Prevalence and association of Human Parvovirus B19V with Hepatitis B and C viruses in Nigeria

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Tables 1: Comparison of subgroups of patients and controls

	HBV infected n=76	HBV HCV co infected n=17	Non HBV/HCV n=44
Mean Age	35.4	31.5	35.5
Gender (M/F)	48/28	13/4	19/25
IgM positives	24 (32%)	6 (35%)	14 (32%)
IgG positives	25 (33%)	8 (47%)	12 (27%)
B19V DNA positives	8 (11%)	3 (18%)	4 (9%)
HBV DNA positives	47 (62%)	10 (59%)	0 (0%)

Table 2: Parvovirus B19V serological and DNA status in HBV infected individuals

	IgG(-) IgM (-)	IgG (+) IgM (-)	IgG(+) IgM(+)	IgG(-) IgM (+)
B19V DNA +ves n=8	2 (25%)	1 (13%)	1 (13%)	4 (50%)
B19V DNA –ves n=68	35 (51%)	14 (21%)	9 (13%)	10 (15%)

Table 3: Parvovirus B19V serological and DNA status in HBV HCV co-infected individuals

	IgG(-) IgM (-)	IgG (+) IgM (-)	IgG(+) IgM(+)	IgG(-) IgM (+)
B19V DNA +ves n=3	1 (33%)	0 (0%)	1 (33%)	1 (33%)
B19V DNA –ves n=14	6 (43%)	4 (29%)	3 (21%)	1 (7%)

Table 4: Characteristic of HBV infected patients segregated according to clinical presentation

Characteristics	Symptomatic HBV patients	Asymptomatic HBV carriers
Mean Age (yrs)	35.5	35.4
Gender (M/F)	11/9	37/19
HBsAg positive	20 (100%)	56 (100%)
Anti HCV positive	3 (15%)	14 (25%)
HBV DNA positive	11 (55%)	36 (64%)
B19V DNA positive	4 (20%)	4 (7%)
B19V IgG positive	4 (20%)	18 (32%)
B19V IgM positive	**7 (35%)**	**17 (30%)**

Table 5: Genotype distribution of identified parvovirus B19V isolates

Sample No	Age (yrs)	Sex	Anti HCV	HBsAg	Genotype
NGA 125	21	F	Neg	Neg	1B
NGA 507	24	F	Neg	Pos	1A
NGA 218	30	M	Neg	Pos	1B
NGA 149	26	M	Pos	Pos	1A
NGA 572	43	F	Neg	Pos	1B
NGA 84	58	F	Neg	Neg	1A
NGA 331	20	M	Neg	Pos	3B
NGA 373	26	M	Neg	Neg	1B
NGA 160	44	F	Neg	Neg	1B
NGA 177	36	M	Pos	Pos	1A
NGA 167	42	F	Pos	Pos	1B
NGA 668	31	M	Neg	Pos	3B

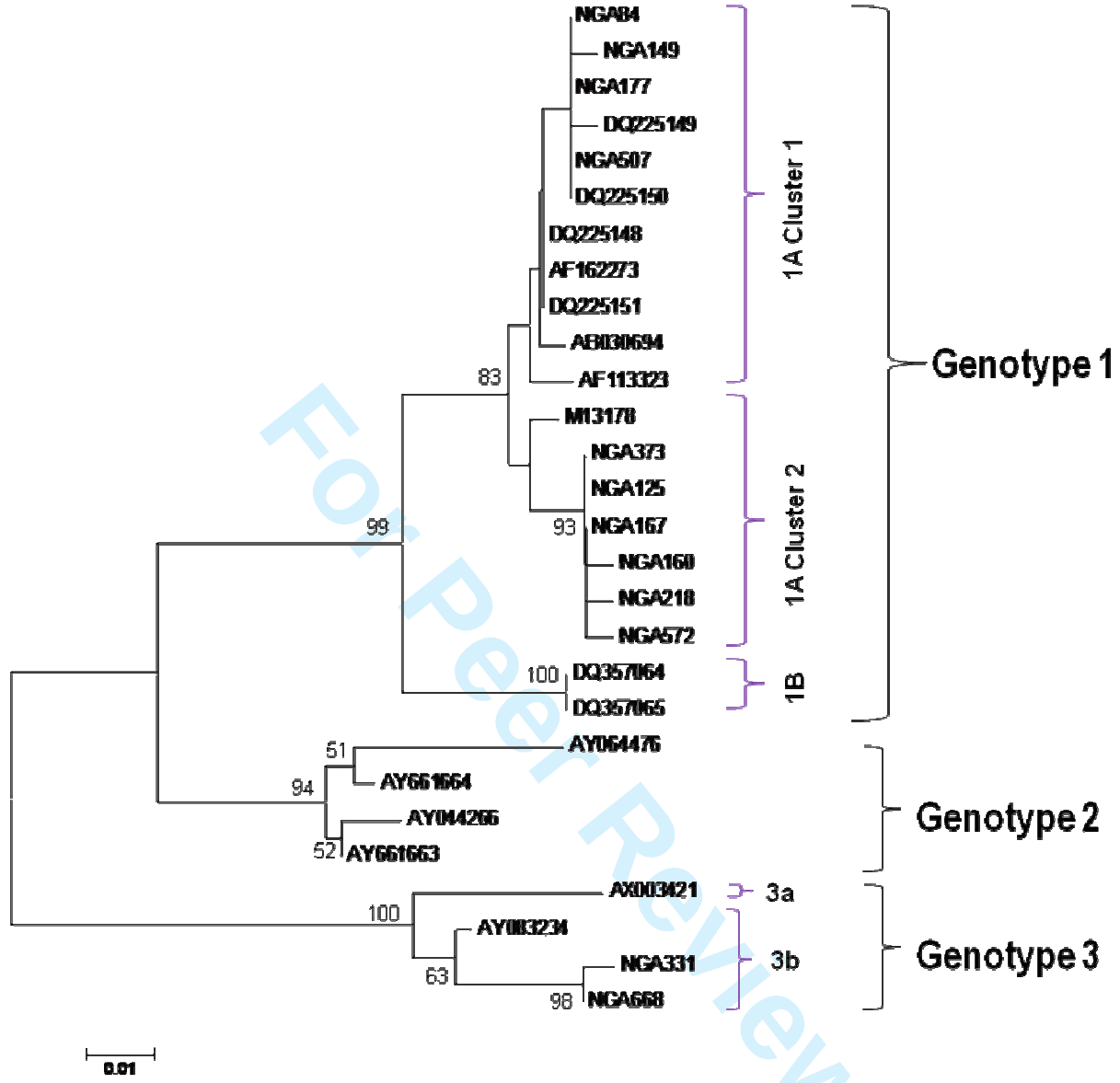

FIG. 1. Phylogenetic tree constructed from partial parvovirus B19 NS1/VP1u region. Twelve B19-positive samples from Nigeria [NGA 668, NGA167,NGA 177,NGA 160, NGA373, NGA 331, NGA84, NGA572,NGA149,NGA218,NGA507 and NGA125] were aligned with comparative reference sequences identified by their GenBank accession numbers : Genotype 1, AB030694,AF113323, AF162273, M13178, DQ225148, DQ225149, DQ225150, DQ225151, DQ357064 and DQ357065; Genotype 2, AY064476, AY044266, AY661663 and AY661664; Genotype 3, AX003421 and AY083234].The phylogenetic tree of a distance matrix [Kimura two parameter method] was created using the neighbor- joining method bootstrap 1000 replicate. Bar, 0.01 nucleotide substitutions per site.

Fig 2a. Nucleotide sequence alignment of 12 Nigerian B19 isolates and 16 reference sequence identifiable by their accession number.

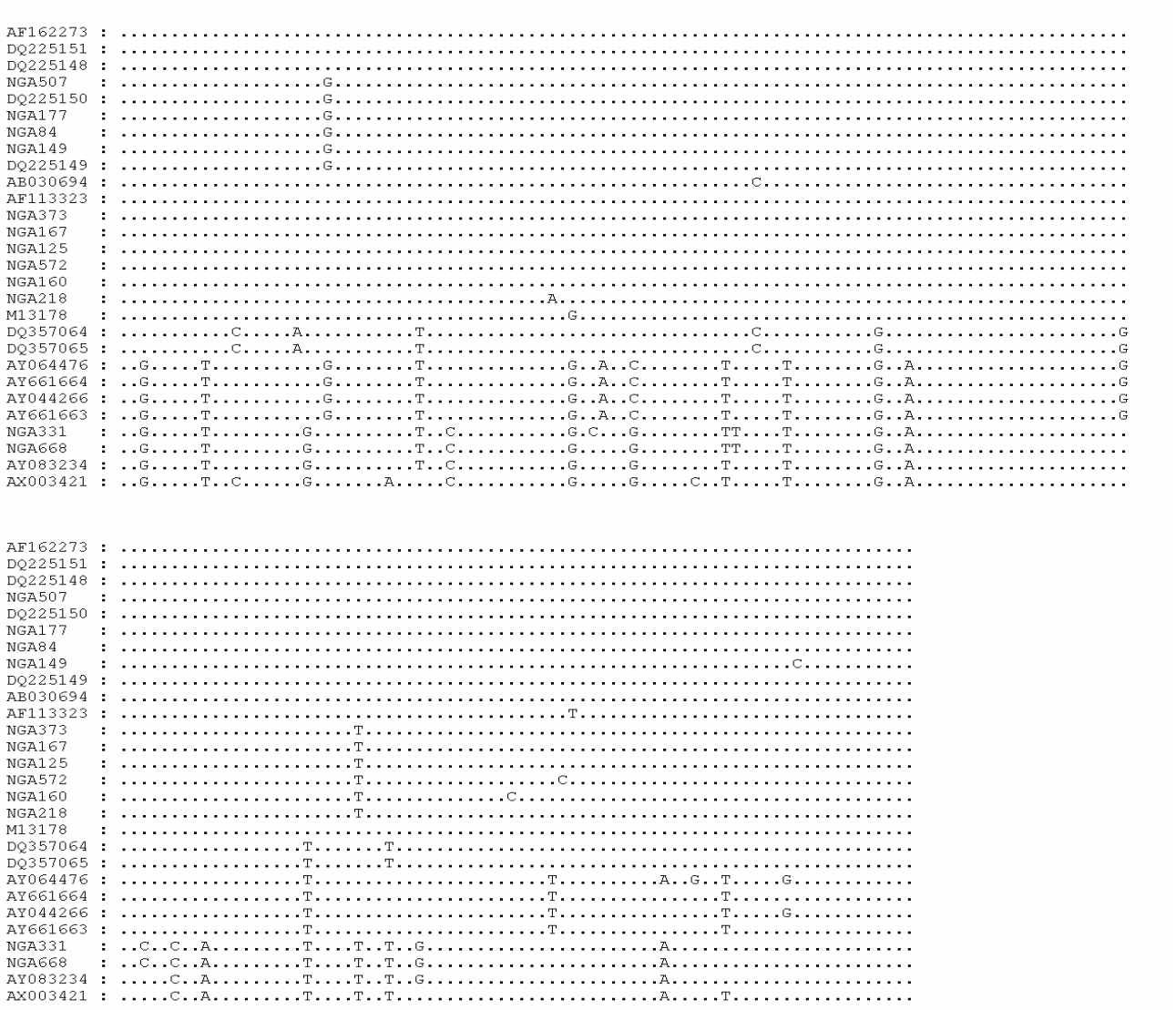

Fig 2b. Alignment of the translated amino acid sequence of NS1 region of 12 Nigerian B19 isolates and 16 reference sequences identifiable by their accession number.

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AY064476 LPVCCVQHIN NSGGGLGLCP HCINVGAWYN GWKFREFTPD LVRCSCHVGA SNPFSVLTCK KCAYLSGLQS FVDYE
AY044266 .......... .......... .......... .......... .......... .......... .......... .....
AY661663 .......... .......... .......... .......... .......... .......... .......... .....
AY661664 .......... .......... .......... .......... .......... .......... .......... .....
DQ357064 .......... .......... .......... .......... .......... .......... .......... .....
DQ357065 .......... .......... .......... .......... .......... .......... .......... .....
NGA373   .......... .......... .......... .......... .......... .......... .......... .....
NGA167   .......... .......... .......... .......... .......... .......... .......... .....
NGA125   .......... .......... .......... .......... .......... .......... .......... .....
NGA572   .......... .......... .......... .......... .......... .......... ....P..... .....
NGA160   .......... .......... .......... .......... .......... .......... .......... .....
NGA218   .......... ....S..... .......... .......... .......... .......... .......... .....
AF113323 .......... .......... .......... .......... .......... .......... .......... .....
M13178   .......... .......... .......... .......... .......... .......... .......... .....
AF162273 .......... .......... .......... .......... .......... .......... .......... .....
DQ225151 .......... .......... .......... .......... .......... .......... .......... .....
DQ225148 .......... .......... .......... .......... .......... .......... .......... .....
NGA177   .......... .......... .......... .......... .......... .......... .......... .....
NGA84    .......... .......... .......... .......... .......... .......... .......... .....
Q225150  .......... .......... .......... .......... .......... .......... .......... .....
NGA507   .......... .......... .......... .......... .......... .......... .......... .....
NGA149   .......... .......... .......... .......... .......... .......... .......... ..H..
DQ225149 .......... .......... .......... .......H .......... .......... .......... .....
AB030694 .......... .......... .......... .......... .......... .......... .......... .....
NGA331   ......E... .....S.... Y......... .......... .......... .......... .......... .....
NGA668   ......E... .......... Y......... .......... .......... .......... .......... .....
AY083234 ......E... .......... .......... .......... .......... .......... .......... .....
AX003421 ......E... .......... .......... .......... .......... .......... .......... .....
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Abstract

Co-infection of parvovirus B19 with hepatitis B virus has been found in patients with acute and chronic hepatitis. The clinical significance of parvovirus B19 in hepatitis B co-infected patients is still controversial. In this study parvovirus B19 antibodies and DNA were investigated in serum samples from 76 patients with HBV infection, 17 with HBV/ HCV co-infection and 44 healthy controls. In the sera from patients with HBV infection, anti-B19V IgM and IgG antibodies were detected in 24/76 (32%) and 25/76 (33%), in 6/17 (35%) and 8/17 (47%) of HBV/HCV co-infected patients, and in 14/44 (32%) and 12/44 (12%) of a non-hepatitis healthy controls respectively. B19V DNA was detected in 8/76 (11%) of patients with HBV infection and in 3/17 (18%) of patients with a HBV/HCV co-infection, and in 4/44 (9%) healthy controls. The occurrence of parvovirus B19 DNA was significantly higher in patients with symptomatic HBV 4/20 (20%) compared to asymptomatic HBV carrier 4/56 (7%) ($p < 0.05$). Ten of the positive B19V DNA sequences belonged to B19V genotype 1 while two belonged to genotype 3. The results of this study showed a significant difference in the prevalence of parvovirus B19 DNA in symptomatic HBsAg-positive as compared to asymptomatic HBsAg positive individuals, however the conclusion that parvovirus B19 infection increased the frequency of liver disease was not supported. Long-term longitudinal studies are, however, required to determine the synergistic effect of parvovirus B19 infection in HBV or HBV and HCV co-infected persons.

INTRODUCTION

Human parvovirus B19 infection is common worldwide and epidemics occur across the Americas, Europe and Asia[Young and Brown 2004]. The virus is transmitted effectively by close contact or blood transfusion. The prevalence of specific IgG antibodies against B19V increases with advancing age: while it is about 15% in children, it increases to approximately 30 to 60% in adults and it exceeds 85% in the elderly population[Henriques et al., 2005].

Human parvovirus B19 is a member of the erythroviruses within the parvoviridae family. It is a small, non-enveloped virus containing a single-stranded DNA of 5600 nucleotides and composed of two capsid proteins, VP1 (84kDa) and VP2 (58kDa), and a non-structural protein, NS1 (77kDa). The two capsid proteins have the same open reading frame and VP1 is identical to VP2 except for an additional 227 amino acids at the NH2-terminus. Over 95% of capsid proteins are VP2, while VP1 accounts for less than 5%. These B19V structural proteins are known to determine the virus tropism and elicit neutralizing antibody responses [Agbandje et al., 1995; Brown et al., 1991; Cotmore et al., 1986]. These neutralizing antibodies are directed at epitopes from both VP2 and the unique region of VP1. Recently, several strains with considerable sequence diversity were discovered, resulting in the identification of three distinct genetic clusters; genotypes 1,2 and 3 are responsible for the majority of human infections worldwide [Servant-Delmas et al., 2009a; Servant-Delmas et al., 2009b].

During the course of B19V infection, a high-titer viremia can appear and last approximately 6–8 days; the viral titer then decreases and viral DNA is detectable for several months by polymerase chain reaction (PCR). Specific IgM antibodies can be determined in parallel to the onset of symptoms (12–17 days after exposure) and decrease between 30 and 60 days later.

Moreover, IgG antibodies appear several days after IgM and remain for several years indicating past infection [Grossebley et al., 1994; Nishida et al., 1997].

Human parvovirus B19 has been associated with a variety of clinical manifestations including erythema infectiosum, hydrops fetalis, aplastic anemia, thrombocytopenia, leukopenia, myocarditis, arthritis, and vasculitis [Bizjak et al., 2009; Booth et al., 2010; Buyukkose et al., 2009]. Recently, hepatic involvement has also been reported in patients with parvovirus B19 infection, especially in patients with fulminant hepatitis or acute hepatitis although the role of this virus as a possible pathogen causing fulminant, chronic or acute hepatitis is still controversial [Hsu et al., 2005; Toan et al., 2006; Wong et al., 2003]. However it is known that the cellular receptor of B19V is the P antigen located on the hepatocytes [Schneider et al., 2008; Soderlund et al., 1997]. Therefore, the virus may play a role in aggravating the liver disease resulting from hepatitis B and C virus infection as reported previously [Hsu et al., 2005].

In Nigeria HBV and HCV infection are endemic with a seroprevalence of approximately 13%-16% and 3%- 5% respectively [Imarengiaye et al., 2006; Mabayoje et al., 2007; Opaleye et al., 2010; Uneke et al., 2005], however the seroprevalence of parvovirus B19 in the general population is yet unknown. Data on parvovirus B19 in Nigeria is limited to one recent study, in which five isolates of B19V DNA were found from patients with rash or fever and negative for both measles and rubella in Nigeria and characterized [Hubschen et al., 2009].

The aim of the present study was to investigate the association of parvovirus B19infection in people with hepatitis B or C infection as compared to persons without hepatitis B or C, as well as symptomatic hepatitis B patients as compared to asymptomatic HBV carriers in Nigeria.

METHOD

Study subjects

Serum samples were collected from patients diagnosed with hepatitis with clinical symptoms and healthy blood donors. In total, 20 samples were collected from symptomatic persons infected with HBV and 56 from asymptomatic hepatitis B carrier while 44 persons negative for HBV and HCV were recruited as controls. Approximately 5ml of blood was drawn intravenously from each individual after informed consent. The study was approved by the ethical committee of Ladoke Akintola University of Technology Teaching Hospital, Osun, Nigeria.

Detection of HBsAg and anti-HCV antibody

Sera were tested for HBsAg and Anti-HCV using a third-generation enzyme-linked immunosorbent assay [ELISA] (Human diagnostics, Wiesbaden, Germany). All tests were performed following manufacturer's instructions.

Detection of anti-B19V antibodies.

Antibodies to human parvovirus B19were detected by using parvovirus B19 IgM and IgG enzyme immunoassay Kits (Parvovirus B19 IgG and IgM, DxSelect ™ FOCUS Diagnostic. Germany). These assays use a recombinant VP1 protein to capture IgG and IgM. Both assays were performed and interpreted according to the manufacturer's instructions.

DNA purification and polymerase chain reaction amplification for parvovirus B19

DNA was extracted from all serum samples using a QIA Amp blood kit (QIAgen, Hilden, Germany) as directed by the manufacturer. The detection of B19V DNA was done by nested PCR (nPCR) using primers specific for the VP1/VP2 coding sequence has been described previously [Bultmann et al., 2003]. All samples testing positive for B19V were confirmed with a second PCR, which was different from the first PCR in order to exclude contamination, using

B19V primers (P5F and P5R for the first PCR and n-P5F and n-P5R for the nPCR) specific for the subgenomic NS1/VP1u region from nt 2355 to 2690 (numbering according to GenBank accession no. AF162273) as previously described [Toan et al., 2006]. The PCR conditions used for the first PCR consist of a 35 cycles of 94^{o}C for 30s, 48^{o}C for 30s and 72^{o}C for 45s and a 40 cycles of 94^{o}C for 30 s, 50^{o}C for 30 s, 72^{o}C for 45 s was used for the nPCR.

Polymerase chain reaction amplification for HBV

The presence of HBV DNA was examined in all samples using a diagnostic PCR approach. Primer pairs designed from the highly conserved regions of the S-gene of the HBV was utilized. A nPCR was also performed for all 120 individuals: First using an outer primer pair HBPr134 (sense) 5'-TGCTGCTATGCCTCATCTTC - 3' and HBPr135 (antisense) 5'- CAGAGACAAAAGAAAATTGG – 3´ genomic DNA was amplified. PCR amplifications were carried out in 25 µl reaction volumes with 5 ng of genomic DNA, 10x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl; Qiagen), 2 mM of dNTPs, 50ng of each primer and 1 U Ampli Taq gold DNA polymerase (Applied Biosystems) on a PTC 200 (Peltier Thermal cycler). Thermal cycling parameters were: initial denaturation at 94 $^{\circ}$C for 2 min, followed by 35 cycles of 30sec at 94 $^{\circ}$C denaturation, 30 sec at 52 $^{\circ}$C annealing temperature, 45 sec at 72 $^{\circ}$C extension, followed by a final extension of 5 min at 72 $^{\circ}$C. Subsequent PCR was performed with an inner primer pairs HBPr75 (sense) 5' – CAAGGTTATGTTGCCCGTTTGTCC - 3' and HBPr94 (antisense) 5'- GGTATAAAGGGACTCACGATG-3'. Thermal cycling parameters remained the same as mentioned above except for the number of cycles that is increased to 40 cycles of amplification. Each PCR product (5 µl) was analysed by electrophoresis in 2% agarose gels. A positive control (HBV plasmid DNA) and negative controls

were integrated to each of these runs to validate the PCR products that yielded a 340bp fragment.

Sequencing and phylogenetic analysis of parvovirus B19

The B19V DNA positive samples were sequenced after using EXO-SAP-IT (exonuclase I and stream alkaline phosphatase enzyme, USB, Cleveland, OH, USA), by incubating 5ul of the PCR product with the 2ul of the enzyme at 37^{o}C for 15min then at 85^{o}C for 15min. The PCR products were sequenced twice using the forward and reverse primers (nP5R and nP5F), for sequencing of a fragment of the NS1/VP1u region from nt 2355 to 2690 (numbering according to GenBank accession no. AF162273). The sequencing was done using dye labeled dideoxy terminators (Big Dye, Applied Biosystems, Foster city, USA) BD and analysed on a ABI PRISM Genetic analyzer 3100 (Applied Biosystems).

B19V genotype analysis

B19V sequences were aligned by using CLUSTAL_W [Thompson et al., 1994] and BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/blast/blast.cgi). The reliability of alignment was additionally checked by using the BioEdit program (Department of Microbiology, North Carolina State University, Raleigh, NC, USA; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic and molecular evolutionary analyses were conducted by the neighbour-joining method using *MEGA* version 4 [Tamura et al., 2007]. Prototype B19V sequences from the GenBank were used as reference sequences (GenBank accession numbers were as follows: genotype 1, AB030694,AF113323, AF162273,

M13178, DQ225148, DQ225149, DQ225150, DQ225151, DQ357064 and DQ357065; genotype 2, AY064476, AY044266, AY661663 and AY661664; genotype 3, AX003421 and AY083234).

Statistical analysis.

Statistical analysis was performed by using the SPSS software (Chicago IL version 15.0). Categorical variables were compared using Fishers exact test.

RESULTS

PCR and ELISA analysis for the presence of B19V DNA and antibodies in patient samples

In order to determine the presence of B19V DNA in peripheral blood samples of 120 Nigerians, nPCR was used for amplifying B19V amplicons from the coding VP1/VP2 and confirmed by a second PCR coding NS1/VP1u regions. The results indicated that B19V genomes were identified in 8 of 76 (11%) patients infected with HBV and 4 of 44 (9%) individuals in the control group. Serum samples taken from the same patients were also tested for IgM and IgG of B19V antibodies. Anti-B19V IgM and IgG antibodies were detected in 24 (32%) and 25 (33%) of 76 samples from patients with HBV infection, 6 (35%) and 8 (47%) of 17 serum samples from patients with HBV/ HCV co-infection, respectively. The presence of B19V IgM and IgG antibodies was detected in 32% and 27%, respectively, among the 44 controls (Table 1). The frequency of anti-B19V IgG(-)IgM(+) was 50% in B19V DNA positive individuals co-infected with HBV, while it was 15% in B19V DNA negative individuals indicating recent infection and a higher level of viremia in individuals with detectable B19V DNA (Table 2). The occurrence of parvovirus B19 DNA was significantly higher in patients with symptomatic HBV 4/20 (20%) compared to asymptomatic HBV carrier 4/56 (7%) ($p < 0.05$) (Table 4).

Nucleotide sequence and phylogenetic analysis

Twelve B19V sequences of the NS1/VP1u region from this study and 16 reference sequences from the GenBank were analysed using the phylogenetic tree method. From this analysis, 10 of the Nigerian samples clustered with B19V genotypes 1 while two clustered with genotype 3. Particularly, 4 samples clustered with genotype 1A while 6 clustered with genotype 1B and the remaining 2 of the Nigerian isolates clustered with genotype 3B (Fig 1). Interestingly 6 sequences from the Nigerian isolates clustered in a different branch within the genotype 1A with a strong bootstrap of 83 at 1000 bootstrap replicate along with a reference sequence GenBank accession number M13178 while the other 4 clustered with the reference sequence with GenBank accession numbers AB030694, AF113323, AF162273, DQ225148, DQ225149, DQ225150 and DQ225151 (Fig 1).

Nucleotide and amino acid divergence in the Nigerian Isolates

The mean genetic distance among the genotype 1 sequence in this study within the 252nt region sequenced was 3% and the maximum genetic distance was 5% between reference sequence DQ357064, DQ357065 and the samples NGA218, NGA373, NGA125, NGA167, NGA160 and NGA572. From the Nigerian isolates some unique nucleotide and amino acid divergences when compared with the 16 reference sequence using pairwise analyses of nucleotide sequences coding for the NS1/VP1 region were identified. In sample NGA149 there was a G to C change at position 2619, there was G2445A in NGA218, A2631G in NGA668, T2449C and A2631G in NGA 331 which translate to an amino acid substitution of W817R and

L852P in this sample NGA331. An L864P mutation was seen in NGA160, C866R in NGA572 and L852P mutation in NGA668 (Fig 2a and 2b). The sequences data from the B19V isolates in this study have been submitted to the gene bank, their accession number is HM030967-HM030987.

DISCUSSION

This study showed that B19V is common in the Nigerian population although the prevalence is low as compare to that in Vietnam and Taiwan in hepatitis B virus infected persons [Hsu et al., 2005; Toan et al., 2006]. It was also found that the prevalence of B19V infection in HBV-infected patients was not significantly higher than that in the healthy control. However, in patients with symptomatic HBV infection as well as those co-infected with HBV and HCV, the B19V prevalence was significantly higher. In this study, the presence of B19V in the healthy population supports the finding in a study where B19VDNA was isolated from the bone marrow of 4/45 [9%] of healthy persons [Cassinotti et al., 1998].

This study also revealed that B19V DNA is found more often in symptomatic Persons infected with HBV when compared with asymptomatic HBV individuals, suggesting that the virus might play a synergistic role in liver disease outcome in those co-infected with HBV, supporting a previous finding from Vietnam [Toan et al., 2006].

Previous studies have suggested that B19V may have a potential role in the pathogenesis of liver disease including fulminant liver failure [Arista et al., 2003; Bernuau et al., 1999b; Karetnyi et al., 1999; Langnas et al., 1995] and post transplantation liver dysfunction [Lee et al., 2002]. This concept has been supported by the detection of B19V DNA in the liver of patients with hepatitis [Bernuau et al., 1999; Drago et al., 1999; Hillingso et al., 1998; Karetnyi et al., 1999;

Yoto et al., 1996]. It has been shown also that there is a significant association between HBV/B19V co-infection and HBV associated hepatocellular carcinoma in adults [Toan et al., 2006].

Published data on parvovirus genotypes in Nigeria is very limited. A study reported the presence of B19V genotypes 1 and 3 from 5 isolates from Nigerian, mostly from rash/fever patients who were negative for both measles and rubella [Hubschen et al., 2009]. In our study the phylogenetic analysis revealed a similar result with HBV infected patients suggesting that the dominating genotype of B19V in Nigeria is genotype 1.

Another interesting finding of the phylogenetic analysis is that the B19V sequences from the Nigerian isolates are more diverse within genotype 1 as they clustered in two different branches within the genotype 1A earlier designated by a study on Vietnamese isolates [Toan et al., 2006]. However further studies on the whole B19V genome would be necessary to unravel the full genetic diversity of the Nigerian B19V isolates.

In conclusion, this study has shown that human parvovirus B19 is associated with symptomatic HBV infection, however, the mechanism of its synergistic effect on symptomatic HBV infection needs to be investigated further.

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