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A Study On Clinical Laboratory Cases Of Acute Febrile Illnesses (AFIS) To Identify The Sero-Molecular Epidemiology Of Orientia Tsutsugamushi, The Etiological Agent Of Scrub Typhus In Surat, South Gujarat, India.

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Abstract
Background: *Scrub typhus (ST), caused by Orientia tsutsugamushi, is an emerging and often under diagnosed cause of acute febrile illness (AFI) in India. Early diagnosis is essential for timely treatment and disease control.*
Objectives: *This study aimed to determine the sero-molecular epidemiology of O. tsutsugamushi among AFI cases in Surat, South Gujarat, and assess the diagnostic performance of Weil-Felix test (WFT), IgM ELISA, and nested PCR (nPCR).*
Methods: *A total of ≈600 blood samples from clinically suspected AFI cases were screened using the pooled WFT. Of these, 346 WFT-reactive samples were further tested by OX-K antigen tube WFT, IgM ELISA and nPCR. Thirteen nPCR-positive samples were subjected to partial gene sequencing, and the sequences were analyzed using NCBI BLAST for strain identification.*
Results: *Out of ≈600 AFI cases, 346 were pooled Weil-Felix test (WFT)screening Rickettsial Infections(RIs) positive. The RIs positive samples were further evaluated, of which 237 cases were positive for ST primary screening OX-K antigen slide WFT. Amongst ST screening positive cases187 were confirm sero-positive by OX-K antigen tube WFT, 126 were positive by IgM ELISA, and 101 were positive by molecular assay nested PCR (nPCR). Sequence analysis of 13 nPCR-product demonstrated more than 95% homology with the Orientia tsutsugamushi Karp strain. All sequences have been deposited in GenBank under accession numbers OR678396–OR678398 and OR766458–OR766467.*
Conclusion: *Scrub typhus is an emerging febrile illness in Gujarat, with Karp-like Orientia tsutsugamushi strains exhibiting subtle local variations, highlighting the need for improved diagnostics, surveillance, and public awareness.*
Keywords: *Scrub typhus (ST); Orientia tsutsugamushi; Acute Febrile Illness (AFIs); IgM ELISA; Nested PCR (nPCR); Weil-Felix test (WFT); Gujarat; Karp strain*

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I. Introduction:

Orientia tsutsugamushi is a gram-negative, obligate intracellular bacterium responsible for scrub typhus (ST), a potentially serious vector-borne disease endemic to regions within the "tsutsugamushi triangle," which includes parts of Asia, the Pacific Islands, and northern Australia. Scrub typhus is a rickettsial zoonotic infection that can lead to life-threatening acute febrile illness (AFI), often presenting as pyrexia of unknown origin (PUO) or Acute undifferentiated febrile Illness (AUFI) in humans. Transmission involves a complex interaction between infected larval mites (chiggers), vertebrate hosts, and human activities i.e., outdoor exposure through farming, gardening, hunting, fishing, camping, trekking, or even pet-walking increases the risk of infection.

In tropics PUO is often caused by infectious diseases i.e., bacterial infection-enteric fever, viral infection-Arbo virus infection, parasitic infection-Malaria, fungal infection. In the world malaria is primarily cause of AFI, but the availability of high through put point of care testing (POCT) i.e., rapid diagnostic test (RDT) and Microscopy will help in early diagnosis, timely treatment, control and management of malaria across the country. This development revealed burden of non-malarial AFI (Joshi *et al*., 2008; Bell, 2012; Acestor *et al*., 2012). A variety of pathogens are responsible for these illnesses, including viruses (such as Dengue, Influenza A, Japanese Encephalitis, and Chikungunya), bacteria (such as Typhoid, Leptospirosis, and

Rickettsial infections including Scrub typhus), and protozoa. Many of these diseases are transmitted through insect bites and are among the common tropical fevers reported in patients from Asian countries (Singhi *et al.,* 2014).

The term scrub typhus originated from the observed prevalence of the disease in scrubland and wasteland areas. In various scientific and regional literatures, it has also been referred to as *chigger-borne rickettsiosis*, *Japanese river fever*, *Kedani fever*, *Akamushi fever*, *tropical typhus*, *flood fever*, *shrub typhus*, and *bush typhus* (Richards & Jiang, 2020).Historically, ST was a disease of major clinical importance, particularly before the advent of antibiotics. It was a significant military health concern during World War II, with thousands of cases reported in the Far East (Xu *et al.,* 2017). These days, it is also categorized as an illness linked to travel, and instances have been documented among returning tourists from endemic areas (Costa *et al.,* 2021). Scrub typhus is a well-recognized cause of acute febrile illness (AFI) and presents a diagnostic challenge due to its non-specific clinical manifestations (Lalchhandama, 2019). It is considered the most common re-emerging rickettsial infection presenting as acute undifferentiated febrile illness (AUFI) (Hazra *et al.,* 2025). Historically, ST held great clinical significance. It was a feared disease in the pre-antibiotic era and emerged as a major military health concern during World War II, particularly in the Far East, where thousands of cases were reported (Xu *et al.,* 2017). Today, it is also recognized as a travel-associated disease, with implications for returning travelers from endemic regions (Costa *et al*., 2021). Scrub typhus and other rickettsial diseases are widely distributed across the tsutsugamushi triangle, encompassing large parts of South and Southeast Asia. However, systematic case reporting and access to reliable diagnostic tools remain suboptimal. In India, despite the established endemicity of scrub typhus in several regions, gaps in surveillance systems and the limited availability of accurate diagnostic assays continue to hinder timely case detection, clinical management, and effective public health response. Therefore, the present study aimed to investigate the sero-molecular epidemiology of scrub typhus in Surat, South Gujarat, to enhance understanding of its local transmission dynamics and to strengthen laboratory-based diagnosis and surveillance. By integrating serological and molecular diagnostic approaches, this study seeks to provide comprehensive evidence to support improved public health strategies for early detection and control of rickettsial infections in the region.

II. Materials And Methods

Study Design and Setting

This study was conducted through field surveillance and a cross-sectional analysis of patient samples, utilizing both serological and molecular assays. It is a prospective cross-sectional study carried out between August 2023 and September 2025 in Surat City, located in South Gujarat, India. The study involved coordinated collection and testing of blood/serum samples from patients presenting with infection associated acute febrile illness (AFI). All laboratory investigations were carried out at facilities affiliated with Veer Narmad South Gujarat University (VNSGU), including the Microbiology Departments of SMIMER and Kholwad College, Surat.

Sample Collection Site & Population

Residual clinical samples were obtained from a network of public and private diagnostic laboratories across Surat, the South Gujarat region. Ethical permission for the use of de-identified, leftover clinical specimens was granted by the respective institutional or departmental authorities.

The study population comprised patients of all ages and sexes who either presented to or submitted samples at these laboratories with symptoms of acute febrile illness (AFI), or were clinically suspected of having common AFI etiologies such as typhoid fever, malaria, chikungunya, dengue, or leptospirosis. Inclusion criteria focused on blood samples from individuals with a documented history of fever and elevated inflammatory markers, including leukocytosis, raised erythrocyte sedimentation rate (ESR), or increased C-reactive protein (CRP) levels, and who were referred for fever panel investigations.

Sample of study

Specimen collection and serological identification methods were described in the journal article published previously (Patil and Naik. 2024). A total of ≈600 non-repeated cases samples and 100 samples of healthy blood donors obtained from a regional blood transfusion center used as negative control were analyzed for the study. All procedures were performed in as per the recommendations in the DHR-ICMR Guidelines for Diagnosis and Management of Rickettsial Diseases in India (DHR-ICMR 2015) and the training manual of the National Centre for Disease Control (NCDC, 2024). From each case, paired blood and serum samples obtained were labeled coded anonymized, and transported to the laboratory at ambient temperature. The serum was separated and aliquoted. Diagnosis of common AFIs was confirmed by suitable laboratory tests routinely employed in our region. The following serological tests were used: Dengue Duo NS1/ IgM Cassette (SD Biosensor Healthcare Pvt. Ltd), Chikungunya (SD Biosensor Healthcare Pvt. Ltd) Widal antigen kit (J. Mitra

and Co. Pvt. Ltd) with titers of O≥80, H≥160 or a four-fold rise in titers, and /or rapid diagnostic test (Oscar Medicare Pvt. Ltd). Malaria was diagnosed with microscopy slides stained and/or antigen detection by rapid diagnostic kits (J. Mitra and Co. Pvt. Ltd).

Diagnostic Methods of Scrub Typhus

Serodiagnosis

Screening by Weil-Felix Test

The diagnosis of scrub typhus in this study was established using the Weil-Felix test (WFT). All samples were subjected to the Weil-Felix slide and tube agglutination test using OX-K antigen (Tulip Diagnostics Pvt. Ltd.;Central research institute (CRI) Kasauli) with quality control measures involving replicates and both negative (Healthy human serum) and positive controls (Tulip Diagnostics Pvt. Ltd available as PROGEN Positive Control REF.:105840001).Titers ≥1:80 were considered suggestive of scrub typhus.

IgM ELISA

ELISA, particularly serum IgM capture assays are probably the most sensitive tests available for rickettsial diagnosis (Saha *et al*., 2018), and IgM antibodies presence, indicate current infection with rickettsial disease. In infection cases with *Orientia tsutsugamushi*, at the end of 1st week IgM antibody is observed, whereas IgG antibodies produce at the end of 2nd week.

Samples from WFT-reactive cases (n=346) were tested using a commercial scrub typhus IgM ELISA serology conducted using two different kits were ELISA-1 ST Detect IgM ELISA system manufactured by InBios International, Inc., USA, and ELISA-2 ST IgM microlisa system of J. Mitra and Co. Pvt. Ltd, Delhi (Patil and Naik, 2024) Absorbance values were interpreted according to the kit protocol. Positivity was defined as per manufacturer's cutoff OD values.

Scrub Typhus Antibody Differentiation Immunochromatographic Test (ICT)

The serum samples were further subjected to qualitative differential detection of ST antibodies. IgM and IgG antibodies using rapid solid phase ICT assay available commercially by J. Mitra Pvt. Ltd., Delhi.

Molecular Diagnosis

According to DHR-ICMR (2015), a ST confirmed case is one, in which Rickettsial DNA is detected either in eschar or whole blood samples by PCR-Polymerase Chain Reaction. PCR gives the result in early days of infection when antibodies were not produced (Saha *et al.,* 2018).

DNA Extraction

Genomic Nucleic acid (DNA) was extracted from 200 µL of blood whole/clot using HiPurA® Multi-Sample DNA Purification Kit (HiMedia) following manufacturer protocols. Whole Blood/Blood clot nucleic acid (DNA) was extracted using HiPurA® Multi-Sample DNA Purification Kit (HiMedia) as described by the manufacturers. After the extraction procedure, the DNA was checked to verify that it was intact and clean of cellular contaminants accomplished by two ways UV spectrophotometry and gel electrophoresis.

Nested PCR (nPCR)

Nested also known as Suicide PCR high sensitivity; used for low-copy samples for the detection of ST DNA and is considered to be more specific and accurate than culture and conventional PCR (Raoult, 2009; Biswal *et al*., 2018). Rickettsial Infection ST Nested PCR (RI-ST nPCR) assay targeting the highly immunogenic, strain-specific; used for genotyping and diagnostics 56-kDa *TSA,* type-specific antigen gene of *O. tsutsugamushi* was performed with extracted DNA by using the optimized protocol (Patil, 2024) of previously described assays by Furuya *et al.,* (1993) and Biswal *et al.,* (2018). Positive control DNA as reference obtained on request from Microbiology department, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh (Biswal *et al.,* 2018) was used in each nPCR run.

The first-round PCR amplification product was, followed by a second-round amplification. Primer sequences, their amplicon sizes and standard protocol for nPCR runs are as shown in table 1 and 2. The selected Oligonucleotide primer sequences of were custom-synthesized from Sigma-Aldrich, Bangalore, India.

Table 1. Oligonucleotide sequences for RI-ST nPCR assay primer (Furuya *et al.*, 1993; Saisongkorh *et al*., 2004)

Name of Primer	Nucleotide Sequences (5' to 3')	Product Size
PCR-1 Outer Primer (OP) Set		
OP-F	TCAAGCTTATTGCTAGTGCAATGTCTGC	
OP-R	AGGGATCCCTGCTGCTGTGCTTGCTGCG	1000 bp
PCR-2 Inner Primer (IP) Set		

IP-F	GATCAAGCTTCCTCAGCCTACTATAATGCC	483 bp
IP-R	CTAGGGATCCCGACAGATGCACTATTAGGC	

Table 2. PCR-1 and PCR-2 reaction mixture preparation and cyclic condition.

PCR-1 Reagent Composition		**PCR-1 Cyclic Condition**	
Master mix	12.5	Initial denaturation	94 °C for 5 min.
Primer forward	01	Denaturation	94°C for 30 sec.
Primer reverse	01	Annealing 20 cycles	57°C for 1 min.
Deionized water	9.5	Extension	70°C for 1 min.
DNA template	01	Final Extension	72°C for 7 min
PCR-2 Reagent Composition		**PCR-2 Cyclic Condition**	
Master mix	12.5	Initial denaturation	94 °C for 5 min.
Primers forward	01	Denaturation	94°C for 30 sec.
Primer reverse	01	Annealing 30 cycles	57°C for 1 min.
Deionized water	5.5	Extension	70°C for 1 min.
DNA template (1st PCR product)	05	Final Extension	72°C for 7 min.

The success of the amplification was confirmed by resolution of the products by electrophoresis on 1.5 % agarose gel (HiMedia Laboratories, India) in 1× Tris EDTA buffer for products of the 56-kDa *TSA* gene. The sizes of the PCR amplified products were determined by comparison with a molecular weight standard (Takara Bio Inc., India) under UV light after ethidium bromide staining. A band of approximately 483 bp was considered indicative of *O. tsutsugamushi*.

PCR Product Sequencing

Thirteen nPCR-positive amplicons were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The purified-PCR products were used for sequencing. Samples for nucleotide sequencing were sent to Genexplore Diagnostics & Research Centre Pvt Ltd, Ahmedabad. Sequencing was carried out in Applied Biosystems 3500/3500xL Genetic Analyzer. Sanger sequencing method (Sanger et al., 1977) and big dye terminator were used for sequencing. The primers (IP-F & IP-R) used for the sequencing were the same as those for PCR. Sequences were determined by bidirectional sequence analysis (Sanger sequencing method). The obtained sequence data was analyzed using bioinformatics tools. Nucleotide sequences were analyzed using the BLAST tool (https://blast.ncbi.nlm.nih.gov/).

Phylogenetic Analysis.

The nucleotide sequences of the 56-kDa gene were verified and aligned by the Bio edit software version 7.0.5.3 to get a contig sequence. With the help of NCBI BLAST analysis software, the sequencing results were thoroughly examined to identify the organism. To build the phylogenetic tree the 56 kDa TSA (partial) nucleotide sequences for prototype and reference strains were obtained from GenBank. Multiple sequence alignment (MSA) and sequence homologies for the evolution analysis were performed and phylogenetic tree was created by the MEGA 11.0 program using neighbor-joining algorithms bootstrapped for 1000 replications (Kumar *et al.,* 2004).

Analysis and Alignment of Nucleotide Sequences.

The sequences were identified by comparison with sequences available in GenBank by using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) to identify strain similarity. All the sequences intra and inter similarities were assessed to found variations in the gene fraction

Scrub typhus, caused by *Orientia tsutsugamushi*, is an emerging vector-borne zoonotic disease in India.While traditionally associated with the Himalayan foothills, South India, and the Northeast.

III. Results And Discussion:

Scrub typhus has re-emerged as a major cause of acute febrile illness (AFI) in India. National surveillance data from the Indian Council of Medical Research–National Centre for Disease Informatics and Research (ICMR–NCDIR) indicate an increase from approximately 32,000 confirmed cases in 2022 to more than 54,000 in 2023 (ICMR–NCDIR, 2023). In response to this rise, the National Centre for Disease Control (NCDC) issued clinical alerts emphasizing early diagnosis and timely administration of doxycycline (NCDC, 2024).

Gujarat has reported increasing numbers of scrub typhus cases in recent years, suggesting its growing public health importance in the state(Patil and Naik,2023;2024; IDSP, 2024.).These results align with previous national reports describing scrub typhus as the predominant rickettsial infection in India, with peak transmission during the monsoon and post-monsoon periods (Krishnamoorthi *et al.,* 2023; Devasagayam *et al.,* 2021). This

study results underscores the significant presence of scrub typhus among acute febrile illness cases in South Gujarat and advocates for the integration of serological and molecular tools to enhance diagnostic accuracy.

Serodiagnosis of Scrub Typhus:

Out of approximately 600 samples of patients presenting with acute febrile illness (AFIs), 346 cases were initially identified as suspected rickettsial infections (RIs) based on a pooled Weil–Felix test (WFT) screening panel. Within this RI-positive group, 237 cases (39.5% of all AFI samples) showed specific reactivity to the OX-K antigen, indicating a likely exposure to *Orientia tsutsugamushi*, the causative agent of scrub typhus. These OX-K–reactive samples were then subjected to confirmatory diagnostic testing using multiple methods. In this follow-up testing, 187 cases (54%) were positive (titer ≥ 80) in the OX-K antigen tube WFT, which provides higher specificity than the initial slide screening. Additionally, 126 cases (36.4%) tested positive for anti-*O. tsutsugamushi* IgM by ELISA, indicating recent infection. Commercially available lateral-flow-format ICTs for the detection of scrub typhus IgG, IgM antibodies and scrub typhus total antibodies were assessed (Table 3).

The ST IgM ELISA has been increasingly adopted as a reliable and adaptable alternative to the indirect immunofluorescence assay (IFA), which is widely regarded as the serological reference standard for the diagnosis of scrub typhus (Thapa *et al.,* 2020). In this study, IgM ELISA was employed to detect anti-*Orientia tsutsugamushi* IgM antibodies and served as the principal confirmatory serological method. When interpreted alongside the WFT results, ELISA provided a more specific assessment of scrub typhus infection. Although a substantial proportion of acute febrile illness cases screened positive by WFT-particularly with the OX-K antigen, the subsequent ELISA testing enabled differentiation of true ST infections from potential cross-reactivity inherent to WFT. Thus, the integration of IgM ELISA with initial WFT screening strengthened diagnostic validity, allowing the study to more accurately characterize the sero-molecular epidemiology of scrub typhus in the region.

Moleculardiagnosis of Scrub Typhus:

Nested PCR (nPCR), which detects pathogen DNA directly, identified 101 cases (29.2%) as positive. Sequence analysis was performed on 13 nPCR-positive samples to characterize the infecting strains.

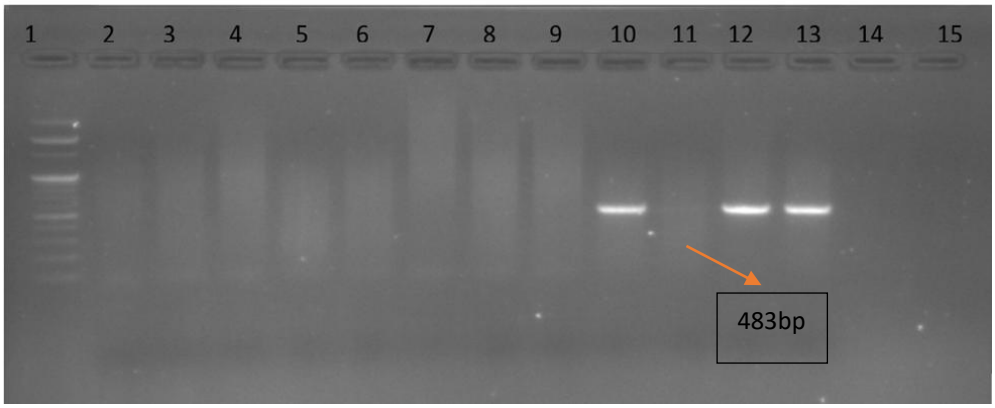

Figure 1. Gel Electrophoresis Image of Nested PCR Products.

Lane 1: 100 bp ladder, **Lane 2**: RC from PCR-1**, Lane 3**:NC(HHB) from PCR -1, **Lane 4**: PC from PCR -1**, Lane 5**:RC from PCR-2 **Lane 6**: NC (HHB) from PCR -2 **Lane 7:** NC-Malaria sample**Lane 8**: NC-Typhoid sample **Lane 9**:NC- Dengue sample**Lane 10**: PC from PCR-2 **Lane 11, 14 and 15**: Negative sample **Lane 12 and 13**: Positive samples

Table 3. Summary of Scrub Typhus Diagnostic Test Results (n = 346 WFT screening-reactive samples)

Diagnostic Test	***Number Positive***	***Percentage (%)***
Weil-Felix screening test (OX-K)	*237*	*68.49*
Weil-Felix Tube Test (OX-K)	*187*	*54.0*
IgM ELISA	*126*	*36.4*
Nested PCR (nPCR)	*101*	*29.2*
ICT For Differential Antibody Detection of ST		
Only IgM Detected	*87*	*25.1*
Only IgG Detected	*04*	*1.16*
Both IgG& IgM Detected	*10*	*2.89*
Total Antibody Detected	*101*	*29.2*

Table 4. Concordance Between Diagnostic Methods

Test Combination	*No. of Samples Positive*	*Interpretation*
WFTT+ IgM ELISA + nPCR	65	High diagnostic confidence
WFTT + IgM ELISA only	89	Possible resolved infection
IgM ELISA + nPCR only	95	Likely early seronegative window for WFT
WFT only	237	Possible false positives
nPCR only	101	Early infection, no antibodies yet

Molecular Typing and Phylogenetics study result:

56-kDa TSA Gene Sequencing:

56-kDa TSA Gene Sequencing is most widely used for molecular typing helps differentiate between *Orientia tsutsugamushi* major strains i.e., Karp, Kato, Gilliam, Kawasaki, and Boryong, among others.

Phylogenetic Analysis:

Molecular phylogeny reveals geographical clustering and frequent recombination events.

Indian strains are closely related to Southeast Asian genotypes but often show novel variants.

To build the phylogenetic tree for our sample sequences the 56 kDa TSA (partial) nucleotide sequences for prototype and reference strains i.e., Karp, Gilliam, Kato, TA763, were obtained from GenBank. Multiple sequence alignment (MSA) and sequence homologies for the evolution analysis were performed and phylogenetic tree was created by the MEGA 11.0 program using neighbor-joining algorithms bootstrapped for 1000 replications (Kumar *et al.,* 2004). From 101 nPCR-positive products obtained,13 purified, clean, single-banded product were selected for sequencing; all sequences were analyzed for sequence similarities with the help of the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). From the BLAST search, we found all sequences had similarities with our targeted organisms (*Orientia tsutsugamushi* strains). All 13 edited sequences were submitted to GenBank (Accession Nos. OR678396–OR678398 and OR766458–OR766467).

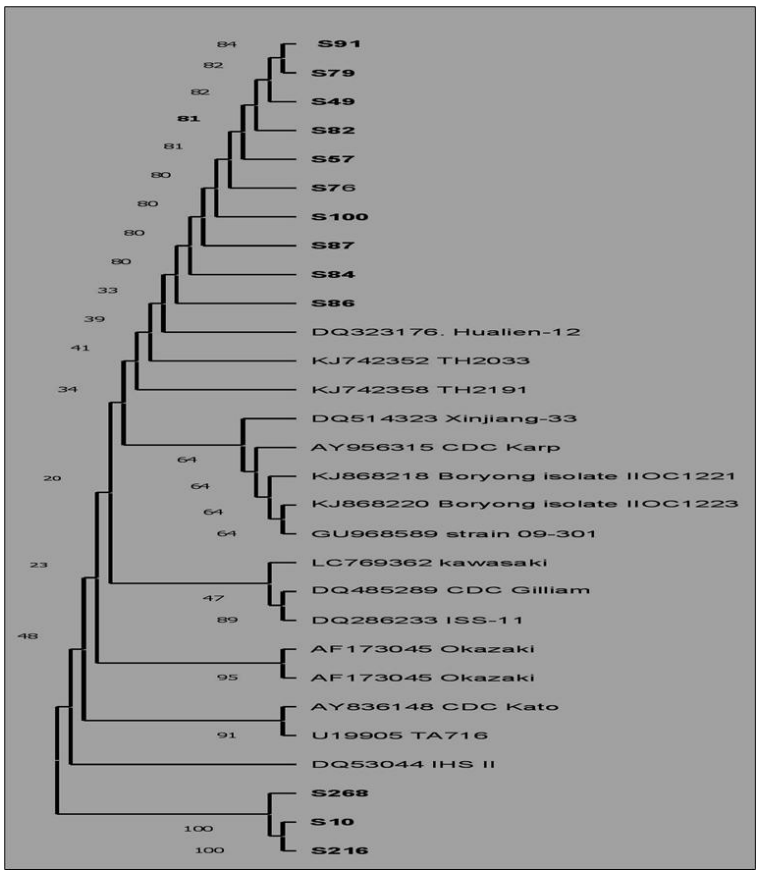

Figure 2. Phylogenetic Tree Based on 56-kDa *TSA* Gene Sequences

Phylogenetic tree of *Orientia tsutsugamushi* constructed on the nucleotide sequences of 56-kDa cell surface antigen gene by the neighbour-joining method, conducted in MEGA11.As depicted in figure 4,the sequences identity no. shown in in bold i.e, S10;S216;S268;S86;S84;S87;S100;S76;S57;S82; S49; S9; S79 were of this study.

All 13 sequences were analyzed for sequence similarities with each other -inter strain and reference strain-intra strain as shown in figure 3 and 4.

Results of Inter strain sequence BLAST Similarity.

Inter-strain sequence similarity of the 56-kDa TSA gene was assessed among 13 *Orientia tsutsugamushi* strains (S1–S13) and visualized as a heatmap (Figure 3).

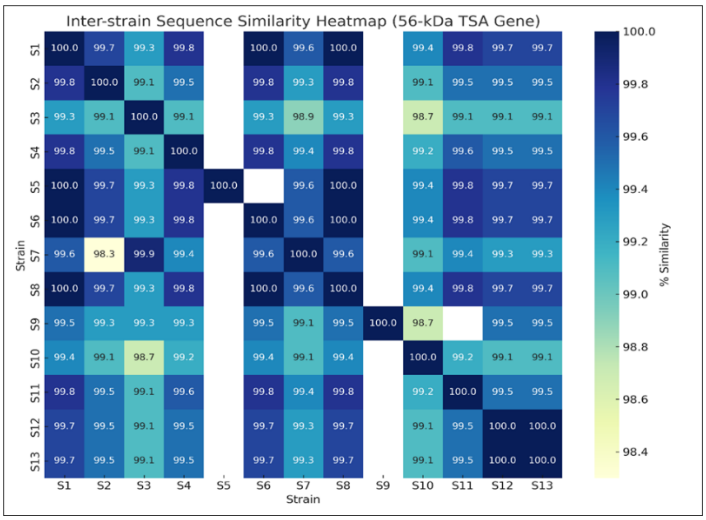

Figure 3. Inter-strain similarity heatmap

Overall, the strains exhibited very high nucleotide similarity, ranging from 98.3% to 100%.

- Most pairwise comparisons showed >99% sequence similarity, indicating strong conservation of the 56-kDa TSA gene among these strains.
- The highest similarities (100%) were observed in several strain pairs, including S1–S5, S1–S6, S5–S6, and S12–S13.
- The lowest similarity detected was 98.3% between S2 and S7, suggesting minor sequence divergence in these strains.
- Overall, the heatmap demonstrates that the majority of strains are closely related at the 56-kDa TSA locus, consistent with high homology with reference Karp-like strains.

These results indicate that the 56-kDa TSA gene is highly conserved across the studied strains, with only minor inter-strain variations.

Results of Intra strain sequence BLAST Similarity

NCBI BLAST results showing top sequence matches for Karp-like strains. Figure 4 shows the top BLAST sequence matches of Karp-like *Orientia tsutsugamushi* strains. The percentages represent sequence identity with reference strains in the NCBI database, indicating close similarity and confirming the Karp-like classification of the sequenced isolates.

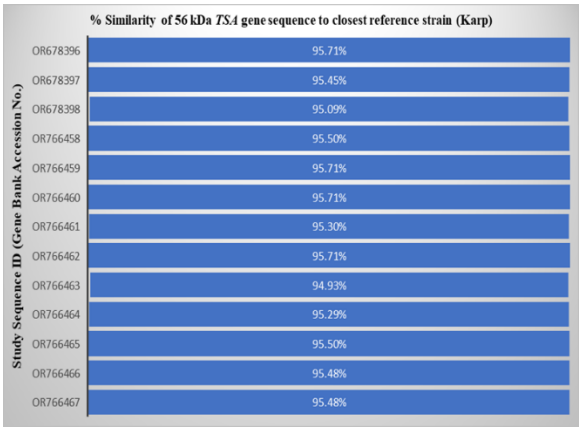

Figure 4. NCBI BLAST Top Sequence Matches and Percentage Identity of Karp-like *Orientia tsutsugamushi* Strains.

High genetic similarity to the Karp strain suggests regional strain predominance. These findings underscore the need for continued surveillance and diagnostic awareness in endemic regions. Scrub typhus was historically underreported in Gujarat.

Orientia tsutsugamushi, the cause of scrub typhus, continues to pose a substantial public health burden in India. Recent national surveillance data reported marked increase in incidence across multiple states (Mohapatra *et al.,* 2024). A recent systematic review of acute febrile illness cases estimated a pooled scrub typhus seroprevalence of 26.4% nationally, underscoring widespread endemicity and persistent diagnostic challenges (Sondhiya *et al.,* 2024). Molecular investigations have further expanded current understanding of transmission ecology, with *O. tsutsugamushi* now detected in non-trombiculid mites, fleas, and adult ticks in endemic regions (Govindarajan *et al.,* 2024).

In Gujarat, recent clinical data indicate a significant disease burden (Patil & Naik, 2024).A 2024 cohort study of IgM-ELISA–confirmed cases reported severe multisystem involvement and a mortality rate of 24%, reflecting substantial morbidity in the central India region (Dahiphle *et al.,* 2024). Despite this, molecular epidemiological data from Gujarat remain limited, highlighting the need for targeted genomic surveillance and enhanced diagnostic capacity.

Strain surveillance and genetic diversity studies across various Indian states have revealed a diverse array of *O. tsutsugamushi* strains, underscoring the necessity for comprehensive strain surveillance. For instance, research in Tamil Nadu and Himachal Pradesh has identified multiple genotypes circulating in these regions (Usha *et al.,* 2016)). This genetic diversity complicates the development of universal diagnostic tests and vaccines. Therefore, establishing a national strain repository and conducting longitudinal molecular surveillance are imperative to monitor strain evolution and inform public health strategies.

While doxycycline and azithromycin remain effective treatments for scrub typhus, emerging concerns about antibiotic resistance necessitate vigilant monitoring. Molecular studies have not only confirmed the efficacy of these antibiotics but also highlighted the importance of detecting potential resistance mutations. This is crucial for timely interventions and the development of alternative therapeutic options.

Despite advancements in molecular diagnostic techniques, their integration into routine public health laboratories in India remains limited. Implementing molecular diagnostics can significantly enhance the accuracy and speed of scrub typhus detection, leading to improved patient outcomes. Considerable investment in infrastructure, training,and diagnostic capacity along with strengthened strain-level surveillances essential for the effective public health management and control of scrub typhus in India.

IV. Conclusion:

This study highlights the significant presence of scrub typhus among AFI cases in South Gujarat and supports the integration of serological and molecular tools for enhanced diagnosis. Scrub typhus is an emerging febrile illness in Gujarat, with increasing case reports and sporadic outbreaks, particularly during the monsoon-post-monsoon seasons. Molecular analysis revealed that circulating Karp-like *Orientia tsutsugamushi* strains show high sequence identity with subtle local variations, indicating strain diversity in the region. Despite growing awareness among healthcare providers, challenges such as underdiagnosis, misidentification, and limited community knowledge persist. Strengthening diagnostic capacity, enhancing vector surveillance, and educating at-risk populations are critical for effective control and prevention of this re-emerging public health threat.

Ethical Considerations

The study utilized residual archived samples collected during routine diagnostic procedures. No additional samples were drawn and all data were anonymized prior to analysis. As per national and institutional guidelines for laboratory-based retrospective studies, formal informed consent was waived. The study was approved by the institutional review/administrative committee.

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in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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