DETECTION OF *Orientia tsutsugamushi* Tsa GENE FROM ACUTE FEBRILE SCRUB TYPHUS FEVER CASES IN WEST BENGAL , INDIA.

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ABSTRACT

Scrub typhus is popularly known as the "tsutsugamushi disease", after the name of its causative agent, Orientia tsutsugamushi. O. tsutsugamushi is a gram-negative, obligate, intracellular bacterium, and scrub typhus is a very rapidly emerging disease that has been neglected for years. Globally, around one billion people are reported to be affected from this disease, and a million of them are said to suffer from it annually. Considering the rampant intensity of scrub typhus, we have applied different analytical tools like DNA extraction, Sandwich ELISA and Nested PCR to detect and investigate the O. tsutsugamushi Tsa gene from the blood samples of the patients collected from the Calcutta School of Tropical Medicine. We have prepared a systematic, statistical observation and critical analyses of the scrub typhus IgM positive patients based on certain parameters like gender, age groups, distribution among the different districts and months etc, which will be highly informative in analyzing the source and distribution of the disease. In addition, it will also be potentially tactful in order to curb further spread of the disease.

Keyword: - *Eschar, DNA extraction, Sandwich ELISA, Nested PCR, IgM*

1. INTRODUCTION

Scrub typhus is an acute, febrile infection caused by the bacterium *O. tsutsugamushi* (also known as Rickettsia). Scrub typhus is also known by other names, such as chigger-borne typhus. It is a zoonotic (sylvatic) disease and the vector is an arthropod, trombiculid mite (*Leptotrombidium deliense*). Humans are accidental or dead-end hosts. Scrub typhus is re-emerging, infectious and rampant in India, especially in the sub-Himalayan belt, such and Jammu-Kashmir and Himachal Pradesh (north), Nagaland (east), Rajasthan (west) and Darjeeling in West Bengal (east). This disease is proven to be more deadly during the monsoons, however in southern India, outbreaks are more during the colder months of the year.

Symptoms of scrub typhus are varied, but the development of a papule at the site of infection is common. The common areas of bite are at the neck, genitalia, or axille. The papule ultimately heals, leaving behind black eschar. Acute fever is common, with temperatures raging from 103-104^{o}F, accompanied by chills, and infection in various parts of the body, most notably the conjunctiva and the intestinal lining. Inflammation of lymphatic nodes are also seen. Around a week later, a macropapular rash emerges first at the trunk and then to the extremities. These symptoms generally disappear in 2 weeks, without any medical assistance. However, various complications may arise, such as intestinal pneumonia and meningoencephalitis, with mortality rates up to 30%.

Mite islands are small areas where the ecosystem is favourable for the growth of mites. When humans walk into these mite islands, they get bitten by the chigger mites (larvae) and get infected subsequently. The larvae feed on the serum of homeothermals once during their life cycle, but adult mites do not feed on humans. The larval stage is both a reservoir and vector for feeding on humans and rodents. Incubation of scrub typhus is around 5-20 days after the

first bite. Rodents belonging to *Rattus sp*. are the reservoir hosts of scrub typhus. Immunity against *O. tsutsugamushi* are effected by antigenic diversity of its agent, where the cross protection are weak, which causes inability to produce an effective vaccine. Protective immunity to homologous strain is from 1-3 years which is high and lasts long, whereas protective immunity to heterologous strain is comparitively shorter and lower (1-3 months). Both humoral and cell mediated immunity are important, where humoral receptors inhibit *O. tsutsugamushi* by blocking its surface components used to adhere to the target cells, and cell mediated immunity produces antigen specific lymphocytes that activates macrophages.

2. TOOLS AND METHODOLOGIES

2.1 DNA extraction from blood samples of patients using QIAmp DNA Minikit®

Components present in the QIAmp Blood Minikit® [Figure 1] are-

-For 50 DNA mini-preps- 50 QIAmp Minispin column, QIAGEN protease, Reagents, Buffers, and 2ml collection tubes.

-For 250 DNA mini-preps- 250 QIAmp Minispin column, QIAGEN protease, Reagents, Buffers, and 2ml collection tubes.

We pipetted out 20 microlitre QIAGENProtease into a 1.5ml MCT (microcentrifuge tube), after which we added 200 microlitre blood sample. Next, 200 microlitre Buffer AL was added and this was vortexed properly. The incubation was done at 56^{o}C for a time period of 10 minutes. The MCT was centrifuged to remove any remaining liquid droplets from the lid. After this, 200 microlitre of 96-100% ethanol was added and vortexed, after which centrifugation was repeated. The mixture was pipetted onto the QIAmp Minispin column and centrifugation was done at 6000 x g for 1 minute. The collection tubes and flow-through were discarded. The column was then placed in a new 200 ml tube and 500 microlitre Buffer AW2 was added. This was put under centrifugation at a speed of 20,000 x g for 3 minutes. Again, the collection tubes and flow-through were discarded and the column placed in a new 1.5 ml MCT. After addition of 200 microlitre buffer AE or dH_2O, this was incubated at room temperature (15-25^{o}C) for 1 minute. For the final step, centrifugation at 6000 x g was carried out to elude the DNA.

Fig-1: The DNA Extraction kit used for the study

2.2 Scrub typhus IgM investigation by Sandwich ELISA method

Preparation of reagents from the given kit [Figure 2]-

1X Wash buffer- The 10X wash buffer is diluted to 1X using Biological or High Grade Water by mixing the given 120 ml of 10X Wash Buffer with 1080 ml of Biological or High Grade water. Then it is stored at room temperature for not more than 6 months. [If any microbial growth is seen, the Wash Buffer must be discarded]

Microtiter wells- The number of wells to be coated are selected. The remaining wells must be sealed properly with desiccant inside the pouch and kept at 2-8^{o}C until ready to use.

The reagents were allowed to reach at room temperature and the samples and controls were diluted to 1:100. Then 100 microlitre of 1:100 samples and controls were applied per well after reading the sample application chart. After this the plate was covered with parafilm or platecover and incubated at 37^{o}C in an incubator for 10 minutes. They were then washed 6 times with the 1X wash buffer, after which 100 microlitre of the enzyme HRP conjugate was added in each well. The plate was again incubated for 30 mins at 37^{o}C. After rewashing the plates 6 times, 150 microlitre of EnWash was applied and incubated for 5 mins at room temperature. Again the plates were washed 6 times and 100 microlitre of liquid TMB was applied and then incubated in the dark for 10 minutes. After this, 50 microlitre of Stop Solution was added in the same manner in which TMB was added. After 1 minute, the OD (450 nm) values were read.

Fig-2: The ELISA kit used for the study

2.3 Identification of Tsa gene by Nested PCR

All the blood samples were collected with full consent of the patients. The EDTA samples were subjected to DNA extraction using the QIAmp DNA Minikit. Purified samples were kept at -20^{o}C. The 626 bp segment gene encoding the 56 kDa protein antigen of the bacterial genome was amplified by nPCR using a modified protocol. It was made up of 2.5 µl of 10X Taq buffer, 0.8 µl of 10 mM dNTP's, 0.5 µl of 25 mM MgCl2, 0.5 µl of 10 picomoles forward and reverse primers, 1.5 units Taq DNA polymerase, and 1 µl of DNA template with volume adjusted to 25 µl with Milli-Q water. The cycling conditions for the first round of nPCR were 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1.5 min, 72°C for 2 min, and a final extension at 72°C for 5 min. First round products (~800 bp) were considered template for second round with initial denaturation at 95°C for 3 min, followed by 25 cycles of 94°C for 30 s, 57°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min. The products were visualized by gel electrophoresis.

3. DATA DISTRIBUTION AND OBSERVATIONS

After performing the ELISA assay on Scrub typhus IgM, it was found that a total of 374 patients' blood samples were analysed to detect Scrub typhus from April to June(15th), 2019 in Calcutta School of Tropical Medicine. Out of these, 113 patients were found to be Scrub typhus positive i.e. the blood of these patients contained the Scrub typhus gene. The results have been analyzed further.

3.1 Age groups

The total number of patients who were found to be Scrub typhus positive were divided into two groups: patients whose age was greater than or equal to 15 years and patients whose age was less than 15 years [Figure 3]. It was observed that majority of the scrub typhus positive patients were in the age group of below 15 years. Out of 113 patients, 25(22%) patients belonged to the age group of greater than or equal to 15 years and 87(78%) patients were in the age group of less than 15 years.

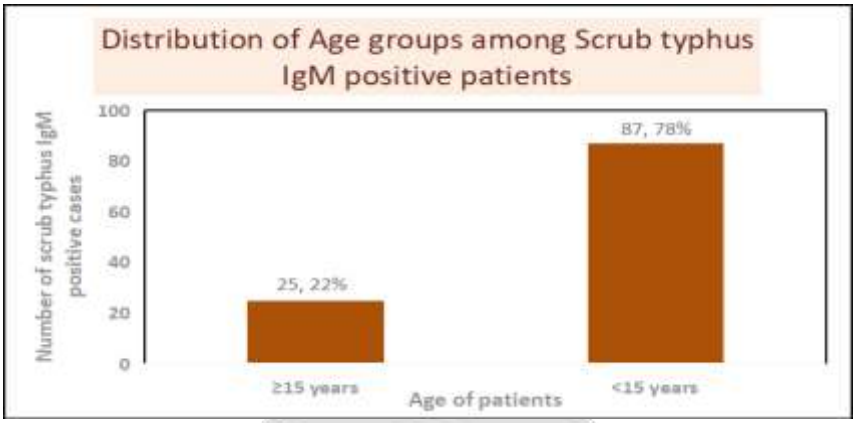

Fig-3: Graph showing the distribution of Scrub typhus positive cases according to age of patients

3.2 Districts

The scrub typhus positive patients were divided into nine groups based on their districts, namely: Kolkata, North 24 Pargannas (N 24 Pgs), South 24 Pargannas (S 24 Pgs), East Midnapore, West Midnapore, Hooghly, Howrah, Murshidabad and Nadia. It was observed that South 24 Pargannas had the highest number of Scrub typhus positive patients (41 patients, 37%) while West Midnapore had the least cases (2 patients, 2%). North 24 Pargannas had the second highest number (18 patients, 16%). Howrah had 17 patients (15%), Kolkata had 11 patients (10%), East Midnapore had 7 patients (6%), Hooghly and Murshidabad had almost the same number of patients (5%) and Nadia had 5 patients (4%) [Figure 4]

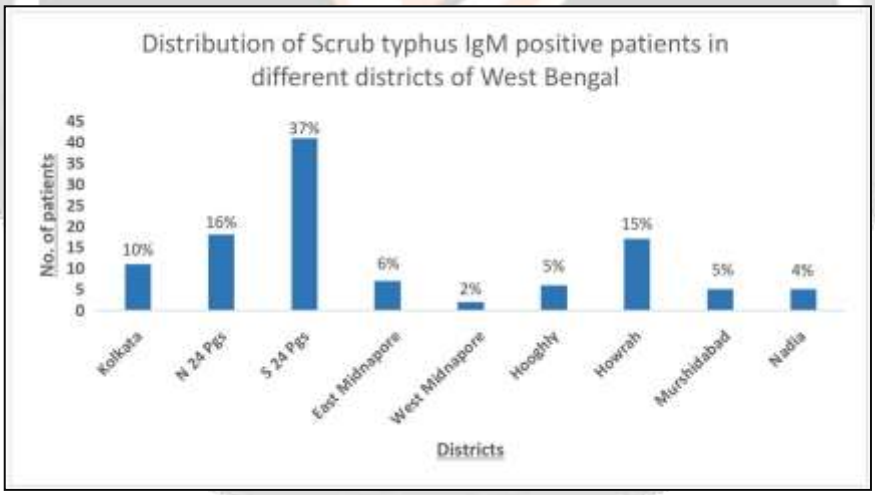

Fig-4: Graph showing scrub typhus distribution among patients belonging to different districts in West Bengal

3.3 Gender

The patients were divided according to their genders, i.e. male and female. Females (66) were observed to suffer more from Scrub typhus as compared to males (46) [Figure 5]

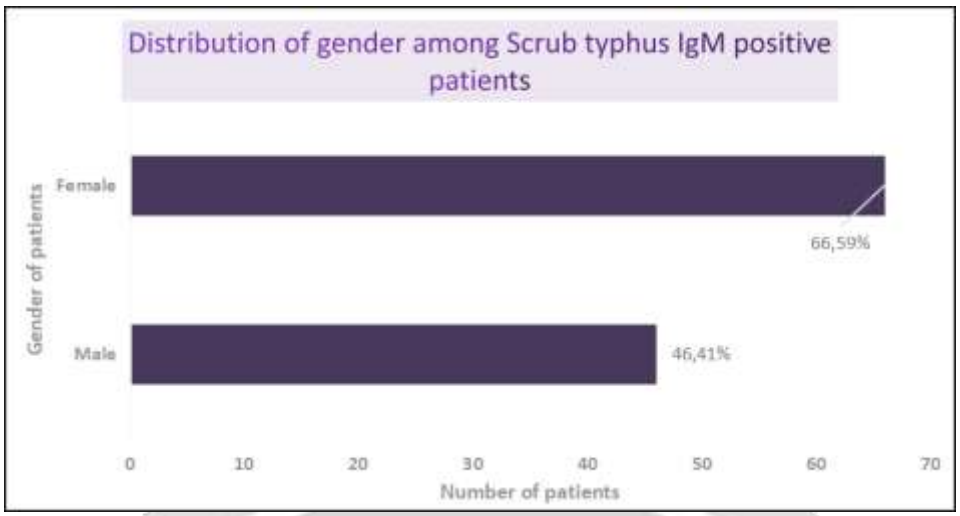

Fig-5: Graph showing the gender distribution of patients affected by Scrub typhus

3.4 Months

The study of Scrub typhus patients was done in the duration from April to June 15th , 2019. The maximum number of cases were reported in the month of May (202 patients, 54%). The months of April (106 patients) had 28% and June (till 15th- 66 patients) had 18% [Figure 6]

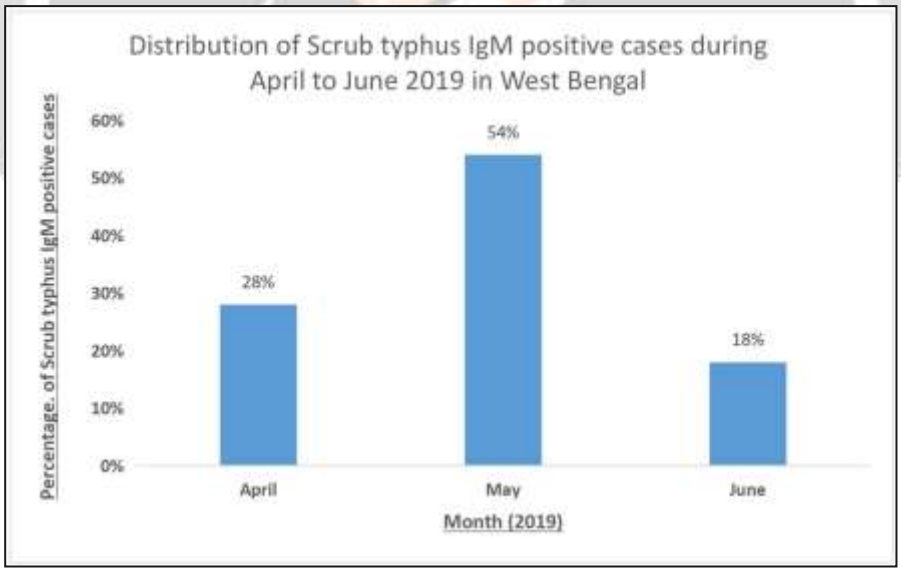

Fig-6: Chart showing Scrub typhus distribution during the months of April to June 2019 in West Bengal

4. CONCLUSIONS

After amplification of gene by Nested PCR , a total of 113 samples were found to be Scrub typhus IgM positive. Out of these 113 samples, 81 samples were amplified through PCR. It was found that 12 out of 81 samples showed clear bands in gel electrophoresis after amplification through Nested PCR.

Thus 12 samples were found to be PCR positive.

5. DISCUSSIONS

After performing the study on Scrub typhus IgM positive cases from different districts of West Bengal, it was found that the maximum cases belonged to rural areas, most notably South 24 Parganas. These may be due to the fact that Scrub typhus occurs in more among persons who engage in occupational or recreational behaviour that brings them into contact with mite-infested habitats like bush and grass [Saha, Chatterji, Mitra, Ghosh, Naskar. Socio-demographic and Clinico-Epidemiological Study of Scrub typhus in Two Tertiary Care Hospitals of Kolkata. Journal of The Association of Physicians of India. Vol 66. May 2018, 24]. When compared with clinical controls, cases were more likely to be involved in farming or gardening activities and spend time resting directly, without a mat, on grass and mud and were also more likely to have no toilets within their houses. [George, Rajan, Peter, Hansdak, Prakash, Antony. Risk Factors for Acquiring Scrub Typhus among the Adults. US National Library of Medicine, National Institutes of Health. Journal List, Vol 10 (3). Jul-Sept 2018]. Also, females were found to be more prone to Scrub typhus infection than males. This may be due to more contact with household mites and rats as compared to males, who work outside the house. Children below the age group of 15 years were also found to be more at risk of being infected with scrub typhus. This may be due to lack of proper care or low immunity due to poor financial conditions or lack of education. Lastly, it was observed that the maximum number of cases were found to be in the months of May and even mid-June had a high number of cases. This is probably due to the fact that this period marks the onset of monsoon, which increases the number of mites and rodents in and around the house. The scrub typhus outbreak starts after the monsoon and rainy season with the peak incidence in July followed by September. This might be because the occurrence of Leptotrombidium deliense is influenced by rainfall, with more chiggers attached to rodents during the wetter months of the year [Gautam, Parajuli, Sherchand. Epidemiology, Risk Factors and Seasonal Variation of Scrub Typhus Fever in Central Nepal. Tropical Medicine and Infectious diseases, MDPI Article. 2nd February, 2019, page 7]. Thus, the data analyzed is in accordance with the findings of other researchers and shows accurate results.

6. REFERENCES

1. Department of Public Health, Ministry of Health, Royal Government of Bhutan, November 2016. National Guideline for Prevention, Treatment and Control of Scrub typhus in Bhutan, 10-40

2. Owen, Punt, Stranford. Kuby Immunology 7th Edition, 660-662

3. UniProtKB-F8RNMO (F8RNMO_ORITS) https://www.uniprot.org/uniprot/F8RNMO

4. InBios, InBios International Inc., Scrub Typhus DetectTM IgM ELISA Protocol, 1, 3-5, 6-7

5. Kelly M. Elkins, in Forensic DNA Biology, 2013. DNA Extraction https://www.sciencedirect.com/topics/neuroscience/dna-extraction

6. QIAamp® DNA Mini Kit (50), DNA Extraction Protocol, 1-2

7. abm, Polymerase Chain Reaction (PCR)- An Introduction, https://www.abmgood.com/marketing/knowledge_base/polymerase_chain_reaction_introduction.php

8. abm, Polymerase Chain Reaction (PCR)-Variations to the System, https://www.abmgood.com/marketing/knowledge_base/polymerase_chain_variation_ system.php

9. Sarma, Chakraborty. Scrub Typhus in Southern Districts of West Bengal. 2017.

10. A review of Scrub typhus (Orientia tsutsugamushi and related organisms) then, now and tomorrow by Luce-Fedrow,Lehman,Kelly,Mullins,Richards. Journal of Tropical Medicine and Infectious disease. Review Article. Published on 17th January, 2018. Page 1-3

11. Scrub typhus, National Health Portal of India (NHP), Govt of India https://www.nhp.gov.in/disease/scrub-typhus

12. Overview of ELISA- ThermoFisher Scientific https://www.thermofisher.com/in/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

13. A review of the global epidemiology of Scrub typhus. Xu, Walker,Jupiter,Melby, Arcari. PLOS, Neglected Tropical Diseases.Published on Nov 3, 2017.

14. Research techniques made simple:Polymerase Chain Reaction (PCR)by Garibyan and Avashia. US National Library of Medicine, National Institute of Health, NCBI PMC. Manuscript availalbe on 14th July, 2017.