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Adenovirus Infection in a Sample of Iraqi Kidney Transplant Recipients: Molecular and Hematological Study

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Abstract

Background Human Adenovirus (ADV) is one of highly prevalent viruses worldwide, after primary infection, it remains latent and then might reactivate in immunocompromised patients. High ADV viremia seen in renal transplant recipients (RTR) with clinical presentations range from asymptomatic viremia to respiratory and gastrointestinal disease hemorrhagic cystitis, graft dysfunction and severe disseminated disease.

Objective The objectives of this study are to determine the rate of occurrence of ADV viremia by quantitative Real time PCR (QRT-PCR) in RTR and correlate them with urine cytology results, renal function tests and patients' hematological parameters.

Methods Seventy-one renal transplant recipients (RTR) were enrolled in this study. Whole blood samples (3 ml) divided into two parts, one part for complete blood picture and differential count and other part from which plasma separated and subjected to viral DNA extraction and then ADV Taqman QRT-PCR analysis for viral load measurement. Five ml urine specimens were collected for Pap-stained urine cytology.

Results Out of 71 RTR, 15 (21.12%) had positive ADV viremia by QRT-PCR, with a mean viral load 4.0 $x10^7$± 1.9 $x10^8$ copies/ml, and 80% (12 out of the 15) of positive viremia patients aged more than 40 years (p=0.011). All of RTRs 15/15 (100%) had symptomatic urinary tract infection (UTI) (p=0.039), and 5 out of 9 patients who had lymphopenia had positive viremia (p=0.007). Pap-stained urine cytology smears showed that 39/71 (55.71%) of the RTRs had positive decoy cells (DC), but there was no significant correlation between ADV viremia and the presence of DC (p=0.107).

Conclusion The present study showed the prevalence of ADV viremia in RTRs, with very high viral load, which is associated with lymphopenia and overt clinical features, this suggests that ADV might be an important cause of morbidity in RTRs.

Keywords Adenovirus, renal transplantation, real-time PCR, urine cytology

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List of abbreviations: ADV = Adenoviruses, CYC = Cyclosporine, DC = decoy cells, IS = Immunosuppressive drugs, MMF = Mycophenolate, QRT-PCR = Quantitative real time polymerase chain reaction, RTR = Renal transplant recipients, TAC = Tacrolimus, UTI = urinary tract infection

Introduction

Since first isolation from adenoid tissue over 60 years ago, human adenoviruses (ADV) belonging to Adenoviridae family, and the Mast adenovirus genus that have made continuous challenges in different clinical manifestations. Adenoviruses are divided into seven species, from A through G [1-3]. It has been reported more than 67 ADV types according to the gene bank for human adenovirus genotype classification [4]. Human ADVs known to cause a number of clinical manifestations, including keratoconjunctivitis, gastroenteritis, hemorrhagic cystitis, and

common cause of upper and lower respiratory tract infections, that produce in vitro cytolysis in these tissues [5].

Adenoviruses play an important role in patients with impaired immune responses, in whom viral diseases cause high morbidity and mortality. In the solid organ transplant recipients (SOT), the primary site of ADV disease is mainly related to the transplanted organ. Some of the clinical presentations occur in lung, liver, renal, and small bowel transplantations including pneumonia, hepatitis, nephritis, hemorrhagic cystitis, enteritis, and less commonly disseminated disease [6].

In renal transplant recipients (RTRs), the most common presentation is acute hemorrhagic cystitis and, to a lesser extent, pneumonia, with a 17% fatality rate [7]. Adenoviruses are included in the pathogens responsible for infections in the immediate post-transplantation period (PTP) as either primary infection or reactivation of a previous infection [8], in less than 2% of ADV cases, which presents as pneumonitis, nephritis, diarrhea, and hemorrhagic colitis or cystitis, the infection can become generalized and cause multiple organ failure [9,10].

Adenovirus infection was shown earlier after kidney transplantation, correlated with low absolute lymphocyte counts, and such patients develop more severe complications and progressive disease, in whom lymphocyte count can be used as a predictor of adenovirus disease and patient outcome [11]. In addition, ADV infection in kidney transplantation could be predicted when there is decoy cells (DC) in Pap-stained urine cytology but less commonly than polyomaviruses [12-14].

Progress in molecular detection methods especially quantitative real time PCR (QRT-PCR) has made the detection and monitoring of adenovirus diseases easily applicable in the clinical practice, in addition real time PCR is available for assessment of risk and accurate rapid diagnosis of invasive ADV infection [15].

In Iraq, several studies had been conducted on viral infections in RTRs, including human cytomegalovirus, BK and JC polyomaviruses, Epstein Barr virus, Human Herpes virus-6 and Parvovirus B19 [16-22]. However, to the best of our knowledge, this study is the first to investigate the prevalence of ADV viremia in Iraqi RTRs using QRT-PCR.

The objectives of the present study are detection of ADV viremia in kidney-transplanted patients by QRT-PCR, Screening of Pap-stained urine cytology smears for viral inclusions, correlate ADV viremia with patients' clinical presentation and renal function tests, and study the relation between the level of ADV viremia and patients' hematological parameters.

Methods

Study Population and Sampling

Cross sectional study conducted from November 2016 to April 2017, seventy-one RTRs were collected from the (Center of Kidney Diseases and Transplantation) in the Medical City of Baghdad. A consent letter obtained from all patients enrolled in the study. This study approved by the ethical committee of the College of Medicine-Al-Nahrain University. The study was conducted in the Microbiology Department at the College of Medicine-Al-Nahrain University.

Clinical parameters (immunosuppressive regimens, acute rejection episodes, transplant renal function, any signs and symptoms, and late complications) obtained from patient's medical records. Two main Standard immunosuppressive regimens were mainly followed in RTRs; either the cyclosporine (CYC), mycophenolate (MMF), and prednisolone, or the regimen that included tacrolimus (TAC) instead of CYC, in addition to MMF and prednisolone. And induction with monoclonal anti-CD25 antibodies (Basilixibam/Daclizumab)

From all 71 RTRs, three ml blood samples collected and divided in to two parts, one part for complete blood picture and differential lymphocytes count, and other part from which

plasma separated and subjected to viral DNA extraction and then ADV Taqman quantitative Real time PCR analysis for viral load measurement. Five ml urine specimens collected and preserved in 95% ethanol 1:1 for Pap-stained urine cytology smear.

Viral DNA Extraction

For viral DNA extraction from the blood samples; Geneius™ Viral Nucleic Acid Extraction Kit III (Geneaid, England) was used. One ml plasma sample used in viral DNA extraction, according to the manufacturer protocol.

Real Time PCR for Measuring ADV Viremia

For the quantitative detection of ADV; Adenovirus [R-gene®Ref.:69-010B France] is a Real-Time test, which is based on the principle of the so-called- "TaqMan" probe. Fifteen µl of Master Mix added into PCR tubes, and 10 µl of the (sample DNA, sensitivity controls, or standards) were added to the master mix. The final reaction volume was 25µl. All components were kept at +2 °C to +8 °C during the PCR preparation. Real time PCR instrument used in this work was7500 Real Time PCR System Applied biosystems (USA). The thermal protocol for Adenovirus R-gene® PCR kit is composed of a two hold steps, a one amplification cycle. The real-time data is collected at the third step of the amplification cycle. The size of the amplified fragment is 138 bp and is located in the Hexon gene coding for hexagonal capsomeres which form the sub-units of the adenovirus capsid protein. An Internal Standard (IC2) is included in the reaction mix controlling the possible inhibition of the PCR reaction. IC2 positive amplification is detected in the HEX fluorophore fluorescence channel. A range of 4 quantification standards is provided with the ADENOVIRUS Rgene®kit ranged from 5000 copies/µl to 5 copies/µl. These quantification standards used to generate a new standard curve in the software provided with the thermocycler. The quantification of Adenovirus genome in unknown samples is extrapolated from this standard curve. At the end of the thermal protocol, the 7500 Real Time PCR System Applied biosystems instrument software automatically calculates the baseline cycles and the threshold. The standard curve is plotted using the data obtained from the defined standards, with the (Y) axis is the Ct-Threshold Cycle, and the (X) axis is the viral DNA copy number. According to the manufacturer instructions, ADV DNA copies was calculated according to the following formula [23]:

$$\text{copy/ml} = \frac{\text{SC x EV}}{\text{IV}}$$

SC = Sample Concentration (copy/µL)
EV = Elution Volume (µl)
IV = Isolation Volume (ml)

Results

Among these 71 RTR; 49 (69.01%) were males, and 22 (30.98%) were females; their mean age was 37.06±12.73 years, ranging between 18 and 63 years, and the mean Post-Transplantation Period (PTP) was 10.44±5.54 months. The mean body weight of the RTRs was 75.54±8.5 Kg, while their mean serum creatinine value was 1.16±0.28, and the mean of their creatinine clearance was 92.08±20.58, and among all the RTRs; 9/71 (12.6%) had lymphopenia. All the RTRs received their allografts from living donors, and out of the 71 RTRs; 32 (45.07%) received their allograft kidney from living related donors, while the remaining 39 (54.93%) received their kidney allograft from living unrelated donors. Regarding the type of immunosuppression, 37/71 (52.11%) were on (CYC) and the reminder 34/71 (47.88%) were on (TAC) regimen.

Depending on the patient's files and questioner at the time of collection, the majority RTRs enrolled in this study had clinical presentations

and complications at the time of sampling, symptomatic urinary tract infections (UTI) were the highest among these presentations as shown in the table (1). Out of the 71 RTRs 40 (56.33%) were hypertensive and 10 (14.08%) were diabetic

Table 1. Clinical presentations of RTRs at the time of sampling

Clinical presentation	No. (%) out of 71 RTRs
UTI	58 (81.69%)
Respiratory disease	39 (54.92%)
Gastroenteritis	36 (50.70%)
Hematuria	23 (32.39%)
Eye infection	15 (21.12%)

Urine cytology smears were Papanicolaou-stained and microscopic examination showed that 39/71 (55.71%) of the RTRs had positive Decoy cells. Figure (1) shows various types of decoy cells among RTRs.

QRT-PCR for detection and quantitation of ADV (a hexon gene which is common to all adenoviral species) in plasma sample (viremia), was positive in 15 out of 71 (21.12%) RTRs. The mean of ADV Viremia was $4.0 \times 10^7 \pm 1.9 \times 10^8$ copies/ml. The standard curve of QRT-PCR included the four quantification standards ranged from 5000 copies/µl to 5 copies/µl. Statistical data in table (2) demonstrated that 80% (12 out of the 15) patients who had positive viremia aged more than 40 years which is statistically significant (p=0.011). There is no significant difference in gender, nevertheless, 9 out of 15 (60%) positive ADV viremia patients were males, while females were 6 out of 15 (40%) ADV positive cases (p=0.395).This study found no significant association between positive viremia and gender type, transplantation period, serum creatinine, creatinine clearance test, and the type of immunosuppression. Regarding the association between ADV viremia and clinical presentations all of RTRs 15/15 (100%) who had positive ADV viremia; had symptomatic UTI, which was statistically significant (p=0.039). Although there were no significant association between ADV viremia and other clinical presentations, however, about 30%, 25% and 23% of those patients who had hematuria, gastroenteritis, and respiratory infections had positive adenovirus viremia, respectively. Pap-stained urine cytology results showed no significant correlation between ADV viremia and the presence of decoy cells (DC), but 73.3% (11 out of 15 positive viremia cases) had positive DCs in urine. On the other hand, (55.6%) 5 out of 9 patients who had lymphopenia (lymphocytes count in the blood below 1.0X109) had positive ADV in plasma, which was statistically significant (p=0.007). In addition, there was a significant association between the value of viral load and lymphopenia in RTRs (p=0.023).

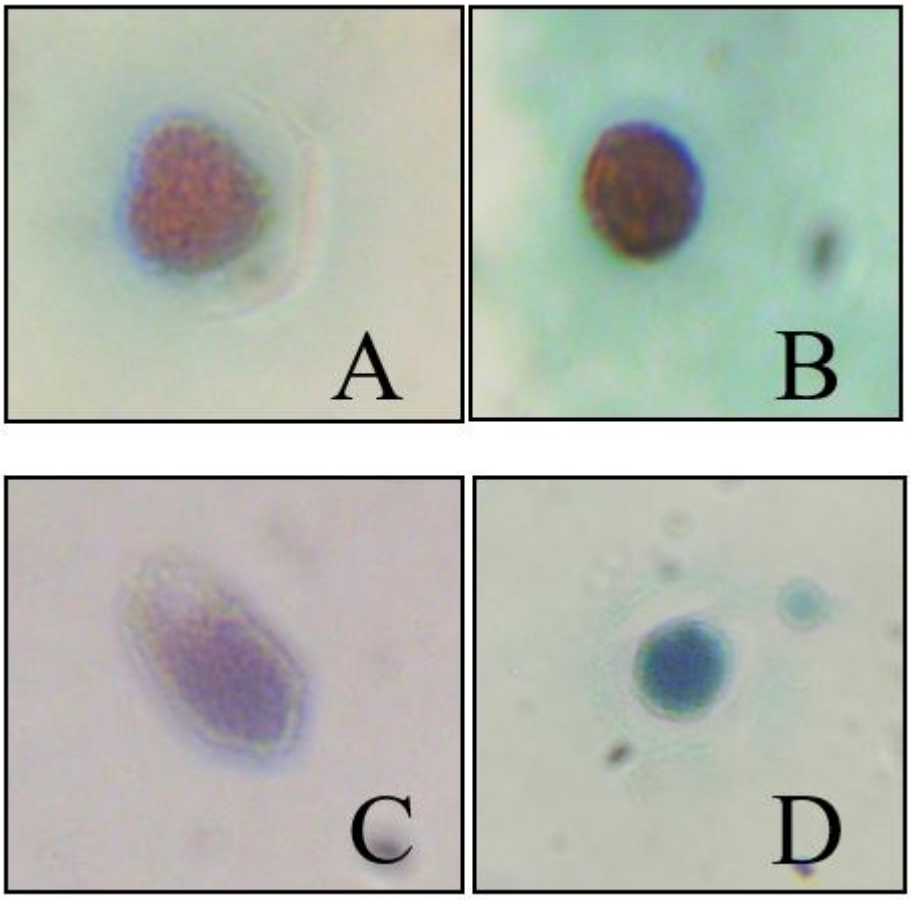

Figure 1. Pap-stained urine cytology smears demonstrate: (A and B) Common ground glass virus-infected (decoy cells (DC)). (C) Common comet-shaped DC. (D) Uncommon clumped variant DC. Magnification power for A (100X), B, C, D (40X)

Discussion

In this study, ADV was investigated in the plasma sample of RTRs using QRT-PCR, and the results showed that more than one-fifth of the RTRs (15 out of 71 (21.12%) had positive ADV viremia in plasma, this frequency is in the range of other studies, that spam between 5 to 22% [24,25]. These results were significantly correlated with RTRs who had low lymphocytes count (lymphopenia). ADV infection in RTRs may be a consequence of a primary infection, reactivation of latent infection or acquired through donor organs, and it is believed that majority of the cases are due to reactivation of latent infection [26]. The mean of ADV viremia was 4.0 $x10^7$ ± 1.9 $x10^8$ copies/ml, which is a very high viral load associated with wide range of clinical manifestations, in which 30%, 25% and 23% of those patients who had hematuria, gastroenteritis, and respiratory infections had positive ADV viremia, respectively. These results are in line with other previous studies on ADV infection in RTRs that showed high mean viral load in immunocompromised patients [27-29]. In immunocompromised patients, the immune escape and persistence of ADV mediated by different mechanisms. Specific viral proteins can block responses to anti-inflammatory and cytolytic cytokines, intrinsic cellular apoptosis, and innate and adaptive cellular immune responses [30]. Moreover, the viral protein E3 can down regulate major histocompatibility complex (MHC) class I molecules, there by affecting antigen presentation and reducing T-cell attack of the infected cells [31,32].

Table 2. Comparisons of the patients with positive ADV viremia and negative viremia including transplants' demographic, clinical and laboratory data

		Positive ADV	Negative ADV	Total	p value
Age	< 40 years	3 (8.6)	32 (91.4)	35	0.011
	≥ 40 years	12 (33.3)	24 (66.7)	36	
Gender	Male	9 (18.4)	40 (81.6)	49	0.395
	Female	6 (27.3)	16 (72.7)	22	
PTP	≤ 1 year	9 (19.1)	38 (80.9)	47	0.568
	> 1 year	6 (25.0)	18 (75.0)	24	
Relatedness	Related	7 (21.9)	25 (78.1)	32	0.980
	Unrelated	8 (21.6)	29 (78.4)	37	
IS Drugs	CYC	9 (24.3)	28 (75.7)	37	0.491
	TAC	6 (17.6)	28 (82.4)	34	
Rejection	No	15 (22.1)	53 (77.9)	68	0.360
	Yes	0 (0.0)	3 (100)	3	
Frequency of transplantation	Once	15 (22.1)	53 (77.9)	68	0.360
	Twice	0 (0.0)	3 (100)	3	
Respiratory Infection	Yes	9 (23.1)	30 (76.9)	39	0.657
	No	6 (18.8)	26 (81.3)	32	
Gastroenteritis	Yes	9 (25.0)	27 (75)	36	0.417
	No	6 (17.1)	29 (82.9)	35	
Eye Infection	Yes	1 (6.7)	14 (93.3)	15	0.122
	No	14 (25.0)	42 (75)	56	
UTI	Yes	15 (25.9)	43 (74.1)	58	0.039
	No	0 (0.0)	13 (100)	13	
Hematuria	Yes	7 (30.4)	16 (69.6)	23	0.184
	No	8 (16.7)	40 (83.3)	48	
Hypertension	Yes	9 (22.5)	31 (77.5)	40	0.747
	No	6 (19.4)	25 (80.6)	31	
Diabetes	Yes	3 (30.0)	7 (70.0)	10	0.458
	No	12 (19.7)	49 (80.3)	61	
Urine cytology	Yes	11 (28.2)	28 (71.8)	39	0.107
	No	4 (12.5)	28 (87.5)	32	
Lymphopenia	Yes	5 (55.6)	4 (44.4)	9	0.007
	No	10 (16.1)	52 (83.9)	62	
Creatinine Clearance	Abnormal	4 (20.0)	16 (80.0)	20	0.884
	Normal	11 (21.6)	40 (78.4)	51	
Serum Creatinine	Normal	9 (19.1)	38 (80.9)	47	0.568
	Abnormal	6 (25.0)	18 (75.0)	24	

PTP: Post-transplantation period, IS: Immunosuppressive, CYC: Cyclosporine, TAC: Tacrolimus, UTI: Urinary tract infection

In addition, studies found this high viral load is associated with many manifestations like pyelonephritis [33,34], hemorrhagic cystitis [35,36], respiratory infections [37-39], and GIT infections [40-42]. However, it should be noted that the high number of patients with upper and lower respiratory tract infections could be mainly because the time of collecting the patients in

this study, which was mainly during the winter season (November to April).

The present study found that 7/15 (46.6%) of RTRs who had positive ADV viremia had hematuria as clinical symptom during collecting the sample this is supported by Hofland et al. 2004, [43] who reviewed 37 cases of ADV hemorrhagic cystitis in kidney transplant recipients, and showed that in RTRs, the most common manifestation is hemorrhagic cystitis. Also, the current study had significant association between ADV viremia and UTI that could be supported by other studies, which found a correlation between adenovirus infection and cystitis and pyelonephritis, with white cell casts in urine [34,44]. Kolankiewicz et al. in 2010 [34] published a case report and a review of the literature in which the patients commonly presented with gross hematuria and dysuria (10/11), fever (9/11), and acute renal failure (9/11), and 27% of the patients had significant graft function impairment after adenoviral nephritis. Rady et al. in 2014 [44] described a case of necrotizing tubulointerstitial allograft nephritis due to adenovirus infection, a significant proportion of patients presented within 8 months of transplant with gross hematuria, dysuria, fever and acute renal failure.

Although there was no statistically significant association of ADV viremia with respiratory infection, nevertheless, 9 out of 15 ADV positive patients had respiratory disease, which was also showed in other studies Watanabe et al. in 2013, in which all patients enrolled in the study had acute respiratory tract infections [37]. Respiratory tract disease ranged from mild upper-tract involvement (URI), typically presenting nonspecific cold like symptoms, to severe pneumonia [39] which agrees with the patients' presentations in this study. In addition, this study found that 9 out of 15 ADV positive patients had gastrointestinal symptoms, mostly diarrhea, although there was no statistically significant association between ADV viremia and GIT disease, another study also showed that GIT infection occurred in RTRs, symptoms ranged from mild diarrhea to hemorrhagic colitis [32].

Results of the current study found a statistically significant association between ADV viremia and low lymphocytes count (lymphopenia) 5 out of 15 (33.3%) (p=0.007) which is supported by the study of Watcharananan et al. in 2011 who showed that early ADV infection appeared to have significantly lower lymphocytes count at several time points compared to those with late infection [11]. This finding underscores the influence of immune recovery during the course of infection ([45,46]. Heemskerk et al. in 2005 found exogenous lymphocyte therapy in the form of donor lymphocyte infusions has been successful in some cases [46]. Cohort of retrospective study of Kim et al. in 2015, found that a low absolute lymphocyte count within 3 months in allogeneic transplantation recipients was significantly associated with poor overall survival, progression-free survival, and mortality [47]. Ison in 2006 [5] demonstrated an association between lymphocyte recovery and recovery from adenoviral infections in RTRs.

On the other hand, there was no significant association between ADV infection and the level of serum creatinine and creatinine clearance (CrCl), a result might exclude the possibility of an associated renal impairment with adenoviremia, and this result is supported by other studies in RTRs that excluded the role of ADV in renal impairment or rejection [48]. Nanmoku et al. in 2016 [49] also showed there is no significant difference was seen before, during, or after disease onset of ADV infection. However, most of the studies reported sporadic cases of renal allograft impairment associated with ADV viremia [13,44,50].

Although the results of present study showed no significant association between ADV viremia and urine cytology findings, however, (73,3%) 11 out of these 15 ADV positive cases had positive decoy cells in Pap-stained urine cytology smears. A result can support the high association of ADV viremia with different urinary, gastrointestinal and respiratory complaints in these RTRs. Studies showed that although it is rare, but adenovirus is one of the viruses that are associated with urinary decoy cells shedding [5]. Viral cytopathic changes (decoy cells), which are typically associated with polyomavirus infection, have been

reported in the urine of RTRs with ADV infection [51,52]. Storsley et al. in 2011 found that four patients demonstrated decoy cells in their urine over the course of a few months, during which time the urine culture and PCR was positive for ADV while BKV virus was negative [53]. Surveillance studies of asymptomatic adult RTRs have shown an incidence of adenoviral viremia by PCR testing of 6.5% and viruria by 11% [5,54]. Asymptomatic viral shedding in the urine makes urinary cultures unreliable in the absence of signs and symptoms of disease activity. The presence of white cell casts with decoy cells on urinalysis may increase the suspicion for ADV infection [54].

In our transplantation center in Baghdad, there is no protocol biopsy for RTRs, and this makes the exact diagnosis of renal pathology extremely difficult. The presence of high adenoviral load in plasma, with high decoy cells shedding, and the significant association of viremia with lymphopenia (both qualitatively and quantitatively) (p=0.007, and 0.023) respectively, all these give strong association between this virus and the patients' clinical manifestations. However, other infections should be excluded.

In conclusion, ADV infection is an important cause of many diseases in RTRs, which is evident by high frequency of this virus with high viral load in these RTRs.

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Authors' contribution

Ahmed: Collection of specimens, DNA extraction, and real time-PCR, writing of the references. Dr. Hussein: Consultant Nephrologist help in providing all patients' data and in selection of patients. Dr. Al-Obaidi: Supervision and performance of viral DNA extraction and real time-PCR run, writing of the manuscript. Dr. Kadhim: Final editing of the manuscript. Dr. Ghazi: Statistical analysis.

Conflict of interest

Authors declare no conflict of interest.

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