DNA PROBES FOR DETECTION AND IDENTIFICATION OF HUMAN PARASITES

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ABSTRACT

DNA methods are providing new approaches for detecting and identifying a spectrum of human parasites. In particular, assays utilizing DNA probes have been shown to be rapid, easy to use under laboratory conditions, accurate and sensitive although logistical problems exist for their application in the field. In this review, a discussion is made of DNA probes now available to identify malaria, African and American trypanosomes, leishmaniae, schistosomes, *Trichinella*, lymphatic filariases, *Onchocerca* and the cestodes, *Echinococcus* and *Taenia*. Techniques for labelling DNA probes by non-radioisotopic methods, a pre-requisite for their use in field, are described and a recent, remarkable advance, the polymerase chain reaction, which can substantially amplify the target DNA of parasite test samples, is highlighted.

INTRODUCTION

DNA hybridization is now recognized as one of the well established and essential tools of modern molecular biology. The application of the technique ranges from the determination of genetic and taxonomic relationships between organisms (including viruses, prokaryotes and a range of eukaryotes including vertebrates). detection and identification of infectious organisms, diagnosis of inherited genetic and transmissible (infectious) diseases, to gaining a greater understanding of the basic biology of cancer and immune deficiencies and the etiology of many other diseases. Here, the effectiveness of the approach in the detection and identification of important human protozoa and helminths is discussed.

For a DNA probe assay to achieve the desired application, the DNA to be analysed should be easy to obtain and the assay should be convenient to use, specific and rapid in order to yield a quantitative result within a short period of time. The probes can either be DNA (genomic), RNA, *c*DNA (obtained from *m*RNA) or an oligonucleotide sequence determined on the basis of an already known sequence of a peptide product. A genomic DNA probe can be obtained by generating fragments of DNA (either by shearing or by restriction endonuclease digestion) and then joining such fragments to either a plasmid or phage vector. Such a library of recombinant plasmids or phages is then propagated into a suitable host bacterium and then screened by direct heterologous or homologous nucleic acid hybridization. The recombinant plasmids or phages of interest are selected and the inserts characterized by mapping and/or sequencing. Highly specific probes can thus be obtained by direct "shot-gun" cloning of genomic DNA although the probe may not be an expression sequence. RNA probes, on the other hand, are single stranded and require higher hybridization temperatures, which improve the speed and specificity of the assay. The disadvantage of RNA probes is that they are generally more susceptible to degradation by enzymes when compared with DNA probes. This disadvantage is generally overcome when the RNA in question is reverse transcribed into complementary DNA and then a *c*DNA, rather than RNA, probe is made. A *c*DNA probe is certain to be an expression sequence provided the sequence is cloned in the correct orientation in an appropriate expression vector. Alternatively, if the amino acid sequence of a protein is known then it is possible to use genetic code to predict the nucleotide sequence of the relevant gene. Such prediction, though, will be an approximation since many amino acids are usually coded by more than one triplet codon. Based upon such predicted nucleotide sequences, a short oligonucleotide probe can be chemically synthesized.

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Such oligonucleotide probes can be either directly used to screen a genomic or *c*DNA library to identify the parent gene or can be cloned into plasmid or phage vectors, the oligonucleotide insert amplified and then used to detect single base pair alterations in the parent gene with the enhanced levels or sensitivity.

The preparation of samples under investigation varies depending upon the nature of the question posed. In situations where it is only necessary to ascertain the presence or absence of a particular sequence, a dot-blot is used. The purified DNA is spotted onto nitrocellulose, nylon filter paper or microscope slide, denatured by treatment with NaOH and fixed to the matrix by baking at 80°C (in case of nitrocellulose filters and microscope slides) or by UV illumination (in case of nylon filters). Such filters are then incubated at 65–70°C in a suitable hybridization medium containing radiolabelled single stranded probe sequences for an appropriate period of time. After hybridization, the filters or slides are then washed in suitable buffer to get rid of unbound radioactivity and are subjected to autoradiography to detect DNA-probe hybrids. If, however, it is required to detect subtle changes in the pre-existing gene/s in the same or related genomes, then a more complicated procedure must be followed. The genomic DNAs in question are first digested independently with suitable restriction enzyme/s, electrophoresed through agarose slab gels to separate the restriction fragments according to their molecular weight, followed by Southern blotting[1] of the fragments onto nitrocellulose or nylon filters. The filters with Southern blotted DNAs are then hybridized with suitably labelled probes and DNA-probe hybrids detected by autoradiography, or by chromogenic or fluorimetric assays.

A suitable label, usually a radio-isotope, is inserted into the probe DNA by a variety of enzymatic methods, involving nick-translation[2], end-labelling[3] or oligolabelling[4]. Much academic and commercial effort is presently being directed to devise the protocols that do not involve the use of radioisotopes. In this respect a modified nucleotide with a biotin molecule attached to the base via a linker arm has been developed[5]. This modified nucleotide can be inserted into the DNA by conventional nick-translation methods. The biotin thus incorporated in double stranded hybridrs can be recognized either by avidin/streptavidin or antibiotin antibodies to which suitable detecting enzymes are attached. Alternative methods of introducing biotin have recently been described. In one approach, a photoactivable group is linked to the biotin molecule via a linker arm (photobiotin)[6,7]. Unfortunately, the sensitivity of biotin-avidin assays is often reduced because of naturally occurring biotin which causes undesirable background exposure. Another method presently under investigation involves direct linking of an enzyme to a DNA probe[8] and subsequent detection of the DNA hybrids by using anti-enzyme antibodies.

DNA probes have immense potential in increasing understanding of the molecular basis of interactions between man and his infectious agents, and DNA analysis is being increasingly applied to many clinical problems on account of its specificity and sensitivity. Such diagnostic methods at the DNA level have the advantage over other identification methods in that the individual genes or parts of genes are studied rather than their expressed products and are, therefore, unlikely to show life-cycle or environmentally-mediated (including host-induced) variation. Progress in the development of nucleic acid-based assays for the diagnosis and epidemiological surveillance of human parasites has been rapid and probes are now available to identify many of the most important species. DNA probes, representing cloned parasite specific DNA sequences which are moderately or highly reiterated in the parasite genome, can be used as diagnostic assays for a parasite species in a given test sample by employing dot-blot hybridization protocols. Alternatively, DNA probes can also be used to assess inter- and intra-specific genetic variation in the DNAs of different and related parasite species by employing restriction enzyme, Southern blot and hybridization protocols. The *c*DNA probes, in addition to their diagnostic potential, can also be used to obtain the eventual protein product and investigate the structural and functional aspects of the protein and the parent gene as well. Thus, any inter- and intra-specific variations detected by DNA and/or *c*DNA probes can be used to devise more specific and sensitive diagnostic protocols and also to understand the evolutionary interrelationships between and within the parasite species.

PROTOZOA

A number of protozoan species occur which are clinically significant to humans. The most important pathogenic genera are *Leishmania*, *Trypanosoma* and *Plasmodium* and these are considered here.

Leishmania

Human leishmaniasis is well recognized as an important public health problem worldwide. Early and accurate identification of *Leishmania* spp is often problematical and this presents difficulties to clinicians and epidemiologists alike. Several non-DNA methods, notably isoenzyme analysis and the use of monoclonal antibodies are available for diagnosis and identification[9–12]. DNA approaches have recently been applied to the problem and a valuable review of the field is available[13]. The most commonly applied DNA identification method involves the use of kinetoplast DNA (*k*DNA). The kinetoplast is a microscopically visible disc, 1–2 μm in diameter, found at the base of the flagellum in all the kinetoplastid protozoa including *Trypanosoma* and *Leishmania*. The kinetoplast contains approximately 10^7 bp of mitochondrial DNA in the form of about 50 maxi-circles which code for mitochondrial genes, and approximately 10,000 to 20,000 highly reiterated mini-circle sequences of 500–2500 base pairs. As a result of the repetitive nature of the *k*DNAs, recombinant probes of *k*DNA origin are highly suitable for diagnostic purposes. Indeed, cloned *k*DNA minicircle probes have been used to distinguish isolates of old world cutaneous *Leishmania* species[14,15], as well as to unambiguously identify single parasites obtained from culture, a clinical lesion or sandfly gut on microscope slides[14]. Further, recombinant probes specific for the *L. braziliensis* complex have also been produced and used in field trials employing Southern blot, dot-blot and microscopic slide *in situ* hybridization methods (figure 1)[13]. The species-specific *k*DNA-sequences for old world *Leishmania* species (*L. major*, *L. tropica*, *L. aethopica* and *L. donovani*) and those from new world isolates (*L. braziliensis* complex), provide promising probes to distinguish single schizodemes and geographically isolated strains of *Leishmanias*[13].

Trypanosoma

Other clinically important members of the kinetoplastid protozoa are the trypanosomes. As with *Leishmania* spp., the *k*DNA of trypanosomes has been exploited to construct diagnostic recombinant probes. Unlike *Leishmania spp*, which have less than 10 major classes of mini-circle sequences, the mini-circles of trypanosomes are more divergent and have up to 300 sequence classes[13]. Nevertheless, mini-circles from each trypanosomatid species have a

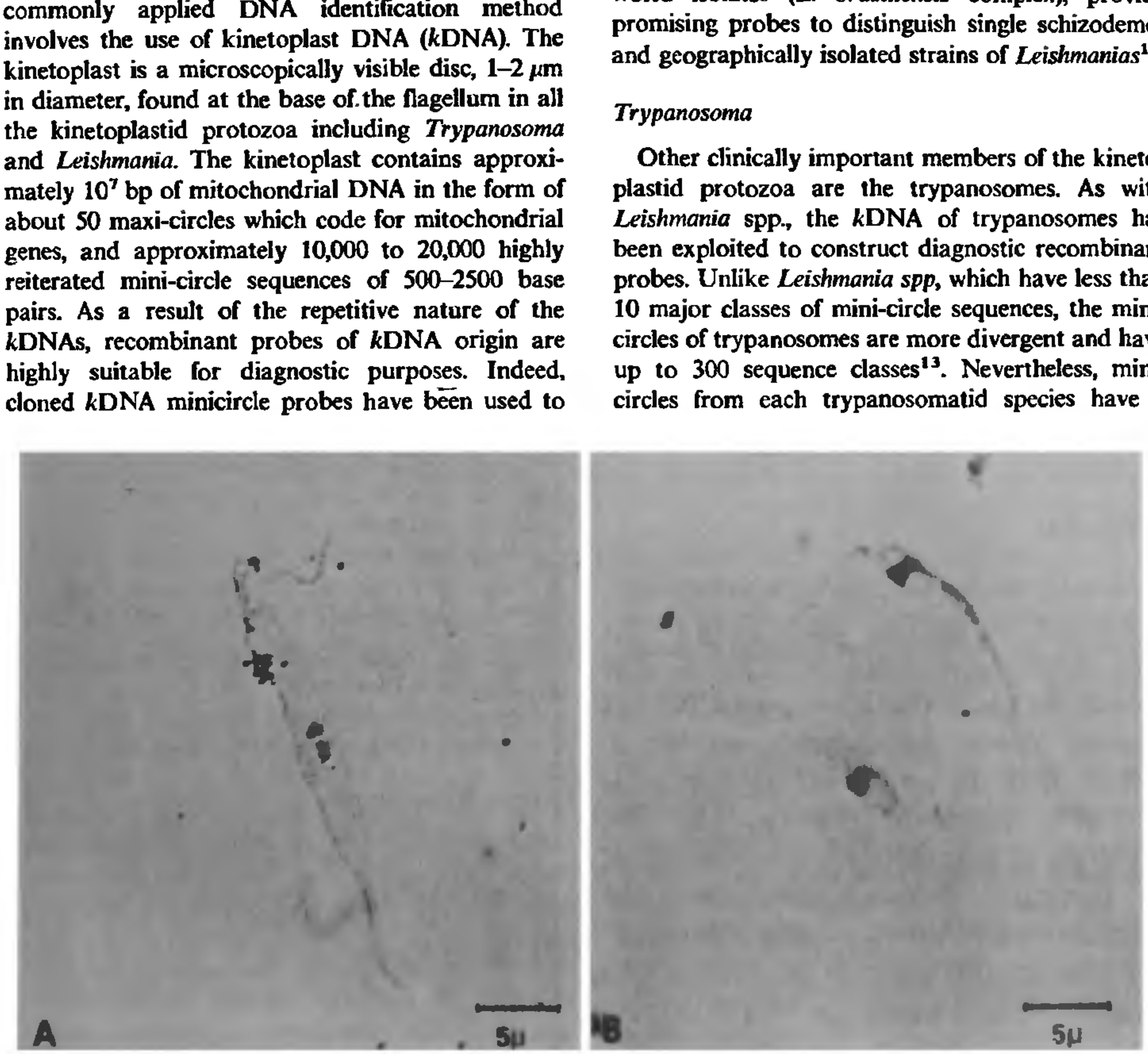

Figure 1A, B. *In situ* hybridization of kinetoplast DNA probes. Both micrographs are promastigotes of *Leishmania m. amazonensis* hybridized with a *k*DNA probe made from homologous organisms. In A the probe was radioactively labelled and has been detected by autoradiography. In B the probe was labelled with biotin and detected by sequential treatment with streptavidin, biotinylated alkaline phosphatase and a chromogenic substrate (after Barker[13]).

conserved region of sequences represented in *T. brucei, T. equiperdum, Leishmania tarentolae* and *T. lewisi*[16-19]. The analysis of percentage of homology, detected by using cloned DNA probes of conserved regions, among the trypanosomatid species may prove useful for taxonomic and epidemiological purposes. Sequencing of such constant mini-circle regions may, for example provide a simple way of classifying the members of the order Kinetoplastida. In a slightly different approach, restriction fragment length polymorphic (RFLP) probes of *T. brucei* have been used to characterize parental and hybrid trypanosome stocks on the basis of their DNA contents and Southern hybridization banding patterns[20]. Furthermore, species- and subspecies-specific trypanosome DNA hybridization probes have been employed recently in detection and identification of trypanosome infections in tsetse flies[21]. In addition, repetitive DNA sequences from three sub-genera of trypanosomes (*Nannomonas, Duttonella* and *Trypanozoon*) of African origin have been cloned and characterized[22]. Restriction fragment length polymorphisms were observed in the DNAs from six cloned *Trypanozoon* isolates using a repetitive probe of homologous origin[21,22]. Using another strategy, total parasite DNA has been used as a probe to detect and identify South American Trypanosomes (*Trypanosoma cruzi, T. rangeli*) in insects[23].

Plasmodium

DNA probes also offer a valuable alternative to current malaria diagnosis[24] which depends principally on the microscopic examination of stained blood films. A DNA probe, specific for *Plasmodium falciparum*, has been developed by isolating a highly repeated 21 base pair DNA sequence from a genomic library of *P. falciparum* DNA (figure 2)[25]. This probe has been used in field studies in Thailand, Brazil and Kenya, employing a simple dot-blot analysis of blood drops taken directly from patients, to detect and identify the parasite. In other similar studies, a repetitive DNA sequence of *P. falciparum* DNA[26] and an RNA probe of the same repetitive DNA sequence of *P. falciparum*[27], have been used in a dot hybridization assay with comparable sensitivity to other contemporary assays for detection of *P. falciparum*. In another approach, a radiolabelled synthetic oligonucleotide was evaluated as a diagnostic probe for *P. falciparum* using blood samples lysed directly on nitrocellulose filters[28] and the results obtained were found to be comparable to the blood smear results.

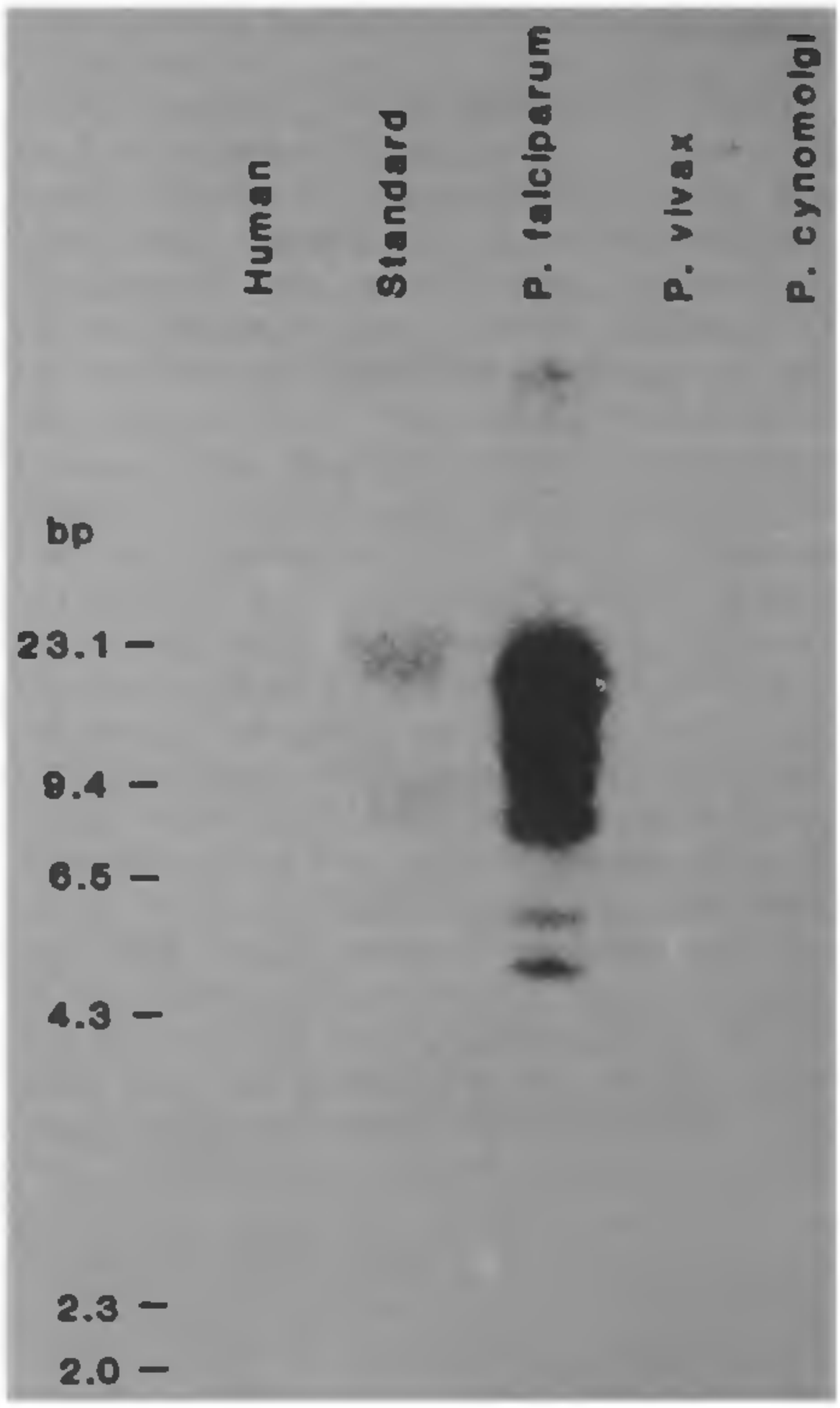

Figure 2. Autoradiograph of species-specific DNA probe (pPF14) hybridized to DraI-derived restriction fragments of genomic DNA of human, *Plasmodium falciparum, P. vivax* and *P. cynomolgi* origin. Each lane contained 2 μg DNA (after Barker *et al*[25]).

In all of the above assay systems the labelling of the probe DNA was carried out by using radioisotope. For wide-spread field application, however, non-radioactive probes will be required to avoid hazards of handling radioactive materials. Towards this end, enzyme-linked synthetic oligonucleotides (previously used successfully in radiolabelled assays) have been used for the detection of *P. falciparum* infections[29]. Here, a 21 bp synthetic oligomer was covalently coupled to alkaline phosphatase for histochemical detection. The conjugate successfully detected purified *P. falciparum* DNA as opposed to host DNA or DNA of other parasitic species at an equal specificity and sensitivity to radiolabelled

probe assays. *P. falciparum*-specific probes have also been used for detection of malarial sporozoites in mosquitoes and further studies are being directed to understand molecular genetics of malaria in order to develop suitable tests for diagnosis of species, subspecies and stages of discrete isolates[30].

HELMINTHS

Using strategies similar to those described above for parasitic protozoa, DNA probes have been developed for identification and characterization of a number of parasitic helminths[31].

Schistosomes

Cloned DNA fragments of the ribosomal RNA gene have been used, in particular, as probes for the identification of the human blood flukes, *Schistosoma* spp. Several strains of *S. mansoni* from Africa and the Caribbean were studied using restriction endonuclease, Southern blot and hybridization analysis of their DNA[32], and it was found that species-, strain- and sex-specific DNA differences existed. Further, polymorphism was observed in the major unit length of the rRNA gene repeat in *S. haematobium* and *S. japonicum*. The ribosomal RNA gene of six closely related species of schistosomes from Africa, *S. haematobium*, *S. mattheei*, *S. intercalatum*, *S. bovis*, *S. currasoni* and *S. margrebowiei* have also been studied. *S. mattheei*, *S. margrebowiei* and *S. haematobium* can be distinguished from the others on the basis of variation in transcribed and non-transcribed spacer regions of this gene[33]. In Senegal, three species of schistosomes occur namely *S. haematobium*, *S. bovis* and *S. currasoni*. Again, the *r*RNA gene has been found to vary in *S. haematobium* and *S. currasoni*[34]. Robosomal RNA gene probes have also been used in dot-blot analysis of DNAs to successfully discriminate between *S. mansoni* infected and uninfected *Biomphalaria glabrata* snails (figure 3)[31].

Nematodes

The identification of nematodes presents problems because of the lack of reliable diagnostic morphological characteristics. However, genomic DNA restriction fragment length differences in repetitive sequences have been used to identify a wide range of nematode species[35]. Furthermore, specific cloned genes have been suggested as a means for identification of nematode strains and a cloned clustered and

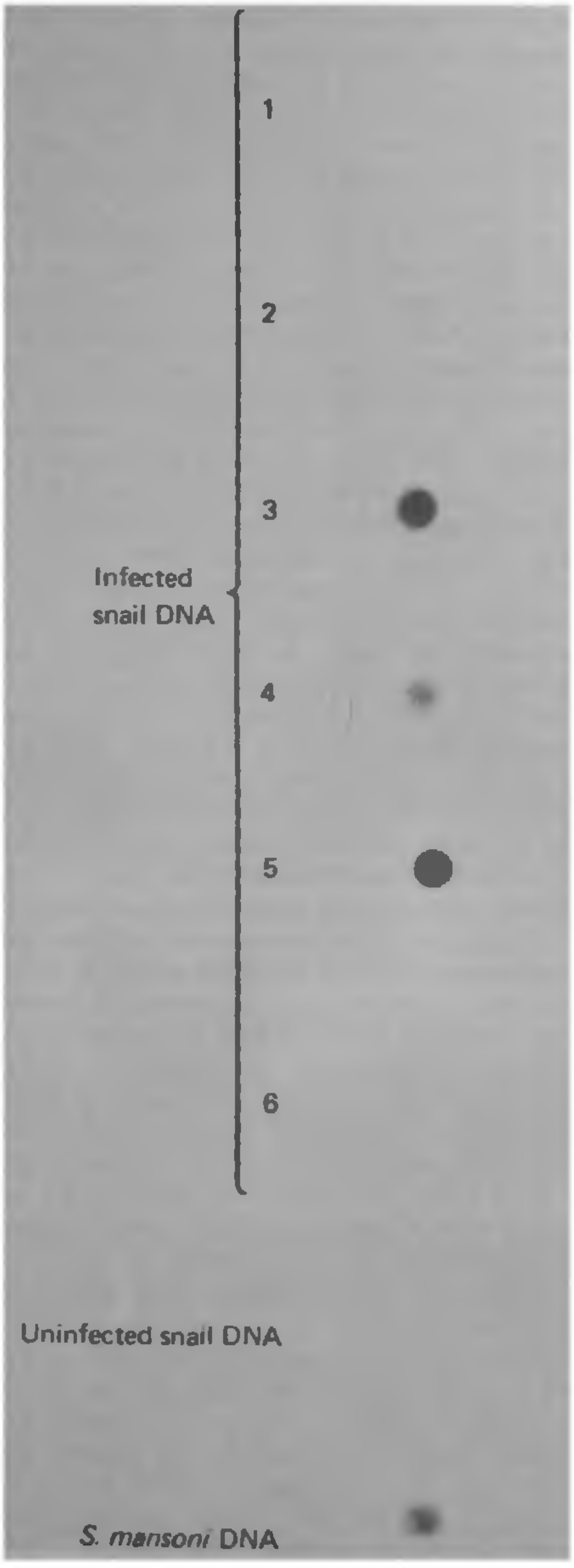

Figure 3. The hybridization of radiolabelled pSM389 to dot-blots of 0.2 μg DNA from *Schistosoma mansoni*, an uninfected snail (*Biomphalaria glaburata*) and 6 infected snails (after Rollinson *et al*[31]).

dispersed repetitive DNA fragment from *Trichinella spiralis* has shown some promise as a diagnostic probe[36]. Also, a *Trichinella*-specific probe has been developed from *T. spiralis* DNA. This probe is repetitive in nature and has been used for identification and comparison of *Trichinella* variants[37]. It was suggested that techniques of molecular characterization could be applied to species and strains of the filarial worms, *Wuchereria* and *Brugia*, in man and mosquitoes[38]. Indeed, various candidate probes have now been developed for differentiation and identification of *Brugia* spp. A cloned, highly repetitive sequence from *B. malayi* with specificity for the genus *Brugia* and with potential to discriminate between *B. malayi* and *B. pahangi* has been used as an extremely sensitive probe for detection of *Brugia* in blood samples[39]. Regions of divergence in this highly repeated *Brugia*-specific probe have been revealed by DNA sequence comparisons and these have been utilized to construct highly species-specific oligonucleotide probes for both *B. pahangi* and *B. malayi*[40]. Such oligonucleotide probes have been used to detect a single microfilaria in crude preparations of DNA from blood or a single L3 larva in crude DNA preparations from mosquitoes[41]. In addition, a highly specific and sensitive DNA probe for *B. malayi* has been proposed as a candidate probe for diagnosis and detection of infected vectors (mosquitoes) in filarial endemic areas during field studies[42]. As an alternative approach, a 900 bp, non-coding spacer region of the *r*RNA gene of *B. malayi* has been successfully used to discriminate *B. pahangi* from *Dirofilaria immitis*[43]. Both the species-specific DNA and oligonucleotide probes could be used to investigate evolutionary inter-relationships of *Brugia* spp. and also to successfully identify a single filarial worm in the infected vector or the vertebrate host[40].

DNA probes have also been developed for the related worm, *Onchocerca* spp., the causative agents of onchocerciasis or river blindness in man[44-47]. Recently, a 153 bp long DNA probe sequence from *O. volvulus* has been cloned[48], which specifically recognizes the west African forest form of *O. volvulus* but does not hybridize to DNAs of west African Savannah or Mexican forms of *O. volvulus*, the simulid blackfly vector or human origins. The progress in the development of DNA probes for *Onchocerca* spp. has been recently reviewed[47], their application in distinguishing different forms of the parasite, and in the development of a simple and sensitive assay to detect *O. volvulus* infections has been highlighted. Such probes may be applicable for epidemiological studies and in monitoring new *O. volvulus* outbreaks within control regions such as the Onchocerciasis Control Programme in west Africa.

Taeniid cestodes

Another important group of parasitic helminths are the taeniid cestodes, representatives of which include *Echinococcus* and *Taenia*. Ribosomal RNA gene sequences from *S. mansoni* have been successfully used to genetically distinguish *E. granulosus*, which causes unilocular hydatid disease in man, from that of related species, *E. multilocularis*, the causative agent of alveolar hydatidosis. In addition, these probes can distinguish intraspecific variants of *E. granulosus* from sheep and horses of UK origin[49]. In an extension to this approach, species- and genus-specific DNA probes of *E. granulosus* origin have been constructed[50] and used to assess intraspecific genetic variation in both the larval and adult stages of *E. granulosus*[51]. Furthermore, both the homologous and heterologous probes have proved very useful in characterizing isolates of *E. granulosus* of sheep, horses/donkeys, goats, humans, cattle, pigs and camels from different geographical locations such as Kenya, Sudan, the Mediterranean littoral, Europe, India and Uruguay[51]. A species-specific probe for *E. granulosus* has been reported and its potential use in identification of *E. granulosus* eggs for field application has also been discussed[50]. In addition, heterologous *r*DNA and homologous sequences have also been applied to assess intraspecific genetic variation in *T. solium*, the cause of human neurocysticercosis. Distinct genetic differences between *T. solium* originating from India, Mexico and Zimbabwe have been observed[52]. In addition, *T. solium*-specific probes have been constructed which can successfully discriminate *T. solium* DNA from that of other taeniid cestodes (figure 4)[53].

FINAL COMMENTS

Recently, it has been pointed out that DNA probes have so far been applied to identify known parasite species and have yet to be used to detect new species[54]. This is in addition to the fact that major logistical problems exist for the wide-spread field use of probes although future strategies for their successful application have been proposed. For the purpose of field application of DNA probes, the major areas of current research involve development

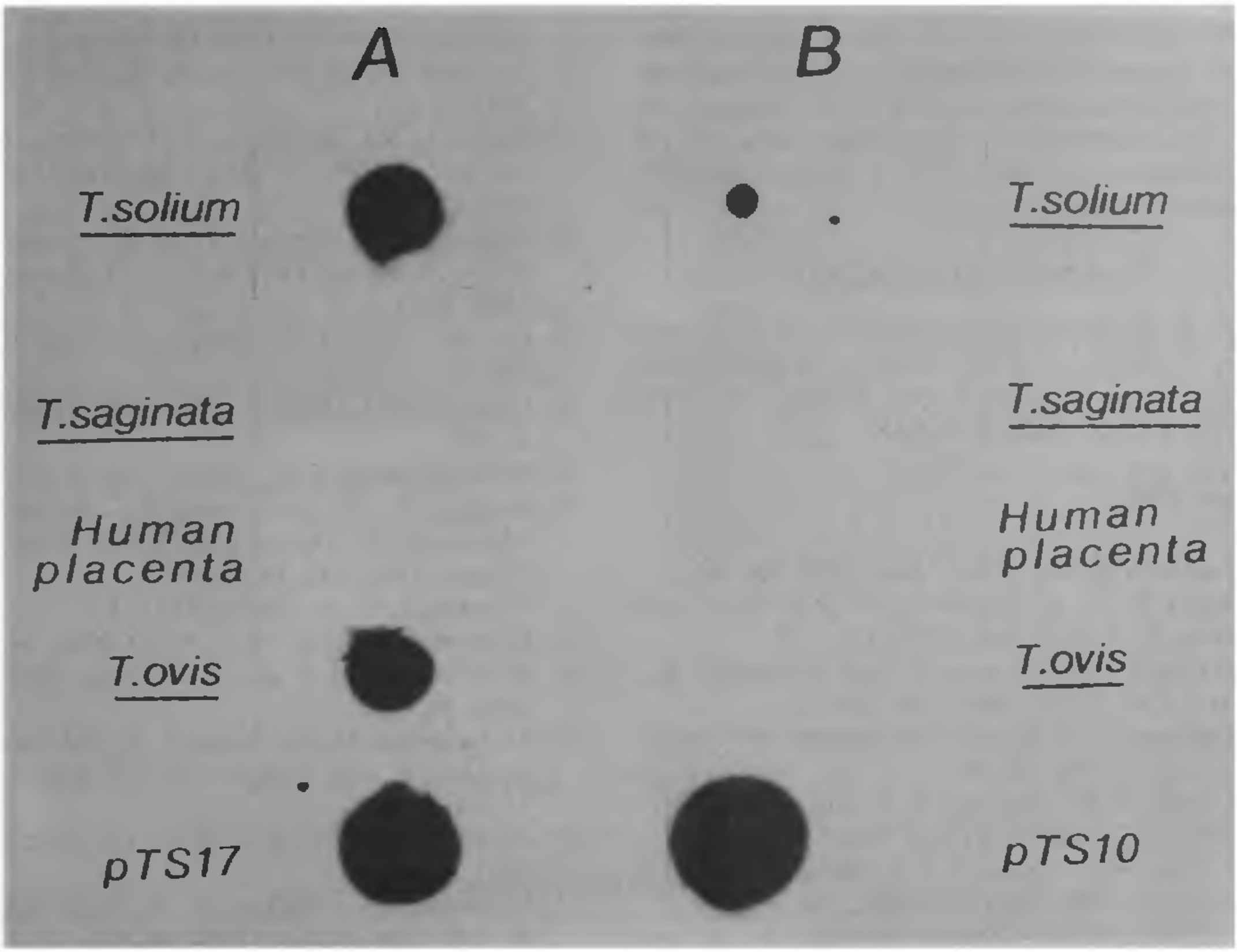

Figure 4. The hybridization of radiolabelled pTS17 (panel A) and pTS10 (panel B) to dot-blotted DNA (parasite and human placenta, 100 nanogram; recombinant plasmids containing *T. solium* genomic inserts, 10 nanogram). Note the specificity of pTS10 and pTS17 (after Rishi and McManus[53]).

of procedures for non-radioactive labelling of specific sequences, suitable hybridization protocols and improved methods for the preparation of target sample DNA.

In a recent advance, a DNA amplification technique called the polymerase chain reaction (PCR) has been used to amplify proviral sequence of the human immunodeficiency virus (HIV-1, directly in DNA isolated from peripheral blood mononuclear cell[55]. Primer pairs from multiple regions of the HIV-1 genome were used to amplify specific regions of the provirus present either as the free episomal form or as the integrated form in patients' chromosomal DNA. The HIV-1 sequences were amplified *in vitro* from 1 μg of genomic DNA of peripheral blood mononuclear cells of both control and HIV-1 infected patients using combination of primer pairs and thermo-resistant DNA polymerase of *Thermus aquaticus*. Such amplified sequences were subsequently detected by hybridizing with HIV-1 diagnostic probes. It has been suggested that above method could be used to complement or replace virus isolation as a routine means of determining HIV-1 infection[55].

Given the sequence organization of a highly species-specific, low copy number human parasite DNA probe, the primer sequences complementary to 3′ and 5′ ends of the whole or part of the probe sequence can be chemically synthesized. Such primers can then be used to amplify the complementary probe sequences in the target DNAs of infected blood or tissue samples by employing the above *in vitro* DNA amplification technique. The approach should permit, in theory, to successfully amplify a single copy probe sequence in the test samples containing as low as one copy of the infective parasite genome. Such amplified target DNA sequences can be detected by using suitably

labelled (preferably non-radioactive) DNA probes. Besides its potential for detecting low copy sequences, the protocol may prove suitable for the diagnosis of very low parasitaemias (even single cells, e.g. of *Plasmodium* or *Trypanosoma*) in field and epidemiological studies.

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ANNOUNCEMENT

GROWTH INHIBITORS

Pre-clinical and clinical evaluation in cancer

The study of growth inhibitory proteins is opening up new avenues in cancer therapy. These inhibitory factors work in balance with growth promoting factors to modulate cell growth and tissue formation; it now appears that loss of response to this system may be of paramount importance in cancer. The three-day international conference to be held during 20–22 March 1989 at the Waldham College, Oxford, has three aims: to provide an up-to-date and thorough review of the biology of growth inhibiting proteins; to examine the lessons learnt from clinical testing of those growth inhibitors that have already been used against cancer; and to discuss the adequacy of present approaches to preclinical testing. For details contact: Dr Renata Duke, IBC Technical Services Ltd., Bath House, 56 Holborn Viaduct, London EC1A 2EX.