Genotype Distribution Pattern, Subgenotope and Subtype of Hepatitis Virus B in General Population from Six Munipios in Timor-Leste

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Abstract

Hepatitis B virus infection (HBV) remains a serious health problem in Timor Leste, but data on its prevalence is still very limited. According to some reports, the prevalence of HBsAg in the general population in Timor-Leste ranges from 6.9% - 8.1%. Previous research on blood donors in the city of Dili also indicated the biodiversity of the HBV genotype and HBsAg subtype. This study aims to determine the distribution patterns of genotypes, subgenotypes and subtypes of HBV in the general population in six municipios present in Timor-Leste. This study was conducted using a Cut Lintang design to find out, genotype distribution pattern, subgenotype and HBV subtype. HBsAg examination is done by immunochromatography method. Determination of genotype, subgenotype and HBV subtype was done by analysis of nucleotide sequence of S gene. The results of 683 subjects from six Munisipio in Timor Leste obtained 69 positive HBsAg. From 69 samples of HBsAg tested positive, genotype C predominant (72.8%), followed by genotype B (25.7%) and genotype A (1.5%). From the subgenotype A1, B3, B7, C1, C2, C7 and C10 there were 3 dominant subgenotypes, subgenotipe C2 (25.8%), followed by subgenotipe C10 (19.7%) and B3 subgenotype (16 , 6). All the major subtypes of HBsAg were found in Timor-Leste, subtype adr (71.2%), followed by ayw subtype (24.2%), adw subtype (3.1%) and ayr subtype (1.5%). Conclusions from this study were three genotypes of HBsAg in the general population of 6 municipios present in Timor-Leste ie genotypes A, B, and C, Subgenotypes A1, B3, B7, C1, C2, C7 and C10, subtype adr, adw, ayw ayr. Genotype C, subgenotype C2, C10 and subtype adr are the most common genotypes, subgenies and subtypes found in the general population at 6 Municipio in Timor-Leste.

Keywords: Genotype, Subgenotype, Hepatitis B Virus Subtype, Timor-Leste.

Introduction

Hepatitis B virus infection (HBV) causes acute hepatitis and chronic liver disease such as chronic hepatitis, cirrhosis of the liver and hepatoma. Globally, individuals exposed to HBV are 2 billion or more and 240 million people with chronic hepatitis B [1]. This condition occurs because viral hepatitis B infection mostly settled in the body. HBV infections result in 500,000 deaths each year. The case of HBV due to liver cancer is the fifth in the world, death from liver cancer of the world's third order [1,2].

Based on the variation of the HBV genome sequence we have identified 10 HBV genotypes (from A to J) HBV have been identified worldwide, referred to as a separate HBV genotype when there is a difference of more than 8% in the entire genome sequence between groups, or more than 4% in all S gene chains, is a subgenotype when there is a 4-8% difference in nucleotides in the entire genome, in a genotype. [3,4,5]. The nine HbsAg subtypes (adw2, adw4, adrq-, adrq +, ayw1-4, ayr) have been defined by two mutually exclusive mutant couples, d / y and w (w1-4) / r, and the general determinants 'a' of HbsAg [5]. Based on the antigenic determinants of HbsAg, there are four major subtypes, namely adw, ayw, adr, and ayr [3,6,7,8].
The subtype difference lies in certain position amino acids for the determination of HBV subtypes. The HBV subtype also shows geographically different distributions and can provide historical information about the migration of the local ancestors. Both genotype and hepatitis B virus subtypes may show differences in virological characteristics and clinical characteristics [9]. Based on studies from several countries, the HBV genotype has an effect on the onset of liver disease and its response to anti-viral drugs.

From several studies also shows that genotype C is a more aggressive HBV, leading to more progressive liver disease with a poorer prognosis than genotype B. Subgenotypes have been clearly identified The HBV / C genotypes have been grouped into six subgenotypes (C1 to C6) [10,11,12,13,14]. HBV / D into six subgenotypes (D1 to D6) [4,14,15] and HBV / B to seven subgenotypes (B1 to B7) [10,13,16,17,18].

As a HBV genotype, the HBV subgenotype appears to be related to their geographic distribution. Distribution patterns of genotypes, subgenotypes and hepatitis B virus subtypes in a geographic area can be observed in various population groups, including among others the general population [6,9,19], acute hepatitis B patient; people with chronic liver disease such as chronic hepatitis B, hepatic cirrhosis and hepatocellular carcinoma; hemodialysis patients [6], and intra-vein drug users [20].

The general population with positive HBsAg is a population group that has an important role in the transmission chain of hepatitis B virus in a population. In a study conducted by Mulyanto [21], particularly with regard to the hepatitis B virus genotype in Dili, was performed on a limited number of samples. Thus, the results of reported studies have not provided representative representation of the genotype distribution pattern of hepatitis B virus in Timor-Leste.

**Material and Method**

This research uses descriptive and explorative cross sectional design. The goal is to find out the distribution patterns of genotype / subgenotype and subtype HBV based on Municipio. Serum samples were obtained from the general population of 6 Municipio in Timor-Leste from October 2016 to January 2017. The study was authorized by the ethics committee of the Ministry of Health of Timor-Leste and approved by the subjects and signed an agreement to participate in the study.

The tested serum samples were tested for HBsAg detection by immunochromatography method. All samples are deviated at -20°C while waiting for serum samples to be sent to ITD (Institutu Tropical Deseases) in Surabaya where samples will be deviated at -80°C.

**Isolation of HBV DNA (by Qiagen Kit)**

HBV was isolated from plasma / serum samples using Protease 20 ul. At first 20 ul protease add 200 μl plasma / serum, add buffer AL 200 ul, add 100% 200 ul ethanol, transfer all liquid to microcentrifus tube to Qiamp mini spin tube about @ 620 ul, then centrifuge 8000 rpm for 1 minute. Add buffer AW1 500 ul then centrifuged 8000 rpm for 1 minute, AW2 centrifuge 500 ul at centrifuge 14000 rpm for 3 minutes, add AE 100 ul buffer. Incubate at room temperature 15 – 25°C for 2 minutes, then centrifuged 8000 rpm for 1 minute. Furthermore, the DNA extraction fluid is stored at -20°C.

**HBV DNA Implications**

HBV DNA implications were performed using part of the S gene with nested polymerase chain reaction (nested PCR) method. In the first round PCR used primary forward P7 (5’-GTG GTG GAC TTC TCT CAA TTT TC-3’; position 256-278) and primary reserve P8 (5’-CGG TAT AAA GGG ACT CAG GAT-3’; position 796 -776) which would result in a 540 base pairs (bp) of DNA fragments. If the first stage of PCR amplification is negative, a second stage PCR
is performed with HBS1 forward primer (5' -
CAA GGT ATG TTG CCC GTT TG - 3';
position 713-694) which will produce a 258 bp
DNA fragment. Amplification was performed
by PCR sprint machine (Thermo USA) in 25 °
of reaction volume, with optimized PCR
conditions with 40 amplification
amplifications, consisting of 94 ° C
pre-denaturation for 7 minutes, denaturation
at 94 ° C for 30 seconds, annealing stage at
53o C for 30 seconds, and extension stage at
72o C for 45 seconds. PCR reaction using PCR Care
System (Qiagen) consists of: Taq -taq (Master
Mix kit) 250 units 12.5 ul, Coralload (10x) 2
ul, P7 2ul, P8 2 ul, H2o 2.5 ul and DNA 3 ul
total 25 ul.

**PCR Product Detection**

PCR product detection was performed with
electrophoresis on 2% agarose gel stained
with ethidium bromide for further
observation using ultraviolet light
illuminators. If PCR product detection is first
round negative, PCR second-round is
performed with the same procedure and cycle
conditions as PCR first round. The PCR
product of round and second round PCR was
positively detected by electrophoresis purified
PCR product.

**Results and Discussion**

**Result**

Furthermore, sequencing of PCR products
using dye-labelled terminators using ABI
prism 310 genetic Analyze, Applied
Biosystems. The HBV genotype analysis was
performed by comparing the S gene gene
obtained in this study with the S genes
published in GenBank using the BLAST
(Basic Local Alignement Search Tool) program.
This program will calculate the homology of
the comparable sequences. The genotype was
determined by homology in the S gene
greater than 96%.

The nucleotide arrangement of the S gene is
then converted to an amino acid sequence.
The subtypes of HBsAg are determined by
the substitution of amino acids 122, 127, 134,
159, 160 and 177. Samples were analyzed for
Polymerase Chain Reaction (PCR), as many
as 69 respondents with HBsAg positive. PCR
analysis showed that forty (59.9%) of the 69
samples were male and 29 (42%) of the
samples of the female individuals, ranging in
age from 20 to 57 years, and the average age
of 30.9 years.

Twelve (17.9%) came from Dili (Tetum), 10
(14.5%) of Aileu (Mambae), 7 (10.2%) of
Liquisa (Tokodede), 11 (15%) of Oecusse
(Baikeno ), 16 (23.2%) of Lautem (Fataluku)
and 13 (18.8%) of Baucau (Makasae).

Hepatitis B virus DNA was detected at 69
(100%). Forty-five (65.2%) of 69 samples
detected hepatitis B virus DNA on PCR first
round of P7-P8 with 2% agarose produced
dNA band 541 base pairs (bp), while 24
(33.3%) samples others detected on a second-
round PCR with HBS1-HBS2 primers
producing DNA bands 259 base pairs (bp).
The result of HBV subtype analysis in Timor-
Leste was found that four known HBsAg
subtypes, the four (adw, adr, ayw and ayr)
were found in Timor-Leste (Table 1).

### Table 1: Distribution Pattern of HBV Subtypes in Timor-Leste

<table>
<thead>
<tr>
<th>Municipio</th>
<th>Total Sample</th>
<th>Subtype HBV n (%)</th>
<th>Adr</th>
<th>Ayw</th>
<th>Adw</th>
<th>Ayr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dili</td>
<td>12</td>
<td>8(66.7)</td>
<td>4(33.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aileu</td>
<td>10</td>
<td>10(100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Liquisa</td>
<td>6</td>
<td>5(83.3)</td>
<td>0</td>
<td>1(16.6)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Oecusse</td>
<td>11</td>
<td>8(72.7)</td>
<td>3(27.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lautem</td>
<td>15</td>
<td>6(40)</td>
<td>7(46.6)</td>
<td>1(6.7)</td>
<td>1(6.7)</td>
<td></td>
</tr>
<tr>
<td>Baucau</td>
<td>12</td>
<td>10(83.3)</td>
<td>2(16.7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>66</td>
<td>47(71.2)</td>
<td>16(24.2)</td>
<td>2(3.1)</td>
<td>1(1.5)</td>
<td></td>
</tr>
</tbody>
</table>

In hepatitis B virus, ayw 16 (24.2%), adr 47
(71.2%) and adw 2 (3.1%) and ayr 1 (1.5%).
Subtypes of adr are found evenly across the 6
existing Municipio Timor-Leste, especially
the southern part. The dominant ayw
subtypes in the eastern and western regions
(except Aileu and Liquisa), the ayr Subtype is
only in the easternmost part of Lautem,
whereas the subtype adw is in Lautem / Fataluku and Liquisa / Tokodede. The results
of genotype and subgenotype analysis in the
study sample showed that of the 10
genotypes of HBV present in the world, 3
(30%) genotypes (A, B, C) were present in
Timor-Leste (Table 2).
The study involving 683 subjects from 6 Municipio in Timor-Leste, of the 2 genotypes (B and C) encountered in Timor-Leste there is no difference in the distribution of the subgenotype. Four subgenotypes in genotype C (C1, C2, C7, C10) were evenly distributed 6 Municipio in Timor-Leste (table 5.6). Among the 4 subgenotypes C is C2 is very dominant 25.8% followed by C10 (19.7%), C1 (13.6%), and C7 (10.6%). Two subgenotypes in genotype B (B3 and B7) spread unevenly 6 Municipio in Timor-Leste (table 5.5). Between 2 subgenotypes B was dominant (16.6%) followed by B7 (12.2%). Subgenotype B3 is distributed almost evenly in 6 Municipio (except in Aileu and Liquisa). Conversely the C2 subgenotype is spread evenly throughout the Municipio except (Lautem).

**Discussion**

Based on different sources of reports, the prevalence of HBsAg in the general population in Timor-Leste ranges from 6.9% - 8.1% [21,22]. HBV infections cause acute, chronic and fulminant hepatitis infections, as well as cirrhosis up to liver cancer. The genetic variation of HBV can be classified into 10 genotypes AJ [23], 42 known subgenotypes in the world and 4 subtypes The main Hepatitis B Surface Antigen (HBsAg), adw, adr, ayw, ayr. Knowledge of the HBV genotype is very important. From a clinical standpoint, there has been considerable evidence suggesting an association between HBV genotypes with clinical manifestations of liver disease and response to antiviral therapy. Epidemiologically, it is known that the spread of HBV in the world differs geographically and ethnically. As an archipelagic country, Timor-Leste has a diverse population of more than 19 ethnic groups. This population diversity is strongly linked to both the human genetic background and the ancient migratory pattern, and is thought to affect the molecular epidemiology of HBV to be depicted in the pattern of genotype distribution and HBV subtypes in Timor-Leste.

To date, reports on the genotype / subgenotipe of HBV in Timor-Leste are still very limited. This study shows the variation in the distribution pattern of the HBV genotype / subgenotipe and the subtypes between the Municipio in Timor-Leste. The most dominant distribution pattern in Timor-Leste genotype C (72.8%), followed by genotype B (25.7%) and genotype A (1.5%). Her genome analysis showed that subgenotype A1 in Municipio Liquisa was identical with A1 subgenotype in Brazil and Kenya. The study involving 683 subjects came from 6 Municipio in Timor-Leste with distribution patterns according to the Municipio ie. Genotype C in Municipio Aileu (100%) south, Liquisa / (83.4%) western, Baucau (83.4%) East, Dili (75%) capital city of Oecusse (72.7%), Lautem / (40%) is East, and Leste genotype B (25.7%) and genotypes A, B and C homology respectively of 98.

**Table 2: Pattern of HBV Genotype Distribution in Timor-Leste**

<table>
<thead>
<tr>
<th>Municipio</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dili (tetum)</td>
<td>12</td>
<td>0</td>
<td>3(25)</td>
</tr>
<tr>
<td>Aileu(Mambae)</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Liquisa(tokode)</td>
<td>6</td>
<td>1(16.6)</td>
<td>0</td>
</tr>
<tr>
<td>Oecusse(Baikeno)</td>
<td>11</td>
<td>0</td>
<td>3(27.3)</td>
</tr>
<tr>
<td>Lautem(Fataluku)</td>
<td>15</td>
<td>0</td>
<td>9(80)</td>
</tr>
<tr>
<td>Baucau(Makasae)</td>
<td>12</td>
<td>0</td>
<td>2(16.6)</td>
</tr>
<tr>
<td>Jumlah Total</td>
<td>66</td>
<td>1(1.5)</td>
<td>17(25.7)</td>
</tr>
</tbody>
</table>

**Table 3: Pattern of HBV Subgenotype Distribution in Timor-Leste**

<table>
<thead>
<tr>
<th>Municipio</th>
<th>A1</th>
<th>B3</th>
<th>B7</th>
<th>C1</th>
<th>C2</th>
<th>C7</th>
<th>C10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dili</td>
<td>12</td>
<td>8,3</td>
<td>16,6</td>
<td>16,7</td>
<td>33,4</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Aileu</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>Liquisa</td>
<td>6</td>
<td>16,6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33,4</td>
<td>16,6</td>
</tr>
<tr>
<td>Oecusse</td>
<td>11</td>
<td>0</td>
<td>27,3</td>
<td>9,1</td>
<td>0</td>
<td>27,2</td>
<td>18,2</td>
</tr>
<tr>
<td>Lautem</td>
<td>15</td>
<td>0</td>
<td>40</td>
<td>90</td>
<td>0</td>
<td>26,6</td>
<td>0</td>
</tr>
<tr>
<td>Baucau</td>
<td>12</td>
<td>0</td>
<td>8,4</td>
<td>16,6</td>
<td>8,3</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>1,5</td>
<td>16,6</td>
<td>12,2</td>
<td>13,6</td>
<td>25,8</td>
<td>10,6</td>
</tr>
</tbody>
</table>

Table 2 shows that 72.8% is included in genotype C, 25.7% is included in genotype B and 1.5% shows genotype A. In the highest genotypes A, B and C homology respectively of 98.
while genotype B is dominant in Municipio Lautem (60%) East, Oecusse (27.3%), Dili (25%) and Baucau (16.6%). And HBV genotype A is only found in the Municipio Liquisa (16.6%). For Municipio Aileu there are exceptions because in the Municipio of the southern city of Dili only genotype C. Municipio Aileu and Liquisa as Municipio with geographical and ethnic proximity with the Municipio Dili appear to exhibit a similar pattern of genotype HBV distribution, which is dominated by genotype C.

Data this further strengthens the notion that geographic and ethnicity as one of the factors influencing the distribution of genetic variants of HBV [4]. This may be due to the high mobility of people from and to the very high Dili Municipio allow for a similarity in the distribution of the HBV genotype between the three Municipio. The HBV genome showed different characteristics in 6 Municipio, of the 7 subgenotypes (A1, B3, B7, C1, C2, C7 and C10) found, subgenotype C2 occupied (25.8%), followed by C10 (19.7%), C1 (13.6%). Subgenotype C2 was predominantly in the southern part of Dili and C10 was dominant in the eastern part of Dili, while HBV subgenotype B3 was found to be dominant (16.6%) and B7 (12.2%) for subgenotype A1 (1.5%). The results of subgenotype B3 and subgenotype B7 are similar to those conducted by Mulyanto in Dili [24]. Subgenotype B3 was found to be dominant in the extreme end of the Eastern City of Dili, a study conducted by Mulyanto [24].

Weifeng [25] in Indonesia and China, said that the B3 subgenotype is widely distributed throughout Indonesia and is called B3 an indigenous subgenotype of Indonesia. The B3 subgenotype was also found in Timor-Leste, especially in 6 Municipio, where the results were illiterate in all the Municipio but this result may illustrate that the people of Timor-Leste and Indonesia may have originated from the same family because it is a land- -Leste and Indonesia. HBV subgenotype B3 has so far been widely reported in Japan, Korea, China, Indonesia, Vietnam and Thailand [26].

The results of subgenotype analysis C2 and C10 in Timor-Leste were evenly distributed. This is no different from the subgenotype C2 distributed throughout Indonesia despite low prevalence [27]. Similarly, subgenotypes C2 and C1 are found in Japanese, Chinese, Vietnamese, Indonesian, Hawaii, Malasya, Franca, Costa Rica, German Vietnamese and Korean [27,28]. The spreading pattern of subgenotypes A1, B3, B7, C2, C1 and C10 of the 6 municipios present in Timor-Leste suggests that the subgenotype may be imported from neighboring Asian countries from Indonesia in the past.

This pattern of distribution provides an indication of the possibility of one of the migration routes of East Timorese ancestors from the Asian plains as long as Malacca, East Nusa Tenggara to Timor-Leste. There is an indication of the distribution pattern of the HBV genome reflecting various migration routes from ancestors in the past and the HBV genes C2 and C10 subgenotypes spread evenly across 6 Municipio in Timor-Leste indicating that the ancestors who migrated in small groups through various migration routes, comes from the same "large group" of "large groups" with the dominant HBV subgenotype C2 and C10. Ten genotypes of HBV present in the world of which 3 (30%) can be found in 6 Municipio in Timor-Leste including genotype A, B and C. C genotype is dominant in the general population with positive HBsAg, followed by genotype B and genotype A.

This result is in line with the results of a study reported Mulyanto et al., 1995, states that 34 HBV isolates from blood donors in Dili are included in genotypes B and C. Mulyanto et al. Research explains that genotype B is dominant, this is the dominant genotype C (72.8%) followed by genotype B (25.7%) and A (1.5%). This is possible because of the sample differences and the characteristics of the sample population taken. The difference is due to the results of this study taken from 6 Municipio in Timor-Leste, while the results reported by Mulyanto et al., Sampling of blood donors in the city of Dili. So it can be said that the genotype of HBV in the general population in 6 Municipio in Timor-Leste, is very dominant with the genotype C.

Although not fully proven because this research is only first done after parting with Indonesia. It was also reported that HBV genotypes B, C and A have been isolated from various countries in the world, namely: Indonesia, Brazil, Filipino china, Japan,
Nigeria, and Canada [29]. For Municipio Aileu there are exceptions because in Municipio Aileu most commonly found is the genotype C. Municipio Dili as a city that has a geographical and ethnic proximity with Municipio Aileu showed a similar pattern of genotype HBV distribution, which is dominated by genotype C. This data is increasingly reinforce the notion that geographic and ethnicity is one of the factors affecting the distribution of genetic variants of HBV[4].Currently there are 4 major subtypes of HBsAg, which are adw, adr, ayw, and ayr based on the amino acid arrangement at position 122 and 160 genes S.

The HBsAg variant also has different geographic and ethnic distributions. Distribution of HBV subtypes found in this study was the most dominant of adr as the most common subtype in Municipio Aileu (100%) to the south, followed by Liquisa (83.4%), Oecusse (72.7%), Baucau (83 , 3%) East, Municipio Dili (66.7%) capital city, while Municipio Lautem (40%) is the eastern part of Dili. While subtypes ayw was found most dominant in Municipio Lautem (46.6%) the easternmost part, followed by Municipio Oecusse (27.3%) and Dili (33.3%). In this study there was also a subtype HBV distribution based on its genotype, ayw is a subtype found in genotype B (19.4%), and genotype A was found subtype adw. The subtype adr is a subtype of genotype C (65.6%), this is similar to research conducted in Indonesia [8]. The results of this study, subtype adw are found in genotypes A and B, this is consistent with that found [30] which says that subtype adw is found in genotypes A, B and C. Meanwhile for the subtypes of ayw in this study more dominant of isolation of genotype B, this is similar to that expressed by [30], that subtypes of ayw1 have been isolated from genotypes A and B. Thus the results of this study indicate that in adult population with HBV infection in Timor-Leste was obtained subtype adr is more dominant than the subtypes ayw, ayr and adw.

**Conclusion**

Conclusions from this study were three genotypes of HBsAg in the general population of 6 municipios present in Timor-Leste ie genotypes A, B, and C, Subgenotypes A1, B3, B7, C1, C2, C7 and C10, subtype adr, adw, ayw ayr. Genotype C, subgenotype C2, C10 and B3 and subtype adr are the most common genotypes, subgenies and subtypes found in the general population at 6 Municipio in Timor-Leste.

**References**


