PN-ABA-947

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 9/20/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Cse

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5 & 3
24 PR

Attachment

UNIVERSIDAD PERUANA CAYETANO HEREDIA
INSTITUTO DE MEDICINA TROPICAL
"ALEXANDER VON HUMBOLDT"
A.P. 5045 - LIMA 100, PERU - TELF. 817551

5.203

THIRD INTERIM PROGRESS REPORT

PROJECT AID 936-5542

OBLIGATION 5361463

"RAPID DIAGNOSIS OF HUMAN LEISHMANIASIS"

January 2, 1987

SCIENTIFIC REPORT
(Period July 1986 - December 1986)

Project AID 936-5542

Obligation Nº 5361463

"Rapid Diagnosis of Human Leishmaniasis"

1. CLONING OF kDNA MINICIRCLE FRAGMENTS INTO BACTERIAL PLASMID VECTORS.

Our previous work using DNA hybridization techniques have demonstrated that parasites causing Andean Cutaneous and Selvatic Cutaneous/Mucocutaneous Leishmaniasis are indistinguishable from L.braziliensis reference strains (1). This fact shows that there is an extent homology between nucleotide sequences of kinetoplast DNA (kDNA) minicircle populations from both andean and selvatic leishmania parasites

On the other hand the growth and mantainance of Leishmania strains from which kDNA is extracted to be used as DNA probes have several disadvantages:

1. It is a slow method as it requires about 15 days to obtain 20 ug of kDNA.
2. The costs are relatively expensive as blood from rabbits is needed to prepare the biphasic medium for their growth. Furthermore the conditions to prepare the medium requires a laminar flow hood to ensure complete sterility.
3. Although we have had no background problems when we used biotinylated DNA probes, we might expect that other groups could find troubles on this subject. As a matter of fact, our routinely kDNA preparation method avoids the use of ultracentrifugation (see first interim report, January 6, 1986). For this reason, there is always a small amount of contaminant nuclear DNA that in case of a large amount of target parasites could increase undesired background.
4. To work with infective parasites requires special security conditions and people with special training.

The reasons mentioned above can be circumvented if specific kDNA fragments could be cloned into bacterial plasmid vectors. DNA sequences that are present in all the Leishmanias from the andean region but not in the selvatic ones could then used to discriminate between both subspecies. Furthermore to growth harmless bacteria containing recombinant DNA plasmids that can be easily amplified, would allow a very rapid, safe and inexpensive method for obtaining minicircle DNA sequences free of nuclear DNA. Thus we started the cloning of

minicircle DNA from different Leishmania isolates.

Kinetoplast DNA fragments obtained by restriction enzyme digestion with MboI where inserted into the BamHI site of the pBR322 according the method described in Maniatis' handbook (2). The LC03 and LC39 isolates, from the jungle and andean regions respectively, were used as DNA sources. Ampicillin resistant colonies that have lost tetracycline resistance phenotype were chosen as possible recombinant DNA candidate clones. Three clones, U2, E9 and E14 showed hybridization when they were used as target to our biotinylated DNA probes (see below). Nevertheless the small yield of true recombinant clones point out to a problem that must exist in our ligation reaction. Meanwhile we decided to look for another plasmid vector as the screening by replica plating is very time consuming. The vectors of the pUC family offer the advantage that the insertion of a DNA fragment in the multiple insertion site inactivates the ability of lactose fermentation. The recombinant colonies are visualized by their colorless appearance (3). Unfortunately our first attempts here in Peru were unsuccessful as the cloning efficiency was very low.

In order to improve our cloning efficiency I and Martin Lopez went to Dr. Dyann Wirth's laboratory (Department of Tropical Public Health, Harvard School of Medicine, Boston). During our 2 months and a half training period we learned a more suitable kDNA preparation technique for cloning purposes, as contaminant nuclear DNA is nearly absent. Furthermore we prepared bacterial competent cells that showed an optimal yield for transformation with pUC vectors. During the last two weeks in Boston we obtained several dozen of candidates, having characterized preliminarily 24 of them by electrophoresis in agarose gels (Fig 1.). According their mobilities and restriction enzyme digestion patterns, some of them seem to contain an insert. They will be further characterized by DNA hybridization techniques. On the other hand we learned how to clone into M13 phage DNA. This will be very important in case that DNA sequencing would be necessary to determine a specific DNA fragment that discriminates between andean and selvatic leishmaniasis

2. USE OF BIOTINYLATED DNA PROBES TO SEARCH FOR RECOMBINANT DNA MOLECULES.

The use of DNA probes labeled with P^{32} are frequently used on crude homogenates (usually fixed to a solid matrix, as nitrocellulose paper) to detect those colonies that harbor recombinant DNA. Unfortunately the use of radioisotopic labeling is restricted in underdeveloped countries for several reasons . We have applied successfully our biotinylated DNA probes to accomplish the same objective as the radioactive ones. Our method, unlike other previously reported (4,5), doesn't need a previous purification step from crude contaminants. We have been able to detect clones containing insert fragments down to about 100 base pairs after a crude Birboim extraction procedure (data not shown). Figure 2 showed the detection of the U2 clone out of several candidates that were Ampicillin resistant, Tetracycline sensitive. That U2 was a true recombinant clone was further confirmed by Southern blot hybridization procedure (Figure 3). In this case we also used biotinylated DNA probes.

REFERENCES:

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2. MANIATIS T., FRISCH E.F. and SAMBROOK J. 1982. Molecular Cloning . A laboratory Manual. Cold Spring Harbor Laboratory.

3. RUTHER U., Koenen M., Otto K. and B. Muller-Hill. 1981. pUR222 a vector for cloning and rapid chemical sequencing of DNA. Nucleic Acids Research 9: 4087-98.

4. POLLICE M. and YANG H. 1985. Use of nonradioactive DNA probes for the detection of infectious bacteria. Clinics in Laboratory Science, 5: 463-73.

5. DORMAN M.A., BLAIR C.D., COLLINS J.K. and BEATY B.J. 1985. Detection of Bovine Herpes virus 1 DNA inmobilized on Nitrocellulose by hybridization with biotinylated DNA probes. Journal of Clinical Microbiology, 22: 990-5.

LEGENDS TO FIGURES

Figure 1. Dot-blot hybridization with Birboim plasmid extracts. Crude plasmid extracts bacteria possibly containing kDNA insertions into the plasmid vector pBR322 were blotted on NC paper and treated with Proteinase K. Hybridization was carried out using biotinylated total L. braziliensis kDNA as probe. 1: Birboim extract of vector pBR322; 2-8: extracts from seven bacterial clones. Number 3 corresponded to a clon containing the largest kDNA insertion (800 bp). 9: pure kDNA from L. braziliensis braziliensis as a control.

Figure 2. Detection of kDNA inserted in a bacterial plasmid vector. A: plasmid extracts from E. coliclones presumably containing a kDNA insert in the plasmid pBR322 were linearized by EcoRI digestion and run in a 1.5% agarose gel (lanes 1,2,4,5 and 6). MboI-cut kDNA from L. mexicana amazonensis and L.braziliensis braziliensis are in lanes 7 and 8 respectively. Lane 4 is EcoRI-cut pBR322 from a Birboim extract. B: Southern blot of gel in A hybridized against a biotinylated total kDNA from L. braziliensis braziliensis. The band in lane 5 corresponds to the clone called UTA2, containing a kDNA insert of approx. 800 bp.

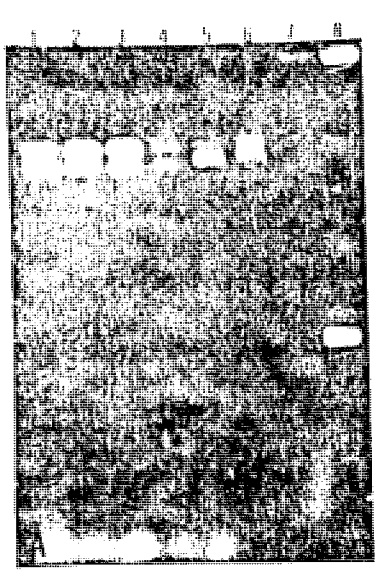
Figure 3. Plasmid DNA electrophoresis in Agarose 1%. Ampicillin resistant Tetracycline sensitive colonies were subjected to DNA ectraction procedures. Lanes 8 and 23 are DNA molecular weight markers (lambda HindIII). Lanes 4,10,19 and 27 are whole pUC12 plasmids. The other tracks are possible recombinant DNA plasmids.

1 4 7

2 5 8

3 6 9

Figure 1



1 2 3 4 5 6 7 8

B

Figure 2

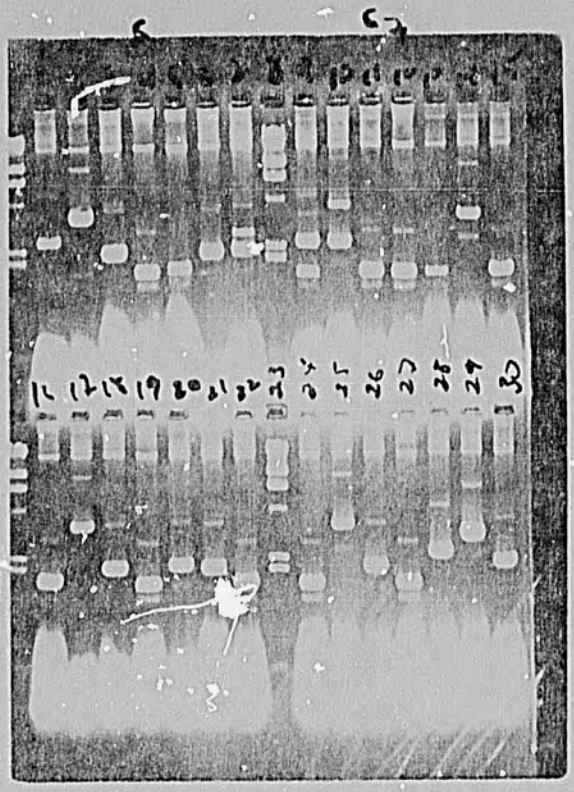


Figure 3