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Colorimetric LAMP PCR kit design for rapid diagnosis of *Bovine coronavirus, Bovine rotavirus* group A, *Escherichia coli* K99+ and *Cryptosporidium parvum* in Neonatal calf diarrhea

Mehmet Demirci [1,a] , Akin Yigin [2,b], Seda Ekici [3,c,*], Hikmet Dinç[4,d]

[1]Kırklareli University, Faculty of Medicine, Department of Medical Microbiology, Kırklareli, Türkiye
[2]Harran University, Faculty of Veterinary Medicine, Department of Genetics, Şanlıurfa, Türkiye
[3]Veterinary Control Central Research Institute, Ankara, Türkiye
[4]Gaziantep Islam Science and Technology University, Faculty of Medicine, Department of Medical Pharmacology, Gaziantep, Türkiye

[a]ORCID: 0000-0002-5411-5925; [b]ORCID: 0000-0001-9670-2426;
[c]ORCID: 0000-0002-7982-5261; [d]ORCID: 0000-0001-7858-5123

*Corresponding Author
E-mail: seda.ergen@hotmail.com

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Abstract

Reducing calf deaths and diseases are important for the future and profitability of cattle herds. Early diagnosis and treatment of calf diarrhea can reduce mortality rates and eliminate the need for cattle imports. It is important to produce local molecular kits based on the colorimetric Loop-mediated isothermal amplification (LAMP) method and to use them in the field, instead of using immunocrotographic tests with low sensitivity. With this study, four important enteric pathogens responsible for calf diarrhea (Bovine coronavirus [BCoV; formerly Betacoronavirus 1], group A Bovine rotavirus [BRV], *Escherichia coli* K99+ and Cryptosporidium parvum) were isolated in farm and field conditions without requiring qualified personnel and complicated devices. It is aimed to design a routine molecular kit based on the colorimetric LAMP method that can be detected in tubes in the range of 15-30 minutes. In addition, it is aimed to reveal the test kit performances by comparing the results of these LAMP PCR kits, which we will design, with in-house PCR and immunochromatographic test kits. A total of 100 calves stool samples were included (these samples were confirmed as positive for BcoV (n:25), BRV group A (n:25), *Escherichia coli* K99+ (n:25) and Cryptosporidium parvum (n:25) by in-house PCR). Nucleic acid (DNA/RNA) isolations from Neonatal Calf stool samples were performed using the commercial isolation kit. Results from LAMP kit and rapid antigen kits were compared according to in-house PCR results. Results of this study, especially *E. coli* K99 diagnostic performance were found to be closer to each other, while performance differences in viral parameters such as BRV and BcoV were found to be significantly different. It was determined that the LAMP PCR method performed better than rapid antigen test. As a conclusion, It is very important for early diagnosis to use the colorimetric LAMP method, which can be detected in separate tubes in 15-30 minutes in farm and field conditions without requiring qualified personnel and complicated devices. We believe that, thanks to early and reliable diagnosis, the calf mortality rates will be greatly reduced, as the infected animals will be treated early.

Keywords: *Bovine coronavirus*, *Bovine rotavirus* group A, *Cryptosporidium parvum*, *Escherichia coli* K99+, LAMP PCR, Neonatal calf diarrhea.

INTRODUCTION

Although the cattle breeding industry, animal facilities, animal care, feeding and herd management have made great progress in our country, newborn calf deaths still continue to be an important problem (Karslı and Evci, 2018). Diarrhea is the most important disease causing death in calves after birth (Mawyl et al., 2015; Fischer 2016; Topal, 2018). It has been reported that 75% of neonatal calf losses are due to diarrhea and diarrhea is the most common disease seen in calves up to three months of age. The most important infectious agents causing diarrhea in Neonatal calves in our country and in the world are bacteria such as *Escherichia coli*, *Salmonella* spp., and *Clostridium perfringens*, viruses such as Bovine rotavirus (BRV) group A, Bovine coronavirus (BCoV), Bovine viral diarhea virus, Bovine calicivirus and Bovine torovirus, parasites such as *Cryptosporidium parvum* and *Giardia intestinalis* (Ok et al., 2009; Cho and yoon, 2014; Meganck ve ark, 2015). It is not enough to carefully control the calves after birth. Since newborn calves are very susceptible to diseases in the first 6 months of their lives, calves should be monitored in the days following their birth. For this purpose, clinical appearances, diarrhea, pneumonia and lethargy scoring are very important for cattle breeding (Batmaz, 2015). Neonatal calf deaths cause productivity and great economic loss. In order to have information about neonatal calf deaths in a farm, it is necessary to know the calf survival rate of the farm. This ratio is found by dividing the number of calves that can live up to a certain period by the number of live births. While this rate is 92.58% in England and 93.60 in the USA, it has been reported that it is between 85-90% in our country, and that the calf loss is 15% of the total 4.5 million calves born throughout the country, which corresponds to approximately 675,000 (TUIK, 2021; Akbaş ve ark, 2017). In order to prevent this economic loss, it is very important to determine the causes of calf deaths, the diagnosis of the factor and the early treatment

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protocols accurately and quickly. Reducing calf deaths and diseases are important for the future and profitability of cattle herds (Glover ve ark, 2013). Early diagnosis and treatment of calf diarrhea can reduce mortality rates, eliminating the need for cattle imports (TUIK, 2021).

In our study, four important enteric pathogens; (Bovine coronavirus [BCoV; formerly *Betacoronavirus 1*], group A *Bovine rotavirus* [BRV], *Escherichia coli* K99+ and *Cryptosporidium parvum*) responsible for calf diarrhea were isolated in separate tubes without the need for qualified personnel and complicated devices under farm and field conditions. It is aimed to design a routine molecular kit based on the colorimetric Loop-mediated isothermal amplification (LAMP) method that can be detected in the range of 15-30 minutes. In addition, The aim of this study was to reveal the test kit performances by comparing the results of these LAMP PCR kits, which we will design, with in-house PCR and immunochromatographic test kits.

MATERIALS AND METHODS

Neonatal Calf stool samples: Ethical committee permission of the study was obtained with the decision of "Veterinary Control Central Research Institute Local Ethics Committee" numbered 2022/23. A total of 100 calves stool samples were included (These samples were confirmed as positive for BcoV (n:25), BRV group A (n:25), *Escherichia coli* K99+ (n:25) and *Cryptosporidium parvum* (n:25) by in-house PCR) Neonatal Calf stool samples were analyzed at Harran University, Faculty of Veterinary Medicine, Department of Genetics.

Detection of pathogens by Total nucleic acid isolations and in-house PCR

Nucleic acid (DNA/RNA) isolations from Neonatal Calf stool samples were performed using the High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. In order to compare the antigen test and LAMP PCR test data, in-house PCR was performed and a total of 100 samples, 25 out of 100 which were positive for each agent, were included in the study. For the detection of pathogens, the primers in Table 1 were ordered (Sentromer Ltd, Istanbul, Turkey) and used by following the in-house PCR method (Cho et al, 2010). A single-step in-house reverse transcriptase PCR method protocol was followed for each pathogen in separate tubes. AMV Reverse Transcriptase XL and AMV-Taq polymerase from one step RT-PCR Kit (Takara, Otsu, Japan) was used according to manufacturer's instruction. The PCR protocol was used; Following 30 minutes of reverse transcription at 50ºC, enzyme activation at 95ºC for 15 minutes, 40 cycles of amplification (30 seconds at 95ºC, 1 minute at 55ºC, 1 minute at 72ºC) and final elongation at 72ºC for 7 minutes. The formed bands were analyzed by examining the bands on 2% agarose gel (Cho et al, 2010).

Table 1. The primers for the detection of target pathogens

Target Pathogen	Primers Name	Oligonucleotide sequence	Reference
Bovine coronavirus **(N)**	BCoVF	CCGATCAGTCCGACCAATC	Cho et al, 2010
	BCoVR	AGAATGTCAGCCGGGGTAT	Cho et al, 2010
Bovine rotavirus **group A (VP6)**	BRVF	ACCACCAAATATGACACCAGC	Cho et al, 2010
	BRVR	CATGCTTCTAATGGAAGCCAC	Cho et al, 2010
Escherichia coli **K99+ (K99)**	K99F	GCGACTACCAATGCTTCTGCGAATAC	Cho et al, 2010
	K99R	GAACCAGACCAGTCAATACGAGCA	Cho et al, 2010
Cryptosporidium **(COWP)**	BB-3F	GCGAAGATGACCTTTTGATTTG	Cho et al, 2010
	BB-4R	AGGATTTCTTCTTCTGAGGTTCC	Cho et al, 2010

Rapid antigen detection from calf stool samples by immunochromatographic method

In order to detect bovine rotavirus, *bovine coronavirus*, *E. coli* K99, *Cryptosporidium parvum*, test positivity in accordance with the manufacturer's directives of Anigen Rapid BoviD-5 Ag (Bionote, Inc. Korea) rapid test kit and Is-Kit 4 antigen rapid test kit (Klonbiotek, Turkey). It was used in stool determined by in-house PCR.

Colorimetric Loop-mediated isothermal amplification (LAMP) method

In order to design a kit based on the colorimetric LAMP method, fasta sequences of specific parameters were obtained from the NCBI database. *Bovine coronavirus* (GenBank accession number: KT318088.1), Bovine rotavirus group A (GenBank accession number: EU873011.1), *Escherichia coli* K99+ (GenBank accession number: M35282.1) and *Cryptosporidium parvum* (GenBank accession number: M59419.1) sequences were used. To desing specific LAMP primer sets, Primer Explorer Ver.5 software (http://primerexplorer.jp/lampv5e/index.html) was used. After the design with the software, 5 different primer sets compatible with each parameter (forward and reverse outer primers (F3 and B3), forward and reverse inner primers (FIP and BIP)) were determined, the appropriate primer set was selected as in-silico and these oligonucleotide primers were used. (Sentromer Ltd, Istanbul, Turkey). Primers used in the design of BRV, BCoV, *E. coli* K99 and C. parvum LAMP PCR kits are reported in Table 2.

In the LAMP PCR study, separate amplification was performed in seperate tubes. Total volume was 25 µL of each reactions (including 4 µL of nucleic acid to the 21 µL LAMP PCR mix), Bsm DNA Polymerase (Thermo Scientific, USA) (8 U/µL) was used according to manufacturer's instructions. 1.6 µM each of the FIP and BIP, 0.2 µM each of F3 and B3 were used for each reactions. For colorimetric results, SYBR Green I dye (Merck GmbH, Germany) was added to the tubes for the reactions. Two different LAMP PCR amplification protocols were performed for each tubes using the T1 PCR System (Bio-Rad, Hercules, CA, USA). These protocols were 65°C for 15 min and 65°C for 30 minutes. The amplification tubes were visually examined under UV light.

Table 2. Primer sets used in the design of BRV, BCoV, *E. coli* K99 and C. parvum LAMP PCR kits

Target pathogen	Name	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Oligonucleotide sequence
BRV	F3	914	932	19	57.11	-7.85	-4.56	0.47	CGGCGTTATTTCCAAATGC
	B3	1072	1089	18	56.31	-6.01	-5.86	0.50	TGGTGGAAAAACTGGTCC
	FIP (F2 and F1c)			42					GCACTGATTCACAAACTGCAGA-TGAACATCATGCAACAGTAG
	BIP (B2 and B1c)			40					TTGCCGACGCAAGTGAAACA-CTGGTATCGCGTATTCTTGT
	F2	942	961	20	55.11	-4.58	-4.07	0.40	TGAACATCATGCAACAGTAG
	F1c	982	1003	22	61.28	-5.90	-5.66	0.45	GCACTGATTCACAAACTGCAGA
	B2	1050	1069	20	56.82	-4.58	-4.41	0.45	CTGGTATCGCGTATTCTTGT
	B1c	1004	1023	20	62.92	-6.69	-4.18	0.50	TTGCCGACGCAAGTGAAACA
BCoV	F3	351	369	19	60.39	-3.57	-5.68	0.53	TCAATTGCTGCCACGATGG
	B3	532	550	19	59.90	-5.55	-5.84	0.63	GAGGGAGTACCGTACCAGG
	FIP (F2 and F1c)			41					GCGACCCAGAAGACTCCGTCA-CTATCTTGGAACAGGACCGC
	BIP (B2 and B1c)			42					CAGGCTGATGTCAATACCCCGG-ACCTAGTCGGAATAGCCTCA
	F2	378	397	20	59.60	-3.31	-7.03	0.55	CTATCTTGGAACAGGACCGC
	F1c	426	446	21	65.80	-7.03	-6.19	0.62	GCGACCCAGAAGACTCCGTCA
	B2	507	526	20	59.14	-4.41	-6.10	0.50	ACCTAGTCGGAATAGCCTCA
	B1c	454	475	22	64.78	-6.08	-7.57	0.59	CAGGCTGATGTCAATACCCCGG
***E. coli* K99**	F3	442	465	24	56.16	-6.00	-4.41	0.33	GGTGGTGCTAATATTAATACTTCA
	B3	631	649	19	55.77	-2.86	-4.21	0.42	ATAGATATGCCCGCAATGT
	FIP (F2 and F1c)			45					CTTTTTTCAGCTGGGCTGAATAGTT-TCACTACGGCTGAATACACT
	BIP (B2 and B1c)			43					CGCTCCGTCTAATGGTGGAT-TATAAGTGACTAAGAAGGATGCT
	F2	467	486	20	57.68	-3.99	-4.13	0.45	TCACTACGGCTGAATACACT
	F1c	511	535	25	62.02	-3.27	-3.12	0.40	CTTTTTTCAGCTGGGCTGAATAGTT
	B2	588	610	23	56.15	-2.31	-5.14	0.35	TATAAGTGACTAAGAAGGATGCT
	B1c	543	562	20	61.26	-6.87	-4.90	0.55	CGCTCCGTCTAATGGTGGAT
C. parvum	F3	813	832	20	55.17	-3.15	-5.70	0.40	TCATTATTGTACCACCCTGA
	B3	1000	1022	23	55.54	-4.11	-4.15	0.35	CCTTTTTATAGGTTTCCTGATCT
	FIP (F2 and F1c)			48					TGTAGCTCCTCATATGCCTTATTGA-TAAGACAAGTCATGAAAGGCTAG
	BIP (B2 and B1c)			42					ACAAGGAAGTCTGGAAACGTG-TGTTGATCCTTTTTGAAGAGC
	F2	833	855	23	57.68	-3.59	-5.26	0.39	TAAGACAAGTCATGAAAGGCTAG
	F1c	873	897	25	61.37	-4.98	-3.15	0.40	TGTAGCTCCTCATATGCCTTATTGA
	B2	979	999	21	56.23	-4.58	-5.09	0.38	TGTTGATCCTTTTTGAAGAGC
	B1c	921	941	21	60.05	-5.00	-5.49	0.48	ACAAGGAAGTCTGGAAACGTG

Analysis of results and evaluation of data

The results of the rapid antigen detection kits (Anigen Rapid BoviD-5 Ag and Is-Kit 4) and the LAMP PCR kit were considered for each parameter and calculations were performed. For sensitivity measurement; True Positive/(True Positive + False Negative) formula, for specificity measurement; True Negative/(True Negative + False Positive) formula, For positive predictive value measurement; For true positive/(True positive+False positive) formula and Negative predictive value measurement; True negative/(True negative + False Negative) formula were used.

RESULTS

Diagnostic test performance data obtained as a result of our study with a total of 100 samples, 25 of which were positive for each agent, are presented in Table 3. Our LAMP PCR design kit performed better than immunochromatographic methods in terms of diagnostic performance in all parameters.

Table 3: Comparison of performance indicators of test kits used for the diagnosis of BcoV, BRV, E.coli K99 and C. parvum

Target Pathogen	Test Performance indicator	BoviD-5	IS-KIT-4	LAMP PCR (30 minute)
BCoV	Sensitivity	92.00%	84.00%	96.00%
	Specificity	90.67%	89.33%	97.33%
	Positive Predictive Value	76.67%	72.41%	92.31%
	Negative Predictive Value	97.14%	94.37%	98.65%
BRV	Sensitivity	84.00%	80.00%	88.00%
	Specificity	88.00%	89.33%	94.67%
	Positive Predictive Value	70.00%	71.43%	84.62%
	Negative Predictive Value	94.29%	93.06%	95.95%
***E. coli* K99**	Sensitivity	92.00%	88.00%	96.00%
	Specificity	94.67%	93.33%	94.67%
	Positive Predictive Value	85.19%	81.48%	85.71%
	Negative Predictive Value	97.26%	95.89%	98.61%
C. parvum	Sensitivity	84.00%	76.00%	88.00%
	Specificity	92.00%	90.67%	93.33%
	Positive Predictive Value	77.78%	73.08%	81.48%
	Negative Predictive Value	94.52%	91.89%	95.89%

BcoV; Among the 25 positive samples that were detected positive by in-house RT-PCR, only one sample was found to have a false negative result with the LAMP PCR kit. Two and four samples were found to be false-negative, respectively, in the BoVID-5 and IS-KIT-4 kits using immunochromatographic methods. While false positive results were detected in two samples with LAMP PCR kit, seven and eight samples were detected as false positive in BoVID-5 and IS-KIT-4 kits, respectively.

BRV; Among the 25 positive samples that were found positive by in-house RT-PCR, three samples were found to be false-negative with the designed LAMP PCR kit, while four and five samples were found to be false-negative in the BoVID-5 and IS-KIT-4 kits, respectively. While false positive results were detected in four samples with the designed LAMP PCR kit, nine and eight samples were found as false positive in BoVID-5 and IS-KIT-4 kits, respectively.

E. coli K99; Among the 25 positive samples that were found positive by in-house RT-PCR, only one sample was found to be false-negative with the designed LAMP PCR kit, while two and three samples were found to be false-negative in the BoVID-5 and IS-KIT-4 kits, respectively. While false positive results were detected in four samples with the designed LAMP PCR kit, four and five samples were found as false positive in BoVID-5 and IS-KIT-4 kits, respectively.

C. parvum; Among the 25 positive samples that were found positive by in-house RT-PCR, three samples were found to be false-negative with the designed LAMP PCR kit, while four and six samples were found to be false-negative in the BoVID-5 and IS-KIT-4 kits, respectively. While false positive results were detected in five samples with the designed LAMP PCR kit, six and seven samples were found as false positive in BoVID-5 and IS-KIT-4 kits, respectively.

Especially *E. coli* K99 diagnostic performance data were found to be closer to each other, while performance differences in viral parameters such as BRV and BcoV were found to be significantly different.

In our kit designed with our study, LAMP PCR processes were tested on the same samples with 2 different temporal protocols of 30 minutes and 15 minutes, following similar master mix and thermal protocol conditions. Test performance data of both temporal protocols are given in Table 4. Decreases in test performances were detected in the 15-minute temporal protocol and it was observed that the shortened time changed the performance indicators of the tests.

Table 4: 15-minute and 30-minute performance indicators of the designed LAMP PCR kit

Target Pathogen	Test Performance indicator	LAMP PCR (15 minute)	LAMP PCR (30 minute)
BCoV	Sensitivity	88.00%	96.00%
	Specificity	96.00%	97.33%
	Positive Predictive Value	88.00%	92.31%
	Negative Predictive Value	96.00%	98.65%
BRV	Sensitivity	80.00%	88.00%
	Specificity	92.00%	94.67%
	Positive Predictive Value	76.92%	84.62%
	Negative Predictive Value	93.24%	95.95%
***E. coli* K99**	Sensitivity	92.00%	96.00%
	Specificity	92.00%	94.67%
	Positive Predictive Value	79.31%	85.71%
	Negative Predictive Value	97.18%	98.61%
C. parvum	Sensitivity	80.00%	88.00%
	Specificity	92.00%	93.33%
	Positive Predictive Value	76.92%	81.48%
	Negative Predictive Value	93.24%	95.89%

DISCUSSION AND CONCLUSION

Infective diarrhea is one of the most important health problems seen in neonatal calves up to the weaning age and is responsible for approximately half of the calf deaths up to this period. This situation directly reduces the profit in livestock farming and negatively affects the future of cattle breeding. In general, the main factors causing infective diarrhea are; It is classified as *E. coli* at 7 days old, *C. parvum* at 7-21 days old, BRV and BCoV at 0-28 days old. Therefore, rotavirus, coronavirus, *C. parvum* and *E. coli* K99+ have been reported as the four most important causes of diarrhea in neonatal calves (Foster and Smith, 2009; Blanchard, 2012; Islam et al., 2022). In our study, a total of 100 samples, 25 of which were positive for these 4 important enteric pathogens responsible for calf diarrhea, were compared with the LAMP PCR method and immunochromatographic test methods in terms of diagnostic performance for target pathogens, and the data showed that LAMP PCR method can be used as a fast, alternative and reliable method.

Calves with neonatal diarrhea diagnosis were determined by clinical, laboratory and pathological results. Diagnosis of the agent; It is detected in an average of 3-4 days in the laboratory environment as a microbiological analysis with the isolation of the agent from blood and stool samples. In addition, since the pathogenicity of the detected agent affects the course of the disease, pathogenicity detection is very important (Bellino et al., 2012; Mendonça, et al., 2021).

Because the course of *E. coli* infections is very rapid, infected calves are often found dead. Therefore, the diagnosis of septicemic and enterotoxemic forms of *E.coli* infections is quite difficult. The diagnosis of enteritis form is possible by examining the clinical signs of stool color, shape, presence of straining and developing hypovolemia symptoms. Since there are many serotypes of *E. coli* infections, it is very important to vaccinate as a preventative for the prevention of the disease. Identification of the causative agent is therefore essential in vaccine selection (Kuliğ and Coşkun 2019; Maier et al., 2022). Pathogen *E. coli* strains isolated from feces and intestinal contents of infected animals can also be detected by direct fluorescent antibody technique and ELISA method. In addition, viral diseases such as rotavirus and coronavirus can be detected by detection of the virus by PCR technique, immunofluorescence method or fluorescence microscopic examination in intestinal cells. *Cryptosporidium* oocysts and *Giardia* cysts are isolated from feces. The intestinal mucosa is examined histopathologically, the developmental stages of cryptosporidiums are found and a definitive diagnosis is made. The smears prepared from stool samples are stained with Ziehl Neelsen, Carbol fuchsin or Giemsa, and oocysts can be seen under the microscope (Bilal, 2007).

In the diagnosis of enteropathogens, it can be easily diagnosed with immunochromatographic test kits. Traditional diagnostic methods have some disadvantages such as long duration, needing experienced personnel and special laboratory materials (Sezer and Akgül 2022). Immunochromatographic test kits, on the other hand, offer many advantages such as being usable in field conditions without a laboratory environment, giving results in a short time, allowing multiple enteropathogens to be examined at the same time with a single test kit, and allowing treatment and protection plans in a short time like 10-15 minutes (Al and Balıkçı, 2012; Özçelik ve Balıkçı 2018).

In recent years, developments in laboratory technologies are very important in terms of rapid detection of pathogens, determination of their genotypes, and new contributions to diagnosis and control strategies. Immunochromatographic tests based on antigen detection developed after these developments are used in the rapid diagnosis of important enteropathogens that can cause calf diarrhea from stool. These tests are frequently preferred

because they are fast, easy and inexpensive, and do not require expert and advanced laboratory conditions, especially in terms of effective treatment without delay (Macit O, 2020). Compared to molecular analyzes such as polymerase chain reaction (PCR) and real-time polymerase chain reaction (real-time PCR) methods, immunochromatographic methods can save time and are simple to perform, making a significant contribution when large-scale testing is required (Gambino et al 2020). However, the sensitivity and specificity of immunochromatographic test kits are always lower than nucleic acid-based molecular methods and may cause false positive or negative results. Although polymerase chain reaction (PCR) and real-time polymerase chain reaction (real-time PCR) are fast methods, they cannot be used in all conditions due to the need for complex devices such as thermal cyclers, complex imaging systems and experienced personnel for reproducing the target region. In recent years, nucleic acid-based molecular techniques have been developed, such as loop-mediated isothermal amplification (LAMP) PCR, which enables the amplification of target regions in isothermal conditions without the need for these devices. Using this technique, it has been ensured that molecular tests can be run in different conditions with only a simple heater, without the need for complicated devices and qualified personnel (Kashir et al, 2020; Carter et al, 2020). These LAMP-based systems, which can be used in the field and do not require a trained technician to operate, have been used at many points in the rapid and reliable detection of SARS-CoV-2 during the COVID-19 epidemic (Marino et al, 2022).

In this period when food is both limited and so valuable (Roubík et al., 2022), it is important to produce local molecular kits based on the colorimetric Loop-mediated isothermal amplification (LAMP) method and use them in the field, instead of using immunocrotographic tests with low sensitivity used at the head of animals in farms.

It is seen that there were limited studies on this topic. But similar to the results of our study, Fan et al and Xie et al, showed that the LAMP PCR design is a reliable and rapid method that can be used for the detection of calves diarrhea (Fan et al, 2012; Xie et al, 2012).

In our study, we compared the LAMP PCR method with the immunochromatographic test method, which is frequently used in the field, and the in-house PCR method, which is performed in the laboratory, for the diagnosis of these 4 factors that are important for calf diarrhea. When the data obtained as a result of our study were examined, it was determined that the LAMP PCR method performed better than both test methods.

As a conclusion, It is very important for early diagnosis to use the colorimetric LAMP method, which can be detected in separate tubes in 15-30 minutes in farm and field conditions without requiring qualified personnel and complicated devices. We believe that, thanks to early and reliable diagnosis, the calf mortality rates will be greatly reduced, as the infected animals will be treated early. For this reason, it is very practical and important to use the LAMP PCR method in the field, which gives reliable results for the reduction of calf mortality.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: M.D., A.Y., Design: M.D., A.Y., Data Collection or Processing: M.D., A.Y., S.E., Analysis or Interpretation: M.D., A.Y., Literature Search: M.D., A.Y., S.E., H.D., Writing: M.D., A.Y., S.E., H.D.

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