Adhesion of *Acanthamoeba* on Scleral Contact Lenses According to Lens Shape

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Purpose. To investigate the adhesion of *Acanthamoeba* to scleral contact lens (ScCL) surface according to lens shape.

Methods. Two strains of *A. polyphaga* (CDC:V062 and ATCC 30461) and one clinical *Acanthamoeba* isolate, were inoculated onto five contact lens (CL): one first-generation silicone hydrogel (SHCL; lotrafilcon B; adhesion control) containing plasma surface treatment; two ScCL (fluorosilicone acrylate) one containing surface treatment composed of plasma and the other containing plasma with Hydra-PEG, and two CL designed with a flat shape having the same material and surface treatments of the ScCL. Trophozoites that adhered to the lens's surfaces were counted by inverted optical light microscopy. Possible alterations of the lens surface that could predispose amoeba adhesion and *Acanthamoeba* attached to these lens surfaces were evaluated by scanning electron microscopy (SEM).

Results. All strains revealed greater adhesion to the ScCL when compared with the flat lenses ($P < 0.001$). The clinical isolate and the ATCC 30461 had a higher adhesion ($P < 0.001$) when compared with the CDC:V062. A rough texture was observed on the surface of the lenses that have been examined by SEM. Also, SEM revealed that the isolates had a rounded appearance on the surface of the ScCL in contrast with an elongated appearance on the surface of the silicone hydrogel.

Conclusions. The findings revealed that the curved shape of the ScCL favors amoeba adhesion.

Keywords: *Acanthamoeba* keratitis, contact lens, scleral contact lens, adhesion

Amoebae of the *Acanthamoeba* genus are aerobic, single-celled, amphizoic protozoa, being widely distributed in nature.[1–3] Although they are considered free-living amoebae, when they come into contact with humans, they can cause opportunistic infections, such as *Acanthamoeba* keratitis (AK).[1,2,4] AK is a complex infection with pathophysiology and treatment not fully understood.[2–4] The main risk factor is the use of contact lenses (CL). It has been observed an increase in the number of cases following the exponential increase in CL wearers, because more than 85% of cases are associated with CL wearers.[1,2,5–7] Epidemiological studies report a global incidence of 2.9 cases per million people and 33 cases per million CL wearers.[6,8]

Adhesion of *Acanthamoeba* to CL may play an important role in the pathogenesis of AK. The factors influencing adhesion include (1) the high percentage of water contents, (2) CL materials, including ionic and nonionic properties, (3) disinfection regimen, (4) frequency of CL disposal, and (5) presence of biofilm.[3,7,9–17] The literature shows that *Acanthamoeba* can adhere to the surface of a variety of CL, such as conventional hydrogel,[9,14,18] silicone-hydrogel (SHCL; first, second, and third generation),[10–12,19] rigid gas permeable (RGP),[14,20,21] and cosmetic CLs (CCL).[22,23]

Particularly, the scleral CL (ScCL) was developed with a new concept of fitting for patients having irregular corneal curvatures. It has also been suggested as a therapeutic option in cases of severe ocular surface disease such as dry eye in Sjögren's syndrome and limbal failure.[24,25] Cases of AK have been diagnosed in ScCL wearers.[26–28] For this reason, we aimed to investigate the adhesion of different *Acanthamoeba* strains to the surface of ScCL, examining whether the lens design and composition could influence this adhesion.

Methods

Ethics and Research Committee

The experimental procedures were carried out after approval by the Research Ethics Committee of the Federal

Table 1. Characteristics of CL and Experimental Groups

Lenses	Type CL	Material	DK/L	Surface Treatment	Experimental Groups
Air Optix Aqua	SHCL	Lotrafilcon B	108	Plasma	Group 1
Esclera	SsCL	Fluorosilicone acrylate	125	Plasma	Group 2.1
Esclera	Flat lens	Fluorosilicone acrylate	125	Plasma	Group 2.2
Medicon Esclera SG	SsCL	Fluorosilicone acrylate	200	Plasma Hydra-PEG	Group 3.1
Medicon Esclera SG	Flat lens	Fluorosilicone acrylate	200	Plasma Hydra-PEG	Group 3.2

CL, contact lens; DK/L, oxygen transmissibility; PEG, polyethylene glycol; SHCL, silicone-hydrogel contact lens; SsCL, scleral contact lens.

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CL

Five types of unworn CL were used in this study: one SHCL Lotrafilcon B (water content 33%), considered a positive control for having proved adhesion to trophozoites[12]; two ScCL fluorosilicone acrylate; and two fluorosilicone acrylate designed flat lenses. The flat lenses were specially developed for the experiments, being composed of the same material as the ScCL, but having a flat design to assess whether the lens shape would predispose adhesion. Lenses characteristics and experimental groups are shown in Table 1.

Acanthamoeba Strains

Three *Acanthamoeba* strains were used in this study, two reference strains: *A. polyphaga* (Centers for Disease Control and Prevention, CDC:V062, genotype T4) and *A. polyphaga* (American Type Culture Collection, ATCC 30461, genotype T4), and a Brazilian strain recently isolated from a patient with AK and ScCL wear (clinical isolate).

Molecular characterization of *Acanthamoeba* clinical isolate was performed by sequencing the PCR fragment of approximately 460 base pairs of the 18S rDNA gene, using forward primer JDP1 and reverse primer JDP2.[29] *Acanthamoeba* clinical isolate belongs to genotype T4 (GenBank accession number: MF576062.1).

Acanthamoeba Culture

Acanthamoeba isolates were cultivated in a tissue culture flask (Greiner, Kremsmünster, Austria) containing 5 mL of protease-peptone, a yeast extract–glucose (PYG) broth medium (Supplementary Material 1), and incubated at 25°C until reaching 90% confluence of trophozoites. After obtaining the monolayer, the number of amoebae was quantified in a Neubauer hemocytometer, and the concentration was adjusted to obtain 5×10^5 trophozoites in 5 mL of PYG broth medium. 1 mL of this suspension (1×10^5 trophozoites) was used in the adhesion experiment.[11,12,23,30–32]

Adhesion Assay of *Acanthamoeba* Trophozoites to CL

Lenses of each type were placed into a six-well plate filled with 5 mL of PYG broth medium. The SHCL was divided in half, and the ScCL as well as the flat lenses were kept whole because it was not possible to divide these lenses owing to their rigidity.[11,12,23,30–32]

Acanthamoeba trophozoites (1×10^5) were added to each well containing different types of CL. The plate was incubated on an orbital shaker (Incu-Shaker, Benchmark Scientific, Sayerville, NJ, USA) (80 RPM) at 25°C for 90 minutes allowing the trophozoites to come into contact with the lens's surface.[11,12,23,30–32] Subsequently, the lenses were rinsed in 5 mL of Page's amoeba saline for 1 minute in the same orbital shaker (80 RPM) at 25°C for 1 minute to remove nonadhered trophozoites. After the washing procedure, with the assistance of sterilized tweezers, the CL were placed in 60 × 15 mm Petri dishes (Greiner, Kremsmünster, Austria) and kept in a humid chamber until counting was performed.[11,12,23,30–32] The entire surface of each lens was directly scanned by an inverted light optical microscope (Eclipse TI-U, Nikon, Tokyo, Japan) at 200× magnification to count *Acanthamoeba* trophozoites adhered to CL. Experiments were performed in triplicate.[11,12,23,30–32]

Scanning Electron Microscopy (SEM) Examination of CL

To evaluate the CL surface, one of each lens type was analyzed by SEM. To evaluate the *Acanthamoeba* adhesion to the CL surface, only amoebae adhered to the surface of ScCL and SHCL were performed. The lenses were subsequently fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 hour at room temperature and then postfixed with 2% osmium tetroxide in the same buffer for 2 hours. The material was dehydrated with successive additions of ethanol at different concentrations, starting with 50%, 70%, 90% ethanol (twice), and finally 100% (three times), for 30 minutes at room temperature. After dehydration, the critical point was carried out in liquid CO_2 (BALZERS CPD 030, Tilbrook, UK), the gluing in the sample holder (stub) using conductive glue, and the metallization through an ion sputtering method of a thin layer of 20 to 30 nm gold plate (LEICA EM SCD 500, Wetzlar, Germany). The material was observed by field emission-SEM Quanta FEG 250 (FEI, Lausanne, Switzerland) at the Center for Electron Microscopy (CEME-UNIFESP). Three images per lens were acquired to evaluate the surface of CL and *Acanthamoeba* adhered to the surface of these lenses.[12,21–23,31]

Statical Analyses

Initially, the data were analyzed using descriptive measures (mean and standard deviation). Means of lens-adherent amoeba counts, by *Acanthamoeba* strains and CL type, were evaluated using a Poisson regression model. When differences in means were verified, distinct groups of means were identified via multiple comparisons with Bonferroni correction. For all statistical tests, a significance level of 5% was used. Analyses were processed using SPSS 20.0[33] and STATA 17.[34]

Results

Adhesion of *Acanthamoeba* Trophozoites to the CL Surface

As shown in Tables 2 and 3, there was an attachment effect between *Acanthamoeba* strains and CL type ($P < 0.001$), indicating that the average number of adhered amoebae per lens type was different according to the strain. Thus, it was observed that the three isolates tested can adhere to the surface of all CL; however, they adhered predominantly to the surface of ScCL when compared with flat lenses, independent of the lens surface treatment.

Additionally, it was verified in the SHCL that the average adhesion of the clinical isolate was higher than that of CDC:V062, which in turn was higher than that of ATCC 30461 ($P < 0.001$). As for the rigid lenses, the average adhesion of the clinical isolate was higher than that of ATCC 30461, which in turn was higher than that of CDC:V062 ($P <$

Table 2. Descriptive Measures of Counts of Amoebae Adhered to the Surface of CL, by *Acanthamoeba* Strains and CL Type

	CL					*P* Value		
Strains	Group 1 SHCL (Control)	Group 2.1 ScCL (Plasma)	Group 2.2 Flat Lens (Plasma)	Group 3.1 ScCL (Plasma + Hydra-PEG)	Group 3.2 Flat Lens (Plasma + Hydra-PEG)	Strain	CL	Interaction Between Strain and CL
CDC:V062								
Mean ± SD	716.9 ± 334.6*	231.6 ± 184.7†	27.8 ± 21.5‡	231.0 ± 150.7†	37.4 ± 31.7‡			
Median	770.0	163.0	22.0	143.0	21.0			
Min.–Max.)	(285.0–1071.0)	(19.0–605.0)	(4.0–62.0)	(78.0–479.0)	(12.0–87.0)			
N	7	7	5	7	5			
ATCC 30461								
Mean ± SD	642.3 ± 617.4†	834.7 ± 508.9*	17.2 ± 10.7‡	857.5 ± 462.9*	76.2 ± 55.2§			
Median	512.0	908.0	15.5	924.5	60.0	<0.001	<0.001	<0.001
(Min.–Max.)	(0.0–1506.0)	(30.0–1447.0)	(6.0–34.0)	(0.0–1348.0)	(26.0–170.0)			
N	6	6	6	6	6			
Clinical Isolate								
Mean ± SD	1332.0 ± 722.0*	1284.3 ± 365.8†	54.8 ± 60.8‡	1363.1 ± 843.9*	340.0 ± 451.3§			
Media	1372.0	1307.5	32.0	1355.0	212.0			
(Min.–Max.)	(546.0–2412.0)	(835.0–1764.0)	(12.0–162.0)	(309.0–2798.0)	(33.0–1127.0)			
N	6	6	5	7	5			

ATCC, American Type Culture Collection; CDC, Center for Disease Control; CL, contact lens; Max., maximum; Min., minimum; PEG, polyethylene glycol; SD, standard deviation; SHCL, silicone-hydrogel contact lens.

Strain effect: Group 1 and Group 2.2: Clinical isolate > CDC:V062 > ATCC 30461; Group 2.1, Group 3.1 and Group 3.2 Clinical isolate > ATCC 30461 > CDC:V062.

Effect of LC on each strain: *, †, §, and ‡ have different averages.

**$P < 0.001$ (descriptive level of the effects of strain, CL, and interaction between strain and CL).

Table 3. Descriptive Measures of Counts of Amoebae Adhered to the Surface of CL, by *Acanthamoeba* Strains and Groups of CL Type (Curvature)

	CL			*P* Value		
Strains	SHCL (Group 1–Control)	SsCL (Group 2.1/Group 3.1)	Flat Lenses (Group 2.2/Group 3.2)	Strain	CL	Interaction Between Strain and CL
CDC:V062						
Mean ± SD	716.9 ± 334.6*	231.3 ± 161.9†	32.6 ± 26.0‡			
Median (Min.–Max.)	770.0 (285.0–1071.0)	157.5 (19.0–605.0)	21.5 (4.0–87.0)			
N	7	14	10			
ATCC 30461						
Mean ± SD	642.3 ± 617.4†	846.1 ± 463.9*	46.7 ± 48.8‡	<0.001	<0.001	<0.001
Median (Min.–Max.)	512.0 (0.0–1506.0)	924.5 (0.0–1447.0)	28.0 (6.0–170.0)			
N	6	12	12			
Clinical Isolate						
Mean ± SD	1332.0 ± 722.0*	1326.8 ± 643.0*	197.4 ± 338.8‡			
Median (Min.–Max.)	1372.0 (546.0–2412.0)	1355.0 (309.0–2798.0)	48.5 (12.0–1127.0)			
N	6	13	10			

ATCC, American Type Culture Collection; CDC. Center for Disease Control; CL, contact lens; Max., maximum; Min., minimum; N, number of lenses; SD, standard deviation; SHCL, silicone-hydrogel contact lens.

Strain effect: Group 1 Clinical isolate > CDC:V062 > ATCC 30461; Group 2.1 and Group 3.1 x Group 2.2 and Group 3.2 Clinical isolate > ATCC 30461 > CDC:V062.

Effect of LC on each strain: *, †, and ‡ have different averages.

**$P < 0.001$ (descriptive level of the effects of strain, CL, and interaction between strain and CL).

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0.001). Figure 1 provides an example of how *Acanthamoeba* adhered to surfaces of different CL in an experiment carried out in triplicate.

SEM Analysis

Analysis of CL Surface. As we can see in Figure 2, all the lenses tested presented roughness on their surface, however, the SHCL (Fig. 2A) presented a rougher surface in comparison to the SsCL and flat lenses (Figs. 2B–2E).

Morphological Analysis of *Acanthamoeba* Trophozoites Adhered to the CL Surface. In this analysis, only the SHCL (Group 1) and the ScCL (Groups 2.1 and 3.1) were investigated concerning the adhesion of the clinical isolate. It was observed on the SHCL (Fig. 3A) that the amoeba had morphological characteristics different from those observed in the ScCL (Figs. 3B, 3C), that is, amoeboid and elongated aspect in the first and rounded and shrunken in the second.

Discussion

Acanthamoeba can adhere to a wide variety of CL available in the customer's market; however, there are no reports in the literature about the adhesion of these amoebae to the ScCL surface, and AK cases associated with the use of these particular lenses have been described in the literature since 2014.[26–28] Thus, the data presented in this study prove that strains of the genus *Acanthamoeba* can adhere to the surface of ScCL, regardless of the surface treatment. The adhesion profiles displayed vary among the different isolates. These data are consistent with other studies found in the literature, in which different species of bacteria, fungi, and even *Acanthamoeba* were able to adhere to the surface of hydrogel,[9,14,18,35–48] SHCL,[10–12,19,36,37,40–43,46–51] RGP,[14,20,21,35] and CCL.[22,23,52,53]

The finding of folds formation on the SHCL surface, in addition to treatment with Plasma, makes these lenses more humid possibly favoring the adhesion of *Acanthamoeba* (Fig. 2A).[10–12,19,30–32] These findings are consistent with the literature, besides the fact that adhesion of the *Acanthamoeba* spp. had a higher preference for SHCL than for hydrogel CL and also when compared between the different generations of SHCL, the greatest adhesion occurred to the first-generation CL.[10–12,19,30–32] Lee et al.[12] and Omana-Molina et al.[19] also noted that first-generation SHCL exhibits a rougher surface compared with other CL, in addition to having small folds that, as a result, they favor contact with the *Acanthamoeba* trophozoites, allowing the cytoplasmic projections (acanthopodia) to firmly attach to the surface of these CL. Furthermore, the amoebae attached to the surface of these CL had an amoeboid and elongated shape, characteristic of trophozoite forms. These considerations described above were also observed in our analyses (Figs. 1, 2A, 3A).

ScCL composed of fluorine silicone acrylate creates a hydrophobic surface. However, treatments have been used to decrease this hydrophobicity, acting as lubricants, as already observed in treatments with plasma applied to the SHCL surface.[10–12,19,30–32] Hydra-PEG also modifies the surface of the CL, making them more humid, prolonging lubricity, and increasing the tear breakup time consequently reducing the formation of deposits on the CL surface.[54–56] However, these ScCL surface treatment strategies (Groups 2.1 and 3.1) may have brought a possible favoring of *Acanthamoeba* strains to adhere to the CL. Although the amoebae had a certain preference for adhering to one lens surface than the other, in our study this was not statistically significant (Table 2). Neither the plasma nor the Hydra-PEG enabled greater fixation of the strains, by making a hydrophobic surface into a more humid surface. This is the first study calling attention to the adhesion of *Acanthamoeba* strains to ScCL with surface treatment promoting hydrophobicity, although studies on lenses without this treatment have to be done to prove this hypothesis.

It was observed in this study that ScCL also presents surface roughness (Figs. 2B, 2D), however, less roughness compared with SHCL (Fig. 2A). This roughness may also predispose *Acanthamoeba* to adhere to these CL surfaces. These findings have already been proven in the literature for both bacterial and *Acanthamoeba* adhesion to the soft CL surfaces.[12,19,22,48,52,53,57,58]

Lee et al.[21] investigated the adhesion of an *Acanthamoeba* strain obtained from a patient who developed AK to the RGP CL. The study revealed that *Acanthamoeba* can adhere to the surface of these lenses, at an average of 223.0 to 428.5 trophozoites per lens. Also, the authors analyzed by SEM the amoebae attachment to the RGP surface, and they observed that the amoebae presented a rounded and shrunken appearance, similar to what we observed on the surface of the ScCL in our study (Figs. 1, 3B, 3C), which may be a characteristic morphology of *Acanthamoeba* spp. when attached to the rigid lens surface, or a shape that represents the transition from trophozoite to a cyst on this type of surface. This finding is also being brought to attention for the first time in the literature.

Comparing the *Acanthamoeba* strains adhesion to the surface of the ScCL with the flat lenses (Table 3), it is noted that the strain's adhesion to the ScCL surface (Groups 2.1 and 3.1) was significantly higher than the adhesion exerted by the same strains to the flat lenses surface (Groups 2.2 and 3.2). It can also be inferred that the ScCL format is predisposing the adhesion. This means that the curvatures projected mainly in the optical zone of these lenses can significantly influence and favor the adhesion of *Acanthamoeba* to these lenses. This finding also described for the first time in the literature opens new horizons for research, because, as observed in this study, it is necessary to research new surface treatments of ScCL to decrease the roughness, cleaning solutions that act on adhesion, mechanical methods of removing parasites adhered in these lenses with peculiar curvatures.

Interestingly, during the counting of the amoebae adhesion, it was noticed that liquids accumulated in the central region of the ScCL's optic zone were exactly where there was a higher concentration of adhered amoebae (data not shown).[59–62] This result could be another factor related to the superior *Acanthamoeba* adhesion to the ScCL, with its design favoring the accumulation of liquids centrally in the optical zone and, because *Acanthamoeba* needs liquids for its locomotion and adhesion, it has in this area an ideal niche for its proliferation.

It is known that ScCL wearers must necessarily fill the ScCL's optical zone with saline solution. It can be said, then, that not only the format, but also the use of the saline solution, is a risk and may interfere with the *Acanthamoeba* adhesion positively, because the accumulation of this solution favors the establishment and proliferation of amoebae in the lenses.[27] This result does not take into account whether the saline solution is contaminated or not by *Acanthamoeba*, especially in wearers who use large bottles of the solution

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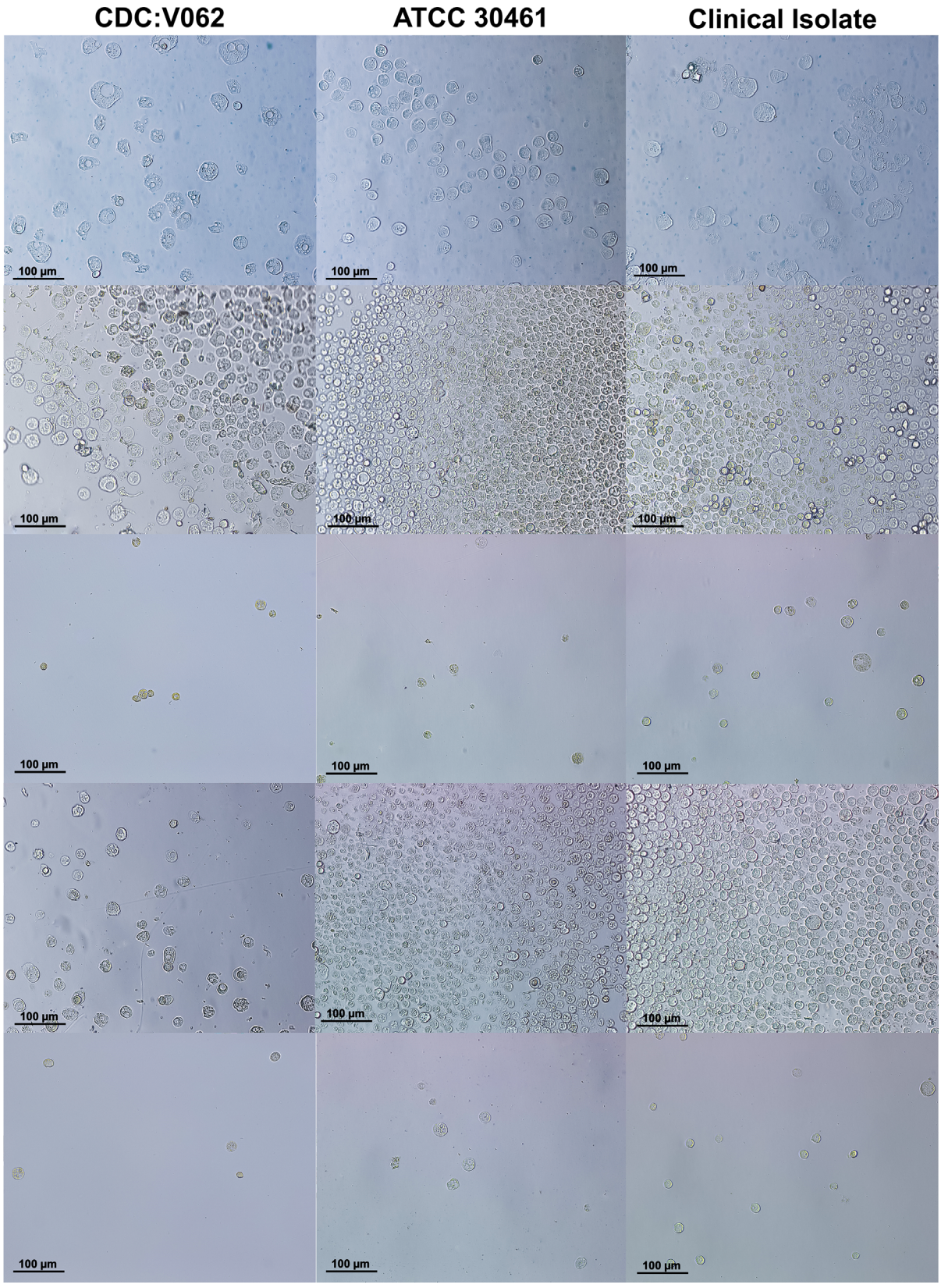

Figure 1. Inverted optical light microscopic images of adherent *Acanthamoeba* trophozoites to different surfaces of CL in an experiment carried out in triplicate. Initial inoculum of 1×10^5 trophozoites/CL. CDC:V062 and ATCC 30461, reference strains of *A. polyphaga*; clinical isolate, isolated from a patient with AK; Group 1, SHCL; Group 2.1, SsCL with Plasma surface treatment; Group 2.2, Flat lenses with Plasma surface treatment; Group 3.1, SsCL with Plasma and Hydra-PEG surface treatment; Group 3.2 Flat lenses with Plasma and Hydra-PEG surface treatment (magnification × 200, Advanced Research Center in *Acanthamoeba*; CEPA-UNIFESP).

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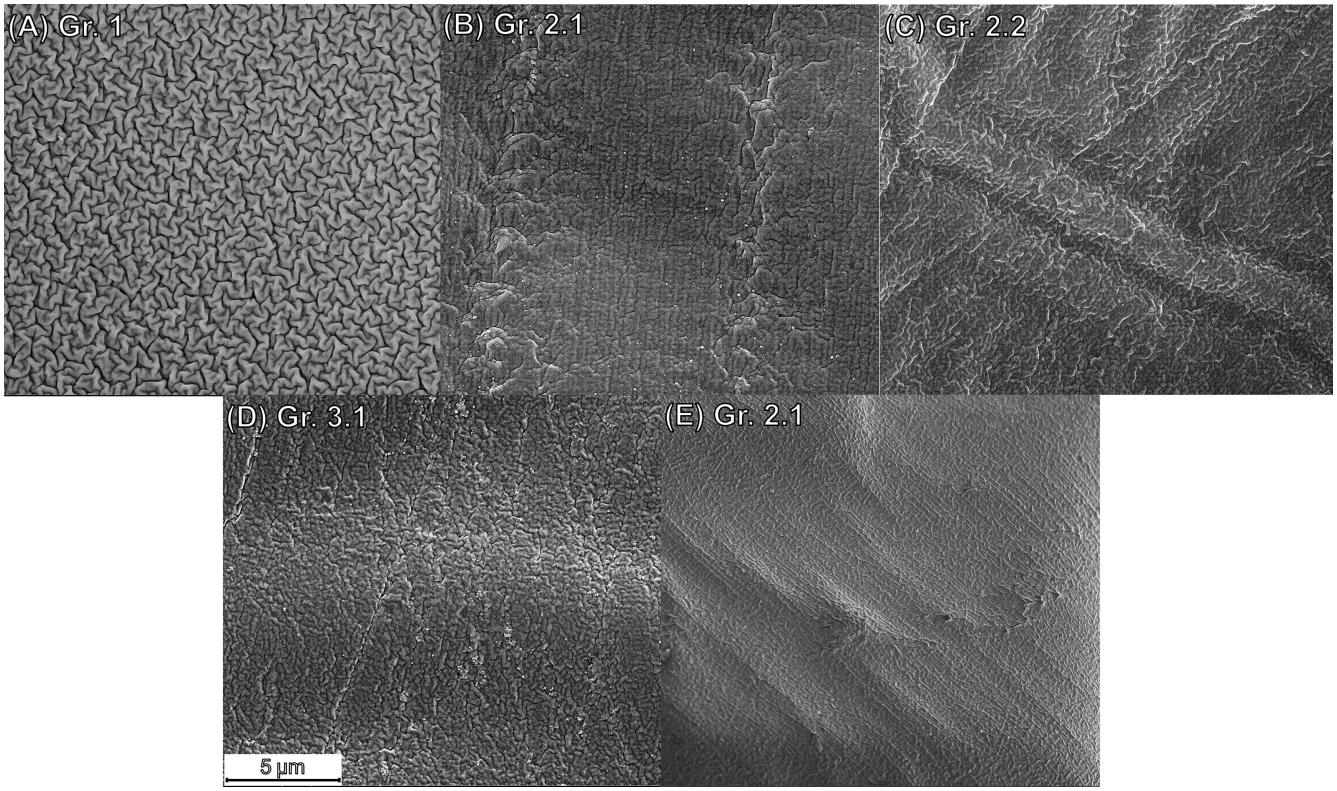

Figure 2. SEM image of CL surface: (**A**; Group 1) SHCL, (**B**; Group 2.1) SsCL (surface treatment with plasma), (**C**; Group 2.2) Flat lens (surface treatment with plasma), (**D**; Group 3.1) SsCL (surface treatment with plasma and Hydra-PEG), (**E**; Group 3.2) Flat lens (surface treatment with plasma and Hydra-PEG) (magnification ×20,000, CEME-UNIFESP).

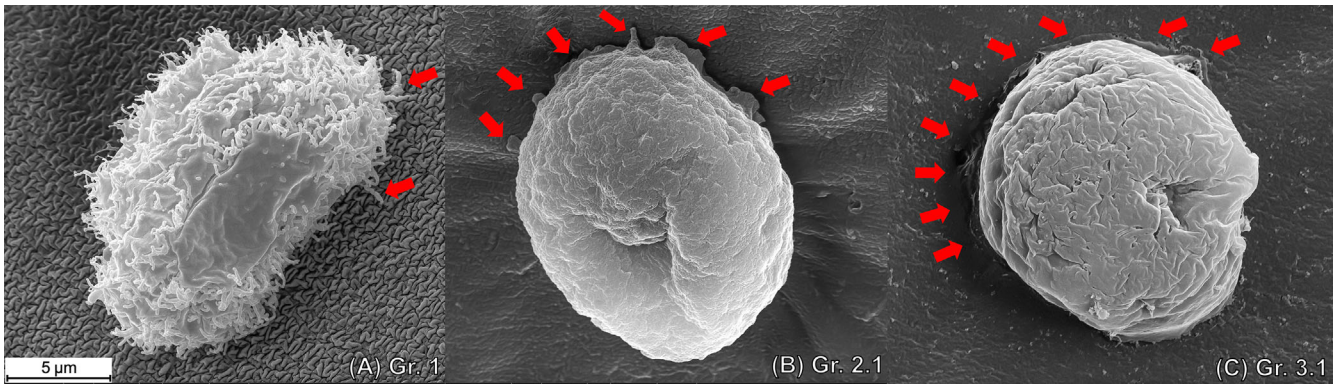

Figure 3. SEM image of the clinical isolate *Acanthamoeba* spp. trophozoites adhered to the SHCL and ScCL surface: (**A**; Group 1) amoeboid trophozoite (clinical isolate) adhered to the SHCL (Group 1), (**B**; Group 2.1) rounded trophozoite (clinical isolate) adhered to the ScCL (surface treatment with the plasma: Group 2.1), (**C**; Group 3.1) rounded trophozoite (clinical isolate) adhered to the ScCL (surface treatment with plasma and Hydra-PEG: Group 3.1). The red arrows indicate the site of the clinical isolate adhesion by the acanthopodia to the lens surface (magnification ×15,000, CEME-UNIFESP).

and not disposable small-volume ones. These hypotheses have already been raised by our group in a previous study.[27]

The present study also investigated the adhesion profile between three *Acanthamoeba* isolates. As shown in Tables 2 and 3, the clinical isolate and ATCC 30461 adhered more to the lens surface than the CDC:V062, except for the SHCL. Bakay et al.[23] evaluated the adhesion of two clinical *Acanthamoeba* strains (*A. castellanii* and *A. hatchetti*) on the surface of five CCL composed of a Hema copolymer (38,5% water), Phemfilcon (55% water), Polymacon (38% water), Polyhema (42% water), and Hema (55% water). They verified that the strains were able to adhere to all lens surfaces. However, as observed in this study, Bakay et al.[23] noticed that the isolates had a greater predisposition to adhere to the surface of some materials (Hema and Polymacon) than others (Phemfilcon and Polyhema), and there were differ-

ences in the adhesion profiles between the isolates to the surface of some copolymers. They also examined the surface of CCL by SEM, and they saw that the colored regions of these CCL were rougher, being the places where the *Acanthamoeba* strains were most attached; the same was observed by Lee et al.[22] These findings are consistent with what was observed on the surface of the CL tested in our study (Fig. 2), in which the roughest surfaces were the ones where the amoebae were most attached.

Given what has been discussed, future studies are needed to more specifically investigate the mechanism involved in the adhesion of *Acanthamoeba* species to the CL surface, thus understanding whether this mechanism may be facilitating the attachment of amoebae to the surface of corneal epithelial cells since this is the initial and crucial stage for the development of AK. It is known that more pathogenic trophozoites express higher levels of acanthopodia and the amoeba binding to corneal epithelial cells is mediated by acanthopodia. As a result, pathogenic strains tend to adhere more easily to corneal surface cells owing to the greatest amount of acanthopodia.[63] Therefore, investigating whether *Acanthamoeba* strains that adhere more to the CL surface, also express higher levels of acanthopodia, may be another way to better understand CL as a vector in the development of AK. In conclusion, the present work shows that the curved shape of ScCL predisposes the higher adhesion to the surface of CL.

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