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A real-time PCR diagnostic method for detection of *Naegleria fowleri*

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ABSTRACT

Naegleria fowleri is a free-living amoeba that can cause primary amoebic meningoencephalitis (PAM). While, traditional methods for diagnosing PAM still rely on culture, more current laboratory diagnoses exist based on conventional PCR methods; however, only a few real-time PCR processes have been described as yet. Here, we describe a real-time PCR-based diagnostic method using hybridization fluorescent labelled probes, with a LightCycler instrument and accompanying software (Roche), targeting the *Naegleria fowleri Mp2Cl5* gene sequence.

Using this method, no cross reactivity with other tested epidemiologically relevant prokaryotic and eukaryotic organisms was found. The reaction detection limit was 1 copy of the *Mp2Cl5* DNA sequence. This assay could become useful in the rapid laboratory diagnostic assessment of the presence or absence of *Naegleria fowleri.*

1. Introduction

Naegleria fowleri, a free-living amoeba, is an oetiological agent of primary amoebic meningoencephalitis (PAM). PAM is a rare but fulminating and mostly fatal disease of the central nervous system (CNS) (Marciano-Cabral, 1988, 2009; Schild et al., 2007; Visvesvara et al., 2007), which predominantly occurs in healthy children and young adults with a history of recent exposure to warm fresh water. *N. fowleri* is thermophilic and can tolerate temperatures of up to 45 °C. Therefore, these amoebae proliferate during warmer months of the year when the ambient temperature is likely to be high (Marciano-Cabral, 1988, 2009; Visvesvara et al., 2007).

The laboratory diagnosis of PAM usually relies on cultivation (Visvesvara, 1999; Schuster, 2002) and on the confirmation of *N. fowleri* in the cerebrospinal fluid (CSF) (Schuster, 2002). In addition, a flagellation test (FT) is routinely used as an ancillary diagnostic technique for *N. fowleri.* In practice, FT needs to be followed by ELISA or another diagnostic method (PCR, RFLP-restriction fragment length polymorphism) after both positive and negative results, because of the incidence of some false negatives (Visvesvara, 1999; De Jonckheere et al., 2001; Behets et al., 2003). ELISA-based diagnostic methods usually provide only late, retrospective and post mortem diagnoses. RFLP is a species-specific diagnostic method based on the different restriction profiles of the genus *Naegleria* (De Jonckheere, 1987; McLaughlin et al., 1988; Milligan and Band, 1988). The advantage of this technique is the possibility of diagnosing not only *N. fowleri* but also other species of the *Naegleria* genus. A detection method using DNA probes was described in the mid 1990s (Sparagano, 1993; Kilvington and Beeching, 1995). In general, molecular methods are very sensitive and may allow the detection of microorganisms which are difficult to identify, and thus these methods are a useful alternative to microscopy and culture. There are drawbacks to most of these techniques, however, such as being time consuming (cultivation), costly (RFLP), providing only late and retrospective diagnosis (ELISA), or providing insufficient results (flagellation test).

PCR diagnostic methods overcome many of these problems, and are useful for the diagnosis of both clinical and environmental specimens. One of the primary advantages is the possibility of isolating DNA from samples without previous cultivation, making these PCR methods less time consuming (MacLean et al., 2004). Also, PCR allows the detection of not only *N. fowleri*, but also enables the differentiation of other species of the *Naegleria* genus. Such specific tests for *N. fowleri* should be recommended to confirm suspected infectious agents in clinical samples.

Currently, the laboratory diagnosis of PAM by PCR is based on conventional PCR methods (Sparagano, 1993; van Belkum et al., 1992; Sheehan et al., 2003; McLaughlin et al., 1991; Kilvington and Beeching, 1995), nested PCR methods (MacLean et al., 2004; Marciano-Cabral et al., 2003; Réveiller et al., 2002) and multiplex PCR methods (Pélandakis and Pernin, 2002). Only a few real-time PCR processes have yet been described (Behets et al., 2006, 2007; Schild et al., 2007; Qvarnstrom et al., 2006; Robinson et al., 2006). In the study of Behets et al. (2007), primers and probe are based on the *Mp2Cl5* gene, but with the use of just a single FAM

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labelled probe. In a previous study, four species of the *Naegleria* genus and four species of the *Acanthamoeba* genus were examined for reactivity with *Mp2Cl5*, and specificity was shown only for *N. fowleri* (Réveiller et al., 2002). The real-time PCR-based diagnostic method developed by Qvarnstorm et al. (2006) uses multiplex PCR with the detection of *Naegleria fowleri* by a HEX (hexachlorofluorescein) labelled probe. Robinson et al. (2006) described a real-time PCR *N. fowleri* detection procedure using primers for *ITS 1* and *ITS 2* noncoding spacers with the intercalating dye SYT09. Schild et al. (2007) utilized a LightCycler instrument for amplification, but primers were designed for the detection of ribosomal-small-unit SSU (18s) r RNA gene, with SYBR Green 1 labelling.

Real-time PCR-based diagnostic methods are advantageous in the rapidity of results and the possibility of monitoring the amplification process in real-time. The goal of our study was to develop a real-time PCR-based diagnostic method for detection of *Naegleria fowleri*, using amplification with a LightCycler instrument and primers and probe based on the *Mp2Cl5* gene. Such an effective laboratory diagnostic method is needed for the confirmation of *N. fowleri* in both clinical and environmental samples. Though the primers used in our real-time PCR procedure were synthesized according to a previous study (Réveiller et al., 2002), the hybridization fluorescent labelled probes are newly designed for detection of the *Mp2Cl5* gene sequence, which produces a protein unique to *N. fowleri*. A specific protein was found to be expressed from a cDNA clone designated *Mp2Cl5*. Northern blot analysis showed that the *Mp2Cl5* mRNA was expressed in pathogenic *N. fowleri* but was not expressed in non-pathogenic *Naegleria* species nor in *Acanthamoeba* (Réveiller et al., 2001). Thus, this real-time PCR assay is a highly sensitive surveillance tool, for the quick detection of an organism that poses a risk to human health.

2. Materials and methods

2.1. Microbial strains and cultures

Naegleria fowleri – ATCC 30214

Naegleria lovaniensis – ATCC 30569

Acanthamoeba sp. and *Hartmannella* sp. – detected in our laboratory by morphology and cultivation, Regional Authority of Public Health, Department of Medical Microbiology, Cesta k nemocnici 1, 975 56 Banská Bystrica, Slovak Republic.

Bordetella pertussis – strain, CNCTC 5268, from the collection of microorganisms of the National Institute of Public Health in Prague

Francisella tularensis – provided by the Department of Epidemiology, Faculty of Medicine, Comenius University

Pneumocystis carinii – confirmed by PCR in our laboratory

Toxoplasma gondii – tachyzoits, provided by The University of Veterinary Medicine in Košice, confirmed in our laboratory by real-time PCR

Streptococcus pneumoniae, *Streptococcus agalactiae*, *Haemophilus influenzae* and *Neisseria meningitidis* – strains provided by a medical microbiology laboratory (HPL Laboratory Ltd., Bratislava).

2.2. Clinical specimens

Cerebrospinal fluid (CSF)-sterile according to bacteriology and virology, real-time PCR for detection of *Naegleria fowleri* was negative; to produce samples for the purpose of this study, the CSF was subsequently spiked with the *Naegleria fowleri* strain and with other organisms that cause CNS diseases and symptoms similar to what are observed in infections caused by *Naegleria fowleri*: *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Streptococcus agalactiae* and *Toxoplasma gondii*.

The bacterial samples used for testing the specificity were prepared by suspending overnight bacterial growth from solid agar medium (5% sheep blood or chocolate agar) to a 0.5 McFarland standard in tryptic soy broth and pipetting 50 μl of the suspension in 0.5 ml of CSF, modified scheme according to the previous study (Kowalski et al., 2006). The sample spiked with *Toxoplasma gondii* was prepared by suspending 10^3 tachyzoits in 1 ml of CSF.

The strains of *Naegleria* spp. were cultured in modified PYNFH medium (ATCC 1034 medium) at 36 °C in 25 cm^2 polystyrene tissue-culture flasks (Greiner Cellstar®). After 4 days culture the flasks were chilled on ice (15 min), cells harvested by centrifugation (5 min, 600*g*). The trophozoites were adjusted to 10^3 cell/ml by using Cyrus I counting chamber and washed with PAS three times (Behets et al., 2007; Qvarnstrom et al., 2006). Consequently the pellets were diluted in parasite-free CSF and directly subjected to DNA extraction.

2.3. Environmental specimens

Water sample-collected from a swimming pool, negative for the presence of free-living amoebas; subsequently spiked with the *Naegleria fowleri* ATCC 30214 strain, the *Naegleria lovaniensis* ATCC 30569 strain and *Acanthamoeba* sp.

2.4. Nucleic acid isolation

After 4 days of culture, DNA was isolated from the reference ATCC 30214 *Naegleria fowleri* strain using the commercial High Pure PCR Template Kit (Roche Diagnostics) according to the manufacturer's protocol. Isolated DNA (5 μl) was then used for each reaction (see below). A sample of deionized water was integrated into each series of nucleic acid isolations to act as a negative control.

Additionally, DNA extractions from several prokaryotic (bacteria) and eukaryotic (some of them ecological relevant) organisms were tested to further explore the specificity of this real-time PCR reaction: *Naegleria lovaniensis* (ATCC 30569), *Hartmannella* sp.; *Acanthamoeba* sp.; *Pneumocystis carinii; Toxoplasma gondii; Bordetella pertussis*; *Francisella tularensis; Streptococcus pneumoniae, Streptococcus agalactiae, Haemophilus influenzae, Neisseria meningitidis.*

2.5. Design of primers and probes

Primers and probes (see Table 1) were designed for the detection of the *Mp2Cl5 Naegleria fowleri* gene sequence. Primers were synthesized by TibMol Biol (Berlin, Germany) according to the study of Réveiller et al. (2002). Probes were designed and synthesized by TibMol Biol (Berlin, Germany).

2.6. Real-time PCR assay

Real-time PCR was performed using a LightCycler PCR instrument, LightCycler software version 3.5.3., and the LightCycler Fast Start DNA Master Hybridization Probes Kit, all from Roche Diagnostics. The kit contains Taq DNA polymerase, $MgCl_2$ solution, PCR-grade water and free nucleotides. Heat-labile uracyl DNA glycosylase (UNG) was used as carry-over prevention.The following

Table 1
Primers and probes sequences.

Forward primer	5′ tct aga gat cca acc aat gg 3′
Reverse primer	5′ gtc ttt gtg aaa aca tca cc 3
Fluorescein probe	caa gat cac ttg ttg aag gct gtc-FL
LC Red 640 probe	LC Red 640 – caa act ctt tgg cct cta ttc ctc tt