PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Development of multiplex PCR for neglected infectious diseases

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Abstract

Scrub typhus, murine typhus, and leptospirosis are widely neglected infectious diseases caused by *Orientia tsutsugamushi*, *Rickettsia typhi*, and pathogenic *Leptospira* spp., respectively. Patients usually present with non-specific symptoms and therefore are commonly diagnosed with acute undifferentiated febrile illness. Consequently, patients face delayed treatment and increased mortality. Antibody-based serological test currently used as gold standard has limitations due to insufficient antibody titers, especially in the early phase of infection. In this study, we aimed to develop multiplex PCR to combine 3 primer pairs that target specific genes encoding 56-kDa TSA of *O. tsutsugamushi*, 17-kDa antigen of *R. typhi*, and LipL32 of *L. Interrogans* and evaluate its performance in comparison to the standard serological tests. Using EDTA blood samples of known patients, the sensitivity and specificity of our multiplex PCR was 100% and 70%, respectively. In addition, the assay was able to diagnose the co-infection of scrub typhus and leptospirosis. The assay may be useful in identifying causative agents during the early phase of these diseases, enabling prompt and appropriate treatment.

Author summary

Scrub typhus, murine typhus, and leptospirosis are diagnosed as acute undifferentiated febrile illness. Diagnostic tests for these diseases depend on antibody detection. However, antibody detection is still limited by its tendency to return negative results during the early phase of aforementioned diseases. In this study, a novel multiplex PCR has been developed for detecting *Orientia tsutsugamushi*, *Rickettsia typhi*, and *Leptospira interrogans* that are simultaneously amplified in a single tube. The results have shown that multiplex PCR could be used as a diagnostic tool for detecting bacteria during the early phase

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of scrub typhus, murine typhus, and leptospirosis, allowing for administration of appropriate treatment.

Introduction

Scrub typhus, murine typhus, and leptospirosis are widely neglected infectious diseases, especially in the tropical and temperate climate regions, caused by *Orientia tsutsugamushi*, *Rickettsia typhi*, and pathogenic *Leptospira* spp., respectively. *Orientia* and *Rickettsia* are obligate intracellular bacteria. *O. tsutsugamushi* is transmitted to human through chigger bite, and *R. typhi* infection is transmitted by inoculation of rat flea's feces on human skin. Pathogenic leptospires are spirochetal bacteria that are mainly transmitted through abraded skin or mucosa after contact with urine of infected animals, contaminated water, or contaminated soil [1–5]. Unfortunately, current diagnostic tools, together with awareness and experience of physicians, have been the limited.

Early clinical manifestations of scrub typhus, murine typhus, and leptospirosis, such as high fever, headache, muscular pain, and anorexia, are non-specific and usually diagnosed as acute undifferentiated febrile illness. These clinical manifestations range from mild, severe, to possibly fatal [6–8]. Eschar caused by chigger bite is a clinical appearance of scrub typhus; however, it is not always present. Moreover, eschar-like lesion can occur in other diseases such as rickettsialpox and anthrax [7, 9, 10]. Macular rash is generally present in murine typhus and scrub typhus as well [11]. The clinical manifestations of leptospirosis are biphasic fever and multiorgan failure in severe cases (Weil's disease) [12]. Due to clinical manifestations of these diseases being recognized as acute undifferentiated febrile illness and subsequently underdiagnosed, a rapid and reliable laboratory investigation is necessary for confirmation and treatment efficiency.

Standard diagnostic tests for scrub typhus and murine typhus, i.e. indirect immunofluorescence assay (IFA), and for leptospirosis, i.e. microscopic agglutination test (MAT), mainly depend on detection of antibodies which often return false negative results in the early phase of diseases. To identify pathogen in the early phase of disease, direct pathogen identification is of vital importance since these bacteria can be found in the bloodstream during the first week after onset [13, 14]. Thus, a new diagnostic test development based on antigen or DNA detection is essential for rapid diagnosis and proper treatment.

Multiplex PCR is a molecular laboratory test used for simultaneous amplification that utilizes different primers in a single tube [15]. It has been applied in diagnosis of several infectious diseases caused by bacteria, fungi, parasites, and viruses [16]. The multiplex PCR is fast and time-saving because it is capable of detecting multiple pathogens at the same time [17]. In this study, we developed multiplex PCR to detect *O. tsutsugamushi*, *L. interrogans*, and *R. typhi* and evaluated its efficiency compared to serological tests. This work selected three target genes encoding 56-kDa type-specific antigen (TSA) of *O. tsutsugamushi*, lipL32 of *L. interrogans*, and 17-kDa antigen of *R. typhi* to identify each bacterium in bacterial cell culture and blood samples of suspected patients.

Materials and methods

Microorganisms

O. tsutsugamushi (Karp, Kato, and Gilliam strains) and *R. typhi* were obtained from Armed Forces Research Institute, Thailand. Pathogenic *L. interrogans* (serovar Pyrogenes, Pomona,

and Bratislava) and non-pathogenic *L. biflexa* serovar Patoc were acquired from Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Thailand.

Other pathogens, consisting of *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella* spp., Dengue virus (serotype1-4), *Plasmodium falciparum*, and *Plasmodium vivax*, were acquired from Department of Microbiology and Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Thailand.

Clinical samples

This study used 83 EDTA blood samples taken from patients presented with acute undifferentiated febrile illness at Armed Forces Research Institute, King Chulalongkorn Memorial hospital, Loei hospital, Takuapa hospital and Chokchai hospital, Thailand. Patients included were over 18 years old, having acute fever (38˚C or higher), exhibiting non-specific symptoms, (headache, muscular pain, anorexia, and rash for 3–5 days), and tested negative for both influenza and dengue antigens. Samples were tested in double-blind experiments and confirmed by IFA, for scrub typhus and murine typhus, and MAT, for leptospirosis. IFA and MAT are antibody detection techniques used as indicator of acute or current exposure; a fourfold increase of antibody titer in paired serum is determined as a positive result [2, 13, 14, 18].

Ethic statement

This study was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Thailand (IRB No. 009/57, 534/57, and 380/59). As we had used human EDTA blood obtained from hospital laboratories that perform serology and molecular analysis, an informed consent document was not required. We have not acquired any patient identification, and the data were analyzed anonymously.

Genomic DNA extraction

Bacterial DNA and blood samples were extracted using PureLink Genomic DNA Mini Kit (Invitrogen, USA). First, bacterial cells were lysed by PureLink Genomic Lysis/Binding Buffer. Then, DNA extraction was carried out according to PureLink manufacturer's instruction. DNA was eluted in 50μl PureLink Genomic Elution Buffer. DNA concentration was measured by UV absorbance at 260/280.

To isolate DNA from blood samples, each blood sample (200μl per sample) was added to 180μl PureLink Genomic Lysis/Binding Buffer. DNA isolation was carried out according to PureLink manufacturer's instruction. Isolated DNA was kept at -20˚C until use.

Primer design

The target genes consisted of 56-kDa TSA gene, 17-kDa antigen gene, and *lipL32* gene were selected for this study. 56-kDa TSA (56K TSA_F and 56K TSA_R) primers were designed from accession no. M33004, using Primer 3 Software. 17-kDa (17K_F and 17K_R) primers followed the research conducted by Webb et al. [19]. LipL32 (LipL32_45F and LipL32_287R) primers were slightly modified from that of Bourhy et al. [20]. Primer sequences and their amplicon sizes are shown in Table 1. The specificity of primers was bioinformatically aligned with databases in National Center for Biotechnology Information (NCBI) using BioEdit software.

Table 1. List of primer sequences.

Primer name	Sequence (5'→3')	Length (bp)	Product size (bp)	references
56-kDa_F	GGC-CAA-GTT-AAA-CTC-TAT-GCT-GAC	24	166	-
56-kDa_R	CAG-CAT-TAA-TTG-CTA-CAC-CAA-GTG-C	25		
lipL32_45F	AAG-CAT-TAC-CGC-TTG-TGG-TG	20	243	[20]
lipL32_287R	CGA-ACT-CCC-ATT-TCA-GCG-AT	20		
17-kDa_F	GCT-CTT-GCA-GCT-TCT-ATG-TT	20	434	[19]
17-kDa_R	CAT-TGT-TCG-TCA-GGT-TGG-CG	20		

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Optimization of singleplex and multiplex PCR

Singleplex PCR condition was initially optimized according to GoTaq Flexi manual instruction (Promega, USA). Multiplex PCR was optimized to ensure appropriate amplification. Extension temperatures of 68˚C and 72˚C were tested in this experiment. Magnesium chloride concentration, ranging from 1.5–4.0 mM, was adjusted to give the most intensity for all PCR products. Annealing temperature, ranging from 55˚C-61˚C, was optimized using a gradient thermal cycler. PCR reaction was carried out in a total volume of 20μl per reaction. Multiplex PCR amplification was performed on Thermalcycler (Applied Biosystems, USA) under the following conditions; 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, 55–61˚C for 1 min, 68/72˚C for 1 min, and final extension at 68˚C for 10 min.

Sensitivity and specificity of multiplex PCR

Limit of detection of multiplex PCR was determined using a tenfold serial dilution, ranging from $5x10^{0}$ to $5x10^{-6}$ ng/μl per reaction, of *Orientia*, *Rickettsia* and leptospiral DNA mixture.

Specificity of multiplex PCR was tested with unrelated pathogens such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella* spp., Dengue virus (serotype1-4), *Plasmodium falciparum*, and *Plasmodium vivax*. The efficiency of this assay was evaluated using masking conditions that mixes *Orientia*, *Rickettsia* or *Leptospira* with other bacteria.

Performance of multiplex PCR

Multiplex PCR was validated in blood samples of acute undifferentiated febrile illness patients compared to serological methods (either IFA or MAT). Diagnostic accuracy of this assay was measured in term of sensitivity, specificity, positive predictive value, and negative predictive value compared to the gold standard methods. The equations for calculating sensitivity, specificity, positive predictive value, and negative predictive value are shown in Table 2.

Gel electrophoresis

After PCR amplification, PCR products were loaded onto 2% agarose gel (Invitrogen, USA) in 0.5X TBE (Tris-borate with EDTA buffer) and separated by horizontal gel electrophoresis for

Table 2. List of the equations for calculating the performance of multiplex PCR.

	Equation
Sensitivity	$\frac{\text{true positive}}{\text{true positive+false negative}}$
Specificity	$\frac{\text{true negative}}{\text{true negative+false positive}}$
Positive predictive value	$\frac{\text{true positive}}{\text{true positive+false positive}}$
Negative predictive value	$\frac{\text{true negative}}{\text{true negative+false negative}}$

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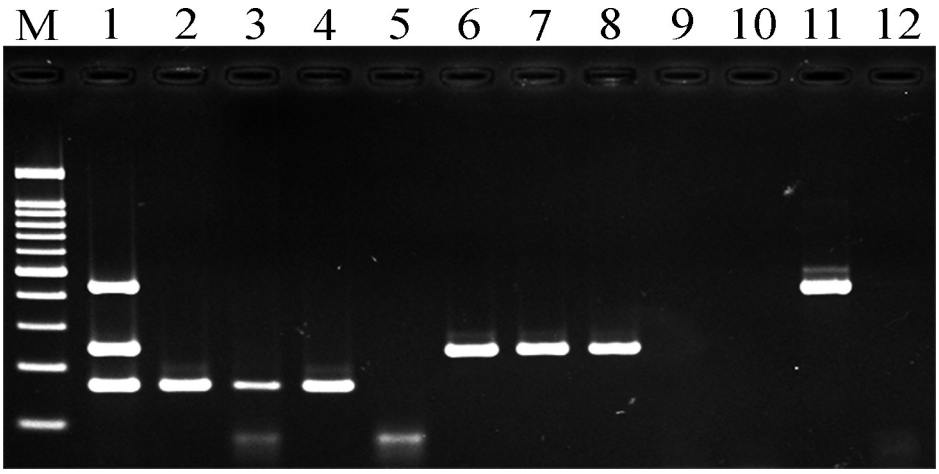

Fig 1. Optimization of singleplex and multiplex PCR. Lane M; 100bp marker, lane 1; DNA mixture derived from *Orientia* (166 bp), *Leptospira* (243 bp), and *Rickettsia* (434 bp) was amplified using multiplex PCR. Lane 2–4; *O. tsutsugamushi* (Karp, Kato, and Gilliam strains), lane 5; negative control, lane 6–8; *L. interrogans* (serovar Bratislava, Pyrogenes, and Pomona), lane 9; *L. biflexa* serovar Patoc, lane 10; negative control, lane 11; *R. typhi*, and lane 12; negative control. Lane 2–12; DNA was amplified using singleplex PCR.

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30 min at 100 volts. Then, agarose gel was stained in 0.5 μg/μl ethidium bromide (Sigma, USA) for 5 min and de-stained in deionized water for 10 min. Amplicons were visualized by UV transilluminator (Biorad, USA).

Results

Optimization of singleplex and multiplex PCR

In this experiment, optimal conditions were initially determined for singleplex PCR and, subsequently, for the multiplex PCR reaction. The results showed different sizes of PCR products containing a 166-bp fragment of *Orientia* DNA, 434-bp fragment of *Rickettsia* DNA, and 243-bp fragment of leptospiral DNA. In singleplex PCR, the 56-kDa TSA primer set was able to detect all strains of *O. tsutsugamushi* (Karp, Kato, and Gilliam strains). 17-kDa antigen primers could amplify DNA of *R. typhi*. LipL32 primers amplified pathogenic leptospiral DNA (serovar Bratislava, Pomona, and Pyrogenes), whereas this primer did not amplify non-pathogenic leptospiral DNA (*L. biflexa* serovar Patoc). (Fig 1).

Multiplex PCR was adjusted to ensure appropriate amplification of each target. The optimal extension temperature at 68˚C gave higher intensity of amplicons than at 72˚C. After magnesium chloride concentrations ranging from 1.5–4.0 mM were tested, the optimal concentration of magnesium chloride was found to be 2.5mM. For annealing temperature optimized using gradient PCR, the optimal annealing temperature of 61˚C was determined (Fig 1).

Specificity of multiplex PCR assay

To determine the specificity of multiplex PCR, we tested three primer pairs with other pathogens, including *E. faecalis*, *S. aureus*, *Salmonella* spp., *K. pneumoniae*, *E. coli*, Dengue viruses, *P. falciparum*, and *P. vivax*. No amplification was obtained from unrelated pathogens (Fig 2A). In addition, individual primer pair was able to amplify each target gene in the masking condition (Fig 2B).

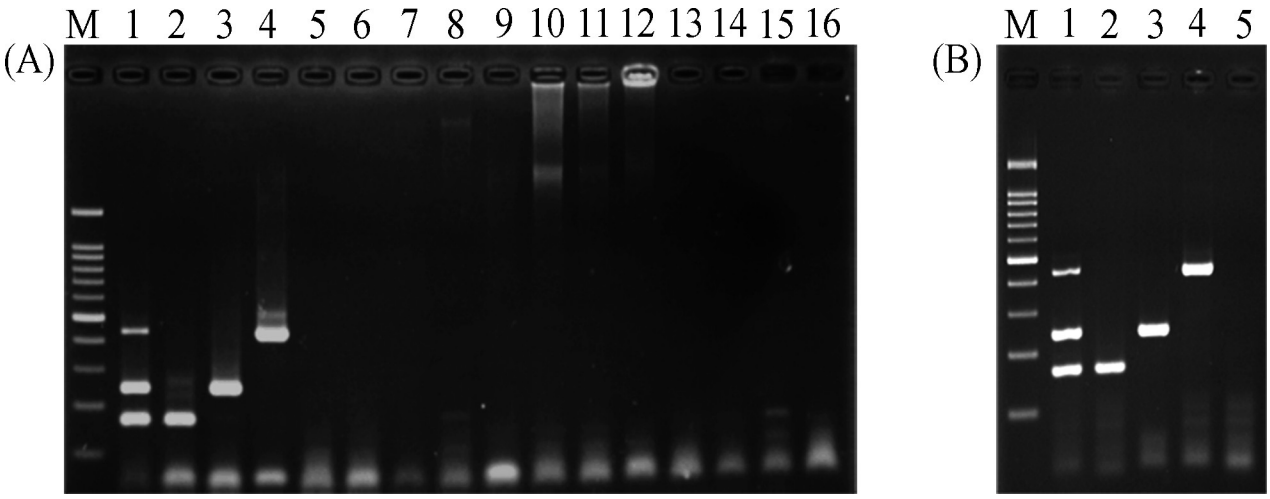

Fig 2. Specificity of multiplex PCR assay. (A) Multiplex PCR was tested with other pathogenic microorganisms. Lane M; 100bp marker, lane 1; Positive control (*Orientia*, *Leptospira*, and *Rickettsia* DNA mixture), lane 2; *O. tsutsugamushi* (166 bp), lane 3; *L. interrogans* (243 bp), lane 4; *R. typhi* (434 bp), lane 5; *E. faecalis*, lane 6; *S. aureus*, lane 7; *Salmonella* spp., lane 8; *K. pneumoniae*, lane 9; Dengue virus I, lane 10; Dengue virus II, lane 11; Dengue virus III, lane 12; Dengue virus IV, lane 13; *P. falciparum*, lane 14; *P. vivax*, lane 15; *E. coli*, and lane 16; negative control. (B) Multiplex PCR was tested in masking condition. Lane M; 100bp marker, lane 1; Positive control (*Orientia*, *Leptospira*, and *Rickettsia* DNA mixture), lane 2; DNA mixture of *O. tsutsugamushi*, *S. aureus*, and dengue virus, lane 3; DNA mixture of *L. interrogans*, *S. aureus*, and dengue virus, lane 4; DNA mixture of *R. typhi*, *S. aureus*, and dengue virus, lane 5; negative control.

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Sensitivity of multiplex PCR assay

In this experiment, we evaluated limit of detection using tenfold serial dilution, ranging from $5x10^{0}$ to $5x10^{-6}$ng/μl per reaction, of mixed DNA and mixed DNA-spiked blood samples. The results have shown that the lowest concentrations of mixed DNA were 0.5pg/μl or 230 copies for *O. tsutsugamushi*, 0.5pg/μl or 106 copies for *L. interrogans*, and 5pg/μl or 4,160 copies for *R. typhi* (Fig 3A). In contrast, the detection limit of mixed DNA-spiked blood samples was 0.5ng/μl for all targets (Fig 3B).

Performance of multiplex PCR assay

To validate efficiency of multiplex PCR assay, we performed multiplex PCR with 83 EDTA blood samples obtained from Thai patients presented with acute undifferentiated febrile illness. Multiplex PCR results were positive in 39 samples (47%), consisting of 22 samples of scrub typhus (26.5%), 11 samples of leptospirosis (13.25%), 5 samples of murine typhus (6.02%), and 1 sample of co-infection between scrub typhus and leptospirosis (1.2%). For comparison, standard serological methods results were positive in 20 samples (24.09%). The results are shown in Table 3 and Fig 4.

Twenty samples were detected by both serological methods and multiplex PCR. Nineteen samples were detected only by multiplex PCR but tested negative by standard serological methods. In addition, 1 in 39 samples exhibited co-infection between scrub typhus and leptospirosis. The diagnostic sensitivity and specificity of multiplex PCR were 100% and 70%, respectively, when serological methods were used as gold standard. Positive predictive value and negative predictive value of this assay were 51% and 100%, respectively.

Discussion

To our knowledge, this work is the first study to combine 3 primer pairs targeting 56-kDa TSA gene of *O. tsutsugamushi*, 17-kDa antigen gene of *R. Typhi*, and lipL32 gene of *L. interrogans*.

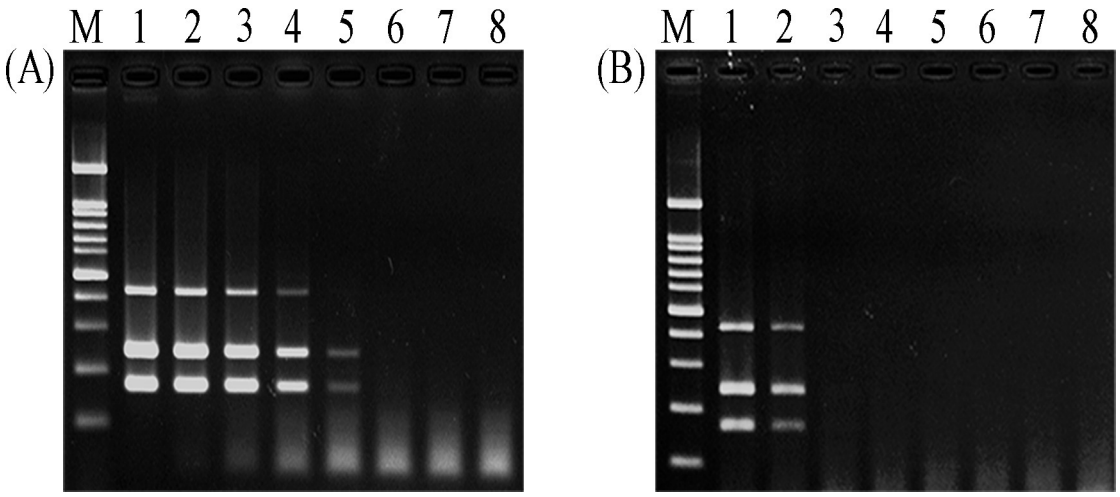

Fig 3. Sensitivity of multiplex PCR assay. (A) Limit of detection of multiplex PCR with a serial dilution of *Orientia*, *Leptospira*, and *Rickettia* DNA mixture, and (B) limit of detection with three bacterial DNA-spiked blood sample in different concentration. In both figures: lane M; 100bp marker, lane 1; 5 ng/μl, lane 2; 0.5 ng/μl, lane 3; 50 pg/μl, lane 4; 5 pg/μl, lane 5; 0.5 pg/μl, lane 6; 50 fg/μl, lane 7; 5 fg/μl, and lane 8; negative control.

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Our results showed that this multiplex PCR assay was able to specifically detect each bacterial DNA target. The primer sets did not cross-react with other bacteria. Moreover, we validated this assay using EDTA blood samples from patients with acute undifferentiated febrile illness. Sensitivity and specificity of the multiplex PCR assay developed in this study were 100% and 70%, respectively.

In the present, standard diagnostic tests for common causes of acute differentiated fever in Thailand are based on antibody detection, such as MAT for leptospirosis and IFA for both scrub typhus and murine typhus. However, these methods require paired sera, extensive labor, and specialized facilities only available at reference laboratories [18, 21]. The delayed diagnosis and treatment may result in severity, complication, and mortality. Therefore, a rapid diagnostic test is necessary for early pathogen detection. Multiplex PCR can be completed within 5 hours, reducing turnaround time for pathogen identification [22, 23]. Previous studies reported conventional PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP) for diagnosis of scrub typhus, murine typhus, and leptospirosis [2, 24]. However, multiplex PCR for simultaneous detection of these three neglected tropical diseases has never been reported. Multiplex PCR is faster and cheaper than conventional PCR for individual disease and serological methods [25]. In addition, this assay is easier and more convenient

Table 3. Comparison of multiplex PCR results and standard serological test results.

Methods/Results			Serological test (either IFA or MAT test)		Total
			Positive	Negative	
Multiplex PCR	Positive	Scrub typhus	13	9	39
		leptospirosis	6	5	
		Murine typhus	1	4	
		Co-infection	0	1	
	Negative		0	44	44
	Total		20	63	83

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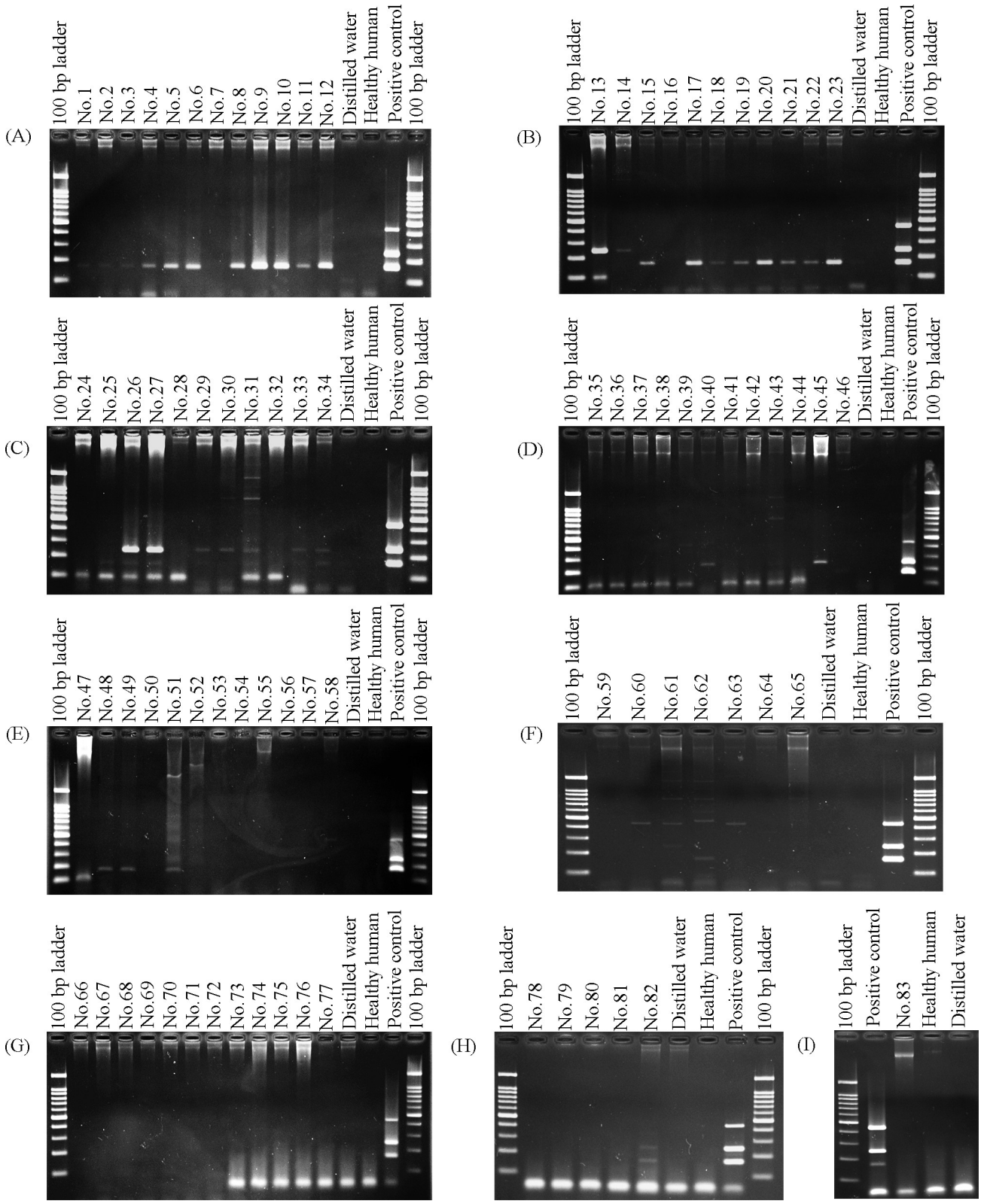

Fig 4. Performance of the multiplex PCR. (A-H) showed the multiplex PCR results when tested with blood specimens from Thai patients presented with acute undifferentiated febrile illness.

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than nested PCR as well as LAMP. Multiplex PCR also reduces the risk of contamination between amplification [22]. Thus, multiplex PCR can be a practical and reliable diagnostic tool for multiple pathogens detection.

Here, we developed multiplex PCR assay that could be used for diagnosis of three neglected diseases in a single tube. Our results have shown that the extension temperature of 68˚C gave optimal result compared to 72˚C. These bacterial DNA are highly AT-rich (65–70%), so the reduction in extension temperature can greatly increase PCR amplification for AT-rich templates [26–29]. On the other hand, extension temperature at 72˚C prevents DNA synthesis at highly AT-rich region [30]. Because having multiple primer pairs in a single tube negatively affect amplification efficiency, further optimization is crucial. Optimal Mg^{2+} concentration is necessary for *Taq* DNA polymerase and specificity of primer-template binding. Inadequate Mg^{2+} concentration decreases PCR product yield and specificity due to incorrect primer-template binding [31]. The Mg^{2+} concentration for our assay was optimal at a final concentration of 2.5 mM. Using a gradient thermal cycler, annealing temperature was optimized for sensitivity and specificity of primer-template binding [32]. Although AT-rich templates require lower annealing temperature [28], 61˚C proved to be optimal for this multiplex amplification.

The limit of detection of our multiplex PCR were 0.5 pg/μl or 230 copies for *O. tsutsugamushi*, 0.5 pg/μl or 106 copies for *L. interrogans*, and 5 pg/μl or 4,160 copies for *R. typhi*. Detection limits of this multiplex PCR were comparable to that of previous studies that detected *O. Tsutsugamushi*, *R. Typhi*, and *L. Interrogans* using singleplex PCR [5, 33, 34]. The limit of detection of multiplex PCR depends on the followings: 1) combination of multiple primers in a single tube that might affect effective amplification; 2) product size, because amplification is more effective in case of smaller product [15, 35]. The higher limit of detection in mixed bacterial DNA-spiked blood samples, 0.5ng/μl, might be a result of PCR inhibitors, such as hemoglobin and other components in blood [36, 37].

Orientia and *Rickettsia* are obligate intracellular bacteria that can be found in buffy coat of blood specimens [2]. However, buffy coat is more difficult to collect than blood samples. Fortunately, leptospires could be found in bloodstream during the acute phase [18]. Previous studies had also reported detection of *Orientia*, *Rickettsia*, and *Leptospira* DNA in blood samples [2, 38]. In this study, we used EDTA blood samples for multiplex PCR validation. Thirty-nine samples (47%) were found to be positive by multiplex PCR, whereas 20 samples (24.09%) were positive by standard serological tests. Nineteen of the 39 samples, which tested positive in multiplex PCR analysis, exhibited negative serological results. These results may be explained by the period of blood collection and the phase of diseases. In the early phase of infection, bacteremia might not induce sufficient antibody level for serological detection [39–41]. Our study suggests that these 19 blood samples were bacteremic and, inferably, collected during the early phase of infection. Consequently, the multiplex PCR was able to detect bacterial DNA in the blood specimens, even though serological tests showed negative results. One sample exhibited dual positive of *O. tsutsugamushi* and *L. interrogans* in multiplex PCR assay, while tested negative in serological analyses. Previous studies had reported co-infection between *O. tsutsugamushi* and pathogenic *Leptospira* spp. in Thai and Taiwanese patients with acute undifferentiated fever [42, 43]. This study suggests that multiplex PCR can be applied in clinical diagnostic test for identifying pathogenic agents that cause scrub typhus, leptospirosis, murine typhus, and co-infections.

This multiplex PCR is a useful tool for early diagnosis of scrub typhus, murine typhus, and leptospirosis. Moreover, the combination of multiplex PCR and serological assays helps increase sensitivity for diagnosis and confirmation. However, sensitivity and specificity determined in this study are only preliminary measurements due to limited sample size. Further study should be performed using a large number of clinical samples.

In conclusion, we developed a novel multiplex PCR assay for identifying causative agents of scrub typhus, murine typhus, and leptospirosis in blood samples. This method is a rapid, sensitive, and specific diagnostic test. The multiplex PCR assay will become useful for the development of better health care and treatment of patients presented with acute undifferentiated febrile illness, particularly in endemic areas of these diseases.

Author Contributions

Conceptualization: Nutchanart Sea-liang, Amornpun Sereemaspun, Kanitha Patarakul.

Formal analysis: Nutchanart Sea-liang.

Funding acquisition: Supaporn Wacharaplusadee, Thiravat Hemachudha.

Methodology: Nutchanart Sea-liang.

Project administration: Amornpun Sereemaspun.

Resources: Kanitha Patarakul, Jariyanart Gaywee, Wuttikon Rodkvamtook, Nattachai Srisawat, Supaporn Wacharaplusadee, Thiravat Hemachudha.

Validation: Nutchanart Sea-liang.

Writing – original draft: Nutchanart Sea-liang.

Writing – review & editing: Amornpun Sereemaspun, Kanitha Patarakul.

References

1. Chikeka I, Dumler JS. Neglected bacterial zoonoses. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2015; 21(5):404–15.
2. Luce-Fedrow A MK, Kostik AP, St John HK, Jiang J, Richards AL. Strategies for detecting rickettsiae and diagnosing rickettsial diseases. Future Microbiology. 2015; 10(4):537–64. https://doi.org/10.2217/fmb.14.141 PMID: 25865193
3. Carr SB, Bergamo DF, Emmanuel PJ, Ferreira JA. Murine typhus as a cause of cognitive impairment: case report and a review of the literature. Pediatric neurology. 2014; 50(3):265–8. https://doi.org/10.1016/j.pediatrneurol.2013.09.017 PMID: 24321542
4. Langoni H, Ullmann LS, Richini-Pereira VB, Ruiz LdS, Sánchez GP, Lucheis SB. Diagnostic methods for the detection of Leptospira spp. in biological samples. Veterinária e Zootecnia. 2017; 24(1):11–20.
5. Paris DH, Dumler JS. State of the art of diagnosis of rickettsial diseases: the use of blood specimens for diagnosis of scrub typhus, spotted fever group rickettsiosis, and murine typhus. Current opinion in infectious diseases. 2016; 29(5):433–9. https://doi.org/10.1097/QCO.0000000000000298 PMID: 27429138
6. Pal M, Hadush A. Leptospirosis: An Infectious Emerging Waterborne Zoonosis of Global Significance. Air & Water Borne Diseases. 2017; 06(01).
7. Rajapakse S, Weeratunga P, Sivayoganathan S, Fernando SD. Clinical manifestations of scrub typhus. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2017; 111(2):43–54. https://doi.org/10.1093/trstmh/trx017 PMID: 28449088
8. Sakamoto N, Nakamura-Uchiyama F, Kobayashi K, Takasaki T, Ogasawara Y, Ando S, et al. Severe murine typhus with shock and acute respiratory failure in a Japanese traveler after returning from Thailand. Journal of travel medicine. 2013; 20(1):50–3. https://doi.org/10.1111/j.1708-8305.2012.00678.x PMID: 23279232
9. Botelho-Nevers E, Raoult D. Fever of unknown origin due to rickettsioses. Infectious disease clinics of North America. 2007; 21(4):997–1011, ix. https://doi.org/10.1016/j.idc.2007.08.002 PMID: 18061086
10. Owen JL, Yang T, Mohamadzadeh M. New insights into gastrointestinal anthrax infection. Trends in molecular medicine. 2015; 21(3):154–63. https://doi.org/10.1016/j.molmed.2014.12.003 PMID: 25577136
11. Blanton LS, Walker DH. Flea-Borne Rickettsioses and Rickettsiae. The American journal of tropical medicine and hygiene. 2017; 96(1):53–6. https://doi.org/10.4269/ajtmh.16-0537 PMID: 27799640

12. Chirathaworn C, Kongpan S. Immune responses to Leptospira infection: roles as biomarkers for disease severity. The Brazilian journal of infectious diseases: an official publication of the Brazilian Society of Infectious Diseases. 2014; 18(1):77–81.

13. Hsu YH, Chou SJ, Chang CC, Pan MJ, Yang WC, Lin CF, et al. Development and validation of a new loop-mediated isothermal amplification for detection of pathogenic Leptospira species in clinical materials. Journal of microbiological methods. 2017; 141:55–9. https://doi.org/10.1016/j.mimet.2017.07.010 PMID: 28756184

14. Theunissen C, Cnops L, Van Esbroeck M, Huits R, Bottieau E. Acute-phase diagnosis of murine and scrub typhus in Belgian travelers by polymerase chain reaction: a case report. BMC infectious diseases. 2017; 17(1):273. https://doi.org/10.1186/s12879-017-2385-x PMID: 28407761

15. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. Multiplex PCR: critical parameters and step-by-step protocol. BioTechniques. 1997; 23(3):504–11. https://doi.org/10.2144/97233rr01 PMID: 9298224

16. Markoulatos P, Siafakas N, Moncany M. Multiplex polymerase chain reaction: a practical approach. Journal of clinical laboratory analysis. 2002; 16(1):47–51. https://doi.org/10.1002/jcla.2058 PMID: 11835531

17. Law JW, Ab Mutalib NS, Chan KG, Lee LH. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Frontiers in microbiology. 2014; 5:770. https://doi.org/10.3389/fmicb.2014.00770 PMID: 25628612

18. Jain M, Nigam R, Malik R. A REVIEW OF LABORATORY TECHNIQUES FOR DETECTING LEPTOSPIROSIS. International Journal of Current Pharmaceutical Research. 2015; 7(3).

19. Webb L, Carl M, Malloy DC, Dasch GA, Azad AF. Detection of murine typhus infection in fleas by using the polymerase chain reaction. Journal of clinical microbiology. 1990; 28(3):530–4. PMID: 2108995

20. Bourhy P, Bremont S, Zinini F, Giry C, Picardeau M. Comparison of real-time PCR assays for detection of pathogenic Leptospira spp. in blood and identification of variations in target sequences. Journal of clinical microbiology. 2011; 49(6):2154–60. https://doi.org/10.1128/JCM.02452-10 PMID: 21471336

21. Watthanaworawit W, Turner P, Turner C, Tanganuchitcharnchai A, Richards AL, Bourzac KM, et al. A prospective evaluation of real-time PCR assays for the detection of Orientia tsutsugamushi and Rickettsia spp. for early diagnosis of rickettsial infections during the acute phase of undifferentiated febrile illness. The American journal of tropical medicine and hygiene. 2013; 89(2):308–10. https://doi.org/10.4269/ajtmh.12-0600 PMID: 23732256

22. Tantibhedhyangkul W, Wongsawat E, Silpasakorn S, Waywa D, Saenyasiri N, Suesuay J, et al. Use of Multiplex Real-Time PCR To Diagnose Scrub Typhus. Journal of clinical microbiology. 2017; 55 (5):1377–87. https://doi.org/10.1128/JCM.02181-16 PMID: 28202789

23. Zhang H, Morrison S, Tang YW. Multiplex polymerase chain reaction tests for detection of pathogens associated with gastroenteritis. Clinics in laboratory medicine. 2015; 35(2):461–86. https://doi.org/10.1016/j.cll.2015.02.006 PMID: 26004652

24. Schreier S, Doungchawee G, Chadsuthi S, Triampo D, Triampo W. Leptospirosis: current situation and trends of specific laboratory tests. Expert review of clinical immunology. 2013; 9(3):263–80. https://doi.org/10.1586/eci.12.110 PMID: 23445200

25. Deshmukh RA, Joshi K, Bhand S, Roy U. Recent developments in detection and enumeration of waterborne bacteria: a retrospective minireview. MicrobiologyOpen. 2016; 5(6):901–22. https://doi.org/10.1002/mbo3.383 PMID: 27397728

26. Adler B, de la Pena Moctezuma A. Leptospira and leptospirosis. Veterinary microbiology. 2010; 140(3–4):287–96. https://doi.org/10.1016/j.vetmic.2009.03.012 PMID: 19345023

27. Liao HM, Chao CC, Lei H, Li B, Tsai S, Hung GC, et al. Genomic Sequencing of Orientia tsutsugamushi Strain Karp, an Assembly Comparable to the Genome Size of the Strain Ikeda. Genome Announcements. 2016; 4(4).

28. Lopez-Barragan MJ, Quinones M, Cui K, Lemieux J, Zhao K, Su XZ. Effect of PCR extension temperature on high-throughput sequencing. Molecular and biochemical parasitology. 2011; 176(1):64–7. https://doi.org/10.1016/j.molbiopara.2010.11.013 PMID: 21112355

29. McLeod MP, Qin X, Karpathy SE, Gioia J, Highlander SK, Fox GE, et al. Complete Genome Sequence of Rickettsia typhi and Comparison with Sequences of Other Rickettsiae. Journal of Bacteriology. 2004; 186(17):5842–55. https://doi.org/10.1128/JB.186.17.5842-5855.2004 PMID: 15317790

30. Su XZ, Wu Y, Sifri CD, Wellems TE. Reduced extension temperatures required for PCR amplification of extremely A+T-rich DNA. Nucleic acids research. 1996; 24(8):1574–5. https://doi.org/10.1093/nar/24.8.1574 PMID: 8628694

31. Settanni L, Corsetti A. The use of multiplex PCR to detect and differentiate food- and beverage-associated microorganisms: a review. Journal of microbiological methods. 2007; 69(1):1–22. https://doi.org/10.1016/j.mimet.2006.12.008 PMID: 17280731

32. Markoulatos P, Siafakas N, Katsorchis T, Moncany M. Multiplex PCR: rapid DNA cycling in a conventional thermal cycler. Journal of clinical laboratory analysis. 2003; 17(4):108–12. https://doi.org/10.1002/jcla.10082 PMID: 12784258

33. Saisongkorh W, Chenchittikul M, Silpapojakul K. Evaluation of nested PCR for the diagnosis of scrub typhus among patients with acute pyrexia of unknown origin. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2004; 98(6):360–6. https://doi.org/10.1016/j.trstmh.2003.10.012 PMID: 15099992

34. Blanco RM, Romero EC. Evaluation of nested polymerase chain reaction for the early detection of Leptospira spp. DNA in serum samples from patients with leptospirosis. Diagnostic microbiology and infectious disease. 2014; 78(4):343–6. https://doi.org/10.1016/j.diagmicrobio.2013.12.009 PMID: 24445157

35. Wei S, Zhao H, Xian Y, Hussain MA, Wu X. Multiplex PCR assays for the detection of Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio vulnificus, and Vibrio cholerae with an internal amplification control. Diagnostic microbiology and infectious disease. 2014; 79(2):115–8. https://doi.org/10.1016/j.diagmicrobio.2014.03.012 PMID: 24731836

36. Janardhanan J, Trowbridge P, Varghese GM. Diagnosis of scrub typhus. Expert review of anti-infective therapy. 2014; 12(12):1533–40. https://doi.org/10.1586/14787210.2014.974559 PMID: 25359599

37. Sonthayanon P, Chierakul W, Wuthiekanun V, Blacksell SD, Pimda K, Suputtamongkol Y, et al. Rapid diagnosis of scrub typhus in rural Thailand using polymerase chain reaction. The American journal of tropical medicine and hygiene. 2006; 75(6):1099–102. PMID: 17172374

38. Budihal SV, Perwez K. Leptospirosis diagnosis: competancy of various laboratory tests. Journal of clinical and diagnostic research: JCDR. 2014; 8(1):199–202. https://doi.org/10.7860/JCDR/2014/6593.3950 PMID: 24596774

39. Karuniawati A, Yasmon A, Ningsih I. Optimizing real-time PCR method to detect Leptospira spp. in human blood and urine specimens. Medical Journal of Indonesia. 2012; 21(1):13–7.

40. Cheemaa PS, Srivastava SK, Amutha R, Singh S, Singh H, Sandey M. Detection of pathogenic leptospires in animals by PCR based on lipL21 and lipL32 genes. Indian journal of experimental biology. 2007; 45(6):568–73. PMID: 17585694

41. Chao CC, Belinskaya T, Zhang Z, Ching WM. Development of Recombinase Polymerase Amplification Assays for Detection of Orientia tsutsugamushi or Rickettsia typhi. PLoS Neglected Tropical Diseases. 2015; 9(7).

42. Lee CH, Liu JW. Coinfection with leptospirosis and scrub typhus in Taiwanese patients. The American journal of tropical medicine and hygiene. 2007; 77(3):525–7. PMID: 17827372

43. Sonthayanon P, Chierakul W, Wuthiekanun V, Limmathurotsakul D, Amornchai P, Smythe LD, et al. Molecular confirmation of co-infection by pathogenic Leptospira spp. and Orientia tsutsugamushi in patients with acute febrile illness in Thailand. The American journal of tropical medicine and hygiene. 2013; 89(4):797–9. https://doi.org/10.4269/ajtmh.13-0402 PMID: 24002486