Detection and identification of Acanthamoeba and other non-viral causes of infectious keratitis in corneal scrapings by real-time PCR and next-generation sequencing-based 16S-18S gene analysis

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ABSTRACT

Acanthamoeba is a free-living amoeba of extensive genetic diversity. It may cause infectious keratitis (IK), which can also be caused by bacteria, fungi, and viruses. High diagnostic sensitivity is essential to establish an early diagnosis of Acanthamoeba-associated keratitis. Here, we investigated the applicability of next-generation sequencing (NGS)-based ribosomal gene detection and differentiation (16S-18S) compared with specific real-time PCR for detection of Acanthamoeba. Two hundred DNAs extracted from corneal scrapings and screened by Acanthamoeba-specific real-time PCR were analyzed using an in-house 16S-18S NGS assay. Of these, 24 were positive using specific real-time PCR, 21 of which were positive using the NGS assay. Compared with real-time PCR; the specificity and sensitivity of the NGS assay were 100% and 88%, respectively. Genotypes identified by the NGS assay included T4 (n = 19) and T6 (n = 2). Fungal and bacterial species of potential clinical relevance were identified in 31 of the samples negative for Acanthamoeba, exemplified by Pseudomonas aeruginosa (n = 11), Moraxella spp. (n = 6), Staphylococcus aureus (n = 2), Fusarium spp. (n = 4), and Candida albicans (n = 1). Conclusively, the 16S-18S assay was slightly less sensitive than real-time PCR in detecting Acanthamoeba-specific DNA in corneal scrapings. Robust information on genotype was provided by the NGS assay, and other pathogens of potential clinical relevance were identified in 16% of the samples negative for Acanthamoeba. NGS-based detection of ribosomal genes in corneal scrapings could be an efficient screening method for detecting non-viral causes of IK, including Acanthamoeba.

Keywords: Denmark, keratitis, NGS, ocular disease, microbiome, parasite infection
INTRODUCTION

Acanthamoeba is a free-living amoeba found primarily in soil and water. The genus exhibits a high degree of genetic diversity, and to date, 20 genotypes have been identified (1). Acanthamoeba can cause infections keratitis (IK), which can lead to blindness if left untreated (2). The overall disease burden of Acanthamoeba-associated keratitis (AK) remains low with an estimated prevalence of 1–9/100,000 according to the Orphanet database (2). However, the incidence has increased dramatically since the first cases of AK were reported in the 1970s (3). This increase is most likely associated with increased clinical awareness, the development of sensitive tests, and an increase in the number of individuals exposed to risk factors.

Acanthamoeba-associated keratitis is often seen in contact lens wearers (4,5). A recent study from Denmark, which included PCR-diagnostic data on cornea scrapings tested at our laboratory, revealed that 89% of AK patients were contact lens users, and 49% and 54% had received corticosteroids and treatment for herpes before the diagnosis was established (6). In Denmark, an Acanthamoeba-positive test result is typically seen in unilateral cases of IK not responding to treatment with chloramphenicol, ciprofloxacin, and/or tobramycin (https://en.ssi.dk/news/epi-news/2016/no-44---2016).

In general, the incidence ranges between 17 to 70 cases per million individuals wearing contact lenses (3). Other events such as corneal surgery, trauma or exposure to contaminated water have also been associated with AK.

An early diagnosis of AK is critical to ensuring a good prognosis (7). However, since IK also can be caused by bacteria, fungi, and viruses with a significant clinical overlap, establishing a diagnosis requires clinical expertise supported by specialized microbiological diagnostics, e.g. real-time PCR, confocal microscopy and/or culture using specialized media (8). This was illustrated in a German multicenter study, in which 172 cases of AK were reported. Only 23% were initially diagnosed as AK. Several other causes were suggested: 48% were first attributed to herpes simplex virus, 25% were thought to be of bacterial origin, and 3% were...
wrongly attributed to fungi (9) While an early diagnosis is critical to the prognosis of AK, an average of 2.8 ± 4.0 months (range, 0–23 months) of delay between symptom debut and the diagnosis has been reported (9).

Since IK can be caused by a variety of microorganisms and often reflects polymicrobial co-infection also including fungi and/or bacteria in addition to *Acanthamoeba* (10,11), a strategy to addressing this situation could be to apply Next Generation Sequencing (NGS) of ribosomal genes in corneal scrapings to screen for all non-viral causes of IK in the early phase of the disease.

In this study, we used a recently developed amplicon-based sequencing assay targeting parasites, fungi, and bacteria based on analysis of nuclear ribosomal genes (16S and 18S) amplified from genomic DNA extracted directly from clinical cornea scrapings (12,13). We compared the results obtained by a well-established real-time PCR for *Acanthamoeba* with the detection and differentiation nuclear ribosomal genes present in cornea scrapings from patients with keratitis in order to i) evaluate the sensitivity of 16S-18S assay compared with real-time PCR, ii) evaluate the robustness of genotyping based on 16S/18S analysis, and iii) explore the potential of comprehensive 16S/18S analysis in terms of simultaneous detection of clinically relevant fungi, parasites, and bacteria as causative agents of keratitis.

**MATERIALS AND METHODS**

**Sampling and reference data**

A total of 200 corneal scrapings collected in 2015 and 2016 were included in this study. These samples had been referred for *Acanthamoeba* PCR at the laboratory of Parasitology, at Statens Serum Institut, Copenhagen, Denmark from patients with suspected IK. Clinicians are advised to take representative and sufficiently large samples in sterile saline water. Samples are usually processed within 24 hours from sampling. DNAs had been extracted using the Qiamp DNA Blood and Tissue Kit (Qiagen). The Taqman-based
real-time PCR assay for *Acanthamoeba* spp. in use in our routine diagnostic laboratory was a single-assay modification of the triplex-assay previously described Qvarnström et al., 2006 (14). All samples had been tested in duplicates by real-time PCR. One water sample (non-template included to test for contamination including between samples) was included and ‘DNA-extracted’ in each run (number of samples extracted per run = 12); these water samples were also included in the real-time PCR and NGS assays. Moreover, a water sample is included in the real-time PCR and in the NGS assay. In the real-time PCR, DNA from an *Acanthamoeba* culture (kindly provided by Marianne Lebbad, Public Health Agency of Sweden, Solna, Sweden) was included as a positive control. No positive controls were included in the NGS assay, since this is a microbe profiling assay and not developed as a diagnostic assay as such. Both real-time PCR-positive (n = 24) and -negative (n = 176) samples were included in this study for validation of the 16S-18S assay. Two of the PCR-positive samples were weakly positive, which means that only one of the two duplicates yielded a signal, and this signal reflected a Ct value of 40 or more.

**16S-18S assay: NGS-based detection and differentiation of nuclear ribosomal genes**

Amplification of nuclear ribosomal genes was performed as previously described (13,15,16). Briefly, the three following primer sets were chosen for 18S rRNA genes: G3F1/G3R1 (GCCAGCAGCCCGTAATTTC / ACATTCTGGCAATGCTTTCGCAG), G4F3/G4R3 (CAGCCGCGTAAATTCCAGCTC / GGTGGTGCCCTTCCGTCAAT) and G6F1/G6R1 (TGGAGGGCAAGTCTGGTGCC / ACGGTATCTGATCGTCTTCGATCCC). G3 and G6 primers target the hyper-variable regions V3-V4, and G4 targets the hypervariable region V3-V5 of the 18S rRNA gene. For 16S rDNA amplification, we used a modified version of the published universal prokaryotic primers 341F/806R, targeting the V3-V4 hyper-variable regions (17). The forward primer had three additional nucleotides attached in the 5’ end (ACTCCTAYGGGRBGCASCAG, 341F3) and the reverse primer had five additional nucleotides attached in the 5’ end (AGCGTGACTACNNGGTATCTAAT, 806R5).

For each primer pair, rDNA was amplified using a short PCR setup as follows: initial denaturation at 95 °C for 3 min was followed by 20 cycles of 95 °C (16S: for 30 sec; 18S: for 1 min), 60 °C for 1 min, and 72 °C for
30 sec; final elongation was carried out at 72 °C (16S: for 7 min; 18S: for 4 min). PCR was performed in a 25 µL volume, using the Extract-N-Amp PCR ReadyMix (Sigma-Aldrich, St Louis, MO, USA) with 0.4µM of each primer and 2 µL template. This PCR is referred to as PCR1. The products from PCR1 were prepared for sequencing by a second PCR (PCR2) using the same PCR program. PCR2 attached an adaptor A, an index i5, and a forward sequencing primer site (FSP) in the 5’ end of the amplicons and an adaptor B, an index i7, and a reverse sequencing primer site (RSP) to the 3’ end of the amplicons (Figure 1). Hence, four PCR products were generated for each sample. DNA was quantified using the Quant-iT™ dsDNA High Sensitive Assay Kit (Thermo Fisher Scientific), and PCR2 products were pooled in equimolar amounts between samples into the pooled amplicon library (PAL). Undesirable DNA amplicons were removed from the PAL by Agencourt AMPure XP bead (Beckman Coulter)-based purification in a two-step process. Firstly, DNA fragments below 300 nucleotides were removed by a 10 µL PAL to 24 µL AMPure beads ratio, following the manufacturer’s protocol and eluted in 40 µL TE buffer. Secondly, large DNA fragments above 1 kbp were removed by 10 µL AM1 to 16 µL AMPure beads ratio. The resulting AMPure beads purified PAL was denoted bPAL. The bPAL was diluted to its final concentration of 11.5 pM DNA with a 0.001 N NaOH concentration, used for sequencing on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA 29122, USA). The library was sequenced with the 500-cycle MiSeq Reagent Kit V2 in a 2 x 250nt setup (Illumina Inc., San Diego, CA 29122, USA).

Data analysis was performed using BION (http://box.com/bion). The pipeline accepts raw sequences and includes steps for de-multiplexing, primer extraction, sampling, sequence- and quality-based trimming and filtering, de-replication, clustering, chimera-checking, reference data similarities and taxonomic mapping and formatting. Non-overlapping paired reads were allowed for analysis.

Identification of Acanthamoeba genotypes

BION automatically assigns a species name to Acanthamoeba-specific ribosomal DNA sequences. According to Martín-Pérez et al. (18), Acanthamoeba genotypes can be robustly grouped based on 18S rDNA.
sequences. Clustered fasta files representing *Acanthamoeba*-specific DNA sequences were downloaded from the BION server and aligned with representative reference sequences downloaded from GenBank. The work by Martin-Péreza and colleagues (18) was used to inform the selection of reference sequences. The alignment was manually edited and phylogenetic analysis of the edited alignment used the neighbor-joining algorithm as implemented in Mega7 (19). *Acanthamoeba*-specific sequences produced by the NGS assay in this study were submitted to the NCBI Database with the following accession numbers: MT919356–MT919376.

**Identification of organisms of potential clinical relevance**

A multitude of organisms have been reported to be involved in IK, including organisms commonly found on the skin (e.g., *Staphylococcus* and *Candida*). Therefore, in the absence of a reference methodology, we reasoned that it would be useful as a preliminary set of observations to define a cut-off for detection of organisms of potential clinical importance using a standardized proportion rather than a standardized absolute number of sequences, given the vast range in sequence read output (see Results section).

Therefore, in samples negative for *Acanthamoeba* where 50% or more of the total output reflecting non-vertebrate DNA (i.e., non-host DNA) could be assigned to a bacterial or fungal genus/species, this genus/species was considered to be of potential clinical relevance. This threshold reflected a conservative approach and was based on the rationale that if at least half of the sequences produced for any given sample could be attributed to one single microorganism, the likelihood of this organism being involved in the keratitis might be considered substantial.

**Ethical considerations**

This study complied with ASM’s Ethical Guidelines (https://journals.asm.org/content/ethical-guidelines). Although the study involved DNA from human clinical samples (corneal scrapings), human DNA was not subject to analysis. Moreover, the samples were anonymized prior to analysis by the 16S-18S assay. Hence,
RESULTS

16S-18S sequence read output

16S and 18S rDNA data was generated from all samples with a range of 3,909 to 381,110 reads per sample (median, 81,848; interquartile range [IQR], 48,219–106,487) (Supplementary Figure 1). Vertebrate DNA (i.e., host DNA) represented a significant proportion of all sequences generated (median, 42,548; IQR, 24,861–64,661). Between three and 74 genera were identified in each sample.

Ability of the 16S-18S assay to identify *Acanthamoeba*-positive samples and usefulness of the obtained sequences for genotyping

A total of 24 samples had been scored *Acanthamoeba*-positive by real-time PCR, of which two samples were considered weakly positive for *Acanthamoeba*. By the 16S-18S assay, *Acanthamoeba* rDNA was detected in 21 samples, all of which had been scored positive by real-time PCR (specificity, 100%). Meanwhile, the 16S-18S assay failed to detect *Acanthamoeba* DNA in three of the samples positive by real-time PCR, two of which were weakly positive (sensitivity, 88%). None of the samples identified as *Acanthamoeba*-negative by real-time PCR were found positive by the 16S-18S assay.

For 16 samples, a result at the species level was returned by the BION. The species identified were *Acanthamoeba palestinensis* (n = 2), *Acanthamoeba hatchetti* (n = 3), *Acanthamoeba polyphaga* (n = 5), *Acanthamoeba castellani* (n = 4), *Acanthamoeba mauritaniensis* (n = 2) and the remaining *Acanthamoeba* spp. (n = 5) could only be identified to a genus level. Based on phylogenetic analysis, a total of 19 sequences reflected genotype T4, and 2 sequences reflected genotype T6.

Overall mapping of bacterial DNA in the samples

The 16S rDNA sequences were analyzed at various taxonomic levels, ranging from phylum to species. The dominant phylum was **Proteobacteria**, which on average represented 67.2% of bacterial reads (range, 0–100%). **Firmicutes** (mean, 16%; range, 0–100%), **Actinobacteria** (mean, 10.3%; range, 0–72.1%), and **Bacteroidetes** (mean, 5.0%; range, 0–59.4%) were also highly represented. Other phyla such as **Acidobacteria** (mean, < 0.1%; range, 0–3.0%), **Fusobacteria** (mean, 0.6%; range, 0–21.4%), **Deinococcus-Thermus** (mean, 0.3%; range, 0–9.3%), **Chlamydiae** (mean, < 0.1%; range, 0–76.2%), **Spirochetes** (mean, < 0.1%; range, 0–3.4%), **Tenericutes** (mean, < 0.1%; range 0–10.1%), **Thermatogae** (mean < 0.1%; range, 0–1.18%), and **Verrucomicrobia** (mean, < 0.1; range, 0–2.8%) were present, but in smaller proportions. The remaining bacterial reads from a diverse set of phyla contributed with very few reads (mean, 0.2%; range 0–9.8%). The most common bacterial genera were **Pseudomonas**, **Acinetobacter**, **Propionibacterium**, and **Streptococcus**, but their relative proportions varied greatly between the samples.

**Organisms potentially causing non-viral IK detected in Acanthamoeba-negative samples.**

In addition to *Acanthamoeba*, several other pathogens that could be involved in the development of IK were detected (Table 1). Fungal and bacterial species of potential clinical relevance were identified in 31 of the samples negative for *Acanthamoeba*; these included *Pseudomonas aeruginosa* (n = 11), *Moraxella* spp. (n = 6), *Staphylococcus aureus* (n = 2), *Fusarium* spp. (n = 4), and *Candida albicans* (n = 1). These were all accounting for 50% or more of the total sequence output reflecting non-vertebrate DNA (i.e., non-host DNA) in *Acanthamoeba*-negative samples.

**DISCUSSION**

In this study, we examined the feasibility of the 16-18S assay as a diagnostic tool for the detection of *Acanthamoeba*-specific DNA in corneal scrapings received due to infectious keratitis (IK).
Our data demonstrate that the 16-18S assay was able to detect *Acanthamoeba*-specific DNA with a sensitivity of 88% and a specificity of 100% compared with *Acanthamoeba*-specific real-time PCR. As an added benefit, the *Acanthamoeba* sequences obtained by the 16S-18S assay served both to confirm the presence of *Acanthamoeba*-specific DNA but also enabled genotyping of the strains based on the sequence output analyzed by BION. Finally, we developed an automated algorithm that identified potentially clinically relevant non-viral causes of IK as represented by at least 50% of the total sequence read count in the sample, and 31 (18%) of the *Acanthamoeba*-negative samples (n = 176) for were positive for a clinically relevant bacterium or fungus.

Reports on NGS-based approaches to detecting and differentiating organisms involved in IK are still very limited. Li et al applied metagenomics to a very limited sample set of paraffin-embedded samples (n = 16) (20). Prashanthi et al., used amplicon-based NGS of the internal transcribed spacer region to characterize alterations in the ocular surface fungal microbiome in fungal keratitis (21). So far, however, no studies have been published on amplicon-based NGS of ribosomal DNA from bacteria, fungi, and parasites in corneal scrapings.

The primers used in the 16S-18S assay are of very limited specificity compared with the primers (and probe) used in the real-time PCR assay, and the fact that the 16S-18S assay was able to detect 21 out of 24 *Acanthamoeba*-positive samples is quite remarkable, especially considering the fact that two of the 24 samples scored positive by real-time PCR were categorized as ‘weakly positive’, and so, the 16S-18S assay identified 21 out of 22 clearly positive samples (95%). No other microorganism which could explain IK was found in these samples using the 16S-18S assay. Unfortunately, we did not have any clinical information on the samples and it is not known to us whether the two faintly positive samples reflected samples from patients who had already been receiving *Acanthamoeba*-targeted treatment prior to sampling, which could explain the very weak signal. None of the three samples testing weakly positive by real-time PCR and negative by the 16S-18S assay were positive for other organisms relevant to IK by 16S-18S analysis;
However, since data from local clinical microbiology laboratories were not available for analysis in the present study, we could not investigate whether these samples were from patients with IK potentially suffering from a viral infection. The sensitivity of the real-time PCR used as a reference in the present study remains unknown. However, about 15% of all samples referred to \textit{Acanthamoeba} PCR and tested by this real-time PCR in Denmark are positive. The ability to establish a diagnosis of AK correctly based on analysis of corneal scrapings not only relies on sufficiently sensitive microbiological methods, but also on the ability to sample appropriately.

Establishing a diagnosis of AK is very difficult under normal circumstances, especially in the later stages of the disease where very few trophozoites are present and the disease is dominated by cysts. It remains to be determined if the sensitivity of this analysis is high enough for routine diagnostics or whether it will remain a supplement to the specific RT-PCR. It is possible that the assay could prove useful in the initial stage of IK, when trophozoites are more abundant than later.

More than 75% (16 samples of 21) was identified to species level providing useful information on the pathogen. Although it is encouraging that the microbiome platform enables species identification, this information should not stand alone, and needs to be supplemented by genotype determination. Table 2 summarizes data from similar studies reflecting \textit{Acanthamoeba} genotypes identified mainly in corneal scrapings, but also in corneal/nasal swabs, contact lens, and contact lens solutions. As seen, T4 is the by far the most common genotype detected, accounting for more than 83% of the cases, followed in prevalence by T3 which accounts for 6% of the cases. As suggested by the distribution of data in Table 2, the data obtained in the present study add support to the claim that T4 is by far the most common genotype involved in human AK (22). Noticeably, we identified T6 in two corneal scrapings, a genotype rarely seen in AK. Walochnik and colleagues identified the T6 genotype when analyzing a 'hyper-virulent' strain of \textit{Acanthamoeba} from a contact lens-wearing patient with keratitis managed in Austria (23). It remains unclear whether the genotypes involved in AK differ in terms of clinical course/severity and susceptibility to
treatment. Given the rarity of the infection, multi-center prospective studies would be appropriate to investigate this.

In this study, we applied a strict criterion as to which of the detected organisms that could be considered as potentially contributors to the development of IK. Hence, only bacteria or fungal species that comprised more than 50% of non-vertebrate sequence reads were included. Still, quite a few genera/species of potential clinical relevance were noticed. Being well-known causes of IK, \textit{P. aeruginosa} was detected in 11 samples and \textit{S. aureus} in two. Several other bacterial species were detected that could potentially be the cause of IK. Unsurprisingly these bacteria mainly comprised bacterial commensals found in the upper airways and as part of the oro-pharyngeal environment and the clinical significance of these findings should be evaluated on a case-to-case basis, just as it should be confirmed using conventional culturing. We did, however, adapt a very conservative algorithm to ensure that the identified bacteria was found at levels that warranted further clinical follow-up. This was also true for fungal species where we found four samples containing \textit{Fusarium} spp. and one sample containing \textit{C. albicans} all of which are potential causes of keratitis and could be of clinical significance.

The availability and cost of this assay is also a concern that needs to be taken into account. In our setup, this assay requires 17.5 technician working hours and 6 molecular bioinformatics working hours. Downstream analysis of the NGS assay output has been automatized so that the freely available software \textit{BION} annotates the sequence results automatically to taxonomic units, which significantly reduces the amount of work related to sequence read analysis.

These findings are encouraging because they demonstrate the ability of the assay to detect and differentiate microorganisms that are usually found using conventional methods. Due to ethical consideration and limitations, this study could not include data from local clinical microbiology laboratories to confirm our findings and validate our algorithm. We suggest developing a prospective study where data from the 16S-18S assay can be compared with data from routine clinical microbiology analyses and other...
investigations used to establish a diagnosis of AK (such as confocal microscopy), as this will be crucial in determining the feasibility of this platform as a frontline screening tool for non-viral causes of IK. The microbiome platform is not dependent on viable bacteria or fungi in order to determine their presence and it is conceivable that this analysis could provide new/additional information. It is not unusual for a patient to have been prescribed topical antibiotics prior to sampling for keratitis and a diagnostic approach that is independent of the viability of pathogens could be of great value. Further studies comparing the 16S-18S platform to standard culturing in this setting would be of great value. Being based on SSU rDNA and not ITS regions, the 16S-18S assay does not have sufficient discriminative power to determine molds at a species levels and subsequent analyses will be needed to make a full identification. The same issue pertains to Streptococcus spp. for which further testing will be needed for identification at a species level.

In conclusion, our study confirmed that the 16S-18S assay is able to detect the presence of Acanthamoeba spp. with a sensitivity of 88% and specificity of 100%. The assay was able to provide valuable information on Acanthamoeba genotypes. Furthermore the assay was able to detect bacterial and fungal pathogens potentially involved in IK; however, further studies are needed to ascertain sensitivity of this analysis.
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**Figure legend**

**Figure 1. Upper panel.** PCR1: Illustration of PCR of the gene target area, using genomic DNA as template. Genomic template DNA was subject to PCR-based amplification with one primer set targeting prokaryotes and three primer sets targeting eukaryotes. Each PCR was run in parallel.

**Middle panel.** PCR2: Illustration of the attachment of the required elements to amplicons for MiSeq sequencing. The products from PCR1 were used as template for the adaptor PCR, where adaptors, barcodes, and sequencing primer-binding sites were added. This was performed in parallel for each of the four primer sets. **Lower panel.** Sequencing: Illustration of the regions sequenced by the MiSeq as RD1 and RD2. See manuscript text for details.
Table 1. Organisms detected in Acanthamoeba-negative samples listed according to frequency and reflecting at least 50% of the sequence reads in the samples in which they were observed.

<table>
<thead>
<tr>
<th>Microbial genus/species</th>
<th>Number of samples in which the organism was observed</th>
<th>Possible interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(S = more likely skin contamination; C = more likely clinical relevance)</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>12</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td><em>(P. aeruginosa = 11; P. fragi = 1)</em></td>
<td></td>
</tr>
<tr>
<td>Moraxella spp.</td>
<td>6</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td><em>(M. cattharalis = 4; M. nonliquefaciens = 2)</em></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>5</td>
<td>S</td>
</tr>
<tr>
<td>Fusarium spp.</td>
<td>4</td>
<td>C</td>
</tr>
<tr>
<td>Malassezia globosa</td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>4</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td><em>(S. mitis = 2; S. dysgalactiae = 1; S. pneumonia = 1)</em></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>2</td>
<td>C</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>Eikenella corrodens</td>
<td>1</td>
<td>C</td>
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</table>
Table 2. A selection of studies showing the distribution of *Acanthamoeba* genotypes identified in *Acanthamoeba*-positive samples such as cornea, contact lenses, contact lens solution, and swabs.

<table>
<thead>
<tr>
<th>Enterococcus faecium</th>
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<th>C</th>
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<tbody>
<tr>
<td>Total</td>
<td>40</td>
<td>C (n = 31), S (n=9)</td>
</tr>
</tbody>
</table>

*Table 2. A selection of studies showing the distribution of *Acanthamoeba* genotypes identified in *Acanthamoeba*-positive samples such as cornea, contact lenses, contact lens solution, and swabs.*
<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Sample material</th>
<th>No. of samples analyzed</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T9</th>
<th>T10</th>
<th>T11</th>
<th>T13</th>
<th>T15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behera et al. (24)</td>
<td>India</td>
<td>Cornea</td>
<td>18</td>
<td>16</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>Rocha-Cabrera et al. (25)</td>
<td>Spain</td>
<td>Cornea</td>
<td>17</td>
<td>17</td>
<td></td>
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<tr>
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