Diagnostic Microbiology and Infectious Disease 95 (2019) 114855

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Mycobacteriology

Utility of multiplex PCR for early diagnosis and household contact surveillance for leprosy

Vinay Kumar Pathak, Itu Singh, Ravindra P Turankar, Mallika Lavania, Madhvi Ahuja, Vikram Singh, Utpal Sengupta *

Stanley Browne Laboratory, The Leprosy Mission Community Hospital, Nand Nagari, Delhi, India, PIN 110093

ARTICLE INFO

Article history:
Received 22 February 2019
Received in revised form 11 June 2019
Accepted 12 June 2019
Available online 20 June 2019

ABSTRACT

Early diagnosis of leprosy is important for limiting the severity of disease, which may lead to disabilities and deformities if not treated timely. Multiplex PCR employing more than one gene, specific to target DNA, is more efficient detection tool. In the present study, slit skin scrapings, blood, nasal swabs and saliva from Paucibacillary (PB) and Multibacillary (MB) cases as well as household contacts of PB cases were tested by multiplex PCR using three different gene targets namely RLEP, *16SrRNA* and *sodA*. We found an increase in overall diagnostic positivity for *M. leprae* DNA detection by M-PCR as compared to individual PCR. In case of nasal swabs using M-PCR the PPV, NPV were 0.5454, 0.8333 respectively. There is remarkable increase in PPV in SSS of PB cases and nasal swabs of HHCs using M-PCR. Conclusively, our finding suggests the utility of M-PCR for early diagnosis and household contact surveillance for leprosy.

1. Introduction

Leprosy is an ancient bacterial disease caused by infection with *Mycobacterium leprae*. The bacillus produces chronic infection that affects mainly peripheral nerve and skin, but it may also affect different sites such as the eyes, mucous membrane of upper respiratory tract, bones, and testes and produces a spectrum of clinical manifestations (Graham et al., 2010; Polycarpou et al., 2013; Walker and Lockwood, 2007). The immunohistopathological classification was described by Ridley and Jopling (1966) and categorized the disease into 2 polar groups, tuberculoid leprosy (TT) and lepromatous leprosy (LL), with 3 intermediate stages, borderline tuberculoid (BT), borderline borderline (BB) and borderline lepromatous (BL) forms. In 1998, for treatment purposes under field condition, WHO classified leprosy patients into 2 broad groups based on number of skin lesions, bacillary index, and nerve involvement (World Health Organization. Action Programme for the Elimination of Leprosy, 1995). Recently, WHO revised the case definitions of PB case: a case of leprosy with 1 to 5 skin lesions, without demonstrated presence of bacilli in a skin smear; MB case: a case of leprosy with more than 5 skin lesions; or with nerve involvement (pure neuritis, or any number of skin lesions and neuritis); or with the presence of bacilli in a slit-skin smear (SSS), irrespective of the number of skin lesions (World Health Organization. Regional Office for South-East Asia and Global Leprosy Programme, 2017).

Leprosy is routinely diagnosed based on the cardinal signs of leprosy, i.e., presence of hypopigmented anesthetic patch, thickened nerve, and presence of acid fast bacilli (AFB) in SSS. Although presence of AFB in skin smear is diagnostic for leprosy, its sensitivity is very low for early diagnosis of leprosy. Furthermore, serological assay based on detection of *M. leprae* specific antigens, phenolic glycolipid 1 (PGL-1) or its synthetic disaccharide (NDO-BSA) and natural disaccharide-leprosy IDRI diagnostic antigen (NDO-LID), employed earlier failed to detect approximately 60% of PB cases of leprosy (Cho et al., 1983; Duthie et al., 2014; Fujiwara et al., 1984). Since level of antibody is associated with intensity of exposure of host to the pathogen, these serological assays were not found to be efficient in detecting majority of PB cases who have not developed significantly high level of antibody as well as could not detect all subclinical infections (Douglas et al., 1988). Another immunological method like T cell immune response as measured by IFNγ production was employed throughout the spectrum of leprosy patients (Geluk et al., 2005; Spencer et al., 2005). PB cases exhibited CMI response, secreting high level of IFNγ after *in vitro* stimulation with *M. leprae* specific antigens; however, as household contacts (HHCs) also showed a similar pattern of IFNγ secretion as that of PB cases, the IFN γ–based assay was found to be of no significance (Martins et al., 2012). Hence, serological and CMI-based tests so far developed failed to develop as diagnostic assays for early detection of leprosy.

Abbreviations: 16S *rRNA*, 16S ribosomal RNA; AFB, acid fast bacilli; CMI, cell mediated immunity; EDTA, ethylenediaminetetraacetic acid; HHCs, household contacts; kDa, kilodalton; MB, multibacillary; M-PCR, multiplex polymerase chain reaction; NDO-BSA, natural disaccharide octyl–bovine serum albumin; NDO-LID, natural disaccharide–leprosy IDRI diagnostic antigen; PB, paucibacillary; PCR, polymerase chain reaction; PGL-1, phenolic glycolipid 1; RLEP, *Mycobacterium leprae* specific repetitive element; *sodA*, superoxide dismutase A; SSS, slit-skin smear; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol.

* Corresponding author. Tel: +91-1122594295 (O), +91-9212761651 (Mob., Delhi).
E-mail addresses: pathakv.vp@gmail.com (V.K. Pathak), itusingh@gmail.com (I. Singh), turankarr83@gmail.com (R.P. Turankar), mallikalavania@gmail.com (M. Lavania), madhviahuja87@gmail.com (M. Ahuja), ravikram.singh10@gmail.com (V. Singh), usengupta2002@yahoo.com (U. Sengupta).

https://doi.org/10.1016/j.diagmicrobio.2019.06.007
0732-8893/

Polymerase chain reaction (PCR) is a very sensitive and specific molecular method for amplification and identification of nucleic acid of a specific organism. Previously, various attempts have been made to detect *M. leprae* DNA in clinical samples of leprosy patients. The clinical samples used not only were skin biopsies but also included different types of specimens such as SSS, nerve twigs, urine, oral/ nasal swabs, saliva, blood, and ocular tissues (Almeida et al., 2004; Caleffi et al., 2012; de Wit et al., 1993; Jardim et al., 2003; Martinez et al., 2006; Rosa et al., 2013; Santos et al., 1993; Shamsi et al., 2007; Turankar et al., 2015). Usefulness of PCR in identification of pure neuritic leprosy has been reported to be sensitive up to 50–70% (Bezerra Da Cunha et al., 2006; Martinez et al., 2014; Rodriguez et al., 2013). Specific and sensitive target is an essential element to develop a diagnostic tool by using PCR-based methods. Woods and Cole have reported that at least 28 copies of a repetitive sequence RLEP are present in an *M. leprae* genome, which make it a more sensitive target than any other single copy gene target. They have reported 73% positivity using RLEP-based PCR in patients with a bacillary index of zero (Woods and Cole, 1990). RLEP-based PCR has been employed by many investigators for diagnosis of leprosy (Jamil et al., 1994; Santos et al., 1993). In previous studies, other gene targets like *16SrRNA* and *sodA* have been employed for viability and detection assay specific for *M. leprae.* They found *16SrRNA*/RLEP RT-PCR *M. leprae* viability assay useful both for short-term experimental purposes and for predicting *M. leprae* viability in biopsy specimens to monitor treatment efficacy, whereas the *sodA*/RLEP RT-PCR *M. leprae* viability assay was limited to short-term experimental research purposes (Martinez et al., 2009).

Multiplex PCR (M-PCR) as an alternative form of PCR employing more than 1 gene specific to *M. leprae* DNA has been used for detection in clinical cases of different categories and HHCs in earlier studies (Banerjee et al., 2010; Sandhika et al., 2016). Chaitanya et al. have reported 75.61% positive detection in indeterminate leprosy by targeting pseudogenes of *M. leprae*, *ML1545*, *ML2180*, and *ML2179* employing M-PCR (Chaitanya et al., 2017). In one of the studies, it was reported that by employing M-PCR in PB case, the percent positivity significantly increased up to 80.3% from 22.2% of AFB positivity for *M. leprae* (Banerjee et al., 2010). As reported in previous finding, the efficacy of conventional PCR can be enhanced by multiplexing the specific targets which are present in multiple copies or present as consensus sequence. We designed our experimentation based on previously reported specific targets for the purpose of developing a diagnostic tool.

In the present study, 4 different types of clinical samples such as slit skin scrapings (SSS), blood, nasal swabs, and saliva from both PB and MB cases as well as from their HHCs were tested by M-PCR using 3 different targets, namely, RLEP, *16SrRNA*, and *sodA*. Furthermore, same clinical samples were tested by individual gene PCR using the same 3 gene targets so as to compare the efficiency of M-PCR over the individual gene PCRs. Hence, the purpose of this study was to develop a better diagnostic tool to detect early leprosy.

2. Material and methods

2.1. Patients and contacts

A total of 91 clinically confirmed cases of leprosy patients (60 PBs and 31 MBs) were included both study groups, and their 50 HHCs were recruited after taking informed consents from patients at 3 different centers of The Leprosy Mission community hospital, India. The procedures of sample collection were in accordance with the standards and guidelines of the Indian Council of Medical Research. The study was approved by "Ethical Committee–The Leprosy Mission Trust India (TLMTI)", meeting held on 17 September 2012. As the present study was designed to find out the efficacy of diagnosing PB cases, the sample size was calculated only for PB leprosy by Yamane method: $n = N / (1 + Ne^2)$, where n = sample size, N = population size, and e = level of precision (at 95% confidence interval, $e = 0.05$). The value of N for PB cases was 73 as reported in the database of annual new case detection at different centers of TLMTI. Applying the above formula, the value of sample size was 61.73.

All of these untreated patients were categorized into PB and MB after examination of their clinical characteristics and SSS positivity for AFB based on guidelines of WHO classification (World Health Organization. Action Programme for the Elimination of Leprosy, 1995). Overall, 60 patients were categorized into PB and 31 into MB. Demographic details of the cases and controls recruited in the study are given in Table 1.

2.2. Clinical samples

SSS, blood, nasal swab, and saliva samples were obtained from each subject and were processed for DNA extraction. After initial processing, PCR was performed on SSS (n 31), blood (n 30), nasal swab (n 30), and saliva (n 31) belonging to MB group, whereas in PB group, SSS (n 60), blood (n 30), nasal swab (n 30), and saliva (n 31) were analyzed. Clinical samples of HHCs used for PCR were SSS (n 30), blood (n 30), nasal swab (n 50), and saliva (n 50). The number and type of clinical samples collected in each category were in concurrence of availability of patients as well as household contacts and their consents.

Furthermore, 30 samples each from nonleprosy cases (patients having other dermatological diseases, tuberculosis) and healthy individuals from nonendemic area were also collected and processed for DNA extraction and were used as negative control group.

2.3. Sample collection methods

SSS samples were collected after taking 4 scrapes of tissue from an incision (5 mm long and 2 mm deep) made with the help of a sterile surgical scalpel blade on the left and right earlobes and skin lesions without any contamination with blood along the skin-slit area. The tissue material thus obtained was collected in microcentrifuge tube containing 70% ethanol, and samples were kept at 4 °C until further processing. Peripheral venous blood (2 mL) was withdrawn from each patient by antecubital venipuncture and collected in EDTA vial and kept at 4 °C. Nasal swab was collected from the surface of the turbinate bone of the nasal septum with sterilized wet cotton swabs soaked in normal saline (0.9% w/v NaCl), and the cotton was stored in dry microcentrifuge tubes and quickly stored at −20 °C. Nonstimulated saliva (approximately 20 mL) was collected from each subject into a sterile container over a period of 5 min. Each collected saliva sample was immediately centrifuged at ≥1800 ×g for 30 min at 4 °C. Supernatant (500 μl aliquot) was collected and then stored at −20 °C after adding 5 μL of preservative (5 mM EDTA, 2 mM Thimerosal).

2.4. DNA extraction

DNA was extracted from SSS and nasal swab using the method described earlier (Jadhav et al., 2001). In brief, SSS collected in 70% ethanol was centrifuged at ≥9000 ×g, and thus, the pellet obtained was air dried. Further extraction method was the same for both SSS and nasal swab which was done by adding lysis buffer (10 mg/mL Proteinase K in 1 M Tris, pH 8.5, and 0.5% Tween 20) to the sample followed by incubation

Table 1
A demographic details of leprosy patients and controls.

Category	Age	Bacillary index	Number of patients
MB	18–40	2–3	20
	40–60	2–4	11
PB	18–40	0	35
	40–60	0	25
HHCs of PB	18–50	-	50
Nonleprosy cases	18–45	-	30
Healthy controls	18–45	-	30

at 60 °C for 16 h. Proteinase K was then inactivated by incubation at 97 °C for 15 min. The lysate was further used for PCR.

The DNA extraction from blood was done using red cell lysis method (Sambrook et al., 1989). In brief, 200 μL of whole blood was mixed with 600 μL red cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, Na_2EDTA, pH 7.4), incubated for 30 min on ice, and then centrifuged at 4500 ×*g* for 10 min at 4 °C. The pellet obtained was treated with sodium EDTA (SE) buffer (75 mM NaCl, 25 mM Na_2EDTA, pH 8), Proteinase K, and 20% SDS and incubated overnight at 37 °C in water bath. Thereafter, the homogenate was mixed with SE buffer and phenol for 10 min and centrifuged at ≥9000 ×*g* for 5 min at 10 °C. The supernatant was collected in fresh tube, and DNA precipitation was done using phenol:chloroform:isoamyl alcohol (25:24:1) method.

The DNA extraction from saliva was done using 10% Chelex (Polgarova et al., 2010). In brief, equal volume of 10% chelex resin was added to tube containing saliva, and the tube was vortexed for a few seconds. The samples were incubated in water bath at 97 °C for 30 min. Thereafter, the samples were centrifuged at ≥9000 ×*g* for 5 min followed by collection of supernatant in fresh tube and used for PCR.

Genomic DNA from *M. leprae,* strain NHDP-63, was kindly provided by the Biodefense and Emerging Infections Research Resources Repository (https://www.beiresources.org/) and was used in all PCR experiments as positive control. Nuclease-free water was used as negative control. The purified genomic DNA of concentration 1×10^{-4} μg/μL (100 pg/μL) was serially diluted up to concentration of 1×10^{-13}μg/μL (0.1 att/μL) and used to test the sensitivity of PCR for target sequence RLEP, genes *16SrRNA* and *sodA*.

2.5. Selection of gene target and primer design

The primer sequences for selected gene target *16SrRNA* and repetitive sequence RLEP were acquired as described in earlier studies (Donoghue et al., 2001; Jadhav et al., 2005). The primer sequence for *sodA* gene was designed in our lab using primer BLAST version 3. The designed primers were obtained commercially by Sigma, USA. The primer sequences are mentioned in Table 2.

2.6. PCR cycling parameters and reaction conditions

The entire clinical samples and serially diluted purified genomic DNA from *M. leprae,* strain NHDP-63, were tested using individual gene PCR as well as M-PCR targeting *16S rRNA*, *sodA*, and RLEP. The DNA extracted from each clinical sample was used in equal quantity for PCR mixture. PCR mix for individual genes was prepared using 2× M-PCR Master Mix (Cat. No. 206143, QIAGEN, Germany), forward and reverse primers (10 μM), and 2 μL of template DNA. The final concentration of Master Mix was made 1× and 0.2 μM for each primer in reaction mix of final volume 20 μL made up with nuclease-free water. M-PCR reaction mix was prepared using 2× M-PCR Master Mix (Cat. No. 206143, QIAGEN, Germany), forward and reverse primer mix (10 μM), and 2 μL of template DNA. The final concentration of Master Mix was made 1× and 0.2 μM for each primer in reaction mix of final volume 20 μl made up with nuclease-free water. Both individual gene PCR and M-PCR were performed with same kind of master mix in order to make equally efficient reaction mixture in terms of enzyme and other constituents.

PCR amplifications were performed using thermal cycler (Corbett Research, Australia). The reaction conditions for individual gene PCR for RLEP were 1 cycle of initial denaturation at 95 °C for 15 min followed by 37 repeats of cycling at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. Termination of reaction was done after final extension at 72 °C for 10 min. Similarly, *16SrRNA* gene was amplified with reaction condition of 1 cycle of initial denaturation at 95 °C for 15 min followed by 37 repeats of cycling at 94 °C for 2 min, 57 °C for 2 min, and 72 °C for 3 min. Termination was done after final extension at 72 °C for 10 min. Amplification of *sodA* gene was performed with reaction condition of 1 cycle of initial denaturation at 95 °C for 15 min followed by 37 repeats of cycling at 94 °C for 30 s, 60 °C for 1.5 min, and 72 °C for 1.5 min. The reaction was terminated after final extension at 72 °C for 10 min. The amplified products were electrophoresed on 2% agarose gel (Tris-Borate-EDTA) at constant voltage (100 V). The gel image was captured using gel documentation system (AlphaImager EC).

The reaction conditions for M-PCR including all 3 targets were 1 cycle of initial denaturation at 95 °C for 15 min followed by 37 repeats of cycling at 94 °C for 30 s, 57 °C for 1.5 min, and 72 °C for 1 min. The reaction was terminated after final extension at 72 °C for 10 min. The amplified products were electrophoresed on 4% agarose gel (Tris-Borate-EDTA) at constant voltage (100 V). The gel image was captured using gel documentation system (AlphaImager EC).

Table 2
Primer sequences for targets.

Gene	Primer orientation	Sequence	Amplicon size
16SrRNA	Forward	5′-CGGAAAGGTCTCTAAAAAATCTT-3′	171 bp
16SrRNA	Reverse	5′-CATCCTGCACCGCAAAAAGCTT-3′	
SodA	Forward	5′-CAGCTGTATGACCAACAGGC-3′	185 bp
SodA	Reverse	5′-TGCGTCTTAGATGTTGCAGC-3′	
RLEP	Forward	5′-TGCATGTCATGGCCTTGAGG-3′	129 bp
RLEP	Reverse	5′-CACCGATACCAGCGGCAGAA-3′	

3. Results

3.1. Standardization with genomic DNA from M. leprae, strain NHDP-63

The standardization of M-PCR showed that, among all 3 targets, RLEP is most sensitive to detect *M. leprae* purified genomic DNA, up to the concentration of 10^{-9} μg/μL, followed by *sodA* (10^{-8} μg/μL) and *16SrRNA* (10^{-6} μg/μL). Similarly, individual gene PCR also showed highest sensitivity for the target RLEP (10^{-8} μg/μL), followed by equal sensitivity (10^{-6} μg/μL) for *sodA* and *16SrRNA* gene targets.

3.2. PCR with clinical samples

3.2.1. Individual gene PCR

All the 4 types of clinical samples, i.e., SSS, blood, nasal swabs, and saliva, were examined with individual gene PCR for these 3 genes, and results are shown in Table 3.

By individual gene PCR, it was found that MB cases showed highest positivity with target RLEP in all kinds of samples except for nasal swabs, which were more positive by targeting gene *16SrRNA*. In PB cases, positivity for RLEP was highest in SSS and saliva samples, and

Table 3
Results showing percentage positivity of clinical samples by individual gene PCR for each individual gene.

Clinical samples	Categories	Percentage (%) positivity by individual gene		
		RLEP	*16SrRNA*	*SodA*
SSS	PB (*n* 60)	51.66	31.66	21.66
	MB (*n* 31)	83.87	70.96	52
	HHCs (*n* 30)	0	0	0
Blood	PB (*n* 30)	46.66	53.33	53.33
	MB (*n* 30)	83.33	60	70
	HHCs (*n* 30)	0	0	0
Saliva	PB (*n* 31)	45.16	35.48	6
	MB (*n* 31)	64.5	41.9	19
	HHCs (*n* 50)	0	0	0
Nasal Swab	PB (*n* 30)	70	76.66	10
	MB (*n* 30)	73	90	13
	HHCs (*n* 50)	10	4	0

positivity for *16SrRNA* was highest in blood and nasal swab samples. Positivity for *sodA* gene was lowest among all 3 genes in all type of samples except blood.

In the context of suitable clinical sample for diagnosing leprosy, both targets RLEP and *16SrRNA* showed highest percent positivity in nasal swabs followed by SSS in both categories, i.e., MB and PB. However, nasal swabs from HHCs also showed positivity for these 2 gene targets. Nasal swabs of HHCs showed positivity of 10% with target RLEP and 4% for *16SrRNA* gene by individual gene PCR. The overall diagnosis of clinical samples of PB cases and their HHCs by individual gene PCR are shown in Table 4.

3.2.2. M-PCR

M-PCR was performed with same clinical samples of PB cases and their HHCs to examine efficiency of M-PCR over the individual gene PCR. PB cases were chosen to perform M-PCR in order to check the efficacy of M-PCR in clinical samples with low bacillary load or in other words early detection of leprosy. Overall detection of *M. leprae* DNA in different types of clinical samples by means of individual gene PCR and M-PCR was compared, and results showed a remarkable increase in sensitivity and specificity of diagnosis using M-PCR. None of the nonleprosy cases and healthy negative controls was found positive for the test. The overall diagnosis of clinical samples of PB cases and their HHCs by M-PCR are shown in Table 5.

A representative image of agarose gel electrophoresis of M-PCR is given in Fig. 1.

In PB cases, sensitivity and specificity for SSS were found to be 0.9333 and 1, respectively [positive predictive value (PPV) 1; negative predictive value (NPV) 0.8823] using M-PCR, whereas using individual PCR, values were 0.5166 and 1 (PPV 1; NPV 0.5084) for RLEP, 0.3166 and 1 (PPV 1; NPV 0.4225) for *16SrRNA*, and 0.2166 and 1 (PPV 1; NPV 0.3896) for *sodA,* respectively. Similar pattern was found in blood samples. In case of nasal swabs, using M-PCR, the sensitivity and specificity were 0.8 and 0.6, respectively (PPV 0.5454; NPV 0.8333), whereas by individual gene PCR, the values were 0.7 and 0.9 (PPV 0.8076; NPV 0.8333) for RLEP, 0.7666 and 0.96 (PPV 0.92; NPV 0.8727) for *16Sr RNA*, and 0.1 and 1 (PPV 1; NPV 0.6493) for *sodA,* respectively. There is a remarkable increase of positive detection up to 40% in nasal swabs of HHCs using M-PCR as compared to individual gene PCR (10%).

Table 4
Results showing overall diagnosis of clinical samples of PB cases by individual gene PCR.

Type of clinical samples	Subjects	Target	Positive	Negative	Positivity (%)	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)
SSS	PB cases (*n* 60)	RLEP	31	29	51.66	0.5166	1	1	0.5084
	HHCs (*n* 30)		0	30	-	-	-	-	
	PB cases (*n* 60)	*16Sr RNA*	19	41	31.66	0.3166	1	1	0.4225
	HHCs (*n* 30)		0	30	-	-	-	-	
	PB cases (*n* 60)	*sodA*	13	47	21.66	0.2166	1	1	0.3896
	HHCs (*n* 30)		0	30	-	-	-	-	
Blood	PB cases (*n* 30)	RLEP	14	16	46.66	0.4666	1	1	0.6521
	HHCs (*n* 30)		0	30	-	-	-	-	-
	PB cases (*n* 30)	*16Sr RNA*	16	14	53.33	0.5333	1	1	0.6818
	HHCs (*n* 30)		0	30	-	-	-	-	-
	PB cases (*n* 30)	*SodA*	16	14	53.33	0.5333	1	1	0.6818
	HHCs (*n* 30)		0	30	-	-	-	-	-
Saliva	PB cases (*n* 31)	RLEP	14	17	45.16	0.4516	1	1	0.7462
	HHCs (*n* 50)		0	50	-	-	-	-	-
	PB cases (*n* 31)	*16Sr RNA*	11	20	35.48	0.3548	1	1	0.7142
	HHCs (*n* 50)		0	50	-	-	-	-	-
	PB cases (*n* 31)	*SodA*	2	29	6	0.0645	1	1	0.6329
	HHCs (*n* 50)		0	50	-	-	-	-	-
Nasal swab	PB cases (*n* 30)	RLEP	21	9	70	0.7	0.9	0.8076	0.8333
	HHCs (*n* 50)		5	45	10	-	-	-	-
	PB cases (*n* 30)	*16Sr RNA*	23	7	76.66	0.7666	0.96	0.92	0.8727
	HHCs (*n* 50)		2	48	4.16	-	-	-	-
	PB cases (*n* 30)	*SodA*	3	27	10	0.1	1	1	0.6493
	HHCs (*n* 50)		0	50	-	-	-	-	-

Table 5
Results showing overall diagnosis of clinical samples of PB cases by M-PCR.

Type of clinical samples	Subjects	Positive	Negative	Positivity (%)	Sensitivity	Specificity	PPV	NPV
SSS	PB cases (*n* 60)	56	4	93.33	0.9333	1	1	0.8823
	HHCs (*n* 30)	0	30	0	-	-	-	-
Blood	PB cases (*n* 30)	26	4	86.66	0.8666	1	1	0.8823
	HHCs (*n* 30)	0	30	0	-	-	-	-
Saliva	PB cases (*n* 31)	17	14	54.83	0.5483	1	1	0.7812
	HHCs (*n* 50)	0	50	0	-	-	-	-
Nasal swab	PB cases (*n* 30)	24	6	80	0.8	0.6	0.5454	0.8333
	HHCs (*n* 50)	20	30	40	-	-	-	-

4. Discussion

Early diagnosis of leprosy is important for limiting the severity of disease by providing early treatment. If not treated early, it may lead to deformities and disabilities. Various approaches were adopted by investigators to detect the presence of bacilli in the spectrum of the disease but could not develop a diagnostic tool up to a gold standard which can identify the disease earlier than finding the cardinal signs identified by a clinician. PCR-based detection added significant improvement in detection of leprosy since 2 decades especially for cases with negative bacillary index and patients who are lacking cardinal signs of the disease. PCR using *M. leprae* specific genes encoding 36 kDa antigen (Kampirapap et al., 1998), 18 kDa antigen (Scollard et al., 1998), 65 kDa antigen (Plikaytis et al., 1990), complex 85 (Martinez et al., 2006), 16S rDNA (Martinez et al., 2009), and repetitive sequence (Truman et al., 2008) has been reported earlier by different investigators as major gene targets for detection of disease. This molecular approach can be made more efficient by targeting gene with high copy number in genome of *M. leprae*. It is evident that sequence RLEP is repeated 28 times in *M. leprae* genome (Martinez et al., 2009). Furthermore, gene targets *Ag85B* (Martinez et al., 2006), *sodA*, and *16SrRNA* (Martinez et al., 2009) have also been employed in conventional and real-time PCR for detection *M. leprae* DNA as well as for assessment of viability of *M. leprae* in clinical samples. In a different study, clinical samples like SSS and blood, and environmental samples were used for comparative analysis of positivity by PCR using RLEP, *16SrRNA, sodA*, and *rpoT* genes and reported highest PCR positivity for RLEP in clinical as well as environmental samples (Turankar et al., 2015).

While standardizing the procedure with *M. leprae* purified genomic DNA strain NHDP 63, we also found RLEP was showing highest sensitivity for detection of *M. leprae* (10^{-9} μg/μL) in purified genomic DNA followed by *sodA* (10^{-8} μg/μL) and *16SrRNA* (10^{-6} μg/μL).

The quality and quantity of the isolated nucleic acid and the PCR product size should also be taken in consideration, as these may affect the amplification procedure generating false-negative results, which depend on type of clinical sample collected and method employed to extract DNA. Thus, a suitable nucleic acid extraction method is also important, as impurities in extract can inhibit the polymerase reaction (de Wit et al., 1991).

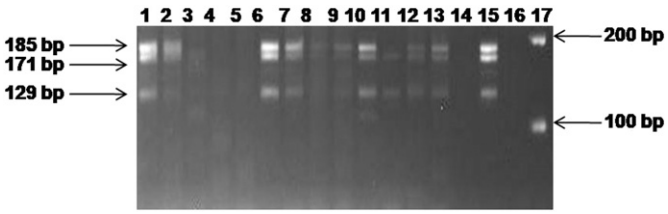

Fig. 1. Multiplex PCR amplifications on 4% agarose gel electrophoresis at constant voltage (100 V) using *M. leprae* specific targets RLEP, *16SrRNA*, and *sodA*. Lane 1–13, clinical samples; lane 15, positive control; lane 16, negative control; lane 17, DNA ladder (100 bp).

In the present study, we examined 4 different types of clinical samples to measure the efficacy of M-PCR over individual gene PCR. The samples collected were SSS, blood, nasal swabs, and saliva and processed by suitable methods as described before.

In the current study, results showed that by individual gene PCR, RLEP is the most suitable target for all kinds of samples. In PB cases, positivity for RLEP was highest in SSS and nasal swab, and positivity for *16SrRNA* was highest in nasal swab samples. Positivity for *sodA* gene was lowest among all 3 genes in all types of samples except blood.

The target RLEP showed higher positivity of 10% in nasal swabs of HHCs compared to 4% positivity of *16SrRNA* gene target by individual gene PCR. This result shows RLEP as a better target as compared to *16SrRNA* for examination of HHCs also. In an earlier study, M-PCR targeting repetitive sequence of *M. leprae* and TTC repeats has been used for early detection of leprosy among close contacts and reported 10.9% nasal swabs positivity in MB HHCs and 1.3% among PB HHCs (Banerjee et al., 2010).

Furthermore, by incorporating more than 1 gene target, detection of *M. leprae* DNA can improve in terms of specificity and sensitivity of the test. M-PCR is reported to enhance the positivity of detection in low–bacillary index cases (Reja et al., 2013). In the present study, M -PCR was performed using 3 targets, RLEP, *16SrRNA*, and *sodA*, specific to *M. leprae*. In our study, M-PCR showed a remarkable increase in overall detection of *M. leprae* DNA in different types of clinical samples as compared to individual gene PCRs. Our results showed that, in PB cases, M-PCR with SSS is the most sensitive (0.9333) and specific (1) test for diagnosing infection of leprosy (Table 5). The PPV and NPV for the test were 1 and 0.8823, respectively, which suggest the efficacy of the test. The test results also showed increase in sensitivity as compared to individual gene PCR. However, with blood samples also, there was similar enhancement of sensitivity using M-PCR as compared to individual gene PCR, that is, from 0.4666 (RLEP), 0.5333 (*16SrRNA*), and 0.5333 (*sodA*) using individual gene PCR to 0.8666 using M-PCR, but it was found to be less sensitive than SSS. These results depict that M-PCR with SSS can be a better choice of molecular tool for diagnosis of leprosy with higher sensitivity and specificity than the conventional PCR.

In earlier studies, detection of *M. leprae* DNA using PCR in nasal swabs samples from HHCs and healthy individuals along with leprosy patients have been reported (Almeida et al., 2004; Beyene et al., 2003; de Wit et al., 1993; Lavania et al., 2012) and showed the positivity in majority of MB patients. However, detection in HHCs was found to be positive approximately up to 10% using gene target RLEP alone (Araujo et al., 2012; Cabral et al., 2013; Klatser et al., 1993) and by targeting RLEP along with TTC repeats for M-PCR (Banerjee et al., 2010). A study for monitoring transmission of *M. leprae* in HHCs of patients reported 16% positivity for *M. leprae* DNA in nasal swabs, and on follow-up, 2 of these nasal swabs positive HHCs became cases (Romero-Montoya et al., 2017). Further, a recent study by Araujo et al.

(2016) using quantitative PCR of RLEP showed 49% positivity in nasal swabs in HHCs of MB cases. Although the present study using M-PCR failed to find any positivity in SSS and blood samples, 40% positivity was noted in nasal swabs of HHCs, which strongly indicated nasal carriage of *M. leprae* even in HHCs of PB leprosy (Tables 4 and 5). Follow-up of these HHCs for 2 years and above for their rate of conversion to clinical cases will indicate the utility of this M-PCR in prediction of clinical leprosy in HHCs.

5. Conclusion

M-PCR can be a better choice of molecular tool for detection of *M. leprae* in clinical samples with higher accuracy than the conventional single-gene PCR. Our findings suggest utility of M-PCR using RLEP, *sodA*, and *16SrRNA* genes for early diagnosis and for surveillance of leprosy household contacts and subclinically infected leprosy endemic population. Attempts are being made to make the test much simpler so that it could be applied in field-based situation.

Ethics approval and consent to participate

The study was approved by the "Ethical Committee–The Leprosy Mission Trust India (TLMTI)" meeting held on 17 September 2012. All participants were recruited after taking informed written consent.

Consent to publish

All the authors have reviewed and given their consent to publish the manuscript.

Availability of data and materials

All the relevant data and materials are presented in the manuscript.

Competing interests

Vinay Kumar Pathak, Itu Singh, Ravindra P Turankar, Mallika Lavania, Madhvi Ahuja, Vikram Singh, and Utpal Sengupta declare that they have no conflict of interest.

Funding

This work was funded by Indian Council of Medical Research, New Delhi, India (Grant No. 5/8/3(4)/2013-ECD-1).

Authors' contributions

All the authors contributed to experiment design and methodology.

Acknowledgement

We acknowledge the infrastructural support rendered by TLMTI to carry out this research work at Stanley Browne Laboratory, The Leprosy Mission Community Hospital, Nand Nagari, Delhi. The authors acknowledge Indian Council of Medical Research, New Delhi, for financial support.

References

Almeida EC, Martinez AN, Maniero VC, Sales AM, Duppre NC, Sarno EN, et al. Detection of *Mycobacterium leprae* DNA by polymerase chain reaction in the blood and nasal secretion of Brazilian household contacts. Mem Inst Oswaldo Cruz 2004;99(5):509–11.

Araujo S, Lobato J, Reis Ede M, Souza DO, Goncalves MA, Costa AV, et al. Unveiling healthy carriers and subclinical infections among household contacts of leprosy patients who play potential roles in the disease chain of transmission. Mem Inst Oswaldo Cruz 2012;107(1):55–9.

Araujo, S., Freitas, L. O., Goulart, L.R., Goulart, I. M., 2016. Molecular evidence for the aerial route of infection of Mycobacterium leprae and the role of asymptomatic carriers in the persistence of leprosy. Clin Infect Dis.1,63(11),1412–1420.

Banerjee S, Sarkar K, Gupta S, Mahapatra PS, Gupta S, Guha S, et al. Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts : a pilot study from India. BMC Infect Dis 2010;10:252.

Beyene D, Aseffa A, Harboe M, Kidane D, Macdonald M, Klatser PR, et al. Nasal carriage of *Mycobacterium leprae* DNA in healthy individuals in Lega Robi village, Ethiopia. Epidemiol Infect 2003;131(2):841–8.

Bezerra Da Cunha FM, Werneck MC, Scola RH, Werneck LC. Pure neural leprosy: diagnostic value of the polymerase chain reaction. Muscle Nerve 2006;33(3):409–14.

Cabral PB, Junior JE, Macedo AC, Alves AR, Goncalves TB, Brito e Cabral TC, et al. Anti- PGL1 salivary IgA/IgM, serum IgG/IgM, and nasal *Mycobacterium leprae* DNA in individuals with household contact with leprosy. Int J Infect Dis 2013;17(11):e1005–10http://dxhttps://doi.org/10.1016/j.ijid.2013.05.011.

Caleffi KR, Hirata RD, Hirata MH, Caleffi ER, Siqueira VL, Cardoso RF. Use of the polymerase chain reaction to detect *Mycobacterium leprae* in urine. Braz J Med Biol Res 2012;45(2):153–7.

Chaitanya VS, Cuello L, Das M, Sudharsan A, Ganesan P, Kanmani K, et al. Analysis of a novel multiplex polymerase chain reaction assay as a sensitive tool for the diagnosis of indeterminate and tuberculoid forms of leprosy. Int J Mycobacteriol 2017;6(1):1–8.

Cho SN, Yanagihara DL, Hunter SW, Gelber RH, Brennan PJ. Serological specificity of phenolic glycolipid 1 from *M. leprae* and use in serodiagnosis of leprosy. Infect Immun 1983;41(3):1077–83.

de Wit MY, Faber WR, Krieg SR, Douglas JT, Lucas SB, Montreewasuwat N, et al. Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues. J Clin Microbiol 1991;29(5):906–10.

de Wit MY, Douglas JT, McFadden J, Klatser PR. Polymerase chain reaction for detection of *Mycobacterium leprae* in nasal swab specimens. J Clin Microbiol 1993;31(3):502–6.

Donoghue HD, Halto J, Spigelman M. PCR primer that can detect low levels of *Mycobacterium leprae* DNA. J Med Microbial 2001;50(2):177–82.

Douglas JT, Steven LM, Fajardo T, Cellona RV, Madarang MG, Abalos RM, et al. The effect of chemotherapy on antibody levels of lepromatous patients. Lepr Rev 1988;59(2):127–35.

Duthie MS, Balagon MF, Maghanoy A, Orcullo FM, Cang M, Dias RF, et al. Rapid quantitative serological test for detection of infection with *Mycobacterium leprae*, the causative agent of leprosy. J Clin Microbiol 2014;52(2):613–9.

Fujiwara T, Hunter SW, Cho SN, Aspinall GO, Brennan PJ. Chemical synthesis and serology of the disaccharides and trisaccharides of phenolic glycolipid antigen from leprosy bacillus and preparation of a disaccharide protein conjugate for serodiagnosis of leprosy. Infect Immun 1984;43(1):245–52.

Geluk A, Klein MR, Franken KL, van Meijgaarden KE, Wieles B, Pereira KC, et al. Postgenomic approach to identify novel *Mycobacterium leprae* antigens with potential to improve immunodiagnosis of infection. Infect Immun 2005;73(9):5636–44.

Graham A, Furlong S, Margoles LM, Owusu K, Franco-Paredes C. Clinical management of leprosy reactions. Infect Dis Clin Pract 2010;18:235–8.

Jadhav RS, Macdonald M, Bjune G, MILEP2 study group. Simplified PCR detection method for nasal *Mycobacterium leprae*. Int J Lepr Other Mycobact Dis 2001;69(4):299–307.

Jadhav RS, Kamble RR, Shinde VS, Edward S, Edward VK. Use of reverse transcription polymerase chain reaction for the detection of *Mycobacterium leprae* in the slit-skin smears of leprosy patients. Indian J Lepr 2005;77(2):116–27.

Jamil S, Wilson SM, Hacket M, Hussain R, Stoker NG. A colorimetric PCR method for the detection of *M. leprae* in skin biopsies from leprosy patients. Int J Lepr Other Mycobact Dis 1994;62(4):512–20.

Jardim MR, Antunes SL, Santos AR, Nascimento OJ, Nery JA, Sales AM, et al. Criteria for diagnosis of pure neural leprosy. J Neurol 2003;250(7):806–9.

Kampirapap K, Singtham N, Klatser PR, Wiriyawipart S. DNA amplification for detection of leprosy and assessment of efficacy of leprosy chemotherapy. Int J Lepr Other Mycobact Dis 1998;66(1):16–21.

Klatser PR, van Beers S, Madjid B, Day R, de Wit MY. Detection of *Mycobacterium leprae* nasal carriers in populations for which leprosy is endemic. J Clin Microbiol 1993;31(11):2947–51.

Lavania M, Turankar RP, Karri S, Chaitanya VS, Sengupta U, Jadhav RS. Cohort study of the seasonal effect on nasal carriage and the presence of *Mycobacterium leprae* in an endemic area in the general population. Clin Microbiol Infect 2012;19:970–4.

Martinez AN, Britto CF, Nery JA, Sampaio EP, Jardim MR, Sarno EN, et al. Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of *Mycobacterium leprae* DNA in skin biopsy samples from patients diagnosed with leprosy. J Clin Microbiol 2006;44(9):3154–9.

Martinez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, et al. Molecular determination of *Mycobacterium leprae* viability by use of real-time PCR. J Clin Microbiol 2009;47(7):2124–30.

Martinez AN, Talhari C, Moraes MO, Talhari S. PCR-based techniques for leprosy diagnosis. From the laboratory to the clinic. PLoS Negl Trop Dis 2014;8(4), e2655. https://doi.org/10.1371/journal.pntd.0002655.

Martins, M.V., Guimaraes, M.M., Spencer, J.S., Hacker, M.A., Costa, L.S., Carvalho, F.M., Geluk, A., van der Ploeg-van Schip, J.J., Pontes, M.A., Gonçalves, H.S., de Morais, J.P., Bandeira, T.J., Pessolani, M.C., Brennan, P.J., Pereira, G.M., 2012. Pathogen-specific epitopes as epidemiological tools for defining the magnitude of *Mycobacterium leprae* transmission in areas endemic for leprosy. PLoS Negl Trop Dis. 6(4)e, 1616. https://doi.org/10.1371/journal.pntd.0001616

Plikaytis BB, Gelber RH, Shinnick TM. Rapid and sensitive detection of *Mycobacterium leprae* using a nested-primer gene amplification assay. J Clin Microbiol 1990;28(9):1913–7.

Polgarova K, Behuliak M, Celec P. Effect of saliva processing on bacterial DNA extraction. New Microbiol 2010;33(4):373–9.

Polycarpou A, Walker SL, Lockwood DNJ. New findings in the pathogenesis of leprosy and implications for the management of leprosy. Curr Opin Infect Dis 2013;26(5):413–9.
Reja AH, Biswas N, Biswas S, Dasgupta S, Chowdhury IH, Banerjee S, et al. Fite-Faraco staining in combination with multiplex polymerase chain reaction: a new approach to leprosy diagnosis. Indian J Dermatol Venereol Leprol 2013;79(5):693–700.
Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis 1966;34(3):255–73.
Rodriguez G, Pinto R, Gomez Y, Rengifo ML, Estrada OL, Sarmiento M, et al. Pure neuritic leprosy in patients from a high endemic region of Colombia. Lepr Rev 2013;84(1):41–50.
Romero-Montoya M, Beltran-Alzate JC, Cardona-Castro N. Evaluation and monitoring of *Mycobacterium leprae* transmission in household contacts of patients with Hansen's disease in Colombia. PLoS Negl Trop Dis 2017;11(1), e0005325http://dx.doihttps://doi.org/10.1371/journal.pntd.0005325.
Rosa FB, Souza VC, Almeida TA, Nascimento VA, Vasquez FG, Cunha Mda G, et al. Detection of *Mycobacterium leprae* in saliva and the evaluation of oral sensitivity in patients with leprosy. Mem Inst Oswaldo Cruz 2013;108(5):572–7.
Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, second edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 1989;61:17–8.
Sandhika W, Adriaty D, Agusni I. Detection of *Mycobacterium leprae* in formalin-fixed paraffin-embedded sample by Fite-Faraco staining and polymerase chain reaction. Procedia Chem 2016;18:231–6.
Santos AR, De Miranda AB, Sarno EN, Suffys PN, Degrave WM. Use of PCR-mediated amplification of *Mycobacterium leprae* DNA in different types of clinical samples for the diagnosis of leprosy. J Med Microbiol 1993;39(4):298–304.
Scollard DM, Gillis TP, Williams DL. Polymerase chain reaction assay for the detection and identification of *Mycobacterium leprae* in patients in the United States. Am J Clin Pathol 1998;109(5):642–6.
Shamsi FA, Chaudhry IA, Moraes MO, Martinez AN, Riley FC. Detection of *Mycobacterium leprae* in ocular tissues by histopathology and real-time polymerase chain reaction. Ophthalmic Res 2007;39(2):63–8.
Spencer JS, Dockrell HM, Kim HJ, Marques MA, Williams DL, Martins MV, et al. Identification of specific proteins and peptides in *Mycobacterium leprae* suitable for the selective diagnosis of leprosy. J Immunol 2005;175(12):7930–8.
Truman RW, Andrews PK, Robbins NY, Adams LB, Krahenbuhl JL, Gillis TP. Enumeration of *Mycobacterium leprae* using real-time PCR. PLoS Negl Trop Dis 2008;2(11), e328http://dx.doihttps://doi.org/10.1371/journal.pntd.0000328.
Turankar RP, Pandey S, Lavania M, Singh I, Nigam A, Darlong J, et al. Comparative evaluation of PCR amplification of RLEP, *16S rRNA*, *rpoT* and *Sod A* gene targets for detection of *M. leprae* DNA from clinical and environmental samples. Int Mycobacteriol 2015;4(1):54–9.
Walker SL, Lockwood DNJ. Leprosy. Clin Dermatol 2007;25(2):165–72.
Woods SA, Cole ST. A family of dispersed repeats in *Mycobactenium leprae*. Mol Microbiol 1990;4:1745–51.
World Health Organization. Action programme for the elimination of leprosy. A guide to eliminating leprosy as a public health problem. Geneva: World Health Organization; 1995http://www.who.int/iris/handle/10665/59680.
World Health Organization. Regional Office for South-East Asia, Global Leprosy Programme. Global leprosy strategy 2016–2020: monitoring and evaluation guide. accelerating towards a leprosy-free world. New Delhi: WHO Regional Office for South East Asia; 2017http://apps.who.int/iris/bitstream/handle/10665/254907/9789290225492.