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RESEARCH ARTICLE

One-Run qPCR Assays for Identification of Domestic Ruminant Abortion: Verification and Application Process

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A B S T R A C T

Infectious pathogens that cause abortions in ruminants result in major economic losses to the global livestock industry. Pathogens associated with abortion have a direct impact on animal and human health. The objective of this study was to develop and validate a quantitative PCR-based diagnostic panel for rapid and simultaneous detection of some important pathogens causing abortion in sheep, goats, and cattle. For this purpose, standard curves were constructed using standard controls of the pathogens (Akabane, Peste des Petits Ruminants, Bluetongue, Bovine Viral Diarrhea, Border Disease, Bovine Herpesvirus, Schmallenberg viruses, *Coxiella burnetii, Listeria monocytogenes, Chlamydophila abortus, Toxoplasma gondii,* and *Neospora caninum*) to develop a one-run qPCR diagnostic panel. These curves were used to determine linearity, reliability, efficacy, and sensitivity of the test panel. Thus, the assay verification was completed, and the results were evaluated. The developed panel was verified on ovine (n=156), caprine (n=17), and bovine (n=15) fetus samples from the field, and causative agents of abortion in the region were identified. The limit of detection (LOD), amplification efficiency (E), regression (R^2), and slope (M) values were determined for optimization and verification studies. The LOD for one-run qPCR assays ranged from 0.45 to 3.24 $\log_{10}$ DNA copies/reaction and 1.33 to 4.64 $\log_{10}$ RNA copies/reaction. The dynamic range for each one-run qPCR assay covered more than five orders of magnitude, and E values ranged between 90 and 100%; R^2 value for all test designs was 0.99; M values also ranged between -3.10 and -3.60. In conclusion, this diagnostic system, which has completed verification studies, can be actively used in routine diagnosis of domestic ruminant abortions, can analyze clinical sample assays within a few hours and can detect all target pathogens simultaneously.

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INTRODUCTION

Infectious abortions in farm animals are considered the leading cause of economic losses in the global food and feed industry (Zeeshan *et al.,* 2023). The reasons for these economic losses can be listed as infertility, stillbirth, fetal deaths, reproductive disorders, decreased milk yield and veterinary care and treatment costs (Sakmanoglu *et al.,* 2021). They are mainly due to the infectious agents responsible for inducing abortions in bovines. These agents are zoonotic or very contagious and have generally been frequently involved in outbreaks on cattle farms, and have been listed by the World Organisation for Animal Health (WOAH) as notifiable animal diseases (Di Bari *et al.,* 2023). The proper application of accurate and reliable means of transportation can lead to effective management of the epidemics by laboratories that conduct the diagnosis of animal disease (Njaa, 2012). This is a matter of great importance for animal and public health (Tomori and Oluwayelu, 2023).

The prevalence of agents causing infectious abortion in each country may vary. Although, Brucella species are reported to be the primary cause of abortion (Sakmanoglu *et al.,* 2021), infectious abortions in domestic ruminants can also be caused by bacterial (Reisberg et *al.,* 2013), viral (Rahpaya *et al.,* 2018) and parasitic (Lin *et al.,* 2000) agents. *Chlamydia abortus*, a common cause of enzootic abortion, has been serologically detected in 2 to 65% of small ruminants (Kaya and Ozturk, 2020). *Coxiella burnetii*, the causative agent of Q fever, is also common, with seropositivity rates reported as 20 and 35% in goats and sheep, respectively (Djellata *et al.,* 2019; Ramo *et al.,*

2022). Viral agents, especially pestiviruses such as BVDV and BDV, are highly associated with abortions in cattle, sheep, and goats, with prevalence rates ranging from 13 to 60%, depending on the host species (Heuer *et al.,* 2007; Tuncer-Goktuna *et al.,* 2016). Although PPRV is primarily known for respiratory and systemic infections, it is increasingly associated with abortions in small ruminants, with detection rates as high as 20% in cases of abortion (Pestil *et al.,* 2020).

Abortions in domestic ruminants usually occur without any warning. In other words, almost no typical differences (except for anomalies) are seen in the fetus and placenta at necropsy (Anderson, 2007). Furthermore, since the etiology of infections in abortions can be complex, the occurrence of multiple infections may be possible. Therefore, it is difficult to predict the specific infectious agent causing the abortion in domestic ruminants (Gouvias *et al.,* 2024). Serological and molecular tests are frequently used in the diagnosis of animal diseases. In serologic tests, which are frequently used in the diagnosis of infectious abortions, it may not always be easy to reach a definitive diagnosis due to lack of an anamnesis, improper collection of samples, and their storage under inappropriate conditions, inadequate serum sample provision, or unconscious use of drugs or vaccines (Sebastiani *et al.,* 2018). In addition, it may be difficult and time-consuming to isolate most bacterial pathogens by conventional methods used in routine diagnostics (Yang and Rothman, 2004). While serological applications require experienced laboratory workers and well-equipped laboratories (Navarro *et al.,* 2009), molecular techniques, which have been widely used in diagnostic laboratories for the last 25 years, allow for accurate and rapid diagnosis of pathogens that cannot be detected serologically (Modise *et al.,* 2023).

Qualitative PCR (qPCR), one of the molecular methods, is known as a satisfactory diagnostic tool for clinical laboratories and research centers due to its rapid diagnostic capability, specificity to the agent, and more practical application and conclusion stages (Sloots *et al.,* 2015). Despite this, most diagnostic laboratories worldwide apply these tests in the form of single-pathogen detection using a single assay (Fulton and Confer, 2012). This approach increases costs, workload and results in longer reporting times. Considering all these limitations of diagnostic methods, in the present study, we aimed to develop a PCR-based diagnostic panel that can detect multiple pathogens simultaneously with a single assay. We believe that such an innovative approach will allow rapid and cost-effective differential diagnosis of pathogens associated with infectious abortion in domestic ruminants. The primary objective of this study was to develop a diagnostic panel with acceptable sensitivity and specificity for the simultaneous identification of key infectious pathogens responsible for abortions in sheep, goats and cattle.

MATERIALS AND METHODS

Ethics approval: This study was conducted with the approval of the General Directorate of Food and Control, granted on January 10, 2024 (Document No E-12760097). The study adhered to international ethical guidelines and was reviewed and approved by the Konya Veterinary Control Institute (Approval No. 2024/03), Konya, Republic of Turkiye.

Target pathogens, positive standards, and primer-probes: In developing the diagnostic panel to identify the most important infectious agents causing abortion in domestic ruminants, the prevalence of the pathogen in the region and the agents overlooked by most diagnostic laboratories were selected as targets. These pathogens were Akabane virus (AKAV), Peste des Petits Ruminants virus (PPRV), Bluetongue virus (BTV), Bovine Viral Diarrhea virus (BVDV), Border Disease virus (BDV), Bovine Herpesvirus virus (BoHV), Schmallenberg virus (SBV), *Coxiella burnetii* (*C. burnetii*), *Listeria monocytogenes* (*L. monocytogenes*), *Chlamydophila abortus* (*C. abortus*), *Toxoplasma gondii* (*T. gondii*) and *Neospora caninum* (*N. caninum*).

Subsequently, standard positives for each pathogen were prepared to assess the precision, sensitivity, linearity, reliability, and efficiency of the diagnostic panel. Synthetic DNA for *L. monocytogenes* (Genbank accession number: PV632091) was obtained from a commercial supplier. Nucleic acids for BVDV, BDV, PPRV, *C. abortus*, *C. burnetii*, and *N. caninum* were obtained from field samples confirmed positive through sequence assays (Genbank accession numbers: PV632088, PV632085, PV589114, PV584245, PV583469, PV632092). The Department of Virology at the Faculty of Veterinary Medicine at Selcuk University provided the BoHV and BTV standards, and the Microbiology Reference Laboratory at the Ministry of Health of the Republic of Turkiye provided the T. *gondii* positive standards. For AKAV and SBV, targeted sequence plasmids were generated using pGEM®-T Easy Vector Systems (Promega), and RNA was transcribed using the MEGAscript® Kit (Ambion) according to the manufacturer's instructions. All these standards were employed throughout the development and verification phases of the diagnostic panel.

The concentrations of the standards were measured three times by DS-11 Spectrophotometer (DeNovix). Nucleic acid copy numbers were calculated using an appropriate formula following Dorak (2007), as shown below:

Copy number RNA (copy/µL) = (Concentration of RNA in ng/µL×6.022×10^{23})/(Fragment length in bp×10^{9}×325);
Copy number DNA (copy/µL) = (Concentration of RNA in ng/µL×6.022×10^{23})/(Fragment length in bp×10^{9}×660).

The primer and probe sets used for the amplification of the target pathogens were selected from reference studies recommended by the World Organisation for Animal Health (Willoughby *et al.,* 2006; Pantchev *et al.,* 2009; Batten *et al.,* 2011; Barry *et al.,* 2019). The sequence specificity of each primer and probe was verified using the Basic Local Alignment Search Tool (NCBI/Primer-BLAST). The information about 13 synthetic oligonucleotides used in the study is presented in Table 1. The primer-probe sets were dissolved in nuclease-free water at 100µmol/µL final concentration, and then they were aliquoted and stored at -20°C till the assays were performed.

Table 1: The nucleotide information of the primer and probe sets used for qPCR assays

Pathohen	Target gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Probe (5´ FAM - 3´ Tamra)	Reference
AKAV	S-segment	TCAACCAGAAGAAGGCCAAGAT	GGGAAAATGGTTATTAACCACT GTAAA	TTACATAAGACGCCACAACCA	Shirafuji *et al.* (2015)
BTV	NSP3	CCTGGACAAGGTCTCGGTAGAA	ATTCAGGACCCCACCCAAAT	CATGCTCGAGGATTGGGTCGTC GT	Hofmann *et al.* (2008)
BDV	5'UTR	CCGTGTTAACCATACACGTAGTA GGA	GCCCTCGTCCACGTAGCA	CTCAGGGATCTCACCACGA	Willoughby *et al.* (2006)
BoHV-1	glycoprotein B	TGTGGACCTAAACCTCACGGT	GTAGTCGAGCAGACCCGTGTC	AGGACCGCGAGTTCTTGCCGC	Abril *et al.* (2004)
BoHV-5	glycoprotein B	ACATCATCTACATGTCGCCCTTC	TTGTAGTAGCCCTCGATTTGCT	ACCGCGAGCACACCAGCTACT	Abril *et al.* (2004)
BVDV	5'UTR	CCTGAGTACAGGRTAGTCGTCA	GGCCTCTGCAGCACCCTATCA	TGCYAYGTGGACGAGGGCATGC	Hyndman *et al.* (1998); Gaede *et al.* (2005)
PPRV	Nucleocapsid	AGAGTTCAATATGTTRTTAGCCTC CAT	TTCCCCARTCACTCTYCTTTGT	CACCGGAYACKGCAGCTGACTC AGAA	Batten *et al.* (2011)
SBV	S-segment	TCAGATTGTCATGCCCCTTGC	TTCGGCCCCAGGTGCAAATC	TTAAGGGATGCACCTGGGCCGA TGGT	Bilk *et al.* (2012)
C. abortus	ompA	GCAACTGACACTAAGTCGGCTAC A	ACAAGCATGTTCAATCGATAAG AGA	AAATACCACGAATGGCAAGTTG GTTTAGCG	Pantchev *et al.* (2009)
C. burnetii	transposase	GTCTTAAGGTGGGCTGCGTG	CCCCGAATCTCATTGATCAGC	AGCGAACCATTGGTATCGGACG TTTATGG	Klee *et al.* (2006)
L. monocytogenes	lap gene	CATGGCACCACCAGCATCT	ATCCGCGTGTTTCTTTTCGA	CGCCTGCAAGTCCTAAGACGCC A	Rodríguez-Lázaro *et al.* (2004)
T. gondii	B1	TCCCCTCTGCTGGCGAAAAGT TTG	AGCGTTCGTGGTCAACTATCGA	TCTGTGCAACTTTGGTGTATTCG CAG	Lin *et al.* (2000)
N. caninum	Nc5	CTGTGCT CGCTGGGACTTC	CGATTTACGACATACGGTGTTCA	CATCGGAGGACATCGCTCACTGA	Barry *et al.*, (2019)

Limit of detection (LOD): Standard positive controls for the pathogens were subjected to 10-fold serial dilutions, and standard curves were generated to validate diagnostic panel design and assess the performance of the qPCR assays. From these standard curves, the limits of detection (LOD), correlation coefficients (R^2), and PCR efficiencies (E) were determined (Table 2), as described previously (WOAH, 2014). Each assay was performed in triplicate on different days. The LOD was defined as the lowest concentration of nucleic acids consistently detected in at least 95% of replicates (Toohey-Kurth *et al.,* 2020). Additionally, the analytical Ct value was calculated based on repeated assays. The most specific and optimal assay conditions were established to ensure precise LOD determinations.

Repeatability and reproducibility: Repeated assays were conducted to assess the repeatability and reproducibility of the test panel. Repeatability was evaluated as a measure of both intra-assay and inter-assay variation. For intra-assay variation, three samples were tested in five replicates. On the other hand, for inter-assay variation, the same three samples were tested across three independent runs. Additionally, the mean, standard deviation (SD), and coefficient of variation (CV) were calculated separately for each sample and used to assess the precision of the test. All these values represented the averages derived from repeated experimental results (Table 3).

Specificity, sensitivity, and diagnostic performance of the assays: The cross-reactivity of the assays was evaluated with all pathogens included in the diagnostic panel, and cross-reactivity control analyses were performed with other panel agents for each pathogen. In addition, various control analyses were performed to evaluate the specificity of these assays, including template control, false positive or false negative controls, and inhibition control. A sample panel (Supplementary Table S1) was prepared to evaluate the diagnostic performance of the assays and calculate the diagnostic specificity (DSp) and diagnostic sensitivity (DSe) according to the WOAH (2014) guidelines. The DSp and DSe values, along with their 95% confidence intervals, were determined using a 2×2 contingency table, as described by Jacobson (1998) and shown in Table 4.

Clinical samples and nucleic acid extraction: Clinical field samples were used to verify the stage of the diagnostic panel. The clinical field samples consisted of 156 ovine, 17 caprine, and 15 bovine fetuses (total 188), which were sent to the Konya Veterinary Control Institute for routine diagnosis between 2019 and 2025. These samples were sent from various farms in provinces where ruminant farming holds significance in Turkiye. They were found negative for *Salmonella sp*., *Campylobacter sp*., *Brucella sp*., and *Leptospira sp*., during routine serological and bacteriological diagnosis. They included the tissues from the lungs, liver, spleen, kidneys, lymph nodes, brain, and placenta. Tissue suspensions were prepared for nucleic acid extraction, as previously described (Oz *et al.,* 2021), and extraction was performed using the IndiMag Pathogen Kit (Indical Bioscience, Germany) following the manufacturer's protocol. The purity and concentration of the extracted nucleic acids were assessed using a DeNovix DS-11 Series spectrophotometer (DeNovix), as described earlier (Oz *et al.,* 2024). DNA and RNA samples were stored at -80°C until further tests. To ensure the reliability and accuracy of the procedure, positive and negative control samples were included at every step of the extraction process.

One-run qPCR workflow and clinical sample assays: Fetal samples were tested simultaneously in a single assay to determine the verification of the diagnostic panel in clinical samples and to observe its performance. Assay verification focused on determining the earliest threshold cycle (Ct), optimal annealing temperature, and highest amplification efficiency to improve diagnostic panels. To this end, melting curves of positive standards were examined to determine whether clinical samples provided the optimal assay conditions to produce specific PCR products during the verification phase. All one-run assay verifications were conducted using Rotor-Gene Q Series software v.2.3.1-Build 49. Viral RNA amplification was performed using the IndiMixJOE kit (Indical Bioscience, Germany) according to the manufacturer's instructions. For

Table 2: Method performance values of qPCR assays

Pathogens	LOD (copies/reaction) [a]	Operating range of the assay (copies/reaction)	Cutoff Ct value[b]	E (%)[c]	R^{2d}	M[e]
AKAV	2.03 log_{10}	3.34-7.34 log_{10}	32	94	0.99	-3.46
BTV	4.64 log_{10}	4.54-8.54 log_{10}	35	94	0.99	-3.34
BDV	1.90 log_{10}	2.00-6.00 log_{10}	34	91	0.99	-3.56
BHV-1	2.55 log_{10}	2.61-6.61 log_{10}	34	90	0.99	-3.60
BHV-5	2.98 log_{10}	3.08-7.08 log_{10}	34	99	0.99	-3.34
BVDV	1.33 log_{10}	1.32-6.32 log_{10}	36	99	0.99	-3.46
PPRV	1.92 log_{10}	2.06-7.06 log_{10}	36	94	0.99	-3.47
SBV	2.30 log_{10}	2.40-6.40 log_{10}	34	98	0.99	-3.37
C. abortus	0.45 log_{10}	0.36-5.36 log_{10}	36	96	0.99	-3.43
C. burnetii	2.47 log_{10}	2.45-7.45 log_{10}	36	93	0.99	-3.49
L. monocytogenes	3.24 log_{10}	3.34-7.34 log_{10}	33	100	0.99	-3.10
N. caninum	0.72 log_{10}	0.71-4.71 log_{10}	35	94	0.99	-3.47
T. gondii	1.30 log_{10}	1.28-6.28 log_{10}	38	93	0.99	-3.50

(a)=LOD: limit of detection (detected in at least 95% of repeated assays); (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.

Table 3: Repeatability and precision values of the one-run qPCR assays

Pathogen	Sample	Intra assay variation[a]		Inter assay variation[b]	
		SD (±)	CV (%)	SD (±)	CV (%)
AKAV	RNA10^{-1}	0.24	1.02	0.30	1.04
	RNA 10^{-2}	0.44	1.64	0.37	1.50
	RNA 10^{-3}	0.46	1.89	0.28	1.09
BTV	Isolate 10^{-1}	0.02	0.09	0.12	0.41
	Isolate 10^{-2}	0.37	1.25	0.03	0.09
	Isolate 10^{-3}	0.24	1.03	0.11	0.38
BVDV	Sample 69	0.20	0.82	0.05	0.21
	Sample 70	0.18	0.69	0.42	1.47
	Sample 75	0.31	1.05	0.27	1.06
BDV	Sample 52	0.08	0.41	0.44	2.00
	Sample 96	0.30	1.20	0.60	2.05
	Sample 164	0.10	0.47	0.18	0.60
BHV-1	DNA 10^{-1}	0.10	0.46	0.02	0.10
	DNA 10^{-2}	0.15	0.62	0.40	1.27
	DNA 10^{-3}	0.12	0.40	0.09	0.31
BHV-5	DNA 10^{-1}	0.12	1.0	0.07	0.43
	DNA 10^{-2}	0.37	1.23	0.22	1.19
	DNA 10^{-3}	0.28	1.20	0.16	1.33
C. abortus	Sample 12	0.20	0.82	0.37	1.48
	Sample 93	0.08	0.84	0.13	0.48
	Sample 160	0.37	1.19	0.10	0.44
C. burnetii	Sample 63	0.52	1.34	0.25	1.17
	Sample 77	0.25	1.31	1.37	1.0
	Sample 109	0.15	0.66	0.45	2.0
	Synthetic NA10^{-1}	0.42	0.70	0.34	0.84
L. monocytogenes	Synthetic NA 10^{-2}	0.51	1.88	0.12	1.10
	Synthetic NA 10^{-3}	0.28	1.94	0.07	1.27
N. caninum	Sample 157	0.15	0.59	0.26	1.14
	Sample 160	0.02	0.08	0.42	1.02
	Sample 166	0.37	1.13	0.05	0.18
PPRV	Sample 60	0.29	1.01	0.09	0.40
	Sample 99	0.24	1.17	0.05	0.24
	Sample 134	0.28	1.60	0.52	1.52
	RNA10^{-1}	0.12	0.43	0.56	1.84
SBV	RNA 10^{-2}	0.52	1.92	0.51	1.70
	RNA 10^{-3}	0.49	1.53	0.54	1.97
T. gondii	DNA 10^{-1}	0.31	1.20	0.26	1.0
	DNA 10^{-2}	0.14	0.49	0.47	1.53
	DNA 10^{-3}	0.48	2.17	0.55	2.01

(a)=Analysis with five replicates of each sample; (b)=Three different assays with each sample; NA=Nucleic acid; SD=Standard deviation of repeatability; CV=Coefficient of variation. Note: The data in the table are the mean of repeated assay results.

amplification of DNA from viral, bacterial, and parasitic pathogens, the LightCycler® 480 Probes Master Kit (Roche Applied Science, Germany) was used according to the manufacturer's protocol. Baseline and threshold settings were adjusted according to the instrument guidelines. Following the development of the diagnostic panel, 188 foetal samples were tested for all pathogens. Nuclease-free water was used as a negative control, and standard controls were included as positive references.

Table 4: The diagnostic specificity and diagnostic sensitivity calculation in the confidence interval at 95% for the one-run qPCR assays

		Authentic status of the samples	
		Known positive	Known negative
qPCR	Positive	TP	FP
	Negative	FN	TN
	Total	TP + FN	TN + FP
	Diagnostic performance	Dse = TP/(TP+FN)	Dsp= N/(TN+FP)

TP=True Positive; FP=False Positive; TN=True Negative; FN=False Negative; Dse=Diagnostic sensitivity; Dsp=Diagnostic specificity. Note: 2×2 table (Jacobson, 1998) was used to calculate diagnostic performance of the assays.

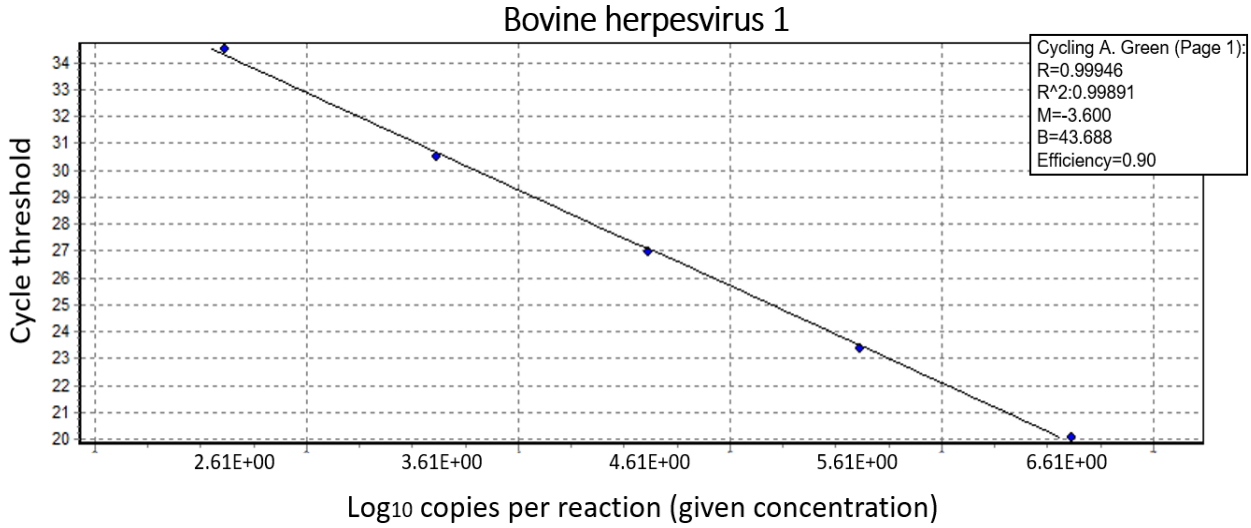
Exogenous internal control analyses: An exogenous internal control (IC) was used during the field sample analyses to demonstrate the accuracy and reliability of nucleic acid extraction and amplification efficiency. The IC was a synthetic RNA template commercially available in the IndiMixJOE kit (Indical Bioscience, Germany), and it was controlled by being applied in the same tube as the field samples in all qPCR analyses.

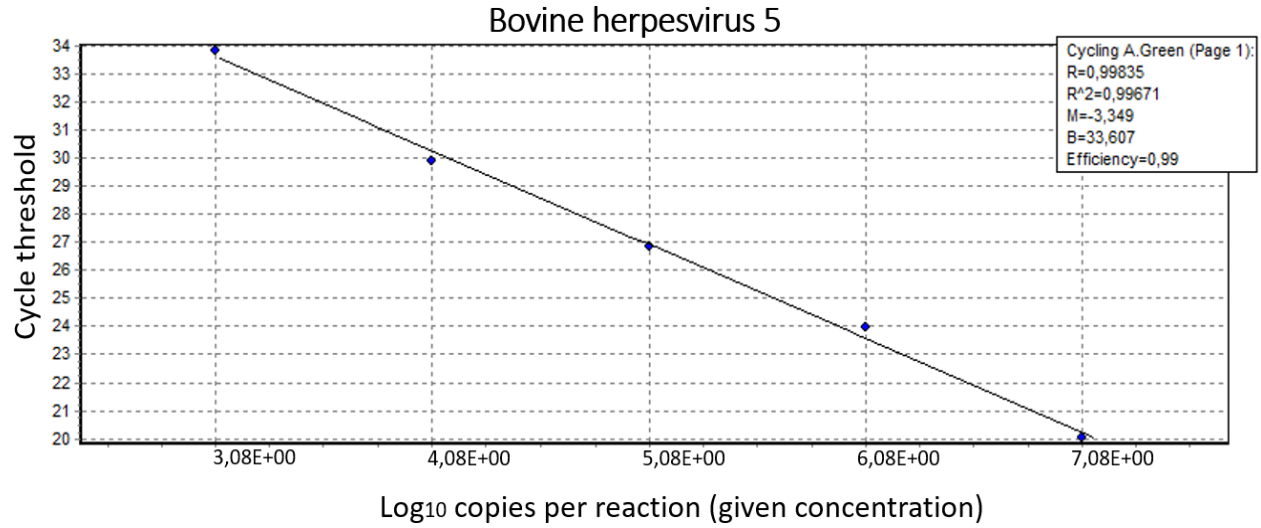
RESULTS

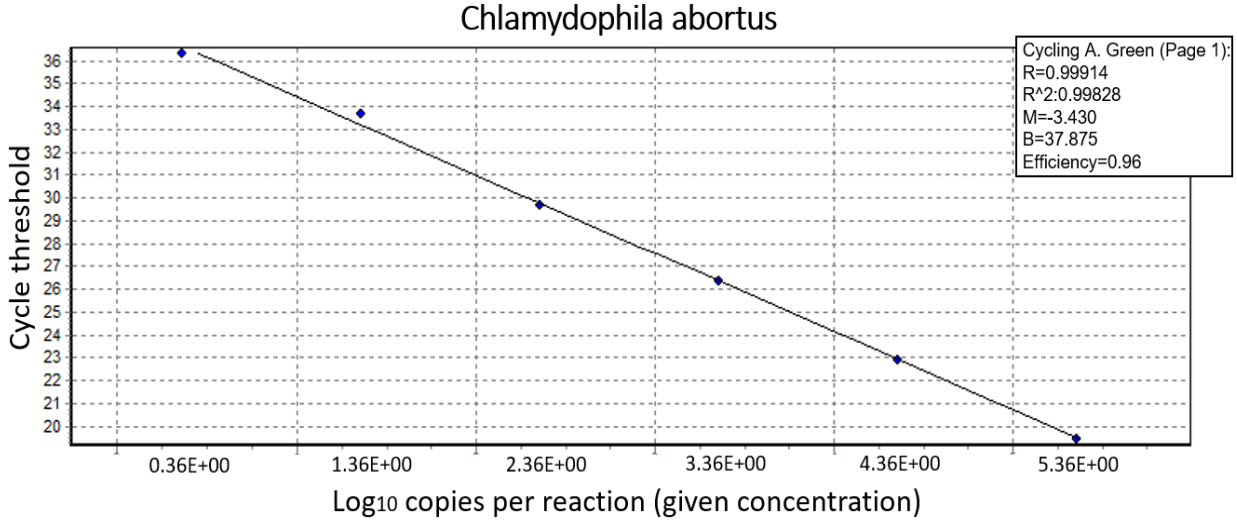
Optimal conditions for one-run qPCR: In all verification assays to evaluate the applicability and optimization of one-run diagnostic panels, the primer and probe concentrations in the reaction mixture were set at 10 and 5µM, respectively. Annealing was performed at 60°C in all PCR assays. Under these concentrations and annealing conditions, higher amplification efficiency and earlier threshold cycle (Ct) values were achieved (results derived from a series of optimization studies for the test panel). The cycling conditions for the RNAs were as follows: reverse transcription at 50°C for 10 minutes, inactivation at 95°C for 2 minutes, followed by denaturation at 95°C for 5 seconds, and annealing/extension at 60°C for 20 seconds, repeated for 40 cycles. For the DNAs, the cycling conditions included: inactivation at 95°C for 10 minutes, denaturation at 95°C for 10 seconds, and annealing/extension at 60°C for 30 seconds, repeated for 40 cycles.

Performance and control of the Detection Panel: To determine the efficiency, linearity, and sensitivity of the developed panels, standard curves were established using 10-fold serial dilutions of standard positives for DNA (Fig. 1) and RNA (Fig. 2). The values derived from the standard curves, including LOD, R^2, E, and Ct, are

presented in Table 2. The detection limits for the developed panel ranged from 1.33 to 4.64 log_{10} RNA copies/reaction and from 0.45 to 3.24 log_{10} DNA copies/reaction (LOD was determined as the minimum level that could be detected in at least 95% of replicate assays). The standard curves of each test covered a linear dynamic range exceeding five orders of magnitude, with R^2 values for all test designs determined as 0.99. The M values ranged between -3.10 and -3.60, and E values ranged between 90 and 100%, indicating sufficient PCR efficiency for copy number quantification. There was no non-specific amplification signal for all primer and probe sets in cross-reactivity and control assays, confirming high specificity for all assays. Exogenous internal controls used in the test of field samples gave stable results and showed amplification patterns consistent with the values given in the IndiMixJOE Kit manual. Successful amplification of the internal control indicated no nucleic acid degradation, presence of inhibitors, or any amplification-related problems.







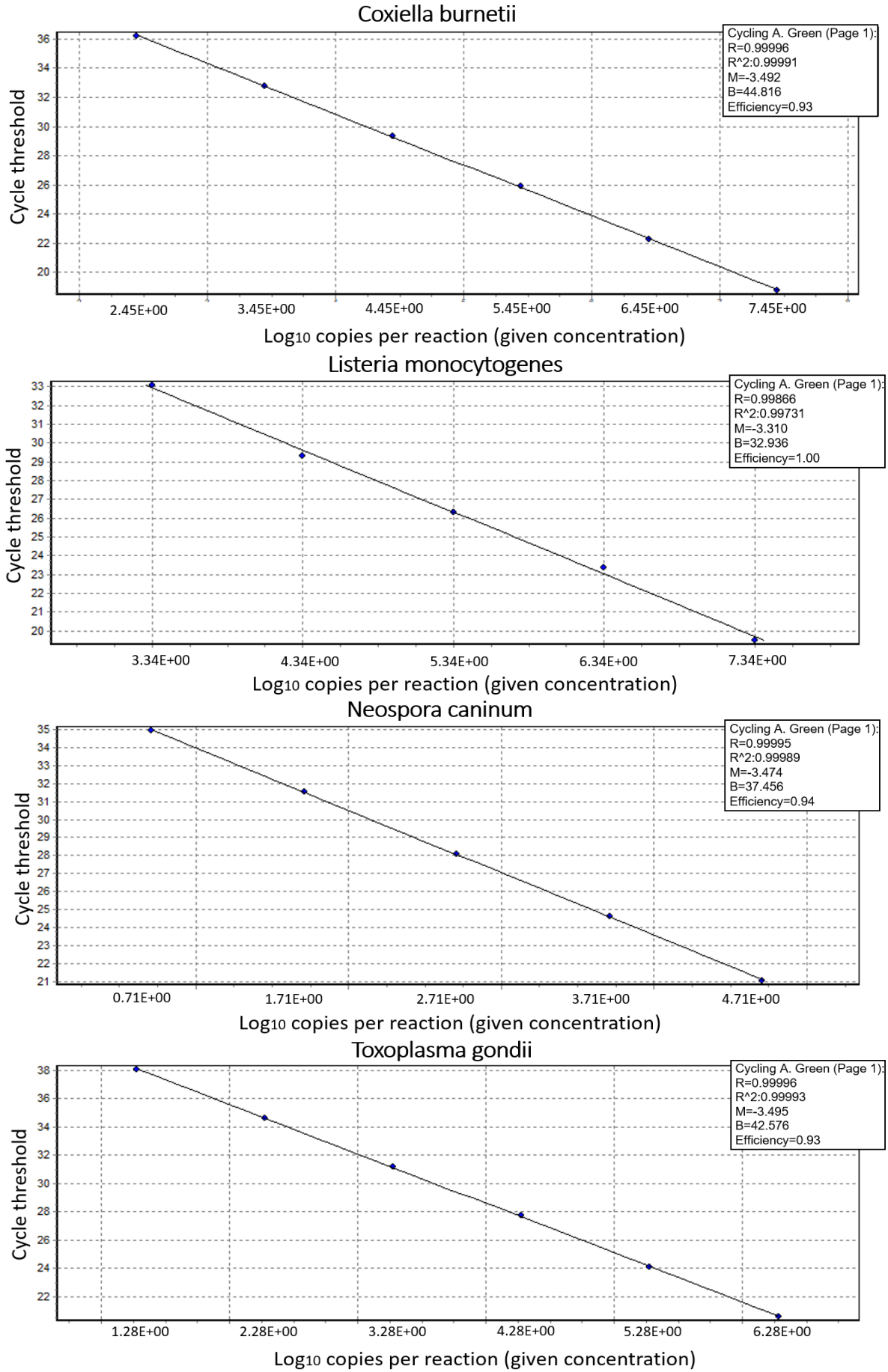
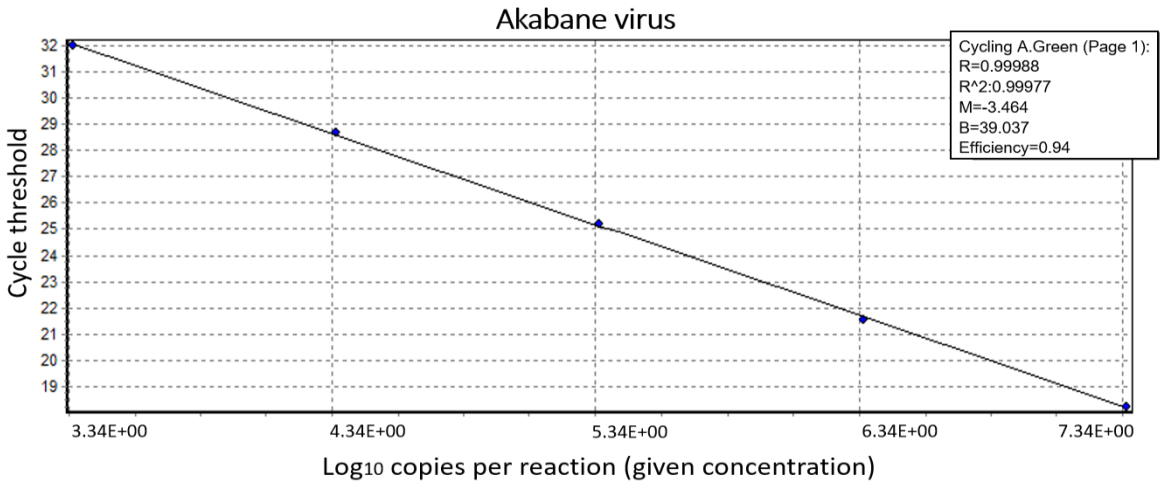
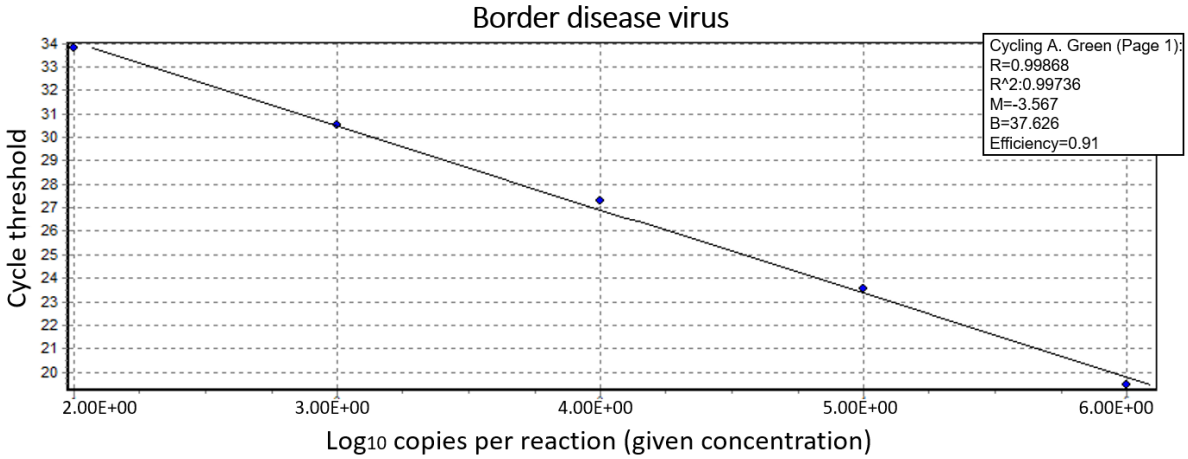
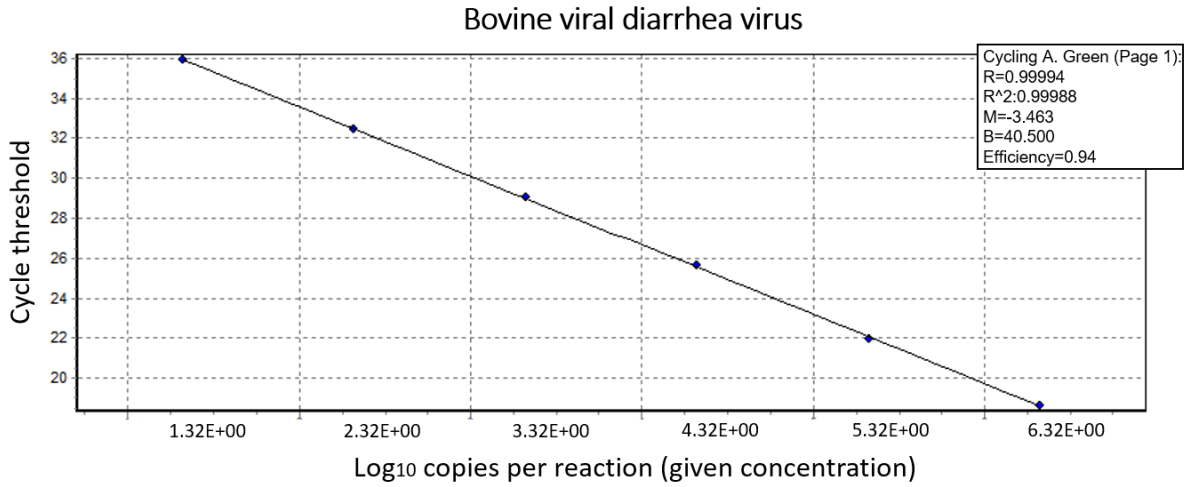
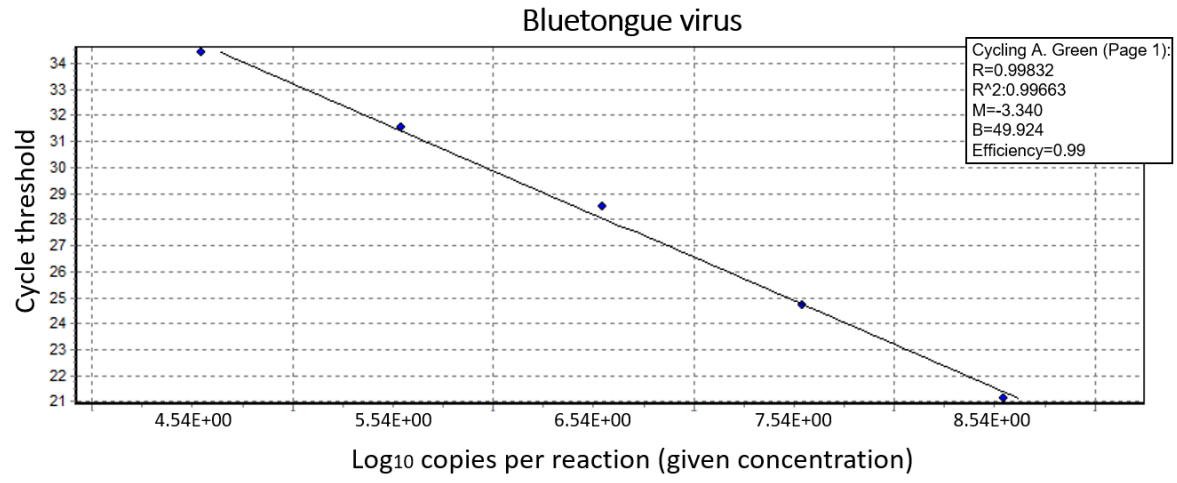


Fig. 1: Standard curves for DNA-based qPCR assays targeting viral (Bovine herpesvirus-1 & 5), bacterial (*Chlamydophila abortus, Coxiella burnetii, Listeria monocytogenes),* and parasitic (*Neospora caninum, Toxoplasma gondii)* agents. Each line represents 10-fold serial dilutions of the DNA templates. The assays were linear from 10^5 to 10^1 and 10^6 to 10^1 template copies; R^2 and reaction efficiency are shown.


Akabane virus
Cycling A.Green (Page 1):
R=0.99988
R^2:0.99977
M=-3.464
B=39.037
Efficiency=0.94
Cycle threshold
Log10 copies per reaction (given concentration)


Border disease virus
Cycling A. Green (Page 1):
R=0.99868
R^2:0.99736
M=-3.567
B=37.626
Efficiency=0.91
Cycle threshold
Log10 copies per reaction (given concentration)


Bovine viral diarrhea virus
Cycling A. Green (Page 1):
R=0.99994
R^2:0.99988
M=-3.463
B=40.500
Efficiency=0.94
Cycle threshold
Log10 copies per reaction (given concentration)


Bluetongue virus
Cycling A. Green (Page 1):
R=0.99832
R^2:0.99663
M=-3.340
B=49.924
Efficiency=0.99
Cycle threshold
Log10 copies per reaction (given concentration)

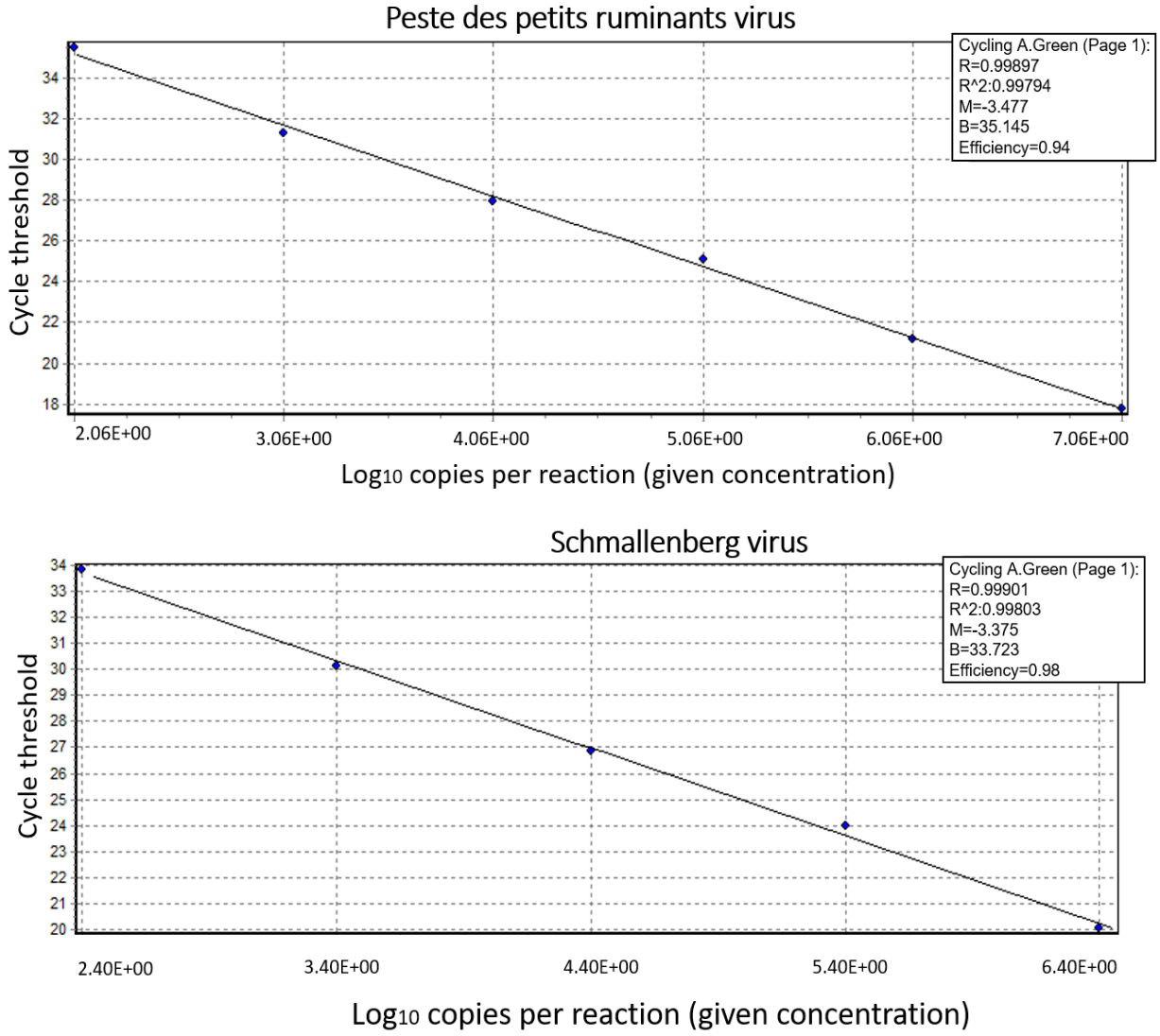


Fig. 2: Standard curves for RNA-based qPCR assays targeting viral (Akabane virus, Bluetongue virus, Border disease virus, Bovine viral diarrhea virus, Peste des petits ruminant's virus, and Schmallenberg virus) agents. Each line represents 10-fold serial dilutions of the RNA templates. The assays were linear from 10^5 to 10^1 and 10^6 to 10^1 template copies; R^2 and reaction efficiency are shown.

Repeatability and reproducibility of the test panel were assessed using coefficient of variation (CV) values calculated from the quantification cycle (Ct) data (Table 3). Additionally, the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were found to be 100% for all tests (Table 5). However, due to the lack of positive field samples for AKAV, SBV, BTV, BoHV, and *L. monocytogenes*, it was impossible to construct a testing setup or calculate DSe and DSp for these pathogens.

Verification of the test panel with clinical samples: As part of the verification studies, all clinical samples were tested for target pathogens. Positive clinical samples had Ct values between 17.21 and 31.66, and negative controls showed no amplification. Table 6 shows the prevalence of agents detected in clinical samples from sheep, goats, and cattle and the co-infections detected in some samples. In addition, sequence analysis data performed to confirm co-infections detected in some field samples have been submitted to the Genebank (accession numbers: PV584243, PV584244, PV583600, PV584198, PV589113, PV599764, PV599765, PV632086, PV632087, PV632089, PV632090, PV632093).

Table 5: Diagnostic performance based on positive and negative predictive values using field samples of the one-run qPCR assays

Diagnostic performance	TP	FP	FN	TN	Dsp (%)	Dse (%)
C. abortus assay	15	0	0	15	100	100
C. burnetii assay	7	0	0	7	100	100
BDV assay	10	0	0	10	100	100
BVDV assay	9	0	0	9	100	100
PPRV assay	10	0	0	10	100	100
N. caninum assay	3	0	0	3	100	100
T. gondi assay	1	0	0	1	100	100

TP=True positive; FP=False positive; FN=False negative; TN=True negative; Dsp=Diagnostic specificity; Dse=Diagnostic sensitivity.

DISCUSSION

Animal disease diagnostics rapid tests have emerged as a pivotal research field over the last several years (Sebastiani *et al.,* 2018; Modise *et al.,* 2023). While there have been some studies on abortions in locally bred ruminants in Turkiye (Tuncer-Goktuna *et al.,* 2016; Sakmanoglu *et al.,* 2021; Deniz and Oruc, 2024), there has been no study that has applied molecular screening tests for the simultaneous detection of bacterial, viral, and parasitic infections in a range of species. In this study, a molecular

diagnostic panel that can identify 13 abortion-inducing pathogens in cattle, sheep, and goats quickly has been presented. The process of verification was according to standard procedures (WOAH 2014; Toohey-Kurth *et al.,* 2020).

Table 6: Verification of the test panel with clinical samples results

Animal Species	Pathogen	Total samples	Positive cases	Percentage (%)
Sheep	*C. abortus*	156	100	64.10
Sheep	PPRV	156	23	14.74
Sheep	BDV	156	21	13.46
Sheep	*C. burnetii*	156	7	4.49
Sheep	*N. caninum*	156	3	1.92
Sheep	*C. abortus* + PPRV	156	4	2.56
Sheep	*C. abortus* + BDV	156	6	3.85
Sheep	*C. abortus* + *C. burnetii*	156	2	1.28
Sheep	PPRV + BDV	156	1	0.64
Goat	BDV	17	7	41.18
Goat	*C. abortus*	17	5	29.41
Goat	*C. burnetii*	17	5	29.41
Goat	*N. caninum*	17	1	5.88
Goat	*C. abortus* + BDV	17	1	5.88
Cattle	BVDV	15	9	60.0
Cattle	*C. burnetii*	15	4	26.67
Cattle	*N. caninum*	15	3	20.0
Cattle	*T. gondii*	15	1	6.67
Cattle	*C. burnetii* + BVDV	15	1	6.67
Cattle	*N. caninum* + BVDV	15	1	6.67

In this study, the validation of the test was completed using various data for measuring major parameters such as test accuracy, sensitivity, specificity, and reproducibility, which form the central theme of this research. These tests were carried out using standard curves generated during the development of a diagnostic panel. The standard curves confirmed the optimized performance of the assays by demonstrating the linearity of the reduction in the number of serial dilutions of positive controls (Table 2). Our findings provide compelling evidence of the high sensitivity and analytical specificity of the quantitative assays. The R^2 values were 0.99, and PCR efficiencies exceeded 90%. The limits of detection (LOD) for the panel ranged from 1.33 to 4.64 $\log_{10}$ RNA copies/reaction and 0.45 to 3.24 $\log_{10}$ DNA copies/reaction. The sensitivity of the test repeatability and reproducibility was assessed using the coefficient of variation (CV) calculated from Ct values (Table 3), and the highest recorded CV value was 2.67%. The positive and negative predictive values were calculated based on the sensitivity and specificity values of the samples. Accordingly, both diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were determined to be 100% (Table 5). These values demonstrate the robustness of the panel and enhance its diagnostic utility, particularly in high-throughput laboratory environments where rapid and accurate decision-making is essential. These performance metrics confirmed that the developed assay conditions were stable and reliable for diagnostic verification. The diagnostic performance of the panel was consistent with the results of other reported multiplex screening tests (Rahpaya *et al.,* 2018; Sebastiani *et al.,* 2018; Modise *et al.,* 2023). For pathogens such as AKAV, SBV, BTV, BoHV, and *L. monocytogenes,* where positive field samples were not detected, diagnostic performance metrics could not be calculated. After the verification of the panel with standard positives, verification studies were also carried out with clinical samples to identify pathogens in the study area simultaneously in a short time and to determine the status of the region in terms of these pathogens. The presentation of the results of clinical field samples may be critical in providing an external perspective for future epidemiological and serological studies. The development of the test panel was the result of many years of validation and repetitive studies in conjunction with routine diagnostics. The institute where the study was carried out is located in the center of Turkiye, in a region where livestock farming is intensive. For this reason, fetal samples are intensively sent to the institute's laboratories. Veterinary diagnostic laboratories with high sample densities need to be able to accurately detect many pathogens in a short time.

In this study, 188 clinical samples that were collected from various ruminant farms were used during routine diagnostics for the verification of the developed assay protocols. Our study focused on abortion cases that were negative for *Salmonella sp., Campylobacter sp., Brucella sp.,* and *Leptospira sp.* Especially considering that *Brucella sp.* is the most critical of the current major abortion causes, investigating the presence of these abortive bacteria primarily with serological methods may be an effective approach in terms of routine workload distribution and cost-effectiveness. The study was planned considering that it would be more effective to focus on other major pathogens after the pathogens in question were determined as serologically negative. Therefore, molecular detection of these critical bacterial pathogens was excluded. *N. caninum, T. gondii, C. burnetii,* and *C. abortus* are the most prevalent bacterial agents that also induce abortion in ruminants (Djellata *et al.,* 2019; Kaya and Ozturk, 2020; Ramo *et al.,* 2022; Di Bari et al., 2023). The panel quickly indicated these critical pathogens, and the findings showed that these agents were widespread in the region. *C. abortus* was detected in more than half of the sheep fetuses in the panel (64.10%), while *C. burnetii*, a zoonotic pathogen, was detected in cattle fetuses (26.67%). Some studies conducted worldwide (Dubey and Lindsay, 2006; Barry et al., 2019; Basso et al., 2022) have reported that parasites such as *N. caninum* and *T. gondii* are commonly detected among pathogens that cause abortion at rates of 13–66% and 5–43%, respectively. In our study, however, these parasites were found at a relatively lower rate (3.72 and 0.53%, respectively). Early diagnosis of bacterial and parasitic pathogens associated with abortion in laboratory diagnostic analysis is essential for immediate control. Due to the high zoonotic susceptibility of *C. abortus* and the possible use of *C. burnetii* as a bioterrorism agent, rapid diagnosis is necessary for the implementation of biosecurity measures in the event of an outbreak (Modise *et al.,* 2023).

Pestiviruses (BDV and BVDV) are a significant threat to most ruminant farms. Persistently infected (PI) animals that develop infection with the virus in utero and become lifelong carriers play an important role in BVDV and BDV transmission. The most effective preventive and control measure is to establish the existence of the agent to identify and eliminate PI animals (Gaede *et al.,* 2005; Willoughby

et al., 2006). In the current research, pestiviruses were found to be the second most common pathogen in cattle (60%) and goat (41.18%) fetuses and the third most common pathogen in sheep fetuses (13.46%). These results are consistent with those of previous studies (Heuer *et al.,* 2007; Tuncer-Goktuna *et al.,* 2016). In addition, PPRV, which is highly contagious for small ruminants and causes abortions, has been detected in sheep fetuses at a rate of 14.74%. This was also consistent with previous studies conducted in the country and the region (Pestil *et al.,* 2020; Oz *et al.,* 2021).

AKAV, BoHV, BTV, and *L. monocytogenes* were not detected in any of the suspected abortion samples. We interpreted the reason for not detecting these agents as "their lower incidence compared to other common pathogens in the region". In addition, no studies have been found in this region regarding these pathogens. For these reasons, studies will continue with the panel to learn the potential status of these pathogens in the region, to continuously monitor them and to provide early diagnosis of possible outbreaks. Co-infections were detected in some samples in the course of the study. Existing co-infections of abortions have previously been reported in local ruminants (Hazlett *et al.,* 2013; Peric *et al.,* 2018; Rahpaya *et al.,* 2018; Song *et al.,* 2021; Modise *et al.,* 2023). Such a molecular panel used to diagnose co-infection would be beneficial for the abortion control and cattle, goats, and sheep epidemiological surveillance.

Conclusions: This article describes the development and verification of a diagnostic panel for rapid and simultaneous diagnosis of principal abortive diseases of ruminants, the pillar of the world livestock economy. The resulting sensitivity profiles demonstrated the utility, reliability, and potency of the panel for conclusive and effective diagnosis. The panel is easy to operate, economical, and highly specific and sensitive. The system is a helpful tool for rapid differential diagnosis of outbreaks, notifiable animal disease, or zoonotic disease, significantly improving their traceability. The limitation of our study is that AKAV, SBV, and *L. monocytogenes,* which are assumed to be less common in the region, were not identifiable in the studied samples. However, it is thought that the routine diagnosis of these pathogens with the developed panel will continue, and early detection of outbreaks will be provided. Another limitation of this study is that some other abortion agents, such as *Brucella sp., Campylobacter sp., Salmonella sp.,* and *Leptospira sp.,* were not included in the one-run qPCR panel. Thus, this system offers a promising alternative to current molecular diagnostic methods, enabling efficient identification of pathogens and contributing to biosecurity and disease control measures.

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Authors contribution: MD, MEO, and SA contributed to the conceptualization, investigation, design, and supervision of the study. MD, MEO, and SA performed the experiments and data analysis. MD, MEO, and SA developed and executed the experimental designs using detailed laboratory methodologies and verification of the analytical method. MD prepared the initial draft of the article, and all authors reviewed and commented on the original version. All authors have read and approved the final version of the manuscript.

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Supplementary Table S1: Sample panel established to determine the diagnostic performance of positive-negative pathogens through qPCR assays

Sample number	Sample	Result	Pathogen
2019_09	Sheep	Positive	*C. abortus*
2019_78	Sheep	Positive	*C. abortus*
2019_114	Sheep	Positive	*C. abortus*
2020_18	Sheep	Positive	*C. abortus*
2020_76	Sheep	Positive	*C. abortus*
2021_25	Sheep	Positive	*C. abortus*
2021_107	Sheep	Positive	*C. abortus*
2021_298	Sheep	Positive	*C. abortus*
2022_45	Sheep	Positive	*C. abortus*
2022_49	Sheep	Positive	*C. abortus*
2023_132	Sheep	Positive	*C. abortus*
2023_279	Sheep	Positive	*C. abortus*
2024_83	Sheep	Positive	*C. abortus*
2024_121	Sheep	Positive	*C. abortus*
2025_10	Sheep	Positive	*C. abortus*
2019_56	Sheep	Negative	*C. abortus*
2019_59	Sheep	Negative	*C. abortus*
2019_100	Sheep	Negative	*C. abortus*
2020_71	Sheep	Negative	*C. abortus*
2020_45	Sheep	Negative	*C. abortus*
2021_27	Sheep	Negative	*C. abortus*
2021_83	Sheep	Negative	*C. abortus*
2021_85	Sheep	Negative	*C. abortus*
2022_03	Sheep	Negative	*C. abortus*
2022_12	Sheep	Negative	*C. abortus*
2023_15	Sheep	Negative	*C. abortus*
2023_28	Sheep	Negative	*C. abortus*
2024_17	Sheep	Negative	*C. abortus*
2024_23	Sheep	Negative	*C. abortus*
2024_38	Sheep	Negative	*C. abortus*
2019_21	Sheep	Positive	PPRV
2019_65	Sheep	Positive	PPRV
2019_100	Sheep	Positive	PPRV
2020_96	Sheep	Positive	PPRV
2020_99	Sheep	Positive	PPRV
2021_02	Sheep	Positive	PPRV
2021_36	Sheep	Positive	PPRV
2021_56	Sheep	Positive	PPRV
2022_63	Sheep	Positive	PPRV
2022_65	Sheep	Positive	PPRV

2022_77	Sheep	Positive	PPRV
2023_27	Sheep	Positive	PPRV
2024_08	Sheep	Positive	PPRV
2024_21	Sheep	Positive	PPRV
2025_02	Sheep	Positive	PPRV
2019_13	Sheep	Negative	PPRV
2019_19	Sheep	Negative	PPRV
2019_29	Sheep	Negative	PPRV
2020_16	Sheep	Negative	PPRV
2020_78	Sheep	Negative	PPRV
2021_51	Sheep	Negative	PPRV
2021_53	Sheep	Negative	PPRV
2021_89	Sheep	Negative	PPRV
2022_15	Sheep	Negative	PPRV
2022_19	Sheep	Negative	PPRV
2022_23	Sheep	Negative	PPRV
2023_10	Sheep	Negative	PPRV
2024_29	Sheep	Negative	PPRV
2024_30	Sheep	Negative	PPRV
2025_26	Sheep	Negative	PPRV
2019_21	Sheep	Positive	BDV
2019_65	Sheep	Positive	BDV
2019_100	Sheep	Positive	BDV
2020_57	Sheep	Positive	BDV
2020_116	Sheep	Positive	BDV
2021_14	Sheep	Positive	BDV
2021_23	Sheep	Positive	BDV
2021_56	Sheep	Positive	BDV
2022_45	Sheep	Positive	BDV
2022_49	Sheep	Positive	BDV
2022_118	Sheep	Positive	BDV
2023_123	Sheep	Positive	BDV
2024_62	Sheep	Positive	BDV
2024_68	Sheep	Positive	BDV
2025_06	Sheep	Positive	BDV
2019_12	Sheep	Negative	BDV
2019_15	Sheep	Negative	BDV
2019_20	Sheep	Negative	BDV
2020_29	Sheep	Negative	BDV
2021_33	Sheep	Negative	BDV
2021_73	Sheep	Negative	BDV
2022_91	Sheep	Negative	BDV
2022_93	Sheep	Negative	BDV
2022_108	Sheep	Negative	BDV
2023_19	Sheep	Negative	BDV
2024_36	Sheep	Negative	BDV
2024_79	Sheep	Negative	BDV
2025_52	Sheep	Negative	BDV
2019_10	Sheep	Positive	C. burnetii
2019_19	Sheep	Positive	C. burnetii
2020_328	Sheep	Positive	C. burnetii
2021_77	Sheep	Positive	C. burnetii
2021_33	Sheep	Positive	C. burnetii
2021_36	Sheep	Positive	C. burnetii
2021_125	Sheep	Positive	C. burnetii
2019_13	Sheep	Negative	C. burnetii
2019_65	Sheep	Negative	C. burnetii
2020_03	Sheep	Negative	C. burnetii
2021_96	Sheep	Negative	C. burnetii
2021_101	Sheep	Negative	C. burnetii
2021_107	Sheep	Negative	C. burnetii
2021_109	Sheep	Negative	C. burnetii
2023_26	Sheep	Positive	N. Caninum
2023_29	Sheep	Positive	N. Caninum
2023_39	Sheep	Positive	N. Caninum
2023_91	Sheep	Negative	N. Caninum
2023_92	Sheep	Negative	N. Caninum
2023_93	Sheep	Negative	N. Caninum
2020_11	Goat	Positive	BDV
2020_18	Goat	Positive	BDV
2021_25	Goat	Positive	BDV
2021_81	Goat	Positive	BDV
2021_90	Goat	Positive	BDV
2022_13	Goat	Positive	BDV
2023_56	Goat	Positive	BDV
2020_106	Goat	Negative	BDV
2020_40	Goat	Negative	BDV
2021_46	Goat	Negative	BDV

2021_49	Goat	Negative	BDV
2021_79	Goat	Negative	BDV
2022_11	Goat	Negative	BDV
2023_23	Goat	Negative	BDV
2021_75	Goat	Positive	*C. abortus*
2021_179	Goat	Positive	*C. abortus*
2021_192	Goat	Positive	*C. abortus*
2022_156	Goat	Positive	*C. abortus*
2022_200	Goat	Positive	*C. abortus*
2020_13	Goat	Negative	*C. abortus*
2020_26	Goat	Negative	*C. abortus*
2021_38	Goat	Negative	*C. abortus*
2022_124	Goat	Negative	*C. abortus*
2023_18	Goat	Negative	*C. abortus*
2019_03	Goat	Positive	*C. burnetii*
2019_10	Goat	Positive	*C. burnetii*
2020_52	Goat	Positive	*C. burnetii*
2021_30	Goat	Positive	*C. burnetii*
2021_95	Goat	Positive	*C. burnetii*
2019_69	Goat	Negative	*C. burnetii*
2019_86	Goat	Negative	*C. burnetii*
2021_14	Goat	Negative	*C. burnetii*
2022_41	Goat	Negative	*C. burnetii*
2023_09	Goat	Negative	*C. burnetii*
2022_53	Goat	Positive	*N. caninum*
2023_369	Goat	Negative	*N. caninum*
2022_71	Cattle	Positive	BVDV
2022_156	Cattle	Positive	BVDV
2023_96	Cattle	Positive	BVDV
2024_84	Cattle	Positive	BVDV
2024_88	Cattle	Positive	BVDV
2024_107	Cattle	Positive	BVDV
2024_152	Cattle	Positive	BVDV
2024_166	Cattle	Positive	BVDV
2024_209	Cattle	Positive	BVDV
2022_71	Cattle	Negative	BVDV
2022_156	Cattle	Negative	BVDV
2023_96	Cattle	Negative	BVDV
2024_84	Cattle	Negative	BVDV
2024_88	Cattle	Negative	BVDV
2024_107	Cattle	Negative	BVDV
2024_152	Cattle	Negative	BVDV
2024_166	Cattle	Negative	BVDV
2024_209	Cattle	Negative	BVDV
2020_09	Cattle	Positive	*C. burnetii*
2021_71	Cattle	Positive	*C. burnetii*
2021_85	Cattle	Positive	*C. burnetii*
2022_51	Cattle	Positive	*C. burnetii*
2020_09	Cattle	Negative	*C. burnetii*
2021_71	Cattle	Negative	*C. burnetii*
2021_85	Cattle	Negative	*C. burnetii*
2022_51	Cattle	Negative	*C. burnetii*
2023_15	Cattle	Positive	*N. caninum*
2023_26	Cattle	Positive	*N. caninum*
2023_98	Cattle	Positive	*N. caninum*
2023_41	Cattle	Negative	*N. caninum*
2024_75	Cattle	Negative	*N. caninum*
2024_83	Cattle	Negative	*N. caninum*
2023_19	Cattle	Positive	*T. gondi*
2024_26	Cattle	Negative	*T. gondi*