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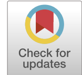
Original Article

Ultrasensitive and rapid diagnostic tool for detection of *Acanthamoeba castellanii*



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ABSTRACT

Acanthamoeba keratitis is a devastating infectious disease of the cornea caused by an opportunistic amoeba, *Acanthamoeba castellanii*. It is poorly recognized, and diagnostic delays can lead to irreversible damage to the vision. The gold standard for diagnosis has been a sample culture that lasts approximately 2 weeks. Nevertheless, the essence of time has led to the need for an accurate and fast technique to detect *A. castellanii* from a sample. We developed both traditional and quantitative real-time-PCR-based methods to detect *A. castellanii* in less than 3 hours and with the sensitivity of one amoeba. Diagnostic laboratories can select the best-suited method for their purposes from 2 comparable methods. The correct treatment can be initiated from the emergency room when the diagnosis has been made quickly within a few hours, hence saving the patient from long-term complications.

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1. Introduction

Corneal opacity causes 1.5 to 2 million cases of new monocular blindness per year (WHO). The leading cause of opacity is infectious keratitis (bacterial, viral, fungal, amoebae), with an incidence of 2.5 to 799 infections per 100,000 [1,2]. Different bacteria are the most common cause of infectious keratitis in general; however, *Acanthamoeba* infections are responsible for 1.96% to 12.5% of infectious keratitis in contact lens wearers [3].

Acanthamoeba castellanii is an opportunistic pathogenic amoeba found in soil and water worldwide. It commonly causes keratitis and, in rare cases, can cause invasive infections, such as granulomatous amoebic encephalitis (GAE). *Acanthamoeba keratitis* (AK) is a sight-threatening and poorly recognized condition with only moderately effective treatment options [4,5]. In particular, wearing contact lenses when swimming in natural waters is considered a high-risk activity. Other risk factors for catching AK are negligence in hygiene when handling eye contact lenses, usage of ophthalmic corticosteroids [6], and perioperative period of ophthalmic operations [4]. There are over 150 million contact lens wearers globally [7], and the yearly sale of contact lenses is approximately 10.1 billion USD emphasizing the vast part of the population belonging to the risk group [8].

In clinical examination, AK resembles viral, bacterial, and fungal keratitis [9–11]. The gold standard for diagnosing AK is culturing the corneal swab sample for approximately 2 weeks [9]. Specialized centers use confocal microscopy, which, when in capable hands, has good sensitivity and specificity; nonetheless, the need for experienced microscopists and equipment limits the usefulness of the method [12]. New approaches based on gene multiplication and detection has been developed, but they have yet to reach widespread use in clinical diagnostics [4,13].

A. castellanii has 8 carbonic anhydrases (CAs, EC 4.2.1.1) belonging to 3 different evolutional families: 3 α-, 3 β- and 2 γ-CAs [14,15]. To date, 8 different families of CAs have been recognized in different organisms [16,17], yet the human genome only contains enzymes belonging to 1 family, α-CAs [17]. CAs are metalloproteases with various roles in fundamental physiological processes in almost all living organisms. CAs catalyze the reversible hydration of carbon dioxide and, for instance, participate in maintaining acid-base homeostasis [18,19]. Recognition of CA-genes of parasites from a sample is arising as a tool to detect different parasites, for example, finding the trichinosis-causing parasite *Trichinella spiralis* from meat samples [20].

Due to the diagnostic challenges, the diagnosis of AK is often delayed, and up to two-thirds of the patients are initially treated with antibacterial eyedrops and the same number with antiviral medication [21]. The approximate delay of the correct diagnosis is 39 days [21]. This emphasizes the need for a sensitive and rapid diagnostic method to optimally provide the result during the patient's visit to the emergency room. In this article, we describe a novel rapid and sensitive PCR-based method and a quantitative real-time PCR (qRT–PCR) method for detecting the β- and γ-*CA* genes of *A.*

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castellanii. We consider these methods promising tools for clinical diagnostics of AK from biological samples containing amoebas with no unspecific signal from the human genome.

2. Materials and methods

2.1. Culture initiation and maintenance

The axenic *A. castellanii* (ATCC ® 30010™) culture was initiated and maintained in T 25 tissue culture flasks (Thermo Fischer Scientific, Waltham, MA, USA) in ATCC Medium 712: PYG (proteose peptone, yeast and glucose) with additives (Sigma–Aldrich, St. Louis, MO, USA) according to the recommendation of the cell provider. The cells were cultured at +25°C with constant temperature monitoring to ensure stable growth conditions. To maintain the culture, the medium was changed twice a week.

2.2. Collecting and lysing the samples

Initially, genomic DNA was isolated from amoeba culture with a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Consequently, the isolated DNA, concentration measured with Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific), was used in testing the primers and optimizing the PCR protocol.

Specific numbers of amoebae were collected in vision control with a light microscope. A borosilicate needle was used to collect the desired number of trophozoites and/or cysts from a petri dish with amoebae in the medium. Approximately 1.2 to 3.5 μL of culture media was contained in 1 amoeba sample. The collected sample was transferred to 100 μL DirectPCR Lysis Reagent (Tail) (Nordic BioSite, Täby, Sverige). Three microliters of proteinase K (20 mg/mL, Macherey-Nagel) were added to the reaction, and the tube was placed in an incubator at +55°C and shook at 200 rpm for 30 minutes. The lysis reaction was terminated by transferring the tube to a heating block at +85°C for 50 minutes.

Human cell samples were collected from buccal mucosa swab samples. They were compiled with sterile loops, which were subsequently dipped in 100 μl DirectPCR Lysis Reagent (Tail) (Nordic BioSite, Täby, Sverige) and thawed. Loops were removed and discarded. As described above, the desired numbers of amoebae were added to the tubes with 3 μL Proteinase K (20 mg/mL, Macherey-Nagel). The lysis reaction was performed similarly to the samples with amoebae only.

2.3. Primer design

Primers for traditional and qRT–PCR were designed using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The most promising primers were selected, manually inspected, and modified to improve their performance. As a result, we obtained 21 and 5 primer pairs for traditional and qRT–PCR, respectively (Table 1).

2.4. Traditional PCR method

The study involved 2 consecutive phases of PCR. First, the crude exclusion of nonworking primers was performed with PCR 1 according to the manufacturer's manual of the master mix 2 x Phusion Flash High Fidelity (Thermo Fischer Scientific, Waltham, MA, USA) with 46.7 ng of isolated genomic DNA per reaction. The non-working primers were excluded, and the functioning primers were chosen for further processes. The amount of genomic DNA was reduced to 2.3 ng per reaction, and again, the nonfunctional primers were excluded. PCR assay 1 was performed with a thermocycler according to the manufacturer's protocol except for 35 cycles and an annealing temperature of +65°C.

In the second phase, we used lysis samples as the template. PCR mix 2 had the same composition as PCR mix 1 apart from 2 μL of a lysed sample as template DNA. There were 6 lysis samples: 1 trophozoite, 1 cyst, 10 amoebae (5 cysts and 5 trophozoites), buccal mucosa, buccal mucosa, and amoebae combined, and hundreds of amoebae. A reaction mix with 2 μL of H_2O as a sample was used as a negative control. The PCR assay 2 was optimized from the basis of PCR assay 1 as a result of the functioning method in a thermocycler with 38 cycles and an annealing temperature of +67°C. The PCR product was visualized in 1.6% agarose gel (Meridian Bioscience Inc., Cincinnati, OH, USA)

Table 1
21 and 5 primer pairs for traditional PCR and qRT–PCR, respectively, constructed for the development of the diagnostic tool. The final primer pairs used in the diagnostic tool are underlined. The primer pair for the reference gene (RG) used in qRT–PCR is also shown.

Primer pair	Forward (5′ - 3′)	Reverse (5′ - 3′)	Target gene (Entry ID)	Product length (bp)
bCA_1	AAACATTGCCAACACGGTCG	GGTCAGAGATCGTACGCCAG	*mitBCA (L8GR38)*	326
bCA_2	CTGGCGTACGATCTCTGACC	AAGGGTCTCCTACCTCGGAC	*mitBCA (L8GR38)*	332
bCA_3	TTCCTAATGACGCGGACAGG	GCAAAGGGTCTCCTACCTCG	*mitBCA (L8GR38)*	249
bCA_4	AAAGCTGGTACTCACCTGCC	AGGTAGTACTCGGCCGTCAT	*cpBCA (L8GLS7)*	476
bCA_5	ATCTTTGACGAGGGCATGGG	AGAAGGAGGTGTACGGACCT	*cpBCA (L8GLS7)*	623
bCA_6	TAATCTGGGTCGGTTCGTGC	TGGGAGAAGGAGGTGTACGG	*cpBCA (L8GLS7)*	418
bCA_7	CCGGAGCTCATCTTTGACGA	GAGAAGGAGGTGTACGGACC	*cpBCA (L8GLS7)*	633
bCA_8	CGAACCACTGTATGGGCTGT	GAGCGAAAGCGATGAGGGAT	*tmBCA (L8H861)*	310
bCA_9	CCTCCCTGGTTGATTCTCGG	CCGGTCTGATGAGCCACTTC	*tmBCA (L8H861)*	499
bCA_10	CGGTACCCTACATTCCCCGA	CACCAGAGCTGAGGCAGTAG	*tmBCA (L8H861)*	352
bCA_11	CGTCAGGTACTCCATCAGGG	GGCAGGATCTTAGCCACGAG	*tmBCA (L8H861)*	358
bCA_12	GTGTGACGTGGAACTTGCTG	GCGAAAGCGATGAGGGATAGA	*tmBCA (L8H861)*	395
bCA_13	CAGTGTGACGTGGAACTTGC	GAGCGAAAGCGATGAGGGATA	*tmBCA (L8H861)*	399
aCA_1	GTGATACCACGCAACGCATC	CCAACCAACACACACACGAC	*aCA (L8GPJ9)*	472
aCA_2	TTGCAAGTTCATCAGCACGC	TCTTTGGGTAGGAAAGCCCC	*aCA (L8H518)*	476
aCA_3	CACACCTCGAAGAAGCAGGT	GAAAGGGGTGGGTACCGTG	*aCA (L8GXK3)*	440
aCA_4	CGGTACCCACCCCTTTCCTC	CCACGAAGATCCAGCCTAGC	*aCA (L8GXK3)*	326
gCA_1	TGCGACATGTAGGACGGAAC	TGGGCGATGAAAGATGGACG	*gCA (L8HK20)*	671
gCA_2	GATCGTTGGCCACTGGGTTA	TGACAAAAAGCTGCGTGTGG	*gCA (L8HK20)*	645
gCA_3	GCCTGTACGATAAGCAGCCT	GAACGATAACAACGCGGGG	*gCA (L8GFM8)*	579
gCA_4	GTGGGCAAGAAGAGCTCCAT	GCACACGTCAGCCACTGTAT	*gCA (L8GFM8)*	419
qgCA_1	CAACAAGCACACTACGCTGG	GTCATACCAGACGGAGGCAC	*gCA (L8HK20)*	130
qgCA_2	CCACTGGGTTACCATCGGTC	CACCAAGGATGGAGTGCCGAT	*gCA (L8HK20)*	125
qbCA_1	GCAGGAACCTCAAGGACGAA	GCTTACGGCAGCAGTGTTTT	*mitBCA (L8GR38)*	128
qbCA_2	GCTTGCTTCCTCCATCTCCC	GTGCTGTGAAGGGGTGAAGA	*mitBCA (L8GR38)*	96
qbCA_3	TTCCTAATGACGCGGACAGG	GAAAACGTTCGCGCTCTTCC	*mitBCA (L8GR38)*	125
18S900 (RG)	GCCCAGATCGTTTACCGTGA	CATTACCCTAGTCCTCGCG	*18s rRNA (L8GGJ8)*	148

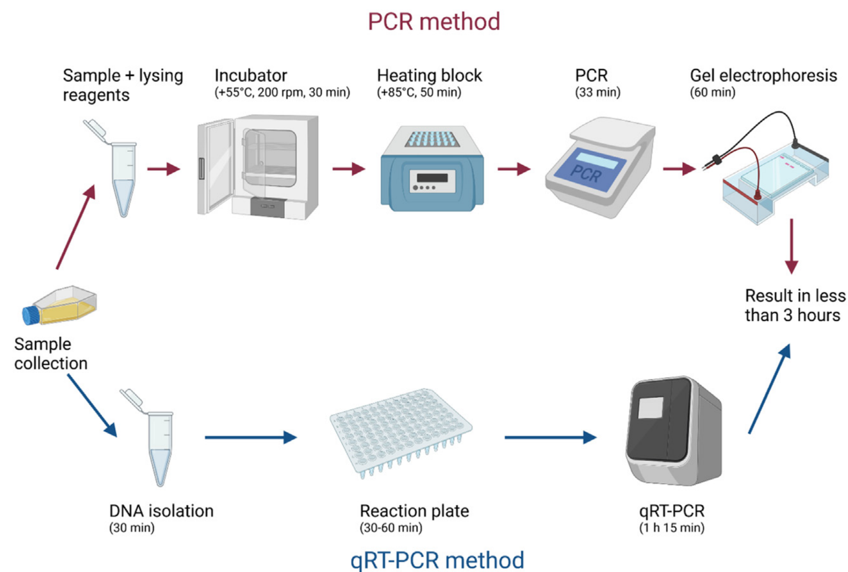

Fig. 1. The workflow for performing PCR (above) and qRT–PCR (below).

with 0.5 μL of Midori Green (Nippon Genetics Europe GmbH, Düren, Germany) at 105 V for 60 minutes. The picture of the gel was taken with Image Lab^{TM} Software (version 6.0, 2017, Bio-Rad Laboratories Inc., Hercules, CA, USA) with GelGreen protocol and otherwise default settings but inverted colors. The obtained gel bands were confirmed using Sanger sequencing by the Tampere Genomics Facility.

2.5. qRT–PCR method

We designed qRT–PCR primers with the NCBI Primer-BLAST tool, as described above. As a reference gene, we used primer pair 18S900 from the 18S rRNA gene, as recommended in Köhsler et al. [22] (Table 1). qRT–PCR was performed in a MicroAmp^{TM} Optical 96-Well Reaction Plate with Barcode (Applied Biosystems^{TM}, Thermo Fischer Scientific) with the reaction mix recommended by the manufacturer of PowerUp^{TM} SYBR^{TM} Green Master Mix (Applied Biosystems^{TM}, Thermo Fischer Scientific). The isolated genomic DNA had varying concentrations between 0.001 ng/μL and 100 ng/μL per well. Instead of a DNA sample, we used 1 μL of H_2O for the no template control and 1 μL of the isolated human genome from buccal mucosa (8.4 ng/μl) for the negative control. The assay was run with a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems^{TM}, Thermo Fischer Scientific) with the program recommended by the manufacturer of the master mix with an annealing and extension temperature of +62°C. Due to the lack of clinical samples, we only generated a standard curve.

3. Results and discussion

We aimed to improve the time-consuming and challenging diagnosis of AK by developing ultrasensitive and rapid diagnostic methods based on both traditional PCR and qRT–PCR (Fig. 1).

For traditional PCR, we identified three equally rapid, sensitive, and accurate primer pairs with the same PCR protocol, hence providing three alternative pairs to use (Table 1). All three primer pairs can detect amoebae from a sample ranging from only one cyst or trophozoite to many thousands without unspecific amplification of human genes (Fig. 2). They can also be used in parallel PCR to verify the achieved result. The protocol includes only five simple steps (Fig. 1) and gives the result in less than three hours. The protocol in traditional PCR can be performed in any diagnostic laboratory without requiring special laboratory equipment. Two primer pairs detected mitochondrial β-CA (Entry ID L8GR38) and one γ-CA (Entry ID L8HK20).

In qRT–PCR, both primer pairs (Table 1) were found to detect *A. castellanii* from a sample with only 0.01 ng of the genome. The whole genome of *A. castellanii* is 42.02 Mb [23] equivalent to 1.9 ng, indicating that the method can recognize only 1 amoeba from a sample. DNA isolation takes approximately 30 minutes, and qRT–PCR takes 1.25 hours. Depending on the pipetting method, the duration from

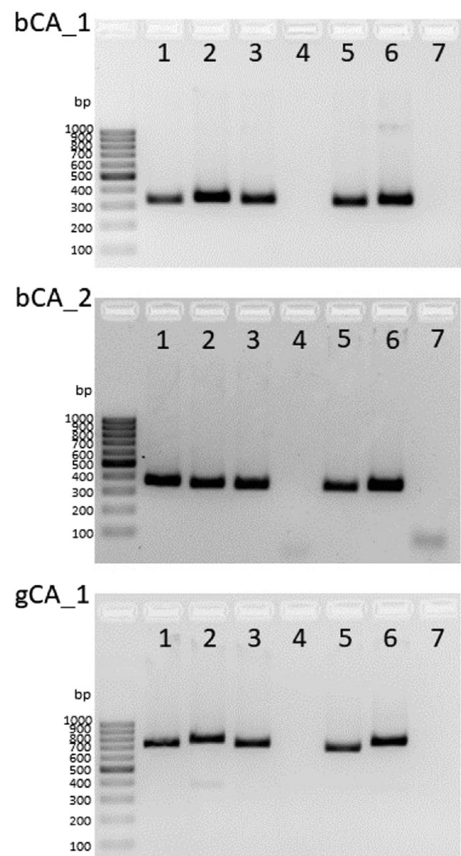

Fig. 2. Traditional PCR results of all three primer pairs used in the diagnostic method visualized in agarose gel (figure cropped with Image Lab^{TM} Software [version 6.0]) . A 1 kbp ladder was used as a standard marker. The lines were assigned as follows: 1) one cyst; 2) one trophozoite; 3) 10 amoebae; 4) buccal mucosa; 5) buccal mucosa and amoebae; 6) hundreds of amoebae; 7) negative control

sample collection to result is approximately 2.5 to 3 hours: the same range as the traditional PCR method.

Several diagnostic methods based on PCR have been developed; however, they have limitations, for instance, time-consuming procedures, the need for DNA isolation before PCR, and a limited ability to detect no less than 10 amoebae in a sample [24–26]. Previously presented most commonly used PCR methods are qRT–PCR or isothermal loop-mediated amplification [27,28] and were designed to detect the 18S ribosomal RNA gene [28]. Previously presented DNA amplification directly from a smear sample was at least as fast as our method and has no requirement for DNA isolation, yet, they do not state the lowest number of amoebae detected or whether they can recognize cysts [13].

At least six commercially available diagnostic kits for detecting AK are all intended for research purposes only. "*Acanthamoeba*_spp 18S ribosomal RNA (18S) gene" (Genesig, Primerdesign, Chandler's Ford, UK), "*Acanthamoeba castellanii* Real-time PCR Kit" (Nzytech, Lisbon, Portugal), "ParoReal Kit Acanthamoeba" (Ingenetix, Wien, Austria) and "HumPCR™Acanthamoeba Detection Kit and HumqPCR-real-time™ Acanthamoeba Real-Time PCR Kit" (Bioingentech Ltd., Concepción, Bío Bío, Chile) provide 18S ribosomal RNA gene -detection based kits; "Techne™ PrimePRO QPCR DNA detection Kit, All Acanthamoeba species" (Thermo Fisher Scientific) does not provide further information on the target gene. They require DNA isolation, and none of the kits provide instructions for performing isolation. They are also more time-consuming than either of our methods; the fastest one promises results in five hours at the minimum. Five are based on quantitative real-time PCR, and the sixth, "HumPCR™Acanthamoeba Detection Kit", uses traditional PCR as our first PCR method. Our qRT–PCR method also requires DNA isolation before the reaction, for which we recommend a commercially available kit. In contrast, our traditional PCR method does not require DNA isolation, removing one error-sensitive step from the protocol.

To summarize, we developed 2 PCR-based methods to detect *A. castellanii* parasites in less than 3 hours, enabling faster diagnosis and initiation of treatment from the emergency room visit. More rapid diagnosis also hinders unnecessary treatment starting with a suspicion of AK, and thus, treatment-related harmful side effects can be avoided. We only had samples from cultured parasites, and the negative control sample was a buccal mucosa sample from a healthy volunteer; thus, our method needs further testing with clinical samples.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Author contributions

SP, MSP and SH designed the experiments; SH conducted the PCR laboratory work; SH and MSP conducted the qRT–PCR laboratory work; SH prepared the first manuscript draft; all authors wrote and finalized the article.

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