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Comparative evaluation of molecular assays for detection of Orientia tsutsugamushi in patients with acute febrile illness in Western Odisha

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Article

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Comparative evaluation of molecular assays for detection of *Orientia tsutsugamushi* in patients with acute febrile illness in Western Odisha

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Abstract: Scrub typhus is an important cause of acute febrile illness in India, but diagnosis may be difficult because molecular detection of Orientia tsutsugamushi and the host antibody response occur over different phases of infection. We compared four molecular assays targeting three genomic regions in 71 hospitalized patients with acute undifferentiated fever in Western Odisha. All patients were tested by IgM ELISA and by TSA-56 quantitative PCR (qPCR), 16S rRNA qPCR, 16S rRNA conventional PCR (cPCR), and GroEL cPCR. Using IgM ELISA as the reference comparator, 16S rRNA qPCR showed the strongest overall performance, with 78.6% sensitivity, 95.3% specificity, 88.7% accuracy and substantial agreement (κ=0.758). TSA-56 qPCR showed 67.9% sensitivity, 95.3% specificity, 84.5% accuracy and κ=0.661. GroEL cPCR showed 78.6% sensitivity, 81.4% specificity, 80.3% accuracy and κ=0.592, whereas 16S rRNA cPCR had the lowest sensitivity (50.0%), accuracy (74.6%) and agreement (κ=0.434). Comparative ROC analysis showed the highest AUC for 16S rRNA qPCR (0.870), followed by TSA-56 qPCR (0.816), GroEL cPCR (0.800) and 16S rRNA cPCR (0.703). These findings suggest that real-time PCR, particularly 16S rRNA qPCR, may provide useful complementary information to serology for scrub typhus detection. The results should be interpreted as agreement with an IgM ELISA comparator rather than definitive diagnostic accuracy.

Keywords: scrub typhus; Orientia tsutsugamushi; TSA-56; 16S rRNA; GroEL; real-time PCR; conventional PCR; IgM ELISA; Western Odisha

1. Introduction

In Odisha, scrub typhus (ST), a significant source of acute fever, is becoming more well acknowledged. In endemic locations, it causes significant morbidity in patients, primarily youngsters, during the rainy season. Previous findings from Odisha have shown significant heterogeneity in the frequency of ST, with a reported incidence of 43% in South Odisha and 3.5% positivity among feverish patients who were not malarial. [1-3] Reducing morbidity and mortality requires early diagnosis and proper treatment.

As molecular techniques directly identify *Orientia tsustsugamushi* (OT) DNA during the acute phase of infection, sometimes prior to the development of a detectable antibody response, they can be used in conjunction with serological approaches. Additionally, PCR-based assays may be more specific in differentiating ST from other tropical febrile disease causes than serological testing. [4–7] However, the genomic target, assay format, bacterial load, timing of specimen collection, previous exposure to antibiotics, and genetic diversity of circulating strains can all affect how well molecular assays perform.

A number of genetic targets have been investigated for OT molecular detection. The 56-kDa type-specific antigen (TSA-56) gene is valuable for molecular epidemiology and strain characterization since it encodes a significant surface antigen and is highly variable among strains. *Orientia* are frequently identified and phylogenetically characterized using the relatively conserved 16S rRNA gene. GroEL has been studied as a potential diagnostic target and encodes a conserved heat-shock protein. [8–10] Assay sensitivity may vary among geographically different populations depending on how well target conservation and sequence diversity are balanced.

Therefore, using IgM ELISA as the reference comparator, the current prospective observational study examined the diagnostic efficacy of molecular tests targeting TSA-56, 16S rRNA, and GroEL in a hospitalized population from Western Odisha.

2. Materials and Methods

2.1 Study population and setting

The study was carried out on patients who came to the General Medicine and Pediatrics in-patient departments of Bhima Bhoi Medical College & Hospital (BBMC&H), a tertiary-care facility in Balangir, Western Odisha, with acute fever. The area is distinguished by a hot and humid environment, with a lot of greenery, and agricultural activities. The institutional Multidisciplinary Research Unit (MRU) conducted molecular experiments.

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2.2 Ethics Consideration & funding:

This study is a part of project entitled Study of Seroprevalence of Scrub Typhus and Comparison of Diagnostic Efficacy among Molecular and Serological Test in a Tertiary Care Hospital of Western Odisha approved by Multi-Disciplinary Research Unit (MRU)- Local Research Advisory Committee (LRAC) (01/ dt.13.10.2022) and Institutional Ethics committee (IEC)- Bhima Bhoi Medical College & Hospital (BBMCH) (31/IEC dt. 7.11.2022). It was funded by Dept. of Health Research (DHR), Govt. of India.

2.3 Sample collection and DNA extraction

The study group consisted of 71 patients with acute undifferentiated fever with a local common fever aetiology that had been ruled out for dengue leptospira, typhoid, malaria, and viral flu after obtaining consent. Written informed consent was obtained from all adult participants and from the parent/legal guardian/legally authorized representative in the case of participants who were minors (<18 years).They are all put through molecular testing and IgM ELISA. IgM ELISA results were used to categorize patients as either positive (28) or negative (43) for ST. Two milliliters of blood were drawn into EDTA tubes for molecular testing, and they were kept at -80 °C until they were processed. A spin-column method (Qiagen, Hilden, Germany) was used to extract DNA. A NanoDrop (DeNovix DS-11 spectrophotometer) was used to determine DNA concentration and 1% agarose gel electrophoresis was used to evaluate DNA quality. The extracted DNA was stored at - 20 °C for molecular studies.

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2.4 Molecular assays

Three genomic targets i.e.TSA-56, 16S rRNA, and GroEL were evaluated using four molecular assays: TSA-56 qPCR, 16S rRNA qPCR, 16S rRNA cPCR, and GroEL cPCR.

2.4.1 TSA-56 qPCR

The Geno-Sen's Scrub Typhus (RG) real-time PCR kit (Genome Diagnostics, India; GenBank accession KP334159.1) was used to test the samples which included OtsuF (5′-AATTGCTAGTGCAATGTCTG-3′) as forward and OtsuR (5′-GGCATTATAGTAGGCTGAG-3′) as reverse primers along with both positive and negative controls. In order to identify genetically varied OT strains, such as strains linked to Karp, Kato, and Gilliam, the test was developed with reference to KP334159.1. To evaluate detection at low target concentrations, five positive controls (P1–P5) with steadily rising Ct values were employed. The manufacturer's instructions were followed for master-mix formulation and cycling conditions. As per the original assay protocol, a Ct value of less than 35 was deemed positive. A CFX96 real-time PCR system with a C1000 Touch thermal cycler (Bio-Rad, USA) was used for amplification.[11]

2.4.2 16S rRNA cPCR

OT-specific 16SrRNA forward primer, (5′GAATTAATGCTGAGTTTGCTTAG-3′) and reverse primer (5′-CTCTCAGACCAGCTACAGATCACA-3′), were created using GenBank sequence L36222 [13,14]. Taq PCR Master Mix (Qiagen, Hilden, Germany) and 10 pmol of each primer were added to a 30-µL reaction mixture to amplify five microliters of extracted DNA (50 ng/µL). 35 cycles of 95 °C for one minute, 61 °C for one minute, and 72 °C for thirty seconds made up the cycling. 220 bp was the anticipated amplicon size.

2.4.3 GroEL cPCR

GroEL primers were designed from a conserved region of the gene and represented isolates from multiple endemic regions. [9,12 & 13] The forward primer was 5′-TTGCTGATGATGTAGACGGA-3′ and the reverse primer was 5′-TGTTCACAACGAGAATTAACTT-3′. Five microlitres of extracted DNA (50 ng/µL) were amplified using Taq PCR Master Mix (Qiagen, Hilden, Germany) and 10 pmol of each primer in a final volume of 30 µL. Cycling consisted of 30 cycles of 95 °C for 20 s, 54 °C for 45 s, and 72 °C for 1 min. Amplification was performed using a CFX96/C1000 Touch thermal cycler (Bio-Rad, USA). Primers were procured from Eurofins, Bengaluru, India. The expected amplicon size was 300 bp.

2.4.4 Gel electrophoresis and 16S rRNA qPCR

The 16S rRNA and GroEL cPCR products, expected to be 220 bp and 300 bp, respectively, were resolved on 1.5% agarose gels prepared in TAE buffer and visualised after electrophoresis at 75 V for 30 min. A 100-bp DNA marker was used to estimate amplicon size. Amplicons were visualised using a gel documentation system. The same 16S rRNA target was subsequently amplified by qPCR with SYBR Green Universal Master Mix (Applied Biosystems) on a CFX96 real-time PCR system, using a set of primer similar to that used for cPCR. (Figure-01)

2.5 Statistical analysis

Data were entered in Microsoft Excel 2016. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, Cohen's kappa, concordance, discordance, McNemar's test, and ROC curve analysis were performed using IBM SPSS Statistics version 21 and Statistics Kingdom. For the qPCR assays, ROC analysis was performed using continuous Ct values. For the cPCR assays, PCR results were analyzed as binary outcomes (positive/negative) based on gel electrophoresis. The Youden index was used to determine the optimal Ct threshold for qPCR assays. IgM ELISA was treated as the reference comparator.

3. Results

3.1 ROC analysis and Ct cut-offs

ROC analysis using continuous Ct values showed good discrimination for both TSA-56 and 16S rRNA qPCR assays against IgM ELISA. The TSA-56 qPCR had an AUC of 0.823 (95% CI: 0.728–0.918), with an optimal Ct threshold of ≤37.65 by the Youden index, corresponding to 71.4% sensitivity and 93.0% specificity, whereas 16S rRNA qPCR had slightly higher discrimination, with an AUC of (0.870; 95% CI: 0.786–0.953) with an optimal Ct threshold of ≤29.85, corresponding to 78.6% sensitivity and 95.3% specificity.[14] (Detailed data are provided in the Supplementary File)

3.2 Comparative ROC performance of all molecular assays

When all four molecular assays were compared using IgM ELISA as the reference standard, the 16S rRNA qPCR had the highest AUC (0.870; 95% CI: 0.786–0.953), followed by TSA-56 qPCR (AUC 0.816; 95% CI: 0.722–0.910), GroEL cPCR (AUC 0.800; 95% CI: 0.703–0.897), and 16S rRNA cPCR (AUC 0.703; 95% CI: 0.599–0.808). Thus, 16S rRNA qPCR demonstrated the greatest discriminatory ability in the comparative ROC analysis. (Figure: 02]

3.3 Diagnostic performance

With sensitivity of 78.6% and specificity of 95.3%, 16S rRNA qPCR also showed the best overall accuracy (88.7%). PPV and NPV were 91.7% and 87.2%, respectively, and there was significant agreement with IgM ELISA (κ=0.758). With significant agreement (κ=0.661), TSA-56 qPCR exhibited the same specificity (95.3%) but a lower sensitivity (67.9%). GroEL cPCR exhibited moderate agreement (κ=0.592), with 78.6% sensitivity and 81.4% specificity. Despite having 90.7% specificity, the 16S rRNA cPCR had the lowest sensitivity (50.0%), accuracy (74.6%), and agreement (κ=0.434). (Table:01)

Table 1. Diagnostic performance of molecular assays compared with IgM ELISA

PCR assay	TP	FN	FP	TN	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Accuracy %	Cohen's κ
TSA-56 qPCR	19	9	2	41	67.9 (49.3–82.1)	95.3 (84.5–98.7)	90.5 (71.1–97.3)	82.0 (69.2–90.2)	84.5	0.661
16S rRNA cPCR	14	14	4	39	50.0 (32.6–67.4)	90.7 (78.4–96.3)	77.8 (54.8–91.0)	73.6 (60.4–83.6)	74.6	0.434

GroELcPCR	22	6	8	35	78.6 (60.5–89.8)	81.4 (67.4–90.3)	73.3 (55.6–85.8)	85.4 (71.6–93.1)	80.3	0.592
16S rRNA qPCR	22	6	2	41	78.6 (60.5–89.8)	95.3 (84.5–98.7)	91.7 (74.2–97.7)	87.2 (74.8–94.0)	88.7	0.758

Note: TP, FN, FP, and TN denote true positive, false negative, false positive, and true negative, respectively. PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

3.4 McNemar's test

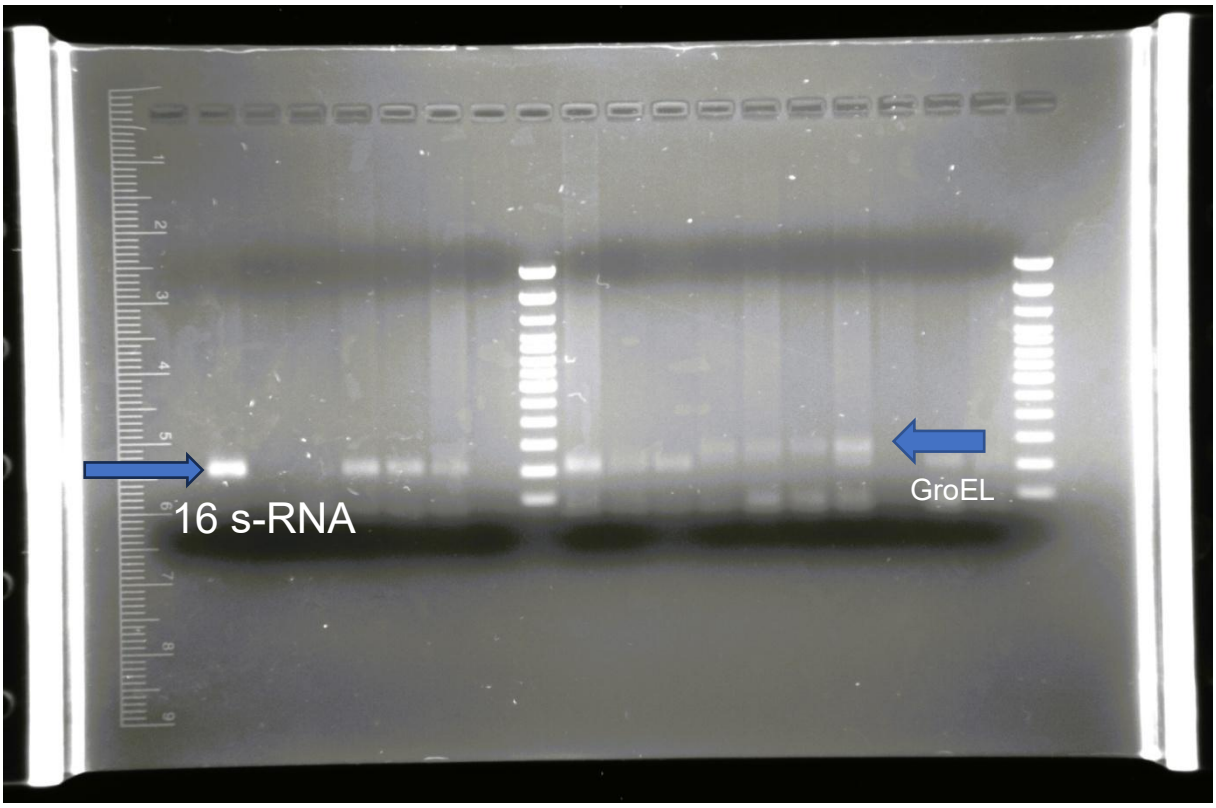
For TSA-56 qPCR versus IgM ELISA, there were 9 ELISA-positive/qPCR-negative pairs and 2 ELISA-negative/qPCR-positive pairs. The difference was not statistically significant by the exact McNemar test (p=0.0654). For 16S rRNA cPCR versus IgM ELISA, 14 ELISA-positive/cPCR-negative pairs and 4 ELISA-negative/cPCR-positive pairs were observed, with a statistically significant difference (p=0.0309). For GroEL cPCR, the discordant pairs were 6 and 8, respectively, with no significant difference (p=0.7905). For 16S rRNA qPCR, there were 6 ELISA-positive/qPCR-negative and 2 ELISA-negative/qPCR-positive pairs, with no significant difference (p=0.2891). (Table:02)

Table 2. McNemar's test comparing molecular assays with IgM ELISA

Comparison	ELISA+/PCR+	ELISA+/PCR−	ELISA−/PCR+	ELISA−/PCR−	Discordant pairs	p-value
TSA-56 qPCR vs IgM ELISA	19	9	2	41	11	0.0654
16S rRNAcPCR vs IgM ELISA	14	14	4	39	18	0.0309
GroELcPCR vs IgM ELISA	22	6	8	35	14	0.7905

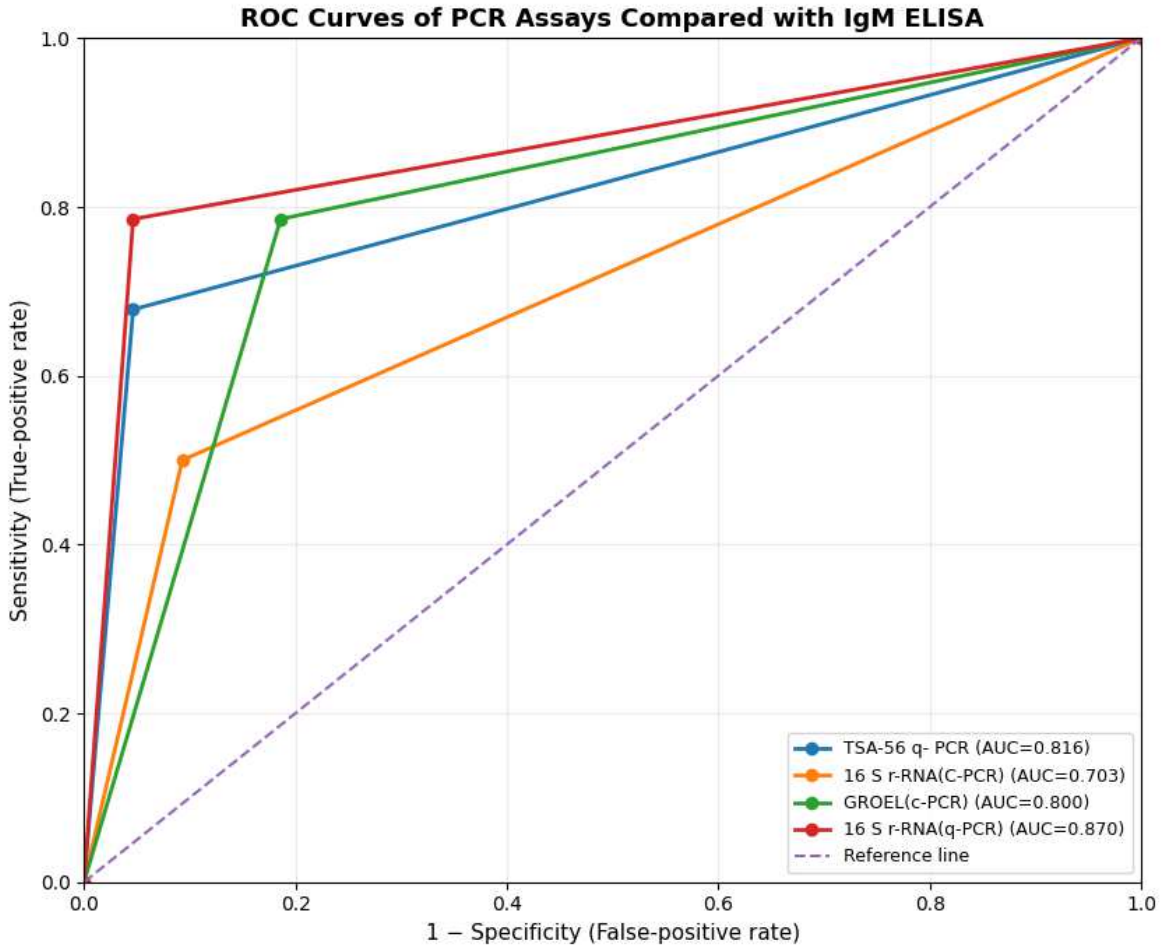
16S rRNAqPCR vs IgM ELISA	22	6	2	41	8	0.2891

Figure 1. Agarose gel electrophoresis of 16S rRNA and GroEL PCR amplicons.



1.5% TAE agarose gel showing PCR products. A 100-bp DNA marker was used for size estimation.

Figure 2. ROC curves of molecular assays compared with IgM ELISA.



4. Discussion

Due to underdiagnosis, inadequate surveillance, overlapping clinical presentation, and poor epidemiological data from several endemic regions, it is still challenging to determine the exact burden of ST, a significant cause of acute undifferentiated febrile illness in India.[15] IgM ELISA results showed that about 16% of probable cases in Balangir were positive, according to HMIS data. [as per study site Hospital database]

The present study compared four molecular assay types targeting three genomic regions, with IgM ELISA as a standard reference. Because IgM ELISA and molecular assays detect different biological markers and may perform differently according to the stage of illness, the present estimates should be interpreted as agreement with the reference comparator rather than as definitive sensitivity and specificity against an independent gold standard.

The degree of agreement between the four molecular assays and IgM ELISA varied. With 78.6% sensitivity, 95.3% specificity, 88.7% accuracy, and significant agreement (κ=0.758), 16S rRNA qPCR demonstrated the best-balanced performance among the molecular tests performed.

With significant agreement (κ=0.661), TSA-56 qPCR demonstrated similar specificity (95.3%) but lower sensitivity (67.9%). GroEL cPCR had a moderate agreement (κ=0.592) with a sensitivity of 78.6% and a specificity of 81.4%. On the other hand, 16S rRNA cPCR had moderate agreement (κ=0.434) and the lowest sensitivity (50.0%) and accuracy (74.6%). In this study population, 16S rRNA qPCR consistently outperformed IgM ELISA.

Complementary data was obtained using the McNemar analysis. IgM ELISA and TSA-56 qPCR (p=0.0654), GroEL cPCR (p=0.7905), and 16S rRNA qPCR (p=0.2891) did not differ statistically significantly. Conversely, there was a statistically significant difference (p=0.0309) between IgM ELISA and 16S rRNA cPCR, indicating a larger discrepancy between the conventional PCR and serological data. Differences in the timing of bacterial DNA detection and antibody formation, as well as the reduced analytical sensitivity of end-point PCR, may be linked to the excess of ELISA-positive/cPCR-negative results.

The performance of 16S rRNA qPCR in current study is broadly consistent with finding of Yun et al. supporting real-time PCR for early detection of ST [13] but contradictory to the finding of Kim et al. [16] Yun et al., examined 148 confirmed cases and found a sensitivity of 91.9% for 16S rRNA qPCR, 87.8% for 16S rRNA cPCR, 81.1% for 56-kDa nested PCR, and 74.3% for 47-kDa qPCR. They also found that patients who did not take antibiotics prior to admission had increased 16S rRNA qPCR sensitivity. [13] The reduced sensitivity demonstrated in this investigation could be attributed to changes in the study population, disease stage, antibiotic exposure, specimen features, bacterial load, and PCR chemistry.

A notable finding was the difference between conventional and real-time PCR targeting the 16S rRNA region. The 16S rRNA qPCR showed substantially better diagnostic performance than 16S rRNA cPCR despite targeting the same genomic region. This suggests that assay format and analytical sensitivity may be important determinants of performance in addition to target selection. Conventional PCR depends on end-point accumulation of amplicon sufficient for visualization on a gel, whereas real-time PCR detects amplification during the exponential phase and can identify lower amounts of target DNA. When appropriately standardized, qPCR can also provide quantitative information. The observed difference is therefore biologically and methodologically plausible.[13&17]

The TSA-56 target warrants particular consideration because the gene is highly variable among *OT* strains and is widely used for molecular epidemiology, strain characterization, and genotyping. [8,9] The sequence variability that makes TSA-56 useful for strain characterization may also influence amplification efficiency when an assay is applied across genetically diverse populations. The relatively lower sensitivity of TSA-56 qPCR in the present study may therefore reflect differences in circulating strains, target sequence variation, bacterial load, specimen characteristics, or assay design.

The present study evaluated TSA-56 in a real-time PCR format for diagnostic detection, whereas previous Indian studies have used TSA-47 KDa q-PCR and 56-kDa nested PCR mostly for sequencing and genotyping. Kumaraswamy et al. characterized the TSA-56 gene among *OT* strains circulating in and around Vellore, South India, highlighting substantial sequence variation among circulating strains. Such genetic heterogeneity is relevant when interpreting the performance of TSA-56–based molecular assays, as sequence variation at primer- or probe-binding regions may potentially influence amplification efficiency and, consequently, diagnostic sensitivity. The sensitivity observed in the present study (**67.9%**) is broadly consistent with the recognized limitations of molecular diagnosis of S. Kannan et al., in a multicentric evaluation of molecular and serological diagnostic methods, demonstrated that the performance of PCR-based assays varies according to the specimen, timing of collection, assay target and reference diagnostic definition. Their findings also emphasize that molecular detection and serological diagnosis identify different stages or aspects of infection and therefore may not show complete concordance. Seetha et al., in a molecular study from Kerala, documented circulating *OT* strains using molecular approaches and demonstrated the usefulness of molecular characterization for understanding the epidemiology of ST in the region. Differences in circulating genotypes may have implications for the performance of assays directed against variable genes such as TSA-56. Trung et al. reported genetic variation in the TSA-56 gene among *OT* from northern Vietnam, reinforcing the concept that TSA-56 is highly informative for molecular epidemiology but is also a genetically variable target. This variability should be considered when interpreting apparently negative TSA-56 PCR results, particularly across geographically distinct populations. [18-21].

The present findings added preliminary clinical evidence on the diagnostic performance of TSA qPCR in Western Odisha. However, because sequencing or genotyping of positive samples was not the primary objective, the relationship between assay performance and local strain diversity cannot be established from the present data.

GroEL cPCR also showed clinically relevant performance, with sensitivity similar to that of 16S rRNA qPCR but lower specificity. Previous investigations have evaluated GroEL as an alternative molecular target and have reported variable diagnostic performance across populations [9,22&23]. Patricia et al. evaluated PCR assays targeting the 56-kDa, 16S rRNA, and *GroEL* genes and reported positivity rates of 27.6%, 20.6%, and 68.2%, respectively, with GroEL PCR demonstrating substantially greater detection than the other two targets. The authors suggested that GroEL may serve as a useful molecular target for improving the diagnosis of ST, particularly when used in combination with serological testing. [9] Similarly, an Indian study from Chhattisgarh reported a sensitivity of 66.6% and specificity of 100% for GroEL PCR, compared with 62.8% sensitivity and 100% specificity for IgM ELISA. The authors concluded that GroEL PCR could complement IgM ELISA, particularly in seronegative cases. These observations are broadly consistent with the present findings and support the potential value of GroEL as a molecular target for OT detection.[22] The differences between published studies and the present findings may reflect variation in study population, disease stage, bacterial load, specimen type, assay design, and local

strain distribution. The present data support GroEL as a potentially useful complementary target rather than as a demonstrably superior target.

Comparison between results of 16S rRNA qPCR (sensitivity- 78.6%, specificity-95.3%, accuracy-88.7%) and 16S rRNA cPCR (sensitivity-50.0%, specificity-90.7, accuracy-74.6%), in the current study revealed that qPCR platform has higher sensitivity, specificity and accuracy than cPCR. This is in accordance with study of Yun et al (16S rRNA qPCR with a sensitivity of 91.9%, 16S rRNA cPCR 87.8%). Better performance of qPCR supports the potential value of real-time amplification in early detection of Scrub typhus.[13]

Discordance between molecular assays and IgM ELISA should also be interpreted in relation to the natural history of ST. PCR detects circulating bacterial DNA, whereas IgM ELISA detects the host antibody response; consequently, the two methods may have different diagnostic windows. Prior antimicrobial treatment may further reduce circulating bacterial DNA and thereby affect PCR sensitivity. Differences between the assays should therefore not automatically be interpreted as failure of one method, particularly when the tests detect different biological markers.

From a diagnostic perspective, IgM ELISA remains a practical and widely used method in routine settings. Molecular assays may provide complementary information, particularly during the early phase of illness when antibody responses may not yet be fully developed. Within the present study population, 16S rRNA qPCR was the most promising molecular assay, while GroEL cPCR may provide an alternative where real-time PCR facilities are unavailable. TSA-56 qPCR showed high specificity and substantial agreement with IgM ELISA and may add value for molecular characterization when combined with sequencing or genotyping.

Several limitations should be considered. First, IgM ELISA was used as the reference comparator rather than culture, paired-serum seroconversion, or a composite reference standard. Therefore, the calculated sensitivity and specificity represent agreement with the study comparator and should not be interpreted as absolute diagnostic accuracy. Second, PCR performance may be influenced by timing of specimen collection, prior antibiotic exposure, bacterial load, specimen type, DNA extraction, and assay-specific factors. Third, the study was conducted in a hospital-based population from Western Odisha, limiting generalizability to other geographical regions with different epidemiological and strain characteristics. Finally, the study did not undertake sequencing or genotyping of positive samples; therefore, the contribution of local strain diversity to assay performance remains unresolved.

5. Conclusion

This study demonstrates substantial variation in the performance of molecular assays targeting different genomic regions of *OT*. Among the evaluated assays, 16S rRNA qPCR showed the most balanced performance and the highest agreement with IgM ELISA, whereas 16S rRNA cPCR performed worse despite targeting the same genomic region. The findings support the potential advantage of real-time PCR over conventional end-point PCR for molecular detection of scrub typhus and highlight the importance of evaluating molecular targets and assay formats in

geographically distinct populations. A combined diagnostic approach incorporating serology and appropriately timed molecular testing may improve detection across different stages of illness. Further multicentric studies using composite reference standards, paired serology, quantitative PCR, and molecular characterization of circulating strains are warranted.

Human Ethics and Consent to Participate declarations: The research was conducted in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration.

Author Contribution declaration: RKP- Concept design, acquisition of data, analysis and interpretation of data, definition of intellectual content, manuscript editing and review. **PMD-**experimental studies, data acquisition, data analysis, literature search, manuscript preparation and editing **BMR**- Conception and design or acquisition of data and manuscript review. **SRM**- Conception and design, acquisition of data, literature search and manuscript review. **AKB**- Conception and design

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