V. DISCUSSION

Blood samples used in this study were from Thai febrile patients in Thailand, who most lived on agriculture and were subjected to high risk of scrub typhus. However, the prevalence of scrub typhus in Thailand has never been reported (84, 85) because of the variability of the manifestation and lack of sensitivity of the laboratory test for confirmation of the disease. In fact , the gold standard for the confirmation of scrub typhus is to isolate organism from the patients (5, 61, 86) and via serological methods which are not only laborious, but also time consuming and the requirement of paired serum for detection of seroconversion. The conventional WF test which is the most common method used in Thailand showed positive seroconversion only 52 out of 300 patients (Table 2). The low sensitivity of WF test due to the too low antibody titer against *O. tsutsugamushi* which is not detectable at the initial stage of hospitalization. In addition, WF test is not a specific serological reaction but a reaction between *O. tsutsugamushi* specific antibody and component of Proteus OX-K. The IFA is the reference standard for serological diagnosis. Although, the IFA has poor sensitivity in the early stage of illness that antibody is too low to be detected, it is still an appropriate technique when antibodies are high enough. Thus, the precise detection of *O. tsutsugamushi* from clinical samples were need for the scrub typhus diagnosis. Integration of molecular diagnostic technique has also been developed for detecting of *O. tsutsugamushi* DNA in blood.

The nested PCR performed in this study was based on the amplification of 56 kDa protein gene of *O. tsutsugamushi* DNA could be detected in 80 out of 84 positive seroconversion patients. Four out of 84 positive seroconversion samples could not amplified by nested PCR. The reason might be due to the clearance of the immune system. The IgM and IgG were usually elevated in day 8-10 after infection whereas the macrophage tried to eliminate *O. tsutsugamushi* but not all because in acute *O. tsutsugamushi* infection, the cellular immunity was suppressed for 7-28 days so *O. tsutsugamushi* in some scrub typhus patients could be cleared by the immune system (87). Some Thai patients usually visit medical care relatively late. Those who visited hospital after 7 days of fever and had high antibody can be diagnosed by IFA and WF test. However, the amplification of Oriential DNA from 40 and 72 patients with negative seroconversion towards *O. tsutsugamushi* by IFA and WF suggested an efficient detection of early infection (Table 4). In this study, *O. tsutsugamushi* DNA could be detected as early as day

3 of fever even before the appearance of specific antibody in the blood by the nested PCR technique.It is indicated that the nested PCR is recommended for scrub typhus diagnosis in early stage of infection especially in the cases which could not be diagnosed by IFA and WF. In addition, nested PCR could detect 100% of scrub typhus before day 5 whereas IFA could diagnose positive seroconversion only 50% of patients on day 5 (Table 5). The PCR is therefore useful for early detection of the infection since the sensitivity and specificity of nested PCR in negative seroconversion was high enough to use in the diagnosis. For the reference standard serological method using paired sera, IFA showed positive seroconversion in 84 out of 300 patients. However, in the early stage, IFA showed positive diagnosis only 52 patients whereas PCR could detect *O. tsutsugamushi* DNA in 76 patients. The sensitivity of PCR, IFA and WF at the early stage of infection in comparing to the reference standard method, IFA were 90.47% (76/84), 61.90% (52/84) and 14.28% (12/84) respectively (Table 6). The paired patient's serum which could not be diagnosed by IFA and WF could be diagnosed by nested PCR. In addition, the assay time of nested PCR method used only single specimen in the assay and the result could be reported in one day whereas IFA and WF test had to obtain paired specimen. Unfortunately, the cost of nested PCR was the highest and not effectiveness (Table 8).

The 56 kDa protein gene of *O. tsutsugamushi* is the major gene specifying antigens. The type specific antigen coding sequences are in variable domain and located on the surface of the organisms. Differentiation of the serotypes is necessary for epidemiological studies. In addition, such differences in pathogenicity are due to the different serotypes of *O. tsutsugamushi* (5, 38). Therefore, the information on serotype distribution in Thailand has not been clear. Serotype identification was originally based on the immunological reaction with polyclonal antibodies. However, these method often shows heterogeneity due to the cross reactivity of antigenic sites with one or more prototypes. It is important to determine the geographic distribution of existing serotype and prevalence of *O. tsutsugamushi* in Thailand. The molecular typing of *O. tsutsugamushi* was carried out by nested PCR. The serotype distribution in Thailand should be re-examined by molecular typing.

In this study, the nested PCR and DNA sequencing were combined for genotype identification. These genotypes accounted for 26% of total isolates. The techniques successfully identified the present of *O. tsutsugamushi* genotypes, which included predominant genotype circulating in different geographic. Among 80 *O. tsutsugamushi* positive samples, it

was found that the Karp genotype was predominant throughout Thailand whereas Kato was found only in the south. No Gilliam genotype was found. The *O. tsutsugamushi* Karp and Kato genotype were confirmed by DNA sequencing. However, the serotype in this study was different from earlier study. The serotypes identified in chiggers in Thailand by serological tests using polyclonal antibodies were Gilliam, Karp and Kato (55). It is also interesting to note that the Gilliam serotype was not found in patients by the PCR technique whereas Gilliam was found in chiggers by serological tests. This might be due to the cross reaction of polyclonal antibody among serotypes in the immunofluorescent assay. In addition, the amino acid composition of type specific antigen of the Gilliam is known to have a high homology with the Karp (88). It appears that the serotype distribution is not clarified by serologic evidence. The use of modern techniques for serotype identification is recommended.

The 56 kDa protein gene of *O. tsutsugamushi* frequently exhibited DNA sequence variation. Nucleotide sequence polymorphism in an antigen specifying gene of *O. tsutsugamushi* could be most apparent among isolates. In this study, analysis of nucleotide sequence polymorphism of 56-kDa protein gene of *O. tsutsugamushi* was performed. The DNA sequence of Karp and Kato genotypes from patients were 93-97% homologous with the reference sequence of *O. tsutsugamushi* Karp and Kato serotype, respectively. None of the sequence variant isolates were detected. In conclusion, the data in this study provide information for prospective studies on *O. tsutsugamushi* DNA sequence and genotype distribution in Thailand which was useful to development of a vaccine against *O. tsutsugamushi* and diagnostic test kit.