A rapid polymerase chain reaction protocol to detect adenovirus in eye swabs

Detecção de adenovírus em "swab" oftálmico empregando protocolo rápido por reação da polimerase em cadeia

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ABSTRACT

Purpose: Viruses of the Adenoviridae family are associated with many clinical syndromes, possessing 50 serotypes. These agents and viruses of the Herpesviridae family are the two major agents responsible for viral conjunctivitis, and a rapid diagnosis is important due to the epidemic character of adenoviral infections. **Methods:** We developed a PCR without DNA extraction for adenovirus using primers that amplify a 300 bp fragment of the hexon capsid protein gene from many serotypes. **Results:** Swab samples from cornea of seven patients with keratoconjunctivitis were analyzed, and one of them was PCR positive for adenovirus. The sequence of this fragment shows a 100% homology with the sequence of adenovirus type 8. **Conclusion:** Sequencing of 300 bp from the hexon gene allows to identify almost all Ad serotypes, including all serotypes related to epidemic keratoconjunctivitis (8,19,37) and almost all serotypes involved with Ad-associated conjunctivitis.

Keywords: Polymerase chain reaction /methods; Adenoviruses, human/isolation & purification; Keratoconjunctivitis/diagnosis; Adenovirus infections, human; Eye infections, viral/diagnosis; Herpesviridae infections

INTRODUCTION

Human adenoviruses (Ads) are non-enveloped icosahedral viruses that contain a 35 kb double-stranded DNA genome. Up-to-date 50 serotypes of Ads have been identified[1-3] and each serotype has a characteristic DNA base composition, size and restriction enzyme map, and these genotype features are reflected in variations of sizes and antigenic properties of the virion proteins and of tissue specificity[4-5]. The hexon protein is considered to be the major subunit of the icosahedral shell of the virus. This protein constitutes a large proportion of the surface of the virus and has been shown to contain determinants for type- and group-specific neutralizing antibodies, although the epitopes involved remain largely undetermined[5-7].

Ads are associated with several clinical syndromes, and most of the population has experienced infection with one or more serotypes by the end of the first decade of life. Ads are a common cause of keratoconjunctivitis and, together with herpes simplex virus (HSV), are the major cause of viral infections of the external part of eye. Ads can cause acute follicular conjunctivitis which, epidemiologically, can occur sporadically or cause disease in large groups of contacts. Ad-associated conjunctivitis are primarily spread by common-use swimming pools and outbreaks usually occur during the summer and are caused by Ad3 and Ad7[8] although infections

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caused by Ads types 9, 15 and 4 are also common[9]. However other types such as 1,2,5,6,10,11,16,17,20 and 22 have also been described[2,8].

Epidemic keratoconjunctivitis (EKC) is highly contagious and is considered a more serious disease. Ads serotypes commonly associated with EKC are serotypes 8, 19 and 37[2,10]. The disease is mainly transmitted through inadequately washed hands and improperly sterilized ophthalmic instruments or solutions[11]. Keratitis begins as the conjunctivitis wanes, and the cornea may remain affected for several months and produce visual disturbance. Although not blinding, these ocular infections may be associated with significant morbidity and economic losses to employer and worker, absence from school, and persistent visual disability due to subepithelial opacities[12]. The onset of conjunctivitis is insidious, frequently bilateral, and preauricular adenopathy is common. Viruses can be isolated readily for at least 9 days after the onset of symptom[9].

Rapid and accurate diagnosis is advantageous to distinguish ocular HSV from Ads infection. Early Ads diagnosis is important for the control of nosocomial outbreaks, which can involve many patients, while herpetic eye infections require prompt acyclovir therapy[1].

Laboratory diagnosis of ocular Ads infection relies on virus isolation in cell culture, which takes about 14 days or in antigen detection assays, including many rapid laboratory methods, such as enzyme immunoassay, immunofiltration, immune dot-blot test[11-13], which can be performed in a few hours, but lack the sensitivity and specificity of virus isolation[1,14]. Other laboratory method is the shell vial technique, which has high sensitivity and specificity, but takes three days to be performed [13]. Polymerase chain reaction (PCR) is a rapid and specific method and has been used for the diagnosis of several viral infections.

We previously developed a PCR for herpesvirus that requires no DNA extraction [15-16]. We adapted this protocol to a PCR for Ads that can identify its DNA with high specificity and within a short period of time. Subsequent sequencing of the amplified DNA fragment can be a powerful tool in the attempt to determine the serotype of Ad, which causes the infection. PCR allied to sequencing can provide the diagnosis of the Ad serotype faster than the isolation-neutralization test.

METHODS

Samples

Seven clinical samples were obtained (conjunctival swabs) from patients with external eye diseases and were in storage when this study was performed. These samples were previously tested for herpesvirus and three of them were PCR positive for HSV and were used as negative controls.

Sample preparation

The eye of the patient was washed with sterile saline and a sterile cotton swab was rubbed onto the conjunctiva and then immersed in Eagle's minimal essential medium supplemented with penicillin, amikacin, amphotericin B and 1% fetal bovine serum. A tenfold dilution of the clinical specimen was heated to 100º C for 10 min and 5 µl were used as DNA template[13].

PCR assay

The adenovirus primers (P1 - 5' GCCGCAGTGGTCTTACA TGCACATC 3', P2 - 5' CAGCACGCCGCGGATGTCAAGT 3') were designed according to the DNA sequence of the hexon region of adenoviruses types 2 and 5. This pair of primers amplifies a fragment of 300 bp from the hexon gene of many serotypes[1]. The PCR reaction was carried out as described elsewhere[17]. The PCR cycle was 1 cycle at 94º C/5min, 35 cycles at 94º C/30s, 55º C/30s, 72ºC/1min and 1 cycle at 72º C/15min. A plasmid containing the hexon gene of Ad was used as a positive control (kindly provided by Prof. Armando Ventura, ICB/USP).

Cloning and sequencing procedure

The fragment of the hexon gene amplified by PCR was purified by PCR Preps System (Promega, USA) and cloned into pUC 18 plasmid (Sure-clone, Pharmacia). The cloned fragment was sequenced in both orientations by the dideoxy chain-termination method[18] using M13 universal primers (fmol DNA Sequencing System - Promega) and (α 32P) dCTP for oligonucleotide labeling. The nucleotide sequences were compared with the sequences present in the GeneBank using the BLASTN program[19].

RESULTS

Of the seven samples studied, six were PCR negative for adenovirus (including the three negative controls) and one was positive (Figure 1, MV1). The amplified DNA fragment of 300 bp was cloned and then sequenced in both orientations. Analysis by BLASTN program of available databases (GeneBank) has pointed out complete homology of the sequenced fragment with Ad type 8 (Figure 2). Only the sample with a clinical hypothesis of Ad infection was positive in Ad PCR.

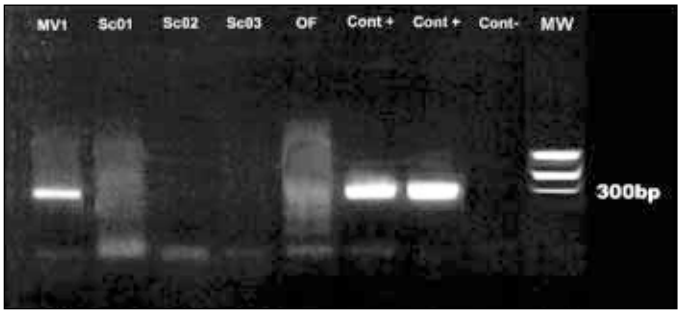

Figure 1 - PCR-amplified products from clinical samples. Swabs samples from patients with keratoconjunctivitis were tested by PCR. The amplified products were fractionated in a 2% agarose gel. MV1, SC01, SC02, SC03 and OF are clinical samples; Cont+ are the positive controls; Cont- is the negative control; and lane MW contains the molecular weight markers

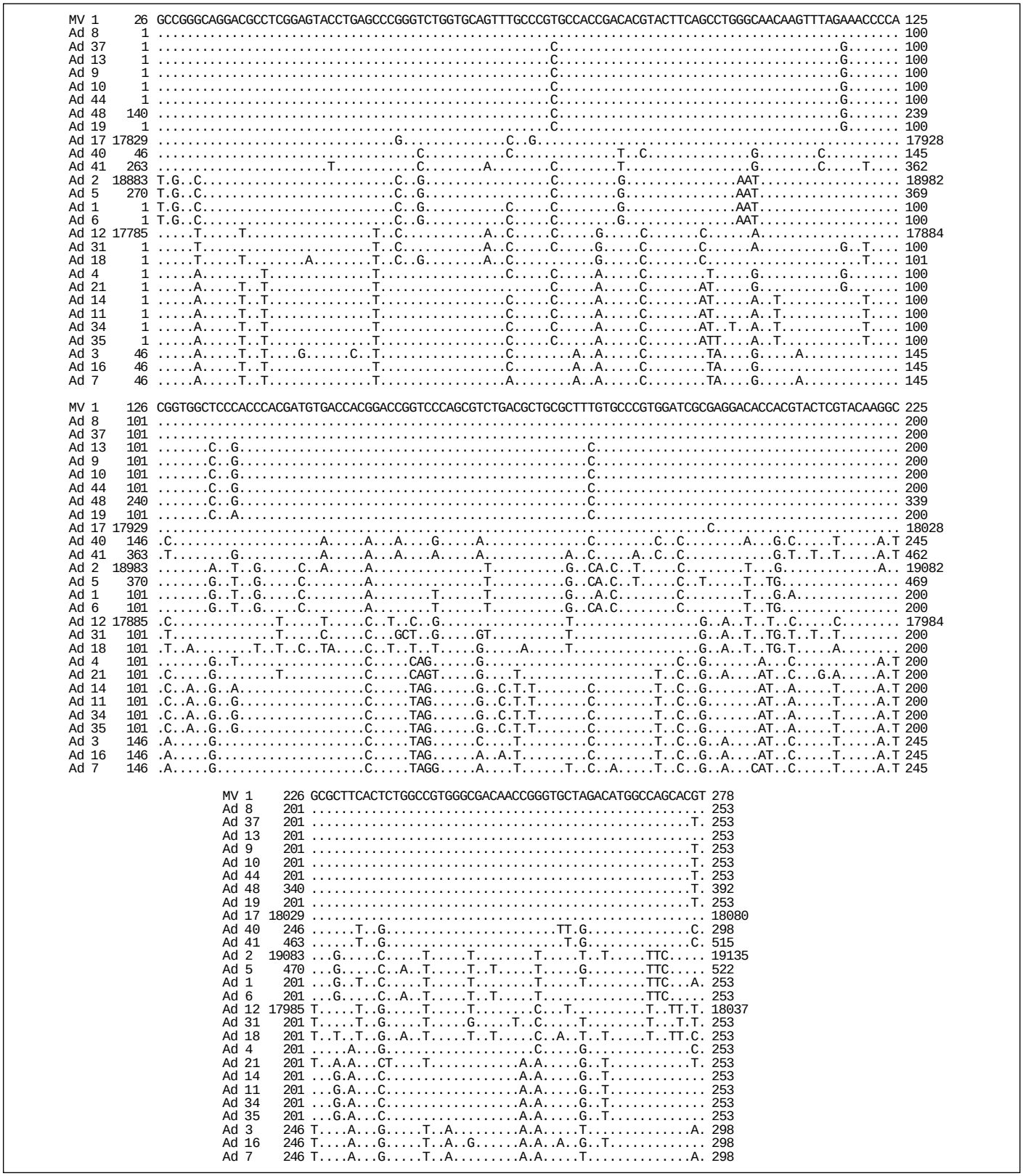

Figure 2 - Alignment of the sequence of the amplified DNA fragment from sample MV1 with those of serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 21, 31, 34, 35, 37, 40, 41, 44 and 48 present in GenBank. Points show regions of identity. These sequences were taken from previous reports as follows (accession number): Ad1 (AF161559.1), Ad2 (J01917), Ad3 (X76549), Ad4 (AF161569.1), Ad5 (J01966), Ad6 (AF161560.1), Ad7 (X76551), Ad8 (AF161561), Ad9 (AF161562.1), Ad10 (AF161563.1), Ad11 (AF161570.1), Ad12 (X73487.1), Ad13 (AF161564.1), Ad14 (AF161571.1), Ad16 (X74662), Ad17 (AF108105), Ad18 (AF161575.1), Ad19 (AF161565.1), Ad21 (AF161572.1), Ad31 (AF161576.1), Ad34 (AF161573.1), Ad35 (AF161574.1), Ad37 (AF161567.1), Ad40 (X51782.1), Ad41 (D13781.1), Ad44 (AF161568.1), and Ad48 (U20821)

DISCUSSION

The PCR assay without DNA extraction has many advantages over phenol-chloroform extraction, which is the standard method to extract viral DNA for PCR assay. The time to perform PCR is shorter than the time for assays in which DNA extraction is required. Moreover, this technique can prevent DNA from contamination of specimens by handling and requires no organic reagents that could inhibit PCR[15].

This protocol can also be used for HSV detection. The use of these two reactions together or in a multiplex Ad/HSV PCR can be a powerful and fast technique for the diagnosis of viral eye infections since it encompasses the two major causative agents of these diseases[1].

Other authors have also shown that preparing DNA for a PCR using a simple lysis buffer is more effective than phenol-chloroform extraction[11]. In our case we have used only a one to ten dilution plus a boiling procedure. This approach is not only faster but also cheaper. Comparing the sequence of the hexon gene fragment amplified by our set of primers with sequences of other human adenoviruses present in the GeneBank, we conclude that this region of the Ad genome can be used to identify almost all Ads serotypes, including all serotypes reported to cause EKC (8,19,37) and almost all serotypes involved in Ads-associated conjunctivitis (1, 2, 3, 4, 5, 6, 7, 11, 15, 16, 17, 20 and 22). However, this fragment is insufficient to distinguish serotypes 9 (reported to cause Ad-associated conjunctivitis), 10, 44 and 48, because their DNA sequence is identical in this region.

The transmission of this easily spread infection can occur through contaminated eye drops, hands and surfaces being important also in nosocomial infections. According to recent studies the cost per infected person is similar to the cost of other nosocomial infections[20].

CONCLUSIONS

We conclude that PCR without DNA extraction followed by sequencing of the amplified fragment can be used for both the diagnosis and the molecular epidemiology of viral eye diseases. This method is highly specific and faster than the isolation-neutralization test, especially if automatic sequencing is used. PCR can also be easily adapted to a multiplex Ad/HSV PCR. Another advantage is the rapid serotyping of the Ad which causes the infection by sequencing the PCR amplified fragment. Certainly, these characteristics are of great advantage for the diagnosis of diseases that can spread easily such as EKC. This approach, however, should be tested in a prospective study to validate its clinical applications.

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RESUMO

Objetivo: Vírus da família Adenoviridae estão associados com muitas síndromes clínicas, sendo conhecidos 50 sorotipos. Vírus desta família e da família Herpesviridae são os maiores responsáveis por conjuntivite viral, sendo um rápido diagnóstico importante devido ao caráter epidêmico das infecções por adenovírus. **Métodos:** Reação em cadeia da polimerase (PCR) para adenovírus foi desenvolvida utilizando iniciadores que amplificam um fragmento de 300 bp do gene da proteína hexon do capsídeo de diversos sorotipos. A reação em cadeia da polimerase foi efetuada sem a etapa de extração de DNA. **Resultados:** Amostras de "swabs" de córneas de sete pacientes com ceratoconjuntivite foram analisadas, sendo que uma amostra foi positiva para adenovírus. O seqüenciamento deste fragmento mostrou 100% de homologia com a seqüência do adenovírus tipo 8. **Conclusão**: O seqüenciamento do fragmento de 300 bp do gene do hexon permite a identificação de quase todos os sorotipos de adenovírus, incluindo aqueles relacionados com a ceratoconjuntivite epidêmica (8,19,37) e todas as amostras associadas com conjuntivite.

Descritores: Reação em cadeia da polimerase/métodos; Adenovirus humano/isolamento & purificação; Ceratoconjuntivite/diagnóstico; Infecções humanas por adenovirus; Infecções oculares virais/diagnostico; Infecções por herpesviridae

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