

Epstein Barr virus genotypes (EBV1/EBV2) in individuals with infectious mononucleosis in the metropolitan area of Belém, Brazil, between 2005 and 2016

Talita Antonia Furtado Monteiro (✉ talitamonteiro859@gmail.com)

Instituto Evandro Chagas <https://orcid.org/0000-0002-2009-4062>

Talita Antonia Furtado Monteiro

Instituto Evandro Chagas

Iran Barros Costa

Instituto Evandro Chagas

Igor Brasil Costa

Instituto Evandro Chagas

Thais Letícia dos Santos Corrêa

Instituto Federal de Educacao Ciencia e Tecnologia do Parana

Beatriz Monteiro Rodrigues Coelho

Escola Superior da Amazonia

Alessandra Alves Polaro

Instituto Evandro Chagas

Amanda Emanuelle Santos da Silva

Instituto Evandro Chagas

Francisco Lúzio de Paula Ramos

Instituto Evandro Chagas

Arnaldo Jorge Martins Filho

Instituto Evandro Chagas

Jose Luiz Furtado Monteiro

Centro Universitario do Estado do Para

Rita Catarina Medeiros Sousa

Universidade Federal do Para

Research article

Keywords: EBNA 3C gene, EBV1, EBV2, EBV1+EBV2, infectious mononucleosis

Posted Date: March 9th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-16300/v1>

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Version of Record: A version of this preprint was published at The Brazilian Journal of Infectious Diseases on July 1st, 2020. See the published version at <https://doi.org/10.1016/j.bjid.2020.06.004>.

Abstract

Background: Two types of Epstein Barr virus (EBV1/EBV2) have been shown to infect humans by causing infectious mononucleosis, their genome being very similar, except for regions of the EBNA genes. This study aimed to describe the EBV genotypes in cases of infectious mononucleosis in the metropolitan region of Belém, Brazil, from 2005 to 2016.

Methods: A total of 8.295 suspected cases with symptoms/signs of infectious mononucleosis (MI) were investigated by doctors of infectious diseases at the Evandro Chagas Institute Health Care Service from January 2005 to December 2016. In 3.0% (251/8.295) of the cases were positive by enzymatic immunoassay were submitted to PCR for EBNA3C region to detect the types of EBV. Biochemical testing involving aspartate aminotransferase, alanine aminotransferase and gamma-glutamyl transferase were realized.

Results: The identification of EBV types by PCR was verified in 30.3% (76/251) of individuals, being 71.1% (54/76) classified as EBV1, 17.1% (13/76) as EBV2 and 11.8% (9/76) as EBV1+EBV2. The number of women infected with EBV1 was higher (61.1% - 33/54) than for men (38.9% - 21/54), most were over 14 years old (66.7%-36/54). The main symptoms/clinical signs observed in EBV1 infection were: cervical lymphadenopathy (64.8%-35/54), fever (63%-34/54), headache (20.3%-11/54), arthralgia (20.3%-11/54) and exanthema (18.5%-10/54). In EBV2 infection, it was also detected in all age groups, with the exception of two groups, with an average age of 24 years. The presence of fever in 76.9% (10/13) with an average duration of 18 days and lymphadenopathy in 53.8 (7/13) were the most relevant signs/symptoms in EBV2. In contrast, EBV1+EBV2 co-infection was more frequent in the £5 year age group, affecting 20.0% (2/10). Women presented 66.7% (6/9) more positive cases. The symptoms involving EBV1+EBV2 co-infection were more related to fever (66.7%-6/9) and cervical lymphadenopathy (55.6% -5/9). The average of enzymatic values according to type of EBV was statistically significant ($p < 0.05$) in individuals with EBV1 infection in those over 14 years of age.

Conclusions: A pioneering study that molecularly identified the genotypes of EBV in 30.3% of cases, with circulation of EBV1, EBV2 and co-infection EBV1+EBV2 in cases of infectious mononucleosis in the northern region of Brazil.

Background

Epstein Barr virus (EBV) belongs to the order *Herpesvirales*, family *Herpesviridae*, subfamily *Gammapherpesvirinae*, genus *Lymphocryptovirus* and species *Human gammaherpesvirus 4* [1]. EBV was the first oncogenic virus described to infect humans [2, 3]. The hexagonal nucleocapsid viral particles were formed with linear, double stranded, enveloped DNA, with a diameter of 180 to 200 nm [4].

Although symptomatic infections with these viruses occur in benign form, EBV has been implicated in the genesis of a variety of lymphoproliferative disorders and severe epithelial neoplasms, such as African Burkitt's lymphoma [5] and the nasopharyngeal carcinoma[6].

In Brazil, several studies have recorded the high frequency of antibodies in the studied populations. Studies conducted by Monteiro et al. (1998) found that at least 70% of the serum samples analyzed in the city of Belém, state of Pará, contain IgG antibodies to EBV, at the outpatient clinic level ranging from 53.8% to 95.6%, or in the community (81.1% to 100%) [7]. Positive rates were expressive, even in the younger age groups. These results demonstrated that active and recent infection (infectious mononucleosis) was detected in 10.6% (25/234) of children and adolescents in northern Brazil [8].

Data from Young and Murray (2003) demonstrated that EBV is present in approximately 90% of individuals and is controlled by the immune system, mainly by cellular immunity, which may make the person more susceptible to virus proliferation and may trigger lymphoproliferative disorders [9, 10].

It is increasingly important to identify the epidemiological characteristics associated with the risk of EBV infection in populations to reduce the clinical conditions associated with possible morbidity and mortality [11].

Cohen (2000) demonstrated that the difference between sequences encoding EBV nuclear antigens (EBNA2, 3A, 3B and 3C) allows the identification of different genotypes with distinct epidemiological characteristics [12].

According to Young et al. (2000) in the human populations, geographic and ethnic factors may influence the distribution of EBV1 and EBV2, changing the detection rates of these viral genotypes in diseases associated with EBV [13]. Type 1 EBV has been more prevalent in western regions, and type 2 is more often described in sub-Saharan Africa and New Guinea than in other parts of the world [14].

The phenotypic difference between EBV1 and EBV2 is more evident during immortalization of B cells in lymphoblastoid cell lines (LCLs) by EBV1 compared to EBV2. This fact reinforces the biological and functional difference between the two viral types, where B cell immortalization in vitro was shown to be more effective by EBV1 [15]. According to Mandell et al. [16] differentiation of genotypes can clarify the different immune responses during viral persistence.

Studies to characterize the circulation of EBV1 and EBV2 types in relation to epidemiological data caused by these agents, such as clinical, demographic (gender, age and origin) and molecular findings in the metropolitan region of Belém, are still lacking. Due to their characteristics of viral persistence, they may induce chronic infections and reactivations in human populations with genetic competence, to possible oncogenic events.

The aim of this study was to detect the types of Epstein Barr virus (EBV1/EBV2) in the clinical cases infectious mononucleosis in the metropolitan area of Belém between 2005 and 2016.

Methods

Patients

Retrospective study involving serum samples collected from January 2005 to December 2016 from 8.295 individuals evaluated by infectious physicians from the Evandro Chagas Institute, Ministry of Health of Brazil (IEC/MS), Reference Center of Infectious Diseases Diagnosis in Northern Brazil, who were clinically suspect of infectious mononucleosis and were confirmed by serological tests.

Laboratory analysis

Serum samples were collected and tested for EBV using an immunoenzymatic assay as well as for biochemical dosages analyses. Peripheral blood mononuclear cells (PBMCs) were separated from the blood sample by Ficoll-Hipaque density gradient centrifugation (Lymphoprep Nycomed Pharma AS, Norway) for use in future PCR tests.

Immunoenzymatic assay (EIA)

EBV screening in samples was carried out using the RIDASCREEN® enzyme immunoassay kit (R-Biopharm, Darmstadt, Germany) which detects IgG and IgM antibodies to viral capsid antigen (VCA), according to manufacturer's guidelines.

Nucleic acid extraction

PBMC DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol.

Identification of the EBV EBNA3C gene

To identify both types of EBV, the primer EBNA-3C was used [17], which due to the primary flanking region can differentiate them by the resulting product: EBV1 153 base pairs (bp) and EBV2 of 246 bp.

Five microliters of the eluted DNA were used for PCR amplification with a primer concentration of 0.05 μ M (EBNA3C1- 5' GCCAGAGGTAAGTGGACTTT 3' and EBNA3C2- 5' TGGAGAGGTCAGGTTACTTA 3', respectively). PCR was performed in 0.5 mL microcentrifuge tubes in a final volume of 25 μ L of mixture containing 0.125 μ L (5 U / μ L) Platinum Taq DNA Polymerase (Invitrogen™, Brazil), 1.5 mM MgCl₂ (Invitrogen™, Brazil), 0.2 mM dNTPs (Invitrogen™, Brazil), 5 μ L of 10X buffer (Invitrogen™, Brazil) and 2 μ L (20 μ M / μ L) of the above mentioned primers. After denaturation of the DNA mold at 94°C / 1 min, PCR cycle conditions (PTC 100 / Peltier Effect Cycle, Thermostable Controller) included 40 cycles of desnaturation at 94°C / 30 sec, annealing at 58°C / 30 sec, extension at 72°C / 1 min, after one final extension cycle at 72°C / 7 min. Water (negative control) and B958 and P3HR1 cell lines (positive EBV1 and EBV2 controls) were used in all PCR tests. The amplified products were submitted to 2% agarose gel electrophoresis using ethidium bromide and visualized with UV illumination.

Biochemical dosages analyses

Biochemical tests such as aspartate aminotransferase (AST; reference: 4-40 U/L), alanine aminotransferase (ALT; reference: 2-41 U/L) and gamma-glutamyl transferase (GGT; reference: 5-55 U/L) were performed on an automated clinical biochemistry analyzer (COBAS INTEGRA clinical PLUS 400 / ROCHE).

Statistical analysis

Results were organized and stored in a database in Microsoft Office Access 2016, *Statistical Package for Social Science* - SPSS 17.0. A *p* value less than 0.05 was considered significant [18].

Ethical considerations

This study was approved by the research ethics committees of the Evandro Chagas Institute (CAAE number 65332717.2.0000.0019, with legal opinion number 2098453, of June 4, 2017).

Results

Serological analysis

A total of 8.295 serum samples was collected from January 2005 to December 2016 and tested for EBV by EIA. In 3.0 % (251/8.295) of these samples, IgM antibodies to EBV were observed. IgG antibody detection was also performed on 162 (64.5%) of the positive samples and all showed negative results for this virus.

Molecular detection

It was possible to identify by PCR the two types of EBV in 30.3% (76/251) of positive samples by EIA using EBNA-3C primer. The classification of these samples by age and sex showed that the age groups, >15-20 years and >30 years were slightly more affected (37,0%- *p*= 0.557) in relation of positivity by PCR (Table 1). Regarding gender, no difference was observed in males (30.6% - 34/111) compared to females (30.0% - 42/140). Comparing the age of the positive cases with sex, it was found that the majority of cases occurred in males aged >30 (38.1%), however, in females it was higher at age >15-20 years (42.8%).

The classification of these positive samples by types showed that 71.1% (54/76) were classified as EBV1, 17.1% (13/76) as EBV2 and 11.8% (9/76) as EBV1+EBV2 coinfection. These PCR positive patients came from the cities of Belém with 76.3% (58/76), Ananindeua with 22.4% (17/76) and Marituba with 1.3% (1/76).

Multiple signs and symptoms were observed in these cases, such as fever in 65.8% (50/76), cervical lymphadenomegaly in 60.5% (46/76), pharyngitis in 19.7% (15/76), arthralgia in 17.1% (13/76) and headache in 9.2% (7/76).

Regarding the number of days that the patient had fever, it was found that 22% (11/50) reported staying for up to 5 days, 16% (8/50) for 10 days, 20% (10/50) for 15 days, 16% (8/50) for 20 days, 16% (8/50) for more than 20 days and 10% (5/50) had no information about time in the epidemiological record.

EBV1 was detected in all age groups, on the other hand, EBV2 was verified at a low or negative level, except at age >10-15, when it presented a much higher value (62.5%) than EBV1 (25.0%). With the exception of age >20-30 all others had at least one case of coinfection. EBV1 got a percentage of 79.1% (34/43) in individuals over 15 years old, with an average age of 22.6 years. The number of women infected with this type was 61.1% (33/54), value higher than the 38.9% (21/54) detected in men. As for the place of origin of individuals infected with EBV1, the frequencies were as follows: Belém city with 75.9% (41/54), Ananindeua 22.2% (12/54) and Marituba 1.9% (1/54).

Regarding the clinical symptoms or signs observed in patients infected with EBV1, the main ones were cervical lymphadenopathy in 64.8% (35/54), fever in 63.0% (34/54), headache and arthralgia in 20.4% (11/54), and exanthema in 18.5% (10/54).

In EBV2 infection, it was also detected in all age groups, with the exception of two groups, with an average age of 24 years. This type was more frequent in males (76.9%- 10/13) than in females (23.1%- 3/13).

Co-infection involving EBV1+EBV2 presented fever and cervical lymphadenopathy as the most frequent symptoms in a percentage of 66.7% (6/9) and 55.6% (5/9), respectively. The most frequent age group was £ 5 years, affecting 20.0% (2/10) of individuals. The average age observed in this group was 21 years. Women showed more positive 66.7% (6/9) cases than in men 33.3% (3/9).

In the assessment of hepatic function, three age groups were used, as they are considered standard in the analysis of these biochemical parameters. Changes in AST in EBV1 infection were confirmed in 14.8% (8/54) of the cases, with results above the reference values (5-40 U/L) with 19.4% (7/36) age >14 years, and for EBV2 it was 7.7% (1/13), with the only case occurring in the same age group (14.2% - 1/7).

For ALT, a value above the reference (2-41 U/L) was also found at age >14 for EBV1 (33.3%- 12/36), showing a significant difference (p-value < 0.05) by the *Wilcoxon* test, as previously observed for AST at this age.

Regarding the GGT, there was no change in the age group from 2 to 5 years, considering that values obtained were within the reference values: (5 - 55 U/L). Changes in the age of 6 to 14 years were observed in relation to EBV1 (9.1%- 1/11) and EBV2 (28.6%- 2/7) and also over 14 years for EBV1+EBV2 using another parameter (12-43 U/L) with values of 41.7% (15/36) and 16.6% (1/6) respectively. Significant difference (p-value < 0.05) was also found for the GGT parameter.

Discussion

Primary EBV infection is usually asymptomatic and may progress to benign lymphoproliferative disease called infectious mononucleosis (IM), especially in late childhood or early adulthood in developing countries [[4](#)].

Infectious mononucleosis is characterized by significant clinical polymorphisms in which factors such as age, immune status and comorbidities have been described as parameters in clinical evolution, which can vary from asymptomatic infection to more severe conditions. It can be evidenced by acute complications, such as multiple organ failure, disseminated intravascular coagulation, ulcer/perforation of digestive tract, coronary artery aneurysm, lymphomas and lymphohistiocytes and EBV-associated hemophagocytosis [[19](#), [20](#)].

Mendoza et al. (2008) confirmed that EBV infection has an incubation period ranging from 4–6 weeks with prodromal symptoms of asthenia, anorexia, headache and chills, which often precede the signs and symptoms of mononucleosis: such as fever (which can reach 39–40 °C) accompanied by pharyngotonsillitis and lymphadenopathy [[21](#), [22](#), [23](#)]. The results obtained in the present study agree with that ones, since the 76 patients with EBV analyzed, fever was the main clinical finding in 65.8% (50/76), cervical lymphadenomegaly in 60.5% (46/76), pharyngitis in 19.7% (15/76), arthralgia in 17.0% (13/76) and headache in 9.2% (7/76).

Regarding the type of EBV infection, there are two different types of EBV [[24](#)]. EBV types are related to variation in the EBNA2 and EBNA3 gene sequences, commonly known as types 1 and 2 [[17](#), [25](#)].

Studies conducted in other countries have demonstrated the predominance of EBV1 infection in China with rates of 76.3%, Argentina in 75.9%, Sweden in 67.0% and Hong Kong 57.0% [[26](#), [27](#), [28](#), [29](#)].

Our findings demonstrated that EBV1 was the most frequent type (82.9%- 63/76) detected by PCR using EBNA 3C gene, in the infectious mononucleosis cases reported in the metropolitan region of Belém, Pará, Brazil, being in 71.1% (54/76) alone and 11.8% (9/76) associated with EBV2. It is worth mentioning that these results, obtained in symptomatic patients, were pioneers in this region. In an investigation conducted with Chinese individuals using the same technique showed slightly smaller results for EBV1 (76.3%- 45/59) [26]. In this context, other studies carried out by Deng et al. (2014) with Japanese patients and by Smatti et al. (2017) in Qatar also describe EBV1 with rates of 73.3% (107/146) and 72.5% (37/51) respectively [[30](#), [31](#)].

Regarding EBV2, it was detected in 28.9% (22/76) of the positive EBV cases, with 17.1% (13/76) alone and 11.8% (9/76) co-infected with EBV1 (Table 1). This value was higher than those described by Deng et al., 2014 [[30](#)] and Correa et al., 2004 [[27](#)] in the Japan (18.5%- 27/146) and Argentina (14.6%- 29/199) respectively. A lower frequency, about 3.5%, was verified

in Qatar by Smatti et al., 2017 [31]. It is important to mention that the positivity observed by EBV2, in the age group > 10–15 was very higher (75%- 6/8), when compared with EBV1 (37.5%- 3/8). However, these cases were detected in different months of these years.

In this investigation we verified that EBV2 infection demonstrated a longer clinical course when compared to EBV1, with the presence of fever on average of 17.6 days (range: 1–90 days), while EBV1 14.7 days (range: 1 to 30 days); the high rate of 62.5% observed to EBV2 in the age group of > 10–15 years was characterized by the presence of fever and lymphadenopathy. The EBV-2 has frequently been associated with special clinical conditions such as patients with weakened immune systems, for example HIV carriers or individuals with oncogenic processes. In vitro, several studies have recorded a low frequency of EBV2 in B lymphocytes demonstrating a reduced or less efficient capacity for replication in cell cultures [[17](#)].

The presence of multiple infections by EBV1 + EBV2 was observed in 11.8% of the positive cases of infectious mononucleosis, where 20.0% (2/10) occurred in children less than five years old (Table 1). This fact emphasize that co-infections are not exclusive to immunocompromised individuals. This association was also verified by Correa et al., 2004, in 10.5% of the healthy individuals participated of a study conducted in Argentina. The co-infection may occur by the simultaneous transmission of both genotypes or by the contact with two people infected with distinct strain [[27](#)].

Another finding that stood out in the present investigation was the occurrence of alterations in transaminases (aspartate aminotransferase - AST, alanine aminotransferase - ALT and gamma-glutamyl transferase - GGT), that varied from 19.4–41.7% (Table 2) for EBV1 in over 14 years old, with expression of these hepatic enzymes, a fact also documented in the studies of Herbing et al. [[32](#)].

It is known that eventually small changes in transaminase enzyme value can occur in normal individuals without infection (less than twice the reference value) while in individuals with EBV-infective mononucleosis, these values can increase 5 to 10 times the reference values and it may evolve for a fulminate hepatitis frame [[33](#)].

In addition to such observations, in this study the mean transferase average was statistically significant in > 14 years of age with infections for EBV1 ($P < 0.05$) when compared to EBV2 and EBV1 + EBV2 co-infection. Similar data have also been documented by Zhang et al. (2018) when compared infectious mononucleosis cases and control ones, as they observed high levels of ALT, AST and GGT only in cases of infectious mononucleosis, indicating that transferases levels should also be observed as a risk alert during infection caused by MI [[34](#)].

Conclusion

Pioneering study, realized in the period of 2005 to 2016 reports the predominance of EBV1, the presence of EBV2 and the co-circulation of both types (EBV1 + EBV2). Also describe relevant clinical

signs/symptoms of lymphadenopathy and fever and expressive enzymatic changes. In addition, knowledge of the main EBV genotypes with local circulation can support the formulation of new clinical approaches, especially in cases of infectious mononucleosis that can evolve with a certain severity. The determination of the types of EBV in the present study allowed for the first time to distinguish the molecular epidemiology and the circulation of these viral agents in individuals from northern Brazil.

Abbreviations

EBV: Epstein-Barr virus; LCLs: lymphoblastic cell lines; AST: aspartate amino transferase; ALT: alanine amino transferase; GGT: gamma-glutamyl transferase; IM: infectious mononucleosis.

Declarations

Acknowledgments

I thank Dra Yvonne Gabbay Mendes for the considerations and revision of this manuscript and Dr. Jones Anderson for the statistical analysis. We are grateful to all the individuals who contributed to the development of this study, especially Antonio de Moura and Dr. Manuel Gomes.

Availability of data and materials

All data generated or analyzed during this study are included in this published study. No other data are available for supplementary materials.

Authors' contributions

The work presented here was carried out as collaboration between all authors. TAFM, IBC, IBC, TLSC, BMRC, AAP, AESS, FLPR, and AJMF carried out most experiments. TAFM and JLFM made contributions to design, data analysis and data interpretation. TAFM and RCMS drafted the manuscript. JLFM and TAFM provided the most financial support. TAFM, AAP and AESS collected and assembled the data. All the authors have given final approval to publish the manuscript.

***Corresponding author**

Correspondence to Talita A. F. Monteiro

Competing interests

There authors declare that there are no competing interests.

Funding

The authors received no specific funding for this work.

Consent for publication

All authors of this article have directly participated in the planning and drafting and all authors listed have read and approved the final version including details and images. The written informed consent for the publication has been obtained from all the authors. The patients, parents, and legal guardians were informed about the publication and had signed informed consent forms.

Ethics approval and consent to participate

All participants provided informed written consent for all study procedures and for the use of their data for scientific evaluation and publication in a blinded form. This study was conducted in accordance with the Declaration of Helsinki, and it was approved by the Evandro Chagas Institute with legal opinion n° 2098453, dated June 4, 2017.

Author information

Affiliations

1- Section of Virology, Evandro Chagas Institute, Ananindeua, Pará, Brazil.

Talita Antonia Furtado Monteiro, Iran Barros Costa, Igor Brasil Costa, Alessandra Alves Polaro, Amanda Emanuelle Santos da Silva¹

2- Federal Institute of Science and Technology of Pará. Brazil.

Thais Letícia dos Santos Corrêa

3- Amazon Metropolitan University Center, Belém, Pará, Brazil.

Beatriz Monteiro Rodrigues Coelho.

4- Unified Medical Care Sector, Evandro Chagas Institute, Ananindeua, Pará, Brazil.

Francisco Lúzio de Paula Ramos.

5- Section of Pathology, Evandro Chagas Institute, Ananindeua, Pará, Brazil

Arnaldo Jorge Martins Filho.

6- State University of Pará, Belém, Pará, Brazil

Jose Luiz Furtado Monteiro

7- Federal University of Pará, Belém, Pará, Brazil

Rita Catarina Medeiros Sousa

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Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

Table 1. Frequency of the EBNA3C gene analyzed by PCR in 251 cases of infectious mononucleosis by age and sex, period 2005 to 2016.

Table 2. Biochemical parameters by age group related to EBV types

Supplementary Files

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