RESEARCH PAPER

Assessment Of Sensitivity Of Probes Targeting Ribosomal Rna Vs Dna In Hansen'S Disease

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Abstract

The relative sensitivity of three *Mycobacterium Leprae* specific probes targeting ribosomal genes sequence has been compared in clinical specimens along with DNA. This analysis was carried out in diluted biopsy homogenates of lepromatous (LL) cases. The overall sensitivity of targeting rRNA vs DNA in specimens from paucibacillary (PB) and multibacillary (MB) was also accessed. The biopsies / slit smears were collected and nucleic acids were extracted by an improved integrated physio – chemical procedure. DNA and RNA were fractionated and measured quantities from similar bacterial populations were blotted on nitro – cellulose membrane. Three probes were synthesized, end – labeled and hybridization with blotted DNA as well as RNA was done. It was observed that rRNA targeting was 10 – 100 fold more sensitive as compared to DNA detection. The sensitivity of targeting rRNA was significantly higher in specimens with low bacillary numbers. These observations indicate greater potential for clinical application for rRNA targeting as compared to DNA for detection of *Mycobacterium Leprae* from various clinical specimens.

Key words:- Diagnosis, rRNA, DNA, Probes, Sensitivity, *M. leprae*.

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Introduction

Hansen's disease is a chronic and disabling infectious disease of man caused by Mycobacterium Laprae. It has a wide clinical spectrum and is operationally classified into paucibacillary (PB) and multibacillary (MB) cases. Treatment of leprosy has drastically changed over the last 10-15 years. Multidrug treatment (MDT) campaigns have led to decline in the prevalence of disease. However , leprosy continuous to be an important public health problem in many parts of the world including India. Diagnosis, treatment as well as controlled strategies have so far been based on indirect evidences and empirical treatment as there is still no easy and acceptable method of *in-vitro* cultivation of its causative organism. The diagnosis of disease is based on clinical, [18] histopathological [4] and bacteriological [11-17] parameters, but these methods have limitations. In paucibacillary cases, especially in the early cases, these methods are not very useful. During the last 5-7 years, several specific gene sequences of M. *leprae* have been identified and gene probes as well as gene amplification techniques (PCR) for identification and detection of M. *leprae* have been developed[19,5]. These include DNA as well as RNA targeting systems. Recent research on molecular diagnosis of leprosy and mycobacterial infections are rapidly moving towards high sensitivity nucleic acid probes amplification tests, particularly focusing on DNA, RNA, MRNA and micro RNA to improve early diagnosis and detect drug resistance cases in leprosy. In mycobacteria there are 2000-5000 copies of RNA (rRNA) per live cell as compared to 1-2 cistons encoding rRNA genes in the DNA. There is thus theoretical advantage of better sensitivity of probes targeting rRNA. rRNA degrades faster than DNA after death and its detection is likely to correlate better with active disease. This study has been carried out to access the comparative sensitivity of targeting rRNA as well as DNA in nucleic acids extracted from biopsies and skin scrapings from leprosy lesions.

Materials and Methods

1. *Clinical Specimens:*

In this study, 130 specimens from untreated leprosy cases (untreated or treated for < 3 months) across the spectrum were studied. These included 56 paucibacillary cases i.e. 3 tuberculoid (TT) , 43 borderline tuberculoid (BT), including 2 relapses and 10 indeterminate (I) and 73 multibacillary cases viz: 22 borderline borderline (BB), 35 borderline lepromatous (BL) and 16 lepromatous (LL) patients. Specimens studied included biopsies (110 specimens) as well as skin scrapings (20 specimens). Fifty controls specimens, which included slit smears

from 30 contacts of leprosy patients and 20 skin biopsies from healthy controls, were also studied.

Biopsies from leprosy patients and healthy controls and contacts:

Biopsies (50 - 100 mg) from active lesions of leprosy patients were collected under aseptic conditions using local anesthesia.

Scrapings from lesions / healthy controls:

After using proper antiseptic precautions, scrapings as for skin smears were taken from the leprosy lesions as well as healthy skin[14] and these were collected in peptone water and processed. All these scrapings were stored at -20^0 C, if not processed immediately.

2. Processing of specimens:

Biopsies were homogenized in T.E. (pH 8.0, 0.01 M Tris, 0.002 M EDTA) buffer. Smears were prepared on circular slides for counting and specimens were further processed for extraction of nucleic acids. These smears were stained with Ziehl-Neelsen stain and examined for acid fast bacilli (AFB) which were counted.[17.]

(a) *Dilution of the specimens from Multibacillary cases:*

Biopsy homogenates form specimens with > 10^5 organisms were diluted 10-10000 fold, counts checked by smear and these preparations were used in the extraction.

(b) *Extraction of nucleic acids from biopsies / scrapings:*

A technique based on the principle of combined application of earlier described techniques[15,2] developed during the study was used.[6] Briefly, the homogenates of the scrapings in TE buffer were frozen-thawed and then enzymatically treated at 37^0 C first with lysozyme (3mg/ml) for 2 hours, followed by proteinase-K (250ug/ml) for 6 hours. To this extract equal volume of phenol-chloroform (1:1) was added and thoroughly mixed. After centrifugation at 8,000 X g for 15 minutes., aqueous layer was removed and equal volume of chloroform: isoamyl alcohol (24:1) was added. This was thoroughly mixed and centrifuged again at 8,000 X g for 15 minutes. To the aqueous materials 2 volumes of chilled ethanol was added and nucleic acids were precipitated. The precipitates were separated by centrifugation at 12,000 X g for 15 minutes., redissolved in lysis buffer (6M guanidine hydrochloride, EDTA 15mM, B-mercaptoethanol 1 mM) and rRNA and DNA were fractionated[8].

(c) *Probes:*

Following probes targeting the ribosomal gene region of *M. leprae* were investigated in the study:

(1) Probe P1: 5'- CACTGGCTTCGGGTGTT 3' 17 mer[6]

(2) Probe P2: 5'- CTTCAAGGCGGATGTCTT3' 18 mer[8]

(3) Probe P3: 5'- ACTCCTGCACCGCAAAAAGCTT 3' 22 mer[3]

(i) *Synthesis and labelling of probes with P32 ATP:*

The probes were synthesized in a Gene Assembler Plus (Pharmacia) using the standard phosphoramadite chemistry as per the technique recommended by the manufacturer. These probes were purified, end labelled with P32 using the method described by Maniatis et al.[12]

(ii) *Denaturation of DNA and blotting:*

The procedure as described by Boddinghaus et al[15] was followed. The denatured DNA were directly applied on nitrocellulose membrane for dot blot analysis by using the dot blot apparatus (BRL, Life Technologies, U.S.A.). After blotting, the nitrocellulose membranes were baked at 80^0C for 2 hours in a vacuum oven (Gallenkamp, U.K.).

(iii) *Blotting of ribosomal RNA:*

The 10-20 ug RNA samples were directly applied on nitrocellulose membrane using dot blot apparatus with vacuum pump. Then nitrocellulose membranes were baked at 80^0C for 2 hours in a vacuum oven (Gallenkamp, U.K.).

(iv) *Hybridization of blotted DNA:*

The hybridization of the dot blot filters was carried out as described by Maniatis et al[12] with some modifications. After baking in step (ii) , the filters were soaked in 2 X SSC (1 X SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% (w/v) SDS for 10 minutes and then kept in hybridization buffer supplemented with tRNA (10mg/ml) at 42^0C for 2-4 hours for pre hybridization. The pre hybridization buffer (same for hybridization also) containing 5X Denhardt (0.1% each of ficoll, polyvinyl pyrrolidone-PVP and bovine serum albumin-BSA in sterile deionised water) and 5 X SSC was prepared. 10ml of pre hybridization buffer containing 50% of formamide (BDH, England) was added to each of the polythene bags. After 2-4 hours of incubation, the pre hybridization buffer was poured off and 0.2 ml of fresh pre hybridization buffer for each square centimeter of the membrane was added and the labelled probe (100 ul, 10^6-10^7 cpm / 50ng) (Specific activity > 3000 ci/m mol) was then added. The incubation was continued at 42^0C for 16-20 hours. When the hybridization process was

completed, the filters were taken out of the bags and quickly immersed in a 4 X SSc , 0.1 % SDS for 1 hour at 42^0C, followed by washing of hybridized membrane with 2 X SSC containing 0.1 % SDS for 1 hour at 50^0C and finally 2^0C below Tm of each of the probes. When washing was completed , the filters were immediately dried and subjected to autoradiography at - 70^0C for the desired length of exposure.

(v) *Hybridization of blotted RNA:*

After baking, the dot blot nitrocellulose membrane / filters were treated with 6 X SSC at room temperature for about 10 minutes and then kept in polythene bags which contained pre hybridization buffer having 6 X SSC and tRNA (10mg/ml). The dot blot filters were immersed in bread box containing 200 ml of same buffer at 46^0C for 2 hours of incubation with continuous shaking in water bath. The pre hybridization buffer was poured off and 0.2 ml of fresh pre hybridization buffer (same as hybridization buffer) for each square centimeter of the membrane was added and labelled synthetic oligonucleotide probe was then added and the incubation continued at 42-46^0C with continuous shaking in a water bath for about 8-12 hours. [6-3] When the hybridization process was completed, nitrocellulose membranes were taken out from the polythene bags and quickly immersed in 4 x SSC containing 0.1 % SDS at 46^0C, 51^0C, 56^0C at 1-2^0C below Tm (Tm for probe P1 = 62^0C, probe P2 = 62^0C, probe P3 = 66^0C) for 15 minutes at each temperature. When washing was completed, the filters were immediately dried and subjected to autoradiography at - 70^0C for the desired length of exposure using appropriate intensifying screens, X – Ray films were developed in an automatic X – Ray Processor (X-O Graph, Genetic Research Instrumentation Ltd. U.K.).

Results

The three oligonucleotide probes used in the study targeted different variable stretches on rRNA gene region and these were compared in a representative number of cases for their sensitivity for detection of DNA vs rRNA extracted from the 10 fold dilutions of biopsy homogenates (Fig.1.). The overall positivity of specimens while targeting DNA vs RNA is detailed in Tables 1 & 2.

The important results were:

(a) Positive signals could be observed at 10-100 fold dilutions (Lower concentration level) with rRNA detection as compared to targeting of DNA (Fig. 1.). The lower limit of positivity for rRNA targeting was about 100-1000 organisms whereas approximately 10^5 organisms were necessary for the getting positive detectable signals, when DNA was the target molecule.

Table 1. Bacterial load and positivity by probes targeting DNA as well as RNA

Paucibacillary Specimens (PB) (n=56)	Probe P1		Probe P2		Probe P3	
$>10^5$ (1)	DNA 1	RNA 1	DNA 1	RNA 1	DNA 1	RNA 1
$<10^5$ (3)	2	3	2	3	2	2
*NM/ND (52)	1	14	2	14	1	4
Multibacillary Specimens (MB) (n=73)						
$>10^5$ (45)	33	29	34	28	26	23
$<10^5$ (8)	2	3	4	4	1	3
*NM/ND (20)	1	7	2	7	0	3

*NM = Not measurable, ND = Not detectable; Number within parenthesis are total specimens.

10^6 10^5 10^4 10^3

RNA.

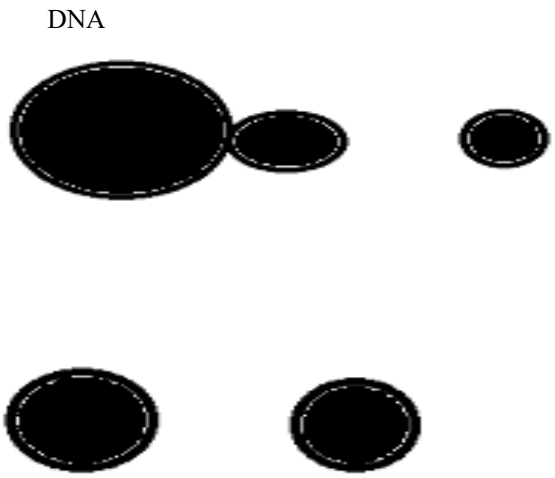

Fig.1 Results of blot hybridization of probes P2 with rRNA and DNA extracted from $10,^6$ $10,^5$ $10,^4$ $10,^3$ and 10^2 organisms – dilutions made from the biopsy homogenate of a lepromatous case.

(b) Below 10^5 bacillary levels (or equivalent DNA) positive signals could be seen by targeting DNA in some of the samples. However the overall positivity drastically decreased as compared to rRNA detection. After accessing this sensitivity range, only undiluted DNA samples were investigated for the remaining specimens. It may be noted that positivity with direct probe hybridization with DNA was marginally higher in smear positive multibacillary cases. However the positivity of DNA detection was very low in smear negative paucibacillary cases. Though there were weak signals in some smear negative paucibacillary and some multibacillary types, the positivity was too low (Table 1). The positivity was slightly higher in targeting DNA detection rather than RNA in specimens with > 10^5 (Table 1.). The positivity rates of DNA vs RNA in these cases (total 56) were: 34 vs 30 with probe P1; 35 vs 29 with probe P2; 27 vs 24 with probe P3; but these differences were statistically significant. The positivity of targeting DNA was much lower than targeting rRNA in specimens with <10^5 or negative cases (total = 73) e.g. 6 vs 27 with probe P1, 10 vs 28 with probe P2, 4 vs 12 with probe P3, respectively. These differences are statistically significant (P values ranging from <0.01 to <0.01).

(c) *Detection of DNA / RNA by probes in the skin scrapings:*

20 skin scrapings from 10 smear negative BT and 10 smear positive BL-LL cases were also studied for hybridization by the same techniques. The results are summarized in Table 2. The trends in scraping specimens were similar to these observed with biopsies.

Table 2. The positivity rates of RNA/DNA detection in the skin scrapings

		Probe 1	Probe 2	Probe 3
Paucibacillary (10)	DNA	0	0	0
	RNA	1	1	0
Multibacillary (10)	DNA	4	4	2
	RNA	5	5	3

Discussion: Advantages in recombinant DNA technology have provided newer techniques and tools for diagnosing several infections with considerably higher sensitivity and specificity than the conventional methods.[21] These tools include specific gene probe / PCR techniques as well as genetically engineered antigens for immunodiagnosis. The need for such techniques is particularly felt for mycobacterial infection and several researchers have made efforts to investigate the Molecular Biology of lepra and Tubercle bacilli. These studies have led to the development of several gene probes and gene amplification techniques for detection of M. leprae. Gene probes are basically of two board types – DNA targeting as well as RNA targeting types. In DNA targeting probes, 10^4 to 10^5 organisms have been considered necessary[1,4] for getting a positive signal. On the other hand, ribosomal RNA targeting probes appear to be more attractive because 2000 – 5000 number of copies ribosomes are estimated to be present per live mycobacterial cell [11,7.] In practice rRNA targeting probes and PCR have been found to be more sensitive for *M.Tuberculosis*[15] . However the situation is difficult in

the case of *M. leprae* which has to be purified from host tissues. We have earlier tried and developed methods for extraction of rRNA and DNA from biopsies[9] using quantitative hybridization of nucleic acids extracted from biopsies of leprosy patients across the spectrum. It was observed that overall rRNA detection is 10 – 100 fold more sensitive than DNA targeting by using probes targeting different stretches of ribosomal gene region (Fig. 1.). Maximum achievable sensitivity was around 10^3 organisms. Considering a maximum of 10 % viability in bacilli derived from leprosy tissues, this sensitivity is around 10^2 live cells. These observations confirm trends reported earlier in cultivable organisms [18,10]. This study indicates that the probes and techniques described earlier are sensitive and on their own can led to detection of *M. leprae* in a good proportion of less bacillated specimens. Such specimens are likely to have bacillary loads of 10^2 to 10^3 organisms which are missed by smear staining but are sufficient for positive signals on rRNA detection. Overall DNA detection appears to be much less sensitive. Additional positive specimens in AFB positive specimens by DNA detection could be due to residual signals in cases who might have had some treatment earlier. Though the probes P1 and P2 are apparently more sensitive than P3, overall trends are similar. The approach may be applicable with skin scrapings also, but more number of specimens need to be tried. However the overall sensitivity achieved with these probes alone is not adequate and is lower than that observed with PCR[11] . It may be desirable to have rRNA targeting PCR system for such cases[7]. Long term studies in these cases from different regions and clinical types will be required. Such studies would be specially important in the emerging scenario of disease with changing clinical types and bacterial load[22]. As a matter of concept, this approach appears to be a workable strategy for detection of *M. leprae* from clinical specimens and is expected to have better relationship with active disease and viability[19]. This approach will have the same advantage for other infectious disease as well.

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