Molecular detection of parasitic protozoa

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SUMMARY

The development of molecular diagnostic methods, particularly those utilizing PCR for the detection of parasitic protozoa will contribute greatly to the identification and control of these pathogens, by increasing speed of diagnosis, specificity and sensitivity, reproducibility and ease of interpretation. PCR methods are not without their problems however, and there is a need for laboratory procedures to be refined before PCR-based assays are accepted as the tools of choice for the routine detection of protozoan parasites. The application of PCR detection to various parasites is discussed.

Key words: Detection, parasites, protozoa, PCR, microscopy, immunodetection.

INTRODUCTION

While microscopy remains the 'gold' standard for the diagnosis of many parasites, it is labour-intensive and requires well-trained microscopists for accurate identification and interpretation, particularly for parasites that are morphologically similar, very small in size or present in very low numbers (Singh, 1997; Table 1). In addition, the diagnostic skills of microscopists can vary greatly from laboratory to laboratory resulting in some infections being misdiagnosed or missed completely.

The *in vitro* cultivation of infectious agents is costly and often very slow, and many protozoan parasites cannot be easily cultured (see Table 1). Immunodiagnostic tests have been developed which offer improvements over microscopy for the detection of protozoan pathogens, but these can be problematic due to cross-reactions with other organisms and inconsistencies in interpretation between laboratories (see Table 1). Some of the limitations of conventional diagnostic detection methods and the advantages of utilizing molecular procedures for the detection of protozoan parasites are summarized in Table 1.

These limitations are well illustrated by considering *Cryptosporidium parvum* which is increasingly recognized as an important cause of diarrhoeal illness in livestock and humans (Fayer, Speer & Dubey, 1997). The parasite cannot be amplified *in vitro* and current laboratory methods for the diagnosis of cryptosporidial infections generally rely on microscopic examination of faecal samples for the presence of *Cryptosporidium* oocysts. However, microscopy is time-consuming, insensitive and requires highly skilled microscopists. The sensitivity of conventional detection methods has been reported to be as low as 50–500000 oocysts per gram of faeces (Weber *et al*. 1991). A number of immunodiagnostic methods have been developed for the detection of *Cryptosporidium*. However, antigenic variability between clinical isolates of *Cryptosporidium* (Griffin *et al*. 1992) can result in some infections remaining undetected and there are conflicting reports as to the sensitivity of immunodetection methods over microscopy (Alles *et al*. 1995; Kehl, Cicirello & Havens, 1995). Flow cytometry coupled with cell sorting methods have been developed and improved in recent years (Vessey *et al*. 1994; Hoffman *et al*. 1997) and have recently been applied to the detection of oocysts in human stool samples (Valdez, *et al*. 1997). However, such procedures are costly, and as flow cytometry relies on the use of fluorescently labelled antibodies, it is still subject to the same problems that apply to immunological detection.

In order to reduce the morbidity caused by protozoan infections, rapid diagnosis is required so that appropriate treatment can be given (CDC, 1995; NSTC, 1995; WHO, 1996). Investigations undertaken during diarrhoeal outbreaks have frequently used limited diagnostic testing that have tended to incriminate agents that are easily identifiable in standard microbiological laboratories, while possibly overlooking other more difficult to detect agents (Thompson, 1994). For example, in 1993, the United States suffered the biggest outbreak of diarrhoea in its history with more than 400000 people in Milwaukee affected with watery diarrhoea due to *Cryptosporidium* infection (WHO, 1996). The outbreak in Milwaukee was initially diagnosed as a viral gastroenteritis, and it was only through careful analysis by the Centres for Disease Control (CDC) in Atlanta that *Cryptosporidium* was diagnosed as the cause of the diarrhoeal outbreak. It is likely that the real cause of such outbreaks are misdiagnosed or go unnoticed (D. Juranek, personal communication; in Morgan & Thompson, 1998).

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Table 1. Diagnosis of parasitic protozoa

Molecular (PCR)	Culture	Immunodiagnosis	Microscopy
Very rapid (cf culture)	Too slow (24 hrs → 1 month); require growth under specific conditions/special media	Rapid	Rapid
Greatest sensitivity and specificity	High cost	Specificity and sensitivity often not as good as DNA. Cross-reactions and inability to detect certain antigenic variants are common problems	Simple
Results easy to interpret and quantitate	Many organisms/strains cannot be cultured	Interpretation difficult due to background interference; inconsistency between laboratories and lack of reproducibility	Lacks sensitivity
Can detect organism before antibodies or antigens produced	Usually need specialist laboratories; risk of infection may require containment facilities	False positives common	Low cost but *labour intensive*
Can detect organisms in latent infections		Quantitation often difficult to perform	Does not differentiate between morphologically similar species/intraspecific variants
Can detect intraspecific differences		Serology cannot differentiate between current and previous infections	Organisms may not be resolved due to small size, and/or low numbers
Can identify organisms that can not be cultured		Identifying current infections may be difficult in individuals in endemic areas	Lack of specific stains
Avoids false positives (major limitation of antibody tests)		High cost of purified reagents	
Not only identification but also characterization of particular isolates, e.g. pathogenicity; sensitivity to drugs etc.		Kits available; easy to use and have a good shelf life	
Need to improve reproducibility			
Can detect organisms in tissue and vectors			
Recent progress in automating and multiple sampling; enhanced cost effectiveness			
More PCR kits coming on to market			

The control and prevention of many protozoan infections also depends upon knowledge of how the aetiological agents survive and are transmitted in different environments. Such information would contribute to the development of effective prevention and control measures but is incomplete for the aetiological agents of many protozoan diseases. Current prevalence data are often lacking or inaccurate and may, in part, be due to variation in diagnostic protocols between diagnostic laboratories. Furthermore, information that is available has usually been collected during outbreak investigations and may not be reflective of conditions during non-outbreak periods. Future studies need to be coupled with the newer, more sensitive diagnostic techniques, that can provide an accurate picture of prevalence in asymptomatic and symptomatic individuals and in non-outbreak as well as outbreak situations. The development of more sensitive assays could also contribute greatly to our understanding of the role of environmental reservoirs such as water and fomites as sources of protozoan infections. Such information is clearly essential to the development of effective prevention and control measures.

New technologies

A number of new technologies such as the electrorotation assay (ERA) and fluorescent-*in-situ* hybridization (FISH) have been developed in recent years which may assist in the diagnosis of protozoan parasites in the future. ERA involves the binding of micro-organisms to antibody-coated particles which are then spun in a rotating electric field. Different micro-organisms have characteristic rotations which can be detected using image analysis technology (Newman, 1995). The method also reportedly differentiates between live and dead organisms, as at low frequencies, viable specimens rotate in the opposite direction to the applied field. Dead protozoans rotate at higher frequencies, but spin in the same direction as the field (Beardsley, 1993; see Goater and Pethig, 1998, this supplement). The technology is currently being miniaturised to create a desktop tester with the area of a Pentium chip and a few millimetres high. Aspects of the chip are currently being tested by Genera Technology, however a marketable item is still a number of years away (Ward, 1997). In addition, this technique still

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suffers from the problems of lack of specificity and cross-reactivity that affect all antibody-based techniques.

FISH is a technique in which fluorescently labelled oligonucleotide probes are hybridized to ribosomal RNA within cells and detected using fluorescent microscopy or confocal-laser scanning microscopy. Depending on the discriminatory power of the target sequence, the technique can identify pathogens to species level and the simultaneous detection of multiple pathogens is possible by using probes labelled with different fluorescent markers (Rochelle, 1995).

Recently, a FISH technique has been developed for the fluorescent labelling of *Cryptosporidium parvum* oocysts (Vesey *et al*. 1998). However, due to background fluorescence problems, immunofluorescent staining was required in addition to FISH in order to detect *C. parvum* oocysts in water samples (Vesey *et al*. 1998).

THE POLYMERASE CHAIN REACTION (PCR)

PCR has provided the basis for the development of a new generation of diagnostics. Sensitivity, ease of use and the ability to analyse large numbers of samples simultaneously make PCR an attractive option for the detection of parasitic protozoa and it is increasingly being used as a diagnostic and epidemiological tool in veterinary and human parasitology. The development of diagnostic assays based on PCR have the added advantage of being able to detect infectious agents directly in clinical or environmental samples without the need to produce large quantities of the agent by *in vitro* or *in vivo* laboratory amplification.

Development of diagnostic PCR primers for protozoan parasites

Diagnostic PCR primers can be developed using a number of different approaches. Sequence information for the 18S rDNA gene is now available for a large number of protozoa and can be retrieved from the rRNA WWW server (http://rrna.uia.ac.be/) (van de Peer *et al*. 1994). Diagnostic primers can easily be designed from this sequence information, however, due to the conserved nature of the 18S gene, the problem of cross-reactions with other organisms can occur. An alternative method is *via* the construction and screening of genomic DNA libraries. However, this method is expensive and time-consuming and requires relatively large amounts of DNA which can be difficult to generate when working with protozoan parasites. Use of the Random Amplified Polymorphic DNA (RAPD) technique allows a much simpler approach to be taken. RAPD or arbitrarily primed PCR (AP-PCR) was independently developed by Williams *et al*. (1990) and Welsh & McClelland (1990). This procedure detects nucleotide sequence polymorphisms in a PCR-assay without the need for previously determined nucleic acid sequence information (see Thompson *et al*. this supplement). As it is PCR-based, it requires only small amounts of material and is therefore suitable for protozoan parasites. Many of the products generated by RAPD-PCR are derived from repetitive DNA sequences (Williams *et al*. 1990). As these sequences are frequently species-specific (Klein-Lankhorst *et al*. 1991), RAPD-PCR is potentially a quick method for developing species-specific diagnostic PCR primers and probes (Crowhurst *et al*. 1991). Diagnostic RAPD bands can be eluted from a gel, reamplified, their specificity tested by hybridization analysis, then subcloned and sequenced; diagnostic PCR primers can subsequently be designed and synthesized from the RAPD sequence (Fig. 1). As this approach circumvents the need for the construction and screening of genomic libraries, RAPD-PCR offers a simple and rapid alternative to conventional techniques and has successfully been applied to the development of diagnostic PCR primers for a number of protozoan parasites (Cere *et al*. 1996; Morgan *et al*. 1996).

Quantitation and automation of PCR-based diagnosis

It is important, particularly for environmental laboratories, to be able to detect pathogens reliably as well as determine the numbers of pathogens present. Quantitative and semi-quantitative techniques have been developed for a number of protozoan parasites (Bretagne *et al*. 1995; Hulier *et al*. 1996; Jenkins, Trout & Fayer, 1997) and new technologies such as the Taqman™ LS-50B PCR detection system (Perkin-Elmer) will assist in the quantitation of protozoan pathogens. PCR is particularly amenable to automation and large throughput processing, and the use of robotic arms and 96 well formats will greatly speed up the detection process in the future.

Problems to be overcome

PCR is not without its problems. Widely recognized limitations of amplification technology include those of false-positive and false-negative results and the difficulty of detecting routinely the wide range of possible pathogens contained in a clinical sample (Vaneechoutte & Vaneldere, 1997).

However, false-positive and false-negative results can be kept to a minimum by adhering to standard procedures for avoiding contamination (Kwok & Higuchi, 1989). In addition, the use of hot-start PCR (Horton, Hoppe & Contitronconi, 1994), can overcome many of these problems. In conventional PCR, non-specific annealing can occur at low

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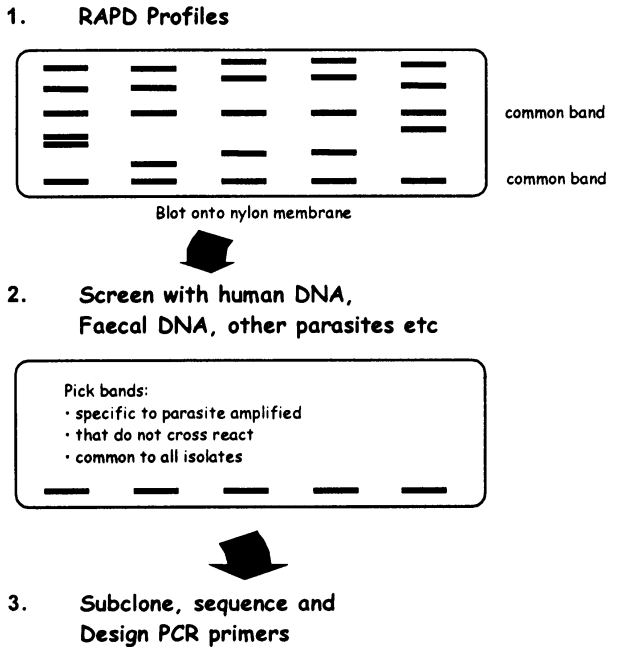

Fig. 1. Description of a method for developing diagnostic PCR primers for parasites using RAPD analysis.

temperatures resulting in aberrant product formation and primer dimer. If these non-specific products are formed early in the PCR-cycle, they will be amplified through the remaining steps. These products reduce specificity by decreasing the amplification of the target signal and compromise sensitivity by causing high background. A thermolabile agent, originally wax or grease, but now usually an antipolymerase antibody, holds the enzyme in an inactive state until the temperature reaches 95 °C, circumventing the amplification of non-specific products and improving the sensitivity and specificity of the PCR. This often results in less starting material being required and partially degraded samples are more likely to be amplified (Barlow & Ewen, 1998). In addition, contamination resulting from previous amplifications can be minimized by the incorporation of Uracil DNA Glycosylase (UNG) for the pre-amplification sterilization of PCR products (Longo, Berninger & Hartley, 1990).

Faecal samples are among the most difficult clinical specimens for DNA extraction and amplification. In order for PCR to be routinely accepted as a diagnostic tool, these problems need to be overcome, and a reliable, fast, standardized method developed for extraction of PCR-amplifiable nucleic acids. Faecal constituents such as bilirubin and bile salts were thought to be the main inhibitors (Widjojoatmodjo *et al*. 1992). However, one group have recently characterized faecal components and identified complex polysaccharides as the key inhibitors (Monteiro *et al*. 1997). Glassmilk extractions on faecal samples did not completely remove inhibition, however, research in our laboratory has shown that boiling faecal samples in 10% polyvinylpolypyrrilidone (PVPP) prior to glassmilk extraction greatly reduced inhibition (Morgan *et al*. 1998*a*). The inclusion of a cationic surfactant Catrimox-14 (TM) (Iowa Biotechnology, Iowa) during extraction of both DNA and RNA has also been shown to eliminate inhibitory substances from faecal samples (Uwatoko *et al*. 1996).

APPLICATIONS

In the following examples, we have tried to illustrate the value and practical potential of applying molecular techniques, principally those based on PCR, for the detection of protozoan parasites. Space constraints have forced us to be selective but we have tried to illustrate the diversity of parasitic protozoan infections to which PCR has been applied, in terms of life cycle and transmission.

Environmental detection of Cryptosporidium

The Milwaukee cryptosporidiosis outbreak coupled with other outbreaks such as the Las Vegas epidemic in 1994 have raised questions about the prevalence and regulation of *Cryptosporidium* in source and drinking water (States *et al*. 1997). In July 1997, water authorities in the US serving more than 10000 began an 18 month monitoring programme for *Cryptosporidium* under the Information Collection Rule (ICR). The currently recommended method for detecting *Cryptosporidium* oocysts in water (Anon, 1993) involves membrane filtration of water samples via yarn-wound polypropylene cartridge filters that retain oocysts. Eluted oocysts are concentrated and purified using Percoll gradients and detected using an immunofluorescence assay (IFA). However, recoveries vary widely (0–80+%) using this technique and it often results in erroneous data, with either false-positives or false-negatives being recorded (Pontius, 1998). A US EPA co-ordinated performance evaluation in January 1995 involving nine 'expert' laboratories yielded recoveries ranging from 0 to 13% (Newman, 1995). Inaccurate assessments will have serious consequences for the water industry if they are used to develop regulatory treatment criteria as implied by the ICR (Pontius 1998).

In order to achieve the acceptable risk defined by the US EPA (one infection per 10000 people), concentrations of *Cryptosporidium* oocysts would have to be $< 0{\cdot}005/100$ L (Haas & Rose, 1994). Highly sensitive PCR-based diagnostics are therefore required. A variety of PCR tests have been developed for the detection of *Cryptosporidium* in both clinical and environmental samples (Morgan & Thompson, 1998). In water samples, sensitive detection of *Cryptosporidium* oocysts has been

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achieved (Johnson *et al*. 1995), down to a single oocyst (Stinear *et al*. 1996). Environmental samples are very inhibitory to PCR however, and it has been reported that the sensitivity of the PCR assay was reduced by as much as 100–1000 fold for oocyst seeded environmental samples compared to purified oocysts (Johnson *et al*. 1995). Although the use of immunomagnetic separation removes this inhibition (Johnson *et al*. 1995; Deng, Cliver & Mariam, 1997), by coupling PCR with immunodetection methods one encounters all the problems associated with antibody detection. Research investigating parameters affecting PCR detection of waterborne *Cryptosporidium* indicates that separation of inhibitory solutes might be sufficient to allow PCR detection of waterborne oocysts for routine application, but this approach will need to be tested on large volumes of water (Sluter, Tzipori & Widmer, 1997).

Recent research has demonstrated that humans are susceptible to infection with at least 2 distinct, apparently host-adapted genotypes of *Cryptosporidium* ('human' and 'cattle') (Awad-El-Kariem *et al*. 1995; Vasquez *et al*. 1996; Carraway *et al*. 1997; Morgan *et al*. 1995; 1997; 1998*a*, *b*; Peng *et al*. 1997; Spano *et al*. 1997; 1998). Increasing population density and land use for animal production enhances the risk of water contamination by oocysts originating from domestic septic tanks and dairy farms. In the event of water contamination, preventative measures to reduce the chance of future spills can be implemented if the source of oocysts can be traced (Widmer, Carraway & Tzipori, 1996). Diagnostic tests which can differentiate between human and animal isolates of *Cryptosporidium* will therefore be of particular benefit in outbreak situations such as the more recent outbreak in British Columbia (Anon, 1996), where determination of the source of infection is important in limiting transmission. Diagnostic primers developed by Morgan *et al*. (1997), which directly differentiate between human and bovine isolates of *C. parvum* on the basis of the size of the PCR product, will assist greatly in understanding the contribution of animal reservoirs and domestic septic tanks to waterborne infection.

Clinical detection of Cryptosporidium

PCR testing is increasingly being applied to clinical detection of *Cryptosporidium* and PCR has been shown to be more sensitive and accurate than immunoassays for the detection of *Cryptosporidium* in faeces (Leng, Mosier & Oberst, 1996). Comparison of PCR with microscopic detection of *Cryptosporidium* in human faecal samples revealed a sensitivity and specificity of 83·7% and 98·9% for microscopy compared to 100% sensitivity and specificity for PCR (Morgan *et al*. 1998*a*). Approximately 17% of the positives detected using the PCR test displayed the 'cattle' genotype, the remaining positives displayed the 'human' genotype (Morgan *et al*. 1998*a*).

In outbreaks of cryptosporidiosis such as those reported from North America, as well as more localized ones in day-care centres, it is likely that the real cause is misdiagnosed or goes unnoticed. Using PCR-based assays provides public health personnel with the most appropriate diagnostic tools in such situations. In addition, as with other enteric protozoan infections such as *Giardia*, high percentages of asymptomatic carriers of *Cryptosporidium* have been reported, particularly in children (Cordell & Addiss, 1994). The role of asymptomatic carriage of *Cryptosporidium* in the spread of infection is not clear, although recent studies suggest it is common (Pettoello-Mantovani *et al*. 1995). Surveillance of high risk population groups such as AIDS patients and children attending day-care using sensitive diagnostic techniques such as provided by PCR, will enable treatment regimes to be instituted early in the infection process thus limiting clinical sequelae and in the case of asymptomatic children, treatment will limit the development of symptomatic infection and its spread.

Entamoeba

Amoebiasis is caused by 2 distinct species, a pathogenic form (*Entamoeba histolytica*) and a non-pathogenic form (*E. dispar*) which are morphologically identical. Although the distinction between these 2 species is of great clinical importance, conventional methods developed for this purpose are time-consuming and labour intensive. A number of PCR tests for the detection of *E. histolytica* have been developed in recent years (Tachibana *et al*. 1992; Acuna-Soto *et al*. 1993). Simple differential detection of *E. histolytica* and *E. dispar* in fresh stool specimens by sodium acetate/acetic acid/formalin concentration and PCR, which can also detect mixed infections even at a 1:10000 ratio, has recently been reported (Troll, Marti & Weiss, 1997). Comparison of PCR and 3 commercially available enzyme-linked immunosorbent assay-based kits (ELISAs) for the detection of *Entamoeba* revealed that 2 of the 3 ELISAs cross-reacted with *E. dispar* and were 100 times less sensitive than PCR (Mirelman, Nuchamowitz & Stilarsky, 1997). Sensitivity, and the ability to discriminate between pathogenic and non-pathogenic species is a powerful advantage of the PCR technique for diagnosing *Entamoeba* infections.

Plasmodium

Four species of *Plasmodium* cause malaria in man: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Conventional diagnosis of malarial infections relies

on laborious microscopic examination of thin and thick blood smears after staining with Giemsa, Firld's stain or Diff Quick (Smits & Hartskeerl, 1995). Newly developed tests such as the QBC method for the fluorescent staining of parasites after an enrichment step of the infected erythrocytes, and the ParaSight®F test for antigen detection by a dipstick are available. However, when parasitaemias are low, particularly in patients with cerebral malaria, it is difficult to detect parasites, and where there is a mixed infection and one species dominates, microscopy fails to detect all the species of parasites present (Singh, 1997). The correct, specific identification of *Plasmodium* is very important, as the choice of antimalarials prescribed depends on the species of malaria parasite.

The development of PCR-based diagnostics for *Plasmodium* spp. has enabled the specific, sensitive and rapid detection of malaria. The majority of PCR tests have been developed for falciparum malaria, although a generic PCR test capable of detecting all *Plasmodium* species has also been developed (Ayyanathan & Datta, 1996). A recent field study in which a total of 410 blood samples from patients in Thailand was analysed, confirmed the superior sensitivity of PCR over conventional diagnosis. Blood samples were analysed by PCR and microscopy for the presence of *P. falciparum*; 53% were positive using the DNA-based assay, while only 32% were positive using conventional microscopic analysis (Seesod *et al*. 1997). A blinded evaluation of the ParaSight®F antigen capture assay (dipstick test) and PCR for the detection of *P. falciparum* infection carried out on 151 febrile travellers revealed that compared with the PCR, the dipstick test had a sensitivity of 88% and a specificity of 97% (Humar *et al*. 1997). The superior sensitivity of the PCR technique was utilized to screen for levels of *P. falciparum* infections that were below the threshold of detection of blood film examination (Roper *et al*. 1996). The results revealed a substantial group of asymptomatic, sub-microscopically patent infections within the population of a Sudanese village present throughout the year, although clinical malaria episodes were almost entirely confined to the transmission season (Roper *et al*. 1996). Reservoir parasite populations were therefore higher than was previously believed in this region, an important finding for understanding the epidemiology of this disease. PCR-RFLP analysis of the dihydrofolate reductase (dhfr) gene has been used to detect pyrimethamine susceptibility of *P. falciparum* in Thailand (Zindrou *et al*. 1996). This method could therefore be used to study the epidemiology of pyrimethamine resistance in *P. falciparum*. In order to study malaria in all geographical locations it is important that specimen collection and DNA extraction for PCR be kept simple. A method for extracting DNA from dried blood spots on filter paper which is capable of detecting one *P. falciparum* and two *P. vivax* parasites/μl of whole blood by nested PCR without compromizing the simplicity of specimen collection or DNA extraction has been reported (Cox-Singh *et al*. 1997).

PCR genotyping also has practical potential for distinguishing a recrudescent from a new infection when treatment studies are conducted in areas with active malaria transmission (Ohrt *et al*. 1997). However, it is advisable not to use a single primer pair in epidemiological field studies for the detection of falciparum malaria since the sensitivity of the PCR can vary between different geographic areas (Jelinek *et al*. 1996).

Tritrichomonas

Tritrichomonas foetus is a sexually transmitted protozoan parasite which causes infertility and spontaneous abortion in cattle (Levine, 1973), and on recent molecular evidence appears to be identical to the intestinal trichomonad of pigs, *T. suis* (Felleisen, 1998). Effective control of trichomoniasis in cattle has been impeded by the insensitivity of traditional diagnostic procedures, which require the isolation and cultivation of the parasite (Ho *et al*. 1994). Diagnosis is also hampered by putative contamination of samples with intestinal or coprophilic trichomonad protozoa which might be mistaken for *T. foetus* (Felleisen *et al*. 1998). An immunohistochemical staining technique has been developed for the detection of *T. foetus* which is relatively specific but which did cross-react with *Trichomonas gallinae* (Rhyan *et al*. 1995). An antigen-detecting enzyme immunoassay (EIA) has also been developed for *T. foetus* (Yule *et al*. 1989). However, while highly sensitive for the detection of antigen derived from cultured organisms, the assay showed poor sensitivity in the detection of antigen in the cervicovaginal mucus of artificially infected heifers, with only 75% of culture-positive samples being considered positive for antigen. In a direct comparison, 23/122 samples from a naturally infected dairy herd gave positive cultures, while only 10/122 samples were considered antigen positive by EIA (Yule *et al*. 1989).

A PCR test for *T. foetus* was developed by Ho *et al*. (1994), which could detect as few as one *T. foetus* organism in culture media or 10 parasites in bovine preputial washings. Analysis of 52 clinical samples showed that 47 (90·4%) of the 52 samples were correctly identified, with no false-positive reactions. In comparison, the traditional cultivation method detected 44 (84·6%) of the 52 samples from *T. foetus*-infected and uninfected bulls. However, hybridization of a chemiluminscent internal *T. foetus* sequence to the amplification product was necessary in order to achieve this sensitivity which adds greatly to the length and cost of the assay. More recently, a

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diagnostic PCR test has recently been developed based on rRNA gene units of *T. foetus* which can directly detect a single organism in diagnostic culture medium or about 50 parasites per ml of preputial washing fluid (Felleisen *et al*. 1998). Documentation and interpretation of results was facilitated by including a DNA enzyme immunoassay for the detection of amplification products which obviated the need for gel electrophoresis. These results indicate that the PCR-based amplification system could be a useful alternative method for the diagnosis of bovine trichomoniasis.

Toxoplasma/Neospora

Neospora caninum is recognized as a frequent cause of foetal abortion and neonatal mortality in cattle, goats and horses as well as causing hind limb paralysis in dogs (Dubey, 1992; Dubey & Lindsay, 1993). It is therefore responsible for significant economic and reproductive losses to the livestock industry. Morphological similarities and serological cross-reactivity between *N. caninum* and the very closely related parasite *Toxoplasma gondii*, have resulted in the frequent misdiagnosis of neosporosis as toxoplasmosis (Ellis *et al*. 1994; Lally, Jenkins & Dubey, 1996). Slight differences in the structure of the tachyzoite organelles known as rhoptries have been detected and these now provide a method for distinguishing *N. caninum* from *T. gondii*, by electron microscopy (Dubey, 1992; Dubey & Lindsay, 1993). In the last few years, diagnosis of neosporosis and toxoplasmosis has been much improved by the development of PCR tests which allow fast and sensitive identification of the parasites (Lally, Jenkins & Dubey, 1996; Yamage, Flechner & Gottstein, 1996; Muller *et al*. 1996). Lally, Jenkins & Dubey (1996), developed a nested PCR reaction based on a *N. caninum* cDNA clone encoding a 14-3-3 protein homologue. The 14-3-3 proteins are a class of proteins which show a high degree of amino acid sequence conservation across several eukaryotic taxa. Using less conserved regions of the *N. caninum* cDNA clone, nested primers were designed for the amplification of a 614-bp *N. caninum* DNA fragment by PCR. The DNA fragment was amplified from *N. caninum* genomic DNA, but not from *T. gondii*, *Sarcocystis muris*, *S. tenella*, or *S. cruzi* genomic DNA. Additionally, the fragment was amplified from DNA prepared from the brains of *N. caninum*-infected mice, but not from the brain of a mouse infected with *T. gondii*. These results suggest that this PCR assay may be useful for the diagnosis of neosporosis.

Trypanosomes

Trypanosomes are the causative agents of major parasitic diseases such as Chagas' disease in South America and sleeping sickness of humans and nagana disease of cattle in Africa. They are transmitted to mammalian hosts by specific insect vectors (Vanhamme & Pays, 1995). Parasitological diagnosis is often difficult because of the low numbers of blood-stream trypanosomes present (Chance & Molyneux, 1995). Numerous PCR tests have been developed in recent years for the detection of trypanosomes (Katakura *et al*. 1997; Masake *et al*. 1997), some of which do not even require DNA purification and with a sensitivity threshold of 1 parasite in 1 ml of blood (Penchenier *et al*. 1996).

PCR diagnosis has also been used to determine the prevalence of various species and subgroups of trypanosomes in their intermediate host, the tsetse fly. For example, in the Sinfra area of Côte d'Ivoire a PCR assay was able to detect mixed infections and facilitated the easy identification of mature trypanosome infections in tsetse flies, providing a reliable estimation of trypanosomiasis challenge (Masiga *et al*. 1996).

Chagas' disease is caused by *Trypanosoma cruzi*, which is predominantly found in South and Central America and Mexico. Chagas' disease has a variable clinical course, ranging from symptomless infection to severe chronic disease with cardiovascular or gastrointestinal involvement or even overwhelming acute episodes (Macedo & Pena, 1998). In the acute phase of Chagas' disease, when the parasitaemia is high, diagnosis can be easily made using conventional parasitological methods. During the chronic phase, due to the low parasitaemia, diagnosis is performed mainly by immunological methods. Conventional serological techniques however, are limited by cross-reactivity with other parasitic diseases, non-standardization of reagents, and the diversity of technical procedures (Gomes, 1997). Methods are being developed to improve the sensitivity and specificity of diagnosis using molecular approaches and PCR tests have been compared with conventional microscopy for the detection of *T. cruzi* (Kirchhoff *et al*. 1996). In this study, eight mice were challenged with *T. cruzi*, and on 31 days over a 380-day period, the ability of PCR to detect parasites in blood was compared with oil immersion microscopic examination. During the acute phase of the infections, parasites were detected on average 3·9 days earlier by the PCR method than by microscopy. Furthermore, the infected mice were consistently positive by PCR during the chronic phase, while parasites were intermittently detected by microscopic examination during that period (Kirchhoff *et al*. 1996). PCR detection of *T. cruzi* has also been applied in epidemiological studies (Silber *et al*. 1997; Wincker *et al*. 1997), but improvements are required to increase sensitivity.

The most serious manifestation of chronic Chagas' disease is a progressive inflammatory cardiomyopathy (Lane *et al*. 1997). However, *T. cruzi* has

not been consistently demonstrated with histological techniques in inflammatory cardiac lesions. A recent study used both PCR amplification of extracted DNA from haematoxylin and eosin-stained tissue scrapings, and *in situ* hybridization to detect the presence of *T. cruzi* in infected murine cardiac tissue sections (Lane *et al*. 1997). Three *T. cruzi*-specific DNA sequences were used: a 122-basepair (bp) sequence localized within the minicircle network (MCS), a 188-bp nuclear repetitive sequence (RS), and a 177-bp sequence within the open reading frame of a gene coding for a flagellar protein (FPS). All 3 sequences were amplifiable from scrapings of murine cardiac tissue. The MCS and RS were detected at 0·167 and 0·24 amastigote DNA equivalents, while FPS was barely detected at 0·24 amastigote DNA equivalents. On the other hand, *in situ* hybridization with all three sequences allowed for the detection of *T. cruzi* amastigotes within the tissue. The MCS and FPS, however, consistently yielded a more intense signal. These results indicate that PCR and *in situ* hybridization may prove useful in establishing the prevalence of *T. cruzi* in human chagasic cardiomyopathy (Lane *et al*. 1997).

Microsporidia

Microsporidia are obligate intracellular spore-forming protozoa which have gained attention as important opportunistic pathogens in the evolving pandemic of HIV infection (Weber & Deplazes, 1995). At least 5 different genera of microsporidia are known to infect humans (Weber & Deplazes, 1995), therefore species- and strain-specific detection is important in determining sources of infection and chemotherapeutic protocols (Sobottka *et al*. 1995; Mathis *et al*. 1997). Detection of these organisms in a clinical laboratory poses significant challenges because of their small size and difficulty in distinguishing them from artifacts with the use of available staining techniques (Hines & Nachamkin, 1996). The situation is further complicated by the fact that clinical specimens of different origin may be submitted for investigation including faeces, urine, sputum, nasal discharges, cerebrospinal fluid and various tissue biopsies. Electron microscopy is the definitive procedure for identification of microsporidia to the species level, however, this is very time-consuming and requires specialized and expensive equipment (Kock *et al*. 1997). Recently, PCR and subsequent restriction fragment length polymorphism (RFLP) analysis (Katzwinkel-Wladarsch *et al*. 1997) and also nested PCR (Koch *et al*. 1997) have been used successfully for species-specific detection of microsporidia in a variety of clinical specimens. PCR amplification and species determination of microsporidia in formalin-fixed faeces has also been achieved (Dowd *et al*. 1998).

FUTURE NEEDS AND OPPORTUNITIES FOR MOLECULAR DIAGNOSTICS

Protozoan parasites such as *Cryptosporidium*, *Giardia* and newly emerging pathogens such as *Cyclospora* constitute important public health problems throughout the world. The development of a multiplex PCR assay which would enable the simultaneous detection of all 3 protozoan parasites would be an extremely useful public health tool, and an attractive, cost-effective procedure for clinical diagnostic laboratories. Previous research by Rochelle *et al*. (1997*a*) on the development of a multiplex test for *Giardia* and *Cryptosporidium* reported that none of the primers examined had the ideal combination of specificity, sensitivity and compatibility with multiplex PCR, and that additional primers need to be urgently evaluated. More recent analysis of a multiplex assay detecting *Cryptosporidium* and *Giardia* heat shock protein (hsp) genes holds promise for the future (Rochelle *et al*. 1997*b*). Another example of the need for such a multiplex PCR assay is in pig production facilities, where both *Isospora* and *Cryptosporidium* have been shown to be important causes of diarrhoea in young piglets, yet the sources of infection remain to be resolved (Christensen & Henriksen, 1994; Xiao, Herd & Bowman, 1994; Otten *et al*. 1996; Olson *et al*. 1997). The rapid detection and discrimination of these 2 parasites in young pigs is essential in terms of treatment and control, and the development of a multiplex PCR assay would be a valuable alternative to tedious and insensitive microscopy.

There are other situations where the availability of sensitive, rapid means of detecting protozoan parasites is required. Surveillance in geographical areas currently free of infections may require sensitive techniques capable of detecting low levels of infection in asymptomatic carrier animals, or in potential hosts in areas bordering endemic regions. For example, the introduction of *Trypanosoma evansi* into Australia from south east Asia (Luckins, 1988) would have enormous economic consequences and thus quarantine authorities regularly screen feral animals in the north of Australia using serological procedures. However, studies in Thailand have found PCR to be more sensitive than both microscopy and serology (Wuyts *et al*. 1995), and PCR techniques may thus prove to be more value in other geographical areas. Similarly, a major limitation in studying the epidemiology of canine visceral leishmaniasis is the inability to identify and count asymptomatic carriers because conventional diagnostic tests are insufficiently sensitive (Berrahal *et al*. 1996). This is very significant because of the zoonotic potential of this disease and the fact that it is an emerging issue in both Europe and the USA. Several groups have now developed PCR-based procedures for the detection of *Leishmania* from dogs and

humans and the results have shown that PCR offers a better gold standard that either *in vitro* or *in vivo* assays for diagnosis, and will be a valuable tool for surveillance (Ashford *et al*. 1995; Mathis & Deplazes, 1995; Berrahal *et al*. 1996). There is also a need for the development of sensitive, PCR-based assays for both equine and bovine babesiosis. They would provide useful screening tools for the detection of carrier animals thus preventing the introduction of these diseases into areas where the respective hosts are currently free of infection and at risk of contracting clinical disease.

FUTURE PROSPECTS FOR MOLECULAR DIAGNOSTICS

Molecular diagnostics are fast, efficient and are more amenable to large through-put processing. Since conventional microscopic parasitological examinations are amongst the most time-consuming procedures in a clinical laboratory, the use of molecular based tests may lead to more cost-effective and clinically efficient use of the diagnostic parasitology laboratory. In the USA, it is estimated that more than 4 million tests for bacteria and parasites are performed annually; thus, 'efficient use of the laboratory in an era of cost containment is critical' (Siegel, Edelstein & Nachamkin, 1990). However, many PCR methods will need improvement before reliable diagnostic methods become available. Clinical applicability will depend on the availability of highly standardized detection systems including methods and ingredients for sample collection, sample preparation and detection, and identification of PCR products (Smits & Hartskkeerl, 1995).

Recent advances using silicon chip technology have the potential to greatly improve diagnosis and detection of pathogens. A team at the University of California, Berkeley has designed a chip to carry out both PCR and electrophoresis. The PCR takes place in a 2 cm-high silicon reaction vessel that sits on top of a flat electrophoresis wafer. Enzymes are added to cut up the DNA and, when the PCR is complete, electric fields draw the fragments into gel-filled capillaries beneath the reaction vessel. Using the Berkeley chip, the team identified *Salmonella* DNA in less than 45 min (Woolley *et al*. 1996; Ward, 1997). Although, there are numerous technical difficulties to be overcome, this type of technology has the potential to greatly speed up the detection of pathogens and because the reaction is more efficient than conventional thermo-cyclers, it will enable more sensitive detection in the future.

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