Detection of Human Pegivirus (HPgV) Infection among Filipino Children with Decompensated Liver Disease Secondary to Biliary Cirrhosis and Liver Transplant Pediatric Patients

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ABSTRACT

Background. Human Pegivirus (HPgV), previously called Hepatitis G virus or GB virus C, is an RNA virus. It can be transmitted vertically (mother to infant), parenterally and sexually. HPgV share common routes of transmission to other viruses such as Hepatitis B virus, Hepatitis C virus and Human Immunodeficiency virus (HIV) thus co-infection is usually observed. Risk groups of HPgV include injection drug users, HIV-positive individuals, multi-transfused patients, hemodialysis patients, hemophiliacs, chronic liver disease patients and organ transplant recipients. The clinical significance of HPgV is not yet established and warrants further studies. Research on HPgV in the Philippines is scarce and has not been updated for over 10 years. There is no published data on HPgV prevalence in Filipino pediatric population specifically among risk groups like multi-transfused children with decompensated liver disease secondary to biliary cirrhosis and liver transplant pediatric patients. The lack of local data warrants conduct of this study.

Objective. To determine the presence of HPgV RNA, HPgV E2 antibody (anti-E2) and HBsAg among Filipino children with decompensated liver disease secondary to biliary cirrhosis (DBC) and liver transplant pediatric patients (LTP).

Methods. Included were 15 children with DBC and 15 LTP recruited from the Section of Pediatric Gastroenterology, Hepatology and Nutrition of the UP PGH. All patients' sera were tested for HPgV RNA by Real Time RT-PCR, HPgV anti-E2 by Enzyme-linked Immunosorbent Assay (ELISA) and hepatitis B surface antigen (HBsAg) by immunochromatographic test. Twenty age and sex matched children with no history of liver disease and blood transfusion served as controls.

Results. All patient and control samples were negative for HPgV RNA. HPgV anti-E2 was detected in 6 of 15 LTP, 5 of 15 DBC and 1 of 20 controls. HBsAg was detected in 2 of 15 LTP, 5 of 15 DBC and 0 of 20 controls. Four patients (two LTP, two DBC) were positive for both HPgV anti-E2 and HBsAg.

Conclusion. This study showed that a proportion of liver transplant patients and those with decompensated biliary cirrhosis are positive for HPgV anti-E2, which indicates that these individuals previously had HPgV infection but is now resolved. Possible source of infection is infected blood from the blood transfusions, infected transplant organ or infected mother. Since routine HPgV screening is not yet recommended for the general population, blood donors and organ donors, the confirmation of exact source of infection may be difficult. Co-infection with HBsAg was also observed in both risk groups which suggests that at some point in time, these children were infected by both HPgV and HBV and also the possibility of simultaneous infection by the two viruses. This study provides preliminary

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data on the proportion of HPgV infection in Filipino children belonging to two of the HPgV risk groups. Studies with a larger and more significant sample size to determine HPgV prevalence as well as studies regarding the pathogenicity of HPgV are warranted. As this may provide basis for routine HPgV screening among risk groups and blood donations in the future.

Key Words: GBV-C, hepatitis G virus, HPgV, Filipino, pediatric, multi-transfused, transplant patients

INTRODUCTION

Human pegivirus (HPgV), formerly called Hepatitis G virus (HGV) or GB virus C, is an enveloped, spherical virus of about 50 nm in diameter belonging to the *Flaviviridae* family.[1] It has a single stranded, positive sense RNA genome of 9.4 kb in length which contains a single long open reading frame (ORF) flanked by 5' and 3' untranslated regions.[2] This open reading frame encodes for two envelope proteins (E_1 and E_2).[3,4] Hepatitis C virus (HCV) is the closest relative of HPgV known to infect humans, they have the same genomic organization yet share only 25% sequence similarity.[5] HPgV predominantly replicates in peripheral blood mononuclear cells, mainly in B and T lymphocytes and bone marrow, and poorly in hepatocytes.[6,7]

HPgV is transmitted sexually, vertically (mother to infant) and parenterally or through exposure to infected blood.[8] It shares common routes of transmission to other viruses such as Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Human Immunodeficiency virus (HIV), thus co-infection is usually observed.[8,9,10] Risk groups for HPgV include parenteral drug users, people living with HIV, men who have sex with men, multi-transfused patients, hemodialysis patients and haemophiliacs.[11,12]

The pathogenic mechanism of HPgV remains unclear. HPgV infection may last for months to years and can be detected by the presence of viral RNA in the blood.[13] Clearance of HPgV is associated by the presence of specific antibodies against HPgV envelope protein E2 (HPgV anti-E2), moreover this is used as a marker for resolved infection.[1,13]

Multiple studies have explored the possible association of HPgV with known clinical diseases. At the early years of the discovery of HPgV, it has been associated to non-A-E fulminant hepatitis but numerous studies designed to confirm this found no epidemiological associations.[6,14,15] As mentioned, HPgV replicates in both T and B lymphocytes, in relation to this, several studies have reported association between HPgV viremia with an increased risk of non-Hodgkin's lymphoma.[13,16,17,18] Beneficial effects of HPgV viremia on HIV disease progression was explored by several groups and found higher CD4 cell counts, lower HIV viral loads and longer AIDs-free survival times for individuals with HPgV infection.[19-22] However, other studies have contradicted these findings and observed no beneficial effects of HPgV infection in individuals living with HIV.[23,24,25]

Tucker et al. reported that HPgV has worldwide distribution and is found in the general population.[26] Prevalence of HPgV viremia in healthy blood donors from developing countries ranges from 5% to 18.9% while in developed countries the prevalence of HPgV ranges from 0.5% to 5%.[1,27] Among the populations at high risk for infection, HPgV prevalence approaches 50%.[6,28,29] There are multiple variabilities in the evaluation of the prevalence of HPgV which includes different age groups, geographical regions, co-morbidities and sample size to name a few. The real burden of HPgV infection is not yet established and up to this day, the clinical significance of HPgV is not yet clear. Currently there is no recommendation regarding the risk for HPgV infection, no country has implemented routine HPgV screening in blood donors.[1]

In the Philippines, there is limited data on the prevalence of HPgV infection. In 2002, Dalmacio et al. published a prevalence data on HPgV infection in various risk groups and found HPgV prevalence in 1.2% volunteer blood donors, 8.0% on chronic liver patients, 3.4% hemodialysis patients and 6.2% multi-transfused patients.[30] Since then, there has been no locally published literature on HPgV. Moreover, there is no published data on HPgV prevalence in Filipino pediatric population specifically among risk groups like multi-transfused children with decompensated liver disease secondary to biliary cirrhosis and liver transplant pediatric patients. The lack of local data warrants conduct of this study.

This is a descriptive study on the presence of HPgV RNA, HPgV E2 antibodies (anti-E2) and HBV surface antigen (HBsAg) among Filipino children with decompensated liver disease due to biliary cirrhosis and liver transplant patients.

METHODS

Study population and Ethics Clearance

In a span of 1 year, Filipino patients aged 0-12 were recruited from the Section of Pediatric Gastroenterology, Hepatology and Nutrition of the Philippine General Hospital (PGH). Controls were recruited from the PGH Laboratory. Study groups included multi-transfused children with decompensated liver disease secondary to biliary cirrhosis, who had at least 2 previous blood transfusions[31] and liver transplant patients, who was at least 6 months post operation. Controls were age and sex-matched children with no history of liver disease and blood transfusion.

Ethics approval was obtained from the University of the Philippines Manila – Research Implementation Development Office (RIDO) and Research Ethics Board (REB). After obtaining a written informed consent/assent from their parents/guardians, they were enrolled in the study. Only the patient, parent/guardian and patient's physician were informed of the results of this study. It was explained to the parent/guardian that due to the lack of established

clinical significance of HPgV infection, there is currently no treatment for it. Appropriate medical care were provided by the physician in charge. The participant or the participant's parent or guardian can withdraw permission from using the blood samples of the subjects anytime.

Blood Extraction and Serum Preparation

Five mL of blood sample was taken from each patient during their routine blood extraction at PGH. Samples for the control group were from residual serum samples from patients with no history of liver disease and blood transfusion obtained from the PGH Laboratory. No additional blood extraction was done to patients in this group. Serum was separated from the whole blood within 6 hours of extraction, by centrifugation and was kept at -80 ˚C until analysis.

RNA Extraction for Real Time Reverse Transcription PCR

Viral RNA was extracted from the serum samples using High Pure Viral Nucleic Acid Kit (Roche) following the manufacturer's instructions.[32] 200 µl serum was mixed with 200 µl binding buffer and 50 µl of proteinase K solution and was incubated for 10 minutes at 72˚C. After the addition of 100 µl of binding buffer, the sample was transferred to a high filter tube and centrifuged for 1 min at 8000 x g. After centrifugation, the filter tube was removed and transferred to a new collection tube, then 500 µl inhibitor removal buffer was added to the upper reservoir of the filter tube and was centrifuged for 1 min at 8000 x g. The same steps were repeated twice using 450 µl wash buffer instead of inhibitor removal buffer. After centrifugation, the assembly was centrifuged for another 10 secs at maximum speed of 13,000 x g to remove any residual wash buffer. The filter tube was then transferred to a nuclease-free, sterile 1.5 ml microcentrifuge tube. Finally, the 50 µl of elution buffer was added to the upper reservoir of the filter tube and then centrifuged for 1 min at 8000 x g to elute the viral nucleic acids. The extracted nucleic acid was stored in -80˚C freezer.

Detection of HPgV RNA virus using HGV Real Time Reverse Transcription PCR kit

HPgV RNA in serum was detected using HGV Real Time Reverse Transcription PCR Kit (Liferiver)(note HPgV was previously called HGV).[33] The Real Time PCR system was Applied Biosystems 7500 Fast Real Time PCR system. The master reaction mix contains Super Mix for the specific amplification of HPgV RNA. The reaction was done in one step real time RT-PCR. HPgV RNA was transcribed into cDNA then a thermostable DNA polymerase was used to amplify the specific gene fragments by polymerase chain reaction. Fluorescence was emitted and measured by the real time system's optical unit. Detection of amplified HPgV DNA fragment was performed in fluorimeter channel FAM with the fluorescent quencher NFQ-MGB. This kit also includes internal control for identification of possible PCR inhibition and an external positive control defined as 1 x 10^7 copies/ml.

Detection of HPgV RNA was performed following the manufacturer's protocol

20 µl master mix containing 18 µl super mix, 1 µl enzyme mix and 1 µl internal control was placed to each Real Time PCR reaction plate well. Separately 5 µl each of RNA sample, positive and negative controls were added to different tubes. The running conditions were 1 cycle at 45 ˚C for 10 minutes, 1 cycle at 95 ˚C for 15 minutes and 40 cycles at 95 ˚C for 15 seconds, 60 ˚C for 1 minute with fluorescence measured at 60 ˚C. 7500 Software v 2.0.6 was used to analyze the obtained Real Time PCR data to determine absence or presence of HPgV RNA.

Detection of HPgV E2 antibody

HPgV E2 antibodies was detected using Human hepatitis G virus antibody (IgG) ELISA kit (BioSource, San Diego CA) following manufacturer's instructions.[34] In this kit, ELISA plate pre-coated with human HPgV E2 antigen, negative control and positive control for HPgV E2 antigen were provided.

10 µl each of the negative control, positive control and serum sample were added to different wells in the ELISA plates, then 100 µl sample diluent was added to each well. A well for the blank, solely sample diluent, was also provided. Plate was covered with adhesive strip and was incubated for 45 minutes at 37 ˚C. Then each well was aspirated and washed with 200 µl wash buffer four times. The plate was inverted and blotted against clean paper towels. 100 µl of Horseradish peroxidase (HRP)-conjugate was added to each well except to the blank well and was incubated for 30 minutes at 37 ˚C. The plate was then washed with wash buffer then inverted and blotted against clean paper towels. After that, 50 µl of Substrate A and 50 µl of Substrate B was added to each well and was incubated for 10 minutes at 37 ˚C. After incubation, 50 µl of stop solution was added to each well. The optical density of each well within 10 minutes at 450 nm was determined.

Interpretation of Result

The calculation of the valence of human HPgV antibody (IgG) was done by comparing the sample well with the control. A cut-off value was defined as the average negative control value plus 0.2. If ODsample < cut-off value then sample is negative for the antibody while if ODsample ≥ cut-off value then sample is positive for the antibody.

Detection of HBV infection

The serum samples were tested for the presence of Hepatitis B antigens using an HBsAg immunochromatographic test. This test utilizes the principle of immunochromatography. Test strips coated with colored polyclonal anti-HBsAg-colloidal gold conjugate complexes was dipped in serum

sample until 1 cm high. Two visible lines in the test strip indicates presence of HBsAg.

RESULTS

Patients and control group

In the span of 1 year, there were a total of 50 patients recruited in this study, 30 patients with liver disease, 15 patients with decompensated liver disease secondary to biliary cirrhosis and 15 post-liver transplant patients, and 20 controls (Table 1). Broken down into the two groups, those with decompensated liver disease, with 6 males and 9 females, were younger (median age: 1 year) compared to liver transplant patients, with 8 males and 7 females, (median age: 3 years). The control group consisted of 10 males and 10 females (median: 2 years).

Table 1. Summary of Patient and Control Group Data

Group	No. of subjects	Age, median, years
DBC	15 (6 males/9 females)	1
LTP	15 (8 males/7 females)	3
Controls	20 (10 males/10 females)	2
Total	50	–

LTP: liver transplant patients
DBC: decompensated liver disease secondary to biliary cirrhosis

Detection of Human Pegivirus (HPgV) RNA

Detection of HPgV RNA in the sera was done to determine active infection of HPgV. Patient and control samples were negative for HPgV RNA (Table 2).

Table 2. Proportion of HPgV RNA, HPgV ANTI-E2 and HBsAG Positive Patients and Controls

Group	No. of Subjects	No. of HPgV RNA (+)	No. of HPgV anti-E2 (+)	No. of HBsAg (+)	HPgV anti-E2 and HBsAg (+)
LTP	15	0	6	2	2
DBC	15	0	5	5	2
Controls	20	0	1	0	0
Total	50	0	12	7	4

Detection of Human Pegivirus E2 Antibody (HPgV anti-E2)

Detection of the presence of the HPgV anti-E2 in the sera was done to determine resolved HPgV infection among the risk groups. HPgV anti-E2 was detected in 6 of 15 liver transplant patients, 5 of 15 decompensated liver disease secondary to biliary cirrhosis and 1 of 20 controls (Table 2).

Detection of Hepatitis B Surface Antigen (HBsAg)

Detection of HBsAg using immunochromatographic test was done to determine infection with hepatitis B virus. HBsAg was detected in 2 of 15 liver transplant patients, 5 of 15 children with decompensated liver disease secondary to biliary cirrhosis and none from the controls (Table 2).

Co-infection of HPgV and HBV

HPgV share common routes of transmission to other viruses such as HBV. For the two risk groups, 2 from each tested positive for both HPgV anti-E2 and HBsAg (Table 2).

DISCUSSION

For more than a decade now, there have been no updates on the prevalence of HPgV infection in the Philippines. There is also no published data on HPgV infection among Filipino children specifically in multi-transfused children with decompensated liver disease secondary to biliary cirrhosis and post-liver transplant patients. In this study, 15 pediatric patients with decompensated liver disease secondary to biliary cirrhosis, 15 post-liver transplant pediatric patients and 20 controls were recruited to determine presence of HPgV infection.

To determine active HPgV infection, the presence of HPgV RNA in the blood was detected by reverse transcriptase PCR (RT-PCR).[13] In this study, all patient and control samples were negative for HPgV RNA which indicates that none were infected with the virus at the time of blood extraction. To determine if these children had previous HPgV infection that resolved, ELISA was done to detect HPgV anti-E2. Several studies have shown that serum samples that are positive for the antibodies are negative for viral RNA and vice versa which makes HPgV anti-E2 a marker for resolved infection.[35] HPgV anti-E2 was detected in 6 of 15 liver transplant patients, 5 of 15 children with decompensated liver disease and 1 of 20 controls (Table 2). This indicates that there was previous HPgV infection in the HPgV anti-E2 positive children. Mechanism of viral clearance is still unclear at the moment and calls for further studies. Children from the two risk groups have had previous blood transfusions. They are at risk for HPgV infection since HPgV can be transmitted parenterally. HPgV is not routinely screened in blood donations and in organ donations thus infection from the transfusions and organ transplant is possible but is not documented. It is also possible that HPgV was transmitted vertically, through transmission of the virus from infected mothers. Serum from the mothers of the HPgV anti-E2 positive children can be tested for HPgV RNA and HPgV anti-E2 to help elucidate possible source of infection. However since collection of samples from the mothers is not within the scope of this study, this can be a recommendation for future studies. The HPgV anti-E2 positive control may have likely got the infection from infected mothers since there is no history of blood transfusion or liver disease.

In this study, HBV infection was determined by HBsAg using immunochromatographic test. HBV infection remains a major public health problem in the Philippines despite vaccination efforts and routine screening in blood donations. An estimated 7.3 million adult Filipinos (16.7%) are infected which makes the country hyperendemic for hepatitis B.[36] Children are also at risk for HBV infection,

most individuals that develop chronic hepatitis B acquire the infection at birth or during early childhood.[36] The risk groups in this study are at risk for HBV infection as well from the multiple blood transfusions and other parenteral routes, infected needles, catheters, exposure to infected fluids and infected organ. HBsAg was detected in 2 of 15 liver transplant patients, 5 of 15 children with decompensated liver disease secondary to biliary cirrhosis and none from the controls (Table 2). For the two risk groups, 2 from each tested positive for both HPgV anti-E2 and HBsAg (Table 2). This suggests that at some point in time, these children were infected by both HPgV and HBV. This also suggests the possibility of simultaneous infection by the two viruses.[30] Possible source of HBV infection of these children is from an infected mother.

Results of this study were relayed to the parent/guardian of the child and to the patient's physician in charge. There is currently no recommended treatment for HPgV infection. There is still no clear evidence that HPgV can cause human disease and there is also no established association with known diseases.[1] Appropriate medical care specifically for those who tested positive for HBsAg was provided by their respective physicians.

This is the first study to report data on the presence of HPgV infection among Filipino children belonging to two of the HPgV risk groups: decompensated liver disease secondary to biliary cirrhosis and liver transplant pediatric patients. However, due to the small sample size, the results is not a good representation of the prevalence rate of HPgV infection among children belonging to the risk groups. Further studies with a larger and more significant sample size are recommended to be able to determine prevalence of HPgV infection among these groups and eventually in other pediatric patients under the other risk groups. In addition, the mothers of the children may also be included in the study since they are possible sources of infection. Studies regarding the pathogenicity of HPgV in Filipino children belonging to the risks groups are also recommended.

CONCLUSIONS

This study showed that a proportion of liver transplant patients and those with decompensated biliary cirrhosis are positive for HPgV anti-E2, which indicates that these individuals previously had HPgV infection but is now resolved. Possible source of infection is infected blood from the blood transfusions, infected organ or infected mother. Since routine HPgV screening is not yet recommended for the general population, blood donors and organ donors, the confirmation of exact source of infection may be difficult. Co-infection with HBsAg was also observed in both risk groups which suggests that at some point in time, these children were infected by both HPgV and HBV and also the possibility of simultaneous infection by the two viruses. This study provides preliminary data on the proportion of HPgV infection in Filipino children belonging to two of the HPgV risk groups. Future studies with a larger and more significant sample size to determine HPgV prevalence as well as studies regarding the pathogenicity of HPgV are warranted. Such studies may provide basis for routine HPgV screening among risk groups and blood donations in the future.

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Statement of Authorship

All authors have approved the final version submitted.

Author Disclosure

All authors declared no conflicts of interest.

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