The *Acanthamoeba*–Fungal Keratitis Study

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• PURPOSE: **To ascertain the incidence of *Acanthamoeba* keratitis and the coexistence of *Acanthamoeba* and fungi in microbial keratitis.**
• DESIGN: **Prospective cross-sectional study.**
• METHODS: **Patients presenting with stromal keratitis were additionally tested for *Acanthamoeba* irrespective of the clinical diagnosis. Culture positivity was the gold standard.**
• RESULTS: **Of the 401 cases included in the study, 40 were positive for *Acanthamoeba* (10%); of these 40, 16 were positive for both *Acanthamoeba* and fungi (4.5% of the study group was *Acanthamoeba* and fungal keratitis positive); 5 were positive for *Acanthamoeba* and bacteria; and 2 had triple infection with *Acanthamoeba*, fungi, and bacteria. Ring infiltrates and stromal edema are frequently associated with *Acanthamoeba* keratitis, as well as in *Acanthamoeba* coinfections. Ring infiltrates in particular were more frequently seen in the *Acanthamoeba* and fungal keratitis group (8/16) and they were often yellowish with hyphate edges (vs ring infiltrates only, which are seen in the patients with *Acanthamoeba* alone). Only 2 patients were contact lens wearers: however, they presented with history of trauma.**
• CONCLUSIONS: ***Acanthamoeba* coinfections are much more frequent and are not restricted to contact lens users. Anticipating coinfections is necessary for establishing a diagnosis as well as for appropriate and timely therapeutic interventions. (Am J Ophthalmol 2019;201:31–36. © 2019 Elsevier Inc. All rights reserved.)**

THE MOST COMMON ASSOCIATION/RISK FACTOR FOR *Acanthamoeba* keratitis is the use of contact lenses, accounting for nearly 85%–88% of reported infections.[1] Contact lens related *Acanthamoeba* keratitis may occasionally be associated with bacterial and fungal coinfections, the frequency being sufficiently rare as to warrant individual case reports. Rumelt and associates[2] described the first coinfection involving *Scedosporium apiospermum* in 2001. This was followed by a similar report by Froumis and associates,[3] once again implicating *Scedosporium apiospermum* and *Acanthamoeba* in a contact lens user. Concurrent coinfections with *Acanthamoeba* species and *Fusarium* have been reported by Lee and associates,[4] Bullock and Warwar,[5] and Lin and associates.[6] Padzik and associates in their series of 26 recalcitrant cases of keratitis in contact lens wearers identified 11 *Acanthamoeba* keratitis cases, 6 of which had coinfections with either bacteria or fungi.[7] Coexistent infections of *Acanthamoeba* with bacteria have been reported by Hong and associates[8] and Sizmas and associates,[9] the former reporting a case of *Pseudomonas aeruginosa* with *Acanthamoeba* and the latter a case of polymicrobial keratitis in a soft contact lens wearer involving 3 pathogens, namely, *P aeruginosa*, *Alcaligenes xylosoxidans*, and *Acanthamoeba*. This propensity for *Acanthamoeba* to coexist with other microbes has generally been attributed to the peculiar and rather unique microenvironment/ecosystem created by the use of contact lenses and the associated paraphernalia, such as storage cases; and cleansing, rinsing, or storage solutions; the use of home-made saline; and the inadequacy of current disinfection systems.

Similar coinfections in noncontact lens users are scarce, with documentation of a case each by Gupta and associates[10] and Lin and associates,[6] respectively. However, in one retrospective analysis of 40 cases of *Acanthamoeba* keratitis, by Rasheed and associates, 34 concomitant fungal coinfections were identified; 16 of these were identified by culture.[11]

In vitro studies have shown that a symbiotic relationship between *Acanthamoeba*, fungi, and bacteria is possible, conferring a biologic advantage to the symbiotic organisms, thereby potentially enhancing pathogenicity as well as survival in adverse conditions. That phagocytosed *Fusarium conidia* can germinate inside *Acanthamoeba* was demonstrated by Nunes and associates.[12] It has also been shown that coculturing fungi with live amoebae or culture supernatant/lysate facilitates fungal growth and enhances survival of amoebae.[13] Although *Acanthamoeba* feeds on bacteria, paradoxically it also supports bacterial endosymbiosis. Though most of the identified symbionts are uncharacterized species, a majority are closely related to known bacterial pathogens and exhibit similar mechanisms of interaction with host cells. The ecosystem and the capacity for integration provided by the amoeba bestow several benefits to the bacteria, such as resistance to digestion, provision of critical nutrients, and regulation of genetic functions.[14–16]

Trauma is a leading cause of microbial keratitis, bacterial and fungal being the most common, with trauma-related *Acanthamoeba* keratitis reportedly a rarity. In the study by Srinivasan and associates, the former 2 accounted for

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approximately 48% each of the culture-positive cases, and the latter 1%.[17] Gopinathan and associates reported *Acanthamoeba* keratitis incidence of 2.4% in a study spanning a decade.[18] However, *Acanthamoeba* and fungi share similar environments and are widely distributed in nature.[19,20] Routine microbiological protocols usually incorporate nonnutrient agar (NNA) with *Escherichia coli* overlay only when *Acanthamoeba* is suspected; this usually hinges on the presence of any 1 of the 5 clinical stages of *Acanthamoeba* keratitis as described by Tu and associates.[21] It is definitely feasible to presume that the likelihood of more than 1 organism being introduced at the time of trauma exists, especially when the injuring agent is organic.

METHODS

THE *ACANTHAMOEBA*–FUNGAL KERATITIS STUDY WAS designed to test the hypothesis that the occurrence of *Acanthamoeba* keratitis and the coexistence of *Acanthamoeba* keratitis with other forms of microbial keratitis (with specific reference to fungal keratitis) are much more prevalent than is currently realized. This prospective diagnostic study was conducted by the Cornea & Refractive Services, and the Microbiology Department of Aravind Eye Hospital, Coimbatore, India. The study was approved by the Internal Review Board of the Aravind Eye Care System. Inclusion criteria were the presence of stromal infiltration, clinical diagnosis of microbial keratitis, the ability for the patient to cooperate for scraping, and cultures of corneal scrapings on at least blood agar and NNA. Patients with age younger than 12 years, paucity of material for scraping, infiltrates that were already responding to therapy, infiltrates less than 1 mm in diameter or too deep for scraping, very thin corneas at risk of perforation, and postkeratoplasty infiltrates were excluded from the study.[22] Suspected secondary infections in viral keratitis and peripheral ulcerative keratitis were also excluded.

After appropriate clinical evaluation of patients with stromal keratitis, which included history taking, review of current medications, vision testing, and slit-lamp evaluation, the patient was taken up for microbiological evaluation. Corneal scrapings were collected using No. 15 sterile surgical blades (Niraj Industries Pvt Ltd, Faridabad, India) to scrape the base and edges of the lesion. After inoculation onto culture plates, additional scrapings were used to prepare smears or mounts for direct microscopic examination. In addition to this routine evaluation, inoculation onto NNA was included for all cases. The identification of isolates from media was performed using classical microbiology techniques. Confocal microscopy was performed where feasible, with the Heidelberg Retina Tomograph 3 (HRT-3 with Rostock Cornea Module; Toshbro Medicals Pvt Ltd, Mumbai, India).

The primary outcome measured was the number of coexistent *Acanthamoeba* and fungal keratitis. The secondary outcomes were the total number of *Acanthamoeba* cases detected, and the correlation between clinical diagnosis and microbiological observations. Culture positivity was the gold standard used for the final analysis.

RESULTS

OVER A PERIOD OF 17 MONTHS, 401 CULTURE-POSITIVE cases (*Acanthamoeba* and/or fungal) were analyzed for the study. Forty of these were culture-positive for *Acanthamoeba* (10% of all culture-positive cases). Of these, 17 were positive for *Acanthamoeba* only (*Acanthamoeba* keratitis [AK] group), 16 positive for *Acanthamoeba* AND fungi (*Acanthamoeba* keratitis and fungal keratitis [AK-FK] group), 5 for *Acanthamoeba* and bacteria (*Acanthamoeba* keratitis and bacterial keratitis [AK-BK] group), and 2 for Acanthamoeba, fungi, and bacteria. A total of 4.5% of the study group was *Acanthamoeba* and fungal keratitis positive. It is to be noted that 2 included subjects were contact lens users, but with a history of trauma. A total of 224 confocal microscopies were performed in the study group. After exclusion of the confocal data done in the *Acanthamoeba*-positive group, an additional 17 fungal culture-positive cases had cysts identified only by confocal microscopy. It is to be noted that several cases that grew only *Acanthamoeba* had hyphae on confocal microscopy. However, in some the density of the stromal infiltration and the associated stromal edema made identification with absolute certainty difficult.

An attempt was made to identify clinical features suggestive of coexistent *Acanthamoeba* and fungal keratitis. The most common feature observed was the presence of a ring infiltrate (8/16 in the AK-FK group, 5/17 in the AK group, and 3/5 in the AK-BK group). It was compared between the groups (AK-FK, AK, AK-BK) using the 2-sample proportion test. The P value of .217 (>.05) indicates that there was no significant difference in presence of a ring infiltrate between the AK-FK and AK group. Similarly, the P values of .696 and .204 (>.05) support the conclusion that there were no differences between the AK-FK vs AK-BK groups and the AK vs AK-BK groups, respectively.

In contrast to the classical grayish ring infiltrate seen in pure *Acanthamoeba* cases (Figure 1A), the ring infiltrate in coinfections was often creamy white or yellowish, and associated with a central patchy stromal infiltration (Figure 1B). Hyphate edges or dot-like infiltrates emanating from the ring infiltrate were common (Figure 1B and C). As the lesion progressed, the ring infiltrate was replaced by a uniform circumscribed infiltration, thereby losing its resemblance to the classical ring infiltrate of *Acanthamoeba* (Figure 1D). Fungal keratitis is usually

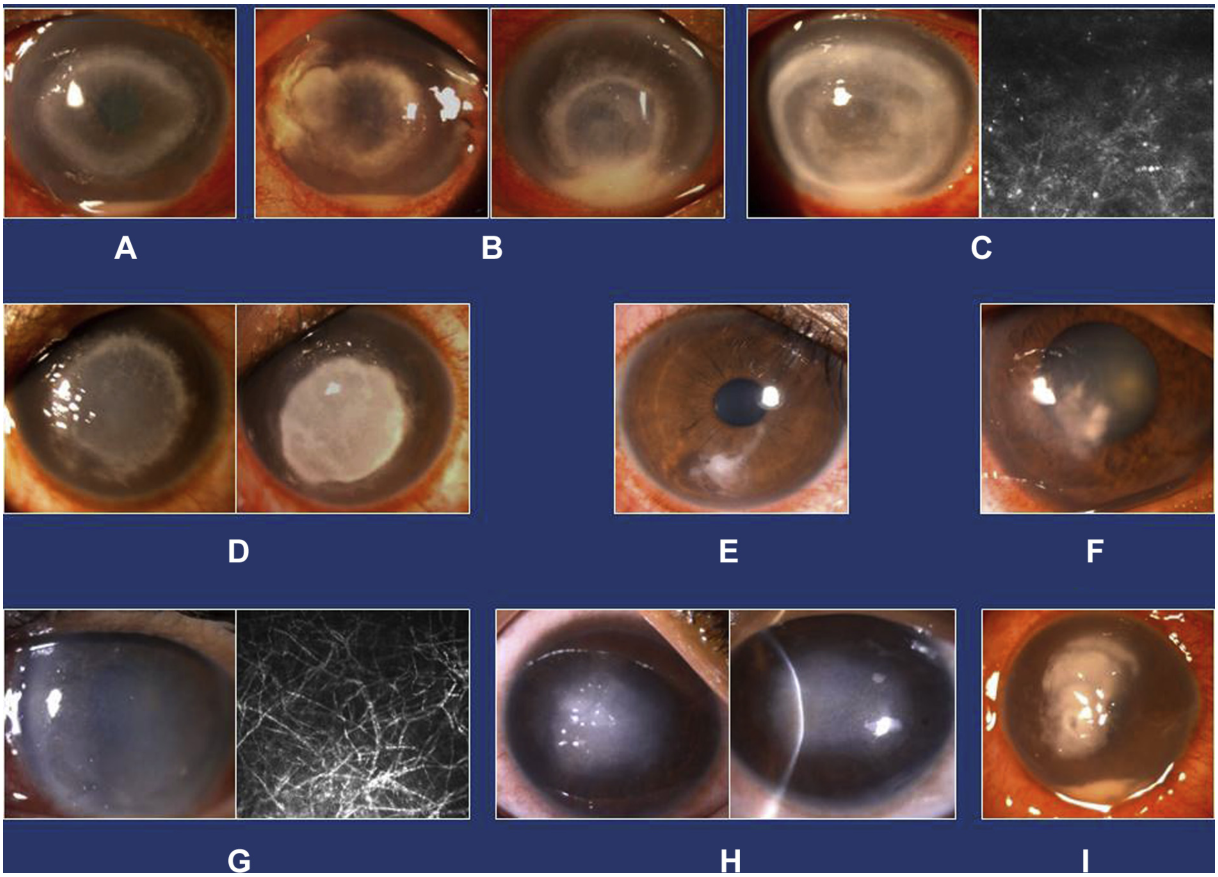

FIGURE 1. (A) Classical ring infiltrate of *Acanthamoeba* keratitis. (B) Ring infiltrates of 2 different patients with *Acanthamoeba*-fungal coinfections. (C) *Acanthamoeba*-positive ring infiltrate with characteristics of fungal keratitis: confocal microscopy reveals both hyphae and cysts. (D) Transitioning of a ring infiltrate into a uniform homogenous infiltrate. (E) Classical fungal keratitis. (F) Stromal edema with typical fungal infiltration. (G) *Acanthamoeba*-fungal coinfection, presenting as ill-defined edematous stromal infiltration: confocal microscopy shows numerous hyphae. (H) *Acanthamoeba* keratitis presenting with edematous stromal infiltration. (I) *Acanthamoeba*-fungal-bacterial triple infection.

described as a raised dry yellowish lesion. Some of our cases could not be distinguished from classical fungal keratitis (Figure 1E). Stromal edema also appears to be a common feature, either associated with an otherwise classical fungal picture (Figure 1F) or associated with an ill-defined patchy infiltration of the stroma (Figure 1G), or as a purely circumscribed edematous infiltration (Figure 1H). Some cases were indistinguishable from bacterial keratitis (Figure 1I).

An attempt was also made to correlate duration of the disease process with clinical observations. The ring infiltrate is supposedly of slow evolution in *Acanthamoeba* keratitis. However in 75% of our cases with *Acanthamoeba* and in 60% of cases of coinfection with *Acanthamoeba* and fungus, the ring infiltrate was seen in a period of 7 days, and all were evident within a month in both groups. *Fusarium* was the most common fungus encountered (accounting for over 50% in the AK-FK group), followed by the hyaline fungi and dematiaceous fungi. Gram-negative bacilli and coagulase-negative staphylococci were the most frequently isolated bacteria.

DISCUSSION

FROM THE DATA IT IS EVIDENT THAT *ACANTHAMOEBA* AND coinfections of *Acanthamoeba* with fungi are more common than anticipated, with 10 % of the study cases being positive for *Acanthamoeba* and 4.5% for coinfection with fungi. However, it would be prudent to refrain from extrapolating these data as a true incidence of *Acanthamoeba* or *Acanthamoeba*-fungal coinfections, until data with longer time frames are obtained. One of the drawbacks of the study is that a number of cases of stromal keratitis that were either fungal or bacterial positive cultures were excluded because NNA had not been incorporated in the microbiological evaluation. Secondly, it was not possible to perform confocal microscopy in all the cases. Because cultures were taken as the gold standard, a number of cases that were positive for *Acanthamoeba* and/or hyphae only via smears or confocal microscopy, were automatically excluded in the final analysis. It is evident that the true incidence of *Acanthamoeba* is much higher than expected.

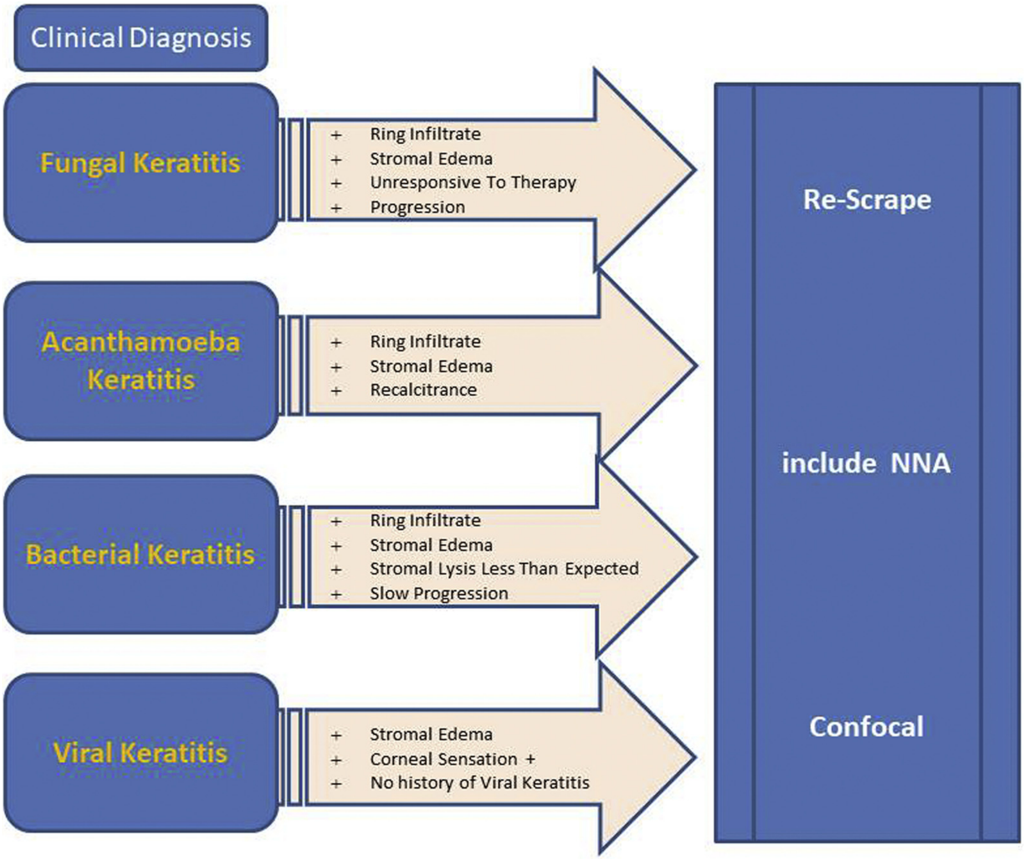

FIGURE 2. Algorithm for diagnosis of coinfections.

Continuing to incorporate NNA as often as possible into routine cultures would result in the evolution of more meaningful numbers.

What seems to be more relevant is that a substantial number of *Acanthamoeba* infections are polymicrobial, with 40% being coinfections with fungi, 12.5% with bacteria, and 5% triple infections. In their entirety, coinfections constituted 55% of all *Acanthamoeba* infections. The data from the study suggest that coinfections seem to be the norm in *Acanthamoeba* keratitis. It does not appear to be a unique association restricted to only contact lens users.

These data have significant clinical implications, as follows: (1) that finding *Acanthamoeba* should automatically elicit a search for other organisms; (2) that many cases of coinfections often resemble bacterial or fungal keratitis, and therefore unresponsive cases should be reevaluated for a potential polymicrobial component; and (3) that ring infiltrates could also suggest a coinfection associated with *Acanthamoeba*.

In the preliminary report of the AK-FK study,[22] we had emphasized the lack of correlation between the clinical diagnosis and the microbiological observations. These interpretations were based on the classical textbook descriptions of fungal, *Acanthamoeba*, and bacterial keratitis. As the study progressed, it was evident that a number of AK-FK cases were ring infiltrates along with the characteristic yellowish dry component of fungal keratitis and hyphate edges. These were automatically classified in the initial phase of the study as fungal keratitis, and therefore the diagnosis of *Acanthamoeba* keratitis clinically was much lower. Since our perceptions and understanding have evolved, we have not attempted to make a similar clinical–microbiological correlation. Figure 1B and C would have been classified as fungal keratitis owing to the presence of hyphate edges and infiltrates, with the characteristic yellowish dry appearance of fungal keratitis. These 3 cases had ring infiltrates too. However, the first 2 grew *Acanthamoeba* and *Fusarium*, while the latter grew only *Acanthamoeba*, although it also had hyphae on confocal microscopy.

It is to be also noted that establishing a coinfection very often necessitated multiple scrapings and cultures, and that only a high index of suspicion based on the aforementioned clinical findings, substantiated by the presence of hyphae or cysts on the confocal imaging, led to the establishment of a definite diagnosis. This was especially applicable to ring infiltrates. Very often either *Acanthamoeba* or fungus grew in the cultures, and the final complete identification occurred only after the keratoplasty button was subject to microbiological processing. Figure 1G shows a case of a patient treated at various centers as *Acanthamoeba* for over

2 months; initial smears and cultures were negative for *Acanthamoeba* and fungi. The confocal images were full of hyphal elements. Repeat cultures, however, were positive for *Acanthamoeba* only. The tissue obtained during keratoplasty finally grew *Aspergillus niger*.

The relevance and the impact of confocal microscopy in this study cannot be overemphasized. In many of the culture-negative cases or where only a single organism was identified, the confocal imaging was of great diagnostic value and ensured that scrapings were repeated. It also facilitated therapeutic decision making.

The management of *Acanthamoeba* and fungal keratitis can be fraught with uncertainty; resistance to therapy and capacity for recurrences are common to both. Given that therapy of both fungal and *Acanthamoeba* keratitis is unpredictable, and with significant treatment failures, the question that we finally consider is whether it is the unidentified polymicrobial nature of the infection that renders medical therapy ineffective. Several workers in the field have emphasized the value of early diagnosis and the initiation of early therapy as important predictors of favorable outcomes in *Acanthamoeba* infections.[1,23] Anticipating coinfections would therefore lead to better diagnostic approaches, especially when confronted with a recalcitrant keratitis. Ascertaining the true composition of the microbial load would perhaps result in better treatment strategies, as appropriate therapy would be initiated earlier. Figure 1H shows the rapid resolution of keratitis in a patient whose cultures grew only *Acanthamoeba*, smears and confocal microscopy being positive for hyphae. Identifying coinfections also reduced the waiting time for therapeutic keratoplasty in some cases that were indolent. Based on our experience from this study, we propose a tentative algorithm that highlights features that could potentially establish the diagnosis of *Acanthamoeba* coinfection(s) (Figure 2).

To summarize, *Acanthamoeba* and *Acanthamoeba* coinfections are more prevalent than known currently. The actual incidence, though much higher than currently quoted, would be more accurately derived with longer periods of study. It is well known that fungal keratitis is more prevalent in the Tropics; therefore, the frequency of *Acanthamoeba*-fungal coinfections could also be higher only in the Tropics. However, since *Acanthamoeba* and fungi inhabit similar environments, there is a definite possibility of coinfections in all traumatic keratitis, irrespective of climate or location. It can be concluded that *Acanthamoeba* coinfections occupy a unique niche in the spectrum of microbial keratitis; that coinfections should be anticipated in cases suspected to be recalcitrant *Acanthamoeba* or in medically unresponsive keratitis; and that a vigorous evaluation comprising repeat microbiological evaluations, confocal microscopy, etc, should be undertaken to establish the diagnosis.

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