MOLECULAR DIAGNOSIS

Detection of *Francisella tularensis* by the polymerase chain reaction

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***Francisella tularensis* is the causative agent of tularaemia. Effective antibiotic treatment of tularaemia is now available, but the early diagnosis of tularaemia remains a problem. Four primers (three pairs) were designed to detect *F. tularensis* by the polymerase chain reaction (PCR), based on the previously published nucleotide sequence of T-cell epitopes of a *F. tularensis* membrane protein. Amplification of purified *F. tularensis* chromosomal DNA with the three pairs of primers resulted in three different products with sizes consistent with those predicted from sequence data (211 bp, 347 bp and 568 bp). The specificity of the PCR was confirmed as no amplification was detected with a range of other bacteria. The sensitivity of the PCR was determined with limiting dilution PCR and viable counts. The preliminary application of the PCR to the detection of *F. tularensis* in aerosols and experimentally infected mice was investigated. Comparison of the results with those from traditional culture indicated that PCR was more sensitive. The animal challenge test showed that, 24 h after inoculation with 15 cfu of *F. tularensis*, 38 (82.6%) of 46 blood samples were positive by PCR, whereas only 22 (47.8%) were positive by culture. The results showed that PCR is a helpful tool for the detection of *F. tularensis* in blood, liver and spleen which should enable the rapid confirmation of clinical diagnoses of tularaemia.**

Introduction

Tularaemia is a zoonotic bacterial disease that occurs endemically between 30° and 71° north latitude [1]. The causative agent, *Francisella tularensis*, can be divided into two main types [2]. *F. tularensis* biovar *tularensis* (Jellison type A) has been found so far only in North America, whereas *F. tularensis* biovar *palaeartica* (Jellison type B) occurs mainly in Asia and Europe and to a minor extent in North America. Type A is associated with ticks and rabbits and is highly virulent in man, whereas type B is found in mosquitoes and rodents and is less virulent for man.

Current diagnosis of tularaemia is usually performed by detecting specific antibody to *F. tularensis* by enzyme-linked immunosorbent assay or agglutination [3, 4]. The antibody response to *F. tularensis* is highly specific, but in-vitro cross-reactions have been reported with *Brucella* spp. [5–9], *Proteus vulgaris* OX19 [10, 11] and *Yersinia* spp. [12]. Although *F.*

tularensis can be isolated from blood [13, 14], diagnosis by this method is notoriously unreliable. In a recent study [14] in the southwest central USA, bacteria were isolated from only 13.2% of serologically confirmed cases. Thus, it is important to develop a sensitive detection system for the organism.

As few as 25 organisms of *F. tularensis* biovar *tularensis* given by aerosol are sufficient to induce tularaemia in man [3]. Non-immune laboratory employees who work routinely with this biovar can eventually acquire the disease [3]. The majority of experiments can be performed with *F. tularensis* live vaccine strain (LVS) (Jellison type B), with riskier experiments on wild-type strains kept to a minimum. The present studies employed two *F. tularensis* strains: the less virulent *F. tularensis* strain 410062 (Jellison type B) was chosen for studies of aerosolised bacteria because of the hazardous nature of *F. tularensis* biovar *tularensis* aerosols, and the highly virulent *F. tularensis* strain 410101 (Jellison type A) was used to challenge Shanghai mice.

The polymerase chain reaction (PCR) has been applied widely in molecular biology, genetics and

Received 25 Aug. 1995; revised version accepted 19 March 1996.

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medical microbiology since its development in 1985 [15] and numerous studies have shown that it is a powerful tool for the detection of specific nucleic acids. The aim of the present study was to develop a PCR-based assay for the sensitive detection of *F. tularensis* based on four primers (three pairs) designed from previously published nucleotide sequence of T-cell epitopes of a *F. tularensis* membrane protein [16].

Materials and methods

Chemicals and materials

All chemicals were of the highest grade commercially available in China. *Taq* DNA polymerase and restriction endonucleases were purchased from Sino-American Biotechnology Company, Beijing.

Bacteria, media and growth conditions

The bacterial strains used in this study are listed in Table 1. *F. tularensis* strains 410062 and 410101 were kindly provided by the national reference laboratory for *F. tularensis*, Beijing. Other strains were purchased from the Institute of Microbiology, Academia Sinica, Beijing. All bacterial strains were grown at 37°C on agar supplemented with sheep blood 10% (Institute of Microbiology and Epidemiology, Beijing) overnight except for *F. tularensis* which was cultured on Francis Medium: Hottinges powder 1.5 g/L, glucose 10 g/L, NaCl 5 g/L, L-cysteine 1 g/L, agar 1.5%, defibrinated rabbit blood 5%, pH 7.3–7.4 for 4 days.

Preparation of PCR templates

Colonies (*c.* 10⁸–10⁹ cfu) were suspended in 10 mM Tris-HCl (pH 8.0) and SDS was added to 5% w/v. DNA was then extracted with phenol-chloroform and precipitated with ethanol. DNA was finally dissolved in distilled water and used as template for the PCR reaction. In addition to the purified DNA, crude extracts of *F. tularensis* were prepared. One colony of *F. tularensis* in 500 µl of phosphate-buffered saline (PBS) was boiled for 10 min and centrifuged at 2500 *g* for 5 min; the supernate was then used as the target for PCR amplification.

PCR of purified DNA and bacterial suspension

Primers for PCR were designed from a previously published sequence of a T-cell epitope of a *F. tularensis* membrane protein [16]. Four primers (P1–P4) were designed for use in three combinations (P1–P2, P2–P3 and P1–P4) to amplify three different length of DNA fragments. The sequences of the primers were as follows: 5' TGG CGA GTG ATA CTG CTT G 3' (primer P1); 5' TAG GAT CCC ATT AGC TGT CCA CTT ACC 3' (P2); 5' GGA ATT CGT TAG GTG GCT CTG ATG AT 3' (P3) and 5' CGC TAA ACC TGC GAT TGA T 3' (P4).

The PCR reaction mix contained 10 mM Tris-HCl (pH 8.4); 50 mM KCl; 1.5 mM MgCl₂; gelatin 0.01%; Triton X-100 0.1%; Tween 20 0.01%; 50 µM each of dCTP, dGTP, dTTP, dATP; 100 pM of each primer and 1 U of *Taq* polymerase. Amplification was performed in a Perkin Elmer DNA Thermal Cycler in 50-µl volumes under the following conditions: pre-denaturation for 10 min at 97°C, followed by the addition of 1 U *Taq* polymerase at 75°C, then 35 cycles consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 1 min at 75°C, followed by 10 min at 75°C. PCR products were detected on ethidium bromide-stained agarose gels (Sigma, 1.5%) in TBE. The identification of the PCR products was confirmed by restriction endonuclease analysis of the products and hybridisation studies.

Restriction analysis of PCR products

The 211-bp, 347-bp and 568-bp products were digested with *Taq*I, *Hind*III, and *Taq*I respectively, under the conditions specified by the manufacturer.

Nucleic acid hybridisation

Nitrocellulose filters (Promega) were pretreated with 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM citric acid) for 2 min, then incubated at 37°C for 2 h. PCR products were denatured by boiling for 10 min, then immersed in an ice-water mixture for 5 min. Filters were spotted with these DNA samples (3 µl), then baked at 80°C for 2 h. Filters were then pre-incubated in formamide 50%, 2 × SSC, 5 × Denhardt's solution,

Table 1. Bacteria used in this study

Bacterial strains	PCR	Bacterial strains	PCR
<i>F. tularensis</i> 410062	+	<i>Morganella morganii</i> 430024	–
<i>F. tularensis</i> 410101	+	<i>Yersinia pseudotuberculosis</i> 410051	–
<i>Pseudomonas pseudomallei</i>	–	<i>Proteus vulgaris</i> 430052	–
<i>Serratia marcescens</i>	–	<i>Proteus mirabilis</i> 430011	–
<i>Brucella abortus</i>	–	<i>Hafnia alvei</i> 45201	–
<i>Providencia stuartii</i> 1.1209	–	<i>Yersinia pestis</i> 410041	–
<i>Enterobacter cloacae</i>	–	<i>Escherichia coli</i> 270011	–
<i>Klebsiella pneumoniae</i> 1.1526	–	<i>Legionella pneumophila</i>	–
<i>Salmonella typhimurium</i> 460691	–	<i>Shigella dysenteriae</i> 480011	–
<i>Vibrio cholerae</i> SM 6	–	<i>Yersinia enterocolitica</i> 52302	–
<i>Bacillus anthracis</i>	–		

sheared and denatured salmon sperm DNA 1 µg/ml, 20 mM EDTA at 42°C for 4 h. Hybridisation was performed in formamide 50%, 2 × SSC, 5 × Denhardt's solution, sheared and denatured salmon sperm DNA 1 µg/ml, sodium sulphate 2.5%, biotin-labelled probe 100 ng/ml. The probe was synthesised with primers P2 and P3 by the PCR method above, with biotin-11-dUTP replacing 25% of dTTP. Filters were washed twice with 2 × SSC, SDS 0.1% and twice with 0.1 × SSC, SDS 0.1% each for 5 min at room temperature. Hybridisation was detected with the AP-Visualization Kit (Beijing Medical University) according to the manufacturer's instructions. Because the 568-bp sequence and pBR322 DNA contain the 347-bp sequence, they should both hybridise with the 347-bp biotin-labelled probe.

Limiting dilution PCR

The sensitivity of the PCR was determined by limiting dilution PCR. *F. tularensis* 410062 colonies were harvested in PBS and then diluted in 10-fold steps to 10⁸. The number of organisms in 100 µl of each dilution was determined by surface agar plate viable counts and 1 µl of each dilution was subjected to PCR. Comparison of the two results enabled the sensitivity of the PCR to be determined.

Detection of *F. tularensis* in aerosols

A stainless steel chamber, with an interior volume of 500 L, was used for the aerosolisation experiments. A built-in rotary drum with a speed of 4 rpm was installed to maintain the stability of aerosols. Two bacterial concentrations were prepared for aerosolisation by diluting stock cultures with PBS containing bovine serum albumen (BSA) 0.01%. Aerosols containing *F. tularensis* 410062 (attenuated strain, not harmful to man) were generated with a TK-2 nebuliser (Factory of Academy of Military Medical Sciences, Beijing) at a pressure of 127.4 KPa and flow rate of 8 L/min for 5 min. PBS was aerosolised for 2 min and sampled as a negative control before each trial. At each bacterial concentration, organisms were sampled with AGI-10 impingers (containing 10 ml of PBS, BSA 0.01%, 50 µl of olive oil) at a flow rate of 8 L/min for 2 min at 0, 10, 20, 40 and 80 min after aerosolisation. The aerosolisation experiments were performed twice. Resulting samples were diluted in 10-fold steps to 10⁸ and the bacterial concentration was estimated by surface viable counts and PCR.

Detection of *F. tularensis* in experimentally infected mice

Specific-pathogen-free Shanghai mice (Experimental Animal Centre, Academy of Military Medical Sciences, Beijing) were inoculated intraperitoneally with *c.* 15 (group 1) and 1.5 (group 2) cfu of *F. tularensis* strain 410101. Mice given the same volume of NaCl 0.9%

solution were used as negative controls. Each day after injection, one mouse in each group was killed and blood, liver and spleen were sampled. Blood was subjected to PCR directly. Liver and spleen were homogenised with a glass tissue grinder, then treated with phenol-chloroform and DNA was precipitated with ethanol. DNA was finally dissolved in 100 µl of distilled water (equivalent to the volume of the original sample). All these experiments were carried out in a level 3 biosafety laboratory of the Institute of Microbiology and Epidemiology, Beijing.

Results

Specificity and sensitivity of PCR assay

Eighteen different types of bacteria genetically unrelated to *F. tularensis* were tested in this assay; all organisms other than *F. tularensis* gave negative results by PCR (Table 1). Sensitivity was determined by limiting dilution PCR. A PCR product could be detected from samples containing 1 cfu of *F. tularensis*/µl.

PCR of purified DNA and crude extracts

With primer pairs P1–P2, P2–P3 and P1–P4, PCR of purified DNA resulted in 568-bp, 347-bp and 211-bp products, respectively (Fig. 1), consistent with previously published sequence data [16]. PCR amplification from crude extracts of *F. tularensis* was also investigated with primer pair P1–P2. No significant difference was observed between the PCR results with bacterial suspension and purified chromosomal DNA.

Identification of PCR products

Restriction endonuclease digestion of the 211-bp, 347-bp and 568-bp PCR products with *TaqI*, *HindIII* and *TaqI*, respectively, produced fragments consistent with the sizes predicted from the published sequence [16].

Fig. 1. PCR of purified DNA and bacteria suspension: Lane 1, pBR322 *HinfI* marker; 2, PCR of purified DNA with primer pair P1–P4; 3, PCR of purified DNA with P2–P3; 4, PCR of purified DNA with P1–P2; 5, PCR of strain 410101 suspension with P1–P2; 6, PCR of strain 410062 with P1–P2.

The 347- and 568-bp products (3–300 ng) hybridised with the 347-bp biotin-labelled probe (Fig. 2).

PCR of aerosol samples

F. tularensis was detected by both culture and PCR (Table 2). The concentration of organisms in the aerosol samples was estimated by limiting dilution PCR (Table 2). For example, when 10^5 dilution was PCR negative and 10^4 was PCR positive (for 1 μ l of sample), the concentration of organisms could be approximately deduced as follows: 1 (i.e. at least one copy of bacterial genome detected) $\times 1000 \times 10^4 = 10^7$

genomes. PCR was approximately 10 times more sensitive than culture (Table 2).

PCR of experimentally infected mice

Two mice died 6 days and 7 days after inoculation with 15 cfu of *F. tularensis*, while one mouse died 7 days after inoculation with 1.5 cfu of *F. tularensis*.

In total, 46 samples were investigated (Table 3), 38 (82.6%) of which were PCR positive, whereas culture was positive for 22 (47.8%). PCR of blood was positive 24 h after inoculation with 15 cfu of *F.*

1	2	3
4	5	6
7	8	9

Fig. 2. Nucleic acid hybridisation with biotin labelled probe: (1) 347-bp product, 300 ng; (2) 347-bp product, 30 ng; (3) 347-bp product, 3 ng; (4) No. 7 pBR322 DNA, 600 ng; (5) No. 16 pBR322 DNA, 600 ng; (6) 211-bp negative control, 300 ng; (7) 568-bp product, 300 ng; (8) 568-bp product, 30 ng; (9) 568-bp product, 300 ng.

Table 2. Detection of *F. tularensis* in aerosols

Time after aerosolisation (min)	Viable count (cfu/ml)*	PCR result at dilution								
		10 ⁰	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
0	6.0 × 10 ⁶	+	+	+	+	+	—	—	...	...
10	1.4 × 10 ⁶	+	+	+	+	—/+ [†]	—	...	...	...
20	4.5 × 10 ⁵	+	+	+	+	—	...	...	...	...
40	4.4 × 10 ⁴	+	+	+	+	—	...	...	...	...
80	9.3 × 10 ³	+	+	+	—	...	...	...	...	...
Control (PBS)	0	—	...	...	...	...	...	...	...	...
Storage suspension	1.2 × 10 ⁹	+	+	+	+	+	+	+	—/+ [†]	—

*The mean value of two separate aerosolisation experiments.

†Discrepant results in the two trials; other results were concordant.

Table 3. Detection of *F. tularensis* in mice

Days after injection	Culture and PCR results with					
	Group 1 (15 cfu)			Group 2 (1.5 cfu)		
	Blood	Liver	Spleen	Blood	Liver	Spleen
1	—/+*	—/+	—/+	—	—	—
2	+	—/+	+	—	—/+	—/+
3	+	—/+	+	+	—/+	+
4	+	+	+	+	—/+	+
5	+	—	—/+	+	—	—/+
6	+	—/+	+	—	—/+	+
7	+	—/+	+	—	—/+	—/+
dead	...	+	+	...	—/+	+

*For discrepant results, first symbol represents culture result and second represents PCR result. Other results were concordant. —, negative results, +, positive result, ..., blood not available from dead animals.

tularensis in all cases. *F. tularensis* was detected in samples from mice inoculated with 1.5 cfu as early as 48 h after injection (Table 3). Samples from control mice were consistently negative for *F. tularensis* by culture and PCR.

Discussion

Little is known about the genetics and molecular biology of *F. tularensis*. The first study on nucleotide sequence analysis of *F. tularensis* was conducted by Sjostedt *et al.* in 1990 [16]; this enabled us and other workers to design primers for PCR [17]. Because the two biovars (Jellison types A and B) of *F. tularensis* both share the TUL4 antigen, PCR of the two strains gave the same result [17]. Primers based on the above sequence were designed to distinguish strains at the genus and species levels.

Because human tularaemia can be treated effectively with antibiotics [18], methods which detect *F. tularensis* early in the disease course may facilitate effective treatment [4]. Laboratory diagnosis of tularaemia is usually by detecting antibodies against *F. tularensis* [3, 4] with, for example, the bacterial micro-agglutination test [19, 20], which may be performed 8–10 days after initial clinical symptoms of tularaemia appear, or enzyme-linked immunosorbent assay (ELISA) [19–22], which may show class-specific antibodies slightly earlier after the onset of illness than agglutination assays. Both methods are too slow to be clinically useful because of the rapid progress of tularaemia. Moreover, the specificity of agglutination and ELISA is often uncertain because cross-reactions have been found with *Brucella* spp. [5–9], *Proteus vulgaris* OX19 [10, 11] and *Yersinia* spp. [12]. The present study developed a PCR-based assay to detect *F. tularensis* in samples of aerosols and laboratory-infected mice and showed that this technique provides a rapid, specific and sensitive method for the detection of *F. tularensis*.

Detection and quantification of airborne *F. tularensis* was achieved by culture and limiting dilution PCR. Viable counts determined by culture were found to underestimate the aerosolised bacterial concentration when compared with the PCR method. The underestimation of *F. tularensis* by culture may be due to the slow growth of *F. tularensis*, the failure of some bacteria to grow on Francis medium or death of organisms during the aerosolisation experiments. Although the non-culturable organisms would not be detected by viable counts, they would still be good targets for PCR. This underestimation is clinically significant because a fraction of bacteria in the aerosols may be viable, yet non-culturable. In the case of *F. tularensis*, viability, but not necessarily culturability, is the measure of infectious potential. Underestimation of viable *F. tularensis* and other

bacterial pathogens (such as *Legionella pneumophila* [23]) can have serious health consequences, because the infectious dose of a pathogenic organism can be very small. This underscores the need for developing sensitive analytical techniques, such as PCR, that can be used in addition to culture methods.

F. tularensis is described as a facultative intracellular parasite, although direct proof of intracellular replication of *F. tularensis* in mononuclear phagocytes is scarce [3]. An animal challenge test [24] showed that when mice were inoculated with *F. tularensis* LVS intravenously, and killed 15 min after injection, $69.9 \pm 5.4\%$ of injected bacteria were recovered from blood, $4.8 \pm 0.3\%$ from liver and $8.6 \pm 0.7\%$ from spleen. The results of the present study showed that organisms were isolated from the blood of nine (64.3%) of 14 mice, two (12.5%) of 16 livers and ten (62.5%) of 16 spleen samples. It is usually thought that the lymph organs (especially liver and spleen) of mice infected with *F. tularensis* contain many bacteria and the reasons for low viable counts from liver cultures are not known. One possibility is the killer ability of liver, unlike spleen (which only concentrates organisms). Another possibility is that organisms may die during the sampling process. Furthermore, inhibitory substances may be released during homogenisation of tissue, hindering the growth of bacteria.

The present study showed that PCR of blood and spleen was positive 24 h after inoculation of mice with 15 cfu of *F. tularensis* (group 1), whereas PCR of blood was positive 48 h after injection of 1.5 cfu of organisms (group 2). It seems that the higher the inoculation dose used, the earlier the detection by PCR. The negative results by PCR may be due to low concentrations of bacteria, inadequate sample preparation, low efficiency of amplification or low resolution of agarose gel electrophoresis. The latter could be improved by Southern blotting and hybridisation.

In conclusion, the present study indicates that PCR can be used for rapid and specific identification of *F. tularensis* in aerosols and experimentally infected mice and, when compared with traditional culture or serology, PCR can be expected to be considerably more reliable and specific in clinical situations.

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