Title
Development of a PCR assay for quantitation of M. tuberculosis in sputum and its possible application in clinical trials of antibiotics against pulmonary tuberculosis

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Development of a PCR assay for Quantitation of *M. tuberculosis* in Sputum and its Possible Application in Clinical trials of Antibiotics against Pulmonary Tuberculosis

by
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A Thesis

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Abstract

I have developed a simple assay for quantitation of *Mycobacterium tuberculosis* in sputum samples using competitive PCR and a colorimetric microtiter well detection format. This assay relies on the co-amplification of a 419 bp *pab* fragment of *M.. tuberculosis* together with a target template *(pab/tet)* engineered by splicing a fragment of the *tet* gene excised from pbr322 between the 5' and 3' ends of the *pab* fragment to create a 380 bp hybrid template amplified with the same primers but readily distinguishable using probes specific for either *pab* or *tet.* I demonstrate the correlation between the results obtained using this assay and quantitative culture. This assay provides quantitative information regarding *M.. tuberculosis* burden in sputum samples containing between $10^3$and 10^7 CFU/ml.

I have also established a pilot clinical study to test the *in vivo* bactericidal efficacy of new drugs for pulmonary tuberculosis. This study involved measuring the sputum CFU/ml of patients receiving either rifampin or isoniazid for one week of treatment. A pilot clinical study involving 15 patients has been carried out and the study is now ready to test new drugs for tuberculosis (augmentin and ofloxacin) using this methodology.

The quantitative PCR assay described here could potentially be used to facilitate the quantitation of mycobacterial burden in sputum samples from patients in a larger clinical trial similar in design to the one described here. However, problems in the scaling-up of the PCR assay to accommodate the large number of samples to be analyzed were encountered. Possible routes to surmount this problem are suggested.

Introduction

Tuberculosis is a chronic infectious disease caused by the intracellular bacterium *M. tuberculosis.* It affects over 55 million people worldwide and is the most common cause of death from any infectious agent (1). In the United States, the incidence of new infections from *M. tuberculosis* declined every year since the beginning of the modern era of antibiotic therapy for this disease in 1952. However, in the last three decades, the prevalence of drug resistant organisms has increased from approximately 2 to 9 percent (2). While in the past most patients with multi-drug resistant tuberculosis acquired the organisms through multiple courses of treatment with anti-tuberculous drugs, recently the direct transmission of multi-drug resistant strains has increased as well (3). This poses a grave threat to current strategies for disease control and makes the development of new anti-tuberculous medications an important research priority.

Treatment of patients infected with MDR strains has proven difficult because there is little data to support the use of specific agents. Although several antibiotics have been shown to be effective *in vitro*, a reliable method for testing new antibiotics against tuberculosis *in vivo* has not been established. In large part, this is due to the fact that, unlike most other infectious agents, tuberculosis is an indolent disease that requires many months of therapy for a cure. Clinical trials of new agents are severely hampered by the long time course to define whether the specific therapeutic intervention is working. In addition, there is no specific and reproducible way to rapidly assess whether a given patient is responding to the course of therapy.

Despite the long time course for definitive cure, previous studies have suggested that a large percentage of *M. tuberculosis* in sputum are eliminated over the first few weeks of therapy. Jindani et al. carried out a study in Africa in a group of 124 patients randomized to receive several individual drugs or combination regimens over a two-week period. They found that single-drug INH treatment resulted in a significant drop in CFU/ml over the first 2

days of therapy with a continual, slower drop over the entire 2 week course. Rifampin, on the other hand, also produced a decline in CFU/ml but this was not as pronounced over the first few days overall was not as significant as with INH. Other drugs tested in their study, such as thiacetazone, demonstrated no significant early bactericidal efficacy compared to patients receiving no treatment(4).

The numbers of patients in the Jindani study were relatively small and therefore difficult to interpret. However, their observation suggests that a similar methodology could be used to test new drugs for *in vivo* efficacy against tuberculosis. If the bacterial counts declined with these novel agents in a similar fashion as with INH or rifampin over the first week of therapy, one could then consider these to be potentially useful agents for long-term therapy against tuberculosis in patients with MDR. Although such a study would not definitively establish the usefulness of these new agents, it could be a screening tool that could then be followed by long-term studies using these novel agents in combination regimens. Given the desperate need for new drugs to treat tuberculosis in the MDR era, such an approach would greatly facilitate the testing of new drugs and provide alternatives in a field that currently relies more on anecdotal reports of cure than on hard scientific evidence to design treatment alternatives. *In vitro* evidence has suggested that amoxicillin/clavulanic acid and several quinolones are effective against tuberculosis (5-8). These later agents have occasionally been used in treating patients harboring MDR strains (9). However, no one has yet proved their efficacy in vivo against *M. tuberculosis* or compared their early bactericidal profile to that of INH or Rifampin.

One problem encountered in trying to measure response to treatment using CFU/ml in sputum samples is the long time course required. Quantitative culture (Q-culture) of tuberculosis in the laboratory can take anywhere from 2-8 weeks. In addition, sputum must be de-contaminated prior to culturing because of the presence of mixed flora that can overgrow the media before the mycobacteria are seen. The sodium hydroxide decontamination procedure kills as many as 70% of the viable mycobacteria in the sample,

making accurate quantitation difficult at best (10). Even with decontamination, it is very frequent to have cultures so overgrown with contaminating bacteria that it is impossible to count the number of mycobacteria.

One possible alternative to standard quantitative culture would be the use of quantitative polymerase chain reaction (Q-PCR). Standard PCR techniques allow only for qualitative analysis of samples and do not provide information as to the relative amounts of target DNA present in the sample. PCR has been extensively studied as a rapid, qualitative assay for detecting tuberculosis in clinical samples thus greatly reducing the time to definitive diagnosis. Several genes have been tested as potential targets in PCR. The most studied target for diagnosis has been the repetitive DNA insertion sequence *IS6110*, which has been shown to be highly specific for the *M. tuberculosis* complex (11, 12) and, under strict quality control conditions, also highly sensitive and specific for diagnosis of pulmonary tuberculosis (13). As a target for quantitation, however, *IS6110* has the disadvantage that its copy number is variable within the genome (14). Other genes specific for the *M. tuberculosis* complex that have been studied as potential targets for PCR are the gene that encodes for protein antigen b (*pab*)(15-17) and *mtb64* (18). Forbes et al compared the sensitivity of *IS6110* vs *pab* for detection of *M. tuberculosis* in sputum and found *IS6110* to be somewhat more sensitive (97.7 % vs 87.2%) (17). However, since *pab* is found only once in each bacterial genome, and it is specific for *M. tuberculosis* complex, it could potentially be a very good target for a Q- PCR assay.

The difficulty in developing a truly quantitative assay using PCR is due to the inherent variability in the amplification process and the uncertainty establishing a correlation between the amount of DNA amplified and the starting material. PCR amplification is only linear within a certain range above which each PCR cycle no longer results in a doubling of DNA molecules. In addition, there is a considerable variability in the PCR output even when the same sample is aliquoted and amplified in several different tubes (19). However, several authors have described modifications to the standard PCR reaction that enable an indirect

determination of the starting material. One such method and the one most generally accepted is that of competitive PCR. Competitive PCR has been used for the determination of HIV burden (20,21,22) and cytokine production (23) among other applications (24). In competitive PCR, a second template present in a known amount is used as an internal standard. This second template is amplified with the same set of primers as the DNA of interest but has an internal modification, such as a small size difference or a distinct restriction site, that allows for differentiation of the product.

This method is based on two assumptions: 1)the reaction is carried out in the linear range and 2) the amplification efficiency of the control and native template is very similar. If these assumptions hold, it is possible to quantitate the starting amount of target DNA present by comparing the relative amount of its template amplified in relation to the competitive template. If several different concentrations of competitive template are amplified with a given sample, ideally one will identify the equivalence point at which the amount of both competitive and native template output is the same. This equivalence point provides the most accurate quantitation possible of the amount of starting material in the sample assuming that the efficiency of amplification of the competitive and native template are the same.

In most instances in which competitive PCR has been used, quantitation of the product has been accomplished using densitometric analysis of Southern blots, fluorometry or other sophisticated quantitation techniques. Unfortunately, this limits the applicability of the method because densitometers and fluorometers are expensive and not widely available in many laboratories. A simplified competitive quantitation assay using microtiter well technology would be easier to perform and could potentially broaden the applicability of quantitative analysis of mycobacterial burden. Microtiter wells are a widely used technology that only requires an ELISA reader, an instrument available in many laboratories.

Quantitation of mycobacteria poses significant technical difficulties and yet could potentially be a very useful tool in mycobacterial research. Unlike HIV and cytokine research in which the DNA of interest can be easily extracted from the sample, *M. tuberculosis* DNA is

notoriously difficult to extract even from bacteria in solution and more so from clinical samples. Nevertheless, a quantitative, non-radioactive and rapid technique for analysis of clinical samples for *M. tuberculosis* DNA could be very useful for a clinical study of new drugs against tuberculosis. In addition, it could provide valuable information for other types of research in which quantitation is necessary.

In summary, the aims of this thesis project were:

1) To develop a rapid, non-radioactive microtiter well based assay for competitive quantitation of mycobacterial burden is sputum samples using PCR.

2) To establish the correlation between the results obtained by standard quantitative culture and the results obtained in 1) above in CFU/ml.

3)To establish a pilot clinical study in a Mexico City hospital to study the response to therapy to standard antibiotic regimens (INH and Rifampin) during the first week of treatment using the quantitative PCR method developed in 1) and 2).

One potential problem with the type of clinical study being proposed here is the recruitment of patients. Despite the increasing number of patients with pulmonary tuberculosis, this is still a relatively uncommon disease in the United States. Establishment of a clinical trial of this kind in a country with a larger disease burden would allow for the recruitment of sufficient numbers of patients to make analysis of the data more meaningful. Attempts to apply molecular biology to research problems in developing nations are often hampered by a lack of attention to streamlining procedures to avoid the use of techniques that are either too complicated or too expensive to be carried out in conditions where limited resources are available. Given that a major goal of this project was to establish a clinical study in Mexico City, the philosophy underlying the design and implementation of the PCR assay was to design it in such a way as to minimize the use of expensive equipment and reagents.

Considerable time and effort where utilized in the initial stages of the project to define the most economical and simple way to carry out the assay.

Methods

Treatment of clinical samples

Sputum from patients diagnosed with pulmonary tuberculosis on the basis of a positive AFB smear were collected from an outpatient chest clinic in Mexico City. Samples were decontaminated and homogenized using standard laboratory techniques.

Briefly, samples were vortexed for 2 minutes with an equal volume of 4% NaOH/100 mM Sodium Citrate solution, 20 mg of N-acetyl-L-Cysteine (Sigma) and 5 ml of glass beads followed by incubation at room temperature for 15 minutes. 30 ml of phosphate buffer (60 mM Na_2HPO_4, 60 mM KH_2P0_4) were added and samples were centrifuged at 4,000*g* in a swinging-bucket rotor to pellet the bacteria. Sediments were resuspended in 2 ml of a 0.25% Tween 80, 0.85% NaCl solution. 100 ul of this was used to prepare serial, ten-fold dilutions for plating on Middlebrook 7H10 plates (duplicate 10^{-1} to 10^{-4} dilutions were plated). 500 ul of the remaining sample was centrifuged at 12,000*g* in a microfuge for 10 minutes. The pellet was resuspended in 500 ul of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), centrifuged again at 12,000g. and resuspended in 50 ul of TE/1% Triton X-100. Samples were stored at -20 ^{o}C.

Recruitment of patients for pilot clinical study

Patients detected as having smear-positive tuberculosis and with no prior history of treatment for tuberculosis were asked to participate in the study. A consent form adhering to UCSF Human Subjects Committee guidelines was used. Inclusion criteria were as follows:

1)Smear positive tuberculosis

2)Greater than 18 years of age

Exclusion criteria were as follows:

1)pregnancy

2)CD4 count less than 250 in HIV positive patients

3)severe underlying disease

4)hemoptysis greater than 25ml

5)evidence or suspicion of extra-pulmonary disease

All patients were hospitalized in the chest service of the Hospital General de Mexico. 12 hour sputum samples (7PM to 7AM) were carried out starting on day #1. After 2 days of sputum collection, patients were started on either rifampin (600mg) or isoniazid (300mg) single-drug treatment for 7 days. The choice of which treatment to give was randomized by placing 10 pieces of paper with the work "rifampin" written on them and 10 with the word "INH" written on them and placing them in 20 individual envelopes. One envelope was selected each day and treatment was started accordingly. Daily 12 hour sputum samples were collected and cultured by the method described above.

Analysis of patient data

The person in charge of de-contamination, culturing and counting of plates was blinded to the treatment received until after all data had been analyzed.

All *M. tuberculosis* culture plates were analyzed at 4 weeks of growth. Plates that appeared to be contaminated were discarded. Plates that had cultures with morphology consistent with *M. tuberculosis* were counted using the dilution plates. Where possible, the dilution plate containing >10 but <100 CFU was used. Acid-fast staining was also done on one colony from each plate to confirm that those colonies being counted were acid-fast.

DNA amplification

Samples were heated at 96 °C for 30 minutes. 25 ul of this extract were added to 425 ul of PCR mix [1.5mM $MgCl_2$, 20 picomoles of each primer, 200 uM each of dATP, dCTP, dGTP, dTTP and 1X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl)]. Primers were those described by Sjobring et al (17). *Pab* 1b (sense): ACCACCGAGCGGTTCGCCTGA *Pab* 2b (antisense): GATCTGCGGGTCGTCCCAGGT. This primer pair amplifies a 419 bp fragment internal to the *pab* gene. Each primer was biotinylated at its 5' end.

The PCR mix with the template DNA was aliquoted into five tubes. To tube one we added 10 ul of H_20. Tubes 2-5 received 10 ul aliquots of dilutions of *pab/tet* internal control at concentrations of 10^7, 10^5, 10^3, 10^1 molecules/ul

The internal control was constructed by excision of a 220 bp Nhe/Nar fragment of the *pab* gene cloned into pbluescript SK- and replacement with a 180 bp Nhe/Nar fragment of the *tet* gene of pbr322 (figure 1). This *pab/tet* internal control plasmid was purified using a plasmid purification column (Quiagen), digested with Bam HI to linearize it and quantified using a Beckman spectrophotometer.

Samples were heated to 94 °C for 5 minutes in a Perkin-Elmer thermal cycler. 2.5ul of *Tae DNA* polymerase (BRL) were then added to each tube and amplification was carried out for 40 cycles consisting of denaturation at 94 °C (1 minute), annealing at 58 °C (2 minutes) and extension at 72 °C (2 minutes). Amplified products were stored at -20 °C until further analysis.

Detection and Quantitation of amplified DNA

Detection and quantitation of PCR product was done in microtiter wells (Immulon 2, Dynatech laboratories). The pab and *tet* probe DNA fragments were prepared by ligating the Nhe/Nar fragments of *pab* or *tet* directly into pbluescript SK- plasmids digested with XbaI/ClaI to provide compatible restriction fragment ends. Single stranded-rescue was then

done using phage VCSM 13 (Stratagene) and the technique previously described for large-scale ssDNA preparation (25). Microtiter wells were coated with ssDNA using a modification of the method described by Kawai et al (26). Briefly, 1.0 ug of ssDNA was dissolved in 50 ul of TE. and then mixed with 50 ul of a solution containing 1.5M NaCl, 300 mM $MgCl_2$ and 300 mM Tris-HCl (pH 8.0). 100 ul of this solution was placed in each microtiter well and incubated for 12-16 hours at 37 °C. Plates were dried (1 hour at 37 °C) washed three times with 1 M NaCl. 100 mM Tris HCl (pH 9.3), 2 mM $MgCl_2$, and 0.1% Tween 20 and then stored at 4 °C until use (up to 1 month).

For each sputum sample, 60 ul of amplified PCR product were boiled for 10 minutes and added to 300 ul of ice-cold hybridization solution (5 X SSC, 0.1% SDS, 200 ug of denatured Salmon sperm DNA) and divided into each of three microtiter wells. Plates were incubated for 2 hours at 50 °C in a shaking water bath and then washed three times in 2X SSC at room temperature. Plates were then incubated for 30 minutes at room temperature in 1% BSA, 1 X PBS, 0.1% Tween with streptavidin-peroxidase (Boehringer-Mannheim) diluted 1/5000, followed by 3 washings in the same buffer. Detection was done using ABTS (Boeringer-Mannheim) substrate as per manufacturers instructions using an ELISA reader at 420nm wavelength(Molecular Devices).

Results

Development of a rapid, non-radioactive microtiter well based assay for competitive quantitation of mycobacterial burden is sputum. samples using PCR and determination of the correlation between the results obtained with this method and standard quantitative culture.

a) **Establishment of conditions and parameters for *pab* fragment amplification from *M. tuberculosis*.** Initial experiments were designed to define the most adequate conditions for amplification of the *pab* fragment. Several variables have been identified as being crucial for the amplification of DNA fragments using PCR. The most important variables are 1) the extraction procedure used to isolate the DNA 2) the concentration of $MgCl_2$ in the reaction buffer and 3) The cycling parameters used. Additional variables which are important for quantitation using PCR include 4) the use of "hot start" vs "cold start" PCR and 5) the number of cycles.

The *pab* fragment primers were those described by Sjobring et al (16) with biotin added to their 5' ends. Amplification of *M. tuberculosis* in solution was done with $MgCl_2$ concentrations of 1.5, 3.0, 5.0 and 10 mM. As can be seen from Fig 1, the most efficient amplification was obtained with 1.5 mM of $MgCl_2$ and all further experiments were done using this concentration.

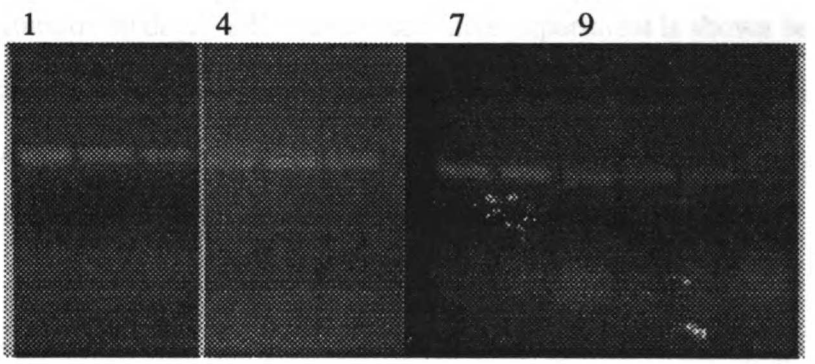

Figure 1. *Lanes 1-3 (1.5mM MgCl2, 10^7, 10^6, 10^5 CFU/ml), lanes 4-6 (3.0mM MgCl2, 10^7, 10^6, 10^5 CFU/ml), lanes 7-9 (5.0 mM MgCl2, 10^7, 10^6, 10^5 CFU/ml), lanes 10-12 (10.0 mM MgCl2, 10^7, 10^6, 10^5 CFU/ml).*

Additional experiments were done to define whether 'Cold" or "Hot" PCR should be used. Several authors have suggested that sensitivity of PCR can be increased if the *Taq polymerase* is added to the reaction mixture after a temperature greater than 60 degrees C has been achieved(27). The theoretical basis for this is that at lower temperatures, the primers are able to bind non-specifically to fragments of DNA that then are primed for DNA synthesis by the *Taq* polymerase. This would allow for non-specific amplification of DNA which would then be amplified in subsequent cycles. Adding the *Taq* polymerase only once the reaction mixture has reached a higher temperature ensures that only specific priming occurs. To avoid the problem of non-specific priming, several companies sell wax beads and other products that separate the enzyme from the reaction mixture at low temperatures. However, these products are expensive and add to the overall cost of the assay, an important point to consider when trying to develop assays widely applicable in countries with limited resources. A more cost-efficient way of achieving the same goal would be to simply add the *Taq* manually to each reaction vessel once the temperature has reached a pre-set limit.

To define whether 'cold' or 'hot' PCR was more effective, I amplified ten fold dilutions of *M. tuberculosis* (10^4, 10^3, 10^2, 10^1, 1.0 and 0.1 CFU/ml) either adding the *Taq* polymerase at room temperature or adding it only after the tubes had reached a steady temperature of 80 degrees C. A representative experiment is shown below. Two additional experiments similar to this were done and all three indicated that the 'Hot start" method improved the sensitivity of the PCR by at least 1 log.

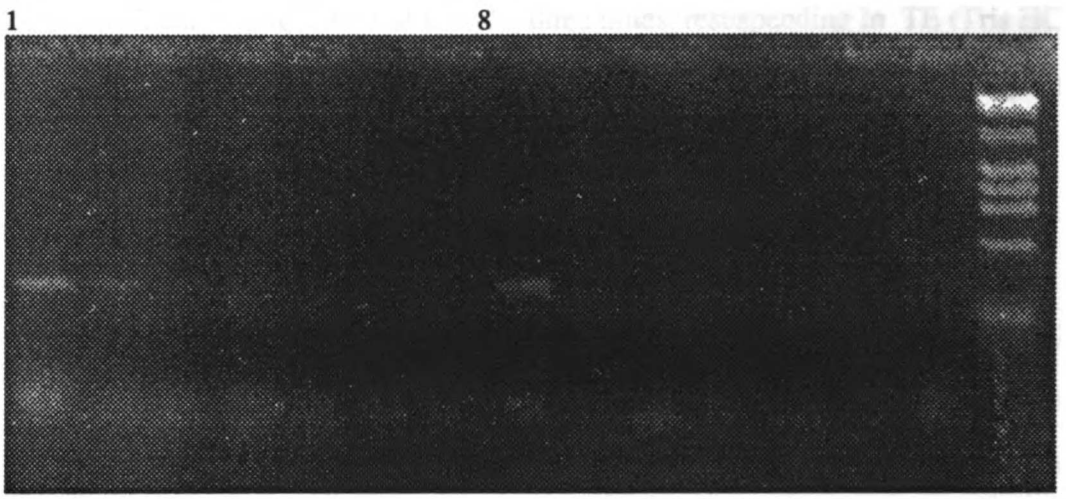

Figure 2. *Lanes 1-6: 10^4, 10^3, 10^2, 10^1, 1.0 and 0.1 CFU/ml amplified with "hot start". Lane 7: negative control. Lane 8-13: 10^4, 10^3, 10^2, 10^1, 1.0 and 0.1 CFU/ml amplified with "cold start."Lane 14: negative control. Lane 15: Phi X 174 marker.*

The cycling parameters used in these prior experiments were: 94 degrees for 1 minute, 58 degrees for 2 minutes and 72 degrees for 2 minutes. I repeated the experiments using $MgCl_2$ at 1.5 mM with 94 degrees for 1 minute, 58 degrees for 1 minutes and 72 degrees for 1 minute for a total of 35 cycles. A slight decrease in the amount of product obtained was noted with the shorter cycles. Therefore, all further experiments were done with 94 (1 minute), 58 (2 minutes) and 72 (2 minutes).

One of the limiting factors in using PCR for *M. tuberculosis* is the difficulty in extracting the DNA from the high lipid content cell wall of the bacterium. Most authors working with PCR for diagnosis of tuberculosis have used variations of the standard phenol:chloroform extraction (with or without proteinase K or lysozyme). However, these methods are tedious and require multiple transfers of the sample to clean eppendorf tubes. For an accurate quantitative assay, this is problematic because each transfer to a new tube leads to an inevitable loss of material. Therefore, I attempted to develop an extraction method that would be both sensitive and require minimal manipulation of the sample.

Three rapid, simple extraction procedures were tested:

1-Centrifuging 500 ul of sample three times, resuspending in TE (Tris HCl 10mM, EDTA 1mM) each time. The final resuspension was done in 50 ul and the sample was boiled for 30 minutes

2-washing with TE twice as in 1) but resuspending in 50 ul TE with 1% Triton X 100 and boiling for 30 minutes

3-centrifuging the 500 ul sample, resupending in 50 ul TE with lysozyme (200mg/ml), incubating at 37 degrees for 2 hours. 30ul of 10N NaOH and 60 ul of 10% SDS were added and samples were boiled for 10 minutes. 70ul of 5N HCl were added samples were then washed once with Chloroform: Isoamyl alchohol (24:1) and precipitated with ethanol. The results of a representative experiment are shown below:

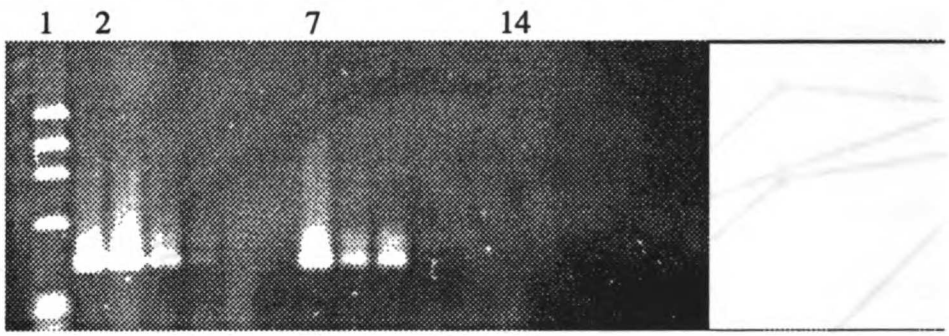

Figure 3. *Lane 1-Phi X174 marker. Lane 2-7 Samples containing 5 X 10^5, 5X 10^4, 5X 10^3 and 5 X 10^2, 50 and 5 CFU/ml extracted with TE?Triton X100. Lane 8-13. Same samples extracted with TE Lane 14-18. Same samples extracted with lysozyme.*

On the basis of these results, all further experiments were done using the TE/Triton X 100 method of extraction.

In quantitative PCR, the total number of cycles is critical because amplification is only linear within a certain range. If the number of cycles is allowed to proceed to the plateau phase at which each cycle no longer results in a doubling of the number of molecules, quantitative results are no longer reliable. In order to define the number of cycles that would give the greatest amplification while still being in the linear phase of the reaction, I amplified concentrations of 10^8, 10^6, 10^4 and 10^2 molecules/ml of purified *pab* and *pab/ tet* plasmid DNA for 30, 35, 40 and 45 cycles. Quantification of the product was achieved using the microtiter hybridization assay.

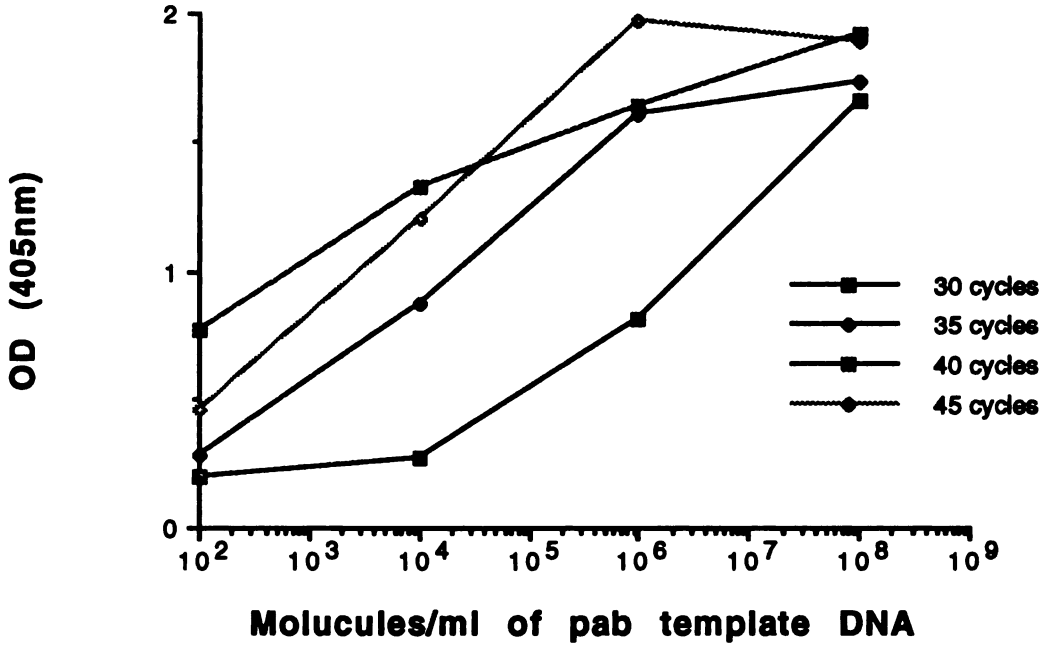

Figure 4A. ***Results of amplification of pab at 30, 35, 40 and 45 cycles.***

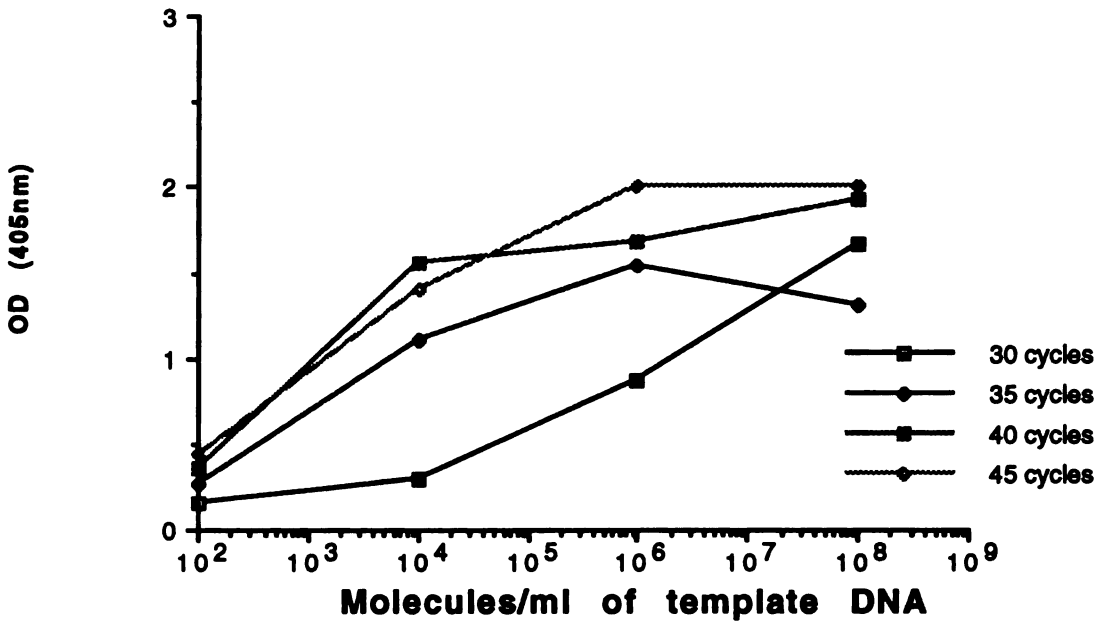

Figure 4B. ***Results of amplification of pab/tet at 30, 35, 40 and 45 cycles.***

As can be seen from Fig 4A and 4B, the amount of product obtained is linear over a broad range of input DNA up to 40 cycles. At 45 cycles, the amount of product detected begins to plateau. These experiments also show that the relative efficiency of amplification of the two templates is very similar, since the signal intensity at equal numbers of cycles is almost identical for the two. Based on these results, all further hybridization experiments were done with 40 cycles of amplification.

b)Development of a microtiter well hybridization assay for capture of PCR product and quantitative analysis.

Several techniques for binding of ssDNA probe to microtiter wells and hybridization of PCR fragments were tested. The Kawai et al (16) method for hybridization of DNA to microtiter wells was chosen as the basis for further experiments because it was the one that required the least number of reagents and least amount of washes. Initial experiments were

done to define the best way of fixing the probe to the microtiter well and the hybridization time that would give the strongest signal.

The Kawai method relies on a incubation in a low salt buffer followed by fixation with UV light to achieve permanent binding of ssDNA to the microtiter well. Other authors (28) have described similar protocols in which fixation is achieved by baking at 37 degrees without UV irradiation. A simple experiment was done to define whether UV irradiation was required to achieve adequate binding of the DNA. 48 Microtiter wells were prepared by placing 1.0 ug of ssDNA *pab* probe on the wells and incubating overnight as described in the methods section. One-half of these wells were fixed by irradiation with UV light at 254 nm using a Stratalinker 2400 apparatus. The other half of the wells were fixed with incubation at 37 degrees for 1 hour. Both series of wells were washed 3 times as described in the methods and stored for 3 days prior to use. PCR was done from samples with known dilutions of *M. tuberculosis* (10^4, 10^3, 10^2, 10^1 10^0 CFU/ml). 20ul of PCR samples were placed in triplicate in each microtiter well. As can be seen from Fig 5, the intensity of the signal was the same for both techniques.

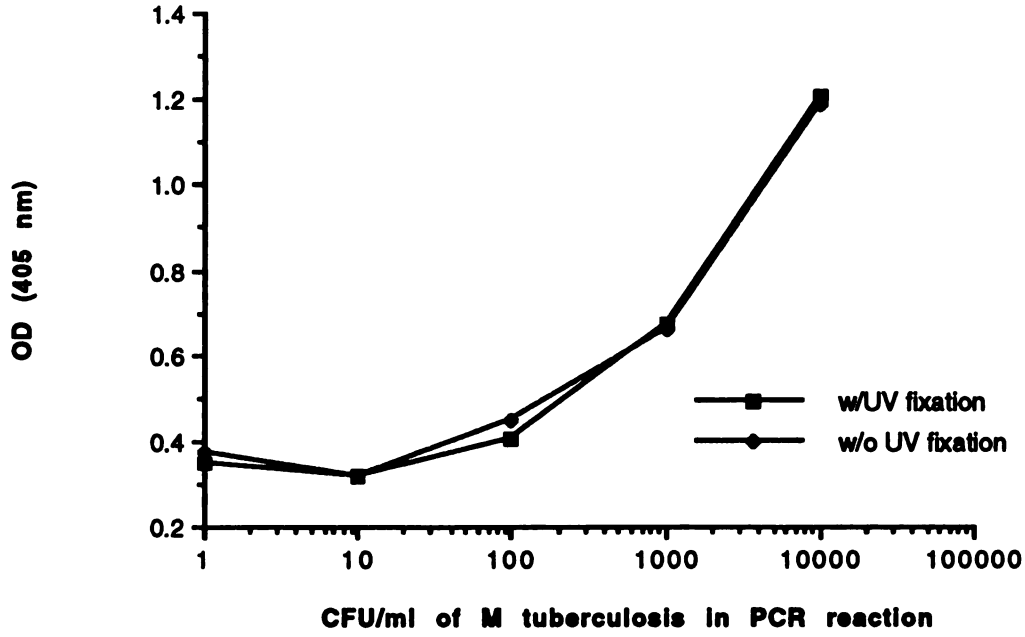

Figure 5. *Comparison of UV vs heat fixation on intensity of hybridization signal on microtiter wells. The x axis shows the [ ] of mycobacteria per ml. Reactions were carried out in a total volume of 100ul.*

On the basis of these results, all further experiments were done by heat fixation. This experiment also illustrates the detection limit of the hybridization assay. I carried out several additional experiments using dilutions of known amounts of *M. tuberculosis* in solution (quantified by standard quantitative culture techniques). A positive signal was defined as being 3 SD above the signal obtained from at least 6 control wells (wells with PCR reaction mix but no *M. tuberculosis* DNA). In most experiments, the limit of detection was between 20-50 CFU (in the experiment shown above, a positive signal was obtained for with 10 CFU but this was not reproducible in other experiments). This was found to be the case even when different *M. tuberculosis* samples were used and with different batches of *pab* ssDNA probe. Figure 6 illustrates the strategy used to construct the competitive template. The identity of this construct *pab/tet* was confirmed by sequencing (data not shown). The size of the *pab/tet*

fragment is 380 bp, making it readily distinguishable on a 2% agarose gel from the *pab* fragment amplified which is 419 pb long.

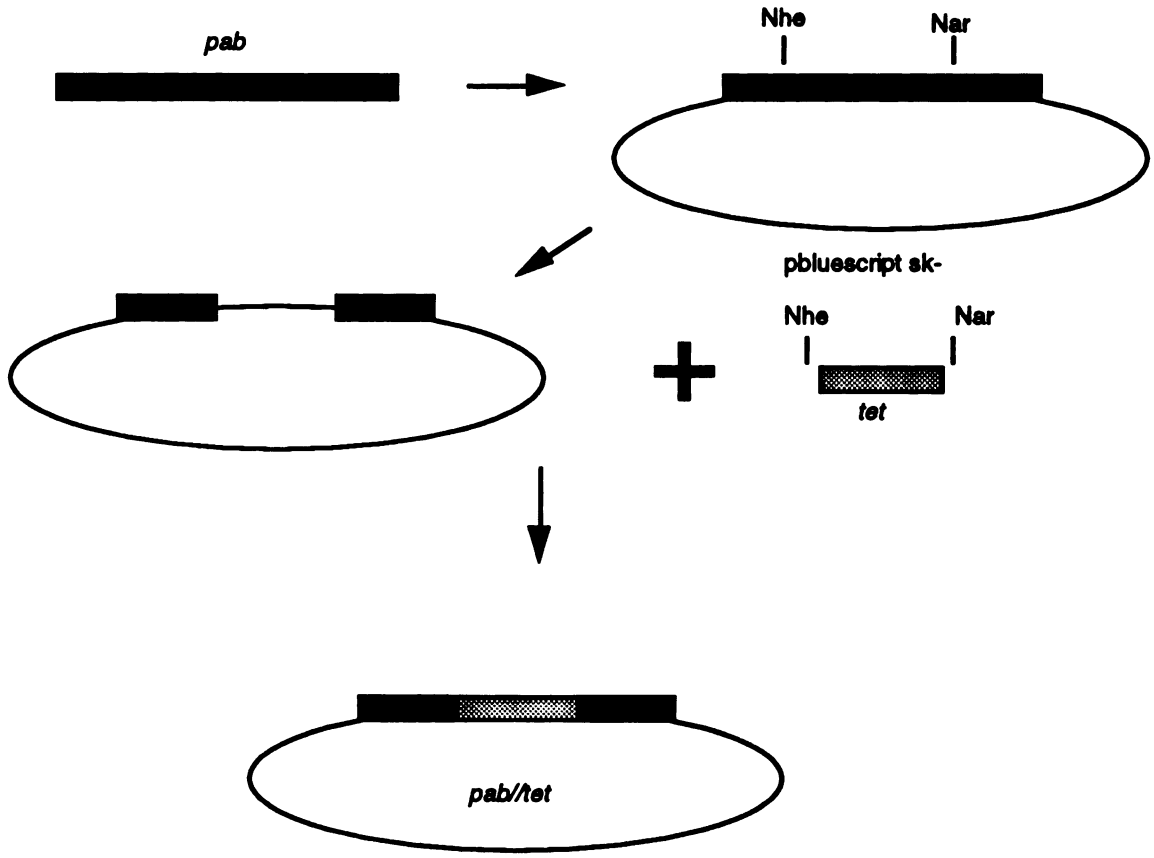

Figure 6. Construction of the *pab*/tet competitive template

Figure 7A and 7B show a representative experiment for quantitation using a known amount of *M..tuberculosis* in solution. A solution containing 5 X 10^6 mycobacteria was amplified in the presence of increasing amounts of *pab/tet* for 40 cycles. The calculated concentration of *M. tuberculosis* by quantitative culture in the experiment represented here was 1 X 10^6. Three other experiments like this were performed and in all cases the result for quantity of mycobacteria obtained by competitive PCR was within 0.5 logarithms of that obtained by culture.

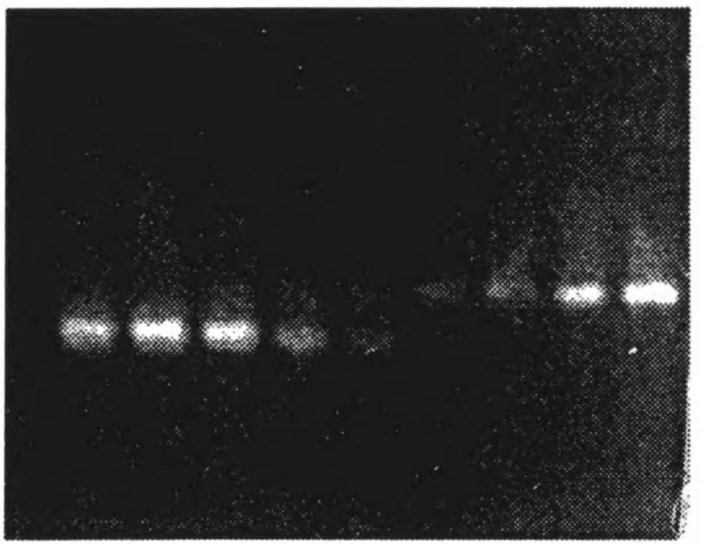

Figure 7A. Competitive amplification of pab and pab/tet. 5 X 10^6. M. tuberculosis in solution were amplified with increasing amounts of pab/tet. Lane 1-9 contain from 1 X 10^4 to 1 X 10^8 molecules of pab/tet plasmid(1 X 10^4, 5 X 10^4, 1 X10^5, etc.)

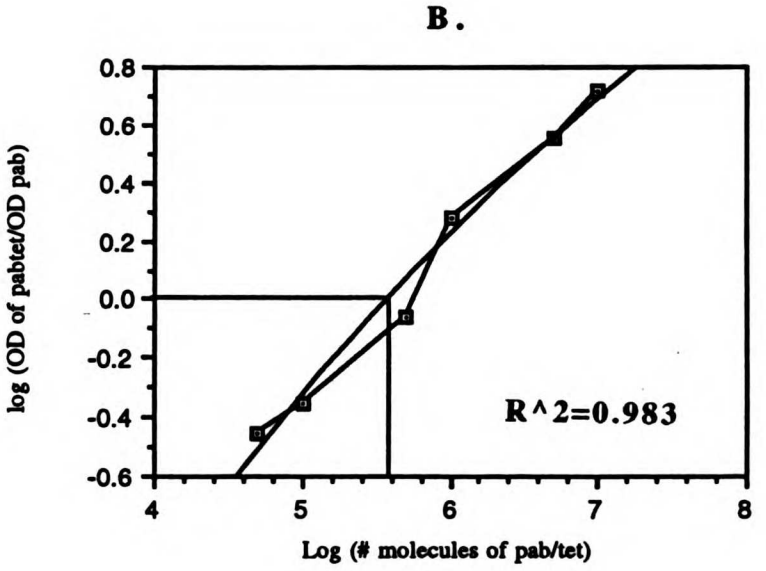

Figure 7B. Quantification of the amount of M. tuberculosis in the starting sample. Each sample shown in 7A was aliquoted into three microtiter wells with pab probe and three with pab/tet probe. Optical density was measured and plotted on the y axis as the log of OD in microtiter plates coated with pab/tet probe/OD of the same sample in microtiter plates coated with pab probe. The x axis is the log of the initial amount of pab/tet template in each tube. The amount of pab template is calculated by extrapolating the 0 point on the y axis (equivalence point for optical densities) with the corresponding point on the x axis.

On the basis of these results using *M. tuberculosis* in solution, I proceeded to test the utility of the assay on clinical sputum samples. Figure 8A and 8B shows the results of quantitation of nine individual sputum samples. Each sample was quantitated in two separate experiments. The results are expressed as the average of the two determinations. In no case was the difference between the two determinations greater than 0.5 logarithms. As can be noted, the results correlate very well with those obtained by quantitative culture. This method gives reproducible results using samples with greater than 10^3 CFU/ml. I attempted to quantify several samples with less than 10^3 CFU/ml using this method but was unable to quantify them reliably.

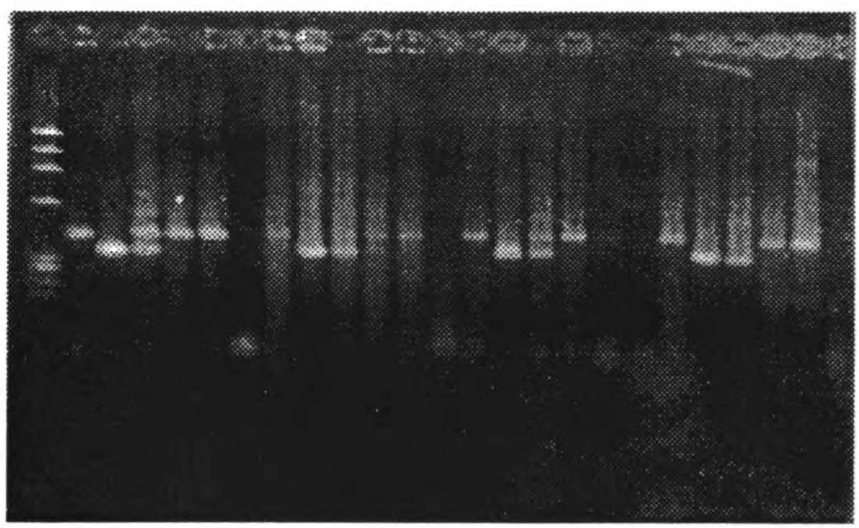

***Figure 8. A.** 2% agarose gel showing the results of competitive PCR for four sputum samples. Lane 1: Phi X 174 marker. Lane 2: sample 1 amplified with no competitive template. Lane 3-6: Sample 1 amplified with 1 X 10^2, 1 X 10^4, 1 X10^6 or 1X 10^8 molecules of pab/tet respectively. Lane 7-negative control. Lane 8-13: sample 2, same order as sample 1. Lane 14-19: sample 3. Lane 20-25: sample 4.*

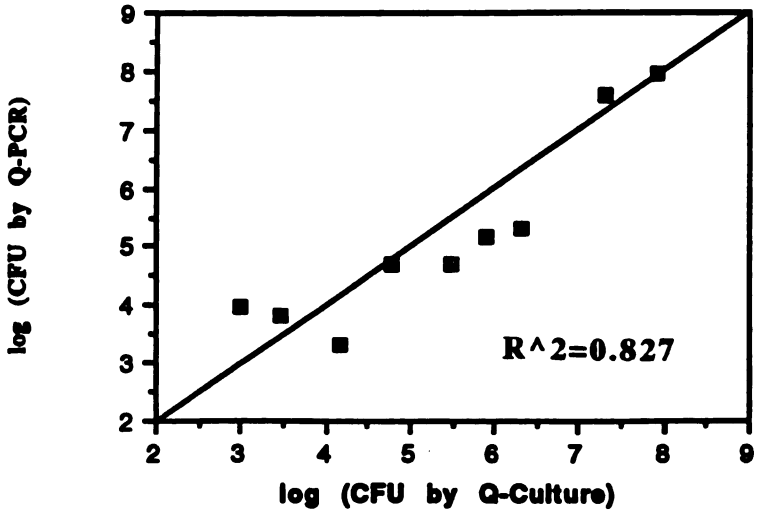

Figure 8B. Quantitation of *M. tuberculosis* burden in sputum samples. 10 sputum samples (samples 1-4 in figure 2A and 6 additional samples) with concentrations of mycobacteria in the 10^3 to 10^7 CFU/ml range were quantified by competitive PCR. Figure illustrates the correlation between Q-PCR and Q-Culture.

3)To establish a pilot clinical study in a Mexico City hospital to study the response to therapy to standard antibiotic regimens (INH and Rifampin) during the first week of treatment using the quantitative PCR Method developed in 1) and 2).

A total of 14 patients were enrolled in the pilot clinical study. Each patient was treated with either rifampin or isoniazid. Treatment was assigned based on opening one of 20 envelopes containing either "INH" or "RIF" written on a piece of paper. The person in charge of processing and quantitating the samples from each patients was not aware of what treatment the patients had received until after therapy was completed.

Due to technical difficulties with the collection and culture of the samples from each patient, only 6 of the 14 patients were analyzed. Patients 1-4 were discarded because a resident strike in the hospital impeded their hospitalization of the full 9 days of the protocol.

Patient 9 had to be removed from the study because of the development of severe hemoptysis on day # 5 of treatment. Patients 12, 13 and 14 had severe overgrowth of non-mycobacterial colonies on the Middlebrook plates and therefore where not amenable to quantitation.

Table 1 shows the results of the INH and Rifampin treated patients overall. Although there was a wide variability in the bacterial counts from day to day, all of the patients except for one of the INH treated patients showed a decrease in the absolute number of CFU/ml between the pretreatment days and the last day of treatment. This becomes more significant if one considers that the pretreatment baseline was the average of two sputum collections.

Day #	Rifampin (n=4) log(CFU/ml)	SD	Isoniazid (n=2) log (CFU/ml)	SD
0	5.68	0.44	4.01	0.82
1	5.42	0.51	3.60	*
2	4.95	0.51	5.53	0.40
3	4.92	1.01	6.25	0.15
4	4.42	0.98	2.91	*
5	4.25	0.58	3.27	0.33
6	4.29	0.81	2.93	*
7	4.49	0.16	3.85	0.29

* N/A because only one data point available.

Table 1. *Analysis of CFU/ml over the course of 7 days of therapy with either rifampin or INH. Day 0 is the mean of 2 pretreatment 12 hour sputum collections. Linear regression analysis were performed on these results. The per day decrease in (log CFU/ml) was as follows. Rifampin: -0.212+/-0.053 (p=0.001). INH: -0.172+/-0.145 (p=0.263).*

As is evident from the table, both the INH and the rifampin group exhibited a decrease in CFU/ml when the CFU/ml for each patient are averaged for each group. The number of patients is too small to attempt statistical analysis with the INH group. The Rifampin treated patients, however do provide statistically significant results. This is due to the fact that although there are only 4 patients, there are 9 timepoints for each patients, which makes the overall results highly significant (p=0.001)

Unlike the Jindani study, I did not include for comparison a group of untreated patients because this would have seemed difficult to justify on ethical grounds. Unfortunately, a large number of the patients for whom data was not analyzed were randomized to the INH than to the Rifampin group. However, my data would seem to contradict the Jindani data to some extent because the results with Rifampin are more striking than what they reported. I think one can conclude from this that:

a)Rifampin appears to have an early bactericidal effect on *M tuberculosis*

b)Results with INH are inconclusive but encouraging.

c)sufficient data has been gathered to establish a justification for beginning a larger study randomizing patients to INH, RIF as well as new drugs such as amoxicillin/clavulanate and ofloxacin to compare the bactericidal efficacy of each in the early treatment of pulmonary tuberculosis.

An initial attempt was made to try to quantify the sputum samples of two of the patients from the rifampin treated group using the quantitative PCR assay developed in order to compare the results obtained with culture and those obtained by PCR. I had a very difficult time preparing enough microtiter plates with ssDNA probe to analyze these patients due to the large number of plates required (approximately 300 total). Microtiter analysis was nonetheless attempted but was unsuccessful on one run. No microtiter wells, either with *pab* or *pab*/tet, showed a reaction. This was probably due to technical mistakes made in dealing with such a large number of samples at once. The experiment was not re-done due to lack of time and the difficulty in obtaining the ssDNA probes (discussed below).

Discussion

The results obtained in this project describe a simple, non-radioactive hybridization assay for detection of the *pab* fragment of *M. tuberculosis* using microtiter wells. The initial experiments were designed to define the most sensitive, cost-effective and least labor-intensive way of extracting the DNA, amplifying with PCR and hybridizing to the microtiter wells. This research was carried out with the ultimate goal of developing an assay that would be reproducible and could be feasibly set up in a laboratory with limited resources. I believe the assay described here fulfills this goal and provides the basis for future research oriented at applying this assay to quantitation and perhaps also diagnosis of *M. tuberculosis* using PCR. The preliminary data presented demonstrate the reproducibility and sensitivity of the quantitative PCR assay over a broad range of sputum CFU/ml. The most important weakness of the assay I have developed is its relative insensitivity. Whereas in other applications PCR has been found capable of detecting as little as a single molecule of template, my assay was only reproducible in detecting between 20-50 CFU (given the tendency of *M. tuberculosis* to clump together, this is probably more on the order of 10^2 molecules of *pab*). However, this sensitivity is within the range that has been described by other authors working the *M. tuberculosis* using much more elaborate assays such as radioactive southern blotting (17). The problem of sensitivity of *M. tuberculosis* detection has been dramatically pointed out by recent studies testing the ability of several research laboratories with expertise in PCR to detect *M. tuberculosis* in sputum samples in a double-blind fashion (13). It is however possible that further work could identify ways to increase the sensitivity of the assay substantially.

A pilot clinical study was established that suggests that both INH and RIF have early bactericidal effects that can be measured using standard quantitative culture. The necessary groundwork has been done to establish the Hospital Infantil de Mexico and the Hospital General in Mexico City as a viable unit for continued clinical research of this kind. Currently,

the clinical project outlined here is continuing and more patients are being enrolled with randomization to INH, RIF, amoxicillin/clavulanate and ofloxacin.

I encountered significant difficulties in attempting to use the Q-PCR method described here for quantitation of the clinical samples from the patients enrolled in the clinical study. The rate-limiting step was the production of enough single-stranded probe to allow for the analysis of a large number of samples. Each microtiter well requires 1.0 ug of ssDNA as probe. Using the standard Maniatis protocol, I was unable to produce more than 75-100 ug of ssDNA from 400-600 ml of starting bacterial culture. The analysis of a single patient required approximately 250 ug of ssDNA (9 days, five samples per day, multiplied times 3 for the number of wells, in duplicate since both *pab* and *tet* probe were needed). Since the protocol for ssDNA production takes 3-4 days to carry out, it became a physically very laborious task to make the number of microtiter wells necessary. Given the limited amount of time available to me, I was only able to attempt the analysis of two patients. For reasons that are unclear but probably related to technical errors made when dealing with such a large number of samples, this hybridization experiment did not work even though *pab* and *pab*/tet bands were clearly visible on an agarose gel. Therefore, I do not at this time have any data to show the application of the Q-PCR method developed to the analysis of the patients from the clinical protocol. Although the preliminary data that were obtained from individual sputum samples are encouraging, several problems still remain to be worked out. The first theoretical problem is that in the clinical study the total volume of sputum being analyzed is much greater than it was in the group of 9 patient samples studied. In the clinical study 12 hour sputum samples are collected and the total volume ranges from 15-45 ml. This large volume of sample is much more difficult to homogenize. Therefore, it is possible that the accuracy and reproducibility of the Q-PCR assay will be reduced with respect to the preliminary data presented here. This problem could perhaps be overcome by slight modifications to the homogenization protocol such as the use of larger bottles, more glass beads or more NALC.

The second problem is the already mentioned difficulty with scaling-up of the microtiter well assay to accommodate the large number of samples. All of the experiments described here were carried out in the laboratory of the *Hospital Infantil de Mexico* in Mexico City. Although this laboratory is fairly well equipped, it does not have many of the amenities common in U.S. laboratories of similar caliber. For instance, bacterial cultures were routinely done in a to and fro motion shaker rather than an orbital shaker which is standard in U.S. molecular biology laboratories. This relatively minor technical point might account for the small yield of single stranded probe since the shaker used allows for less mixing and aeration and therefore potentially less bacterial growth.

However, if this method is to be applicable to the type of clinical study being proposed here and potentially to other uses in which quantitation of mycobacteria is desired, it is possible that no amount of modifications to the protocol will make it technically feasible to produce enough ssDNA in anything short of an industrial laboratory. Therefore, it may be worthwhile to invest more time in modifications to the hybridization protocol used in order to make it more practical.

One potential solution to the problem posed by the large amount of ssDNA necessary would be to modify the characteristics of the probe to allow for using a smaller amount of DNA per microtiter well. For example, instead of using a single *pab* or *tet* DNA fragment inserted in the plasmid as was done here, one could use a smaller probe fragment inserted in the plasmid in the form of a repetitive sequence cassette. Such an approach was tested by Kawai et al (26) in a microtiter assay format for PCR fragment detection. In their study, they compared the relative sensitivity for detection of ng quantities of PCR of a single fragment probe and a 56 repeat unit probe. They found the repeat probe capable of reliably detecting 0.5 ng of input PCR product whereas the single unit probe only detected 5 ng of DNA. Although they did not specifically address the issue of decreasing the amount of DNA used per microtiter well using the repeat probe, their results suggest that one could maintain the same level of sensitivity of the assay with 1/10 the amount of single stranded probe. Such a

modification would make the large-scale use of the quantitative assay much more feasible. In addition, a repetitive probe could also increase the sensitivity of the assay, making it possible to accurately quantitative less than 10^3 CFU/ml, which is the limit of sensitivity that I found with the assay as describe here (although in this case obviously the increased sensitivity of the assay and decreasing the amount of probe would probably not be achievable at the same time).

Another possible modification to the assay that would obviate the need for large-scale ssDNA production would be to use streptavidin coated plates and bind the PCR product directly to them. A probe directed against either the *pab* or the *tet* internal fragment could then be used if this probe had a second reporter molecule, such as digoxigenin, attached to it. This approach would obviate the need for large scale quantitated of ssDNA probe although it would probably increase the cost to some degree by requiring the use of two reporter molecules.

Several factors contribute to make the quantitation of mycobacteria in sputum samples difficult. The lack of homogeneity of the samples despite the use of mucolytic agents and the tendency of mycobacteria to clump together contribute to making even quantitative culture unreliable. In addition, PCR requires efficient extraction of the DNA which is particularly difficult with mycobacteria given the high lipid content of the cell wall. Available techniques for DNA extraction all require manipulation with the necessary loss of starting material. This is probably especially important in an assay such as ours in which the competitive template is added as a free plasmid in solution, probably making it much more available for amplification than the native template. Therefore, the conditions for amplification of the competitive template and the native template are not equal. As mentioned above, quantification of mycobacteria using this method was not reproducible with concentrations less than 10^3 CFU/ml. While the reason for this is unclear, it is evident that at lower concentrations, aliquoting a reproducible amount of mycobacteria into each tube becomes less reliable.

Recently, Kolk et al published their results using a gene fragment inserted into *M. smegmatis* as the competitive template for quantitative PCR (29). Their approach holds promise in that both the native and control template need to be extracted in order to be

amplified and therefore should theoretically be more accurate than an assay in which the competitive template is added as the free plasmid. However, they used *IS6110* as the target for amplification which, although somewhat more sensitive because of the higher copy number, potentially creates a source of inaccuracy given that not all mycobacteria contain the same number of copies of this repetitive element. O approach would be to try to combine the two systems; transform *M smegmatis* with a the *pab/tet* plasmid developed here and use dilutions of the this mycobacterium as the control instead of the free plasmid in solution. However, this approach has technical challenges of its own since DNA is easier to extract from *M. smegmatis* than from *M. tuberculosis* and it would be difficult to guarantee that the *M. smegmatis* strain used contained only one copy of the inserted plasmid.

I think one clear advantage of the method describe in this thesis is that it is a micro-titer based assay that does not require the use of radiation or of densitometric scanning. Kolk et al used direct visualization of the competitive and the native template on agarose gels as their method for estimating the relative amounts of the starting material. The microtiter assay described here provides actual numeric data and therefore is potentially more accurate and amenable to automation. ELISA readers are widely available in many laboratories and could perhaps give this assay wider applicability in a variety of clinical settings.

While in other systems quantitative PCR has been shown capable of distinguishing two or three fold differences in starting material, I believe that this is not a realistic expectation for mycobacteria given the considerations described above. The ability to distinguish one-log differences in mycobacterial concentrations is nevertheless useful for many applications. For example, it might be useful in determining the infectivity of sputum from a given patient in a more accurate manner than the current standard of acid-fast positivity. Currently, patients that are acid-fast negative are considered to be non-infectious. However, given the current increasing prevalence of multi-drug resistant strains, it would be useful to have a method that could be used to more clearly define which patients require continued isolation and which do not. In addition, one potential advantage of the Q-PCR assay is that it would allow for

simultaneous analysis of all the samples from a single patient on the same day, therefore decreasing inter-assay variability.

The Q-PCR assay developed here could also provide a means to follow clinical response in patients at high risk for multi-resistant strains who are being treated either with conventional therapy or experimental therapies. Currently, the methodology for testing patients for multi-drug resistant strains involves plating bacteria on media plates impregnated with each individual drug. However, in the patients what matters is the in vivo synergistic effect of all drugs being used which is not necessarily the same as the sum of the *in vitro* response to each individual drug. Serial quantitation of sputum samples over the first few days of therapy in patients with suspected multi-drug resistant strains could help to identify those patients who require more additional drugs and those who do not. The data presented in this study show that at least in some patients, 2-3 log decreases in CFU/ml do occur over the first week of therapy with a single agent. It would seem reasonable to expect that this drop would be even more dramatic if a similar study were to be done in patients receiving multiple drugs.

In addition, it could offer a convenient and rapid way to assay for the burden of mycobacteria in air or other environmental samples and could therefore be a useful tool in infection control. It is conceivable that the sensitivity of the assay could be increased for other samples in which the factors mentioned for sputum are not a concern, for example CSF, pleuritic fluid or other body fluids in which homogenization does not pose such a daunting problem.

In summary, I have attempted to develop a simple, non-radioactive quantiative PCR assay for the analysis of *M. tuberculosis* in sputum with the aim of applying it to a clinical study of bactericidal efficacy of drugs against *M. tuberculosis*. I have successfully generated a competitive PCR assay that accurately quantifies *M. tuberculosis* in solution as well as in sputum. I have also successfully established a pilot clinical study to compare the bactericidal efficacy of rifampin with INH from patients in Mexico City. Although this study is

continuing, at this time I have insufficient data on INH. However, the rifampin data do show a significant decrease in bacterial counts over the first week of therapy. This clinical study will now begin to randomize patients to INH and rifampin as well as amoxicillin/clavulanate and ofloxacin. Significant technical issues still need to be resolved in order to reliably apply the Q-PCR assay developed here to the patients in the clinical study given the large number of samples that need to be analyzed.

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