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

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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.⁷ 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 *Taq* DNA polymerase, has provided a valuable approach in the diagnosis of infectious diseases.^{9–11} 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.* 5×10^6) 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 13 000 *g*, and the pellet resuspended in 0.5 ml of lysis buffer. Tissue sections, cut from a block fixed in formalin followed by paraffin

Fig. 3. Analysis of PCR products amplified from clinical samples obtained from four patients suffering from scrub typhus disease. A, PCR products were separated by electrophoresis on an agarose 1% w/v gel and stained with ethidium bromide. DNA size markers comprised a ϕ x174 *Hae*III digest (lane 1). DNA templates were isolated from paraffin-embedded skin biopsy sections (patient no. 1, lanes 2 and 3), peripheral whole blood (patient no. 2, lanes 4 and 5; patient no. 3, lanes 8 and 9; patient no. 4, lanes 10 and 11), and tissue homogenates (patient no. 2, lanes 6 and 7). PCR products were obtained with primers 1 + 3 (lanes 2, 4, 6, 8 and 10) and primers 2 + 3 (lanes 3, 5, 7, 9 and 11). B, Southern blot of the agarose gel shown in A. Following hybridisation with the 538-bp probe labelled with α [32 P]dCTP.

also used as substrates for PCR amplification. Amplification was observed with DNA from peripheral whole blood, tissue homogenates and paraffin-embedded skin biopsy sections (fig. 3A). To confirm the specificity of the amplified DNA fragments, a 538-bp DNA probe obtained directly from *R. tsutsugamushi* was used for Southern blot analysis. As expected, hybridisation was detected to both the 538-bp and 109-bp fragments amplified from the clinical samples (fig. 3B) since the 109-bp fragment is contained within the 538-bp fragment (fig. 1).

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.¹⁵ The

deduced amino-acid sequence of this protein shows a high degree of homology to heat shock protein 60.⁷ 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*, *Triticum aestivum* (wheat), *Saccharomyces cerevisiae* and man.⁷ 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*.¹⁶ *R. tsutsugamushi* consists of several subtypes that exhibit different antigenic properties, and these different epitopes have been used for serological examination. Apart from immunological studies,^{8, 17, 18} genetic analysis has also been used to investigate relationships between strains of *R. tsutsugamushi*.¹⁵ 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.

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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

Edited by K. J. TOWNER, E. BERGOGNE-BEREZIN and C. A. FEWSON. 1991. ISBN 0-306043902-6. Plenum Press, New York. Pp. 451. \$105.

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 which, perhaps, is 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.

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