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Standardization and Evaluation of an In-house Multiplex Real-time Polymerase Chain Reaction for Simultaneous Detection of Pathogenic Species of *Brucella*, *Rickettsia,* and *Leptospira* in Patients with Acute Febrile Illness

Nithiyanandan Saravanan[1], Prashanth Rajendiran[1], Sathish Sankar[1]*, Mageshbabu Ramamurthy[1], Kumaran Vadivel[1], Ranganathan Babujanarthanam[2], Mary V. Jesudason[3], George Varghese[4], Mercy John[4], Mathew Mazhavancheril[4], Rajarajeshwari Mangalakumar[5], Balaji Nandagopal[1], Gopalan Sridharan†[1]

Abstract

Background and Objectives: Acute febrile illness is caused by a wide range of etiologies. Brucella, Rickettsia and Leptospira as a cause of acute febrile illness have not been well studied. Identification by culture is laborious and diagnosis is often based on serological tests. We aimed to develop an in-house multiplex real-time PCR assay for the simultaneous detection of Brucella, Rickettsia and Leptospira and evaluate on samples collected from patients presenting with acute febrile illness. **Methods:** Samples (n=1101) were collected from patients presenting with acute febrile illness from different regions. An inhouse multiplex real-time PCR was developed for the specific detection of Brucella species, Rickettsia species and pathogenic species of Leptospira. The assay was evaluated on clinical samples. IgM ELISA was carried out on randomly selected samples (n=178). **Results:** The detection limit of the multiplex real-time PCR assay was 6.3, 43.7 and 1.2 genome copies per 10 µl of PCR input for Brucella, Rickettsia and Leptospira respectively. Among 1101 samples, Leptospira was positive in 1.36% samples and Rickettsia was positive in 0.36% samples. Among random samples, 37.1% of samples were positive for Brucella IgM, 19.6% were positive for Rickettsia IgM and 11.2% were positive for Leptospira IgM. **Interpretation and Conclusions:** The study showed a high seroprevalence of Brucellosis and Rickettsiosis among the samples. The in-house multiplex real-time PCR assay will be a useful tool in the syndromic diagnosis for a specific and comprehensive laboratory diagnostic testing.

Keywords: Acute febrile illness, *Brucella*, *Leptospira*, Multiplex real-time polymerase chain reaction, *Rickettsia*

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Introduction

Acute febrile illness is a common presentation and has a wide range of etiologies.[1] Differential diagnosis of acute febrile illness is difficult when it is based only on clinical features because most of the illnesses show non-specific symptoms such as the sudden onset of fever, headache, and malaise.[2] Pathogen-specific tests are expensive, and therefore, patients are often managed with empirical treatment resulting in unnecessary and/or overuse of antimicrobials thereby adding to a global burden of antimicrobial resistance.[3] The major bacterial cause of acute febrile illness and its prevalence remains poorly characterized in many parts of India. Emerging and reemerging bacterial infections, especially enteric fever, brucellosis, rickettsial infections, and leptospirosis as a cause of acute febrile illness, are often underestimated due to the non-availability of diagnostic resources in many health-care settings and expensive molecular assays. A syndromic diagnostic approach is needed for specific and comprehensive laboratory diagnostic assays.

Brucella, *Rickettsia,* and *Leptospira* are important groups of pathogens transmitted by animals causing mild-to-severe infections in humans.[4] These pathogens can be associated with significant morbidity and mortality especially in people with occupational risks such as animal exposure and dwelling in rural communities. Clinical presentation is very protean and often presents as an acute febrile illness with symptoms such as fever, malaise, and headache.

These pathogens are obligate intracellular bacteria, so a very low number of pathogens circulating in the blood and bacterial isolation from blood are difficult. Serological diagnosis is often

[1]Sri Narayani Hospital and Research Centre, Sri Sakthi Amma Institute of Biomedical Research, Vellore, Tamil Nadu, India
[2]Department of Biotechnology, Nano and Energy Biosciences Laboratory, Thiruvalluvar University, Vellore, Tamil Nadu, India
[3]Department of Microbiology, Sri Narayani Hospital and Research Centre, Vellore, Tamil Nadu, India
[4]Department of Microbiology, Pushpagiri Institute of Medical Sciences, Thiruvalla, Kerala, India
[5]Department of Microbiology, King Institute of Preventive Medicine and Research, Chennai, Tamil Nadu, India.
†Deceased February 24, 2019
Corresponding Authors: Dr. Sathish Sankar, Sri Sakthi Amma Institute of Biomedical Research, Sri Narayani Hospital and Research Centre, Sripuram, Vellore - 632 055, Tamil Nadu, India. Phone: +91-416-2206335, Email: sathish3107@gmail.com/Dr. Ranganathan Babujanarthanam, Nano and Energy Biosciences Laboratory, Department of Biotechnology, Thiruvalluvar University, Serkadu, Vellore - 632 115, Tamil Nadu, India. Phone: +91-9787919191. Email: babukmg@gmail.com

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relied on, although it suffers because of low sensitivity and specificity and cross-reactions between species. This complicates correct diagnosis and delay treatment thus increasing morbidity and mortality. Because an effective treatment regimen is available for these infections, a clinically relevant specific identification of these pathogens using molecular techniques is warranted. In this study, we developed an in-house multiplex real-time polymerase chain reaction (PCR) assay for the simultaneous detection of pathogenic species of *Brucella*, *Leptospira,* and *Rickettsia* from clinical samples. The assay was evaluated for the detection of the three pathogenic groups in patients with acute febrile illness as a syndromic diagnosis.

Materials and Methods

Subject Selection

In this cross-sectional study, blood samples were collected from patients of all age groups presenting with acute febrile illness [Table 1]. The primary study center, Sri Narayani Hospital and Research Centre (SNHRC) is a 300-bed multispecialty hospital serving in the rural area of the Vellore district. The samples from SNHRC (n = 536) were collected during the period from May 2017 to November 2018. In addition, we collected samples from three other centers. An upgraded Primary Health Centre (PHC, Ussoor) at a nearby rural area of Vellore district caters to patients from rural and tribal areas of Vellore district; 184 samples were obtained during the period of mid-August 2018 to mid-November 2018 from here. The required permission to collect samples from PHC was obtained through the Department of Public Health, Vellore (District office), and Directorate of Public Health and Preventive Medicine, Chennai (Head office). King Institute of Preventive Medicine and Research, Guindy, Chennai, is a medical research institute and reference center. A total of 120 archived serum samples collected from patients with acute febrile illness at different periods were obtained for our study. Pushpagiri Institute of Medical Sciences [PIMS], Thiruvalla, Kerala, is a tertiary care teaching hospital. Samples (n = 261) collected as part of their routine investigation of patients with acute febrile illness were used for our study. To note, the samples from PIMS were collected during the monsoon season and post-monsoon floods (July 2018–November 2018) in Kerala. The month-wise distribution of cases of acute febrile illness is shown in Figure 1. Samples were collected after obtaining the consent or assent from the patient or guardian, respectively, and clinical pro forma was filled in. Ethical approval from the Institutional Review Board had been obtained for the study.

Table 1: Samples tested from four different study centers

Study centers	*Samples collected (n)*	*Nature of sample*
Sri Narayani Hospital and Research Centre	536	Whole blood*
Upgraded PHC, Ussoor	184	Whole blood*
King Institute of Preventive Medicine and Research	120	Serum
Pushpagiri Institute of Medical Sciences	261	Serum

*Buffy coat was separated from the whole blood and used for PCR

Sample Processing

From SNHRC and PHC centers, venous blood was collected in two parts, one for blood culture in the BacT-Alert system and the other was collected in a Vacutainer containing EDTA. Buffy coat was separated from the blood as described previously.[5] Positive BacT-Alert system culture bottles were processed for bacterial identification and antimicrobial susceptibility testing using standard methods.[6] Buffy coat was suspended in phosphate buffer saline (pH 7.3) and plasma samples were stored at –20°C until use. In KIPM and PIMS centers, only serum was collected. DNA was extracted from buffy coat/serum using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Multiplex Real-time PCR

An in-house multiplex real-time PCR was developed to detect pathogenic species of *Brucella*, *Rickettsia,* and *Leptospira* on samples collected from patients presenting with acute febrile illness. The target genes were selected based on the literature that has demonstrated high sensitivity and specificity in clinical samples. The multiplex real-time PCR cocktail contained primers and probes specific for *Brucella*, *Rickettsia,* and *Leptospira* [Table 2]. The primers and probes were designed using IDT oligo design software and the sequences were cross-checked by BLAST analysis for specificity with respective pathogens. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal amplification control. DNA constructs (250 bp) spanning the amplification region were commercially synthesized (Eurofins, India) for all three pathogens. These constructs were used to establish a lower limit of detection as well as to serve as positive control templates for all real-time PCR assays.

The plasmid constructs were serially diluted 10-fold in TE buffer (pH 8.0) in the concentration range of 10^{-1}–10^{-12}. Each dilution was tested in triplicates by real-time PCR. Appropriate negative controls were used replacing the template with water and included as every third sample. The PCR runs were validated only if the controls were satisfactory. Amplification shown in the highest dilution (least concentration) in at least two of the triplicates tested at each dilution was taken as the lower limit of detection as genome copies per microliter. The calculation for the plasmid copy number for the pathogens tested was according to standard methods as described previously.[5]

The real-time multiplex PCR amplification was performed as a 25 µl reaction using 1x QuantiTect Multiplex PCR NoROX master mix (Qiagen, Hamburg, Germany). DNA extract (10 µl), forward and reverse primers (1.5 pmol each), and probes (0.5 pmol each) were used with initial 15 min 95°C activation step followed by 2-step cycling of denaturation at 94°C for 60 s and annealing/extension at 60°C for 60 s (50 cycles). Appropriate negative controls replacing the template with water were included in every third sample. The cutoff for real-time PCR endpoints was determined as amplification within the 40th cycle with a typical sigmoid amplification curve as described previously.[7]

Test for IgM

To determine the prevalence of IgM for *Brucella*, *Rickettsia,* and *Leptospira* on the samples, 178 samples were randomly selected using statistical methods. The samples collected from SNHRC,

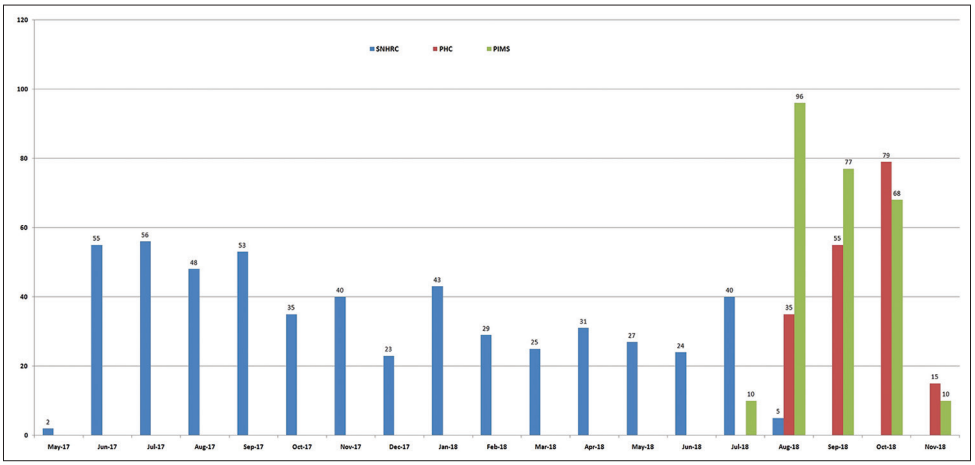

Figure 1: Month-wise distribution of acute febrile illness cases

Table 2: List of primers and probes used in the study

Pathogens	*Forward primer*^	*Reverse primer*^	*Probe*^
Brucella species	CAGTCAGACGTTGCCTATTG	GCGACCGATTTGATGTTTG	6-FAM-AAATCTTCCACCTTGCCCTTG-BHQ1
Rickettsia species	ACCGTCGCAAATGTTCAC	TTTGTTCAGGGTCTTCGTG	HEX-TCTTTCCATTGTGCCATCCAG-BHQ1
Leptospira species	TGGAGACTTAGTAAGCGACG	TTTGGCGATTTGGTCAGG	CY5-TTGGTTTGATACTTGGATCCGTG-BHQ2
GAPDH#	GGAAACTGTGGCGTGATG	CGTTCAGCTCAGGGATG	TexasRed-AGTAGAGGCAGGGATGATGTT-BHQ2

#Internal amplification control. ^The oligonucleotide sequences are given as 5' to 3' position. FAM, HEX, Cy5, and Texas red are the fluorescent dyes; BHQ1 and 2 are quencher dyes. The target gene, nucleotide position, and the GenBank accession number for each pathogen are given below: *Brucella: Brucella* BCSP31 gene encoding a 31-KDa cell surface protein; NCBI accession No. M20404; nucleotide position: 847–992. *Rickettsia: Rickettsia raoultii* strain IM16 genome gltA gene encoding citrate (Si) synthase; NCBI accession No. CP019435; region: 1285902.1287209; nucleotide position: 72–163. *Leptospira: Leptospira interrogans* serovar Grippotyphosa strain RTCC2808 LipI32 (lipl32) gene encoding outer membrane protein; NCBI accession No. JN886738; nucleotide position: 327–440. GAPDH: Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 3, mRNA; NCBI accession No. NM_001289745.2; nucleotide position: 746–844

KIPM, and PIMS were included for the random table generation. Samples from PHC were not included. The IgM prevalence was studied on these samples using commercial IgM ELISA assay for *Brucella* (NovaTec Immundiagnostica, GmbH, Germany), *Rickettsia* – typhus group (TG) (Fuller Laboratories, Fullerton, California, USA), *Rickettsia* – spotted fever group (SFG) (Fuller Laboratories, Fullerton, California, USA), and *Leptospira* (Panbio diagnostics, Gyeonggi-do, Republic of Korea). The assays were carried out according to the manufacturers' instructions and all the kits are CE certified. IgM ELISA assay kits for *Brucella*, *Rickettsia* SFG, and *Rickettsia* TG contained anti-IgG (RF-adsorbent) in the sample dilution buffer for the specific detection of IgM class antibody. IgM ELISA kits for *Leptospira* did not contain RF-adsorbent.

The results of *Brucella* IgM assay were expressed in NovaTec units (NTUs). A titer of 11 NTU or more was considered positive. Samples with equivocal titers (9–11 NTU) were not considered. The results of TG and SFG of *Rickettsia* were expressed as index values. A value of more than 1.1 was considered positive, samples with equivocal titers (0.9–1.1) were not considered. Test results for *Leptospira* IgM were expressed as Panbio units. A unit of more than 11 was considered positive and samples with equivocal titers (9–11) were not considered.

Statistical Analysis

All the data were entered into an MS-Excel spreadsheet and used for statistical analysis. Mid-*P* values at 95% confidence interval, Fisher's exact test, Chi-square test, and test for significance were calculated using Epi Info 7 statistical program (CDC, USA) and Social Science Statistics (https://www.socscistatistics.com/). $P < 0.05$ was considered statistically significant.

Results

Patients presenting with acute febrile illness were recruited for our study during the period from May 2017 to November 2018. Among the patients recruited from SNHRC (n = 536), males were 340 and females were 196. The age ranged from 12 days to 91 years (median = 45). Among the patients recruited from PHC (n = 184), males were 80 and females were 104. The age ranged from 2 to 77 years (median = 25). Among the patients recruited from PIMS (n = 261), males were 162 and females were 96 (data not available for 3), age ranged from 1 to 85 (median = 43.5) years. Among 1101 patients, 451 patients had a moderate fever with 100°F–102°F; 68 patients had high-grade fever with temperature more than 102°F, and 562 patients had a mild fever (<100°F) at the time of presentation (data for others are not available). The majority of them had an undulating fever (88.5%) and a few had prolonged fever (8.5%). Appearance of rashes (erythematous/macular/maculopapular/purpuric) was recorded in 13 (1.2%) patients.

Out of 536 samples collected from SNHRC, 106 samples were positive for blood culture. The majority were coagulase-negative *Staphylococcus* (n = 55) followed by *Escherichia coli* (n = 16) and *Klebsiella* species (n=6).

The in-house real-time PCR assay had a detection limit of 6.3, 43.7, and 1.2 genome copies per 10 µl of PCR input for *Brucella*, *Rickettsia,* and *Leptospira,* respectively. This indicated the high assay sensitivity of the real-time PCR assay in terms of a lower limit

of detection. The positive findings of multiplex real-time PCR assay were concordant with the results of the repeat testing as uniplex assays. The samples that were positive for multiplex real-time PCR but negative on repeat testing in uniplex testing were considered negative for the pathogen. Among 1101 samples tested by multiplex real-time PCR, *Leptospira* was positive in 15 (1.36%) samples, *Rickettsia* was positive in 4 (0.36%) samples, *Brucella* was positive in 1 (0.1%) sample. The real-time PCR amplification curve for a sample positive for *Leptospira* is shown in Figure 2. The real-time PCR positive samples for either of the three pathogens were all negative in blood culture. The distribution of positive cases among different samples is shown in Table 3.

Among the 178 random samples tested by IgM ELISA, 66 (37.08%) samples were positive for *Brucella*, 42 (23.59%) samples were positive for *Rickettsia,* and 20 (11.23%) samples were positive for *Leptospira*. The number of positives by IgM ELISA is shown in Table 4. The number of IgM positives for the TG was higher compared to the SFG of *Rickettsia*. Seven patients were positive for both TG and SFG *Rickettsia* IgM. Close contact with animals (canine, feline, cattle, and poultry) was recorded in eight and nine patients among TG and SFG *Rickettsia* IgM positives, respectively.

Apparent symptoms such as the sudden onset of fever, rigors, myalgias, and headache were observed in all IgM-positive cases. Gastrointestinal symptoms that include nausea, vomiting, diarrhea, and abdominal pain were observed in a majority of the patients with IgM positives. Typical clinical presentation of *Leptospira* such as conjunctival suffusion though observed in three patients, none were seen in patients positive by either real-time PCR or IgM ELISA. Among 17 *Leptospira* IgM positives, four had close contact with animals (canine and cattle). The clinical presentation among IgM positives for the three pathogens is shown in Table 5. *Brucella* IgM-positive cases were seen in all age groups and indicate that all age groups are affected. Close contact with animals was recorded in 20 patients (canine, feline, and poultry) out of 66 positives. Three had prolonged fever and 56 had an undulating or relapsing fever (data for five patients were not available). IgM positives for *Leptospira* and *Rickettsia* SFG were seen in all age groups except septuagenarians. TG of *Rickettsia* was higher in the age group of 31–40 years of age. No statistical difference was observed among different age groups who were IgM positives (χ^2 = 8.67, P = 0.73). The age-wise distribution of IgM-positive cases is shown in Figure 3.

Details on the duration of fever were analyzed among IgM positives. A higher number of patients with IgM positives had a fever for 4–7 days but the difference in the fever duration with relation to IgM positive rates was not statistically significant (χ^2 = 0.84, P = 0.36). The difference between males and females among *Brucella* IgM positives was not statistically significant (χ^2 = 2.89, P = 0.41). Similarly, no significant difference was observed between males and females among IgM positives of *Leptospira* (χ^2 = 0.21, P = 0.64), *Rickettsia*-TG (χ^2 = 0.48, P = 0.48), and *Rickettsia*-SFG (χ^2 = 0.33, P = 0.55).

The analysis on the distribution of IgM-positive cases within the study period showed a high seroprevalence of brucellosis

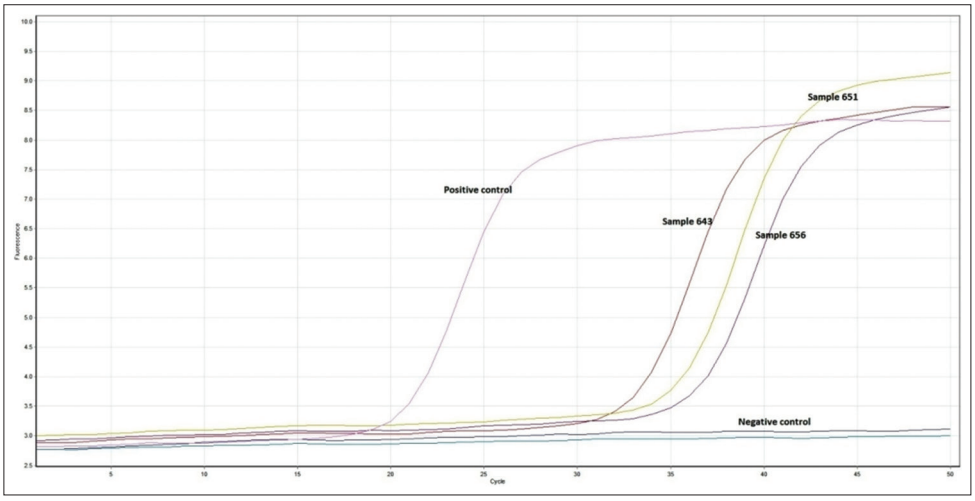

Figure 2: The real-time polymerase chain reaction amplification curve for a sample positive for *Leptospira*

Table 3: Number of pathogens positive by PCR on samples collected from four different centers (SNHRC, PHC, KIPM, and PIMS)

Study centers	*Number of positives by PCR n (%) [Mid-P, 95% CI]*		
	Brucella	*Rickettsia*	*Leptospira*
Sri Narayani Hospital and Research Centre (*n*=536)	-	3 (0.56) [0.14–1.5]	1 (0.18) [0.009–0.91]
Upgraded Primary Health Centre, Ussoor (*n*=184)	-	1 (0.54) [0.03–2.6]	-
King Institute of Preventive Medicine and Research (*n*=120)	1 (0.83) [0.04–4.04]	-	-
Pushpagiri Institute of Medical Sciences (*n*=261)	-	-	14 (5.36) [3.1–8.6]

Table 4: Number of positives by IgM ELISA on random samples (*n*=178) generated from three different centers (SNHRC, KIPM, and PIMS)

Study centers	*Number of positives by IgM ELISA n (%) [Mid-P, 95% CI]*			
	Brucella	*Rickettsia (typhus group)*	*Rickettsia (spotted fever group)*	*Leptospira*
Sri Narayani Hospital and Research Centre (*n*=143)	64 (44.75) [36.75–52.97]	25 (17.48) [11.91–24.37]	16 (11.18) [6.76–17.77]	17 (11.88) [7.32–17.99]
King Institute of Preventive Medicine and Research (*n*=27)	2 (7.41) [1.26–22.38]	-	1 (3.7) [0.18–16.94]	1 (3.7) [0.18–16.94]
Pushpagiri Institute of Medical Sciences (*n*=8)	-	-	-	2 (25) [4.42–61.17]

Table 5: The clinical presentation among IgM positives

Descriptions		*Number of IgM positives*			
		Brucella (n=66)	*Leptospira (n=20)*	*Rickettsia TG (n=25)*	*Rickettsia SFG (n=17)*
Age groups (years)	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
0–10	16 (8.98)	6 (10)	1 (5)	1 (4)	3 (17.64)
11–20	13 (7.30)	3 (5)	1 (5)	2 (8)	3 (17.64)
21–40	37 (20.78)	20 (33.33)	4 (20)	8 (32)	3 (17.64)
41–60	51 (28.65)	18 (30.0)	5 (25)	6 (24)	3 (17.64)
>60	55 (30.89)	18 (30.0)	8 (40)	8 (32)	5 (29.41)
Age median		45	56	50	40
Gender					
Male	117 (65.73)	41 (62.12)	15 (75)	15 (60)	10 (58.82)
Female	55 (30.89)	24 (36.36)	5 (25)	10 (40)	7 (41.17)
Fever temperature (°F)					
<100	63 (35.39)	28 (42.42)	7 (35)	13 (52)	8 (47.05)
100–102	66 (37.08)	26 (39.39)	8 (40)	10 (40)	5 (29.41)
103–105	9 (5.06	5 (7.57)	1 (5)	0	2 (11.76)
Fever duration					
<3 days	66 (37.08)	24 (36.36)	5 (25)	9 (36)	5 (29.41)
4–7 days	59 (33.15)	29 (43.94)	10 (50)	12 (48)	9 (52.94)
8–14 days	7 (3.93)	4 (6.06)	6 (30)	1 (4)	4 (23.52)
More than 15	6 (3.37)	2 (0.03)	1 (5)	2 (8)	1 (5.88)
Unknown/not recorded	40 (22.47)	6 (9.09)	1 (5)	1 (4)	1 (5.88)
Clinical presentations					
Myalgia	79 (44.38)	31 (46.97)	11 (55)	15 (60)	9 (52.94)
Gastrointestinal symptoms	73 (41.01)	20 (30.30)	7 (35)	8 (32)	9 (52.94)
Cough	64 (35.95)	22 (33.33)	10 (50)	10 (40)	6 (35.29)
Chills	44 (24.72)	20 (30.30)	4 (20)	4 (16)	5 (29.41)
Headache	40 (22.47)	19 (28.79)	7 (35)	7 (28)	7 (41.17)
Rigor	8 (4.49)	6 (9.09)	0	0	2 (11.76)
Renal insufficiency	36 (20.22)	7 (10.60)	1 (5)	4 (16)	3 (17.64)
Breathing difficulty	35 (19.66)	17 (25.76)	4 (20)	9 (36)	2 (11.76)
Loss of appetite	14 (7.86)	5 (7.57)	2 (10)	1 (4)	0
Arthralgia	5 (2.81)	2 (0.03)	0	1 (4)	0
Profuse sweat	4 (2.25)	1 (1.51)	0	0	0
Rash	3 (1.68)	2 (0.03)	0	0	1 (5.88)
Hematuria	2 (1.12)	1 (1.51)	0	0	0
Conjunctival suffusion	1 (0.56)	1 (1.51)	0	0	0
Pulmonary edema	1 (0.56)	1 (1.51)	0	0	0
Pedal edema	1 (0.56)	0	0	1 (4)	0
Retro-orbital pain	1 (0.56)	1 (1.51)	0	0	1 (5.88)
Petechia	1 (0.56)	1 (1.51)	0	0	1 (5.88)
Eschar	1 (0.56)	0	0	0	0

and rickettsiosis among the samples collected from patients between June and January followed by a considerable decline till June of the following year [Figure 4]. The IgM seroprevalence of leptospirosis was variable during the entire study period. Among the 178 samples, 20 samples were positive for *Leptospira* IgM. Of these, one sample was positive by both real-time PCR and IgM ELISA; three samples were positive only by real-time PCR and 19 samples were positive only for IgM. *Brucella* was positive in one sample by real-time PCR and 66 samples were positive for IgM. The one real-time PCR-positive sample was negative by IgM ELISA. Four samples were positive for *Rickettsia* by real-time PCR and 35 samples were positive for any of the two groups of *Rickettsia* (TG and SFG) IgM. None of the samples was positive by both PCR and IgM ELISA.

Discussion

Brucellosis, rickettsiosis, and leptospirosis are important emerging public health problems but are highly underreported in India. A multiplex real-time PCR assay was developed for the simultaneous detection of *Brucella*, *Rickettsia,* and *Leptospira* and evaluated in patients with acute febrile illness. We found the

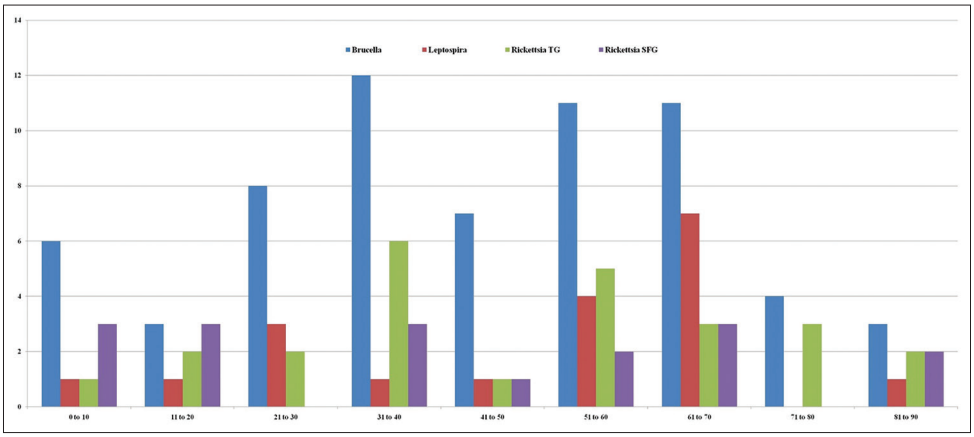

Figure 3: Age-wise distribution of IgM positives among acute febrile illness cases

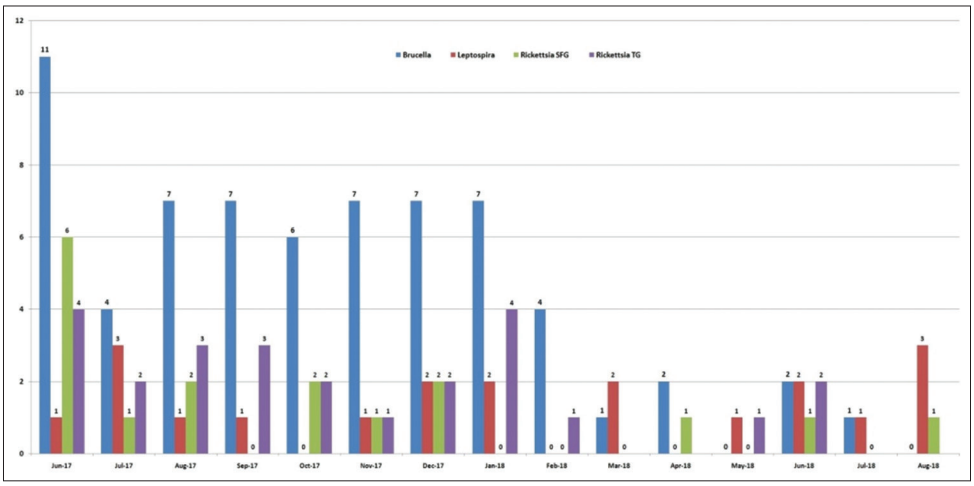

Figure 4: Month-wise distribution of IgM positives among acute febrile illness cases

prevalence of the three pathogens in less than 2% of the samples tested. We found a high seroprevalence of IgM for *Brucella* (37.1%) followed by *Rickettsia* (23.6%) and *Leptospira* (11.2%).

Brucellosis is a significant public health burden in India causing a socioeconomic loss for livestock species and humans. In an estimate on the economic losses and health impact of brucellosis in India, a median loss of 220 billion rupees in livestock[8] and the annual median losses due to human brucellosis were estimated to be 627.5 million rupees.[9] In the high-risk group of patients, occupational exposure or consumption of unpasteurized milk was the major risk factor.[10-12] In the nearby state of Karnataka, an overall prevalence of brucellosis among veterinary health care workers was 7.04%. The prominent clinical symptoms observed were intermittent fever (71.62%) followed by joint pain and body aches.[13] Brucellosis as a cause of febrile illness has been investigated by culture, serum agglutination test, and ELISA. About 3–5% of the samples were positive by culture, agglutination, and in-house indirect ELISA, but none of them was positive by IgM.[14] Compared to blood cultures and serum agglutination, IgM and IgG ELISA be promising in the diagnosis of *Brucella*.[15]

In our study, the samples (n = 1101) were subjected to real-time PCR and randomly generated samples (n = 178) were subjected to IgM testing by commercial IgM ELISA. Real-time PCR was positive in one sample and IgM was positive in 66 samples (37.1%) by IgM ELISA. The accuracy indices of the assay could not be established due to low positivity by real-time PCR assay. The one sample positive by PCR could be not detected by ELISA and may indicate recent infection or maybe carrying too low a bacteremia to produce detectable antibodies. The 66 samples positive by IgM ELISA could not be detected positive by PCR and this may indicate a recent infection with too low a bacterial load. The buffy coat samples are a suitable clinical specimen for the detection of *Brucella*.[16,17] We earlier showed the detection of *Brucella* (1%) as a cause of acute febrile illness using conventional PCR targeting the omp2 gene from DNA extracted from buffy coat samples.[18] In this study, we chose to amplify the BCSP31 gene that codes for 31-kDa cell surface protein amenable to the multiplex real-time PCR format from buffy coat samples. Our in-house developed real-time PCR assay was able to detect <10 genome copies for a PCR reaction indicating a high assay sensitivity and the target primer-probe sequences were genus-specific indicating high assay specificity. The BCSP31 target gene has been shown previously to be highly genus-specific among many other target genes[19-21] and suitable for molecular detection of *Brucella* species in clinical specimens.

Reports from different parts of the country indicate a remarkable increase in the incidence of rickettsial infections warranting timely diagnosis and treatment.[22,23] Rickettsial infections often present with petechial rashes after the onset of

fever. However, skin rashes sometimes appear late or invisible in patients with dark skin.[24,25] In a seroepidemiological study in the northeast part of India, antibodies to SFG of rickettsiae and TG of rickettsiae were found in 14 and 4.2% of individuals, respectively.[26] In a recent seroepidemiological study in North-Central India, a prevalence of 26% by both ELISA and gold standard immunofluorescence assay was reported. Eschar was reported in only 3% of the positive cases.[27]

In our study, we found 4 (0.36%) samples positive for *Rickettsia* among 1101 samples by genus-specific real-time PCR that amplifies all rickettsial species. The IgM ELISA each specific to the SFG and TG was individually tested with randomly generated 178 samples. A total of 17 (9.44%) were positive for IgM for *Rickettsia*-SFG and 25 (14%) were positive for IgM for *Rickettsia*-TG and nine showed positive for both the groups. The results of PCR did not corroborate with the IgM ELISA and may indicate a low bacterial load. Among the different target genes, gltA citrate synthase gene is highly useful for the specific detection of typhus and SFG of rickettsiae,[28,29] and buffy coat was reported as a highly suitable specimen for the detection of *Rickettsia*.[30] The genus-specific real-time PCR assay for *Rickettsia* was able to detect less than 45 genome copies for a PCR reaction.

The genus *Leptospira* includes pathogenic species including *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira santarosai*, and *Leptospira weilii*, as well as non-pathogenic such as *Leptospira biflexa* and *Leptospira meyeri*, and opportunistic pathogens such as *Leptospira broomii*, *Leptospira fainei,* and *Leptospira inadai*. Identification of pathogenic *Leptospira* species from clinical samples is highly desirable for appropriate patient management. Among the outer membrane proteins of *Leptospira*, LipI32 is abundant and highly conserved among pathogenic species.[31] LipI32 is a highly reliable target for PCR assays to detect pathogenic *Leptospira* on clinical samples.[32,33] In our study, the real-time PCR assay targeting LipI32 was able to detect down to 15 genome copies per PCR reaction indicating a very good assay sensitivity. The positive findings of multiplex real-time PCR assay were concordant with the results of the repeat testing as uniplex assays. A total of 15 (1.36%) samples were positive by real-time PCR and 20 (11.2%) samples were positive by IgM ELISA. Among the 1101 samples, one sample collected from Vellore was positive and 14 samples collected from Kerala were positive indicating a high prevalence of leptospirosis in the state of Kerala. The samples from Kerala were collected during the monsoon and post-monsoon floods, while the samples from Vellore and Chennai were collected over different periods.

Although a decreasing seroprevalence of leptospirosis has been reported in North India based on IgM testing with modified Faine's criteria,[34] a high proportion of cases has been reported in several parts of the country.[35,36] Leptospirosis along with scrub typhus is an important etiology of acute febrile illness in many parts of India.[37,38] In a country-wide multicenter study on 1564 febrile illness patients, leptospirosis was identified in 7% of samples with marked high incidence in North and North-East India.[39] In our study, of 1101 samples tested by real-time PCR, *Leptospira* was predominant and positive in 15 (1.36%) samples followed by *Rickettsia* (0.36%) and *Brucella* (0.1%). Among the 178 random samples tested by IgM ELISA, *Brucella* was predominant with 66 (37%) samples positive followed by *Rickettsia* (17.48%) and *Leptospira* (11.23%).

Details on the duration of fever until sample collection were analyzed among IgM positives. The number of positives was higher in patients with <3 days of fever and gradually decline with increasing days of fever. This indicates that the IgM ELISA is useful as a diagnostic tool during the acute phase of the illness. Patients who were positive for IgM with fever more than 2 weeks of illness could be due to the prolonged persistence of IgM over an extended period.

Five patient samples showed IgM positivity for both *Leptospira* and *Rickettsia*; 15 were positive for both *Rickettsia* and *Brucella;* and five patients showed IgM for both *Leptospira* and *Brucella*. Three patient samples showed IgM positivity for *Leptospira* along with the two groups of *Rickettsia* and six patient samples showed IgM positivity for *Brucella* with two groups of *Rickettsia*. This indicates concurrent infections of these pathogens in patients with acute febrile illness. The study warrants the use of multiplex real-time PCR assay for the detection of these three important pathogens to be considered as part of the acute febrile illness etiologies.

In our study, we developed an in-house multiplex real-time PCR assay to detect seven bacterial agents in three tubes in blood samples collected from patients presenting with acute febrile illness. The study showed a high prevalence of brucellosis and leptospirosis. The study confirms the usefulness of the multiplex real-time PCR assay as a routine laboratory test for rapid and specific detection of the pathogens and therefore suitable for inclusion as a part of comprehensive workup to detect infections.

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