Short-Form Paper:

Comparative accuracy of the InBios Scrub Typhus Detect™ IgM Rapid Test for the detection of IgM antibodies using conventional serology.

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Abstract

This study investigated the comparative accuracy of a recombinant p56 kDa type-specific antigen-based rapid diagnostic test (RDT) for scrub typhus for the detection of IgM antibodies using conventional serology, in well-characterized serum samples from undifferentiated febrile illness patients. The RDT showed high specificity and promising comparative accuracy with 82% sensitivity and 98% specificity for samples defined positive at the IgM IFA positivity cutoff titer of ≥1:1,600, versus 92% and 95% at ≥1:6,400, respectively.

Short report (<1,500 words)

There is an urgent need for inexpensive accurate point-of-care rapid diagnostic tests (RDT) for scrub typhus. Clinical diagnosis on admission is rendered difficult due to the non-specificity of presenting symptoms, like fever and skin rash. The presence of an eschar at the mite inoculation site is a valuable diagnostic clue when found in combination with a positive RDT result, with a positive predictive value (PPV) and negative predictive value (NPV) of 84.9% and 93.0% respectively, but unfortunately the varying occurrence of this lesion, especially in endemic settings limits this approach (1). Currently, three modalities are used for the diagnosis of scrub typhus; culture, nucleic acid and antibody detection. Culture from patient samples is insensitive, laborious and expensive; nucleic acid detection is accurate in the early phase of infection, but sensitivity falls with fever duration beyond 9 days (2). Antibody detection, traditionally by indirect immunofluorescence (IFA), requires skilled technicians and expensive equipment, and is limited by the problem of background titers in endemic settings, antigen selection and standardization (3). The rigorous use of paired serum samples with a four-fold or greater antibody titer rise required as diagnostic endpoint, has overcome some issues, but confounding factors include pre-existing antibodies and cross-reactivity. Attempts at improving the gold standard have included combining all modalities into the scrub typhus infection criteria (STIC) proposed for diagnostic assay validations (2). However, the single admission endpoint titer conundrum is not yet adequately resolved. Recent Bayesian Latent Class Modeling (LCM) data have highlighted the low specificity of admission and paired dynamic IFA IgM titers with low convalescent titers, such as a ≥4-fold rise to ≤1:800 (1), (*pers comm* Cherry Lim).

An affordable, accurate point-of-care RDT that demonstrates a positivity cut-off at the population background antibody titer could potentially replace the admission IFA and impact patient management positively by guiding administration of specific treatment. More data on the variation of endemic background cut-off titers between geographical regions is required, and more sensitive RDTs (i.e. RDTs that provide a positive result at a lower antibody titer) might be better in non-endemic regions and less sensitive RDTs that are positive at higher cutoff titers are more useful in endemic regions. A

57 comparative analysis of an RDT in an endemic region for scrub typhus has shown improved specificity
58 using IgM over total antibody, while maintaining sensitivity (4). IgM is produced immediately after
59 pathogen exposure, with a shorter half-life in blood and lymphatics than the more pathogen-specific
60 IgG, which is produced later and provides a long-lasting response dependent on the pre-existing
61 exposure of the individual (5). Although IgG can persist for a long time and is thought to be more
62 specific in paired samples, it can be associated with higher RDT false positivity rates in endemic areas
63 where the population is continuously exposed. Two important questions remain unresolved; what is
64 the longevity of IgM and IgG in human scrub typhus, and which isotype appears earlier in naïve and
65 exposed populations? Non-human primate timecourse studies have shown that IgM and IgG can
66 appear almost simultaneously in cynomolgus macaques (6, 7).
67
68 In this study we evaluated a new commercial immunochromatographic-based RDT based on *O.*
69 *tsutsugamushi* recombinant p56kD type-specific antigen (TSA) of Karp, Kato, Gilliam and TA716
70 strains (Scrub Typhus Detect IgM Rapid Test, InBios International Inc., Seattle WA, USA). Two RDT
71 prototype versions with different antibody IgM detection modalities were tested using either polyclonal
72 (pAb) or monoclonal (mAb) secondary antibodies.
73 The InBios RDT tests were performed from the same batch and lot (Part #800231 and Lot #NB273/52
74 respectively) using serum samples (10 uL serum per test strip) according to the manufacturer's
75 instructions. Previously characterized admission serum samples (total n=100) were used, collected
76 from febrile illness patients enrolled into ethically and Institutional Review Board (IRB) fully approved
77 prospective 'causes-of-fever' studies performed in Udon Thani, NE Thailand (2000 to 2001; n=85) and
78 Kathmandu, Nepal (2008 to 2011; n=15) (8, 9). The included samples consisted of confirmed scrub
79 typhus cases (n=21) meeting any of the previously defined stringent scrub typhus infection criteria
80 (STIC); culture-positivity and/or admission IgM antibody titer of >1:12,800 and/or a ≥4-fold rising IgM
81 IFA antibody titer and/or positivity for ≥2 out of 3 PCR gene targets. Murine typhus cases with paired
82 dynamic serology and/or qPCR positivity (n=23) and dengue cases with NS1 antigen positivity (n=5)

were included. The other cases represented patients with undifferentiated febrile illness (n=51) with negative test results for scrub typhus, murine typhus and NS1 antigen tests (2, 10).

The IFA used pooled *O. tsutsugamushi* Karp, Kato, Gilliam antigens to detect IgM antibodies with IFA slides produced by the Australian Rickettsial Reference Laboratory (Geelong, Australia). Patient sera were serially 2-fold diluted from 1:100 to 1:25,600 and the endpoint was determined by two experienced staff members as the highest titer displaying specific fluorescence (11). Three independent laboratory technicians read the developed RDTs blinded to each other's results and the majority interpretation was final. Sensitivity and specificity were calculated using a range of IFA endpoint cutoff titers for positivity and binomial 95% confidence intervals (95% CI) were calculated. Kappa statistics were calculated for inter-reader variation. Statistical analysis and logistic regression (Figure 1) were performed using Stata/IC software (Version 13.0, Statacorp, College Station, TX, USA) and plotted using R version 3.1.1 (available on www.r-project.org).

The challenges of a point-of-care RDT are comparable to that of a single titer admission IFA, albeit with a simplified procedure and clear-cut endpoint. In this study, we did not attempt to estimate the classic diagnostic accuracy of the InBios RDT– this would require a prospective study design – but rather attempted to define the antibody titer associated with optimal RDT performance in a set of characterized samples, as its comparative accuracy. Hence, we assessed the agreement of RDT positivity rates against a range of samples with pre-defined different IgM IFA admission titers. Table 1 summarizes the assay results and the respective sensitivity and specificity values at the different IFA cutoff titers.

The proportion of RDTs with a positive result at each IFA IgM titer increased with higher IFA IgM titers (test for trend; $p<0.001$). All of the RDTs (100%) provided negative results at IFA titers of ≤1:400 and all of the RDTs (100%) provided positive results at titers of ≥1:25,600. The results show that both versions of the new test identifies the same number of positive samples identified by IFA when the reciprocal antibody cut-off titer is high, thereby giving 100% sensitivity. Although the tests agree well if

the sample has high antibody levels, the new test misses some of the IFA-positive samples at low cut-off titers with subsequent reduction of sensitivity. Plotting the proportion of positive RDTs per different IFA titers delineates a sigmoidal relationship, with increasing proportions of RDT positivity at titers ≥1:3,200 (Figure 1). This response was comparable with the previously assessed PanBio RDT (12). There was minimal difference between the two secondary antibody versions in terms of proportion positive at the different IFA titers. The interpretation of bands was perceived more difficult due to weaker, paler and more smeared bands in positive samples using RDTs based on mAbs compared to RDTs based on pAbs, which was reflected by a marginally higher kappa, 0.97 vs 0.93 for the high and low antigen density versions respectively (data not shown).

The RDTs assessed in this study were specific and sensitive for the detection of high IFA titer samples only. This RDT would therefore be expected to perform well in endemic areas where a higher background antibody titer would be expected in the population, as low titers would result in a negative RDT result and higher titers detected with good diagnostic accuracy.

It is noteworthy that a >64-fold difference in IgM antibody concentration exists between the samples with titers of 1:400 and >1:25,600. In samples with a low IFA titer (≤1:400) the RDT results were generally negative, contributing to a high specificity. Currently, it is not known if the antibodies in serum samples with an IFA titer of 1:400 may be different from the antibodies in samples with a 1:25,600 titer, either in affinity or target. However we have shown in recent Bayesian LCM analyses, that paired dynamic IFA IgM titers with low convalescent titers, such as a ≥4-fold rise to ≤1:800 contribute to low specificity of the IFA assay, and as such a higher endpoint cut-off positivity titer needs to be considered (1), (Lim C, *pers comm*).

The choice of an IFA positivity cut-off endpoint titer at 1:400 over 1:1,600 to 1:6,400 results in a stepwise improvement of the InBios RDT diagnostic accuracy, with sensitivities (95% CI) from 52% (32-71), over 82% (57-96) to 92% (64-100) while retaining a specificity of ≥94% (Table 1). The size of the study dataset did not allow for in-depth and detailed analyses, however it can be safely assumed that the positivity cut-off titer of the InBios RDT lies around the 1:1,600 – 1:3,200 titer range.

The RDT under evaluation, may have benefited from the inclusion of antigenically disparate recombinant immunodominant p56kDa antigens from four *O. tsutsugamushi* strains, three more than the PanBio IgM RDT and one more than the reference IFA; the additional *O. tsutsugamushi* TA716 strain. A study limitation is that the benefit of a broader antigen spectrum covered by the RDT would have gone unnoticed in the current evaluation as anti-TA716 antibodies would not have been detected by the IFA used (based on Karp, Gilliam and Kato strains), which would have increased the RDT false positive rate, due to false negative IFA results.

In conclusion, the InBios RDTs tested here show promising performance characteristics for use in endemic zones where the admission IgM IFA positivity cutoff titer would lie around a titer of 1:1,600 – 1:3,200. The RDT assay based on polyclonal endpoint detection is preferable for a prospective evaluation. Attention should be given to understanding why the RDTs are negative at ≤1:400 and how this can be improved to develop tests for non-endemic zones.

Acknowledgements

We are grateful for the assistance of the clinical teams during the collection of sample specimens for this study. This work was supported by the Wellcome Trust of the United Kingdom. The RDTs assessed in this study were provided by InBios International Inc.

Table 1. Summary of characterized patient admission serum samples and rapid diagnostic test (RDT) performance in this study. The results are stratified by their IFA IgM antibody positivity titers (horizontal rows) with corresponding InBios RDT diagnostic accuracies reported separately for IgM detection modalities, polyclonal (pAb) versus monoclonal antibodies (mAb). Although the number of characterized samples with confirmed scrub typhus was 21/100, more samples had low IFA IgM positivity, i.e. of all samples, if IFA cutoff titer was chosen at ≥1:400, then 14 of these were true positives (TP) and 13 were false negatives (FN) - with rising IFA IgM titers, the rate of FN decreased.

IFA reciprocal cutoff titer	Samples with IFA IgM titer cutoff (N/100)	RDT Version*	RDT results				Sensitivity % (95% CI)	Specificity % (95% CI)
			TP (N)	FP (N)	FN (N)	TN (N)		
≥400	27	pAb	14	2	13	71	52 (32-71)	97 (90-100)
		mAb	14	3	13	70	52 (32-71)	96 (88-99)
≥1,600	17	pAb	14	2	3	81	82 (57-96)	98 (92-100)
		mAb	14	3	3	80	82 (57-96)	96 (90-99)
≥6,400	13	pAb	12	4	1	83	92 (64-100)	95 (89-99)
		mAb	12	5	1	82	92 (64-100)	94 (87-98)
≥25,600	11	pAb	11	5	0	84	100 (72-100)	94 (87-98)
		mAb	11	6	0	83	100 (72-100)	93 (86-97)

Footnote: *RDT version; pAb=polyclonal secondary antibodies; mAb=monoclonal secondary antibodies. N = number. TP=True positive RDT; FP = false positive RDT; FN=False negative RDT; TN=true negative RDT. CI = confidence interval.

Figure 1. Relationship between IFA titer and rapid diagnostic test (RDT) positivity

This figure shows the proportion of positive RDTs at different IFA titers plotted on a logarithmic scale. Logistic regression was used to describe the sigmoidal relationship between RDT positivity and IFA titer, illustrating the low proportions of RDT positivity below IFA titers of 1:3,200. No significant difference between the two RDT versions was observed (secondary polyclonal antibody detection in red and monoclonal in blue).

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