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How trypanosomes tolerate drugs that once killed them

Three compounds are used to treat and prevent animal trypanosomiasis, a disease that kills cattle, sheep and goats in many developing countries. All three compounds—diminazene, homidium and isometamidium—have been used for more than 30 years. The high costs of developing such compounds make it unlikely that new trypanocides will be marketed in the near future unless they act against a broad range of protozoan parasites. Until new trypanocides are developed, the efficacy of the compounds in current use must be maintained to keep domestic livestock healthy in the extensive areas where trypanosomiasis is endemic.

Scientists researching parasite drug-resistance at ILRAD are working to help maintain the efficacy of the trypanocides now available by developing biochemically based diagnostics that will rapidly quantify drug sensitivity in trypanosome field populations.

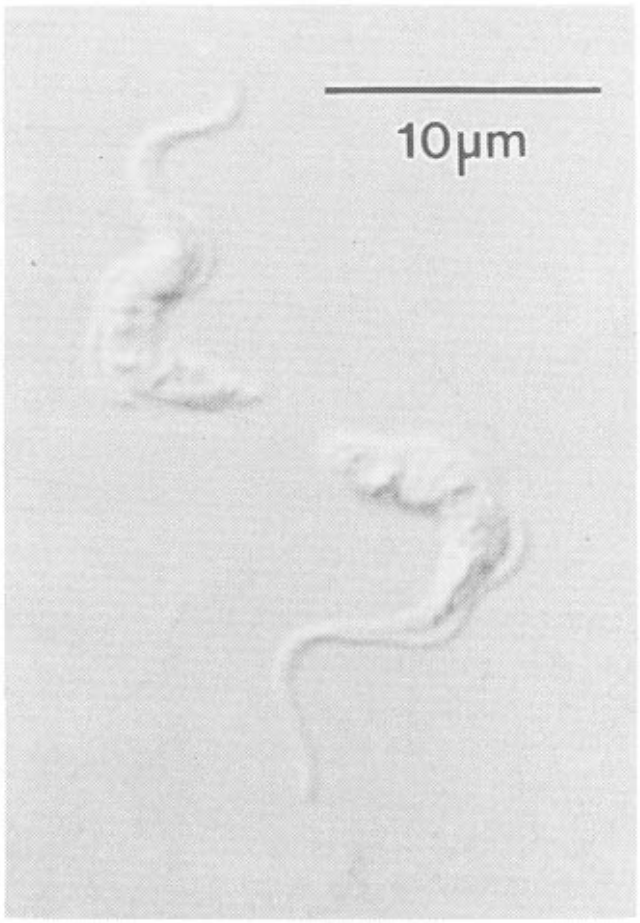
Such diagnostics will enable disease control workers to make optimal use of trypanocides. To develop such diagnostics, however, researchers must first understand the molecular basis of drug action and drug resistance in trypanosomes

ILRAD scientists are focusing their research on isometamidium because this is the most common prophylactic used for trypanosomiasis and thus the first compound against which parasites in the field are likely to develop resistance. Results of earlier studies on this drug demonstrated several possible actions: inhibition of RNA polytnerase, DNA polymerase and mitochondrial type II topoisomerase, and polyamine metabolism.

The following report summarizes preliminary results obtained by scientists from ILRAD and the Israel Institute of Technology (Technion) who are conducing a collaborative project to elucidate the molecular basis of parasite drug resistance.

ANY FOREIGN COMPOUND other than food that interacts with the physiology of an organism, affecting its structure or function, is a drug. Some drugs activate bodily processes, others injure or destroy disease-causing organisms that invade the body. Drugs developed against bacteria, viruses and parasites are targetted to destroy these pathogens while causing minimum harm to the cells of the mammalian host.

All organisms live in the midst of toxic compounds. The latter include pollutants such as PCBs and dioxin; heavy metals in the environment such as cadmium, lead and mercury; toxins produced by an organism's own metabolism; simple or complex poisons produced by organisms to gain ecological or environmental advantage over other organisms; and novel compounds synthesized by man for use as pharmaceutical agents, insecticides and pesticides. To survive exposure to these toxins, all living things have evolved mechanisms for minimising their potentially lethal impact.



Two single-celled trypanosome parasites obtained from mammalian blood visualized by light microscopy using the differential difference contrast technique.

The ability of pathogenic organisms to tolerate drugs that previously killed them is an increasing concern in medical and veterinary science. Hospitals, for example, are now facing a resurgence of post-operative infections by *Staphylococcus*, which have become resistant to standard antibiotics. Such drug resistance has recently been sensationalized in the lay press with stories of 'super-bugs' malevolently adapting themselves to formerly potent drugs.

Although a particular concern today, the development of drug resistance is not a new phenomenon. It is a consequence of two biological facts: the existence of biochemical pathways that are able to detoxify foreign compounds (xenobiotics), and the process of natural selection. In this process, individuals of a species possessing traits adapted to their environment will reproduce more than other individuals and thus their descendants, inheriting these traits, will come to dominate the population.

Small organisms, such as unicellular parasites, have the advantage of very short generation times, which means that beneficial mutations, such as drug resistance, can be selected for quickly in a parasite population. In man and other mammals, which may reproduce only every few years or decades, selection is necessarily slower. Being multicellular and highly complex, however, mammals have their own advantages: cellular plasticity and redundancy allows for a range of ways of minimizing the impact of toxic compounds.

Any infection treated with a drug is an unsupervised experiment in evolution. The toxicity of the drug acts as a selection pressure, potentially increasing the numbers of individuals able to tolerate it. If the effect of the drug is overwhelming, the infection will be cleared, but if the drug levels administered are low or the dosing is infrequent, tolerance to the drug may evolve in the infecting organism. Strategies to reduce levels of drug resistance include ensuring that dosing is sufficient to clear an infection (hence a doctor's advice to complete a course of drugs even after symptoms subside) and the use of combinations of drugs, known as sanative pairs, each of which acts on the target organism differently, thus ensuring that at least one compound will clear the infection. (It is improbable that an individual cell would develop tolerance to both compounds.)

The problem of drug resistance in trypanosomiasis

Animal trypanosomiasis is a wasting disease that causes major losses in cattle productivity in sub-Saharan Africa. The disease is caused by infection with the trypanosome, a single-celled parasite transmitted by the bite of a bloodsucking tsetse fly. The method most commonly used to control animal as well as human (sleeping sickness) trypanosomiasis is drug treatment, which is administered both to prevent and to treat the disease.

The potential for the development of drug resistance in wild populations of trypanosomes is exacerbated by the small number of compounds used against the parasite, the long and widespread use of these compounds and the similar chemical structures of the drugs. The latter means that a mechanism that evolves to tolerate one drug may well be effective against others. This complicates use of the sanative pair strategy. An equally important problem is that drugs administered in the field are often adulterated or given in insufficient doses; this exposes trypanosomes to sublethal levels of the drug, which encourages the emergence of tolerant parasite populations.

Several research projects are being conducted at ILRAD to investigate the appearance and prevalence of parasite resistance to trypanocidal drugs in the field and the laboratory. Central to understanding trypanosome drug resistance are on-going experiments designed to determine the precise mechanism(s) that enable some trypanosomes to resist the normally lethal effects of trypanocides.

Mechanisms of drug resistance

Pathogens employ several mechanisms to tolerate drugs. Some parasites that are fully sensitive to a drug may, for example, appear to be resistant because they evade contact with the drug by occupying a part of the host that is inaccessible to the compound. Drug tolerance may arise through changes in the interactions of the drug and its target organism. Pathogens, for example, may become tolerant of the presence of drugs upon alteration of the cell membrane so that it admits less drug into the cell, thus keeping cell drug concentrations below a lethal threshold for the pathogen. Other cell mechanisms may be harnessed to export drug compounds immediately after they enter the cell

Drug tolerance may also develop as a result of conversion of a drug into a less toxic form. Finally, properties of an intracellular target of a drug may be altered, making the target less susceptible to the drug (a case of moving the molecular goal posts).

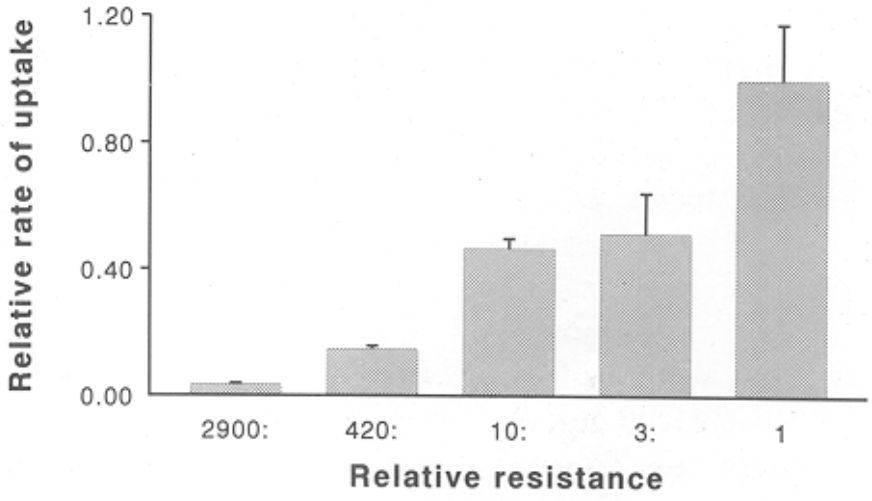
Drug resistance in trypanosomes

Three species of trypanosomes—*Trypanosoma congolense, Trypanosoma vivax* and *Trypanosoma brucei brucei*—account for most trypanosome infections in domestic animals. *Trypanosoma congolense* is an important pathogen of African cattle. The drug most frequently used to combat infection with this species is Samorin®, isometamidium chloride. Samorin uptake by *T. congolense* populations has been investigated at ILRAD with the aim of determining the mechanism employed by the parasite to take up the drug and how this mechanism may relate to parasite sensitivity or resistance to the drug.

A panel of five trypanosome populations were selected on the basis of their apparent resistance to Samorin in cattle and mice. Among these populations, there was a 3000-fold difference in sensitivity to Samorin between the most resistant and most sensitive. When the same panel of trypanosomes was tested in a laboratory culture system developed at ILRAD, identical patterns

of resistance and sensitivity were observed. This discounts the possibility that resistance is due to trypanosomes 'hiding' from the drug in the host body. Resistance must therefore be intrinsic to the trypanosome.

The uptake of Samorin was investigated using two approaches. In the first, the fluorescence property of Samorin was used, since it emits light when illuminated with light of a particular colour. The emitted light changes when the molecule enters a trypanosome cell. Using a sensitive instrument called a fluorimeter, drug entry into the parasites can be measured by this change. In a second series of experiments, Samorin labelled with the radioactive carbon-14 atom was used to measure drug entry into parasite cells.



The five populations of T. congolense *used in the resistance studies varied by almost 3000-fold between most and least resistance. Using the most sensitive type as reference, the rate of uptake of the drug is shown by the height of the bar. The rate of uptake is clearly inversely related to the resistance.*

The rate at which the drug entered cells was found to depend on the concentration of drug in the surrounding solution. The rate varied, however, in a special way. If the speed of drug entry increased without limit, with increasing concentration of drug, then a simple diffusion across the cell membrane could be assumed. However, in the experiments the rate levelled off to a limiting value, a situation indicating a finite number of transporter molecules present in the cell membrane that are responsible for entry of the drug. Most importantly, the level of this limiting value, the maximum possible rate at which cells can take up the drug, differed between the types of trypanosomes investigated. The more resistant the trypanosome, the lower this maximum rate.

Possible reasons for this result are that the drug-resistant parasites have fewer numbers of these transporters or that their transporters function more slowly than those in drug-sensitive parasites. Whatever the cause, the effect of the limited rate of drug uptake in resistant parasites is a lower concentration of drug within the cell, which may enable other parasite mechanisms to cope with the presence of the drug without being overwhelmed.

These results have clearly shown that the resistance or sensitivity of populations of *T. congolense* to Samorin is related to properties of transport molecules in the cell membrane. The work is being continued to identify and characterize the transport molecules, with the aim of developing rapid diagnostic tests for resistant parasite populations in the field.

In the longer term, knowledge of the underlying mechanisms of drug resistance in trypanosomes may be used to develop treatments that will revert resistance to susceptibility, and in this way restore the efficacy of overworked trypanocides.

This article is based on a report by Jon Wilkes. Andrew Peregrine, who helped write the introduction, is head of ILRAD's chemotherapy research project. Dan Zilberstein, a visiting scientist from the Department of Biology of the Technion Israel Institute of Technology, originated and continues to collaborate on the work reported here.

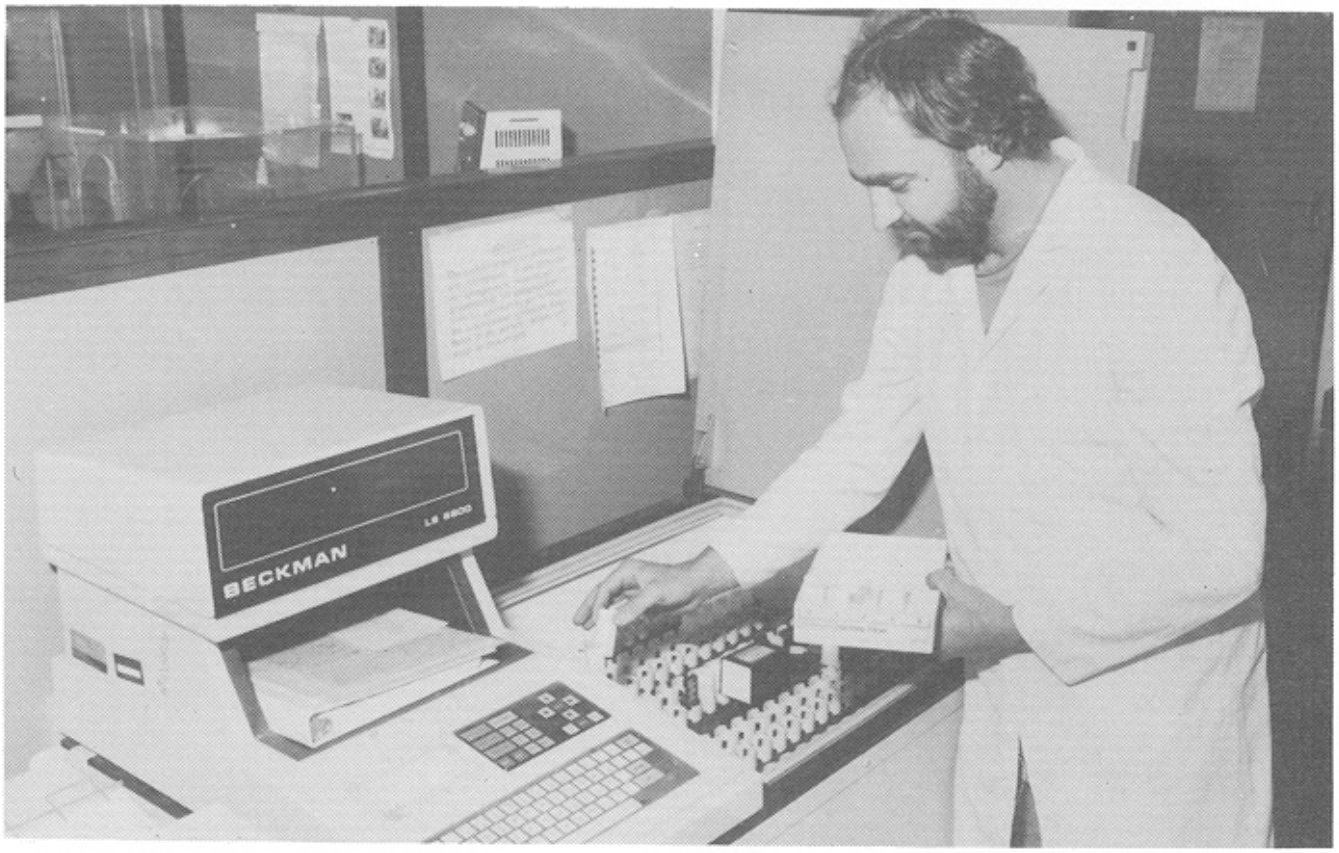
The following article, on ILRAD's use of RAPD technology to distinguish trypanosomes, is based on a report and work by John Waitumbi, Mohamed Dirie, Peter Gardiner and Noel Murphy.

History of the Israel-ILRAD Collaborative Project

In 1990 a scientist from the Technion-Israel Institute of Technology, Dr. Dan Ziberstein, spent three months at ILRAD conducting collaborative studies of the transport of nutrients in leishmania and trypanosome parasites. While discussing with ILRAD colleagues the best approach to developing a simple and reliable assay for quantifying levels of drug resistance in trypanosome populations, Dan suggested using the fluoresence properties of Samorin® to investigate its interactions with trypanosomes. He measured the degree of Samorin's fluorescence first when the drug was placed in solution and then when trypanosomes were added to the drug solution. The intensity of fluorescence displayed by the drug molecule when in solution and when inside the parasite differed greatly. This exciting observation suggested a new way of quantifying the interaction of Samorin and its target parasite.

This experiment demonstrated not only that the intensity of Samorin's fluorescence increases dramatically when the molecule enters trypanosomes but also (1) that this change is specific to trypanosomes and (2) that the intensity of Samorin's fluorescence also differs significantly between parasite populations that are resistant and susceptible to Samorin. Using this approach as well as radio-labelled Samorin, Technion and ILRAD scientists went on to demonstrate that Samorin is taken into trypanosomes by a specific protein in the plasma membrane.

The collaborating scientists also wanted to know which molecule inside the trypanosome was interacting with Samorin and thus causing the increase of the drug's fluorescence. At ILRAD, Dr. John Wilkes further characterized this interaction and found that Samorin interacts solely with a single molecule inside trypanosomes and that the affinity of Samorin to this molecule is high, resembling that of receptors and hormones in mammalian cells. Dan Zilberstein then used this information to pull out this protein using affinity chromatography. This parasite molecule is now being characterized at the Technion. The information gained in this collaborative research effort will be used to elucidate further the mechanism of resistance to Samorin in trypanosomes.



Dr. Jon Wilkes places into a scintillation counter vials holding different populations of trypanosome parasites that have been incubated with the anti-trypanosome drug Samorin®. The drug has been labelled with radioactive molecules. The scintillation counter measures the amount of radioactivity in each vial. This tells the scientist how much drug has been taken up by the different parasite populations. Drug-resistant populations have evolved ways of reducing their drug uptake.

RAPD fingerprinting of trypanosomes

CHARACTERIZING and diagnosing trypanosome infections in animals is complicated by the existence of many disease-causing subgenera, species and subspecies of the genus *Trypanosoma,* in addition to the occurrence of several apparently harmless trypanosome populations. Three subgenera—*Nannomonas, Duttonella* and *Trypanozoon*—cause large, widespread losses in livestock productivity throughout the tropical and subtropical regions of the world. Members of the Nannomonas subgenus cause the greatest ruminant losses due to trypanosomiasis in sub-Saharan Africa; five genetic types of parasites have been identified within this subgenus. Two subspecies in the *Trypanozooon subgenus—Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gumbiense*—cause life-threatening human diseases. Complicating the picture still further are mixtures of parasites of the same or different subgenera, species or subspecies infecting an animal simultaneously.

What is needed by both scientists and animal disease control workers are systems for quickly and efficiently distinguishing among the many trypanosome populations. Such systems would enable workers to make accurate diagnoses, to obtain sound epidemiological data and to optimize the use of chemotherapy to prevent and treat trypanosomiasis.

SEVERAL TECHNIQUES are used to distinguish the different populations of trypanosomes that infect people and their domestic animals. Antigen-trapping enzyme-linked immunosorbent assays (antigen-ELISAs) are a cost-effective technique for diagnosing infections in the field (see the October 1988 issue of *ILRAD Reports).* Other technologies, based on the binding of complementary single strands of DNA (deoxyribonucleic acid), identify trypanosomes with unparalleled sensitivity (January *1991 ILRAD Reports).* The latter technologies have the further

advantage that DNA is more robust and stable than the proteins and antibodies used in the monoclonal antibody-based antigen-ELISAs. For the most part, however, the DNA-based technologies have been restricted to laboratory work because they require use of radioactive chemicals.

The standard polymerase chain reaction technique

The polymerase chain reaction technique is used to amplify specific regions of an organism's DNA to levels where the DNA fragment can be identified. The technique generally requires a pair of short pieces of single-stranded DNA composed of 20 nucleotide bases, called oligonucleotides, which are specifically synthesized for the reaction. Each paid of oligo-nucleotides is specific for the section of the genomic DNA of the organism being analysed. These oligonucleotides under particular conditions will bind to their complementary sequences in the genomic DNA and act as 'primers' that initiate copying of a specific region of the DNA of the organisms under study.

Two primers are used—one for each strand of the DNA double helix. The DNA strands of the double helix are copied, or synthesized, in opposite directions. Only a short region of the genome is amplified. Each round of amplification effectively doubles the copy number of the DNA region being amplified. After only 30 rounds of amplification, the copy number of the specific sequence increases over one million-fold. This amount of DNA is easily seen after staining with a DNA-binding reagent when the reaction products are separated by electrophoresis in a gel.

Several recent developments are now making these technologies attractive for field as well as laboratory use. These include an increasingly widespread use of the polymerase chain reaction (PCR) technique, the advent of new systems for detecting parasite DNA that require no radioactive labelling and are more sensitive and reliable than those used in the past, and increasing cost-effectiveness of the technologies. The DNA based technologies are thus likely in the future both to improve diagnosis of infections and to advance epidemiological studies.

The PCR technique is used to amplify a specific region of an organism's DNA to levels where the DNA fragment can be identified (see the box on this page and the October 1991 *ILRAD Reports).* Although powerful, PCR requires that a pair of short pieces of single-stranded DNA be synthesized specifically for each DNA sequence to be detected by subsequent amplification in the reaction. Moreover, to generate these primers, known as oligonucleotides, DNA of the relevant genes must first be sequenced. In addition, use of the PCR with a pair of primers for a specific DNA sequence does not produce different reaction fragments—seen in a gel as DNA bands of different sizes—and thus cannot distinguish isolates of the same species of trypanosome.

SCIENTISTS have now developed more refined systems for identifying organisms on the basis of stretches of their DNA. The breakthrough was made by two American groups that first identified closely related organisms using genetic 'fingerprints' produced by amplifying DNA of the organisms with arbitrary primers. This technique, soon dubbed 'RAPD' for 'random amplified polymorphic DNA' (the method is also, less euphoniously, known as AP, for 'arbitrary primer'-PCR), uses single primers of arbitrary sequence of only 10 nucleotides in length rather than the longer (20-nucleotide) pairs of primers for specific sequences used in the standard PCR method. Because of their shorter sequences, the arbitrary primers used in the RAPD technique will bind to many stretches of DNA in an organism's genome—that is, to the many sites that have a complementary, or nearly complementary, sequence of nucleotide bases.

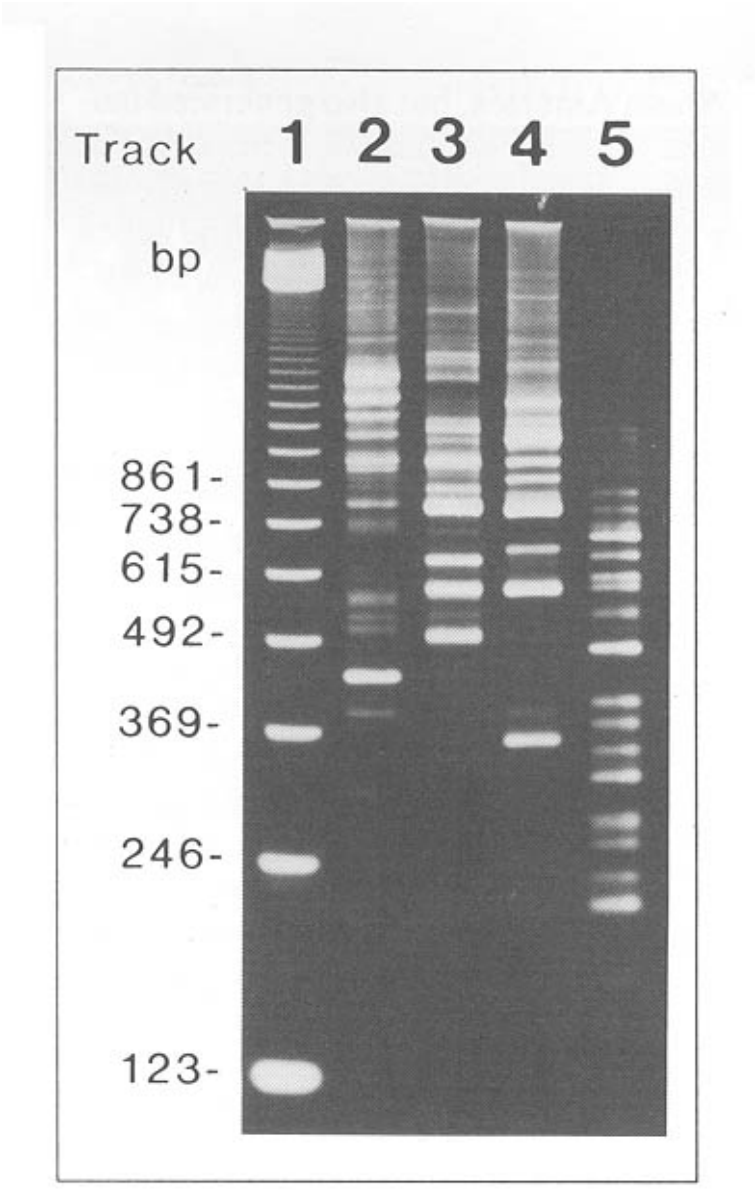
As organisms evolve, fragments of their DNA may be deleted or inserted or changed through genetic mutation. The arbitrary primers point up such differences among closely related populations by generating different-sized PCR products, which appear as different-sized bands on a gel, or by generating products with DNA from one population (bands) but no products with DNA from another population (no bands). The differences are easily demonstrated by running the DNA amplified from different organisms alongside each other in gels (see the figure on this page). The degree to which two organisms are related is indicated by the degree to which the fingerprint or pattern of bands produced in a RAPD reaction are similar.

The RAPD technique thus overcomes some limitations of the standard PCR reaction: it enables scientists to generate a fingerprint of the genome of an organism that can be used to identify the organism rapidly, even when only a few of the organisms are present in samples being analysed.

The main advantages of the RAPD method are that a single short primer is sufficient to differentiate many different organisms and that to produce the primer the experimenter needs no prior information about DNA sequences in the organism of interest. The technique can generate fingerprints of different populations of the same species, which can then be used to distinguish the populations from each other. Due to the complexity of trypanosomiases, this advantage is particularly important in detailed studies of trypanosome infections in the field. For example, individual parasite populations that are particularly virulent could be followed and their influence on the epidemiology of the disease in the field monitored.

SCIENTISTS at ILRAD have used the RAPD technique to determine differences among and within species of trypanosomes. The fingerprints produced from DNA of members of three trypanosome subgenera —*Nannomonas, Trypanozoon,* and *Duttonella*—have been examined after using different arbitrary 10-mer oligonucleotides to amplify DNA from these organisms. Of the primers tested, one designated ILO 525, with a nucleotide base sequence of 5' CGGACGTCGC 3', distinguished the three subgenera from each other and also produced identifiable fingerprints for the different parasite populations within each of the subgenera.

The different fingerprint patterns, or polymorphisms, generated using this primer allowed scientists easily to differentiate five previously identified subtypes of the *Nannomonas* group: *Trypanosoma simiae* and the Kilifi-, Tsavo-, savannah- and West African forest/riverine-types of *Trypanosoma congolense.* Although *T. simiae* is morphologically indistinguishable from other members of the *Nannomonas* subgenus, most of which are pathogenic in cattle, *T. simiae* is the only one of the subgroup to be particularly pathogenic in pigs.



The different DNA products of four Nannomonas isolates—Trypanosoma congolense *Kilifi (lane 2), forest/riverine (West African), (lane 3) and savannah (lane 4), and* Trypanosoma simiae *(lane 5)—generated using the RAPD method, separated by electrophoresis in a gel and stained with a dye that binds to the DNA and allows it to be visualized with ultraviolet light.*

RAPD fingerprints of *T. simiae* populations differed markedly from the fingerprints of the other members of the *Nannomonas* group. For all the *T. congolense* parasites analysed, oligonucleotide ILO 525 generated fingerprints that not only unequivocally identified the species but also revealed polymorphisms among the different isolates of the same subspecies.

Disadvantage of RAPDs

A disadvantage of using the RAPD technique to identify trypanosomes is that the parasites must be purified from host nucleate cells: any contamination by host DNA will alter the fingerprint patterns.

This separation is achieved by passing host blood samples over DEAE-cellulose columns. To be effective, this technique requires relatively that the host have high degrees of parasitaemia to provide sufficient numbers of parasites.

Modifications of this technique or new procedures for isolating trypanosomes need to be developed before the RAPD technique can reach its full potential in diagnosis and epidemiological studies of trypanosomiasis.

THE FINGERPRINTS produced using primer ILO 525 divided members of the *Trypanozoon* subgenus into three major groups: (1) *Trypanosoma brucei brucei and T. b. rhodesiense,* (2) *T. evansi* and (3) *T. b. gambiense.*

Group one comprised *T. b. brucei* populations from East and West Africa, which do not infect humans, and *T. b. rhodesiense,* which is found in several areas in Africa and does infect people. Although the fingerprints did not distinguish *T. b. brucei* from *T. b. rhodesiense,* the profiles in group one were sufficiently different to identify different isolates. The inability of the RAPD method to differentiate *T. b. brucei* and *T. b. rhodesiense* lends support to results of DNA hybridization and isoenzyme comparisons that suggest that these two subspecies are components of a single species group and that *T. b. brucei* can potentially infect people.

Group two comprised all isolates of *T. evansi,* which are morphologically similar to the other members of the *Trypanozoon subgenus* but are transmitted globally in tropical and subtropical regions not by tsetse but by other biting flies. Their distinction from other *Trypanozoon* isolates was indicated by the absence of one DNA band in the fingerprint and the presence of a larger, less intense band. Analysis of the sequences of the two DNA bands showed them to differ from each other. To date, the RAPD technique has not revealed qualitative differences among the *T. evansi* isolates. More oligonucleotides need to be tested to identify arbitrary primers that will reveal differences in the fingerprints of these *T. evansi* isolates.

Group three comprised *T. b. gambiense* isolates from West Africa. This organism causes human sleeping sickness. The RAPD technique**,** again carried out with ILO 525, generated two sorts of DNA fingerprints for the *T. b. gambiense* isolates tested. Both sorts of fingerprints lacked the two bands that distinguish the other two *Trypanozoon* groups. This division of *T. b. gambiense* into two types agrees with earlier subdivisions of isolates of this organism that were made on the basis of different variant surface glycoprotein genes possessed by the two types.

Application of the RAPD technique using *T. vivax* isolates of the *Duttonella* subgenus from South America and Africa has confirmed results of previous analyses and facilitated differentiation of isolates that in the past could be accomplished only by employing a combination of several relatively tedious techniques. Primer ILO 525 was again found to be the most useful in distinguishing these parasites. The RAPD method not only placed all non-Kenyan *T. vivax* into one group containing widely distributed isolates from Africa and South America, but also generated isolate-specific patterns that may, in the future, enable scientists to identify regions of the genome encoding genes responsible for the expression of different traits, such as resistance to drugs and an ability to infect rodents, to induce haemorrhagic disease and to be transmitted by tsetse.

The similarities observed between the West African and South American (Colombian) *T. vivax* populations supports the generally held hypothesis that the South American parasites are derived from tsetse-transmitted West African trypanosomes. The number of South American parasites sampled so far, however, is insufficient to determine the degree of heterogeneity among the South American isolates and whether this parasite was imported from Africa on one or on several occasions.

IN SUMMARY, use of the single oligonucleotide primer ILO 525 at ILRAD in the RAPD technique—rather than pairs of primers needed to identify each different parasite population in the standard PCR technique—has enabled scientists to differentiate the different subgenera, species and individual isolates of trypanosomes from Africa and South America. This advantage greatly simplifies and extends the possibilities in studies of the epidemiology of trypanosomiasis.

In the future, scientists may use this technique to identify genes expressing traits of importance. Like other PCR based methods, the RAPD method uses very small quantities of DNA (billionths of a gram) and therefore can be used to identify infecting organisms even when samples contain as few as a hundred parasites.

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ILRAD was founded in 1973 to conduct research into better ways of controlling livestock diseases. The current primary goal of the Laboratory is to develop safe, effective and economical methods to control two parasitic diseases that severely constrain animal production in Africa:trypanosomiasis, transmitted to animals by the bite of a tsetse fly, and East Coast fever, a virulent form of theileriosis, transmitted to cattle by ticks. An international staff of about 50 scientists conducts basic research much of it aimed at the development of vaccines in the fields of biochemistry, cell biology, electron microscopy, epidemiology, genetics, immunology, molecular biology, pathology, parasitology and the socio economics of animal disease control

ILRAD is one of 18 international agricultural research centres sponsored by the Consultative Group on International Agricultural Research (CGIAR). The secretariat of the CGIAR is located in the World Bank headquarters in Washington D.C. The CGIAR is an informal umbrella organization of 40 national governments, international organizations and private foundations that together provide about US$300 million annually to the 18 centres for research., training and advisory services. The CGIAR aims to help farmers in developing countries increase their production of staple food crops livestock fish and trees in ways that improve the nutrition and well-being of low-income peoples and the management of natural resources.