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A new molecular approach to the diagnosis of small ruminant morbillivirus with EvaGreen based assay

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Abstract: The present work describes a new method that is anticipated to contribute to the global control and eradication strategies of small ruminant morbillivirus (SRMV), which negatively affects sheep and goat breeding in many countries where outbreaks emerge. Described assay was developed to detect SRMV by targeting the fusion glycoprotein gene. In the study, the EvaGreen based real-time RT-PCR method is compared with real-time RT-PCR based on TaqMan probe, which is recommended as the diagnostic test by World Organisation for Animal Health (OIE). Detection limit, normalized fluorescence values, and sensitivity of the methods were evaluated in the analysis. The lowest limit of detection obtained was approximately 12 RNA copies per reaction corresponding to a C_T value of 36.79. According to the results, it was observed that the developed in-house method has a specific, sensitive, and powerful detection limit as much as the gold standard test. As a result, it was concluded that the proposed method, which was developed as a more cost-effective and easily optimized analysis, could provide an advantage for detecting SRMV in diagnostic laboratories.

Keywords: EvaGreen, fusion glycoproteingene, low-cost diagnosis, real-time RT-PCR, SRMV

1. Introduction

Peste des petits ruminants (PPR), a systemic disease of small ruminants, has devastating effects on the livestock industry with a high fatality rate [1,2]. The causal virus of PPR called peste des petits ruminants virus (PPRV) has been renamed recently as small ruminant morbillivirus (SRMV) in the *Morbillivirus* genus under the *Paramyxoviridae* family [3]. SRMV has a negative-stranded 16 kilobase RNA genome that encodes six structural and two nonstructural proteins [4]. While a single serotype of SRMV was reported in studies, it was determined that fusion glycoprotein (F) and nucleoprotein (N) genes were genetically grouped into four different lineages in partial sequence analysis [5–8].

SRMV disease is included in the transboundary animal diseases list by the World Organisation for Animal Health (OIE) because it is contagious and spreads very rapidly. The meeting held in Abidjan, Cote d'Ivoire in 2015 with the participation of the Food and Agriculture Organization (FAO) and the OIE, was aimed at the global eradication of SRMV by 2030 [2,8]. Due to the high morbidity and mortality rates [9], rapid and accurate diagnostic tools need to be developed to control and eliminate SRMV disease [7,10].

SRMV is not characterized by a single pathognomonic clinical symptom and can be confused clinically with many different diseases such as bluetongue and capripox [11]. Therefore, the diagnosis of the disease remains difficult without a complementary laboratory test, especially in a new epidemic case. A clinically suspected case of SRMV can be confirmed using a range of assays, including virus isolation, AGID, ELISA [9, 12], and immunochromatography [13]. However, more specific and sensitive diagnosis of SRMV became feasible with the molecular techniques developed in recent years. Among the molecular techniques performed, the quantitative reverse transcription polymerase chain reaction (qRT-PCR) method has been preferred by veterinary diagnostic laboratories in the diagnosis of viral diseases due to its fast and high sensitivity [10,14,15].

EvaGreen (EG) dye is a green fluorescent nucleic acid dye that becomes fluorescent when bound to dsDNA and the dye is essentially nonfluorescent by itself but becomes highly fluorescent upon binding to dsDNA [16]. Availability and cost-effectiveness [17], fluorescent signal strength, increased precision, and production of a more linear decay graph can be regarded as the main advantages

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of the EG dye in the qPCR analysis [18]. EG-based PCR studies that identify many pathogenic agents, including human, animal and plant viruses, have been performed successfully [19–21]. Although two SYBR Green I based qRT-PCR studies have been reported for the detection of SRMV [22,23], a study that uses EG dye for the diagnosis of SRMV has not been reported yet.

The F glycoprotein of morbilliviruses, which has a trigger function in viral replication, has a highly conserved gene sequence antigenically [24,25]. Moreover, the F glycoprotein makes a significant contribution during the development of a specific and protective immune response against SRMV [26]. Previous studies have shown that conventional RT-PCR targeting a fragment of the gene encoding the F glycoprotein can be used to successfully detect the genetic material of SRMV from clinical samples [27]. Furthermore, it has also been reported that these amplified F glycoprotein genome sequences are highly suitable for molecular epidemiological research and SRMV genotyping [6].

In this study, a highly conserved sequence portion of the SRMV F glycoprotein gene (lineage IV) was detected by an EG-based qRT-PCR test and the performance of the method was evaluated.

2. Materials and methods

2.1. Collection of tissue specimens

Animal tissue material consisted of 703 tissue samples (spleen, lung, lymph node, liver, and internal organs of aborted fetuses) from 218 animals (sheep, goat and fetuses) sent from Konya province and its surrounding (Aksaray, Antalya, Isparta, Niğde province) to Konya Veterinary Control Institute with suspicion of PPR between years 2016 and 2018. The fetal tissues included in the study were pooled and used for viral nucleic acid extraction. Information on tissue material isdisplayed in Table 1. All tissue samples were stored at –85 °C before molecular diagnosis. SRMV detection from all field samples was performed directly with the gold standard (N-protein gene-based qRT-PCR) method [10,15]. Furthermore, virus isolates were used in addition to SRMV positive clinical samples to evaluate and improve the EG-based qRT-PCR assay in this research (Table 1).

2.2. Cell culture

For viral propagation, SRMV nucleic acids positive goat and sheep lung samples (line 1–4 in Table 1) were homogenized using tissue rupture (Qiagen, Valencia, CA) in 10% PBS with antibiotics. The homogenates were centrifuged at 3000 g for 15 min at 4 °C to obtain supernatants. The supernatants were inoculated to Vero cell line and maintained in DMEM (Sigma-Aldrich, Darmstadt, Germany) enriched with 5% fetal bovine serum and antibiotics (Sigma-Aldrich, Catalog No: A5955). The cells were incubated at 37 °C and controlled regularly for observation of cytopathic effect (CPE). CPE positive cultures obtained after three passages were confirmed with SRMV specific conventional RT-PCR [27] and qRT-PCR [15] methods.

2.3. Primer design

Specific primer pairs of the F glycoprotein gene region of SRMV (using sequences of Nigeria 75/1, Benin/10/2011, KN5/2011, Turkey_1996, and Turkey2000 strains; with respective GenBank accession numbers of X74443, KR781449, KM463083, FR667647, NC_006383) were designed to develop the EG-based in-house method. Reference SRMV sequences were aligned with MAFFT [28] and were edited with AliView v1.26 [29] manually before primer design using Primer3-v.0.4.0 software [30]. The designed primers corresponded to nucleotide positions 954>973 (FwdSRMV_F: 5'-TCATTGATGAATTCCCAAGC-3') and 1048<1027 (Rev SRMV_R: 5'-GTCTCCTTATTTGCAAGTCTGA-3') in the gene sequence (Figure 1). The primer pair targets a highly conserved region of the F glycoprotein gene within the specific sequence reported by Forsyth and Barrett (1995). The size of the F glycoprotein gene fragment targeted by the primer pair is 95 bases (bp).

2.4. Nucleic acid extraction and RT-PCR assay

Viral nucleic acid was extracted from the field samples and CPE positive culture samples using the robotic extraction method (QIAcube, Qiagen, Hilden, Germany) with the RNeasy Mini Kit (Qiagen). The concentration and purity of the nucleic acid samples were measured by a NanoDrop device (DeNovix, Wilmington, DE, USA). Fglycoprotein gene-based conventional RT-PCR detection [27] was carried out using a LifeECO Thermal Cycler (Bioer Technology, Hangzhou, China) with OneStep RT-PCR Kit (Qiagen). The analysis was carried out in 25 µL reaction mixtures containing 5 µL 5X RT-PCR buffer, 5 µL 5X Q solution, 1 µL OneStep RT-PCR enzyme mix, 10 mmol dNTPs, 1 µL (10 pmol/µL) forward-reverse primer, 8 µL nuclease-free water, and 3 µL template RNA. The optimized RT-PCR condition was as follows: reverse transcription (RT) 50 °C for 30 min; PCR initial activation 95 °C for 15 min; followed by 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min and extension at 72 °C for 2 min; with a final extension step at 72 °C for 10 min. Amplification products were loaded and visualized on a 1% agarose gel with GelRed (Biotium, Fremont, CA, USA).

2.5. Optimization of the qRT-PCR assays conditions

EG-based qRT-PCR assay was standardized on a Qiagen Rotor-Gene Q (5PLEX HRM, Qiagen) using AgPath-ID One-step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) supplemented with EG dye (Biotium). The analysis was carried out 20 µL reaction mixtures containing

Table 1. Evaluation of SRMV F glycoprotein gene detection of the EvaGreen qRT-PCR assays. For this purpose, samples 1–32 were used from the South Central Anatolia Region and the Western Mediterranean Region in Turkey. Information on the references used to develop the analysis is registered inline 40–47.

No	Sample particulars	EvaGreen qRT-PCR[a]		TaqMan qRT-PCR[b]		EG modified qRT-PCR[c]		RT-PCR[d]
		C_T value	NF value	C_T value	NF value	C_T value	NF value	
1	Virus isolate-1 from goat lung tissue-1	19.94	0.72	24.60	0.26	20.29	0.88	Pos
2	Virus isolate-2 from goat lung tissue-2	23.70	0.64	26.77	0.25	25.34	0.70	Pos
3	Virus isolate-3 from goat lung tissue-3	24.41	0.62	28.23	0.24	25.07	0.78	Pos
4	Virus isolate-4 from ovine lung tissue-1	24.73	0.62	29.03	0.22	25.96	0.68	Pos
5	Goat bronchial lymp node-1	22.14	0.53	23.04	0.27	20.05	0.70	Pos
6	Ovine bronchial lymp node-1	12.71	0.83	14.42	0.31	12.37	0.99	Pos
7	Ovine bronchial lymp node-2	15.98	0.83	16.94	0.31	15.23	0.98	Pos
8	Ovine bronchial lymp node-3	18.81	0.78	20.30	0.28	18.23	0.93	Pos
9	Ovine mesenteric lymp node-1	23.12	0.70	24.61	0.27	22.64	0.84	Pos
10	Ovine mesenteric lymp node-2	27.76	0.58	30.08	0.22	27.89	0.68	Pos
11	Ovine mesenteric lymp node-3	30.26	0.48	33.21	0.17	31.52	0.42	Neg
12	Ovine mesenteric lymp node-4	22.00	0.70	25.00	0.26	22.91	0.83	Pos
13	Ovine mesenteric lymp node-5	26.02	0.60	29.53	0.22	27.07	0.68	Pos
14	Goat mesenteric lymp node-1	27.08	0.58	30.72	0.21	28.88	0.58	Pos
15	Goat mesenteric lymp node-2	27.53	0.57	31.24	0.21	30.01	0.49	Pos
16	Goat oral lesions-1	24.49	0.65	25.96	0.25	22.56	0.85	Pos
17	Goat oral lesions-2	15.54	0.82	16.52	0.30	15.14	1.00	Pos
18	Ovine spleen-lung tissues-1	18.05	0.78	19.41	0.27	17.28	0.96	Pos
19	Ovine spleen-lung tissues-2	21.18	0.73	22.75	0.27	20.93	0.89	Pos
20	Ovine spleen-lung tissues-3	25.71	0.61	27.48	0.25	25.06	0.78	Pos
21	Ovine spleen-lung tissues-4	29.38	0.51	33.47	0.16	29.89	0.53	Neg
22	Ovine spleen tissue-1	20.99	0.74	23.62	0.27	21.08	0.89	Pos
23	Ovine spleen tissue-2	24.50	0.65	26.97	0.25	24.78	0.78	Pos
24	Ovine spleen tissue-3	28.52	0.54	32.35	0.17	28.54	0.63	Pos
25	Goat lung tissue-4	18.06	0.79	20.24	0.29	18.73	0.93	Pos
26	Goat lung tissue-5	27.47	0.60	28.75	0.24	25.93	0.75	Pos
27	Goat lung tissue-6	30.63	0.44	32.08	0.20	28.73	0.63	Neg
28	Ovine lung tissue-2	25.21	0.64	24.89	0.32	23.60	0.80	Pos
29	Goat foetus tissues-1	25.50	0.64	27.16	0.31	26.69	0.73	Pos
30	Goat foetus tissues-2	29.23	0.51	32.19	0.19	30.95	0.46	Neg
31	Goat foetus tissues-3	32.16	0.32	36.33	0.05	34.14	0.22	Neg
32	Sheep foetus tissues-1	30.70	0.42	37.23	0.05	32.10	0.37	Neg
33	SRMV free goat tissue-1	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
34	SRMV free goat tissue-2	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
35	SRMV free goat tissue-3	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
36	SRMV free goat tissue-4	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
37	SRMV free goat tissue-5	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
38	SRMV free goat tissue-6	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
39	SRMV free goat tissue-7	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg

Table 1. (Continued).

40	SRMV Nig 75-1 (Lin I-F gene)/Vaccine strain*	12.45	0.81	16.56	0.31	13.35	0.90	Pos
41	SRMV Benin 2011 (LinII)/RNA extracts**	18.82	0.76	25.76	0.25	20.03	0.88	Pos
42	SRMV Kenya 2010 (Lin III)/RNA extracts**	33.19	0.23	35.65	0.08	31.56	0.38	Pos
43	SRMV (Lin IV-F gene)/Field isolate***	21.85	0.54	23.84	0.27	20.16	0.72	Pos
44	CDV/Vaccinestrain, NobivacPuppy DP, Intervet	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
45	BDV/Field isolate****	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
46	BVDV/Field isolate****	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
47	BTV/Vaccine strain*	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg
48	Negative control (nuclease-free water)	No C_T	No NF	No C_T	No NF	No C_T	No NF	Neg

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a = In-house assay, b = Gold standard qRT-PCR assay, c = Gold standard assay modified with EG, d = F glycoprotein gene RT-PCR assay, C_T= Threshold cycle values, NF = Normalized fluorescence values.

5 µL RNA, 10 µL 2X RT-PCR Buffer, 0.8 µL 25X RT-PCR Enzyme Mix, 1 µL 20X EG dye, 0.8 µL forward-reverse primer (10 pmol/µL), and 1.6 µL nuclease-free water. The optimized qRT-PCR conditions were as follows: reverse transcription (RT) 45 °C for 10 min; PCR initial activation 95 °C for 10 min; followed by 40 cycles of denaturation at 95 °C for 15 sec, and primer annealing-extension at 60 °C for 15 s. The fluorescence measurement was reported at the end of each cycle. A melt curve analysis was performed to verify the specificity of the PCR products (at a rate of 0.2 °C per s and collecting fluorescence data continuously on the 90–65 °C ramp).

In addition, the gold standard method [15] and its EG modified optimization were performed under the conditions described above using the same One-step RT-PCR Kit and real-time PCR cycler for comparative analysis. Although the EG modified qRT-PCR assay reaction mixture was performed with the same volume of the reagents, 0.4 µL probe (5 pmol/µL) was used instead of 20X EG dye in the TaqMan probe-based assay. In both assays (TaqMan probe-based and EG modified) described here, the primer pair reported in the gold standard test [15] was used.

2.6. In vitro transcribed RNA

SRMV samples from a case in Turkey (year 2017) were generated using the F gene-based RT-PCR method [27]. These RT-PCR amplicons were cloned into the pCR2.1-TOPO vector (Invitrogen, Paisley, UK) according to the manufacturer's instructions. For in vitro transcription using Megascript T7 kit (Thermo Fisher Scientific, Waltham, MA, USA), 1 µg linearized product was used and transcribed RNA was digested to remove the remaining DNA regions. Samples were purified with RNeasy Cleanup Kit (Qiagen) according to the manufacturer's instructions. Then the concentration of purified RNA samples was quantified by a NanoDrop device (DeNovix).

2.7. Analytical specificity and sensitivity

SRMV strains representing all lineages (I–IV),were used as the reference and control strains for the developed assay. Also, canine distemper virus (CDV), border disease virus (BDV), bovine viral diarrhea virus (BVDV), and bluetongue virus (BTV) was used for specificity of the in-house method. Furthermore, to investigate the possibility of nonspecific amplification, seven nucleic acid extractions from goat tissue previously determined to be SRMV free were further analyzed. The sources of all RNA extracts, vaccine strains, and field strains used to develop in-house assay are displayed in Table 1.

In order to determine the analytical sensitivity of the test, a standard curve was constructed from ten-fold dilutions from transcribed RNA and all dilutions were assayed in duplicates. The extraction of RNA from each dilution and newly developed EG-based qRT-PCR assay were applied as described above. The ten-fold diluted RNA standard was tested repeatedly for assessing the reproducibility of the test. The intraassay variations and interassay variations were recorded according to duplicate or triplicate experiment results using Rotor-Gene Q series v. 2.3.1-Build 49 (Qiagen) software. RT-PCR also was performed to compare analytical sensitivity.

3. Results

3.1. Diagnostic performance of the EG-based qRT-PCR

The performance of the newly developed assay was investigated for assessing its performance. The in-house assay was compared to both the TaqMan probe-based

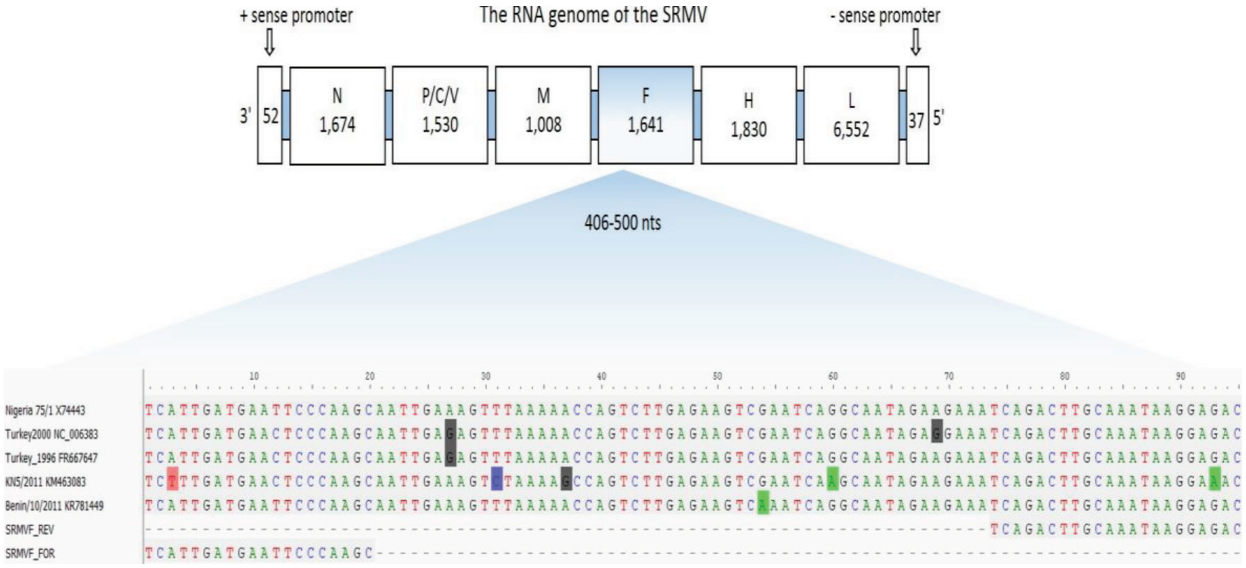

Figure 1. Position of constructed primer pair on the SRMV genome.

gold standard method [15] and the EG modified qRT-PCR analysis of this method. The newly developed assay was also compared to conventional RT-PCR [27] targeting the same sequence. SRMV nucleic acids were detected positive in the same 32 (14.7%) samples in each of the EG-based qRT-PCR, TaqMan probe-based qRT-PCR and EG modified qRT-PCR methods. The threshold cycle (C_T) values obtained in these qRT-PCR analyzes are shown in Table 1. Also, 26 (12%) of the same samples were found positive with conventional RT-PCR analysis (Table 1). The diagnostic performance of the in-house method has been evaluated in terms of effectiveness and PCR signal strength of EG. The normalized fluorescence (NF) values obtained in the comparative qRT-PCR analyses described above displayed that a more precise and strong fluorescence signal was obtained in EG-based analyses, especially in late signals (late cycles). NF values ranged from 0.32 to 0.83, from 0.05 to 0.31, and from 0.22 to 0.99, respectively (Table 1). Additionally, it was shown that all lineages (I–IV) of SRMV could be detected by the new, optimized method (four lineages of SRMV were displayed line 40–43 in Table 1).

3.2. Specificity

The specific amplification signal from the SRMV positive specimens was characterized by creating a melting peak at 78.67 ± 0.30 °C (Figure 2). The melt peak analysis showed that the in-house assay produced an amplification signal only with SRMV specific nucleic acid, and no primer-dimers or nonspecific amplification was observed. The assay performed is high-throughput and specific. Moreover, no amplification signal occurred with the other related viruses (CDV, BVDV, BDV, and BTV), no template control (NTC), and SRMV free 7 goat samples (Table 1).

3.3. Creation of standard curve and detection limit of the assay

A series of ten-fold dilutions starting from 2.8×10^{14} copies were prepared to create a standard curve and the relationship between C_T values and RNA dilutions were analyzed. The concentration of RNA was 15 ng/µL, and the created standard curve that displayed linearity over the entire range of quantification covered a linear range of eight orders of magnitude. Regression correlation was detected with a coefficient of determination (R^2) of 0.9995, the reaction efficiency of 90%, and the slope of –3.575. While the lowest detection limit was found 2.2×10^1 copies/µL in conventional RT-PCR (figure not shown), it was obtained approximately 12 copies/reaction with corresponding to C_T value of 36.79 ± 0.5 in qRT-PCR (Figure 3). The EG qRT-PCR assay proved more sensitive than conventional RT-PCR assay.

3.4. Reproducibility

The intraassay and interassay reproducibility of the in-house assay was detected based on the C_T values corresponding to the RNA standard ranging from 2.2×10^7 copies/µL to 2.2×10^0 copies/µL. Intraassay and interassay standard deviation (SD) and coefficient of variation (CV) values were calculated after the experiments and shown in Table 2. Also, the deviation ($R^2 = 0.9995$) indicated that the test was reproducible (Figure 3).

4. Discussion

The present work describes a high-throughput sensitive EG-based qRT-PCR test that targets the highly conserved near-center sequence of the SRMV F glycoprotein gene. The in-house test was evaluated with clinical samples and cell culture supernatant representing SRMV lineage

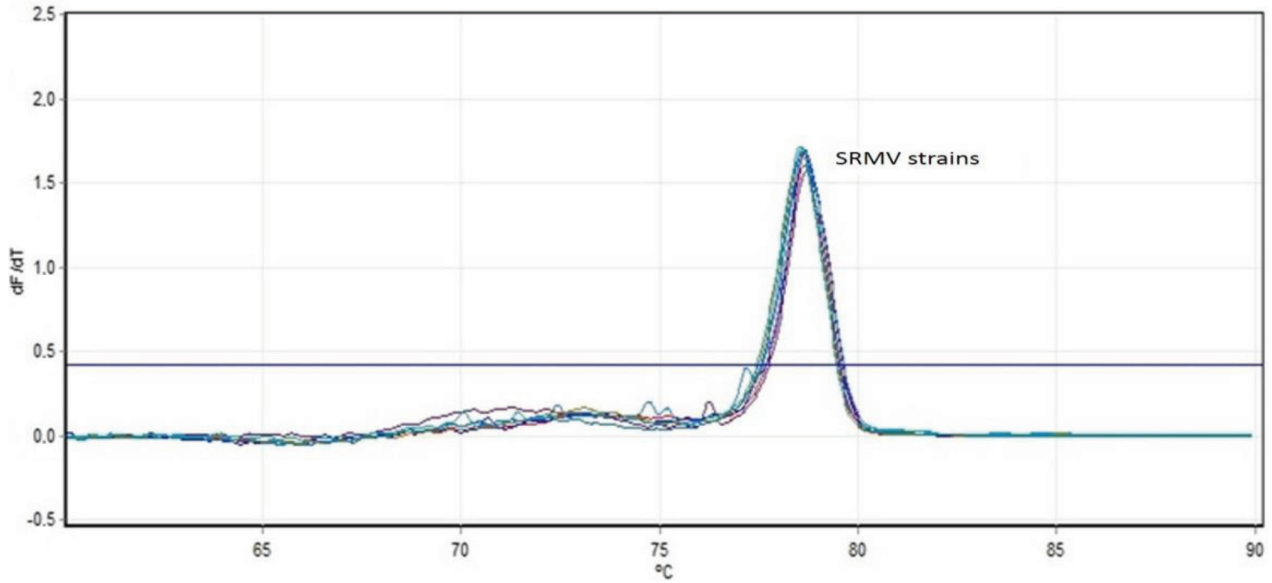

Figure 2. Melting curve analysis of EvaGreen qRT-PCR.

Table 2. Intraassay variability and interassay reproducibility of the EvaGreen qRT-PCR assay.

RNA copies/µL		2.2×10^7	2.2×10^6	2.2×10^5	2.2×10^4	2.2×10^3	2.2×10^2	2.2×10^1	2.2×10^0
A	C_T	11.96	15.72	19.26	22.64	26.34	30.06	33.53	36.64
	SD (±)	0.16	0.07	0.05	0.09	0.26	0.43	0.38	0.40
	CV%	1.33	0.44	0.26	0.40	0.98	1.43	1.13	1.1
B	C_T	11.98	15.87	19.39	22.80	26.46	30.18	33.80	36.79
	SD (±)	0.12	0.26	0.25	0.28	0.28	0.37	0.55	0.52
	CV %	1	1.63	1.29	1.22	1.05	1.23	1.63	1.41

A = Intraassay variability, B = Interassay variability, C_T = Mean constant threshold cycle values, SD = Standard deviation of constant threshold cycle values, CV = Coefficient of variation.

IV sampled from the South Central Anatolia Region and the Western Mediterranean Region in Turkey. EG qRT-PCR test, which can be useful in clinical diagnosis, has been standardised by evaluating its efficiency, specificity, reliability and reproducibility.

PPR, which is a threat to sheep and goat production, is thought to limit food safety and elevate poverty, since it cannot be controlled and eradicated, especially in developing countries [31]. For this purpose, FAO and OIE have developed a strategy aimed at controlling and eliminating epidemics by 2030. This strategy consists of four coordinated phases. These are (a) evaluation of the epidemiological characteristics, (b) control (the aim is to inhibit the transmission of SRMV, performing mass vaccination), (c) eradication, and (d) posteradication [8]. PPR has been historically confused with other similar clinical diseases, such as bluetongue, foot and mouth disease, and capripox [11], therefore laboratory diagnosis is necessary to discriminate other diseases with similar clinical signs. For this purpose, many different molecular-based diagnostic techniques have been developed recently. For instance, two different SYBR Green I-based qRT-PCR assays have been published for the specific diagnosis of SRMV [22, 23]. These methods are qRT-PCR methods which may pose certain possible disadvantages of SYBR Green I dye, such as toxicity, amplification inhibition, and tendency to promote nonspecific amplification when used above a certain threshold concentration, or are characterized by a delay in C_T [32].

As a noncovalent dsDNA intercalating dye, EG is not sequence-specific [16, 17]. The main advantages of this new generation dye are its high availability, cost-effectiveness, nonmutagenicity, and noncytotoxicity, as well as hydrolytical and thermal stability [18,33]. Due to

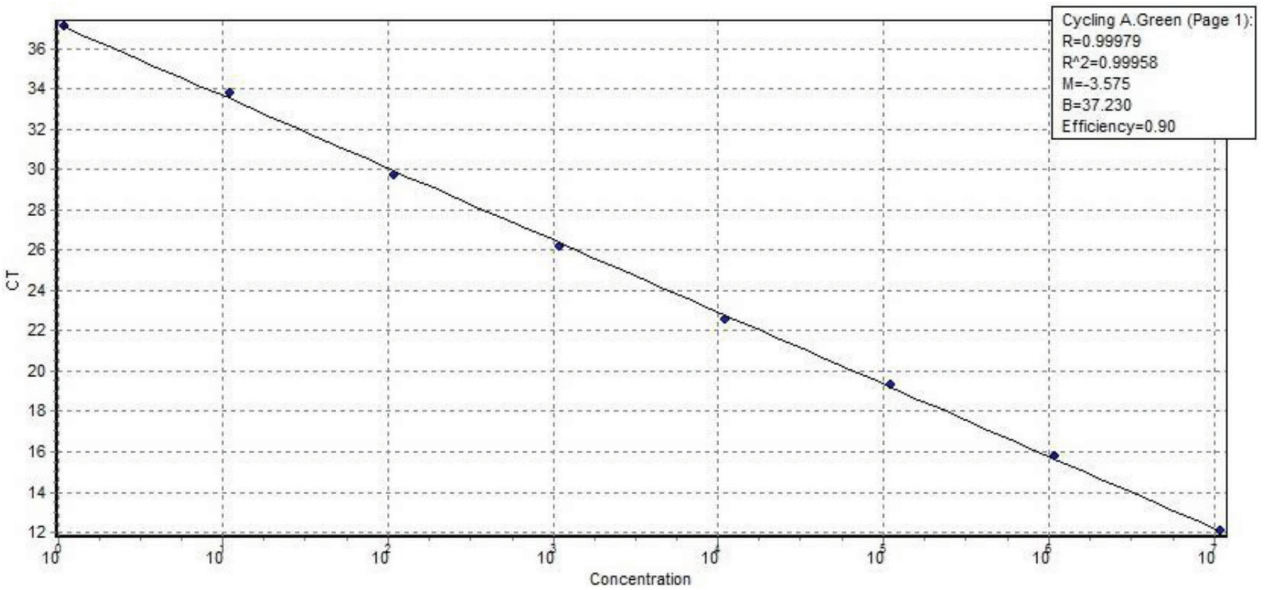

Figure 3. Linearity of the standard curve, the detection limit of the assay was 12 copies/reaction with a corresponding C_T value of 36.79.

these potential advantages, EG dye has increasingly being used in many new diagnostic applications, including qRT-PCR and HRM methods.EG-based qRT-PCR assays have been developed for the specific, sensitive, and low-cost detection of many viral agents that cause infections in domestic animals. On the other hand, utilization of the EG dye for the diagnosis of SRMV has not been reported yet. It has been reported that EG-based assays would provide significant benefits in epidemiological and etiological studies as well as sporadic outbreaks [34–36]. In agreement with the similar literature, the in-house method is very specific for SRMV detection in goat and sheep tissues (lung, lymp node, oral lesion, and spleen).

In the present study, an in-house method was compared with the gold standard assay (by using a TaqMan probe and probe-free EG modification) to determine and compare the SRMV detection capacity in the clinical samples. In comparative analyses, it was determined that the EG-based method detected strong positive and weak positive samples 2 to 5 C_T earlier, respectively. Similarly, the fluorescence signal values of this method were recorded higher (these values were displayed in lines 6 and 31 in Table 1). The test specificity was monitored by melting curves. SRMV F glycoprotein gene sequence-specific amplification was determined by establishing a consistent melting peak at 78.67 ± 0.30 °C (Figure 2). No primer-dimer or nonspecific products were observed. Besides, the assay was examined for specific signal amplification with a number of pathogens clinical and genetically related to SRMV, and according this data, amplification signal did not occur with any one of the viruses (this result was also confirmed by conventional PCR). According to these results, it has been observed that EG-based molecular detection is effective, high-throughput and sensitive. As a result, it is suggested that the in-house assay is specific for routine molecular detection of the SRMV F glycoprotein gene and can provide an efficient diagnosis.

The target detection limit, which is a critical parameter in PCR methods, depends on various factors such as analyte, enzymatic processes, properties of the applied dye or probe, and the optical detection system used [17]. Thus, a standard curve was constructed with synthetic RNA standards to determine the limit of detection of the EG qRT-PCR assay. It was observed that the standard curve containing eight linear size ranges reveals linearity over the entire quantification range and these data were considered applicable for accurate quantification of SRMV. The lowest limit of detection obtained was approximately 12 RNA copies per reaction corresponding to a C_T value of 36.79 (Figure 3). In the molecular diagnosis of SRMV, EG-based assay is as sensitive as other qRT-PCR assays [14,15,22].

The SD and CV values calculated based on C_T values obtained by the analysis of RNA standards, were displayed in (Table 2). The detected coefficient of determination based on the C_T values and deviation ($R^2 = 0.9995$) revealed that this newly developed assay is reproducible similar to previously reported methods [14,15].

The in-house method was sufficient to identify SRMV in small ruminant specimens but also should be tested on field samples collected from other susceptible species. Also, to increase the usability of this newly developed

test, further research should be conducted using different endemic site samples representing SRMV lineages I, II, and III.

An EG-based qRT-PCR assay with 100% specificity and sensitivity for the molecular detection of the SRMV Fglycoprotein gene was introduced in the present study. The improved sensitivity is likely to be sourced from the physicochemical properties of the EG dye, which has little nonspecific intercalation and inhibitory effect for qPCR analysis [33]. Low-cost EG dye was used for the proposed test [17,33–36], which enabled the rapid and specific detection of SRMV Fglycoprotein gene in clinical specimens, without any requirement for sequence optimization of specific oligonucleotide probes. Sequence-specific oligonucleotide probe-based assays have higher cost per reaction than EG-based assays [17]. The results of this study indicate that low-cost EG dye has a highly efficient and effective testing capability.

In conclusion, the cost-effective SRMV diagnostic method is considered to be useful for the control and eradication strategy that continues to be implemented in PPR endemic developing countries. Specific, rapid, and reproducible diagnosis of suspected clinical specimens of SRMV, one of the transboundary diseases of global control and eradication studies, is very important. The EG-based assay targeting the F glycoprotein gene is a high-throughput sensitive method for the detection and quantification of SRMV nucleic acids. Since EG has the advantages of low cost and fluorescent signal strength, it could be used effectively in diagnostic laboratories.

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Conflict of interest
The authors declare that they have no conflict of interest.

Contribution of authors
Experiments were performed by all authors. MEO, ET, OA, MD and ATU designed the study, provided general supervision, and prepared the manuscript. All authors have read and approved the submission of the manuscript.

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