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EVALUATION OF IGM ELISA, WEIL FELIX TEST AND INDIRECT IMMUNOFLUORESCENCE ASSAY IN THE DIAGNOSIS OF SCRUB TYPHUS.

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ABSTRACT Scrub typhus is an acute febrile illness caused by Orientia tsutsugamushi. There are reports of resurgence of scrub typhus in different parts of India including Kerala. Many cases go undiagnosed due to the lack of knowledge regarding the diagnostic test to be used. This study was undertaken at Pushpagiri Institute of Medical Sciences and Research Centre, Tiruvalla in the Department of Microbiology from January 2014 to September 2015, to assess the validity of various tests in diagnosing scrub typhus. A total of 94 samples were subjected to IgM ELISA, Weil Felix (WF) test and Indirect Immunofluorescence assay(IFA). The agreement between ELISA, IFA and Weil Felix test was assessed by κ (kappa) coefficient of Cohen using SPSS version 17. The sensitivity and specificity of ELISA with respect to IFA was 100% and 94.67% respectively, and that of IFA with respect to ELISA was 82.61% and 100% respectively. The sensitivity and specificity of WF test with respect to ELISA was 43.48 and 100% respectively and that of WF test with respect to IFA was 52.63% and 100% respectively. Although IgM ELISA is highly sensitive, it cannot be relied upon as the sole diagnostic test due to high false positivity. WF test is not a sensitive test, but when positive it is rather specific. IFA has high sensitivity and specificity, but is unsuitable for moderately equipped laboratories. Hence, we suggest that scrub typhus could be diagnosed using a combination of IgM ELISA and WF test and these tests should be included in the panel of serological tests ordered for patients presenting with undifferentiated febrile illnesses.

KEYWORDS : Scrub typhus, IgM ELISA, Weil Felix test, IFA

INTRODUCTION

Scrub typhus, also known as 'tsutsugamushi' fever orJapanese River disease, is an acute febrile illness caused by *Orientiatsutsugamushi* which is transmitted by the larvae of Leptotrombidium mites(chiggers) (Saifudheen, Jose, Gafoor, Sajeeth Kumar, & Veena, 2012).It is a publichealth problem in Asia where about 1 million new cases are identified annually and 1billion people may be at risk of the disease.(Watt & Parola, 2003). Although it was known in Chinasince the 3rd century A.D, the first case of scrub typhus was described in Japan in1810.(Harden VA, 1993). It gained importance during the Second World War when thousands ofsoldiers were affected among the Allied troops(Groves MG, Harrington KS, 1994)In India, there are reports of resurgence of scrub typhus from Northern, NorthEastern, Western and Southern states(Mahajan et al, 2006), including Karnataka and Tamil Nadu. Keralabeing heavily dependent on them for food grains, fruits and vegetables, there isincreased chance of transport of mites attached to rodents.(Chrispal et al., 2010), (Razak, Sathyanarayanan, Prabhu, Sangar, & Balasubramanian, 2010).

It presents clinically as a non specific febrile illness accompanied byprodromal symptoms. The severity variesfrom subclinical to severe illness with multiple organ system involvement, which canbe serious enough to be fatal, unless diagnosed early and treated.(Saifudheen et al., 2012)

The mainstay in the diagnosis of scrub typhus is serology. Weil Felix test isthe oldest test which is still in use. It is inexpensive but lacks sensitivity andspecificity, so it is used only as a screening tool.(D J Kelly, Wong, Gan, & Lewis, 1988). Enzyme Linked ImmunosorbentAssay (ELISA) utilizes *O. tsutsugamushi* derived recombinant antigen mixture forthe detection of IgM antibodies in the patients'serum.(Gurung, Pradhan, & Bhutia, 2013a). Indirectimmunofluorescent assay(IFA) was long considered to be the gold standard, but thelack of standardization of endpoints and of the antigen strains used in the test has resulted in considerable confusion.(Daryl J Kelly, Fuerst, Ching, & Richards, 2009). Polymerase Chain Reaction (PCR) assay is auseful tool for detecting scrub typhus, but the high resource cost and trainingrequired make it impractical in many endemic areas.(Koh, Maude, Paris, Newton, & Blacksell, 2010)

Scrub typhus is grossly underdiagnosed in India due to its nonspecific clinicalpresentation, limited awareness and lack of diagnostic facilities. In Kerala, first cases of scrub typhus were reported fromThiruvananthapuram district in year 2000. Since then, scattered cases have beenreported every year , not only from Thiruvananthapuram, but also from the Malabarregion including the districts of Kozhikode, Kannur, Malappuram, Palakkad,Wayanad(Abhay Kumar Sharma, 2013). It is still not known if scrub typhus is a newcomer. It is possible that it existedin the state but went unnoticed as many physicians might have been treating their patients with the commonly used antibiotics like doxycycline and azithromycin, towhich scrub typhus responds, without considering it in their differential diagnoses. To add on, there is a confusion regarding the diagnostic test to be used.

In our hospital, a fair proportion of the non specific febrile illnesses are suspected to be due to scrub typhus. Hence, we conducted this study to assess the validity of various tests in diagnosing scrub typhus.

MATERIALS AND METHODS

This study was conducted at Pushpagiri Institute of Medical Sciences and Research Centre, Tiruvalla in the Department of Microbiology from January 2014 to September 2015. Human ethical clearance from the Institution Ethics Committee and patient consent was obtained prior to the start of the study.

All scrub typhus IgM ELISA positive (InBios Scrub Typhus Detect IgM ELISA System) serum samples (n=23) during the study period were included. In addition, 71ELISA negative samples (calculated by taking the expected specificity of *Orientia tsutsugamushi* IFA as 96% and that of Weil Felix test as 97%, at a significance level of 5% and power of 80) were also included in the study.Samplesseropositive for leptospirosis, dengue, malaria and enteric feverwere excluded from

the study.Thus,a total of 94 samples were subjected to Weil Felix test using MICROPATH antigens/febrile antigen kits (Omega Diagnostics Ltd, Scotland, United Kingdom)and*Orientia tsutsugamushi* Indirect immunofluorescence assay(IFA)using IFA kit- Fuller laboratories, California, USA.

Statistical Analysis

The data generated from the results were analysed using Microsoft excel. The sensitivity, specificity, positive predictive value, negative predictive value of the tests with respect to each other was calculated. The agreement between ELISA, IFA and Weil Felix test was assessed by κ (kappa) coefficient of Cohen using SPSS version 17. A Kappa statistic of < 0.2 was considered to indicate a "poor" strength of agreement, 0.21-0.40 was "fair", 0.41-0.60 was "moderate", 0.61-0.80 was "good".

RESULTS

A total of 23 Scrub typhus IgM ELISA positive samples and 71 ELISA negative samples) were subjected to both *Orientia tsutsugamushi* IFA and Weil Felix test, comparison of which is detailed in Table no:1.

Table: 1- Year wise comparison of IgM ELISA positive results with respect to IFA and WF

Year	Total Number of Scrub typhus IgM ELISA Positives	ELISA Positive IFA Positive Weil Felix OX K Positive	ELISA Positive IFA Positive Weil Felix OX K Negative	ELISA Positive IFA Negative Weil Felix OX K Negative	ELISA Positive IFA Negative Weil Felix OX K Positive
2014	9	5	2	2	0
2015	14	5	7	2	0

ELISA vs IFA

Comparison of scrub typhus IgM ELISA and IFA were performed on all the 94 samples,. (Table 2). The validity of the tests were analysed with respect to each other(table: 3)and their agreement using Kappa(κ) analysis was estimated.(table 7)

Table: 2-Comparison of ELISA vs IFA (n=94)

ELISA Results	IFA Results	
	Positive	Negative
Positive	19	4
Negative	0	71

Table:3- Validity of ELISA and IFA with respect to each other(n=94)

Parameters	ELISA	IFA
Sensitivity	100%	82.61%
Specificity	94.67%	100%
Positive Predictive Value(PPV)	82.61%	100%
Negative Predictive Value(NPV)	100%	94.67%

ELISA vs Weil Felix test

Both scrub typhus IgM ELISA and WF test were also performed on all the 94 samples,. The results of the same were compared.(Table 4).

Table:4-Comparison of ELISA vs Weil Felix test (n=94)

WF test Results	ELISA Results	
	Positive	Negative
Positive	10	0
Negative	13	71

IFA vs Weil Felix test

The results of IFA vesrus WF were compared.(Table 5). The validity of Weil Felix test was analysed with respect to IFA and ELISA (table: 6) and the overall agreement between the three tests were estimated using Kappa(κ) analysis (table 7).

Table: 5- Comparison of IFA vs Weil Felix test (n=94)

WF test Results	IFA Results	
	Positive	Negative
Positive	10	0
Negative	9	71

Table:6- Validity Of Weil Felix Test

Parameters	With Respect to ELISA	With Respect to IFA
Sensitivity	43.48%	52.63%
Specificity	100%	100%
Positive Predictive Value(PPV)	100%	100%
Negative Predictive Value(NPV)	84.52%	88.75%

Table:7- Overall Agreement between ELISA, IFA and Weil Felix test

Parameters	Kappa statistics	Strength of association
ELISA & IFA	0.878	Good
ELISA & WF test	0.537	Moderate
IFA & WF test	0.638	Good

DISCUSSION

Scrub typhus is a zoonotic disease which presents as a non specific febrile illness and is clinically indistinguishable from other febrile illnesses. It was till recently thought to be a militarily important disease of the past, confined to the North Eastern part of the world. But,there have been reports on outbreak of scrub typhus in the recent past from various parts of our country. In India, the burden of rickettsiosis is under estimated as there is lack of both community based studies and availability of specific laboratory tests. Rickettsiosis should be differentiated from other infections like meningococcemia, brucellosis, malaria, viral illness and typhoid fever as there is an overlap of clinical features. A high index of suspicion is required to diagnose rickettsiosis especially in endemic areas.

There are several studies on rickettsial infections conducted in Tamil Nadu and Pondicherry in South India,(Vivekanandan, Mani, Priya, & Singh, 2010)(Kamarasu et al., 2007)but studies from Kerala are very few.

Early diagnosis of scrub typhus is crucial as delayed administration of effective antibiotics such asdoxycyclinecan lead to fatal complications likeARDS, pneumonitis,meningoencephalitis, shock or multiple organ dysfunction syndrome (MODS) which can inturn lead to death of the patients.

The gold standard test for diagnosis of scrub typhus is PCR. But it cannot be performed in moderately equipped laboratories due to the high cost involved and trained personnel required. There is also confusion regarding the ideal sample required for doing PCR. In our study we tested serumsamples using ELISA, IFA and Weil Felix test. According to previous studies done, ELISA and IFA have got comparable sensitivities and specificities, whereas WF test is inferior to both ELISA and IFA.

In our study, the sensitivity, specificity, positive predictive value(PPV) and negative predictive value(NPV) of ELISA with respect to IFA was 100%, 94.67%, 82.61% and 100% respectively. The sensitivity, specificity, PPV and NPV of IFA with respect to ELISA was 82.61%, 100%, 100% and 94.67% respectively. The sensitivity, specificity, PPV and NPV of WFtestwith respect to ELISA was 43.48%, 100%, 100% and 84.52% respectively and the sensitivity, specificity, PPV and NPV of WF test with respect to IFA was 52.63%, 100%,100% and 88.75% respectively.

Out of the 13 cases which were negative for OX-K antigen by Weil Felix test , nine of them were both ELISA and IFA positive, ie; more number of cases were positive by ELISA and IFA than by WF test. This finding was comparable to a study conducted in 2014 (Usha et al., 2014)and 2013 (Gurung, Pradhan, &Bhutia, 2013), where more number of scrub typhus cases were reported by ELISA and ICT (Immuno Chromatographic Test) tests than by Weil-Felix test. Similarly, in a study conducted at Tirupati, among the tested 280 febrile patients, 158 (56.42%) were found to be positive by Weil-Felix test, whereas 163 (58.21%) were positive by ELISA.(Usha et al., 2014)Therefore, Weil Felix test alone should not be relied upon.

In several regions of the world, especially in the developing countries, Weil-Felix test is used as the initial test in documenting the presence of rickettsial infections. But Weil-Felix test may be negative during the early stages of the disease because agglutinating antibodies are detectable only during the second week of illness. In our study we used single acute phase sera from patients with febrile illness attending the hospital. This may be the reason for the low sensitivity of WF test.Isaac et al. has demonstrated that the sensitivity of Weil-Felix test is 49% and specificity96%.(Isaac, Varghese, Mathai, J, & Joseph, 2004). In another

study by Prakash et al., Weil-Felix test showed specificity of over 98% and a sensitivity of about 43%.(Prakash, Abraham, & Mathai, 2006)Both the results are comparable to that obtained in our study.

Inspite of its low sensitivity, WF test still has a role in our setting due to the low cost and the ease with which it can be performed.Weil-Felix test was found to be promising as a screening test for scrub typhus in correlation with clinical features in a hospital setting where gold standard tests are not available. Therefore, WF test should always be performed in conjunction with a serological test of higher sensitivity like ELISA or IFA. When WF test is positive, it can also be used to differentiate scrub typhus from typhus fever and spotted fevers.

In our study, the sensitivity and specificity of ELISA was 100 % and 94.67% respectively. Study by Issac et al., showed that the sensitivity and specificity of ELISA was 90% and that by Prakash et al., has showna sensitivity of 86.5%.(Prakash et al., 2006). In a study done at Goa Medical College, out of the 44 patients who presented with undiagnosed febrile illness, 15 (34%) were found to be positive for IgM antibodies against *O. tsutsugamushi* by ELISA(Narvencar et al., 2012).George M Vargheseet al, in a study at Vellore,diagnosed scrub typhus in 47% of the patients with undiagnosed febrile illness using IgM ELISA(Isaac et al., 2004).Therefore, as ELISA based systems have got high sensitivity and specificity, it is helpful for detection of IgM antibodies where labs are moderately equipped. The one disadvantage of using ELISA is that the results have to be interpreted with caution as false positive reactions are possible in cases of malaria, dengue, enteric fever and leptospirosis. (Prakash et al., 2006) This can be overcome by raising the cut off value, but there is lack of consensus regarding the gold standard reference assay to determine diagnostic cut-offs. Also, higher cut-off would lead to false negatives thereby resulting in missing of many cases.—(Saraswati, Phanichkrivalkosil, Day, & Blacksell, 2019)

According to our study, IFA has got comparable sensitivity and specificity to ELISA. Of the total 208 samples collected, IFA positivity was reported in 19 cases(9.1%) at a titre of 1:64. In a study conducted by Kim et al, out of 135 patients who were clinically suspected to havescrub typhus, 118 patients gave a positive result with indirect immunofluorescent IgM assay at a titre of 1:10.(Kim et al., 2006) In a study done in Thailand, 30 children were diagnosed to have scrub typhus during a 2 year study period using IFA *O. tsutsugamushi* IgM at a titre>1/400.(Sirisanthana, Puthanakit, & Sirisanthana, 2003) Similarly in Thailand, 20 children were diagnosed with scrub typhus during a six month period using IFA.(Chanta & Suwalee Chanta, 2005)In a study done in China,(Zhang et al., 2010) at a titre of 1:32, 98% of the clinically suspected cases of scrub typhus (104 patients) were confirmed using IFA. The sensitivity of IFA in our study is higher when compared to that reported by Ching et al (78%)(Ching et al, 2001), whereas it is lower to that reported by Zhang et al from China (94%)(Zhang et al., 2010). The specificity of IFA in our study was 100% which is comparable to that done in China(Zhang et al., 2010) and also by Brown et al.(Brown, Shirai, Rogers, & Groves, 1983)

Another finding in our study was that, three patients who were ELISA negative, turned out to be IFA positive, but on examining the history and further evaluating the patients, it was found that two of them were confirmed cases of autoimmune disease and one patient was diagnosed with dermatomyositis. Hence, these three patients were excluded from our study. This suggests that there are high chances for IFA to give false positive reactions. Also, the interpretation of IFA is subjective. A positive reaction is seen as small sharply defined fluorescent rod forms dispersed within each antigen spot, both intracellular(cytoplasmic) and extracellular (Figure:1). A negative reaction is seen as counterstained (red) cells alone or fluorescence different from that seen in the positive control wells(Figure:2).

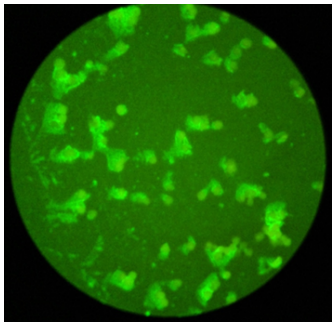

Figure:1- Positive IFA

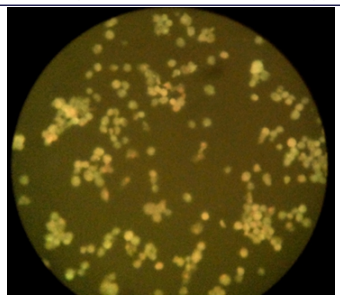

Figure:2- Negative IFA

Inspite of the good sensitivity and specificity of IFA,it is quite unsuitable in our settings, due to the high cost involved, the requirement of a fluorescent microscope, the need for trained personnel and the subjectivity of interpretation . There is also a delay of several days between the onset of the illness and the increasedantibody titer, and a positive reaction is often detected onlyafter the acute illness has resolved. Diagnosis can be difficult in the early stage of illness when the antibody titers are not yet high enough to be detected. Also, in many previous studies, a clear justification for the methodology and cutoff used was not provided, and it is likely that differences in approach evolved naturally on the basis of local antigenic strains and the pretest odds of disease, depending on the local level of scrub typhus endemicity. This variation makes it difficult to compare seroprevalence rates between studies and, more seriously, raises questions about which IFA positivity cutoff value is the most appropriate for the diagnosis of acute scrub typhus infection.

In view of increasing tourism and migration oflabourersinto our state, scrub typhus must be considered as a differential diagnosis in patients presenting with acute febrile illness and appropriate tests ordered to rule out the possibility. It is not only a re-emerging disease of northern and southern parts of Kerala, but there are cases in the central region as well, which might have been missed due to limited awareness on the available diagnostic tests, in addition to lack of clinical suspicion and immediate response to the commonly used antibiotics like doxycycline and azithromycin.

A high index of suspicion and routine testing is required to diagnose the disease. Weil-Felix test is not a very sensitive test but when positive, it is rather specific test.(Mahajan et al, 2006) IgM ELISA has got a higher sensitivity, but when positive,it must be interpreted with caution duetothe high chances of false positivity. Therefore, a combination of these two tests would be appropriate for the diagnosis of scrub typhus in moderately equipped laboratories.

CONCLUSION

Laboratory diagnosis should be strengthened for proper and early treatment of the cases to prevent morbidity and mortality due to scrub typhus. Scrub typhus can be diagnosed in moderately equipped laboratories using a combination of IgM ELISA and WF test. Hence, we suggest that *Orientia tsutsugamushi* IgM ELISA and WF test should be included in the panel of serological tests ordered for patients presenting with undifferentiated febrile illnesses.

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