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ORIGINAL PAPER

Effectiveness of sampling methods employed for *Acanthamoeba* keratitis diagnosis by culture

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Abstract

Purpose This retrospective, observational study was designed to evaluate the effectiveness of the sampling methods commonly used for the collection of corneal scrapes for the diagnosis of *Acanthamoeba* keratitis (AK) by culture, in terms of their ability to provide a positive result.

Methods A total of 553 samples from 380 patients with suspected AK received at the Parasitology Section of the Public Health Institute of Chile, between January 2005 and December 2015, were evaluated. A logistic regression model was used to determine the correlation between the culture outcome (positive or negative) and the method for sample collection. The year of sample collection was also included in the analysis as a confounding variable.

Results Three hundred and sixty-five samples (27%) from 122 patients (32.1%) were positive by culture. The distribution of sample types was as follows: 142 corneal scrapes collected using a modified bezel

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needle (a novel method developed by a team of Chilean corneologists), 176 corneal scrapes obtained using a scalpel, 50 corneal biopsies, 30 corneal swabs, and 155 non-biological materials including contact lens and its paraphernalia. Biopsy provided the highest likelihood ratio for a positive result by culture (1.89), followed by non-biological materials (1.10) and corneal scrapes obtained using a modified needle (1.00). The lowest likelihood ratio was estimated for corneal scrapes obtained using a scalpel (0.88) and cotton swabs (0.78).

Conclusion Apart from biopsy, optimum corneal samples for the improved diagnosis of AK can be obtained using a modified bezel needle instead of a scalpel, while cotton swabs are not recommended.

Keywords *Acanthamoeba* · Keratitis diagnosis · Contact lens · Eye pathogens

Introduction

Acanthamoeba keratitis (AK) is a rare sight-threatening corneal infection with an increasing incidence over the past few decades largely attributed to the increasing number of contact lens wearers [1]. Because of the ability of *Acanthamoeba* to encyst in host tissues, AK requires extensive and prolonged treatment; the early diagnosis and initial therapy (within 1 month of onset) remain the most important prognostic factors [2]. Clinically, AK presents significant diagnostic challenges and is frequently misdiagnosed as bacterial and herpetic keratitis in its early stage or as fungal keratitis in the advanced stage [3]. Moreover, AK is associated with polymicrobial infection or coinfection with herpes simplex virus in 10–23% of cases [4].

Presently for the accurate diagnosis of AK, a combination of both in vivo confocal microscopy and in vitro cultivation is recommended [5, 6]. Although corneal scrapings, typically obtained using a 21-gauge fine-needle or Kimura scalpel, and biopsies are the optimal reported material for the diagnosis of AK by culture, these procedures are associated with poor sensitivities, ranging from 6.7 to 57% [3, 4, 7]. This could be explained, in part, by the fact that *Acanthamoeba* infection results from degradation of an underlying stromal matrix and deep penetration into the cornea [8]. Therefore, a deeper scraping is required to recover amoeba [9]. In addition, the test specimens are available in limited quantity, thus reducing the probability of getting a cultivable specimen [10]. Nevertheless, the quality of the sample is one of the most important factors influencing the sensitivity of the laboratory diagnosis; there are limited reports on how "corneal scrape" is obtained or rarely demonstrated how the samples are transported to the laboratory. Furthermore, the details regarding the diverse sample types (biopsies, corneal scrapes, contact lens, and contact lens paraphernalia) and culture procedures are also generally unavailable [11].

Therefore, the present investigation was initiated to develop and standardize a highly effective sampling method to obtain optimum corneal scrapes, which could improve the sensitivity of the diagnosis of AK by culture. For the present study, we were able to design and validate a novel sampling technique using a modified bezel needle for the optimum collection of corneal scraping for the effective diagnosis of AK in Chile and evaluated its ability in providing a higher likelihood ratio for the positive result by culture. This is the first study that has analyzed a large number of samples and also the first in providing data on the diagnosis of AK in Chile.

Materials and methods

Data source

The data for this retrospective observational study were retrieved from the Laboratory of Parasitology, the Public Health Institute of Chile and the only national reference center for the diagnosis of AK in the country. A total of 553 samples (corneal scrapes, biopsies, and lens paraphernalia) from 380 patients with suspected AK, which were received for the diagnosis at 33 hospitals between January 2005 and December 2015, were evaluated in the study. The number of patients investigated ranged from 20 to 46 patients per year, with an average of 35. Of these, 238 (62.6%) were female and 142 (37.4%) were male.

Samples received for culture

Since there are no standardized protocols for sample collection, the corneal tissue samples received at the ISP were diverse, in terms of both the method of

sample collection and the presentation. Besides clinical specimens, contact lens paraphernalia, such as lens cases and solutions, were also collected for investigation.

Clinical specimens

The types of clinical samples collected were categorized as:

(a) Corneal scrapes collected using a modified bezel 21-gauge (21G) needle (see below), (b) corneal scrapes collected using a scalpel, (c) lens paraphernalia, including contact lens, lens cases, and/or lens solutions, (d) cotton swabs, commonly used for diagnosis of bacteria, and (e) tissue samples obtained and/or send in other forms, including biopsies or corneal scrapes deposited onto pieces of gauze, immersed in solutions or else.

The samples were distributed as follows: 142 corneal scrapes obtained using modified bezel needle; 176 corneal scrapes collected using a scalpel; 155 non-biological materials, including contact lens and contact lens paraphernalia; 30 corneal swabs; and 50 biopsies with different clinical presentations.

In 60.2% (n = 230) of the cases, only one type of sample was available per patient. The preferred sample collection method in these cases was corneal scrape, performed both using a scalpel (36.1%, n = 83) and a modified bezel needle (33.5%, n = 77), along with contact lens paraphernalia (17.8%, n = 41), biopsies (7.4%, n = 17), and cotton swabs (5.2%, n = 12). In 32.7% (n = 125) of the cases, two types of samples were available per patient, a combination of corneal scrapes and lens paraphernalia being the most frequent (65.6%, n = 82). However, three or four types of specimens per patient were available in only 7.1% of the cases (n = 25).

Modified bezel needle technique

Modified bezel needle technique, a novel technique for the collection of a corneal scrape, was developed by a team of Chilean cornea specialists headed by Dr. Rodrigo Donoso. According to the team, the use of a modified bezel 21G needle instead of a Kimura scalpel or unmodified needle facilitates effective collection of sample particularly for culture.

This technique utilizes a hypodermic 21G needle (0.8 mm × 40 mm) consisting of a precision tip with 45–90-degree bend, forming a small loop. The tip of the needle can be easily and quickly bent with the help of a pair of scissors (Fig. 1). The corneal sample was collected by scraping the leading edge of the corneal ulcer with the help of a bent tip, taking care while moving deeper into the stroma, also ensuring the sample inside the bezel of the needle. Then, the needle was placed back in its cover and immediately sent to the laboratory for further processing.

Culture

Samples were inoculated onto non-nutrient agar plates overlaid with a suspension of live *Escherichia coli* (NNA-*E. coli*). The method used for inoculation depends on the sample type and its presentation. Scalpels or needles containing the corneal scrapes were directly inoculated into the NNA-*E. coli* medium and left stuck in the center of the NNA-*E. coli* plate, and receptacles were dropped into Page's amoeba saline buffer (142 mg/mL Na_2HPO_4, 136 mg/L KH_2PO_4, 4 mg/mL $MgSO_4x7H_2O$, 4 mg/mL $CaCl_2 \times 2H_2O$, 120 mg/mL NaCl), to prevent the loss of sample [12]. Corneal scrapes or biopsies received in liquid media were inoculated immediately onto the center of NNA-*E. coli* plate. However, when corneal tissue was received onto a cloth or gauze, the samples were placed onto the surface of the NNA-*E. coli* plate, as hard as possible, without disturbing the media. The same procedure was followed for cotton swabs and contact lens. The lens was usually received and immersed in the solution; this solution is spread onto the surface of the culture plate. Cleansing solutions were also processed in the same way. Contact lens cases were washed with Page's buffer, and the liquid was spread onto the surface of the NNA-*E. coli* plate. All samples were processed within 72 h of sampling and incubated at 37 °C and examined daily under a conventional light microscope for the presence of *Acanthamoeba* species. Diagnostically, plates were considered positive when characteristic *Acanthamoeba* cyst was visualized. Plates with no signs of growth after one week were considered negative.

Data analysis

A logistic regression model was used to analyze the nested data to determine a possible correlation between the results (positive or negative) obtained

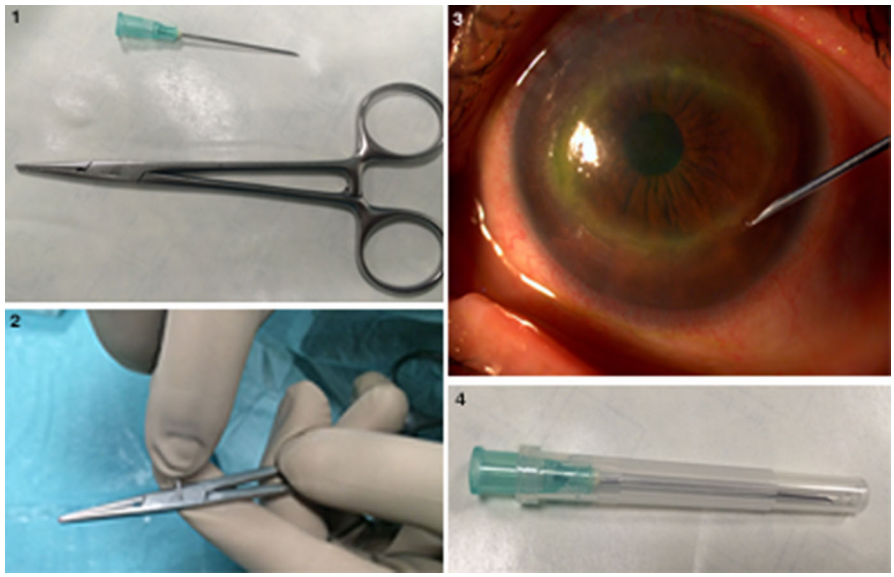

Fig. 1 Modified bezel needle method. The tip of a 21G needle is bent with the aid of a pair of scissors (1, 2) to form a spoon-like ending. The lesion is scraped (3), and the needle along with the tissue is sent to the laboratory inside its own cap (4)

by culture, the sample type and year of collection. This model considers the possible existence of intra-individual correlations in those cases where more than one sample is available from the same patient. The year of collection was also included as a variable to evaluate a possible inter-operator variability. The statistical analyses were performed by Rodrigo Villegas (PhD in Statistics) using Stata v13.

Results

A total of 151 (27.3%) samples from 122 (32.1%) patients were found positive for AK by culture. Of these, 74 (60.7%) were females and 48 (39.3%) were males. The percentage of positive patients per year ranged from 5 to 55.2%, with an average of 31.7 ± 12.9 patients. The patients' demographics, distribution according to positive cases, and study periods are presented in Table 1.

The distribution of samples according to sampling method per year and the percentage of positive cultures obtained from each patient are shown in Table 2.

According to logistic model analysis, the best sampling methods that can be employed to obtain the highest likelihood ratio for the positive result by culture using the generalized estimation equations are shown in Table 3.

Considering the modified bezel needle corneal scrape as the reference category, the samples from biopsies exhibited the significant highest positive likelihood ratio (1.89, $p = 0.054$, IC 95%), followed by contact lens paraphernalia (1.10, $p = 0.682$, IC 95%). However, lower likelihood ratios for the positive results were estimated for corneal scrapes taken using a scalpel (0.88, $p = 0.612$, IC 95%) and cotton swabs (0.78, $p = 0.551$, IC 95%).

Furthermore, the highest likelihood ratio for the positive result by culture was significantly correlated with the year of sample collection (odd ratio 0.92, $p = 0.029$, IC 95%).

Discussion

The incidence of AK, a rare but severe sight-threatening corneal disease, has been steadily increasing over the past years, mostly associated with the use of contact lens [8]. Several studies have suggested that the early diagnosis is the key to a successful treatment outcome [2, 13, 14]. We and others strongly recommend considering AK in all contact lens wearers with suspicious keratitis, particularly, when patients are presented with intense pain out of proportion to the observed injury [3]. The sensitivity of corneal culture which is the gold standard for the diagnosis of AK is highly affected by the quality of the sample and, therefore, by the sampling method [15]. Given the

Table 1 Distribution of total patients, positive, and negative cultures obtained during the study period. Gender distribution and percentage of positive cultures by year are also shown

Year	Total number of patients			Positive			Negative		
	Female	Male	Total	Female	Male	Total	Female	Male	Total
2005	15	14	29	7	9	16 (55.2%)	8	5	13
2006	34	19	53	10	5	15 (28.3%)	24	14	38
2007	30	16	46	9	9	18 (39.1%)	21	7	28
2008	23	14	37	7	4	11 (29.7%)	16	10	26
2009	23	15	38	10	2	12 (31.6%)	13	13	26
2010	15	14	29	4	6	10 (34.5%)	11	8	19
2011	29	14	43	6	4	10 (23.3%)	23	10	33
2012	24	6	30	6	3	9 (30.0%)	18	3	21
2013	18	10	28	9	4	13 (46.4%)	9	6	15
2014	12	8	20	1	0	1 (5.0%)	11	8	19
2015	15	12	27	5	2	7 (25.9%)	10	10	20
Total	238	142	380	74	48	122	164	94	258
	62.6%	37.4%		60.7%	(39.3%)	32.1%			67.9%

Table 2 Samples from each category analyzed per year. The percentage of positive cultures obtained from each of them is indicated in parenthesis

Year	Sampling method					Total
	Modified needle	Scalpel	Lens paraphernalia	Cotton swab	Biopsy	
2005	12 (50%)	15 (33%)	13 (54%)	3 (0%)	5 (40%)	48 (42%)
2006	21 (24%)	29 (31%)	21 (33%)	5 (20%)	0 (0%)	76 (29%)
2007	13 (31%)	24 (33%)	28 (29%)	1 (100%)	3 (67%)	69 (3%)
2008	15 (33%)	19 (16%)	14 (21%)	1 (100%)	5 (60%)	54 (28%)
2009	17 (18%)	16 (13%)	22 (27%)	0 (0%)	2 (100%)	57 (23%)
2010	11 (36%)	12 (25%)	15 (20%)	1 (0%)	7 (29%)	46 (26%)
2011	19 (32%)	17 (12%)	11 (18%)	5 (20%)	60 (18%)	60 (18%)
2012	7 (14%)	11 (27%)	10 (30%)	2 (0%)	10 (40%)	40 (28%)
2013	13 (31%)	13 (38%)	8 (25%)	3 (0%)	4 (75%)	41 (34%)
2014	5 (0%)	11 (9%)	3 (0%)	2 (0%)	4 (25%)	25 (8%)
2015	9 (11%)	9 (22%)	10 (30%)	7 (29%)	2 (0%)	37 (22%)
Total	142 (27.5%)	176 (24.4%)	155 (28.4%)	30 (20.0%)	50 (38.0%)	553 (27.3%)

Table 3 Estimated odds ratio and 95% confidence intervals obtained from the logistic regression model

Corneal scraping by modified bezel method was used as the reference category (1)

Asterisks show statistically significant *p* values

	Odds ratio	Robust SE	*z*	$P > \|z\|$	[95% CI]	
Sample type						
Scalpel (2)	0.884	0.216	− 0.510	0.612	0.548	1.425
Lens paraphernalia (3)	1.104	0.266	0.410	0.682	0.689	1.769
Cotton swab (4)	0.775	0.331	− 0.600	0.551	0.335	1.791
Biopsy (5)	1.892	0.626	1.930	0.054*	0.990	3.617
Year	0.920	0.035	− 2.180	0.029*	0.854	0.099
Constant	0.546	0.148	− 2.230	0.026	0.321	0.930

absence of studies in this regard, the present investigation was undertaken to evaluate the effectiveness of the sampling methods usually employed for AK diagnosis in providing a positive result by culture.

This retrospective observational study comprised of 553 samples from 380 patients with suspected AK, collected over a period of eleven years. To the best of our knowledge, this is the first study that has analyzed a large number of samples and is the first in providing data about the diagnosis of AK in Chile.

As shown in Table 1, we obtained an average of 11 new cases per year, representing 31.7 ± 12.9% of positive patients per year. The scarce data published so far and the lack of well-standardized methods make it tricky to make comparisons between studies. A reference center from Austria reported 154 new cases of AK over a period of 20 years, indicating an average of 7.7 new cases and 54.6% of positive samples per year [1]. Nevertheless, unlike Chile, there are several other laboratories in Austria where *Acanthamoeba* diagnostics has been established, so the prevalence in the country cannot be extracted. On the other hand, Wagner et al. recorded 3.3% of positive patients after analyzing 550 samples collected in a period of 30 years [16]. This low percentage in comparison with ours could be explained at least in part by the fact that they used the culture plates with heat killed *E. coli*, a practice that according to our experience provides less positive cultures than live bacteria. In Iran, Hajialilo et al. analyzed 138 corneal scrapes and contact lenses during a 5-year period at the reference laboratory and reported 13% of patients infected with *Acanthamoeba*, which is also lower than our result. In this case, amoeba culture was grown at room temperature instead of 37 °C [17].

Interestingly, the year in which the samples were analyzed was significantly correlated with the final result ($p > 0.029$). Since the methods have not changed during the study period, this correlation can be explained only by the fact that several operators were in-charge of the analysis during this period. The sensitivity of the AK diagnosis by culture is influenced by human factors at all the steps, and our findings underline the importance of the experience and necessity of proper training of the personnel in order to get reliable results [18]. With the aim of controlling and measuring this confounding factor, all the negative cultures at the ISP are further analyzed using PCR. This would minimize the risk of false-negative results as well as check the skills of the personnel and implement remedial actions if needed.

Another critical factor affecting the sensitivity of the diagnosis using culture is the type of sample. The samples received at the ISP mostly consisted of corneal tissue, contact lens paraphernalia, and cotton swabs. The corneal tissue samples were obtained by scraping, either with a modified bezel needle, scalpel or by biopsy. Except for the modified needle, which was mostly used in our study, the type of clinical samples received at the ISP did not differ from those reported by other authors and diagnostic services [1, 16, 17].

Considering the corneal scraping obtained using the modified needle method as the reference, the positive likelihood ratio for all of the above-mentioned categories was evaluated (Table 3). As expected, the highest positive likelihood ratio corresponded with biopsy samples. A biopsy involves the resection of a part of the corneal tissue, increasing the probability of getting cultivable amoeba. Nevertheless, a biopsy cannot be considered as a reference method due to its invasive nature. Regarding corneal scrapes, the possibility of getting a positive result is lower when scalpel or conventional needles (odd ratio: 0.88) were used as compared to the modified needle. This may be attributed to their straight and flat surface, which does not facilitate efficient deep scrapping and has an associated risk of the tissue falling down [11]. In contrast, the modified needle with a spoon-shaped end facilitates ease of sampling, can go deeper into the stroma, and retains the collected tissue specimen efficiently. Several experts in the field of corneal research in the country have already adopted this technique. It is also being taught to the medical students at the Corneal Unit of the Salvador Hospital and National Center of Ocular Trauma (Santiago, Chile). Our data further support the utility of this technique, which has been used until now based solely on personal experience and observations.

Cotton swabs, which are usually employed for the investigations of bacterial infections, showed the lowest positive likelihood ratio (0.78). This could be attributed to the fact that *Acanthamoeba* penetrate the cornea and are usually not found on the corneal surface; thus, superficial swab samples often remain negative [3, 19]. Nevertheless, Wagner et al. [16] obtained a similar number of positive cultures from conjunctival swabs than from corneal scrapes (three of

each). In light of our findings, this result could be explained by a relatively high number of conjunctival swabs compared to corneal scrapes, which may also explain the low percentage of positive samples found in their study (3.3%). Unfortunately, the number of samples from each type analyzed in the study is not mentioned and therefore a reliable conclusion could not be drawn.

Corneal tissue samples are often accompanied by contact lens, lens cases, and/or lens liquids. The probability of a positive culture from these contact lens paraphernalias resulted in a higher likelihood ratio as compared to the reference category (ratio 1.10), probably because of the high affinity of *Acanthamoeba* for contact lens, which serves as a source of infection [8, 20]. Several studies have demonstrated that contact lens and the storage cases from healthy people can be contaminated with amoeba with contamination rates ranging from 1 to 65.9% depending on the geographic regions [21–24]. It has also been demonstrated that contact lens from AK patients are almost always colonized with amoeba [25]. Therefore, a positive result for any of these materials is inconclusive by itself, but it may justify the management of the patient with compatible signs and symptoms. In our study, 19 lens cases from patients with suspected AK were found to be positive for *Acanthamoeba* even though the corneal tissue was negative. This could be attributed to a very superficial scraping, which would not be able to remove any cultivable amoeba [9].

Although all the above findings on the effectiveness of each sampling technique are in agreement with our experience at the laboratory, only the positive likelihood ratio for biopsy samples was close to the statistical significance ($p > 0.054$), which could be attributed to the uneven distribution of samples among the categories. The corneal scrapes and non-biological materials accounted for 85.5% of the total samples ($n = 473$), whereas cotton swabs and biopsies only corresponded to 14.5% ($n = 80$). Nevertheless, we decided to include all the data to represent a real scenario of the AK diagnosis in Chile, emphasizing the lack of standardized methods for AK sampling.

This study confirms the influence of the sampling method on the sensitivity of the diagnosis by culture, as shown by the different likelihood ratios for a positive result obtained for each sample type. The determination of likelihood ratios is a simple way to compare the performance of invasive sampling methods which otherwise could not be compared. The absence of more than one corneal sample per patient reflects the invasive nature of the procedures employed for its obtention, so it would not be possible to direct compare the performance of corneal scrape taken by scalpel versus modified needle, for example. Even if it were possible, the uneven distribution of amoeba into the stroma makes it possible to obtain different culture outcomes from the same patient [26].

In conclusion, the present study confirms the effectiveness of the modified bezel needle technique to obtain optimum corneal scrapes for the diagnosis of AK by culture, and the limited value of the cotton swabs for this purpose. Furthermore, in light of these findings and our own experience, we strongly recommend clinicians to request the patients to provide their contact lens paraphernalia for better investigation, while avoiding cotton swabs as sampling method for AK diagnosis [8].

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Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony, or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval For this type of study, formal consent is not required.

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