Direct Detection of Human Adenovirus in Hemorrhagic Cystitis patients from Babylon Province by Real-Time PCR technique

Naeem Rahman Al-Jeburi, Azhar Amran Al-Tahab, Ghanim Aboud Al-Mola

Department of Biology, College of Science, University of Babylon

ABSTRACT

Hemorrhagic cystitis (HC) is consider of the important complications of lower urinary tract symptoms that include dysuria, hematuria, and hemorrhage. In hemorrhagic cystitis, short-term hematuria can also be seen in bladder infection as a result of viral infection. Viral cystitis represents another form of non-bacterial UTI touching adult and children. Adenovirus types 11 and 21, polyomavirus BK, and CMV viruses also can causes of hemorrhagic cystitis. This study was aimed to use of Real-Time PCR technique as molecular method for direct detection HAdVs from urine samples of hemorrhagic cystic patients. The result of present study was found that 132 samples (66%) have positive bacterial culture were excluded, and the other 68 samples (34%) give negative bacterial culture were included as a viral cause of hematuria. All negative bacterial culture cases was classified as 2 (2.9%) patients with glomerulonephritis, 13 (19.11%) chronic renal failure, 27(39.70%) U.T.I., 12(17.64%) kidney transplantation 14(20.58%) cystitis. All urine patients 68 with 20 urine samples of control were analyzed by Real-Time PCR technique for detection HAdVs. It was found only 4(6%) as a positive result. In conclusion, this study report the design, development and application of real-time PCR assay for rapid, specific, and highly sensitive detection of group B Adenovirus as causative agents in hemorrhagic cystitis.

INTRODUCTION

Hemorrhagic cystitis is characterized by painful hematuria due to hemorrhagic inflammation of the urinary bladder mucosa. Hemorrhagic cystitis, temporary hematuria can be seen in bladder infection as a result of viral infection [9]. Viral infection is defined as a presence of an identifiable viral organism with inflammatory symptoms. In addition the disease can also occur as a complication of cyclophosphamide, ifosfamide and radiation therapy. [1]. The symptoms of lower urinary tract infection include hematuria, genital or lower abdominal pain, urgency, frequency (secondary to inflammatory response and irritation of the bladder wall), pyuria, and hematospermia. In rare instances of prostatic abscess, the obstructive voiding symptom and urinary retention can be found. [2]. Viral cystitis represents another form of non-bacterial UTI affecting adult and children. Adenovirus types 11 and 21, Cytomegalovirus, polyomavirus BK, and herpes simplex viruses can cause irritative voiding symptoms, hemorrhagic cystitis and even urinary retention or obstruction urinary. In non-immunized or immunosuppressed children, herpes zoster cystitis presents similarly. Immunosuppressed children undergoing kidney or bone marrow transplantation, or those receiving chemotherapy are especially susceptible to viral cystitis, including those caused by cytomegalovirus and adenoviruses 11, 21, and 35 and associated with glomerulonephritis and pyelonephritis patients. [3]. Diagnosis of viral UTI is more challenging because viruses are small organisms, and they cannot be visualized with even the best optical microscope. The culture of viruses may take up to 14 to 28 days, and often it is too late to treat a patient with disseminated multi organ viral infections at that time [10]. Thus, molecular and immunofluorescence techniques are used more commonly [4,5]. Therefore, in this study, we developed a rapid, sensitive TaqMan probe-based real-time PCR molecular technique targeting a highly conserved region of hexon gene of the HAdVs, has been developed for the detection and of HAdVs virus in urine specimen.

Corresponding Author: Naeem Rahman Al-Jeburi, Department of Biology, College of Science, University of Babylon

E-mail: dr.naeem72@yahoo.com
MATERIALS AND METHODS

Patients:
This study was performed on two hundred bloody urine and blood specimens were collected from 200 patients which selected patients to eliminate other causes of hematuria, the patients group comprised 149 male and 51 female age group 4 -46 years, who attending to different hospitals in Babylon governorate include Al-Hilla Teaching Hospital, Marjan Teaching Hospital, Babylon Hospital for Maternity and Children over period between April 2014 to December 2014. Questionnaires were used to obtain information from the patients itself and parents or guardians accompanying the patients to hospital. Information included signs and symptoms of illness (hematuria slight pink to frank bright red blood with or without blood clots, dysuria (painful urination), burning with urination., urinary frequency, urinary urgency, urinary incontinence (involuntary loss of urine, etc.).

Collection of Samples:
Two hundred urine samples, varied in volume from 20 ml to 50 ml per patient were collected in disposable sterile containers and centrifuged at 3500 rpm for 30 minutes then examined under light microscope to determine grade(mild, moderate and severe) of hematuria and divided to three parts and distributed in eppendrof tubes to perform bacterial culture by inoculated of urine samples on to culture blood agar and MacConkey agar. Than incubated at 37°C for 24hours. After that, the negative bacterial culture urine samples were used in Real-Time PCR technique for HAdVs.

Viral genomic DNA extraction:
Viral genomic DNA was extracted from lip urine specimen by using (Genomic DNA extraction kit, Bioneer, Korea). 200µL urine specimen was transferred in 1.5 ml micro centrifuge. Then, viral DNA was extracted according to kit instructions. The purified DNA was eluted in 50µL elution buffer provided with kit and store at -20°C, then used for preparation of Real-Time PCR master mix reaction. The extracted viral DNA was checked by Nanodrop spectrophotometer.

Real-Time PCR:
Real Time PCR was performed for detection of Human Adenovirus in DNA from urine samples by using the primers and probe specific for hexon gene and this technique was carried out according to method described by [12] the primers and probe were designed by this study using conserved region of hexon gene in HAdVs (GenBank: AB330092.1). These primers were provided by Bioneer Company. Korea as showed in following table(1):

Table 1: Hexon primer and Hexon probe

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexon primer F</td>
<td>ATAAAGCAGCACTGTGGATGG</td>
<td>94bp</td>
</tr>
<tr>
<td>Hexon primer R</td>
<td>TGTGTAGTTGCGGACATC</td>
<td></td>
</tr>
<tr>
<td>Hexon probe</td>
<td>FAM-ACGTAGCCCAATGCAACATGACCA-TAMRA</td>
<td></td>
</tr>
</tbody>
</table>

The Real-Time PCR amplification reaction was done by using (AccuPower® DualStar™ qPCR PreMix kit Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as following table(2):

Table 2: RT-PCR master mix components

<table>
<thead>
<tr>
<th>PCR master mix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>5µL</td>
</tr>
<tr>
<td>Forward primer (20pmol)</td>
<td>1µL</td>
</tr>
<tr>
<td>Reverse primer (20pmol)</td>
<td>1µL</td>
</tr>
<tr>
<td>TaqMan probe (25pmol)</td>
<td>2µL</td>
</tr>
<tr>
<td>DEPC water</td>
<td>11 µL</td>
</tr>
<tr>
<td>Total</td>
<td>20µL</td>
</tr>
</tbody>
</table>

The qPCR master mix reaction components that mentioned in table (3.6) above were added into qPCR tube containing (TaqMan probe premix). Then all strips tubes vortex for mixing the components and centrifuged for 3000rpm for 3 minutes in Exispin centrifuge, after that transferred into Miniopticon Real-Time PCR thermocycler. Real-Time PCR thermocycler conditions was set according to primer annealing temperature and RT-PCR TaqMan kit instructions by Miniopticon Real-Time thermocycler system as in the following table(3):
qPCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification of Human Adenovirus Hexon gene in Real-Time PCR cycle number.

**Results:**

Two hundred bloody urine specimens were collected from patients and subjected for culturing on blood agar and MacConkey agar. After incubation the samples at 37°C for 24-48 hour, it was found that 132 samples (66%) give positive bacterial culture were excluded from the study samples, and 68 samples (34%) give negative bacterial culture (No. growth) which might belong to other causes. Were these samples enrolled in the study as a viral cause of hematuria for detection of adenoviral infection Figure(4.1). All negative culture cases was diagnosed by urology physician and classified as: two (2.9%) patients underwent glomerulonephritis, 13 (19.11%) chronic renal failure, 27 (39.70%) urinary tract infection, 12 (17.64%) kidney transplantation, 14 (20.58%) cystitis. The distribution of these study groups are listed in table 4.

![Figure 1: Patients group according to causative factor of hematuria](image)

<table>
<thead>
<tr>
<th>Study group</th>
<th>No. of patients</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulonephritis</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>Chronic Renal Failure</td>
<td>13</td>
<td>19.11</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>27</td>
<td>39.70</td>
</tr>
<tr>
<td>Renal transplantation</td>
<td>12</td>
<td>17.64</td>
</tr>
<tr>
<td>Cystitis</td>
<td>14</td>
<td>20.58</td>
</tr>
</tbody>
</table>

Real-Time PCR technique was done by using specific primers and probe were designed in this studied via using the complete sequence of human adenovirus hexon gene (Gen Bank: AB330092.1) from NCBI GeneBank data base and Primer3 plus online and provided through (Bioneer company, Korea). To confirm further presence of adenovirus and to exclude the possible contamination with other viruses. All urine patients 68 suffering hematuria with 20 urine samples of control (no hematuria) were analyzed by RT-PCR for detection HAdVs. It was found that 4 (6%) positive result from 68 total patients suffering hematuria. Figure (2),(3),(4)and (5).
Fig. 2: Percentage of HAdvs by using RT-PCR technique

Fig. 3: Real-Time PCR amplification plot for hexon gene in adenovirus positive and negative hematuria patient samples by using Taq Man probe (FAM dye) reaction.

Fig. 4: Real-Time PCR endpoint analysis for adenovirus in positive hematuria patient samples. Where Unkn: unknown patient samples, NTC: Non template control, and Neg Ctrl: negative control.
Due to the serious morbidity of viral infections, we planned to conduct a study to detect HAdVs infection in hemorrhagic cystitis and consequently, determine the frequency rate of the infection in the viral hemorrhagic cystitis cases in patients attending to some of hospitals in Babylon governorate. Apart from which, we would also like to identify the clinical features pertaining to HAdVs hemorrhagic cystitis which might help us in differentiating it from other causes of viral hemorrhagic cystitis and hence, assist us in diagnosis and managing these cases. In this study, the study was aimed to use of Real-Time PCR technique as molecular method for direct detection HAdVs from urine samples of hemorrhagic cystic patients [11]. The Real-Time PCR assay, applicable to clinical specimens, was developed that could detect all known serotypes of human adenovirus with high sensitivity and allow for serotype determination by sequencing analysis. [13] PCR showed a decreased time to detection and an increased sensitivity compared to culture. Culture has been considered the “gold standard” for laboratory diagnosis of AdV; however, detectable replication typically requires 3 days to 3 weeks, varying with the specimen source and with the concentration of virus in the specimen. Furthermore, culture requires viable virus [6]. In the present study, all negative bacterial culture growth 68 specimen bloody urine and 20 control from healthy person were also tested by PCR to screen for asymptomatic shedding of adenovirus. The results of present study showed that a well characterized set of primers that amplify part of the hexon gene was used. In study done by [7] who said that Initially, when urine samples were tested by PCR without extraction, sensitivity was very low, suggesting that many urine samples contained inhibitory substances. When methods to purify DNA from such sources were tested, sensitivity markedly increased. The main reasons for introduction of molecular diagnostics are rapidity and the possibility to detect uncultivable or difficult-to-culture viruses [14]. Also in the field of viral cystitis, more molecular diagnostic assays have been introduced for detection of the causative viruses. [8]. The present study agreement with [15] who said, the value measured during real-time PCR analysis was used as an indicator of the viral load found in the urine sample. All patients with positive PCR for adenovirus had mainly hematuria symptoms with hemorrhagic cystitis ranging from mild to severe hematuria.

REFERENCES


Fig. 5: Real-Time PCR threshold cycle (CT) of adenovirus in positive and control samples. Where, positive samples CT:29.57-33.83 and NA: Non amplification as control samples.

Dissuasion:

Due to the serious morbidity of viral infections, we planned to conduct a study to detect HAdVs infection in hemorrhagic cystitis and consequently, determine the frequency rate of the infection in the viral hemorrhagic cystitis cases in patients attending to some of hospitals in Babylon governorate. Apart from which, we would also like to identify the clinical features pertaining to HAdVs hemorrhagic cystitis which might help us in differentiating it from other causes of viral hemorrhagic cystitis and hence, assist us in diagnosis and managing these cases. In this study, the study was aimed to use of Real-Time PCR technique as molecular method for direct detection HAdVs from urine samples of hemorrhagic cystic patients [11]. The Real-Time PCR assay, applicable to clinical specimens, was developed that could detect all known serotypes of human adenovirus with high sensitivity and allow for serotype determination by sequencing analysis. [13] PCR showed a decreased time to detection and an increased sensitivity compared to culture. Culture has been considered the “gold standard” for laboratory diagnosis of AdV; however, detectable replication typically requires 3 days to 3 weeks, varying with the specimen source and with the concentration of virus in the specimen. Furthermore, culture requires viable virus [6]. In the present study, all negative bacterial culture growth 68 specimen bloody urine and 20 control from healthy person were also tested by PCR to screen for asymptomatic shedding of adenovirus. The results of present study showed that a well characterized set of primers that amplify part of the hexon gene was used. In study done by [7] who said that Initially, when urine samples were tested by PCR without extraction, sensitivity was very low, suggesting that many urine samples contained inhibitory substances. When methods to purify DNA from such sources were tested, sensitivity markedly increased. The main reasons for introduction of molecular diagnostics are rapidity and the possibility to detect uncultivable or difficult-to-culture viruses [14]. Also in the field of viral cystitis, more molecular diagnostic assays have been introduced for detection of the causative viruses. [8]. The present study agreement with [15] who said, the value measured during real-time PCR analysis was used as an indicator of the viral load found in the urine sample. All patients with positive PCR for adenovirus had mainly hematuria symptoms with hemorrhagic cystitis ranging from mild to severe hematuria.

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