Investigation of Adenoviruses in Patients with Conjunctivitis by Various Methods

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ABSTRACT

Adenoviruses are the most common causative agents of epidemics of community acquired and nosocomial conjunctivitis. Adenoviruses are resistant to physical and chemical factors and they can spread easily. Due to these factors, rapid and accurate diagnosis is very important in adenoviral eye infections. In this study, it was aimed to investigate adenoviruses from conjunctival swab samples of 50 patients suspected of adenoviral conjunctivitis by two different cell culture and PCR methods. Conjunctival swab samples from patients suspected of adenoviral conjunctivitis were taken between March 2012- December 2012. Adenovirus DNA was investigated by real-time PCR method in specimens. Samples were inoculated on conventional HEp-2 cell culture and HEp-2 shell-vial cell culture. Virus isolation in conventional HEp-2 cell culture was confirmed with an immunofluorescence kit and a monoclonal antibody kit. Adenovirus DNA was found positive in 23 (46 %) of 50 conjunctival samples by real-time PCR. Virus isolation in conventional HEp-2 cell culture was shown in 19 (38 %) samples by immunofluorescence method and 13 (26 %) samples by monoclonal antibody method. Results obtained from Argene immunofluorescence kit were considered as positive results for conventional HEp-2 cell culture method. Shell-vial culture could be performed in 24 swab samples and 17 of samples were found positive. These 17 samples that found positive in shell-vial culture were also positive with PCR and conventional HEp-2 cell culture methods. Additionally, four samples were found positive by PCR but adenovirus could not be isolated by two cell culture methods. Two samples that were found positive with PCR and conventional HEp-2 cell culture methods were found negative by shell-vial cell culture. Considering HEp-2 cell culture confirmed with immunofluorescence method as gold standart, sensitivities and specifities were found as 100 % and 87 % respectively for PCR, 89 % and 100 % respectively for shell-vial cell culture. The cell culture method is still important in adenoviral eye infections but can not provide rapid diagnosis. In adenoviral eye infections, it is considered that real-time PCR is a more useful method that provides results in the shortest period of time with high sensitivity.

Key Words: *Adenovirus, cell culture, conjunctivitis, PCR*

Konjonktivitli Hastalarda Adenovirüsün Çeşitli Yöntemlerle Araştırılması

ÖZET

Adenovirüsler gerek toplum kökenli, gerek nozokomiyal konjonktivit salgınlarında en sık etken olan ajanlardır. Fiziksel ve kimyasal etkenlere dirençli virüslerdir ve kolayca bulaş görülebilmektedir. Bundan dolayı adenoviral göz enfeksiyonlarında hızlı ve doğru tanı çok önemlidir. Bu çalışmada, adenoviral konjonktivit düşünülen 50 hastanın konjonktival sürüntü örneklerinden iki farklı hücre kültürü ve PCR yöntemleriyle Adenovirüs araştırılması amaçlandı. Konjonktival sürüntü örnekleri Mart 2012 - Aralık 2012 tarihleri arasında alındı. Sürüntü örneklerinde Adenovirüs DNA gerçek zamanlı PCR yöntemiyle araştırıldı. Örnekler konvansiyonel HEp-2 ve HEp-2 shell-vial kültürlerine ekildi. Konvansiyonel HEp-2 hücre kültüründe doğrulama bir immünofloresan kiti ve bir monoklonal antikor kiti kullanılarak sağlandı. Gerçek zamanlı PCR yöntemiyle 50 hastanın 23'ünde (%46) Adenovirüs DNA pozitif bulundu. Konvansiyonel HEp-2 hücre kültüründe immünofloresan yöntemi ile 19 (%38), monoklonal antikor yöntemi ile 13 (%26) hastada Adenovirüs gösterildi. İmmünofloresan kiti ile doğrulanmış olan sonuçlar konvansiyonel HEp-2 hücre kültürünün pozitif sonuçları olarak kabul edildi. Shell-vial hücre kültürüne yalnız 24 hasta örneği ekilebildi ve 17 hastada Adenovirüs izole edildi. Bu yöntemle pozitif bulunan 17 örnekte PCR ve konvansiyonel HEp-2 hücre kültürü yöntemleriyle de Adenovirüs gösterildi. İlaveten, PCR yöntemi ile Adenovirüs DNA saptanan 4 örnekte ise her iki hücre kültürü yöntemiyle de virüs izole edilemedi. PCR ve konvansiyonel HEp-2 hücre kültürü yöntemleriyle pozitif bulunan iki örnekte ise shell-vial hücre kültürü yöntemiyle Adenovirüs izole edilemedi. İmmünofloresan kiti ile doğrulanan konvansiyonel HEp-2 hücre kültürü altın standart olarak kabul edilerek PCR ve shell-vial hücre kültürü yöntemlerinin duyarlığı sırasıyla %100 ve %89, özgüllüğü ise PCR için %87 ve shell-vial hücre kültürü yöntemi için %100 olarak bulundu. Sonuç olarak, hücre kültürü adenoviral göz enfeksiyonlarında halen önemini korumakla birlikte hızlı tanıyı sağlayamamaktadır. Gerçek zamanlı PCR yöntemi, yüksek duyarlığı ve daha kısa sürede sonuç vermesi bakımından daha kullanışlı bir yöntemdir.

***Anahtar kelimeler**: Adenovirüs, hücre kültürü, konjonktivit, PCR*

INTRODUCTION

Adenoviruses cause a variety of infections such as conjunctivitis, respiratory tract infections, genitourinary infections and gastroenteritis (1,2). They consist of 57 serotypes have been defined so far and all serotypes have been consantrated in seven species A-G (3,4). Adenoviruses have been major factors of conjunctivitis by the rate of 15-70 % worldwide (5). Adenoviral eye infections are clinically seen as follicular conjunctivitis, pharyngoconjunctival fever and epidemic keratoconjunctivitis (6). In general; serotypes 1-11, 15, 16, 17, 19, 20, 22 cause conjunctivitis, serotypes 2, 3, 4, 5, 7, 8, 10, 11, 19, 21, 22, 29, 34, 37, 53, 54, 56 cause epidemic keratoconjunctivitis and serotypes 3, 4 and 7 cause pharyngoconjunctival fever (5,7,8). Outbreaks of adenoviral infections are common (9). Due to remaining infectious at room temperature for weeks and high level of resistance to environmental factors, transmission of adenovirus may occur easily (9,10). As can be transmitted from person to person, transmission may occur through water, objects and medical devices. Therefore nosocomial adenovirus infections can be seen (10).

Adenoviral infections are mainly diagnosed by direct methods such as cell culture, nucleic acid detection and antigen detection (10,11). Indirect diagnosis based on the serological methods are mainly preferred in epidemiological researches instead of diagnosing adenoviral infections because of their low sensitivity rates (11). Rapid diagnose in adenoviral eye infections, especially in outbreaks of nosocomial conjunctivitis is very important and essential for confirmation of clinical diagnosis, limiting the spread of infection, implementation of appropriate protective measures and prevention of inappropriate treatment (12). Promptly isolation of infected individuals is critically important for control of outbreaks in both community acquired and nosocomial outbreaks (13). In this study, it was aimed to investigate adenovirus from conjunctival swab samples of patients suspected of adenoviral conjunctivitis by cell culture and PCR methods.

MATERIALS AND METHODS

This study was performed in Virology Laboratory, with approval of Local Ethics Committee approval (06/05/2010) and supported by Scientific Research Projects Department (TSU-11-3645 code number). Total of 50 patients admitted to Ophthalmology Clinic whom suspected of adenoviral conjunctivitis between March 2012- December 2012 were included in this study. Two sets of conjunctival swab samples using plastic handle with a Dacron swab were taken from each patient. All samples were hold in viral transport medium (Vircell, Spain) in -70 °C until the day of study. One of the viral transport medium was used in

Table 1. Comparison of real-time PCR, conventional HEp-2 cell culture and shell-vial culture methods

Number of samples	PCR	Conventional HEp-2 cell culture	Shell-vial culture*
27	-	-	*
17	+	+	+
4	+	-	-
2	+	+	-

*Shell-vial culture could be performed in only 24 samples.

cell culture and the other one was used in real-time PCR per patient. Nucleic acid isolation procedure was performed using EZ1 Virus Mini Kit v2.0 (Qiagen, Germany) according to the manufacturer's recommendations. Isolation procedure was applied in the isolation device EZ 1 Advanced (Qiagen, Germany). Amplification procedure was performed in Rotor-Gene Q5 plex (Qiagen, Germany) device using amplification kit (Realstar Adenovirus PCR Kit 1.0, Altona Diagnostics, Qiagen, Germany) according to the manufacturer's recommendations for detection of adenovirus DNA in all samples. After the completion of reaction, the target regions and internal control curves were compared for each patient during the evaluation process. The viral loads of samples were evaluated and curves remaining over the threshold line were considered positive.

All samples were cultivated in conventional HEp-2 cell lines and incubated in 5 % CO2 incubator at 37 °C. All steps of cell culture were performed in a laminar flow cabinet that cleaned with 10% hypochlorite and sterilized with ultraviolet light. The cells were examined daily with an invert microscope. All samples with/without cytopathic effect were evaluated eight days after inoculation. Cell suspensions were prepared from all samples and adenovirus antigen was investigated by immunofluorescence method and monoclonal antibody method using two different commercial kits. The immunofluorescence kit was Argene (Argene, France) and the monoclonal antibody kit was Vircell (Vircell, Spain). All steps were performed according to their manufacturers' recommendations. Even if one cell emitting apple green fluorescence was seen with fluorescence microscope, the test was considered positive during the evaluation process. The shell-vial tubes containing HEp-2 cells (Vircell, Spain) were used for isolation of adenovirus in shell-vial cell culture. All steps were performed in a laminar flow cabinet that cleaned with 10% hypochlorite and sterilized with ultraviolet light. Isolation in shell-vial culture tubes could be performed only 24 samples. The shell-vial tubes cultivated and incubated for three days in 5 % CO2 incubator at 37 °C. At the

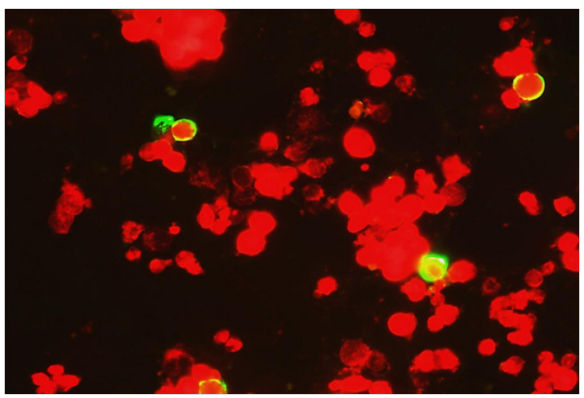

***Figure 1**. Positive result with Argene immunofluorescence kit in conventional HEp-2 cell culture*

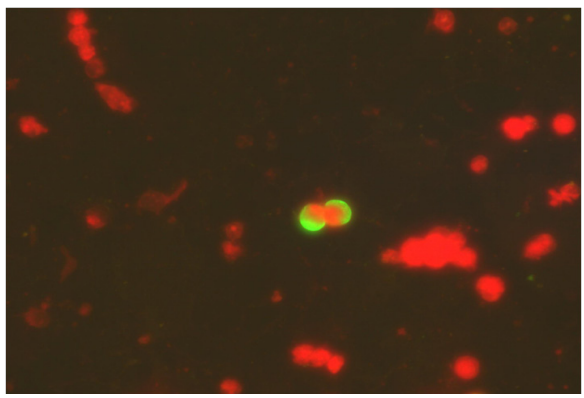

***Figure 2**. Positive result with Vircell monoclonal antibody kit in conventional HEp-2 cell culture*

***Table 2**. The sensitivity and specifity percentages of real-time PCR and Shell-vial culture*

Method	Sensitivity (%)	Specifity (%)
Real-time PCR	100	87
Shell-vial Culture	89	100

end of this time, adenovirus antigen was investigated by using monoclonal antibody kit (Vircell, Spain) according to the manufacturer's recommendations. Even if one cell emitting apple green fluorescence was seen with fluorescence microscope, the test was considered positive during the evaluation process. Conventional HEp-2 cell culture method confirmed with Argene immunofluorescence kit was considered as gold standard and sensitivity and specifity of other two methods were calculated.

RESULTS

Adenovirus DNA was detected in 23 (46%) of 50 conjunctival swab samples by real-time PCR method. In conventional HEp-2 cell culture method, 19 (38%) sample was positive with Argene immunofluorescence kit and 13 (26%) sample was positive with Vircell monoclonal antibody kit (Figure 1, Figure 2). Results obtained from Argene immunofluorescence kit were considered as positive results for conventional HEp-2 cell culture method. Consequently, 23 samples were positive by real-time PCR and 19 of them were isolated in conventional HEp-2 cell culture method. Shell-vial culture was performed only 24 of 50 conjunctival swab samples. In this method, 17 (70,8 %) of 24 samples were positive by using monoclonal antibody kit (Figure 3). All of 17 samples that were positive by shell-vial culture were also found positive by conventional HEp-2 cell culture and real-time PCR methods. Comparison of real-time PCR, conventional HEp-2 cell culture and shell-vial culture methods are shown in Table 1. In real-time PCR method, sensitivity and specifity was found 100% and 87% respectively. In shell-vial culture method, sensitivity and specifity was found 89% and 100% respectively (Table 2).

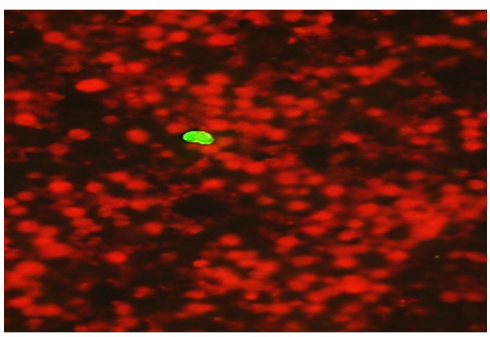

***Figure 3**. Positive result with monoclonal antibody kit in shell-vial culture*

DISCUSSION

Adenoviruses are the most common agents of community-acquired and nosocomial viral conjunctivitis (14). Transmission is seen via contaminated fingers, shared goods, medical devices and swimming pools and a wide range of outbreaks can ocur in schools, workplaces and hospitals (15). Ocular adenovirus infections are seen as outbreaks especially in conditions of poor hygiene, crowded places and in tropical climate areas (16). Although limited data has been reported about incidence and epidemiology of ocular adenovirus infections in United States and European countries, over a million cases have been reported by national surveillance studies in Japan annually (17,18). In our country, there have been no enough study about the epidemiology of ocular adenovirus infections.

Ocular adenovirus infections are usually diagnosed by clinical appearance and laboratory diagnosis is rarely consulted (19). In order to confirm the diagnosis, antigen detection and molecular methods from conjunctival swab samples have been implemented in virology laboratories (11,20). Taking clinical samples at early phase of infection and cell richness are increasing sensitivity of laboratory diagnosis. After having conjunctival swab samples, they have to be kept in a cold environment (2-8°C) and sent quickly to the laboratory (20). Previous studies have shown that factors like taking samples in the late phase of infection and storing samples in improper conditions can decrease the number of positive results (21). Wölfel et al. (22) have shown that applying a local anesthetic before sampling was increasing the number of cells from conjunctiva. In the same study, three different transport mediums were compared and it has been shown that the medium containing only sodium chloride gave better results than other two commercial transport mediums in

virus isolation and in quantitative PCR (22). In this study, conjunctival swab samples were taken from patients suspected of adenoviral conjunctivitis without any anesthetic drug implementation in the acute phase and samples were hold in commercial available viral transport mediums in -70 °C until the day of study.

In order to ocular adenovius infections can be seen as outbreaks, rapid and reliable diagnostic methods are needed (22,23). When considering clinically adenoviral conjunctivitis, confirmation the diagnose by laboratory is helpful in terms of taking hygienic precautions immediately and determining of the epidemiological importance of the infection (9). Nowadays, cell culture methods and molecular methods are used for confirmation of the diagnose in many virology laboratories (9,24). Virus isolation in cell culture has been gold standard for the diagnose of ocular adenovirus infections (25). Adenoviruses have been isolated best in human epithelial cell lines like A549, HEp-2 and HeLa (20). Therefore, the cell culture was considered as the gold standard diagnostic method in this study. The time needed to obtain positive results after cultivation have been reported as 3-29 days by several studies (26). Temperature and pH alterations, humidity, virus concentration of the sample, properties of the medium, status and viability of the cell type can affect the isolation of the virus although adenoviruses are stable (27). A previous study showed that adenovirus isolation can be depressed by other pathogens in the sample and the cell culture results can be affected (27). After isolation in the cell culture, confirmation is done by using immunofluorescence, hemagglutination inhibition or nötralization tests (28). In this study, immunofluorescence and monoclonal antibody tests were applied to confirm virus isolation on the eighth day of inoculation to all samples with/without cytopathic effect in conventional HEp-2 cell culture. The immunofluorescence kits' results were considered as positive results for conventional HEp-2 cell culture method, its' positivity rate was found 38%. Vircell monoclonal antibody kit could not found all positive samples found by Argene kit, its' positivity rate was 26 %.

Shell-vial culture method provides faster results than conventional HEp-2 cell culture method, but this method is more expensive (26). Especially using epithelial cell lines like HEp-2 and HeLa in shell-vial culture provides faster results in the diagnose of adenoviruses (29). Percivalle et al. compared shell-vial culture with conventional cell culture method in patients with adenoviral conjunctivitis and they found equal sensitivity rates (12). Kowalski et al. compared shell-vial culture with conventional cell culture method in patients with adenoviral conjunctivitis and reported that efficacy rates of the two methods were equal. It was reported that shell-vial culture could be preferred due to getting faster results (30). In the same study, three samples that were positive by conventional cell culture method could not be found as positive in shell-vial culture (30). In our study, although HEp-2 cell lines were used in both methods, two samples that were positive by conventional cell culture method could not be found as positive in shell-vial culture and this is a remarkable result.

Quantitative real-time PCR method is commonly used by many laboratories for diagnose of viruses in recent years (11). Obtaining faster results and detecting genetic material even if low virus levels are advantages of this method and it's highly effective in terms of taking treatment and preventive measures in the early stages (30,31). The detection and amplification of viral genome provides high sensitivity if viral load in the sample is too low to be isolated in cell culture methods and if the virus is non-infectious (10). Recent studies have shown that PCR method is more sensitive than cell culture methods in adenoviral conjunctivitis (3,8,12,25,27). Percivalle et al. investigated adenovirus by PCR and conventional cell culture methods and reported that sensitivity rates of PCR was 88,4 % and was 76,9 % of cell culture. The specifity rates of two methods were both found as 100% (12). In our study, the sensitivity and specifity was found as 100 % and 87 %, respectively for real-time PCR and shell-vial culture. Wölfel et al. compared real-time PCR and rapid cell culture method in conjunctival swab samples and reported that real-time PCR was more favorable due to giving faster results and higher sensitivity in ophthalmological screening (22). In another study, the real-time PCR method was recommended for screening in adenoviral conjunctivitis outbreaks (32). Damen et al. emphasized of the importance of real-time PCR in both research and diagnostic protocols (2). In our study, adenovirus was detected by real-time PCR in 23 of 50 conjunctival swab samples but all of them could not be isolated in cell culture methods. This result may be due to various factors like samples waiting in -70 °C for months and real-time PCR can also detect the genome of non-viable virus.

Currently, conventional cell culture method is still important but obtaining results in a long time, requirement of experienced personnel and equipment are the disadvantages of this method. Shell-vial culture method provides

faster results and can be preferred if there are suitable conditions. We have limitations because this this was a single-center study, typing of adenoviruses could not be done, local anesthetic was not implemented in the sampling process and shell-vial cell culture method could be performed in only 24 samples due to limited research budget. Compared with cell culture methods, real-time PCR provides faster results and has high sensitivity rates. In conclusion; it has been concluded that using real-time PCR is more appropriate than other methods for the diagnose of ocular adenovirus infections.

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