Detection of Adenoviruses from clinical samples in bone marrow transplant patients by nested PCR (polymerase chain reaction)

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ABSTRACT

Adenoviruses are recognized as common human pathogens that are responsible for a wide variety of infectious syndromes. Bone marrow transplant patients are prone to life threatening opportunistic infections like adenoviruses. The nested polymerase chain reaction has provided an alternative, sensitive diagnostic method for detection of Adenoviruses. In this study we developed PCR from hexon genes as rapid diagnostic method of Advs infections on different clinical samples. Adenovirus infections was defined as the presence of DNA in the blood, urine, stool, or respiratory lavage from bone marrow transplant patients. Two sets of primers (Group specific primers and internal primers) were required to optimize the PCR protocol. This highly sensitive method could detect different types of Advs in two separate sets of PCR. Therfore,DNA amplification in BMT patients would be valuable screening way to evaluate bone marrow transplant recipients. Early detection of Advs by PCR assay is important to asymptomatic infections or preventing aggressive antiviral thearapy.

Keywords: Adenovirus; Nested PCR; bone marrow transplant (BMT); clinical sample

INTRODUCTION

Human Adenoviruses (HAdvs) are known as a family of Adenoviridae [1]. which can cause variety of human disease. These microorganisms divided into six species, A-F with 51 serotypes. The new Adenovirus species has been isolated from gastroenteritis patient type G with serotype 52 [2]. Adenoviruses as viral pathogens produce major complicated problems in immunocompromised or transplant patients. Rapid and proper diagnosis of HAdvs infection is critical and controversial in bone marrow transplant recipients. DNA amplification method is preferentially diagnostic technique to apply for clinical samples [3]. In this study we developed nested PCR from hexon genes to detect different HAdvs from stool, urine, respiratory lavage in bone marrow transplant recipients. The PCR assay has provided significant advantages in sensitivity over antigen detection or culture methods. Thus, with the primer sets used in amplification process proved capability of rapid identification the human Adenoviruses species [4]. Here, we collected clinical specimens from bone marrow transplant recipients, then extracted DNA to identify the infected peoples .

DNA extraction from blood, serum, cell suspension

In this method we used QIA Gen Kit, Germany, then transferred sample into a 1.5 ml eppendorf tube so 200ul from cell suspension poured into 200ul PBS. Added 25 ul QIA Gen protease stock solution and 200ul buffer into the sample. Then mixed immediately by vortex for 15 seconds. Incubated at -70° c for 10 min. For serum, blood, plasma the amount of 210ul isopropranol was added to the cell suspension and mixed by vortex. Placed a QIA amp spin column in a 2ml collection tube. Applied the mixture from above step to the column without moistening the rim, closed the cap, centrifuged at 8000rpm. After several washing steps, DNA was eluted with 200 µl of preheated buffer or distilled water. For urine DNA extraction, the viral RNA Kit and in DNA preparation in feces the diluted sodium chloride

were used, and sterilized by filtration (0.22µM filter).

PCR primers

Two Adenovirus primers were chosen from hexon genes. These primers made a PCR fragment with 134 base pairs.[5,6,7,8,9] Then two pairs primers, one pair as general primers or group specific and the others as internal to amplify two fragments. One fragment with 300 bp and these products were used for second PCR to make a 243 bp fragment. The sequence of the primers as follows:

5‘ATG-ACT-TTT-GAG-GTG-GAT-CCC-ATG-GA3‘	26mer
5‘GCC-GAG-AAG-GGC-GTG-CGC-AGG-TA 3‘	23 mer
Primer sequence in PCR to amplify a 134bp fragment	
5‘GCC-GCA-GTG-GTC-TTA-CAT-GCA-CAT-C3‘	25mer
5‘CAG-CAC-GCC-GCG-GAT-GTC-AAA-GT3‘	23mer
General primers(Group specific Primers) to make a 300bp fragment	
5‘CCA-GGA-CGC-CTC-GGA-GTA-CCT3‘	21mer
5‘TGG-AAG-CCA-TAT-CAA-GCA-CAC3‘	21mer
Internal primers produce a 243bp fragment	

Table 1. The PCR components

Primer AdenoQCR4R	50 pmol/µl each	2.0 µl
Primer AdenoQCR1	50pmol/µl each	2.0 µl
Dinucleotide Triphosphate(dNtp)	1.5*mM(mixed)	8.0µl
10x PCR buffer(15 mMMgcl_2)	15mMmgcl,500mMKCL,100mMtris,0.1%gelatin	5.0 µl
Taq polymerase	5u/µl (Boehringer manheim)	1.3 µl
Double Distilled Water	DDW	26.7 µl

*stock solution of dNTP(contained 1.5mM each deoxynucleotide triphosphate dNTP in strile distilled water

Table 2. PCR PROGRAMME TABLE

First cycle	94° c	10min
Second cycle	94° c	45second
Annealing tempreture	55° c	20 second
Extension	74° c	45 second

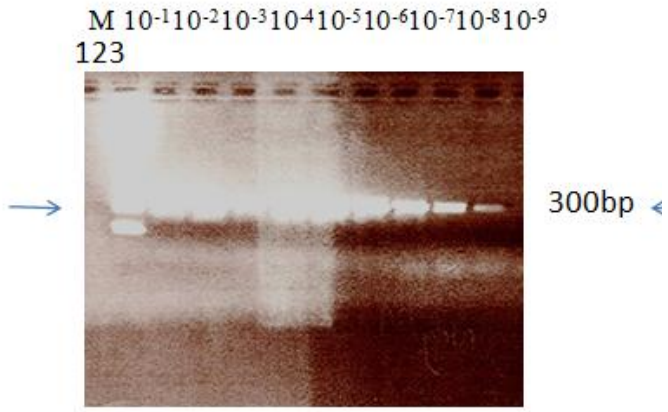

Figure 1. The different DNA dilution in first column is marker(123bp DNA Ladder), second column 10-1,10-2,10-3,10-4,10-5,10-6,10-7,10-8,10-9 negative control. The first PCR with 300bp is detected.

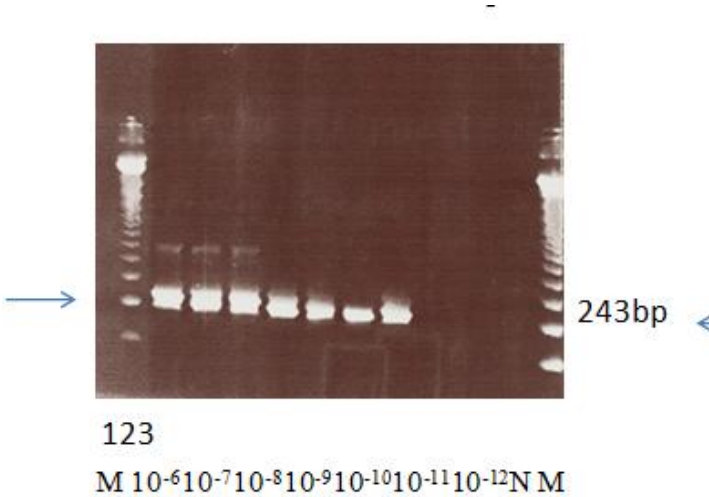

Figure 2. The nested PCR with 243 bp in the right,second column 10^{-6},10^{-7},10^{-8},10^{-9},10^{-10},10^{-11},10^{-12}, negative control.(two 123bpDNA marker).

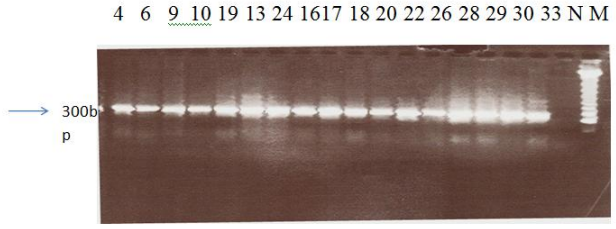

Figure 3. General PCR (300bp) of different types of Adenoviruses from left to right
Ade Type 4,6,9,10,19,13,24,16,17,18,20,22,26,28,29,30,33,negative control,123bp DNA marker.

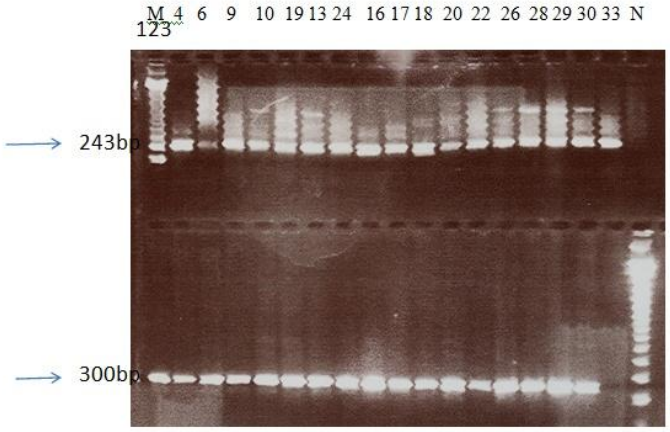

Figure 4. In the figure ,upper part, nested PCR from pcr product 243bp detected.
The first column is marker and the second Ade Type 4,6,9,10,19,13,24,16,17,18,20,22,26,28,29,30,33,negative control.
The lower part the same as above with 300bp in general PCR.

Reaction mixture is shown in table 1. The PCR with two groups specific primers was performed. It made 300bp with all groups of Adenoviruses (A-F). The nested PCR was done from PCR product of Ad2,3,5,7,21,25,32, with using the internal primers. The number of cycle was 30. The nested PCR program was the same as above unless the 45° c for the annealing temperature.

Determination of PCR sensitivity

We extracted DNA and cleaned with purification Kit . Then diluted from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}. The PCR was run with the general primers and diluted DNA. (figure 1)This figure demonstrates the PCR sensitivity.

To apply the method to detect different types of Adenoviruses, DNA extracted from cell culture supernatant (by phenol-chlroform isoamylalcohole), two PCR , first in general primers to make a fragment with 300bp, then using the PCR product directly in the nested PCR to make a 243bp fragment. Different types including 4, 6, 9, 10, 13, 19, 24, 16, 17, 18, 20, 22, 26, 28, 29, 30, 33 were detected. (Figure 3, 4)

MATERIALS AND METHODS

Clinical samples were collected by bone marrow transplant unit(BMTU) in Essen University Clinic Centre(Germany). Different samples, urine, stool, respiratory lavage from eight transplant recipients in different times were prepared. All the samples sequentially collected before and after a months of transplant. Then the results were evaluated. Totally eighty four samples were selected from eight patients. The clinical materials were cultured in KB cells (human aliquates were cultured into carcinoma cells in MEM media) stored in -20° freezer. To make positive viral samples, one ml of Ad2,Ad14,Ad5,Ad25,Ad21,Ad7,Ad4,Ad3,and 2ml of Ad32,Ad37,ad8,Ad31,were added into KB cells. For negative control only MEM culture media and uninfected cells were used.

Sample Preparation

Before amplification, Adenovirus DNA should be free of capsid or contamination. To 100 µl of sample (already aliquot into 100 µl) 25 µl of lysis buffer and 1µl of proteinase K(25mg/ml) was added. Incubate in 50° c water bath for one hour. Then heated at 65° c for 20 min to inactivate proteinase K. An equal volume of phenol chloroform isoamylalchol was added. For one to two minutes vortexed. Centrifuged at 12000 in two minutes.The upper layer removed and added an equal volume of chloroform-isoamylalchohol. Then vortexed and centrifuged as above. Removed the upper layer and or low in virus load, DNA should be concentrated with ethanol method. Added 1/10 volume of 3M sodium acetate, ph 5.2. Added 2 volume of ethanol(100%) in -20° c, mixed and centrifuged in 12000g for 30 min. in 4° c. Removed supernatant , washed the pellet twice with ethanol 70% (-20° c).

PCR

The nested PCR in 30 cycles with denaturation temperature 94°C and annealing tem.55° C in first run but 45° C in second run detected all different types of Adenoviruses .The sensitivity of two PCR with the serial dilution of DNA were determined. We prepared different clinical specimens from bone marrow transplant unit of eight transplanted patients.

RESULTS

Two PCR designed in this study to detect human Adenoviruses. The hexon gene was chosen as the target as more is known about this gene than any other Adenoviral gene. With two primers Adenovirus QCR4R 5‘ATG-ACT-TTT-GAG-GTG-GAT-CCC-ATG-GA 3‘ , 5‘GCC-GAG-AAG-GGC-GTG-CGC-AGG-TA 3‘ from hexon gene could detect a 134 base pair fragment of four serotypes of Adenovirus (Ad2 ,Ad5 ,Ad14 ,Ad25). There was no amplification in negative control(cells without adding virus) or in specificity control (herpes simplex virus added to the cells) . Two sets of primers , one as group specific and the other internal primers were designed . The general primers 5‘ TTG-ACA-TCC-GCG-TGC-TG 3‘ ,TAT-CAT-TTT-GAC-CTT-GAG-C- 3‘detected a 300bp fragments of all groups of Adenoviruses (A-F) .The internal primers 5‘ CCA-GGA-CGC-CTC-GGA-GTA-CCT 3‘ , TGG-AAG-CCA-TAT-CAA-GCA-CAC 3‘ were made a 243 bp fragments of PCR product in first run .

Table 3. Patient results from eight BMT recipients.

Patient name ZM. Date of birth 04/09/1956 . Transplanted on 29/07/1997

Start date and end date of sample preparation	Bronchoalvoalar lavage	urine	stool	serum
25/07/97	negative	---------	--------	--------
28/07/97	----------	posirive	--------	--------
29/07/97	----------	negative	--------	--------
31/07/97	----------	----------	negative	positive
04/08/97	----------	positive	negative	negative
08/08/97	positive	----------	---------	--------
11/08/97	positive	negative	---------	negative

Table 3a

Patient name WE. Date of birth 11/12/46 transplanted in 28/07/97

Start date and end date of sample preparation	BAL	urine	stool	serum
18/07/97	positive	positive	negative	--------
24/07/97	negative	negative	--------	--------
31/07/97	positive	----------	---------	--------
04/08/97	----------	positive	negative	--------
07/08/97	negative	negative	--------	--------

Table 3b

Patient name PTH. date of birth 23/03/55 transplanted 04/08/97

Start date and end date of sample preparation	BAL	urine	stool	serum
28/07/97	neg	neg	---------	--------
08/08/97	neg	pos	---------	neg

Table 3c

Patient name AD. Date of birth 24/07/52 transplanted 30/07/97

Start date and end date of sample preparation	BAL	urine	stool	serum
15/07/97	pos	pos	neg	-------
18/07/97	-------	------	pos	-------
22/07/97	pos	pos	-------	-------
24/07/97	neg	neg	-------	-------
27/07/97	neg	neg	-------	-------
29/07/97	-------	-------	neg	-------
04/08/97	pos	neg	neg	-------
11/08/97	pos	-------	-------	-------

Table3d

Patient name BH .date of birth 30/05/48 transplanted 15/07/97

Patient Start date and end date of sample preparation	BAL	urine	stool	serum
08/07/97	pos	neg	neg	------
15/07/97	pos	pos	neg	------
21/07/97	pos	-------	neg	-------
27/07/97	neg	neg	-------	-------
04/08/97	pos	neg	neg	-------
11/08/97	-------	-------	neg	-------
18/07/97	------	pos	-------	--------

Table3e

Patient name KG .date of birth 30/01/50 transplanted 24/07/97

Patient Start date and end date of sample preparation	BAL	urine	stool	serum
18/07/97	neg	pos	neg	-------
21/07/97	pos	--------	-------	--------
27/07/97	-------	pos	--------	--------
29/07/97	--------	--------	neg	----------
04/08/97	pos	neg	---------	----------
09/08/97	--------	--------	neg	----------
11/08/97	pos	--------	---------	----------

Table3f

Patient name BE .date of birth 30/08/51 transplanted 05/08/97

Patient Start date and end date of sample preparation	BAL	urine	stool	--------
29/07/97	pos	pos	neg	--------
04/08/97	pos	neg	--------	--------
11/08/97	pos	neg	neg	--------

Table3g

Patient name HE .date of birth 20/02/41 transplanted 28/07/97

Patient Start date and end date of sample preparation	BAL	urine	stool	serum
27/07/97	pos	neg	neg	--------
29/07/97	-------	--------	neg	--------
04/08/97	neg	neg	------	--------
11/08/97	pos	neg	neg	--------

Table3 h

One type specific PCR performed in all positive specimens . Using specific primers type 31 could detect none of positive samples. Figures 5, 6, 7,8 have shown the above positive results on gel. We added Adenovirus 2 in all negative urine samples as inhibitory control. Then extracted DNA by viral RNA kit. All negative urine specimens were positive . Figure 8 represented the results. None of type specific primers for Ad1,2,4,5 were positive in bone marrow patients.

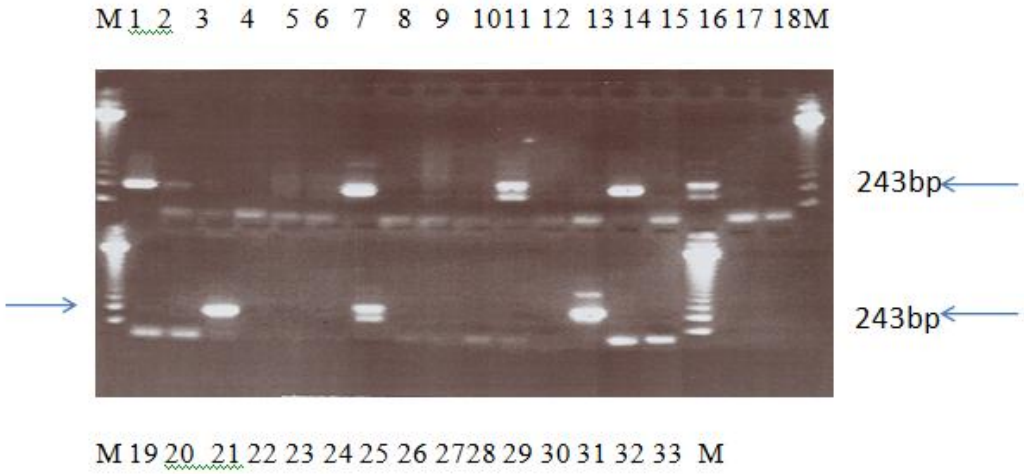

Figure 5. PCR from clinical samples in BMT patients. First column 123bp DNA Ladder, No.1 positive urine sample in ZM, No.2Positive urine sample with faint band, No.3 negative urine sample in AD, No.4 negative urine sample in WE,No.5 negative urine sample in AD,No.6 negative urine sample in PTH,No.7 positive urine sample inKG,No.8 negative urine sample in ZM,No.9 negative respiratory sample in KG, No.10 negative respiratory sample in ZM,No.11positive respiratory sample in KG,No.12negative respiratory sample in AD,No.13.negative respiratory sample in WE,No.14.positive respiratory sample in HE,No.15.negative respiratory sample in AD,No.16.positive urine sample in BH by adding Ad25 ,No.17.negative respiratory sample in BH,No.18.negative urine sample in BH,No.19.negative urine sample in HE,No.20.negative urine sample in BH,No.21 positive stool sample in AD,No.22 negative stool sample in BH,No.23.negative stool sample in BH,No.24.negative stool sample in different date in BH,No.25.positive stool sample plus Ad25in BH,No.26.negative stool sample in WE,No.27.negative stool sample in HE,No.28.negative stool sample in BE,No.29.negative stool sample in AD,No.30.negative stool sample in KG,No.31Ad2positive control,No.32.negative control,No.33.negative control for second PCR.

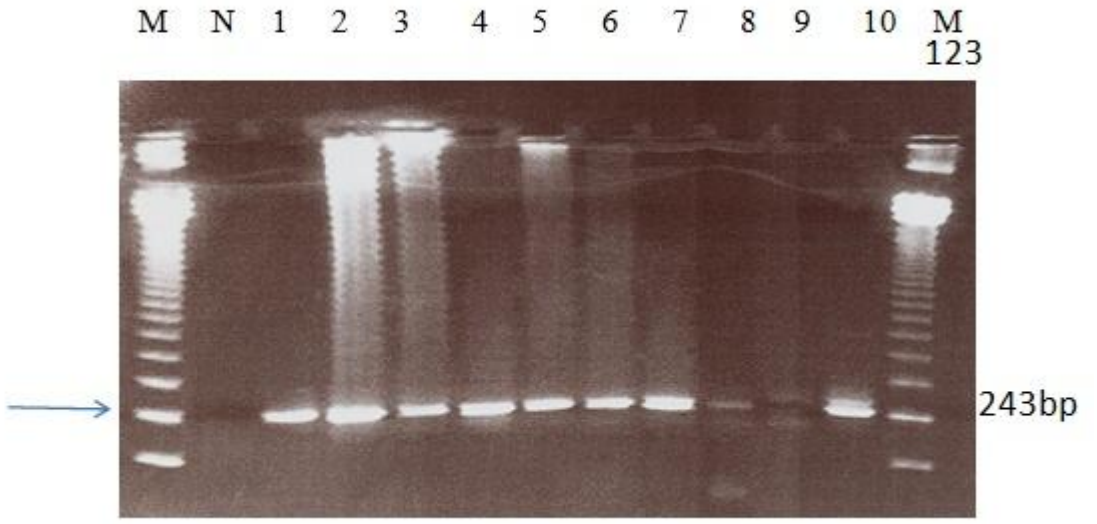

Figure 6. PCR from urine positive samples.No.1 positive BH urine sample,No.2. positive AD urine sample,No.3.positive WE urine sample,No.4 positive KG urine sample,No.5.positive AD urine sample in different date,No.6 positive ZM urine sample,No.7positive BH urine sample,No.8positive BE urine sample,No.9 negative AD urine sample,No.10 positive urine sample in KG.

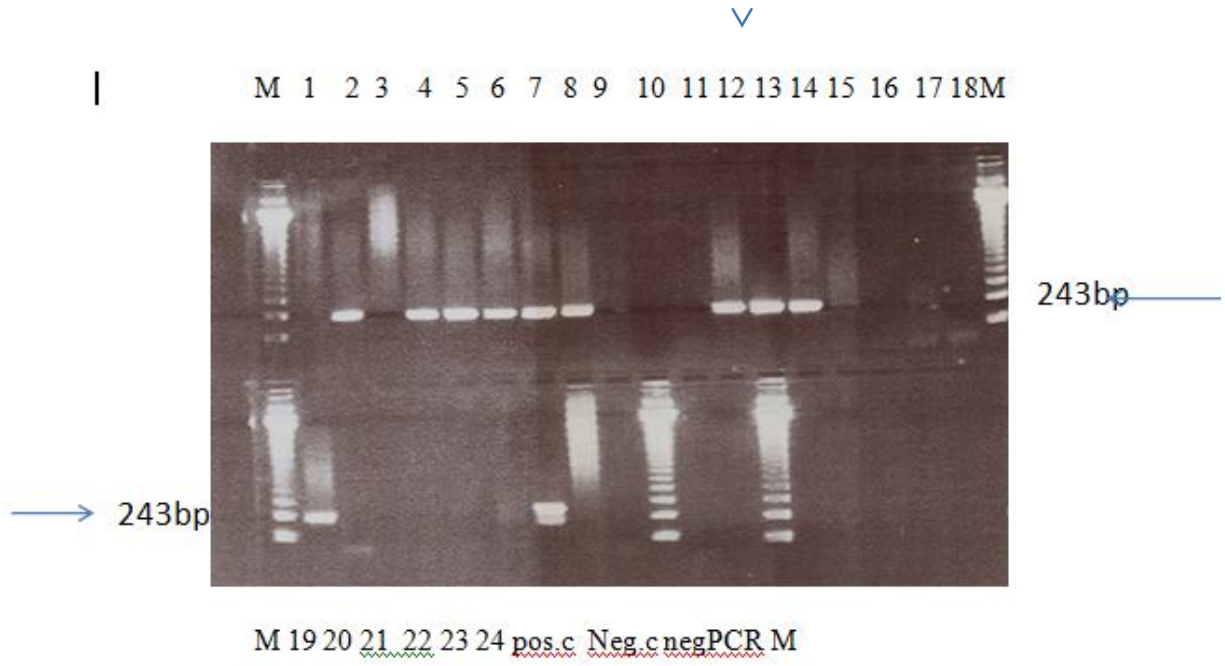

Figure 7. PCR from PCR product in different clinical samples, in lower side Before marker negative result then negative control, positive control.

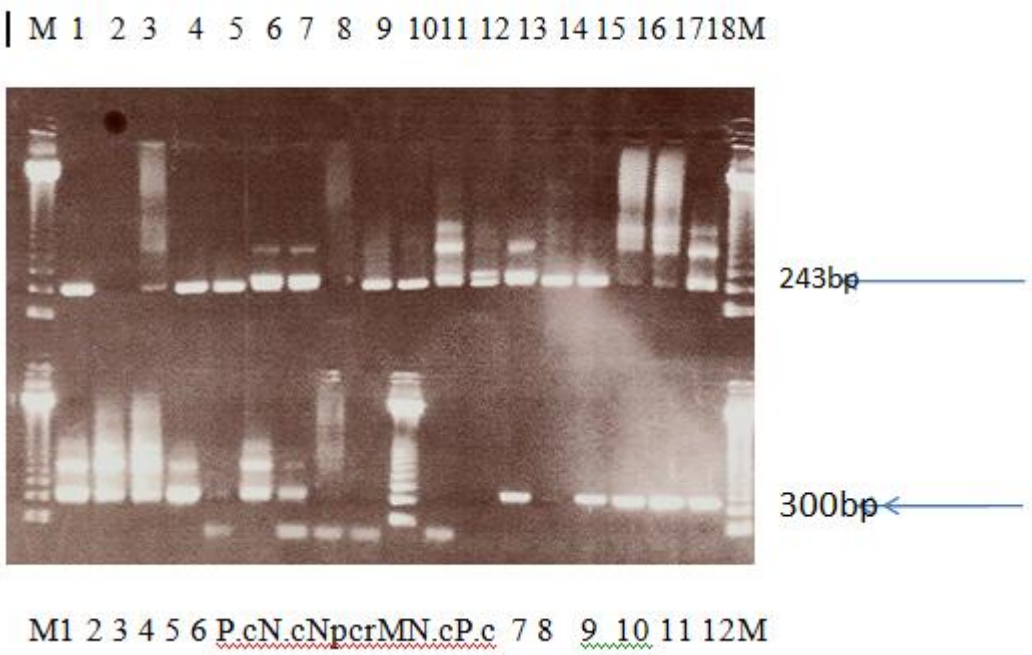

Figure 8. The upper side is the nested PCR with 243bp positive specimen in clinical patient samples ,in lower side before marker ,negative PCR then neg.control and pos.control.plus negative results which added Ad2 to the samples to make it positive in first PCR as inhibitory control stored on ice. It could be stored in -70° c. For diluted specimens or small volume.

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DISCUSSION

There are overall sequence homology less than 25% between the 6 species of Adenoviruses [10]. Most of the studies are based on amplification of highly conserved region of Adenovirus genome. The sequence information of hexon gene in different types of Adenoviruses explains the 50% nucleotide homology. The incidence of Adenovirus infection following the bone marrow transplantation was high in first 6 months. [11]. The patients with Advs infection are susceptible to multiple opportunistic co-infections which contribute severe disease. Infection is spread through aerosolized droplets with breathing or nosocomially [12].The risk factors in post bone marrow transplant Advs infectivity could be types of immunosuppressive therapy, and transplantation from unrelated donors [13]. The highly progress of molecular diagnostic techniques of viral infections which are the gold standard methods used in the lab. These techniques are the most efficient way to identify the viral agents in making quick decisions of therapeutic measures for avoiding the transmission of the disease [14]. There are several mechanisms may explain the viral infections in immunocompromised peoples like reactivation of the virus or transmission from donor to recipient [15]. To our knowledge , none of our positive results were strain Adv31 which others reported an outbreak of Adv31 infections in 2003 with nosocomial origin. Adenoviruses produce nosocomial infections which can be controlled by using adequate disinfection of instruments or using gloves to examine patients. Even in swimming pools with inadequate chlorination can cause infections [16]. Although in this study , the presence of virus in asymptomatic feature in spite of inhibitors to taq polymerase in urine samples, two steps PCR detected all positive specimens. The importance of early diagnosis is establishing the procedures of the steroid therapy [17]. The present of Adenoviral DNA in stool samples allows the early differentiation of gastroenteritis infections or graft-versus-host-disease(GVHD)in transplant patients. The hexon gene makes the large amount of the virus surface and it has been shown to contain the group specific and the type

specific antigenic parts of the gene.(18) The two steps PCR which described here with small PCR product without storage problems of clinical samples ,thus offers significant improvement in speed and sensitivity in comparing with conventional methods. Using the sensitive methods such as PCR in diagnosis could be helpful to distinguish different stages of infection by quantitate the genome copies in our experiments [19]. The nested PCR provides a unique method to detect previously unrecognized Adenoviruses even presence of one copy of Advs. is shown. This method are not able to support the association between clinical syndromes and various human Ads. Serotypes because real time PCR with cloning fragment overlapping internally control to known Ads.types [20].There are various reports of Advs in immunocompromised transplant recipients. Most of these infections were types 2,5,11,12,31,35 but in our experiments none of the positive samples were Advs 31. This type of Adenovirus needs more investigation to prevent infections in bone marrow transplant patients. Conclusion

In our study, the optimized PCR detected different types of Advs in clinical specimens. This highly sensitive diagnostic method described even presence
One copy of viruses in the early identification of transplant patients.

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