International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319 – 6505, Impact Factor: SJIF: 5.995
Available Online at www.journalijcar.org
Volume 6; Issue 7; July 2017; Page No. 4534-4538
DOI: http://dx.doi.org/10.24327/ijcar.2017.4538.0532

Research Article

COMPARATIVE EVALUATION OF ELISA, PCR AND MICRO-IFA IN DIAGNOSIS OF SCRUB TYPHUS

Anshu Gupta*[1]., DigVijay Singh[2]., Santwana Verma[3]., Anil Kanga[4] and Sanjay Mahajan[5]

[1,2,3,4]Department of Microbiology, Indira Gandhi Medical College, Shimla
[5]Department of Medicine, Indira Gandhi Medical College, Shimla

ARTICLE INFO

Article History:

Received 9th April, 2017
Received in revised form 11th May, 2017
Accepted 17th June, 2017
Published online 28th July, 2017

Key words:

Scrub typhus, ELISA, IFA,
PCR, 56kDa antigen

ABSTRACT

Background: Scrub typhus is a rickettsial infection which is caused by Orientia tsutsugamushi and transmitted by the bite of chigger. It is an important cause of acute undifferentiated febrile illness. Delay in diagnosis may prove to be life threatening.
Objective: To compare IgM ELISA and PCR for 56kDa antigen with Micro IFA-IgM in diagnosis of scrub typhus and perform IgG ELISA, MicroIFA-IgG for prevalence of scrub typhus.
Material and Methods: There were 327 cases clinically suspected of scrub typhus over a period of 1 year. Commercially provided kits for IgM ELISA (In Bios Inc. USA), IgG ELISA (In Bios Inc. USA), IgM IFA (Fuller Laboratories, USA), IgG IFA (Fuller Laboratories, USA) and PCR for 56kDa antigen (Roche, Indianapolis, IN) were used. Analysis was done using statistical software Epi-info version 7(7.1.1.0).
Results: Out of 327 clinically suspected scrub typhus cases, 227 were IgM and/or IgG ELISA positive. Out of 177 IgM ELISA positive cases, 174(98.3%), 151(85.3%), 129(73%) and 17(9.6%) cases were positive for IgM IFA, IgG IFA, IgG ELISA and PCR respectively. The sensitivity of IgM ELISA and IgG ELISA as compared to IgM IFA and IgG IFA was 95% and 89% respectively. The specificity of IgM ELISA as compared to IgM IFA was 93%. The sensitivity and specificity of nested PCR was 9.6% and 100% respectively.
Conclusion: Good specificity and low sensitivity of PCR using plasma needs further evaluation for optimizing the type of sample required for PCR. In resource limited settings, IgM ELISA seems to be a good alternative method for serological diagnosis.

INTRODUCTION

Scrub typhus is a zoonotic disease caused by an obligate intra-cytoplasmic bacterium, *Orientia tsutsugamushi* and transmitted by the bite of chigger. It has now been excluded taxonomically from genus *Rickettsia* because its 16S rRNA gene sequences revealed that it is located away from rickettsial cluster[1]. It is a public health problem in Asia, where about 1 million new cases are identified annually and 1 billion people may be at risk for this disease. Mortality occurs in 30% to 50% of untreated cases[2]. The disease has shown a seasonal pattern with maximum positivity from September to November. The basic pathology of scrub typhus is vasculitis with its main target cells being monocytes. The early classical manifestation includes fever, rash, lymphadenopathy and eschar. Eschar has been considered pathognomic in various studies but its variable occurrence in different geographical region is a concern. Its presence therefore is not essential for the diagnosis of scrub typhus. Progression of severe scrub typhus may manifest as acute respiratory distress, meningo-encephalitis and acute renal failure[3]. The rapid progression to fatal complications and associated mortality calls for improved diagnostic services and prompt initiation of treatment. Clinical diagnosis of scrub typhus is very difficult because of its vague presenting features. In 2003, 96 cases of fever of unknown origin were reported in Himachal Pradesh which were diagnosed to be scrub typhus.[4] According to the laboratory data, there were 6455 positive cases in Himachal Pradesh in last 5 years till 16th June 2015, of which 129 deaths occurred. *O. tsutsugamushi* is difficult to grow and requires Bio Safety Level 3 (BSL 3) facilities[5]. Serology is the mainstay of diagnosis in most laboratories and can be done by various tests. IgM ELISA is a rapid and sensitive method with sensitivity ranging from 93% to 97% and specificity from 91% to 95%.[6] Indirect Immunofluorescence Assay (Micro-IFA) can quantify either IgG or IgM antibodies independently and is considered as a gold standard. The sensitivity and specificity of IFA is 95% and 88% for IgM antibodies and 90% and 100% for IgG antibodies respectively.[7] It is useful in diagnosis of both acute cases and seroepidemiology[6].

*Corresponding author: **Anshu Gupta**
Department of Microbiology, Indira Gandhi Medical College, Shimla

However, it requires the use of a fluorescent microscope and a skilled observer. Polymerase Chain Reaction (PCR) of eschar material is more sensitive than the buffy layer of blood and results are available in 24 hours. It may be positive in patients on antibiotics.[8] However PCR requires expensive equipment and infrastructure. Sensitivity depends on sample type and timing.

MATERIAL AND METHODS

Informed consent was obtained from all the patients or their guardians. Ethical clearance was taken from the institute's ethical committee.

Samples

Five ml of venous blood (3ml serum tube+ 2ml EDTA tube) under all aseptic conditions was collected from 327 patients clinically suspected of scrub typhus from June 2015 to May 2016.

Serology

Serum was separated by centrifugation at 2500g for 10 min and the clots were stored at -20˚C till further analysis. An indirect ELISA that detects IgM and IgG antibodies to *Orientia tsutsugamushi* was performed in all serum samples. The absorbance was measured at 450 nm. A titer of 0.468 OD for IgM ELISA and 0.5 OD for IgG ELISA was taken as positive. The serum samples from patients were subjected to IgM IFA and IgG IFA. The IFA slides were precoated with four scrub typhus strains (Gilliam, Karp, Kato, and Boryong) propagated in L292 cells. Serum samples of all the patients were put at dilutions of 1:64, 1:128, 1:256, and 1:512 for IgM IFA and 1:128, 1:256, 1:512 and 1:1024 for IgG IFA . The antigen-antibody reaction was visualized using a fluorescent microscope. A positive reaction was visualized as small green fluorescent rods in the background of counterstained red cells. A titer of ≥64 units for IgM IFA and ≥128 units for IgG IFA were taken positive.

PCR

DNA was extracted from 200 µl of plasma using the AuPreP GENbt DNA Extraction kit as per manufacturer's instructions. The purified DNA was aliquoted and amplification was carried out by real- time format. The gene coding for 56kDa antigen of *O. tsutsugamushi* was amplified by PCR and the primer sequences used are given below:

Forward primer: 5'-
AACTGATTTTATTCAAACTAATGCTGCT-3'.
Reverse primer: 5'-
TATGCCTGAGTAAGATACRTGAATRGAATT-3'.
Probe: 6FAM-
TGGGTAGCTTTGGTGGACCGATGTTTAATCT-TMR.

According to $N_1V_1=N_2V_2$, the volume of master mix used per reaction was 10 µl. Forward and reverse primer 1 µl each, Probe 0.8 µl and distilled water 2.2 µl per reaction to make a total volume of 15 µl. To 15 µl volume, 5 µl of the extracted DNA was added to make a total reaction volume of 20 µl. The PCR profile was determined with the following steps: Denaturation at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s and final incubation at 65˚C for 1min in a thermocycler. The assay was run on StepOne Real-Time PCR System along with a positive and negative control. The Ct value was determined for the samples positive for PCR.

Statistical analysis

We computed the sensitivities and specificities, the positive and negative predictive values for all the 5 tests. Statistical analysis was done using statistical software Epi-info version 7(7.1.1.0).

RESULTS

Enzyme linked Immunosorbent Assay (ELISA)-

Out of 327 clinically suspected scrub typhus cases, 227(69.4%) were IgM and/or IgG ELISA positive. One hundred cases were negative for both IgM and IgG ELISA. Out of 227 cases, 179(78.8%) were positive for IgG ELISA and 177 (77.9%) cases were positive for IgM ELISA. A total of 129(56.8%) were positive both for IgM and IgG ELISA. Out of 177 IgM ELISA positive cases, 174(98.3%) cases were positive for IgM IFA, 151(85.3%) were positive for IgG IFA, 129(73%) cases were positive for IgG ELISA and 17(9.6%) cases were positive for PCR. Out of 179 IgG ELISA positive cases, 178(99.4%) cases were positive for IgG IFA, 137(76.5%) by IgM IFA, 129(72%) by IgM ELISA and 17(9.4%) cases by PCR. Out of 50 cases negative for IgM ELISA, 50(100%) cases were positive for IgG ELISA, 49(98%) cases positive for IgG IFA, 9(18%) cases positive for IgG IFA and none positive for PCR. Out of 48 cases negative for IgG ELISA, all 48(100%) cases were positive for IgM ELISA, 46(95.8%) cases were positive for IgM IFA, 22(45.8%) cases positive for IgG IFA and 1(2.1%) positive for PCR.

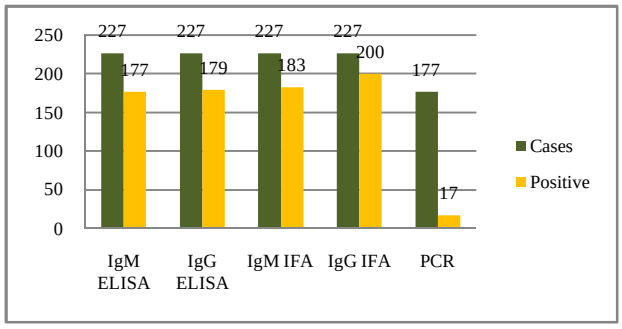

Chart 1 Comparing positivity by using different serological tests and PCR in ELISA (IgM/IgG) positive cases (n=227)

Micro-Immunofluorescence Asaay (Micro-IFA)

Out of 227 cases positive for IgM and/or IgG ELISA, 183 (80.6%) cases were positive for IgM IFA whereas 200(88.1%) were positive for IgG IFA. Out of 129 cases positive for both IgM and IgG ELISA, 128 (99.2%) cases were positive both for IgM and IgG IFA whereas out of 100 cases negative for both IgM and IgG ELISA, 9(9%) cases were positive by IgM IFA while all 100 cases were negative by IgG IFA.Out of 183 IgM IFA positive cases, 174(95%) cases were positive for IgM ELISA, 157(85.7%) cases were positive for IgG IFA,137(74.8%) cases were positive for IgG ELISA and 17(9.2%) cases were positive for PCR. Out of 200 positive IgG IFA cases, 178(89%) were positive for IgG ELISA, 157(78.5%) cases positive for IgM IFA, 151(75.5%) cases for IgM ELISA and 17(8.5%) cases positive by PCR. Out of 44 cases negative for IgM IFA, 43(97.7%) cases were positive for IgG IFA, 42(95.4%) cases were positive for IgG ELISA, 3(6.8%) cases positive for IgM ELISA and none positive for PCR wheras out of 27 IgG IFA negative cases, 26(96%) cases

were positive each for IgM ELISA and IgM IFA, 1(3.7%) case positive for IgG ELISA and none was positive for PCR.

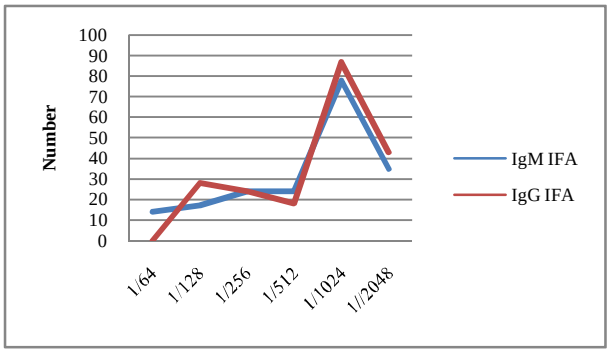

Chart 2 Various dilutions of IgM IFA and IgG IFA in scrub typhus positive cases

Polymerase Chain Reaction (PCR)

Out of 177 IgM ELISA positive cases, 17 (9.6%) cases were positive by PCR. Out of 17 PCR positive cases, all 17(100%) cases were positive for IgM ELISA, IgM IFA and IgG IFA whereas 16(94.1%) cases were positive for IgG ELISA. Out of 160 PCR negative cases, all 160(100%) cases were positive for IgM ELISA, 157(98.1%) cases were positive for IgM IFA, 134(83.75%) cases were positive for IgG IFA and 113(70.6%) cases were positive for IgG ELISA.

Table 1

	IgG ELISA +	IgG ELISA-	Total
IgM ELISA +	129	48	177
IgM ELISA -	50	0	50
Total	179	48	227

	IgM IFA +	IgM IFA-	Total
IgM ELISA +	174	3	177
IgM ELISA -	9	41	50
Total	183	44	227

	IgG IFA +	IgG IFA-	Total
IgM ELISA +	151	26	177
IgM ELISA -	49	1	50
Total	200	27	227

	PCR +	PCR-	Total
IgM ELISA +	17	160	177
IgM ELISA -	0	0	0
Total	17	160	177

Total number of cases positive for scrub typhus by any diagnostic method (ELISA/IFA/Nested- PCR) was 236. (227 cases positive by IgM and/or IgG ELISA + 9 cases positive by IgM IFA). Hence, the clinical features, Biochemical, hematological parameters and radiological investigations were studied in 236 cases.

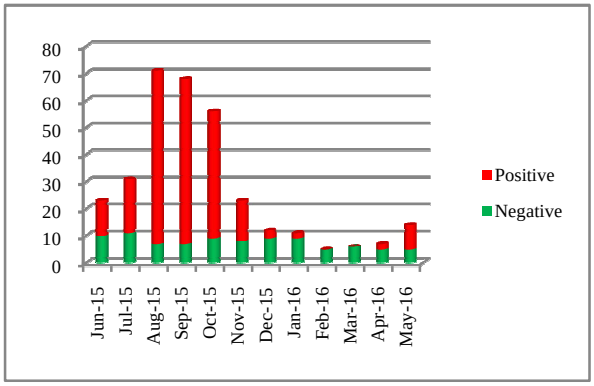

Chart 3 Seasonal Distribution of Scrub typhus positive cases (n=236)

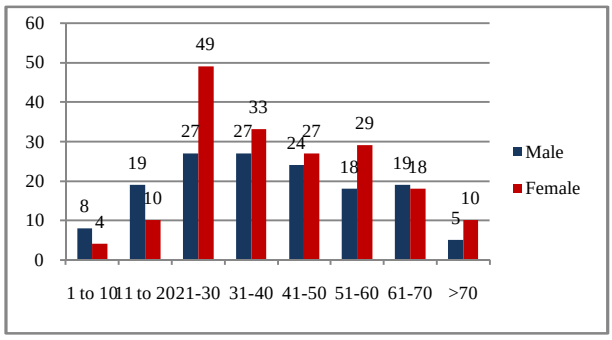

Chart 4 Age and Sex Distribution of Scrub typhus positive cases (n=236)

Fever >39˚C was seen in all 236 patients. The duration of the fever at the time of diagnosis ranged from 5-20 days with mean duration of 12 days. Rash was seen in 35% (82/236) cases. The rash was mostly maculopapular and was predominantly found on the trunk. Eschar was seen in 33(14%) cases with chest and extremities being the most common location. Lymphadenopathy was reported in 84(35%) cases. Icterus was seen in 64(27%) cases. Pulmonary manifestations were noted in 148(62.7%) cases with most patients presenting with cough 100(42%). Two cases (1%) progressed to Acute Respiratory Distress Syndrome (ARDS). Hepatomegaly (21%) and Splenomegaly (15%) was noted in some patients. Acute kidney injury evidenced by increased urea and creatinine was seen in 226(95%) cases. Features of encephalitis were seen in 23(10%) cases. Myocarditis was seen in 16(5%) cases whereas 7 cases of pericardial effusion occurred.

Biochemical and Haematological Parameters

Haematological abnormalities noted in patients of scrub typhus were anaemia (64%, 151/236), leucocytosis (37%, 87/236), thrombocytopenia (32%, 76/236) and increased transaminase levels (69%, 163/236). Pancytopenia was noted in (75%, 15/20). Total of 56 patients were treated with azithromycin while doxycycline was used to treat 198 patients. Both doxycycline and azithromycin was given to treat 28 patients. 230(97.4%) patients were cured and discharged while 6(2.5%) died.

Evaluation of Pcr Positive Samples

Out of 177 cases who presented with fever duration of 7 or less than 7 days, 174 were positive for IFA IgM while 17 were positive for PCR. The mean duration of fever in PCR positive cases was 6 days with a minimum of 1 day. Eschar was positive in only 1 PCR positive case. Most common presentation in PCR positive cases was cough with chest pain along with increased transaminase and alkaline phosphatase levels. All the PCR positive cases responded to treatment except 1who died.

Sensitivity and Specificity of various tests

The sensitivity of IgM ELISA, IgG ELISA, Micro- IFA IgM and Micro- IFA IgG was found to be 75%, 76%, 81% and 85% respectively. The specificity of all the tests was 100%. The sensitivity of IgM ELISA and IgG ELISA as compared to IgM IFA and IgG IFA was 95% and 89% respectively. The specificity of IgM ELISA as compared to IgM IFA was 93%. The specificity of IgG ELISA as compared to IgG IFA was

99.2 %. The sensitivity and specificity of PCR was 9.6% and 100% respectively.

Table 2 Diagnostic Evaluation of Serological and Molecular Method

Tests	IgM ELISA	IgG ELISA	IgM IFA	IgG IFA	PCR
Sensitivity	75%	76%	81%	85%	9.6%
Specificity	100%	100%	100%	100%	100%
PPV	100%	100%	100%	100%	100%
NPV	61%	61.4%	67.4%	72%	35%

DISCUSSION

Scrub typhus is a seasonal disease with high incidence in the post monsoon season, probably because of the spurt in the growth of scrubs. This creates a favorable environment for the increase in mite population. Total of 186 patients presented in the months of September to November. This seasonal pattern should alert the physicians to consider scrub typhus in the list of differential that commonly includes dengue and malaria during these months. Age group of 21- 60 years was the most commonly affected age group with predominance of females of reproductive age group as females make the major working population in Himachal Pradesh. Empirical therapy may be used in areas where no specific testing facilities exist especially in seriously ill. Rapid defervescence after antibiotic therapy is so characterstic that in itself can be considered as a diagnostic test. Scrub typhus is potentially treatable and therefore the morbidity and mortality associated with it can be reduced if diagnosed early. Doxycycline is considered as a drug of choice for treatment of scrub typhus[9]. The diagnosis of scrub typhus has traditionally been based on the assessment of the antibody titer in the serum samples obtained during the acute and convalescent phases of illness. However, it takes several weeks to confirm the diagnosis through serologic testing for establishing a ≥4-fold rise in titer. The 56-kDa type-specific antigen (TSA) is best suited for diagnostic testing, as it is the major immunodominant surface protein, containing both group- and strain-specific epitopes, and it is abundant in patient sera. IFA uses fluorescence-labeled anti-human immunoglobulin to detect antibodies in the serum of the patient that have bound to immobilized bacterial antigen on a slide.

The results of PCR are available within 2-3 hours, and this can greatly help to guide proper patient management. Plasma was used to perform quantitative detection by PCR. PCR assay was based on specific primers derived from the 56-kDa major outer membrane protein antigen of *O. tsutsugamushi*. PCR assays, either conventional or real-time, targeting the 56 kDa gene, 47 kDa gene, *16 S rRNA* and *groEL* gene have been explored and reported to have specificity approaching 100%[3]. Sensitivity of the nested PCR assays using 56 kDa or the *16 S rRNA* genes can be as low as 22.5% to 36.1%[10]. Real-time PCR assays show a better sensitivity ranging from 45%[11] to 82%[12]. It's been shown in a study from Thailand that *O. tsutsugamushi* DNA can to be detected by the nested PCR technique as early as day 3 of the fever phase; this is before the appearance of specific antibodies in the blood[13]. However, in our study, *O. tsutsugamushi* DNA could be detected in the blood by PCR as early as the first day of fever onset. Kim *et al*. retrospectively evaluated the accuracy of conventional PCR targeting the 16S rRNA gene (16S C-PCR) for diagnosing scrub typhus and found an increased sensitivity of 87.0% and specificity of 100% compared with those obtained with other targets and is thus a simple and clinically useful method with good diagnostic accuracy.[14]

In a study conducted by Prakash JAJ *et al*, only 9(10.3%) samples tested positive both for IgM ELISA and Nested PCR with a sensitivity of 58% and 100% specificity by LCA analysis[15]. The low yield in PCR could be due to the presence of heme, a known inhibitor of PCR in the sample[16]. The improved sensitivity obtained by Kim *et al*, also could be due to the volumes (50µl) used for extracting DNA[17] in contrast to 200 µl used in our study, as recommended by manufacturer. High rate of false negative PCR results whose IgM titers were high may be due to clearance of bacteria by the immune system[18]. The use of 2 sets of primer for 56kDa antigen detection in cases of Nested PCR has improved specificity.

To conclude, good specificity and low sensitivity of PCR using plasma as sample needs further evaluation of this test using blood, serum, buffy coat and eschar biopsy samples for optimizing the type of sample required for PCR. New technique for DNA extraction like Magnetic bead extraction may be evaluated comparing the conventional methods of DNA extraction for PCR. Since antigen detection tests have low sensitivity/ specificity and require biopsy specimens, in the clinical setting, serological assays are the mainstay of diagnosis[3] as they are simple and easy to perform[19]. IgM ELISA for scrub typhus has high diagnostic accuracy and is less subjective than the IgM IFA. We suggest that the IgM ELISA may be used as an alternative reference test to the IgM IFA for the serological diagnosis of scrub typhus.

References

1. Ohashi N, Fukuhara M, Shimada M, Tamura A. Phylogenetic position of Rickettsia tsutsugamushi and the relationship among its antigenic variants by analyses of 16S rRNA gene sequences. *FEMS Microbiol Lett*. 1995; 125(2-3):299-304.
2. Bakshi D, Singhal P, Mahajan SK, Subramaniam P, Tuteja U, Batra HV. Development of a real-time PCR assay for the diagnosis of scrub typhus cases in India and evidence of the prevalence of new genotype of *O. tsutsugamushi*. *Acta Tropica* 2007; 104(1): 63-71.
3. Peter JV, Sudarsan TI, Prakash JA, Varghese GM. Severe scrub typhus infection: Clinical features, diagnostic challenges and management. *World J Crit Care Med*. 2015; 4(3): 244-250.
4. Sharma A, Mahajan S, Gupta ML, Kanga A, Sharma V. Investigation of an outbreak of scrub typhus in the Himalayan region of India. *Jpn J Infect Dis* 2005; 58: 208-10.
5. Luksameetanasan R, Blackshell SD, Kalambaheti T, Wutheikanum V, Chierakul W, Chueasuwanchai S *et al*. Patient and sample related factors that affect the in vitro isolation of *Orientia tsutsugamushi. Southeast Asian J Trop Med Public Health* 2007; 38: 91-96.
6. Land MV, Ching WM, Dasch GA, Zhang Z, Kelly DJ, Graves SR *et al*. Evaluation of commercially available recombinant protein enzyme linked Immunosorbent assay for detection of antibodies produced in scrub typhus Rickettsial infections. *J Clin Microbiol* 2000; 38: 2701-5.
7. Phetsouvanh R, Blackshell SD, Jenjaroen K, Day NPJ, Newton PN. Comparision of Indirect Immunoflorescence Assays for diagnosis of scrub

typhus and murine typhus using venous blood and finger prick filter paper blood spots. *Am J Trop Med Hyg* 2009; 80: 837-840.
8. Kaore NM. Laboratory Diagnosis of Scrub Typhus. *J K Science* 2010; 12:72-5.
9. Sheehy TW, Hazlett D, Turk RE. Scrub typhus. A comparison of chloramphenicol and tetracycline in its treatment. *Arch Intern Med.* 1973; 132(1):77-80.
10. Fournier PE, Siritantikorn S, Rolain JM, Suputtamongkol Y, Hoontrakul S, Charoenwat S, *et al*. Detection of new genotypes of *Orientia tsutsugamushi* infecting humans in Thailand. *Clin Microbiol Infect* 2008; 14:168-73.
11. Allen AC, Spitz S. A Comparative Study of the Pathology of Scrub Typhus (Tsutsugamushi Disease) and Other Rickettsial Diseases. *Am J Pathol*. 1945; 21:603-681.
12. Rathi NB, Rathi AN, Goodman MH, Aghai ZH. Rickettsial diseases in central India: proposed clinical scoring system for early detection of spotted fever. *Indian Pediatr*. 2011; 48:867-872.
13. Paris DH, Blacksell SD, Newton PN, Day NP. Simple, rapid and sensitive detection of Orientia tsutsugamushi by loop-isothermal DNA amplification. *Trans R Soc Trop Med Hyg*. 2008; 102:1239-1246.
14. Kim DM, Park G, Kim HS, Lee JY, Neupane GP, Graves S, Stenos J. Comparison of conventional, nested, and real-time quantitative PCR for diagnosis of scrub typhus. *J Clin Microbiol*. 2011; 49:607-612.
15. Prakash JAJ, Kavitha ML, Mathai E. Nested polymerase chain reaction on blood clots for gene encoding 56 kDa antigen and serology for the diagnosis of scrub typhus. *Indian Journal of Medical Microbiologists.* 2011; 29(1):47-50.
16. Liu YX, Cao WC, Gao Y, Zhang JL, Yang ZQ, Zhao ZT, *et al*. *Orientia tsutsugamushi* in eschars from scrub typhus patients. *Emerg Infect Dis* 2006; 12:1109-12.
17. Kim DM, Yun NR, Yang TY, Lee JH, Yang JT, Shim SK, *et al*. Usefulness of nested PCR for the diagnosis of scrub typhus in clinical practice: A prospective study. *Am J Trop Med Hyg* 2006; 75:542-5.
18. Saisongkorh W, Chenchittikul M, Silpapojakul K. Evaluation of nested PCR for the diagnosis of scrub typhus among patients with acute pyrexia of unknown origin. *Trans R Soc Trop Med Hyg.* 2004; 98:360-366.
19. McDade JE. Rickettsial diseases. In: Hausler WK, Sussman M, editors. Topley & Wilson's Microbiology & Microbial Infections. London: Arnold; 1998. pp. 995-1011.

How to cite this article:

Anshu Gupta *et al* (2017) ' Comparative Evaluation Of Elisa, Pcr And Micro-Ifa In Diagnosis Of Scrub Typhus', *International Journal of Current Advanced Research,* 06(07), pp. 4534-4538. DOI: http://dx.doi.org/10.24327/ijcar.2017.4538.0532

****