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The molecular epidemiology of Hepatitis B in the Indigenous people of northern Australia

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ABSTRACT AND KEYWORDS

Background and Aim

The hepatitis B surface antigen was first described in the blood of an Indigenous Australian man, yet little is known about its molecular epidemiology in this population, in which it is endemic. We aimed to determine the clinical and molecular epidemiology of HBV in Indigenous people from northern Australia.

Methods

Following ethics approval and informed consent, we obtained blood specimens and clinical details from Indigenous adults known to be infected with hepatitis B virus, and who were born and raised in Indigenous communities in northern Australia. Hepatitis B virus genotypes were determined in isolates with sufficient Hepatitis B virus DNA by PCR by sequencing of the polymerase/surface gene.

Results

Between June 2010 and June 2012, 65 patients were recruited from six different regions of northern Australia. Thirty two patients (49%) were hepatitis B e antigen positive and 48% were hepatitis B e antibody positive. No patients were found to be co-infected with hepatitis C virus or HIV. Of the 49 samples with sufficient viral load for genotyping, 100% were infected with genotype C4, previously only reported from two Indigenous Australians. All isolates had wild-type polymerase gene sequences, despite 14 currently or previously receiving antiviral treatment. The canonical

sG145R vaccine-escape variant was detected in the surface antigen of virus from two patients.

Conclusions

The exclusive HBV genotype in this ancient population is genotype C4. Whole genome sequencing and clinical follow up of this cohort are in progress, with the aim of exploring the clinical significance of these findings.

Word count 250

Key words - Hepatitis B, Indigenous, Genotype

Accepted Article

TEXT

Introduction

Hepatitis B virus (HBV) affects over 2 billion people worldwide with 350–400 million people chronically infected (1). It is a leading cause of morbidity and mortality in the form of liver cirrhosis and hepatocellular carcinoma (HCC). In Australia, the overall prevalence of HBV is estimated to be low (0.8-0.9%) (2, 3). However, HBV is endemic in Indigenous communities in the Northern Territory (NT) of Australia, with estimates of HBV seroprevalence ranging from 5%–20% (4-10). The predominant route of transmission in this setting is likely to be perinatal. The “Top End” of the NT of Australia has a significant Indigenous population (26% of residents) of whom 30% live in remote or very remote communities sparsely distributed over an area of 500 000km² (11). Universal hepatitis B vaccination at birth has been in place for Indigenous children since 1988 and all children since 1990 in the NT. Despite this there are concerning reports from small studies suggesting reduced hepatitis B vaccine efficacy in fully vaccinated Australian Indigenous populations (12, 13).

There are currently 10 genotypes of HBV (A-J) described in the literature based on sequence divergence of more than 8%, and within these multiple sub-genotypes based on sequence divergence of 4-8% (14). There is a distinct geographical distribution of genotypes and sub-genotypes described to date which is continually being updated (14). Northern Australia is in close proximity to South East Asia, where genotypes B & C predominate. Evidence supporting the importance of HBV genotype for the natural history of chronic HBV infection with regard to progression of disease, risk of HCC and response to treatment, continues to emerge in the worldwide literature (15-17). Patients infected with genotypes B or C generally

remain hepatitis B e antigen (HBeAg) positive well into their 3rd or 4th decade, whereas patients infected with genotypes A2 or D generally convert to HBeAg negativity by 20 years of age (18). Genotypes B and C have been associated with more rapidly progressive liver disease, and progression to HCC is more common in people infected with HBV genotype C than those infected with other HBV genotypes (19). Specific mutations in the pre-core (PC) and basal core promoter (BCP) regions of the HBV genome have been associated with a higher risk of progression to cirrhosis and liver cancer (15).

Despite the first description of hepatitis B surface antigen or the “Australia antigen” being from the blood of an Australian Indigenous man, little is known about the HBV genotypes prevalent in this population. HBV genotypes from five Australian Aborigines from Queensland were characterised by Sugauchi et al in 2001 (20); three were genotype D-4 and two classified as a novel variant genotype C. This was the first report of the sub-genotype now referred to as C4. McIntosh et al reported two Australian Aborigines with genotype D HBV in New South Wales (21) but the sub-genotype was not determined. The aim of the present study was to determine which HBV genotypes/sub-genotypes are present in Indigenous Australians in the Top End of the NT, to explore clinical correlates and to look for the existence of vaccine escape mutants.

Methods

Patients

Ethical approval for the study was obtained from the Human Research Ethics Committee of Northern Territory Department of Health and Menzies School of Health Research (HREC-09/105). Patients were recruited through the Royal Darwin

Hospital Liver Clinic Service which is based in the capital city Darwin but has regular outreach clinics to numerous remote Indigenous communities distributed across 500,000km² in the NT (**Figure 1**). We approached patients who had previously been diagnosed with HBV infection and referred to our clinic. Informed consent was obtained and blood and clinical details collected from HBV positive patients who met the following criteria: born and spent at least the first 5 years of their life in the NT; 18 years of age or over; thought to have acquired their infection in the first 5 years of life.

Information about location of birth and early life, mother's birth location, risk factors for viral hepatitis, treatment and current liver disease were recorded as well as the results of routine blood tests for full blood count, electrolytes, renal function, liver function, coagulation profile, hepatitis C virus (HCV) and HIV status. Cirrhosis was considered to be present if at least one abnormality was present on both imaging and blood tests. For imaging this could include: splenomegaly; enlarged portal vein diameter; and relatively enlarged caudate lobe of the liver. For blood assays, this could include: international normalised ratio (INR) >1.3; platelet count <150 x 10⁹/L; albumin <35 g/dL without alternative explanation; or serum bilirubin >37µmol/L.

Serological analysis assays

All serology was carried out at the NT Government pathology service laboratory. HBV, HCV and HIV testing was conducted using commercial assays: currently Abbott ARCHITECT, previously Abbott AXSYM assays (Abbott Laboratories, Chicago, IL, USA).

HBV-DNA quantitative analysis

Hepatitis B viral loads were carried out by the Victorian Infectious Diseases Reference Laboratory (VIDRL) and determined using Abbott Realtime HBV Assay; dynamic range 15-1x10⁹ IU/mL (Abbott Laboratories).

Genotype determination

HBV DNA was extracted from 200µL serum using the QIAamp DNA Minikit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. To determine genotype and sub-genotype, sequence data was obtained for the reverse transcriptase (RT) region of the HBV polymerase gene (and corresponding overlapping region of the S gene). HBV DNA was amplified with the primers Seq2 5'-TTG GCC AAA ATT CGC AGT C -3' (nt 300-318 (numbering from *EcoR1* site)) and 2996 5'- GCG TCA GCA AAC ACT TGG C -3' (nt 1175-1193), which amplify a 893bp segment of the RT region. Amplification conditions were as previously described (22). PCR products were sequenced using the PCR primers as above. Mutation analysis was carried out using SeqHepB free-ware (23) at VIDRL. To screen for basal core promoter (BCP)/ pre-core (PC) mutations, HBV DNA was amplified with the primers PC5 5'- TCG CAT GGA GAC CAC CGT GA -3' (nt 1604-1623) and PC2 5'- GGC AAA AAC GAG AGT AAC TC -3' (nt 1940-1959). Amplification, sequencing and analysis were carried out as above.

Serotype determination of HBsAg

Serotype was deduced from the nucleotide sequence for amino acid positions 122, 127 and 160 of the surface antigen protein as described elsewhere (24) .

Phylogenetic analysis

Alignment of 549bp from the catalytic domains A-E of RT region, corresponding to amino acids 67 to 226 of the overlapping surface gene, was carried out using ClustalW (Bioedit) (25) for the 49 samples, and compared to 15 sequences obtained from Genbank (2 previously published sub-genotype C4 sequences (Accession numbers AB0704, AB0705), 12 sequences from sub-genotypes C1, C2, C3 and C5 and one sub-genotype B1 sequence (Accession number AB0289) used as an out-group). The phylogenetic tree was constructed using the Neighbour-Joining method, and the evolutionary distances were computed using the Kimura 2-parameter method with bootstrapping of 1000 replicates. Phylogenetic analysis was conducted in MEGA5 (26).

Statistical analysis

Data were entered into a Microsoft Access 2007 database (Microsoft, Redmond, WA) and analysed using STATA version 10 (Statacorp, TX). Results were presented as mean \pm SD for normally distributed parameters and median \pm IQR for non-normally distributed parameters. Bivariate analyses were performed using the Student's t-test or (for non-parametric data) Mann-Whitney U-test, with p-values of <0.05 considered significant.

Results

Between June 2010 and June 2012, 65 patients were recruited from six different regions of the NT (**Figure 1**). Sixteen had an insufficient viral load for genotyping. Of the remaining 49, phylogenetic analysis revealed 100% were infected with genotype C4, clustering with the previously published C4 sequences (**Figure 2**). All sequences were determined to be serotype *ayw3* HBV.

Demographics and patient characteristics are presented in **Table 1(27)**, laboratory markers and clinical details are presented in **Table 2**. The majority (86%) of patients were born and raised (and were therefore assumed to have acquired HBV) in the same location as where their mother was born, and of the remaining 14%, 11% did not know their mothers' place of birth, 1.5% were born within the NT and 1.5% in Western Australia. Fourteen patients had received or were receiving nucleos(t)ide analogue antiviral treatment for HBV. Ten were on current therapy and four women had received therapy during the perinatal period for prevention of mother to child transmission that had since been ceased.

Half of the cohort were HBeAg positive, and these patients were significantly younger than those who were HBeAg negative (29.6 years vs 41.4 years, $p=0.0002$, Table 2). When stratified by age group the percentage of individuals who were HBeAg positive is presented in **Figure 3**. No patients were co-infected with HCV (n=58 tested) or HIV (n=52 tested).

Twenty percent of patients had evidence on abdominal ultrasound and blood tests to suggest cirrhosis but only one patient had had a liver biopsy and 41% of patients had never had an ultrasound. No patients had evidence of decompensated cirrhosis at the time of enrolment.

Of those patients with sufficient viral load to determine their sub-genotype as C4 (n=49), 18 (37%) were HBeAg negative and 31 (63%) HBeAg positive. All isolates had wild-type polymerase gene sequences. The canonical sG145R vaccine-escape variant was detected in the surface antigen of virus from two patients. Analysis of the

PC and BCP region was possible in 44 samples. The classical PC mutation G1896A was present in 14 (32%) of these patients. BCP mutations G1764A/C1766T or A1762T/G1764A were present in 26 individuals (59%) of those with available sequence. This is presented with respect to HBeAg status in **Figure 4**.

Discussion

In the largest study of HBV molecular epidemiology in Indigenous Australians performed to date, we have determined that the exclusive HBV genotype present in Indigenous people tested in the Top End of the NT of Australia is the unusual sub-genotype C4. This has previously only been identified in two other individuals (20), both of whom were Indigenous Australians from Queensland. This genotype also appears to be present in the small numbers of central Australian Indigenous people who have had a genotype documented as part of clinical care (personal communication, Dr Lloyd Einsiedel, 2012). It is intriguing and surprising that this appears to be the only genotype present in our study population as the communities included are very widely distributed across a large geographical area. The strong concordance in the place of birth for mother and child in our population suggests that permanent movement and admixture of language groups across this region has been limited. There are other reports in the literature of predominant genotypes within isolated, remote and Indigenous populations such as in Nigeria (28) and Inuit populations (29) but none to our knowledge of exclusivity. The absence of co-infection is consistent with a predominant perinatal route of transmission and low rates of intravenous drug use in this population.

Despite 22% of patients in this remote setting having received antiviral treatments, sequencing of the polymerase gene found no evidence of drug resistant variants.

This is particularly relevant given the potential challenges involved in rolling out HBV antiviral treatment to remote Indigenous communities. These data suggest that potential concerns regarding poor adherence (30) and consequently widespread emergence of resistance are unfounded.

On analysis of the PC and BCP regions, the G1896A and either G1764A/C1766T or A1762T/G1764A mutations were found frequently. These polymorphisms have been increasingly associated with more severe hepatitis, liver cirrhosis, HCC and acute fulminant hepatitis (31-33). In the HBeAg negative group there was only one patient who did not have HBV with some combination of these mutations. A1762T/G1764A has been associated with an increased risk of HCC (34) and was present in 51% of samples. The presence of wild type 1896 sequence and BCP A1762T/G1764A in a cohort of Taiwanese genotype C patients was associated with the highest risk of HCC (adjusted hazard ratio of 2.99) (35); 22% of our patients fit this profile. Previous evidence from the NT has demonstrated high HCC incidence in Indigenous Australians, similar to that observed in South East Asia, as well as documenting later presentation and poorer prognosis than in non-Indigenous Australians (36-38).

Genotype C HBV is associated with an aggressive phenotype and worse prognosis than other genotypes in terms of delayed HBeAg seroconversion, progression to cirrhosis and development of HCC (35, 39-43). This is the first description of a cohort of people with genotype C4 chronic HBV infection and it appears that C4 also has the genetic polymorphisms consistent with a poorer prognosis. Although HBeAg negative individuals were significantly older ($p=0.0002$) than HBeAg positive individuals, the interquartile range for HBeAg positive individuals extends from 24 to

42 years with the range up to 53 years. Chu and Liaw in their review of the implications of delayed HBeAg seroconversion in the context of HBV acquired in childhood define “delayed seroconversion” as the persistence of eAg positivity after the age of 40 years. They suggest that this situation has been associated with a significantly higher risk of progression to cirrhosis and warrants earlier consideration of antiviral or immunomodulatory therapy (44). In our sub-genotype C4 cohort 35% of those aged 40-49 and 33% of those aged 50-59 remained HBeAg positive, demonstrating ‘delayed’ HBeAg seroconversion using this definition (figure 3). Overall in an endemic context similar to ours it would be expected that less than 10% of individuals with chronic HBV would remain HBeAg positive over the age of 40 years (44). The implications for morbidity and mortality in our setting where C4 is the exclusive sub-genotype could be significant and pose multiple clinical dilemmas; should we move towards individualized genotype guided therapy i.e. earlier commencement in HBeAg positive individuals with sub-genotype C4 and in our circumstances should our general guidelines be modified towards earlier treatment, minimal use of interferon and earlier screening based on our emerging knowledge of the C4 sub-genotype. These concerns warrant further investigation and clarification.

All our isolates are HBsAg serotype ayw3 based on aa sequence at positions 122, 127 and 160. Concerns have been raised regarding the efficacy of hepatitis B vaccine in Indigenous Australians (9, 13, 45, 46) which is derived from serotype *adw* virus. There is also some evidence to raise concerns about HBV vaccine efficacy in the Gambia where there is also a mismatch between vaccine (serotype *adw*) and endemic HBV which is genotype A-2, serotype ayw-4 (47, 48). We hypothesise that our unusual sub-genotype could be a contributory factor in the observed lack of vaccine efficacy which, if proven, could point to the need to consider using a

modified vaccine in our region. Further epidemiological and virological studies to investigate this question are planned.

Due to inherent bias in clinical recruitment methodology the study population is relatively well with a low proportion of individuals on treatment or with evidence of established cirrhosis. However, given that only 59% of patients had a documented abdominal ultrasound, and only one patient had undergone liver biopsy (non-invasive assessment of hepatic fibrosis was unavailable in the NT during the study period), cirrhosis is likely to have been underdiagnosed in this population. Low levels of alcohol use were reported, with 72.3% of individuals reporting complete abstinence; although some degree of reporting bias is likely, it is important to recognise that alcohol is prohibited in most remote communities where patients were recruited.

In order to extend our knowledge of the impact of this sub-genotype of HBV on Aboriginal peoples of the Top End of the NT, we are now planning full genome sequencing on all isolates as well as further phylogenetic and phylogeographical analyses. We plan for long-term follow-up of this cohort to further evaluate the clinical progression of individuals living with this sub-genotype, to better target therapeutic interventions and optimise clinical approaches to these communities.

In conclusion we have determined that the exclusive sub-genotype found across a wide range of Indigenous Australian communities living in the NT is C4, which appears to have similar characteristics to other C genotypes in terms of prolonged persistence of HBeAg positivity and the genetic characteristics that have been associated with earlier progression to cirrhosis and higher incidence of HCC. This has potentially significant consequences for public health and clinical protocols and

guidelines in our setting and further supports the importance of being aware of both individual and regional genotype patterns in planning optimal management strategies.

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FIGURE LEGENDS

Figure 1

Map of the Top End of the Northern Territory demonstrating the main place of residence during the first five years of life for individuals recruited. ★ = people recruited from this community.

Figure 2

Phylogenetic tree analysis of 49 polymerase sequences from this study compared with the 2 published C4 sub-genotype sequences and sequences from other C sub-genotypes. The optimal tree is shown, bootstrap values shown next to the branches. The lengths of the horizontal bars represent the number of base substitutions per site. The out-group consisted of the sub-genotype B1 sequence.

Figure 3

Percentage of HBeAg positive individuals stratified by age group.

Figure 4

Visual representation of the distribution of pre-core (PC meaning G1896A) and basal core promoter (BCP meaning G1764A/C1766T or A1762T/G1764A) mutations with respect to e antigen status for the 49 C4 hepatitis B samples. Yellow = eAg positive, green = eAg negative, red = mutation present, blue = mutation not present, grey = no sequence available.

TABLES

Median age	37.5 years (IQR 28-46)												
Male	33 (51%)												
Indigenous status	65 (100%) Indigenous Australian												
Home region*	<table border="0"> <tr> <td>East Arnhem Land</td> <td>22 (33.9%)</td> </tr> <tr> <td>West Arnhem Land</td> <td>10 (15.4%)</td> </tr> <tr> <td>Tiwi Islands</td> <td>6 (9.2%)</td> </tr> <tr> <td>Darwin urban</td> <td>2 (3.1%)</td> </tr> <tr> <td>Katherine region</td> <td>12 (18.4%)</td> </tr> <tr> <td>Daly River region</td> <td>13 (20.0%)</td> </tr> </table>	East Arnhem Land	22 (33.9%)	West Arnhem Land	10 (15.4%)	Tiwi Islands	6 (9.2%)	Darwin urban	2 (3.1%)	Katherine region	12 (18.4%)	Daly River region	13 (20.0%)
East Arnhem Land	22 (33.9%)												
West Arnhem Land	10 (15.4%)												
Tiwi Islands	6 (9.2%)												
Darwin urban	2 (3.1%)												
Katherine region	12 (18.4%)												
Daly River region	13 (20.0%)												
Mother's place of birth = home region	56 (86%)												
Alcohol use	Nil = 47 (72.3%) Hazardous [†] = 9 (14%)												
Diabetes mellitus	14 (21.5%)												
Hepatitis B genotype	C4 = 48 (100% of those genotyped)												
eAg status	Positive 32 (49%) Negative 32 (49%)												
eAb status	Positive 31 (48%) Negative 33 (51%)												
HBV DNA viral load (IU/ml), Median (IQR)	9680 (415 – 1.1x10 ⁸)												
Evidence of cirrhosis[‡]	13 (20%)												
Childs class	<table border="0"> <tr> <td>A</td> <td>9 (14%)</td> </tr> <tr> <td>B</td> <td>4 (6%)</td> </tr> <tr> <td>C</td> <td>0</td> </tr> </table>	A	9 (14%)	B	4 (6%)	C	0						
A	9 (14%)												
B	4 (6%)												
C	0												
Currently receiving nucleos(t)ide analogue treatment for HBV	10 (15%)												

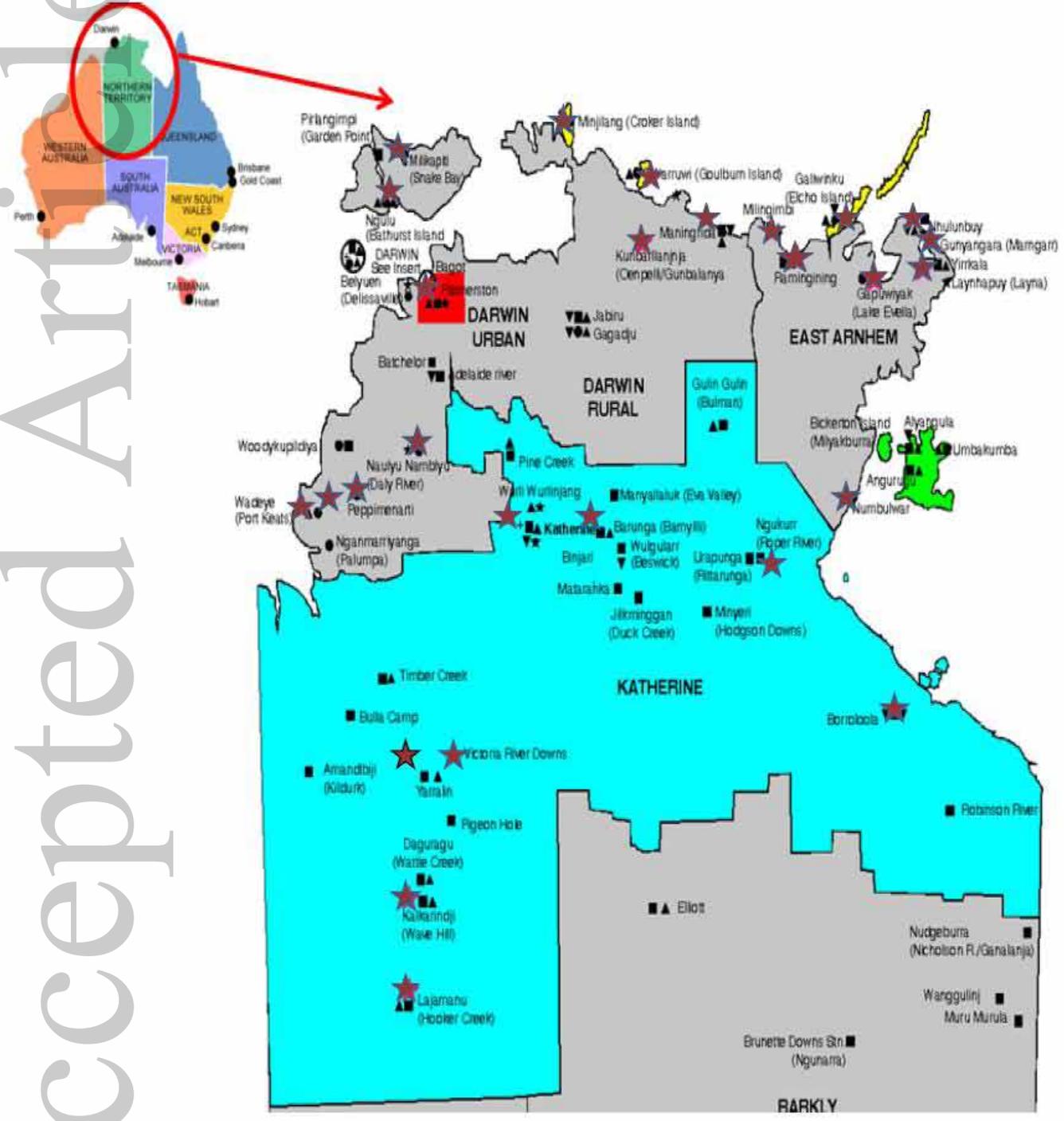
Table 1 – Baseline characteristics of participants (n=65)

n=65, values are number and (percentage) unless otherwise stated * main residence during first five years of life [#]ultrasound evidence and at least one biochemical marker (bilirubin>37, albumin<35, INR>1.3, platelets<150) of cirrhosis [^]Hazardous = >4 standard drinks/day(27)

Table 2 – Clinical details of eAg positive compared with eAg negative participants.

	eAg positive (n=32)	eAg negative (n=32)	P value
Age(years)	29.6 [24-42, 19-53]	41.4 [36-49, 25-67]	0.0002
ALT (U/L)	35 [17-55, 12-327]	23 [18-34, 8-1462]	NS
Serum bilirubin (umol/L)	4.5 [4-7, 2-77]	6.5 [5-11, 2-87]	0.01
Albumin (g/L)	41 [36-44, 24-50]	43 [40-45, 28-51]	NS
INR	1.0 [1-1.1, 0.8-3.0]	1.0 [1-1.1, 0.8-2.2]	NS
Platelet count (x10⁹/L)	220 [187-280, 91-479]	231 [179-287, 101-430]	NS
Serum Creatinine (umol/L)	72 [62-83, 43-593]	71 [62-83, 37-276]	NS
HBV DNA viral load (IU/ml)	1.14x10 ⁸ [1.99x10 ⁷ -2.07x10 ⁸ , 4.22x10 ³ -2.27x10 ⁹]	395 [80-1149, 15-7729938]	<0.001
Evidence of cirrhosis	7 (22%)*	6 (19%)*	NS

n=64 values are median [IQR, range] unless otherwise stated * absolute number (percentage of whole group)



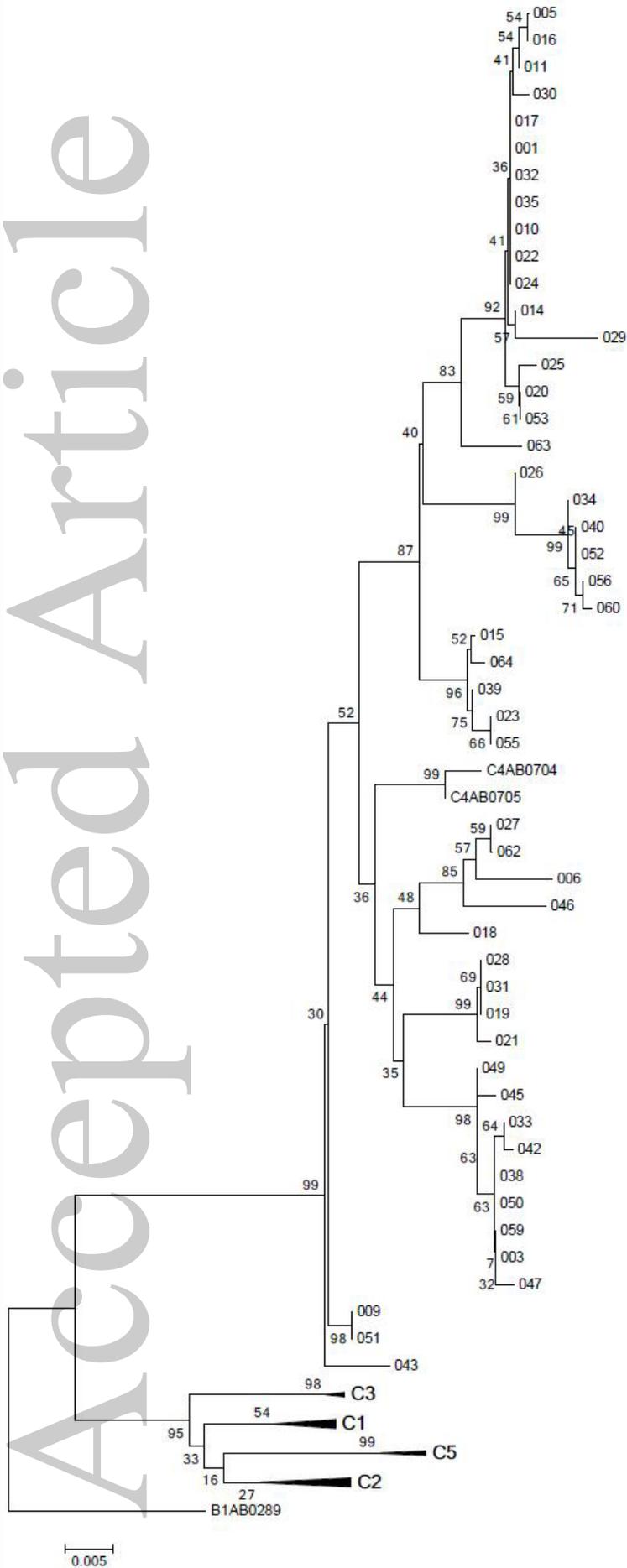
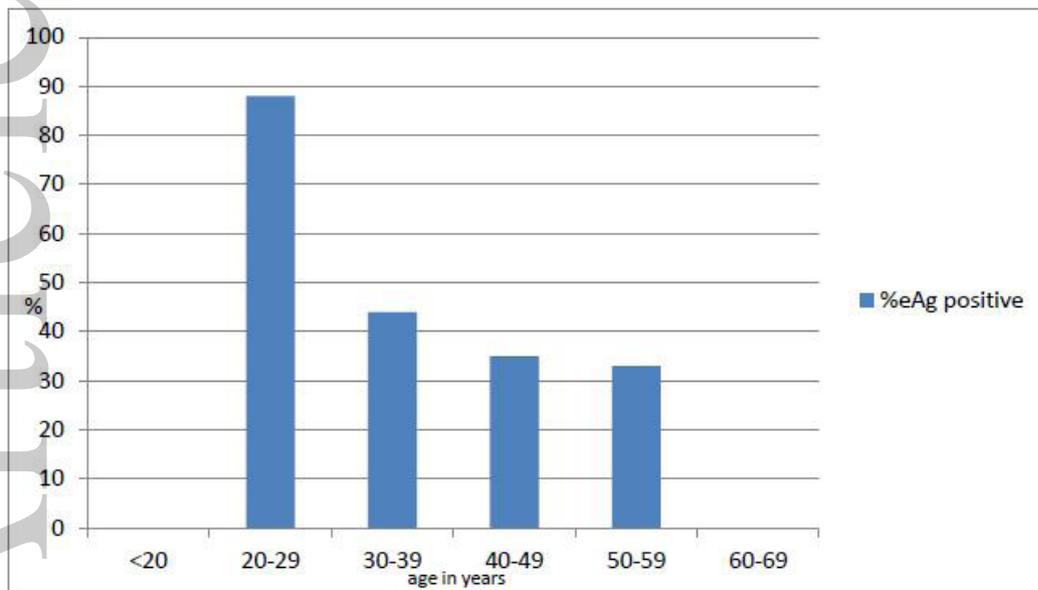


Figure 3



jgh_12177_f3.JPG

