Serological and molecular analysis of parvovirus B19 infection in Mayan women with systemic lupus erythematous in Mexico.

Análisis serológico y molecular de la infección por parvovirus B19 en mujeres mayas con lupus eritematoso sistémico en México.

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**Introduction**

Systemic lupus erythematosus (SLE) is a chronic inflammatory systemic autoimmune disease of unknown etiology, caused by the interaction of genetic and environmental factors that contribute to the production of autoantibodies against self-antigens. The disease has a worldwide distribution and predominantly affects women. Asian countries such as China, Hong Kong, Philippines and Japan, has reported more cases, and others like the United States, France, Spain, UK, and some regions of Australia, has presented increase in patients. Several studies have been conducted in patients from different populations (Asian, European, American), but few in Mexican population.

Mexico has an admixed Mestizo population with a genetic pool from the Amerindian and the Spanish. The ancestry data derived from the HapMap project, which included Mexicans, shows that the Yucatan mestizos are the only ethnic group with Amerindian ancestry that are geographically distant from other Amerindian groups. On the other hand, Mexican individuals with SLE appear to have a more severe disease than European, a lower age of onset and a higher frequency of disease activity flares. Also, it has been reported that the prevalence of SLE in Yucatan (0.7%) is slightly higher than the national prevalence (0.6%).

Environmental factors such as bacterial, parasitic, fungal and viral infections have been associated with the pathogenesis of the disease in genetically predisposed patients. It has been reported that various viruses and bacteria can produce superantigens which, through mechanisms such as adjuvant effect (bystander) and molecular mimicry, induce activation of autoreactive T and B lymphocytes. Viral particles in infected B lymphocytes can lead to the production of autoantibodies and cytokines such as IFN-α, contributing to autoimmune and inflammatory mechanism. Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human T-lymphotropic virus 1 (HTLV-1), and Parvovirus B19 (PV-B19) have been linked to the pathogenesis of SLE.

Human PV-B19, which was identified in 1975 by Yvonne Cossart and her colleagues, is a small single-strand DNA virus (22-24 nm diameter) that causes a variety of diseases in humans. Its icosahedral capsid is composed of two identical structural proteins, VP1 (83 kDa) and VP2 (58 kDa), except for an additional fragment of 277 amino acids at the amino terminus of VP1. This unique VP1 region is external to the capsid, with many linear epitopes and phospholipase A2 activity (PLA2), which causes cytotoxicity and infectivity. PV-B19 also has the nonstructural protein NS1 (77kDa) involved in its transcription and translation. Three genotypic variants of PV-B19 have been identified: genotype 1 has a worldwide distribution; genotype 2 has been detected in patients from several European countries, the United States and Brazil; genotype 3 is most frequently in Africa and less frequently in other geographical areas.

PV-B19 is transmitted by respiratory aerosol spread from individuals acutely infected, or by parenteral transmission via blood transfusion and blood products. The virus replicates in the erythroblasts in the bone marrow, which express the blood group P antigen or globoside (Gb4), the alpha5beta1 integrin, and the Ku80 protein. Viral replication, leading to viremia on day 6, appears to be important in most clinical manifestations. Most infections are asymptomatic or have mild clinical pictures, but when the infection is associated with age-influenced clinical disorders or immune and hematologic status, it presents a wide variety of clinical manifestations that may be confused with systemic autoimmune diseases such as rheumatoid arthritis (RA), progressive systemic sclerosis, Sjögren’s syndrome (SS), vasculitis or SLE. PV-B19 infection may be misdiagnosed as a new onset SLE, but at the same time, can both occur simultaneously in some patients.

PV-B19 is a ubiquitous virus, distributed worldwide, which can infect any age group. Primary infection usually occurs in childhood and adolescence. Seroprevalence (presence of specific IgG denoting past infection) increases with age. In industrialized countries, it is estimated from 2% to 10% in children under five years, that could increase up to 50% at the age of 15 and in adults it varies from 40% to 70%. By the age of 70, it becomes 80% to 100%. Japan and Germany have reported high infections rates in pregnant women.

In Mexico, there are few clinical and epidemiological studies of PV-B19. Tapia et al., determined IgM and IgG in 128 people from groups considered at high risk of infection with PV-B19, and healthy people of all age groups and both sexes, in the Infectious Diseases Hospital at la Raza Medical Center. The results showed the presence of infection especially in women (63.2%) of 25-44 years (48.4%), with exanthems, habitual abortion, and anemia in immunocompromised patients or hematologic disorders. In 61 patients (47.6%), higher IgG antibodies were found, and only 4 of them had IgM. Vera et al., conducted a preliminary prospective study of 102 pregnant women in two rural towns of Yucatan, Mexico, founding a seroprevalence of 5.9% of IgM and 11.8% of IgG; confirming the presence of PV-B19 infection in these populations.

Several studies have been focused on the diagnosis of PV-B19 infection, but the relationship of PV-B19 with established SLE has not been studied in Mayan population of Mexico. Our objective was to perform a preliminary serological and molecular analysis of PV-B19 infection in women of the Mayan population with established SLE and healthy women. IgM and IgG anti-PV B19, presence of viral DNA and viral load were evaluated in both groups.

**Materials and Methods**

**SLE patients**

Sixty-six SLE women of Mayan origin, were recruited at the Rheumatology outpatient of the Agustin O’Horán and ISSSTE Regional Hospital, Yucatan. Diagnosis was established according American College of Rheumatology (ACR) criteria, and disease activity was evaluated by SLEDAI score. SLE women reported having different times with the disease. Sixty-six healthy women of the same origin with no history of autoimmune or infectious diseases as controls were studied. None of them were receiving any treatment. All women included gave their informed consent, according to the Declaration of Helsinki. The study was approved by the Research Ethics Committee of the Agustin O’Horán Hospital of Yucatan (CIE-008-1-11). All women gave 10 mL of venous peripheral blood (without anticoagulant) in one collection to obtain serum.

**IgM and IgG anti-PV-B19**

Two commercially available ELISA kits for detection of anti-B19 IgM (EIA-3504) and anti-B19 IgG (EIA-3503) (DRG Instruments GmbH, Germany) were used. Microtiter wells as a solid phase are coated with recombinant Parvovirus B19 antigen (VP1 proteins). Diluted serum from patients and controls, and ready-for-use...
controls, are pipetted into these wells. During incubation Parvovirus B19-specific antibodies of positive serum and controls are bound to the immobilized antigen. Subsequently, the specific human anti-IgG or anti-IgM conjugated to horseradish peroxidase (HRP) is added. The reaction is visualized by adding tetramethylbenzidine (TMB) which generates a blue color. The enzymatic reaction is stopped by addition of sulfuric acid solution (H₂SO₄), which develops a yellow color. The color intensity is proportional to antibody concentration. Reading was performed at a wavelength of 450 nm in an ELISA reader (model BioTek® ELx800), and antibody concentration was determined by the following formula:

Antibody concentration= (Abs) (10)/ CO

Where:

Abs= sample absorbance
10= constant to compare absorbance (cut-off and control samples)
CO= Cut-off control mean absorbance

Antibody concentration is expressed in DU (DRG units, exclusive measure of supplier used to have a parameter measurement of immunoglobulins), taking the absorbance cutoff control as reference. Each assay was performed in duplicate using the positive, negative and cutting controls, contained in the kit. Results were interpreted as follows: IgM positive >11DU, IgG positive >12 DU, IgM negative <9 DU, and IgG negative <8.5 DU, respectively.

DNA isolation

DNA extraction was performed on IgM and IgG positive sera from patients and controls by saturated phenol method[39]. This procedure was based on the classical phenol/chloroform extraction method using 200 µL of serum samples. Solution of chloroform-isooamyl alcohol (24:1) was added to separate protein, and the DNA was precipitated with 100% ethanol and 7.5 M ammonium acetate for 24 h at -20°C. The DNA pellet was washed twice with 70% ethanol, dried in the oven at 37°C for 1 h, and then resuspended in 30 (L of ultrapure water. After incubation of 20 min at 56°C, the DNA was quantified in a spectrophotometer (Nanodrop DU, IgM negative <9 DU, and IgG negative <8.5 DU, respectively

Viral load

IgM and IgG positive samples were quantified by real time PCR using the same primers and probe, described above. Viral load was quantified using a standard curve assay with different copy number of the plasmid containing the fragment of 168 bp of the NS1 gene (4,099bp). The number of copies of the 6-point standard curve was determined using the URI Genomics & Sequencing Center software (http://cels.uri.edu/gsc/cndna.html). This calculation was based on the assumption that the average weight of a base pair (bp) is 650 Daltons. This means that one mole of a bp weighs 650 g and that the molecular weight of any double stranded DNA template can be estimated by taking the product of its length (in bp) and 650. The inverse of the molecular weight is the number of moles of template present in one gram of material. Using Avogadro’s number, 6.022x10²³ molecules/mole, the number of molecules of the template per gram is calculated. The number of copies of template was estimated by multiplying by 1x10⁶ to convert to ng and then multiplying by the amount of template (in ng). The formula used, starting from an initial concentration of 1ng, was:

\[(1 \text{ ng} \times 6.022 \times 10^{23}) / (4099 \times 1 \times 10^{9} \times 650) = 2.25 \times 10^{4} \text{ copies.}\]

Five serial dilutions at 10 were included (Table 1). Each sample and standard curve were tested by triplicate. Amplification conditions were as follows: 2 min pre-heating at 50°C, 10 min polymerase activation at 95°C, following by 50 cycles of denaturation (95°C for 15 s), primer annealing (55°C, 40 s), extension step of 20 s at 72°C, and a final holding stage at 60°C for 30 sec. Amplification reaction was performed in the StepOne™ Real-Time PCR System.
equipment (Applied Biosystems), using the Maxima Probe/ROX master mix (Thermo Scientific), primers (0.5 µM), and probe (0.1 µM). The number of copies in the samples was calculated with the StepOne software taking into account the average of CT values obtained with respect to the standard curve. Viral load are expressed in copies per milliliter of serum (cps/mL).

Statistical Analysis
Wilcoxon matched-pairs signed rank test was used to assess the significance of any difference in values of IgM and IgG, and viral load (cps/mL) among SLE patients and control subjects (p <0.05). Correlation analysis was done using the Pearson correlation coefficient. In all comparisons, the level of significance was p <0.05, using the Graph Pad Prism 5 software.

Results
Characteristics of SLE patients and controls
The average age of the patients and controls was 39.03 and 38.18 years, respectively. The average time with disease in patients was 9 years (Table 2). All female patients were under treatment, 56.1% of them had active disease determined by SLEDAI (>4).

Levels of IgM and IgG anti PV-B19
Antibodies were detected in 42.4% (28/66) of SLE patients and 53.0% (35/66) of controls. We found 14.3% (4/28) of patients and 11.4% (4/35) of controls with no significant levels of IgM (p= 0.7922). On the other hand, 82.1% (23/28) of patients and 82.9% (29/35) of controls showed IgG, but significantly higher levels were detected in patients (p= 0.0353) (Table 3 and Fig. 1). Only one patient and two controls have both IgG and IgM. We found 58.3% (14/24) of patients with disease duration of 4 years or more presented IgG, but no correlation was observed. Correlation was also not observed in those who presented less than 4 years (41.7%, 10/24) (Fig. 2). Association analysis of IgG and IgM with SLEDAI was performed but no correlation was observed between IgM levels and disease activity (SLEDAI >4). However, IgG levels showed significant negative correlation in patients with lower disease activity (SLEDAI <4) (Fig. 3).

Detection of PV-B19 DNA and viral load
The sequenced fragment of NS1 protein, used as positive control, was analyzed by BLAST and showed 100% of homology with reported sequences of NS1 gene of PV-B19 in GenBank[33-45]. (Fig. S1). The sequence was registered in the GenBank (BankIt1994458 Human KY680313). PV-B19 DNA of genotype 1 was detected in 86.4% (57/66) and 81.8% (54/66) of patients and controls, respectively (Table S1). Viral load was quantified in 28/66 SLE patients and 30/66 healthy controls, which were positive for IgM and IgG (Table S2). We found that 67.9% (19/28) of patients presented viral load: 10.7% (3/28) with IgM and 57.1 % (16/28) with IgG. It was also found viral load in 80.0% (24/30) of the controls: 13.3% (4/30) with IgM and 66.7 % (20/30) with IgG, respectively (Table 4). Viral load was no detected in patients with IgM (1/28), IgG (7/28), or both (1/28), neither in controls subjects with IgG (5/30), or IgM and IgG (1/30) (Table S2). No correlation of IgM or IgG with viral load was found in both groups; however, viral load was significantly higher in the controls with IgG (Fig. 4).
A graph representing the number of copies of the standard curve is shown with the CT values of a sample (Fig. 5).

Discussion
During viral infection the humoral immune response is crucial to limit infection. In immunocompetent individuals, viremia begins 6 days after infection and decreases days later with the presence of antibodies against VP1 and VP2 proteins. In acute infection IgM antibodies are detectable in the first 3 days of the infection and undetectable between 60 and 90 days, but it could remain elevated between 3 and 6 months[46-48]. Some authors report that acute PV-B19 infection may trigger its onset or exacerbate preexisting SLE[49,50].

This is the first report on PV-B19 infection in women with established SLE of the Mayan population in Mexico. IgM antibodies against VP1 were detected in patients with confirmed SLE who showed disease activity (SLEDAI >5), and had an average of 9.8 years with the disease. Although IgM levels in the patients were not different from the controls, and showed no correlation with the
disease activity, probably due to the duration of the disease and the treatment, data suggest recent infection in our patients and seems to correlate with the reactivation of the disease. This supports that PV-B19 infection is associated with confirmed SLE as etiopathogenic factor, and it corresponds to what was suggested by Ramos et al.\(^5\) 

IgG is detected days after IgM, indicating resolution of infection and past or chronic infection, providing lifelong immunity.\(^4\),\(^7\),\(^8\). In our study, high levels of IgG antibodies were detected in SLE patients, supporting past or chronic infection. Unlike the data reported by Pugliese et al.,\(^5\) who found a significant correlation between IgG anti-PV-B19 and SLE, we found no correlation between IgG and SLEDAI; the inverse correlation observed between IgG with low disease activity (SLEDAI <4), is probably due to high IgG values in a single patient, which we consider not representative. Our data suggest that IgG levels appear to increase in SLE patients likely due to support therapy for chronic PV-B19 infection, since all of our patients were being treated with anti-inflammatory drugs, corticosteroids and immunosuppressive agents. In this regard, it has been described that the use of corticosteroids, immunosuppressive agents, and biological therapies may increase the risk of viral infection in SLE patients and PV-B19 infection could become chronic or severe on them.\(^5\),\(^3\). However, longitudinal studies are required to confirm this.

PV-B19 genotype 1 was detected in SLE patients and controls, and viral load was quantified in those patients and controls with high levels of IgM or IgG antibodies. We found no correlation between IgM or IgG antibodies and viral load in both groups; however, higher viral load was found in controls confirming presence of PV-B19 in the region, and supporting the prevalence of infection in the Mayan population. IgG antibodies and viral load in patients seem to support the chronic infection associated with immunosuppression by therapy. Viral load was not detected in some patients with IgM, IgG or both. In this regard, it has been reported that viremia disappears at day 10 post infection, whereas IgM (10-12 days) and IgG (14 days) start to synthesize. In this stage, viral particles are not detected, indicating that the reason for not detecting viral DNA in some of our patients and controls with high titer of IgG and/or IgM, could be that those individuals were in day 12 of infection, when virus is not present. However, longitudinal studies are required to confirm this.

In our control women, no clinical symptoms suggestive recent infection or illness were observed, but IgM and IgG antibodies, as well as presence of DNA and viral load were detected. Despite the differences in sample size and populations studied, the

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Table 4. Serum viral load (cps/mL) in SLE patients (n = 28) and controls (n = 30) with IgM or IgG, analyzed by qPCR and ELISA, respectively, as described in Material and methods.

<table>
<thead>
<tr>
<th>Viral load/antibodies</th>
<th>SLE patients(%)</th>
<th>Healthy controls(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cps/mL (+) IgM (+)</td>
<td>3/28 (10.7)</td>
<td>4/30 (13.3)</td>
</tr>
<tr>
<td>cps/mL (+) IgG (+)</td>
<td>16/28 (57.1)</td>
<td>20/30 (66.7)</td>
</tr>
<tr>
<td>cps/mL (+) IgM (+)</td>
<td>1/28 (3.6)</td>
<td>-</td>
</tr>
<tr>
<td>cps/mL (+) IgG (+)</td>
<td>7/28 (25.0)</td>
<td>5/30 (16.7)</td>
</tr>
<tr>
<td>cps/mL (+) IgM/IgG (+)</td>
<td>1/28 (3.6)</td>
<td>1/30 (3.3)</td>
</tr>
</tbody>
</table>
data support the IgM and IgG seroprevalence found by Vera et al., and confirm the circulation of the virus in the Mayan population. Viral load was not detected in some controls with IgG or IgM/IgG antibodies. In this regard, it has been reported that immunocompetent individuals produce antibodies that effectively eliminate viremia within a few days of infection, and the infection is often not developed, is asymptomatic or has mild clinical manifestations (like the common cold), which seems to agree with what we found in our controls. On the other hand, there have also been rare cases of chronic PV-B19 infection in healthy individuals, with involvement of the central nervous system, causing nonspecific symptoms such as fatigue, fever, arthralgia and myalgia, which may hinder the diagnostic. None of our controls women manifest some of these symptoms, however, longitudinal studies are needed to evaluate the association of PV-B19 infection with neurological, autoimmune or hematological disorders in the immunocompetent Mayan population.

We conclude that the high prevalence of PV-B19 in Yucatan, and the presence of IgM, IgG, and viral load in Mayan women with established SLE suggest that PV-B19 infection could be an environmental factor to trigger or reactivate SLE. However, longitudinal studies and a large sample are required to confirm the association of PV-B19 with the development of SLE, as well as the effect of immunosuppressive therapy on the resurgence of the virus.

Acknowledgements:
This work was supported by CONACYT (National Council of Science and Technology), grant FONSEC SALUD 2010-1-139788

Founding:
This work was supported by CONACYT (National Council of Science and Technology), grant FONSEC SALUD 2010-1-139788.

Conflict of interest:
The authors declare no financial or commercial conflict of interest.

References


Table S1. Presence/absence of viral DNA and IgM or IgG in SLE patients (n = 66) and controls (n = 66) analyzed by qpcr and elisa, respectively, as described in material and methods.

<table>
<thead>
<tr>
<th>SLE Patients</th>
<th>Presence Absence</th>
<th>IgM (DU)</th>
<th>IgG (DU)</th>
<th>Healthy control</th>
<th>Presence Absence</th>
<th>IgM (DU)</th>
<th>IgG (DU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>A</td>
<td>2.60</td>
<td>2.20</td>
<td></td>
<td>A</td>
<td>3.40</td>
<td>3.20</td>
</tr>
<tr>
<td>Control</td>
<td>A</td>
<td>1.80</td>
<td>1.40</td>
<td></td>
<td>A</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table S2. Serum viral load (cps/ml) in sle patients and controls with igm or igg analyzed by qpcr and elisa, respectively, as described in material and methods.

<table>
<thead>
<tr>
<th>SLE Patients</th>
<th>Presence Absence</th>
<th>IgM (DU)</th>
<th>IgG (DU)</th>
<th>Healthy control</th>
<th>IgM (DU)</th>
<th>IgG (DU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>A</td>
<td>3.40</td>
<td>3.20</td>
<td></td>
<td>3.40</td>
<td>3.20</td>
</tr>
<tr>
<td>Control</td>
<td>A</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
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