Original article

Design and construction of a positive control for molecular diagnosis of *Coxiella burnetii*

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Background: Bacterial infections are one of the most important issues in public health and could lead to death in many countries. Molecular methods for detection of these serious infections are fast and reliable, but there is a problem for the diagnosis of some cases of infectious agents, like high-virulence bacteria or rarely founds bacteria. Therefore, designing plasmid DNA construct as positive control is a smart way to solve the diagnosis problem in these cases.

Objectives: The aim of this work was to design and generate a construct to be used as positive control for amplification in the molecular detection assay of *Coxiella burnetii (C. burnetti)*.

Methods: A plasmid construct for *C.burnetii* and 2 sets of primers were designed. The designed construct contains a specific region of the *com1* gene for the diagnosis of *C.burnetii*. Finally, the designed construct was used as a template in a nested – polymerase chain reaction (PCR) reaction to validate its usefulness.

Results: PCR reaction was performed and 2 specific bands at 580 and 300 bp were observed in 2% agarose gel electrophoresis stained by KBC power load. Also, the sensitivity of the reaction was determined to be about 2.1×10^{-5} ng/μL of DNA.

Conclusion: The designed construct could be used as a positive control for a fast and reliable diagnosis of *C.burentii*. Further, this method can similarly be used for other microorganisms.

Keywords*:* Positive control constructs, *Coxiella burnetii*, molecular detection, nested-PCR.

Today, bacterial infections are a major problem in public health. Therefore, it is very important to specifically and accurately detect these infections. There are many methods available to detect the bacteria. Some of them are molecular-based which works on the genomic area and some of them are serological that works on antibodies. [1-3] Among these methods, the molecular ones are preferred because they are fast with a high accuracy. Molecular methods detect a specific area in the genomic region or signature plasmids, so false detection or vague results is minimized. However, there are many molecular diagnosis methods such as polymerase chain reaction (PCR)[4], DNA microarray [5], and loop-mediated isothermal amplification (LAMP).[6] However, the main problem with this approach is genome preparing.

For example, the preparation of genome is challenging for *Coxiella burnetii (C.burnetii),* a gram-negative bacterium that caused Q fever in humans and animals.[7] There is no standard strain for *C. burnetii* in Thailand, and isolation or culturing these bacteria is almost impossible because they require special conditions for culture in the microbial media [8] at level 3 biosafety conditions. [9] In the current study, we designed a construct for the molecular detection of *C.burnetii* that can be used as a positive control for PCR reaction. In this work, we chose conserved regions of *com1* which encoded 27 kDa outer membrane proteins as a target gene to be used for *C.burnetii* diagnostics by nested-PCR. This vector and the specially designed primers can be used as a PCR positive control for an accurate and safe diagnosis of *C.burnetii.*

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Materials and methods
Vector designing:
In this study, a specific vector was designed by snap gene software (Figure 1). First, the *com1* gene encoding 27 kDa outer membrane protein (GenBank:

AB004711.1) from the genomic area of *C.burnetii* was chosen as a target for detection. Then, sequences retrieved from NCBI/EMBL were organized and aligned using 6 mega blast software. After that, *com1* gene segment was amplified by PCR reaction, purified and ligated into a TA-vector (pTG19-T PCR cloning Vivantis) and transformed into cloning host cells (*Escherichia coli* DH5α). Thereafter, recombinant bacteria were cultured in LB media, and plasmid extraction was performed according to Gene-all plasmid extraction kit. The extracted plasmids were diluted 1:50. Then these plasmids were used as template for PCR amplification.

Primer designing and PCR amplification:

Two sets of specific primers were designed by oligo analyzer online software. These primers were used to amplify the *com1* gene in the nested-PCR assay. Primer sequences and amplicon sizes a shown in Table 1. To validate the selected gene segments and primers, the PCR was performed in a volume of 5 µl as follows: 1µl DNA template (21 ng/µl), 0.5 µM (0.25 µl) from each forward and reverse primers, 2.5 µl master mix of Taq DNA polymerase (amplicon

PCR kit, Denmark) and 1 µL of sterile water. PCR assay with two sets of primers was performed separately. Nested PCR for *com1* with F_1 and $R_{-1-}com1$ and F_2 and R_2-*com1* were performed. PCR program was set into thermocycler device as follows: initial denaturation at 94°C for 5 min and 30 cycles of denaturation (95°C, 45 sec), annealing (53°C, 45 sec), extension (72°C, 45 sec) and a final extension at 72°C for 5 min. After PCR amplification, PCR products were visualized on 2% agarose gel electrophoresis stained with KBC power load stain (Kosar Biotech Company, Iran) and the specific bands were observed under UV illumination.

Sensitivity and specificity:

To test sensitivity, a serial dilution of the plasmid was prepared (from 2.1 ng/µL until 2.1×10^{-6}ng/µL) and PCR was performed according to the previous protocol. Also, four gram-negative bacteria (*Vibrio Cholerae, Pseudomonas Aeruginosa, Salmonella typhi* and *Enterotoxigenic Escherichia coli (ETEC)*) were used in the PCR to test the specificity of the designed primers.

Figure 1. Schematic representation of construct designed by Snap Gene software.

Table 1. Target region primers and amplicon size in this work.

Primer name	Target gene	Sequence	Amplicon size
F_1-com	*com1*	5'GACATACAAAGCATCGTCCAC 3'	
R_1-com		5'GCAGTTCTTTGAAGACAACG 3'	300bp
F_2-com	*com1*	5'GGAATAGATCGTGAAGAACC 3'	
R_2-com		5'GAGCTGAGCAACATTTAATCC 3'	580bp

Results

Template preparing and monoplex PCR amplification:

The recombinant plasmid was extracted and purified (Figure 2A). To evaluate the plasmid vector and primers, PCR assay was performed. Results in 2% agarose gel electrophoresis showed single bands for each gene target at 300 bp and 598 bp for *com1* gene (inner and outer), respectively (Figure 2B).

Nested PCR assay:

After PCR assay, we performed a nested-PCR reaction using the plasmid construct as template and F_1 and R_1- *com1* via F_2 and R_2- *com1* primers. Results showed two bands at 300 and 598 bp in 2% agarose gel electrophoresis (Figure 2B).

Sensitivity and specificity:

The specificity of the reaction was examined with four gram-negative bacteria, *Vibrio Cholerae, Pseudomonas Aeruginosa, Salmonella typhi* and *Enterotoxigenic Escherichia coli (ETEC)* (Figure 3A). Also, the sensitivity of the reaction was evaluated using serial dilution of the plasmid from 2.1 ng to 2.1×10^{-5}ng. According to Figure 3B, the sensitivity of this assay was approximately 2.1×10^{-5}ng, which was equivalent to 3.2×10^{4} copies of the target gene.
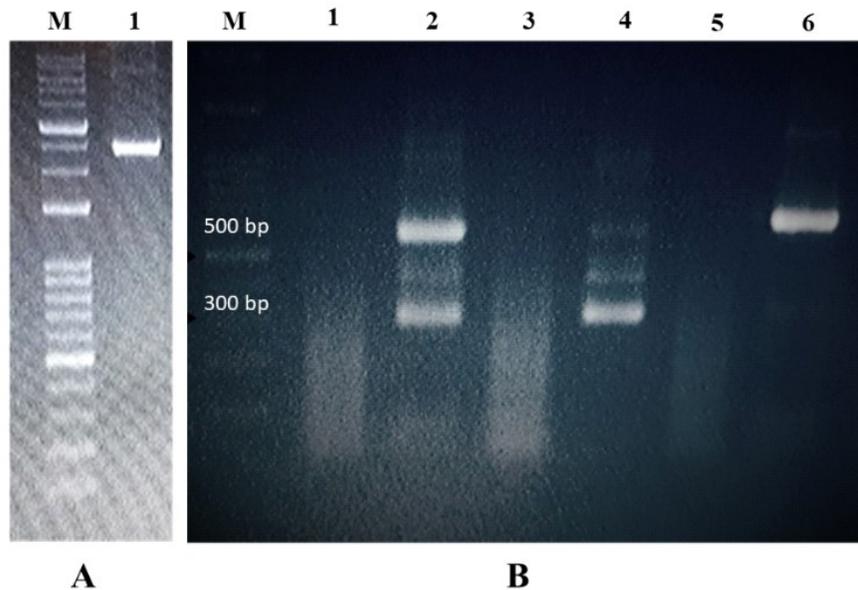
Figure 2. (A) Plasmid extraction in 2% agarose electrophoresis gel stained by KBC power load. Lane M. 100 to 10000 bp DNA ladder (Smobio, DM3200), lane 1. Extracted plasmid. (B) Analysis of monoplex and nested-PCR amplification in 2 % agarose electrophoresis gel. lane M. 100 to 10000 bp DNA ladder (Smobio, DM3200), lane 6. *com1* with F_1 & R_1 primers at 580 bp, lane 5. Negative control, lane 4. *com1* with F_2 & R_2 primers at 300 bp, lane 3. Negative control, lane 2. Nested-PCR a dual band on 598 and 300 bp for com1 gene, lane 1. Negative control.
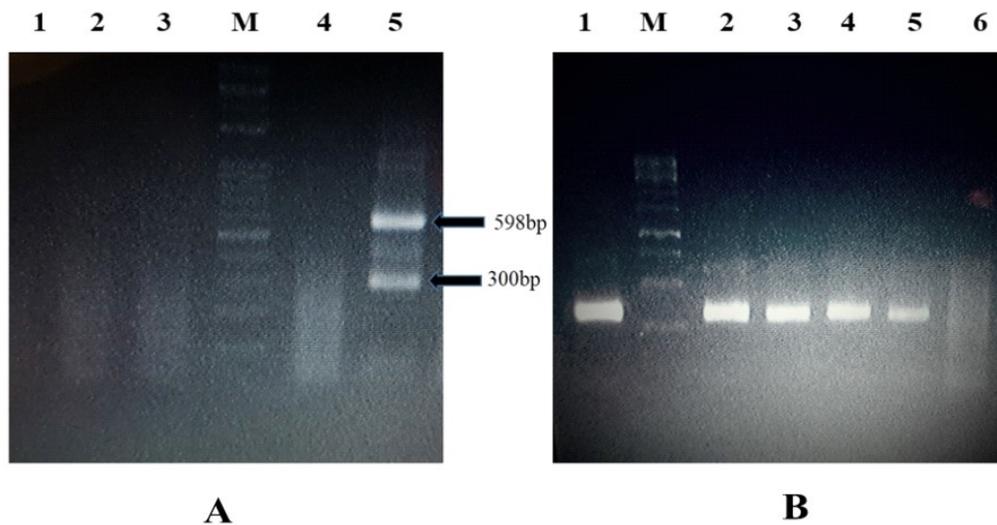
Figure 3. (A). Specificity test of primers in 2% agarose electrophoresis gel stained by KBC power load. Lane 1. PCR reaction by *Salmonella typhi,* lane 5. Nested-PCR reaction, lane 4. PCR reaction by *V.cholerae* genome, lane M, 100 to 10000 bp DNA ladder (Smobio, DM3200), lane 3. PCR reaction by *Enterotoxigenic Escherichia coli (ETEC),* lane 2. PCR reaction by *Pseudomonas Aeruginosa.* (B). Sensitivity test in 1.5% agarose electrophoresis gel stained by KBC power load. Lane 1. 2.1 ng, lane M. 1KB DNA ladder (Thermo fisher, SM0311), Lane 2. 2.1×10^{-1} ng, lane 3. 2.1×10^{-2} ng, lane 4. 2.1×10^{-3} ng, lane 5. 2.1×10^{-4} ng, lane 6. 2.1×10^{-5} ng.

Discussion

Synthetic constructs can be used for different applications. They can serve as subunit vaccines to immunize animals and humans against bacteria or serve as positive control for the detection of microorganisms that are pathogenic or rare in nature. Also, positive controls constructs were used for various aims. For example, in some clinical tests, they may be useful for biomedical assays because they are non-pathogenic and the laboratory staff can work in a safe environment. In some cases, like the Q fever agent, which are categorized as BSL3 bacteria, it is hard and dangerous to work with such bacteria. Q fever in humans may result in hepatitis, acute endocarditis, vascular infections, and lymphadenitis.[10] *C.burnetti* is high-risk, highly virulent bacteria and can survive a long period in nature. Therefore, a simple and safe diagnosis method for this agent is necessary.[4, 11] This bacterium can be found in milk and other dairy products from infected live stocks, so it can cause food safety and other major health problem. For this, reagents for a rapid quantification assay for this microbe are essential in animal husbandry centers. There are several methods for the rapid detection of this bacterium, some of them are serological-based and some of them are molecular-based assays. In the serological methods, antibodies such as IgM and IgG

were used for diagnosis, but these methods are time-consuming because antibodies are detected only after 2 - 3 weeks from the onset of the disease.[12] Therefore, molecular methods are preferred for fast and simpler detection of most infections. Molecular detection methods in genomic regions of several bacteria utilize plasmids with such regions. For example, to differentiate *C.burnetii* strains, the molecular diagnosis was based on plasmids because the strains have a special plasmid such as (QPH1, QpRS, QpDG, QpDV).[13] In the previous study, De Bruin and colleagues, have detected *C.burnetti* using multiplex real time-PCR and *IS1111, icd* and *com1* were chosen as the target in their work.[14] Also, our research team is currently exploring a new diagnosis method for *C.burnetii*. We developed a new multiplex-nested PCR for this bacterium and the target genes in the study were *com1* and *IS1111*.[15] The main problem in molecular methods is the accessibility to the genome or the standard strain of the organism. In some cases like *C.burnetii* in some country such as Islamic Republic of Iran, genome or standard strain of the bacteria is inaccessible, so the development of new approaches for molecular detection and molecular typing for this kind of organism is vital. In this field, one smart solution is to design and produce positive control construct for target

amplification. These are some plasmid vectors which consist of some specific genomic region of the related pathogen. These constructs are used to ensure that the reaction components like primers, enzymes and the devices are working correctly. Another advantage of these kinds of constructs is that they are safe and may assist the development of new diagnosis methods for dangerous pathogens requiring increased biosafety handling. Laboratories with biosafety levels three and four are expensive to operate and maintain, therefore using these constructs for hazardous pathogens such as viruses and bacteria is an economical approach in this field. In the current study, we suggested a new approach to solving this problem. For this, we designed a plasmid vector that contains special areas of the *C.burnetti* genome which include the *com1* gene. Then special primers for these areas were designed and finally, this construct can be used as positive PCR control for an experiment in suspected contaminated cases. In this field of detection, Caasi DR, *et al*. designed PCR positive controls for *Barley yellow dwarf virus*, *Soilborne wheat mosaic virus*, *Triticum mosaic virus,* and *Wheat streak mosaic virus.* [16] Also, Pourmehdi N, *et al*. studied the detection of *Yersinia pestis* and *Francisella tularensis* by multiplex PCR using synthetic construct.[17] In another study, Menard JP, *et al*. designed a synthetic plasmid construct for the quantification *Lactobacillus* species, *G. vaginalis, etc.,* by qPCR method. [18] In a previous study, Sohni Y, et al. produced a control for the amplification of Bacillus anthracis by cloning synthetic segments into a plasmid vector. [19]

Conclusion

Due to the lack of standard microbial strain for some microbes, cloning conserved regions of their genomic segments into a plasmid vector or designing and producing synthetic constructs with sequence information from the public database could be useful for the diagnosis of rare microorganisms. These constructs could be designed to identify multiple pathogens at the same time, which should assist in the timely and accurate identification of bacterial infection.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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