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The Polymerase Chain Reaction (PCR), along with hybridization to chemiluminescent DNA probes, was used to detect *Mycobacteria* potentially present in patient specimens from the Mycobacteriology Laboratory at Walter Reed Army Medical Center (WRAMC). DNA from specimens were prepared by two different methods, and used in the PCR amplifications. Primers were evaluated using both known and unknown (blinded) samples. With known samples, the genus specific primers detected 74% of *Mycobacteria* positive samples, while the *M. tuberculosis* primers were able to amplify *M. tuberculosis* DNA in 59% of the positive samples. With the blinded samples, the genus specific primers were able to detect Mycobacterial sequences in 3 of 8 (37.5%) of the *Mycobacteria*-containing samples. There were no *M. tuberculosis* containing specimens in the blinded samples, and all samples were negative for *M. tuberculosis* by PCR.

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David C. Fisher 18 May, 1993

PI - Signature Date

INTRODUCTION

Mycobacterial infections are being recognized as being a major problem in patients with compromised immune systems, such as those with AIDS (Collins, 1992). The two most common Mycobacterial species found in AIDS patients are *M. tuberculosis* and *M. avium*, though opportunistic infections caused by other Mycobacterial species have been reported. While infection by *M. avium* seems to be an opportunistic infection, occurring in the late stages of clinical AIDS (Collins, 1992), infection by *M. tuberculosis* takes often occurs much earlier in the course of the disease. Indeed, there appears to be a synergistic effect between the two types of infection, since the onset of tuberculosis can accelerate the onset of clinical AIDS, reducing the survival time to less than eight months (Horsburgh and Silik, 1989). For these reasons, it is very important that Mycobacterial infections in HIV infected patients be diagnosed as rapidly as possible.

Detection of Mycobacteria in clinical samples is hampered by the slow growth rate of the bacteria (from 4 to 8 weeks to grow a culture of *M. tuberculosis*), and by the difficulty in differentiating different species of acid-fast bacilli in samples (Sommers and Good, 1985). Shortening the interval required to diagnose Mycobacterial infection, and to differentiate among Mycobacterial species is clearly of major importance. One method that has been used is the Polymerase Chain Reaction (PCR), using primers specific for Mycobacterial DNA sequences (Böddinghaus, et al, 1990; Kolk, et al, 1992; Shawar et al, 1993; Soini et al, 1992; Thierry, et al, 1992).

In last years report, we described our efforts to improve the detection of *M. tuberculosis* and *M. avium* in clinical samples by finding new species specific sequences, and by using computer analysis to design new primers for amplification of these sequences. In the following report, we describe our results using these primers, our analysis of a number of blinded test samples from WRAMC, and our efforts to develop a primer set for the detection of *M. avium* in clinical samples.

MATERIALS AND METHODS

Materials:

All materials for DNA amplification were purchased from Perkin Elmer (Norwalk, CT). Agarose was purchased from Gibco/BRL (Gaithersburg, MD). Oligonucleotides used for PCR primers and hybridization probes were synthesized on a Applied Biosystems 380B DNA synthesizer. DNA labeling kits (Genius™ 6) were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were reagent grade or better.

Methods:

Source of Clinical Samples: All samples were obtained from the Microbiology laboratory at the Walter Reed Army Medical Center. Most samples supplied were sputum samples that had already been cultured for the detection and speciation of *Mycobacteria*. One hundred Bronchoalveolar Lavage (BAL) samples were also supplied by WRAMC for use as blinded samples. In the latter case, though the samples were cultured, the results of the culturing were not supplied to us until after we had tested the samples by PCR for the presence of *Mycobacteria* and *M. tuberculosis*.

Preparation of Clinical Samples: DNA was isolated from Clinical samples by the method of Boom et al (1990). Briefly, in this method, the sample is added to a lysing solution containing 9 M guanidinium isothiocynate and vortexed in the presence of glass beads. The high concentration of the guanidinium salt lyses the cells and causes the DNA to bind to the glass. The beads are then washed with a second guanidinium containing buffer, with ethanol and finally with acetone. The beads are dried, and the DNA eluted with water or TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

DNA from clinical samples were also prepared by the method of Thierry et al (1992). In this procedure, 0.5 to 1 ml. of sample was pelleted and resuspended in 200 µl of lysis buffer (0.1 M NaOH, 2 M NaCl, and 0.5% SDS) and incubated at 95°C for 15 minutes. The sample was then extracted twice with phenol-chloroform, and ethanol precipitated. The DNA was resuspended in 100 µl of water, and 10 µl aliquots were used for the amplification reactions.

Amplification and Detection of Mycobacterial DNA in Clinical Samples: Mycobacterial DNA was amplified in a standard 100 µl reaction mix containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, approximately 2 mM $MgCl_2$ (depending on the optimal $MgCl_2$ determined for the primer set used), 1 mM in each dNTP, 1 µM in each primer, 25U/ml AmpliTaq® DNA polymerase, and either 0.1 ng control DNA, or 5-10 µl of DNA from a clinical sample. The timing and temperatures of the thermal cycling reactions were dependent on the primer set used. In all cases, controls were run that were -DNA and that contained purified *M. avium* and *M. tuberculosis* DNA.

One tenth volume of each reaction was electrophoresed on a 1.8% agarose gel, and stained with 0.5 µg/ml ethidium bromide. After staining, the samples were transferred to nylon filters by the method of Southern (1975). Oligonucleotide probes were 3' end labeled with digoxigenin-11-dUTP (DIG-dUTP) by tailing with terminal deoxynucleotide transferase, following the instructions in the Genius™ 6 kit. Briefly, approximately 100 pmole of the oligonuicleotide was tailed in a 20 µl reaction mix containing 200 mM sodium cacodylate, 25 mM Tris-HCl (pH 6.6), 0.25 mg/ml BSA, 5 mM $CoCl_2$, 50 µM Dig-dUTP, 500 mM ATP, and 2.5 U/µl terminal transferase. After an incubation of 15 minutes at 37°C, the reaction was put on ice and stopped by the addition of EDTA. The reaction products were ethanol precipitated in the presence of glycogen. Hybridization was carried out as described in the Boehringer Mannheim Genius™ System Users guide for Filter Hybridization, though at different temperatures and formamide concentrations, depending on the oligonucleotides used as probes.

RESULTS

During the past year, three types of studies were performed: 1) Continued testing of the primers on known samples to improve our detection efficiency; 2) Testing of unknown samples from Walter Reed Army Medical Center (WRAMC) samples to determine the actual efficiency of our detection methods (testing blinded samples); and 3) Trying to find primer sets for the efficient detection of *M. avium*. The results of these studies are summarized below.

Known samples were supplied by the Walter Reed Army Medical Center, though several tissue samples from *M. tuberculosis* infected monkeys were supplied by Dr. J. Burris at the National Institutes of Health. In all cases, DNA was extracted from the samples as described above, and the samples were amplified by PCR, using either the *Mycobacteria*-specific genus primer set described in last years report (based on the sequence of 16S rRNA), or one of two *Mycobacteria tuberculosis* specific primer sets derived from the sequence of the *M. tuberculosis*-specific insertion element, IS6110 (Shawar et al, 1993). More than 100 samples were tested with the genus primer set, while more than 160 samples were tested for *M. tuberculosis*. Some samples were tested multiple times to examine means to improve the sensitivity of the experiment. The results of these experiments are summarized in Table 1 and Table 3. Using the *M. tuberculosis* probe, 60% of the samples that were culture positive were also found to contain *M. tuberculosis* when tested by PCR. 95% of the samples that were negative by culture were also negative by PCR. Finally, eighteen different samples were *Mycobacteria* positive by culture, though the species was not identified. Of these, 3 were *M. tuberculosis* positive by PCR. These results are examined statistically in Table 3. In order to understand the terminology used in this table, certain terms need to be defined. A true positive is a sample in which *M. tuberculosis* was detected both by culture and by PCR. Likewise, a true negative is a sample that is *M. tuberculosis* negative, both by culture and PCR. A false positive is a sample that is *M. tuberculosis* negative by culture, but positive by PCR, while a false negative is a sample that is positive by culture but negative by PCR. The formulas used for calculating the sensitivity, specificity, positive and negative predictive value, and efficiency are given in Table 3. The sensitivity is the percentage of *M. tuberculosis* containing samples that were positive by PCR, while the specificity is the percentage of negative samples that were found to be negative by PCR. Positive predictive value is a measure of the value of a positive result by PCR on a sample, while negative predictive value is measures the value of a negative PCR result. Finally, efficiency is the percentage of the samples that were predicted correctly by PCR. These analyses show that while the sensitivity of the *M. tuberculosis* probe was only about 60%, the specificity and positive predictive value are both in the ninety percent range, and the overall efficiency is about 70%. The negative predictive value is rather low at just over 50%.

When the same analyses are performed with samples tested by the genus probe, we see that approximately 75% of the culture positive samples were also PCR positive, while two-thirds of the culture negative samples were also PCR negative. Only 9 of the 108 samples tested with the genus probe proved to be culture negative for *Mycobacteria*, meaning that the sample size is too small to be significant. The small sample size is reflected in the statistical analyses of these data, where the negative predictive value obtained is only 18%. However, the other values look much better, with sensitivity and efficiency of approximately 75%, a positive predictive value of over 98%, and a specificity of over 80%.

Results for the blinded bronchoalveolar lavage samples are shown in Table 2, with the calculated values shown in Table 4. 66 BAL samples were tested with the *Mycobacterial* primers and probes, while 37 samples were tested with the primers and probes for *M.*

tuberculosis. 8 BAL samples were *Mycobacteria* positive, according to culture results. Of these, 3 were detected by PCR. Of the 58 culture negative samples, 35 were also PCR negative. 37 BAL samples were tested with the *M. tuberculosis* primers and probe, and none were found to be either culture or PCR positive. The calculated results of these studies are shown in Table 4. For the *M. tuberculosis* probe, the sensitivity and positive predictive values are not interpretable, since none of the samples tested contained *M. tuberculosis*. Since no *M. tuberculosis* was found by PCR in any samples, the specificity, negative predictive value, and efficiency were all 100%. With the genus probe, the results were less than acceptable. While the specificity and negative predictive value were reasonable, with values of about 60% and 87.5% respectively, the sensitivity, positive predictive value and efficiency were all much less than that seen with the known samples. Some reasons for this will be discussed later.

During the past year, several different primer sets were used in our attempts to identify *M. avium* in clinical samples. The majority of these were based on the sequence of the *M. avium* specific insertion sequence, IS901(Krunze et al, 1991) or IS902(Moss et al, 1992), an almost identical insertion sequence that is also *M. avium*-specific. Computer analysis of these sequences was used to design suitable primers and oligonucleotide probes. However, when tested, none of the primer sets proved to be sufficiently specific or sensitive, either amplifying *M. tuberculosis* DNA, or only being sensitive to about 100 fg of *M. avium* DNA. Another sequence used was the intergenic region between the 16S and 23S ribosomal RNA genes (Frothingham and Wilson, 1993), which shows a great deal of variation among species, and among copies of the gene in a given species. In this case, the strategy was to design a set of primers that would amplify any Mycobacterial DNA, using specific probes to distinguish among the different species. Unfortunately, these probes proved to be less sensitive than the IS901 primer/probe combination, meaning that this approach was also not suitable for the detection of *M. avium* in clinical samples.

CONCLUSIONS

In the last year, we have obtained some encouraging results, and have also found some problems with our means of detecting Mycobacterial infections by the use of PCR. As stated in last years report, our results obtained for both the genus and the *M. tuberculosis* primer sets are generally similar to specificities obtained by other workers using PCR to detect Mycobacteria. Shawar et al (1993), using primers from the same insertion sequence correctly identified 55% of the *M. tuberculosis* samples by EtBr stained gels, and 74% by hybridization. Soini et al (1992)detected 56% of *M. tuberculosis* containing samples on gels. It should also be emphasized that the sensitivities, specificities, positive predictive values, and efficiencies were all reasonably good, with the specificities and positive predictive values all being in the 90% range. There are two possible explanations for the low negative predictive values obtained for the known samples. The first is that, in the case of the genus probe, only 9 of 108 samples tested were *Mycobacteria* negative. Since the negative predictive value is the number of true negatives divided by the sum of the true and false negatives, the relatively small number of negative samples will make the negative predictive value look smaller than it probably should be. It is also possible that, since the PCR assay is theoretically so sensitive, it was able to detect *Mycobacteria* in samples that are culture negative.

The results from the blinded bronchoalveolar lavage are somewhat troubling. In the case of the samples tested with the genus primer set, the relative number of false negatives was high, while there were a large number of false positives. However, most of the false positives were generated from samples that were prepared on one or two days. Since the

false positives generated on these days were quite strong, it seems probable that a contamination problem arose during the preparation of those samples. If the results from those suspect samples is removed from the data, the sensitivity stays the same at 37.5%, the specificity rises to 74.5%, the positive predictive value goes up to 20%, the negative predictive value remains at 87.5%, while the efficiency increases to 69%. While these values are still lower than desired, they are quite a bit closer to that seen with the known samples. It is impossible to draw any conclusions from the results of the *M. tuberculosis* assays on the BAL samples, since none contained *M. tuberculosis*. All that can be said is that the PCR assays on these samples were 100% accurate, since no samples were PCR positive for *M. tuberculosis*. Ideally, we would want to repeat the assays on another set of blinded samples, with two changes from the experiments reported here. First of all, we would want a number of samples to be *M. tuberculosis* positive, allowing evaluation of that set of primers. Secondly, we would like the culture results to be presented quantitatively, so that we would know, not only if *Mycobacteria* were present in the sample, but also how many were present per milliliter. This would aid in our evaluation of the sensitivity of the PCR assay.

The PCR assay for *Mycobacteria* appears to be less sensitive when using clinical samples than would be expected, based on the results of dilutions of purified Mycobacterial DNA. Possibly, there is something present in the clinical samples that inhibits the PCR reaction. One way to test this hypothesis would be to seed a relatively large sputum sample with a known amount of *Mycobacteria*, and look at the effect of different preparation methods on the efficiency of the PCR reaction. It is also possible that, upon storage of the samples, the sensitivity of the reaction is lowered. A similar phenomenon was reported by Shawar et al (1993), who noted that long term storage of clinical samples at 4°C greatly decreases the sensitivity of the PCR assay. Although our samples were stored at -20°C, the samples used for the blinded samples were up to two years old, which could lower the sensitivity of our PCR reactions. Again, this is something that needs to be examined, probably by storing aliquots of a sample known to be *Mycobacteria* positive at -20°C, and preparing DNA the from samples at different times and testing them with PCR.

We still have not been able to find a set of primers and probes that allow the detection of *M. avium* in clinical samples. Though we have tried a number of different probes based on the *M. avium*-specific insertion sequence IS901, as well as probes based on the 16s rRNA sequence and on the intergenic region between the 16s and 23s rRNA genes, none have worked to our satisfaction. A number of these proved to be specific for *M. avium*, though none had the sensitivity required for efficient detection of *M. avium* in clinical samples. We believe that finding *M. avium* specific primers now would require starting a search for *M.; avium* specific genes, which would be a long term project.

Finally, in a recent report, Noordhoek et al (1994) sent out sputum samples that had been spiked with known amounts of *M. bovis*, a species in the *M. tuberculosis* complex, to seven different laboratories using PCR for the detection of *M. tuberculosis*. The laboratories were asked to use PCR on IS6110 to detect the presence of *M. tuberculosis* in the samples (*M. bovis* contains a single copy of IS6110 (Fomukong et al, 1992)). The results obtained from the seven laboratories fell into two groups. In the first, the sensitivity was not very good, though the laboratories had few, if any, falsely positive results. In the second set of laboratories, the sensitivity of detection was very high, but with a correspondingly high level of false positives. With our known samples, we are squarely in the first group, with sensitivity that is not as good as we would want it, but with few false positives. Clearly, more work needs to be done to increase the sensitivity of detection of *Mycobacteria* without losing specificity. We believe that it would be worthwhile to extend these studies so that more blinded samples could be tested. In addition, either sputum or Bronchoalveolar Lavage samples could be spiked with known amounts of *M. tuberculosis*

so that the sensitivity of detection in clinical samples could be evaluated. In this way, we should be able to learn more about the limits of detection of *Mycobacteria* in clinical samples in our hands, as well as learning more about our ability to detect *M. tuberculosis* in clinical samples. In addition, we believe that the effect of storage of samples, as well as the effect of different preparation procedures should be examined. Finally, we believe that it would be useful to construct a plasmid containing a part of IS6110. This plasmid could then be used to spike clinical samples so that the effect of inhibitors on the PCR reaction could be examined without having to grow *M. tuberculosis* in culture.

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Table 1. Summary of PCR Results obtained with Known Samples

Primer set	Number samples tested	Number Culture positive	PCR and Culture positive	Number Culture Negative	PCR and Culture Negative	Number species not known	PCR positive when species not known	% Culture positive and PCR positive	% Culture negative and PCR negative
Genus	108	99	73	9	6			74%	67%
M. tb.	168	82	48	51	48	18	3	59%	94%

Table 2. Summary of PCR Results Obtained with Unknown (Blinded) Samples

Primer set	Number samples tested	Number Culture positive	PCR and Culture positive	Number Culture negative	PCR and Culture negative	% Culture positive and PCR positive	% Culture negative and PCR negative
Genus	66	8	3	58	35	37.5%	60%
M. tb.	37	0	0	37	37	0%	100%

Table 3 Calculated Parameters from Known Test Samples

	Genus Probe	M. tb probe
Sensitivity	73.6	59.7
Specificity	83.3	91.2
Positive Predictive Value	98.5	93.0
Negative Predictive Value	17.9	53.4
Efficiency	74.2	70.3

Definitions:

$$\text{Sensitivity} = \frac{tp}{(tp + fn)} \times 100$$

$$\text{Specificity} = \frac{tn}{(tn + fp)} \times 100$$

$$\text{Positive Predictive Value} = \frac{tp}{(tp + fp)} \times 100$$

$$\text{Negative Predictive Value} = \frac{tn}{(tn + fn)} \times 100$$

$$\text{Efficiency} = \frac{(tp + tn)}{(tp + tn + fp + fn)} \times 100$$

where tp stands for true positives, tn for true negatives, fp for false positives, and fn for false negatives.

Table 4. Calculated Parameters from Unknown (Blinded) Test Samples

	Genus Specific Primer Set	M.tb. Primer Set
Sensitivity	37.5	*
Specificity	60.3	100
Positive Predictive Value	11.5	*
Negative Predictive Value	87.5	100
Efficiency	57.6	100

Sensitivity, specificity, PPV, NPV, and Efficiency are calculated as described in Table 3.
* No *M. tuberculosis* containing samples were present.