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Paucibacillary Leprosy: Reappraisal using Ziehl-Neelsen staining of slit skin smears and *16S rRNA* Real Time Polymerase Chain Reaction of nasal swabs

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Summary

Background: Leprosy is diagnosed by cardinal signs. Classification of paucibacillary (PB) and multibacillary (MB) leprosy is based on the number of skin lesions and nerve involvement.

Objective: The study was conducted to determine the role played by acid fast bacilli (AFB) positivity of slit skin smear (SSS) in clinical classification.

Methodology: SSSs and nasal swab smears (NSSs) were stained by Ziehl-Neelsen staining. RNAs was extracted from 120 NSSs. NSSs and SSSs were examined by microscopy and *M. leprae* viability in NSS was determined by real time polymerase chain reaction (RT-PCR). NSSs from 50 healthy individuals were used as controls.

Results: Group-A PB cases, classified simply by presenting with <5 patches, showed the presence of AFB in NSSs (7·5%) and SSSs (15%). Group-B PB cases,

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classified on the basis of a negative skin smear were AFB negative. Control group NSSs were AFB negative. RT-PCR of NSSs of PB cases of Group A and Group B were 65% and 87% positive for viable *M. leprae* respectively. All NSSs were positive for *16S rRNA* gene with variable copy numbers. It was noted that 3 SSSs negative AFB cases from Group-A were positive for AFB in NSS. It was also observed that 6 SSSs positive AFB (Group-A) cases were negative for AFB in NSS. It was interesting to note that none of the patients in Group-A was AFB positive in both NSS and SSS. However, all of these patients were positive for *16S rRNA* qPCR in NSSs.
Conclusion: Our findings strongly suggest immediate inclusion of AFB staining of SSS for classification of leprosy.

Keywords: PB leprosy cases, AFB, *M. leprae*, Real Time PCR, BI negative, 16S rRNA gene target, Z-N staining

Introduction

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae* which primarily involves skin, peripheral nerves and mucous membranes. Based on the clinical, bacteriological, histological and immunological manifestations, the disease has been classified on a scale of five group spectrum.[1] The treatment regimens for leprosy were initially designed to treat leprosy cases based on the above classification. However, for convenience of administration of multidrug therapy (MDT) in the leprosy eradication programme (NLEP) under field conditions, the disease has been classified into only two types: paucibacillary (PB) and multibacillary (MB) leprosy wherein MDT is administered for 6 months to PB and 12 months to MB cases.[2] According to this classification any patient having a bacteriological index (BI) value ≥ 2 was given the MB drug regimen and patients having BI value < 2 were given the PB drug regimen.

However, it was later determined that skin smear positivity alone would be an indicator for categorizing a patient as MB[3] and hence every skin smear positive patient was administered the MB drug regimen. Later still, due to the lack of availability of skin smear staining facilities in field situations and in order to cover the remote areas under NLEP, where staining facilities were not available, the classification was further simplified by counting only the number of lesions. Any patient with < 5 lesions is designated as PB and patients having ≥ 5 lesions is classified as MB.[4]

It is well established that *M. leprae* infection is noted very early in the nose before the involvement of skin and nerve.[5] Further, *M. leprae* is known to be excreted in the environment through nasal discharge of infected populations.[6,7] Modern molecular methods have a great potential for detection and identification of *M. leprae* in tissues because they are more sensitive than the conventional methods.[8] The *M. leprae* specific repetitive element (RLEP) was found to be capable of detecting *M. leprae* DNA in 73% of patients with 0 BI.[9] Furthermore, real-time polymerase chain reaction (RT-PCR) was found to be more sensitive for the detection of *M. leprae* DNA sequences from specimens than conventional PCR, and it also gave a quantitative result.[10] Therefore, the present study was done to determine whether the clinical classification of PB, based on number of lesions alone is reliable or whether skin smear staining should be added to determine the appropriate MDT regimen. In addition, the present study also investigated the presence of viable *M. leprae* in the nose of PB patients by quantitative real time qPCR.

Materials and methods

ETHICS STATEMENT

Informed consents were obtained from all eligible participants. The protocol for this study was reviewed and approved by the TLM Ethics Research Committee on 22nd December 2016.

SUBJECTS

New PB leprosy patients were enrolled from the Outpatient Department of Dermatology at the All India Institute of Medical Sciences (AIIMS), Safdarjung Hospital and TLM Hospitals at Shahdara (Delhi), Purulia (West Bengal), Naini (Uttar Pradesh) and Muzaffarpur (Bihar). All PB patients were enrolled in the study after obtaining their informed consent.

A total of 120 PB patients and 50 healthy hospital staff were enrolled as healthy controls (HC).

At AIIMS and Safdarjung hospitals (Group-A) PB patients were classified initially by number of patches followed by collection of skin smears and nasal swabs. At TLM hospitals nasal swabs were collected from PB patients who were classified by number of patches and AFB negative skin smear (Group-B). 50 hospital staff comprised the control group (Group-C).

SLIT SKIN SMEAR (SSS)

Routine SSS samples were made from the lesions and were stained by Ziehl-Neelsen (Z-N) staining method as described earlier.[11]

Nasal swab smear (NSS): Per nasal swabs (Sterile Pure Viscose Swab, HIMEDIA Catalog no. PW043) were used for the collection of nasal swab samples. The protocol described earlier by Lavania *et al.*[12] was used. In brief, swabs were dipped in normal saline immediately prior to use, and passed through the base of the turbinate until the posterior wall of the nasopharynx was encountered. The nasal swabs were obtained separately from both nostrils of the PB cases and controls. Swabs collected from both the nostrils were initially used for NSS before transferring the same to a tube containing RNA Later (Sigma). The swabs thus collected were maintained at 4°C and transported at 2–8°C to the laboratory. The collected nasal swabs were stored at 4°C for further analysis. Positive and negative controls were kept for quality controls while performing the polymerase chain reaction (PCR) assay.

Reference *M. leprae g* DNA (strain Thai53) was used as a positive control which was procured from BEI Resources, and plain normal saline soaked swab was used as the negative control for PCR.

Z-N Staining of NSS and SSS

Smears prepared on glass slides from the nasal swabs (NSs) and SSSs were allowed to dry before staining by Z-N staining method. Every SSS and NSS stained smear was examined under oil immersion field in a binocular microscope by two senior laboratory technicians and one leprosy expert for presence of AFB and for determination of bacterial index (BI). BI was calculated based on Ridley's logarithmic scale.[13]

RNA extraction and DNase treatment procedure

RNA extraction of NSS samples was done by using Trizol (TRI) reagent and glycogen mixture according to a standard laboratory protocol.[14] Briefly, the homogenized sample in solution containing 800 μl TRI Reagent and 200 μl glycogen, was centrifuged at 12,000 x g for 10 min at 4°C. The elimination of genomic DNA was performed by the acid phenol extraction method. After the extraction the aqueous phase was re-extracted with 200 μl of chloroform to remove traces of phenol. RNA was extracted from the upper aqueous phase using 500 μl of cold isopropanol. The pellet was washed with 70% ethanol and stored at − 80°C freezer or processed by drying the pellet followed by dissolution of the pellet in diethyl pyrocarbonate treated water. The total RNA suspension was treated with DNase I (Cat No: M0303S, NEB). The reaction was setup on ice by adding and mixing of all DNase components in 0·6 μl micro centrifuge tube and incubated at 37°C for 10 minutes followed inactivation by adding 1 μl of 0·5 M EDTA at 75°C for 10 minutes. The complete removal of gDNA from RNA samples was confirmed by using a PCR assay. The concentration of the RNA sample was determined on a UV Spectrophotometer (Shimadzu Inc. Japan). Total RNA was reverse transcribed (RT) into cDNA. The RT reaction was performed according to manufacturer's instruction and all cDNA samples were stored at − 20 °C until further use.

Real Time PCR amplification

Initially the standard curve was prepared with a 7-fold dilution of *M. leprae* cDNA from 1ng/reaction to 1fg/reaction. cDNA was amplified using *16S rRNA* gene on real time PCR (Rotor Gene-Q Qiagen USA). Briefly, 2X of Brilliant III SYBR Green Q PCR Master Mix (Agilent Technologies, Cat No.: 600882), 200 nM concentration of each of the forward and reverse primers of *16S rRNA* gene and 200 ng of cDNA as templet (total of 25 μl reaction mix) was amplified in Rotor-Gene Q with cycling conditions as 95°C for 10 min (initial denaturation and activation of enzyme) followed by 37 cycle of 95°C for 30s, annealing at 60°C for 60s and extension at 72°C for 30s. Positive and negative controls without template were run with every amplification.

Results

NSS AND SSS FROM PB PATIENTS

AFB Positive: Out of 40 PB cases in Group A where classification of cases was done simply by the number of patches, SSS of six (15%) were positive for AFB and BI of skin smears ranged from 0·6+ to 3+). In addition, of these 40 patients, three (7·5%) had AFB positive NSS (Table 1).

ZN staining of NSS showed presence of both extracellular and intracellular AFB (Figure 1).

However, NSS from all BI negative PB cases recruited at both Group A and Group B hospitals and all HCs were negative for AFB (Table 1).

16S rRNA positive NSS: The viability assessment of *M. leprae was* performed by RT-PCR using *16S rRNA* gene target from NS. The cut-off Ct value for qPCR was taken as average Ct> 30. The amplification of the target gene was verified with gel electrophoresis which was negative for DNA amplification. RT-PCR of NSs showed the presence of 65% and

Table 1. Acid fast staining of SSS, NSS and RT PCR of NSS from PB patients.

Group	Number of patients/ controls	Number AFB positive in NSS (%) [BI range 0·6 to 2+]	Number AFB positive in SSS (%) [BI range 0·6 to 3+]	Number positive (%) (range of *16S rRNA* copy number)
*Group-A	40	3(7·5%)	6(15%)	26(65%) (2×10^4 to $7{\cdot}91x \times 10^6$)
#Group-B	80	0	0	70 (87%) ($1{\cdot}02 \times 10^2$ to $3{\cdot}42x \times 10^5$)
Group-C	50	0	ND	0%

*Paucibacillary (PB) cases classified by number of patches only; # PB cases classified by number of patches and APB negative skin smears; AFB = Acid fast bacilli; Group-A = All India Institute of Medical Sciences + Safdarjung Hospitals; Group-B = The Leprosy Mission Hospitals; Group-C = healthy control; PCR = Polymerase chain reaction; ND = Not done: BI = Bacterial Index; NSS = Nasal swab smear; SSS = Slit skin smear.

87% viable *M. leprae* in PB cases in patients from Group A and Group B respectively. All NSS from controls were negative for presence of any viable bacilli. All NSS samples were positive for *16S rRNA* gene of *M. leprae* with copy numbers varying from a minimum of $5{\cdot}85X10^4$ to a maximum of $3{\cdot}48X10$.[5] It was noted that three cases from AIIMS and Safdarjung hospital that were negative for AFB in the skin smear were positive for AFB in NSS (BI 0·6+ to 2+). It was also observed that six cases from AIIMS and Safdarjung hospital that were negative for the AFB in the nose, were found to be positive for AFB in the skin smear (BI range 0·6+ to 3+) (Table 2).

It was interesting to note that none of the patients in the group was found to be positive for AFB in both NSS and SSS.

Discussion

Leprosy transmission is still continuing and it has been estimated that 63% of total leprosy cases of the world are living in India.[15] Since 1995 onwards[4] classification of leprosy has been based on number of patches and any patient having <5 patches is being classified as PB leprosy and is administered 6-month regimen of MDT. Although it was realised that the only

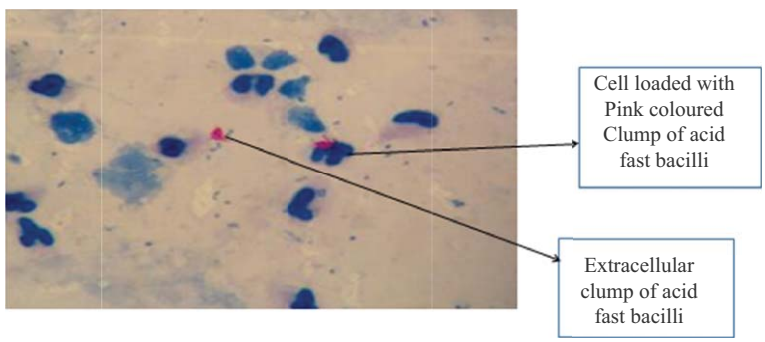

Figure 1. Nasal swab smear from a PB case showing presence of extracellular and intracellular AFB, (Z-N Stain X 1000).

Table 2. Acid fast staining of NSS, SSS and qRT PCR of *16S rRNA* gene of *M. leprae* in nasal swab samples of PB patients.

Case	Age	Sex	Hospital	Patches	Type	AFB from Nasal swab	AFB from Slit skin smears	qRT-PCR-Ct Values	*16S rRNA* gene copies in nasal swab
Case 1	35	M	AIIMS	3	PB	0·6+	Neg	21·8	$8{\cdot}99 \times 10^4$
Case 2	38	M	AIIMS	4	PB	1+	Neg	22·42	$5{\cdot}85 \times 10^4$
Case 3	18	M	Safdarjung	4	PB	2+	Neg	22·4	$5{\cdot}94 \times 10^4$
Case 4	54	M	Safdarjung	4	PB	Neg	2+	21·55	$1{\cdot}07 \times 10^5$
Case 5	36	M	Safdarjung	3	PB	Neg	1·6+	20·48	$2{\cdot}24 \times 10^5$
Case 6	33	F	Safdarjung	1	PB	Neg	0·6+	19·84	$3{\cdot}48 \times 10^5$
Case 7	50	M	Safdarjung	3	PB	Neg	1·6+	22·25	$6{\cdot}61 \times 10^4$
Case 8	27	M	Safdarjung	3	PB	Neg	0·6+	20·02	$3{\cdot}08 \times 10^5$
Case 9	31	M	AIIMS	5	PB	Neg	3+	20·52	$2{\cdot}17 \times 10^5$

important criterion of skin smear positivity for categorizing a patient as MB but still this single meaningful criterion,[3] one of the cardinal signs of leprosy, was discontinued from the programme due to non-adherence to the quality of Z-N staining of skin smears at the field level.[4] However, as this is a very important criterion for making a decision in categorization of a case from PB to MB for the suitability of duration of drug regimen of a patient to either 6 months or 1 year, the present study was carried out to find out the percentage of SSS positives present in PB patients classified by number of patches. It could be seen from Table 1 that 15% of those patients should have been classified as MB but have been classified as PB and would have been treated with 6 months MDT regimen under the programme. This clearly indicates the importance of SSS staining for AFB for estimation of BI in the classification of leprosy and for inclusion of patients into a proper drug regimen for adequate therapy. This might be one of the reasons for relapse and spread of *M. leprae* infection in the community due to inadequate treatment of those BI positive PB classified cases. Considering the above the leprosy control programme could initiate introduction of SSS staining at the field level. As the vertical set up of the control programme has been now integrated with the general health services it would not be a difficult proposition because SSSs collected from the field could be easily be transported to the PHC or District laboratory where Z-N staining is a routine procedure for diagnosis of tuberculosis (TB). Leprosy staff can easily be trained by the TB technicians on staining procedures for establishment of SSS staining under the programme. Further, NSSs of patients from Group B hospitals wherein PB classification was performed after determination of BI and all BI negative patients from Group A hospitals, were negative for AFB. This clearly indicates that determination of BI is a very crucial investigation for screening the leprosy population discharging AFB in the environment. The importance of the respiratory tract for exit of bacilli has been well documented earlier.[6,16–19]

Advanced molecular techniques such as absolute quantification by real time PCR was performed using *16S rRNA* gene of *M. leprae* in nasal cavity of all PB cases. It was noted that while 65% of NS samples where BI was determined later after obtainment of NS samples in Group A and BI negative Group B NS samples were 87% positive for *16S rRNA*. This finding of higher percentage positivity in nose for viable *M. leprae* from BI negative patients compared to that of BI positive patients is very difficult to explain. However, it could be reasoned that as the method of sample collection from nostrils is not a well standardised

procedure therefore, the results are expected to yield variations. Further, from the present study it could be noted that whatever the status of BI, nose remains positive for viable *M. leprae* (Table 2). However, as treatment is known to make bacterial negativity earliest in the nose[20,21] an adequate treatment with full course of MDT is expected to stop AFB discharge from nose and thus might check the risk of transmission. In spite of this a future cohort study will be required to establish the presence of viable bacilli in nasal discharge of PB patients while on treatment. The finding of viable *M. leprae* by *16SrRNA* gene qRT-PCR in NSS of BI negative PB patients can be explained by the use of more sensitive PCR based molecular technique than the simple visual observation of Z-N stained AFB under microscope.

From the above, it could be emphasised that SSS staining for AFB for presence of *M. leprae* should be reintroduced in the leprosy control programme for adequate MDT regimen and to check transmission of disease in the community.

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