·Clinical Research·

Evaluation of patients with dry eye disease for conjunctival *Chlamydia trachomatis* and *Ureaplasma urealyticum*

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Abstract

• **AIM: To determine the possibility of the development of dry eye disease (DED) as a result of persistent infection with *Chlamydia trachomatis* and *Ureaplasma urealyticum* in the conjunctiva of patients.**

• **METHODS: This study was conducted on 58 patients of age range 20-50y, diagnosed with DED confirmed by Schirmer I test and tear breakup time. The non-dry eye control group included 27 subjects of the same age. Ocular specimens were collected as conjunctival scrapings and swabs divided into three groups: the first used for bacterial culture, the second and third taken to detect *Chlamydia trachomatis* and *Ureaplasma urealyticum* by direct fluorescent antibody (DFA) assay and polymerase chain reaction (PCR) method.**

• **RESULTS: *Chlamydia trachomatis* was detected in 65.5% and 76% of DED patients by DFA and PCR methods respectively. *Ureaplasma urealyticum* was found in 44.8% of DED infected patients using the PCR method. Both organisms were identified in only 37.9% of DED patients found to be infected. Control subjects had a 22% detection rate of *Chlamydia trachomatis* by DFA assay versus a 7% detection rate by PCR; while *Ureaplasma urealyticum* was detected in 3.7% of the controls by PCR method. The conjunctival culture revealed that gram positive microorganisms represented 75% of isolates with coagulase negative Staphylococci the most common (50%) followed by *Staphylococcus aureus* (20%), whereas gram negative microorganisms occurred in 25% of cases, isolating *Moraxella* spp. as the most frequent organism.**

• **CONCLUSION: Our results tend to point out that *Chlamydia trachomatis* and *Ureaplasma urealyticum* were detected in a moderate percentage of patients with DED, and could be a fair possibility for its development. PCR is more reliable in detecting *Chlamydia trachomatis* than DFA technique. The presence of isolated conjunctival bacterial microflora can be of some potential value.**

• **KEYWORDS:** dry eye disease; conjunctiva; *Chlamydia trachomatis*; *Ureaplasma urealyticum*; direct fluorescent antibody; polymerase chain reaction
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INTRODUCTION

Dry eye syndrome is a common disorder that results from decreased tear production, excessive tear evaporation or abnormality in mucin or lipid components of the tear film[1]. Dry eye disease (DED) is the most common eye disease, affecting 5%-6% of the population[2]. Approximately 1 out of 7 individuals aged 65y to 84y reports symptoms of dry eye often or all of the time[3]. In order to address the problem, the International Dry Eye Workshop (DEWS) defines dry eye as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface[4]. Indeed, DEWS has recognized dry eye as a disturbance of the lacrimal functional unit, an integrated system comprising the lacrimal glands, ocular surface (cornea, conjunctiva and meibomian glands) and lids, and the sensory and motor nerves that innervate them. Dysfunction of any component of the lacrimal functional unit may lead to ocular surface disease, related to inflammation and increased tear film osmolarity[3]. DED is a growing public health problem and one of the most frequent reasons for seeking ophthalmological intervention. Various terms have been used to describe DED including keratoconjunctivitis sicca and, more recently, dysfunctional tear syndrome suggesting that the name more accurately reflects pathophysiological changes. The definition of DED

which includes etiology, pathophysiology, and symptoms was recently improved in the light of new findings about the role of tear hyperosmolarity and ocular surface inflammation in dry eye and its effect on visual function [5]. Inflammation is a central feature of ocular surface disease. Conjunctival inflammation is manifested by infiltration of inflammatory cells and upregulated expression of immune markers[5]. In dry eye, a chronic inflammatory reaction, possibly subclinical, is generated at the ocular surface, which can result in vital dye staining of the cornea and conjunctiva [6]. The accumulation of inflammatory molecules at the ocular surface of dry eye patients, accompanied by a stagnant tear film and decreased level of mucins, can lead to destruction of epithelial tight junctions, and result in sloughing of the ocular surface epithelia [7]. Dry eye is often associated with ocular surface conditions such as anterior blepharitis, meibomian gland disease, keratitis, where alterations in the concentration and type of bacteria present have been reported [8]. Such disorders, among others, have been associated with several gram-positive and gram negative bacteria, including coagulase negative Staphylococci (CNS), *Staphylococcus aureus*, *Streptococcus* spp., *Bacillus subtilis*, *Rhodococcus* sp., *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Haemophilus aegyptius*, and *Klebsiella* spp. [9-11]. The production of lipases and toxins by many of these colonizing bacteria may induce ocular surface cellular damage and destabilization of the lipid layer of the tear film contributing to tear film instability, inflammation, and symptoms of significant ocular irritation. Similar symptoms commonly occur in dry eye, without evidence of purulent exudative infection [12-13]. This causes activation of inflammatory cells including T-lymphocytes by immune system of body. T-cells release cytokines which causes inflammation of ocular surface and glands, thereby resulting in abnormal tears and dry eye symptoms. An increase in osmolarity of the aqueous layer is suggested as a global feature of DES and is known to trigger inflammation, damaging the ocular surface [14]. In addition to the DEWS definition of DED emphasizing the role of inflammation in the pathogenesis of this disease, which is reflected in the therapeutic strategies that have been used recently to treat DED. Some long-term clinical manifestations of inflammation, as conjunctival hyperemia, edema, and insignificant infiltration, are shared by both DED and chronic conjunctivitis, which is clinically indistinguishable. Furthermore, chronic conjunctivitis may possibly result in DED [15]. Chronic inflammation of the conjunctiva also may be caused by persistent infection leading to the development of DED. *Chlamydia trachomatis* and *Ureaplasma urealyticum* are the most common pathogenic microorganisms capable of persisting in tissues of human body for long periods and causing chronic low-grade nonspecific inflammation. Evidences of conjunctival localization with possible development of conjunctivitis have been reported for these pathogens[16-17].

The aim of our present work was to determine the possibility of the development of DED as a result of persistent infection with *Chlamydia trachomatis* and *Ureaplasma urealyticum* in patients' conjunctiva.

SUBJECTS AND METHODS

Subjects The study adhered to the tenets of the Declaration of Helsinki and was approved by Ethics Committee of Research Institute of Ophthalmology, Giza, Egypt. All patients were informed regarding the procedure with written consent. This study was carried out after obtaining the approval from the Ethical committee of Research Institute of Ophthalmology, Giza, Egypt.

The study included 58 patients, with age range 20y to 50y, and admitted to the outpatient clinic of the Research Institute of Ophthalmology.

The subjects enrolled for the study were complaining of all or one of the following symptoms: eye dryness, foreign body and/or sand sensation, watering of eyes and conjunctival discharge. They were clinically diagnosed as DED, with a Schirmer's I test of 15 mm or less and tear film break-up time (BUT) of 8s or less.

The patients excluded from the study enrollment were those having acute conjunctivitis, a history of refractive surgery or Reiter's syndrome. In addition, those who were currently on antibiotics or anti-inflammatory agents administration were excluded. Patients wearing contact lenses were also excluded from the study. Smoking was also included in the exclusion criteria. The non-dry eye control group included 27 subjects of the same age.

Methods All patients underwent thorough slit lamp biomicroscopic examination by an ophthalmologist. Demograpic details including age, clinical history, duration of the disease and associated findings were recorded onto a standard clinical history form. To assess the severity of the disease, Schirmer's I test and tear BUT tests were performed. Schirmer's I test was performed according to standard procedures, briefly: Schirmer test measures aqueous tear production easy to perform (but may be subjected to errors). Strips of filter paper, called Schirmer strips are placed on the lower lid inside the tarsal conjunctiva. The patient is allowed to blink normally and the tear strip is scored according to the degree it wets in 5min. There are two ways to perform this test: 1) without topical anesthesia (Schirmer test I) which evaluates the ability of the ocular surface to respond to surface stimulation; 2) under topical anesthesia (Schirmer test II) which evaluates basal tear secretion. In our study we performed the Schirmer test I method. Patients with tear soaking less than 10 mm are considered to have clinical DED and less than 5 mm wetting are considered severe DED.

BUT measures the interval in seconds between a complete blink and the appearance of the first break in the tear film. Average BUT values were calculated from three repeated measurements. A drop of fluorescein dye was instilled into the eyes and the tear film was observed under the slit lamp biomicroscope [15,18]. The readings of these repeated measurements were used to calculate the average BUT values.

Ocular Samples Conjunctival scrapings were taken by the ophthalmologist under all aseptic precautions from both eyes, and used for bacteriological culture, direct fluorescent antibody (DFA) assay and polymerase chain reaction (PCR). Five minutes after instillation of local anesthetic to the eye, each patient had a conjunctival scraping taken from over the tarsal conjunctiva. The material obtained were divided into 3 groups to be inoculated onto the surface of agar plates; spread on a slide and fixed with 70% cold methanol; and placed in nutrient broth, for the culture, DFA and PCR respectively.

Bacterial Culture The obtained material was inoculated onto blood agar, chocolate agar and MacConkey's agar media. The inoculation technique consisted of multiple "C" shape streaks on the culture plate to localize the site of implantation of the conjunctival scrape on the agar media. The inoculated blood and chocolate agar plates were incubated in CO_2 incubator at 37℃, and MacConkey's agar plate was incubated aerobically at 37 ℃. All plates were inspected for growth daily for 5d. If no growth occurred, plates were discarded as negative. Culture positive growth was identified by their colony morphology, Gram staining and further identified by relevant biochemical tests [19].

Direct Fluorescent Antibody Assay

Preparation of the sample slides Conjunctival scrape smears were rubbed firmly and evenly over the entire coated well of a multispot glass slide (flow lab. USA). The smear was allowed to dry and then fixed immediately by covering the area with methanol, which was allowed to evaporate. Fixed smears were stored at 2℃-8℃ to be stained within 7d of collection (immuno-fluorescent staining)[20]. The *Chlamydia trachomatis* direct fluorescent monoclonal antibody reagent kit (Syva UK) was used, according to the manufacturer's instructions and as the method described by Thomas *et al* [21].

Preparartion of control slides Positive and negative control slides (included in the test kit) were stored at 2℃-8℃. On the staining day, the sealed slides were left at room temperature for at least 5min, before removing it from its foil pouch.

Immunofluorescent staining procedure The monoclonal antibody reagent, control and patient slides, were allowed to reach room temperature before use. Conjunctival scrape smears and control slides were covered with 25 microliters of fluorescein-isothiocyanate- (FITC)-conjugated monoclonal antibody for 30min at room temperature in a dark, humidified chamber. After being washed in phosphate buffer saline (PBS) and twice in distilled water, they were allowed to air dry. A drop of mounting fluid was added to the center of each slide well and a cover slip was placed on top of the drop.

Reading of the test The slides were read using a fluorescence microscope (Leitz), ×1000 magnification. The elementary bodies (EB) appeared as individual pin-points of medium to bright apple-green fluorescence. To ensure specimen adequacy, at least 10 intact columnar epithelial cells should appear on the slide. A Specific pattern was considered as small, pin point, apple- green loci of fluorescence, located intra or extracellularly or large moderate bright green loci of fluorescence, intracellularly which corresponds to individual cells and intracellular inclusion bodies, respectively [22]. At least 10 EBs with contrasting reddish brown back ground of counterstained cells were identified on the positive specimen [23]. For performance verification of the staining procedure and reading, both control slides were stained and read in parallel with each series of patient's slides. The positive control slide was used as a reference in evaluating EBs on the patient specimens.

Polymerase Chain Reaction

Sample collection and DNA extraction DNA from all conjunctival swabs was extracted within one month of receipt. Briefly, DNA was extracted from each conjunctival swab using QIA amp DNA Micro extraction kit from Qiagen respectively according to manufacturer's instructions. QIA shredder was also used to harvest the lysate.

DNA amplification The primers used in this study, their sequence, product size and references are: 1) *Chlamydia trachomatis*, 144 bp [24]: CT1:CCT/GTG/GGG/AAT/CCT/GCT/GCT/GAA,CT4:GTC/GAA/AAC/AAA/GTCATCCAGTA/GTA; 2) *Ureaplasma urealyticum*, 429 bp [25]: U5:5-CAA TCT GCT CGT GAA GTA TTA C-3,U4:5- ACG ACG TCC ATA AGC AAC T-3.

Conditions for *Chlamydia trachomatis* The primers used were derived from highly conserved regions of the published DNA sequences for the major outer membrane protein (MOMP) of *Chlamydia trachomatis* serovars[26-28]. All serovars produced the same intensity 144-bp fragment [24]. *Chlamydia trachomatis* is classified into 15 distinct serovars based on antigenic variation of the ompA genes that encode the organism's MOMP. The 15 different serovars display well-documented and unique tissue tropisms. Serovars A, B, Ba, and C are the causative agents of trachoma, the most common infectious cause of blindness worldwide. Serovars D-K are a major cause of oculogenital infection worldwide but are not associated with blinding trachoma. Inclusion conjunctivitis is the most common form of an eye infection

caused by *Chlamydia trachomatis* serovars DK. Serovars L1, L2 and L3 cause lymphogranuloma venereum [24]. In brief, 2 μL of DNA extracts was processed in a 30 μL reaction volume containing PCR buffer [10 mmol/L Tris (pH 9.0), 50 mmol/L KCl, 0.01% gelatin], 200 μmol/L deoxynucleoside triphosphates, 2.5 mmol/L $MgCl_2$, 0.5 μmol/L each primer, and 1 U of Taq polymerase. Amplifications were carried out in a master cycler. The first cycle, consisting of a 5-min denaturation at 94℃, was followed by 35 cycles each of 30s at 94℃, 45s 56℃, and 1min at 72℃, with a final extension for 10min at 72℃. The PCR products were visualized in 2% agarose gels containing 0.5 μg of ethidium bromide/mL.

Conditions for *Ureaplasma urealyticum* Reaction mixture was prepared in a PCR tube by combining the reagents as follows: PCR master mix 12.5 μL, DNA template 2.5 μL, Primer 1 MgpaW1 2 μL, Primer 2 MgpaR 2 μL H_2O 6 μL, total volume 25 μL. PCR tubes were then placed in a thermal cycler and PCR amplification was done according to the following program.

Temperature cycling program: step 1, initial denaturation at 94℃ for 2min. Step 2, 50 cycles of (step 2.1: denaturation at 94℃ for 30s, step 2.2: annealing at 56℃ for 1min and step 2.3: elongation at 72℃ for 45s). Step 3, final extension at 72℃ for 5min. A positive PCR test should yield a 429 bp DNA fragment that would appear as an intense band on ethidium bromide stained 2.0% agarose gel.

Statistical Analysis Data were analyzed using IBM SPSS advanced statistics version 22 (SPSS Inc., Chicago, IL, USA). Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. Kappa test was used to evaluate agreement between two diagnostic methods. If the PCR was considered as the gold standard then sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for the MFA. All tests were two-tailed. A P-value <0.05 was considered significant.

RESULTS

The study included 58 patients, with age range 20y to 50y. The non-dry eye control group included 27 subjects of the same age. It is shown that 50 (86.2%) of the total number of DED patients (58) are below 40y of age, while only 8 (13.8%) were above 40y. Control group subjects were almost the same age as DED patients. By statistical analysis, there was no significant age difference between patients and controls (P=0.573). It was noted a statistical significance for *Chlamydia trachomatis* by PCR, in relation to age groups among the DED patients, where out of the 50 patients below 40 years of age, 40 (80%) were infected with *Chlamydia trachomatis* as detected by PCR, while in half (50%) of the patients who were above 40y, were found to be positive for *Chlamydia trachomatis* by PCR, P=0.066. On the contrary, no significance was observed when *Chlamydia trachomatis* was diagnosed by DFA among DED patients and its relation to the age groups. *Chlamydia trachomatis* was found at a similar percentage in patients below and above 40y (68% and 50% respectively). There was no preponderance of the organism in either group of patients, P=0.320. Similarly, *Ureaplasma urealyticum* had no predilection in either age group, where they were detected in 48% and 25% in DED patients, below and above 40y, respectively. No statistical significance was observed, P=0.276. Interestingly, of the DED patients below 40y of age, 22 (44%) were co-infected with both *Chlamydia trachomatis* and *Ureaplasma urealyticum* detected by PCR, while none of the DED patients who were above 40y of age were infected with both organisms. *Chlamydia trachomatis* in association with *Ureaplasma urealyticum* was not identified in the group above 40y of age. Thus a statistical significance was found for both organisms detected by PCR in relation to age, P= 0.019. *Chlamydia trachomatis* was detected in 44 (75.9%) out of 58 DED patients by PCR, in comparison to only 2 cases (7.4%) out of 27 non DED control group. The results obtained showed significant difference between the patients and control groups, P<0.001 (Table 1). By using the DFA test in comparison to the PCR method, positivity of *Chlamydia trachomatis* immunefluorescence was found in 38 (65.5%) DED patients versus 6 (22.2%) control non DED group. By statistical analysis, there was a significant difference between patients and control group, P<0.001 (Table 2). There was a statistical significant difference noted between DED patients and control groups in the *Ureaplasma urealyticum* percentage of detection, P<0.001. Of all the 58 DED patients, 44.8% were found to be infected with *Ureaplasma urealyticum*, while only 1 (3.7%) patient of the 27 non DED control group was infected with *Ureaplasma urealyticum* (Table 3). Table 4 shows the positivity of both organisms (*Chlamydia trachomatis* and *Ureaplasma urealyticum*) in DED infected patients. Interestingly, of all 58 DED infected patients, 22 (37.9%) were co-infected with *Chlamydia trachomatis* and *Ureaplasma urealyticum*, while none of the control non DED group were found to be infected with both organisms concomitantly. Statistical analysis showed a significance of difference between patients and control groups, P<0.001. Table 5 shows the agreement between DFA and PCR methods for *Chlamydia trachomatis* among the DED group of patients. The percentage of positive and negative agreement between both tests for *Chlamydia trachomatis* was 65.5% and 24.1%, respectively, Kappa was significant 0.754. In addition, none of the patients were PCR negative and DFA positive, while 10.3% were PCR positive and DFA negative for *Chlamydia trachomatis*. The difference between results of the two tests is significant, P<0.001. The conjunctival culture isolates from the total number of patients

Table 1 *Chlamydia trachomatis* in DED patients by PCR *n* (%)

CT PCR	Group		Total
	Patients	Control	
Positive	44 (75.9)	2 (7.4)	46 (54.1)
Negative	14 (24.1)	25 (92.6)	39 (45.9)
Total	58 (100)	27 (100)	85 (100)

CT: *Chlamydia trachomatis*. χ^2=34.769, *P*<0.001.

Table 2 *Chlamydia trachomatis* in DED patients by DFA *n* (%)

CT DFA	Group		Total
	Patients	Control	
Positive	38 (65.5)	6 (22.2)	44 (51.8)
Negative	20 (34.5)	21 (77.8)	41 (48.2)
Total	58 (100)	27 (100)	85 (100)

CT: *Chlamydia trachomatis*. χ^2=13.831, *P*<0.001.

Table 3 *Ureaplasma urealyticum* in DED patients by PCR *n* (%)

UU PCR	Group		Total
	Patients	Control	
Positive	26 (44.8)	1 (3.7)	27 (31.8)
Negative	32 (55.2)	26 (96.3)	58 (68.2)
Total	58 (100)	27 (100)	85 (100)

UU: *Ureaplasma urealyticum*. χ^2=14.375, *P*<0.001.

Table 4 Positivity of both *Chlamydia trachomatis* and *Ureaplasma urealyticum* in DED patients by PCR *n* (%)

CT_UU PCR	Group		Total
	Patients	Control	
Both positive	22 (37.9)	0 (0)	22 (25.9)
Others	36 (62.1)	27 (100)	63 (74.1)
Total	58 (100)	27 (100)	85 (100)

CT: *Chlamydia trachomatis*; UU: *Ureaplasma urealyticum*. χ^2= 13.818, *P*<0.001.

Table 5 Agreement between DFA and PCR methods for *Chlamydia trachomatis* among DED patients %

CT DFA		CT PCR		Total
		Positive	Negative	
Positive	Count	38	0	38
	% within CT DFA	100.0	0.0	100.0
	% within CT PCR	86.4	0.0	65.5
	% of total	65.5	0.0	65.5
Negattive	Count	6	14	20
	% within CT DFA	30.0	70.0	100.0
	% within CT PCR	13.6	100.0	34.5
	% of total	10.3	24.1	34.5
Total	Count	44	14	58
	% within CT DFA	75.9	24.1	100.0
	% within CT PCR	100.0	100.0	100.0
	% of total	75.9	24.1	100.0

CT: *Chlamydia trachomatis*. κ=0.754, *P*<0.001.

(58), of the DED group, depicted that gram-positive and gram-negative organisms were isolated in 43 (74.1%) and 15 (25.8%) patients, respectively, where 50 (86.2%) of the DED group patients who were less than 40y had positive bacterial growth isolated from their conjunctival culture; contrary to the DED patients who were above 40y, who had a positive bacterial growth isolated in only 8 (13.8%) cases. Gram positive bacteria were the predominant organisms isolated in DED patients below 40y of age in comparison to those above 40y of age, 88.4% and 11.6%, respectively. Similarly gram negative organisms were isolated in 80% of DED patients below 40y of age versus 20% gram negative isolates in DED patients above 40y. However, by statistical analysis, there was no significant difference between the results of the conjunctival culture in relation to the age group. No statistical significance was noted between *Chlamydia trachomatis* detected by PCR and conjunctival culture among DED patients, P=0.018; while on the contrary there was a significant correlation between conjunctival culture and *Chlamydia trachomatis* detected by DFA among the DED patients, P=0.074. On the other hand, gram positive and gram negative bacteria were isolated from conjunctival culture at a closely similar percentage (44.2% and 46.7%, respectively) in DED patients infected with *Ureaplasma urealyticum* by PCR method, indicating that there was not a significant difference observed between conjunctival culture and *Ureaplasma urealyticum* detected among DED group of patients, P=0.868. The results obtained also showed that there was no significant correlation observed between the conjunctival culture and both organisms (*Chlamydia trachomatis* and *Ureaplasma urealyticum*) detected by PCR, P=0.296.

If you considered PCR as gold standard then the sensitivity of DFA was 86.4%, specificity was 100%, PPV was 100%, NPV was 70.0%, accuracy was 89.6%.

DISCUSSION

Patients with complaints of foreign body sensation, hyperemia and conjunctival infilteration is associated with chronic infectious conjunctivitis. Since mild conjunctivitis is associated with DED [29], therefore chronic conjunctivitis would be associated with DED and clinically proven by Schirmer test and tear BUT. This chronic infectious conjunctivitis could be caused by *Chlamydia trachomatis* and *Ureaplasma urealyticum*.

Certain exclusion criteria were defined and our patients were selected accordingly. The risk factors for DED including age, history of refractive surgery, contact lens, systemic disease, occupational risk were among these exclusion criteria and this was for the reason of defining the etiology of DED in these patients to an infectious agent (*Chlamydia trachomatis* and *Ureaplasma urealyticum*) and not the above mentioned risk factors.

Chlamydia trachomatis and *Ureaplasma urealyticum* are considered chronic infectious agents persisting in tissues for long periods, causing a chronic inflammation[30-32].

According to the DEWS, the pathogensis of DED is inflammatory in nature [33] with the clinical picture of chronic inflammation including conjunctival hyperemia, edema and

infiltration, is presented by both DED patients and chronic conjunctivitis as well. Chronic inflammation of the conjunctiva may be caused by a persistent and latent infection which ultimately leads to the development of DED.

As there are some evidences that *Chlamydia trachomatis* and *Ureaplasma urealyticum* are possibly implicated in conjunctivitis [34-35], our aim was to observe a possible association between these two pathogenic organisms and the studied patients, who were clinically diagnosed as DED.

There is now an increased recognition by clinicians that DED is a common disorder characterised by dryness and damage of the ocular surface. It affects quality of life, including aspects of physical, social and psychological functioning, because it induces ocular discomfort, burning sensation, light sensitivity, visual disturbances or even corneal erosions and infections. DED is also known as keratoconjunctivitis sicca, dry eye syndrome and dysfunctional tear syndrome [4].

Demographic characteristic of the patients regarding age group showed that 86.2% of the DED patients were below 40y of age and considered as young adults. This was also observed in a study by Boiko *et al* [15] whose DED group patients were in the average of 35y; but Moss *et al* [36] reported that the prevalence of dry eye was 14.4% in subjects aged 48y to 91y and they observed that the prevalence of the disease doubled after the age of 59. In addition, the Beaur Dam population based study found the DED prevalence rate to be 14% in adults 48 to 91y of age[36].

The Definition and Classification Subcommittee of the International Dry Eye Workshop (DEWS) of 2007 has somewhat modified this definition. DEWS determined that dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance and tear film instability, with potential damage to the ocular surface [33]. The DEWS definition also states that dry eye is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface. These features lead to the dry eye cascade of visual degradation, epithelial cell damage and discomfort.

Most individuals with this condition are female, ages 30 and older. According to the Women's Health Study, the prevalence of dry eye affects more women as they age [37]. Although the prevalence increases in men, too, it doesn't keep pace with the presence of dry eye in women. Also, women who used hormone replacement therapy (HRT) had a 69% greater risk of developing dry eye syndrome [37]. If estrogen therapy was combined with progesterone/progestin, the risk went up another 29% [37]. The risk of dry eye increased 15% for every three-year interval that the women remained on HRT [37]. Many patients develop a dry eye condition over years and decades before it is recognized.

The most recent concept in dry eye pertains to the role of inflammation. Opinions vary as to whether inflammation initiates or occurs in the middle of the dry eye cycle. Still, once inflammation begins, damage can occur to the ocular structures. This, in turn, perpetuates and intensifies the signs and symptoms of dry eye. No matter what the cause, we must break the cycle in this cascade. Inflammation of the ocular surface influences the stability of tear fluid, which is measured by tear BUT. The average BUT in our studied DED patients was 4-5s in comparison to controls which was 10-11s. Uchida and Imanaga [29] reported that 54 cases with DED and complicated with mild conjunctivitis had a mean BUT of 3.54. Nearly the same timing was also observed by Boiko *et al* [15].

Dry eye was verified by the Schirmer test which showed reduced tear production in the DED group patients, with an average of 8 to 9 mm in comparison to the control group which was 17 to 20 mm. An average reading of 8 mm and 15 mm, was reported by Boiko *et al* [15], for DED and control groups, respectively. Ohashi *et al* [18] suggested that a combination of dry eye symptoms and suggestive findings on Schirmer (<10 mm wetting after 5min) would verify clinical dry eye. To confirm a diagnosis of dry eye, certain tests are required in the clinical setting, including tear film stability which can be assessed with fluorescein tear BUT test. Patients with BUT less than 3s are classified as clinical dry eye.

The study deals with the possible etiology role of *Chlamydia trachomatis* and *Ureaplasma urealyticum* in the development of dry eye. Conjunctival chlamydia infection is manifested by the clinical picture of chronic slowly progressive inflammation with the progression of DED after a latent period of about 2-3y [38]. The average duration of the eye affliction in our patients was reported within the range of 4-5y, with a gradual increase in the severity of symptoms over time. This long duration and the clinically asymptomatic picture is a characteristic of latency of infection.

There is an association between DED and *Chlamydophila pneumoniae* in patients with follicular conjunctivitis which proves localization of the bacteria in the conjunctiva [39]. A similar connection with *Chlamydia trachomatis* was observed as a causative agent for chronic conjunctival inflammation[40]. Chronicity is a key role of *Chlamydia trachomatis* in the pathogensis of DED. The persistence of the pathogenic organism, and the changes that occur in its morphology characterized by change in epitope expression elucidates the changes that occur in conjunctival cellular morphology during latent infection [41]. The prevalence of *Chlamydia trachomatis* in a population, its persistence and tendency towards chronic inflammation [42], all these are similar factors in the pathogensis of conjunctivitis and trachoma, where in the latter there is additional scarring with lymphocytic infiltration [43]. Interestingly these are the same processes that take place in DED caused by infection, but in addition there

is a decrease in tear production and change in tear composition due to goblet cells and lachrymal gland changes[41].

The prevalence of *Chlamydia trachomatis* in our DED patient group was assessed by two different methods, DFA and PCR. There was a significant increase of detection rate of *Chlamydia trachomatis* in the conjunctiva of DED patients than in the control non dry eye groups, by DFA and PCR (65.5% *vs* 22.2%) and (75.9% *vs* 7.4%), respectively, $P<0.001$, Tables 1, 2. In a study done by Boiko *et al* [38], they examined the conjunctiva of 150 patients with DED by DFA for *Chlamydia trachomatis* infection. The infection rate was reported to be 63.3% as mono or mixed infection with *Mycoplasma and Ureaplasma*. They reported that Chlamydia infection of the conjunctiva is one of the causes artificial dry eye. Simalrly, Boiko *et al* [38] found an increased prevalence of dry eye in patients testing positive for chlamydia conjunctivitis. However, Krasny *et al* [44] noted that patients who were successfully treated for chronic follicular conjunctivitis due to *Chlamydia trachomatis* demonstrated improvement in their dry eye condition. They suggested that ocular surface infection could possibly predispose to dry eye, rather than dry eye predispose to infections in a patient. We are strongly inclined towards their former suggestion given that *Chlamydia trachomatis* infection is associated with high prevalence in Egypt and the cause of trachoma disease which is endemic in Egypt, followed by chlamydial conjunctivitis, which is one of the most common causes of conjunctivitis and this could explain the high frequency of detection rate of *Chlamydia trachomatis* in DED patients who were less than 40y of age in comparison to those who were above 40y of age, by the PCR method (80% *vs* 50%), $P=0.066$. Boiko *et al* [15] reported a similar finding, where they observed that DED patients were infected with *Chlamydia trachomatis* in 63.2% of them, detected by DFA method. They concluded that *Chlamydia trachomatis* was detected with high frequency in the conjunctiva of young adults (25-45y) with DED, and this was related to increased risk of urogenital infection in this group[45].

Another organism, *Ureaplasma urealyticum*, was investigated in our study, which is also associated with urogenital disease in young adults[32,45]. The mycoplasmataceae family can cause conjunctival damage and there is evidence that *Ureaplasma urealyticum* is associated with chronic conjunctivitis and DED[35]. Conjunctival scrapes were examined by PCR method for evidence of *Ureaplasma urealyticum*, in our DED patient group, there was a statistical significant increase of detection rate for *Ureaplasma urealyticum* in the DED group in comparison to the non dry control group (44.8% *vs* 3.7%), $P<0.001$, Table 3. Our results was in close agreement with that reported by Boiko *et al* [38], where they detected *Ureaplasma urealyticum* in 35.3% of DED patients by DFA method. Boiko *et al* [15] detected a slightly higher rate for *Ureaplasma urealyticum* in the DED group (42.1%) also by DFA technique. Although *Ureaplasma urealyticum* is associated with DED in young adults [35], this was not observed in our results, as there was no statistical significant difference for detection of *Ureaplasma urealyticum* in DED patients below 40y or above 40y, $P=0.276$.

Both *Chlamydia trachomatis* and *Ureaplasma urealyticum* were detected by PCR in an increased rate in the DED group patients, than those in the control group (37.9% *vs* 0.0%) respectively, $P<0.001$, Table 4. In addition, both organisms were detected in higher frequency in young patients of the DED group, who were below 40y of age, more than those above 40y of age, $P=0.019$.

Urogenital disease can be associated with conjunctival damage and 65% of cases were more than one organism (*Chlamydia trachomatis*, Mycoplasma and *Ureaplasma urealyticum*), as observed by Boiko *et al* [15]. Similarly, they reported this high frequency of polymicrobial infection in their young adult DED patients group. Clamydial urogenital infection is associated with *Chlamydia trachomatis* ocular disease [46], thus it is possible that urogenital disease caused by both *Chlamydia trachomatis* and *Ureaplasma urealyticum* can be associated with chronic conjunctivitis and DED, with the natural transmission of infection to the conjunctiva is *via* the blood [22].

In a trial to evaluate the PCR method as an additional technique, and comparing it with the DFA method, also applied by other authors, for the study of prevalence of *Chlamydia trachomatis* in the DED group patients, we observed a significant agreement between both methods, $P<0.001$, Table 5. The DFA method is subject to the issue of non specific fluorescence, reliance on the judgment of the observe which is subjective rather than objective. Although we applied strict criteria for a specificity pattern and this helped in the limitation of false positive results, and this explains the significant agreement between both methods.

Among the risk factors observed for DED, is the conjunctival flora, which we investigate in our study.

In a recent review by Miller and Lovieno [47], they noted the ocular surface harbors a diverse group of microorganisms, with *Staphylococcus* species being the primary commensals recovered from the conjunctiva, besides other bacteria including gram negative *e.g. Pseudomonas*, *Acinetobacter* and *Moraxella*. A number of studies have addressed the ocular surface microbial load and changes in dry eye patients. Albietz and Lenton [48] concluded that the ocular surface of DED patients had a great bacterial load compared to healthy patients. Graham *et al* [49] also reported increased load of CNS as normal flora, in dry eye patients.

Our study showed a statistical significance between gram positive and gram negative bacteria isolated from the conjunctiva of DED group patients, with *Chlamydia*

trachomatis detected in them by PCR and DFA, 83.7% *vs* 53.3%, P=0.018 and 72.1% *vs* 46.7%, P=0.074. There was no significant association between gram positive and gram negative bacteria isolated and *Ureaplasma urealyticum* detected by PCR in DED group patients 44.2% and 46.7%, respectively, P=0.868; in addition no correlation was found between the isolation of gram positive and gram negative bacteria in DED patients who had both organisms detected in their conjunctiva by PCR, P=0.296.

Previous studies have shown that conditions causing dry eye including anterior blepharitis and meibomian gland dysfunction, are associated with a variety of gram positive and gram negative bacteria.

The inflammation associated with DED, would promote colonization of bacteria in the conjunctiva, whether non pathogenic or opportunistic, in addition to *Chlamydia trachomatis* which causes the development and maintenance of inflammation. Thus *Chlamydia trachomatis* could assume both a primary role in pathogenesis of the disease and a secondary role in increasing the bacterial colonization and consequently the microbial load associated in DED patients. This was in agreement with our results which showed a stronger association of bacteria with *Chlamydia trachomatis* positive DED patients than those with *Ureaplasma urealyticum* detected by PCR. This was among the focus of our study to elucidate the pattern of microbial growth in DED patients and evaluate its relation with the two studied organisms. Investigation of the microbial load in the non dry eye group was not performed and this could be considered a limitation of the study.

In conclusion, our results tend to point out that *Chlamydia trachomatis* and *Ureaplasma urealyticum* were detected in a moderate percentage of patients with DED, and could be a fair possibility for its development. PCR was more reliable, in detecting *Chlamydia trachomatis*, than DFA technique. The presence of isolated conjunctival bacterial microflora could be of some potential value.

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