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COMPARISON BETWEEN DIFFERENT METHODS FOR DIAGNOSIS OF *ACANTHAMOEBA* INFECTION AND ISOLATION OF GENOTYPE T9 FROM A CONTACT LENS CASES

By

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Abstract

Acanthamoeba genus is a medically important free living amoeba causing serious humans infections. Amoebic keratitis (AK) is a sight threatening infection of cornea caused by *Acanthamoeba* pathogenic genotypes, which prevalence remarkably increased in developed countries. The study compared different methods for diagnosing AK and identified *Acanthamoeba* genotypes by molecular examination in contact lens wearers (CLWs). Patients were 79 clinical corneal swaps (CS) and 15 samples from contact lens storage cases (CLSC). Clinical CSs were divided into four groups; GI: 20 patients suffering from chronic corneal ulcers, GII: 15 patients with traumatic ulcers, GIII: 24symptomatic CLWs and GIV: 20asymptomatic control individuals. CLSC were provided from apparently healthy asymptomatic CLWs (15). Swabs and solution samples were underwent microscopic and staining examination, cultivation on non-nutrient agar (NNA) plates and PCR molecular analysis. Sequencing and genotyping of PCR- positive samples were performed.

The results showed that *Acanthamoeba* parasites were detected in 3.8% of CS and 6.7% of CLSC samples. The highest significantly positive results were by culture (3.8%) followed by Giemsa and trichrome stains (2.5%) and lastly direct microscopy (1.3%) of CS samples. Only one positive sample (6.7%) was detected in CLSC by all methods, but without statistical significance. Sensitivity of PCR compared to culture was 25%.

Acanthamoeba parasites in CS were from subgroup II with 12.5% detection rate in CLWs, but the positive case from CLSC was from subgroup I with 6.7% detection rate. This study confirmed different risk factors in association with AK in CLWs. Genotype determination for *Acanthamoeba* positive case by PCR revealed homology with *Acanthamoeba* genotype T9 isolate ICS20.

Key words: *Acanthamoeba*, Amoebic keratitis, AK, Contact Lens, CLSC, Genotype T9.

Introduction

Acanthamoeba is one of the ubiquitous free-living amoebae (FLA) of worldwide distribution (Aghajani *et al,* 2016). It exists as either a vegetative trophozoites or dormant cysts (Gomes *et al,* 2016). Under environmental stress conditions, *Acanthamoeba* phenotype switched from trophozoites into cysts stages (Rezaeian *et al,* 2008). *Acanthamoeba* species have been isolated from soil, air, drinking water, swimming pools, sewage, eyewash solutions, contact lenses, dialysis and dental treatment units (Cateau *et al,* 2014). *Acanthamoeba* pathogenic genotypes can cause different infections in man resulting in fatal granulomatous amoebic encephalitis (GAE) and AK which is a sight threatening infection to the cornea (Visvesvara, 2013). *Acanthamoeba* keratitis usually starts by pain, photo-phobia and lacrimation and progresses leading to ring ulcers, corneal opacity and corneal perforation (Lorenzo-Morales *et al,* 2015). The AK prevalence remarkably increased in developed countries, due to increased CLWs number, accurate diagnosis and disease worldwide awareness (Dart *et al*, 2009).

Identification of *Acanthamoeba* spp. depended mainly on morphological characters (Page, 1988). Molecular methods, especially PCR based on analysis of the diagnostic fragment 3 (DF3) region of 18s rRNA genes are recently used for identification. To date, 20 *Acanthamoeba* genotypes (T1-T20) have been established and accepted (Behera *et al,* 2016). Genotype T4 is the commonest and abundant pathogenic isolate from clinical cases (Khan, 2006; Maciver *et al,* 2013). Other genotypes; including T2, T3, T5, T6, T11 & T15; were isolated and related to clinical manifestations (Lorenzo-Morales *et al*, 2011; Omaña-Molina *et al*, 2016).

The present study aimed to compare the molecular biology with the conventional methods of *Acanthamoeba* detection, also to ascertain the isolation and identification of the *Acanthamoeba* genotypes from AK patients at our institution.

Materials and Methods

Study population: This is a cross sectional study included 79 randomly selected individuals (45 females & 34 males) of different age groups, 46 from Outpatient Clinic of Ophthalmology, Menoufia University Hospitals and 33 individuals from Ophthalmology Hospital in Shebin El-Koum. The study was carried out from January 2014 to April 2015. Written informed consents were obtained from all participants. They were divided into four groups; G1: 20 patients suffering from resistant corneal ulcer not responding to medical treatment for more than 2 weeks, GII: 15 patients with traumatic ulcers, GIII: 24 symptomatic CLWs and GIV: 20 asymptomatic control individuals. The study was carried out on CS. Also, 15 samples from CLSC were provided from apparently healthy asymptomatic CLWs. All participants underwent an ophthalmic examination by the ophthalmologist.

A structured questionnaire: It included demographic data, complaint and risk factors as trauma, exposure to contaminated water, history of previous keratitis and socioeconomic standard(SES) In case of CLWs, signs of over-use and incompliance to CL hygiene (including sleeping in contact lenses, inadequate cleaning of lenses with contact lens solution, & frequency of changing contact lenses…etc.) were investigated.

Specimen collection: Corneal swabs using sterilized cotton swab were taken under complete aseptic conditions before giving any antibiotic therapy. Three swabs were obtained from each patient. One was suspended in Page's amoeba saline (PAS) for direct microscopic and staining examination, the second was cultivated on non-nutrient agar (NNA) plates and the third was put in a sterile Eppendorf tube with 200µl PBS and preserved at -20°C for subsequent DNA extraction. Laboratory procedures were done at Parasitology Department laboratories, Menoufia University. Solution samples (15) from CLSC were collected in sterile tubes. Swabs from CLSC inner surfaces were taken for Biofilms examination. Each Biofilm swab was mixed well with the corresponding lens solution and left for 1-2 h before managing. The solution samples underwent the same methods of examination as CS samples.

Microscopic examination: Wet mount Giemsa (Ithoi *et al*, 2011) and Trichrome stained slides (Garcia and Bruckner, 1997) from saline suspension of swabs and CLSC solutions were microscopically examined for cysts and trophozoites by oil immersion lens.

Cultivation of specimens: Corneal swabs and CLSC solutions were cultivated according to Schuster *et al.* (2002) and Lorenzo-Morales *et al.* (2015) on 1.5% non-nutrient agar (Agar No.1, Oxoid, Thermoscintific) prepared in PAS overlaid with thin layer of live *Escherichia coli* after cooling. Plates were incubated at 28±2^{o}C, and daily examined for *Acanthamoeba* growth up to 2 weeks by a light microscope (10x & 40x objectives). Identification of organisms from positive culture plates was accomplished by direct examination and/or Giemsa-staining to characterize cysts morphology (shape, size, features of ectocysts & endocysts) and trophozoites (acanthopodia & pseudopodia). Cysts were measured by using an ocular micrometer scale and morphological characteristics into subgroups I, II, or III based on the criteria given by Pussard and Pons (1977).

Molecular analysis: DNA extraction from samples was performed by the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA amplification was done using *Acanthamoeba* genus-specific primers, amplify a fragment of approximately 500bp of the ASA.S1 region of the 18s rRNA gene. Specific primer pair used in this study was the forward primer JDP1; 5-GGCCCAGAT CGTTTACCGTGAA and the reverse primer JDP2; 5-TCTCACAAGCTGCTAGGGGAG TCA (Schroeder *et al*, 2001). DNA amplification reaction was done (Booton *et al*, 2004); each tube contained 25µl total volumes composed of: 5uL template DNA, 1µl of each primer, 12.5µl PCR Master Mix (DreamTaq Green PCR Master Mix, Thermo Fisher Scientific), and 5.5µl sterile deionized water. Amplification was done by a thermocycler (PerkinElmer Cestus, Norwalk, CT). The process began with an initial denaturation step at 95°C for 7min, followed by 40 cycles of denaturation at 95°C for 1min, then primer annealing at 55°C for 1 min, and extension at 72°C for 2min. The final extension occurred at 72°C for 15min. For negative control, distilled water was added instead of DNA and positive control DNA was kindly provided by Dr. Omnia Sobhy, Faculty of Medicine, Ain Shams University. A 100-1000 base pair (bp) ladder was used as a DNA size marker (Gene Ruler TM, Fermentas, Thermo Fisher Scientific). DNA amplified products were then electrophoresed using 3% agarose gel stained with ethidium bromide (0.5µg/ml) and then visualized under UV illumination.

Sequencing and genotyping: PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and sequenced in both directions. Sequencing of ASA.S1 region of the 18s rRNA gene was performed using approximately 20-25ng of PCR purified product, primers JDP1 or JDP2 and Big Dye Terminator technology. Genetic analysis was done on 3500 genetic DNA analyzer (Applied Biosystems, Thermo Fisher Scientific, USA). Following genotyping, the Basic Local Alignment Search Tool (BLAST) of the US National Center for Biotechnology Information (NCBI) was used to identify similar sequences to the present result. The sequences obtained by BLAST were aligned in MEGA 6 software program (Tamura *et al*, 2004) using the ClustalW method, and phylogenetic tree was constructed by neighbor joining algorithm based on evolutionary distances calculated from maximum composition likelihood method estimated with 1,000 bootstrap samplings (Tamura *et al*, 2013).

Statistical analysis: SPSS, version18, (SPSS Inc., Chicago, IL, USA) program was used. Data were computerized and analyzed. The $\chi 2$ and Fisher's exact tests examined the relation between qualitative variables. Values were considered significant if probability value was less than 0.05 ($P<0.05$) and highly significant when P value was 0.001. Sensitivity, specificity, and diagnostic accuracy of different diagnostic methods were calculated in comparison to the gold standard test. Odds ratio (OR) and 95% confidence intervals were computed to assess relation between risk factors and *Acanthamoeba* infection in contact lens user.

Ethical consideration: The ethical approval was obtained from the Committee of Research, Publications and Ethics of Faculty of Medicine, Menoufia University. All procedures were explained to the patients and written informed consents were obtained.

Results

Detection of *Acanthamoeba* infection in CS & CLSC by different diagnostic methods: Total 94 samples were examined, 79 CS & 15 samples from CLSC. Out of 79 CS, the highest positive cases were detected by culture in NNA media, *Acanthamoeba* spp. was identified in 3 clinical samples (3.8%). Direct microscopic examination detected one positive case (1.3%) and 2 positive cases (2.5%) by Giemsa and trichrome stains while none positive was detected by PCR (0%). Only one positive sample for *Acanthamoeba* spp. (6.7%) out of 15 CLSC samples was detected by all examinations methods. No significant difference was recorded among different methods of examinations for CS and CLSC samples except for PCR technique ($P <0.05$) (Tab. 1) (Fig.4).

Table 1: Detection of *Acanthamoeba* infection in CS and CLSC by different diagnostic methods

Method	CS (79)			CLSC (15)			Analysis	
	Positive	Negative	Positive %	Positive	Negative	Positive %	χ2	P
Direct microscopy	1	78	1.3%	1	14	6.7%	1.766	0.184
Giemsa stain	2	77	2.5%	1	14	6.7%	0.698	0.404
Trichrome stain	2	77	2.5%	1	14	6.7%	0.698	0.404
Culture	3	76	3.8%	1	14	6.7%	0.255	0.614
PCR	0	79	0.0%	1	14	6.7%	5.323	0.021

Positive samples were detected on patients using contact lenses (GIII); rate was 12.5% by culture. Staining methods detected 2 positive cases (8.3%), but without significance difference between examination methods and positive cases (p > 0.05) (Fig.1).

Table 2: Distribution of demographic features of the examined CL cases in relation to *Acanthamoeba* infection.

Risk factor		*Acanthamoeba*				Total		Odds ratio	95% confidence interval		P value
		+ve (3/24)		-ve (21/24)							
		No.	%	No	%	No	%		Upper	Lower	
Sex	M	1	33.3%	5	22.7%	6	24.0%	1.700	0.126	22.873	1.00
	F	2	66.7%	17	77.3%	19	76.0%				
Age group	15-<30	0	0.0%	17	81.0%	17	70.8%	0.069	0.006	0.769	0.026
	>30-45	3	100.0%	4	19.0%	7	29.2%				
SES	High	1	33.3%	3	14.3%	4	16.7%	3.000	0.203	44.359	0.44
	Moderate	2	66.7%	18	85.7%	20	83.3%				
Residence	Rural	0	0%	2	9.5%	2	8.3%	33.0	2.248	484.45	0.011
	urban	3	100%	19	90.5%	22	91.7%				

Culture was considered as a gold standard test. The highest values were of Giemsa and trichrome stain, they showed one false-negative sample that was positive by culture yielding 75% sensitivity, 100% specificity and 99% accuracy followed by microscopy, which showed 2 false-negative samples with 50% sensitivity, 100% specificity and 98% accuracy. Lowest results were by PCR method which gave 3 false-negative samples with 25% sensitivity, 100% specificity and 97% accuracy.

Direct microscopy and stained smears of positive samples showed *Acanthamoeba* trophozoites characterized by its' irregular shape, centrally placed single nucleus, large, dense nucleolus, many cytoplasmic contractile vacuoles and fine, tapering, and thorn-like acanthopodia arising from the body surface. Cysts showed wrinkled or smooth ectocysts and endocysts that varied in shape, being stellate or spherical according to species. Cysts had one nucleus with central dense nucleolus (Figs.2 & 3).

Cysts of *Acanthamoeba* subgroup I had rounded smooth ectocysts that were clearly separated from the endocysts. Ectocyst and endocyst were joined by radiations forming star-shaped structure with a mean diameter of more than 20µm. Cysts were subgroup II had ectocysts and endocysts; either close together or widely separated. Ectocysts were wrinkled or smooth and endocysts were polygonal or round with a mean diameter of less than 18µm. Most of *Acanthamoeba* were of subgroup II (3 positive clinical cases from symptomatic CLWs) and the positive isolate from CLSC was of subgroup I.

Demographic criteria in CLWs regarding *Acanthamoeba* infection: *Acanthamoeba* was significantly detected among ages >30-45with (P=0.026). Infection was detected in female more than males. Two out of 3 positive cases (66.7%) were females. Also, 2 out of 3 (66.7%) were detected in moderate SES. Sex and the SES had no significant relation with *Acanthamoeba* infection (P> 0.05). All positive 3 cases for *Acanthamoeba* infection were detected among urban patients (P= 0.011) (Tab.2).

Table3: Distribution of CL hygiene and water exposure risk factors in relation to *Acanthamoeba* infection in symptomatic CLWs (N=24).

Risk factor		*Acanthamoeba*				Odds ratio	Confidence interval 95%		P
		+ve (3)		-ve (21)					
		No.	%	No.	%		lower	upper	
Hygiene awareness	good	0	0%	18	85.7%	0.053	0.005	0.605	0.015
	poor	3	100%	3	14.3%				
Hand washing	+ve	1	33.3%	21	100%	0.030	0.002	0.445	0.011
	-ve	2	66.7%	0	0%				
Regular CLSC cleaning	+ve	1	33.3%	20	95.2%	0.025	0.001	0.572	0.032
	-ve	2	66.7%	1	4.8%				
Rinsing CL with tap water	+ve	2	66.7%	1	4.8%	40.000	1.749	914.787	0.032
	-ve	1	33.3%	20	95.2%				
Rinsing CLSC with tap water	+ve	3	100%	2	9.5%	26.667	2.178	326.453	0.008
	-ve	0	0%	19	90.5%				
Multipurpose solution	+ve	3	100%	2	9.5%	26.667	2.178	326.453	0.008
	-ve	0	0%	19	90.5%				
Special solution	+ve	0	0%	16	76.2%	0.088	0.008	0.954	0.041
	-ve	3	100%	5	23.8%				
Showering + CL wear	+ve	2	66.7%	1	4.8%	40.00	1.749	914.787	0.032
	-ve	1	33.3%	20	95.2%				

Distribution of risk factors among CLWs to infection: There were significant associations between poor awareness, negligence of hand washing before handling CL, irregular cleaning of CL and *Acanthamoeba* infection (P=0.015, 0.011& 0.032 respectively). Water exposure risk factors in CL wearers showed significant association between showering while wearing CL, rinsing CL or their cases with tap water, using multipurpose solution for cleaning CL, wothout use special solution & positive cases (P=0.032, 0.008 & 0.041 respectively) (Tab.3).

Table 4: Distribution of other CL-related risk factors in relation to *Acanthamoeba* in symptomatic CLWs

Risk factor		*Acanthamoeba*				Odds ratio	Confidence interval 95%		P
		+ve (3)		-ve (21)					
		No.	%	No.	%		lower	upper	
Sleeping +CL wear	+ve	2	66.7%	5	23.8%	6.400	0.474	86.343	0.194
	-ve	1	33.3%	16	76.2%				
Corneal trauma	+ve	3	100%	4	19.0%	14.400	1.300	159.513	0.026
	-ve	0	0%	17	81.0%				
Purpose of CL wear	Optical	2	66.7%	17	81.0%	0.471	0.034	6.568	0.521
	Cosmetic	1	33.3%	4	19.0%				
Frequency of application	Daily	2	66.7%	15	71.4%	0.800	0.061	10.562	1.000
	Occasionally	1	33.3%	6	28.6%				
Use of expired CLs	+ve	1	33.3%	6	28.6%	1.250	0.095	16.503	1.000
	-ve	2	66.7%	15	71.4%				
Dust exposure	+ve	3	100%	5	23.8%	11.333	1.048	122.549	0.041
	-ve	0	0%	16	76.2%				
Topical steroid use	+ve	2	66.7%	0	0%	33.000	2.248	484.447	0.011
	-ve	1	33.3%	21	100%				

Acanthamoeba infection was significantly detected in cases with dust exposure history (P=0.04), daily using CL than occasional ones and sleeping wearing CL (66.7%), but without significant association (P >0.05). Other risk factors as the purpose of CL wear and the use of expired CL, without significance (P>0.05) but, using topical steroid was significant (P=0.011) (Tab. 4).

Genotyping was done for nucleotide from *Acanthamoeba* positive case. The partial nucleotide sequences of ASA.S1 region of the 18S rDNA gene aligned using ClustalW. Sequence homology search for *Acanthamo-*

eba spp. in the National Center for Biotechnology (NCBI) showed homology with genotype T9 isolate ICS20. The present sequence generated was submitted to the Genbank database (accession number KR 270798) and designated as strain NA-2015.

Phylogenetic tree reconstructions using the neighbour-joining method & MEGA6 software program placed the present *Acanthamoeba* spp. within genotype 9 with 71% similarity to *Acanthamoeba* genotype T9 isolate ICS20 and 58% similarity with *A. astronyxis* isolate: IK-HD191 (Fig. 5).

Discussion

Acanthamoeba keratitis is an ulcerative disease of the cornea which can cause severe ocular damage, ending in complete loss of vision (Lorenzo-Morales *et al*, 2015).

In the present study, the *Acanthamoeba* was detected in 3.8% of corneal swabs and 6.7% of CLSC samples. In Egypt, the prevalence of AK was 5.26% & 27.37% in corneal swabs and scraping respectively (Aboul-Magd *et al*, 2016). Also, *Acanthamoeba* was identified in 32/260 (12.3%) of cases with infectious keratitis (Taher *et al*, 2018). The low detection rate in the current work might be attributed to corneal swabbing. These results agreed with Anisah *et al.* (2005) who reported that swabbing was an insensitive technique for isolation and detection of amoeba. Also, Vemuganti *et al.* (2000) reported that the trophozoites were in the anterior stroma, but the cysts in the deeper one.

In the present study, the highest significant positivity was by culture (3.8%) followed by Giemsa and trichrome stains (2.5%), microscopy (1.3%) and lastly PCR (0%) of CS samples. Only one positive case (6.7%) was detected in CLSC by all methods, but without significance. Wanachiwanawin *et al*. (2012) reported positive rate of 15.3% for direct microscopy and 46.1% for culture. Niyyati *et al*. (2009) reported that corneal scrapes examination from AK patients was negative and culture was positive in 14.3%. The obstacles of *Acanthamoeba* detection by direct smear was due to small corneal samples with few parasite and required technical expertise (Qvarnstrom *et al*, 2006). Also, the antibiotics pre-treated patients have a very rare parasite (Lorenzo-Morales *et al*, 2015).

In the present study, culture was used as a gold standard test to detect *Acanthamoeba*. Direct smear showed 2 false-negative samples with sensitivity 50% and Giemsa stain showed one false-negative sample with 75% sensitivity. These results agreed with Boggild *et al.* (2009) who found that direct smear had the poorest diagnostic sensitivity (33-55%) and Giemsa-stained smear showed 55% sensitivity, Giemsa stain differentiated nuclear and cytoplasm, without staining cysts' outer wall (Behera and Satpathy, 2016).

In the present study, trichrome stain revealed *Acanthamoeba* in 3/94 specimens with 75% sensitivity and 100% specificity. El-Sayed and Hikal (2015) reported that Modified trichrome was the most consistent stain for *Acanthamoeba* cysts and superior to Giemsa stain with high rank (56%).

Molecular diagnosis improved AK diagnosis by amplifying *Acanthamoeba* DNA and detected scanty organisms in clinical cases (Laummaunwai *et al*, 2012). In the present study, genus-specific primers pair; JDP1 (forward) and JDP2 (reverse) were used for PCR amplification of *Acanthamoeba*-specific nuclear small subunit ribosomal RNA; 18S rRNA gene segment or the *Acanthamoeba* specific amplimer-S1 (ASA-S1), which were well accepted (Gatti *et al*, 2010; El-Sayed *et al*, 2014;Tawfeek *et al*, 2016).

In the current study, one positive *Acanthamoeba* case (6.7%) out of 15 CLSC samples was detected by PCR and all positive three corneal samples detected by culture were PCR-negative. PCR sensitivity (using JDP primers) compared to culture was 25%. But, Wanachiwanawin *et al.* (2012) reported positivity rate of 92.3% for conventional PCR, & 100% for real-time PCR. Aboul-Magd *et al.* (2016) reported that the highest significantly positive cases were obtained by PCR in both swabbed (5.26%) and scraped (27.37%) samples. Taher *et al.* (2018) reported PCR positivity of 12.3%.

Regarding the same primer pair, low sensitivity was obtained by Boggild *et al.* (2009), assumed that it might be due to the PCR inhibitor in the corneal tissue or low volume of corneal sample. Schroeder *et al.* (2001) attributed the low PCR sensitivity to presence of mature resistant cysts in positive sample, whereas samples with trophozoites or immature cysts were PCR positive. Likewise, Goldschmidt *et al.* (2008) found that the PCR-false-negative results might be due to the high resistance of *Acanthamoeba* cysts to reagents exposed DNA or insufficient DNA material from corneal samples. Application of local anesthesia before taking corneal samples inhibited Taq polymerase or act as PCR inhibitors (Laummaunwai *et al,* 2012; El-Sayed *et al,* 2014).

The current results showed that all *Acanthamoeba* infected cases were detected among CLW and CLSC, with the detection rate of 12.5% & 6.7% respectively. In Egypt, *Acanthamoeba* infection was detected in 81 % of CLW (El-Sayed *et al,* 2014). This association between AK & CLWs was proven once upon a time by others (Ibrahim *et al,* 2009; Gupta and Aher 2009). Wanachiwanawin *et al.* (2012) diagnosed AK in 62.5% of CLWs and in 37.5% of non-contact lens wearers (NCLW). Ghamilouie *et al.* (2014) reported that 5.6% of keratitis patients were *Acanthamoeba* positive in all the contact lens wearers. Also Aboul-Magd *et al*. (2016) reported that *Acanthamoeba* infection was higher in CLWs (34.48%) than NCLW (16.21%) but without significance. This association may be due to trauma in the corneal epithelium during manipulation of contact lens and transmission of *Acanthamoeba* trophozoites to the eye (Ibrahim *et al,* 2009). Also, chronic hypoxic stress on corneal epithelium by continuous use of CL led to edema and significant thinning of corneal epithelium (Liesegang, 2002).

In the present study, the *Acanthamoeba* parasites among CLWs were from group II with detection rate 12.5% and the only one positive identified case from CLSC was from group I with detection rate of 6.7%. Casero *et al.* (2017) reported that *Acanthamoeba* isolates from CL demonstrated phenotypic differentiation, where 82% of them were group II & 18% group III. Besides, Walochnik *et al.* (2015) reported that group II was the predominant pathogenic clinical isolates. Buchele *et al.* (2018) recorded that *Acanthamoeba* isolate identified by cyst morphology belonged to group II.

This study called attention to the risk factors associated with AK among CLWs. Regarding demographic criteria, *Acanthamoeba* infection was significantly detected in cases their age group >30-45 including all positive cases, (P=0.026). But, Taher *et al.* (2018) reported that *Acanthamoeba* parasite was significant in age group ≥21 to 25 years.

In the present study, *Acanthamoeba* was detected in females more than males, 2 out of 3 positive cases (66.7%) were females. This agreed with Walochnik *et al.* (2015) and Taher *et al.* (2018). On the contrary, Ibrahim *et al.* (2009) revealed that the females incidence were less than males. The association of *Acanthamoeba* infections among females may be attributed to usage of contact lens for cosmetic purpose in youth and refusal of wearing glasses (Mahittikorn *et al,* 2017). Also, females usually use cosmetics as eye mascara, which could coat the CLs surfaces, allowing bacteria and/or *Acanthamoeba* to adhere (Srinivasan *et al,* 2015).

In this study, there was significant association between different risk factors related to CL hygiene and water exposure and positive cases for *Acanthamoeba* parasite. Lack of awareness about CL hygiene was significantly associated with AK in 34.4% of cases (Taher *et al,* 2018). This might result from moderate to low SES.

The CDC sent a strong message about the risk of exposure to water sources on development of AK (Legarreta *et al,* 2013). Also, Evyapan *et al.* (2015) pointed out swimming and showering while wearing CL and lack of hand washing were an important risk factor for acquiring AK due to the forward regression analysis. Gomes *et al.* (2016) reported that high detection rate of *Acanthamoeba* in CLWs not washing hands before handling, or showering while wearing CL and in patients not cleaning the CL cases. Carnt *et al.* (2018) confirmed these risk factors and proved the importance of CL and hand hygiene, avoidance of CLs exposure to contaminated water, use of effective CL disinfection solutions, or use of disposable CLs in reducing the AK incidence.

Acanthamoeba genus was divided into 20 different genotypes (T1 to T20) based on the variation of ribosomal RNA nucleotide sequences (Fuerst *et al,* 2015). Each one displayed 5% or more sequence variations between different genotypes (Corsaro *et al,* 2015). Genotypes T3, T4, T5, T6, T10, T11, T13, & T15 cause human *Acanthamoeba* keratitis (Siddiqui and Khan, 2012).

In the current study, genotype determination was done for nucleotide sequence of positive cases. Partial nucleotide sequences of ASA.S1 region of the 18S rDNA gene aligned using ClustalW. The isolated sequ ence in NCBI revealed homology with *Acanthamoeba* genotype T9 isolate ICS20. The results agreed with Schroeder *et al.* (2001); Booton *et al.* (2002) and Kilvington *et al.* (2004) who found that the strains isolated from lens storage case and soil were both *A. comandoni* of genotype T9, widely reported as nonpathogenic. In this study, strain was isolated from contact lens storage case of an asymptomatic contact lens wearer without pathogenicity evidence. Orosz *et al.* (2018) elucidated the identification of *Acanthamoeba* isolate belonging to T8 in corneal sample and fluid from contact lens storage case.

In Egypt, studies reported different environmental prevalent *Acanthamoeba* genotypes. Lorenzo-Morales *et al.* (2006) identified 5 genotypes in freshwater sources in the Nile Delta, which were T1, T2, T3, T4 & T7 genotypes. Hassan *et al.* (2012) in Alexandria isolated *Acanthamoeba* from the hydraulic systems of both hemodialysis and dental units. Al-Herrawy *et al.* (2014) identified six *Acanthamoeba* species from 10 different swimming pools in Cairo; *A. polyphaga*, *A. castellanii*, *A. rhysodes*, *A. mauritaniensis*, *A. royreba* and *A. triangularis*. Tawfeek *et al.* (2016) also detected three isolates; T4, T3, & T5 from environmental sources.

Conclusion

The culture method proved to be standard test for *Acanthamoeba* species. It is reliable, cheaper and sensitive than either direct DNA extraction or PCR. There are obstacles with PCR to detect *Acanthamoeba* especially in corneal swab. The important risk factors that predispose to AK are related to the contact lens misused. This study confirmed different risk factors association with AK in CLWs. Genotype determination for *Acanthamoeba* positive case by PCR revealed homology genotype T9 isolate ICS20. Health education regarding proper CL hygiene and dangers of tap water exposure is important. To our knowledge, this is the first time that *Acanthamoeba* genotype T9 is isolated from CLSC in Menoufia Governorate, Egypt.

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Explanation of figures

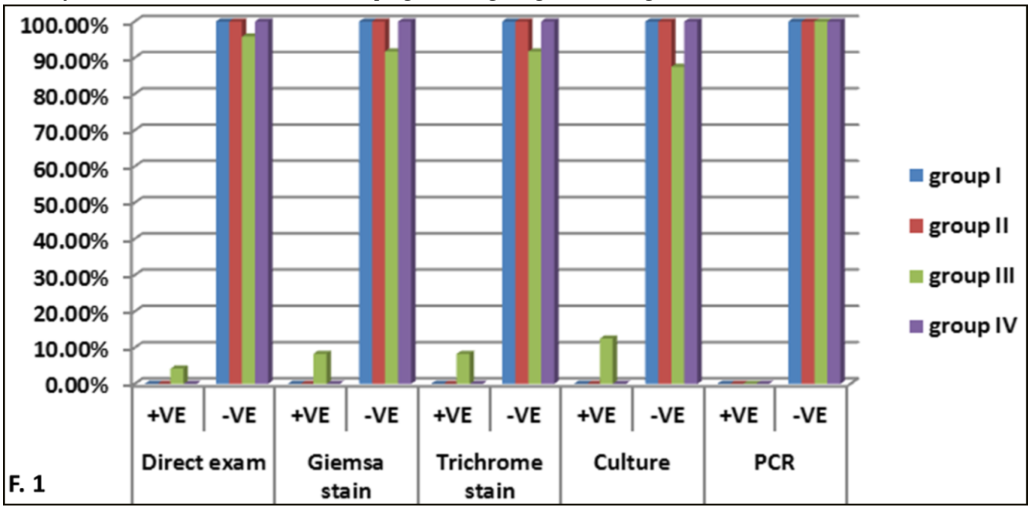
Fig. 1: Distribution of *Acanthamoeba* infection in different groups examined by different techniques

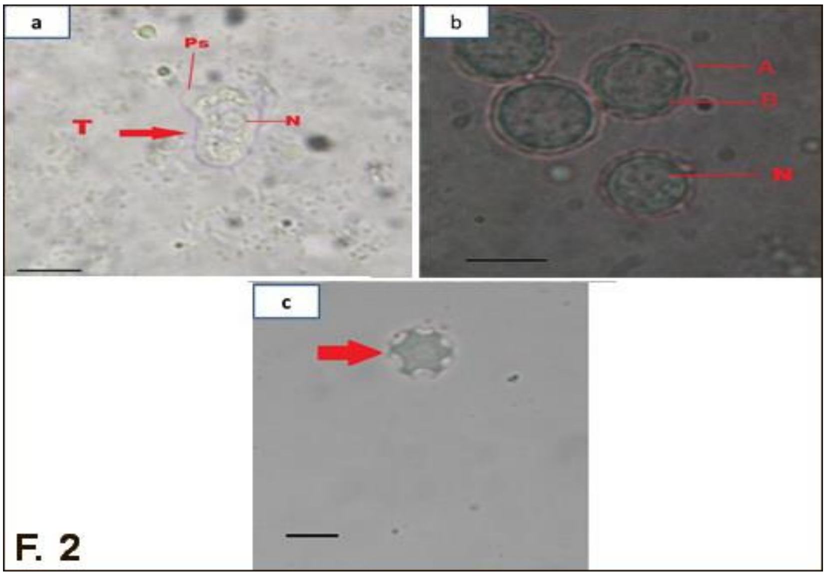
Fig. 2: Direct wet mount m\smear (x1000), Scale bar 20um. a: *Acanthamoeba* trophozoite (T), N=nucleus, Ps= pseudopodium. b- Cysts of subgroup II, A= ectocyst, B= endocyst, N=nucleus. C- Cyst of subgroup I (red arrow)

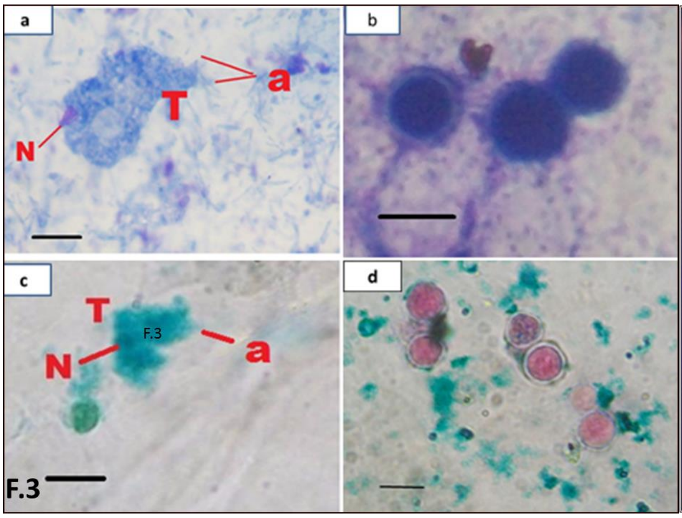
Fig. 3: *Acanthamoeba* trophozoite **(a)** and cysts **(b)** with Giemsa stain. *Acanthamoeba* trophozoite **(c)** and cysts **(d)** with trichrome stain (x1000). T= trophozoite, N= nucleus, a= acanthopodia. Scale bar =20 µm.

Fig. 4: Agarose gel electrophoresis of PCR products, Lane 1=DNA ladder (100-1000 bp), Lane 2= +ve control, Lane 3= -ve control, Lane 4= -ve sample, Lane 5= +ve sample, and Lanes 6, 7 and 8 = -ve samples.

Fig. 5: Phylogenetic tree including strain NA-2015. Evolutionary distances were computed using Maximum Composite Likelihood method. Phylogenetic analysis conducted in MEGA6 software program using Neighbor-Joining method.






a
b
c
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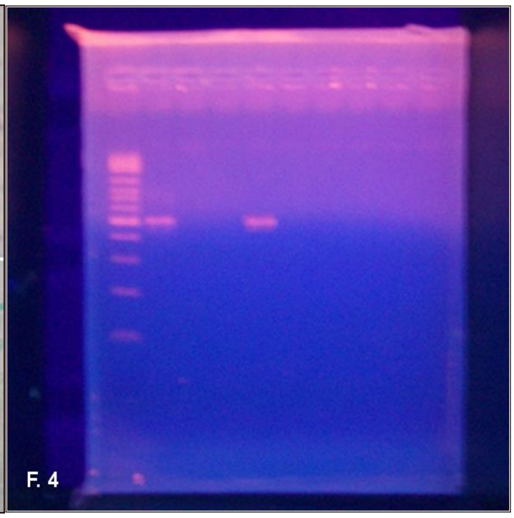
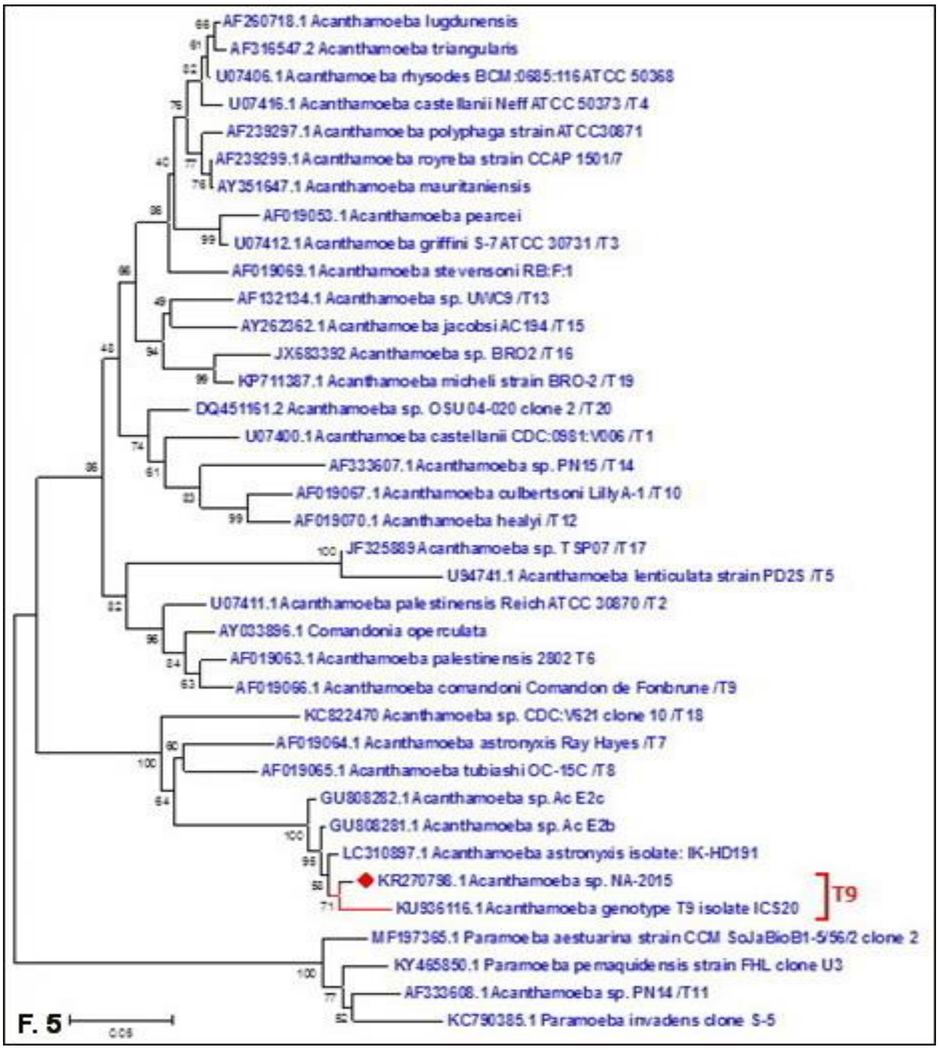
N
T
a
F.3


F. 4


AF260718.1 Acanthamoeba lugdunensis
AF316547.2 Acanthamoeba triangularis
U07406.1 Acanthamoeba rhysodes BCM:0685:116 ATCC 50368
U07416.1 Acanthamoeba castellanii Neff ATCC 50373 /T4
AF239297.1 Acanthamoeba polyphaga strain ATCC30871
AF239299.1 Acanthamoeba royreba strain CCAP 1501/7
AY351647.1 Acanthamoeba mauritaniensis
AF019053.1 Acanthamoeba pearcei
U07412.1 Acanthamoeba griffini S-7 ATCC 30731 /T3
AF019069.1 Acanthamoeba stevensoni RB:F:1
AF132134.1 Acanthamoeba sp. UWC9 /T13
AY262362.1 Acanthamoeba jacobsi AC194 /T15
JX683392 Acanthamoeba sp. BRO2 /T16
KP711387.1 Acanthamoeba micheli strain BRO-2 /T19
DQ451161.2 Acanthamoeba sp. OSU 04-020 clone 2 /T20
U07400.1 Acanthamoeba castellanii CDC:0981:V006 /T1
AF333607.1 Acanthamoeba sp. PN15 /T14
AF019067.1 Acanthamoeba culbertsoni Lilly A-1 /T10
AF019070.1 Acanthamoeba healyi /T12
JF325889 Acanthamoeba sp. T SP07 /T17
U94741.1 Acanthamoeba lenticulata strain PD2S /T5
U07411.1 Acanthamoeba palestinensis Reich ATCC 30870 /T2
AY033896.1 Comandonia operculata
AF019063.1 Acanthamoeba palestinensis 2802 T6
AF019066.1 Acanthamoeba comandoni Comandon de Fonbrune /T9
KC822470 Acanthamoeba sp. CDC:V621 clone 10 /T18
AF019064.1 Acanthamoeba astronyxis Ray Hayes /T7
AF019065.1 Acanthamoeba tubiashi OC-15C /T8
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GU808281.1 Acanthamoeba sp. Ac E2b
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T9
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KY465850.1 Paramoeba pemaquidensis strain FHL clone U3
AF333608.1 Acanthamoeba sp. PN14 /T11
KC790385.1 Paramoeba invadens clone S-5
F. 5
0.05