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Improving sensitivity of single tube nested PCR to detect fastidious microorganisms

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ABSTRACT

Single Tube Nested PCR (ST-nPCR) is of value to clinical laboratories with limited settings for the detection of fastidious microorganisms. The detection sensitivity of ST-nPCR is dependent on ensuring minimal leftovers of outer primers during the second round of the reaction. In this work, we investigated various approaches to optimize the performance of outer primers, including decreasing outer primer concentrations; using antisense oligonucleotides to block outer primers; using chemically modified inner primers; and using Q5 Taq polymerase that lacks 5′-3′ exonuclease and strand displacement capabilities. These solutions were tested on *C. abortus* and *C. psittaci*, which are both fastidious intracellular bacteria that are difficult to diagnose.

The best obtained result was by using Q5 Taq polymerase. A detection limit with a range between 0.1 and 1 ag was achieved, which corresponds to a range between 0.2 and 2 copies of the plasmid positive control. This level of sensitivity is comparable or even better than the sensitivity achieved by TaqMan probe based real-time PCR assays. The assay was validated using 70 veterinary clinical samples from small ruminant abortions and 10% of these samples gave positive results.

In conclusion, sensitivity of ST-nPCR to detect fastidious microorganisms can be improved by using Taq polymerases that lacks 5′-3′ exonuclease. The proposed assay is affordable and applicable to a wide range of fastidious pathogens and can be suitable for laboratories with limited settings.

1. Introduction

Improving sensitivity and specificity of PCR for the detection of microorganisms is one of the major challenges in diagnostic microbiology. While modern laboratories use real-time PCR as a sensitive method for microbial detection, many laboratories, especially those with resource-limited settings are still using nested PCR (nPCR) as a powerful technique in terms of sensitivity and specificity [1, 2]. Nested or semi-nested PCR assays involve the running of a first PCR using an outer primers set. Next, an aliquot of the amplified product serves as the template in the running of a second PCR using an inner set of primers [3, 4, 5, 6]. Typically, the first round with low number of cycles (10–20) serves to boost the scarce amount of the natural DNA template to generate a sufficient amount of a synthesized template for the second round. In contrast to single round PCR, nPCR is a very sensitive assay to detect extremely low copy number target DNA. In terms of specificity, nPCR reduces the possibility to produce non-specific products as compared with single round PCR with high number of cycles ($\geq$40) and this is simply because the chance to

amplify misprimed non-specific sequences generated during the first round is very low as these non-specific sequences do not contain the binding sites of the inner primers.

Although the nPCR technique has detected various microbial agents due to its superior performance, it is prone to contamination and artifacts. These problems are mainly due to contamination during the pipetting process of the amplified material generated in the first round so as to prepare the template for the second round [7]. To overcome this problem, a modified nPCR assay was developed where the two rounds of amplification take place in the same tube and without the need to open the PCR tube after the first round [8]. This system was designated close-nested PCR or single tube nested PCR (ST-nPCR) [9, 10]. ST-nPCR can detect different fastidious microorganisms with a significant improvement in sensitivity and specificity [11, 12, 13, 14, 15].

The success of ST-nPCR is highly dependent on minimizing the interference between the two sets of primers. Some early reports proposed a physical approach to separate the two sets of primers in ST-nPCR [16]. However, the most commonly used approach to minimize

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this interference is by designing the two sets of primers with differential annealing temperatures. Typically, the first round is performed at a high annealing temperature (high temperature round) and uses outer primers with relatively high melting temperature (Tm). The second round (low temperature round) is performed at a low annealing temperature and uses nested primers that have lower Tm to amplify an internal segment of the amplicon generated by the first round [9, 17, 18]. In theory, ST-nPCR is an attractive solution to reduce handling time and to prevent cross contamination of conventional nPCR. It has frequently been shown, however, that the detection sensitivity of ST-nPCR is lower than the nPCR [19, 20, 21]. The main technical challenge to ensure high detection sensitivity of ST-nPCR is to ensure a good activity of the outer primers during the high temperature round, and to halt this activity during the low temperature round. Residuals of outer primers during the low temperature round can generate mispriming artifacts and reagents exhaustion. Another undesired activity of outer primers residuals is the ability of their polymerizing strand to hydrolyze the bound inner primer through the 5′-3′ exonuclease activity of Taq polymerase enzyme (TaqMan activity). To our knowledge, this activity of outer primers in ST-nPCR has not been investigated previously.

Several attempts were proposed to overcome the problem of outer primers residuals. One of the solutions is by lowering the concentration of the outer primers. A ST-nPCR targeting the internal transcribed spacer (ITS1) region was developed to diagnose ovine induced abortions by *Toxoplasma gondii* [22]. In this assay the authors used 0.01 μM of outer primers and 0.4 μM of inner primers to achieve 0.1pg detection sensitivity. In another ST-nPCR, a concentration of 0.5 mM of inner primers and 0.005 mM of outer primer were used for the diagnosis of *Coxiella burnetii*. The assay sensitivity was 5 fg and a 10–100 fold enhancement over the conventional PCR was achieved [14]. However, using outer primers in minimal concentrations is very tricky and can lead to inefficient boosting of the first amplicon at the high temperature round. To be able to use outer primers at high concentrations and without any non-specific amplification, *Brisco et al.* developed an approach named "antisense PCR". This technique employs antisense probes that have complementary sequences to the outer primers and have 5′ and 3′ tags that hybridize to the excess amount of the outer primers to inhibit their binding to the target template during the low temperature round [23]. In the antisense PCR, the challenge is to design the two antisense probes in a way that they only hybridize and inhibit the outer primers under the low temperature round. Although antisense primers can improve the sensitivity of ST-nPCR, the antisense PCR approach requires extra primers and more effort to optimize the reaction conditions. Therefore, there is a need to come up with more simple solutions to avoid the activity of the outer primers during the low temperature round.

The aim of this study was to develop a sensitive ST-nPCR assay using *C. abortus* as a model to validate the test. Different critical parameters were investigated to overcome the problem of outer primers' residuals, including annealing temperatures; primers concentrations and cycling conditions. In addition, several modifications of the ST-nPCR were investigated to minimize the effect of outer primer undesired activity during the low temperature round, including the use of complementary antisense probes; the use of chemically modified inner primers and the application of different classes of Taq polymerases in the reaction.

2. Materials and methods

2.1. Samples

C. abortus was selected as the target pathogen because of the difficulty of its culturing and its low abundance in clinical samples [24]. *C. abortus* field strain MM-12-2013 was obtained from the Central Veterinary Laboratory (CVL) Ministry of Agriculture to construct the plasmid positive control.

The DNA samples of the following nine bacterial strains were used to examine the specificity of the ST-nPCR: (*Brucella. melitensis* bv.1 str. 16M, *Brucella. abortus* str. 544, *Bacillus cereus, Bacillus thuringiensis, Campylobacter fetus* subsp. *fetus, Campylobacter fetus* subsp. *venerealis,* avian pathogenic *Escherichia coli* (APEC), *Salmonella typhimurium* and *C. abortus* vaccine strain namely (Ovax Clamidia, Fatro, Italy)). Some of these bacteria were selected because they are frequently associated with abortion of small ruminants and we want to rule out any possible interference.

Seventy clinical samples, which were collected in 2015 from vaginal swabs of aborted ewes and does were used to validate the developed ST-nPCR assay. The samples were collected and processed by the CVL teams within the first 3 days post abortion. Briefly, the vaginal swabs were collected and transported on ice to the CVL and processed immediately upon arrival or stored at −80 °C. Total DNA was isolated from vaginal pellet using QIAamp DNA Mini Kit, Cat # 51304 (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.2. Primers

Cloning primers, ST-nPCR primers and antisense probes were designed and tested using the Oligo 7 software. The designed primers were synthesized by Hy-labs, Rehovot. The antisense probes were designed by Syntezza, Jerusalem. These primers were purified by standard desalting. All primers and probes are shown in Table 1.

2.3. DNA polymerases

Two classes of DNA polymerases were used. The first is Taq DNA Polymerase High Purity (Cat # HTD0078), produced by the Hy-labs company. The second is the Q5 High Fidelity-DNA Polymerase (Cat # M0491S) from New England BioLabs (Ipswich, Massachusetts).

Table 1. Sequences of primers and probes designed in this study. The capital letters at both ends of antisense oligonucleotides refer to 5′ and 3′ tags, which prevent the outer primer extension. The asterisks refer to a chemically modified phosphorothioate bond.

Primer name	Sequence (5′-3′)	Tm (°C)	Amplicon size (bp)
Cloning primers			
POMP-cloning F	TGCTGATAAACTCACCATTGTC	53.0	838
POMP-cloning R	CTCCTACCCATTACGGTTATCAA	53.9	
ST-nPCR primers			
Chlamydia out F	CGGCACCTAAAGGCGGAGCTATTTGCA	67.9	490
Chlamydia out R	CCACCTGAGGAAGGCGTCTGTAACGTA	66.3	
Chlamydia in F	GGTGAATGTAGCTTAACCGC	57.8	369
Chlamydia in R	TGCTTCTAAAGTGACACCATCT	56.5	
Antisense oligonucleotides			
Chlamydia out F A1	AATtgcaaatagctccgcctttTCC	59.3	–
Chlamydia out R A1	TTGtacgttacagacgccttGGA	59.1	
Chlamydia out F A2	AATtgcaaatagctccgccAAA	56.8	
Chlamydia out R A2	TTGtacgttacagacgccAAG	56	
Chlamydia out F A3	AATtgcaaatagctccCGG	54.3	
Chlamydia out R A3	TTGtacgttacagacgGGA	54	
Chlamydia out F A4	AATtgcaaatagctGGC	49.1	
Chlamydia out R A4	TTGtacgttacagaGCG	50	
5′-phosphorothiated inner primers			
Chlam F In M	C*G*G*TGAATGTAGCTTAACCGCT	57.4	372
Chlam R In M	T*T*T*TGCTTCTAAAGTGACACCATCT	54.9	
Sheep and goat internal control markers			
Sheep F	TGTAGGGTATTAAACTGCTTGAC	59	961 (Sheep)
Goat F	CCACTCCACAAGCTTACAGAC	61	
Universal R	GTGGCTGGCACGAGATTTACCAAC	67	1428 (Goat)

2.4. Marker selection and in silico validation

The POMP 90A/91A gene of *C. abortus* and *C. psittaci* was selected as a marker to design the primers for the ST-nPCR (Table 2). Reference sequences for this gene were retrieved from the National Center of Biotechnology Information (NCBI). To validate the specificity of this marker to *C. abortus* and *C. psittaci*, these sequences were used as a query for the Basic Local Alignment Search Tool (BLAST) against all genomic sequences (excluding the two species of *Chlamydophila*) deposited in the NCBI Genbank (as of August 2018). To validate conservation of the selected marker among all *C. abortus* and *C. psittaci* strains, these sequences were used as a BLAST query against all genomic sequences of these two species in the NCBI database (as of August 2018).

2.5. Construction of positive control

To construct a positive control template, a genomic DNA from the *C. abortus* field strain MM-12-2013, was obtained from (CVL) and amplified using POMP-cloning F and POMP-cloning R primers that span the whole locus. The reaction mixture consisted of: 0.6 units of thermostable Taq DNA polymerase (Hy labs), 1X Taq reaction buffer, 2.5 mM of MgSO$_4$, 0.2 mM of each dNTPs (Cat # DNTp 10-1KT) produced by Sigma company, 0.2 μM of forward primers, 0.2 μM of reverse primers and 1 μl of the DNA template. The total volume of the reaction was 25 μl. Amplification was performed using Applied Biosystems 2720 thermocycler. The cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s followed by a final extension at 72 °C for 10 min. The 838 bp amplified product was cloned in the pGEM-T vector (Cat # A1360) produced by the Promega company, and the construct was transformed into *Escherichia coli* DH5α.

2.6. Plasmid extraction

Transformed DH5α colonies were screened by PCR for the right insert size. One of the verified colonies was incubated in 10 ml LB provided with 12.5 μl of 50 mg/ml Ampicillin, with shaking overnight at 37 °C. The plasmid extraction was performed using NucleoSpin® Plasmid (NoLid) plasmid extraction kit following manufacturer's instructions (Macherey-Nagel). To test assays sensitivity, extracted plasmids containing the specific marker were 10-fold serially diluted, starting from 10 ng DNA, which corresponds to $2.4*10^9$ copies of the plasmid and reaching 1 ag of DNA which corresponds to 0.2 copies of the plasmid. Plasmid copy number was calculated using the following formula:

Number of copies = (amount [ng] * 6.022 x 10^{23})/(length [bp] * 1 x 10^9 * 660).

2.7. Sequencing of the extracted cloned plasmid

The plasmid containing the selected insert was sequenced using the SP6 Promoter Primer (TATTTAGGTGACACTATAG) (Cat # Q5011, Promega) and the T7 Promoter Primer (TAATACGACTCACTATAGGG) (Cat # Q5021, Promega). Sanger sequencing was performed at the Hereditary Research Laboratory/Life Science Department/Bethlehem University (Bethlehem).

Table 2. Selected Locus (for POMP 90A/91A gene) of *C. abortus* and *C. psittaci* for the study.

Organism	Strain	marker	Coordinates	Accession number(s)
C. abortus	S26/3	Pmp 90/91	322496–323458	NC_004552.2
C. psittaci	6BC		710780–711744	NC_015470.1

2.8. ST-nPCR

The outer and inner primers of ST-nPCR reaction are shown in Table 1. Unless otherwise specified, all ST-nPCR reactions were performed in 25 μl of reaction mixture containing 2.5 mM MgSO$_4$, 1X Taq PCR buffer, 0.2 mM of each dNTP and 0.6 units of Taq DNA polymerase (Hy labs). The PCR program started with 2 min of denaturation at 94 °C. The high temperature round consisted of 15 cycles of denaturation at 94 °C for 20 s, primer annealing at 70 °C for 20 s and extension at 72 °C for 25 s, and 5 min of incubation at 56 °C were added to activate the antisense probes when they were used. The low temperature round consisted of 40 cycles of denaturation at 94 °C for 20 s, primer annealing at 56 °C for 20 s and extension at 72 °C for 30 s. The ST-nPCR ended with a final extension step at 72 °C for 3 min.

2.9. Antisense oligonucleotides

Antisense oligonucleotides were designed as in [23]. These oligonucleotides carried 5′ and 3′ tags, each consisting of three nucleotides, which prevented the outer primer extension on both the template strand and the hybridizing oligonucleotide molecule in the low temperature round amplification. In this study, four different antisense pairs, namely, A1, A2, A3, and A4 with different melting temperatures were designed and tested. Unless otherwise specified, the reactions were performed in 25 μl of reaction mixture containing 2.5 mM MgSO$_4$, 1X Taq PCR buffer, 0.2 mM of each dNTP and 0.6 units of Taq DNA polymerase (Hy labs). The PCR program started with 95 °C for 2 min. The amplification consisted of 30 cycles of denaturation at 94 °C for 15 s, gradient annealing that ranged between 73 °C-57 °C or between 61 °C-45 °C and extension at 72 °C for 40 s. The reaction ended with a final extension step at 72 °C for 3 min.

2.10. ST-nPCR protocol using 5′- phosphorothioate inner primers

The 5′- phosphorothioate inner primers (Table 1) were manufactured by Syntezza (Jerusalem). In these primers, the first three phosphodiester bonds were modified by substituting the non-bridging oxygen with a sulfur atom. This type of modification is a frequently used method to reduce the sensitivity of oligonucleotides to extra and intracellular nucleases [25].

2.11. ST-nPCR protocol using the Q5 Taq polymerase

The Q5 Taq polymerase is a high fidelity, thermostable DNA polymerase with 3′-5′ exonuclease activity. However, this polymerase lacks both the 5′-3′ exonuclease activity and the strand displacement activity; being suitable for avoiding the effect of any excess of outer primers in the second low temperature cycling round. Unless otherwise specified, the reactions were performed in 25 μl of reaction mixture containing 5X Q5 reaction buffer, 5X Q5 high GC enhancer, 0.2 mM of each dNTP, 0.5 units of Q5 high fidelity DNA polymerase (NEB, Cat # M0491S).

2.12. Analytical specificity

The analytical specificity of the developed ST-nPCR assay to distinguish target from non-target DNA was validated using a panel of DNA samples from different pathogens associated with abortion of small ruminants or that may share the same environment in the farm.

2.13. Validation assay

The newly developed assay was validated by testing 70 DNA samples from sheep and goat abortion specimens collected by the CVL in 2015. The samples were tested for sheep and goat internal control markers to

confirm their suitability for PCR. The mitochondrial DNA (D-loop region +500 bp) was used to design primers for the internal control markers (Table 1).

3. Results and discussion

3.1. Effect of outer primer on ST-nPCR

To explore the effect of the outer primers concentration on the performance of the inner primers in ST-nPCR, five different concentrations (0.0, 0.01, 0.05, 0.1, 0.5 μM/each primer) of the outer primers were tested in duplicate. In addition to generating undesired amplicons (Figure 1-A), increasing the concentrations of the outer primers reduced the amplification efficiency of the inner primers (Figure 1-B). A possible explanation for this behavior is that the leftover outer primers from the high temperature round can degrade the annealed inner primers through the 5′-3′ exonuclease activity (TaqMan effect) of the regular Taq polymerase. *Da Silva et al.* observed a similar reduction in ST-nPCR sensitivity when increasing outer primers concentration. They attributed this

behavior to a competition between outer primers and inner primers for the same targets [19].

It is clear from the previous experiment that preventing the negative effect of the outer primers, during the low temperature round, by calibrating their concentrations is a tricky solution. Therefore, there is a need to have a robust solution to get rid of or to impede the activity of the outer primers after the first round.

3.2. Solution 1: chemical modification of inner primers

To minimize the 5′-3′ exonuclease activity that may degrade the annealed inner primers, chemically modified inner primers were produced by replacing the phosphodiester bonds between the first three nucleotides at the 5′ terminus with phosphorothioate bonds. This modification is supposed to render the inner primers more resistant to exonuclease activity and thus may reduce degradation of the annealed inner primers. The obtained results showed some improvement in amplification yield; but no improvement was noticed in detection sensitivity (Figure 2-B). In addition, the modified inner primers tend to
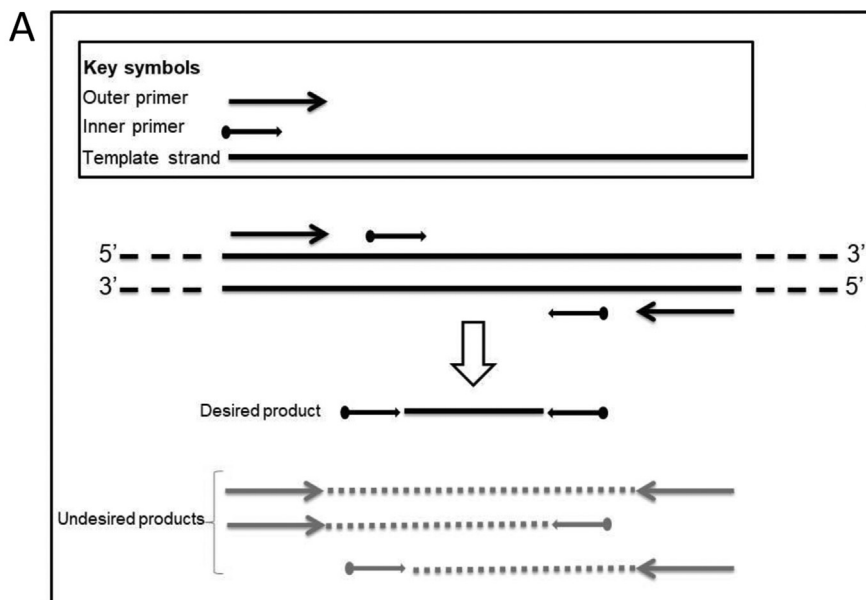

Figure 1. A. Possible amplicons of ST-nPCR: A schematic diagram showing the desired amplicons for close nested PCR using two pairs of outer primers and inner primers along with the potential undesired amplicons that may arise by combinations of outer and inner primers. B. Effect of outer primers residuals: 2% agarose gel electrophoresis results of conventional ST-nPCR using different concentration of outer primers. The POMP 90A/91A gene of *C. abortus* (369 bp) was amplified using five concentrations of outer primers (0.0 μM, 0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM) respectively and 0.2 μM of inner primers. The reactions were carried out in duplicate (marked as 1 and 2) and 1 pg of positive control was used. Lane N: template control. Lane M: 100 bp DNA ladder (GeneDireX). Please see Supplementary PowerPoint file for the corresponding original image.
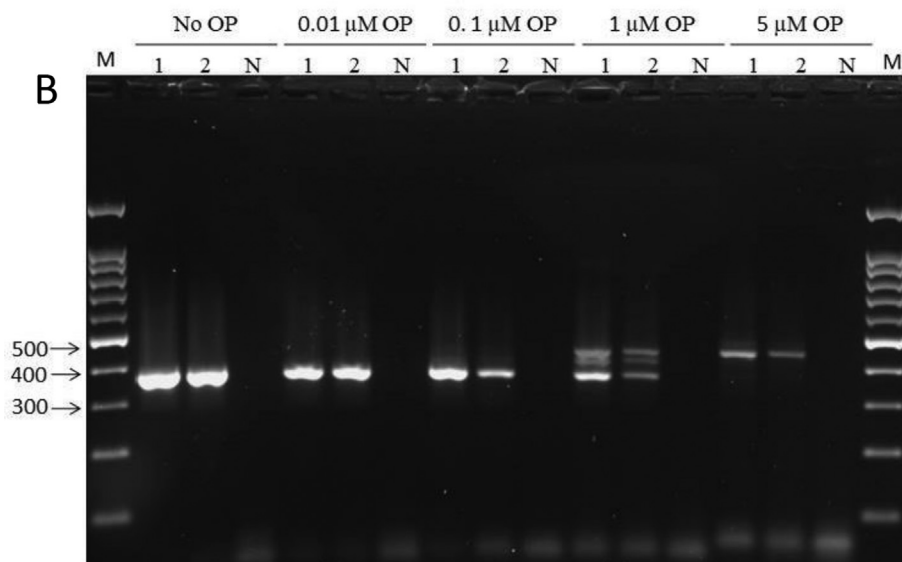
Figure 2. Comparison of the respective sensitivities of ST-nPCR with modified ST-nPCR: 2% agarose gel electrophoresis results for the amplification of POMP 90A/91A gene of *C. abortus* (369 bp) from diluted plasmid positive control. Lanes 1–7 in each gel represent the positive control plasmid with different number of copies (2.4×10^5, 2.4×10^4, 2.4×10^3, 2.4×10^2, 2.4×10^1, 2.4, 0.2) respectively. Lane N: no template control. Lane M: 100 bp DNA ladder (GeneDireX). **A.** Results of ST-nPCR using 0.01 μM of the outer primers and 0.4 μM of the inner primers. Please see Supplementary PowerPoint file for the corresponding original image. **B.** Results of ST-nPCR using modified inner primers. The modification was performed by replacing the phosphodiester bonds between the first three nucleotides at the 5′ terminus with phosphorothioate bonds. The amplification was done by using 0.01 μM of the outer primers and 0.4 μM of the inner modified primers. Please see Supplementary PowerPoint file for the corresponding original image. **C.** Results of ST-nPCR using the Q5 Taq polymerase and 0.01 μM of the outer primers and 0.4 μM of the inner primers. Please see Supplementary PowerPoint file for the corresponding original image.
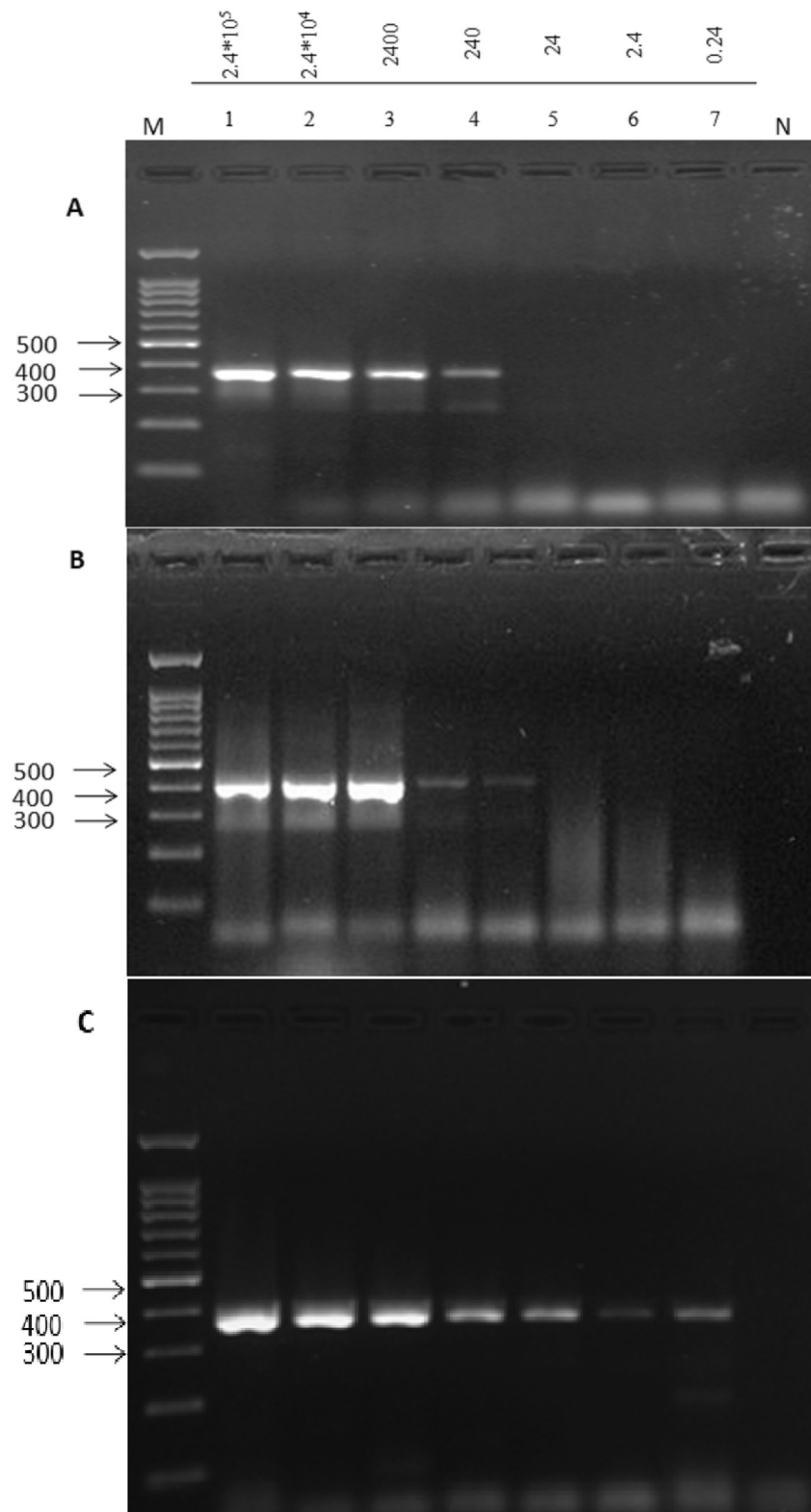
produce more smears than the unmodified primers. Repeating the experiment with higher annealing temperatures decreased the detection sensitivity.

The phosphorothioate modification was used to design antisense probes for gene silencing. The aim of this modification is to decrease the degradation sensitivity of these probes by cellular nucleases [26]. In addition, this modification was used to protect primers in an allele-specific PCR assay. However, these 3′ phosphorothioate modified primers were found to be degraded by Taq polymerases that contained the 3′-5′ exonuclease activity [27]. Although little is known about 5′

phosphorothioate modifications, and in the light of the previously discussed verifications, our results could indicate that the phosphorothioate modification was not efficient to resist the 5′-3′ exonuclease activity of the conventional Taq polymerase, so the outer primers product was able to degrade annealed inner primers decreasing the assay sensitivity. In addition, the phosphorothioate modification seems to affect the physicochemical properties of the primers and thus the annealing behavior. Therefore, this solution seems to be tricky and requires further investigations to understand the behavior of these modified primers.

3.3. Solution 2: a 5′-3′ exonuclease deficient Taq polymerase

As an alternative solution to block degradation activities of the growing chain of the outer primers on the inner primers, a thermostable DNA polymerase that lacks 5′-3′ exonuclease activity was tested. The Q5 Taq polymerase which is a high-fidelity thermostable DNA polymerase that lacks both the 5′-3′ exonuclease and the strand displacement activities, was selected. The use of Q5 Taq polymerase significantly increased the detection sensitivity by at least 100–1000 fold compared to the conventional ST-nPCR protocols (Figure 2-C). The experiment was repeated several times with a consistent detection limit down to 0.1 ag which corresponds to 0.2 copies of the plasmid.

This level of assay detection sensitivity is comparable or slightly better than the sensitivity achieved by real-time PCR assays [28]. This significant improvement in the detection sensitivity can be attributed to the lack of the 5′-3′ exonuclease activity and the lack of strand displacement activity of Q5 Taq polymerase, which can eliminate the inhibitory effects caused by the excess leftover of the outer primers. To further test this hypothesis, Q5 Taq polymerase was used with different concentrations of outer primers. It can be seen that increasing the concentration of outer primers is still causing depletion of inner primers amplicon (Figure 3). Further investigations are needed to understand the mechanism of Q5 Taq polymerase in ST-nPCR and to evaluate the contribution of the 5′-3′ exonuclease deficiency in the observed substantial improvement in the detection sensitivity.

3.4. Solution 3: antisense oligonucleotides

Antisense oligonucleotides to control the annealing of the outer primers during the lower temperature round were then tested. The idea of using antisense oligonucleotides to improve ST-nPCR was adapted from [23]. Antisense oligonucleotides contain 5′ and 3′ tags that prevent the outer primers from generating non-specific amplifications during the low temperature round by blocking any leftovers of these primers [23]. The design and test of the inhibitory effect of 4 sets of antisense oligonucleotides (A1, A2, A3, and A4) that have gradually decreasing Tm (A1 pair: ~59 °C; A2 pair: ~56 °C; A3 pair: ~54 °C; A4 pair: ~50 °C) were carried out. The inhibitory effect of the four pairs of antisense oligonucleotides on the outer primers under different annealing temperatures was first tested. In this experiment, the amplification was based only on the outer primers and with the exception of the annealing temperature, all other parameters were fixed. As expected, the antisense oligonucleotides with higher Tm achieved more efficient inhibition of the outer primers than antisense oligonucleotides with lower Tm (Figure 4). This is an expected behavior as higher Tm of the antisense oligonucleotides will ensure more favorable and thermodynamically stable annealing of these oligonucleotides with the complementary sequence of the outer primers.

Based on the above experiments, antisense oligonucleotides A2 and A3 were further examined, as their inhibitory activities are more compatible with the range of the lower temperature round of inner primers. Before testing the antisense oligonucleotides in a ST-nPCR reaction, any possible effect of these oligonucleotides on the inner primers was examined and we used A3 as a model. Different concentrations of A3 were added to a PCR reaction that contained the inner primers alone. As can be seen in Figure 5, the antisense pair A3 has no effect on the amplification efficiency of the inner primers. When tested in ST-nPCR reaction, it was noticed that A3 oligonucleotides improve the detection sensitivity in comparison with regular ST-nPCR, however, they could not block the excess of free outer primers (Figure 6-C). On the other hand, when testing A2 oligonucleotides under the same conditions a slight improvement in the specificity was observed (Figure 6-
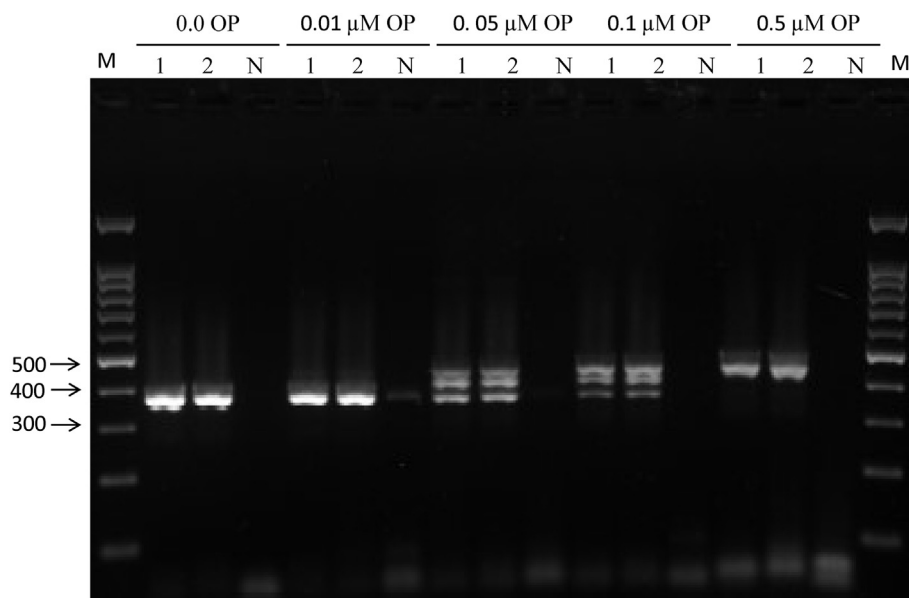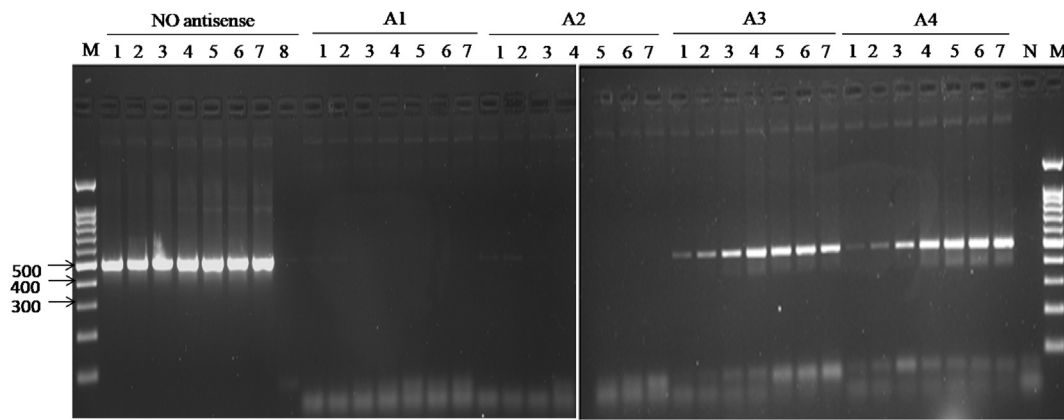

Figure 3. ST-nPCR using the Q5 Taq polymerase and different concentration of outer primers: 2% agarose gel electrophoresis for the POMP 90A/91A gene of *C. abortus* (369 bp) amplified using different combinations of final concentrations of outer primers (0.0 μM, 0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM) respectively and 0.2 μM of inner primers. The reactions were carried out in duplicate (marked as 1 and 2) and 1 pg of positive control was used. Lane N for no template control. Lane M for 100 bp DNA ladder (GeneDireX). Please see Supplementary PowerPoint file for the corresponding original image.

Figure 4. Analysis of the behavior of four tested antisense pairs combined with the outer primers by electrophoresis. 10 ng of DNA was amplified for 30 high-temperature cycles (outer primers cycles produced a 490-bp band) using gradient annealing temperature (73 °C, 70 °C, 67 °C, 63 °C, 60 °C, 58 °C and 57 °C) to test the behavior of antisense primers. The first 1–7 lanes were amplified using outer primers alone, Lane 8: No template control. 0.1 μM of each antisense pair and outer primers were used (A1, A2, A3, A4). Lane N: no template control for all antisense mixes. Lane M: 100 bp DNA ladder (GeneDireX). Please see Supplementary PowerPoint file for the corresponding original image.
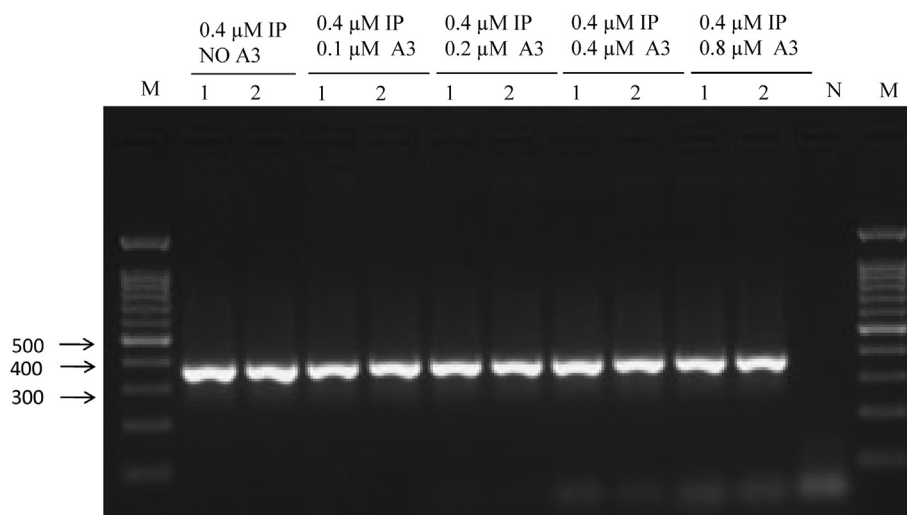

Figure 5. Analysis of the behavior of different concentrations of antisense 3 A3 combined with the inner primers by electrophoresis: 10 ng of DNA was amplified in duplicate for 30 low-temperature cycles (inner primers cycles). The amplification was done using different concentrations of the antisense 3 primer (0.0 μM, 0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM) and 0.4 μM of inner primers. Lane N: no template control. Lane M: 100 bp DNA ladder (GeneDireX). Please see Supplementary PowerPoint file for the corresponding original image.

B). The use of antisense oligonucleotides is an interesting method to control outer primers; however, this approach of improving the reaction can be cumbersome and requires a lot of optimization. In addition, designing such a PCR with two additional long primers to the 2 outer and 2 inner primers would increase the cost of the reaction that is originally meant to be an affordable solution for laboratories with limited resources.

3.5. Validation of test specificity

The ST-nPCR conditions with Q5 Taq polymerase and 0.01 μM of the outer primers and 0.4 μM of the inner primers, as in (Figure 2-C), was adopted to perform validation and field samples screening. The specificity of the adopted ST-nPCR was tested against a wide range of bacterial strains and showed no cross reaction (Figure 7).

To validate the assay, 70 veterinary clinical abortion samples from goat and sheep were tested. To ensure that the samples are appropriate for PCR amplification, they were examined using internal control markers for both sheep and goat (data not shown). Of the 70 samples, 69 were positive either for sheep or goat DNA. Of the tested 69 samples, 7 samples (10.1%) were found to be positive by our ST-nPCR (Figure 8). These results are in good agreement with results observed

by other studies. *C. abortus* prevalence, tested using recombinant enzyme linked immunosorbent assay (rELISA) and cell culture, in female goats of commercial milking farms sampled in Guanajuato, Mexico, was 4.87 % (n = 246) and the prevalence among animals in individual farms ranged between 3.44 and 13.51 % [29]. In Turkey, *C. abortus* was identified in 9.86 % of aborted fetuses from sheep and goat using cell culture and PCR [30]. In Algeria, 7.2 % of ewes were seropositive and 33.3 % of sheep flocks had at least one seropositive ewe when testing frozen fetal and placental tissues of aborting ewes using a *C. abortus*-specific indirect ELISA kit [31]. On the other hand, serological examination of the sheep using the ID ScreenTM *C. abortus* indirect multi-species antibody ELISA in Belgium, revealed a low seroprevalence rate of 0.68% [32]. The dissimilarity in percentages could be attributed to several factors including the climatic conditions and farming practices. In addition, it is important to recall that these studies followed different sampling methodologies and diagnostic strategies. Finally, the sample number tested in this work is relatively small and it was used for a preliminary screening purpose. Therefore, future work with the veterinary services-Ministry of Agriculture to screen a large number of samples from different geographical locations is planned to draw a clearer picture about the epidemiology of this pathogen in Palestine.
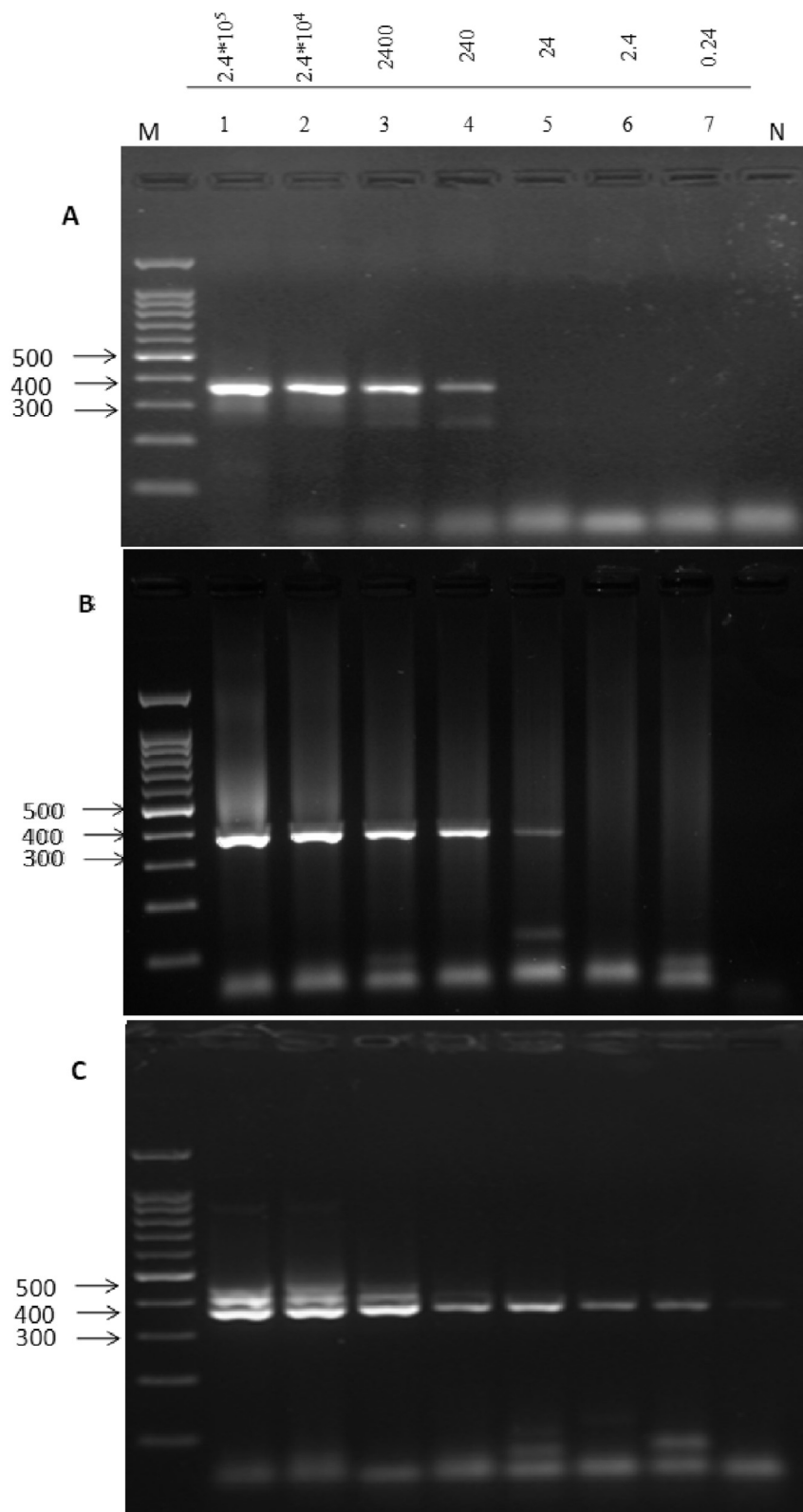
Figure 6. Comparison of the sensitivities of ST-nPCR and ST-nPCR performed by Q5 Taq polymerase using antisense A2 and A3 primers: 2% agarose gel electrophoresis results for the amplification of POMP 90A/91A gene of *C. abortus* (369 bp) from diluted positive control. Lanes 1–7 in each gel represent the positive control plasmid with different number of copies (2.4×10^5, 2.4×10^4, 2.4×10^3, 2.4×10^2, 2.4×10^1, 2.4, 0.2) respectively. Lane N: no template control. Lane M: 100 bp DNA ladder (GeneDireX). **A.** Results of ST-nPCR using 0.01 μM of the outer primers and 0.4 μM of the inner primers. Please see Supplementary PowerPoint file for the corresponding original image. **B.** Results of ST-nPCR performed by Q5 Taq polymerase using 0.2 μM of outer primers and 0.4 μM of inner primers and 0.4 μM of antisense primer A2. Please see Supplementary PowerPoint file fo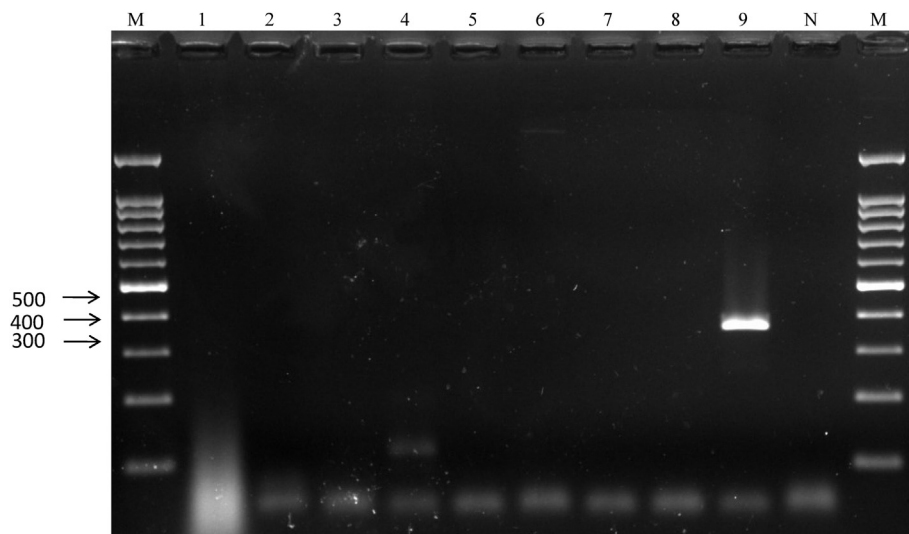r the corresponding original image. **C.** Results of ST-nPCR were performed by Q5 Taq polymerase using 0.2 μM of outer primers and 0.4 μM of inner primers and 0.4 μM of Antisense primer A3. Please see Supplementary PowerPoint file for the corresponding original image.

Figure 7. Agarose gel results for testing specificity of the ST-nPCR: Samples from different pathogens that are associated with abortion of small ruminants or that may share the same environment in the farm were analyzed. The amplification was performed by Q5 Taq polymerase for POMP 90A/91A gene of *C. abortus* using 0.4 μM of the outer primers and 0.4 μM of the inner primers. Lane 1: Avian pathogenic *Escherichia coli* (APEC); Lane *2: Bacillus thuringiensis;* Lane *3: Bacillus cereus;* Lane 4: *Campylobacter fetus* subsp. *fetus;* Lane *5: Campylobacter fetus* subsp. *venerealis;* Lane 6: *Brucella abortus* str. 544; Lane 7: *Brucella melitensis* bv. 1 str. 16M; Lane *8: Salmonella typhimurium*; Lane 9: Ovax Clamidia (a vaccine strain of *C. abortus)*; Lane N: no template control. Lane M: 100bp DNA ladder (GeneDireX). Please see Supplementary PowerPoint file for the corresponding original image.
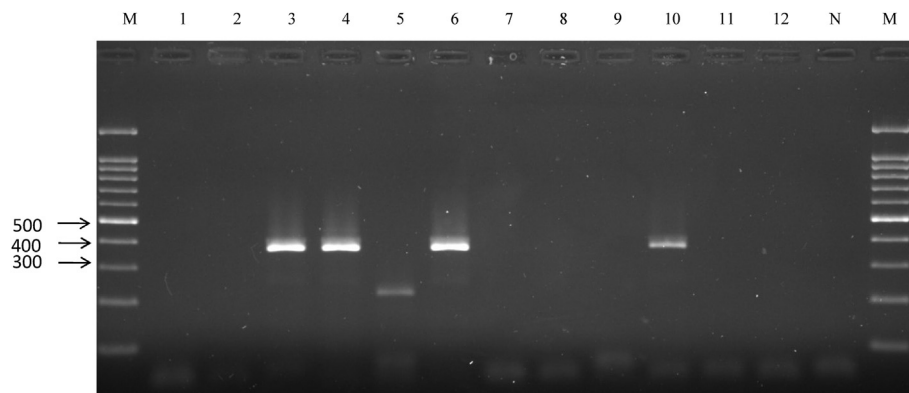

Figure 8. A representative gel of *C. abortus* testing using the developed assay: Clinical veterinary samples were analyzed by ST-nPCR. Lanes 1–12: random veterinary clinical samples. Lane N: no template control. Lane M: 100 bp DNA ladder (GeneDireX). Please see Supplementary PowerPoint file for the corresponding original image.

4. Conclusion

In theory, the idea of boosting the PCR reaction sensitivity by using two sets of primers, in two rounds, in the same vessel seems very promising. Unfortunately, the translation of this idea from the theoretical level to the experimental level is not an easy task and can face various obstacles, including optimization of various parameters such as concentrations of the different primers and the optimal melting temperature for the two rounds of amplification.

In this study, various suggestions to enhance the ST-nPCR sensitivity and specificity were exploited. A detection limit with a range between 0.1 and 1 ag (corresponds to a range between 0.2 and 2 copies of the used plasmid positive control) for the detection of *C. abortus* was achieved. Sensitivity was found to be enhanced by using Q5 Taq polymerase that lacks 5′-3′ exonuclease activity, while specificity could be enhanced by designing and applying the best outer primers complementary antisense oligonucleotides. These techniques, if appropriately adapted and translated, can help to improve the diagnosis of *C. abortus*, especially in laboratories that have limited resources.

Declarations

Author contribution statement

Diala Shatleh-Rantisi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Asmaa Tamimi: Performed the experiments.

Yaqoub Ashhab: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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