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Diagnosis of Rickettsioses in Clinically Suspected Cases of Rickettsial Fever by Real-Time PCR in a Tertiary Care Centre

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ABSTRACT

Background: Rickettsial infections are zoonotic diseases transmitted between mites, ticks, or fleas and animals, particularly rodents. Despite being underdiagnosed, they are a significant cause of Fever of Unknown Origin (FUO) globally, including India. Timely diagnosis is crucial as untreated cases can have fatality rates as high as 30-35%, emphasizing the need for rapid diagnostic methods. This study aims to diagnose clinically suspected cases of rickettsial fever by utilizing both the traditional Weil-Felix serological test and the more advanced PanR8 real-time PCR.

Methods: This cross-sectional study was conducted between December 2017 and June 2019 at Bangalore Medical College. A total of 100 blood samples were collected from clinically suspected rickettsial fever patients. Serological diagnosis using the Weil-Felix test and molecular confirmation via PanR8 real-time PCR targeting the 23S rRNA of *Rickettsia* species was performed. Data analysis was done using descriptive statistics to interpret prevalence and symptom correlations.

Results: Out of 100 samples, 37% showed positivity for OX-19 and OX-2 antigens in the Weil-Felix test. In the real-time PCR, 7% of samples tested positive for *Rickettsia* species, with a $p<0.05$, showing a significant correlation between PCR and Weil-Felix results in high-titer cases. The majority of patients (100%) had a fever, 46% had a rash, and hepatosplenomegaly was observed in 20%.

Conclusion: The study demonstrated the utility of both Weil-Felix test and PanR8 real-time PCR for diagnosing rickettsial infections. The PanR8 real-time PCR improved diagnostic accuracy and reduced turnaround time, proving valuable in clinical settings.

Key-words: Bengaluru; Rickettsial infections; Weil Felix test; Panrickettsia real-time PCR

INTRODUCTION

Rickettsial infections have been rampant all over the world (except Antarctica) for ages. Rickettsial diseases are zoonoses where human beings are inadvertently involved in a chain of transmission between trombiculid mites (chiggers), ticks or fleas, and animals (most commonly rodents) [1].

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Among the major groups of rickettsioses, scrub typhus, murine flea-borne typhus, Indian tick typhus, and Q fever are widely reported in India [2]. These infections are debilitating and difficult to diagnose, which makes them more attention-worthy. Fatality rates as high as 30%-35% have been observed in untreated cases. The variable and nonspecific presentations of this infection have often made it difficult to diagnose clinically. With proper, timely diagnosis, they are often easily treated, with less morbidity and mortality. Rickettsial infections are attributed to being one of the essential causes of Fever of Unknown Origin (FUO). There is a need to differentiate it from other common febrile illnesses like enteric fever, malaria, and dengue for appropriate treatment [3].

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Complications such as jaundice, renal failure, pneumonitis, acute respiratory distress syndrome (ARDS), septic shock, myocarditis, meningoencephalitis, and multi-organ failure make the early diagnosis and prompt antibiotic therapy of rickettsial infections a must [4]. Underdiagnosed and misdiagnosed rickettsial infections are significant public health problems. Apart from causing morbidity and mortality, they add to the financial burden of the patient. Microbiological diagnosis of rickettsiosis is usually established by serology, as isolation in cell culture or animals is difficult and dangerous for laboratory personnel, and immunohistochemistry is not widely available [3].

As IgM increase takes 15–26 days, serological diagnosis is usually retrospective, thus reducing the clinical impact of diagnosis [3]. Moreover, species identification is limited by cross-reactions. Considering the uncertainties and difficulties with the available serological tests, serology is considered less significant as an adequate marker for rickettsial illnesses. Cases of confirmed rickettsioses have been described even without an increase in rickettsial antibody titer.

The diagnosis of rickettsial infections relied heavily on the Weil Felix (WF) test for decades in the past. WF test detects antibodies to various Proteus species, which contain antigens with cross-reacting epitopes to antigens from members of the genus Rickettsia [5]. Weil-Felix test is not considered a sensitive and specific test. Still, due to the lack of availability of definitive tests in India, they can be helpful when used and interpreted in the correct clinical context [6]. This test should be done after 5-7 days of onset of fever [4]. Either a fourfold rise in agglutinin titre in paired sera (better) or a single titre of more than 1:80 is considered diagnostic for rickettsial infections [7]. However, baseline titres need to be standardized for each region. Despite all its drawbacks, the Weil-Felix test still serves as a valuable and cheap diagnostic tool for laboratory diagnosis of rickettsial disease. It can be used as a screening test, which detects more cases than misdiagnosed ones [8,9]. Few studies have demonstrated that the sensitivity of Weil-Felix was 30% at a breakpoint titre of 1:80, but the specificity and positive predictive value were 100% [10]. Hence Weil-Felix test is still not entirely obsolete but has to be interpreted in the correct clinical context, especially in resource-poor settings.

IgM and IgG ELISA techniques, particularly immunoglobulin M (IgM) capture assays, are probably the most sensitive tests available for rickettsial diagnosis. The presence of IgM antibodies indicates a recent infection with rickettsia [11]. But, lack of region-specific standard cut-off titres and the need for equipment and skilled workforce limit their usage. The Immunofluorescence assay (IFA) has been the "gold standard" in rickettsial disease diagnostics for decades [6-8]. However, the lack of standardization, variable cut-off titres for endemic regions, the requirement for paired sera, high cost, and subjective endpoints are causes for concern [12].

Several PCR assays targeting different rickettsial genes have been developed for rapid diagnosis of rickettsiosis [13]. While some PCR assays targeted several species (Panrickettsia), others were designed to detect only a single rickettsial species (species-specific) [14]. As several rickettsiae can be responsible for the same clinical syndrome, a broader spectrum for diagnosis is warranted. Traditional PCR techniques lack the sensitivity to diagnose infection when there are low numbers of rickettsiae in peripheral blood mononuclear cells. The development of a real-time PCR specific for Rickettsial diseases is useful in overcoming the defects of serology and conventional PCR. Panrickettsia assays have utilized conserved sites in the 17-kDa lipoprotein precursor antigen gene, outer membrane protein (*ompA* and *ompB*), 16S rRNA, and citrate synthase (*gltA*) genes. In contrast, species-discriminating assays have targeted the 16S rRNA, *sca4*, *ompB*, *gltA*, and *ompA* genes [15]. This study utilizes the Panrickettsia (PanR8) real-time PCR to diagnose suspected cases of Rickettsial diseases. The PanR8 real-time PCR detects 16 Rickettsia species (*Rickettsia akari*, *R. amblyommii*, *R. australis*, *R.canadensis*, *R. conorii*, *R. felis*, *R. honei*, *R. massiliae*, *R. montana*, *R. parkeri*, *R. rhipicephali*, *R. sibirica*, *R. slovaca*, *R. rickettsii*, *R. prowazekii* and *R. typhi)*. The species-specific real-time PCR detects a single species, as in the case of RRi6 real-time PCR assay for detecting *R.rickettsii* only and not others [16].

It is essential to diagnose rickettsial infections rapidly and correctly to ensure prompt antibiotic therapy, shorter course of the disease, and lower morbidity and mortality. There is a need for physicians and health care workers in India to be aware of the clinical features, available diagnostic tests and their interpretation, and the therapy of these infections. This study proposes presumptively diagnosing clinically suspected cases of

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Rickettsial fever by Weil Felix test and confirming the diagnosis of Rickettsioses using real-time PCR.

MATERIALS AND METHODS

Research Design- This study employed a cross-sectional design and was conducted at the Department of Microbiology, Bangalore Medical College and Research Institute (BMC&RI) in Bengaluru between December 2017 and June 2019. The cross-sectional design was chosen as it allows for the analysis of data collected from a population at a specific time. The study involved 100 blood samples obtained from clinically suspected cases of rickettsial fever, characterized by either acute undifferentiated febrile illness lasting ≥5 days with or without an eschar or fever of <5 days with an eschar, associated with clinical symptoms such as headache, rash, hepatosplenomegaly, lymphadenopathy, and multi-organ involvement (liver, lung, kidney), with or without a history of tick exposure.

Inclusion Criteria

- Clinically suspected cases of rickettsial fever.
- Patients presenting with acute undifferentiated febrile illness for ≥5 days or with a fever of <5 days but with an eschar.
- Patients exhibiting clinical features such as headache, rash, hepatosplenomegaly, lymphadenopathy, and involvement of multiple organs (liver, lung, kidney).

Exclusion Criteria

- Patients with confirmed non-rickettsial infections were excluded. These included cases with laboratory-confirmed enteric fever, malaria, dengue, leptospirosis, bloodstream infection, urinary tract infection, respiratory infection, and tuberculosis.

Sample Collection- After obtaining clearance from the institutional ethics committee and securing informed consent from the participants, patient history and clinical examination details were documented. Routine investigations were conducted as per the patient's admission chart.

Blood samples were collected at the time of admission:

- **EDTA Blood** (3-5 ml) for Real Time PCR.
- **Plain Blood** (3-5 ml) for the Weil-Felix test.

The Weil-Felix test was performed on acute samples, and if available, a convalescent sample was collected after two weeks of treatment. The test was conducted using the MICROPATH PROTEUS OX-K, OX-19, and OX-2 kit (Omega Diagnostics Ltd.), following the manufacturer's protocol. A titre of 1:80 or more was considered indicative of Rickettsial infection.

DNA Extraction and Real-Time PCR- DNA was extracted from the aliquoted plasma samples using Qiagen Blood Mini Kits per the manufacturer's protocol. The extracted DNA was processed through Real-Time PCR targeting Rickettsia species using a pan-Rickettsia real-time PCR assay focused on the 23S rRNA gene. The extracted DNA was stored at -80°C if immediate processing was not feasible, and repeated freeze-thaw cycles were avoided to maintain sample integrity.

The real-time PCR setup involved:

- Mastermix: 12.5 µl Sso Advanced Universal Probes Supermix (BIO-RAD).
- Forward Primer: 2.5 µl.
- Reverse Primer: 2.5 µl.
- Probe: 1 µl.
- Nuclease-Free Water: 1.5 µl.
- Extracted DNA: 5 µl.

Table 1 shows the primer and probe sequences used in the real-time PCR assays for detecting *Rickettsia* species. The forward primer (PanR8_F) sequence is 5'-AGC TTG CTT TTG GAT CAT TTG G-3', while the reverse primer (PanR8_R) sequence is 5'-TTC CTT GCC TTT TCA TAC ATC TAG T-3'. Additionally, the probe (PanR8_P) sequence is labeled with fluorescence (Fl) at the 5' end and a black hole quencher (BHQ1) at the 3' end: 5'-CCT GCT TCT ATT TGT CTT GCA GTA ACA CGC CA-3'. This table emphasizes the molecular tools used in the detection process, particularly highlighting the specificity of the primers and probe used in the real-time PCR assay to target the 23S rRNA of *Rickettsia* species.

Table 1: Primer and Probe sequence

Primer	Primer sequence (5'-3')
PanR8_F	AGC TTG CTT TTG GAT CAT TTG G
PanR8_R	TTC CTT GCC TTT TCA TAC ATC TAG T
PanR8_P (probe)	Fl-CCT GCT TCT ATT TGT CTT GCA GTA ACA CGC BHQ1

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The PCR cycling conditions were as follows:

- Initial denaturation at 95°C for 8 minutes.
- Forty-five cycles of denaturation at 95°C for 5 seconds, followed by extension at 60°C for 30 seconds.

PCR was performed on the BIO-RAD CFX96 Real-Time System C1000 thermal cycler, and results were analyzed using CFX 96 Manager software.

Statistical Analysis- Data collected from the study were analyzed using descriptive statistics. Results were presented in numbers and percentages to describe the prevalence and characteristics of rickettsial fever among the study population. The statistical analysis was conducted to identify trends in the occurrence of the disease and its correlation with the clinical symptoms, which helped evaluate the effectiveness of diagnostic methods like the Weil-Felix test and Real-Time PCR. The descriptive approach allowed the researchers to present a detailed picture of the findings without requiring complex inferential statistics.

RESULTS

Table 2 compares the clinical manifestations of rickettsial infections across multiple studies. In all the studies, 100% of the cases presented with fever. However, variations are observed in other symptoms. Eschar, a key diagnostic marker, was absent in the present study, whereas other studies recorded up to 7%. These variations highlight the diverse clinical presentations of rickettsial infections, underscoring the challenge in diagnosis and the need for reliable laboratory confirmation.

Table 2: The clinical profile of rickettsial infections in various other studies and the present study

Table Symptoms	Rathi *et al.* [1,7]	Chunchanur *et al.* [12]	Sankar *et al.* [17]	Thomas *et al.* [20]	Nawab *et al.* [21]	Present study (%)
Fever	100	100	100	100	100	100
Rash	83	40	29.7	54.2	83.3	46
Headache /myalgia	-	26	53.5	-	16.7	15
Nausea /vomiting	-	23	40.4	-	36.6	17
Hepatosplenomegaly	99	51	18.9	87	70	20
Lymphadenopathy	41	-	20.3	21.8	3.3	0
Eschar	7	06	2.7	5.7	6.7	0
Convulsions	28	26	4	19.1	-	13
Altered sensorium	-	10	2.7	-	-	07
Suspected exposure to ticks	80	-	-	-	-	11

Table 3 has shown a comparative analysis of Weil-Felix test results from various studies, focusing on the positivity rates of the OX-19 and OX-2 antigens. The present study reported a combined positivity rate of 37%, which is higher than several previous studies, which showed a range of 39.3% for OX-2 and 8.1% for OX-19. Sankar et al. reported slightly lower positivity rates, with 13.1% for OX-19 and 12.1% for OX-2. The data suggests that while the Weil-Felix test remains helpful in diagnosing rickettsial infections, the positivity rates can vary widely depending on the study population, geographical region, and period.

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Table 3: Weil Felix results in comparison to other studies

Study	Year	OX-19 and OX-2 positive results
Sanap *et al.*[16]	2017	OX-19- 10.52% and OX-2- 13.81%
Sankar *et al.*[17]	2017	OX-19- 13.1% and OX-2- 12.1%
Dias *et al.*[18]	2014	OX-2- 88.23% and OX-19- 19.60%
Mittal *et al.*[19]	2012	1999-04: 39.3%-OX-2, 8.1%-OX-19
		2005-09: 27.5%-OX-2, 6.8%-OX-19
Chunchanur *et al.*[26]	2018	24%(OX-19 and OX-2 combined)
Kamarasu *et al.*[29]	2006	4.6%(OX-19 and OX-2 combined)
Varghese *et al.*[30]	2014	27.5%(OX-19 and OX-2 combined)
Sudhindra *et al.*[31]	2017	17.3% (OX-19 and OX-2 combined)
Present study	2018	37%(OX-19 and OX-2 combined)

Table 4 compares the real-time PCR results from the present study with other international and Indian studies. The present study found that 7% of the samples tested positive using PanR8 real-time PCR, which is relatively low compared to other studies. The present study's findings establish the existence of rickettsial species other than *Scrub typhus* in the Bangalore region, emphasizing the need for region-specific diagnostic approaches.

Table 4: The real-time PCR result of the present study as compared to various other International and Indian studies

Publication Yr/ place	Authors	Sample size	Type of PCR	Results	Remarks
International studies					
2012/ CDC, Georgia, USA	Kato *et al.*[9]	223 (banked DNA extracts tested for rickettsia by nested PCR)	Pan R8 Real-time PCR	41 (18.4%) tested positive	CDC-developed PCR, considered better than nested real-time PCR, results in <2hrs
2015/ CDC, Georgia, USA	Zemtsova *et al.*[22]	87(Blood and tissue samples from rickettsia infected laboratory animals)	SYBR green-based real-time PCR	34 (39%)	Better sensitivity than semi-nested PCR, nested PCR or conventional PCR
2005/ Seoul	Choi *et al.*[23]	100 seropositive samples*	Nested real-time PCR	71 (71%)	-
2011/ Switzerland	Giulieri *et al.*[3]	16 specimens from 13 different patients	Duplex real-time PCR detecting Pan rickettsia and species-specific	3 (in total) (18.75%)	Good sensitivity for at least 10 DNA copies per reaction and good reproducibility
2015/ Tunisia	Znazen *et al.*[24]	79 whole blood seropositive samples* for rickettsia	Duplex quantitative real-time PCR detecting	5 (in Pan rick PCR) (6.3%)	-

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			Panrickettsia and species-specific		
Indian studies					
2012/ Vellore	Prakash *et al.*[25]	58 (Skin biopsy samples from rash)	Nested PCR	34- SFG** (58.6%)	This PCR detects *gltA*, 17 kDa lipoprotein antigen gene, *ompA* and *ompB*
2017/ Chandigarh	Zaman *et al.*[26]	51	*ompA* PCR	3 (5.9%)	-
Present study		100	Pan R8 (Panrickettsia) real time PCR	7 (7%)	Study establishes the existence of rickettsial species other than Scrub typhus in and

***Seropositive samples refer to samples positive for Rickettsia immunofluorescence assay; **Spotted fever group*

Out of the 100 samples, 58 patients were in the age group 0 to 10 years, 22 patients were in the age group 11 to 20 years, 9 patients were in the age group 21 to 30 years, 3 patients were in the age group 31 to 40 years, 5 patients were in the age group 41 to 50, 3 patients were in the age group 51 to 60 years. Male preponderance (57%) was observed. Fever and rash were the most common symptoms in the patients included in the study, fever being the most consistent symptom (100%) present in all study population and rash being the second most common (46%) symptom. Hepatosplenomegaly was recorded in 20%. None of the enrolled patients had lymphadenopathy or eschar. Nausea/vomiting (17%), convulsions (13%) and headache (15%) were the other common symptoms recorded in the patients in this study. 11% of patients were suspected to have been exposed to ticks based on the history elicited from them (exposure to shrubs, high grass, leaf litters, poultry or animal farming, etc.)

Weil Felix Slide agglutination (Fig. 1) titre correlated with the tube agglutination titre for most samples. A titre of 1:80 or more for OX-K is suggestive of Scrub typhus infection and a titre of 1:80 or more for OX-19 and OX-2 is suggestive of Rickettsiae other than Scrub typhus (Spotted fever group and Typhus fever group). 37 samples showed high titre agglutination for OX-19 and OX-2 combined and less titre for OX-K suggestive of Rickettsial infections other than Scrub typhus (Spotted fever and Typhus groups). This indicates that the other 63 samples showed either higher titre for OX-K than OX-19 and OX-2 or were negative for OX-19 and OX-2 combined. Fever and rash were the major symptoms recorded in patients who were positive for OX-19 and OX-2 high titre combined, followed by hepatosplenomegaly.

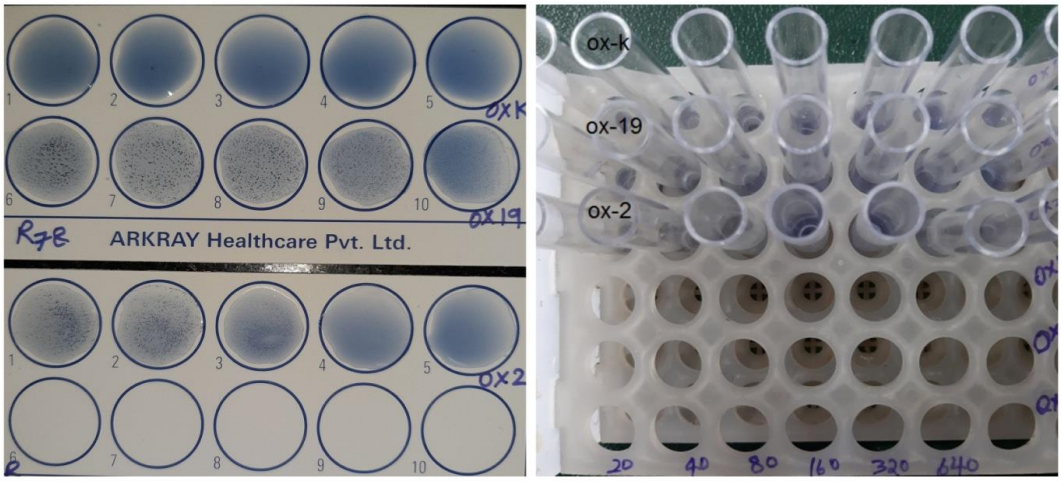

Fig. 1: Weil Felix Slide and Tube Agglutination Method

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All the 100 samples were subjected to Real-time PCR. A total of 7 samples tested positive in the Real-time PCR in the present study. This study considered a Ct Value of <40 positive for Real-time PCR. The amplification plot of one of the positive test samples (Ct value-29.55), which is almost comparable to the positive control used in the run, is shown in Fig. 2. These 7 Real-time PCR positive samples showed a titre of 1:80 or more for OX-19 and OX-2 combined.

Considering PCR results among those patients in whom Weil Felix test was positive (OX 19 and OX 2 >1:80), PCR positivity was 19% (7 out of 37 samples).

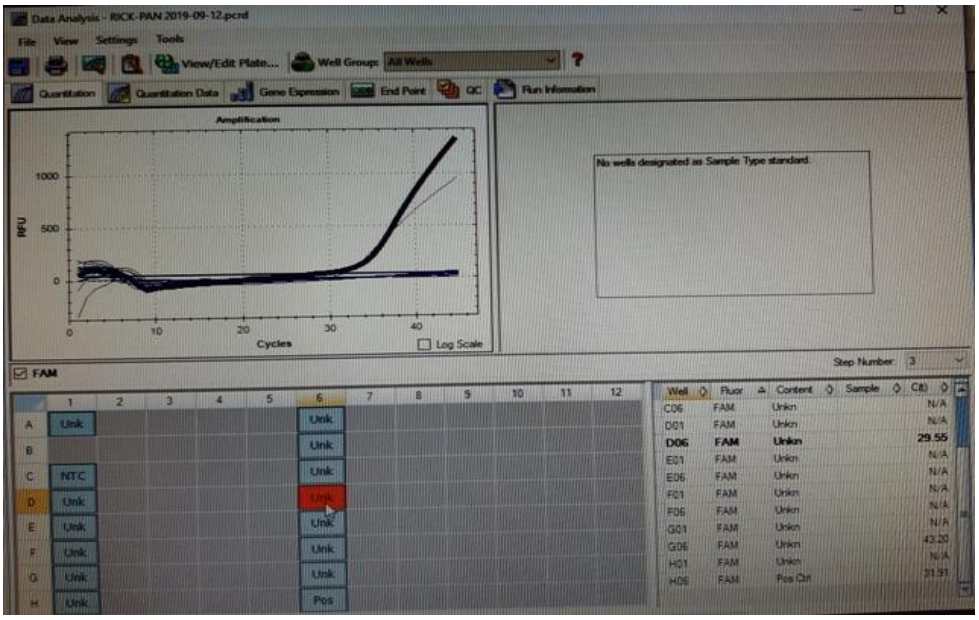

Fig. 2: Amplification plot of positive test sample along with positive control

DISCUSSION

Rickettsial infections are among the oldest and re-emerging infectious diseases [21]. These infections have been present in communities for centuries but have been undetected or incorrectly detected often because of low clinical suspicion, lack of proper diagnostic facilities or cross-reactivity among available serological tests [22].

Higher mortality rates have been associated with delayed identification and delayed antibiotic therapy. With correct and rapid identification, these infections respond tremendously to antibiotic therapy. Rapid identification of these infections is now possible with the development of group-specific (Pan R8) and species-specific (RRi6) real-time PCR, which gives results within 2 hrs of sample processing.

The majority (80%) of the study population was in the 0-20 year's age group. The study conducted by Nimboor et al showed 35.2% of patients in the age group below 13 years [23], a finding similar to our study. A study by Znazen *et al.* showed that ages 5 to 29 is the most commonly affected [24]. A survey conducted by Sanap et al showed a predominance of suspected cases in the age group below 10 years [25].

A mean age of 35 years was shown in one of the studies conducted by Sankar *et al.*[17]. Our study findings were consistent with the findings of other studies.

Fever and rash were the major symptoms documented during admission in this study. There were no patients with the findings of lymphadenopathy or eschar. The patients with suspected exposure to ticks either gave a history of exposure to shrubs, high grass, leaf litter, poultry or animal farming, mushroom farming, or contact with wet wood dumps. The clinical profile of the study population was observed to be in concordance with other studies. Weil Felix results, compared to other studies, have been compiled in our study. The results of the Weil Felix test for OX-19 and OX-2 combined in this study are comparable to studies done by Chunchanur *et al.*[12], Zaman *et al.*[26] and Schrader *et al.*[27] .

All the 100 samples collected were subjected to Pan R8 (group-specific) Real-time PCR for the final diagnosis. 7 samples tested positive in real-time PCR. Considering PCR results among those patients in whom Weil Felix test was positive (OX 19 and OX 2 >1:80), PCR positivity was 19% (7 out of 37 samples). The results of real-time PCR were available within 2 hrs of processing the sample [27,28].

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Among these 7 cases, all (100%) had complaints of fever, 5 (71.4%) had complaints of rash, 1(14.3%) had complaints of headache, 1(14.3%) had complaints of nausea/vomiting, 1 (14.3%) had a history of exposure to ticks, 3(42.8%) patients had hepatomegaly and 3(42.8%) patients had convulsions.

The real-time PCR result of the present study has been compared with the results of various other International and Indian studies. This study is the first to diagnose rickettsioses in clinically suspected cases of rickettsial fever using Panrickettsia (PanR8) real-time PCR in and around Bengaluru. The low positivity rate in our study could be due to PCR inhibitors in blood [28], late presentation to our hospital and delayed sample collection, loss of DNA due to prior antibiotic treatment or external factors, or gene polymorphism of targeted gene [29] in the organism.

This study highlights the role of the Weil Felix test (OX-19 and OX-2 combined) in detecting clinically suspected cases of rickettsial infections and its confirmation using Panrickettsia real-time PCR. The Panrickettsia real-time PCR improves the detection of rickettsial organisms by reducing the turnaround time to <2 hrs compared to 1-2 days for traditional nested PCR or DNA sequencing [30]. This allows clinicians to promptly start treatment of suspected rickettsial infections, reducing the chances of severe and fatal complications [31].

CONCLUSIONS

The study demonstrated the utility of both the Weil-Felix test and PanR8 real-time PCR for diagnosing rickettsial infections. The PanR8 real-time PCR improved diagnostic accuracy and reduced turnaround time, proving valuable in clinical settings. This study highlighted the prevalence of rickettsial infections in Bangalore, with significant findings supporting the use of PanR8 real-time PCR in conjunction with the Weil-Felix test for early and accurate diagnosis. The PCR technique's quick turnaround time enhances its value for clinical diagnosis, allowing prompt treatment to prevent severe complications. The study contributes to understanding rickettsial infections in South India, specifically in Bangalore, by establishing a diagnostic framework using the Weil-Felix test and PanR8 real-time PCR. It underscores the importance of rapid diagnosis to improve patient outcomes and decrease mortality rates in resource-limited settings.

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