Research in Molecular Medicine (RMM)
Volume 6, Issue no. 4, DOI 10.18502/rmm.v6i4.4801
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Research Article

Simple and Rapid Detection of *Yersinia pestis* and *Francisella tularensis* Using Multiplex-PCR: Molecular Detection of *Yersinia pestis* and *Francisella tularensis*

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Abstract

Background: *Yersinia pestis* and *Francisella tularensis* cause plague and tularemia, which are known as diseases of the newborn and elderly, respectively. Immunological and culture-based detection methods of these bacteria are time-consuming, costly, complicated and require advanced equipment. We aimed to design and synthesize a gene structure as positive control for molecular detection of these bacteria.

Materials and Method: Conserved regions of each bacterium were determined. A fragment containing the *fopA* and *caf1* genes (conserved genes of *F. tularensis* and *Y. pestis*, respectively) was artificially synthesized, cloned into the pUC57 vector (pUC-fopA-caf1), transformed into *E. coli* DH5α, and used in a multiplex PCR assay. The sensitivity of this assay was examined by serial dilution of the extracted plasmid, whereas the specificity was examined using genomes of *Escherichia coli, Salmonella typhi, Enterobacter aerogenes*, *Vibrio cholerae* as templates. Finally, PCR products were analyzed in agarose gel electrophoresis.

Results: As expected, our analysis showed a clear dual band in the size range of 107 bp to 176 bp, confirming the presence of *fopA* and *caf1* genes. Another 351 bp band was detected due to amplification being dependent on the forward primer of *fopA* and the reverse primer of *caf1*. Optimization of the PCR protocol reduced the amplification of this 351 bp band. The sensitivity of this assay was determined to be 36×10^{-3}ng/µl and the selectivity test confirmed the specificity of this method is appropriate for the detection of target genes.

Conclusion: This multiplex PCR method could be used in research laboratories for identification of these important pathogens.

Keywords: *Francisella*, *Yersinia*, Multiplex PCR, Detection, Positive Control Sample

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Editor-in-Chief:
Dr. Alireza Rafiei

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1. Introduction

Several bacterial agents cause serious infections, lead to many deaths around the world and thereby pose a serious threat to public health (1). Counteracting these infections

imposes high costs on governments. Rapid and timely detection of these diseases assists in controlling the spread of infections and reducing heavy expenses (2). *Francisella tularensis* and *Yersinia pestis* are two biological agents that are responsible for high mortality rates. *F. tularensis* and *Y. pestis* cause tularemia and plague, respectively, and are both aerobic gram-negative coccobacillus species (3, 4). They can easily get transmitted between people and disperse across a population. Moreover, *F. tularensis* can cause infection when present even at low doses in aerosol (5, 6).

Several methods are currently used for detection and identification of *F. tularensis* and *Y. pestis*. The best growth media for *F. tularensis* are cysteine-enriched broths and blood- or chocolate-supplemented agars. On the other hand, *Y. pestis* is able to grow on routinely-used culture media, such as sheep blood agar or selective media like MacConkey and Eosin Methylene-blue agars (1). However, growth rate on these media is slow, and colony formation requires over 24 to 48 h on enriched media. Immunoassays such as micro-agglutination or ELISA could be used alternatives (7-9). Yet, these diagnostic tests are time consuming, have lower sensitivity and specificity, and cannot test for the presence of multiple agents simultaneously. Molecular detection methods can help us quickly assess samples with possible microbial infection. PCR is an easy method that can be used as a rapid detection test. PCR can also be targeted toward different regions like the *fopA* gene of *F. tularensis* encoding one of the outer membrane proteins or the F1 antigen (*caf1* structural gene) (10-12). In addition, multiplex PCR assays can detect many pathogens in only a few hours with high sensitivity and specificity (13). Complicated immunological and culture-based detection tests require microbiology laboratories with specific equipment and facilities. Moreover, due to the lack of the presence of standard microbial strains for some bacteria like *F. tularensis* and *Y. pestis*, a standard genome is not readily available for use in molecular methods. Thus, a new strategy to overcome these limitations is necessary. To this end, designed plasmids containing the targeted regions of these bacteria can provide a solution (14).

Aiming to develop a molecular detection kit, we designed here a new plasmid construct containing the conserved *fopA* and *caf1* (F1 capsule antigen) genes from *F. tularensis* and *Y. pestis,* respectively, to be used as positive control. For this purpose, a multiplex PCR method was established, and the specificity and sensitivity of the assay were determined. When used as positive control, the method we developed here can allow quick and simultaneous molecular identification of these bacteria in clinical laboratories.

2. Material & Methods

2.1. Construct and primer design

Two genes, *fopA* and *caf1*, are located in conserved regions in genomes of *F. tularensis* and *Y. pestis*, respectively. These genes were therefore selected as targets for plasmid construct design. Sequences of these genes from different bacteria species were collected from NCBI databases, and aligned. Conserved regions of each gene were identified by performing bioinformatics analyses. These regions were chosen for inclusion in our construct, and suitable primers were designed for these regions (Table 1) by using Gene Runner, OligoAnalyzer and primer blast tool of NCBI. Then, a construct, including the conserved region of each target, was designed along with restriction sites for validation tests. The construct was artificially synthesized and cloned into pUC57 (named pUC-*fopA-caf1*) as shown in Figure 1. For this purpose, the sequence was cloned into *HindIII* and *BamHI* site of pUC57 using common cloning methods, and finally transformed into competent *E. coli DH5*α for use in subsequent steps.

TABLE 1: Target region primers and amplicon sizes used in this study.

Target organism	Target region	Primer sequence		Length	Tm	GC%	Amplicon size
Francisella	fopA	F	5′-GCGCTTTGACTAACAAGGAC	20	40.3	46.7	107 bp
		R	5′-CTACACCTAAGTACCACTGGC	21	37	49.2	
Yersinia	caf1	F	5′-CCGTTATCGCCATTGCATTATTT	23	49.3	46.6	179 bp
		R	5′-CAAGAGTAAGCGTACCAACAAG	22	40.5	47.9	

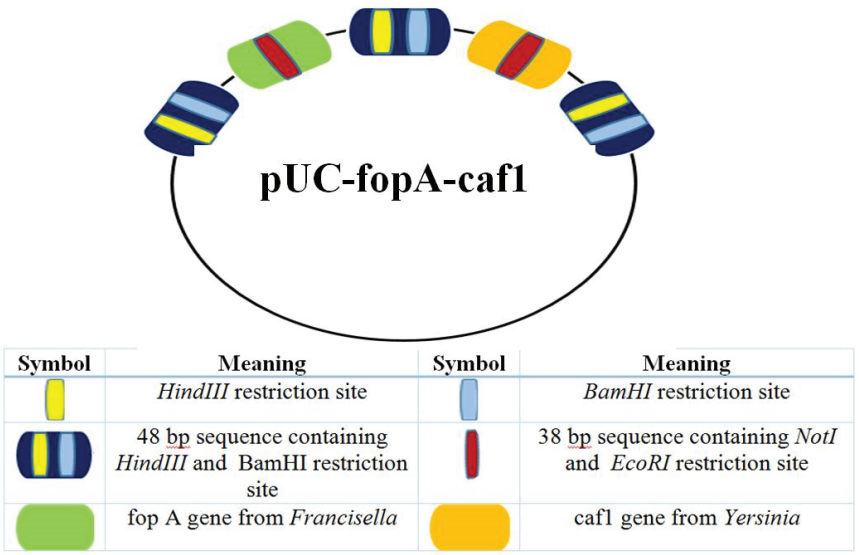

Figure 1: Schematic representation of the designed construct pUC- fopA- caf1.

2.2. Monoplex PCR reaction

First, *E. coli DH5*α cells containing the new plasmid (pUC- *fopA*- *caf1*) were incubated overnight at 37 ˚C in LB broth. Plasmid was extracted using the Gene All Kit (Pishgam Company, Iran) and confirmed using 1% agarose gel electrophoresis. Next, monoplex PCR reaction was used to confirm the correct function and specificity of each primer. At this stage, the final volume for each reaction was 5 µL, which included 2.5 µL master mix of Taq DNA polymerase (Ampliqon PCR Kit, Denmark), 2.5 µM (0.5 µL) of each primer, 0.5 ng/µL (1 µL) of plasmid as template and 0.5 µL of distilled water. The following PCR protocol was used: initial denaturation at 94 ˚C for 5 min followed by 20 cycles, each including denaturation (95 ˚C, 10 sec), annealing (62 ˚C, 10 sec), extension (72 ˚C, 10 sec) and final extension (72 ˚C, 5 min).

2.3. Multiplex PCR reaction and optimization

After the initial confirmation of the primers, the multiplex PCR was performed in a similar way to that of monoplex PCR, except that the primers for *fopA* and *caf1* were added to the PCR mixture. Eventually, PCR products were examined using 2% agarose gel electrophoresis and fluorescence staining on KBC power load (Kawsar Biotech Company, Iran). In order to reduce the amplification of the 351 bp fragment, we optimized the multiplex PCR protocol by using the following two PCR protocols:

1. Initial denaturation at 94 ˚C for 5 min, followed by 10 cycles, each including 94 ˚C for 5 sec, 62 ˚C for 5 sec, 72 ˚C for 5 sec, and a final extension at 72 ˚C for 5 min.
2. Initial denaturation at 94 ˚C for 5 min, followed by 10 cycles, each including 94 ˚C for 3 sec, 62 ˚C for 3 sec, 72 ˚C for 3 sec, and a final extension at 72 ˚C for 5 min. PCR products were analyzed using 1% agarose gel electrophoresis.

2.4. Sensitivity and specificity

In order to measure the sensitivity of primers, serial dilutions of the plasmid from 10^{-1} to 10^{-10} were prepared and used as templates in the multiplex PCR reaction. On the other hand, to confirm the specificity of primers, 10^{-2} diluted genomic DNA of four bacteria species, including the enterotoxigenic *Escherichia coli (*ETEC*), Salmonella typhi, Enterobacter aerogenes*, *Vibrio cholerae* was prepared and used as template in the multiplex PCR reaction. Finally, the PCR products were analyzed using 2% agarose gel electrophoresis.

3. Results

Recombinant DNA plasmid (pUC-*fopA*-*caf1*) was extracted from *E. coli DH5*α. The plasmid was confirmed to be extracted correctly (as shown in Figure 2-A) and used as template in mono and multiplex PCR reactions. The monoplex PCR reaction confirmed the presence of individual fragments of specific primers designed for *fopA* (107 bp) and *caf1* (176 bp) (Figure 2-B). Similarly, in the multiplex PCR reaction, the following three fragments were observed after amplification: a 107 bp (*fopA*) fragment, a 176 bp (*caf 1*) fragment and another 351 bp fragment which is the result of amplification by forward and reverse primers of *fopA* and *caf 1*, respectively (Figure 3-A). In order to remove the 351 bp amplicon, we optimized the PCR protocol by reducing the cycle incubation time from 10 sec to 5 or 3 sec. As a result, amplification of the two amplicons, *fopA* and *caf1*, increased (Figure 3-B and C).

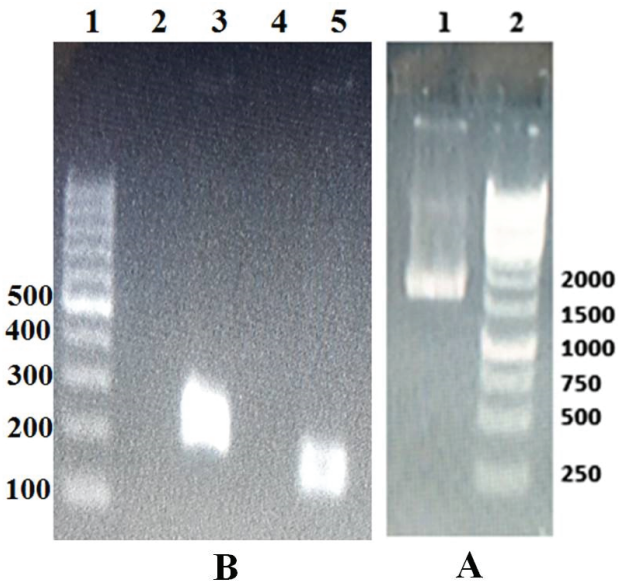

Figure 2: (A) plasmid extraction: (1) extracted plasmid, (2) 1 kb DNA ladder. (B) Analysis of the monoplex PCR product using 2% agarose gel electrophoresis. (1) 100 bp DNA ladder, (2) negative control, (3) *Y. pestis* (176 bp), (4) negative control, (5) *F. tularensis* (107bp).

In order to examine the specificity of the method, multiplex PCR reaction was performed for four bacterial genomes including the genomes of enterotoxigenic *E. coli (*ETEC*), S. typhi, E. aerogenes*, and *V. cholerae*. The results showed that primers are indeed specific for *fopA* and *caf1* (Figure 4-A). The concentration of the target plasmid was measured to be 36 ng/µl. The plasmid was diluted from 36×10^{-1} ng/µl to 36×10^{-10} ng/µl by serial dilution, and then used as template in multiplex PCR. The analysis of the multiplex PCR products showed that the amplification was performed up to 36×10^{-3}ng/µl concentration (Figure 4-B).

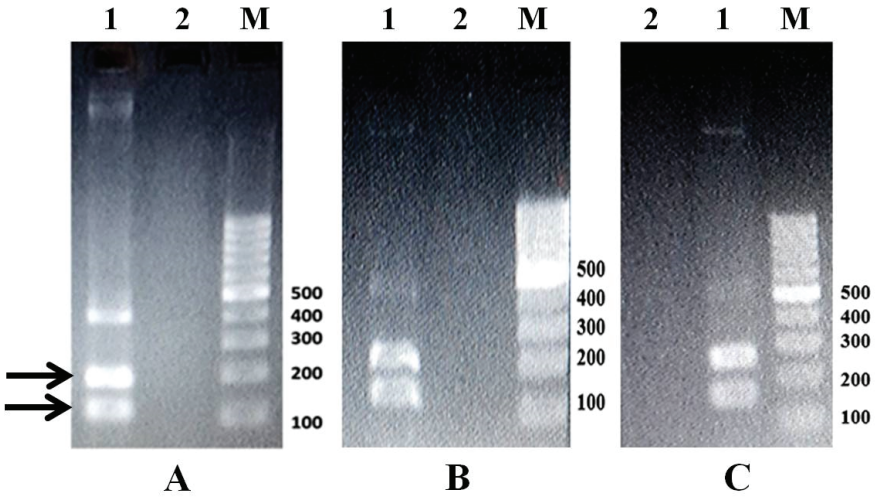

Figure 3: The optimization of multiplex PCR product at 10 (A), 5 (B) and 3 (C) seconds of incubation cycles. (1) Multiplex PCR for *F. tularensis* (107 bp) and *Y. pestis* (176 bp); 351 bp fragment for amplification of both genes. (2) Negative control. (M) 100 bp DNA ladder.

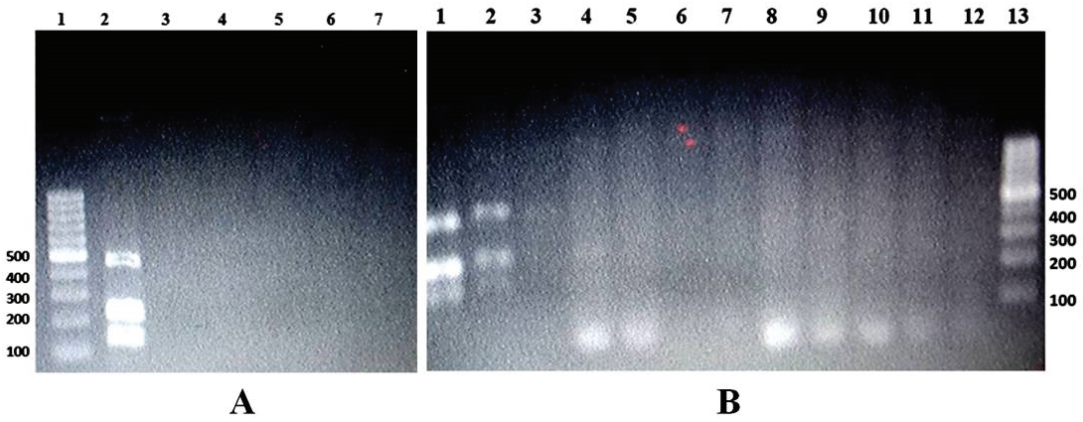

Figure 4: (A) Specificity of primers was examined by multiplex PCR using four different genomes as template. (1) 100 bp DNA ladder. (2) multiplex PCR for *F. tularensis* (107 bp) and *Y. pestis* (176 bp), 351 bp fragment for consecutive amplification of both genes. (3) Enterotoxigenic *Escherichia coli* (ETEC) (4) *Salmonella typhi*, (5) *Enterobacter aerogenes*, (6) *Vibrio cholera*, (7) negative control. (B) Sensitivity of primers was examined by multiplex PCR of plasmid serial dilutions: (1) 36 ng/µl, (2) 36 $\times 10^{-1}$ ng/µl, (3) 36 $\times 10^{-2}$ ng/µl, (4) 36 $\times 10^{-3}$ ng/µl, (5) 36 $\times 10^{-4}$ ng/µl ,(6) 36 $\times 10^{-5}$ ng/µl, (7) 36 $\times 10^{-6}$ ng/µl, (8) 36 $\times 10^{-7}$ ng/µl, (9) 36 $\times 10^{-8}$ ng/µl, (10) 36 $\times 10^{-9}$ ng/µl, (11) 36 $\times 10^{-10}$ ng/µl, (12) negative control, (13) 100 bp DNA ladder.

4. Discussion

There are several deadly and pathogenic microbial agents (15). Timely, rapid and correct diagnosis of these pathogens are essential in delivering proper health services and reducing the mortality rates (16). The National Institutes of Health (NIH) and Centers for Disease Control and Prevention (CDC) have classified *F. tularensis* and *Y. pestis* into category A as dangerous pathogens with potential to cause high mortality and morbidity rates in domestic animals and human populations. Advanced laboratories are

required for the detection and identification of these pathogens (3, 17, 18). Different methods are available for detecting microbial agents. Some of these methods are culture-dependent with infection risks, whereas some of them are time-consuming immunoassays (19, 20), requiring advanced equipment. Correct identification of these pathogens depends on culturing and genome extraction, for which level 3 laboratories and specialized researchers are necessary. On the other hand, molecular detection of these bacteria provides a rapid and simple alternative. A smart strategy for identification of these bacteria without the aforementioned limitations is the design of plasmids containing the conserved genes as positive controls. In 2004, Raoult used this strategy for identification of four pathogens including the smallpox virus, *B. anthracis*, *F. tularensis* and *Y. pestis* (14). The sensitivity of this method was determined to be about 1 to 100 DNA copies. In 2009, Henrickson and co-authors used multiplex PCR and RT-PCR enzyme hybridization assays to design recombinant plasmids as positive controls and the limit of detection (LOD) of their DNA assay was about 10^2 to 10^3 copies/ml (2). In 2018, Rohani reported the first tularemia case in humans since 1980 from the Kurdistan Province in Iran. Real-time PCR was used for molecular detection of the *ISFtu2* and *fopA* gene with amplicon sizes of 97 and 87 bp. They used the DNA of *F. tularensis* subsp. *holarctica* NCTC 10857 as a positive control (21). In 2017, Mostafavi studied rodents from the Kurdistan Province to assess the status of *Y. pestis* and *F. tularensis* outbreak. For this purpose, the chromosomal *yihN* and the pMT1-borne *caf1* genes from *Y. pestis* and the *tul4* and *fopA* genes from *F. tularensis* were selected as positive controls, cloned into pUC57, and used in real-time PCR (22). In another study, the *ISFtu2* and *pla* plasmid genes were used for the molecular identification of *F. tularensis* and *Y. pestis*, respectively (23).

In this work, we developed a rapid and specific multiplex PCR assay by designing a novel recombinant plasmid for identification of *F. tularensis* and *Y. pestis,* based on the detection of *fopA* and *caf1* genes , respectively. The sensitivity of the assay was calculated as 36×10^{-3}ng/µl. Due to the lack of standard microbial strains for some bacteria species, alternative molecular detection methods can be used such as cloning the conserved regions of genome into a plasmid and using them as positive control. These constructs can be used for detecting multiple pathogenic agents. Thus, the detection becomes faster than simple molecular tests. As different bacteria have various strains, species and sub-species, it is important to find and utilize highly conserved regions in their genome when developing these constructs. Another important aspect is that designed primers must be specific for a certain organism. As demonstrated in this study, methods allowing the molecular detection of multiple bacteria simultaneously may be used as rapid and cheaper alternatives to traditional detection methods.

5. Conclusion

In this study, we synthesized a new construct containing conserved genes of *F. tularensis* (*fopA* gene) and *Y. pestis* (*caf1* gene). This construct can be used as positive control in order to detect the presence of these important pathogens in clinical specimens. In clinical studies, we encounter with a sample which is suspicious, so at first time it requires a treatment and then it will use as target in molecular detection procedure. We estimate that our plasmid construct design strategy here can be used to detect the presence or absence of mentioned agents in samples with possible microbial infection with high rates of success (more than 90%). The method we have developed in this study can be readily used in research laboratories, and further studies are required to investigate its utility in clinical laboratories.

Acknowledgement

The authors would like to thank the research council of Malek Ashtar Universities of Technology (MUT) for the financial support of this investigation.

Conflict of Interests

The authors declare no conflict of interest**.**

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