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Scrub typhus in Sri Lanka – beyond the stethoscope

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Introduction

Scrub typhus, or tsutsugamushi disease, is an acute febrile illness in humans caused by infection with *Orientia tsutsugamushi* (OT) following a bite of an infected mite vector of the genus Leptotrombidium[1]. Scrub typhus is endemic in the Asia-Pacific region, extending from Afghanistan to China, Korea, the islands of the western Pacific and Indian Oceans, and northern Australia[2,3]. This endemic region is often referred to as the tsutsugamushi triangle, and hosts approximately 1 billion people[4]. The vectors can be found in a variety of ecological conditions, from the mountainous regions of northern India to the tropical climates of the Malay Peninsula and Indonesia[2]. Trans-ovarial transmission of OT within vectors appears to be essential to maintenance of the agent in nature; thus, the mite serves as both the vector and the reservoir[2]. Transmission of the etiologic agent to vertebrate hosts occurs during feeding of the larval or "chigger" stage of mites[2]. While Orientia is vertically maintained in Leptotrombidium mite populations, it may be transmitted horizontally from mites to vertebrate hosts[2]. The transmission to humans is incidental. Currently, there is no vaccine against scrub typhus[5].

Scrub typhus became more familiar during World War II, when soldiers deployed to endemic regions were affected in great numbers[2]. Scrub typhus was considered the most devastating and threatened ground attack by an unseen enemy during the world wars, carrying high morbidity and mortality. However, the incidence of scrub typhus died down after the introduction of tetracyclines and chloramphenicol. Similar to other countries in the tsutsugamushi triangle where scrub typhus is endemic the documented history of scrub typhus in Sri Lanka dates back to the Second World War[5]. However, its interest re-emerged during the last few decades due to increasing incidence of the illness. During the past decade we found patients presenting with serious complications such as encephalitis, myocarditis, pneumonitis and multi-organ involvement [6,7,8,9,10,11,12]. The incidence of the disease seems to be based on human activity, and ecological and climatic factors. Research since World War II has highlighted dramatic antigenic variation among strains of OT[2]. Today, more than 20 antigenically distinct strains of OT have been reported, including the initially serologically distinguished prototypic strains Karp, Gilliam, and Kato[13]. The disease mimics several other tropical febrile illnesses, and can vary from mild to fatal disease, with reported mortality rates of 35%-50% during the pre-antibiotic era[14,15] to 7-9% currently, in different geographical regions[5]. Detection of the eschar; the site of the bite of the mite assists in the early diagnosis and treatment, thus preventing development of serious complications. However the occurrence of this sign is highly variable and depends on the host, geographical region, and possibly the bacterial strain[16]. Furthermore, great inter-strain variability in virulence has been shown in mouse models. However, it is not clear whether virulence for mice can be directly applicable to humans[2]. Although, antigenic variation may result in a spectrum from very mild to fatal disease[2], the mortality due to scrub typhus can be reduced dramatically by early diagnosis and treatment[17,18,19]. Although relapses and reinfections are known to occur[20] this is poorly investigated. However such relapses are also likely to respond to treatment with current anti-rickettsial antibiotics such as doxycycline and azithromycin[2].

Until recently the diagnosis of these infections were mainly on clinical grounds and scrub typhus was supported by the Weil Felix test; a test considered non-specific and obsolete today. Therefore in June 2008, in collaboration with the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, we established IFA based rickettsial disease diagnostics in Sri Lanka. Our initial collaborations with CDC identified Gilliam, Karp, and Kato serotypes as the dominant serotypes in Sri Lanka similar to that is documented in many other geographical locations. Therefore antigens of these serotypes were employed for the disease confirmatory IFA test in Sri Lanka. The IFA-IgG titre of 1:128 was recommended as the diagnostic cut off titre for acute rickettsioses including that for scrub typhus by the CDC as for other endemic regions. Although IFA test was considered the gold standard test for the diagnosis of rickettsial infections, we realised that the CDC recommended cut off titre of 1:128 was not helping the diagnosis of acute rickettsial

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infections in Sri Lanka. This was later identified as due to high background sero-prevalence of IgG antibodies against scrub typhus and spotted fever group (SFG) rickettsioses in many geographic locations of the country. Although various combinations of antigenically distinct dominant serotypes and genotypes have been increasingly documented in regional countries, we were not aware of the prevailing *O tsutsugamushi* serotypes and genotypes in Sri Lanka. Identification of serotypes and genotypes of OT circulating in Sri Lanka is considered important in order to improve the diagnostic accuracies as well as for development of vaccines against scrub typhus.

The current IFA based diagnostics against scrub typhus is time consuming, expensive and require trained experts for carrying out the diagnostic procedures and for interpretation. Furthermore, it is impossible for accurate diagnosis when a new or unknown serotype is found[20,21]. Therefore, it was needed to identify *Orientia tsutsugamushi* serotypes and genotypes in Sri Lanka and to identify or develop a rapid diagnostic test such as immune-chromatographic card tests that could be utilised bed side in the diagnosis of scrub typhus in Sri Lanka.

Methods, results and discussion

In order to address the problem encountered with IFA-IgG cutoff titres in the diagnosis of acute rickettsial infections in Sri Lanka, the clinical and laboratory database of Rickettsial Disease Diagnostic and Research Laboratory (RDDRL), Faculty of Medicine, University of Kelaniya, was retrospectively analyzed in relation to the known duration of illness at the time of sampling and whether the patients responded favourably to anti-rickettsial antibiotics. Out of 478 samples that had been analysed for rickettsioses by November 2010, the date of collection in relation to illness and follow up serology was known in 261. Fifty six had been sent on or before the 7th day of illness with a mean of 5 days, and 205 were sent after the 7th day with a mean of 19 days. Of the 146 suspected SFG infections, only 3 responders of 25 patients had titres $\leq$ 1/128 with less than 7 days of illness while all 9 with titres $\geq$ 1/256 responded (false negative with 1/256 cut-off was 12%, false positive was 0%). For illness that was greater than 7 days, the false negative and positive rates at $\geq$ 1/256 were 5% (3/59) and 11.3% (6/53). For the 115 suspected ST infections false negative and positive rates with $\geq$ 1/256 cut-off at less than 7 days of illness were (2/14) and 0% (0/8) respectively while with illness greater than 7 days, false negative and positive rates were 2% (1/51) and 0% (0/42). Therefore, we concluded that in an endemic setting, when the sample is obtained > 7 days of illness, a single $\geq$ 1/256 titre is diagnostic for all scrub typhus (ST) infections and 90% of SFG infections. However, for either SFG or ST, if the sample is obtained $\leq$ 7 days of illness, an IgG titre of $\leq$ 1/128 requires a follow up sample in the diagnosis. Most patients who had a 1/128 titre (the cut-off which is recommended by the CDC in the presumptive diagnosis of acute rickettsioses) in a sample obtained after the 7th day of illness had no clinical rickettsioses[22].

One drawback in our results was that we were unaware of the spectrum of SFG and ST rickettsial organisms causing disease within our geography and whether higher titres against autochthonous SFG or ST antigens might be obtained than with *R. conorii* or *Orientia tsutsugamushi* Karp. This is because it was known that some rickettsial species are known to induce delayed immunological responses and therefore, a delayed rise in IgM and IgG titres[14,16,18]. Therefore, we recommended that while initial higher IgG titers against rickettsioses seem to be more accurate in the diagnosis of acute rickettsioses, the need for further studies in order to identify the spectrum of rickettsioses in the country. Consequently, another study to look at concomitant changes in IFA-IgM titres was considered important to clarify uncertainties of mixed IFA-IgG titres against SFG, murine typhus, or ST in the diagnosis of acute rickettsioses in the country. However, cost was the major drawback in additional evaluation of IgM titres in the diagnosis of rickettsioses in our setting.

With view to address above deficiency and in order to identify the strains of OT causing clinical illness, a preliminary survey was carried out in two scrub typhus prevalent areas along the western coastal belt of Sri Lanka. Ragama and Balapitiya, which are endemic for OT and probably having similar ecological characteristics were selected for this study. Adult patients and children who were admitted with an acute febrile illness and presumed to be having acute scrub typhus based on presence of an eschar and other supporting clinical features were recruited. Eschar biopsies and buffy coat samples collected from patients who were confirmed having OT by IFA were further studied by real time PCR (Orientia 47 kD) and nested PCR (Orientia 56 kD) amplification. DNA sequences were obtained for 56 kD gene amplicons and phylogenetic comparisons were analyzed using currently available data in GenBank [Neucleotide substitution per 100 residues, 1000 Bootstrap Trials]. Twenty eschar biopsies (Location 1,19, Location 2,1) and eight buffy coat samples (Location 1,6, Location 2,2) examined by real time PCR revealed Orientia amplicons in 16 samples. DNA sequences were obtained for the 56 kD gene amplicons in 12 eschars

and 4 buffy coat samples. The genotypes of the Location1 samples revealed that, 7 exhibiting close homology with JP1 [distantly related to UT177 Thai (Karp related)], five had close homology with Kato strain, two had close homology with JGv and JG AF [Distantly related to Kawasaki M63383] and one had close homology with Gilliam strain. The Location 2 strain was closely related to Kuroki-Boryong L04956, the genotype which is distributed in far eastern Asia. Similar to other patients in the cohort this patient also had never travelled out of Sri Lanka. This is the first time, the identification of *O. tsutsugamushi* genotypes were carried out in Sri Lanka and we observed all three main OT genotypes in the country, and the majority fell into Thai Karp related clade. These results demonstrate great antigenic diversity of OT in the studied areas of Sri Lanka. However, it is interesting to note the close homology with the Kuroki-Boryong organism in one patient from study site 2 (Ragama) who, like the others, had never travelled out of Sri Lanka[23]. Although we were not in a position to comment on clinical severity of this infection among this small cohort of patients, a 2011 study in Korea comparing the clinical severity between Boryong and Karp genotypes suggested that eschars and rashes were found in 97% and 94% of the patients infected with the Boryong cluster compared to 73.7% and 68.4% of the patients infected with the Karp cluster, respectively, suggesting clinical variation among infections with different serotypes of OT[24]. However, in a previous study conducted in the same geographical region, there was a high eschar rate (89%) that is in keeping with the above findings[25]. Our ongoing studies are further focusing on genotypes of scrub typhus in many other geographical locations in the country.

Accuracy and rapidity of a test to diagnose *Orientia tsutsugamushi* infection is important in clinical practice which assists early management with appropriate treatment. The clinical diagnosis is complicated with similar clinical symptoms with other acute febrile illnesses including leptospirosis, murine typhus and haemorrhagic fever with renal syndrome. The confirmatory diagnosis of scrub typhus even with the gold standard IFA test is challenging as it has more than twenty serotypes. The identification of high genotypic variability of OT in Sri Lanka and the detection of far eastern serotypes such as Kuroki-Boryong may suggest probable underperformance of current IFA test which utilizes Karp, Kato and Guillium strains. However, inclusion of antigens in an IFA test to cover all likely serotypes is not feasible. Furthermore, the current IFA test is costly, time consuming and needs expertise in the diagnosis. Therefore, with view to further simplify the diagnosis of scrub typhus in Sri Lankan endemic setting, in collaboration with Immune Med, Korea, the improved ImmuneMed Scrub Typhus Rapid Diagnostic Test (RDT) was developed and validated for its performance and investigated for its ability to discriminate scrub typhus from seventeen other febrile aetiologies such as hemorrhagic fever with renal syndrome, leptospirosis, and murine typhus[26].

Based on the fact that 56 kDa of surface antigenic protein of Orientia tsutsugamushi has antigenicity and diagnostic value, genes encoding the fragment of 56 kDa protein from the major serotypes, including Gilliam (tsg56, GenBank AY335819), Karp (tsa56, GenBank AY956315), and Kato (tst56, GenBank M63382), were amplified by polymerase chain reaction (PCR). The antigenic region was selected from the 56-kDa outer membrane protein gene which showed more than 30% amino-acid sequence homology with each other at 56-kDa outer membrane proteins of *O. tsutsugamushi* prototype Gilliam, Karp and Kato to make the chimeric 56-kDa protein. The amplified DNAs were connected in series and cloned into protein expression vector (pET-22b+). The cloned DNA was expressed in *E. coli* as a fusion protein. This fusion antigenic protein (cr56, 103 kDa) which is produced, isolated and purified in a single process can be used in diagnosing scrub typhus.

In addition to Gilliam, Karp, and Kato, the gene encoding 56 kDa protein (kr56, GenBank AF302990) from *O. tsutsugamushi* Kangwon strain and the gene encoding 21 kDa protein (r21, GenBank AM494475) from *O. tsutsugamushi* Boryong, were amplified by PCR and cloned into protein expression vector (pET-30a) to improve the sensitivity of the scrub typhus diagnosis. Each cloned DNA was expressed in *E. coli* and purified to use in the scrub typhus diagnosis and each protein was added to cr56. The expressed 3 antigens were purified using His-bind Resin (Novagen, Cat No. 69670-4) and dialyzed with potassium phosphate buffer. After formulating each antigen as 2 mg/mL, the mixed antigens were applied to the test line of the immunochromatographic test.

The sensitivity at the base of each IgM and IgG indirect immunofluorescent assay (IFA) in Korean patients was 98.6% and 97.1%, and the specificity was 98.2% and 97.7% respectively. The sensitivity and specificity for retrospective diagnosis at the base of IFA in Sri Lanka was 92.1% and 96.1%. ImmuneMed RDT was not reactive to any serum from seventeen diseases including hemorrhagic fever with renal syndrome (n = 48), leptospirosis (n = 23), and murine typhus (n = 48). ImmuneMed RDT shows superior sensitivity (98.6% and 97.1%) compared with SD Bioline RDT (84.4% at IgM and 83.3% at IgG) in Korea. The retrospective diagnosis of ImmuneMed RDT exhibits 94.0% identity with enzyme-linked

immuno-sorbent assay (ELISA) using South India patient serum samples. These results suggest that this RDT can replace other diagnostic tests and is applicable for diagnosis of scrub typhus in Sri Lanka. This rapid and accurate diagnosis will be beneficial for diagnosing and managing scrub typhus.

Conclusions

We conclude that in IFA-IgG based diagnosis of rickettsioses in the endemic setting of Sri Lanka, when the serum sample is obtained > 7 days of illness, a single ≥ 1/256 titer is diagnostic for all scrub typhus infections and 90% of SFG infections. However, for either SFG or ST, if the sample is obtained ≤ 7 day of illness, an IgG titer of ≥ 1/128 requires a follow up sample in the diagnosis. Most patients who had a 1/128 titer (the cutoff which is recommended by the CDC in the presumptive diagnosis of acute rickettsioses) in a sample obtained after the 7th day of illness had no clinical rickettsioses. For the first time in Sri Lanka we observed all three main OT genotypes in the country, and the majority fell into Thai Karp related clade demonstrating great antigenic diversity of OT in the studied areas of Sri Lanka. However, we also noted the existence of a far eastern serotype Kuroki-Boryong organism in one patient who, like the others, had never travelled out of Sri Lanka suggesting the need for inclusion of such organisms in the diagnostic pool of scrub typhus in the country. For the rapid diagnosis of scrub typhus in the country, the ImmuneMed Rapid Diagnostic test which contained common and far Eastern serotype showed sensitivity and specificity of 92.1% and 96.1%, and the test kit was not reactive to any serum from seventeen diseases including hemorrhagic fever with renal syndrome, leptospirosis, and murine typhus. These results suggest that this RDT could replace other diagnostic tests and is applicable for diagnosis of scrub typhus in Sri Lanka.

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