ELISA-BASED COLORIMETRIC DETECTION OF *RICKETTSIA TSUTSUGAMUSHI* DNA FROM PATIENT SERA BY NESTED POLYMERASE CHAIN REACTION

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Abstract. A rapid diagnostic system for scrub typhus was established using colorimetric detection of nested polymerase chain reaction (PCR). This system relied on binding the amplified DNA via a sequence in one of oligodeoxyribonucleotide to the DNA-binding protein GCN4 coated on the well of a micotiter dish. The primer pairs used for the nested PCR were designed on the basis of the homologous nucleotide sequence of the gene that encodes the 56 kDa antigen of serovariants. With this colorimetric PCR, diagnosis can be performed easily from serum samples of patients before the antibody titer increases or in the early stage of the disease. Furthermore, these positive results are able to be confirmed by pathogenic isolation.

INTRODUCTION

Rickettsia tsutsugamushi is a very small coccobacillus and an obligate intracellular parasite of human beings transmitted via chigger mites. The antigenic types of this rickettsia referred to as Gilliam, Karp and Kato strains are commonly recognized, and thus frequently called the prototype strains. Yet, additional antigenic types such as Shimokoshi (Tamura *et al*, 1984), Kawasaki (Yamamoto *et al*, 1986) Kuroki (Ohashi *et al*, 1990; Yamamoto *et al*, 1989) and Boyong (Kim *et al*, 1993) were isolated in the last decade. These variants are distinguishable from each other by serological cross-tests with strain-specific polyclonal or monoclonal antibodies. The serotype variation of the rickettsia depend on the antigenicity of an immunodominant 56 kDa major protein, which was demonstrated to be a type-specific antigen, located on the rickettsial outer membrane (Murata *et al*, 1986; Ohashi *et al*, 1992).

The immunofluorescence assay (IFA) technique has been widely employed for its clinical identification. However, the process of cultivating and isolating the antigenic *R. tsutsugamushi* for IFA is quite complicated and time consuming. Besides, the diagnostic method is somewhat of limited used in the early stage of the illness, when the serum antibody titer of the patient happens to be too low to measure (Sugita *et al*, 1993).

It has been reported that PCR can be used to detect the DNA sequence corresponding to this antigenic 56 kDa protein from blood clots (Furuya *et al*, 1992, 1993; Kawamori *et al*, 1993; Sugita *et al* 1993). Although the sensitivity of the PCR is excellent, the procedures for detection of the products of PCR are not well suited to routine equipment in many laboratories and to mass screening, as they generally require gel electrophoresis, Southern blot or specific DNA microplate hybridization. We describe here a nested colorimetric PCR, which is an ELISA-based test, is applicable to the detection of *Rickettsia* DNA from serum of patients in the early stage of the illness.

MATERIALS AND METHODS

Bacterial strains and media

R. tsutsugamushi Gillam, Karp and Kato, courtesy of Okinawa University, were propagated respectively in L929 cells as positive controls for the PCR.

Flasks (with 150 cm^2 culture surface) with fully grown L929 cells were infected with suspensions of *R. tsutsugamushi* at 5 moi. After 1 hour at 37°C, the monolayer was washed with minimal essential medium containing 2% fetal calf serum in an atmosphere of 5% CO_2 and 95% air (Chen *et al*,

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1992). The infected monolayer were harvested when greater than 70% of the cell were infected. The isolation of *R. tsutsugamushi* from lymphocytes and cell cultures were performed as described by Shieh *et al* (1995).

Clinical samples

Serum for the PCR was taken obtained from a 40 year old farmer who was having skin rash, fever, and chill, symtoms typical of the acute phase of scrub typhus. The IgGAM antibody titer to *R. tsutsugamushi* is 40 at first bleeding, 5 days after onset. After tetracycline treatment, the symptoms of this patient subsided and he went home. Therefore the second bleeding for the confirmational IFA could not be done and the case remains dubious. The detection of antibody titer against *R. tsutsugamushi* were performed according to Chen *et al* (1992).

Furthermore, sera were obtained from another four patients with low IFA titers or without secondary bleeding. For comparison, serum sample was also obtained from a patient with Q fever, which showed a titer for phase II antigen up to 2640.

For PCR amplification, template DNA was isolated from culture suspension of each of Gilliam, Karp and Kato strains or from patient's sera. 300 µl of the specimens were incubated with lysozyme at 1 mg/ml in 1 × TE buffer (50 mM Tris-HCl, pH 9.0, 1mM EDTA) and 0.5% SDS for 30 minutes at 37°C. Following digestion with protease K at 2 mg/ml, for 30 minutes at 55°C, the DNA was purified by repeated phenol-chloroform extraction and then followed by ethanol precipitation. The DNA pellets were resuspended in 10 µl of 1 × TE buffer (pH 7.5).

Polymerase chain reaction

Two pairs of primers were synthesized for the nested PCR. In the first PCR amplification, a pair of 5'-AGTACTATTAAATATTAGTATAC-3' (501-523, primer a) and 5'-TAAAGTCAATACC-AGCATAATTCTT-3' (1207-1186, primer b) were synthesized to generate 707 bp DNA fragments. In the second case, 5'-biotin-ATTGTCATACCC-GCAGCCAATGT-3' (673-688, primer c) and 5'-GGATGACTCATTGTTGTCGTTGCCGTTTTCAG-3' (875-853, primer d) were the pair for a 203 bp DNA fragment instead, plus a 5'-biotin label and a 3'-GCN4 protein binding site (5'-GGATGACT-CATT-3').

100 µl of PCR mixture contained 50 mM KCl, 10 mM tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 250 uM each dNTP, 5 uM of primers, 1.25 U of Taq DNA polymerase (Promega) and 2 µl of DNA template. The target DNA was first amplified by PCR with oligo primers a and b. The mixture was denatured at 94°C for 1 minute 30 seconds, annealed at 55°C for 1 minute 30 seconds, and then chain was extended at 70°C for 1 minute 30 seconds in a thermal cycler. (Perkin-Elmer Cetus). This cycle was repeated 30 times. For the second PCR amplification, 5 µl of the above PCR product were amplified by a similar PCR cycle but with oligo primers c and d. The mixture was denatured at 94°C for 1 minute, annealed at 37°C for 1 minute, and then extended at 70°C for 1 minute. The cycle was repeated for 30 times,

Colorimetric detection of PCR product

The colorimetric assay of the PCR product was carried out according to Kemp *et al* (1990). In brief, it relies on the binding of the amplified DNA to its binding protein GCN4 from *Saccharomyces cerevisiae*, which is precoated on the wells of the microtiter dish (AMRAD Corporation, Ltd, Kew, Australia). The DNA products of the second PCR contained a 3'-GCN4-recognition site, could be captured by binding to GCN4 immobilized in wells of microtiter dish (Kemp *et al*, 1990). On the other hand, the products of PCR contained 5'-biotin label which allowed detection with avidin conjugated to horseradish peroxidase, followed by reaction with chromogenic substrate as a standard ELISA reaction.

RESULTS

The specific primers for DNA amplification used in this study were selected on the basis of the homology with the nucleotide sequence of a gene encoding the antigenic 56 kDa protein in seven sero-variants (Gilliam, Karp, Kato, Shimokoshi, Kato, Kawasaki and Boryong) of *R. tsutsugamushi* (Fig 1). Agarose gel electrophoresis showed that amplification of DNA of expected size was ob-

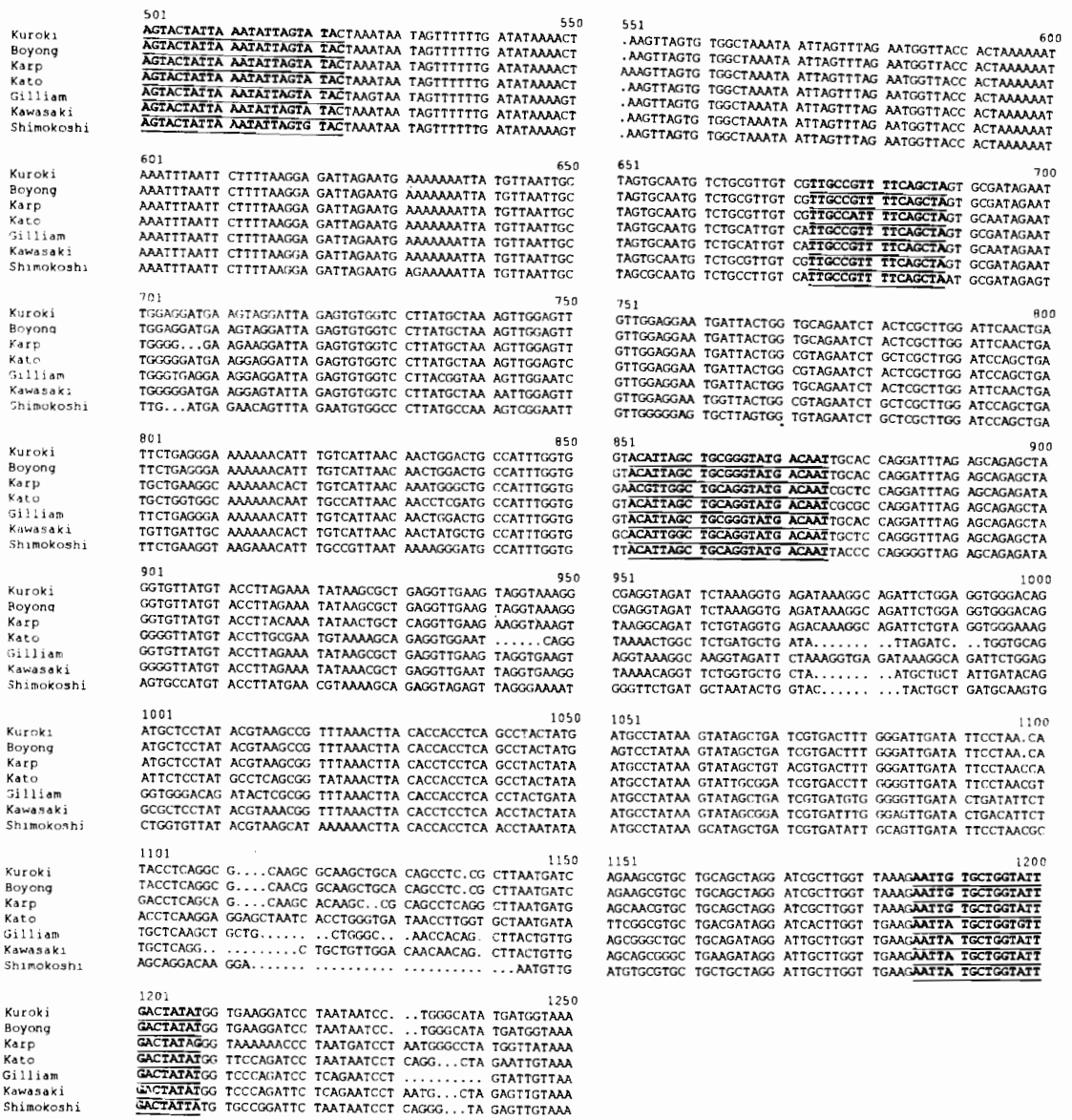

Fig 1–Alignment showing the positions and nucleotide sequences of primers (a, b, c and d, as indicated by bold letter and underline) from the 56 kDa protein corresponding gene of the seven strains. The DNA sequence data were run on a micro VAXII (Devereux *et al*, 1984) with UWGCG SEQUENCE ANALYSIS SOFTWARE (University of Wisconsin Genetic Computer Group). The program Stringsearch was used to search the individual sequence of the seven strains; whereas Pileup were performed to alignment the nucleotide sequences.

served from Gilliam, Karp and Kato strains tested (Fig 2). This 203 bp DNA fragment was also generated from the serum sample of a 40 year old patient (Fig 3, lanes 2, 3 and 4); no DNA amplification was observed from the patient with Q fever (Fig 3, lane 1). These results indicate that the primers we describe here are specific to *R. tsutsugamushi*, within the limits of the testing done.

The various volumes of the PCR product amplified from the serum of the 40 year old patient, are shown in Fig 4. Samples 1-5 were analyzed by the colorimetric assay. The color reaction was clearly visualized within 30 minutes of incubation with

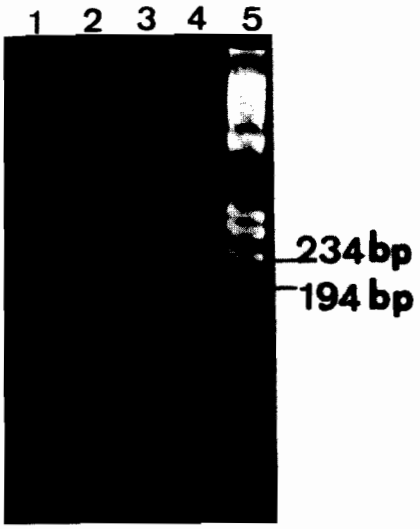

Fig 2--Agarose gel electrophoresis (1.5%) of amplified DNA by nested PCR with primers a, b, c and d for strains Gilliam (lane 2), Karp (lane 3), Kato (lane 4) and uninfected cell (lane 1). Lane 5 contained a DNA size marker (HT, Biotechnology Ltd), and the size fragments were 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 271 bp, 234 bp, 194 bp, 118 bp and 72 bp, only 234 and 194 bp are indicated.)

the substrate, and the optical density of the reaction at 450 nm was measured. Five µl (5 ng) or PCR products could be detected by this colorimetric assay (Fig 4, sample 5). However up to 20 µl (20 ng) of PCR product could be observed by agarose gel electrophoresis (Fig 3, lane 2).

There was negligible background from a PCR mix incubated with a serum sample from a Q fever patient and without substrate DNA (Fig 4, samples Q and 6). Furthermore, with template DNA from cell cultures of Gilliam, Karp and Kato strains, ODs of 0.836, 0.929 and 0.592 were obtained, respectively (Fig 4, samples G, Kp and Kt).

Serum samples with low antibody titers obtained from four suspected patients were also examined by colorimetric PCR in early stage of onset. All four samples were positive (Table 1). These results were then confirmed by pathogenic isolation with cell cultures.

DISCUSSION

The symptom of *R. tsutsugamushi* disease appear after 1 to 2 week-latent period. Because the antibody titer against *R. tsutsugamushi* is not high enough to be detected at the initial stage of hospitalization, the timely serodiagnosis of this disease is difficult sometimes. Here we have presented a nested colorimetric PCR for direct diagnosis of serum samples from suspected patients in the acute phase of the illness.

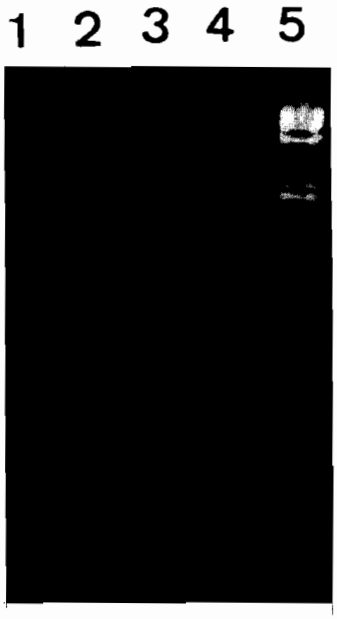

Fig 3–Agarose gel electrophoresis (1.5%) of amplified DNA by nested PCR with primers a, b, c and d with template DNA from the patient of suspected with scrub typhus. Lanes 2, 3 and 4 are with 20, 15 and 10 µl of the template DNA; lane 1, with 20 µl of the template DNA from a patient with Q fever. Lane 5, DNA size marker the same as Fig 2, lane 5.

The application of PCR is expected to be good for the diagnosis of this disease (Furuya *et al*, 1992, 1993; Kawamori, *et al*, 1993). The specific primers for DNA amplification were selected on the basis of the nucleotide sequence of a gene encoding the 56 kDa protein of *R. tsutsugamushi*, which has been suggested as being specific and sensitive for PCR detection. Although the primers used were practical for diagnosis of the strains isolated from Japan, in our cases, an additional lower molecular weight band was also seen (Shieh *et al*, 1995).

Primers a, b, c and d we selected have high homology with known serovariants including the

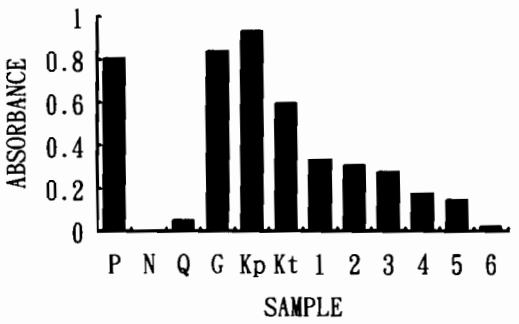

Fig 4–Use of the colorimetric PCR to detect the specific DNA of *R. tsutsugamushi*. Sample G, Kp and Kt: 25 µl of the PCR product from the cell culture of Gilliam, Karp and Kato strains. Sample Q: 25 µl of the PCR products from derived from the serum of the patients with Q fever. Sample 1-6: 25, 20, 15, 10, 5 and 0 µl of PCR products derived from the serum of the 40 year old patient, respectively. Sample P and N: positive and negative control of the colorimetric reaction.

Boyong strain isolated from Korea. The specific 203 bp DNA fragments of Gilliam, Karp and Kato strains were amplified by PCR, but sequences of Q fever were not. DNA isolated from other rickettsial pathogens such as *R. typhi, R. porowazekii, R. conorii* and *Coxiella burnettii* (data not shown) were not amplified by PCR. These indicate that the primers we used are specific for the diagnosis of *tsutsugamuchi* disease.

As many as 295 patients may have suffered from *R. tsutsugamushi* infection in Taiwan in 1994 alone. Nevertheless, it remains unknown whether there are serovarient strains in Taiwan. Therefore it is important for pathogen identification to be done. Here we present a colorimetric PCR method which could specificly detect the pathogens from sera of suspected patients with low IFA titers.

An enzyme-linked immunosorbent assay (ELISA) microplate hybridization method for *R. tsutsugamushi* detection has been reported (Sugita *et al*, 1993). This assay is quite sensitive, highly specific and reliable, but the protocol is time consuming since the PCR products obtained from whole blood sample had to be passaged through a sephadex G-50 column, the specific DNA probe had to be immobilized on the microplate, and the hybridization had to be carried out in the microplate wells for performing the enzymatic color reaction. Here we have described the application of a novel detection system for serum, which is an ELISA-based test, which can be readily adapted to diagnostic laboratories. The routine detection of *R. tsutsugamushi* from serum of suspected patients by this colorimatric PCR method concomitantly with IFA and pathogenic isolation are in progress.

Table 1

Diagnosis of *R. tsutsugamushi* patients by antibody titers, colorimetric PCR (CPCR) and pathogenic isolation (PI).

Patient No.	Days of illness	Titers[a]	CPCR	PI
35	6	0	0.408	+[b]
73	5	40	0.815	+
100	4	320	1.074	+
101	5	0	0.628	+
Control[c]		nd[d]	0.000	nd

a) Antibody titers of patient sera against *R. tsutsugamushi* protostrains. The titers were expressed as the highest dilution of the serum. Antigens were prepared from infected L929 cell culture.
b) Pathogen isolation with positive results.
c) Serum from healthy donor.
d) Not done.

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