Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.36, 2015

Rapid Identification of Human Adenoviruses and Cytokine Estimation among Patients with Epidemic Keratoconjunctivitis in Babylon Governorate, Iraq

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Abstract
One hundred seventy five conjunctival swabs were collected from patients with keratoconjunctivitis. All swabs were cultured on Blood agar and MacConkey agar , 82 positive bacterial culture isolates were excluded and 93 negative bacterial culture isolates were taken as a study samples. All 93 swabs were subjected to rapid immunochromatography test (IC) test. The results of rapid identification revealed that 79 specimens (84.9%) were found positive for human adenoviruses , whereas 14 specimens (15.1%) were found negative for human adenoviruses. In addition , the results indicated that the positive rate of the IC test for specimens obtained within 1–5 days after of the onset of illness 59 (74.7%) were significantly higher than that for specimens obtained within 6–10 days after of the onset of illness 20 (25.3%) at P-value < 0.05.Tear samples were also collected from both (30) patients and (25) controls to estimate the tear levels of some immune parameters Interleukine-6 (IL-6), Interlukine-8 (IL-8), and tumor necrosis factor alpha (TNF-α) by ELISA (Enzyme linked Immunosorbent Assay) method .The cytokine profile showed that there is a significant increase (P<0.05) in the mean tear concentration of each (IL-6, IL-8 and TNF-α) between EKC patients and control groups , and the result showed that IL-8 was the predominant cytokine released and followed by IL-6 and TNF-α .
Keywords: Adenovirus, Keratoconjunctivitis, Immunochromatography test, Cytokine

Introduction

Epidemic keratoconjunctivitis (EKC) is a contagious infectious disease that at the most implicates the surface of the eye . EKC is caused by adenoviruses that are highly resistant to environmental influence and transmitted from person to person by way of infectious secretions , mainly as tear fluids (Aldloch *et al.,* 2010). Human adenoviruses are divided into seven groups (A-G), comprised of 57 serotypes/types of human adenovirus have been reported , that based on immunological distinctiveness and sequence (Walsh *et al .,* 2009) .

EKC occurs mainly in adults and is typically caused by HAdV-8, -19 , and -37, it also has been occurring in ophthalmology clinics , hospitals , camps , military bases and industrial plants . HAdVs can remain viable for several weeks on wash basins and towels and is transmitted by contaminated ophthalmic solutions and instruments and by the sharing of bathrooms (Ruuskanen *et al.,* 2009) . Several types belonging to the HAdV-D, were caused of keratoconjunctivitis including type HAdV-53 and HAdV-54 (Nakamura *et al .,* 2012).

Adenoviruses initiate both innate and adaptive immune responses. The most important host defense against many viruses, including adenoviruses, is cell-mediated immunity. Fatal adenovirus infections occur most commonly in immunocompromised patients, especially those with defects in cell-mediated immunity. Fatal adenovirus infections occur most commonly in immunocompromised patients, especially those with defects in cell-mediated immunity (Kojaoghlanian *et al.,* 2003) .

Conjunctival exudates in EKC contain a range of leukocytes representing both innate and acquired immune responses, along with angiogenic factors and proliferating endothelial cells. True conjunctival membranes can and do form in EKC because the presence of macrophages , T lymphocytes and activated dendritic cells, which suggested activation of an acquired immune response (Chintakuntlawar and Chodosh, 2010) .

The immune response of the corneal epithelium utilizes a variety of different substances to arise an attack on organisms invading the ocular surface. Cytokines and chemokines are produced and endogenously released by corneal epithelial cells to direct and indirect recruit and activate immune cells of both innate and adaptive immunity (Dartt *et al.,* 2011).

Cytokines play a role in maintaining the integrity of the normal cornea. They take part in the regulation of the inflammatory and immunological reactions of the ocular surface (Fodor *et al.,* 2006) . Many kinds of cytokines are present in tears to maintain the condition of the ocular surface (Carreño *et al.,* 2010).

Aim of Study

The present study aimed to detect of HAdVs in conjunctival swabs of patients with keratoconjunctivitis by using an immunochromatography test (IC) test and detection of some interleukins profile (IL-6, IL-8, and TNF-α) in

tears of patients which involved in the immune response against HAdVs .

Materials and Methods

Clinical Samples

Conjunctival swabs used in this study were collected from one hundred and seventy five patients randomly selected patients with age groups ranging from four to sixty seven years from both sexes admitted to Ophthalmic Units in some Hospitals of Babylon Governorate, Iraq were diagnosed by an experienced ophthalmologist, patients were examined under slit-lamp and a consent was obtained from each patient. All specimens were collected during a period from August 2013 to July 2014 and subjected to rapid immunochromatography test (IC) test.

In addition, tear samples were collected without stimulation from the lower tear meniscus without touching the lid margin or the conjunctiva .The duration of the collection was in all cases exactly 2 minutes . The collected tear samples were frozen without centrifugation and stored at ($-80C^0$) for immunological study (determine some interleukin concentrations) .In addition to patients , twenty five tear samples were collected from healthy subject and were involved as a control group; also their age range was approximately matched to patients.

Immunochromatography test (IC)

The immunochromatography test was performed according to the manufacturer's instruction (SA Scientific^{TM} , San Antonio, TX, USA). Provided four drops (approximately 150 µL) of the specimen were placed into the specimen well of the kit . The results were read within 15 minutes . Some positive results may be observed in as short as 30 seconds depending on the concentration of antigen . The test was considered to be positive if two colored lines appear and the test was considered negative if only one colored line appears in the C (control) area. Colored lines that appear after 15 minutes were not diagnosed and excluded.

Estimation of Cytokines in Tear

There are different cytokines enrolled in the present study , the assay principle and methods are performed at room temperature according to manufacturer's instructions (BioSource Europe SA, Nivelles, Belgium). Calculation of the results and interpretation were done according to the specific standard curve of each interleukins , including (IL-6 , IL-8 and TNF-α) .

Statistical Analysis

Data were performed using commercially available software (SPSS *version19*) and continuous variables were presented as means and standard deviations (mean ± S.D) . A P-value ≤ 0.05 was considered as statistically significant .

Results

Among the 175 cases of keratoconjunctivitis , it was found that 82 specimens showed positive culture (bacterial isolates) were excluded from the study samples, and 93 specimens showed negative culture (no growth) were taken as a study samples .

Positive human adenoviruses antigen was detected in 79 out of 93 conjunctival specimens by immunochromatography test (IC) test (Table1). Data from table (1) indicate that the positive rate of the IC test for specimens obtained within 1–5 days after of the onset of illness 59 (74.7%) were significantly higher than that for specimens obtained within 6–10 days after of the onset of illness 20 (25.3%) ($P < 0.05$) .

Tear samples were collected within 2 min. ranged from (10-100 µl) from normal control and patients with keratoconjunctivitis. In normal controls, the mean level of IL-6 was 112.79 pg/ml (SD 33.032). The IL-6 concentrations were significantly higher than this in tears of patients with epidemic keratoconjunctivitis 305.44 pg/ml (SD 58.14) ,the result showed that a significant increase at ($P < 0.05$) of concentration in all patients after comparison with control (Table 2) .

The result of IL-8 measurement showed that the mean of IL-8 was 170.97 pg/ml (SD 57.21) in normal controls. In tears of patients with epidemic keratoconjunctivitis was 635.73 pg/ml (SD 90.84) were significantly higher than the control group. The result of this study showed that significant increase at ($P < 0.05$) of concentration in all patients after comparison with control (Table 3) .

Statistical analysis for TNF-α revealed a significant increase at ($P<0.05$) of concentration in tears of the control group when compared with the patients group at mean value 95.10 pg/ml (SD 50.82) and 139.75 pg/ml (SD 61.33), respectively (Table 4) .

Discussion

Adenovirus is a common ocular pathogen that frequently induces epidemic keratoconjunctivitis . Therefore ,

several rapid diagnostic tests have been developed Uchio *et al.,* (1997).This means that physicians, medical staff and family members can be informed prospectively about the expected spread and clinical form of the illness.

Immunologic tests are more rapid , they rely on the presence of a threshold amount of reactive adenoviral antigen within the sample. This may vary considerably with eye swabs , based on the time course of the infection at which the specimen is taken and the amount of adenovirus infected epithelial cells have a major influence on the antigen content of the eye swab (Kinchington *et al.,* 1994).

Kimura *et al.,* (2009) , stated that a possibility of eyes are contagious during the incubation period of adenoviral conjunctivitis in some situations , these results suggest that the possibility of the eye acting as source for spreading infection during the incubation period by analyzing the presence of adenovirus using the immunochromatography (IC) test. An eye cannot spreading infection during the incubation period before the onset of adenoviral conjunctivitis because the minimal interval of sample collection before onset was one day. However , if conjunctival scraping could be obtained within one day before the onset in contralateral eye , adenovirus antigen might be detected by laboratory tests. In addition the incubation period of the contralateral eye was 7-12 days in most cases . However , the presumed incubation period may extend to more than 14 days .

In this study, it was observed that IC test was useful, easier to be conducted , IC test has the added advantage of providing faster result and for early diagnosis of epidemic keratoconjunctivitis. Correctly identifying patients may reduce the spread of disease and limit the toxicity and antibiotic resistance associated with unnecessary empiric treatments.

These findings are consistent with those reported by Uchio *et al.,*(1997) who stated that IC is a more rapid and easier test and it has high specificity and the detection of HAdVs antigen by this simple and rapid method will serve physicians as a useful tool for early diagnosis and prevention of adenoviral conjunctivitis.

Many kinds of cytokines are present in tears to maintain the condition of the ocular surface and twenty-five cytokines and chemokines were detected in tears from healthy subjects (Tuominen *et al.,* 2001 ; Carreño *et al.,* 2010). Chemokines such as IL-8 are cause leukocyte chemotaxis with a high degree of specificity. Because of existing evidence that polymorphonuclear neutrophils infiltrate the corneal stroma in EKC (Tsai *et al.,* 1992) . This study focused attention on neutrophil-specific interleukins such as IL-6, IL-8 and TNF-α . This group of soluble molecules plays an extremely important role in corneal immunology.

The results showed that IL-8 was predominant interleukin implicated in EKC cases followed by IL-6 and TNF-α in the mean levels (305.44 ± 58.14, 635.73±90.84 and 139.75±61.33) respectively using an ELISA assay. In addition, it was seen that a marked increase in the release of IL-6, IL-8 and TNF-α in active EKC cases when compared to controls tears, and the concentrations of IL-6, IL-8 and TNF-α were significantly greater in tears of patients with EKC at P-value < 0.05 when compared to controls.

Chodosh *et al.,* (2000) stated that the IL-8 play a potential role in the pathogenesis of adenovirus induced subepithelial infiltrates (SEI). Here, present results consistent with the result reported by Rajaiya and Chodosh, (2006) were demonstrated that adenovirus infection of corneal cells in patients with EKC induces them to produce chemokines including IL-8, which is an α chemokines that strongly and selectively induces chemotaxis and degranulation of neutrophils with a long period of action. Also, IL-8 is a CXCL8 chemokine that induced by a variety of stimuli, including TNF, IL-1, bacterial and viral infection (Huffmann *et al.,* 2002). IL-6 and IL-8 were considered that normal constituents of the human tear fluid and both of them could have a role in the maintenance of the ocular surface homeostasis (Fodor, 2009).

On the other hand , The results of this study showed that IL-6 concentration levels were significantly higher in patients than in control groups. Cubitt *et al.,* (1995) have pointed out that keratocytes connect with one another through gap junctions and play an important role in host responses to adenovirus infection through the production of cytokines and chemokines , including IL-6 and both human corneal epithelial cells and keratocytes can synthesize IL-6 and that production of this cytokine is enhanced in both cell types after exposure to either IL-lα or TNF-α .

Furthermore , findings of the current study showed that significantly increase in release of TNF-α when compare with control in tears of patients with EKC. Kopp and Ghosh , (1995) **;** Xue *et al.,* (2002) stated that tumor necrosis factor (TNF-α) was induced during corneal inflammation, suggesting that regulation of synthesis of cytokines affects inflammation in the eye. Nuclear factor (NF)-κB protein is one of the major transcription factors which correlated with the over expression of proinflammatory cytokines such as TNF-α, which mediate inflammation and a strong immune response, therefore contribute to corneal damage.

Conclusions

The rapid diagnosis (IC test) is very necessary to provide physicians with information about the prognosis of epidemic keratoconjunctivitis disease at the patients first visit and the rapid test avoid expensive , unnecessary and ineffective antibiotic therapy. On the other hand, Cytokine profile measurement (IL-6, IL-8, and TNF-α) indicates the level of IL-8 was significantly higher in tear of patients with EKC than among controls followed by IL-6, and TNF-α, which all play an important role in the pathogenesis of corneal and conjunctival

inflammation and these results considered that IL-8 have a significant effect on the pathogenic process of the EKC.

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Table (1): Immunochromatography test to detect HAdVs in keratoconjunctivitis specimens according to the days of specimens collection after the onset of infection

Days of specimens collection after onset	Immunochromatography test		Total %
	Positive %	Negative %	
1 – 5*	59 (74.7)	9 (64.3)	68 (73.1)
6 – 10	20 (25.3)	5 (35.7)	25 (26.9)
Total	79 (84.9)	14 (15.1)	93 (100)

*** The days before onset in the contralateral eye**

Table (2): Concentration of interleukin-6 (IL-6) pg/ml for epidemic keratoconjunctivitis patients and control

Groups	IL-6 Mean (pg/ml) ± S.D	P-value
Patients (n = 30)	305.44 ± 58.14	P < 0.05*
Healthy Control (n= 25)	112.79 ± 33.032	

S.D = Standard Deviation , * Significant

Table (3): Concentration of interleukin-8 (IL-8) pg/ml for epidemic keratoconjunctivitis patients and control

Groups	IL-8 Mean (pg/ml) ± S.D	P-value
Patients (n = 30)	635.73 ± 90.84	P < 0.05*
Healthy Control (n= 25)	170.97± 57.21	

S.D = Standard Deviation , * Significant

Table (4): Concentration of tumor necrosis factor-α (TNF-α) pg/ml for epidemic keratoconjunctivitis patients and control

Groups	TNF-α Mean (pg/ml) ± S.D	P-value
Patients (n = 30)	139.75 ± 61.33	P < 0.05*
Healthy Control (n= 25)	95.10 ± 50.82	

S.D = Standard Deviation , * Significant

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