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Making genes visible

ILRAD scientists are harnessing a new technique for synthesizing large amounts of DNA in long-term research to improve the health of domestic livestock. The practical applications of

this technique—called the polymerase chain reaction (PCR) —are already changing the nature of ILRAD's research on theileriosis (East Coast fever) and trypanosomiasis (sleeping sickness), diseases that cause farmers in developing countries ruinous food and economic losses.

The protozoan parasites that cause East Coast fever and trypanosomiasis have held back tropical agriculture throughout this century. Attempts to develop vaccines or improved diagnostics and treatments for the diseases have been made difficult largely because the parasites are too small to distinguish and manipulate in conventional ways. Now, with the advent of the polymerase chain reaction (PCR), scientists can 'see' their quarry by producing large quantities of parasite DNA. In addition, by making the DNA of parasites—as well as that from their animal hosts—in quantities that make it easily visible, PCR has already greatly improved standard procedures for copying DNA sequences (cloning), for determining the order of bases in DNA (sequencing) and for producing large quantities of molecules of interest (expressing genes). An immediate practical benefit of PCR has been the development of superior methods for identifying parasites and distinguishing among similar parasite populations, methods that will improve diagnosis of the diseases they cause.

More important in the long term, perhaps, is that the new technique is helping ILRAD scientists to understand the molecular mechanisms that underlie East Coast fever and trypanosomiasis. When these mechanisms are better understood, novel control methods may be developed. Vaccines, for example, may be made to improve the quality of an animal's immune response to infection, and thus help to prevent disease from developing. The immune response could also be improved by use of genetically engineered molecules that mimic disease control mechanisms found in livestock breeds that tolerate parasite infections. Or therapeutic drugs might work to undermine the ability of parasites to proliferate in their hosts, and thus interrupt the parasite life cycle. It is hoped that such future novel control methods, tailored for widespread application in the field, will help bring an end to the deprivations these diseases continue to cause farmers and pastoralists in many areas of the developing world.

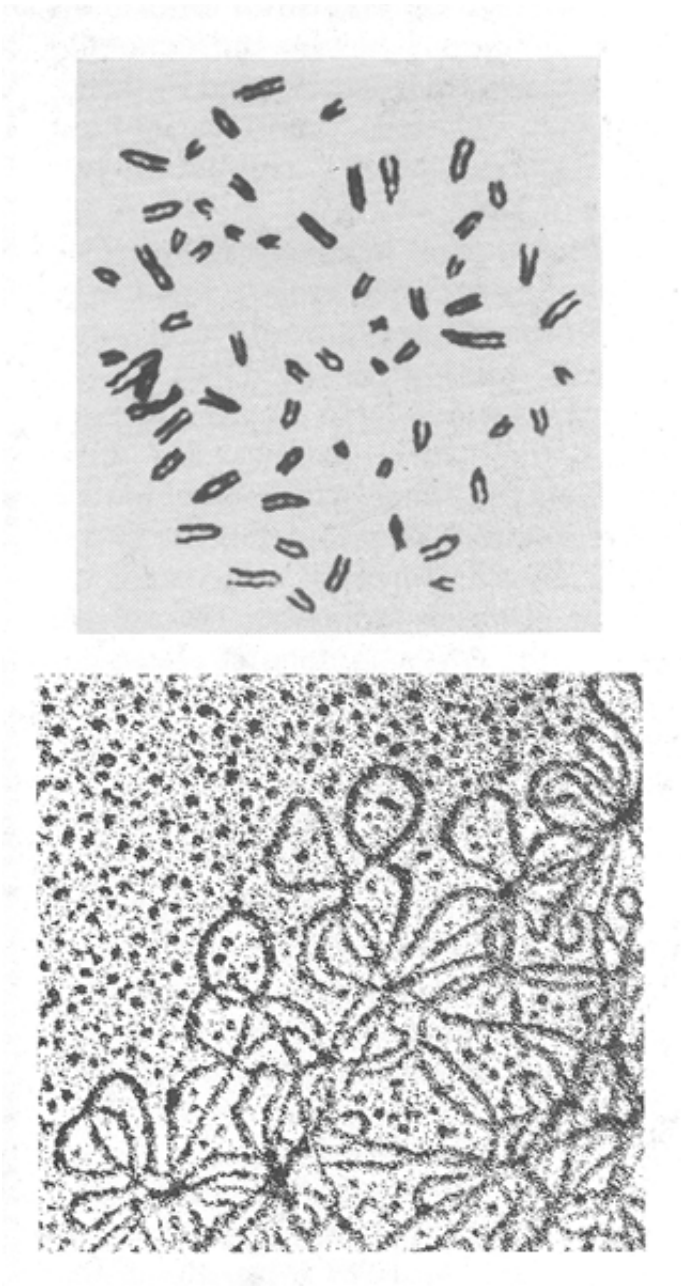

FIGURE 1. *(a) The sixty chromosomes enclosed within the cell nucleus of domestic cattle. Compressed in each chromosome is a single long double-stranded molecule of DNA. (b) Networks of coiled DNA that occur in the kinetoplast structure, which lies outside the nucleus, of the trypanosome parasite. Along the length of the threadlike DNA, the order of a sequence of nucleotides carries the genetic code which, read consecutively, directs the formation of an entire organism and governs its biological functions. The discrete functional units of DNA—the sentences—are genes, which direct protein synthesis. Remarkably, the language of DNA—the genetic code—is nearly identical for all forms of life, from bacteria to humans. Like great works of literature, the text carried on DNA molecules is dense with layered meanings. Reading the full text is a daunting proposition, requiring different kinds of expertise and levels of understanding. All the letters of the text may be decoded, for example, by determining the order of the sequence of base pairs in the genome of an organism—a total of some billion base pairs in the single-celled trypanosome and 3 billion in the human. On another level, the words and sentences formed by these letters—the codons and genes, respectively—must be distinguished from nonsense ('junk') stretches of DNA. The mystery of how genes are regulated may then begin to be unravelled. Finally, perhaps, scientists will begin interpreting the meanings and seeming ambiguities of this*

ancient biological text.

Life before PCR

The study of biology underwent a radical change in 1944. In that year, Oswald Avery and his colleagues at the Rockefeller Institute determined that the 'transforming principle' responsible for heredity was a particular molecule located in the chromosomes of the cell nucleus (Figure 1). The molecule was deoxyribonucleic acid, soon to be known as DNA. Subsequently, a series of far-reaching discoveries at the molecular level, along with development of powerful enzymological and chemical techniques, gave rise to DNA technology and the new field of genetic engineering. The recent discoveries and techniques, particularly those of the last two decades, since the discovery of the first restriction enzyme in 1970, have transformed biology into an experimental as well as descriptive science, a discipline based as firmly on the laws of chemistry and physics as it is on the laws of evolution.

The most important developments in the new 'molecular biology' stemmed from the historic 1953 postulation by James Watson and Francis Crick, then working at the Cavendish Laboratory in Cambridge University, that the DNA molecule consists of two intertwined and complementary polynucleotide chains. 'It was promptly proposed that the two strands of the double helix should be regarded as a pair of positive and negative templates, each specifying its complement and thereby capable of generating two daughter DNA molecules with sequences identical to those of the parental double helix[1] (Figure 2).

[1]J.D. Watson, J. Tooze and D.T. Kurtz, *Recombinant DNA: A Short Course,* New York: Scientific American Books, 1983, p. 19.

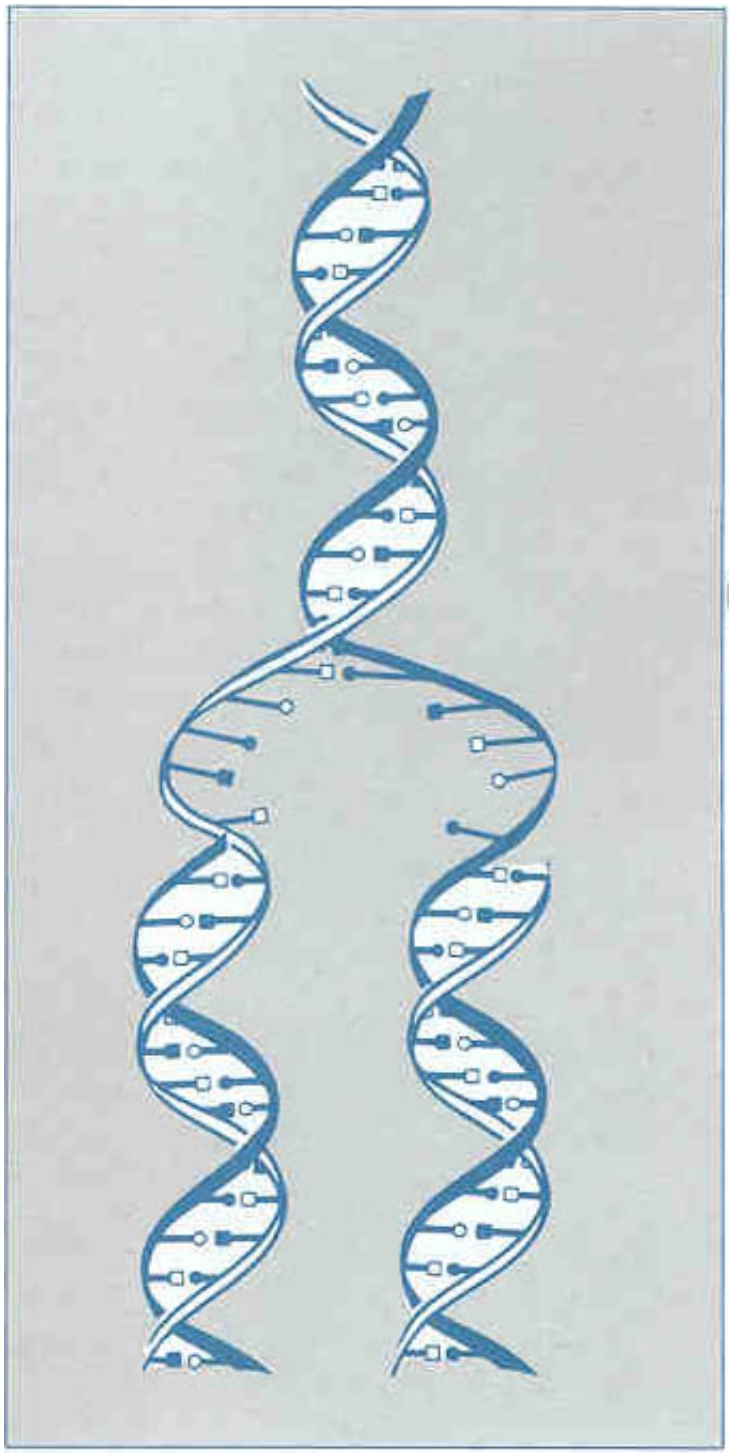

FIGURE 2. *Schematic diagram of a DNA molecule as it replicates in the cell nucleus.*

By June 1966, the entire genetic code had been deciphered following the discovery in 1961 by Sydney Brenner and Francis Crick, at Cambridge, that each of the twenty amino acids that are the building blocks of proteins is specified by a unit of three successive nucleotides in a gene, called a codon.

A problem in subsequent genetic studies was the great length and fragility of DNA molecules, which made the molecules difficult to manipulate. This was solved in 1970, when Hamilton Smith and colleagues at Johns Hopkins—following studies in Geneva by Werner Arber and Stewart Linn demonstrating that certain bacterial enzymes break down foreign DNA—isolated the first 'restriction enzyme'. These enzymes act as precise molecular scissors, cutting DNA molecules wherever there occurs a particular sequence of the four nitrogenous bases that make up the variable part of a DNA

molecule. The four bases are adenine [A], guanine [G], thymine [T] and cytosine [C]. In double-stranded DNA, base A always pairs with T and G with C. The pairs are known as base pairs.

Many different kinds of restriction enzymes, each with its own specific cleavage site, have now been found. Use of restriction enzymes make it possible to cut DNA into smaller, identifiable pieces. The various fragments generated when DNA is cut by a restriction enzyme can be easily separated by size using a technique called agarose gel electrophoresis. The first 'restriction map', showing the sites on viral DNA molecules that are attacked by bacterial restriction enzymes, was produced in 1971 by Daniel Nathans, a colleague of Hamilton Smith at Johns Hopkins. Knowing the locations of the restriction sites cleaved by these enzymes enabled scientists for the first time to identify significant regions of DNA.

A subsequent landmark in molecular biology occurred in 1972 when Arthur Kornberg, of Stanford University, used the joining enzyme DNA ligase to link together DNA fragments produced by restriction enzymes, thus producing the first recombinant molecule. Methods developed in the early 1970s to clone specific DNA sequences in plasmids—circular, extrachromosomal DNA molecules derived from bacteria—and then to isolate these cloned genes from normal bacterial genes, ushered in the era of widespread use of recombinant DNA technology.

Studies using restriction maps and restriction fragments also paved the way for the development of new methods for determining the sequence of bases that make the DNA molecule. The first of these rapid sequencing methods was developed by Fred Sanger, of the British Medical Research Council Laboratory of Molecular Biology, in 1977. (Twenty years previously, Sanger had also been the first to determine the sequence of amino acids in a protein molecule.)

Once the sequence of bases that make up a gene is determined, it is easy to deduce the sequence of amino acids in the protein that the gene specifies. In fact, scientists sequencing proteins today often find it simpler to work indirectly by determining the sequence of bases that codes for the protein than to work directly on the protein itself.

The recent advent of the polymerase chain reaction, first unveiled in 1985, is now dramatically increasing the pace of molecular biology studies.(See the boxed story of the discovery of PCR) This new DNA replication procedure multiplies a few copies of a given DNA sequence directly and exponentially. Using PCR, millions of copies of any selected DNA fragment can be synthesized in a matter of hours. The quantity of DNA produced in an amplification is large enough to be easily visualized and manipulated, and this greatly simplifies analysis of the genetic material.

How PCR works

The polymerase chain reaction exploits polymerase, an enzyme that synthesizes DNA in the cell, and the phenomenon of 'complementarity', that is, that any two single-stranded DNA molecules with complementary nucleotide sequences will, under the right conditions, bind together to form a spiralling double-stranded molecule, the famous double-helix.

The recipe for making DNA is re markably simple (Figure 3). A piece of double-stranded DNA is mixed with the four chemical building blocks o DNA—the bases A, G, T and C. An enzyme, DNA polymerase, is added to the mixture; given one strand of DNA, this polymerase will assemble the complementary strand. Two 'oligonucleotides' are added. These are synthetic strands of DNA, each typically about 20 bases in length that are complementary to regions at either end of the target DNA sequence. These DNA fragments act as primers in the replication of the DNA: they anneal to opposite

ends of the target DNA sequence and direct synthesis in opposite directions. The length of the DNA product is defined by the distance between the primers.

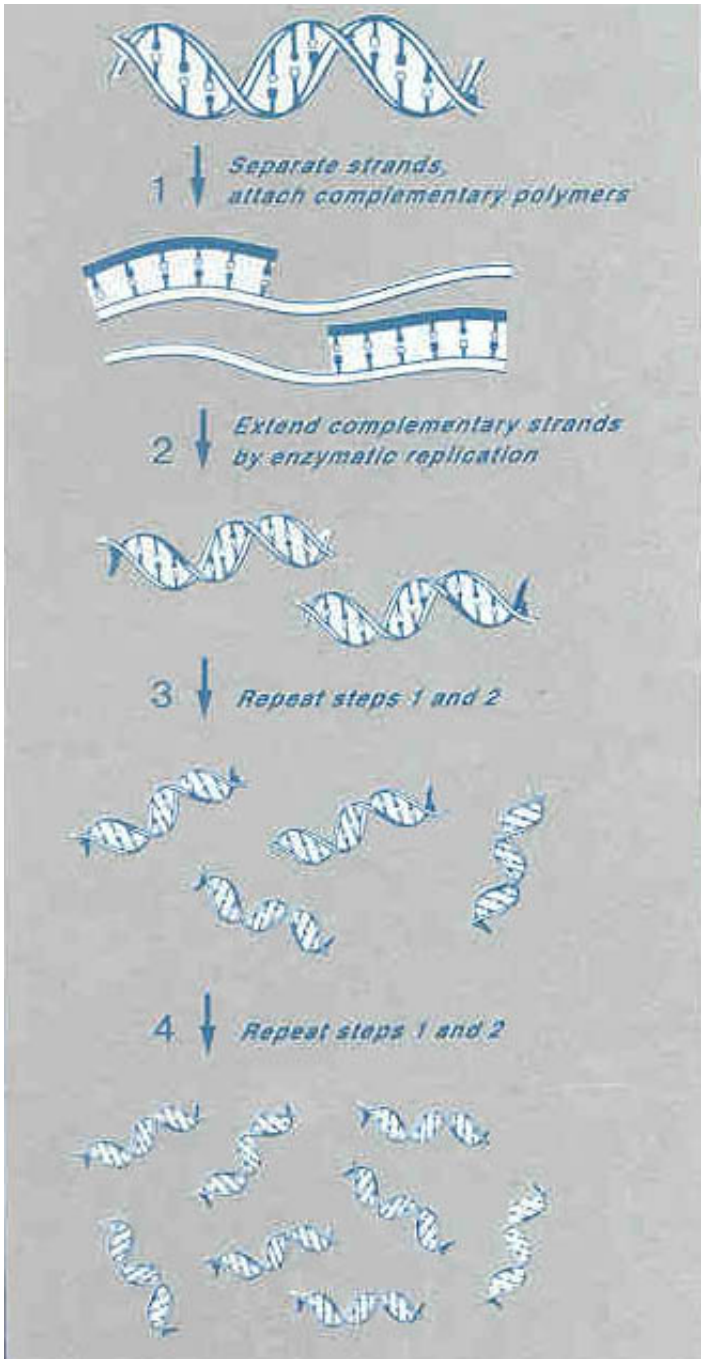

FIGURE 3. *Steps in the polymerase chain reaction. (From Figure 4, Mapping the Human Genome, Lawrence Berkeley Lab. 1989.)*

To start the reaction, a machine is used to heat and cool this mixture in cycles lasting a few minutes each. The first heating, to 95 °C, separates the double-stranded DNA into two single strands. The temperature is then reduced to 45–65 °C to allow the primers to find and bind to their complementary sequences. Finally, the temperature is again raised, this time to about 70 °C to allow the DNA polymerase to extend each primer into a new DNA strand.

The next cycle of heating separates the copies from the original strands and *both* sets become templates for a new round of DNA synthesis. As a result of this doubling, the target DNA fragment multiplies exponentially—in a chain reaction. This procedure is usually repeated for 25–35 cycles; starting with just a few copies of a DNA segment,

30 cycles typically produces more than a million copies, allowing the DNA fragment to be easily detected, analysed and manipulated.

In practice, no universal protocol exists for PCR. Parameters such as the priming temperature for the oligonucleotides, the extension time for the polymerase and the particular buffer used have to be tailored for specific pairs of primers. Moreover, several variations of this standard protocol are used for different purposes.

PCR in research on trypanosomiasis

Diagnosis of trypanosomiasis

Established methods of diagnosing trypanosomiasis and theileriosis rely on direct observation of parasites, detection of serological responses, inoculation of potentially infective material into test animals and, in some cases, on immunological methods for antigen detection. None of these methodologies is ideal.

Whatever method is employed, definitive diagnosis of *infection* inevitably relies initially on the detection of parasites. Successful detection, in turn, depends in part on the relative abundance of parasite material. In the case of theileriosis, parasite detection *per se* is usually not difficult, except where a carrier state is involved (described below). The major diagnostic problem for this disease is thus characterization of the infecting parasite, that is, defining the parasite's relationship with other subspecies.

Diagnosis of trypanosomiasis infection, on the other hand, has been problematical. Such diagnosis has relied largely on light microscopy to reveal parasites in the peripheral blood of the mammalian host. However, methods based on direct observation of trypanosomes are unreliable because the number of parasites circulating in the blood of a host fluctuates markedly during the course of an infection; thus, few or no trypanosomes may be present in any given sample. Moreover, the most sensitive of the direct observation methods are unsatisfactory for identifying parasite species and subspecies.

Researchers also need a sensitive and reliable means of detecting trypanosomes in the tsetse fly vector, particularly to assess levels of parasite challenge in epidemiological surveys and to monitor the status of control programs. The detection method currently used involves tedious procedures for the careful dissection and microscopic examination of the flies. Furthermore, use of this method does not necessarily reveal the species of the infecting trypanosome.

Established DNA-based methods to detect trypanosomes involve (1) preparing DNA from mammalian blood samples before 'spotting' onto a suitable membrane and probing and (2) spotting the abdominal contents of tsetse flies onto a nylon membrane before probing. DNA methodologies incorporating PCR amplification of trypanosome genes have greatly increased the sensitivity of tests used to detect trypanosomes in both the host and the fly. These tests are particularly useful for detecting low-level infections. The polymerase chain reaction is also being used to generate large quantities of pure DNA for increasing the speed, reliability and ease of sequencing long stretches of target parasite DNA.

DNA probes are now available for all important species of trypanosomes. The probes in current use are labelled with radioisotopes so that their hybridization with the DNA in samples being examined (such as PCR products) can be visualized. This labelling technique works well in the laboratory but is impractical for field work. Therefore, before DNA probes can be used in the field, simpler and safer ways of labelling them must be introduced. This is now being investigated at ILRAD (see the January 1991

issue of *ILRAD Reports).*

DNA-based techniques for diagnosing trypanosomiasis used in conjunction with the polymerase chain reaction have thus improved both trypanosome detection and characterization, but current use of these techniques remains largely restricted to sophisticated laboratories. The fast pace of biotechnological development, however, makes it probable that new DNA-based biotechnologies will continue to improve the diagnosis of trypanosomiasis while the technologies also become less complex, enabling their use in small as well as large laboratories and in the field. (See the boxed article on page 5 for a discussion of how PCR is already 'democratizing' DNA work.)

Mapping the bovine genome

Most cattle in Africa, including East African and European breeds, are highly susceptible to trypanosomiasis, but certain indigenous breeds in West Africa are known to resist the disease. ILRAD scientists would like to know how the West African cattle tolerate trypanosome infections when other breeds succumb so quickly. In the few years since its arrival at ILRAD, the polymerase chain reaction has become a central tool in the Laboratory's research into the mechanisms of resistance to trypanosomiasis, known as trypanotolerance. To learn more about these resistance mechanisms, ILRAD scientists are investigating the genetics of trypanotolerance. The major work in this area is a project to 'map the bovine genome'[2]. The term 'genome' refers to all the DNA sequences—the complete set of genetic instructions—of an individual.

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[2]ILRAD's bovine genome mapping project is an international collaborative endeavour. Working with ILRAD are gene mappers from the Commonwealth Scientific and Industrial Research Organisation, Australia; ETH, Zurich; the Hebrew University of Jerusalem, Israel; and Texas A & M University, USA.

A genetic map comprises a set of readily detectable DNA sequences that are spread throughout the genetic material of an organism. These distinctive base sequences can act as landmarks along the chromosomes, marking the general location of a gene or DNA sequence of interest. To be useful in mapping, the markers must be polymorphic, that is, they must differ among individuals.

Like all mammals, a cow carries two sets of genetic information—in the form of pairs of matching chromosomes—in the nuclei of its cells. One chromosome in each pair is inherited from the mother and the other from the father. In the series of cell divisions (meiosis) that gives rise to sperm or eggs (germ cells), paired chromosomes repeatedly recombine by exchanging DNA segments of equal length. As a result, each chromosome that is transmitted in a germ cell is generally a mix of segments from the two parental chromosomes.

This genetic recombination means that an animal will often possess two versions of a given polymorphic marker. By identifying which versions of the marker were present in the parents of the animal and which were inherited by the offspring, it may be determined which of the four sets of parental DNA contributed the two markers to the offspring. In this way, the inheritance of markers can be traced through several generations. Scientists can use this information to determine which markers lie near which DNA segments carrying genes of interest.

Night puttering

Kary B. Mullis recalls that he 'ran into' the polymerase chain reaction one night in April 1983

as he drove to a cabin retreat in northern California[3]. Mullis was working at the California biotechnology company Cetus, where he had been hired in 1979 to synthesize oligonucleotide probes. Having recently become `successfully unemployed' due to automation of this process, Mullis found himself with `a fair amount of time available to think and putter'.

[3]Kary B. Mullis, The unusual origin of the polymerase chain reaction, *Scientific American,* April 1990, pp. 56-65.

As he drove to Mendocino County this night, Mullis was mentally toying with a new base sequencing technique. He decided the technique would be more definitive if he used two oligonucleotides rather than one. He conducted his thought experiment over and over until jolted by a realization that his mock reaction would double the number of DNA targets in a sample. He stopped the car to confirm his arithmetic on paper: just 20 cycles of this technique, each of only a few minutes duration, would indeed yield over a million copies of a DNA sample.

On his return to Cetus on Monday, Mullis immediately began devising a way to test his hypothesis. A few months later, he ran a one-test-tube experiment and successfully amplified a selected fragment of plasmid DNA. Cetus unveiled the polymerase chain reaction in 1985. Three years later, the technique was greatly improved by use of an enzyme obtained from a bacterium that lives in hot springs. Because the enzyme withstands the high temperatures reached in the reaction, it could be added just once, at the start of the reaction, rather than during each cycle.

Today, the number of possible applications of PCR is so large that most remain unexplored. But new fields are already emerging. In medicine, the technique is being used in 'gene therapy' to locate in an individual or fetus an aberration in perhaps a single pair of nucleotides that will later cause disease. In `molecular' archaeology and evolutionary biology, the long arm of PCR is enabling scientists to analyse DNA from Egyptian mummies and million-year-old plant and animal fossils and to chart the evolution of species with more confidence. And in forensic medicine, minute amounts of DNA at the scenes of crimes are sufficient to establish 'genetic fingerprints' of criminals.

Ideal genetic landmarks

The goal of ILRAD's bovine genome mappers is to identify an array of markers spanning the entire genome that will act as reference points for locating the gene or genes that regulate trypanotolerance. The first essential component is therefore a large number of highly polymorphic markers. The polymerase chain reaction has given the ILRAD mappers a means of identifying a particularly fruitful class of markers called microsatellites. These are segments of DNA that contain multiple copies of simple nucleotide repeats.

Microsatellites are common in the bovine genome and are highly polymorphic in the number of copies of the repeated motif they contain; in one genome a microsatellite may contain a stretch of DNA in which two nucleotides are repeated 20 times, while in another the same nucleotide pair is repeated 30 times. This makes microsatellites ideal markers for a genetic map. The polymerase chain reaction has enabled scientists to visualize the microsatellites for the first time.

The inheritance of a particular bovine microsatellite can be detected by using PCR to amplify genomic DNA—prepared from small blood samples or even single hairs—of members of a family group. By examining the base sequences lying on either side of a microsatellite, primers can be designed for the PCR that will amplify only that microsatellite from among tens of thousands of microsatellites in the original samples of genomic DNA. The length of this target microsatellite in parents and offspring is then determined by electrophoresis and the pattern of inheritance can be deduced.

Knowing the order in which individual markers appear along the chromosomes dramatically increases the confidence with which a genetic map may be used. One way to determine this order is to examine the frequency with which a group of markers recombines during reproduction. The less frequently recombination occurs between two markers, the closer the markers are on a chromosome. Until the arrival of PCR, however, the only way to examine the frequency with which a set of polymorphic markers recombines in cattle was to note the inheritance of such markers over several generations in large families of closely related animals. Such an undertaking is necessarily expensive and time-consuming. The PCR provided an elegant solution to this problem.

Because the polymerase chain reaction is remarkably sensitive, it is able to amplify microsatellites from a single germ cell (gamete). Such amplification makes it possible to determine which one of the four possible combinations of two polymorphic markers occurs in single sperm or eggs. Sperm and eggs are readily available in essentially unlimited quantities. Examining by PCR the chromosomes in a set of sperm produced from a single animal on a single occasion, for example, allows scientists to follow the chromosomes through a large number of meioses, which gives estimates of the frequency with which a set of polymorphic markers recombines. These estimations are both more accurate and much cheaper than those that can be determined from following chromosomal inheritance, from parents to calves. As noted, the frequency of recombination tells one the approximate distance between two microsatellites: the higher the frequency of recombination, the further apart are the microsatellites.

Application of the polymerase chain reaction has thus transformed microsatellites into powerful genetic markers whose relative positions in the bovine genome can be determined. The ILRAD mappers calculate that a panel of up to about 200 relatively evenly spaced polymorphic microsatellites will enable them to name confidently which parent or grandparent of an animal has transmitted a particular segment of DNA to its offspring. This has several important implications. First, using PCR and extensive pedigrees of resistant-susceptible crossbred cattle, scientists may be able to locate the approximate positions of the genes controlling the traits of resistance and susceptibility to trypanosomiasis. Then scientists might be able to identify the genes and the protein products of the genes that control these traits. This knowledge will help elucidate the mechanisms of resistance and, at the very least, provide a practical and rapid means of identifying resistant animals for use in livestock breeding programs.

PCR in research on East Coast fever

Epidemiological studies

Detecting carrier animals

In studies of the epidemiology of East Coast fever, ILRAD scientists are using PCR to detect animals that are carriers of the causative parasite, *Theileria parva*. Carriers are animals that have recovered from an infection, and are therefore immune, but continue to carry *T. parva* and thus provide a source of infection for the tick vector, which in turn is able to infect susceptible animals.

The carrier state has long been recognized in African buffalo infected with *T. parva*. Recent work by Dadson Kariuki, of the Kenya Agricultural Research Institute, has demonstrated the existence of a *T. parva* carrier state in a high proportion of cattle in certain districts of Kenya. It seems, therefore, that carrier animals significantly affect the epidemiology of East Coast fever. However, the typically few parasites found in the blood of carrier animals has made their identification difficult. The great sensitivity of PCR makes this an excellent tool for detecting carriers.

Democratizing the double helix

One result of using PCR in the mapping and sequencing of the human genome, one of the chief scientific endeavours of the decade, 'may be a transformation of the sociology of the genome project ...' Because of the easy access to DNA landmarks provided by PCR, small labs will be able to achieve a kind of parity with big ones, coordinating their work with the major projects unfolding in the national labs.

`Indeed, one of the most far-reaching effects of PCR during [this decade] may not be at the level of research results at all, but in opening up new opportunities for workers whose ability to work at the level of DNA was previously limited. As Rainey of Berkeley's Vertebrate Museum points out, until PCR came along, "unless you had biomedical-scale grants, you generally couldn't sequence DNA". The same may be true in fields other than Rainey's [developmental biology], and, if so, the most profound effect of PCR may be to democratize the double helix.'

—Tim Appenzeller, Democratizing the DNA sequence, *Science,* Vol. 247, p. 1032.

Until recently, the most reliable way to demonstrate whether an animal was a carrier or not was allow ticks to feed on the animal and then to demonstrate whether the ticks were able to transmit parasites to another host. This demonstration, however, is impractical for the large-scale sampling needed in epidemiological surveys.

To detect *T. parva* in blood using PCR, ILRAD scientists developed two sets of primers, one derived from a repetitive sequence of DNA in the parasite genome and the other derived from a gene that codes for a 67-kilodalton surface antigen of the sporozoite form of the parasite.

The first experiments were carried out using purified parasite DNA. Later, with the introduction of PCR at ILRAD, parasite amplification products were obtained from the equivalent of 1µ1 of whole cattle blood subjected to minimal processing and from cattle blood spotted onto nitrocellulose and stored at ambient temperature for three weeks before processing.

Theileria parva amplification products were obtained from cattle infected with four different stocks of the parasite. Interestingly, no amplification of parasite DNA occurred when using blood samples obtained from cattle infected with a fifth stock, *T. parva* Muguga; this result agrees with demonstrations in tick transmission experiments that *T. parva* Muguga does not induce a carrier state.

The amplified DNA products are detected by agarose gel electrophoresis followed by staining with a fluorescent marker, ethidium bromide, and visualization of the DNA bands under ultraviolet light. The DNA gel can then be transferred by Southern blotting onto a membrane and probed with an oligonucleotide that will hybridize only with the amplified region, thereby confirming the identity of the PCR product.

Using PCR technology to detect carrier animals has several advantages over other detection assays. For example, PCR is more sensitive than microscopic examination of Giemsa-stained blood samples for the piroplasm form of the parasite. PCR is also easier to apply when analysing many samples. Use of the conventional indirect fluorescent antibody (IFA) test for detecting antigens of the schizont form of *T. parva* suffers from problems of cross-reactivity, particularly with *Theileria laurotragi*. In addition, the IFA test does not reliably distinguish current infections from previous ones. *Theileria parva* PCR primers, on the other hand, do not amplify DNA from *T. taurotragi* or *Theileria mutans* and moreover only amplify DNA sequences from animals undergoing active infections.

The polymerase chain reaction has also been used at ILRAD to detect the schizont stage of the parasite in biopsied material taken from the lymph nodes of cattle (opening

up the possibility of early diagnosis of infection) and to detect the sporozoite form of the parasite in infected tick salivary glands. PCR has thus already become a useful addition to the techniques available for detecting *T. parva* in both its mammalian host and its tick vector.

Identifying parasite stocks

The existence of many stocks of *T. parva* greatly complicates the epidemiology of East Coast fever, as well as decision-making regarding which disease control method(s) should be used in which areas. To better distinguish among stocks of *T. parva,* scientists at ILRAD in collaboration with Basil Allsopp, of Cambridge University, have developed a pair of oligonucleotide primers that amplifies a 390-base-pair section of a repetitive region in the parasite genome designated TPR1.

The primers made to amplify TPR1 are relatively conserved, that is, the base sequences of the primers are the same in most stocks of the species. The sequence of the amplified region, however, is highly polymorphic among *T. parva* stocks. Using the TPR1 primers in the polymerase chain reaction, the TPR1 segment of several *T parva* stocks was amplified. The amplified products were then cloned into plasmid vectors and sequenced. By comparing the DNA sequences of these cloned segments, which are conserved within stocks and variable among stocks, the researchers could then design synthetic oligonucleotides specific for individual stocks and groups of stocks.

In this way, oligonucleotide probes have been developed that hybridize specifically with each of three *T. parva* stocks: Muguga, Marikebuni and Uganda. The ability to identify Marikebuni reliably is particularly useful to researchers of East Coast fever: this stock has been extensively characterized in terms of its cross-protection with other stocks and is currently being employed in vaccination trials at the Kenya coast.

Probes that identify *T. parva* stocks in this 'plus/minus' fashion may be used, for example, in monitoring vaccination trials. Use of such probes circumvents the need to analyse parasite DNA by restriction fragment length polymorphism, which involves expensive enzymes, agarose gels and Southern blotting. The probes may also be used to enhance the sensitivity and specificity of PCR assays used to detect carrier animals and as markers in experiments designed to identify a sexual cycle in *T.parva*.

Joining the molecular bandwagon: advantages and disadvantages

Use of specific DNA probes for identifying diseases by hybridization of the probe to DNA from the infected host has been commonplace for, several years in many laboratories (see the January 1991 *ILRAD Reports*). However, before Mullis' discovery, DNA probes had a major disadvantage: Though exact, they were insensitive, that is, thousands of sample pathogen cells had to be collected before a probe would detect them: With the PCR techniqiue, the DNA in a tiny original sample (even a single cell,) can be amplified.

As a diagnostic tool, PCR has advantages over classical diagnostic techniques, such as use of highly specific monoclonal antibodies to detect pathogens directly. The latter approach is susceptible to cross-reactions with antigens other than the target. Moreover, detecting anti-bodies to a particular pathogen indicates that the individual was exposed to the pathogen but not that the pathogen is still present. DNA probes detect the actual presence of an organism.

In addition, to visualize small amounts of DNA without PCR amplification necessitates labelling DNA probes with radioactive material, which can then be detected by making an autoradiograph. Radioactive labelling is expensive, hazardous and somewhat unstable. Because PCR yields large amounts of DNA—typically microgram quantities—more user friendly methods, such as primers with fluorescent or luminescent labels are possible. Simplest of all, the DNA can. be stained with a fluorescent marker and viewed under

ultraviolet light.

Speed and ease are not the only advantages PCR has over cloning and restriction-fragment analysis. Those methods, require abundant, high-quality DNA. In contrast, PCR can work with almost any genetic starting material, no matter how scanty or degraded

The very sensitivity that makes PCR so useful, however, also threatens to render it useless. The primers are particular about the sequence they will bind to but do not distinguish sample DNA from exogenous DNA that has contaminated the sample. Particularly where target DNA-levels are low, this can lead to false positive results. To prevent this, work to set up PCR reactions should take place in a separate room from analysis of the PCR products Vessels, surfaces and equipment must be kept scrupulously clean. Good experimental design also helps, with use of appropriate negative controls containing no DNA to ensure that the source of the amplified DNA is indeed the sample

A further drawback to the use of PCR at present is the expense: commercial PCR machines cost as much as US$10,000, although cheap 'homemade' machines, such as those that use immersion, heaters for heating and tap water for cooling, can also be used. The thermostable DNA polymerase needed for the reaction is also expensive: the fee charged. by the best source of this enzyme averages US$2 per amplification. As the technology, becomes more widely used; however, this cost should drop.

Determining the existence of a sexual cycle in *T. parva*

Morphological evidence suggests that *T. parva* undergoes sexual reproduction in its tick vector, *Rhipicephalus appendiculatus.* Whether the parasite does or does not have a sexual phase has important implications for the development of vaccines against *T. parva* because the existence of genetic recombination could undermine a vaccine's stability, that is, the duration and area of its effectiveness.

Development of plus/minus oligonucleotide probes, along with the availability of PCR technology, now makes it possible to carry out experiments designed to confirm the presence of sexual reproduction in *T. parva.* One such experiment is under way at ILRAD. Two parasite stocks, *T. p. parva* Muguga and *T. p. parva* Uganda, for which plus/minus oligonucleotides derived from TPR1 repetitive sequences are available, were injected into cattle at the same time. Ticks were then fed on these animals and collected.

It is believed that a single kinete form of the parasite is derived from a single zygote—the cell produced by the union of male and female gametes in reproduction, before the cell undergoes division. Evidence also suggests that when a tick ingests parasites with a blood meal, a single parasite kinete infects a single tick acinar cell, which is located in the tick's salivary glands. DNA of sporozoite forms of the parasite that developed from single-infected acini was amplified using PCR, after which the DNA products were probed with the oligonucleotides specific for the Muguga and Uganda stocks.

A positive reaction with both oligonucleotides would indicate that the sporozoites had derived from a hybrid Muguga-Uganda zygote. These sporozoites can be used to infect bovine lymphocytes at sporozoite:lymphocyte ratios that ensure that only a single sporozoite infects a given lymphocyte. The lymphocytes can then be cultured to allow analysis of other polymorphic markers.

In recent preliminary experiments, ILRAD researchers produced sporozoites from single-infected acini in tick salivary glands and used PCR to amplify DNA from the sporozoites. The parasite DNA reacted with both the Muguga and the Uganda oligonucleotides. It thus appears that hybrid Muguga-Uganda zygotes are produced in ticks, demonstrating that sexual reproduction does indeed take place in *T. parva.* The sporozoites derived from these zygotes are now being analysed in greater detail.

Vaccine development studies

Cattle that survive infection with *T. parva* are thereafter immune to East Coast fever for long periods. This suggests that it may be feasible to control the disease by immunizing livestock against the parasite. The cells responsible for acquired immunity in vertebrates are white blood cells known as lymphocytes. The two major classes of lymphocytes are B cells, which produce antibody, and T cells, which are responsible for diverse kinds of immune responses. At least three subpopulations of T cells exist. Members of one of these populations, cytotoxic T cells, help protect animals against infection by binding to and killing foreign cells and host cells infected with pathogens.

Cytotoxic T-cells are known to help protect cattle against East Coast fever. As part of an effort to develop new strategies for the development of vaccines against this disease, a team of ILRAD scientists has used the polymerase chain reaction to study receptor molecules located on the surface of cytotoxic T-cells that recognize the target cells to be destroyed.

The T-cell receptor is a cell-surface molecule that recognizes antigenic peptide molecules comprising about 8 or 9 amino acids. For T-cell recognition of target cells to occur, the foreign peptides must be bound to a particular molecule produced by the host animal and located on the surface its cells. This host molecule is known as an MHC molecule because it is encoded by a gene located in an area of the mammalian genome designated the major histocompatibility complex (MHC). Knowledge of how the T-cell receptor recognizes and interacts with the combined parasite peptide/host MHC molecule should provide clues in the search for parasite antigens that can be used in vaccines against East Coast fever.

T-cell receptors are similar in structure to antibodies. Each T-cell receptor is composed of two chains, an alpha and beta chain (TCR I) or a gamma and delta chain (TCR II). Each chain in turn is composed of a variable and a constant region. T-cell receptors are not soluble proteins, like antibodies, but are rather expressed in small numbers on the cell surface.

Like antibodies, T-cell receptors show great diversity in their variable region. It is estimated that the total potential combinations of alpha and beta chains enables the bovine immune system to produce many billions of different T cells, a quantity presumably sufficient to deal with any antigentic challenge.

The messenger RNA coding for T-cell receptors is expressed in small quantities, but when converted into complementary DNA (cDNA) by the reverse transcriptase enzyme, the message can be amplified using PCR. This allowed ILRAD scientists to begin analysing the elements of the T-cell-receptor repertoire that are directed against cells infected with *T. parva*.

The first source of messenger RNA used in this work came from two cytotoxic T lymphocyte clones that were grown *in vitro,* were restricted by the same MHC molecules and were specific for the same *T. parva* strain. Primers used to amplify the cDNA of receptors of cytotoxic T-cells were derived from sequences of previously characterized bovine alpha and beta cDNA, which ILRAD staff cloned from peripheral blood lymphocytes. The combination of primers used in this set of experiments allowed PCR amplification of the entire variable region of alpha and beta chains from these T-cell clones.

Expansion of elements of the T-cell repertoire can be monitored *in vivo* following challenge with an antigen. Using PCR, ILRAD scientists are cloning T-cells obtained from efferent lymph of immune animals and sequencing the genes that code for receptors on these T-cells. This approach is enabling the scientists to analyse the

extent and types of cytotoxic T-cells deployed against *T. parva.* This information will lead to a better understanding of immune responses against the parasite.

Reaping the harvest of molecular biology

The polymerase chain reaction is certain to continue to play a major role in ILRAD's biological research—molecular and otherwise. It is startling that a technique still in its infancy has become essential to so many of the institute's research projects. As scientists learn more subtle and powerful ways to use the technique, its contributions to biomedical and veterinary science will also proliferate, perhaps starting an acceleration in research progress not unlike the exponential power of the chain reaction itself.

Studies of a trypanosome enzyme that may prove useful as an antigen (Ph.D.Thesis)

The parasite *Trypanosoma congolense* causes the disease trypanosomiasis. Infection begins when trypanosomes are injected into the blood of a mammal by a tsetse fly as it feeds on the animal. In the animal host and the tsetse fly vector, the parasites pass through several developmental stages in their life cycle.

As part of ILRAD's research into improved methods of controlling trypanosomiasis, scientists are investigating parasite components that might be attacked without harming cells of the host animal. In this area, biochemical studies were conducted on the activity of proteolytic enzymes of the parasite. These enzymes break down protein molecules into smaller fragments. The studies were designed to determine where these enzymes are located within the parasites and whether proteolytic enzymes occur only in particular stages of the parasite's life cycle, in which case they are known as 'developmentally regulated', or whether they occur in all stages.

An experiment was conducted to examine the activity of proteolytic enzymes in different developmental forms of *T. congolense.* The results indicated that in bloodstream and metacyclic forms of the parasite, enzyme activity occurs inside the lysosome, a cell structure rich in enzymes that functions in intracellular digestion; in epimastigote insect forms, however, enzyme activity occurs outside the lysosome, in the cytosol of the cell (the soluble portion of the cytoplasm).

A cysteine protease (so-called because the enzyme contains the amino acid cysteine in its active site) was purified from the lysosome-like organelles of bloodstream forms of *T. congolense* and from suspensions of ruptured parasites. Rabbit antibody to the cysteine protease was prepared and used to determine where the protease is located in the parasite. The polyclonal antibody raised against the purified enzyme was labelled with markers. Viewed in the electron microscope, the markers appeared in parasite structures resembling lysosomes. It was also found in the flagellar pocket, through which the parasite engulfs nutrients, and in other structures that are part of the parasite's endocytotic (nutrient uptake) network.

The presence of the cysteine protease within the flagellar pocket suggests that direct contact is possible between the enzyme and host animal and, furthermore, may result in pathologic degradation of important host proteins. Interestingly, collaborative studies with Dr. Edith Authie (ILRAD) suggest that trypanotolerant N'Dama cattle make antibodies against this enzyme during the course of infection, whereas cattle that are susceptible to the disease do not.

Fragments of .the trypanosome gene that codes for the cysteine protease were isolated, cloned and partially sequenced. The full gene was found to be at least 1.6 kilobases long. Messenger RNA was isolated from all four life cycle stages of the