

Scrub Typhus and Leptospirosis: The fallacy of Diagnosing with IgM Enzyme Linked Immunosorbant Assay

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Abstract

Introduction: Leptospirosis and scrub typhus are important causes of acute febrile illness in India. IgM Enzyme Linked Immunosorbant Assay (ELISA) is the most common diagnostic modality used for their diagnosis. The common epidemiology of both the diseases creates an opportunity of dual infections with these diseases. Therefore, the objective of the study was to detect and evaluate the cases of serological and molecular dual infections.

Methodology: A cross sectional diagnostic study from October 2013 to October 2015 was conducted on 258 patients with acute febrile illness. All the samples were subjected to IgM ELISA for scrub typhus and leptospirosis. The samples that were positive for both scrub typhus and leptospirosis were subjected to serological tests for other infections. They were also subjected to PCR assays to find out the cases of molecular dual infections.

Results: A total of twenty serum samples were positive by IgM ELISA for leptospirosis while thirty five serum samples were positive by IgM ELISA for scrub typhus. Among these, ten samples were positive for both the serological tests. These dual positives were additionally positive by serology for some other infections [Dengue (n = 2), *Mycoplasma pneumoniae* (n = 1), Malaria (n = 1), *Chlamydia pneumoniae* (n = 6), Typhoid (n = 2) and *Legionella pneumophila* (n = 1)]. Only one case of molecular dual infection was confirmed.

Conclusion: The possibility of serological co-infections should be investigated in endemic areas. In a case of serological dual infection, since there are high chances of serological cross reactivity, molecular confirmation should be sought for. The choice of therapy in inconclusive cases should include those drugs that cover for both the infections.

Keywords: Scrub typhus; Leptospirosis; Serology

Introduction

Scrub typhus is an important cause of acute febrile illness with a yearly incidence of around one million in the South East Asian region [1]. Although, there are isolated reports from the African region but the disease is geographically restricted to the Asian and Australian continent [2]. Leptospirosis is another important cause of febrile illness with a widespread global distribution [3]. The global annual incidence of severe leptospirosis is estimated to be 350,000–500,000 [4]. Both these infections present with nonspecific clinical features making it very difficult to distinguish them on clinical grounds alone. Eschar has been shown to be pathognomic of Scrub typhus but a low prevalence has been reported from most Indian studies [5]. Even though both these diseases are endemic in India, they are less commonly reported from New Delhi and surrounding areas [6-10]. The gold standard for diagnosis of scrub typhus is Immunofluorescence assay for IgM antibodies while that for leptospirosis is Microscopic agglutination test (MAT) [11,12]. Most laboratories in India diagnose both scrub typhus and leptospirosis using IgM ELISA. Both these diseases are zoonotic with rodents acting as the reservoir. They have a common seasonal pattern too. Their common epidemiology creates an opportunity of dual infections with these diseases [13]. Dual infection has been reported from Thailand and Taiwan [13,14]. Only three case reports of serological dual infection have been reported from India [15-17]. Most studies have relied on serologic tests, and the possibility remains that co-infection is due to cross-reactivity between serological tests, or new infection by one pathogen in the background of past infection by

another pathogen. It is therefore, possible that many people diagnosed with leptospirosis in the past may actually be cases of scrub typhus and vice versa. Molecular assays are known to be more specific and therefore, the percentage of molecular co-infection in these cases of serological co-infections should be determined. The objective of the study was to estimate the prevalence of serological and molecular dual infections and evaluate them for possible cross reactivity.

Methodology

A cross sectional diagnostic study was designed whereby a total of 258 patients with acute febrile illness were included in the study. The time period of the study ranged from October 2013 to October 2015. A brief clinical history of all the patients was taken. The serum samples received from these patients were subjected to IgM ELISA for both scrub

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typhus and leptospirosis (Scrub typhus IgM Indirect ELISA, Inbio Inc, USA; Leptospirosis IgM Indirect ELISA, Panbio, Australia). The samples that were positive for both scrub typhus and leptospirosis were subjected to serological tests for other infections. The tests for following infections were conducted: Dengue, malaria, enteric fever, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila*. Malaria antigen was detected using immunochromatographic test (ICT) using monoclonal anti-pan specific p LDH antibody and anti- P.f pLDH antibody (Advantage MAL CARD, J. Mitra and Co. Pvt. Ltd, New Delhi, India). ICT positive for either *Plasmodium vivax* or *Plasmodium falciparum* was taken as positive. Dengue virus was diagnosed using Anti Dengue IgM μ capture ELISA kit (NIV, Pune, India). Enteric fever was diagnosed using Widal test with titres greater than 1:80 for O or H antigen taken as positive. Commercial IgM Indirect ELISA was used to detect *Mycoplasma pneumoniae* (Anti *Mycoplasma pneumoniae* IgM ELISA, Euroimmun Medizinische Labordiagnostika, Lubeck, Seekamp), *Chlamydia pneumoniae* (Anti *Chlamydia pneumoniae* IgM ELISA, Euroimmun Medizinische Labordiagnostika, Lubeck, Seekamp) and *Legionella pneumophila* (Serion ELISA classic *Legionella pneumophila* 1-7 IgM) using kit instructions.

The patients that were serologically positive for both the diseases were further evaluated by performing PCR assays on their whole blood samples. Nested PCR assay for scrub typhus targeting the 56kDa gene to get a 483 bp band was used using conditions described by Furuya et al [18].

The primer sequences that were used are given below: Outer primer set P55: 5'-TCA AGC TTA TTG CTA GTG CAA TGT CTGC- 3' P34: 5'-AGG GAT CCC TGC TGC TGT GCT TGC TGCG- 3' Inner primer set P10: 5'-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3' P11: 5'-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3' The PCR amplification mixture (50 μ l volume) contained 1.25 units of Taq polymerase, Taq buffer (1.5 Mm MgCl₂, 50 mM KCl, 10m M Tris-HCl), 200 μ M of dNTPs, 10 pmol of primers (p55 and p34 in the first reaction, p10 and p11 for the second reaction) and 5 μ l of the extracted DNA. 5 μ l of the primary PCR product was used for the nested reaction. For both PCR runs, the amplification protocol consisted of denaturation of template at 94°C for 30 sec, annealing at 55°C for 2 min followed by extension at 70°C for 2 min for 35 cycles in a thermal cycler. The 483 bp PCR product from a known positive blood sample was cloned (pGEM-T easy vector). The recombinant plasmid was used as the positive control. Autoclaved double distilled water was used as negative control.

PCR for leptospirosis was performed on whole blood samples targeting the rrs gene to get a 331 bp product as described by Merien F et al with some modifications [19]. The oligonucleotide primers used were lep I, 5' -GGCGGCGCGTCTITAAACATG - 3' and lep II, 5' - TTCCCCCATTGAGCAAGATT- 3'. Amplification of DNA was performed in a total volume of 25 μ l. The reaction mixture consisted of 1.5 mM MgCl₂, 20 pmole each oligonucleotide primer, 200 μ M each dATP, dTTP, dCTP, and dGTP, one unit of Taq DNA polymerase and 50-100 ng of template. The PCR cycle consisted of initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1.5 min and extension at 72°C for 1.5 min. A final extension at 72°C for 5 min was carried out to complete the polymerization of the entire amplified fragment. The 331 bp PCR product from a standard culture strain *L. interrogans* was cloned (pGEM-T easy vector). The recombinant plasmid was used as positive control. Autoclaved double distilled water was used as negative control.

PCR products from both the PCR assays were electrophoresed

in 1.5% agarose gel containing ethidium bromide (0.5 μ g/ml) and visualized using a gel documentation system (BioRad, USA).

To confirm whether IgM IFA for scrub typhus gave the same results as that of IgM ELISA, all the samples positive for dual infections were also tested for IgM Immunofluorescence assay (Fuller Laboratories, USA) for scrub typhus. End point titres up to 1: 512 were calculated. The criteria for positivity as recommended by kit literature were kept at 1: 64. In the absence of MAT, similar confirmation could not be done for leptospirosis.

Data analysis: The clinical and laboratory parameters of patients diagnosed with scrub typhus and leptospirosis were expressed as percentage. The results were categorised into three subsets for further analysis: Scrub typhus cases (IgM ELISA for scrub typhus+, IgM ELISA for leptospirosis-, n = 25), Leptospirosis cases (IgM ELISA for scrub typhus-, IgM ELISA for leptospirosis+, n = 10) and dual positives (IgM ELISA for scrub typhus+, IgM ELISA for leptospirosis +, n = 10). Fisher's exact test was employed to analyse the clinical and laboratory parameters in the three subsets and a p-value for each parameter was calculated. The data was analysed with Stata version 12.1.

Results

A total of twenty serum samples were positive by IgM ELISA for leptospirosis while thirty five serum samples were positive by IgM ELISA for scrub typhus. Among these, ten samples were positive for both the serological tests. 88% of scrub typhus cases presented in the month of September, October and November while the maximum frequency of leptospirosis cases (80%) was seen from August to November (Figure 1).

There was no statistically significant difference between the clinical and laboratory parameters of the three subsets. Splenomegaly, pulmonary manifestations and thrombocytopenia were more common in scrub typhus cases. Acute Kidney Injury and icterus were more common in leptospirosis cases. Rash, eschar, headache and myalgia were more common in dual positives (Table 1).

The ten samples that dual positive were further analysed. These samples were additionally positive for Dengue (n = 2), *Mycoplasma pneumoniae* (n = 1), Malaria (n = 1), *Chlamydia pneumoniae* (n = 6), Typhoid (n = 2) and *Legionella pneumophila* (n = 1). One sample was positive for a total of five co-infections. Three samples were positive for a total of four infections. Four samples were positive for a total of three infections while two samples were positive for only two infections (Table 2). All the samples that were positive serologically for both scrub typhus and leptospirosis were also positive by IgM IFA for scrub typhus (Figure 2). On molecular analysis of the serological dual positives, three

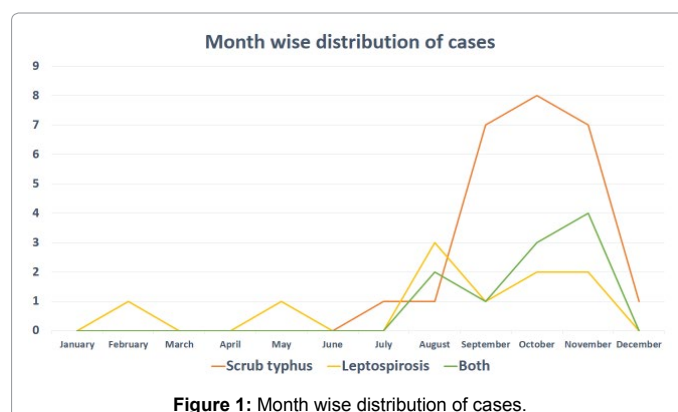


Figure 1: Month wise distribution of cases.

Clinical features	Scrub typhus cases (n = 25)	Leptospirosis cases (n = 10)	Dual positives (n = 10)	p value (Fisher exact test)
Eschar	4 (16%)	0	3 (30%)	0.149
Conjunctival suffusion	1 (4%)	0	1 (10%)	0.694
Myalgia	5 (20%)	2 (20%)	5 (50%)	0.214
Headache	4 (16%)	3 (30%)	5 (50%)	0.134
Rash	4 (16%)	1 (10%)	4 (40%)	0.304
Lymphadenopathy	7 (28%)	2 (20%)	2 (20%)	1.00
Icterus	5 (20%)	4 (40%)	2 (20%)	0.523
Hepatomegaly	11 (44%)	4 (40%)	5 (50%)	1.00
Splenomegaly	6 (24%)	1 (10%)	2 (20%)	0.882
Pulmonary manifestations	20 (85%)	5 (50%)	6 (60%)	0.190
Acute Kidney Injury	6 (24%)	6 (60%)	4 (40%)	0.116
Neurological abnormalities	6 (24%)	2 (20%)	3 (30%)	0.904
Cardiological abnormalities	6 (24%)	2 (20%)	1 (10%)	0.877
Leucocytosis	10 (40%)	3 (30%)	6 (60%)	0.429
Transaminitis	16 (64%)	4 (40%)	5 (50%)	0.364
Thrombocytopenia	14 (56%)	2 (20%)	4 (40%)	0.133

Table 1: Clinical features of cases diagnosed by serology.

S/N	Dengue	Mycoplasma	Malaria	Chlamydia	Typhoid	Legionella	PCR	IFA titre
1.	Negative	Negative	Negative	Positive	Positive	Negative	Negative	512
2.	Positive	Negative	Negative	Positive	Negative	Positive	Negative	512
3.	Negative	Positive	Negative	Positive	Negative	Negative	Negative	128
4.	Negative	Negative	Negative	Negative	Negative	Negative	Negative	128
5.	Negative	Negative	Negative	Positive	Positive	Negative	Scrub typhus and Leptospirosis	256
6.	Negative	Negative	Negative	Positive	Negative	Negative	Scrub typhus	128
7.	Positive	Negative	Negative	Negative	Negative	Negative	Negative	128
8.	Negative	Negative	Negative	Positive	Negative	Negative	Scrub typhus	256
9.	Negative	Negative	Positive	Negative	Negative	Negative	Negative	256
10.	Negative	Negative	Negative	Negative	Negative	Negative	Negative	64

Table 2: Result of other serological reactions and molecular assays in serological dual positives (Scrub typhus and leptospirosis).

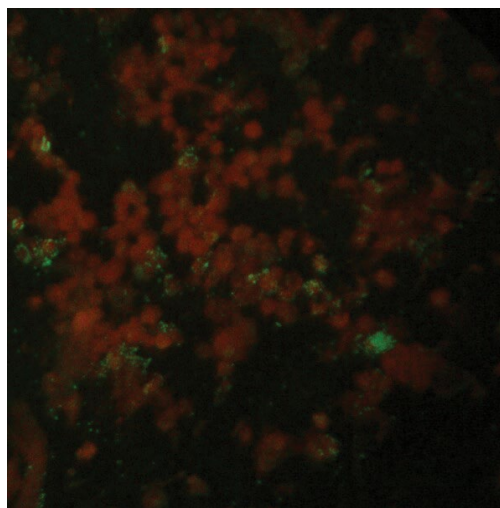


Figure 2: Immunofluorescence assay for scrub typhus showing antigen antibody reaction (green fluorescent forms) in the back ground of counterstained red cells.

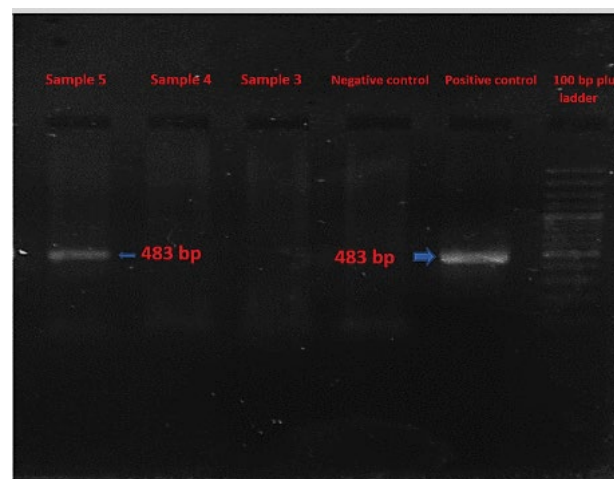


Figure 3: Nested PCR assay for scrub typhus.

samples were positive for scrub typhus PCR (Figure 3) while one sample was positive for leptospirosis PCR (Figure 4). One sample among these was positive for both the PCRs (Figure 5).

Discussion

Both scrub typhus and leptospirosis are neglected tropical diseases

with similar presenting features. Therefore, making a distinction between two diseases is difficult on clinical grounds alone. In a study conducted in New Delhi from 2005-2009, the seropositivity for scrub typhus in suspected patients was found to be 16.05% [20]. Other studies conducted in different parts of India reported prevalence rates of scrub typhus ranging from 30.8% to 46% [21-24]. The seropositivity of leptospirosis in the Delhi region has been reported between 3.22 to 42.6 per cent [6,8-10]. In a recent study conducted by our hospital from 2000-2010, seropositivity for leptospirosis was found to be 26.9% [6].

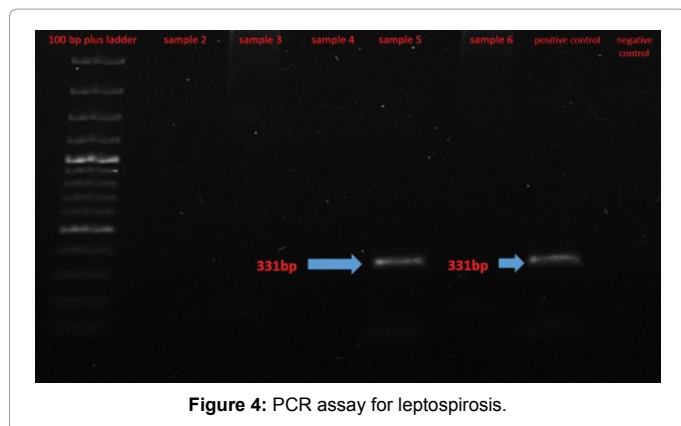


Figure 4: PCR assay for leptospirosis.

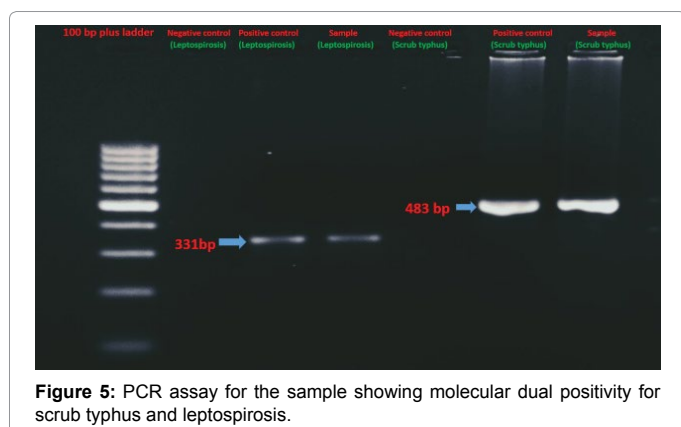


Figure 5: PCR assay for the sample showing molecular dual positivity for scrub typhus and leptospirosis.

Scrub typhus is a seasonal disease with high incidence in the post monsoon season. The incidence of scrub typhus probably increases in this season because of the increase in growth of scrubs which enables the increase in mite populations. Most of the scrub typhus patients presented in the months of September to November. Similar observations were recorded in other studies [23,25]. The incidence of leptospirosis increases after the rainy season because of the ensuing waterlogging caused. Several epidemics of leptospirosis have been reported during the monsoons [6,10,26].

The absence of statistically significant difference between the clinical and laboratory parameters of three subsets reaffirms the fact that, clinical distinction between scrub typhus and leptospirosis is very difficult. Although, eschar is usually diagnostic of scrub typhus but it is uncommon (9.5-45%) in Indian settings [21,25,27]. Eschar was noted in 30% of cases in dual infections, while it was noted in 16% of scrub typhus cases. Headache, myalgia and rash were more commonly noted in cases of dual infections. Headache and myalgia are common entities in both the diseases but rash is uncommonly seen in both scrub typhus and leptospirosis [28]. Acute Kidney Injury and icterus were more common in leptospirosis compared to the other subsets. Icterus is present in only 5-10% of leptospirosis cases but its presence signifies a severe progression. Renal failure in both scrub typhus and leptospirosis is non-oliguric and recovers completely. The incidence of renal failure in scrub typhus ranges from 18% to 66.4% [22,29,30] while it is 16 to 40% in cases of leptospirosis [31]. Pulmonary manifestations in the form of dyspnoea and cough were more commonly seen in the cases of scrub typhus. Recent studies from India show an increase in the incidence of pulmonary manifestations in cases of scrub typhus [22,25]. Lung

involvement has known to occur in 20 to 70% of leptospirosis cases [32,33]. Both scrub typhus and leptospirosis can affect the nervous system. The incidence of meningoencephalitis in cases of scrub typhus ranges from 9.5% to 23.3% [22,25,30] while it is seen in only 10-15% of cases of leptospirosis [34].

IgM ELISA has extensively been used for diagnosis of scrub typhus and leptospirosis in low resource settings. IgM antibodies for scrub typhus appears as early as 4 days after fever and stays for more than 120 days [35]. The sensitivity and specificity of the commercial IgM ELISA kit used for scrub typhus has shown to be around 85.3 % and 95.5% respectively in a previous study [36]. IgM antibody in leptospirosis begins as early as first week of illness and may take months or years to decrease [31]. The sensitivity and specificity of the kit used for leptospirosis has shown to be 52.3% and 66.4% respectively [37]. The possibility of cross reactivity has been a major issue with ELISA [38,39]. So, a patient diagnosed with leptospirosis on the basis of IgM ELISA may turn out to be falsely positive with antibodies against scrub typhus cross reacting with antigen for leptospirosis and vice versa. In any case, even if the clinical features are pointing more towards one of the two diseases, sending the samples for testing of both diseases makes good sense. Also, Rheumatoid factor which is an IgM antibody against the Fc portion of IgG, is known to interfere with Indirect ELISA and may cause false positivity. Several cross reactions of leptospirosis has been reported with Dengue, Hepatitis E, Enteric fever etc. in the past [6,40-42]. With samples in our study showing serological positivity to as many as five infections, there is a strong chance that the serological dual positivity is because of cross reactivity.

To check, whether the issues faced with ELISA could be resolved by using the reference standard for diagnosis of scrub typhus i.e., IFA, all patients with serological dual reactions were subjected to IgM IFA. All the ten patients were also positive for IgM IFA and no definite correlation with the end point titres could be drawn. Therefore, IFA did not give any advantage over IgM ELISA in the evaluation of serological dual positives. In a recent publication, Lim et al. questioned the accuracy of a serological gold standard for diagnosis of scrub typhus [43].

Most of the previous reports of co-infection have used IgM ELISA. Whether these reported cases are actually co- infection or cross reactivity of antibodies is a point of contention. In a study conducted in Thailand, of the 82 patients with serological dual infection, only 5 had PCR assay positive for both scrub typhus and leptospirosis. In our study, of the 10 patients with dual infection, only 1 was positive for both by PCR assays. This indicates that there might be gross over reporting of dual infections in many studies.

Since the possibility of cross reactivity and serological dual positivity can't be ruled out with ELISA based tests, it is better to send the samples for both the diseases. If a patient of scrub typhus is diagnosed wrongly as leptospirosis, because of the cross reactivity and is treated accordingly, the results can be catastrophic. The treatment of choice for severe leptospirosis is I.V penicillin or I.V ceftriaxone while that for scrub typhus is doxycycline. In the cases of dual serological infections, where the possibility of cross reactivity cannot be ruled out, doxycycline is a safer bet to start as mild leptospirosis also responds to doxycycline. In patients with severe manifestations, both I.V Beta lactams and doxycycline can be initiated and deescalated after results are available.

We conclude that, the possibility of serological co-infections should be investigated in endemic areas considering the high prevalence noted in our study. In a case of serological dual infection, since there are high

chances of serological cross reactivity, molecular confirmation should be sought for. The choice of therapy in inconclusive cases should include those drugs that cover for both the infections. There is a need for better serological assays and future research works should be directed towards meeting this deficit.

The limitation of the study was that, MAT could not be performed for confirmation of cases of leptospirosis. Also, in many cases the samples for molecular diagnosis were sent late in the course of illness or after initiating antibiotic therapy, which would have hampered the sensitivity of PCR assays.

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