Acanthamoeba keratitis: improving the Scottish diagnostic service for the rapid molecular detection of Acanthamoeba species

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Acanthamoeba species are responsible for causing the potentially sight-threatening condition, Acanthamoeba keratitis, which is commonly associated with contact lens use. In this report, we highlight the challenges faced using conventional laboratory identification methods to identify this often under-reported pathogen, and discuss the reasons for introducing the first national service in Scotland for the rapid and sensitive molecular identification of Acanthamoeba species. By comparing culture and molecular testing data from a total of 63 patients (n=80 samples) throughout Scotland presenting with ocular eye disease, we describe the improvement in detection rates where an additional four positive cases were identified using a molecular assay versus culture. The testing of a further ten patients by confocal imaging is also presented. This report emphasizes the importance of continuing to improve clinical laboratory services to ensure a prompt, correct diagnosis and better prognosis, in addition to raising awareness of this potentially debilitating opportunistic pathogen.

INTRODUCTION

Acanthamoeba keratitis is an uncommon but sight-threatening disease of the cornea caused by free-living protozoan amoebae belonging to the genus Acanthamoeba (Lorenzo-Morales et al., 2013). This painful, often unilateral eye disease may eventually progress to blindness and is associated with contact lens use but can also be diagnosed in non-contact lens wearers. Acanthamoeba species can be found in a variety of different environments including recreational and drinking water, soil and air (Gianinazzi et al., 2009; Legarreta et al., 2013; Rivera et al., 1991). Trophozoites and highly robust cysts can persist for years and are often resistant to drug treatments.

Global increases of Acanthamoeba keratitis occurred during the 1970s and 1980s with links to contact lens use in over 85% of cases, which is consistent with recent data from America and Canada (Stehr-Green et al., 1989; Fraser et al., 2012; Page & Mathers, 2013). In addition, non-commercial saline solutions have been implicated as potential risk factors (Newton et al., 1986). The UK experienced an increase in cases during the 1990s which coincided with increased use of soft contact lenses (Illingworth et al., 1995).

There is a lack of recent robust data on the prevalence of Acanthamoeba keratitis in Scotland, but based on a study by Seal et al. (1999), the incidence was 149 per million soft contact lens wearers compared with much lower incidence rates of around 18 to 21 per million in the rest of the UK (Radford et al., 2002). Presently, there are approximately 3.7 million contact lens wearers in the UK (British Contact Lens Association, http://www.bcla.org.uk/).

An early, accurate diagnosis is crucial for achieving a better prognosis. However, conventional techniques to detect Acanthamoeba species may lack sensitivity, which is likely to result in the true prevalence being underestimated. The challenges of an early diagnosis are compounded by the ability of Acanthamoeba to mimic non-specific clinical symptoms of viral, bacterial and fungal ocular pathogens (Hammersmith, 2006). To add to the complexity of managing Acanthamoeba keratitis, the treatment regime is aggressive and sometimes lengthy, emphasizing the importance of a correct diagnosis to prevent unnecessary treatment being administered to those who are uninfected. The issue of recurrence and the potential for the disease to become progressive support the need for a timely, accurate diagnosis.

Protocols in the UK for diagnosing Acanthamoeba infections are not standardized and depend upon individual practices adopted by local health boards. The method most commonly used to detect Acanthamoeba species
from ocular samples remains culture, which requires non-nutrient agar and stocks of Escherichia coli. The cultivating of Acanthamoeba species may take up to 10 days and the lack of sensitivity using this technique leads to the potential for a significant number of true positive cases being misdiagnosed, which impacts on the timely administration of treatment.

There have been several developments in the diagnosis of this disease using a variety of technologies. Molecular detection assays aimed at identifying ocular pathogens are advantageous in that they are rapid, highly sensitive and specific, requiring small quantities of clinical specimen (Tavarnati et al., 2013). The sensitivity of culture is determined by the ability to obtain at least one viable organism from the cornea but as molecular detection is based upon nucleic acid amplification rather than live organism, this eliminates concerns of pathogen viability during sampling or transportation. Another useful tool is confocal microscopy (Auran et al., 1994; Pfister et al., 1996), which is non-invasive and permits the rapid visualization of ocular structures of the cornea and surrounding nerves. In contrast to conventional microscopy where the image can be observed directly and all points in the specimen are imaged parallel, a confocal microscope optimizes illumination for a single point only (Guthoff et al., 2009). However, the actual organism causing the infection cannot be discriminated using this method and laboratory confirmatory testing is necessary.

Validating and verifying diagnostic assays for use in clinical services in line with the UK Accreditation Service requirements can be challenging, particularly for Acanthamoeba, which is still a relatively rare pathogen. Also, the quantity of material available for testing is often very small which restricts the ability to compare with other methods. In addition, comparing with culture as the ‘gold standard’ is not optimal due to its varied sensitivity. In this report, we compare confocal imaging, molecular detection and culture wherever sufficient sample was available to assess the potential of offering an improved detection service for patients as part of the diagnostic remit within the Scottish Parasite Diagnostic and Reference Laboratory (SPDRL), Glasgow, UK.

**METHODS**

A total of 90 samples were received from 73 patients presenting with ocular eye disease to local ophthalmology departments within seven Scottish health boards. Acanthamoeba testing was not requested for patients who presented with well-defined corneal infiltrates as these are suggestive of bacterial infections. Patients with all other infiltrates were tested for the presence of Acanthamoeba species. More importantly, patients with epithelial changes including punctate erosions or pseudodendrites, which can be present during the early stages of the disease in the absence of infiltrates, were suspected of having Acanthamoeba keratitis and, therefore, samples were taken.

All corneal samples for culture and molecular testing were obtained using 22 gauge (green) needles. Sample types included corneal scrapes (n=61), contact lens (n=15), contact lens fluid (n=8), eye swab (n=5) and tear fluid (n=1).

Patients were divided into two groups; the first group included 80 samples from 63 patients that were all subjected to molecular detection by the SPDRL, Glasgow Royal Infirmary, Scotland. Fifty-one of the 80 samples were also cultured in accordance to local standard operating procedures within local microbiology laboratories. The remaining 29 samples were not cultured, either because this was not performed routinely within the microbiology laboratory, or due to there being insufficient material to culture as a result of the difficulty in sampling material from the eye or a lack of patient compliance with sampling protocols. In the second group, 10 samples taken from a further 10 patients presenting with corneal infiltrates were subjected to molecular detection with or without culture and confocal imaging.

**Confocal imaging.** Confocal imaging was performed at the Ophthalmology Department, Gartnavel Hospital, Glasgow, Scotland where appropriately trained staff were available on-site to perform the technique using the Confoscan series confocal microscope (Nidek).

**Sampling and transportation for molecular detection and culture.** To perform a corneal scrape, topical proxymethacaine eye drops were applied to the patient’s eye to act as anaesthesia. At least one green (21 gauge) needle was used to remove as much of the epithelium as possible at the site of clinical pathology. Increased quantities of epithelium were removed using a further fresh needle if necessary to maximize the possibility of isolating Acanthamoeba DNA and to reduce the infection load. For molecular detection, all samples were taken using needles which were swirled in 500 μl proteose-peptone glucose transport broth (PPG broth) supplied by SPDRL (Sams Research Services Ltd, Argyll, UK) in screw-capped Eppendorf tubes. Needles were disposed of after sampling to eliminate the need to send sharp implements, in accordance with local health and safety policies. The PPG broth was supplied on request and samples were sent to SPDRL via local transport systems. The volume of broth was limited to 500 μl to maximize the recovery of DNA from a very small sample size whilst being sufficient to allow the sample to be deposited into the broth from the needle. Samples for culture were forwarded to local microbiology laboratories for processing in accordance with local microbiology standard operating procedures (UK Standards for Microbiology Investigations (SMI, https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/391169/B_216.pdf), Bacteria by B2; Investigation of bacterial eye infections which has been merged with SMI B52). Briefly, corneal tissues were sampled using needles and the samples were added to non-nutrient agar plates usually within ophthalmology clinics. The plates were transported to the local microbiology laboratory. They were then seeded with Escherichia coli and incubated at 30 °C before being examined daily using an inverted microscope for 5 days, then again on day 7 and day 10 before being discarded. The presence of Acanthamoeba species was noted by the formation of tracks along the bacterial layer.

**Sample processing.** On receipt of corneal scrapings at the SPDRL, 200 μl transport broth containing the sample was transferred to a clean Eppendorf tube. If contact lens solution was received, 200 μl of the solution was transferred to a clean tube. Contact lenses sent in fluid were prepared in the same way as for contact lens fluid. If the contact lens was sent dry, it was flushed in 500 μl PPG broth. Then, 200 μl of the broth was removed and transferred to a clean tube. DNA was extracted using QIAamp DNA mini spin columns (Qiagen, QIAGEN Ltd, UK) following the manufacturer’s instructions, with DNA eluted into a final volume of 200 μl kit buffer.

The DNA from each sample was amplified in duplicate based on the assay described by Riviere et al. (2006) with modifications. Amplification of the human albumin gene was also included as an internal control (Pongers-Willemsen et al., 1998) (Table 1).

http://jmm.sgmjournals.org
The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s and 40 °C for 5 s in a reaction volume of 25 μl. A positive control (Acanthamoeba castellanii, reference 1501/1A Culture Collection of Algae and Protozoa) was run with each assay and a negative control substituting DNA for water was also included. Initial validations were performed that included testing samples which were positive for ocular pathogens other than Acanthamoeba species including adenovirus, herpes simplex virus, varicella zoster and chlamydia. All samples tested negative, as expected, using the Acanthamoeba-specific assay. Tenfold serial dilutions of the reference strain of A. castellanii in PPG broth were also examined to confirm the sensitivity of the assay. Riviere et al. (2006) reported sensitivities as low as ten trophozoites or cysts which were confirmed by SPDRL (data not shown) and have also been reported by Thompson et al. (2008).

### RESULTS

#### Molecular detection only

A total of 29 samples were subjected to PCR only. Twenty-seven were PCR-negative and two were PCR-positive. The PCR positive samples, both corneal scrapes, were obtained from two different patients.

#### Molecular detection versus culture

A total of 51 samples were subjected to both PCR and culture (corneal scrapes, n = 36; swab, n = 1; contact lens, n = 9; contact lens fluid, n = 5) (Table 2). An additional four positives from four different patients were detected using the molecular assay compared with culture (Table 2).

Of the 46 samples where Acanthamoeba was not detected either by culture or PCR, 14 grew organisms other than Acanthamoeba species (Table 3). No mixed infections were detected.

### Table 1. Sequences of Acanthamoeba-specific oligonucleotides and internal control oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthamoeba forward primer (TaqAcF1)</td>
<td>CGACCAGCGATTAGGAGACG</td>
</tr>
<tr>
<td>Acanthamoeba reverse primer (TaqAcR1)</td>
<td>CCGACGCCAAGGACGAC</td>
</tr>
<tr>
<td>Acanthamoeba probe (TaqAcP1)</td>
<td>FAM-TGAATACAAAAACACCACCATCGGCGC-BHQ2</td>
</tr>
<tr>
<td>Albumin forward primer</td>
<td>TGAACATACGTCCCCAAGAGTTT</td>
</tr>
<tr>
<td>Albumin reverse primer</td>
<td>CTCCTITTCTCAGAAAGTGTGCATAT</td>
</tr>
<tr>
<td>Albumin probe</td>
<td>VIC-TGCTGAAACATTACCTTCCATGCAGA-TAMRA</td>
</tr>
</tbody>
</table>

#### Molecular detection versus confocal imaging and culture

Samples from a separate group of ten patients deemed to be at high risk of Acanthamoeba keratitis were subjected to both confocal imaging (examples of results shown in Fig. 1) and molecular detection. Five of those samples were also subjected to culture. The remaining five samples were not cultured due to insufficient material. A comparison of confocal imaging, molecular amplification and culture to assist with a diagnosis of Acanthamoeba keratitis is shown in Fig. 2.

### DISCUSSION

Firstly, this report highlights the importance of improving diagnostic services at a local and national level whilst raising awareness of Acanthamoeba keratitis. In view of the supporting data, the SPDRL now offers a molecular identification service to all users throughout Scotland to detect Acanthamoeba species directly from ocular specimens. Feedback from hospital users described the challenges in maintaining a culture service specifically for this pathogen, which included issues around detection sensitivity, lengthy turnaround times for reporting results and the labour intensive procedure. Health board protocols often required microbiology laboratory staff to be present within ophthalmology departments to permit the addition of the sample directly to culture media. Cultures can take days to weeks.

### Table 2. Molecular detection versus culture of Acanthamoeba species

<table>
<thead>
<tr>
<th>Acanthamoeba culture-negative</th>
<th>Acanthamoeba culture-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-negative n = 46</td>
<td>PCR-positive n = 4</td>
</tr>
<tr>
<td></td>
<td>Acanthamoebaculture-</td>
</tr>
<tr>
<td></td>
<td>positive n = 1</td>
</tr>
</tbody>
</table>

### Table 3. Pathogens other than Acanthamoeba species cultured from corneal tissue

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium species</td>
<td>2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1</td>
</tr>
<tr>
<td>Gram-negative bacillus</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>3</td>
</tr>
<tr>
<td>Yeast</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus mitis</td>
<td>2</td>
</tr>
</tbody>
</table>
to become positive and sensitivity can be variable. Mathers et al. (1996) reported the screening of 217 patients where there were no positive cultures despite cytology being positive for Acanthamoeba species in 20% of patients (n=43). Similarly, Lehmann et al. (1998) described the amplification of Acanthamoeba species DNA in 16 corneal epithelial samples despite only 10 (53%) of these samples being successfully cultured. More recently, a study by Yera et al. (2007) described the screening of patients deemed to be at risk or to have clinical signs of Acanthamoeba keratitis. Of the 15 samples which were positive by molecular detection, only one was positive by culture. This lack of positives resulting from reduced sensitivity of conventional techniques combined with pathogen rarity creates challenges when maintaining staff competencies in recognizing positive Acanthamoeba cultures.

Our data are supportive of those described by Thompson et al. (2008) who validated the Rivière et al. (2006) real-time PCR assay by comparing the results with culture, resulting in the identification of four additional positives using a PCR assay. Other groups have also assessed molecular-based tools including gel-based PCR assays (Schroeder et al., 2001), real-time PCR assays (Qvarnstrom et al., 2006) and loop-mediated isothermal amplification (Lek-Uthai et al., 2009; Ge et al., 2013; Yang et al., 2013). Our data support the use of molecular tools for identifying this pathogen, particularly as only one of five PCR-positive samples produced Acanthamoeba in culture. The carrier rate of Acanthamoeba species in contact lens wearers with no symptoms of Acanthamoeba keratitis has not been examined in the Scottish population and would require further investigation to assess the likelihood of false positives using more sensitive methods. However, it is assumed that the laboratory findings in this report describing additional PCR-positive samples are genuine, taking into account the clinical suspicions of an Acanthamoeba species

Fig. 1. (a) Corneal infiltrate around a corneal nerve; this is pathognomonic of Acanthamoeba keratitis. (b) Round hyper-reflective bodies present in a patient with corneal infiltrate and Acanthamoeba-positive DNA.

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**Fig. 2.** Comparison of confocal imaging, molecular amplification and culture to assist with a diagnosis of Acanthamoeba keratitis.
keratitis diagnosis in this patient group. Another consideration to support the introduction of the molecular assay is that the time taken to process samples significantly improved from days or weeks to within 24 h.

We describe the testing of contact lenses and contact lens fluid as alternatives to corneal scrapes. Since Acanthamoeba species access ocular tissue by first attaching to the contact lens, identifying this organism from a contact lens or within lens fluids may be suggestive of involvement of this pathogen, which could therefore assist with a diagnosis in conjunction with clinical findings. However, it should be noted that isolating a pathogen in non-clinical material is not direct evidence for its role in the disease process, as shown by Devonshire et al. (1993) who demonstrated Acanthamoeba species from 3.3% of contact lens cases in asymptomatic contact lens wearers. Advantages of testing contact lenses and/or contact lens fluid are the ease of sampling, which might be preferable for certain patients, and greater sample volume. The possibility of analysing tear fluid, which contains antibodies against Acanthamoeba surface proteins, is being evaluated as excessive tearing in patients is a feature of the disease and results in non-invasive samples which are easily collected. Corneal tissue testing remains the optimal choice since identifying this pathogen directly from tissue is conclusive evidence for its involvement in the disease process and an additional benefit is that the removal of the cornal layer decreases the pathogen load.

The first report of using confocal microscopy to assist with a rapid, non-invasive Acanthamoeba keratitis diagnosis was over 20 years ago by Chew et al. (1992). An association with reflective round bodies has been found in up to 85% of cases clinically presenting as Acanthamoeba keratitis (Mathers et al., 1996; Parmar et al., 2006). Sensitivities of around 90 to 100% have been described using confocal microscopy compared with much lower sensitivities of 0 to 50% using culture (Mathers et al., 1996; Parmar et al., 2006; Kanavi et al., 2007; Tu et al., 2008; Vaddavalli et al., 2011). Supporting laboratory evidence is still required as it is not possible to state with certainty if the round structures are definitely Acanthamoeba cysts or trophozoites. In a study by Mathers et al. (2000), 23% of PCR-positive samples lacked round structures, reflecting our data. It is perhaps possible that the formation of extensive scar tissue prevents accurate visualization of these structures. In contrast, we also identified PCR-negative samples from cases which had round structures. Confocal imaging is subjective and structures thought to be Acanthamoeba can be difficult to differentiate from leukocytes or epithelial nuclei (Villani et al., 2014). The experience of the observer is a key factor in achieving an accurate diagnosis, which is evidenced by Hau et al. (2010) who report that observers with variable or no experience demonstrated a sensitivity of up to 55% compared with culture. Further work is required to examine this technique on a much larger sample size combined with in-depth training to conclude the usefulness of this subjective technique.

This report highlights the importance of collaborations between clinicians and laboratory scientists combining skills and expertise to evolve laboratory services. Offering molecular detection of Acanthamoeba species directly from clinical specimens removes the need for lengthy, laborious, insensitive culturing of samples resulting in an improved, accurate, timely diagnosis of this potentially sight-threatening disease and a better prognosis. Since access to molecular expertise and specialized equipment is limited to certain microbiology laboratories, offering this test nationally on one site allows clinicians to access this improved service irrespective of the patient’s residency within Scotland. In addition, it permits the collection of essential prevalence and epidemiological data which are currently lacking, and provides the potential to gather novel data on possible seasonal and geographical fluctuations whilst ensuring a robust collection of materials for future developmental projects. As knowledge on the pathogenicity of Acanthamoeba is lacking, further studies are ongoing at SPDRL using molecular tools to provide an insight into the genotypes and associations with antimicrobial resistance in a bid to gain a fuller understanding of this potentially sight-altering pathogen.

ACKNOWLEDGEMENTS

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REFERENCES


A molecular detection service for Acanthamoeba species


