Misdiagnosis of toxoplasma infection by PCR: fears unfounded

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Abstract

A nested PCR based diagnostic assay for the detection of toxoplasmosis was devised in 1990 and was used successfully among a battery of tests for the clinical diagnosis of *Toxoplasma gondii* infection since 1991. However, it was reported that the assay produced false positive diagnoses with *Nocardia asteroides* infection. Investigation of this phenomenon showed that although cross reactivity with some unrelated organisms may be observed when altered conditions are employed, the assay does not lead to misdiagnosis if performed under the appropriate, stringent conditions.

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Difficulties in the diagnosis of human toxoplasmosis by conventional and serological methods, especially in AIDS and organ transplant patients, led to the development of nucleic acid based diagnostic assays. Several diagnostic tests based on the polymerase chain reaction (PCR) have been devised in the last few years, with the most widely used amplifying parts of either the B1 gene¹ or the major surface antigen gene SAG1,^{2,3} previously known as p30 gene, of *Toxoplasma gondii*. The assay devised in this laboratory² is a nested PCR for the SAG1 gene in which the first amplification generates a 914 base pair (bp) fragment that is used as the target for the amplification of a 522 bp fragment in the second PCR; the PCR products may be visualised either directly on agarose gels or after Southern blotting and hybridisation. This assay has been successfully employed in conjunction with other tests to diagnose toxoplasmosis since 1991,⁴ but a recent report⁵ indicated that it gives false positive results with the phylogenetically unrelated intracellular bacterium *Nocardia asteroides*. In preliminary studies, we investigated this cross reactivity and confirmed that it occurred with some species of *Nocardia*, *Mycobacterium*, and *Actinomyces* and argued that, although these organisms yield PCR products, this did not pose any problems with the diagnostic utility of the assay since sound clinical practice warrants that PCR should not be the only basis of diagnosis for toxoplasmosis.⁶ In this technical note we report the findings of our detailed investigations of this cross reactivity.

Methods

POLYMERASE CHAIN REACTION

Genomic DNA was isolated from the species listed in table 1 using a proteinase K and

Table 1 Cross reacting species and sizes of the PCR products

Species	Approx size of PCR products (bp)	Hybridisation at:	
		60°C	65°C
<i>Toxoplasma gondii</i> (RH strain)	522	+	+
<i>Nocardia</i> spp			
<i>N. asteroides</i>	450	+	-
<i>N. brasiliensis</i>	600, 475, 450, 250	+	-
<i>N. farcinica</i>	750, 650, 425, 400	+	-
<i>N. otitidis-caviarum</i>	600, 475, 250	+	-
<i>Actinomyces</i> spp			
<i>A. naeslundii</i>	Smear	+	-
<i>A. bovis</i>	100	+	-
<i>A. denticola</i>	285	+	-
<i>A. odontolyticus</i>	Smear	+	-
<i>Mycobacterium</i> spp			
<i>M. tuberculosis</i> (7 clinical isolates)	450	+	-
<i>M. avium</i>	No product	NA	NA
<i>Plasmodium falciparum</i>	No product	NA	NA

bp, base pairs; NA, not applicable.

Figure 1 A representative 2% wt/vol agarose gel showing second PCR products from *Toxoplasma gondii* (lane 2), *Nocardia asteroides* (lane 3), *Mycobacterium tuberculosis* (lane 4), and *Actinomyces denticola* (lane 5). Lane 6 is a control without DNA and lanes 1 and 7 are molecular weight markers (Gene Ruler™ 100 bp DNA ladder; MBI Fermentas) containing the following fragments (in base pairs): 1000, 900, 800, 700, 600, 500, 400, 300, and 200.

CTAB extraction method.⁷ The nested PCR for the *T. gondii* SAG1 gene was performed using the conditions and the oligonucleotide primers described earlier.² The PCR products were visualised on 2% (wt/vol) agarose gels in TAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (5 µg/ml). Normally, 15 µl of the PCR product was mixed with 3 µl gel loading buffer (0.25% wt/vol bromophenol blue, 0.25% wt/vol xylene cyanol FF, 30% vol/vol glycerol) and applied on the gel; electrophoresis was performed at 5 volts/cm of gel.

SOUTHERN HYBRIDISATION

Southern hybridisation was carried out using standard methods.⁸ Briefly, any second PCR

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A

1 ACACCGACGG AGAACCACCTT CACTCTCAAG TGCCCTAAAA CAGCGCTCAC
 51 AGAGCCTCCC ACTCTTGCCT ACTCACCCAA CAGGCAAATC TGCCAGCGG
 101 GTACTACAAG TAGCTGTACA TCAAAGGCTG TAACATTGAG CTCCTTGATT
 151 CCTGAAGCAG AAGATAGCTG GTGGACGGGG GATTCTGCTA GTCTCGACAC
 201 GGCAGGCATC AAACCTACAG TTCCAATCGA GAAGTTCCCC GTGACAACGC
 251 AGACGTTTGT GGTGCGTTGC ATCAAGGGAG ACGACGCACA GAGTTGTATG
 301 GTCACGGTGA CAGTACAAGC CAGAGCCTCA TCGGTCGTCA ATAATGTGCG
 351 AAGGTGCTCC TACGGTGCAG ACAGCACTCT TGGTCTGTC AAGTTGTCTG
 401 CGGAAGGACC CACTACAATG ACCCTCGTGT GCGGGAAAGA TGGAGTCAAA
 451 GTTCTCTCAAG ACAACAATCA GTACTGTTC GG

B

1 GGTGGAACAC GTCGGGGTGA CCGTCGGGCA CGGGCATCAA TTTTGTGCG
 51 AGGCTCACGG CGTTCTCCCC ATTTCTCTCT CGCGTGTACC TCAGGCTCAC
 101 CCTTGAGTCC CGGTGCGAAT CATGCCAACG CCGTGGCGGA AACACCAATC
 151 GCAGCACGCT TGCTAACTCT GCGAATGGCG TCTTCACAAC ACCCGGTACG
 201 CTCGGTTGGG TGTCCAAGAC CTACGTCGGC TCGCGGGTCC GCCAACTGCG
 251 TAACGAGCGC GGGTTTCAGC AGGCCGCGCT GGCCAGATG CTGGAGATCT
 301 CGCCGAGCTA TCTGAACCAG ATCGAACACG ACGTCCGGCC GCTGACCGTG
 351 GCCGTGCTGC TGCGCATCAC CGAAGTGTTC GGGGTGGACG CGACGTTCTT
 401 TGCCTCCCA

C

1 ACGGCCACCA ATGGCGCATC GGACAAGGCT TCAAATGGTG GCAGGAAACA
 51 GTGCTTCGGC AACCAAAACC CCGCCGCGCG ACAAGTATTG CAGCACGGAC
 101 ACCAGCACTC CTGGGAGGTC CAGACCAGCG CTCAACGGAT CCGCATCTCC
 151 TCCTCGACGT TCGATTGCGG ATCCGACTGG TACCGCGACG CCCACGGCCA
 201 AGACGCCAAA CGCGGCGGCC TGACCTACCT ACTCCGCGAC GGCGAAGTCT
 251 CCCGAATGAG CCTCGTCTGA TGCGCGGACC CCGAAGTTCA TAGCGCCACC
 301 GGTAGCGGGC GCTTCCCGAG ACATTCCCTC TTGGAGGCGC ACCGTGCCGA
 351 TGCCTGACTT TCACGGCGAT TGCGCGCCCG AACCGTTCTGA CATTGCCCAA
 401 GACGCCTATG GCGTCTTCAG CGCATGGTGG TCCGCGACA CCGACGTGCT
 451 CCAGGCCGTC TACTCCGGAC AACACCAACT CCCCAGCCCC CGCACCTCGC
 501 AGTGCGCGG AGGAGTGATC GGCGCGCTGG CACGGTTCTG GTGGGGCGC
 551 CCCGTCCTTC AGGACTCGAA ACGCCTGCAC GTCCCTGCCG CAGCCGACGT
 601 CGCCACCACT TCCGCGAGAC TCCTGTGGG CAGCGCCCGT CCTGGCTTCT
 651 CACCGAAGGC GACGCCACCA ATCTGAAGGA CGCGCAAGCC CGGTTGAACG
 701 AACTGCT

D

1 TGGAGACGGT GTCGTAAGAC ACGGACGCGT GCGCACCGAA GGGAGAAGGC
 51 ACCGTGGAAC GGCCTGACGA ACAGGCGATC GAGGCCGTCA AGCTCTACTA
 101 CCAGCAGGGC TTGAGCCAGG CCGAGGTTGC CTCCCGTATG GGTCTGTGCG
 151 GCCCCACGGT TGCCAAGCTT CTTGCCACAG GCCGCGATCG CGGCTTCGTG
 201 ACCATCGAGA TCCATGATCC GCGCGAGGAC GCCTCG

Figure 2 Sequences of the second PCR product from *T. gondii* (A), *Mycobacterium tuberculosis* (B), *Nocardia farcinica* (C), and *Actinomyces denticola* (D). The two PCR primer sequences (20 bases each) are not shown. The GenBank accession numbers for the sequences are: *Mycobacterium tuberculosis*, AF 133644; *Nocardia farcinica*, AF133645; and *Actinomyces denticola*, AF 133646. The *Toxoplasma gondii* sequence is a part of the SAG1 sequence (GenBank accession number, X14080, M23658).

products generated by the putative cross reacting species were separated by electrophoresis on an agarose gel and transferred onto Hybond-NTM nylon membrane (Amersham Pharmacia-Biotech). Probe preparation, hybridisation, and detection of hybrids was performed as described by the manufacturer of the DIG DNA labelling and detection kit (Boehringer Mannheim). Briefly, the membranes were incubated for six hours at 60°C (or at 65°C) in the hybridisation solution (5×SSC, 0.1% wt/vol N-laurylsarcosine, 0.02% wt/vol sodium dodecyl sulphate (SDS), 1% blocking reagent provided in the kit; SSC is 0.15 M NaCl and 0.015 M Na₃ citrate), followed by six hours in fresh hybridisation solution containing the probe, which was the 522 bp second PCR product of *T. gondii* labelled using digoxigenin (DIG). Membranes were then washed at the hybridisation temperature twice (five minutes each) in 2×SSC containing 0.1% SDS and twice for five minutes each and once for 15 minutes in 0.1×SSC containing 0.1% SDS.

CLONING AND SEQUENCING OF PCR PRODUCTS

The PCR products were purified from agarose gels using the QIAquick gel extraction kit (Qiagen) and cloned into the pGEM[®]-T easy vector as recommended by the supplier (Promega Corporation). Recombinant plasmids were purified using the QIAprep spin miniprep kit (Qiagen) and sequenced at the core sequencing facility in the School of Animal and Microbial Sciences (The University of Reading) using Thermosequase[™] and an ALF express[™] DNA sequencer as recommended by the supplier (Amersham-Pharmacia Biotech).

Results and discussion

In the nested PCR assay developed in this laboratory,² use of *T. gondii* DNA as the target generated the expected products (that is, a 914 bp and a 522 bp product following the first and second PCR, respectively) but in the other species examined this was not the case (table 1; fig 1).

With most of the other organisms, the first PCR either did not yield a detectable product or the product was seen as a faint smear. The second PCR with these species generated products which ranged from 100 to 750 bp in length and in some cases only a smear was observed. Our results for *Nocardia asteroides* (table 1 and fig 1) are different from those reported by McHugh *et al.*,⁵ who showed that the size of the PCR product was the same as that of *T. gondii*.

One explanation for the difference in the size of the PCR products in these two studies is that it may reflect differences in the strains used. In the present study we used a type culture of *N. asteroides* (NCTC 11293), while McHugh *et al.* used a clinical isolate identified as *N. asteroides*. It is also possible that McHugh *et al.* used an *N. asteroides* DNA preparation that may have been contaminated with either *T. gondii* DNA or a *T. gondii* PCR product. In a recent comparison of PCR assays (including ours) for detection of *T. gondii* and the related coccidian parasite *Neospora caninum*, Ellis⁹ noted that

false positive results were observed regularly and that they were caused by contamination of reactions with previously amplified DNA. During the course of the present study, similar problems were encountered and strict precautions for avoiding such contamination had to be taken. The results of this study therefore indicate that although PCR products may be generated by organisms other than *T gondii*, these vary greatly in size and may be differentiated easily on agarose gels.

In order to investigate the nature of the PCR products obtained from cross reacting organisms, we used two approaches. First, we performed Southern blotting and hybridisation to the 522 bp PCR product from *T gondii* so as to increase the sensitivity of detection and to obtain insight into the extent of similarity between the PCR products of the different species. The results (table 1) showed that at the stringent hybridisation temperature of 65°C,² routinely used for our clinical investigations, only the *T gondii* PCR product hybridised; however, at conditions of lower stringency (at a hybridisation temperature of 60°C) all the PCR products hybridised. Therefore the results indicate that there is little sequence similarity between the PCR products of *T gondii* and those of the cross reacting species; consequently, if hybridisation at stringent conditions is used for diagnostic purposes, false positives are not evident.

The second approach used to investigate the nature of the PCR products from the cross reacting organisms was cloning and nucleotide sequence determination of the second PCR products obtained from *Mycobacterium tuberculosis*, *Actinomyces denticola*, and *Nocardia farcinica*. The DNA sequences obtained from these PCR products and that from *T gondii* are shown in fig 2. Comparisons of these DNA sequences confirmed that, at the nucleotide level, there was no significant similarity between the various PCR products and the nucleotide sequence of the SAG1 gene of *T gondii*.¹⁰

In conclusion, therefore, although the SAG1 based nested PCR assay generates PCR products from DNA of some other species under certain conditions, it remains effective for the clinical diagnosis of toxoplasmosis if carried out under the stringent conditions described earlier.² In any case, the results may be confirmed by performing hybridisation of the PCR products with the toxoplasma probe to substantiate the identity of the PCR products obtained. However, it is important to ensure that adequate measures are taken to avoid any contamination with *T gondii* DNA or PCR products, or both, as such contamination will inevitably result in false positive diagnoses. Our findings emphasise the need for strict adherence to published protocols when using molecular methods for clinical diagnosis.

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