UDC 79.841.95:577.2.08:579.252.5:619:616.98-076
https://doi.org/10.15407/biotech11.04.068

DEVELOPMENT OF RECOMBINANT POSITIVE CONTROL FOR *Francisella tularensis* DETECTION BY qPCR

O. B. Zlenko
A. P. Gerilovych

National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" of the National Academy of Agrarian Sciences of Ukraine, Kharkiv

E-mail: oksana.ceratium@gmail.com

Received 20.05.2018

The aim of the work was to construct and test the recombinant positive control for *F. tularensis* detection by real-time polymerase chain reaction (qPCR). The molecular TA-cloning of pTZ57_F/R plasmid ligated with *tul4* gene PCR product into DH5α *E. coli* was provided. The minimal detection level in a qPCR was one copy number per reaction. The obtained positive control was highly sensitive, specific and safe to be used in the tularemia laboratory diagnostics.

Key words: recombinant positive control, qPCR, tularemia, molecular cloning.

Francisella tularensis (McCoy and Chapin, 1912; Dorofeev, 1947) is a gram-negative non-sporulating zoonotic, intracellular, obligate aerobe pathogen and the causative agent of the illness tularemia. *F. tularensis* is a natural foci disease that occurs in lagomorphs (rabbits and hares), and in rodents, especially microtine rodents (such as voles, vole rats and muskrats). A wide range of other mammals and several species of birds also can be infected. Among domestic animals, hunting cats and dogs are able to act as a carrier of the bacterium. It can be spread also by insects, were the most important vectors are ticks: 13 species of them that belong to 4 genera *Amblyomma*, *Dermacentor*, *Haemaphysalis,* and *Ixodes* can carry the bacteria [1, 2]. It is transmitted to humans by several ways, including direct contact with infected material or inhalation of infected aerosols, ingestion of contaminated food or water, arthropod bites. Human-to-human transmission is not known to be reported [3].

The inhaled minimum infectious dose is 10–50 colony forming units. *F. tularensis* is considered to be a dangerous potential biological weapon because of its extreme infectivity and ease of dissemination. "Unusual" tularemia outbreaks in war-torn or crisis-afflicted regions which for years had appeared to be free of the disease give rise to speculation that these epidemics may have been artificially triggered [3–5]. Thus, monitoring of *F. tularensis* outbreaks is highly relevant today, especially in high conflict areas as Ukraine. Also, development of native high-quality test systems for tularemia is a question of first priority. The diagnosis of tularemia often relies upon the demonstration of an antibody response to *F. tularensis* or the direct culturing of the pathogen. Established tularemia ELISAs and confirmatory Western blot assays are mostly based on lipopolysaccharide (LPS) -antigen reactions, which are time and cost consuming and give false-positive results with other bacteria, for example *Brucella* genera. As *F. tularensis* is a fastidious, slow-growing organism, culture is often not the preferred diagnostic method for it when a rapid result is required. In addition, handling live *Francisella* poses a risk to laboratory personnel due to the highly infectious nature of this pathogen. PCR is the main method for direct and rapid detection of *F. tularensis* and requires positive controls for valid results. Further, recombinant positive controls are often used to provide additional biosafety and biosecurity measures [1, 6].

Materials and Methods

The plasmid was designed using the Clone Manager 9 (Scientific and Educational Software, USA). For the real-time PCR assay we used the FT-FP tul4 primer system: For: CAGCATACAATAATAACCCACAAGG; Rev: TCAGCATACTTAGTAATTGGGAAGC; Probe: TTACAATGGCAGGCTCCAGAAGGTT [7] with an amplicon product of 103 bp. These primers were checked using BLAST online service for specific annealing. The *F. tularensis* subsp. *holarctica* vaccine strain 15 NIIEG was used as a positive DNA template. The final volume of the reaction mixture was 25 µl and was comprised of AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA) 1,25 U/50 µl, PE-Buffer 1X, dNTP mix 0,2 mM, $MgCl_2$ 1,5 mM, primer F 10 pM, primer R 10 pM, probe 5 pM, with 5 µl of purified template DNA to give the desired genome copy number per reaction volume and 5 µl pf PCR-grade water to the no-template-control reactions. Thermal cycling was performed using the following program: one cycle at 95 °C for 5 min for Hot-Start Tag activation, followed by 40 cycles at 95 °C for 15 s for denaturation, 60 °C for 30 s as annealing, 72 °C for 20 s for extension, and final elongation at 72 °C for 10 min. A fluorescence reading was taken at every 72 °C step.

To ensure inserts of appropriate size we used the M13 F/R primer system. Our reaction mixture contained 12.5 µl of Maxima Hot Start PCR Master Mix (2X) (Thermo Fisher Scietific, USA), 1 µl of 10 pM M13 forward primer, 1 µl of 10 pM M13 reverse primer, 5 µl of template DNA and PCR-grade water up to a total volume of 25 µl. Thermal cycling was performed using the following program: one cycle at 95 °C for 5 min for Hot-Start Tag activation, followed by 30 cycles at 96 °C for 30 s for denaturation, 50 °C for 20 s as annealing, 72 °C for 60 s for extension. Results reading was done in 2% agarose gel.

PCR products were purified using two different kits: The Monarch PCR & DNA Cleanup Kit (NEB, England) and Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scietific, USA), according to the manufacturers' instruction. The concentration of DNA was determined usind a NanoDrop spectrometer DeNovix DS-11 (Wilmington, USA). The PCR product was subcloned with a vector to insert ratio of 1:5 using the InsTAclone PCR TA cloning Kit (Thermo Scientific, USA). Ligation was carried out overnight at 16 °C. The product was transformed into *E. coli* DH5α chemically competent cells, which were allowed to recover 1 h at 37 °C in Luria broth. Competent cells were produced using the adapted and modified $CaCl_2$ method of Mandel and Higa [8]. Pelleted bacterial strains from 25 ml cultures were resuspended with gentle pipetting in 10 ml of ice-cold 0.1 M $CaCl_2$ (formulated in de-ionized water and autoclaved) and incubated on ice for 20 min. The bacteria suspension was pelleted at 3800 g at 4 °C for 10 min followed by gentle resuspension in 5 ml of 0.1 M $CaCl_2$ + 15% (v/v) glycerol and stored in 100 µl aliquots at −80 °C. Transformed cells were screened for gene insertion using the blue-white method and confirmed by PCR mentioned above. Plasmids were isolated using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA).

Results and Discussion

It was constructed a plasmid with a 103 bp fragment of the *tul4* gene ligated into the pTZ57R/T vector. The complete sequence of the new plasmid is 2990 bp in length (Fig.1). The vector pTZ57R/T encodes an ampicillin resistance and the *lacZ* gene, which were used as selective markers for *E. coli* DH5α clones. We screened 10 white single *E. coli* colonies by PCR using *tul4* specific primers FT-FP (Fig. 2) and the M13 F/R primer system which generate a PCR product of 103 bp (Fig. 3).

The colonies #1 and #2 showed positive results with PCR products of 257 bp. It was used ligated pTZ57F/R plasmid without insert as positive template.

Extracted plasmid minipreps from clones #1 and #2 were sequenced (Eurofins,

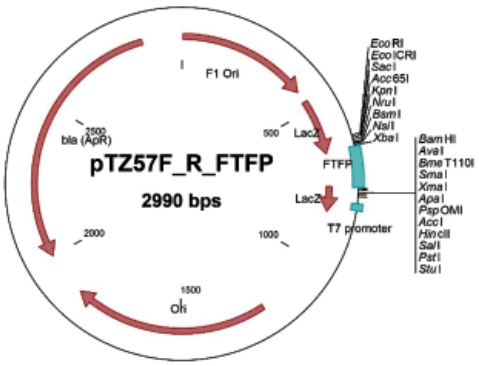

Fig. 1. **Plasmid vector pTZ57F_R_FTFP**

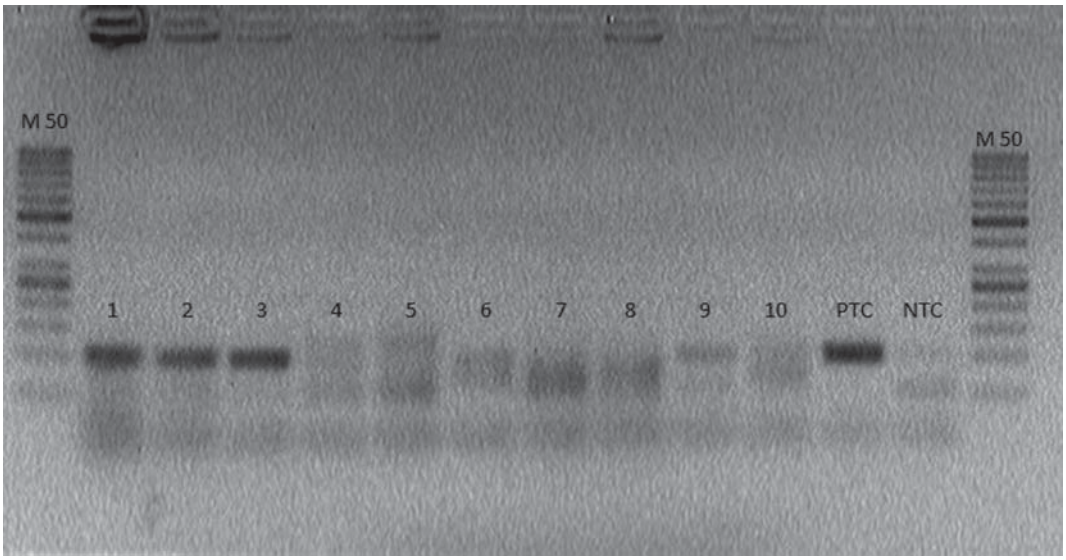

Fig. 2. **Screening the *tul4* positive clones using FT-FP primers**

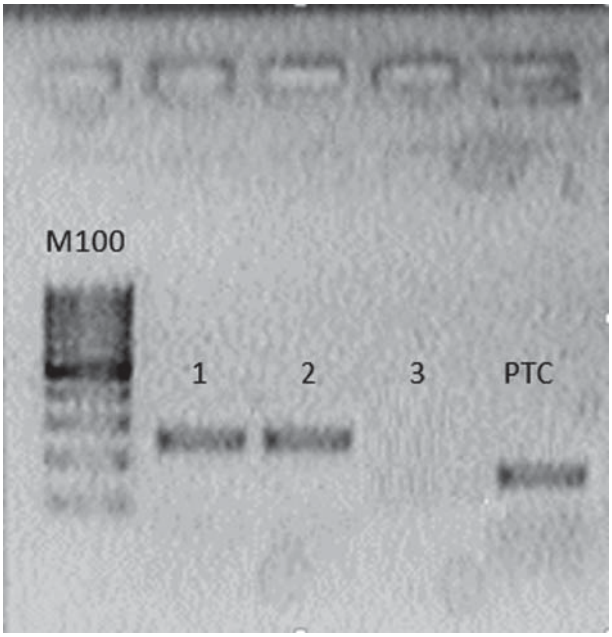

Fig. 3. **Screening the *tul4* positive clones using M-13 mers**

Germany) with M-13 primer system. Sequence results has shown that colony #1 sequence is in accordance with *tul4* gene sequences.

Thus, the colony #1 miniprep was chosen for further studies of sensitivity detection. The lowest threshold of detection was 1 copy number per reaction.

It has been developed the highly-sensitive recombinant positive control for detection of *F. tularensis* in conventional and a real-time polymerase chain reaction. The minimum of detection is 1 copy number per reaction.

This work was funded by the Ukrainian-German biosecurity initiative on zoonosis risk management at the EU external borders. We thank Dr. Roman Wolfel, Dr. Heiner von Buttlar, Dr. Julia Schwarz, Angela Duerr from Institute of Microbiology, Munich, Germany and GIZ for project support.

Also, we thank the Mechnikov Anti-Plague Research Institute of the Ministry of Health of Ukraine for provision of *F. tularensis* subsp. *holarctica* vaccine strain 15 NIIEG.

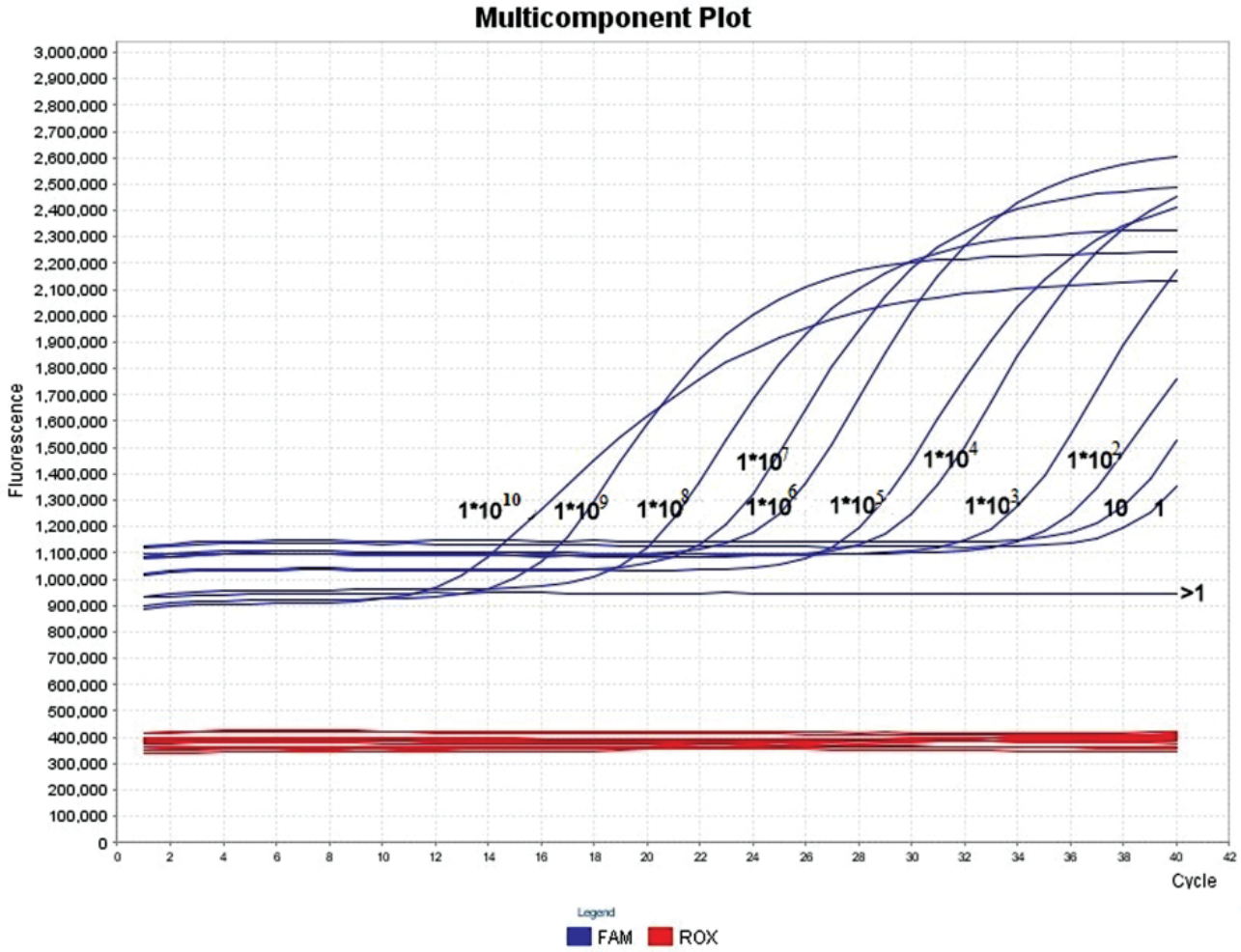

Fig. 4. **The results of pTZ57F_R_FTFP minimal copy number detection through PCR**

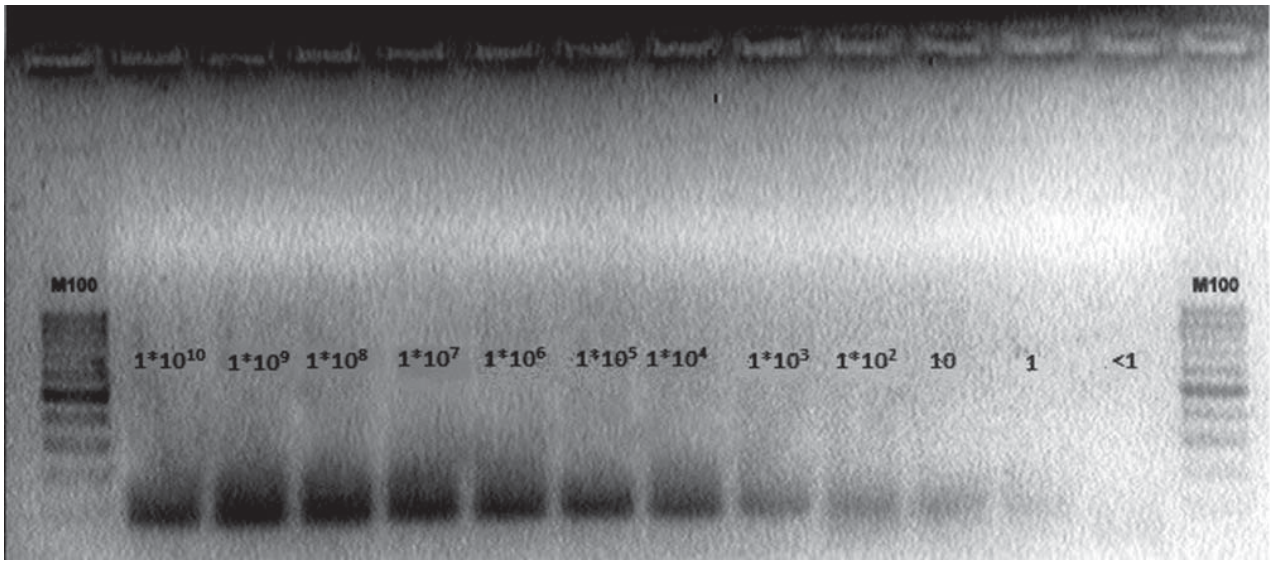

Fig. 5. **The results of pTZ57F_R_FTFP minimal copy number detection (electrophoresis in agarose gel)**

REFERENCES

1. *Hestvik G., Warns-Petit E., Smith L.A., Fox N.J., Uhlhorn H., Artois M., Hannant D., Hutchings M. R., Mattsson R., Yon L., Gavier-Widen D.* The status of tularemia in Europe in a one-health context: a review. *Epidemiol. Infect*. 2015, V. 143, P. 2137–2160. https://doi.org/10.1017/S0950268814002398
2. *Pearson A.* In Zoonoses — Biology, Clinical, Practice and Public health control. Chapter 24. Tularemia. *Oxford University Press*. 1998, P. 303–312. https://doi.org/10.1093/med/9780198570028.003.0031
3. *Oyston P., Sjostedt A., Titball R.* Tularemia: bioterrorism defense renews interest in Francisella tularensis. *Nat. Rev. Microbiol*. 2004, 2 (12), 967–978. https://doi.org/10.1038/nrmicro1045
4. *World Organization for Animal Health*. Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.1.22. Tularemia. *OIE*. 2008, P. 361–366.
5. *Buzard G., Baker D., Wolcott M.J., Norwood D.A., Dauphin L. A.* Multi-platform comparison of ten commercial master mixes for probe-based real-time polymerase chain reaction detection of bioterrorism threat agents for surge preparedness. *Forensic Sci. Int*. 2012, 223 (1–3), 292–297. https://doi.org/10.1016/j.forsciint.2012.10.003
6. *Mandel M., Higa A.* Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol*. 1970, 53 (1), 159–162.
7. *Hightower J., Kracalik I. T., Vydayko N., Goodin D., Glass G., Blackburn J. K.* Historical distribution and host-vector diversity of *Francisella tularensis*, the causative agent of tularemia, in Ukraine. *Paras. Vectors*. 2014, V. 7, P. 453–458. https://doi.org/10.1186/s13071-014-0453-2
8. *Reintjes R., Dedushaj I., Gjini A., Rikke T., Cotter J. B., Lieftucht A., D'Ancona* F., *Dennis D. T., Kosoy M. A., Mulliqi-Osmani G., Grunow R., Kalaveshi A., Gashi L., Humolli I.* Tularemia Outbreak Investigation in Kosovo: Case Control and Environmental Studies. *Emerg. Infect. Dis*. 2002, 8 (1), 69–73. https://doi.org/10.3201/eid0801.010131

РОЗРОБЛЕННЯ РЕКОМБІНАНТНОГО ПОЗИТИВНОГО КОНТРОЛЮ ДЛЯ ДЕТЕКЦІЇ *Francisella tularensis* ЗА ДОПОМОГОЮ qPCR

О. Б. Зленко, А. П. Герілович

Національний науковий центр «Інститут експериментальної та клінічної ветеринарної медицини» НААН України, Харків

E-mail: oksana.ceratium@gmail.com

Метою роботи було конструювання та випробування рекомбінантного позитивного контролю для виявлення *F. tularensis* під час проведення полімеразної ланцюгової реакції в режимі реального часу (qPCR). Здійснено молекулярне ТА-клонування плазміди pTZ57_F/R з ампліконом гена *tul4* і подальшу її трансформацію в компетентні клітини *E. coli* DH5α. Мінімальна діагностична кількість плазміди в реакції становила одну копію. Отриманий позитивний контроль є високочутливим, специфічним і безпечним для використання в лабораторній діагностиці туляремії.

Ключові слова: рекомбінантний позитивний контроль, qPCR, туляремія, молекулярне клонування.

РАЗРАБОТКА РЕКОМБИНАНТНОГО ПОЛОЖИТЕЛЬНОГО КОНТРОЛЯ ДЛЯ ВЫЯВЛЕНИЯ *Francisella tularensis* С ПОМОЩЬЮ qPCR

О. Б. Зленко, А. П. Герилович

Национальный научный центр «Институт экспериментальной и клинической ветеринарной медицины» НААН Украины, Харьков

E-mail: oksana.ceratium@gmail.com

Целью работы было конструирование и испытание рекомбинантного положительного контроля для выявления *F. tularensis* при проведении полимеразной цепной реакции в режиме реального времени (qPCR). Осуществлено молекулярное ТА-клонирование плазмиды pTZ57_F/R с ампликонами гена *tul4* и ее дальнейшая трансформация в компетентные клетки *E. coli* DH5α. Минимальное диагностическое количество плазмиды в реакции составляло одну копию. Полученный положительный контроль является высокочувствительным, специфичным и безопасным для использования в лабораторной диагностике туляремии.

Ключевые слова: рекомбинантный положительный контроль, qPCR, туляремия, молекулярное клонирование.