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DEVELOPMENT OF DNA PROBES FOR *M. TUBERCULOSIS*

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Summary. **Attempts were made to develop DNA probes for *M. tuberculosis*. Random library of *M. tuberculosis* was constructed in plasmid pGEM -4. Selection of recombinant clones was made by hybridisation with ^{32}P labelled *M. tuberculosis* probe. Ten recombinant clones were selected on the basis of strong signals from the random library. These 10 clones named $pTRC_{1-10}$ were subjected to tests for specificity and sensitivity. On this basis, $pTRC_4$ was chosen and this is also, useful in restriction fragment length polymorphism (RFLP) studies.**

Introduction

Tuberculosis has been reported with increasing frequency in patients with acquired immunodeficiency and other immunocompromised patients'. Diagnosis of tuberculosis is time consuming and depends on isolation of the pathogen, which takes up to eight weeks. Direct microscopy lacks sensitivity. The bactec system, even though it has reduced the time needed for detection, is still growth dependent[2].

In recent times attention has been focussed on developing sensitive and specific tests for detecting *M. tuberculosis*. Recombinant DNA technology, a powerful tool, has opened up the possibility of developing specific DNA probes which can help in the detection of *M. tuberculosis* and also to differentiate between the different strains of mycobacteria. The technique is therefore of potential value for basic and epidemiologic studies of tuberculosis. There have been many reports of specific DNA probes for diagnosis of *M. tuberculosis* using the Polymerase Chain Reaction (PCR), latest technique in the recombinant DNA field[3,4,5,6]. Even though the sensitivity of the tests using PCR is 100% the specificity is only 62.5% compared to culture results[7]. Other reports too have not shown better specificity because in this kind of approach proper primers have to be used. As of today, there is no single immuno-diagnostic test or tests based on molecular approaches which could be recommended as a confirmatory test for tuberculosis Here we report the isolation of a DNA probe from a *M. tuberculosis* library made in plasmid pGEM. This probe has been tested for specificity as a whole probe since this probe has also been found to be useful in restriction fragment length polymorphism (RFLP) studies with various strains of mycobacteria. The interesting feature of this probe is that it gives a different RFLP pattern with drug resistant strains.

Material and Methods

Mycobactetial strains : Reference and clinical isolates of *M. tuberculosis*, reference strains of atypical mycobacteria and non-mycobacterial species were from the Bacteriology Department of the Centre.

Preparation of mycobacterial DNA : Mycobacteria were cultured in Sauton-tween medium. D-glycine was added to a concentration of 1mg per ml when the cells were in log phase to enhance lysis. After a drop in density, the cells were harvested by centrifugation and lysed with sodium dodecyl sulphate (SDS). DNA was purified by a phenol-chloroform-isoamyl alcohol procedure.

Digestion with restriction endonucleases : Reaction mixtures containing 1 to 2 ug of DNA and 4 to 10 units of enzyme were incubated at 37°C overnight.

Cloning into pGEM-4 : DNA from *M. tuberculosis* $H_{37}R_v$ was digested with Rsa-1 and ligated with a

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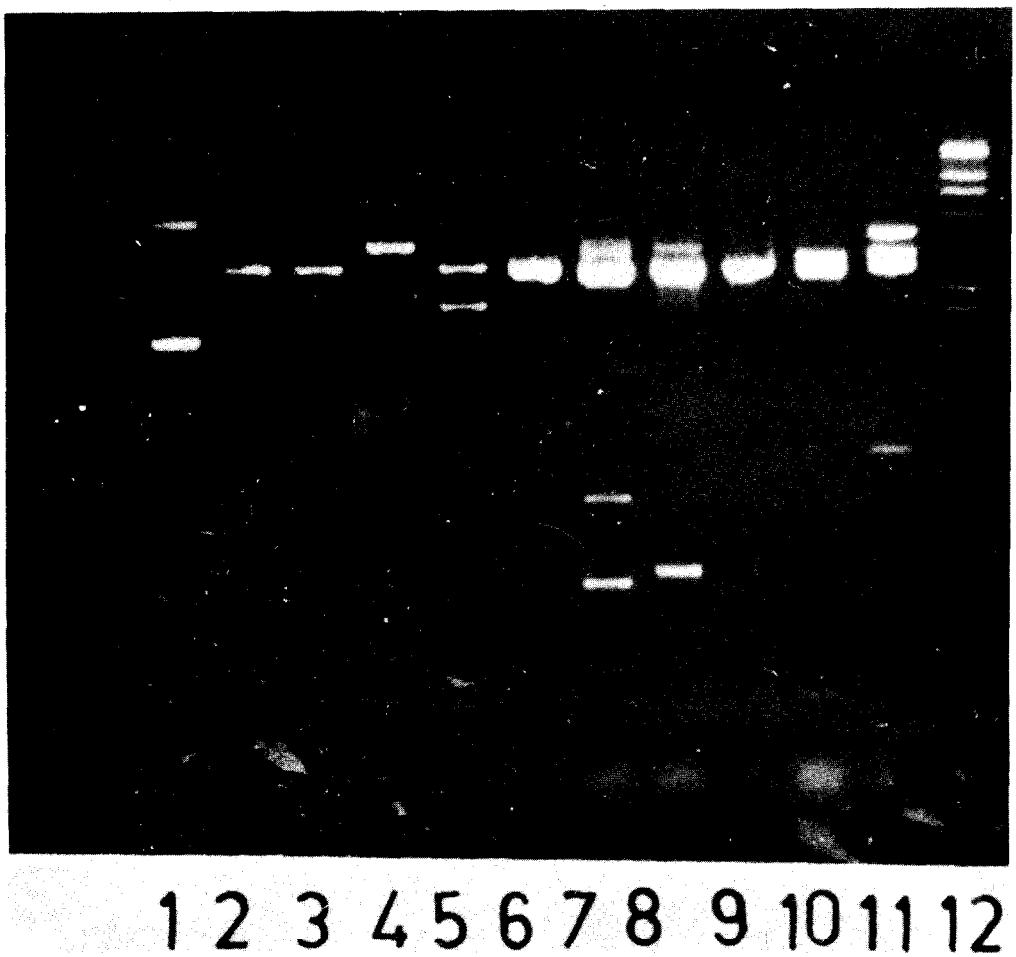

Fig. 1. The 10 recombinant pGEM clones were purified by $CsCl_2$ density gradient and run on 1% agarose gel. Lane 1 pGEM; Lane 2 to Lane 11 pTRC 1 to pTRC 10 and Lane 12 *M. wt* marker.

Hinc II digest of pGEM-4. *Escherichia coli* HB 101 were transformed and plated. The recombinant colonies appearing on the Ampicillin plates were screened with 32_p labelled *M. tuberculosis* $H_{37}R_v$. The various recombinants which lighted up strongly were picked up, grown in log phase cultures of *E. coli* HB 101 cells. DNA from recombinant pGEM plasmids was isolated by alkaline lysis method. To excise the insert DNA, the DNA from recombinant plasmids was digested with EcoRI and Hind III which cleave at the opposite ends of poly linker region. The sizes of the excised fragments were determined by agarose gel electrophoresis.

Labelling DNA probes : Total chromosomal DNA and recombinant DNA were labelled with ^{32}P d CTP or ^{32}P d ATP(3000m Ci/mmol) by nick translation. Specific activity of the probes was about 10^8 cpm/ug of DNA and approximately 10^6 to 10^7 cpm was used in each hybridisation.

Slot blot hybridisation : DNA was denatured in 0.4 N NaOH at room temperature for 10 minutes, neutralised with an equal volume of 2M Tris HCl, pH (7.0) and loaded on a slot blotter (minifold II Schliecher & Schuell Inc. Keene N.H.) with Gene screen membrane (DuPont NEN Research product, Boston, Mass.). The pre-hybridisation solution consisted of 10% dextran sulphate, 1% SDS & 1M sodium chloride and 100ug of denatured salmon sperm DNA per ml. Labelled probe DNA was added to the hybridisation solution, Membranes were hybridised at 65°C and washed as follows. 2X SSC (2X) and 0.5% SDS at room temperature for 5' 2X SSC & 1.0% SDS for 1/2 hour twice and 0.2 X SSC & 0.1% SDS for 1/2 hour.

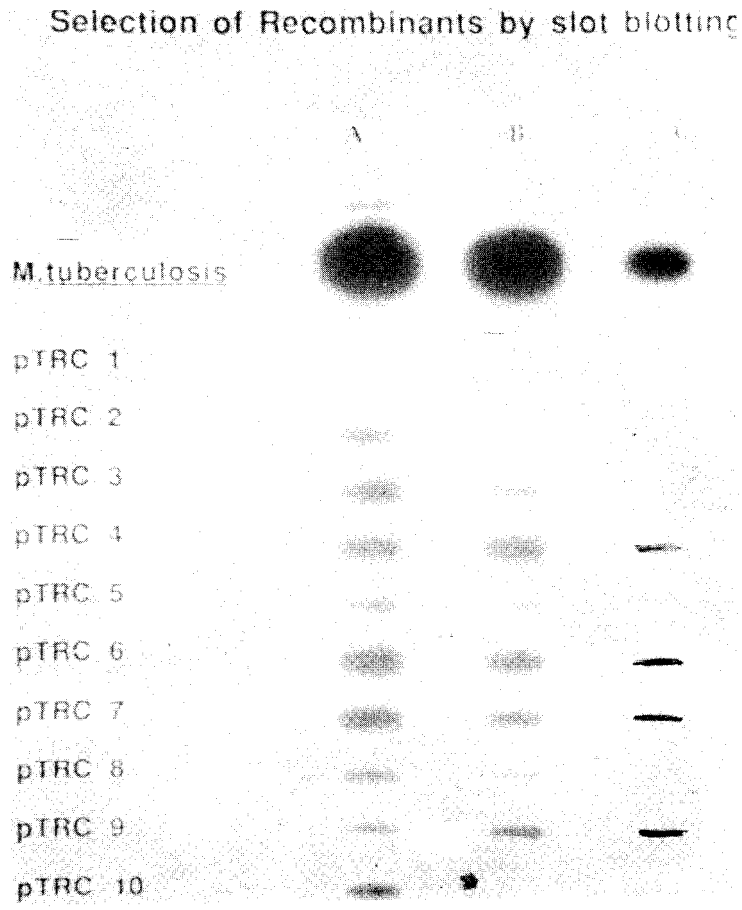

Fig. 2. The 10 recombinant clones from the pGEM library were spotted in the slot blot in order of decreasing concentration as shown above. The 1st row represents standard ***M. tuberculosis*** DNA. The rest of the 10 rows represent the 10 recombinant clones numbering 1-10. The probe used for hybridisation was ^{32}P labelled- *M. **tuberculosis*** DNA.

Southern blot hybridisations : Digests were electrophoresed on 0.8% agarose gels containing ethidium bromide and photographed. DNA fragments were denatured and transferred to Gene screen plus membrane (DuPont, NEN Research products, Boston, Mass.) by using either a modified Southern transfer method as described by Transblot method (Trans vat 80 Hoeffer Scientific Instruments, San Francisco).

Auto-radiography : Auto-radiographs were prepared by exposing the hybridised blots for varying lengths of time at 70°C to Kodak x-omat AR film and cronex lightning screens.

Results

The criterion used in screening the pGEM recombinant colonies was selection of those clones which give strong signals when hybridised with ^{32}P labelled *M. tuberculosis.* A total of 10 such clones with strong signals was chosen and further tested for specificity and sensitivity using slot blot hybridisation. The sizes of these 10 recombinant clones are shown in Fig 1.

Slot blot hybridisation : The 10 recombinant clones, standard strain of *M. tuberculosis* or non tuberculosis strains were spotted in the slot blot in order of decreasing concentrations (2.0ug,

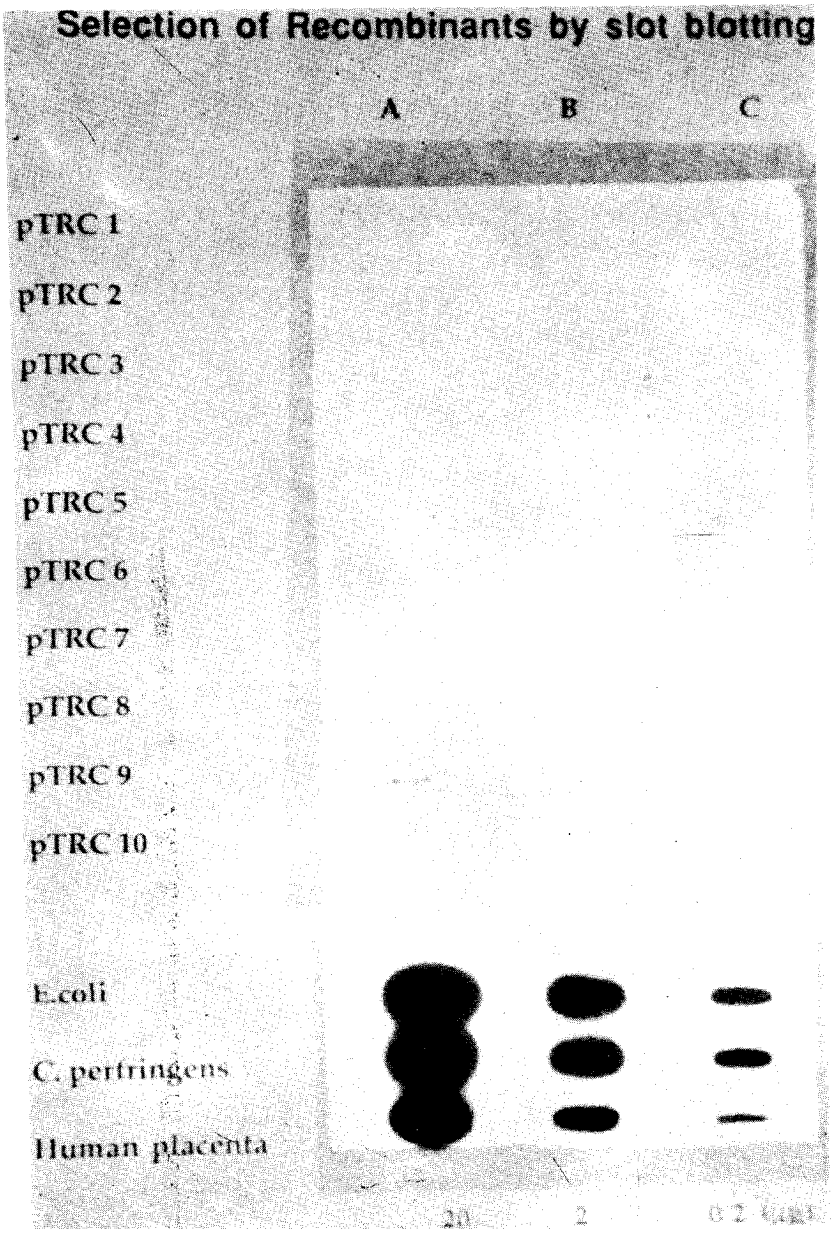

Fig. 3. The 1st 10 rows represent the 10 pGEM recombinant clones at three different concentrations. The 11th, 12th and 13th rows show *E.coli, Clostridium perfringenes* and human placental DNA. The probe used for hybridisation was ^{32}P labelled pooled DNA from *E. coli, Clostridium perfringenes* and human placenta.

0.2ug, 0.02ug). These were hybridised with ^{32}P labelled *M. tuberculosis.* Those clones which lighted up strongly at the lowest concentration were chosen for further evaluation (Fig 2). A duplicate of the above slot blot was hybridised with a non mycobacterial probe (^{32}P labelled DNA of a pool of *E. coli, Clostridium pefringenes,* and human placenta). Those recombinant clones which reacted least with non-mycobacterial DNA were chosen for further work. (Fig 3) Among the clones chosen according to the above criteria, one clone- $pTRC_4$ is promising. As a further step, the specificity was studied using DNA from *E.coli, E.coli Kk6, Bacillus subtilis, Proteus mirabilis,*

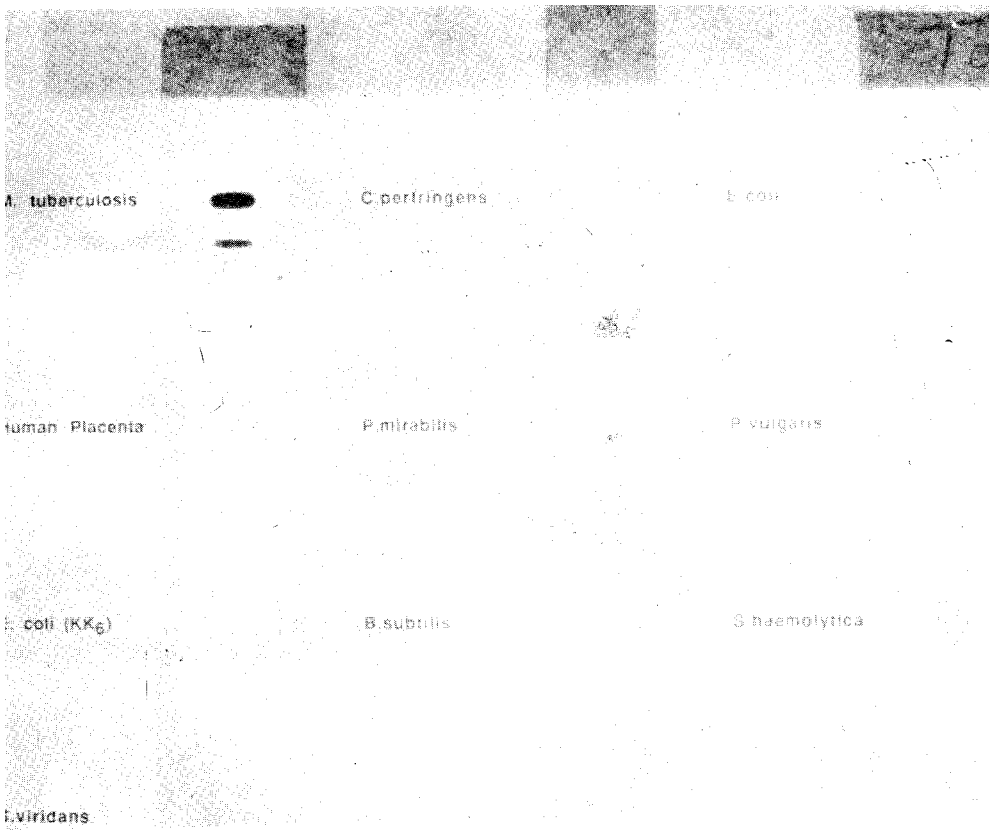

Fig. 4. Various non mycobacterial species were spotted in the slot blot at 4 different concentrations (20.0, 2.0, 0.2 & 0.02 ug) probe - ^{32}P labelled $pTRC_4$

Proteus vulgaris, Clostridium perfringenes S. hemolytica and human placenta. The DNA from these species were spotted in concentrations ranging from 20, 2.0, 0.2, and 0.02 ug of DNA. $pTRC_4$ did not cross react with any of the non-mycobacterial DNA used except with human placental DNA, at the highest concentration used i.e. 20 & 2 ug (Fig. 4). The mycobacterial fragment of $pTRC_4$ is 2.2 kb in size and when it was further subjected to restriction digestion with various enzymes, gave sites only for Sal-I.

$pTRC_4$ was restriction digested with EcoR I, Hind III and Sal I and run on 1% agarose gel electrophoresis. The gel was Southern transferred to Gene screen membrane. Three lanes were hybridised with ^{32}P labelled *M. tuberculosis*, ^{32}P labelled $pTRC_4$ and ^{32}P labelled human placental DNA respectively. The individual fragments did not cross react with $pTRC_4$ (Fig. 5). The individual fragments are being sequenced for further evaluation of their sensitivity and specificity.

The potentiality of this clone in RFLP studies and specificity was evaluated. The DNA from various strains isolated from patients were restriction digested with Pst I and Sal I and run on 1.0% agarose gel, Southern transferred on to Gene screen membrane and probed with 32 P labelled $pTRC_4$ (Fig.6). Except BCG & *M. tuberculosis* none of the atypical mycobacteria reacted with the probe. The various atypical mycobacteria used were *M. kansasii, M. avium, M. gordonae, M. phlei, M. xenopi, M. chelonei, M. fortuitum, M. chitae, M. bovis BCG* and BCG strain. The interesting feature of the probe is that it distinguishes the drug resistant strain from the other strains of mycobacteria.

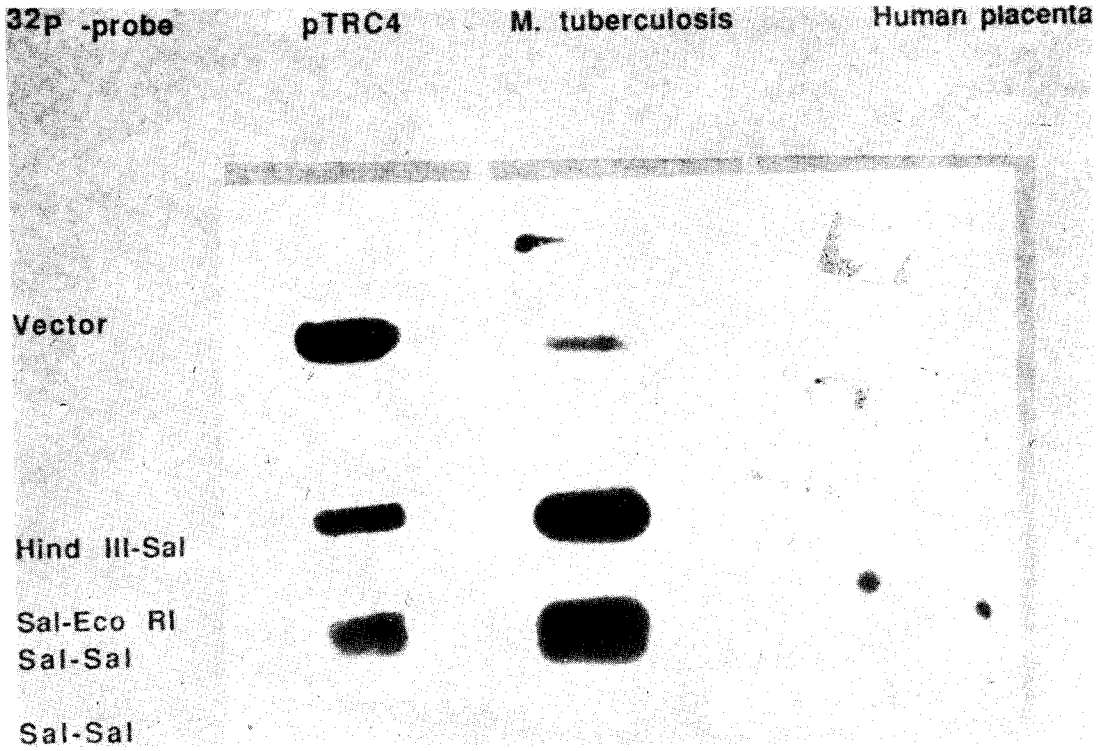

Fig. 5. pTRC$_4$ clone restriction digested with three agents.(triplicate) and probed with ^{32}P pTRC$_4$, ^{32}P labelled *M. tuberculosis* and ^{32}P labelled human placental DNA.

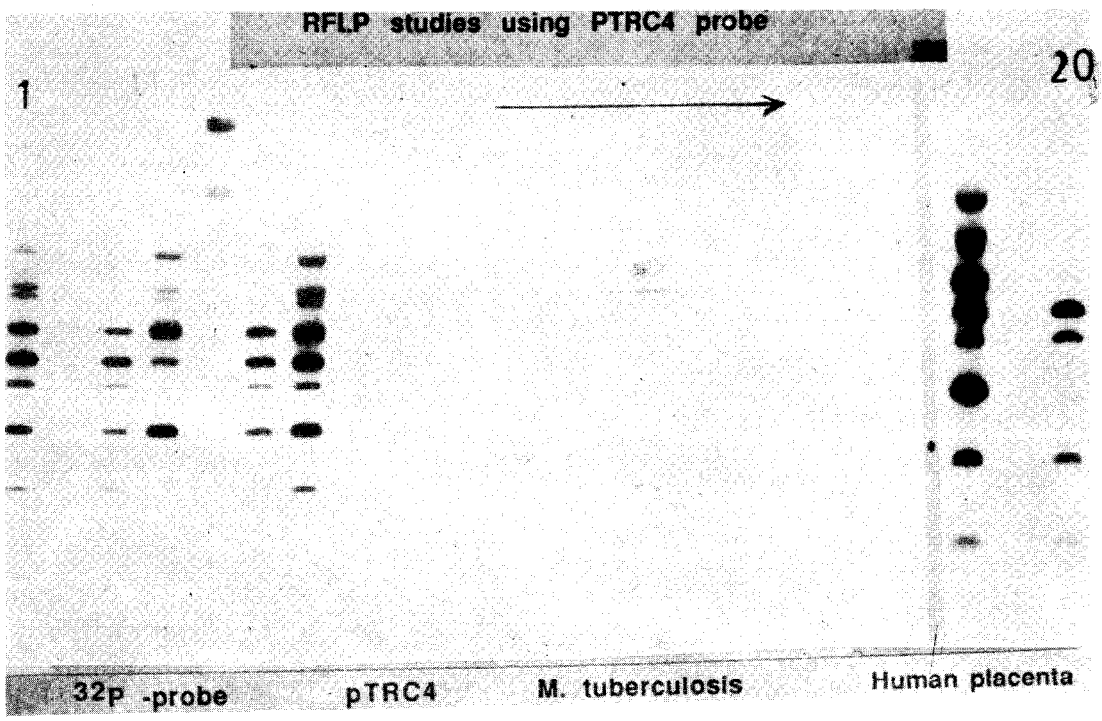

Fig. 6. DNA from clinical isolates of *M. tuberculosis* and atypical mycobacteria restriction digested with Sal1 and Pst1 run on 1% agarose gel, Southern transferred and probed with 32$_p$ labelled pTRC$_4$. Lanes l-7 clinical isolates of *M. tuberculosis.* 8. *M. kansasii* 9. *M. avium* 10. *M. phlei* 11. *M. simiae* 12. *M. smegmatis* 13. *M. gondonae* 14. *M. chelonei* 15 *M. fortuitum* 16. *M. chitae* 17. *M. xenopi* 18. *M. bovis* BCG. 20. Rifampicin resistant clinical isolate.

Discussion

We have isolated 10 recombinant clones from a library of *M. tuberculosis* in pGEM. These were selected on the basis of their strong hybridisation signals with ^{32}P labelled *M. tuberculosis.* These clones were evaluated for their specificity and sensitivity by doing a slot blot hybridisation with non mycobacteria DNA and DNA from *M. tuberculosis* at concentrations from 20 ug to 0.002ug. Out of these 10 clones, 4 seem to be promising with regard to specificity and sensitivity. Among these four clones one, $pTRC_4$, is also a repetitive fragment, helpful in studying the restriction fragment length polymorphism (RFLP) with various strains of mycobacteria.

Several repetitive sequences have been described in the genome of members of the mycobacterium tuberculosis complexes[8,9,10,11,12.] From the reported size and map of the clones, ours seems to be different.

The data presented indicate that $pTRC_4$ has potential as a diagnostic probe for identification of *M. tuberculosis.* The $pTRC_4$ clone is being subcloned. Nucleotide sequencing is in progress to facilitate PCR amplification of target DNA and thereby increase the sensitivity of the probe. The subclones also would be used in RFLP studies with mycobacterial strains.

The present study also indicates that $pTRC_4$ is repeated around ten times in the genome of various strains of *M. tuberculosis.* The repetitive fragment did not show cross hybridisation with DNA from atypical mycobacterial strains used except *M. bovis BCG* which showed similar pattern as *M. tuberculosis.* The interesting feature of this clone is that it has given different banding pattern with drug resistant isolates of *M. tuberculosis.* Since there is RFLP between the normal and drug resistant strains which has been detected with this probe, further investigations could be carried out to find out whether this difference has arisen due to mutation. Mycobacterium has been a challenge because many facts about its virulence, pathogenicity and other properties have been a mystery in spite of the long years of work on it.

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