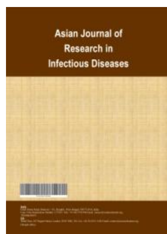

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Usefulness of Nested PCR Assay for the Molecular Diagnosis of Human Rickettsial Infection: A Study in Bangladesh

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Authors' contributions

This work was carried out in collaboration among all authors. Author ST performed Conceptualization, Methodology, Validation, Supervision, Writing- reviewing and Editing, Investigation, and software of the manuscript. Author MB helped in Conceptualization, Methodology, Validation, writing- reviewing and Editing, Investigation, and software the manuscript. All authors have contribution in Manuscript writing. All authors read and approved the final manuscript.

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ABSTRACT

Background: Rickettsial infections are re-emerging arthropods born worldwide zoonotic disease caused by Rickettsia, which is responsible for spotted fever and typhus fever. The diagnosis of a rickettsial illness is important for appropriate antibiotic treatment.
Aims: The study aimed to determine the diagnostic accuracy and clinical usefulness of using nested polymerase chain reaction (PCR) by comparing nested PCR, ELISA, and Weil-Felix (WF) tests.

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Methodology: This was a prospective type of cross-sectional study. A total of 135 clinically suspected rickettsial infection cases were enrolled. Peripheral blood was taken to detect gltA, 17 kDa lipoprotein antigen gene (17 kDa), ompA, and ompB gene of Rickettsia by nested PCR. ELISA and Weil-Felix tests were done to compare with nested PCR.
Results: Out of 135 cases, we detected Rickettsia in 70(51.85%) cases by nested PCR assay ($p<0.01$), 33((24.4%) by Weil- Felix test, 34 (25.18%) by ELISA. Only 26.66% of cases were PCR positive, which were negative by both ELISA and Weil-Felix test. Fifteen (11.11%) cases were positive by all three tests. Among 70 PCR positive rickettsia cases most frequently detected gene was ompB 42(60%), followed by 17kDa 34(48.58%); gltA 21(30%), and ompA 3(4.28%). Multiple gene combinations (ompB, 17kDa and gltA) detected in 98.57 % cases.
Conclusion: Nested PCR assays showed the highest rate of detection of rickettsia cases than ELISA and Weil-Felix test. Multiple gene combinations (ompB, 17kDa, and gltA) showed the highest positivity. Therefore, diagnosis of rickettsial infection can be confirmed by PCR assay, and clinicians can plan appropriate treatment for these patients.

Keywords: Rickettsia; nested PCR; ELISA; weil felix test; rickettsial genes.

1. INTRODUCTION

Rickettsial infections are re-emerging arthropod born worldwide zoonosis caused by the genus Rickettsia in the order Rickettsiales. Rickettsia is responsible for spotted fever and typhus fever, having a predilection for vascular endothelium. The family Rickettsiaceae comprises of two genera Rickettsia and Orientia [1]. Rickettsiae are tiny obligate intracellular Gram-negative bacteria in the host cell cytoplasm, transmitted by arthropod vectors such as ticks, fleas, mites, and lice [2].

A modern classification based on whole-genome analysis divides the species of the genus Rickettsia into four groups: spotted fever group (*R. rickettsii*, *R. conorii, R. parkeri*, and several others,) typhus group (*R. prowazekii and R. typhi*), ancestral group (*R. bellii and R. canadensis*, not known to be pathogenic), and transitional group (*R. akari, R. australis, and R. felis*) [3]. Acute rickettsial infection generally presents with non-specific flu-like symptoms [4]. More characteristic symptoms are a rash and an inoculation eschar but are not always observed [5]. The diagnosis of a rickettsial illness is essential for appropriate antibiotic treatment to be given promptly. Traditionally, the diagnosis of a rickettsial disease has been based on serological tests. Weil-Felix assay cheap and widely available serological method. Of the serological tests, the indirect Immunofluorescence assays (IFA) are the gold standard for detecting IgM and IgG antibodies level against rickettsiae. Still, at least two weeks apart, they require an acute and convalescent-phase sample of blood to make a definite diagnosis [5-7]. ELISA (Enzyme-linked immunosorbent assay) is more sensitive, faster than the IFA for detecting the low level of antibody [8] and many serum samples can be tested simultaneously, and results can be processed and recorded directly into a computerized database [9]. Culture techniques can be used for diagnosis and are very sensitive but restricted to reference laboratories because it is a potentially hazardous and technically demanding method that must be performed at a biosafety level 3 and requires staff capable of maintaining living host cells (animal mouse models or embryonated eggs) or cell cultures (Vero, L929, HEL, XTC-2, or MRC5 cells) [10,11]. PCR assays can be very useful because the infection can be detected before seroconversion or positive culture has occurred [12]. Modifications of the PCR technique led to its improvement and development of the nested PCR (nPCR) technique, which increased the sensitivity of PCR down to the level of detecting 1–10 genomic equivalents per reaction [13]. The five genes usually targeted by PCR for detection and diagnosis are citrate synthase *glt*A*,* gene D *sca*4, the 17kDa lipoprotein precursor antigen gene 17kDa, and genes for outer membrane proteins A and B *omp*A and *omp*B [14]. The combination of three sequential PCR assays (*omp*A, *omp*B, and *glt*A) achieved 100% sensitivity [15]. Detection of rickettsial DNA in a clinical specimen via amplification of a specific target by PCR assay (considered as laboratory-confirmed) along with clinical evidence of fever and one or more of the following: rash, eschar, headache, myalgia, anemia, thrombocytopenia, or any hepatic transaminase elevation should be considered as confirmed case of rickettsial fever [16].

So this study was designed to diagnose clinically suspected cases of rickettsial infection precisely to help the clinician plan appropriate antibiotic treatment to prevent complications and mortality.

2. MATERIALS AND METHODS

2.1 Patient Selection and Test Strategy

We conducted a prospective type of cross-sectional study on patients suffering from a possible rickettsial infection. We enrolled clinically suspected cases of rickettsial fever (fever ≥10 days along with one or more of the following: rash, eschar, headache, myalgia) attending the department of Internal Medicine, Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Medical College Hospital (DMCH). This study was carried out from a period of March 2014 to February 2015. The study was approved by the institutional review board (IRB) of Bangabandhu Sheikh Mujib Medical University (BSMMU). All study subjects provided informed written consent for research and publication. Peripheral blood samples were collected for nested PCR, Weil-Felix test, and ELISA. Laboratory works were performed in the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Shahbagh, Dhaka.

2.2 Weil-Felix

Weil-Felix tests were performed for agglutination assay to see the anti rickettsial IgM antibody, which cross-reacts with the *Proteus vulgaris* OX-19 and OX-2 and *P. mirabilis* OX-K strains (PROGEN, Proteus antigen suspension for Weil-Felix test, 058-83/1110/AE/VER-01, Tulip Diagnostic (P) Ltd.Goa-403 722, India). A single Weil-felix titer 1:80 was considered a positive result.

2.3 ELISA

ELISA were performed for IgM using ELISA kit (Delta Biological S.R.L.via Nicaragua 12-14 00040 Pomezia Italy, DBE-043, 13, eric 101-z). Optical densities (OD) of each microtiter well were measured using an ELISA plate spectrophotometer with a 450 nm measuring filter and a 650 nm reference filter. The result was measured by calculating the antibody index [AI=Sample O.D/ Cut off serum mean O.D) X 10]. Antibody index >11 was interpreted as a positive result (Interpretation as per kit). The mean cut of O.D was 1which is used for measuring antibody index.

2.4 Nested PCR Assay

Whole blood samples were used for the nested PCR. DNA was extracted by using Qiagen DNA extraction kit from EDTA blood (QIA amp DNA Mini and Blood Mini Handbook, April 2010). Nested PCR assay was performed using specific primers targeting genes of citrate synthase (gltA), 17 kDa protein (htrA), outer membrane protein A (ompA), and external membrane protein B (ompB). The test was done as described previously by Furuya and others [17] with some modification. Details about PCR primer pairs and the size of the amplicons (bp) are shown in Table 1.

Table 1. Details of PCR primer pairs, size of the amplicons (bp)

Gene target	Primer name	Primer sequence (5'–3')	Amplified fragment(bp)
GltA	F R1RpCS.1258n1-	GGG GAC CTG CTC ACG GCG G	381
[18,19]	RpF2-RpCS.896	ATT GCA AAA AGT ACA GTG AAC C	
	CS.877p	TTC TCA ATT CGG TAA GGG C	
	R2-RpCS.1233n	GCG ACG GTA TAC CCA TAG C	338
17 kDa	F1-R17122	CAG AGT GCT ATG AAC AAA CAA GG	378.
[20,21]	R1- R17500	CTT GCC ATT GCC CAT CAG GTT G	
	F2- Tz 15	TTC TCA ATT CGG TAA GGG C	
	R2- Tz 16	ATA TTG ACC AGT GCT ATT TC	246
OmpB [19]	F1-Rc.rompB.4362p	GTCAGCGTTACTTCTTCGATGC	475
	R1 F2Rc.rompB.4496p-	CCGTACTCCATCTTAGCATCAG	
	Rc.rompB.4836n	CCAATGGCAGGACTTAGCTACT	
	R2-Rc.rompB.4762n	AGGCTGGCTGATACACGGAGTAA	267
OmpA [22]	F1-Rr190k.71p	TGG CCA ATA TTT CTC CAA AA	650
	R1 -R F2-Rr190 k.7	TGC ATT TGT ATT ACC TAT TGT	
	R2-Rr190k.602n	TGAGT GCA GCA TTC GCT CCC CCT	532
	1pr190k.720n	AGT GCA GCA TTC GCT CCC CCT	

The PCR amplification mixture contained 10 µl master buffer composed of a mixture of PCR buffer, MgCI, and deoxy nucleoside triphosphate (Texas Bio Gene Inc, USA) 0.2 µl taq polymerase (Geneaid Biotech Ltd, Taiwan). 1 µl of (30 pmol) of each gene-specific primers in each step, 2.5 µl of extracted DNA were added in first amplification and 2 µl amplified DNA of first-round was added in second amplification. The PCR was carried out in an automated DNA thermal cycler (Applied biosystems 2720). The reactions were carried out for 35 cycles in both the first and second amplifications. The PCR products of the first PCR were run in 2% agarose gel stained with ethidium bromide and visualized under the UV transilluminator. Sample was scored as positive when a PCR product of 381 bp in case of gltA, 378 bp in case of 17kDa, 475 bp in case of ompB, 650 bp in case of ompA, could be detected in first-round PCR. In nested PCR, samples were scored as positive when a PCR product of 338 bp in gltA, 246 bp in 17kDa, 267 bp in case of ompB, and 532 bp in case of ompA, were detected. Cross-contamination was avoided as the DNA extraction, preparation of PCR master mix, the addition of DNA template, thermal cycling, and detection of the PCR product was performed in separate rooms using dedicated labware.

3. RESULTS AND DISCUSSION

Out of 135 patients, Nested PCR were positive in 70 (51.85%) cases followed by ELISA 34, (25.18%; $p<0.01$) and Weil- Felix test, 33 (24.4%; $p<0.01$) (Fig. 1).

Among 135 study population, 15 (11.11%) cases were positive by all three tests, 9 (6.66%) cases were positive by both Weil-Felix test and PCR, 10 (7.4%) cases were both ELISA and PCR positive, 4 (2.96%) cases were both Weil-Felix and ELISA positive, but PCR negative, 5 (3.7%) cases were only Weil-Felix positive, 5 (3.7%) cases were only ELISA positive, and 36 (26.66%) cases were only PCR positive (Table 2). A total of 70 (51.85%) cases detected rickettsial DNA using OmpB gene (60%), 17kDa gene (48.58%), gltA gene (30%), and OmpA gene (4.28%) by nested PCR assays (Fig. 2).

Most of the cases 25 (35.71%) were positive by only ompB gene followed by only17 kDa gene 19 (27.13%); gltA, 17kDa and ompB gene 7 (10 %) ; gltA and ompB gene 6 (8.58 %) ; ompB and 17 kDa gene 4 (5.71%) ; gltA and 17 kDa gene 3 (4.29 %) ; only gltA gene 3 (4.29 %) ; gltA ,17kDa and ompA gene (1.43%); gltA ,ompA gene (1.43 %) and only ompA gene (1.43 %). It also revealed that the combination of ompB, 17kDa, and gltA gene positivity in 98.57 % of total PCR positive Rickettsia cases (Table 3). Performance characteristics of the Weil-felix test and ELISA were done considering PCR as the gold standard. The sensitivity and specificity of Weil-felix test were 34.28% and 86.15 **%,** respectively, and the sensitivity and specificity of ELISA were 35.71 % and 86.15 **%,** respectively (Table 4).

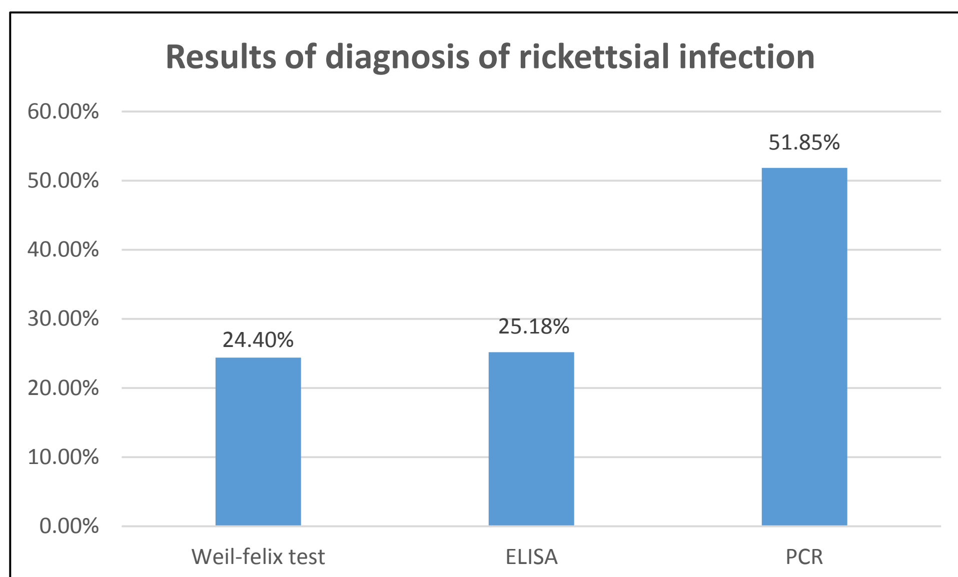

Fig. 1. Results of Weil-Felix test, ELISA, and PCR for diagnosis of rickettsial infection in study population

Table 2. Distribution of Weil-Felix test, ELISA and PCR positivity among study population (n= 135)

Methods	Positive No.	%
Only PCR positive	36	26.66
Only ELISA positive	5	3.7
Only Weil-Felix positive	5	3.7
PCR, ELISA and Weil Felix positive	15	11.11
PCR and ELISA positive	10	7.4
PCR and Weil-Felix positive	9	6.66
ELISA and Weil-Felix positive	4	2.96

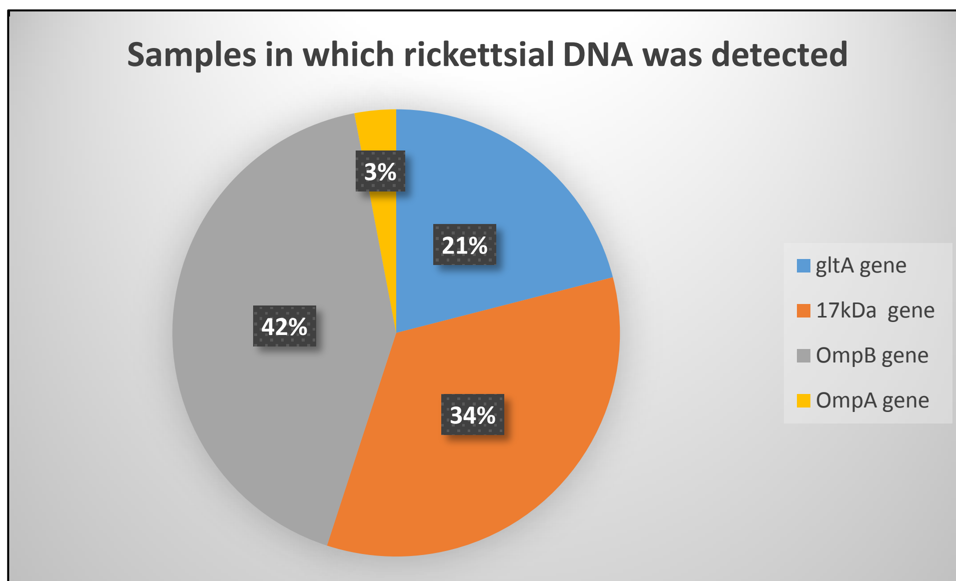

Fig. 2. Number of samples in which rickettsial DNA was detected using nested PCR methods targeting rickettsial genes

Table 3. Distribution of rickettsial genes among nested PCR positive cases (n=70)

Gene detected	Positive No.	%
gltA ,17kDa and ompB	7	10
gltA,17kDa and ompA	1	1.43
glt A and ompB	6	8.58
gltA and 17 kDa	3	4.29
gltA and ompA	1	1.43
ompB and 17 kDa	4	5.71
Only 17kD a	19	27.13
Only ompB	25	35.71
Only ompA	1	1.43
Only gltA gene positive	3	4.29
Total	70	100

Note: All four genes could not be detected by PCR in 65 (48.15%) cases

The diagnosis of rickettsial infection has traditionally been based on assessing the antibody titer in the serum samples obtained during the acute and convalescent phases of illness. It takes several weeks to confirm the diagnosis through serologic testing for establishing a 4-fold or greater titer increase. The delay in administering effective antibiotic treatment to some patients can lead to enhanced complications or mortality [4]. A rapid and precise diagnosis is necessary for the proper medical management of rickettsial infection. The PCR

Table 4. Performance characteristics of Weil-felix test and ELISA considering PCR as a gold standard (n=135)

PCR	Weil-felix		ELISA	
	Positive No. (%)	**Negative No. (%)**	**Positive No. (%)**	**Negative No.(%)**
Positive(n=70)	24(34.28) (TP)	46(65.72) (FN)	25(35.71) (TP)	45(64.29 (FN)
Negative(n=65)	9(13.84) (FP)	56(86.16) (TN)	9 (13.84) (FP)	56(86.16) (TN)
Sensitivity	34.28%		35.71 %	
Specificity	86.15 %		86.15 %	

Note TP=True positive, TN=True negative, FP=False positive, FN =False negative

assay is a useful tool for facilitating the diagnosis of infectious diseases that are caused by fastidious or slowly growing microorganism [13].

The nested PCR method is 100 times more sensitive than performing single PCR for detecting *Rickettsia* DNA [13-15]. In this study, we used the whole blood for nested PCR assay by targeting *Rickettsia* specific primers citrate synthase (gltA), 17 kDa protein (htrA), and outer membrane protein B (ompB) outer membrane protein A (ompA) antigen. Weil-Felix assay is the most easily used, cheap, and widely available serological method to test for rickettsial infection and continues to be used in many developing countries as a first-line diagnostic test. This test lacks both sensitivity and specificity [4-6]. In this study, 33 (24.4%) cases were positive by Weil-Felix tests and shows the sensitivity of Weil-Felix test was 34.28 % and the specificity was 86.15% as PCR assay considered as laboratory-confirmed case [16].

Among 135 study populations, IgM ELISA were positive in 34 (25.18%) cases, and the sensitivity and specificity of IgM ELISA were 35.71% and 86.16%, respectively, PCR assay considered laboratory-confirmed case [16].

A study in India showed that the sensitivity and specificity of IgM ELISA were 91% and 100% respectively using spotted fever and scurb typhus specific kits [23]. Another study was conducted in Bangladesh in 2010 in six different tertiary care hospital using IgM ELISA specific for *R. typhi* and *O tsutsugamushi* which showed that 66.6% seropositivity for *R. typhi* and 23.7% for *O tsutsugamushi* respectively [24].

Low sensitivity and specificity of IgM ELISA in this study may be due to the reason that in primary infection there is a rapid rise in IgM antibodies within eight days, whereas secondary or re-infection is characterized by a sharp increase in IgG levels, with a variable IgM response [25]. Lower specificity also may be due to IgM ELISA specific for *R conorii* used in this study as the actual prevalence and distribution of species throughout the country is not known. ELISA positive cases are suggestive of spotted fever group (SFG) Rickettsiae whereas *R typhi* and *O. tsutsugamushi* may be missed out by ELISA.

Among 135 study population nested PCR showed positive results in 70 (51.85%) [12] cases, and the most frequently detected gene was ompB 42(60%) followed by 17kDa 34(48.58%); gltA 21(30%) and ompA in 3(4.28%) cases. A study was conducted in Spain in 2012 which revealed that using nested PCR assays, the detection of rickettsial DNA was 75% for ompB, 50% for gltA, and 25% for ompA from EDTA treated blood and that the highest sensitivity (100%) was obtained by combining three nested PCR assays for ompA, ompB, and gltA gene [15]. The current study revealed that among 70 Rickettsia positive cases, 98.57% were positive by nested PCR with multiple gene combinations of ompB, 17kDa, and gltA, and only nested PCR positive cases were 36 (26.66%) which were not positive by ELISA and Weil-Felix test. So nested PCR assay should be done to confirm the diagnosis and reveal the cases that can be missed by ELISA & Weil-Felix test. The failure to detect *Rickettsia* DNA in the negative control suggests that the PCR test has high specificity.

In this study IgM ELISA were conducted once. But follow up ELISA were done in those cases which were positive by both ELISA and nested PCR. At the end of 7 days antibiotic therapy only 2 patients remain IgM ELISA positive. On 9th th days of treatment one of them became ELISA negative and another patient lost to follow up. At the end of 7-days antibiotic therapy, 13 nested

PCR positive cases remain positive. Among them 7 cases became nested PCR negative on 9th days of treatment and 4 patients became negative at 11th day after treatment. Another 2 patients were lost to follow up. Forty-five nested PCR positive cases were ELISA negative because only *R. conori* specific IgM ELISA were performed in this study as the actual prevalence and distribution of species throughout our country is not known and for that reason R *typhi* & O. *tsutsugamushi may* be missed out. So the ELISA positive cases in this study is suggestive of SFG Rickettsiae.

Therefore, judicious use of nested PCR to evaluate patients with suspected rickettsial infection, and especially for the serologically unconfirmed fatal cases, could be a specific diagnostic tool for the detection of rickettsial infection.

4. CONCLUSION

In conclusion, the results of our study suggest that nested PCR of the whole blood and multiple gene combinations (ompB, 17kDa, and gltA) is a reliable method for diagnosing rickettsial infection.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL AND CONSENT

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by institutional review board (IRB). Written informed consent was obtained from the patients for research and publication of this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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