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In situ Hybridization in the Histological Diagnosis of Early and Clinically Suspect Leprosy[1]

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ABSTRACT

The present study tests the utility of the *in situ* hybridization procedure for *M. leprae* rRNA in the histological diagnosis of early leprosy and clinically suspect leprosy, both diagnostically demanding situations. The histological confirmation obtained with routine histopathology (Haematoxylin-Eosin staining for studying morphologic alterations and Fite-Faraco staining for demonstration of acid-fast bacilli) were 32% for early leprosy and 25% for clinically suspect leprosy. With performance of the *in situ* hybridization on the histologically unconfirmed cases, the positivity rates obtained were 58.8% and 55%, respectively. The results of the study confirm the utility of the procedure in the diagnostically difficult situations of early and suspect leprosy, and it is proposed that the procedure be employed in situations of clinical doubt.

RÉSUMÉ

Cette étude a eu pour but d'évaluer l'utilité de l'hybridation *in situ* des ARNr de *M. leprae* dans le diagnostic histologique de la lèpre précoce ainsi que de la lèpre cliniquement suspecte, des situations toutes deux très exigeantes en terme de diagnostic. La confirmation histopathologique obtenue par les techniques et lectures de routine (Hémalun-éosine pour étudier les lésions et le Fite-Faraco pour la mise en évidence des bacilles acido-résistants) a été de 32% pour la lèpre précoce et de 25% pour la lèpre cliniquement suspecte. Avec la mise en œuvre de l'hybridation *in situ* des cas non confirmés par l'examen histologique, les taux de positivité furent de 58,8% et de 55%, respectivement. Les résultats de cette étude confirment l'utilité de la procédure d'hybridation *in situ* dans les situations diagnostiques difficiles des lèpres précoces ou suspectes et il est proposé que cette procédure soit employée en cas de doute clinique.

RESUMEN

El presente estudio prueba la utilidad del procedimiento de hibridación *in situ* del rRNA de *M. leprae* en el diagnóstico histológico de la lepra temprana y de la enfermedad bajo sospecha clínica, ambas situaciones difíciles de diagnosticar. La confirmación histológica obtenida por histopatología de rutina (hematoxilina-eosina para estudiar las alteraciones morfológicas y Fite-Faraco para la demostración de bacilos ácido-resistentes) fue del 32% para la lepra temprana y 25% para la lepra en sospecha clínica. En la hibridación *in situ* de los casos histológicamente no confirmados, las tasas de positividad obtenidas fueron del 58.8% y 55%, respectivamente. Los resultados del estudio confirman la utilidad del procedimiento en las situaciones de difícil diagnóstico de la lepra temprana y de la lepra bajo sospecha, y se propone que el procedimiento sea empleado en situaciones de incertidumbre clínica.

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Leprosy is a chronic mycobacterial disease caused by *Mycobacterium leprae*. The diagnosis of the disease is primarily clinical and is dependent on the presence of two cardinal signs ([8]), namely, the presence of typical skin lesions with hypoaesthesia, and/or the presence of acid-fast bacilli (AFB). In the established form of the disease, the diagnosis is relatively simple, more particularly so when additional characteristic features such as typical skin lesions, cutaneous infiltration, and nerve thickening are present ([23]). In the early stages, however, the cardinal signs are yet to manifest and the diagnosis becomes problematic. There is thus the situation, more often encountered in areas of endemicity, where the diagnosis of leprosy cannot be made due to the absence of cardinal signs, yet the diagnosis of leprosy cannot be precluded as the lesion is too visually suggestive—a condition often referred to as "suspect leprosy" ([24, 25]). In such situations, the clinician resorts to histopathology to help resolve clinical doubt.

Routine histopathology requires for the confirmatory diagnosis of early leprosy, (i) the presence of infiltration within dermal nerves, or (ii) the presence of AFB, both of which are not uniformly present ([27]). Consequently, the histological confirmation of clinically early or suspect leprosy is possible only in a low percentage of cases. One way to augment the histological diagnosis is to use immunohistochemical (IHC) procedures which demonstrate the presence of mycobacterial antigens ([20]). As AFB are sparse in early forms and are rarely demonstrable in tissue sections despite serial sectioning, immunostaining, being a sensitive technique, can be used to demonstrate the presence of mycobacterial antigens in AFB negative specimens thereby pointing to the aetiology of the non-specific pathology seen ([1, 21]). There have, however, been some reservations expressed regarding the specificity of the procedure, particularly when anti–BCG is employed as the antigen-detecting antibody. *In situ* hybridization (ISH), first described by Pardue and Gall ([11]), is a powerful technique for demonstrating organism-specific nucleic acid sequences in tissue sections, and with the advent of non-radioactive and highly sensitive systems of labelling and detection ([19]), has become more amenable for diagnostic application on routinely processed paraffin sections ([2]). Nucleic acids specific to a variety of pathogens have been demonstrated in tissues using *in situ* hybridization ([18]). The present study tests the applicability and the usefulness of the procedure in the histological diagnosis of early and clinically suspect leprosy.

MATERIALS AND METHODS

Patients were selected from the those attending the out-patient department of the Institute. Only cases *not* having a history of prior consultation or treatment were chosen and these were clinically categorized using defined criteria ([4, 23]). The categories chosen were defined as: (i) indeterminate (Idt) leprosy, where the lesions were flat with vaguely defined margins and showing clearly demonstrable hypoaesthesia, (Hypoaesthesia was tested using the pin prick method.) (ii) Early Borderline Tuberculoid (BT) is leprosy where the lesions were flat with margins defined in parts, and hypoaesthesia are clearly demonstrable. [(i) and (ii) constituted the clinical category of early leprosy.] (iii) Clinically "suspect" leprosy is leprosy in which the lesions were visually suggestive of leprosy showing the features in categories (i) and (ii), but *without* clearly demonstrable hypoesthesia.

The clinical history and features were recorded, and slit-skin smears from the lesions were performed to examine for the presence of AFB ([26]). Incisional skin biopsies of the lesions were taken in 10% buffered formalin after taking written consent, and the tissue specimens were processed for routine histopathologic examination, i.e., for paraffin wax embedding, and subsequent Haematoxylin-Eosin staining for studying morphologic alterations, and Fite-Faraco staining for demonstrating AFB ([28]). Sections which, on such examination, showed a non-specific pathology (in the form of a mononuclear cell infiltration usually in the upper or mid-dermis, in perivascular and periadnexal locations), and which were negative for AFB, were subjected to the *in situ* hybridization procedure. Glass slides coated with organosilane (APES, Sigma Cat. No. A3648) were used such that the tissue specimens would remain adherent to the slides during the harsh conditions necessitated during the use of the *in*

situ hybridization procedure. Apart from the test specimens, each set of slides stained contained a positive control in the form of a histologically AFB-positive specimen. The negative control consisted of the same AFB positive specimen on which the same procedure was performed as with the other specimens but without the probe. A section of clinically normal skin from a healthy contact of a BT patient was additionally included.

The probes used were oligonucleotides, with the 18-mer (nucleotide length) oligonucleotide targetting the 16s rRNA of *M. leprae*, and the 20 mer targeting the 5s rRNA of *M. leprae*, and bearing the sequences: 5′ CTT CAA GGC GGA TGT CTT 3′ and, 5′ GGC TAC GGC GGG GGA CTC AA 3′, respectively.

The 16s rRNA targeting probe is recognized to be specific to *M. leprae* and has been used by several workers. The 5s rRNA targeting probe was tested as an additional option.

The probes were procured commercially from a firm [Bioserve Biotechnologies (India)] to whom the desired sequence was provided. The oligonucleotides were synthesized using phosphoramidite chemistry and the product was verified by 12% polyacrylamide gel electrophoresis and optical density measurements. The oligos, which were supplied in a lyophilized state, were reconstituted in 600 µl of deionised water to give a concentration of 119 picomoles/µl in the case of the 16s rRNA targeting probe, and a concentration of 112.8 picomoles/µl in case of the 5s rRNA targeting probe. Oligonucleotides procured from the firm are currently being used in various studies at the Institute, either as primers for PCR studies targeting *M. leprae* and *M. tuberculosis* specific sequences, or as probes in membrane hybridizations (Southern and Dot Blot).

The probes were labeled with digoxigenin using the 3′ end-labelling method of oligolabelling, employing the enzyme terminal deoxynucleotide transferase (Roche Molecular Biochemicals, Cat. No, 1362372) which attaches a single molecule of digoxigenin to the 3′ end of the probe. Probe labeling was checked by direct detection on nylon membranes using the labeled control oligonucleotides provided in the kit as the standard (Fig. 1A). Probe specificity was checked by dot-blot hybridization using nylon membranes (Roche, Cat. No.1209299). Armadillo-derived *M. leprae* DNA was used as the target at a concentration of 0.25 ng/µl (vol. = 1 µl/spot) and the labeled oligoprobe at a final concentration of 1 ng/µl (vol. = 1 µl/spot) (Fig. 1B).

The *in situ* hybridization procedure was performed as detailed below. Section pretreatments: (i) sections were dewaxed, rehydrated and treated with 0.2N HCl to permeabilize the sections to the probe; (ii) proteolysis was performed with 500µg/ml of pepsin in 0.2N HCl at 37°C for 20 min, the proteolysis stopped with 2% glycine, and the sections subsequently post-fixed with 4% paraformaldehyde for 20 min; (iii) acetylation with acetic anhydride in ethanolamine (0.2%) was done to quench free amino groups which helped reduce nonspecific signals; (iv) pre-hybridization was done using the hybridization mix in which the probe was not added, for a period of 2 hr at 42°C. The hybridization mix was prepared for a final volume of 3000 µl (3ml) for each set of slides with 50 µl being employed per tissue section.

The individual constituents of the hybridization mix and their final concentrations were as follows:

• Denhardt's Reagent:	5× (1 × Denhardt's Reagent = 0.02% Ficoll + 0.02% BSA + 0.02% polyvinyl pyrrolidine)
• SSC	5× (SSC = Standard sodium citrate)
• SDS	0.5% (SDS = Sodium Didoceyl Sulphate)
• EDTA	1 mM (EDTA = Ethylene Diamine TetraAcetate)
• Tris HCl (pH7.5)	40 mM
• Formamide	25%
• Carrier tRNA	250 µg/ml
• Dextran Sulphate	10% (w/v)
• PROBE	1 µg/ml
• De-ionized H_2O	to make up vol.

Target denaturation was achieved by application of heat at 95°C for 3 to 4 min, which was followed by immediate, rapid cooling with ice.

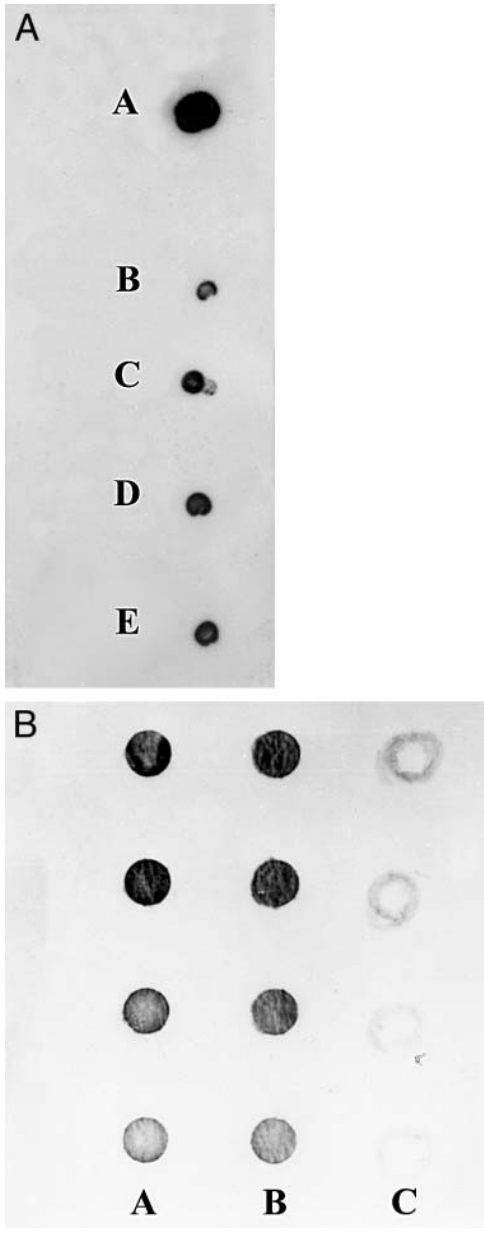

Fig. 1. (A) Confirmation of labeling of probes by direct detection on nylon membranes. A = Labeled control oligo; B, C = Labeled 16s r RNA targeting probe; D, E = Labeled 5s r RNA targeting probe. (B) Confirmation of specificity of 16s and 5s targeting probes by membrane hybridization using armadillo-derived *M. leprae* DNA as target. Target amounts = 1µg, 0.5 µg, 0.25 µg and 1µg. A = Dilutions of 16s r RNA targeting probe; B = Dilutions of 5s r RNA targeting probe; C = Negative control.

Continuing the hybridization proceedure: (v) Hybridization was carried out at a final probe concentration of 1 µg/ml of hybridization mix, at 42°C, applied overnight (15 hr). (vi) Post hybridization washes were done with Standard Sodium Citrate (SSC, of strengths 2×, 1× and 0.5×) containing 0.1% sodium dodecyl sulphate (SDS), over a time period of 45 min. (vii) Visualization was done using the enzyme alkaline-phosphatase and with NBT/BCIP (Nitro-Blue-Tetrazolium/Bromo-Chloro-Indoyl-Phosphate) as the substrate chromogen. (viii) Two percent Neutral Red was used as the counterstain, and, DPX, a synthetic resin, was the mounting medium employed.

RESULTS

Twenty-five cases each from the clinical categories of early leprosy (Idt = 15, BT = 10) and suspect leprosy, were included in the study. The cases were all adults (age ranges 15 to 50 yrs for both categories), predominantly male, and all negative for AFB on skin smear examination. Routine histopathology confirmed the diagnosis of leprosy (all histologically Indeterminate) in 8 (32%) of the cases of early leprosy with AFB being seen in 2 cases; in clinically suspect lesions, histological confirmation was possible in 5 (20%) cases with AFB being seen in a solitary case. *In situ* hybridization performed in the remaining cases with the 16s rRNA-targetting probe yielded positive signals in 10/17 (58.8%) cases from the early leprosy category and in 11/20 (55%) of the clinically suspect cases (The Table). The positivity rates obtained with the 5s rRNA-targeting probe were closely similar with positive signals being seen in 9/17 (52.9%) cases of early leprosy and in 12/20 (60%) cases of suspect leprosy.

Positive signals, viewed as deep blue precipitates of the NBT/BCIP-Alkaline Phosphatase reaction, were easily discernible against the red background of the Neutral Red counterstain, and were found to be mostly located among the cells of the dermal infiltrates (Figs. 3, 4, 5). The morphology of the tissue specimens was fairly well preserved and the signals could be located in the context of tissue pathology. Background and non-specific staining were minimal, and the latter was usually seen in the cells of the basal epidermis, eccrine glands,

THE TABLE. *Data resulting from routine histopathology and* in situ *hybridization study of early and clinically suspect leprosy.*

16s rRNA targetting probe

Clinical category	No. of cases	Diagnosis of leprosy (routine HP)	% HP positive	Nos. *IS*H tested	Nos. *IS*H positive	% *IS*H positive
Early leprosy (Idt, BT)	25	8	32	17	10	58.83
Clinically suspect leprosy	25	5	20	20	11	55

5s rRNA targetting probe

Clinical category	No. of cases	Diagnosis of leprosy (routine HP)	% HP positive	Nos. *IS*H tested	Nos. *IS*H positive	% *IS*H positive
Early leprosy (Idt, BT)	25	8	32	17	9	52.9
Clinically suspect leprosy	25	5	20	20	12	60

and occasionally in the nuclei of the inflammatory cells. Dark-blue thick NBT/BCIP crystal precipitates were at times seen, but could be easily distinguished from the specific signals by virtue of their morphology and bizarre locations. Any ambiguous staining seen was treated as a negative result.

The specificity of the signals was supported by the control results—positive results in the positive control, and absence of signals in the negative control and in normal skin. The specificity of the probes had already been confirmed by membrane blotting as described earlier.

DISCUSSION

There has been a decline in the number of leprosy cases worldwide ([36]), and concomitant with it there has been a rise in the fraction of both early cases and of clinically suspect cases. Both are situations where diagnostic difficulties are encountered. Routine histopathology's help in these situations is rather limited, a fact being borne out by several studies ([10, 24, 34]). In a well documented epidemiological study conducted in Malawi, the histological confirmation of suspected cases of leprosy varied from 29% to 58%, with the percentage confirmation being strongly dependent on the degree of clinical suspicion involved. The clinical categories in the present study were chosen by clinicians using defined criteria, and clinical suspicion was accorded by more than one clinician, which helped maintain a degree of homogeneity in the series of cases studied. The observed histopathological confirmation rates in the present study are slightly lower, which is not unusual given a different environmental situation.

As stated earlier, immunohistochemical staining procedures which demonstrate mycobacterial antigens can be used to augment the diagnosis, particularly in situations where AFB are not demonstrable ([1, 14, 21]). The contribution made by the use of these procedures is noteworthy but limited, with immunostaining positivity in AFB negative specimens averaging about 35% in the studies quoted above. This leaves a significant number of cases diagnostically unconfirmed. *In situ* hybridization offers a very high specificity and a high sensitivity, and, as stated earlier, has been put to widespread use in the diagnosis of bacterial and viral infections; in some situations, it is clearly more sensitive than immunohistochemistry ([30]). The procedure has, however, not been tested on tissue sections for its diagnostic utility in leprosy and there is a general perception that the procedure is not sensitive enough to contribute to the diagnosis of leprosy, particularly the paucibacillary forms

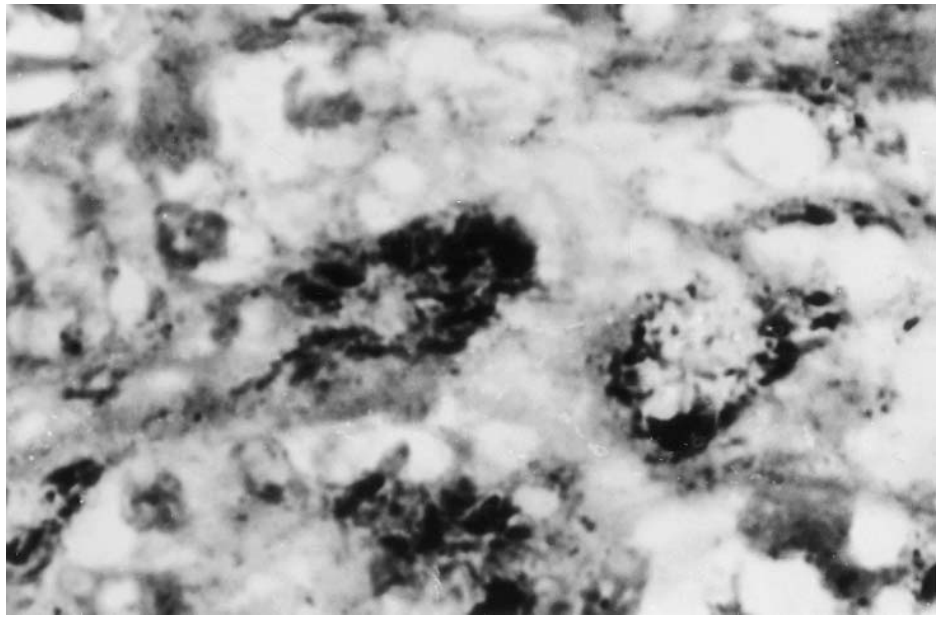

FIG. 2. Microscopic appearance of a positive control specimen. Intense positive signals of *in situ* hybridization are seen in the macrophages infiltrating the dermis of an AFB positive LL specimen. (Detection system—Alkaline phosphatase—NBT/BCIP. Counterstain: Neutral Red. ×670).

([3, 12]). In the present study however, positive signals could be obtained in 58.8% of cases of early leprosy and in 55% of cases of clinically suspect leprosy where the histological features were nonspecific. Viewed in the context of expectations, the positivity rates would appear high. Though the relatively high positivity rate could be attributable to

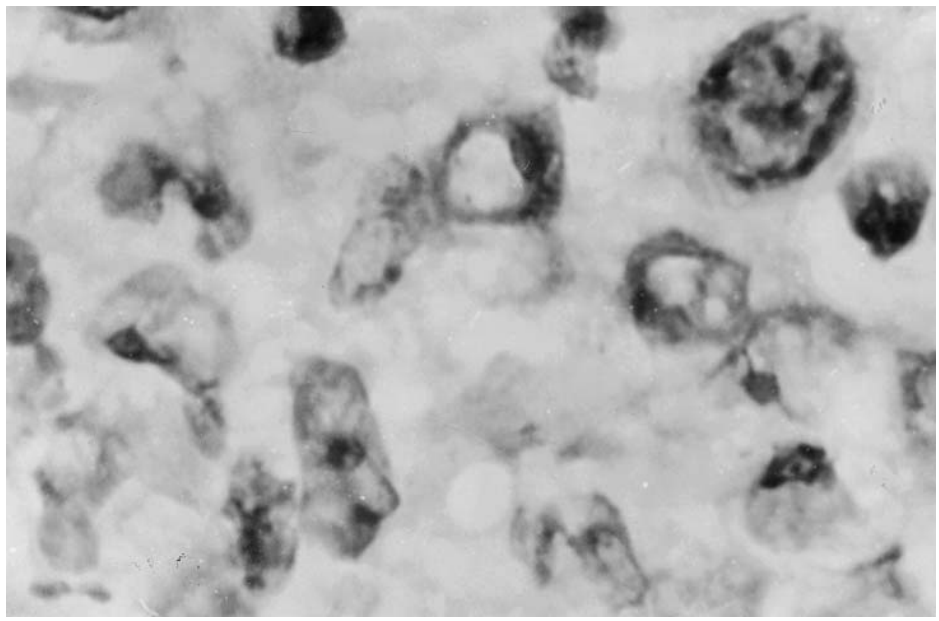

FIG. 3. Appearance of a negative control where the hybridization procedure was performed without the probe. (Detection system—Alkaline phosphatase—NBT/BCIP. Counterstain: Neutral Red. ×670).

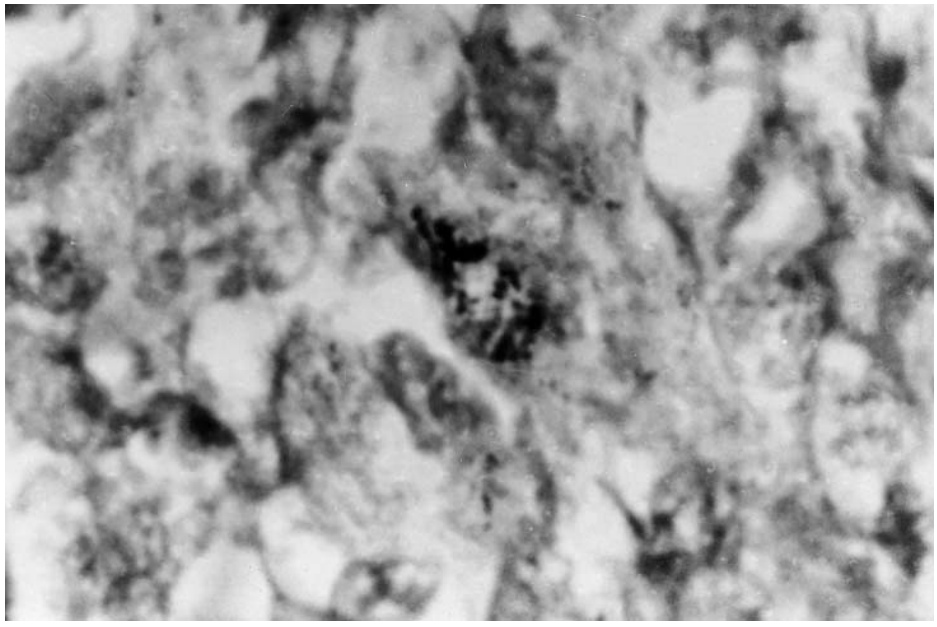

FIG. 4. Positive hybridization signals seen in the upper derma infiltrate of a tissue specimen from a case clinically diagnosed as indeterminate leprosy where routine histopathology was nonspecific and AFB absent. (Detection system—Alkaline phosphatase—NBT/BCIP. Counterstain: Neutral Red. ×670).

a certain extent to a hospital-based bias in the selection of cases, high positivity rates with the *in situ* hybridization procedure are not unknown. Positivity rates of the order seen in the present study have been reported in varied situations. Investigators have reported the detection of *M. avium* subspecies *paratuberculosis* in 40% of diseased tissues

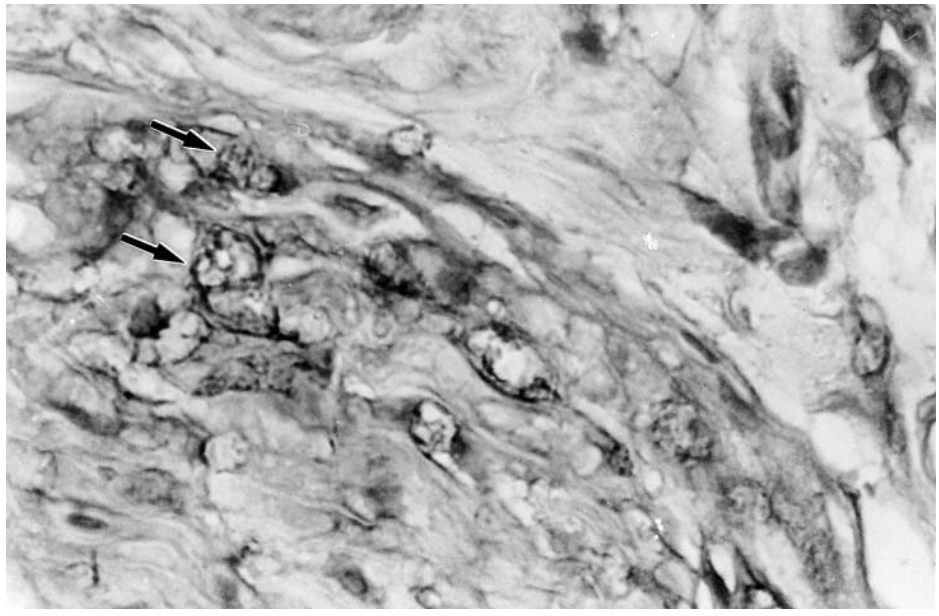

FIG. 5. Positive hybridization signals are seen in the subperineurial region of a dermal nerve in a case of clinically suspect leprosy. Routine histopathological examination revealed a non-specific pathology and AFB were absent. (Detection system—Alkaline phosphatase—NBT/BCIP. Counterstain: Neutral Red. ×468).

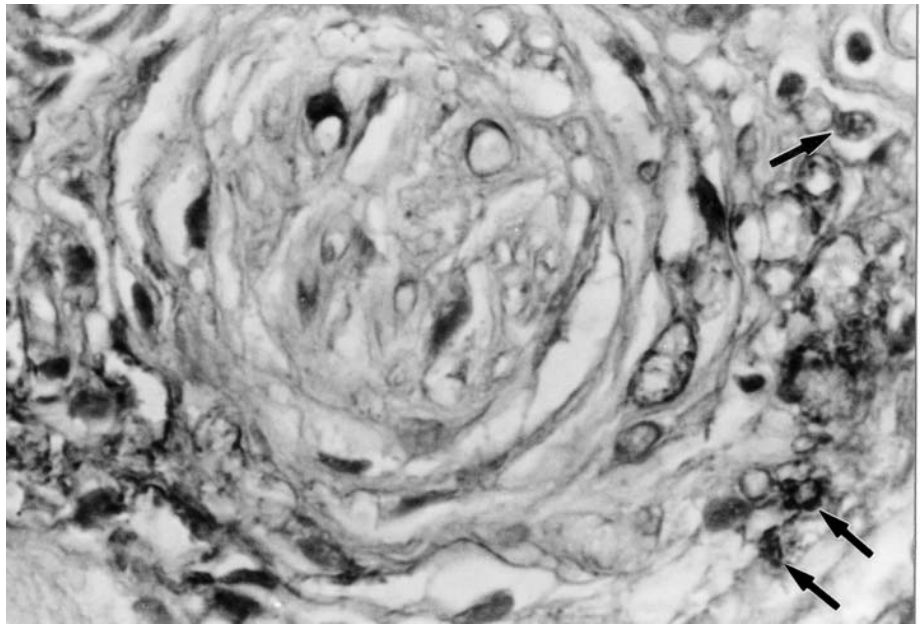

FIG. 6. Positive hybridization signals seen in the perineural infiltrate of a skin biopsy specimen in a case clinically diagnosed as early leprosy; acid fast bacilli were not demonstrable. (Detection system—Alkaline phosphatase—NBT/BCIP. Counterstain: Neutral Red. ×670).

from patients with Crohn's disease using *in situ* hybridization ([15]); a more recent study reported a positivity of 73% in similar cases and in whom, additionally, granulomas were absent ([32]). Cytomegalovirus DNA could be seen in 9 of 13 colonoscopic biopsies which were histologically negative ([29]), and Human Papilloma Virus was demonstrable in 8/11 histologically normal tissue of patients with genital cancer ([17]). Though exact parallels cannot be drawn from these studies, it cannot be denied that the *in situ* hybridization procedure is sensitive enough to give good positivity rates and that it works in histologically negative situations.

Oligonucleotides were chosen as the probes because they penetrate well into tissues. The particular oligonucleotides used in the study were chosen from a panel of *M. leprae* targeting probes bearing sequences complementary to those encoding parts of the 18kDa protein ([35]), 36kDa protein ([13]), 16s rRNA ([5, 16]), and 5s rRNA of *M. leprae*, and whose specificity was confirmed using dot-blot hybridization. Each of these oligonucleotides were developed for use as primers in procedures employing the polymerase chain reaction (PCR), which held great promise in the diagnosis of paucibacillary states where the causative pathogen was not demonstrable. Several studies have been performed and replicated on a variety of specimens ranging from mouse footpad biopsies, to biopsies of skin lesions from patients, either in the form of frozen sections or paraffin-embedded sections. Importantly, the procedure could be performed on ethanol-fixed and neutral-buffered formalin fixed tissue specimens. Detection limits have varied between 1 and 100 bacilli, and the positivity rates in biopsy specimens from paucibacillary leprosy with a BI of 0 have varied between 35% and 73%. Thus, from a purely diagnostic point of view, PCR on material extracted from tissues would be simpler and more sensitive compared to the *in situ* procedures. The constraints lie in the distinct possibility of false positive results due to external contamination and in the lack of structural correlation, both of which are obviated with use of *in situ* procedures.

The probes targeting 16s rRNA and 5s rRNA were found to perform the best, producing strong, consistent signals during standardization, and were therefore used in the present study. The 16s rRNA of *M. leprae* is known to bear sequences specific to

the organism, which has been exploited as an identification tool in several studies ([16, 22, 33]); the 5s rRNA-targeting probe which performed equally well provides an additional option for use.

Digoxigenin was chosen as the label because of its high sensitivity, which approaches that of radioactive labels ([31]). The 3′-end labelling and tailing methods of oligonucleotide labeling were tried and both the methods were found to perform equally well despite the latter's avowed higher sensitivity; the 3′-end labeling procedure was eventually employed. The detection system employed—the alkaline-phosphatase NBT/ BCIP system—is recognized to be the most sensitive ([6]), and brief dips in alcohol and xylene, which are necessary subsequent steps, did not wash away the blue-black end product. The color of the end product precluded the use of Haematoxylin (the ideal) as the counterstain, and 2% Neutral Red was used in its stead.

In addition to the categories of specimens chosen for the study, the *in situ* hybridization procedure was performed on histologically confirmed cases to get an idea of the overall sensitivity of the procedure. Of the ten cases studied, 2 cases were negative by ISH, but in both the cases the histologic diagnosis was based on morphological alterations, and not on the presence of AFB. The sensitivity of the procedure is therefore high and the specificity was indicated by the absence of signals in the negative controls as well as in normal skin.

Some aspects of the *in situ* hybridization procedure that were brought to the fore during the performance of the study deserve mention. A major consideration is the technical difficulty wrought by the length of the entire procedure, which extends into the third day. Occasionally, the procedure itself may fail, or the tissue may be either lost or suffer gross disruptions in its morphology. These are related to the necessarily miniscule volumes of the reagents employed during the entire procedure, and to the relatively harsh conditions which prevail during the performance of target denaturation, and during the pre-hybridization and hybridization steps of the procedure. Nevertheless, these encumbrances should not be a deterrent to the effective use of the procedure, as the yields more than compensate for the efforts expended. The procedure is well worth considering for use in situations of clinical doubt.

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