ORIGINAL ARTICLE

Association between Guillain-Barré syndrome and Herpes virus family members, A Multiplex PCR study

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ABSTRACT

Background: *Guillain-Barré syndrome is a serious autoimmune peripheral neuropathy, in which the immune system attacks healthy nerve cells. The reason for this condition is unknown; however it is usually triggered by an associated infection, gastrointestinal or a respiratory, and to a less extent vaccination. Several infections have been encountered preceding Guillain-Barré syndrome: Campylobacter jejuni, Influenza, Cytomegalovirus, Epstein-Barr virus, Mycoplasma pneumonia and HIV.* ***Objectives:*** *The aim of this research is to evaluate the etiological correlation between CMV, EBV & HHV-6 infection and Guillain Barre syndrome.* ***Methodology:*** *Peripheral blood samples were collected from 30 patients attending the clinic of Neurology Department, as well as 20 control subjects, to identify the presence of the CMV, EBV & HHV-6 viral genome by Real-time multiplex RCR.* ***Results and Conclusion:*** *This study revealed that CMV UL 32 gene was significantly higher among studied cases as compared to controls and thus may be an attributable etiological agent for triggering the disease.*

INTRODUCTION

Guillain-Barré syndrome (GBS) is a rare, however serious autoimmune peripheral neuropathy (APN), in which the immune system attacks healthy nerve cells in peripheral nervous system. It was delineated by French physicians operating within the Sixth Army camp throughout the First World War. Its spectrum of presentation ranges from weakness, numbness, tingling to even paralysis. The reason for this condition is unknown; however it is triggered by an associated infectious illness, usually gastrointestinal disease or a respiratory infection as *Campylobacter Jejuni* or respiratory viral infection.[1,2]

Guillain-Barre syndrome is rare, affecting only about 0.4 to 2 in 100,000 Americans, according to the "National Institute of Neurological Disorders and Stroke". Males are affected more than females. The incidence rises with age; there is a minor peak among young adults. There is no cure for this syndrome, however treatment will diminish the severity of symptoms and shorten the length of the disease.[3,4]

The precise etiology of GBS is unknown. According to the statistics of the Centers for Disease Control and Prevention (CDC), more than half of individuals with GBS have developed it shortly after they have been infected with diarrheal disease or a respiratory tract infection. This implies that the disorder could also be triggered by associated improper immune response to the previous infection.[5]

Infections either bacterial or viral and to a less extent vaccination; are among triggers of GBS. Cross-reactivity between infectious and neural epitopes has been well demonstrated.[6]

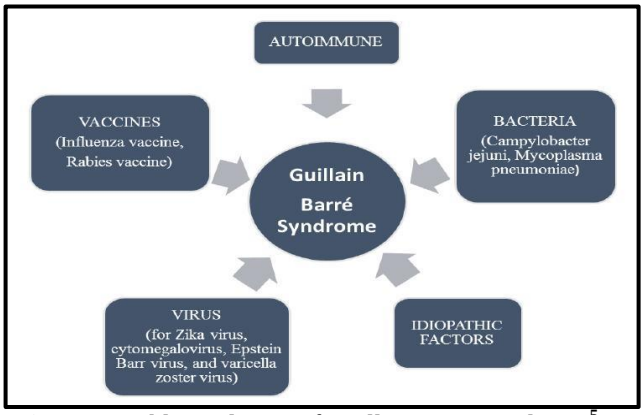

Fig. 1: Possible etiologies of Guillan Barre Syndrome[5]

Several infections have been associated with GBS: *Campylobacter jejuni* , Influenza, Cytomegalovirus, Epstein-Barr virus, *Mycoplasma pneumonia* and HIV.[7]

CMV is classified as the main viral cause of an infection preceding GBS, present in ten to fifteen percent of patients. The association between CMV and GBS was well-known since long time. The first case of GBS occurring in association with CMV infection was first reported in a renal transplant recipient in 1970. Second case was reported by Bale et al. of an active CMV infection inducing GBS. It is difficult to distinguish between primary and reactivation of infection, which is an important issue; because about

half of the immunocompetent subjects in developed countries have serological evidence of CMV exposure.[8]

CMV associated GBS presents a distinct entity occurring more in females with young age group showing severe initial course and respiratory difficulties, they often develop severe sensory loss and bilateral facial palsy. Delayed recovery is usually seen with elevated liver enzymes. Anti GM2 antibodies are significantly present in CMV associated GBS more than in controls. Diagnosis of CMV associated GBS is made according to positive serum and CSF fluid CMV PCR, the typical albuminocytologic dissociation of CSF analysis, and progressive neurological manifestations.[9]

EBV is the most common causal infectious agent associated with the mild form of GBS, and five to twenty eight percent of all GBS cases due to EBV were described as mild diseases. Moreover, severe forms of GBS complicated by respiratory failure or quadriplegia were associated with primary EBV infection. Therefore, infectious mononucleosis could be easily overlooked in patients with the mild form of GBS. The diagnosis of GBS could be missed in patients having initial symptoms of mild distal weakness and parasthesia, unless clinical neurological examination is accurately performed. [10]

Primary HHV-6 infection usually occurs in infants and it represents the most common cause of fever-induced seizures in children aged ranging from six months to two years. Acute infection is rare in immunocompetent adults but it may present as mononucleosis like illness. Post-transplant GBS has usually been related to infection and to a less extent to neurotoxicity of conditioning regimens. Approximately seventy five percent of patients have signs of infection shortly before diagnosis of GBS. A strong indication that the virus is actively replicating within the CNS is the detection of HHV-6 DNA in CSF. During the clinical course of GBS, an active HHV-6 infection in the CNS suggests also a coexisting infection might be associated with GBS and not only preceding one. [11]

Isolation of the virus or recognition of viral gene products is needed for the definitive diagnosis of herpesvirus infection. Improved serologic assays are becoming available, also the application of immunoblot technology. However, PCR DNA amplification technique has proved most successful in the diagnosis of Herpesvirus infections of the CNS, particularly when applied to CSF.[7]

The aim of this research is to evaluate the etiological correlation between CMV, EBV & HHV-6 infection and Guillain Barre syndrome, and also to investigate the possible relation between the viral load of CMV, EBV and HHV-6 and the severity of the disease according to Hughes grade scale for assessing functional motor deficits.

METHODOLOGY

Study population:

Peripheral blood samples (2 ml blood on EDTA, plasma) were collected from 30 patients attending the clinic of the Neurology Department at El Hadara University Hospital and diagnosed as Guillain barre syndrome according to Brighton criteria. Also samples were collected from 20 control subjects, apparently health persons, to identify the presence of the CMV, EBV & HHV-6 viral genome in their peripheral blood.

Data were collected from our patients including medical history, clinical and neurological evaluation using Brighton's criteria, as well as disability evaluation by Hugh's scale. Informed consent was obtained from all cases included in the study. This study was approved by the Ethics committee, Faculty of Medicine, Alexandria University.

Multiplex PCR Amplification:

DNA was isolated from 200 µl of plasma using the QIAamp DNA blood mini kit (QIAGEN GmbH, QIAGEN strasse 1,40724 Hilden, Germany) according to manufacturer 's instructions following the blood DNA purification protocol.[12]

The multiplex real-time PCR detects a wide array of viruses with broad dynamic range, reproducibility, good lower limit of detection (sensitivity) and specificity when tested with heterologous viruses. The cytomegalovirus UL 32 gene codes for large structural phosphoprotein that participates in last steps of viral maturation, HHV 6 U67 gene is present as only one copy per virus genome that is considered as possible DNA packaging protein, and the EBV BHRF1 gene is a highly conserved sequence that has an anti-apoptotic function. PCR amplification was performed with a thermal cycler (Applied Bio-system One Step).[13,14]

For real time PCR assay, 25 µl reaction mixture was prepared as follows: 12.5 µl Maxima SYBR Green/ROX qPCR Master Mix, forward and reverse primers 1 µl each, 5.45 µl of DNA extract made up to 25 µl with water. Thermal cycling conditions included an initial cycle of 50ºC for 2 minutes and 95ºC for 10 minutes followed by 40 cycles of 95ºC denaturation for 15 seconds, optimized annealing temperature 60ºC for 30 seconds and extension step at 72ºC for 30 seconds. This is followed by melting point analysis consisting of 95ºC for 30 seconds followed by cooling to 60ºC for 60 seconds at a rate of 0.2ºC/s with continuous fluorescence acquisition. Positive CMV DNA samples had a cycle threshold (CT) less than 40, and melting temperature (Tm) of 86ºC for CMV, 60ºC for HHV6 and 86ºC for EBV.

Table 1: Oligonucleotide primer sequence for UL32, U67 and BHRF1 genes

Target	Nucleotide sequence (5'-3')	Function	Tm
UL 32	F: TGCAGTTTGGTCCCTTAAAG R: AAGAATCCTCACCTGGCTTA	Codes for large structural phosphoprotein and participates in last steps of viral maturation.	86ºC
U 67	F: AAGCTTGCACAATGCCAAAAAACAG R: CTCGAGTATGCCGAGACCCCTAATC	Present as only one copy per virus genome and it is possible DNA packaging protein.	60ºC
BHRF1	F: GGA GAT ACT GTT AGC CCT G R: GTG TGT TAT AAA TCT GTT CCAAG	A highly conserved sequence that has an anti-apoptotic function.	86ºC

RESULTS

All patients included in the study were in the age range (9 – 80) years with a mean of 43.10 ± 17.97 and a median 42 years. The age of control subjects ranged from 21 to 63 years with a mean of 37.15 ± 12.11 and a median of 33.5 years. There were no statistical significant differences between the two groups according to age (p=0.201). Out of the total 30 patients male sex represented 56.7 % of patients (n=17) and 50 % of control (n=10), while female sex represented 43.3 % of patients (n=13) and 50 % of control (n=10). There were no statistical significant differences between the two groups according to sex (p=0.643).

Table 2: Comparison between the two studied groups according to demographic data

	Cases (n=30)		Control (n=20)		Test of sig.	p
	No.	%	No.	%		
Sex						
Male	17	56.7	10	50.0	χ^2= 0.125	0.643
Female	13	43.3	10	50.0		
Age (years)						
Min. – Max.	9.0 – 80.0		21.0 – 63.0		t= 1.296	0.201
Mean ± SD.	43.10 ± 17.97		37.15 ± 12.11			
Median	42.00		33.50			

χ^2: Chi square test **t: Student t-test** p: p value for comparing between the two groups

All patients enrolled in the current study (30 patients -100 %) were presenting with upper & lower limb (UL and LL) weakness (n=30), while 93.3% of patients were presenting with UL and LL numbness (n=28). On the other hand, 16.7% of patients were presenting with bulbar symptoms (n=5), while 6.7% of patients were presenting with sphincteric symptoms (n=2).

Table 3: Distribution of the studied cases according to clinical presentation (n=30)

	No.	%
UL & LL weakness		
Absent	0	0.0
Present	30	100.0
UL & LL numbness		
Absent	2	6.7
Present	28	93.3
Bulbar symptoms		
Absent	25	83.3
Present	5	16.7
Sphincteric symptoms		
Absent	28	93.3
Present	2	6.7

About 90% of patients had antecedent upper respiratory tract (URT) infection symptoms before onset of GBS symptoms (n=27), while only 10% of patients had no antecedent URT symptoms before onset of GBS symptoms (n=3).

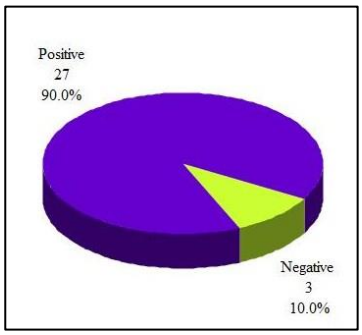

Fig. 2: Distribution of the studied cases according to URT symptoms.

As for the nerve conduction study, out of the total number of 30 patients, 80% of patient had AIDP (n=24), 13.3% of patient had AMSAN (n=4) and 2% of patient had AMAN (n=2).

Table 4: Distribution of the studied cases according to NCS (n=30)

NCS	No.	%
AIDP	24	80.0
AMSAN	4	13.3
AMAN	2	6.7

Regarding the total number of 30 patients, 30% of patients with GBS were positive for BHRF1 gene of EBV (n=9), while 30% of control were positive for BHRF1 gene of EBV (n=6). No significant statistical correlation between presence of EBV DNA in the blood and occurrence of GBS in our study.

On the other hand, 10% of patients with GBS were positive for U67 gene of HHV 6 (n=3), while 5% of control were positive for U67 gene of HHV 6 (n=1). This difference is not significant with p value 0.641. No significant statistical correlation between the presence of HHV6 DNA in the blood of patients and occurrence of GBS in our study.

Moreover, 26.7% of patients with GBS were positive for UL32 gene of CMV (n=8), while none of the control subjects was positive for UL32 gene of CMV (n=0). This difference is significant with p value 0.015; CMV may be a trigger of GBS in patients in our study.

Table 5: Comparison between the two studied groups according to the genes of different Herpes viruses

	Cases (n=30)		Control (n=20)		χ^2	p
	No.	%	No.	%		
BHRF1 gene for EBV						
Positive	9	30.0	6	30.0		1.000
Undetermined	21	70.0	14	70.0		
U67 gene for HHV 6						
Positive	3	10.0	1	5.0	0.408	^{FE}p= 0.641
Undetermined	27	90.0	19	95.0		
UL32 gene for CMV						
Positive	8	26.7	0	0.0	6.349*	0.015*
Undetermined	22	73.3	20	100.0		

χ^2: Chi square test **FE:** Fisher Exact
p: p value for comparing between the two groups
*: Statistically significant at $p \leq 0.05$

As regards the correlation between the viral load and the severity of GBS symptoms for positive cases. The severity of symptoms was assessed according to the Hughes grade scale of disability with the majority of cases with the grade 4 (confined to bed or chair bound). The r_s and p value were 0.077 and 0.786 respectively, so there was no significant correlation between the viral load and the severity of GBS symptoms in our study. This may be attributed to small number of the studied group.

Table 6: Correlation between viral load and severity for positive cases

	Viral load	
	r_s	p
Hughes scale (severity)	0.077	0.786

r_s: Spearman coefficient

DISCUSSION

Guillain-Barre is a syndrome characterized by fulminant autoimmune polyradiculoneuropathy which is considered to be acute and in most cases severe in nature. It is the most common cause of acute or subacute generalized paralysis and is also known as Landry-Guillain-Barré-Strohl syndrome and acute inflammatory demyelinating polyneuropathy (AIDP). Global annual incidence is reported to be 0.6–2.4 cases per 100,000 per year. Although the etiology of GBS is not completely understood, it is believed to be due to autoimmune reaction to infection, stimulating anti-ganglioside antibodies production being the trigger in the majority of cases. Symptoms appear 1–3 weeks after an acute infectious process in most cases. Many organisms thought to be involved like; *Campylobacter jejuni* (diarrhea), *Mycoplasma pneumonia*, *Haemophilus influenzae*, cytomegalovirus, Epstein-Barr virus and influenza. Although administration of outmoded anti-rabies vaccines and A/New Jersey (swine) influenza

vaccine was considered to be associated with a slight increase in GBS incidence, the new influenza vaccines appear to confer risk of less than 1 per million and are relatively safe.[15,16]

The aim of the current study was to evaluate the etiological correlation between CMV, EBV & HHV-6 infection and Guillain Barre Syndrome. Evaluating the possible relation between the viral load of CMV, EBV and HHV-6 and severity of the disease according to Hughes grade scale for assessing functional motor deficits was also our aim. The demographic data of the studied group revealed that the age range was (9-80) years with a mean of 43.10 ± 17.97 and a median 42 years. The age of control subjects ranged from 21 to 63 years with a mean of 37.15 ± 12.11 and a median of 33.5 years. There were no statistically significant differences between the two groups according to age (p=0.201). These results were comparable to the study of Fokke *et al.* [17] that enrolled patients with age range (36-66) years with a median of 53 years. As regards sex, male sex represented 56.7 % of patients (n=17) and 50 % of control (n=10), while female sex represented 43.3 % of patients (n=13) and 50 % of control (n=10). There were no statistically significant differences between the two groups in our study according to sex (p=0.643). These results were not far from Fokke *et al.* [17] results that were 55% for male sex and 44% for female sex denoting slight increase in the prevalence of GBS in males.

On the other hand, another finding of our study is that the prevalence of antecedent URT symptoms was 90% and only 10% for gastroenteritis. These results were in accordance with the results of Sadek et al. [18] in which antecedent infections were found in 33 patients (66%), of them, 25 (76%) had upper respiratory tract infection, gastroenteritis was present in seven patients (21%) while urinary tract infection was present in only one patient (3%). Similarly, the study of Greene et al. also reported that the most common associated symptoms were URT symptoms followed by GI symptoms, however, the percentage of URT symptoms was significantly lower than our results, only (50%). Furthermore, Zhang et al. [19] also reported that URT symptoms were significantly common among patients with GBS; moreover, they found more than 55% of the patients had a respiratory tract infection proof before the onset of symptoms of GBS.

The most common clinical presentation in our study was upper and lower limb weakness 100% (n=30), while upper and lower limb numbness 93.3% (n=28). The less common symptoms were bulbar symptoms and sphincteric symptoms with prevalence of 16.7% (n=5) and 6.7% (n=2) respectively.

Dimachkie et al. [20] showed that acroparesthesia with little objective sensory loss are the most common initial symptom of GBS. Severe radicular back pain or neuropathic pain affects most cases. Within a few days, weakness ensues commonly in a symmetric "ascending pattern". Most patients present initially with leg weakness and arm weakness (32%) or selective proximal and distal leg weakness (56%) often spreading to the arm while some have onset of weakness in the arms (12%). This prevalence of weakness was significantly lower than found in the current study.

However, Asiri et al. [21] who aimed to determine the pattern of muscle weakness in patients with GBS, had results that was not far from the current study. They found that in 80% of patients, muscle weakness started in lower limbs while at presentation four limb weaknesses was the most frequent (96%). They also found that the upper extremity weakness was mainly distal in 73% of patients, while lower extremity weakness was mainly proximal in 68%. Another finding of their study was that weakness in extremities associated with cranial nerve involvement occurred in 72% of patients. Trunk muscles were involved in 34%. Various modes of spread of muscle weakness were seen in their study but the ascending variety was the most common occurring in 78% of patients and it was characterized by upward spread.

Uncini and his colleagues [22] aimed to study clinical and nerve conduction features in GBS associated with Zika virus infection. They found that AIDP was diagnosed in 70% of patients. Moreover, 40% of nerves of AIDP patients showed a prevalent distal demyelinating involvement.

This study concluded that the prevalence of BHRF1 gene for EBV was the same in both patients with GBS and the healthy subjects (30% both). The prevalence of U67 gene for HHV6 was also comparable in both patients and healthy subjects (10% and 5% respectively).

The prevalence of UL32 gene for CMV was significantly higher in patients with GBS than the healthy subjects (26.7% and none of healthy control subjects respectively).

On the other hand, Taheraghdam et al.[23] used ELISA to detect serum antibodies for CMV and EBV. They concluded that CMV-IgM was positive only in serum of one patient (3.3%) but CMV-IgG were positive in 29 patients (96.7%). As for EBV-IgG was detectable in 27 patients (90%) and none with EBV-IgM. Moreover, the results done by Armin et al. [24] were four cases, (26.6%) had evidence of past CMV infection (with positive serum CMV-IgG). One patient was diagnosed with recent infection (positive serum CMV-IgM and IgG) and 2 cases (13.3%) with active infection (positive CMV PCR and CMV-IgM, and IgG in serum sample). Although CMV DNA was not detected in the CSF of any patient with GBS, IgM antibody had positive results in CSF in 2 cases. Seven patients (46.6%) had past EBV infection with positive results for serum IgG and negative IgM or PCR. Four patients (26.6%) had positive results for IgG in CSF,

and one (6.6%) of them had positive result for IgM, revealing recent EBV CNS infection.

Wahren et al [25] partially agreed to our results as they found the mean serum antibodies titer to EBV virus capsid antigen (EBV-VCA) was higher in GBS patients. Nine of the 15 GBS patients (60%) displayed a four-fold variation in antibody titer to EBV-VCA during the course of their disease. The mean titers against CMV and HSV were also slightly higher in GBS patients compared with controls. The CSF antibody titers against EBV were higher in the beginning of the disease compared with the values during convalescence. No measurable CSF antibody titers against CMV were found. Tam et al [26] found that the prevalence of EBV infection in patients with GBS was 33% which was comparable to our results. Similarly, Kaneko et al [27] found that the prevalence was as high as 56%.

Although 10% of patients with GBS were positive for U67 gene of HHV 6 (n=3), while 5% of control were positive for U67 gene of HHV 6 (n=1) in the current study, Merilli et al found that the prevalence of HHV6 was 33 % significantly higher than our results. [28]

In this study we also investigated the possible correlation between the viral load and the severity of GBS symptoms and found that there was no significant impact of the viral load on the severity of the disease, this can be attributed to the limited number of the studied group.

CONCLUSION

Finally, Molecular techniques can be a good diagnostic tool added to the traditional diagnosis of GBS that is usually made on clinical basis; further research should be done to confirm the benefit of the use of antiviral drugs to shorten the hospital stay and improve the outcome especially in CMV triggered GBS patients.

Conflicts of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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