The rapid and sensitive detection of the plant parasitic nematode *Globodera pallida* using a non-radioactive biotinylated DNA probe

Paul R. BURROWS

Department of Entomology and Nematology, AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, UK.

SUMMARY

This work demonstrates the first effective use of a non-radioactive biotinylated DNA probe to detect the presence of *Globodera pallida* DNA bound to a nitrocellulose filter. The sensitivity of the technique in this application has been improved, facilitating the detection of a single *G. pallida* infective second stage juvenile. The rapid preparation of crude nematode DNA samples coupled with a high concentration of biotinylated probe during hybridisation makes the detection process very quick. Non-specific staining of the sample dots is not a problem even when probing impure DNA samples. The significance of this research in the development of simple diagnostic kits for the routine identification of agriculturally important species, races and pathotypes of plant parasitic nematodes is discussed.

RÉSUMÉ

Détection rapide et sensible du nématode phytoparasite Globodera pallida *par utilisation d'une sonde de DNA non radio-active marquée à la biotine*

Le présent travail a trait à la première utilisation efficace d'une sonde de DNA non radio-active, marquée à la biotine, pour caractériser la présence du DNA de *Globodera pallida* fixé sur un filtre de nitrocellulose. Lors de cette mise au point, la sensibilité de la technique a été améliorée, permettant ainsi la détection d'un seul juvénile de 2e stade de *G. pallida*. La rapidité de la préparation des extraits bruts du DNA du nématode, couplée à la forte concentration de la sonde marquée à la biotine lors de l'hybridation, conduisent à une technique de détection très rapide. Dans le cas d'échantillons relatifs à un DNA non purifié la coloration non spécifique de certaines zones de l'échantillon ne cause aucun problème. Une discussion porte sur les conséquences de la présente recherche pour la mise au point d'un système simple d'identification routinière des espèces, races et pathotypes de nématodes d'importance agronomique.

Many species of plant parasitic nematodes pose a significant threat to agriculture worldwide. Accurate and reliable identification of these microscopic pests is fundamental to many aspects of their control and management. Diagnosis of species based on morphology is often difficult as the characters used show considerable intraspecific variation. Moreover, the identification of many morphologically identical nematode races or pathotypes is impossible using physical characteristics alone.

Molecular biology offers a number of exciting new approaches that are revolutionising parasite identification, but with a few notable exceptions these techniques have been largely neglected by plant nematologists. Digestion of plant parasitic nematode DNA with restriction endonuclease enzymes followed by agarose gel electrophoresis has revealed useful restriction fragment length polymorphisms (RFLPs). RFLPs have been shown to differentiate several nematode genera, species and races (Curran, Baillie & Webster, 1985; Burrows & Boffey, 1986; Burrows, 1988). However, it is the use of cloned specific DNA hybridisation probes coupled with dot blot assays that promise to facilitate the greatest advances in parasite diagnosis (Barker *et al.*, 1986; Rollinson, Walker & Simpson, 1986; Ole-Moiyio, 1987; Burrows & Perry, 1988). For routine identification of plant parasitic nematodes, specific DNA probes have an important advantage over RFLPs in that they can give a positive or negative assay for nematode species, race or pathotype without the need for restriction digestion of the DNA or time consuming electrophoresis. The search for diagnostic DNA probes for agriculturally important nematodes is still in its infancy. Even so, it seems that a major impediment to the wider acceptance and use of this technique for routine diagnostics will be the need to detect probes using radioisotopes. Radiolabelled probes are expensive, hazardous to work with and once labelled the activity decays within a few weeks necessitating replacement or frequent relabelling. The full potential of specific DNA probes for the identification of plant

parasitic nematodes will only be realised when suitable non-radioactive methods for the detection of bound probes are developed or adopted. Specific DNA probes can be visualised by a number of different non-radioactive methods (Gilliam, 1987) but they are often considered to be too insensitive. Although this is generally correct, one particular group of techniques based on biotin is very promising.

Biotin-11-dUTP is a nucleotide analogue that may be incorporated into a probe by nick translation in the same way as a radionucleotide. Hybridisation of a biotin labelled probe with nematode DNA bound to a nitrocellulose filter can be detected by conjugating a preformed complex of avidin and alkaline phosphatase to the biotin. Enzyme catalysed production of an insoluble blue pigment at the site of hybridisation reveals the presence of the probe.

This work details modifications to a basic biotin technique and its subsequent use to visualise DNA from the potato cyst nematode (PCN) *Globodera pallida* using a *G. pallida* specific DNA probe (Burrows & Perry, 1988) in dot blot assays. The sensitivity of the method in this application has been improved and non-specific background staining has been almost eliminated even when using crude DNA preparations.

Materials and methods

Nematode populations

Two populations of potato cyst nematodes *Globodera rostochiensis* Rol (Woburn) and *G. pallida* Pa 4 (New Leake), were multiplied on the susceptible potato cv. Arran Banner in glasshouse pot cultures. Eggs and second stage juveniles (J2) were collected and prepared for DNA extraction as detailed (Burrows & Boffey, 1986).

Extraction and preparation of DNA

Nematode DNA was extracted and purified as previously reported (Burrows & Perry, 1988). A 100 µl pellet of packed *Globodera* eggs and J2 yielded 50-100 µg of clean DNA. DNA prepared in this way was a good substrate for many of the enzymes such as restriction endonucleases, ligases, kinases and polymerases commonly used in molecular biology.

Crude DNA preparation

This method results in comparatively impure nematode DNA. However, samples may be prepared from very few nematodes in as little as 30 min.

Globodera eggs and J2 (10 µl packed pellet or a single cyst) were washed twice with DNA extraction buffer (EB) (0.1 M Tris·Cl pH 8.5, 0.1 M NaCl, 0.05 M EDTA) and transferred to a centrifugal micro-homogeniser (Biomedix®) (Hearse, 1984). The nematodes were pelleted in the homogeniser tube in a microcentrifuge, resuspended in 20 µl EB, and homogenised on ice for 1 min.

The homogeniser was centrifuged briefly (1 sec) to clear the polypropylene pestle of cell debris, and 180 µl EB, 30 µl SDS (20 % W/V in EB) and 20 µl proteinase K (25 mg/ml, Sigma) were added. The tube was capped, the contents gently but thoroughly mixed and then incubated at 55 °C for 25 min.

The nematode homogenate was extracted once with an equal volume of phenol chloroform 1 : 1, and the aqueous phase adjusted to 2 × SSC (SSC : 0.15 M NaCl, 0.015 M Na_3 citrate. 2 H_2O, pH 7.0) with approximately 12 µl 20 × SSC stock. DNA samples prepared in this way could be used directly in dot blot assays.

Dot blot

Aliquots of purified *G. pallida* DNA or crude DNA preparations from either *G. pallida* or *G. rostochiensis* were diluted to 500 µl with 2 × SSC, denatured by boiling for 7 min in a waterbath and cooled on ice.

A 9 × 12 cm sheet of nitrocellulose paper was washed in distilled water, until it was uniformly hydrated, incubated in 20 × SSC for 10 min and then assembled into a 96 well dot-blot microfiltration manifold (Bio-rad). The denatured *Globodera* DNA samples (500 µl) were applied and allowed to filter passively through the nitrocellulose membrane. The filter was removed from the dot blot manifold, air dried and baked for 1 h at 80 °C. At this stage filters could either be used immediately or stored desiccated at 4 °C for up to 6 months.

Incorporation of biotin or radio-label into the *G. pallida* specific DNA probe

A *G. pallida* specific DNA probe (Burrows & Perry, 1988) incorporated into the plasmid cloning vector pUC-9 was labelled by nick-translation with either the nucleotide analogue Biotin-11-dUTP (GIBCO BRL) or [α^{-32}P] dATP (Amersham). Unincorporated radionucleotide was separated from the probe by chromatography through a sephadex G 50 column; however, excess Biotin-11-dUTP was not removed.

Detection of *G. pallida* DNA with a biotinylated DNA probe

Maximum economy of solutions and reagents was achieved by performing the steps of prehybridisation, hybridisation, filter blocking and staining in sealed plastic bags only slightly larger than the filters. Washing the filters required larger volumes of solution and could be carried out satisfactorily in plastic trays.

Nitrocellulose filters or filter strips were placed in a plastic bag containing prehybridisation fluid (50 % V/V

Formamide, 5 × SSPE, [SSPE : 0.18 M NaCl, 0.01 M $NaHPO_3$ pH 8.3, 1 mM EDTA], 5 × Denhardt's (without the BSA), 0.1 % SDS, 100 µg ml^{-1} sheared and denatured salmon sperm DNA : use 100 µl per cm^2 of filter) and incubated at 42 °C for 2-4 h. Prehybridisation solution was replaced with hybridisation fluid (4 parts prehybridisation solution, 1 part 50 % dextran sulphate : use 25 µl per cm^2 of filter). The probe was heat denatured and added to the solution to give a final concentration of 200 µg ml^{-1}. The bag was resealed and incubated overnight with gentle shaking, at 37 °C. After hybridisation the filters were washed for 3 × 1 h in 300 ml 6 × SSC at 65 °C.

Washed filters were blocked using 4 % w/v BSA in incubation buffer (0.1 M Tris·Cl, 0.5 M NaCl pH 9.0 : use 200 µl per cm^2 filter) for 1 h at 40 °C. A preformed complex of avidin and the chromogenic enzyme alkaline phosphatase was then conjugated to the biotin label by incubating the blocked filters with avidin-AP (GIBCO BRL) (avidin-AP 1 µm ml^{-1}, 1 % BSA in incubation buffer : use 40 µl per cm^2 of filter) at 10 °C for 30 min. The filters were washed at room temperature 2 × 20 min in incubation buffer containing 1 % BSA, 1 × 20 min in incubation buffer and finally rinsed briefly in staining buffer (0.1 M Tris·Cl pH 9.5, 0.11 M NaCl, 0.01 M MgCl). Bound alkaline phosphatase was detected by staining the filters in a freshly made solution of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (0.35 mg NBT ml^{-1}, 0.20 mg ml^{-1} BCIP in staining buffer : use 200 µl per cm^2 filter). Colour was allowed to develop in the dark for 5 min to 2 h, but filters may be left to stain for much longer (up to 3 or 4 days) providing background colour does not form on the nitrocellulose. Overdevelopment was the greatest single cause of false positives. Once developed the filters were rinsed in distilled water and allowed to air dry.

Rapid detection of *G. pallida* DNA with a biotinylated probe

Filters were incubated in prehybridisation fluid (50 % Formamide, 5 × SSPE, 5 × Denhardt's, 250 µg ml^{-1} sheared and denatured salmon sperm DNA : use 100 µl per cm^2 filter) for 30 min at 45 °C. Prehybridisation solution was quickly replaced with hybridisation fluid (45 % Formamide 5 × SSPE, 1 × Denhardt's, 250 µg ml^{-1} sheared and denatured salmon sperm, biotin labelled probe 500 µg ml^{-1} : use 25 µl per cm^2 filter) that had been heated at 75 °C for 25 min during prehybridisation and then cooled on ice. Filters were incubated in the hybridisation mix for 30 min at 45 °C and then washed at room temperature with brisk shaking 2 × 3 min in 2 × SSC 0.1 % SDS, 2 × 3 min in 0.2 × SSC 0.1 % SDS, and 2 × 10 min in 0.1 × SSC 0.1 % SDS.

Blocking was performed as in the full protocol except the filters were incubated at 45 °C for 20 min. The avidin-AP complex (avidin-AP 2 µg ml^{-1}, 1 % BSA, in incubation buffer : use 40 µl per cm^2 of filter) was conjugated to the biotin at 10 °C for 20 min and the filters then washed 3 × 10 min in incubation buffer containing 1 % BSA at room temperature. Bound alkaline phosphatase was visualised as before.

Detection of *G. pallida* DNA with a radio-labelled DNA probe

Prehybridisation, hybridisation, washing and autoradiography were carried out as previously reported (Burrows & Perry, 1988) except that autoradiography was performed using two intensifying screens (Rapid X®, Genetic Research Instrumentation Ltd).

Results

Detection of *G. pallida* DNA with a biotinylated DNA probe

The ability of the biotin labelled *G. pallida* specific DNA probe used here to detect the presence of *G. pallida* DNA in dot blot assays was demonstrated (Fig. 1 A). Cross reaction with the morphologically similar species *G. rostochiensis* was not observed and no colour developed over the control dots.

Sensitivity of the full biotin protocol

The detection threshold of *G. pallida* DNA using this technique was expressed in two ways. Results from the first method, based on the weight of *G. pallida* DNA (ng) applied to the filter, are shown in Fig. 1 A. Serial dilutions of purified *G. pallida* DNA demonstrate that the detection level under these conditions was approximately 0.08 ng (80 pg) of *G. pallida* DNA per mm^2 of filter (dot surface area = 7 mm^2). This is only slightly less sensitive in the most dilute samples than the equivalent filter incubated with ^{32}P labelled *G. pallida* probe (Fig. 1 E). However, hybridisation and detection of the radiolabelled probe was not optimised and the sensitivity could have been improved considerably.

The second method of expressing detection threshold was by determining the number of *G. pallida* eggs and J2 needed to detect a positive result : the DNA was prepared from a predetermined number of *G. pallida* (Fig. 1 C) and *G. rostochiensis* (Fig. 1 D) eggs and J2 by the crude DNA extraction method. The full biotin protocol is capable of detecting as little as one *G. pallida* egg or J2 per mm^2 of filter in dot blots (Fig. 1 C). This result indicates that with an appropriate dot-blot manifold capable of delivering the DNA in a dot 1 or 2 mm^2 at the surface of the nitrocellulose a single *G. pallida* J2 could be detected.

Efficient extraction of DNA from one egg or juvenile would not appear to be a problem using the centrifugal

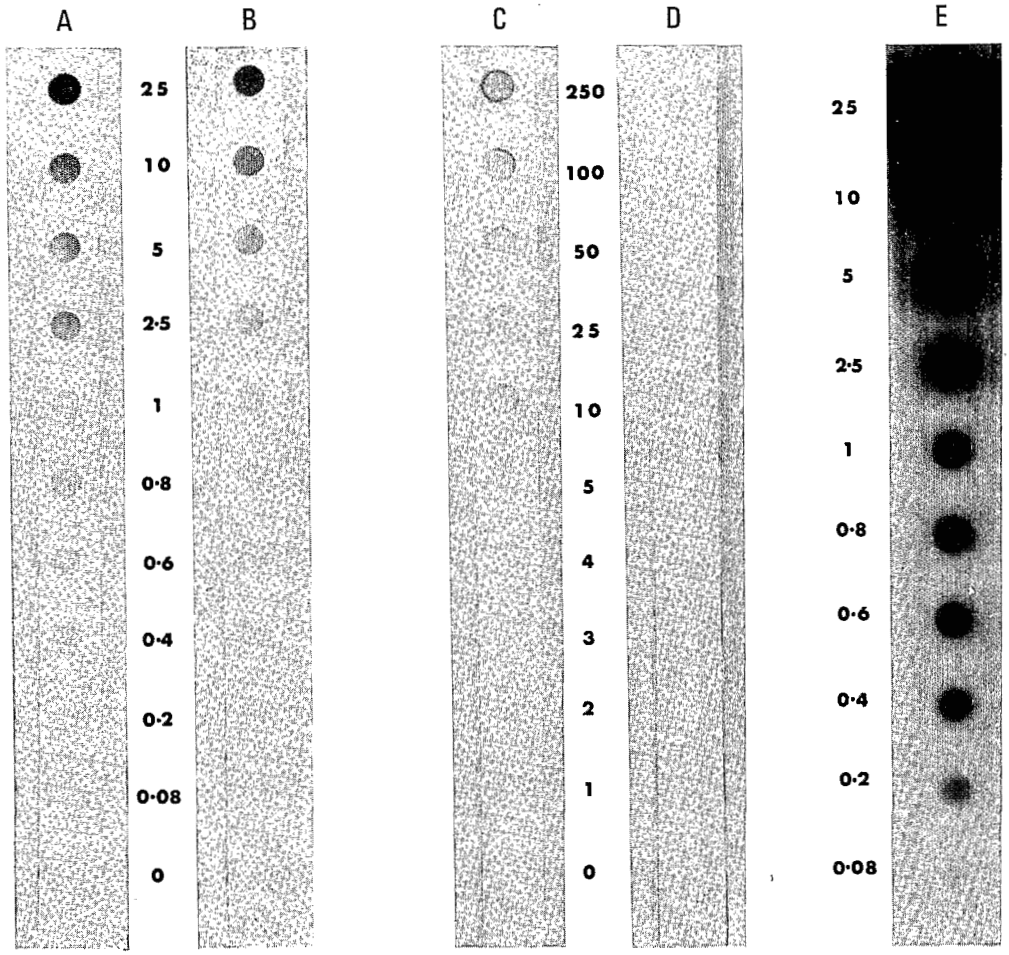

Fig. 1. Dot blots of *G. pallida* and *G. rostochiensis* total DNA. (A) and (B), amount of *G. pallida* DNA (ng) per mm^2 of filter visualised with a biotin labelled *G. pallida* specific DNA probe — A : using the full biotin protocol; B : using the rapid biotin method; C and D : crude DNA preparations from a predetermined number of *G. pallida* or *G. rostochiensis* eggs and J2 visualised using the full biotin protocol. Number of eggs and J2 per mm^2 of filter — C : *G. pallida;* D : *G. rostochiensis;* E : Amount of *G. pallida* DNA (ng) per mm^2 of filter detected with the ^{32}P labelled *G. pallida* specific DNA probe.

micro homogeniser system which is extremely economic with the samples. The *G. rostochiensis* filter (Fig. 1 D) was included to establish the amount of non-specific staining of the sample dots that may be expected when using Biotin with rather impure DNA samples. It demonstrates that with this technique very little non-specific staining occurs.

Rapid biotin method

Fig. 1 B shows a similar filter to Fig. 1 A but the *G. pallida* DNA has been visualised using the rapid biotin method. This result is encouraging as it is only marginally less sensitive than the full protocol. However, slightly more background colour was evident on the nitrocellulose filter which under some circumstances may mask more dilute samples of DNA. When sufficient nematode material is available (three or more *G. pallida* J2) the rapid method may be used with confidence.

Crude DNA preparation

Crude DNA preparation proved to be extremely

useful in this investigation (Fig. 1 C, D). It facilitated the fast and efficient extraction of DNA from a small number of nematodes with minimum handling losses. The single phenol/chloroform step removed enough protein and cellular debris to avoid high levels of non specific staining of the sample dots.

Discussion

The identification of species, host races and pathotypes of plant parasitic nematodes using cloned specific DNA probes is potentially an extremely powerful and versatile technique. DNA based diagnostics has a number of inherent advantages over other methods of characterising nematodes. The developmental stability of DNA means that it is free from influence of host or the environment and this circumvents much of the variation usually associated with morphology. Moreover, unlike many electrophoretic protein profiles and antigenic or enzymatic traits, DNA probes are independent of stage in the nematodes' life cycle.

The separation of two plant parasitic nematodes using a DNA probe coupled to a sensitive non-radioactive detection system is a significant step towards the development of simple diagnostic kits for the identification of agriculturally important nematodes. This work demonstrates the modification and subsequent use of an efficient biotin labelling technique (Hearse, 1984) to differentiate *G. pallida* from the morphologically similar species *G. rostochiensis*. Biotin labelled DNA probes have been used with some success to detect the parasites of man or animals (Barker, 1987), but this is the first application of such an approach to plant parasitic nematodes.

Sensitive probes are an advantage when working with organisms as small as most plant parasitic nematodes, and the great sensitivity of this technique can be explained in part by the highly repetitive nature of the *G. pallida* probe. DNA probes derived from highly repetitive DNA families will have proportionally more target sequences per genome than those directed at single or low copy sequences. This seems to imply that for the sake of adequate detection levels DNA probes must be limited to moderately or highly repetitive DNA sequences. Although this is not necessarily a disadvantage, the emerging technology of the polymerase chain reaction (PCR) (Mullis & Faloona, 1987) now offers an exciting and potentially extremely simple way to amplify a specific low copy DNA sequence in complex mixtures such as unfractionated genomic DNA. PCR is a three step reaction that may be cycled *in vitro* by sequentially raising and lowering the incubation temperature. Each cycle takes approximately eight minutes and doubles the number of targetted sequence. As more cycles are performed the number of copies increases exponentially. PCR raises the possibility of detecting minute quantities of nematode DNA in whole host tissue or root system preparations. This is sure to find wider application beyond diagnostics.

Further improvements to the detection of nematode DNA with biotinylated DNA probes may be achieved by using avidin complexed with a polymer of alkaline phosphatase. This simple modification is being investigated to facilitate the attachment of many more chromogenic groups per probe. It has been used with some success to detect as little as 1 pg of target DNA immobilised on nitrocellulose (Leary, Brigati & Ward, 1983).

The hydrolysis of BCIP in the presence of NBT by alkaline phosphatase yields a fine grained blue pigment that is highly insoluble in aqueous and organic solvents. Although this provides a sensitive means of visualising biotinylated probes the blue colour is effectively permanent and prevents reprobing suitable filters with different probes. Other enzymes or substrate systems are available that give more soluble products but these tend to be less sensitive. A sensitive enzyme/substrate combination that allows repeated probing of a single DNA blot would be a valuable addition to the DNA taxonomists' armoury.

Non-specific staining is a common problem when biotinylated probes are used to detect crude DNA samples. The rapid and reproducible visualisation of impure *G. pallida* DNA without detrimental levels of background demonstrates the value of this method. Furthermore, treatment of the filters with proteinase K to remove much of the protein that is responsible for the non-specific staining of samples may lead to the development of effective and rapid squash blotting techniques for root knot nematode (*Meloidogyne* spp.) and cyst nematode females.

Thus, the use of molecular biology to identify plant parasitic nematodes is only just beginning. Specific DNA hybridisation probes that exploit differences in DNA sequences between nematode species, races and pathotypes avoid many of the variations associated with phenotype. Biotinylated DNA probes offer a cheap, non toxic and stable alternative to ^{32}P labelling. For diagnostic purposes the sensitivity is sufficient to reveal repetitive sequences in crude DNA preparations equivalent to one plant parasite nematode. This level of detection is particularly important for statutory control and quarantine regulations where the presence of even a single nematode is significant. Future developments such as PCR, enzyme polymers and substrate systems that allow multiple probing of single blots will extend the application of non-radioactive detection systems.

This work is an important step towards the production of diagnostic kits for agriculturally important plant parasitic nematodes. Such kits will use small amounts of nematode material, give a reliable result within a few hours and require little operator skill or knowledge of nematode morphology.

REFERENCES

BARKER, D. C. (1987). DNA diagnosis of human Leishmaniasis. *Parasitol. Today,* 3 : 177-184.

BARKER, D. C., GIBSON, L. J., DENNEDY, W. P. K., NASSER, A. A. A. A. & WILLIAMS, R. H. (1986). The potential of using recombinant DNA species specific probes for the identification of tropical Leishmania. *Parasitology,* 91 : 5139-5174.

BURROWS, P. R. (1988). The use of nucleic acid probes to identify plant parasitic nematodes. *Proc. Brighton Crop Protect. Conf., Pests & Dis., Brit. Crop Protect. Counc., Nov. 1988,* 2 : 811-820.

BURROWS, P. R. & BOFFEY, S. A. (1986). A technique for the extraction and restriction of total DNA from *Globodera rostochiensis* and *G. pallida* second stage juveniles. *Revue Nématol.,* 9 : 199-200.

BURROWS, P. R. & PERRY, R. N. (1988). Two cloned DNA fragments which differentiate *Globodera pallida* from *G. rostochiensis. Revue Nématol.,* 11 : 441-445.

CURRAN, J., BAILLIE, D. L. & WEBSTER, J. M. (1985). Use of genomic DNA restriction fragment length differences to identify nematode species. *Parasitology,* 90 : 137-144.

GILLAM, I. C. (1987). Non radioactive probes for specific DNA sequences. *Trends Biotech,* 5 : 332-334.

Accepté pour publication le 11 août 1989.

GREGERSEN, N., KOCH, J., KOLVRAA, S., PETERSEN, K. B. & BOLUND, L. (1987). Improved methods for the detection of unique sequences in southern blots of mammalian DNA by non-radioactive biotinylated DNA hybridisation probes. *Clinica Chimica Acta,* 169 : 267-280.

HEARSE, J. D. (1984). Microbiopsy metabolite and paired flow analysis : a new rapid procedure for homogenisation, extraction and analysis of high phosphates and other intermediates without errors form tissue loss. *Cardiovascular Res.,* 18 : 384-390.

LEARY, J. J., BRIGATI, D. J. & WARD, D. C. (1983). Rapid and sensitive colourimetric method for visualising biotin labelled DNA probes hybridised to DNA or RNA immobilised on nitrocellulose : Bio-blots. *Proc. natn. Acad. Sci. USA,* 80 : 4045-4049.

MULLIS, K. B. & FALOONA, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Meth. Enzymol.,* 155 : 335-350.

OLE-MOIYIO, O. K. (1987). Trypanosome species-specific DNA probes to detect infection in Tsetse flies. *Parasitol. Today,* 3 : 371-374.

ROLLINSON, D., WALKER, T. K. & SIMPSON, A. J. G. (1986). The application of recombinant DNA technology to problems of helminth identification. *Parasitology,* 91 : 553-671.