Parasitol Res
DOI 10.1007/s00436-007-0749-7

ORIGINAL PAPER

Demonstration of *Balamuthia* and *Acanthamoeba* mitochondrial DNA in sectioned archival brain and other tissues by the polymerase chain reaction

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Received: 9 July 2007 / Accepted: 30 August 2007
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Abstract Granulomatous amoebic encephalitis (GAE) is a usually fatal disease caused by the free-living amoebae *Balamuthia mandrillaris* and *Acanthamoeba* spp. The intent of this study was to determine if the polymerase chain reaction (PCR) could be used retrospectively to detect amoeba mitochondrial 16S ribosomal ribonucleic acid gene deoxyribonucleic acid (DNA) in confirmed archival tissue sections from GAE cases stored in our laboratories for 1 to 34 years. The DNA was extracted from deparaffinized sections, and appropriate primer sets for each of the two amoebae were used for DNA detection. Indirect immunofluorescent staining (IIF) of tissue sections was used as the standard for identification of amoebae against which the PCR results were compared. Sixty slides from a total of 56 cases were processed by PCR for amoeba 16S DNA. In 28 (47%) slides, there was agreement between the IIF and PCR results. In 41 of the slides (52%), no DNA was detected after PCR. In one slide (1%), the PCR and IIF results did not agree. While PCR supported IIF findings in about half of the slides, there are significant limitations in amoeba DNA identifications in formalin-fixed brain tissues. Degradation of amoeba DNA because of formalin fixation was probably a factor in limiting valid results.

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Introduction

Granulomatous amoebic encephalitis (GAE) caused by the free-living amoebae *Balamuthia mandrillaris* and *Acanthamoeba* spp. is an indolent disease with an extended prodromal period. It mimics several other diseases of the central nervous system: meningeal tuberculosis, brain tumor, viral encephalitlis, neurocysticercosis, and acute disseminated encephalomyelitis. In examining brain tissue from GAE-infected individuals, pathologists may be unfamiliar with the amoebae and either fail to recognize or misidentify them or mistakenly identify them as macrophages. Because of the mimicry, imprecise symptomatology and the rarity of cases of GAE that occur in the community, the disease profile remains unfamiliar to most clinicians and pathologists. Diagnoses are often made postmortem. Until recently identification of amoebae in hematoxylin–eosin (H&E) or immunofluorescent-stained (IIF) brain sections was the chief means of diagnosis. More recently, the polymerase chain reaction (PCR) has been employed as an adjunct in the diagnosis of acanthamoebiasis (MacLean et al. 2007; Shirwadkar et al. 2006; Yagi et al. 2007) and balamuthiasis (Tavares et al. 2006; Shirwadkar et al. 2006; Yagi et al. 2005).

We estimate ~400 published and unpublished cases of GAE in humans and animals worldwide, with about 150 and 250 cases of balamuthiasis and acanthamoebiasis, respectively. Because of the problems inherent in recognizing GAE, it is likely that other cases have occurred, which were either undiagnosed or misdiagnosed.

PCR has been used to detect *Balamuthia* and *Acanthamoeba* mitochondrial 16S ribosomal ribonucleic acid (rRNA) gene deoxyribonucleic acids (DNAs) from amoebae in culture, in clinical specimens (brain tissue and

cerebrospinal fluid [CSF]) and in environmental samples (Booton et al. 2003a,b,c; Dunnebacke et al. 2004; Yagi at al. 2005, 2007). The technique is sufficiently sensitive to detect DNA from less than a single *Balamuthia* amoeba per reaction mixture, as demonstrated in unfixed CSF samples spiked with lysed amoebae (Yagi et al. 2005). PCR has been used in several laboratories for detection of amoeba mitochondrial DNA in fresh as well as formalin-fixed tissues (MacLean et al. 2007; Shirwadkar et al. 2006; Tavares et al. 2006; Yagi et al. 2005; Yagi et al. 2007).

In this study, we had a unique opportunity to test a collection of archival brain tissue slides from individuals confirmed as having GAE by IIF staining of brain tissue sections from as far back as 1973. Thus, we were able to test the efficacy of PCR in a truly retrospective study of amoeba-infected brain tissue.

Materials and methods

Archival material The archival slides, from the USA and abroad, were among those submitted to the Centers for Disease Control and Prevention (CDC) and the California Department of Public Health (CDPH) for assistance in diagnosis of suspected amoebic encephalitis cases. The presence of amoebic infection was confirmed by examination of H&E-stained slides; specific identification of amoebae as *Acanthamoeba* spp. or *Balamuthia* was accomplished by fluorescein isothiocyanate (FITC)-staining of sections after treatment with rabbit antiamoeba antibodies (Visvesvara et al. 1990). Unstained formalin-fixed, paraffin-embedded brain tissue sections (Table 1) were deparaffinized and scraped from the slides with a scalpel for DNA extraction. Lysis buffer was added to the tissues from slides. When clinical samples were available for PCR, lysis buffer was added to CSF sediment collected by centrifugation, to unfixed (fresh frozen) brain tissue, and to positive-control amoebae, followed by vortexing, etc., as previously described (Yagi et al. 2005).

The number of slides used for extraction varied and depended on their availability. In most cases (~90%), two slides were available for extraction of DNA; one slide was available for the remaining cases. Sections on some slides had been immunostained with FITC for amoeba identification after deparaffinization and before DNA extraction. The immunofluorescent staining did not interfere with subsequent PCR steps. PCR was done primarily upon the brain but also on other tissues when such were available. Fresh frozen brain tissue was available from two patients, and DNA was extracted and compared with that from formalin-fixed sections on slides. CSF samples for PCR were available from five cases.

Primer sets Two primer sets for *Balamuthia* 16S rRNA gene DNA were used, one ("long") producing an amplicon of 1,075 bp (Booton et al. 2003a,b,c):

- 5′Balspec16S (5′-CGCATGTATGAAGAAGACCA-3′)
- 3′Balspec16S (5′-TTACCTATATAATTGTCGATA TACCA-3′) and the second ("short") producing an amplicon of 230 bp:
- 5′Balspec16S (5′-CGCATGTATGAAGAAGACCA-3′)
- Bal16Sr610 (5′-CCCCTTTTTAACTCTAGTCATAT AGT-3′)

For *Acanthamoeba* DNA, the primer set produced an amplicon of 161 bp (Foreman et al. 2004):

- Aca16Sf1010 (5′-TTATATTGACTTGTACAGGTGCT-3′)
- Aca16Sr1180 (5′-CATAATGATTTGACTTCTTC TCCT-3′)

DNA extracted from each slide was amplified with both the *Balamuthia* and *Acanthamoeba* primer sets.

Polymerase chain reaction The PCR procedure used was the same as previously reported (Yagi et al. 2005). Amplification was done in 40 cycles: denaturation (10s at 94°C), annealing (5s at 58°C), and primer extension (15s at 72°C) using a LightCyclerz® System (Roche Diagnostics, Indianapolis, IN).

Amoeba DNA for positive controls Positive controls of DNA extracted from axenically grown amoebae (Schuster 2002) were run with all samples tested. These consisted of DNA extracted from two *B. mandrillaris* isolates (CDC: V188 and CDC:V194), or from *Acanthamoeba castellanii* (ATCC 30010, Neff strain). Pure water (Sigma, St. Louis, MO) was used as a blank. Because of the known ability of formalin to "damage" DNA strands, Restorase® DNA polymerase (Sigma) was used on extracted DNA from 38 (63%) of the 60 slides in an effort to repair sites in strands that might have been modified by fixation or other chemical exposures. The enzyme was used preamplification according to the manufacturer's instructions.

Comparison: IIF and PCR Although amoebae could be identified in the H&E-stained sections, it can be difficult to distinguish between *Acanthamoeba* and *Balamuthia* in such sections. Staining by IIF is highly specific for the two genera of amoebae. Thus, the gold standard employed in this study for detection and identification of amoebae was IIF. All slides were subjected to IIF upon receipt at the two laboratories, and extra slides were filed away as part of the archive. The PCR results were compared with the IIF

Table 1 Comparison of results from immunostaining (IIF) and PCR detection of *Balamuthia* and *Acanthamoeba* mitochondrial 16S DNA in formalin-fixed slide sections of brain and other tissues

Number	Year	Source[a]	Tissue[b]	IIF results	PCR results	IIF/PCR agreement
1	2006	Spain	Human	*Acanthamoeba*	No DNA detected	
2	2006	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
3	2005	Portugal	Human	*Balamuthia*	*Balamuthia*	Agreement
4	2005	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
5	2005	India	Human	*Acanthamoeba*	*Acanthamoeba*	Agreement
6	2004	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
7	2004	USA	Human	*Acanthamoeba*	No DNA detected	
8	2003	USA	Brain	*Balamuthia*	*Balamuthia*	Agreement
9			Kidney	No fluorescence	No identification	Agreement
10			Lung	No fluorescence	*Balamuthia*	Disagreement
11	2003	USA	Human	*Acanthamoeba*	No DNA detected	
12	2003	USA	Canine	*Balamuthia*	No DNA detected	
13	2003	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
14	2003	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
15	2003	Peru	Human	*Balamuthia*	No DNA detected	
16	2003	Peru	Human	*Acanthamoeba*	No DNA detected	
17	2003	USA	Human	*Acanthamoeba*	No DNA detected	
18	2003	USA	Human	*Acanthamoeba*	No DNA detected	
19	2003	USA	Human	*Acanthamoeba*	No DNA detected	
20	2003	USA	Human	*Acanthamoeba*	*Acanthamoeba*	Agreement
21	2002	USA	Human	*Balamuthia*	No DNA detected	
22	2002	USA	Gorilla	*Acanthamoeba*	No DNA detected	
23	2002	USA	brain	*Balamuthia*	*Balamuthia*	Agreement
24			adrenal	No fluorescence	No DNA detected	
25			lung	No fluorescence	No DNA detected	
26	2001	USA	brain	*Balamuthia*	*Balamuthia*	Agreement
27	2001	USA	Human	*Acanthamoeba*	*Acanthamoeba*	Agreement
28	2000	USA	Human	*Acanthamoeba*	*Acanthamoeba*	Agreement
29	2000	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
30	2000	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
31	2000	USA	Human	*Balamuthia*	No DNA detected	
32	2000	USA	Human	*Acanthamoeba*	No DNA detected	
33	2000	USA	Human	*Acanthamoeba*	No DNA detected	
34	1999	USA	Human	*Balamuthia*	No DNA detected	
35	1999	USA	Human	*Balamuthia*	No DNA detected	
36	1999	USA	Human	*Balamuthia*	No DNA detected	
37	1999	USA	Human	*Balamuthia*	No DNA detected	
38	1999	USA	Bovine	*Acanthamoeba*	*Acanthamoeba*	Agreement
39	1999	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
40	1999	USA	Gorilla	*Balamuthia*	*Balamuthia*	Agreement
41	1999	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
42	1999	USA	Human	*Acanthamoeba*	*Acanthamoeba*	Agreement
43	1997	USA	Mouse	*Balamuthia*	No DNA detected	
44	1995	USA	Human	*Acanthamoeba*	*Acanthamoeba*	Agreement
45	1994	Peru	Human	*Acanthamoeba*	No DNA detected	
46	1993	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
47	1993	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
48	1993	Peru	Human	*Balamuthia*	No DNA detected	
49	1993	USA	Human	*Balamuthia*	No DNA detected	
50	1992	Thailand	Human	*Balamuthia*	No DNA detected	
51	1992	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
52	1992	Mexico	Human	*Balamuthia*	No DNA detected	
53	1992	USA	Human	*Acanthamoeba*	*Acanthamoeba*	Agreement
54	1991	Australia	Human	*Balamuthia*	No DNA detected	
55	1990	Japan	Human	*Balamuthia*	No DNA detected	

Table 1 (continued)

Number	Year	Source[a]	Tissue[b]	IIF results	PCR results	IIF/PCR agreement
56	1990	USA	Human	*Balamuthia*	No DNA detected	
57	1989	USA	Human	*Balamuthia*	*Balamuthia*	Agreement
58	1984	USA	Mouse	*Acanthamoeba*	No DNA detected	
59	1982	USA	Gorilla	*Balamuthia*	*Balamuthia*	Agreement
60	1973	USA	Gibbon	*Balamuthia*	No DNA detected	

[a] Country from which tissue was obtained
[b] Human tissue unless otherwise specified

results, with either: (1) agreement noted between the two techniques (Table 1) or (2) no agreement by virtue of no DNA being detected after PCR. Tissues on all slides in the study were formalin fixed, and the sections were cut from paraffin blocks at the hospital pathology laboratory where the patient was admitted during the acute phase of the disease, or if the patient died, tissue was obtained at autopsy, if performed. Slides representing 56 cases and spanning a time period from 1973 to 2006 were subjected to PCR. Two of the cases (slides 8–10 and 23–25) provided multiple tissue sections (lung, kidney, adrenal gland) in addition to the brain. PCR was done in a blinded manner on coded slides, without prior knowledge of the identity of the amoebae by the PCR investigator. Two cases provided fresh frozen brain tissue (represented by slides 8–10 and 26), and four cases provided CSF (represented by 4, 8–10, 11, 20), which were tested by PCR to affirm amoeba identities (data not included).

Results

Amoeba DNA The 230- and 160-bp primer sets were specific for *Balamuthia* and *Acanthamoeba* DNAs, respectively. DNA extracted from sections on each slide and amplified with the "short" *Balamuthia* primer set gave strong bands for *Balamuthia* mitochondrial DNA in the 230-bp region (Fig. 1), whereas the primer set ("long") giving the 1,075-bp amplimer either produced no band or a very faint band (data not included). The 1,075-bp amplimer worked well with fresh brain tissue from two of the cases (reviewed in part in Yagi et al. 2005). *Acanthamoeba* mitochondrial DNA was detected in sections from acanthamoebiasis cases using the 161-bp primer set (Fig. 2).

Of the slides positive for *Balamuthia* and *Acanthamoeba* by IIF that are listed in Table 1, there was agreement between the IIF results and the PCR identification in 28 (47%) of the 60 extracts, and no DNA was detected in 31 of the 60 (52%) slides. The treatment of the DNA samples

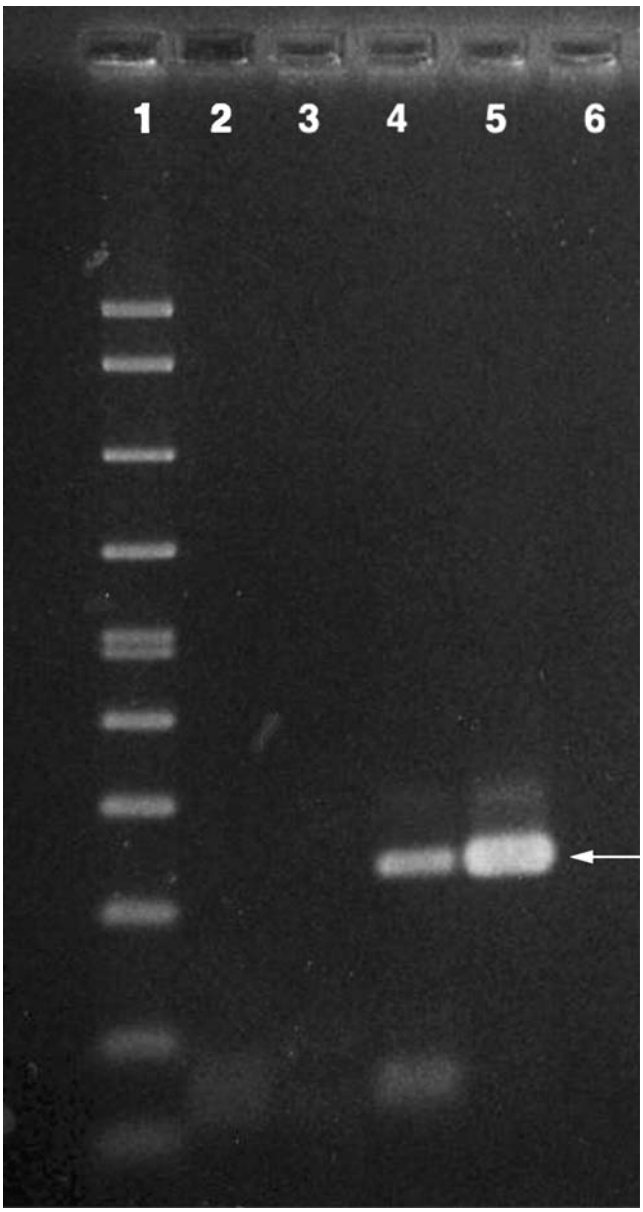

Fig. 1 PCR gel for *Balamuthia* mitochondrial DNA (230-bp primer set). *1* Molecular marker. Bands at ~230 bp (*arrow*). *2* Brain tissue from confirmed acanthamoebiasis case (slide 8). *3* Kidney tissue from a balamuthiasis case (slide 6). *4* Lung tissue from a balamuthiasis case (slide 7). *5* Positive control: *Balamuthia* mitochondrial DNA. *6* Brain tissue from confirmed acanthamoebiasis case (slide 3)

with Restorase® DNA polymerase enzyme to repair damaged DNA strands did so for 4 of the 38 DNA (11%) extracts treated (i. e., amoeba DNA that originally went undetected produced a band after treatment). The DNA polymerase was not used on the other 22 slides because their DNA had previously been extracted and amplified.

In two *Balamuthia* cases listed in Table 1, unstained slides with tissues in addition to the brain were available for testing. These included the kidney and lung from one case (slides 9 and 10) and adrenal gland and lung tissues from a second case (slides 24 and 25). With the exception of the slide of lung tissue (slide 25) that was positive by PCR but negative by IIF, the other three slides were negative for *Balamuthia* by IIF as well as PCR.

Discussion

GAE caused by free-living amoebae *Balamuthia* and *Acanthamoeba* is a relatively rare disease. Cases date from the 1960s when they first appeared in the medical literature. Today, GAE cases are often regarded as "emerging infections," but they are only in the sense that more physicians, pathologists, and laboratorians have become aware of their existence. A retrospective review of cases from neuropathology archives would doubtlessly uncover more cases and give a more realistic picture of their prevalence. Even today, with increasing numbers of papers appearing about GAE, it remains an enigma to most clinicians.

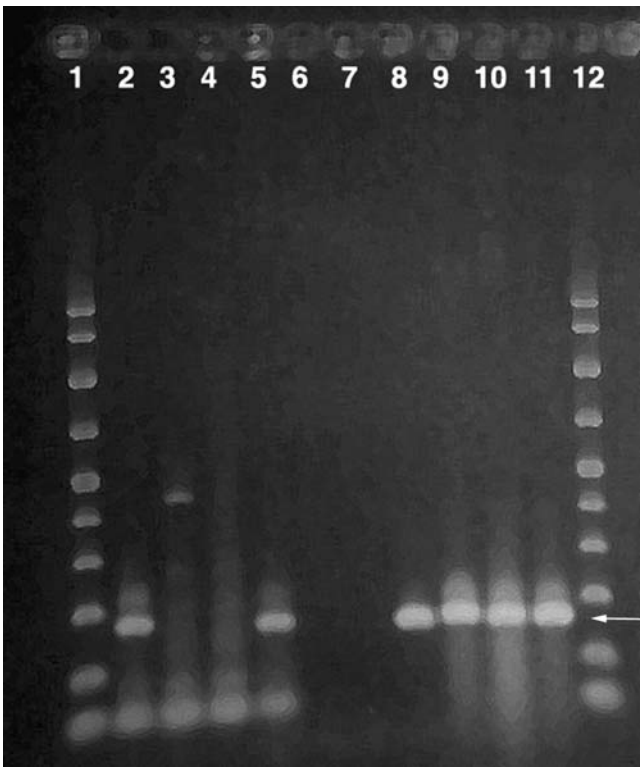

Fig. 2 PCR gel for *Acanthamoeba* mitochondrial DNA (161-bp primer set). *1* Molecular marker. Bands at ~161 bp (*arrow*). *2* Brain tissue from acanthamoebiasis case (slide 8). *3* Kidney tissue from a balamuthiasis case (slide 6). *4* Lung tissue from a balamuthiasis case (slide 7). *5* Brain tissue from acanthamoebiasis infection (slide 3). *6* DNA extracted from *Balamuthia* amoebae in culture. *7* Negative control (Sigma water). *8* Positive control: *Acanthamoeba* sp. (V021) mitochondrial DNA. *9* Positive control: *Acanthamoeba* sp. (V029) mitochondrial DNA. *10* Positive control: *Acanthamoeba castellanii* (Neff strain) mitochondrial DNA. *11* Positive control: *Acanthamoeba polyphaga* (A1) mitochondrial DNA. *12* Molecular marker

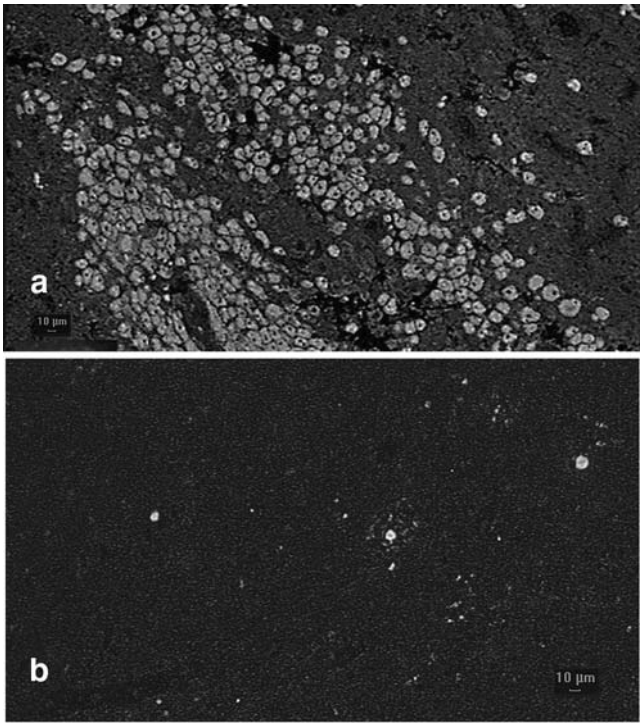

Fig. 3 Two sections of brain tissue from two different balamuthiasis patients. Immunofluorescent staining for *Balamuthia* was used on both sections. The *bar* in both micrographs indicates 10 µm. **a** Section showing numerous amoebae in a perivascular location in brain tissue. The perivascular accumulation of amoebae is generally typical of most cases of amebic encephalitis. Centripetal movement of amebae into the brain parenchyma can also be seen in this micrograph. **b** Section of brain tissue showing very few amoebae in perivascular areas and brain parenchyma

In this study, we have shown, with important qualifications, that amoeba mitochondrial DNA extracted from archival slides of formalin-fixed brain and other tissues can be detected using PCR methodology. Only one previous attempt at a retrospective analysis of *Balamuthia* cases was made (Taratuto et al. 2001). This was based on histopathologic examination of 19 slides from a series of 1,100 deceased pediatric patients with intracranial lesions. Four slides (21%) were found to be positive for balamuthiasis. In this study, we have used PCR on archival slides representing 56 confirmed cases of balamuthiasis and acanthamoebiasis in both humans and other mammals. The overall success in identification of amoebic DNA in

brain tissue was 47%. No DNA was detected in 31 slides (52%). In 1 slide out of 60, the IIF and PCR results did not agree. Successful identification of amoebae in brain tissues occurred in every instance, except one (slide 10, lung tissue), when extracted DNA was available for amplification. In those slide sections where DNA was not detected, we believe it was due to structural modification of the DNA probably as a result of formalin fixation.

The PCR technique and formalin fixation of cells PCR as practiced in this study is based on extraction of amoeba mitochondrial 16S rRNA gene DNA from host tissues. The host tissues were paraffin-embedded 5-μm sections mounted on slides. Two slides were used for most extractions. In extracting the DNA, however, it was less a matter of the number of slides that were available and more a matter of size and number of sections on a slide and the number of amoebae present in the section. Tissue sections can vary greatly in the number of amoebae present, as seen in Fig. 3a and b. The integrity of DNA strands is a concern when dealing with DNA extracted from formalin-fixed tissues destined for PCR testing (Ferrer et al. 2007; Karlsen et al. 1994; Schild et al. 2007). DNA stability can be a function of duration of time in formalin (Ferrer et al. 2007; Karlsen et al. 1994); the longer the exposure, the less useful the DNA strands will be for amplification.

In a variety of tissues, Karlsen et al. (1994) have shown that 8 h in formalin will inhibit amplification. Additional time in formalin further decreases the possible amplification length to less than or equal to 200 bp. In another study, prolonged formalin exposure of brain tissue rarely yields useful DNA for PCR (Ferrer et al. 2007). *Naegleria fowleri* amoeba 18S rRNA DNA gene in mouse brain, however, was able to tolerate greater than or equal to 1 year in phosphate-buffered saline-buffered formalin (Schild et al. 2007). Precise information was not available to us on the duration of formalin fixation of brain tissue. In general, fresh brain tissue obtained at autopsy that is destined for H&E and immunofluorescent staining—and needed for rapid diagnostic testing—is fixed for a short period of time, while the whole brain is immersed in formalin for 5–7 days for subsequent studies.

It was to be expected that brain tissue sections from patients who died of GAE would contain amoeba DNA by PCR. Other organs have been reportedly involved in *Balamuthia* infections, including kidneys and adrenal glands (Anzil et al. 1991; Rodriquez et al. 1997), thyroid (Candenedo-Gonzalez and Gamboa-Dominguez 2000), and lungs (Martinez and Visvesvara 1997). In this study, lung tissue from one case (slide 10) was positive by PCR but negative by IIF. We note that slides from the brain of the same patient varied widely in the numbers of amoebae present, as seen in Fig. 3a and b.

Thus, it is possible that amoebae may be absent in a section, although they were present in large numbers in previous sections. In such cases, IIF may turn out to be negative, whereas the PCR technique, which uses DNA from several slides and is presumably more sensitive, gives a positive result.

How trustworthy is a diagnostic technique that is "right" about half of the time? The application of PCR to archival materials is not without drawbacks. Compared to immunostaining, the standard against which PCR was measured, successful identifications were made in 47% of the tests. The problem, however, seems not to be with the PCR technique per se but rather with the way the tissues may have been treated. In many recent cases in our laboratories when brain and CSF were available for testing, PCR has been 100% successful in detecting or not detecting amoeba DNA in positive or negative cases, even in formalin-fixed tissue sections (Yagi et al. 2005; Shirwadkar et al. 2006). Additionally, conclusions from PCR determinations have been supported by clinical information, laboratory data, and postmortem studies. However, PCR testing becomes problematic in the study of retrospective tissue samples.

Balamuthia DNA was more consistently amplified by the shorter (230-bp) primer set than by the longer primer pair (1,075 bp) in fixed tissue specimens. This may be a result of structural distortion of long DNA strands so that only short base pair lengths were available for binding and amplification by the short primer set. Shorter primer sets are definitely better than the longer primer sets in the amplification. For example, Booton et al. (2003c) encountered difficulty in demonstrating *Balamuthia* DNA from formalin-fixed brain tissue by PCR with the 1,075-bp primer set. They used a modified primer set producing an amplicon of 500 bp. This altered primer set, in conjunction with seminested PCR, detected *Balamuthia* DNA, while only a faint band was produced by conventional PCR.

In contrast to fixed material, amoeba DNA in fresh or fresh-frozen brain tissue and CSF from recent cases of balamuthiasis showed good amplification with the 1,075-bp amplicon (Yagi et al. 2005, 2007). This was less of a problem with the *Acanthamoeba* primer set (161 bp). Fixation and amoeba density in tissue sections are not under the control of the PCR operator and can be the reason for inability to detect DNA as listed in Table 1.

Balamuthia DNA from formalin-fixed brain tissue sections was detected in a balamuthiasis case from Spain (Tavares et al. 2006), in several cases from the USA (Yagi et al. 2005), and in two cases from California and from India (unpublished data). Similarly, *Acanthamoeba* DNA was detected in fixed brain tissue sections of a fatal case of acanthamoebiasis (Shirwadkar et al. 2006) and from brain tissue and CSF of a patient with systemic lupus (Yagi et al. 2007).

In conclusion, the use of PCR in retrospective studies of formalin-fixed tissue specimens should be regarded with a degree of skepticism. It should be remembered that PCR is not the sole basis for diagnoses of suspected GAE cases. Prospectively or retrospectively, supportive clinical and laboratory results are essential in reaching a diagnosis, including neuroimaging scans, patient serum titers for amoeba antibodies, and results from lumbar puncture and symptomatology. Our study indicates that DNA polymerase treatment can be of help in repairing the damage to DNA caused by formalin fixation, but even then, its efficacy in damage repair is limited.

Acknowledgments The research was supported in part by a grant from the Emerging Infectious Diseases Program of the Centers for Disease Control and Prevention (U50/CCU915548-09) to Dr. Carol Glaser of the California Department of Public Health, Richmond, whom the authors thank for her encouragement and support. We thank Rama Sriram (CDC) for her technical expertise and assistance. The research performed in this study was in compliance with the laws of the USA and the authors' respective institutions.

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