Diagnosis of typhus infection with *Rickettsia tsutsugamushi* by polymerase chain reaction

Y. SUGITA, T. NAGATANI, K. OKUDA*, Y. YOSHIDA† and H. NAKAJIMA

Departments of Dermatology and *Bacteriology, Yokohama City University, School of Medicine, 3–9 Fukuura, Kanazawa-ku, Yokohama 236 and †Kanagawa Prefectual Public Health Laboratory, Yokohama, Japan

Summary. Two sets of oligonucleotide primers were used to amplify the genomic DNA of *Rickettsia tsutsugamushi*, the causative agent of scrub typhus (tsutsugamushi disease), by the polymerase chain reaction. Each set of primers amplified 538-bp and 109-bp products, representing part of a gene encoding a possible major 58-kDa immunogenic protein, from whole genomic DNA extracted from *R. tsutsugamushi* strains Karp, Kato, Gilliam, Kuroki and Kawasaki. No amplification was observed from *R. sibirica*, *R. rickettsii*, mouse and human genomic DNA. DNA amplification was observed from crude lysates of peripheral whole blood, tissue homogenates and paraffin-embedded skin biopsy sections obtained from patients with scrub typhus disease. Southern blot analysis demonstrated the specificity of the amplified DNA fragments following hybridisation with a DNA probe generated from *R. tsutsugamushi* strain Karp. By means of this procedure, a rapid and sensitive diagnosis of scrub typhus disease can be made during the acute stage of this infection.

Introduction

Scrub typhus disease is a febrile illness found in many areas, but especially in Asia, and is sometimes fatal. *Rickettsia tsutsugamushi*, the causative agent of scrub typhus disease, is transmitted by infected mites. Several components of this bacterium are recognised by sera from immunised animals, and immunological investigation has revealed that *R. tsutsugamushi* can be divided into several sub-types that exhibit different epitopes that may play an important role in the immune response.1-4

The diagnosis of scrub typhus disease is based on its clinical features and serological tests. The existence of specific antibodies against bacterial proteins has been used for diagnosis, but the sensitivity and specificity may not always be reliable. Immunogenic proteins are conserved in scrub typhus rickettsiae.5,6 The deduced amino-acid sequences of the 58-kDa antigen were found to be homologous to heat shock protein 60, which shows a high degree of homology and antigenic cross-reactivity in many species.5 Despite the high homology of the amino-acid sequence, a distinct antigenicity of the 58-kDa protein was reported,7 suggesting that the protein could be a useful diagnostic reagent or potential vaccine component.

The polymerase chain reaction (PCR),8 employing specific oligonucleotide primers and *Tag* DNA polymerase, has provided a valuable approach in the diagnosis of infectious diseases.8-11 This powerful method can amplify the DNA from a single bacterium and detect the presence of pathogens in a clinical sample. In this paper, the development of a PCR test for the specific detection of DNA fragments encoding the 58-kDa protein of *R. tsutsugamushi* is described.

Materials and methods

*Bacterial strains and DNA preparation*

*Rickettsiae* were grown in mouse fibroblasts (L cells). Infected cells were provided by the Kanagawa Prefectural Public Health Laboratory (Yokohama, Japan). DNA from *R. tsutsugamushi* (strains Karp, Kato, Gilliam, Kuroki and Kawasaki), *R. sibirica* and *R. rickettsii* was isolated by lysis of infected mouse L cells (c. 5 × 10⁹) in 0·5 ml of lysis buffer containing: proteinase K 0·5 mg/ml, SDS 2% w/v, 10 mM Tris-HCl, pH 8·0, and 10 mM EDTA. Chemicals were purchased from Bethesda Research Laboratories (Gaithersburg, MD, USA).

Clinical samples were from four patients who had the typical features of scrub typhus disease, such as generalised rash, fever, headache and malaise. All four patients showed high titres of anti-*R. tsutsugamushi* antibodies in serological tests. Peripheral whole blood, tissue homogenates and paraffin-embedded skin biopsy sections from each patient were used as DNA sources. Whole blood (0·5 ml) from a patient was incubated with 0·5 ml of double concentration lysis buffer. Tissue fixed with formalin was homogenised in saline, centrifuged for 30 min at 13000 g, and the pellet resuspended in 0·5 ml of lysis buffer. Tissue sections, cut from a block fixed in formalin followed by paraffin
Polymerase chain reaction (PCR)

Two sets of oligonucleotide primers were selected, based on the established nucleotide sequence of the 58-kDa antigen of *R. tsutsugamushi*.7 The primers had the following sequences, numbered according to Stover and colleagues:8 primer 1, (372–394), 5'-GTACATGGGATCAATGTCGTAA-3'; primer 2, (801–824), 5'-GTTCATCTAATGGAGACGCCG-3'; primer 3, (909–888), 5'-CAAGTTAAAATTTTAGAATC-3'. Primer sets 1 + 3 and 2 + 3 were used to amplify 538-bp and 109-bp DNA fragments, respectively (fig. 1). A separate set of primers, 5'-GGTGGCGATCAATGTCGTAA-3' and 5'-GGAAATAGCCAGG-3' (Takara Shuzo Co., Kyoto, Japan) was used for amplification of a 345-bp fragment of the human β-globin gene. DNA fragments were generated by *Tag* DNA polymerase in a model PHC-2 PCR processor (Techne, Cambridge) with 40 cycles, each consisting of denaturation for 1 min at 94°C, followed by annealing for 1.5 min at 58°C, followed by extension for 1 min at 72°C. Amplification products were visualised after electrophoresis of 5-μl portions on NuSieve GTG agarose (FMC Bioproducts, Rockland, ME, USA) 3.5% gels containing ethidium bromide 0.5 μg/ml.

Southern blot analysis

PCR products obtained from clinical samples were electrophoresed on agarose 1% gels and transferred to a nitrocellulose filter by capillary transfer.18 Pre-hybridisation and hybridisation were performed at 42°C for 2 h and 16 h, respectively, as described.18 The DNA probe for Southern blot analysis was generated by PCR from *R. tsutsugamushi* strain Karp by means of oligonucleotide primers 1 and 3. Sequencing of this fragment by the dideoxynucleotide chain termination method19 showed a sequence identical to the published data (results not shown). This DNA fragment was labelled with α[32P]dCTP by random priming14 and used as the probe.

Results

DNA isolated from infected mouse L cells containing rickettsial genomic DNA was used as a substrate for PCR. As expected, primers 1 + 3 and 2 + 3 generated 538-bp and 109-bp DNA amplification products, respectively, with all strains of *R. tsutsugamushi* tested, but no specific amplification was observed with DNA from *R. sibirica* or *R. rickettsii* (fig. 2a). No detectable amplification occurred with the primers designed for *R. tsutsugamushi* from *Escherichia coli*, mouse or human genomic DNA, although it was possible to amplify a 345-bp β-globin gene DNA fragment from human DNA with the specific set of primers described in Materials and methods (fig. 2b).

DNA samples extracted from clinical specimens obtained from patients with scrub typhus disease were...
segments targeted for amplification were the 538- and 109-bp regions of the \( R.\) tsutsugamushi gene encoding a 58-kDa protein that forms a major antigen recognised by affinity-purified antibodies.

Discussion

PCR was used to attempt specific DNA amplification from control strains of \( R.\) tsutsugamushi and clinical samples obtained from patients with scrub typhus disease (tsutsugamushi disease). The DNA segments targeted for amplification were the 538- and 109-bp regions of the \( R.\) tsutsugamushi gene encoding a 58-kDa protein that forms a major antigen recognised by affinity-purified antibodies.

The deduced amino-acid sequence of this protein shows a high degree of homology to heat shock protein 60. The oligonucleotide primers were selected to have a low degree of homology to the gene encoding heat shock protein 60 from \( E.\) coli, Mycobacterium leprae, Coxiella burnetii, Trichinella abortus (wheat), Saccharomyces cerevisiae and man. The results indicated that the primers were specific for all strains of \( R.\) tsutsugamushi, but not other rickettsiae such as \( R.\) rickettsii and \( R.\) sibirica.

These results support a previous report, based on immunological analysis, suggesting that \( R.\) tsutsugamushi is unique among members of the genus Rickettsia. \( R.\) tsutsugamushi consists of several sub-types that exhibit different antigenic properties, and these different epitopes have been used for serological examination. Apart from immunological studies, genetic analysis has also been used to investigate relationships between strains of \( R.\) tsutsugamushi. Sequencing or analysis of restriction fragment length polymorphisms (RFLPs) of amplified DNA fragments may be useful in further investigations of this species.

\( R.\) tsutsugamushi DNA was detected by PCR in all clinical samples obtained from patients in an acute stage showing typical features of scrub typhus disease. The pathogen might even be detectable earlier, perhaps during the incubation period, because of the high sensitivity of the PCR technique. However, since this method also amplified DNA from blood obtained from patients who had been treated successfully with antibiotics (results not shown), it appears that non-viable \( R.\) tsutsugamushi may also be detected by PCR. Thus, the high sensitivity of the PCR technique may mean that it is not suitable for treatment evaluation, although it may be useful for a rapid diagnosis of the disease itself. When considered in conjunction with the DNA hybridisation analysis, which confirmed the specificity, this procedure seems to be a powerful method for diagnosing scrub typhus disease at an early stage, with high sensitivity and specificity compared with serological tests. In combination with proper treatment, the procedure may help to prevent serious morbidity in patients with this disease.

References

5. Oaks EV, Stover CK, Rice RM. Molecular cloning and expression of \( R.\) tsutsugamushi genes for two major

ANNOUNCEMENT

The Winter Meeting of the PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

will be held at the QUEEN ELIZABETH II CONFERENCE CENTRE, LONDON on 6–8 JANUARY 1993

Provisional programme

**Wednesday, 6 January.** Symposium—"Diarrhoeal disease—current concepts and future challenges."

**Thursday, 7 January a.m.** Symposium—"New developments in sexually-transmitted diseases"

(jointly with the STD Discussion Group)

p.m. Free papers and posters.

**Friday, 8 January a.m.** Free papers and posters.

Further information may be obtained from: Mrs Jacqui Edwards, Pathological Society of Great Britain and Ireland, 2 Carlton House Terrace, London SW1Y 5AF. Tel: (071) 976 1260

BOOKS RECEIVED

The Biology of Acinetobacter


This book grew from the Second International Workshop on *Acinetobacter* held in Paris in September 1990. In their Preface, the editors write that they "...decided not simply to publish the proceedings of the Workshop, but to commission a series of review articles...", and that the book comprises "these reviews, together with a number of articles that expand on the research talks presented at the Workshop." The strengths and weaknesses of the book flow, almost inevitably, from this mode of construction.

Put simply, the book is a Commonwealth of Independent Articles, not a Union. Its extent is huge, covering taxonomy, typing, epidemiology, virulence factors, clinical role, antibiotic resistance mechanisms, genetic organisation, metabolism and industrial applications. Some of the constituent articles are comprehensive, up-to-date reviews; others describe an author's own results on selected strains supplemented with a few comments on the behaviour of the genus as a whole. The contents list does not distinguish between these types of article, which is an inconvenience to the reader. Another inconvenience is that the "new" taxonomy, advocated by the editors in their introductory chapter, is not used by all the contributing authors. The editors note this problem with, perhaps, understandable when one considers the chaotic history of the genus. Less forgivable are the enclaves of "old" taxonomy that remain in articles that otherwise use the new. Surely the editors could have exerted some central authority here—likewise, on one of their own number, who writes on p. 97 "Imipenem is theoretically not inactivated by β-lactamases...", followed, on p. 99, by "Although β-lactamases capable of inactivating imipenem, produced by *Pseudomonas maltophilia*..." Ah, for the days of the ruthless use of the editorial pencil!

But these quibbles about presentation and editing should not detract from the wealth of information contained in the book, nor from its timelessness. There are rich seams of information for those who care to dig a little. The book will provide an invaluable reference source to anyone undertaking research on almost any aspect of this increasingly important genus. Such workers should certainly buy a copy. Those with a more general interest in, for example, the clinical importance of the species, would be advised to peruse a library copy.

D. M. LIVERMORE