Evaluation of Combined Rapid Immunoglobulin M and Immunoglobulin G Lateral Flow Assays for the Diagnosis of Leptospirosis, Scrub Typhus, and Hantavirus Infection

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ABSTRACT

Objective: Leptospirosis, scrub typhus, and hantavirus infection are commonly identified as causes of acute undifferentiated fever in rural parts of Asia. Although the characteristic presentations of these infections are well described, many of them present with nonspecific manifestations. Diagnosis is usually made by combined history of exposure, clinical features and positive antibody detection. The development of rapid antibody detection assay, using an immunochromatographic test (ICT) for the diagnosis of multi-diseases, has provided tools for more accurate diagnosis and appropriate antibiotic treatment of the acute undifferentiated fever syndrome.

Methods: We evaluated the diagnostic performance of a commercially available combined rapid ICT for the diagnosis of leptospirosis, scrub typhus, and hantavirus infection, using archived blood samples from 434 patients with laboratory-confirmed leptospirosis (131) or scrub typhus (128), and from patients with other causes of fever as the negative control (175). Polysaccharide of nonpathogenic *Leptospira patoc*, a chimeric recombinant protein cr56 and two other recombinant proteins, r21 and kr56, from different serotypes of *Orientia tsutsugamushi*, and 21kDa species-specific antigen and recombinant CNP antigen derived from the Soochong virus were used as antigens for the diagnosis of leptospirosis, scrub typhus, and hantavirus infection in the combined ICT used in this study.

Results: For the diagnosis of leptospirosis; in acute phase, the sensitivity and specificity of the ICT detection of IgM/IgG were 38.2% (95% CI, 29.9- 46.5%), and 99.0% (95% CI 97.9-100%); while in convalescent phase, the same were 84.6% (95%CI, 77.1- 92.0%), and 96.2% (95%CI, 92.5- 99.8%), respectively. For scrub typhus, in acute phase, the sensitivity and specificity of the ICT detection of IgM/IgG were 71.9% (95% CI, 64.1- 79.7%), and 97.4% (95% CI 95.6 - 99.2%); while in convalescent phase, the same were 84.6% (95%CI, 74.8- 94.4%), and 90.2% (95%CI, 85.3- 95.1%) respectively. For hantavirus infection, nine patients had detectable IgM for hantavirus infection. All these cases were diagnosed as scrub typhus by indirect immunofluorescent assay.

Conclusion: The performance of this combined ICT for leptospirosis and scrub typhus were comparable to those published data of other ICTs. However, the rapid test for the diagnosis of leptospirosis, using antigen detection, is needed. Hantavirus infection was not detected in this study population.

Keywords: Immunochromatographic assay; leptospirosis; scrub typhus; hantavirus (Siriraj Med J 2020; 72: 253-258)

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INTRODUCTION

Acute undifferentiated febrile illness is a leading cause of hospital visit among adults in rural areas of South–East Asia. The common causes of such fever include scrub typhus, murine typhus, leptospirosis, and hantavirus infection.[1,2] Leptospirosis is a worldwide zoonotic disease, caused by pathogenic members of the genus *Leptospira*.[3] Human infection occurs through the direct or indirect exposure to the organism excreted in the urine of both wild and domestic mammals. Scrub typhus is the most common rickettsial infection in the Asia- Pacific region. It is caused by *Orientia tsutsugamushi*, an obligate intracellular Gram-negative bacterium.[4] Human accidentally infected through the bite of the infected chiggers. Over a billion people are at risk of scrub typhus and approximately one million cases occur annually.[4,5] In Thailand, scrub and leptospirosis are the main causes of acute undifferentiated fever, after dengue infection is excluded.[1,6]

Hemorrhagic fever with renal syndrome (HFRS) is caused by various hantaviruses in the genus Hantavirus of the family Bunyaviridae. Seoul virus (Seoul orthohantavirus, SEOV), the only species of the genus Hantavirus that is found to be globally spread as hantavirus, is a common cause of HFRS. HFRS is a viral zoonosis transmitted by rodents.[7] To date, only 2 patients with hantavirus infection have been reported in Thailand.[8,9] However, the incidence of HFRS could be underestimated in Thailand due to the unavailability of a diagnostic test.

Although the characteristic clinical presentations of leptospirosis, scrub typhus, and HFRS are well described, many patients present with protean and nonspecific symptoms and signs.[2] Consequently, the diagnoses of either of these infections are usually made by combination of a history of exposure, well recognized symptoms and signs, and positive antibody detection.[5,6] In addition, hantavirus infection and leptospirosis can share similar clinical and exposure risks.[10] The availability of rapid antibody tests using an immunochromatographic test (ICT) has provided tools for point-of-care serologic testing. ImmuneMed AFI Rapid® is one such commercially available rapid ICT for the qualitative detection of both IgM and IgG antibodies to hantavirus, *O. tsutsugamushi*, and *Leptospira spp*. in a patient's serum, plasma, or whole blood. In this study, we conducted the study to determine the diagnostic performance of this assay, using the stored serum/ plasma samples of patients who presented with an acute febrile illness caused by leptospirosis, scrub typhus, or other diagnoses.

MATERIALS AND METHODS

Patients with leptospirosis or scrub typhus

Blood samples (n=259) were collected from patients (male : female = 2 :1), aged 15- 84 years old (median age 45 years old) who presented with acute febrile illness at four hospitals in Thailand between January 2000 and December 2018. Three hospitals are located in the northeastern region of the country (Maharaj Nakhon Ratchasima Hospital, Loei Provincial Hospital and Banmai Chaiyapod Hospital, Burirum Province). In these hospitals, blood samples were collected as part of the epidemiological and clinical studies of patients with acute undifferentiated fever.[6,11,12] Included in these studies were adult patients (>18 years) who presented with acute fever (oral temperature, >38.0°C for less than 15 days) in the absence of an obvious focus of infection. Blood samples were also collected from patients, using the same inclusion criteria, at Siriraj Hospital, Bangkok, Thailand. All of these clinical studies were conducted after the approval of the Ethical Review Subcommittee, Public Health Ministry of Thailand and the Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University (Si 014/2019). All patients provided the informed written consent before enrollment to the study. Blood samples were collected on the day of admission, and/or during convalescence or after discharge from the hospital. Plasma was divided into that needed for immediate use and a leftover sample was stored at –70°C.

Non-scrub typhus and non-leptospirosis samples

Blood samples (n= 175) from patients with other tropical febrile illnesses (laboratory-confirmed) were collected from the same hospitals as the patients with leptospirosis and scrub typhus. All of these samples were tested by indirect immunofluorescent assay (IFA) and were shown to be negative for *O. tsutsugamushi* and *Leptospira*. The diagnoses of patients in this control group were dengue infection in 59 patients; zika virus infection in 16 patients; influenza A or influenza B in 26 patients; murine typhus in 60 patients; other bacterial infections, such as Escherichia coli septicemia, melioidosis, and salmonellosis in 8 patients; and *Plasmodium falciparum* malaria in 6 patients.

The IFA assay for the laboratory confirmation of leptospirosis, scrub typhus, and murine typhus was performed as described previously.[6,11,12] The *Leptospira interrogans*, serovar autumnalis; pooled *O. tsutsugamushi* from Karp, Kato and Gilliam strains; and Rickettsia typhi (Wilmington strain) were used as the antigens for the detection of IgM and IgG antibodies for the

diagnosis of leptospirosis, scrub typhus, and murine typhus respectively. Samples with inconclusive IFA results such as suspected co-infection or low antibody titers were not included in this study.

Combined ICT for leptospirosis, scrub typhus, and hantavirus infection

Polysaccharide of nonpathogenic *Leptospira patoc*, a chimeric recombinant antigen cr56 and two other recombinant antigens, r21 and kr56, from various serotypes of *O. tsutsugamushi*, and 21kDa genus-specific protein and recombinant CNP protein derived from the Soochong virus of the genus Hantavirus were used as the antigens for the detection of IgM/ IgG for the diagnosis of leptospirosis, scrub typhus, and hantavirus infection respectively.[13-15] The ImmuneMed AFI Rapid® (ImmuneMed, Inc., South Korea) test was performed according to the manufacturer's instructions. In brief, approximately 3 μL of serum was applied to the ICT sample well, and then approximately 7 drops (300 μL) of the sample diluent was added into the sample well immediately. Results of the assay were interpreted visually at 15 minutes, as either negative if only the control band was stained or as positive when both the test and control bands were clearly stained (Fig 1).

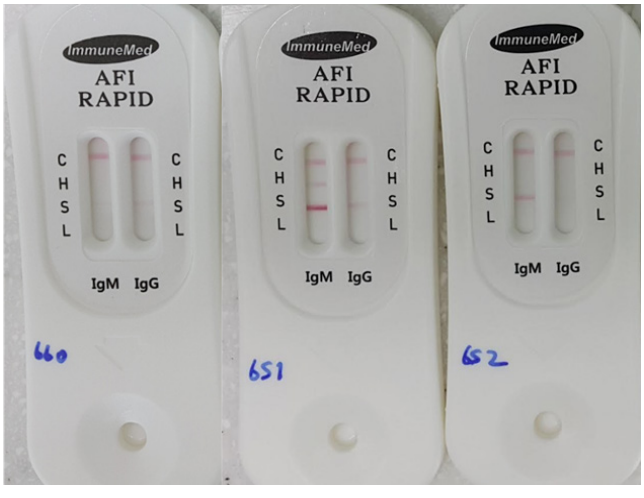

Fig 1. Examples of the visually interpretation of results from three patients with negative result (no 660, left panel), positive band for both scrub typhus IgM, IgG and hantavirus IgM (no 651, middle panel), and positive scrub typhus IgM (no 652, right panel). C, H, S, L represented control, hantavirus, scrub typhus, and leptospirosis respectively.

Data analysis

The diagnostic performance was determined by comparing the IgM and IgG ICT results with the result from the IFA for each patient. The diagnostic criterion for the reference IFA assay for scrub typhus and leptospirosis was either an IgM or IgG IFA assay titer ≥ 1:400 at an acute phase sample or a four-fold increase between paired acute and convalescent phase samples. Inconclusive results were considered negative in the statistical analysis. A two-by-two table was constructed, in which the IFA results, as a gold standard test were cross-tabulated with the ICT assay result to calculate the percentage of true-positive, false-positive, false-negative, and true-negative results. The standard diagnostic accuracy indices of the sensitivity and specificity with 95% confidence intervals (CIs) were calculated, using the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Overall there were 434 patients included in this study, with the median duration of fever was 4 days (ranged from 3 to 14 days). Of these 193 patients' convalescent samples were available. The median duration between acute and convalescent periods of 10 days (IQR, 6 to 14 days). Among 131 patients with laboratory-confirmed leptospirosis, convalescent samples were available in 91 patients. In acute- phase samples, the sensitivities of the ICT tests for the detection of IgM and IgG antibodies for the diagnosis of leptospirosis were 37.4% (95% CI, 29.1- 45.7%), and 9.2% (95% CI 4.3-14.1%) respectively (Table 1). The specificities of IgM and IgG were 99.0% (95%CI, 97.9- 100%), and 100% (95%CI, 88.2-100%) respectively. False positive IgM/IgG antibody detection was found in only 3 patients (in 2 patients with scrub typhus, and 1 patient with murine typhus) among 303 acute patients. The sensitivities of the test were improved to 84.6% in the convalescent phase, with false positive IgM/IgG antibody detection in 4 patients (3 patients with influenza infection) among 104 convalescent patients.

Among 128 patients with IFA-confirmed scrub typhus, convalescent samples were available in 52 patients. The sensitivities of the ICT tests for the detection of IgM and IgG antibodies against *O. tsutsugamushi* in the acute- phase samples were 68.0% (95% CI, 55.9- 76.1%), and 41.4% (95% CI 32.9- 49.9%) respectively. In acute-phase, the specificities of IgM and IgG antibodies against *O. tsutsugamushi* were 97.4% (95%CI, 95.6-99.2%), and 99.7% (95%CI, 99.1-100%) respectively. False positive IgM/IgG antibody detection was found in 8 patients (in 7 patients with leptospirosis and in 1 patient with *S. aureus* bacteremia) among 306 acute patients. The sensitivities of this ICT test was improved as 84.6% when the test was performed using convalescent samples. In the convalescent samples, false positive IgM/ IgG antibody detection was found in 14 patients, comprising; 12 patients with leptospirosis (3 of them also had a positive IFA for scrub

TABLE 1. Sensitivities and specificities of the ICT for the diagnosis of leptospirosis and scrub typhus in acute and convalescent samples.

	Acute phase samples			Convalescent phase samples		
	IgM	IgG	IgM/IgG	IgM	IgG	IgM/IgG
Leptospirosis	49/131	12/131	50/131	77/91	43/91	77/91
Others	3/303	0/303	3/303	4/104	0/104	4/104
Sensitivity, %	37.4	9.2	38.2	84.6	47.3	84.6
95%CI	29.1-45.7	4.3-14.1	29.9- 46.5	77.1-92	37-57.5	77.1-92.0
Specificity, %	99.0	100	99.0	96.2	100	96.2
95%CI	97.9-100	88.2-100	97.9-100	92.5- 99.8	NA	92.5- 99.8
Scrub typhus	87/128	53/128	92/128	44/52	32/52	44/52
Others	8/306	1/306	8/306	14/143	1/143	14/143
Sensitivity, %	68.0	41.4	71.9	84.6	61.5	84.6
95%CI	55.9-76.1	32.9-49.9	64.1-79.7	74.8-94.4	48.3-74.7	74.8-94.4
Specificity, %	97.4	99.7	97.4	90.2	99.3	90.2
95%CI	95.6-99.2	99.1-100	95.6-99.2	85.3-95.1	97.9-100	85.3-95.1

typhus), and 2 patients with influenza infection, among 143 convalescent patients. Details of the sensitivities and specificities of IgM/IgG for the diagnosis of leptospirosis and scrub typhus, in the acute and convalescent samples are shown in Table 1.

For hantavirus infection, overall there were 9 patients with a positive IgM test, comprising 2 patients who were positive in both acute and convalescent samples, 4 patients who were negative in the acute phase but positive in the convalescent sample, and 3 patients who were positive in only acute samples. None of the acute and convalescent tested positive for IgG. All patients who had positive IgM for hantavirus infection also had positive IgM for scrub typhus. All the patients were empirically treated with oral doxycycline and became afebrile during follow-up. As an example, one woman among the positive patients to hantavirus but who was negative to dengue IgM/IgG had fever for 5 days with a normal white blood cell count, but increased in atypical lymphocytes, and thrombocytopenia. This patient fully recovered after the treatment with doxycycline. Only an acute- phase sample was available in this patient. Her IFA IgM/ IgG antibody titers against *O. tsutsugamushi* were 1:800 and 1:200, respectively. These positive hantavirus IgM samples were retested by another IFA for the detection of hantavirus infection at the ImmuneMed laboratory, Korea. None of them was confirmed hantavirus infection with the second IFA.

DISCUSSION

Among patients with non-malaria fever, leptospirosis and scrub typhus were diagnosed in approximately 10% to 30% of them.[1,6,7] The awareness and an early diagnosis of both leptospirosis and scrub typhus has impact on choice of antibiotic treatment during the acute phase of illness. Empirical treatment with oral doxycycline is considered to be the most cost-effective strategy for the initial treatment of patients with clinically suspected leptospirosis or scrub typhus.[16] However, in the absence of rapid and reliable laboratory tests for both diseases, misdiagnosis and delayed appropriate patient management occur frequently. Consequently, there is an urgent need for a more accurate and easy to perform point-of-care leptospirosis and scrub typhus diagnostics.

For the syndromic approach of acute fever, this rapid ICT for the simultaneous detection of IgM/IgG for the diagnosis of leptospirosis, scrub typhus, and hantavirus infection demonstrated comparable sensitivity and specificity to the previously reported individual ICTs for either leptospirosis[17] or scrub typhus.[18]

The results of this study confirmed that in the acute phase of leptospirosis (less than 7 days of illness), serological diagnosis using either IgM/IgG detection is not sensitive. The antibody against *Leptospira spp*. only become detectable in the late acute phase of the disease.[17] Molecular diagnosis by either conventional or real- time polymerase chain reaction is the most common laboratory test for the confirmation of leptospirosis in this early phase. The main use of serological testing is for confirmation of a diagnosis of leptospirosis when the convalescent sample is available. Therefore, an ICT for the detection of IgG/IgM against *Leptospira spp*. alone might not be cost- effective for routine implementation. Thus rapid point- of- care for antigen detection is urgently needed for the early diagnosis of leptospirosis.

The assay demonstrated better sensitivity for the diagnosis of scrub typhus than leptospirosis in the acute phase of infection. *O. tsutsugamushi* re-infection is not uncommon in endemic areas of scrub typhus.[5] The pattern of antibody response to Orientia re-infection mimics that found in those who have had re-infection by dengue virus. Therefore, the detection of both IgM and IgG antibodies at the same time provided higher sensitivity than the assays for IgM or IgG alone.

For the diagnosis of either leptospirosis or scrub typhus, both IgM and IgG could be detected in more than 80% of samples collected after day 6 to day 14 of fever. False- positives may be caused by many factors, including cross-reactivity between antibodies among *O. tsutsugamushi, Leptospira spp.*, or other pathogens, such as influenza, or by the persistence of an antibody following recovery from previous scrub typhus or leptospirosis. For hantavirus infection, only IgM was detectable in 9 patients. However, all of them were diagnosed with scrub typhus by IFA and as all patients recovered after doxycycline treatment, further investigation at the ImmuneMed laboratory, confirmed that all of these samples were not hantavirus infection. Thus cross reaction of hantavirus infection and scrub typhus was the most likely explanation of results found in this study.

We did not calculate the positive and negative predictive values for this ICT because we used the stored samples collected from various hospitals, at different periods of time. As a result, the proportion of samples from patients with leptospirosis, scrub typhus, and other diagnoses did not represent the true prevalence of these diseases among patients with acute fever in Thailand. Overall, it would be more cost effective to implement this ICT for the simultaneous diagnosis of either leptospirosis or scrub typhus (or hantavirus infection) than the application of individual ICTs for these diseases sequentially. Although the determination of the diagnostic performance of a newly developed assay is commonly performed by using well stored samples, prospective clinical studies are needed to determine the more accurate diagnostic performance of the assay as discrepancies between stored samples and prospective studies with the same ICT assays may exist. More studies are also needed to confirm that hantavirus infection is rare in Thailand.

CONCLUSION

For the syndromic approach to acute fever, this rapid ICT for the simultaneous detection of IgM/IgG for the diagnosis of leptospirosis, scrub typhus, and hantavirus infection, demonstrated comparable sensitivity and specificity to the previously reported individual ICTs for either leptospirosis or scrub typhus in endemic areas. However, we did not detect hantavirus infection in this study, and a rapid test for the diagnosis of leptospirosis, using antigen detection, is still needed.

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Conflict of Interest: Professor Yoon-Won Kim advised the scientific contribution when ImmuneMed AFI Rapid® has been developed.

Abbreviations

HFRS: Hemorrhagic fever with renal syndrome, ICT: Immunochromatographic test, IFA: Indirect immunofluorescent assay

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