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Comparison of Direct Immunofluorescence (DIF) Method and Giemsa Staining with PCR Method for Detection of *Chlamydia trachomatis* in Patients with Follicular Conjunctivitis

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Abstract

Background: *Chlamydia trachomatis* is the most common cause of chronic follicular conjunctivitis. As a rapid diagnosis is important in the reducing the long-term squeal of the diseases, the objective of this study was to compare the three methods, direct immunofluorescence (DIF), staining and PCR, for detection of *Chlamydia trachomatis* in patients with follicular conjunctivitis.

Material and methods: Overall 90 patients with conjunctival were enrolled in this study smears were prepared for DIF and Giemsa staining. PCR amplification after Extraction performed using CT1 and CT5 primers designed from *Omp1* gene.

Results: Of the 90 patients, 28 (31.1%) were positive by DIF and 13 (14.4%) by Giemsa staining; and 35 patients (38.8%) showed positive results in PCR. Sensitivity, specificity, predictive positive value, and negative predictive value of DIF in comparison to PCR respectively were calculated as 88.33, 100, 100 and 88.70. Sensitivity, specificity, predictive positive value and negative predictive value of DIF in comparison to PCR respectively were calculated as 61.40, 100, 100 and 71.42. Therefore, sensitivity and negative predictive value of DIF are significantly higher than Giemsa staining.

Conclusion: DIF is more sensitive and more reliable than Giemsa staining for detection of *Chlamydia trachomatis* in the conjunctiva samples of patients with follicular conjunctivitis.

Keywords: Outer membrane protein; Follicular conjunctivitis; *Chlamydia trachomatis*

Introduction

Chlamydia species are the most common microbial cause of chronic conjunctivitis that among different species *C. trachomatis* is the major cause of chronic follicular conjunctivitis.

This bacterium is responsible for three clinical syndromes: trachoma, adult inclusion conjunctivitis, and neonatal conjunctivitis. The terms of chronic follicular conjunctivitis is assigned for the inflammation of conjunctiva that covers the surface of eyes. If conjunctivitis lasts for more than 16 day it defined as chronic follicular conjunctivitis. The eyes infection in sexually active adults and in new-born that acquired the infection through child birth, are the most prevalent [1,2]. However, patients are not aware of their illnesses until the disease prompts the symptomatic stage.

Due to infection of majority of people in the world WHO organization prompt some effort to control the disease by 2020 [3]. Consequently, to achieve this goal the early detection of Chlamydia is the main concern in better recovery from diseases and successful treatment.

Although the ocular infections with this bacteria are well characterized, detection of *C. trachomatis* is very difficult. In many studies, different methods are used for detection of ocular infection: cell cultures, direct enzyme immunoassay (EIA) and fluorescent antibody (DFA). The Gold standard is cell culture but it is time-consuming and difficult. Another method is staining of Chlamydia inclusion with Giemsa which has not a high sensitivity; so, detection of Chlamydia with DFA and EIA assays are more prevalent because we don't need a live bacteria in clinical specimen. Also hybridization of nucleic acid (detection of rRNA) and PCR recently become popular for detection of bacteria [4,5].

There are few studies about incidence of Chlamydia in conjunctivitis. One study performed by Malathi et al. [6] from India, about 328 ocular swabs were examined in which 16 cases (4.9%) were positive for presence of Chlamydia by PCR method [6].

Because most of studies were conducted on genital specimen and few studies were on ocular specimen, the aim of this study was to compare different diagnostic methods, including Giemsa aining, DIF and PCR of *omp1* gene for detection of Chlamydia in conjugatival specimens of patients with follicular conjunctivitis that were referred to Farabi hospital in 2012 and also to evaluate the sensitivity and

specificity of Giemsa staining and DIF methods as common assays for detection of chlamydia in comparison with PCR method.

Material and Methods

This was a descriptive study of 90 patients with follicular conjunctivitis that were referred to the Laboratory of Farabi Hospital in 2012. Patients with an index of chronic conjunctivitis as it was confirmed by optometrist were included in this study, then specimen was taken with specialist using Kimura spatula or Dacron swab [5]. All patients were asked to fill the information and sign the consent form.

The specimen was stained with Giemsa immediately after sampling and the inclusion body was observed near the epithelial cells [7]. In order to perform the Direct Immunoflurecence Assay, specimens were fixed with methanol on slide and then stained with monoclonal IgG Ab conjugated with fluorescein according to manufacture instructions (Product name: *C. trachomatis* DFA kit; Code: PL.1010; Manufacturer: Pro-Lab Diagnostics, Canada). The elementary bodies were observed as green fluorescence under microscope [8]. Then they were stocked in classical 2SP transport medium (0.2 M sucrose-0.02 M phosphate; QUELAB Laboratories Inc., Montréal, Canada) and kept in -20 refrigerator for further analysis. After that the extraction of DNA was performed using Bioneer extraction kit (AccuPrep® Genomic DNA Extraction Kit (Bioneer, Alameda, CA).

To amplify OMP1 region, two pair primer was designed with following sequences:

CT1: GCCGCTTTGAGTTCTGCTTCCTC

CT5: ATTTACGTGAGCAGCTCTCTCAT

PCR mixtures were prepared in a volume of 25 µl, which contained 2.5 µl PCR buffer, 50 nM of each primer, 1.5 mM MgCl, 20 µM from each dNTP, 1U Taq DNA polymerase, and 50 ng DNA sample. The mixtures were placed in a thermo cycler (Eppendorf, Hamburg, Germany). PCR amplification was performed under the following conditions: initial denaturation at 94°C for 6 min followed by 35 cycles of denaturation at 94°C for 30 sec. Annealing temperature was 55°C for 30 sec and extension at 72°C for 30 sec, final extension at 72°C for 7 min [8]. Negative and positive controls were used in all reactions that *Chlamydia trachomatis* strain 886-ATCC VR was considered as positive. Control. PCR products were visualized by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and examined under UV illumination.

Data statistical analysis was carried out using statistical package for special sciences (SPSS)- software version 18. Software for comparison analyses of categorical data were done by Chi square test and Fisher exact test. P values equal to or less than 0.05 were considered to be significant.

Results

In this study, overall 90 patients with chronic conjunctivitis were enrolled (41 women (45.5%) and 49 men (54.5%)). The age range of most patients was between 20-29 years old (mean=48.5 and SD 28). The most prevalent symptoms (give % of each in parentheses) of patients were redness of eye (xx%), swelling of eyelid (xx%) flocculation of conjunctiva (xx%) and foreign body sensation (xx%). Among these patients, 18 cases (20%) had a history of UTI.

To compare the three basic diagnostic methods; Giemsa staining, DFA and PCR, at first the specimens were Giemsa stained. Then we performed a DIF assay. Finally the PCR method was applied.

Form total of 90 patients with symptom of chronic Conjunctivitis the presence of Chlamydia was detected in 13 patients (14.4%) with Giemsa staining method, 28 patients (31.1%) with DIF method, and 35 patients (38.8%) with PCR of the *omp1* gene. The positive PCR product size of specimen of patients with chronic Conjunctivitis using CT1 and CT5 primers was about 1200 bp which were visualized with ethidium bromide after electrophoresis (Figure 1).

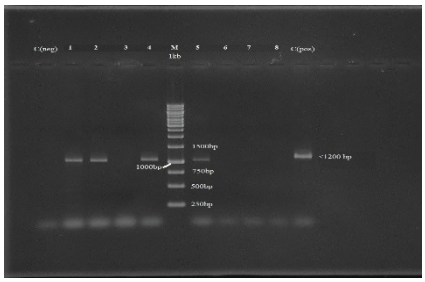

Figure 1: PCR product of *omp1* gene: c(neg): negative control; c(pos) positive control; 1 kb marker; 1,2,4 positive sample; 3,6,7,8 negative samples.

Thus, the sensitivity, specificity, positive and negative predictive value of Giemsa staining in comparison with PCR were clarify percentages comma and decimal point all to same precision 61.4%, 100%, 100% and 71.42% respectively. and the sensitivity, specificity, positive and negative predictive value of DIF in comparison with PCR were 88.3%, 100%, 100% and 88.70% respectively. We use p-value and Fisher test to evaluate the sensitivity, specificity, positive and negative predictive value of two Giemsa staining and DIF that they were 0.0009, 1.0000, 1.0000, and 0.0047 respectively (Table 1).

Diagnosis	Sensitivity	Specificity	Sensitivity	Specificity
	Giemsa		DIF	
Positive predictive value	61.40%	100%	88.30%	100%
Negative predictive value	71.42%	100%	88.00%	100%

Table 1: Comparison of DIF and Giemsa with PCR method for ssdetection of *C. trachomatis*.

Table 1 demonstrates the comparison of Giemsa staining and DIF tests with PCR in specificity, sensitivity, positive and negative predictive value of which the sensitivity and negative predictive value were totally significant whereas the positive predictive values and specificity did not contain any significant values.

Discussion

Comparison of these diagnosis tests demonstrated that EIA and DFA assays are less sensitive than culture, but another hand PCR had a high sensitivity. The high sensitivity of PCR in different specimen like

male genital tract swab and female endocervix swab, urine specimen of symptomatic and asymptomatic patients has been proved [4,5]. Due to sensitivity and specificity of PCR based methods for rapid detection of chlamydial infections in comparison with time-consuming culture methods, it can be concluded that the former methods are reliable for identification and detection of this bacterial species [9]. In many studies (references) various proliferation genes were introduced for diagnosis with PCR of Chlamydia. These plasmid and chromosomal genes such as major outer membrane proteins (MOMP) ribosomal RNA (rRNA) and cysteine rich proteins [10], which MOMP genes are most specific for detection of *C. trachomatis*.

The purpose of the study was to compare was to compare different diagnostic methods, including Giemsa staining, DIF and PCR of *omp1* gene for detection of Chlamydia in conjugative specimen of patients with follicular Conjunctivitis and also to evaluate the sensitivity, specificity and predictive value of Giemsa staining and DIF methods as common assays for detection of Chlamydia in comparison with the PCR method. Chlamydia infection is the most common cause of ocular infection in many studies it is confirmed that *C. trachomatis* is the most cause of chronic follicular Conjunctivitis and the main bacterial cause of neonatal conjunctivitis ("In many studies…"). In our study the age range of patients were between 20-30 years old and we mostly used the specimen of adult chronic follicular conjunctivitis and ultimately, we detected the bacteria in about 14.4% of specimen with Giemsa staining, 31.1% by DIF. Because most of people are more sexually active in these range of age, these results suggest that the genital tract infections may be a major source of Chlamydia ocular infections. The sign of genital infection was observed in 20% of people. Because the Chlamydia infections mostly don't have any symptoms, there was the possibility of asymptomatic Chlamydia infection in other patients.

In overall the prevalence of this infection in different reports is very controversial, that is, it is reported between 20% up to 90% according to age ranges [11]. The incidence of infection was about 1 in 44000 people in the age range of 15-44 years old and 1 in 100,000 in all the population of Bristol in 1981 [12].

We compared the Giemsa staining and DIF methods with PCR. The positive results of *C. thracomatis* with Giemsa staining, DIF and PCR for *omp1* gene were 14.4%, 31.1% and 38.8% respectively.

There are different methods for laboratory diagnosis of *Chlamydia trachomatis* infections. The differences between these methods are based on sensitivity, specificity, positive and negative predictive values. In laboratories with minimum equipment, Giemsa staining of smear for observing the intra cytoplasmic inclusions is mostly used. This method has good results for neonatal specimens but on the other hand, it has less sensitivity in adults' specimen; so, although it has a low sensitivity, just because of feasibility and accessibility, Giemsa staining method is one of the most used diagnosis tests. The sensitivity and specificity of DIF method were 70% and 90% respectively in comparison with microbial culture. Samples used for this diagnosis method has a 10 to 20 columnar and/or squamous metaplastic according to criteria mentioned by Tullo et al. The DIF method has70% to 100% sensitivity than culture method and its specificity is more than 87% to 99% and is the unique method that can assess the quality of specimen. The accepted sample for DIF test is specimen with about 10 to 20 columnar and/or squamous metaplastic [13]. The incidence of Chlamydia in conjunctivitis patients with PCR method is compatible with Isob (what is Isob isobutyrate??) which reported that from 38 swabs of conjugative, 18 specimens (47.3%) were positive for *C. trachomatis*. also they confirmed that the rate of ocular infection of Chlamydia is compatible with genital diseases [14]. Palayekar et al. compared DIF staining with Giemsa staining and concluded that the rate of positive results with the DIF test was more than that with Giemsa staining (16.8% versus 10%) That, result [15] supports our results [15]. Haller et al. [16] showed that PCR could be a good alternative for culture and that DIF was a useful for quick diagnosis as we concluded in this study. Lin et al. [17] declared that 38% scraping specimens were positive by immunofluorescent staining and 29% were positive by Giemsa staining so they came up with high prevalence in positive results with Gimmesa staining and DIF staining than our results [17]. In overall the studies on comparison of Giemsa staining and DIF was performed on genital specimen. There are a few reports about follicular Conjunctivitis caused by Chlamydia. Soltanzadeh et al. [18] declared that the low incidence of Chlamydia conjunctivitis in neonatals was due to the low prevalence of sexually transmitted infection in Iran [18]. Because patients who enrolled in this study were examined by specialist it justify our high prevalence. Given that PCR was the most sensitive method for detection of *C. trachomatis* in clinical specimen that was confirmed in reports [16], We compare the Gimmesa staining and DIF methods with PCR. The positive results of *C. thracomatis* with Giemsa staining, DIF and PCR for *omp1* gene were 14.4%, 31.1% and 38.8% respectively. In conclusion, the DIF method with 88.33% specificity in comparison with Giemsa (61.4%), specificity was higher and had a significant p-value. Also, the negative predictive value of DIF was significantly higher than Giemsa and DIF (71.42% vs 88.70% vs in comparison of 71.42% and 88.70%) the DIF was significantly higher but about the positive predictive value of these two method there was no statistically significant relevant. One study which conducted on cervical specimen demonstrate that the sensitivity and specificity of PCR were about PCR had a 100% sensitivity and specificity whereas the enzyme immunoassay just had a 58.8% sensitivity but and 100% specificity which is compatible with that it was aligned with results in our study [19]. the same results obtained from Wu et al. demonstrated that that the sensitivity and specificity of EIA were 87% and 97.7% and the sensitivity and specificity PCR were 95.6% and 97.7% respectively, that the percentage of specificity of EIA assay was higher than our results (87% and 95.6% for sensitivities and 97.7% and 98% respectively for specificities [20]. Also, the CDC (Centers for Disease Control) in 2002 advised that it is better not to use less specific methods such as Giemsa, EIA and iodine for detection of inclusion body of *C. trachomatis* [21].

According to studies DIF has a higher sensitivity and negative predictive value for detection of Chlamydia follicular conjunctivitis in comparison with Giemsa staining and so, in laboratories that don't access to PCR which is the more sensitive, it is better to use DIF method as an alternative method for detection of *Chlamydia trachomatis* infections. But using less sensitive methods for detection of conjunctivitis disease may lead to unnecessary hospitalization or on the other side the false negative result may lead to progression of disease and irreversible outcomes.

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