The Pediatric Infectious Disease Journal Publish Ahead of Print

DOI: 10.1097/INF.0000000000002099

Acute Encephalitis Syndrome in Gorakhpur, Uttar Pradesh, 2016: Clinical and Laboratory Findings

Mahima Mittal[1], MD, Vijay Bondre[2], PhD, Manoj Murhekar[3], MD[#], Hirawati Deval[2] PhD, Winsley Rose[4], MD, Valsan Philip Verghese[4], MD, Mahim Mittal[1], MD, Gajanan Patil[2], MSc, R. Sabarinathan[3], BE, Jeromie Wesley Vivian Thangaraj[3], MPH, K. Kanagasabai[3], MSc, John Antony Jude Prakash[4], MD, Nivedita Gupta[5], MD, Manish Gupte[5], PhD, and Mohan D Gupte[6], MD

[1]BRD Medical College, Gorakhpur, Uttar Pradesh, India

[2]ICMR-National Institute of Virology, Gorakhpur Unit, Uttar Pradesh, India

[3]ICMR-National Institute of Epidemiology, Chennai, Tamil Nadu, India

[4]Christian Medical College and Hospital, Vellore, Tamil Nadu, India

[5] Freelance statistician

[6] Indian Council of Medical Research, New Delhi, India

[#]Address for correspondence: National Institute of Epidemiology, Indian Council of Medical Research, R-127, Tamil Nadu Housing Board, Ayapakkam, Chennai - 600070, India, Email: mmurhekar@nieicmr.org.in

Abbreviated title: Scrub typhus etiology of AES, Gorakhpur, India

Running title: Acute Encephalitis syndrome, Gorakhpur, 2016

Financial Support: Indian Council of Medical Research, New Delhi

Acknowledgements: The authors gratefully acknowledge the members of the expert group on Research-Cum-intervention project on AES/JE for their inputs on the study findings. Cooperation extended by Drs Anita Mehta, S.K. Srivastava and Bhoopendra

Sharma, Department of Pediatrics, BRD Medical College, Gorakhpur is gratefully acknowledged.

Abstract

Background: Seasonal outbreaks of acute encephalitis syndrome (AES) with high fatality have been occurring in Gorakhpur, Uttar Pradesh, India for several years. We conducted investigations during the 2016 outbreak, to identify the etiology.

Methods: We included 407 hospitalized AES patients with CSF pleocytosis (>5 cells/cmm) in our study. These patients were clinically examined; their blood and CSF samples were collected and investigated for scrub typhus (ST), Japanese encephalitis virus (JEV), dengue virus and spotted fever group of rickettsia (SFGR) by serology and/or PCR.

Results: Of the 407 AES patients, 266 (65.4%), 42 (10.3%) and 29 (7.5%) were diagnosed to have ST, JEV and dengue respectively. Four patients were diagnosed to have SFGR infection. A significantly higher proportion of scrub typhus patients with AES had hepatomegaly, splenomegaly, and facial edema. The common hematological and biochemical abnormalities among ST positive patients include thrombocytopenia, raised liver enzymes and bilirubin levels. The case fatality ratio was significantly higher among ST negative AES patients (36.2% vs 15.2%, $p<0.05$).

Conclusion: ST accounted for approximately two third of the AES case-patients. Efforts are required to identify the etiology of AES case-patients that are negative for ST, JE and dengue fever.

Key words: Scrub typhus, Acute Encephalitis Syndrome, Gorakhpur, India

Introduction

Outbreaks or sporadic cases of acute encephalitis syndrome (AES) occur frequently in several Indian states. According to India's National Vector-borne Disease Control Program (NVBDCP), more than 60,000 AES cases were reported during 2010–2016 with 8 India states (Assam, Uttar Pradesh, West Bengal, Odisha, Tamil Nadu, Karnataka, Manipur, and Tripura) accounting for most cases (1).

In Uttar Pradesh, seasonal outbreaks of AES with high fatality have been reported from Gorakhpur division since 1978 *(2)*. Available data indicate that annually about 2000 AES patients are admitted in BRD Medical College (BRDMC) – the only tertiary care hospital in Gorakhpur division, with case fatality ratio (CFR) ranging between 20–30% *(2)*. Japanese encephalitis virus (JEV) encephalitis accounted for half of the AES cases admitted during 2005 *(3)*. However, the number of JEV cases have declined substantially, after introduction of JE vaccine in the Gorakhpur division in 2006 *(4)*. Between 2008-2012, JE accounted for <10% of AES cases admitted at BRDMC (5). In the past, samples from AES patients were tested for viral and non-viral agents including herpes simplex, enteroviruses, Chandipura, measles, mumps, dengue, varicella, parvovirus, West Nile, malaria, and typhoid (National Institute of Virology, unpublished data), but the etiology remained largely unknown. Studies focusing on AES patients in the State of Uttar Pradesh, have documented that cases with unknown etiology accounted for 61% of cases in 2007 (6), 41.6% in 2011–2012 (7), 59% in 2013–2014 (8). Investigations conducted in 2014 (9) and 2015 indicated the possibility of scrub typhus (ST) *(10)*. A case-control study revealed higher odds of IgM antibodies against *Orientia tsutsugamishi* in AES patients compared with healthy controls (*11*), further suggesting the role of scrub typhus in the etiology of AES. We present the findings of investigations conducted during 2016 among AES patients

admitted at BRDMC, Gorakhpur.

Patients and Methods

The study was approved by the institutional ethics committee of the National Institute of Epidemiology, Chennai.

Case definition:

A case of AES was defined as an acute onset of fever with change in mental status and/or new onset of seizures, excluding simple febrile seizures in a person of any age *(12)*.

Data collection:

During 2016, AES cell was established at BRDMC, Gorakhpur. All AES cases hospitalized during August–October 2016 were enrolled in the AES cell prospectively. These case-patients were clinically examined; their clinical and demographic details were collected. Three ml of blood was collected and tested for routine hematologic and biochemical investigations. CSF was collected within 24 h of admission. An aliquot of blood, serum and CSF were stored for further investigations. Information about the outcomes of hospitalized patients in terms of death, discharged and left against medical advice (LAMA) were collected from the case records.

Laboratory investigations:

During August–October 2016, a total of 1242 AES case-patients were admitted in BRDMC. CSF was examined in 1122 and 1037 had CSF pleocytosis (>5 cells per cmm) *(13)*. We listed the AES patients with CSF pleocytosis and selected a total of 407 for etiological investigations. This included 150 patients each from August and September and 107 from October, selected randomly. We also conducted minimally invasive autopsy from 5 deceased patients; their brain, spleen and liver samples were subjected to molecular studies.

Sera were tested for presence of IgM antibodies against *Orientia tsutsugamushi* (OT) by commercial enzyme linked immunosorbent assay (ELISA) (Scrub typhus Detect, InBios International Inc., Seattle, USA), Japanese encephalitis (National Institute of Virology (NIV), Pune, India) and dengue (NIV, Pune, India). CSF was tested for presence of IgM antibodies against JE and OT.

Although, for Inbios Scrub typhus Detect IgM ELISA, an optical density (OD) value of >0.5 was found to have 93% sensitivity and 91% specificity (14) and has been used in several studies conducted in India (15, 16, 17, 18), we decided to estimate the cut-off for local area. To determine the cut-off of OD values for IgM antibodies against OT in serum, we plotted the frequency distribution of OD values for 389 AES patients included in the study. The OD value corresponding to the anti-mode was considered as the cut-off (data not presented). To determine the cut-off for OD values of IgM antibodies against OT in CSF, we considered 366 AES patients for whom the IgM OD values for both serum and CSF were available. We estimated the regression equation between OD values of IgM antibodies against OT in serum and CSF. Using this equation, we calculated the cut-off for OD value for CSF corresponding to the cut-off for serum OD (data not presented). Thus, an OD value of >0.76 for serum and >0.224 for CSF was considered as positive.

PCR for OT and spotted fever group of rickettsia:

Genomic DNA from CSF and whole blood was extracted. The PCR was carried out using an outer PCR primer set OTs 56KF (5'-ATA GAA TTG GGT GAG GAA GGA GGA TTA GAG-3') and OTs 56KR (5'-AGT CAA TAC CAG CAC AAT TCT TTA ACC A-3') amplifying 504 base pair from 56-kDa type-specific antigen (TSA) gene of Gilliam, Karp, Kato, Kawasaki, and Kuroki strains (19). An internal forward primer OTs56KnF (5'-GTT GGA ATC GTT GGA GGA ATG ATT ACT GG-3') was

used along with OTs56KR to amplify 456 base pair product in semi-nested PCR. The PCR profile for both PCR was determined with the following steps: denaturation at 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 s, 58˚C for 45 s, and 72˚C for 1 min, with a final extension at 72˚C for 7 min. β-actin house keeping gene specific forward (5'-CCACACCTTCTACAATGAGC- 3') and reverse (5'-ACAGCCTGGATAGCAACGTA-3') primers were used as control to authenticate the nucleic acid extraction and PCR reactions due to non-availability of OT bacteria DNA as positive controls. These primers amplify 189 bp fragment of human β-actin gene and are used in molecular diagnosis to verify that the PCR conditions are optimum, and are thus known as amplification controls (20-22).

For detection of members of spotted fever group of rickettsia (SFGR), generic 23S-5S intergenic spacer (IGS) based primers RCK/23-5-F (5'-GAT AGG TCR GRT GTG GAA GCA C-3') and RCK/23-5-R (5'-TCG GGA YGG GAT CGT GTG TTT C-3') were used to amplify 377 base pair product from multiple species of Rickettsia (23). The semi-nested PCR on the first PCR amplified product amplifies a 327 base pair using RCK/23-5N1F (5'-TGT GGA AGC ACA GTA ATG TGT G-3') and RCK/23-5N1R (5'-TCG TGT GTT TCA CTC ATG CT-3') primers. The PCR conditions used for amplification of 23S-5S IGS fragments were as follows: 95°C for 10 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 65°C for 1.5 min, and a final cycle of 65°C for 7 min for primary PCR and 95°C for 10 min, 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1.5 min, with a final cycle of 72°C for 10 min (for the nested reaction).

All primers used in the study were synthesized by Integrated DNA Technologies (Coralville, Iowa, United States). The PCR products were analyzed on 1.5% agarose gel and the PCR reactions amplifying expected product size of 327 bp in nested PCR

were gel purified using *QIAquick Gel Extraction* Kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced using the dye terminator method (BigDye Terminator sequencing kit, Applied Biosystems, Foster City, CA) with an ABI Prism 377 DNA sequencer (Applied Biosystems) as described earlier (24).

Data analysis

We considered the diagnosis of scrub typhus when any of the following tests had a positive result: IgM antibodies against OT in serum and/or CSF, PCR for OT in blood and/or CSF. A patient found positive for more than one etiology was considered as co-infected. We described the clinical and laboratory findings by calculating medians, inter-quartile ranges and proportions. The clinical and laboratory findings of scrub typhus negative patients and those infected with *O. tsutsugamushi* alone were compared using χ^2 test . P value of <0.05 was considered as statistically significant. Statistical analysis was performed using STATA version 13 software.

Results

All case-patients were from rural area. Majority (n=188) of the patients were aged between 61-179 months (46.2%) and 51% (n=207) were males (Table-1). Most AES case-patients were from four districts of Gorakhpur (Gorakhpur, Kushinagar, Deoria and Maharajgunj) and Basti (Sant Kabir Nagar and Siddhartha Nagar) division of Uttar Pradesh.

Of the 407 AES patients, 266 (65.4%), 42 (10.3%) and 29 (7.5%) were diagnosed to have ST, JE and dengue respectively. Four patients were diagnosed to have SFGR infection (Table-2). Brain autopsy from one patient was positive for the outer membrane protein A, specific for SFGR. Among the 266 ST AES patients, co-infection was detected in 36 patients: 23 were positive for dengue; 10 were positive for JEV (4 patients had IgM antibodies against JEV in serum as well as CSF, 4

patients had IgM antibodies in serum, 3 patients had IgM antibodies in CSF alone); 1 was positive for JE and dengue while 2 were positive for SFGR.

Among the 36 patients with co-infections, the diagnosis of scrub typhus was based on PCR (n=10, of these 7 also had IgM antibodies against OT in CSF and serum; 2 had IgM antibodies in either CSF or serum and 1 was negative), presence of IgM antibodies in serum and CSF (n=18), and presence of IgM antibodies in serum or CSF (n=8). Overall, 105 (25.8%) patients were negative for all etiologies tested.

Besides fever, common presenting symptoms in scrub typhus AES patients included seizures, altered sensorium, vomiting and headache (Table -1). The median duration from fever onset to CNS manifestations and CNS manifestations to hospitalization was 6 (IQR: 4-8) and 1 (IQR: 1-3) days respectively. A significantly higher proportion of ST positive AES patients had hepatomegaly, splenomegaly, and facial edema (Table 1). The common hematological and biochemical abnormalities among ST positive patients include thrombocytopenia, raised liver enzymes and bilirubin levels (Table 1). The median Glasgow Coma Score (GCS) at the time of admission was 8 (IQR: 8-10). None of the parents of the 42 children with JEV infection could recall a history of JEV vaccination.

Most AES patients received intravenous azithromycin (n=392, 96.3%). Ninety-two patients (22.6%) died while 11 (2.7%) left against medical advice (LAMA). The case fatality ratio (CFR) was significantly higher among ST negative AES patients (51/141=36.2% vs 35/230=15.2%, p=0.000). Among the ST AES patients, the CFR was comparable among those infected only with OT and those co-infected with JE or dengue. (35/230, 15.2% vs 6/36=16.7% p=0.823).

Discussion

Earlier investigations conducted during 2014 and 2015 indicated high sero-positivity to *O. tsutsugamushi* among AES patients (9, 10). AES patients also had higher odds of having IgM antibodies against *O. tsutsugamushi* as compared to healthy controls (11). Our study provides additional evidence in support of ST as the etiology of AES. We found OT PCR positivity in CSF and/or blood in 20% of AES patients. We also found high prevalence of IgM antibodies against OT in CSF, in excess of 60%. Besides scrub typhus, JEV and dengue virus was the etiology in 10% and 7.5% AES patients respectively.

The etiology of AES outbreaks observed in our series was different than the recent reports from the State of Uttar Pradesh as well as other parts of country. In a cohort of 921 AES patients from Lucknow, Uttar Pradesh, JEV was the commonest causative agent, accounting for 16.2% of the cases; followed by Dengue virus (10.8%), HSV (9.3%), measles virus (8.9%) and mumps virus (8.7%) and VZV (4.4%) (7). In the eastern state of Odisha, viral etiology was ident

ified in 91 (17.2%) of the 526 AES cases. HSV, measles and JEV were the commonest viral etiologies (25). In Central India, of the 183 AES patients investigated, viral etiology was detected in 31 (16.9%), non-encephalitic illness in 15 (8.2%), bacterial meningitis in 9 (4.9%), tubercular meningitis in 5 (2.7%) and cryptococcal meningitis in 2 (1.1%) patients. No etiology was detected in 79.7% of the patients (26). None of the patients from these three cohorts were investigated for scrub typhus etiology. On the other hand, in a study from Assam *O. tsutsugamushi* contributed to 20.3% of the 511 AES patients investigated (27).

Conventionally, ST is considered to be an occupational disease affecting mainly adults *(28)*. In Gorakhpur division, ST positive AES case-patients were from rural

areas with disease mainly among the pediatric age group suggesting that the infection is associated with living conditions. The case fatality ratio among AES patients was high. Since 2014, intravenous azithromycin is recommended as empiric treatment for hospitalized AES patients. More than 96% of AES included in our study were administered azithromycin. Lower adverse outcomes among ST AES patients indicate better prognosis as compared to ST negative AES patients, possibly on account of azithromycin use. Although the efficacy of azithromycin in the treatment of complicated scrub typhus is largely unknown, a study conducted by Jang et al found that the outcomes of Azithromycin therapy were comparable to those of doxycycline therapy in patients with complicated scrub typhus (29). Our findings are consistent with that study (29).

In ST patients, early administration of appropriate antibiotics is the key for better outcomes. In Gorakhpur, ST positive AES patients were hospitalized 7.5 days after onset of fever. Earlier studies indicated that ST accounted for 18% (95% CI: 13.3-23.3) of febrile patients attending peripheral health facilities in the region *(30)*. These findings make a case for a potential role of administering doxycycline empirically to all suspected ST patients attending peripheral health facilities and thereby prevent the progression of ST patients to AES patients. Further studies are required to generate evidence about the usefulness of this strategy.

The presence of eschar is considered pathognomonic of scrub typhus and it has been reported that eschar incidence varies from 7% to 97% in endemic areas (28). There are conflicting findings about the association between presence of eschar and disease severity. Sonthayanon et al reported that scrub typhus patients with eschar had greater severity, possibly on account of higher bacterial load in blood (31). On the other hand, Kim et al reported absence of eschar as a predictor of severity (32). None of the scrub

typhus positive patients in our study had eschar. Absence or low incidence of eschar was also reported in earlier studies among scrub typhus patients presenting with AES (10) as well as febrile illness (30) from Gorakhpur. Low incidence (9.6%) of eschar was also reported in scrub typhus patients from In sub-Himalayan belt in India (33). Further studies are needed to understand the reasons for absence of eschar among scrub typhus patients in Gorakhpur as well as its relation with severity.

In our study, no etiology was identified in about 25% of AES patients. The case fatality among these patients was also significantly higher. This observation calls for urgent efforts to identify the etiology of AES patients with negative etiology for scrub typhus, dengue and Japanese encephalitis.

Our investigation had certain limitations. First, we included patients meeting the case definition of AES. Since the WHO case definition for AES is not very specific, there is a possibility of including patients with meningitis, encephalopathy, acute disseminated encephalomyelitis (ADEM) besides cases of encephalitis in the study. Second, we included patients admitted to only one tertiary health facility in the region. BRDMC is the only referral hospital for the region, and hospitalized AES patients are more likely to be serious and have higher case fatality than those treated in other facilities. Third, we investigated AES patients for a limited number of etiologies. The choice of etiological agents investigated was based on the findings of investigations conducted earlier *(10)*. Fourth, it was not possible to perform autopsies on more patients, as the patients' relatives declined consent. Fifth, we could not conduct neuro-imaging of AES patients due to logistical reasons. Information about outcome was obtained from the records and we did not clinically evaluate AES patients neuro-cognitive sequelae at the time of discharge. Lastly, about 14% of AES patients infected with *O. tsutsugamushi* were co-infected; mostly with dengue and

JEV. While the high rate of co-infection suggests false positive results with the serological tests for these viruses, in hyper-endemic areas co-infections are frequently reported (34).

In conclusion, our findings indicate that ST is an important cause of AES in Gorakhpur region, accounting for nearly two third of the AES case-patients. Besides continuing azithromycin administration to AES patients empirically, it is necessary to generate data about the usefulness of empiric doxycycline administration to febrile patients attending peripheral health facilities. Efforts are also required to identify the etiology of AES case-patients that are negative for ST, JE and dengue fever.

References

1. National Vectorborne Disease Control Program. Ministry of Health and Family Welfare, Government of India. Statewise number of AES/JE cases and deaths from 2010–2017, 2017. http://nvbdcp.gov.in/Doc/je-aes.pdf, [cited 2017 Dec 30].
2. Mittal M, Kushwaha KP. AES: Clinical Presentation and Dilemmas in Critical Care Management. J comm Dis 2014; 46: 50-65
3. Parida M, Dash PK, Tripathi NK, Ambuj, Sannarangaiah S, Saxena P, Agarwal S, Sahni AK, Singh SP, Rathi AK, Bhargava R, Abhyankar A, Verma SK, Rao PV, Sekhar K. Japanese Encephalitis Outbreak, India, 2005. Emerg Infect Dis. 2006;12:1427-30.
4. Ranjan P, Gore M, Selvaraju S, Kushwaha KP, Srivastava DK, Murhekar M. Decline in Japanese encephalitis, Kushinagar District, Uttar Pradesh, India. Emerg Infect Dis. 2014;20:1406-7.
5. Ranjan P, Gore M, Selvaraju S, Kushwaha KP, Srivastava DK, Murhekar M. Changes in acute encephalitis syndrome incidence after introduction of Japanese encephalitis vaccine in a region of India. J Infect. 2014;69:200-2.
6. Bhatt GC, Bondre VP, Sapkal GN, Sharma T, Kumar S, Gore MM, Kushwaha KP, Rathi AK. Changing clinico-laboratory profile of encephalitis patients in the eastern Uttar Pradesh region of India. Trop Doct. 2012;42:106-8.
7. Jain P, Jain A, Kumar A, Prakash S, Khan DN, Singh KP, Garg RK, Kumar R, Kumar GA. Epidemiology and etiology of acute encephalitis syndrome in North India. Jpn J Infect Dis. 2014;67:197-203.
8. Gupta S, Shahi RK, Nigam P. Clinico-etiological Profile and Predictors of Outcome in Acute Encephalitis Syndrome in Adult. International J Scientific study 2016; 3: 78-83

9. Pulla P. Disease sleuths unmask deadly encephalitis culprit. Science. 2017;357:344.
10. Murhekar MV, Mittal M, Prakash JA, Pillai VM, Mittal M, Girish Kumar CP, Shinde S, Ranjan P, Oak C, Gupta N, Mehendale S, Arora R, Gupte M. Acute encephalitis syndrome in Gorakhpur, Uttar Pradesh, India - Role of scrub typhus. J Infect. 2016;73:623-626
11. Mittal M, Thangaraj JWV, Rose W, Verghese VP, Kumar CPG, Mittal M, Sabarinathan R, Bondre V, Gupta N, Murhekar MV. Scrub Typhus as a Cause of Acute Encephalitis Syndrome, Gorakhpur, Uttar Pradesh, India. Emerg Infect Dis. 2017; 23:1414-1416.
12. World Health Organization. Acute Encephalitis Syndrome. Japanese encephalitis surveillance standards. January 2006. From WHO-recommended standards for surveillance of selected vaccine-preventable diseases. WHO/V&B/03.01. Available from: http://apps.who.int/iris/bitstream/10665/68334/1/WHO_V-B_03.01_eng.pdf [accessed 30.12.17].
13. Seehusen DA, Reeves MM, Fomin DA. Cerebrospinal fluid analysis. Am Fam Physician. 2003; 68:1103-8.
14. Blacksell SD, Tanganuchitcharnchai A, Nawtaisong P, Kantipong P, Laongnualpanich A, Day NP, Paris DH. Diagnostic Accuracy of the InBios Scrub Typhus Detect Enzyme-Linked Immunoassay for the Detection of IgM Antibodies in Northern Thailand. Clin Vaccine Immunol. 2015;23:148-54
15. Varghese GM, Janardhanan J, Trowbridge P, Peter JV, Prakash JA, Sathyendra S, Thomas K, David TS, Kavitha ML, Abraham OC, Mathai D. Scrub typhus in South India: clinical and laboratory manifestations, genetic variability, and outcome. Int J Infect Dis. 2013;17:e981-7

16. Bhargava A, Kaushik R, Kaushik RM, Sharma A, Ahmad S, Dhar M, Mittal G, Khanduri S, Pant P, Kakkar R. Scrub typhus in Uttarakhand & adjoining Uttar Pradesh: Seasonality, clinical presentations & predictors of mortality. Indian J Med Res. 2016;144:901-909.
17. Sharma N, Biswal M, Kumar A, Zaman K, Jain S, Bhalla A. Scrub Typhus in a Tertiary Care Hospital in North India. Am J Trop Med Hyg. 2016; 95:447-51.
18. Sood AK, Chauhan L, Gupta H. CNS Manifestations in Orientia tsutsugamushi Disease (Scrub Typhus) in North India. Indian J Pediatr. 2016; 83: 634-9.
19. Jado I, Escudero R, Gil H, Jiménez-Alonso MI, Sousa R, García-Pérez AL, Rodríguez-Vargas M, Lobo B, Anda P. Molecular method for identification of Rickettsia species in clinical and environmental samples. J Clin Microbiol. 2006;44:4572-6.
20. Betsou F., Beaumont K, Sueur JM, Orfila J. Construction and evaluation of internal control DNA for PCR amplification of Chlamydia trachomatis DNA from urine samples. J. Clin. Microbiol.2003;41:1274–1276
21. Hoorfar J, Cook N, Malorny B, Wagner M, De Medici D, Abdulmawjood A, Fach P. Making internal amplification control mandatory for diagnostic PCR. J. Clin. Microbiol. 2003;41:5835
22. Hoorfar J., Malorny B., Abdulmawjood A., Cook N., Wagner M., Fach P. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J. Clin. Microbiol. 2004;42:1863–1868.
23. Kakumanu ML, Ponnusamy L, Sutton HT, Meshnick SR, Nicholson WL, Apperson CS. Development and Validation of an Improved PCR Method Using the 23S-5S Intergenic Spacer for Detection of Rickettsiae in Dermacentor

variabilis Ticks and Tissue Samples from Humans and Laboratory Animals. J Clin Microbiol. 2016;54:972-9.

24. Bondre VP, Jadi RS, Mishra AC, Yergolkar PN, Arankalle VA. 2007. West Nile virus isolates from India: evidence for a distinct genetic lineage. J Gen Virol. 2007;88 (Pt 3):875-84.
25. Rathore SK, Dwibedi B, Kar SK, Dixit S, Sabat J, Panda M. Viral aetiology and clinico-epidemiological features of acute encephalitis syndrome in eastern India. Epidemiol Infect. 2014;142:2514-21.
26. Joshi R1, Mishra PK, Joshi D, Santhosh SR, Parida MM, Desikan P, Gangane N, Kalantri SP, Reingold A, Colford JM Jr. Clinical presentation, etiology, and survival in adult acute encephalitis syndrome in rural Central India. Clin Neurol Neurosurg. 2013 ;115:1753-61.
27. Khan SA, Bora T, Laskar B, Khan AM, Dutta P. Scrub Typhus Leading to Acute Encephalitis Syndrome, Assam, India. Emerg Infect Dis. 2017;23:148-150.
28. Rahi M, Gupte MD, Bhargava A, Varghese GM, Arora R. DHR-ICMR Guidelines for diagnosis & management of Rickettsial diseases in India. Indian J Med Res. 2015;141:417-22.
29. Jang MO, Jang HC, Kim UJ, Ahn JH, Kang SJ, Jung SI, Shin HY, Park KH. Outcome of intravenous azithromycin therapy in patients with complicated scrub typhus compared with that of doxycycline therapy using propensity-matched analysis. Antimicrob Agents Chemother. 2014;58:1488-93.
30. Vivian Thangraj JW, Mittal M, Verghese VP, Kumar CPG, Rose W, Sabarinathan R, Pandey AK, Gupta N, Murhekar M. Scrub Typhus as an Etiology of Acute Febrile Illness in Gorakhpur, Uttar Pradesh, India, 2016. Am J Trop Med Hyg. 2017 Aug 14.

31. Sonthayanon P, Chierakul W, Wuthiekanun V, et al. Association of high Orientia tsutsugamushi DNA loads with disease of greater severity in adults with scrub typhus. J Clin Microbiol. 2009;47:430–434.
32. Kim DM, Kim SW, Choi SH, Yun NR. Clinical and laboratory findings associated with severe scrub typhus. BMC Infect Dis. 2010;10:108.
33. Mahajan SK, Rolain JM, Kashyap R, Bakshi D, Sharma V, Prasher BS et al. Scrub typhus in Himalayas. Emerg Infect Dis 2006; 12: 1590-2
34. Sapkota S, Bhandari S, Sapkota S, Hamal R. Dengue and Scrub Typhus Coinfection in a Patient Presenting with Febrile Illness. Case Reports in Infectious Diseases, 2017; 6214083,
35. Ahmad S, Dhar M, Bhat NK et al. Rare Co-infection of Malaria, Scrub Typhus and Dengue virus in an Immunocompetent Patient. Int J Curr Microbiol App Sci 2015; 4: 295-297

Table 1: Clinical presentation of Scrub typhus positive and negative AES patients, Gorakhpur, Uttar Pradesh, India, 2016.

	Scrub typhus patients		Scrub typhus negative patients (n=141)
	Number (%) infected with *O. tsutsugamushi* alone (n=230)	Number (%) with mixed infection (n=36)	Number(%)
Age group (in months)			
6-11	2 (0.9)	1 (2.8)	6 (4.3)
12-23	16 (7.0)	1 (2.8)	13 (9.2)
24-60	96 (41.7)	12 (33.3)	46 (32.6)
61-179	103 (44.8)	20 (55.6)	65 (46.1)
>=180	13 (5.7)	2 (5.6)	11 (7.8)
Median age [IQR]	61.5 [36-120]	81 [48-120]	72 [36-108]
Male sex	114 (49.6)	17 (47.2)	76 (53.9)
Symptoms			
Fever	230 (100.0)	36 (100.0)	141 (100.0)
Altered Sensorium	160 (69.6) *	28 (77.8)	117 (83.0)*
Seizures	204 (88.7)	25 (69.4)	124 (87.9)
Vomiting	107 (46.5) *	19 (52.8)	40 (28.4) *
Headache	32 (13.9)	5 (13.9)	19 (13.5)
Abdominal pain	35 (15.2)*	7 (19.4)	12 (8.5)*
Diarrhea	11 (4.8)	2 (5.6)	7 (5.0)
Median [IQR] duration (in days) between fever onset and CNS manifestations	6 [4-8]	6 [4-7]	3 [2-6]
Median [IQR] duration (in days) between CNS manifestations and hospitalization	1 [1-3]	2 [1-3]	1 [1-2]
Median [IQR] duration (in days) between CNS fever onset and hospitalization	7.5 [6-10]	7.5 [5.5-10]	5 [3-8]
Physical examination			
Hepatomegaly	100 (43.5)*	10 (27.8)	37 (26.2)*
Peri-orbital edema	76 (33.0)*	5 (13.9)	20 (14.2) *
Splenomegaly	24 (10.4)*	2 (5.6)	5 (3.5)*
Jaundice	10 (4.3)	1 (2.8)	2 (1.4)
Rash	12 (5.2)	0 (0.0)	8 (5.7)
Eschar	0 (0.0)	0 (0.0)	0 (0.0)

Neurological examination			
Consciousness			
Drowsy/Somnolent	180 (78.3)	22 (61.1)	103 (73.0)
Irritable/agitated	17 (7.4)	9 (25.0)	12 (8.5)
Normal/Alert	23 (10.0)	4 (11.1)	11 (7.8)
Unresponsive/comatose	10 (4.3)	1 (2.8)	15 (10.6)
Median [IQR] GCS at admission	8 [8-10]	8 [8-11]	8 [7-9]
GCS <=8 at admission	134 (58.3)*	18 (50.0)	99 (70.2)*
Tone			
Normal	80 (34.8)	15 (41.7)	37 (26.2)
Hypotonia	9 (3.9)	2 (5.6)	3 (2.1)
Hypertonia	141 (61.3)	19 (52.8)	101 (71.6)
Reflexes			
Normal	42 (18.3)	10 (27.8)	25 (17.7)
Decreased	4 (1.7)	1 (2.8)	1 (0.7)
Increased	181 (78.7)	24 (66.7)	110 (78.0)
Absent	3 (1.3)	1 (2.8)	5 (3.5)
Plantar reflexes			
Flexor	22 (9.6)	5 (13.9)	12 (8.5)
Extensor	185 (80.4)	30 (83.3)	108 (76.6)
Asymmeteric	21 (9.1)	0 (0.0)	15 (10.6)
No response	2 (0.9)	1 (2.8)	6 (4.3)
Neck rigidity	14(6.1)	3(8.3)	7(5.0)
Hematological investigations			
Total leukocyte count/ul, median [IQR]	13200 [9800-18000]	12550 [9500-18900]	13700 [9000-16800]
Number with TLC >11,000	157 (68.3)	23 (63.9)	91 (64.5)
Platelet count /ul, median [IQR]	105000 [60,000-159,000]	80,000 [60,000-129,000]	184,000 [100,000-280,000]
Number with platelet count <150,000/ul	167 (72.6)*	28 (77.8)	60(42.6)*
Biochemical investigations			
AST in IU/l, median [IQR]	117.8 [65.3-225.5]	128.1 [70.1-263.9]	57.6 [36.2-113.0]
Number with raised AST>100 IU/l	125 (54.3)*	23 (63.9)	37(26.2)*
ALT in IU/l, median [IQR]	81.7 [48.2-145.4]	104.3 [68.1-186.0]	40.8 [25.2-80.5]
Number with raised ALT>100 IU/l	97 (42.2)*	20 (55.6)	25 (17.7)*
Blood glucose in mg/dl, median [IQR]	82 [70-99]	93 [71-116]	90 [76-115]
Serum bilirubin in g/dl, median [IQR]	0.63 [0.41-1.1]	0.87 [0.54-1.7]	0.54 [0.42-0.79]
Number with bilirubin >1.5 g/dl (%)	38 (16.5)*	8 (22.2)	10 (7.1)*
Serum urea in mg/dl, median [IQR]	34.5 [28.4-48.4]	36.8 [25.6-46.2]	34.6 [25.1-46.5]

Number with serum urea >40 mg/dl (%)	84 (36.5)	14 (38.9)	48 (34.0)
Serum creatinine in mg/dl, median [IQR]	0.78 [0.61-0.99]	0.68 [0.56-0.93]	0.76 [0.66-0.90]
Number with serum creatinine>1.2 mg/dl	29 (12.6)	3 (8.3)	15(10.6)
CSF examination			
CSF appearance – clear, lack of gross purulence/blood, Number	230(100.0)	36 (100.0)	141(100.0)
CSF cell count, in cells/ul, median [IQR]	24 [16-43]	20 [16-39]	16 [10-30]
CSF protein in mg/dl, median [IQR]	106.5 [69.5-141.5]	115.8 [75.0-141.7]	67.8 [45.2-118.0]
Number with CSF protein <=100 mg/dl	106 (46.1)*	15 (41.7)	98 (69.5)*
Outcome			
Alive	191 (83.0)	30 (83.3)	83 (58.9)
Death	35 (15.2)*	6 (16.7)	51 (36.2)*
Left Against Medical Advise	4 (1.7)	0 (0.0)	7 (5.0)

**P<0.05, clinical and lab findings were compared between patients positive for O. tsutsugamushi alone and negatives*
TLC: total leukocyte count
AST:Aspartate transaminase
ALT: Alanine transaminase
IQR: interquartile range
Figures in round and square bracket indicate percentages and IQR respectively

Table2: Laboratory investigations conducted among AES patients, BRD Medical College, Gorakhpur, India, 2016

Etiologic agent	Test	Type of specimen	No. Tested	No. Positive	%
O. tsutsugamushi	IgM ELISA	Serum	389	229	58.3
	IgM ELISA	CSF	374	227	60.7
	PCR	Blood	389	66	17.0
	PCR	CSF	374	32	8.6
	IgM or PCR	Serum/blood or CSF	407	266*	65.4
Japanese Encephalitis virus	IgM ELISA	CSF	375	28	7.5
	IgM ELISA	Serum	390	34	8.7
	IgM ELISA	CSF or Serum	407	42	10.3
Spotted fever group of rickettsia	PCR	CSF or Serum	389	4	1.0
Dengue virus	IgM ELISA	Serum	389	29	7.5

**36 patients were con-infected: 23 positive for dengue, 10 for JE, 2 positive for SFGR and 1 positive for both JEV and dengue.*