AD-A248 259

(2)

AD_____

MIPR NO: 91MM1512

TITLE: POLYMERASE CHAIN REACTION BASED DIAGNOSTIC ASSAYS FOR PNEUMOCYSTIS CARINII

PRINCIPAL INVESTIGATORS: Timothy J. O'Leary, Ph.D.
Melanie Cushion

CO-INVESTIGATORS: Cynthia Wright, Thomas Fanning, and Mark Tsai

CONTRACTING ORGANIZATION: Armed Forces Institute of Pathology
Department of Cellular Pathology
Walter Reed Army Medical Center
Washington, DC 20307

DTIC
ELECTE
MAR 24 1992
D

REPORT DATE: March 1, 1992

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

1 March 1992 Final Report (2/1/91 - 1/31/92)

Polymerase Chain Reaction Based Diagnostic Assays for Pneumocystis Carinii

MIPR No. 91MM1512

63105A ✓

Timothy J. O'Leary, Ph.D.; Melanie Cushion; Cynthia Wright; Thomas Fanning; and Mark Tsai

3M263105DH29.AD.012

WUDA335719

Armed Forces Institute of Pathology
Department of Cellular Pathology
Walter Reed Army Medical Center
Washington, DC 20307

U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland 21702-5012

Approved for public release; distribution unlimited

The objectives of this study are to develop polymerase chain reaction assays for the identification and quantitation of Pneumocystis carinii infections in the lungs, solid organs, bronchioloalveolar lavage fluids and blood of rats and humans. Work during the initial project period consisted of implementing qualitative reactions capable of identifying the organism in humans and rats, developing the quantitation necessary for using the assay in rats, investigation the efficacy of the assay in following the development and treatment of Pneumocystis carinii infections in the lungs and BAL fluids of rats, and attempting to implement the assay in a sensitive and reliable way in blood. With the exception of implementing the assay in a sensitive and reliable manner in blood, all the objectives for the first project period have been met.

Diagnosis; HIV; RA I; PCR; P. Carinii; Lab Animals; Rats

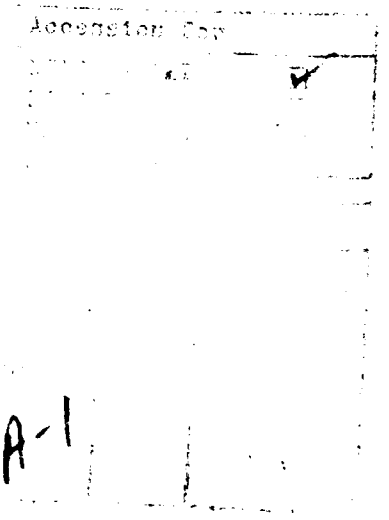
Unclassified Unclassified Unclassified Unlimited

FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.



REPORT TO THE ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

POLYMERASE CHAIN REACTION ASSAYS FOR DIAGNOSIS OF PNEUMOCYSTIS CARINII INFECTIONS (MIPR91MM1512)

PRINCIPAL INVESTIGATORS: TIMOTHY J.O'LEARY
MELANIE CUSHION

COINVESTIGATORS: CYNTHIA WRIGHT
THOMAS FANNING
MARK TSAI

OBJECTIVES:

The objectives of this study are to develop polymerase chain reaction assays for the identification and quantitation of *Pneumocystis carinii* infections in the lungs, solid organs, bronchioloalveolar lavage fluids and blood of rats and humans. Work during the initial project period consisted of implementing qualitative reactions capable of identifying the organism in humans and rats, developing the quantitation necessary for using the assay in rats, investigation the efficacy of the assay in following the development and treatment of *Pneumocystis carinii* infections in the lungs and BAL fluids of rats, and attempting to implement the assay in a sensitive and reliable way in blood. With the exception of implementing the assay in a sensitive and reliable manner in blood, all the objectives for the first project period have been met.

BRIEF OUTLINE OF THE EXPERIMENTAL DESIGN AND METHODOLOGY

92-07494

92 3 2 057

Sasco Sprague-Dawley (SSD+), (O'Fallon colony, Room OM1, Omaha, Nebrasca) were used for this investigation. The rats were maintained in barrier-isolated conditions, and fed a normal diet. Some of the rats were maintained for various lengths of time (see below) without immunosuppression, while others were immunosuppressed with methylprednisolone. Some of the rats were treated for various lengths of time with the anti-*Pneumocystis* drugs pentamadine and trimethoprim-sulfamethoxazole, others were withdrawn from immunosuppression, and others remained immunosuppressed for the entire course of the study. Rats were divided into groups of eight rats each for these experiments. Rats were sacrificed either at the end of a treatment period or the end of the study (see below), and the lungs were removed and lavaged. The bronchioloalveolar lavages were then aliquotted and frozen, while the lungs were fixed in ethanol. The livers, spleens, testes, brains and a sample of bone marrow were also taken and fixed in ethanol.

Only a subset of these specimens were analyzed using PCR; the lungs on all animals were analyzed however, and the bronchioloalveolar lavage fluids and blood specimens are now in the process of analysis.

PCR Methodology for Rats:

Ethanol-fixed lung tissue was picked up with a sterile needle and placed on a clean microscope slide, minced, and homogenized with a fresh razor blade. The tissue was then transferred into a microfuge tube and suspended in 100 uL of buffer containing 20 ug proteinase K. The tissue was digested in this buffer at 52 C for at least 1 hour or until the lyste cleared; the lysate was then heated to 95 C for 10 minutes (to destroy proteinase K activity) prior to storage at -20 C.

A 0.5 uL sample of each lysate was utilized in a PCR mix containing 10 mM tris-HCl,(pH 8), 50 mM KCl, 3mM MgCl2, 0.5 mM dNTP's, 0.5 uM of each primer and 1 U *Taq* polymerase. The sample was overlayed with 25 uL mineral oil, denatured for 2 min at 94 C, and then subjected to 30

cycles consisting of 94 C (1 minute), 60 C (50 sec) and 72 C (50 sec).

Each set of PCR assays included the lung tissue of rat 1245 and a blank sample as positive and negative controls, respectively. Assays of rat beta-globin and *Pneumocystis carinii* DNA were performed in separatte reaction tubes, but at the same time under the same conditions from PCR through Southern blot analysis.

Oligonucleotide primers and probes were synthesized on an Applied Biosystems DNA synthesizer.

Gene	Primer/probe	Sequences
globin	Primer 1	5' GGTGCACCTAACTGATGTTG 3'
	Primer 2	5' GCTTGTCACAGTGGAGTTCAC 3'
	Probe	5' GATAATGTTGGCGCTGAGGCCC 3'
PCP	PAZ102-E	5' GATGGCTGTTTCCAAGCCCA 3'
	PAZ102-H	5' GTGTACGTTGCAAAGTACTC 3'
	PAS102-L2	5' ATAAGGTAGATAGTCGAAAG 3'

A 10 uL aliquot of the PCR product was electrophoresed for 1-2 hours on a 2% agarose gel, then blotted overnight on a sheet of nylon hybridization membrane, air dried, then blocked with Oncor Membrane Blocking Solution. Hybridization was carried out at 45 C in 5 ml Hybrisol III containing 4 pmole of 32P labeled probe. After hybridization, the blot was washed 3 times at room temperature in 100 ml washing solution containing 0.2X SSC and 0.1% SDS. The blot was air dried and then exposed to a Kodak XAR-5 filem for 0.5 to 1 hour to obtain authoradiograms for densitometry measurement.

Densitometry was carried out on a Leitz TAS image analysis compauter, and the assay results expressed as the ratio of *Pneumocystis*/globin. Each ratio was then adjusted to its relative value with respect to that of tissue #1245, which was assigned to be 100 for every reaction. In this way, we compensated for differences in incorporation of radioactive dATP into the probes.

Measurements on other tissues and BAL fluids were caried out in a similar manner, as were measurements on human tissue, which utilized probes and primers applicable to human PCP and tissue, respectively.

Brief Summary of Results on Rats with Pneumocystis Carinii Infections: PCR Assessment of Pulmonary Pneumocystis Load in Normal, Immunocompromised and treated Rats

The following statistical summary refers to experiments in which pulmonary tissues were examined for *Pneumocystis carinii* infection using a quantitative PCR technique in which signals generated by PCR for *P. carinii* ribosomal DNA were compared with those generated by PCR for rat globin, and the autoradiographic signals measured by densitometry and compared. In a related experiment, similar measurements were made from bronchioloalveolar lavage (BAL) fluid from the same animals.

Initial experiments:

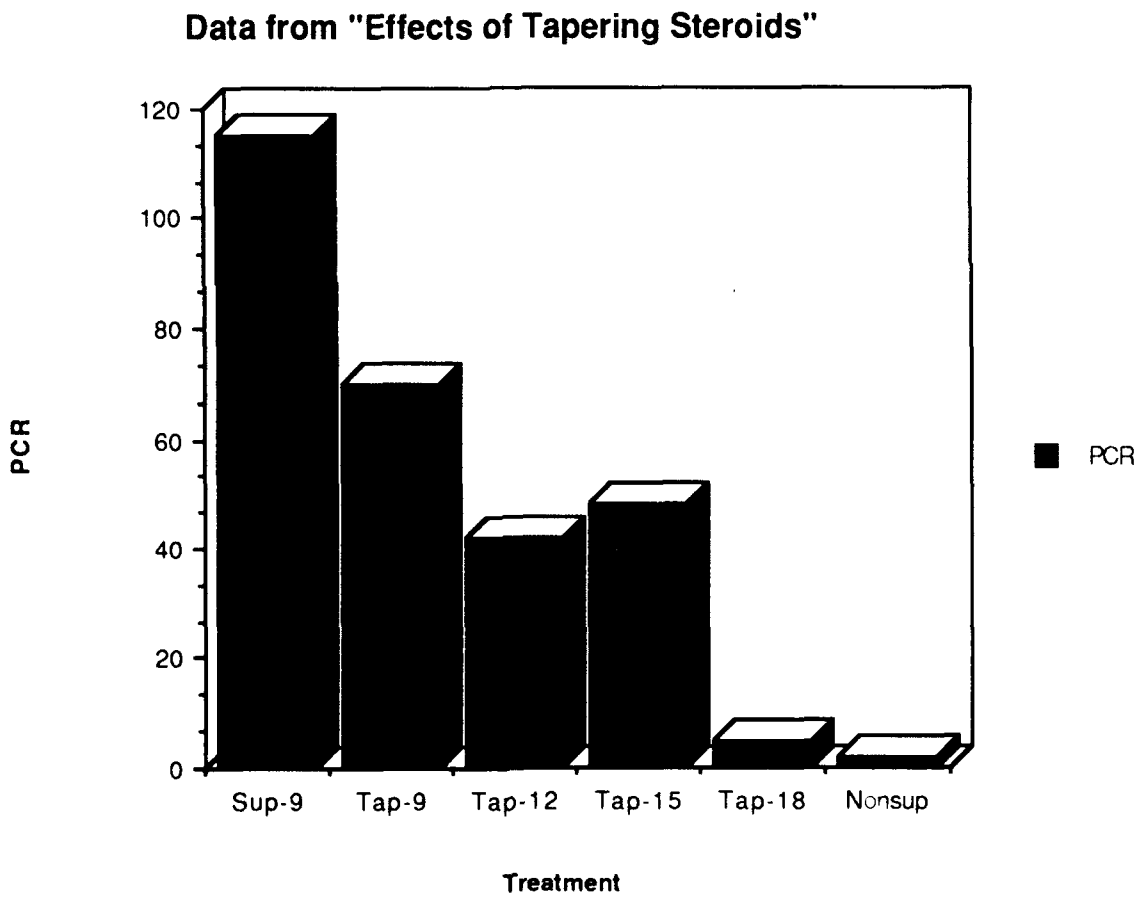
The signals were measured in a series of rats which were maintained in a non-immunosupppressed state for periods ranging from 3 weeks to 18 weeks. The rats were then sacrificed, and lung tissue pooled and utilized for PCR. A low PCR signal was found for many of the rats, but not all. Since the obtained signals were not normally distributed, comparison among groups for this and all further investigations was carried out using the Wilcoxan rank sum method. No differences were detected in the PCR signals from any of the non-immunosuppressed groups.

Immunosuppression time course experiments:

Animals were immunosuppressed with steroids for periods ranging from three to twelve weeks. Because of small numbers of animals for which assay could be performed in the three and 7.5 week groups (these animals had been used to perfect the assay procedure), no analysis was performed on these groups. There was no significant difference between the PCR signal ratios obtained in animals which had been suppressed for 9 and 12 weeks.

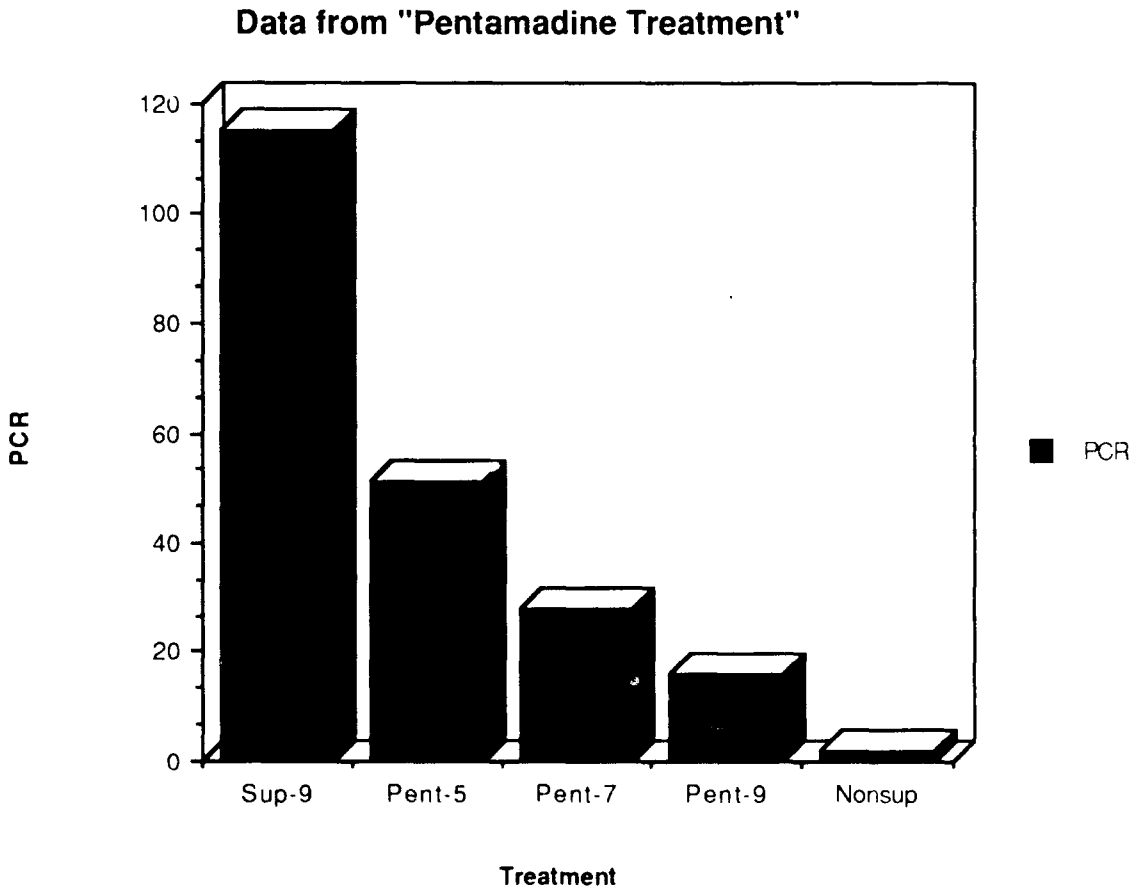
Tapering experiments:

Animals which had been immunosuppressed for six weeks were then tapered off their steroid doses. Although the average PCR signal was somewhat lower for animals which had been suppressed for six weeks then tapered for three, as compared to those which had been suppressed for nine weeks, the difference was not statistically significant (p = 0.16). There was a statistically significant reduction in the PCR signal for animals which had been withdrawn from steroids for six and nine weeks, however (p=.04 and p=.003 respectively).



Treatment with pentamadine:

The signals for animals which had been maintained on steroids but treated with pentamadine were compared both with animals which had been treated with steroids but not treated with antibiotics, and with animals which had not been immunosuppressed. Although the average PCR signal was lower for animals which had received 5 doses of pentamadine than for animals which had been immunosuppressed but not treated, the difference was not statistically significant (p=0.13). The difference between immunosuppressed treated and untreated animals was significant, however, when 7 or 9 doses of pentamadine has been received (p=.007 and p=.0006 respectively).

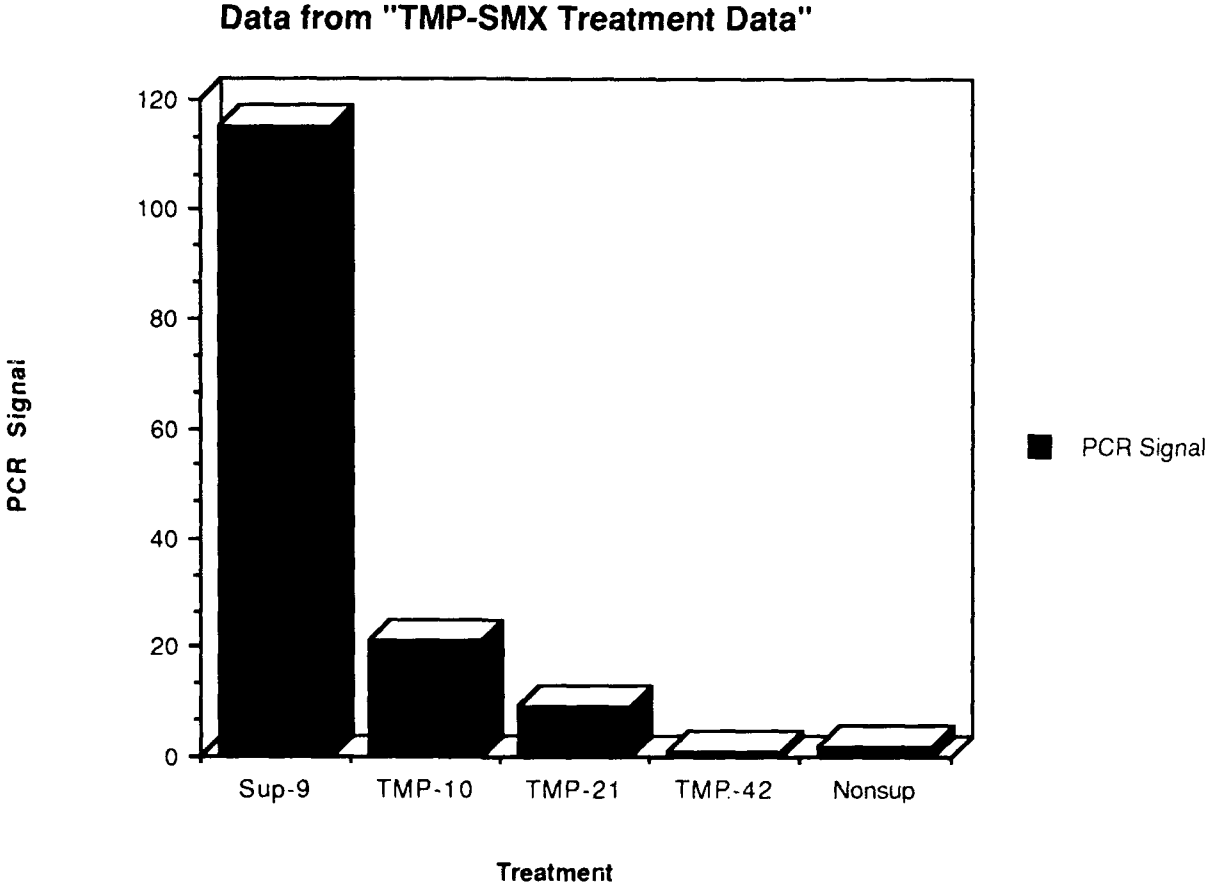


The differences in signals among the three pentamadine treated groups were not statistically significant. We attribute the lack of statistical significance in these comparisons to the relatively small numbers of animals in each group.

We also compared the animals which were immunosuppressed but treated with pentamadine with animals which were never immunosuppressed. There was a statistically significant difference in the PCR signals between the control animals and animals which had been treated with 5 ($p=.0003$), 7($p=.0003$) and 9 ($p=.004$) doses. These results clearly indicate that the treatment course employed does not reduce the level of *P. carinii* infection to normal levels, although it does reduce it significantly below the levels of immunosuppressed and untreated rats.

Treatment with trimethoprim-sulfamethoxazole:

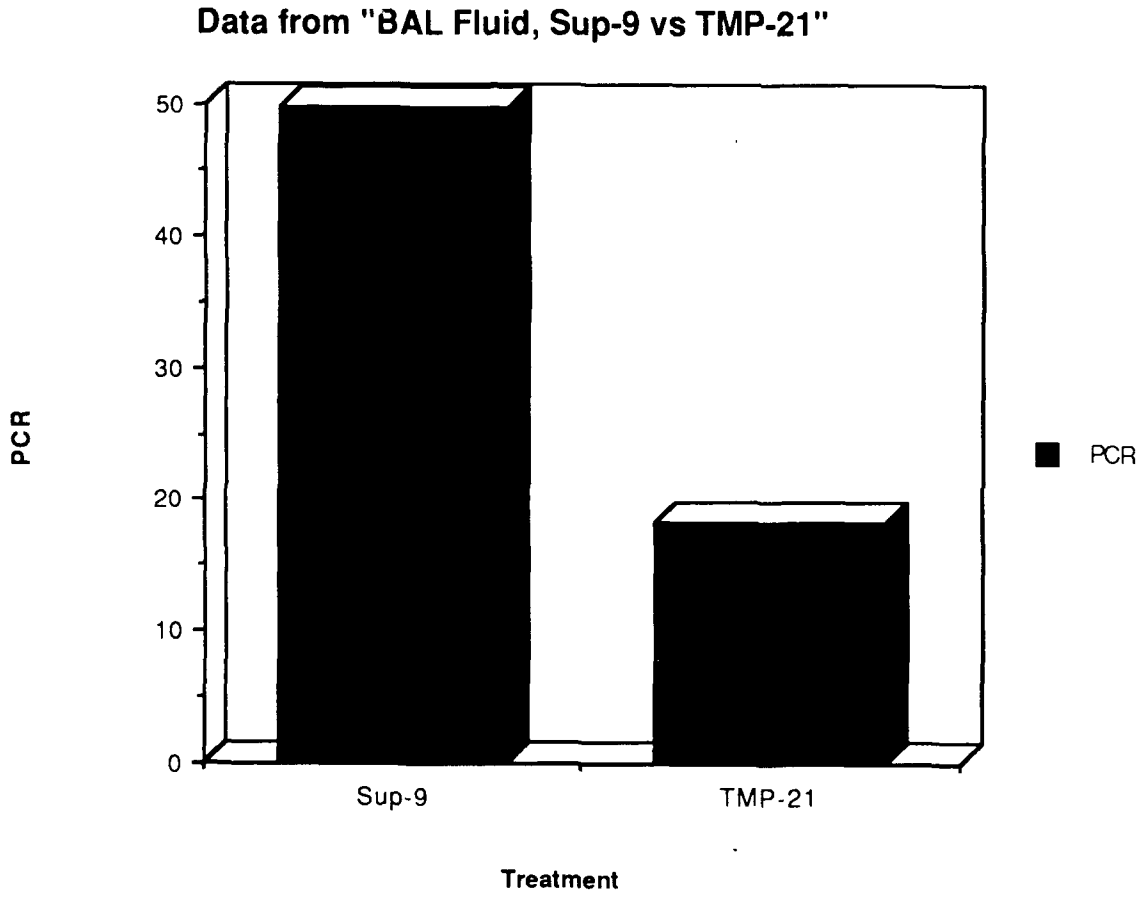
Rats were treated with trimethoprim-sulfamethoxazole (TMP-SMX) for 10, 21 and 42 days prior to sacrifice, and the results compared with those for immunosuppressed untreated and non-immunosuppressed animals. There was a statistically significant reduction in PCP PCR signal for all three groups of TMP-SMX treated animals ($p=.001$ at 10 days, $p=.0001$ at 21 days, and $p=.002$ at 42 days).



Although animals treated for 10 and 21 days had a significantly higher signal than did controls (p=.04 and p=.01 respectively), animals treated for 42 days did not have a significantly higher PCR signal than did animals which had never been immunosuppressed.

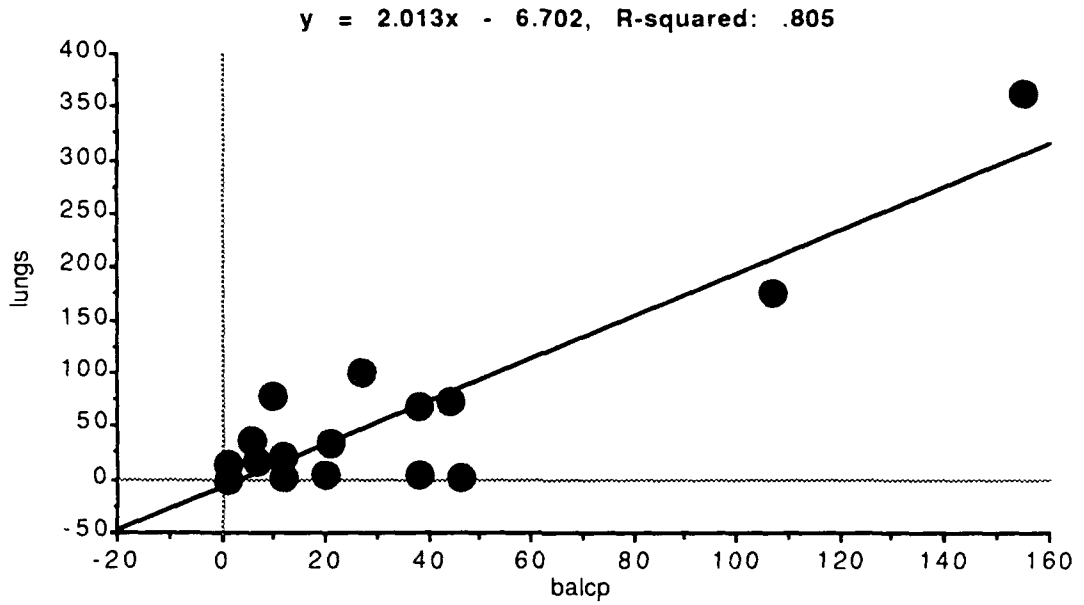
Experiments on BAL fluid:

BAL fluid was taken from all animals, but PCR experiments have only been performed on two groups of 8 animals each. The differences between the PCR signals from cell pellets prepared from the BAL fluid were statistically significant between animals which had been immunosuppressed for nine weeks, and those who were immunosuppressed but treated with 21 days of trimethoprim-sulfamethoxazole.



There was a strong linear correlation between the PCR signals from the cell pellets and the PCR signals obtained from whole lungs.

PCR Signal of Lungs as Function of PCR Signal in BAL Cell Pellet



There was a correlation between the PCR signals from either lung or BAL fluid and the logarith of the counts of cysts or nuclei in lung homogenates (r=0.61 for the PCR signal from lung vs the logarithm of the number of counted nuclei). The variance among animals in the results was less for the PCR method than for the nucleus or cyst counting methods, particularly for animals which had been immunosuppressed. As a result, we see no reason to prefer the manual counting of nuclei or cysts to the PCR method for quantitating the extent of *P. carinii* infection. Nevertheless, these results are very preliminary, since they are based on only 12 samples, and a considerable amount of experimental data should be accumulated before these results are considered definitive.

COMPARISON WITH HISTOLOGY:

WE have prepared sections from a number of these rat lungs. Based on a comparison of PCR results and classical histology, we conclude that the PCR method will be considerably more sensitive for diagnosis.

CONCLUSIONS:

The experiments clearly demonstrate that the extent of *P. carinii* infection can be determined using PCR assay of the lungs, and that the efficacy of treatment can be assessed in the same manner. They strongly suggest that PCR analysis of PCR fluid reflects the extent of infection in the lung, and can also be used to follow treatment. Further experiments are required to establish this with certainty in rats, and experiments in humans will, of course, also be required.

Brief Summary of Results on Rats with Pneumocystis Carinii Infections: Results in Nonpulmonary Sites

The results on nonpulmonary sites are not as complete as those based on pulmonary infections. The results briefly are as follows:

We have assessed a number of animals who had been immunosupressed for 9 weeks for the presence of PCP in their testes and livers. Only a small minority (about 15 %) showed evidence of such infections. Work on bone marrow is yet to be begun.

We have spent considerable effort on developing assay methodology appropriate for blood. As yet this has not turned out. Presently, the amount of whole blood which we can assay is to small to allow a useful blood test, since approximately 1000 organisms/ml would have to be present to have a fifty percent chance of detection. Work continues on improving our assay.

Brief Summary of Results On Humans:

We have demonstrated that our assay is capable of detecting pneumocystis carinii infection in humans. Although the assay is not as sensitive as is immunohistochemical assay for formalin-fixed, paraffin-embedded tissue, we believe that it will be up to three orders of magnitude more sensitive when fresh tissue is used for the PCR assay.

We have validated this assay on approximately 20 cases of PCP infection in formalin-fixed, paraffin-embedded tissue, and also on 5 patients with and without PCP infection whose frozen lung and spleen tissue were provided by Major David Anderson of the Walter Reed Army Institute of Research.

PUBLICATIONS:

No publications have yet resulted from this research. The results on rats are now publishable, however, and both an abstract and a paper are in preparation.