity of the xMAP assay, using the biotin-labelled PCR products of each virus being separated by probe-coupled beads, was studied. No cross reactivity was observed similar to 100% analytical specificity reported in the assay for simultaneous detection of seven enteric viruses of humans.[11] The results of a previous investigation in which 40 clinical specimens were screened were totally consistent with those obtained using the traditional PCR approach where 18 of the tested specimens were positive.[20] In a second investigation, 52 clinical samples were analyzed for the presence of either rabbit hemorrhagic disease virus or rabbit rotavirus using either the xMAP test or enzyme-linked immunosorbent assay. The xMAP test demonstrated excellent specificity by not reacting to any other infections.[21] Coefficients of variation for intra-assay and inter-assay comparisons should be less than 11.00% and 7.00%, respectively, indicating that an assay is reproducible and stable. The CV between replicates of this newly devised test was determined to be within a reasonable range. The devised test also demonstrated excellent specificity by not reacting to any closely similar diseases.

In the present study, total number of 200 samples were screened by real-time PCR and xMAP assays and 29 samples were found positive for one or the other virus in both assays. The results obtained by these two methods were 100% consistent. These findings suggested that the developed multiplex assay for screening of targeted viral pathogens was "fit for purpose".

The authors have compared the results of real-time PCR and bead-based multiplex assay for the respiratory virus panel detection and found that concordance between them was 94.10 -100%; whereas, the developed assay and real-time RT-PCR showed 100% corroboration for these viral pathogens detection.[22] A newly developed multi-plexed Luminex-based assay can be completed in less than 24 hr, from sample preparation to receiving the results. It can detect multiple viral infections simultaneously. Hence, it fulfils the criteria of quick, sensitive and specific diagnostic method for detection of viral gastroenteritis caused by BoCV, BTV and BRV, either alone or in combination. More specialized probes can be added to this assay in order to find other viral or bacterial causes of gastroenteritis or other disorders. It is recommended that further studies are mandatory regarding identification of enteric viruses in Indian dairy herds through incorporating more numbers of samples from different geographic locations. Utilizing molecular diagnostic technology to the fullest extent is essential for virus surveillance and monitoring which will eventually contribute to the creation of early and effective preventive and control measures minimizing financial losses in the cattle industry.

The newly developed bead-based Luminex xMAP assays were powerful diagnostic tools for the quick detection and differentiation of enteric viruses in livestock because they were more quick, highly sensitive, specific and effective with excellent reproducibility and repeatability. Further, the increased multiplexing by expanding the present assay could reduce the cost. The newly developed MAGPIX assay could be used to determine the prevalence of these diarrhea-associated viruses by testing fecal samples.

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Conflict of interest

The authors declares no conflict of interest.

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