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Development of a Novel Real-Time PCR-Based Test Panel System for Detection of Infectious Abortion in Domestic Sheep

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ABSTRACT

The etiologic factors of abortion in sheep can be complicated by infections with more than one pathogen. This makes it difficult to identify the pathogen involved in abortions. In this study, we developed a real-time PCR-based diagnostic panel that can analyze many infectious agents simultaneously and allow faster diagnosis of some important infectious agents in ovine abortions. For this purpose, some important pathogens that are frequently detected in the study region and cause abortion in sheep (Bluetongue virus, Border disease virus, Peste des petits ruminant's virus, *Chlamydophila abortus*, *Coxiella burnetii*, *Neospora caninum* and *Toxoplasma gondii*) were selected as targets. In order to evaluate the diagnostic test panel, standard curves of positive standard nucleic acid samples were first generated, and the analysis processes checked. According to the test results, the panel proved to be highly sensitive and specific. All target pathogens were also detected within a few hours. On the basis of results obtained, it can be concluded that the developed system can be used as a diagnostic panel with a broad detection of infectious agents in sheep abortion.

Key words: Sheep abortion, Diagnosis, Infectious agents, Real-time PCR, Verification.

INTRODUCTION

Abortion in domestic sheep is one of the most important causes of economic losses worldwide. Most infectious agents causing abortions in sheep are known to be zoonotic or are on the list of notifiable animal diseases of the World Organization for Animal Health (WOAH) (Njaa 2012; Liu et al. 2014; Di Bari et al. 2023; Tomori et al. 2023). Rapid and effective diagnosis of these pathogens, which affect public health and have a devastating impact on sheep flocks by causing epidemics, is important for managing and controlling epidemic diseases, limiting the spread of infections and preventing zoonotic diseases, thus protecting public health (Menzies 2011; Díaz Aparicio 2013). Rapid and accurate laboratory diagnosis plays a central role in the effective and efficient control of infectious outbreaks; thus, the development of molecular diagnostic methods has become the focus of diagnostic laboratories (Lin et al. 2000; Gaede et al. 2005; Klee et al. 2006; Willoughby et al. 2006; Hofmann et al. 2008; Pantchev et al. 2009; Batten et al. 2011; Barry et al. 2019). However, it appears that the current molecular methods used by many laboratories worldwide are not manageable, but time-consuming or costly.

In cases of abortion caused by multiple infectious agents, the use of molecular methods, in contrast to conventional diagnostic tests, is extremely important to minimize the loss of offspring and to take appropriate control measures as soon as possible (Clothier and Anderson 2016; Wolf-Jäckel et al. 2020; 2021). In the last 30 years, molecular-based diagnostic methods have become important tools in veterinary laboratory workflows. Real-time PCR is actively used in clinical laboratories and research institutes to provide results in less time and faster than conventional PCR (Yang and Rothman 2004; Watzinger et al. 2006; Sloots et al. 2015). The purpose of the study was to develop a diagnostic method with high sensitivity and specificity that can simultaneously and rapidly detect some of the most important pathogens in sheep abortion. For this purpose, a real-time PCR-based diagnostic panel was developed for the simultaneous detection of several important infectious abortion pathogens, i.e., *Chlamydophila abortus* (*C. abortus*), *Coxiella burnetii* (*C. burnetii*), Bluetongue virus (BTV), Border disease virus (BDV), Peste des petits ruminant's virus (PPRV), *Neospora caninum* (*N. caninum*) and *Toxoplasma gondii* (*T. gondii*).

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MATERIALS AND METHODS

Ethical approval

This research was carried out with the permission of the General Directorate of Food and Control, Türkiye, dated 10.01.2024 and numbered E-12760097.

Materials and pathogens

The study was carried out at the Microbiology Laboratory of the Republic of Türkiye, Ministry of Agriculture and Forestry, Konya Veterinary Control Institute. The study material consists of sheep fetus samples (n=32) sent to the Laboratory of Molecular Microbiology of the provinces within the area of responsibility of the institute (Fig. 1). It will be more appropriate to indicate these places on a map. These sheep samples (brain, kidneys, spleen, lungs and liver) were selected from those evaluated as *Brucella spp.* negative samples by the Bacteriology Laboratory of the Institute. Some of the main pathogens that cause abortion in sheep (Bluetongue virus, Border disease virus, Peste des petits ruminants virus, *Chlamydophila abortus*, *Coxiella burnetii*, *Neospora caninum* and *Toxoplasma gondii*) were selected in the analyzes as target pathogens.

Nucleic acid extraction procedure

The suspected organs of the sheep fetus (brain, kidneys, spleen, lungs, and liver) were stored in sterile tubes at -80°C until nucleic acid extraction. About 30-40mg of tissue samples were placed in 2mL of sterile Eppendorf tubes and 200µL of nuclease-free water were added and completely homogenized using a micro tissue homogenizer. The homogenates were centrifuged at 4,000rpm for 10min at +4°C and the supernatants were used for nucleic acid extraction. Nucleic acid extraction was performed using an automated extraction device (QIAcube, Qiagen, Germany) according to the procedure of the QIAamp cador Pathogen Mini Kit (Qiagen, CA) (Kreizinger et al. 2015). Subsequently, all extraction products were stored at -20°C until real-time PCR analysis.

Analytical sensitivity and specificity of diagnostic panels

To design diagnostic panels and evaluate the performance of PCR, all standard nucleic acid positive samples for the target agent were obtained from reference laboratories. Standard curves for the targeted agents were generated after 6 tenfold serial dilutions (1×10^0–1×10^6 copies/reaction) of the positive standard samples and a series of verification studies after real-time PCR analyzes using the same PCR protocol and heat cycles. Therefore, important parameters such as the limit of detection (LOD), the correlation coefficient (R^2) and the PCR efficiency (E) of all pathogens were determined. For one step real-time PCR assay, 5µL nucleic acid standard aliquots of the corresponding six 10-fold dilutions were added to the reaction mix. The linear regression line is given by the formula: Cycle Threshold (Ct) = M [$\log_{10}(x)$] + B, where M is the slope of the standard curve, B is the y-intercept, and x is the standard quantity. The amplification efficiency (E) was also calculated with the M for each linear regression line, using the formula: $E = [10^{(-1/M)}] - 1$. Also, each standard loaded was calculated to assess linearity, using the formula: log10(x) = [Ct − B]/M. All the calculations were performed in the Rotor-Gene Q series v.2.3.1-Build 49 software (WOAH 2014; Toohey-Kurth et al. 2020). During these studies, dozens of experiments were performed, and the most specific and effective heat cycles and PCR protocols were established. This ensured the efficiency and precision of the test. The PCR conditions and PCR protocols under which standard curves are generated and all verification is performed are described in detail in the section Analysis of clinical samples.

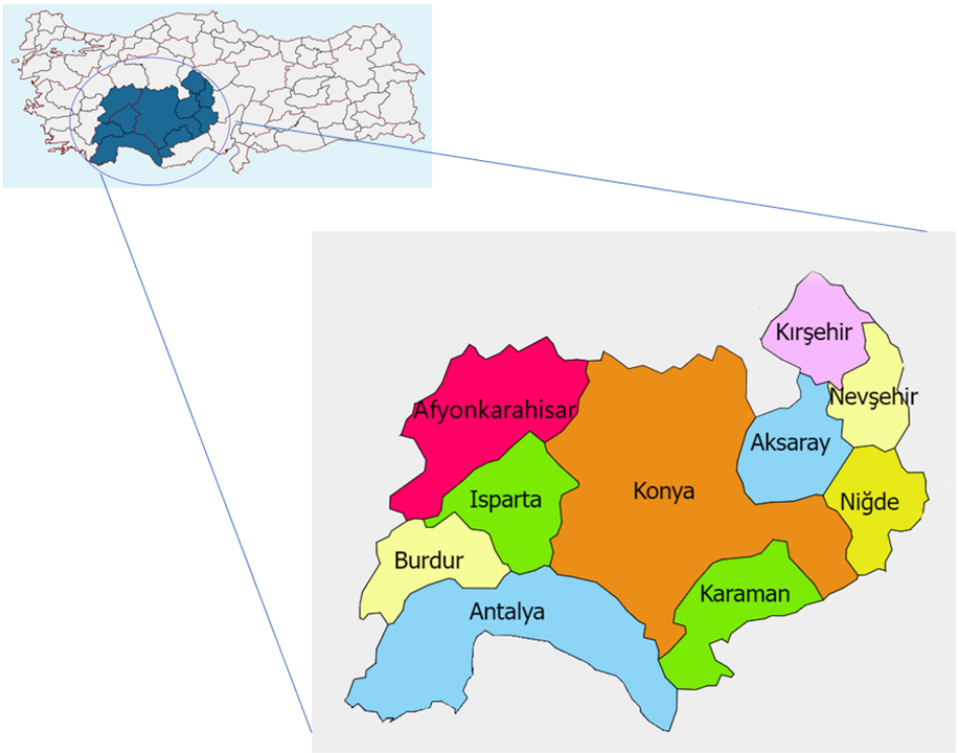

Fig. 1: The map of Türkiye showing the location of provinces where fetus samples were collected. Map showing the location of the study area in the Mediterranean and Central Anatolia.

Analysis of clinical samples

After completion of the verification studies, the developed panel was started to be used in the routine analysis of field samples. First, 32 *Brucella spp*. negative fetus samples were analyzed for all targeted pathogens. The sets of primer and probe recommended by WOAH, were used in all verification studies and in the diagnosis of field samples (Clothier and Anderson 2016; Tsuchiaka et al. 2016; Rahpaya et al. 2018; Wolf-Jäckel et al. 2020). Standard positive controls from reference laboratories were used in the preparation of diagnostic panels to verify and standardize the tests. In the real-time PCR process, BTV, BDV, and PPRV were performed as real-time RT-PCR under the same reaction conditions and in a single run. Furthermore, *C. abortus*, *C. burnetii*, *N. caninum*, and *T. gondii* were tested simultaneously and in a single-run real-time PCR assay. In this way, all samples were analyzed and displayed simultaneously with the generated test panels. Nuclease-free water was used as a negative control in the tests. The AgPath ID one-step RT-PCR kit (Thermo Fischer, USA) was used in real-time RT-PCR and the 480 Probe Master kit (Roche, USA) was used in real-time PCR assay. All analyzes were performed according to the kit procedures and all primers were added at a final concentration of 10µM and the probes were 5µM (Information on the primers and probes can be found in Table 1). When field samples were monitored, separate test tubes were taken for each pathogen and a mixture was prepared according to the kit procedure. We added 20µL from the prepared mixtures and 5µL from the template nucleic acids to the test tubes and subjected them to real-time PCR and real-time RT-PCR.

The analyses were performed on the Qiagen Rotor-Gene Q (5PLEX HRM, Qiagen). The AgPath-IDTM one-step RT-PCR reagent kit (Thermo Fisher) was used to perform a one-run real-time RT-PCR panel according to the kit instructions. The cycle conditions were as follows: reverse transcription at 60°C for 10min, inactivation at 95°C for 10s, followed by denaturation at 95°C for 10s and annealing and extension at 60°C for 20s for 40 cycles replicates. For DNA amplification of bacteria and protozoa, the LightCycler® 480 Probes Master kit (Roche Applied Science) was used for amplification with real-time PCR in one-run according to the manufacturer's protocol. The cycle conditions were as follows: Inactivation at 95°C for 10min, followed by denaturation at 95°C for 10s and binding and extension at 60°C for 30s, for 40 replicates. In both assays, fluorescent signals were obtained during the ligation and extension steps. The baseline and threshold were established according to the instrument procedure (Qiagen Rotor-Gene Q).

RESULTS

Analytical sensitivity and specificity of the detection panel

All analysis was performed on Qiagen Rotor-Gene Q (5PLEX HRM, Qiagen). The sensitivity values (LOD, Cutoff Ct value, R^2, E and M) of the one-step diagnostic panel, created for the detection of some pathogens that cause abortion in domestic sheep were determined. The limit of detection (LOD) established per reaction based on the DNA copy number of all pathogens ranged from 1 to 100 copies. The limit of detection (LOD detected in at least 95% of replicate analyzes) for the created panels ranged from 1.88-4.53 log10 RNA copies/reaction for real-time RT-PCR. And for real-time PCR, it ranged from 0.49-2.61 log10 DNA copies/reaction. The coverage of the calibration curves for each test was within a linear dynamic range of more than five orders of magnitude. All R^2 values were determined to be 0.99. The E values were in the range of 87-96%. The analysis showed that all primer and probe sets detected the target pathogen and there was no cross-reaction. This means that all analyses are very specific (the assay performance values of the real-time PCR panels are shown in Table 2).

Table 1: Sequence of primer-probe sets

Pathogen	Target gene	Primer/probe sequence (5'-3')	Amplicon size (bp)	References
Bluetongue virus	NSP3	Fwd: CCTGGACAAGGTCTCGGTAGAA Rev: ATTCAGGACCCCACCCAAAT Probe: FAM-CATGCTCGAGGATTGGGTCGTCGT-BHQ1	96	Hofmann et al. (2008)
Border disease virus	5'UTR	Fwd: CCGTGTTAACCATACACGTAGTAGGA Rev: GCCCTCGTCCACGTAGCA Probe: VIC-CTCAGGGATCTCACCACGA-NFQ-MGB	155	Willoughby et al. (2006)
Peste des petits ruminants virus	Nucleocapsid	Fwd: AGAGTTCAATATGTTRTTAGCCTCCAT Rev: TTCCCCARTCACTCTYCTTTGT Probe: FAM-CACCGGAYACKGCAGCTGACTCAGAA-Tamra	132	Batten et al. (2011)
Chlamydophila abortus	ompA	Fwd: GCAACTGACACTAAGTCGGCTACA Rev: ACAAGCATGTTCAATCGATAAGAGA Probe: FAM-TAAATACCACGAATGGCAAGTTGGTTTAGCG-Tamra	82	Pantchev et al. (2009)
Coxiella burnetii	Transposase	Fwd: GTCTTAAGGTGGGCTGCGTG Rev: CCCCGAATCTCATTGATCAGC Probe: FAM-AGCGAACCATTGGTATCGGACGTTTATGG-Tamra	295	Klee et al. (2006)
Toxoplasma gondii	B1	Fwd: TCCCCTCTGCTGGCGAAAAGT Rev: AGCGTTCGTGGTCAACTATCGATTG Probe: FAM-TCTGTGCAACTTTGGTGTATTCGCAG-Tamra	98	Lin et al. (2000)
Neospora caninum	Nc5	Fwd: CTGTGCT CGCTGGGACTTC Rev: CGATTTACGACATACGGTGT TCA Probe: FAM-CATCGGAGGACATCGCTCACTGA CTG-BHQ1	85	Barry et al. (2019)

Table 2: Assay performance values

Pathogens	Assay	LOD [a] (copies/reaction)	Cutoff Ct value [b]	E (%) [c]	R^2 [d]	M [e]
Bluetongue virus	Real-time RT-PCR	4.53 log_{10}	36	95	0.99	-3.42
Border disease virus	Real-time RT-PCR	1.88 log_{10}	36	88	0.99	-3.85
Peste des petits ruminants virus	Real-time RT-PCR	1.94 log_{10}	37	89	0.99	-3.56
Chlamydophila abortus	Real-time PCR	0.49 log_{10}	38	93	0.99	-3.35
Coxiella burnetii	Real-time PCR	2.61 log_{10}	37	94	0.99	-3.43
Neospora caninum	Real-time PCR	0.81 log_{10}	38	96	0.99	-3.39
Toxoplasma gondii	Real-time PCR	1.52 log_{10}	38	94	0.99	-3.61

(a)=LOD: limit of detection (detected in at least 95% of repeated analyses). (b)=Cutoff Ct value: last positive Ct value created by the standard curve (c)=E: % reaction efficiency. (d)=R^2: regression value. (e)=M: slope. Ct=Threshold Cycle. Real-time PCR: Quantitative polymerase chain reaction. Real-time RT-PCR: Reverse transcription quantitative polymerase chain reaction.

Table 3: Results of real-time PCR assays and Ct value of positive samples

Sample #	BTV	BDV	PPRV	*C. abortus*	*C. burnetii*	*N. caninum*	*T. gondii*
1	Negative	Negative	Negative	Positive (Ct 26.72)	Negative	Negative	Negative
2	Negative	Positive (Ct 28.13)	Negative	Negative	Negative	Negative	Negative
3	Negative	Negative	Negative	Positive (Ct 24.15)	Negative	Negative	Negative
4	Negative	Negative	Negative	Positive (Ct 26.42)	Negative	Negative	Negative
5	Negative	Negative	Negative	Negative	Negative	Negative	Negative
6	Negative	Negative	Negative	Positive (Ct 28.18)	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Positive (Ct 25.66)	Negative	Negative
8	Negative	Positive (Ct 22.94)	Negative	Negative	Negative	Negative	Negative
9	Negative	Positive (Ct 28.83)	Negative	Negative	Negative	Negative	Negative
10	Negative	Negative	Negative	Positive (Ct 20.53)	Negative	Negative	Negative
11	Negative	Negative	Negative	Positive (Ct 21.10)	Positive (Ct 29.36)	Negative	Negative
12	Negative	Negative	Negative	Positive (Ct 27.94)	Negative	Negative	Negative
13	Negative	Negative	Negative	Positive (Ct 22.34)	Negative	Negative	Negative
14	Negative	Positive (Ct 19.24)	Negative	Negative	Negative	Negative	Negative
15	Negative	Negative	Negative	Negative	Negative	Negative	Negative
16	Negative	Negative	Negative	Positive (Ct 20.38)	Negative	Negative	Negative
17	Negative	Negative	Negative	Negative	Negative	Negative	Negative
18	Negative	Negative	Negative	Positive (Ct 28.98)	Negative	Negative	Negative
19	Negative	Negative	Negative	Positive (Ct 25.18)	Negative	Negative	Negative
20	Negative	Negative	Negative	Negative	Negative	Negative	Negative
21	Negative	Negative	Negative	Positive (Ct 23.72)	Negative	Negative	Negative
22	Negative	Positive (Ct 21.49)	Negative	Negative	Negative	Negative	Negative
23	Negative	Negative	Negative	Negative	Positive (Ct 27.21)	Negative	Negative
24	Negative	Positive (Ct 23.95)	Negative	Positive (Ct 26.71)	Negative	Negative	Negative
25	Negative	Negative	Negative	Positive (Ct 23.13)	Negative	Negative	Negative
26	Negative	Negative	Negative	Negative	Negative	Negative	Positive (Ct 28.53)
27	Negative	Negative	Negative	Positive (Ct 28.71)	Negative	Negative	Negative
28	Negative	Negative	Negative	Positive (Ct 20.18)	Negative	Negative	Negative
29	Negative	Negative	Negative	Positive (Ct 25.38)	Negative	Negative	Negative
30	Negative	Negative	Negative	Positive (Ct 23.92)	Negative	Negative	Negative
31	Negative	Positive (Ct 21.88)	Negative	Negative	Negative	Negative	Negative
32	Negative	Negative	Negative	Negative	Negative	Positive (Ct 29.14)	Negative

Ct=Threshold Cycle.

Clinical samples test results

During the optimization process of the assays, standard positive controls were used to determine the limit of detection (LOD), standard curve, and standardization of the threshold cycle values (Ct), as well as to validate the detection panels. The Ct of the reference positive controls were found to be 18.60, 21.13, 15.20, 16.10, 21.73, 21.08 and 25.33, respectively.

After panel verification studies, field samples were tested to determine the targeted agents on the diagnostic panels (RT-PCR and real-time PCR). The Ct values of the positive field samples ranged from 19.24 to 29.36 (results of real-time PCR assays and the Ct value of the positive sheep samples shown in Table 3). No amplification was detected in the negative controls. The results of the field samples were evaluated as follows: BDV in 7(21.9%); *C. abortus* in 18(56.26%), *C. burnetii* in 3(9.4%), *N. caninum* in 1(3.1%) and *T. gondii* in 1(3.1%) of the samples. Furthermore, *C. abortus* and *C. burnetii* were found in one fetus samples and BDV and *C. abortus* were detected in one case simultaneously as a co-infection. No causative pathogen was identified in 4 fetuses (Table 3).

DISCUSSION

This study reports on the development and validation of a one-run real-time PCR assay for the simultaneous detection and differentiation of 3 viruses, 2 bacteria and 2 parasites in sheep abortions. During the planning phase of the study, panels were created by selecting abortive pathogens that were widely circulating in the region. This system includes a one-run real-time RT-PCR analysis for viruses and a one-step real-time PCR analysis for bacteria and parasite agents. These panels allowed for the diagnosis of many agents in 1 hour simultaneously.

Diagnostic panels enable the simultaneous detection of many pathogens and are designed to allow new target pathogens to be added with minor revisions depending on the laboratories' needs. During the development of the tests, different pathogens were included in the diagnostic panel and analyzed simultaneously with the targeted pathogens in the same run. Repeated studies showed that the added agents and targeted pathogens could be carried out in the same run and gave confirmed results. In this regard, it can be assumed that the diagnostic panel can be easily developed and revised for different laboratories or different regions. A search in the literature revealed that there were other studies similar to the diagnostic panel (Rahpaya et al. 2018; Sebastiani et al. 2018; Sunaga et al. 2020; Shibanuma et al. 2023). These studies were found to develop as panels for the diagnosis of abortion, diarrhea, and respiratory infectious diseases in cattle, sheep, goats, pigs, and chickens. However, there is no study comparable to our study to examine these pathogens in sheep abortions.

Abortion in sheep is a pathological event that affects productivity and progeny depending on the spread of possible infectious agents and causes economic losses on farms. To prevent the spread of infectious agents throughout the flock and, in some cases, even across entire regions, effective control measures must be implemented and therefore accurate and rapid diagnostic methods must be used (Di Bari et al. 2023; Modise et al. 2023). Although abortion rates of 3-5% can be expected in a healthy herd, if this rate increases, the cause of the underlying pathology must be investigated. Contagious infectious agents are the main risk factors. Many of the agents found in animals are zoonotic (Tsuchiaka et al. 2016; Sebastiani et al. 2018). Rapid and effective diagnosis of infectious agents is also important for the implementation of protection and control strategies for human and animal health (Njaa 2012; Kahn 2006). *C. abortus*, *C. burnetii* and *T. gondii*, which were included in the diagnostic panel, are zoonotic pathogens that affect human and animal health (Esteban-Redondo and Innes 1997; Gorgani-Firouzjaee et al. 2022). In Türkiye, domestic ruminant abortions are frequently associated with brucellosis. *Chlamydophila abortus* can be considered one of the other most serious causes of abortion (Borel et al. 2006; Dubey and Lindsay 2006; Raeghi et al. 2011). We found *C. abortus* in more than half of the samples used in the study. This shows that *C. abortus* is circulating at a high rate in the region. Other zoonotic pathogens, *C. burnetii* and *T. gondii*, were detected in a lower number. Identifying these zoonotic pathogens through rapid laboratory diagnosis tests and reporting them to authorities will contribute to the adoption of urgent control measures. It requires urgent biosecurity measures, especially considering the high zoonotic risk of *C. abortus* and the fact that *C. burnetii* is a potential bioterrorism agent (Díaz Aparicio 2013; Modise et al. 2023). Additionally, mixed infections were also detected in sheep using this simultaneous diagnostic method. The data are one of the biggest advantages of the diagnostic panel and show the potential of our test to detect mixed infections. Very little literature reports cases of co-infection (Kishimoto et al. 2017; Peric et al. 2018; Song et al. 2021). We interpret this result as an indication of the small number of systems similar to this panel and the examination of very few pathogens in low-abortion cases.

On the other hand, viruses such as BTV and PPRV, which are on the WOAH list of notifiable animal diseases, occur in epidemics and have a devastating impact on herds. BTV and PPRV were not detected in any sample in the study. Since the study was carried out in the winter months and there are no culicoides in the environment, it could be the reason for the negative results of BTV (Federici et al. 2019; Daif et al. 2024). The Ministry of Agriculture and Forestry of the Republic of Türkiye is conducting vaccination studies against sheep and goat plague (PPR) in Türkiye (except in the Thrace region). Vaccination efforts will continue until eradication is complete in Türkiye. For this reason, we can interpret that nucleic acid from PPRV was not detected (FAO 2015).

According to the results of the simultaneous analyzes, Border disease was detected in most samples. The detection of BDV was considered important information, which was found to be a common problem in adult sheep in the study area. BDV and bovine viral diarrhea virus (BVDV), which belong to the pestivirus genus, are a common problem in the study area and in Türkiye farms. Following fetus infection, these two pathogens can lead to the birth of persistently infected (PI) sheep that continuously carry the virus throughout their life. PI animals are very important for the spread of BVDV and BDV. For this reason, the diagnosis of diseases and the removal of PI animals from the herd are the only protective and precautionary measures (Gaede et al. 2005; Willoughby et al. 2006; Broaddus et al. 2007; Heuer et al. 2007). *N. caninum* was found in 1 fetus sample. *N. caninum*, which is quite common worldwide in cattle and dogs, was found in a small number in the study, so it could be due to the fact that the pathogen is relatively rare in sheep flocks (Dubey et al. 2007; Barry et al. 2019).

This panel is highly specific, as all target pathogens were correctly identified with no cross-reactivity between species, and no fluorescent signal was detected in the DNA of non-target pathogens. Another advantage of the Diagnostic Panel is that it can be adapted to different real-time PCR devices. Furthermore, this panel is compatible with other real-time PCR platforms and is easy to test and interpret. It could become a potential tool for easy implementation in any public health and veterinary diagnostic laboratory, even on a low budget. This diagnostic system is believed to be a frequently preferred tool by veterinary diagnostic laboratories for monitoring sheep abortions, due to its simple, fast, reliable, high accuracy and cost-effective results in the future.

Conclusion

This study reports on the development and validation of a diagnostic panel that provides a rapid and simultaneous diagnosis of important zoonotic and notifiable abortus agents of sheep, which are an important source of income for developing countries. Pathogens analyzed consist of agents that circulate frequently throughout the country but are ignored by most veterinary diagnostic laboratories. Using real-time PCR analysis, 7 abortion pathogens could be detected simultaneously in a single run. Using standard curves, precision profiles were observed on the basis of the efficiency and reliability values of the test. The developed diagnostic panels were found to be accurate, precise, acceptable, and reliable.

As a result, this diagnostic panel is able to quickly and easily identify a wide spectrum of pathogens in a single run compared to conventional tests that detect only one pathogen. Taking into account the different etiologies of abortion in domestic sheep, this real-time PCR test panel allows us to take immediate treatment and prevention strategies in one-run. This detection system is a candidate method for a simpler, faster, and more comprehensive alternative testing method than conventional tests. This diagnostic panel will help veterinary diagnostic laboratories detect the factors in a short time and take urgent control measures.

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REFERENCES

Barry R, Nissly RH, Feria W, Thirumalapura N, Tewari D, Jayarao BM and Kuchipudi SV, 2019. A probe-based real-time PCR assay for the detection of *Neospora caninum* in clinical samples from cattle. Veterinary Parasitology 269: 2–6. https://doi.org/10.1016/j.vetpar.2019.04.002

Batten CA, Banyard AC, King DP, Henstock MR, Edwards L, Sanders A, Buczkowski H, Oura CC and Barrett T, 2011. A real-time RT-PCR assay for the specific detection of Peste des petits ruminants virus. Journal of Virological Methods 171(2): 401–404. https://doi.org/10.1016/j.jviromet.2010.11.022

Borel N, Thoma R, Spaeni P, Weilenmann R, Teankum K, Brugnera E, Zimmermann DR, Vaughan L and Pospischil A, 2006. Chlamydia-related abortions in cattle from Graubunden, Switzerland. Veterinary Pathology 43(5): 702–708. https://doi.org/10.1354/vp.43-5-702

Broaddus CC, Holyoak GR, Dawson L, Step DL, Funk RA and Kapil S, 2007. Transmission of bovine viral diarrhea virus to adult goats from persistently infected cattle. Journal of Veterinary Diagnostic Investigation 19(5): 545–548. https://doi.org/10.1177/104063870701900514

Clothier K and Anderson M, 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 85(5): 933–938. https://doi.org/10.1016/j.theriogenology.2015.11.001

Daif S, El Berbri I and Fassi Fihri O, 2024. First molecular evidence of potential culicoides vectors implicated in bluetongue virus transmission in Morocco. Parasites Vectors 17(1): 71. https://doi.org/10.1186/s13071-024-06167-y

Di Bari C, Venkateswaran N, Fastl C, Gabriël S, Grace D, Havelaar AH, Huntington B, Patterson GT, Rushton J, Speybroeck N, Torgerson P, Pigott DM and Devleesschauwer B, 2023. The global burden of neglected zoonotic diseases: Current state of evidence. One Health 17: 100595. https://doi.org/10.1016/j.onehlt.2023.100595

Díaz Aparicio E, 2013. Epidemiology of brucellosis in domestic animals caused by Brucella melitensis, Brucella suis and Brucella abortus. Revue scientifique et Technique (International Office of Epizootics) 32(1): 43–60.

Dubey JP and Lindsay DS, 2006. Neosporosis, toxoplasmosis, and sarcocystosis in ruminants. The Veterinary Clinics of North America. Food Animal Practice 22(3): 645–671. https://doi.org/10.1016/j.cvfa.2006.08.001

Dubey JP, Schares G and Ortega-Mora LM, 2007. Epidemiology and control of neosporosis and *Neospora caninum*. Clinical Microbiology Reviews 20(2): 323–367. https://doi.org/10.1128/CMR.00031-06

Esteban-Redondo I and Innes EA, 1997. Toxoplasma gondii infection in sheep and cattle. Comparative Immunology, Microbiology and Infectious Diseases 20(2): 191–196. https://doi.org/10.1016/s0147-9571(96)00039-2

FAO, 2015. Global strategy for the control and eradication of PPR. Avaliable at: https://www.woah.org/app/uploads/2021/03/ppr-global-strategy-2015-03-28.pdf (accessed 10 march 2024).

Federici V, Goffredo M, Mancini G, Quaglia M, Santilli A, Di Nicola F, De Ascentis M, Cabras P, Volpicelli C, De Liberato C, Satta G, Federico G, Leone A, Pisciella M, Portanti O, Pizzurro F, Teodori L and Savini G, 2019. Vector competence of Italian populations of culicoides for some bluetongue virus strains responsible for recent northern African and European outbreaks. Viruses 11(10): 941. https://doi.org/10.3390/v11100941

Gaede W, Reiting R, Schirrmeier H, Depner KR and Beer M, 2005. Nachweis und spezies-spezifische differenzierung von pestiviren mit der real-time RT-PCR (Detection and species-specific differentiation of pestiviruses using real-time RT-PCR). Berliner und Munchener tierarztliche Wochenschrift 118(3-4): 113–120.

Gorgani-Firouzjaee T, Kalantari N and Ghaffari S, 2022. Molecular identification and genotyping of Toxoplasma gondii isolated from sheep and cattle in northern Iran. Veterinary Research Forum 13(3): 371–378. https://doi.org/10.30466/vrf.2021. 138408.3080

Heuer C, Healy A and Zerbini C, 2007. Economic effects of exposure to bovine viral diarrhea virus on dairy herds in New Zealand. Journal of Dairy Science 90(12): 5428–5438. https://doi.org/10.3168/jds.2007-0258

Hofmann M, Griot C, Chaignat V, Perler L and Thür B, 2008. Blauzungenkrankheit erreicht die Schweiz (Bluetongue disease reaches Switzerland). Schweizer Archiv fur Tierheilkunde 150(2): 49–56. https://doi.org/10.1024/0036-7281.150.2.49

Kahn LH, 2006. Confronting zoonoses, linking human and veterinary medicine. Emerging Infectious Diseases 12(4): 556–561. https://doi.org/10.3201/eid1204.050956

Kishimoto M, Tsuchiaka S, Rahpaya SS, Hasebe A, Otsu K, Sugimura S, Kobayashi S, Komatsu N, Nagai M, Omatsu T, Naoi Y, Sano K, Okazaki-Terashima S, Oba M, Katayama Y, Sato R, Asai T and Mizutani T, 2017. Development of a one-run real-time PCR detection system for pathogens associated with bovine respiratory disease complex. The Journal of Veterinary Medical Science 79(3): 517–523. https://doi.org/10.1292/jvms.16-0489

Klee SR, Tyczka J, Ellerbrok H, Franz T, Linke S, Baljer G and Appel B, 2006. Highly sensitive real-time PCR for specific detection and quantification of Coxiella burnetii. BMC Microbiology 6: 2. https://doi.org/10.1186/1471-2180-6-2

Kreizinger Z, Szeredi L, Bacsadi Á, Nemes C, Sugár L, Varga T, Sulyok KM, Szigeti A, Ács K, Tóbiás E, Borel N and Gyuranecz M, 2015. Occurrence of *Coxiella burnetii* and Chlamydiales species in abortions of domestic ruminants and in wild ruminants in Hungary, Central Europe. Journal of Veterinary Diagnostic Investigation 27(2): 206–210. https://doi.org/10.1177/ 1040638714563566

Lin MH, Chen TC, Kuo TT, Tseng CC and Tseng CP, 2000. Real-time PCR for quantitative detection of *Toxoplasma gondii*. Journal of Clinical Microbiology 38(11): 4121–4125. https://doi.org/10.1128/JCM.38.11.4121-4125.2000

Liu Q, Cao L and Zhu XQ, 2014. Major emerging and re-emerging zoonoses in China: a matter of global health and socioeconomic development for 1.3 billion. International Journal of Infectious Diseases 25: 65–72. https://doi.org/10.1016/j.ijid.2014.04.003

Menzies PI, 2011. Control of important causes of infectious

abortion in sheep and goats. The Veterinary Clinics of North America. Food Animal Practice 27(1): 81–93. https://doi.org/10.1016/j.cvfa.2010.10.011

Modise BM, Mpoloka SW, Settypalli TBK, Hyera, J, Natale A, Ceglie L, Gcebe N, Marobela-Raborokgwe C, Viljoen GJ, Cattoli G and Lamien CE, 2023. A novel multiplex qPCR-HRM assay for the simultaneous detection of four abortive zoonotic agents in cattle, sheep and goats. Scientific Reports 13(1): 12282. https://doi.org/10.1038/s41598-023-39447-1

Njaa BL, 2012. Kirkbride's Diagnosis of Abortion and Neonatal Loss in Animals. John Wiley and Sons, Oxford, UK.

Pantchev A, Sting R, Bauerfeind R, Tyczka J and Sachse K, 2009. New real-time PCR tests for species-specific detection of *Chlamydophila psittaci* and *Chlamydophila abortus* from tissue samples. Veterinary Journal 181(2): 145–150. https://doi.org/10.1016/j.tvjl.2008.02.025

Peric L, Sabadi D, Rubil I, Bogdan M, Guzvinec M, Dakovic Rode O, Kaic B, Tabain I and Vilibic-Cavlek T, 2018. Imported brucellosis and Q-fever coinfection in Croatia: a case report. Journal of Infection in Developing Countries 12(6): 499–503. https://doi.org/10.3855/jidc.10151

Raeghi S, Akaberi A and Sedeghi S, 2011. Seroprevalence of Toxoplasma gondii in sheep, cattle and horses in Urmia north-west of Iran. Iranian Journal of Parasitology 6(4): 90–94.

Rahpaya SS, Tsuchiaka S, Kishimoto M, Oba M, Katayama Y, Nunomura Y, Kokawa S, Kimura T, Kobayashi A, Kirino Y, Okabayashi T, Nonaka N, Mekata H, Aoki H, Shiokawa M, Umetsu M, Morita T, Hasebe A, Otsu K, Asai T, Yamaguchi T, Makino S, Murata Y, Abi AJ, Omatsu T and Mizutani T, 2018. Dembo polymerase chain reaction technique for detection of bovine abortion, diarrhea, and respiratory disease complex infectious agents in potential vectors and reservoirs. Journal of Veterinary Science 19(3): 350–357. https://doi.org/10.4142/jvs.2018.19.3.350

Sebastiani C, Curcio L, Ciullo M, Cruciani D, Crotti S, Pesca C, Torricelli M, Sebastianelli M, Felici A and Biagetti M, 2018. A multi-screening fast qPCR approach to the identification of abortive agents in ruminants. Journal of Microbiological Methods 148: 12–17. https://doi.org/10.1016/j.mimet.2018.03.009

Shibanuma T, Nunomura Y, Oba M, Kawahara F, Mizutani T and Takemae H, 2023. Development of a one-run real-time PCR detection system for pathogens associated with poultry infectious diseases. The Journal of Veterinary Medical Science 85(4): 407–411. https://doi.org/10.1292/jvms.22-0482

Sloots TP, Nissen MD, Ginn AN and Iredell JR, 2015. Rapid identification of pathogens using molecular techniques. Pathology 47(3): 191–198. https://doi.org/10.1097/PAT.0000000000000241

Song J, Hu X, Li X, Chen Y, Yan X, Zhu W, Ding Y and Zhou J, 2021. *Rickettsia burnetii* and *Brucella melitensis* co-infection: a case report and literature review. BMC Microbiology 21(1): 270. https://doi.org/10.1186/s12866-021-02323-x

Sunaga F, Tsuchiaka S, Kishimoto M, Aoki H, Kakinoki M, Kure K, Okumura H, Okumura M, Okumura A, Nagai M, Omatsu T and Mizutani T, 2020. Development of a one-run real-time PCR detection system for pathogens associated with porcine respiratory diseases. The Journal of Veterinary Medical Science 82(2): 217–223. https://doi.org/10.1292/jvms.19-0063

Tomori O and Oluwayelu DO, 2023. Domestic animals as potential reservoirs of zoonotic viral diseases. Annual Review of Animal Biosciences 11: 33–55. https://doi.org/10.1146/annurev-animal-062922-060125

Toohey-Kurth KL, Mulrooney DM, Hinkley S, Lea Killian M, Pedersen JC, Bounpheng MA, Pogranichniy R, Bolin S, Maes R, Tallmadge RL, Goodman LB and Crossley BM, 2020. Best practices for performance of real-time PCR assays in veterinary diagnostic laboratories. Journal of Veterinary Diagnostic Investigation 32(6): 815–825. https://doi.org/10.1177/ 1040638720962076

Tsuchiaka S, Masuda T, Sugimura S, Kobayashi S, Komatsu N, Nagai M, Omatsu T, Furuya T, Oba M, Katayama Y, Kanda S, Yokoyama T and Mizutani T, 2016. Development of a novel detection system for microbes from bovine diarrhea by real-time PCR. The Journal of Veterinary Medical Science 78(3): 383–389. https://doi.org/10.1292/jvms.15-0552

Watzinger F, Ebner K and Lion T, 2006. Detection and monitoring of virus infections by real-time PCR. Molecular Aspects of Medicine 27(2-3): 254–298. https://doi.org/10.1016/j.mam.2005.12.001

Willoughby K, Valdazo-González B, Maley M, Gilray J and Nettleton PF, 2006. Development of a real-time RT-PCR to detect and type ovine Pestiviruses. Journal of Virological Methods 132(1-2): 187–194. https://doi.org/10.1016/j.jviromet.2005.10.007

WOAH, 2014. Validation guideline, chapter 3.6.3: development and optimization of nucleic acid detection assays. Available at: https://www.woah.org/fileadmin/Home/eng/Health_standards/aahm/current/GUIDELINE_3.6.3_NAD_ASSAYS.pdf (Accessed 10 march 2024).

Wolf-Jäckel GA, Hansen MS, Larsen G, Holm E, Agerholm JS and Jensen TK, 2020. Diagnostic studies of abortion in Danish cattle 2015–2017. Acta Veterinaria Scandinavica 62(1): 1. https://doi.org/10.1186/s13028-019-0499-4

Wolf-Jäckel GA, Strube ML, Schou KK, Schnee C, Agerholm JS and Jensen TK, 2021. Bovine abortions revisited—enhancing abortion diagnostics by 16S rDNA amplicon sequencing and fluorescence in situ hybridization. Frontiers in Veterinary Science 8: 623666. https://doi.org/10.3389/fvets.2021.623666

Yang S and Rothman RE, 2004. PCR-based diagnostics for infectious diseases: uses, limitations and future applications in acute-care settings. The Lancet: Infectious Diseases 4(6): 337–348. https://doi.org/10.1016/S1473-3099(04)01044-8