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ORIGINAL ARTICLE

Detection of the presence of Epstein–Barr virus and cytomegalovirus using polymerase chain reaction in patients with chronic kidney disease

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Abstract:

Background: Chronic kidney disease (CKD) is an advanced and irreversible renal failure that contributes to increased mortality worldwide. Patients who undergo dialysis are more susceptible to developing infections due to their general condition. Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) are associated with oral and systemic manifestations. The most modern and accurate technique to detect the presence of these viruses is polymerase chain reaction (PCR), which can predict viral reactivation even before the onset of symptoms and identify subclinical infections. This study aimed to detect the presence of Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) in patients with chronic kidney disease (CKD) undergoing hemodialysis using real-time polymerase chain reaction (PCR). **Methods:** An epidemiological and observational case-control study was conducted. Two groups were considered (case group: patients on hemodialysis from the Nephrology Services of the Hospital das Clínicas of UFPE and Hospital Maria Lucinda and control group: healthy patients without CKD from the Department of Stomatology of the UFPE to evaluate the viruses presence using the real-time PCR technique. **Results:** Of the hemodialysis patients (n=54), 29 (53.70%) tested positive for CMV and only 10 (18.51%) for EBV, while in the healthy group (n=55), eight (14.54%) were positive for EBV and none for CMV. In neither group were systemic characteristics or oral manifestations observed due to the presence of viruses. **Conclusion:** The EBV and CMV viruses have a higher prevalence in hemodialysis patients. However, these patients did not present oral or systemic manifestations.

Keywords: Infections; Oral Manifestations; Renal dialysis; Real-Time Polymerase Chain Reaction; Viruses.

INTRODUCTION

Chronic kidney disease (CKD) occurs due to structural and/or functional kidney alterations that cause a decrease in the glomerular filtration rate. As the disease progresses, there is a greater accumulation of toxic metabolites, triggering uremia, which, if left untreated, can lead to death. Therefore, these patients require substitutive therapies to ensure their survival, among which hemodialysis is the most common treatment[1,2].

The dialyzer is the main equipment in dialysis, in which the exchange of substances between the blood and dialysis fluid occurs, ultrafiltration and retrofiltration[3]. Despite significant technological advances, this method is still not completely free of complications. Contamination may occur during the reuse of dialyzers, exposing patients to the risk of blood infections[4].

Uremia or uremic syndrome is a term used to describe abnormalities that occur in renal failure. It comprises a clinical triad of thrombocytopenia, anemia, and acute kidney injury. It can be diagnosed when the renal glomerular filtration rate is ≤15 ml/min[5,6]. Compromise of the body causes a failure of the immune system, making patients more prone to infection, and can also mask the signs and symptoms of infection, further aggravating their clinical condition.

Herpesviruses, especially Cytomegalovirus (CMV) and Epstein-Barr virus (EBV), are responsible for frequent clinical infections and dysfunction of transplanted organs. These viruses have characteristics that allow them to escape immune control in the replication and latency phases to establish infection and dissemination in immunocompromised people[7]. Complications arising from immunosuppression may be responsible for the most significant morbidity and mortality observed after organ transplantation[8].

EBV is generally an asymptomatic infection in childhood and typically manifests itself as symptoms of infectious mononucleosis in late primary infection[9]. It is associated with various malignancies, including post-transplant lymphoproliferative disorder, nasopharyngeal carcinoma, Hodgkin's lymphoma, and non-Hodgkin's lymphoma. Studies have also shown the presence of the virus in oral lesions such as gum inflammation, severe epithelial dysplasia, and oral squamous cell carcinoma [10]. CMV remains the most common infection affecting patients undergoing organ transplantation. Maintenance of this infection complicates the clinical picture of immunologically compromised patients and, in some cases, results in transplant loss and/or patient death[11]. In the oral region, CMV has been recognized to cause lesions, usually in the form of deep aphthous ulcers with painful symptoms that affect the quality of life of the patient[12,13].

A reliable test for viral identification is required to make an accurate diagnosis. In transplant recipients, real-time polymerase chain reaction (PCR) viral load testing has been proven to be an excellent modality for EBV and CMV detection[11]. Using whole blood or plasma, viral nucleic acids are extracted, amplified, and compared with known standards[14]. Therefore, the increase in viral load detected by PCR correlates with the probability of the patient developing clinical symptoms of the disease, contributing to both a preventive and therapeutic approach[11].

It is fundamental to monitor the presence of these viruses in hemodialysis patients, as being the main candidates for transplantation, they also present an immunocompromise resulting from uremic syndrome and can develop serious aggravation due to this viremia. Therefore, the objective of this study was to detect the presence of EBV and CMV viruses in patients with CKD undergoing hemodialysis using real-time PCR.

MATERIALS AND METHODS

Study design

An epidemiological and observational case-control study was conducted between March and November 2016. This study was approved by the Research Ethics Committee of the Universidade Federal de Pernambuco (opinion number: 1,445,608). Two groups were considered: hemodialysis patients (cases) and systemically healthy individuals (control). Patients of both sexes and aged >18 years were included in the study.

The patients in the case group came from the Nephrology Services of Hospital das Clínicas da Universidade Federal de Pernambuco (HC-UFPE) and Hospital Maria Lucinda. All hemodialysis patients were invited to participate at the time of the visit. The control group included patients who declared themselves systemically healthy and came to the Stomatology Clinic of UFPE for their first dental visit for evaluation, pairing the groups in relation to sex and age.

An informed consent form was obtained from all patients; they filled out information regarding their identification, medical data, and current disease history. Then, a researcher trained in phlebotomy collected 3 ml of blood from each patient, which was stored in a freezer at -20°C until DNA extraction. The equipment used for RT-qPCR was QuantStudio™ 5 Real-Time PCR or Applied Biosystems 7500 Real-Time PCR.

Oral examination

The patients were evaluated through intra and extra oral exams, besides the anamnesis, by a trained team, with undergraduate and graduate students and professors. If the patient presented a lesion, details such as its color, consistency, location, symptomatology, size, and period of evolution were noted.

Isolation of DNA

DNA was extracted from whole blood using a Wizard Genomic DNA Purification Wizard Extraction Kit (Promega Bio Sciences, LLC. San Luis Obispo, CA, USA), according to the manufacturer's recommendations, was made from 300 µl of whole blood, and the final product was quantified using a NanoDrop-2000 (Thermo Fisher Scientific, Wilmington, USA).

Amplification of CMV- and EBV-related genes

The gene sequence corresponding to the catalytic subunit of DNA polymerase (pol) present in the CMV genome (human herpesvirus 5 strain NANU complete genome) available in GenBank (KU550090.1) was used as a template for the design of two new specific oligonucleotides that flank a conserved region of CMV pol that includes a stretch of TaqMan primers and probes ringing, as shown in Table 1. For amplification of the pol region, the primers: 1-P1F CMVpolcloning (5'-GCCAGCCTCTATCCTTCCATCATCATC-3') and P1R CMVpolcloning (5'-CGCTGTCCGTCCGTAGATG-3') were used, and the PCR product had a fragment of 586 bp.

Table 1. Oligonucleotides designed from the genome of human herpes virus *5 strain NANU gb KU550090.1* specific for the CMV polymerase gene.

Oligonucleotides	Sequence (5' – 3')
PF CMV*po*/TaqMan	5'- CATGCGCGAGTGTCAAGAC -3'
PR CMV*po*/TaqMan	5'- ACTTTGAGTGCCATCTGTTCCT -3'
TaqMan probe	5'-TGCGCCGTATGCTGCTCGACA-3'

The gene sequence corresponding to the 177,320 bp EBV genome (human herpesvirus 4 DNA, complete genome strain: YCCEL1) available in GenBank: AP015016.1 (www.ncbi.nlm.nih.gov) was used as the template for the design of two new specific oligonucleotides that flank a 404 bp region that includes a previously designed TaqMan primer and probe annealing section, as shown in Table 2.

Table 2. Specific oligonucleotides designed from the genome of human herpesvirus 4 strain.

Oligonucleotides	Sequence (5' – 3')
PF EBVTaqMan	5'- CCCAACACTCCACCACACC -3'
PR EBVTaqMan	5'- TCTTAGGAGCTGTCCGAGGG -3'
EBV TaqMan probe	5'- ACACTACACACACCCACC -3'
P1F EBVcloning	5'- CAAATGTAAGAGGGGGTCTTC-3'
P1R EBVcloning	5'- CTCCTCTTCTTGCTGGACG-3'

The PCR reaction was performed in a final volume of 25 ml containing 0.4 mM of each dNTP, 1.6 mM of each primer related to EBV and CMV (Tables 1 and 2), 2.5 U of Platinum Taq DNA Polymerase (Invitrogen), buffer containing 20 mM Tris-HCl (pH, 8.4) or 50 mM KCl, 2 mM $MgCl_2$, and 100 ng of template DNA extracted from a positive CMV and EBV sample. The reaction was performed with a BIOMETRA thermal cycler programmed for denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 50 s, 52°C for 50 s, and 72°C for 120 s, and a final step at 72°C for 10 min. The amplified products were analyzed with 1% agarose gel electrophoresis in 0.05X TBE buffer (8.9 mM Tris; 8.9 mM boric acid, 2 mM EDTA), stained with ethidium bromide, and visualized using an ultraviolet light transilluminator. The amplified gene fragments were removed from the gel and purified with the "GFX DNA and Gel Band Purification" kit (GE Healthcare) before the cloning step.

Cloning and sequencing of the CMV and EBV genes in pGEMTeasy

The PCR products of the CMV and EBV genes were excised and purified from agarose gel, which was subjected to an A-tailed treatment step. The reaction was carried out in a final volume of 10 ul containing 5 U of Platinum Taq DNA polymerase (Invitrogen), buffer containing 20 mM Tris-HCl (pH 8.4) or 50 mM KCl, 2 mM $MgCl_2$, and 0.2 mM of deoxynucleotide dATP and incubated at 70°C in a thermoblock programmed for 30 min. pGEM-T Easy (Promega) was used as a cloning plasmid vector. The combination of plasmid vector/PCR fragments was calculated according to the manufacturer's instructions and treated with T4 DNA ligase (Promega) at 3 U/ul. After the ligation step, plasmid DNA was used to transform chemocompetent bacteria (*Escherichia coli* strain top 10). Colonies were selected in culture plates with ampicillin/IPTG/X-Gal following the instructions in the technical manual of the pGEM-T and pGEM-T Easy Vector Systems Kit and inoculated in 5 ml of Luria-Bertani culture medium for 16 h at 37°C to perform plasmid DNA extraction. Plasmid miniprep preparations were prepared using a QIAprepSpin Miniprep Plasmid DNA Purification Kit (Qiagen). The plasmid DNA preparations were submitted to the ABI Prism 3100 Genetic Analyzer (Applied Biosystems), an automated sequencing reaction, performed at the Technological Platforms Center (NPT) of CPqAM, to evaluate the identity and integrity of the cloned sequences. The obtained plasmid constructs were used as positive standards for RT-PCR to diagnose CMV and EBV.

Determination of the viral load of CMV and EBV

The plasmodium constructs obtained in the above step were used to prepare a standard curve to determine the absolute viral load of CMV. Five points were used in serial dilutions of the standard curve, starting at 106 ng/µL. In RT-PCR, in addition to the primers and probes listed in Table 1, the Universal master MIX TaqMan (Thermo Scientific) was used. The reaction had a final volume of 25 ul (12.5 ul of master mix, 0.9 ul of primers F and R, 0.625 of TaqMan probe, 6 ul of H_2O, and 5 ul of DNA template). The cycling program was as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 58°C for 1 min. Efficiency analysis and concentration calculations were performed using SDS software (scientific terms).

Statistical analysis

After collection, data were tabulated in Microsoft Excel and analyzed with SPSS 20.0 program for descriptive statistical analysis. Continuous variables were compared using non-parametric tests, Mann-Whitney U test to compare two categories and the Kruskal-Wallis test to compare more than two groups. If the chi-square test was unable to be used, the likelihood ratio test was used to compare the proportions. A significance level of 95% ($p<0.05$) was considered statistically significant in all cases.

RESULTS

A total of 109 patients were divided into two groups: control (55) and hemodialysis (54), with a mean age of 46.5±12.8 years (range, 18-73 years).

In the hemodialysis group, 10 patients (18.51%) were positive for EBV, with a mean viral load of 196.4 copies/ml. In the case group, eight patients (14.54%) were positive for EBV, with a mean viral load of 34.7 copies/ml. However, there were no statistically significant differences when the mean (p=0.615) or viral load (p=0.328) between the two groups were compared, as seen in Table 3.

According to Table 4, the hemodialysis group sex (p=0.019) showed a statistically significant result with a higher mean EBV viral load for females (431.6±297.0) compared to males (39.7±54.1). However, this difference was not observed in the control group. Regarding hemodialysis time (p=0.588), no statistically significant differences were observed in relation to the viral load of EBV.

As shown in Table 5, the control group did not present any individuals with viral load for CMV, while 29 patients in the hemodialysis group were positive for CMV, with a mean viral load of 265,282.8 copies/ml. Regarding CMV in the hemodialysis group, no statistically significant differences were observed in relation to the time of hemodialysis (p=0.091) or sex (p=0.425), as shown in Table 6.

DISCUSSION

Hemodialysis patients are known to have a low immune system and a greater chance of developing various oral and systemic alterations. Despite the need to evaluate the presence of some herpes viruses when dialysis patients undergo renal transplantation, this monitoring hardly occurs during the years of dialysis, and little is known about whether the immune deficiency of this patient would influence the increase in viral load compatible with the development of oral alterations related to viruses, such as EBV and CMV, in dialysis patients.

Aiming to detect the presence of EBV and CMV by real-time PCR technique in healthy patients and hemodialysis treatment, the results of this study were statistically significant for CMV, and the prevalence of this virus was higher in the hemodialysis group. However, in relation to EBV, although the hemodialysis group had a higher prevalence and a higher mean viral load than the control group, the results were not statistically significant.

In our study, of the 54 patients in the hemodialysis group, 29 (53.70%) had a viral load for CMV and only 10 (18.51%) for EBV. The higher prevalence of CMV in the hemodialysis group may be due to several blood transfusions to which these patients are submitted or exposed during hemodialysis procedures[15]. A higher prevalence of CMV can also occur in transplant recipients, as observed in the study by Bal et al.[16], which linked the long

Table 3. Distribution of case and control groups according to EBV.

Variables	Group				Total		*p*-value[1]	OR	95% CI	
	Hemodialysis		Control							
	n	%	n	%	n	%				
EBV										
Positive	10	18.5	8	14.5	18	16.5	0,615	1.36	0.48	3.69
Negative	44	81.5	47	85.5	91	83.5				
Total	**54**	**100.0**	**55**	**100.0**	**109**	**100.0**				

[1] Fisher's Exact Test; *Statistically significant (p<0.05).

Table 4. EBV viral load descriptive measures according to the variable sex and treatment time in the hemodialysis group and control group.

Group	Variable	N	Media	SD	Min.	Max.	*p*-value
Hemodialysis	**Sex**						
	M	6	39.7	54.1	1.6	137.5	0,0192
	F	4	431.6	297.0	123.4	834.0	
	Total	10	196.4	268.3	1.6	834.0	
	Treatment time						
	Up to 12 months	3	87.8	74.2	2.5	137.5	0.5881
	Between 13 to 24 months	3	263.8	212.7	22.5	424.4	
	Over 24 months	4	227.4	405.5	1.6	834.0	
	Total	**10**	**196.4**	**268.3**	**1.6**	**834.0**	
Control	**Sex**						
	M	4	22.9	24.9	3.0	59.1	0.1492
	F	4	46.5	30.1	15.8	75.5	
	Total	**8**	**34.7**	**28.5**	**3.0**	**75.5**	

[1-] Kruskal-Wallis non-parametric test; [2-] Mann-Whitney non-parametric test, * Statistically significant (p<0.05); SD=Standard deviation.

Table 5. Descriptive measures of viral load according to viral load.

Group	Viral load	N	Media	SD	Min.	Max.	*p*-value
Viral load EBV	Hemodialysis	10	196.4	268.3	1.6	834.0	0.328
	Control	8	34.7	28.5	3.0	75,5	
Viral load CMV	Hemodialysis	29	265,282.8	41,922.1	214,259.0	396,821.0	

[1-] Mann-Whitney non-parametric test, * Statistically significant (p<0.05); SD=Standard deviation.

Table 6. Descriptive measures of CMV viral load according to the variables sex and treatment time in the hemodialysis group.

Variable	N	Media	SD	Min.	Max.	*p*-value
Hemodialysis time						
Up to 12 months	12	251,953.4	30,449.1	214,259.0	324,644.0	0.0911
Between 13 to 24 months	3	242,228.7	15,991.5	227,705.0	259,366.0	
Over 24 months	14	281,648.1	48,850.6	224,398.0	396,821.0	
Total	29	265,282.8	41,922.1	214,259.0	396,821.0	
Sex						
M	17	273,318.6	49,018.7	222,776.0	396,821.0	0.4252
F	12	253,898.7	27,154.0	214,259.0	308,705.0	
Total	29	265,282.8	41,922.1	214,259.0	396,821.0	

[1-] Kruskal-Wallis non-parametric test; [2-] Mann-Whitney non-parametric test, * Statistically significant (p<0.05); SD=Standard deviation.

duration of dialysis, preoperative induction therapy, and post-transplant regimens with the development of virus infection.

Regarding the oral cavity, the presence of EBV can cause infectious mononucleosis and oral hairy leukoplakia and is associated with several types of lymphoid and epithelial malignancies in the head and neck region [10,17,18]. However, in our study, no patient in the case group had oral lesions or systemic alterations compatible with this virus, which would be consistent with the low viral load found in these patients (range, 1.6-834 copies/ml).

Regarding CMV, the most common type of oral manifestation is deep aphthous ulcer[12]. In our study, the patients did not have oral or systemic lesions characteristic of the disease, probably due to the low viral load for both EBV and CMV.

The severity of many diseases triggered by herpesviruses has been shown to correlate with viral load. Quantifying this load through laboratory tests, such as real-time PCR, is extremely useful for studying the presence of a virus and the role of viral reactivation or persistence in disease progression. Therefore, quantitative CMV and EBV load tests are important for diagnosis, infection management, and antiviral therapies[19].

Another widely used test to detect viral infections is the serological test (IgG and IgM)[20,21]. Enan et al.[22] reported that due to the frequent reactivation of the virus and the increase or decrease in antibody levels, this test would not support a true diagnosis of infection in immunosuppressed patient populations. Therefore, serology has a limited diagnostic value in transplant patients. In the above study[22], CMV was detected by real-time PCR in 32/98 patients (32.7%), a higher rate than the IgM result of 6.1%. This considerable variation between tests is due to the higher sensitivity and specificity of real-time PCR for detecting viral DNA compared to serological tests.

Real-time PCR testing allows for less labor-intensive and more sensitive monitoring of viral loads, improving accuracy, reducing the time needed for amplification and quantification of viral load, and demonstrating efficiency in detecting infections among transplant recipients[19,23].

In the present study, the mean viral load was 196.4 copies/ml for EBV and 265,282.8 copies/ml for CMV in the hemodialysis group, and the viral loads were considered low according to the detection limit used in our study. The results of our study are consistent with the study by Enan et al.[10], which considered the presence of CMV in viral loads as low (23,076 copies/ml), with the absence of clinical symptoms of the disease.

Although we obtained a higher presence of EBV and CMV in hemodialysis patients compared to the control group, the study did not show a great variation in the mean viral load in each group, and was considered low for both groups studied, not characterization of systemic disease and not even oral lesions compatible with both viruses. Future studies with a larger number of participants are necessary to reaffirm the evidence presented in the literature, which confirms the need to detect these viruses in patients with chronic renal failure.

Through its analyses, the present study verified a higher prevalence of EBV and CMV in hemodialysis patients, although they did not present with systemic and oral manifestations characteristic of viral diseases. This factor is consistent with the viral load, which is considered low for both viruses.

CONFLICTS OF INTEREST

None.

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