I. INTRODUCTION

Scrub typhus is an acute infectious disease that occurs when humans are bitten by *Leptotrobidium* mites harboring the etiological agent *Orientia tsutsugamushi*. Typical symptoms include fever, headache, rash, eschar and lymphadenopathy. Clinical manifestations of this infection often mimic other tropical infections such as leptospirosis, murine typhus, malaria, dengue fever and viral hemorrhagic fever and cannot be differentiated in the early period. Thus the occurrence is frequently classified as pyrexia of unknown origin (PUO) (1). Clinical diagnosis is based on the history of the individual patient and the characteristics of patient population. However, differentiation of scrub typhus from other acute febrile illness is difficult due to the similarities of symptoms (2). Chloramphenicol and tetracycline antibiotics have demonstrated marked therapeutic efficacy against the disease (3). Unfortunately, fatalities occur each year because of misdiagnosis and consequent erroneous choice of antibiotics. There are variations in *O. tsutsugamushi* pathogenicity to human; however, ignorance persists as to the proper diagnostic methods.

Scrub typhus accounts for up to 23% of all febrile episodes in endemic areas of the Asia-Pacific region (2). Therefore, a highly sensitive and specific detection for *O. tsutsugamushi* is necessary. The gold standard method for *O. tsutsugamushi* detection is the cell culture technique. Unfortunately, the culture technique is time consuming and labour intensive. It takes 45 days to complete the test and requires access to specialized facilities. Currently, laboratory diagnosis of the disease employs immunological techniques such as Weil Felix (WF) test, the passive hemagglutination assay (PHA), indirect immunofluorescence (IF) test, indirect immunoperoxidase (IIP) test and enzyme link immunosorbance assay (ELISA). The WF test has been widely used for its clinical identification in hospitals of tropical countries especially in Thailand. This method is easy to perform. In contrast, the IF, IIP and ELISA are quite complicated and time consuming (4, 5). In addition, these immunological methods have limitations for early detection of illness, because the serum antibody concentration in the patient can be too low.

The recently developed polymerase chain reaction (PCR) technology allows the detection of even the smallest amounts of specific nucleic acids from clinical samples, by

repeated amplification steps of respective target sequence. The PCR has been shown in general to be highly sensitive for detection of pathogens in clinical samples (6).

Differentiation of *O. tsutsugamushi* serotypes is necessary in epidemiologic studies to establish the regional prevalence of the organism. Serotype classification was based originally on serological recognition of antigen epitope on the major outer membrane protein located on the Oriential surface. The IFA was the reference serological method. At first, it could identify three serotypes of *O. tsutsugamushi*; Gilliam, Karp and Kato. However, the serotyping of these organisms has limitations, due in particular to the commercial unavailability of the panels of antisera for typing. In addition, the immunological technique is not definitive clear because some of monoclonal antibodies show cross-reaction with some heterologous types (7, 8).

Developed serotyping methods for *O. tsutsugamushi* are based on the analysis of the nucleotide variation of major membrane protein gene, which is responsible for the antigenic variation of the protein. The 56 kDa protein, the predominant constitutional protein in *O. tsutsugamushi,* is located on the Oriential surface and shows type specific antigenicity. The serotyping methods relying on sequencing are known as nested polymerase chain reaction (PCR) and nucleotide sequencing.

Nested PCR, a two-step PCR using 2 pairs of primer, has been used for serotyping. The first amplification is carried out using universal primers whereas the second amplification is carried out using specific serotype primers. The primer design is based on the homology of nucleotide sequence of the gene encoding the 56-kDa major outer membrane protein of *O. tsutsugamushi*. The serotype of *O. tsutsugamushi* is identified by comparing the fragment patterns of amplified *O. tsutsugamushi* PCR products with the reference strain. The nucleotide sequencing is used to determine the nucleotide sequence polymorphism of 56-kDa protein gene and the molecular epidemiology. Moreover, the detailed nucleotide sequence polymorphism studies may show the variation in clinical isolates.

In this study, the 56-kDa major outer membrane protein gene of *O. tsutsugamushi* from clinical samples in Thailand was amplified by nested PCR which used two pairs of universal primers. For genotyping, the first step was amplified for positive detection and genotyping was continued by using serotype specific primers in second PCR. The genotype pattern was analyzed by comparison with its own reference serotype. The genomic region was

amplified directly by PCR from extracted DNA and sequenced by the dye-terminator method of ABI(Perkin-Elmer, Applied Biosystems, USA). The sequence, combined with the 56-kDa major protein gene sequence of *O. tsutsugamushi* obtained from the GenBank database, was analyzed by using a computer programme for classification into specific genotypes. Analysis of the derived nucleotide sequences was performed by NCBI-BLAST network service of the National Center for Biotechnology Information, Bethesda , Maryland, USA.

The nucleotide sequence polymorphism of 56-kDa major protein gene was characterized so that it could be useful for prospective molecular epidemiologic studies and diagnostic test kit development.

Aims of the study are as follows:

1. To identify serotype-specific of *O. tsutsugamushi* in Thailand.
2. To determine nucleotide sequence of the gene encoding 56-kDa protein in predominant type of *O. tsutsugamushi* in Thailand.
3. To determine nucleotide sequence polymorphism of the gene encoding 56-kDa protein which are useful for possible future use in vaccine development and the development of a diagnostic test kit.