

The Relatedness between Hepatitis B Virus from Non-Papuan Blood Donors in Jayapura and the Papuan Clusters

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The genotypes (A-H) and subtypes (*adw2*, *adw4*, *adrq-*, *adrq+*, *ayw1-4*, *ayr*) of HBV show distinct geographical distributions, which have been associated with anthropological history. The novel finding of the HBV subgenotypes C6 and D6 from Papuans formed a specific cluster distinct from the previous HBV subgenotypes C1-C5 and D1-D5. In this study we determined the most recent genotype-subtype patterns of the HBV from non-Papuan blood donors who live in Jayapura and their phylogenetic relatedness, especially with the Papuan clusters. Fifteen HBsAg-positive serums were obtained from non-Papuan blood donors including from people in Java (46.7%), Maluku (26.7%), Sulawesi (20%) and East Nusa Tenggara (6.7%). S gene of all HBV serum isolates were partially sequenced and analyzed. Most HBV isolates (53.3%) were classified as genotype B, followed by genotype C (26.7%) and D (20.0%). The subtype *adw2* (33.3%) was predominant, followed by *adrq+* (26.7%) and *ayw1/ayw2* (20.0%). All HBV isolates with subtype *adw2* and *ayw1* belonged to genotype B, while *adrq+* belonged to genotype C and *ayw2* belonged to genotype D. The most predominant HBV genotype-subtype (B/*adw2*) was consistent with the ethnic background (mostly from Java people). Nevertheless, based on the phylogenetic relatedness, many non-Papuan isolates (40%) were classified into HBV/C6 and HBV/D6 of the Papuan clusters. Other isolates were classified into HBV/C1, HBV/B3 and HBV/B7. In conclusion, many HBV isolates from non-Papuans in Jayapura belonged to the Papuan clusters, but others had different genotype-subtype patterns with frequencies dependent on ethnicity.

Key words: hepatitis B virus, genotypes, subtypes, phylogenetic relatedness

Hepatitis B virus (HBV) infection is a serious global health problem. In 2000, Core Working Party for Asia-Pacific Consensus on Hepatitis B and C reported that Indonesia belonged to the moderate-to-high hepatitis B endemic region (Khan *et al.* 2004). Carrier rates among blood donors ranged from 2.1 to 9.5% in 11 large cities and even higher at 17.5% in Jayapura, the Papua Province (Sastrosoewignjo *et al.* 1991).

Eight genotypes (from A to H) of HBV have been identified worldwide, based on a divergence of 8% or more of the complete genome, and more than 4% at the level of the S gene (Okamoto *et al.* 1988; Norder *et al.* 1994; Magnius and Norder 1995; Stuyver *et al.* 2000; Arauz-Ruiz *et al.* 2002; Kramvis *et al.* 2005). Nine HBV subtypes (*adw2*, *adw4*, *adrq-*, *adrq+*, *ayw1-4*, *ayr*) were defined by two mutually exclusive determinant pairs, *d/y* and *w(w1-4)/r*, and a common determinant 'a' of HBsAg (Magnius and Norder 1995; Kramvis *et al.* 2005). The HBV isolates of different genotypes and subtypes show different geographical distributions, virological characteristics, and possibly clinical outcomes (Kao *et al.* 2000; Orito *et al.* 2001; Kao 2002; Kidd-Ljunggren *et al.* 2002). They could also provide historical information on the migration pattern of the ancestor of local population (Magnius and Norder 1995; Orito *et al.* 2001).

The subgenotypes have been identified for certain HBV genotypes. HBV/C has been classified into six subgenotypes (C1 to C6) (Sugauchi *et al.* 2001; Huy *et al.* 2004; Chan *et al.* 2005; Sakamoto *et al.* 2006; Lusida *et al.* 2008), HBV/D into six subgenotypes (D1 to D6) (Norder *et al.* 2004; Bozdayi *et al.* 2005; Banerjee *et al.* 2006; Schaefer 2007; Lusida *et al.* 2008) and HBV/B into seven subgenotypes (B1 to B7) (Sugauchi *et al.* 2001; Nagasaki *et al.* 2006; Sakamoto *et al.* 2006; Sakamoto *et al.* 2007; Nuraeny *et al.* 2008). As HBV genotypes, the HBV subgenotypes seem to be associated with their geographical distributions.

Previous studies of genotype-subtype distributions of HBV in Jayapura, especially those from Papuans, reported that C/*adr* was predominant (Sastrosoewignjo *et al.* 1991; Mulyanto *et al.* 1997; Lusida *et al.* 2008). In 1997 Mulyanto assumed that the Papuan ancestor came from New Caledonia (HBV/C3), where *adr* is largely found. Besides, based on anthropological origin, Papuans resemble Australian Aborigines, because the mainlands of the two tribes were one continent before it was separated 7000 years ago (Sugauchi *et al.* 2001). However, Sugauchi *et al.* (2001) found the C/*ayw3* isolates from Australian Aborigine (HBV/C4), which had a different HBV subtype from that of Papuans. Recently, Lusida *et al.* (2008) reported that most HBV isolates from Papuan blood donors in Jayapura belonged to the novel C subgenotype (HBV/C6), separated from the previous HBV C subgenotypes, including the New Caledonia (HBV/C3) and Australia clusters (HBV/C4). They also reported that other Papuan isolates belonged to the novel HBV D subgenotype, namely D6.

The aim of this study was to determine the most recent genotype-subtype patterns of HBV based on part of the S gene in HBsAg-positive blood donors from non-Papuans who live in Jayapura, the Papua Province. Their

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phylogenetic relatedness was also analyzed, especially with HBV of Papuan clusters.

MATERIALS AND METHODS

Collection of Field Samples. Serum samples were taken from non-Papuan blood donors who visited the Blood Transfusion Unit-Indonesian Red Cross in Jayapura from July 2006 to September 2006, and were screened for HBsAg by using the immunochromatography method (entebe HBsAg strip, Hepatika Laboratory, Mataram). All serum samples were stored at -20 °C until transported to the Institute of Tropical Disease in Surabaya, where they were stored at -80 °C. Ethical clearance of this study was obtained from the ethics committee of the Faculty of Medicine, Airlangga University in Surabaya, Indonesia. All blood donors signed an agreement to participate in this study.

Viral DNA Extraction, PCR Amplification and Sequencing. Genomic DNA of HBV was extracted from serum samples by using the DNAzol reagent (Invitrogen) following the manufacturer's guidelines. The extracted DNA was used as a template for the amplification of the respective gene regions. PCRs were performed with the PCR mastermix (Fermentas). The reactions contained 25 µL PCR mastermix, 10 mL DNA and 0.5 µL of each primer with a concentration of 20 pmol µL⁻¹, in a total reaction volume of 50 µL. The thermocycling conditions included a 5-min denaturation step of 94 °C; followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. Partial S gene was amplified in the first round by using primers P7 (5'-GTG GTG GAC TTC TCT CAA TTT TC-3', nt 256 to 278) and P8 (5'-CGG TAW^[A/T] AAA GGG ACT CAM^[A/C] GAT-3', nt 796 to 776). If the first round PCR was negative, therefore the second round PCR was performed by using primers HBs1 (5'-CAA GGT ATG TTG CCC GTT TG-3', nt 455 to 474) and HBs2 (5'-AAA GCC CTG CGA ACC ACT GA-3', nt 713 to 694) (Lusida *et al.* 2003). PCR products were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI Prism 310 Genetic Analyzer (Perkin Elmer).

Analysis of Sequence. Sequence analysis was performed with Genetyx-Mac version 9 (Software Development Co., Ltd., Tokyo, Japan). The HBV genotype of the samples was determined based on the homology percentage of >96% in the S gene (Magnius and Norder 1995; Arauz-Ruiz *et al.* 1997). After aligning the sequences of the HBV nucleotide and converting it into amino acid sequences, the HBV subtype of the samples was assigned by analyzing the amino acid substitutions at positions 122, 127, 134, 159, 160, 177 in the S gene (Okamoto *et al.* 1988; Norder *et al.* 1994; Kramvis *et al.* 2005). The phylogenetic tree was constructed by means of Unweighted Pair Group Method using the clustering of Arithmetic averages (UPGMA).

RESULTS

A total of 15 HBsAg-positive serum was obtained from non-Papuan blood donors (14 male and 1 female; age 18-52). The blood donors were people from Java (46.7%), Maluku (26.7%), Sulawesi (20%) and East Nusa Tenggara (6.7%) living

in Jayapura. A sequence analysis of part of the S gene of HBV serum isolates was performed (Table 1).

Table 1 Ethnicity of non-Papuan blood donors in Jayapura

Ethnicity of non-Papuan blood donors	Number
Java	7
Sulawesi	3
Maluku	4
East Nusa Tenggara	1
Total	15

The distribution of the HBV genotypes among the 15 serum in non-Papuans indicated that most isolates (53.3%) were classified into genotype B, followed by genotype C (26.7%) and D (20%) (Table 2).

The HBV subtypes were determined by aligning 15 HBV sequences from non-Papuans and 70 reported HBV sequences (A-H genotypes) including HBV of the Papuan clusters, at the amino acid positions 117-180 of the S gene (Fig 1). By analyzing amino acid substitutions at positions 122, 127, 134 and 160, it was found that the subtype *adw2* (33.3%) was predominant, followed by *adr* (26.7%), and *ayw1/ayw2* (20%) (Table 2). All isolates with *adr* subtype had alanine (A) at position 159 and valine (V) at position 177 in HBV, and this combination is considered important for the *q* determinant (Fig 1).

All HBV isolates with *adw2* and *ayw1* belonged to genotype B, subtype *adr(q+)* belonged to genotype C, and *ayw2* to genotype D (Table 2). No *Cladrq-* isolates were found here.

The genotype subtype patterns of HBV from various ethnicities of non-Papuan blood donors obtained in Jayapura, were summarized in Table 3. HBV B/*adw2* was predominant in Javanese, followed by B/*ayw1* or *Cladrq+*. HBV B/*adw2* was also more than B/*ayw1* in Sulawesi people. HBV D/*ayw2* was commonly found in Moluccans, followed by *Cladrq+*, but only HBV *Cladrq+* was found in East Nusa Tenggara people.

The phylogenetic analysis of part of the S region (nt 503 to 694) of the HBV genome revealed that many HBV isolates (40%) from non-Papuans (Javanese and Moluccans) were classified into Papuan clusters, such as HBV/C6 (6NP, 27NP and 49NP) and HBV/D6 (7NP, 25NP, 26NP). Some isolates from Javanese and Sulawesi people (30NP, 37NP and 57NP) were classified into HBV/B3. Two isolates from Javanese (9NP and 18NP) were classified into HBV/B7 (Alor, East Nusa Tenggara). One isolate from East Nusa Tenggara people

Table 2 HBV genotype and subtype distribution patterns from non-Papuan blood donors in Jayapura

HBV Genotypes/subtypes	Number of cases in non-Papuan blood donors
B/ <i>adw2</i>	5
B/ <i>ayw1</i>	3
<i>Cladrq+</i>	4
D/ <i>ayw2</i>	3
Total	15

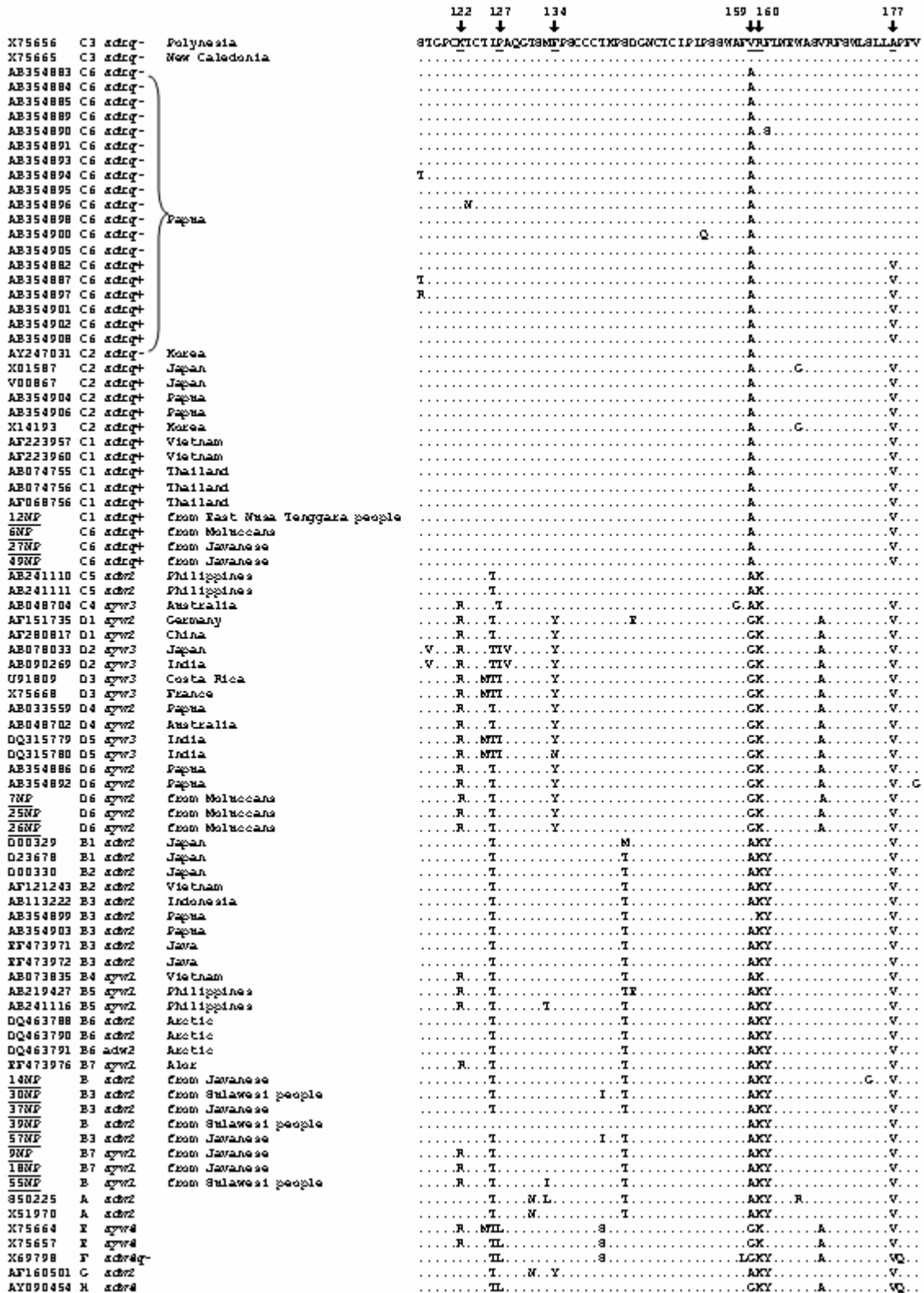


Fig 1 Alignment of amino acid sequences of HBV isolates from 15 non-Papuan blood donors in Jayapura, Indonesia (code: NP) and 70 reported sequences of genotypes A to H including the Papuan clusters, C6 and D6 (Lusida *et al.* 2008). The sequences correspond to amino acids 117 to 180 of the S gene.

Table 3 HBV genotype-subtype patterns from various ethnics of non-Papuan blood donors in Jayapura

Ethnics of non-Papuan blood donors	No. of cases				Total
	B/adw2	B/ayw1	C/adrq+	D/ayw2	
Java	3	2	2		7
Maluku			1	3	4
Sulawesi	2	1			3
East Nusa Tenggara			1		1
					15

(12NP) was classified into HBV/C1 (South East Asia). Some HBV/B isolates from Javanese and Sulawesi people (14NP, 39NP and 55NP) were not definitely classifiable on the basis

of the partial S gene sequencing alone. All HBV/C isolates were from non-Papuans separated from Far East Asia (HBV/C2), New Caledonia-Polynesia (HBV/C3), Australia (HBV/C4) and the Philippines (HBV/C5) clusters (Fig 2).

DISCUSSION

Papua island is in the eastern part of Indonesia with Papuans as its native people. Beside the natives, many other ethnic groups in Indonesia, like Javanese, Sumatrans, Moluccans, and people from Sulawesi and East Nusa Tenggara descent live in Papua. Here, HBV isolates from non-Papuans who live in Jayapura, Papua province, were presented.

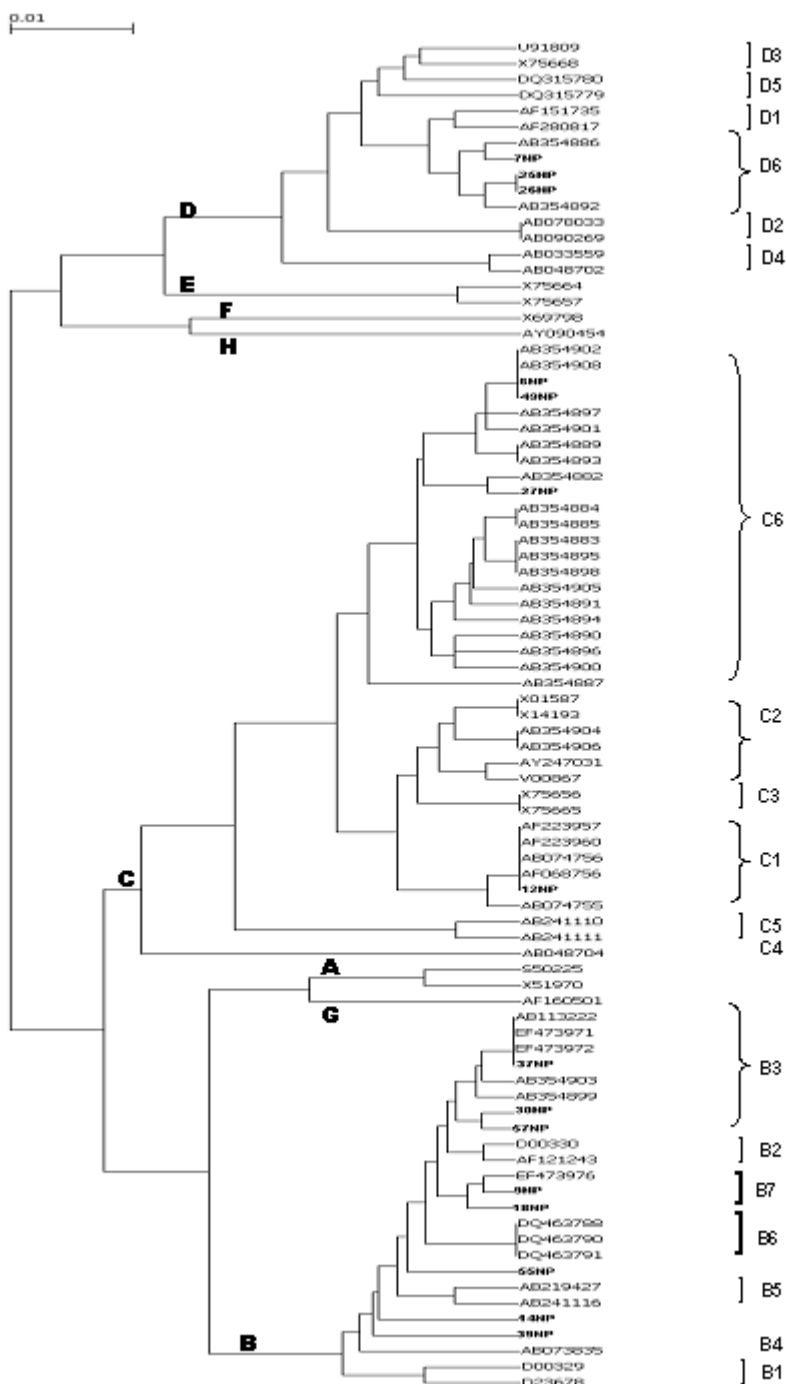


Fig 2 Phylogenetic analysis of HBV isolates obtained from 15 non-Papuan blood donors (code: NP) compared with the reported of all genotypes and subgenotypes of HBV, including the subgenotypes C6 and D6 of the Papuan clusters (Lusida et al. 2008); on the basis of the partial S genes (503-694). The genotypes are indicated on the branches and the subgenotypes are on the right.

This study demonstrated that most (53.3%) HBV isolates from non-Papuan blood donors were classified into genotype B, followed by genotype C (26.7%) and D (20%) (Table 2). No HBV isolates were found with genotypes A, E, F, G or H from the non-Papuans. Each HBV genotype has characteristic geographic distribution. In some cases, differences in the distribution of the genotypes can be found within a single country, as has been observed in China, India, USA and Indonesia as well. The genotype distributions can be influenced by the ethnic background and the country origin of the individual carriers of the virus (Kramvis *et al.* 2005). Indonesia is a country that has more than 300 ethnic groups and over 250 languages (Sofro 1982). It is characterized by its national motto - “*Bhinneka Tunggal Ika*” or “unity in diversity”. The diversity in this country can now be assessed also in the light of the HBV genotypes. In the western part of Indonesia, the HBV genotype B was predominant, while the HBV genotype C was predominant in the eastern part of Indonesia, especially in Papua (Sastrosowignjo *et al.* 1991; Mulyanto *et al.* 1997; Usuda *et al.* 1999; Lusida *et al.* 2008). The results revealed that most predominant HBV genotype of non-Papuans isolates was genotype B. The samples were mostly obtained from people of the western part of Indonesia (Javanese) (Table 1 and 3). HBV genotype B was also found more in Sulawesi people, which was consistent with the report of Sastrosowignjo *et al.* (1991). Like other regions in most developing countries where vertical transmission is predominant, the distributions of HBV genotypes-subtypes in Papua seemed to be dependent on ethnicity.

The subtype *adw2* (33.3%) was the most predominant in all HBV isolates from non-Papuan blood donors, followed by *adrq+* (26.7%) and *ayw1/ayw2* (20%) (Table 2). Each HBV subtype markedly varied in different geographical regions in Indonesia. The most predominant HBV subtype in the western part of Indonesia, such as Java, is *adw* (Mulyanto *et al.* 1997). Beside that, they concluded that Sulawesi was the mixed HBV subtype zone, including *adw*. It was consistent with our results that the subtype *adw2* was mostly obtained from Javanese, followed by Sulawesi people (Table 3). Nevertheless, it was different with the predominance of the *adr* subtype in Papuans. Sastrosowignjo *et al.* (1991) showed that 50% of 14 HBV isolates from blood donors in Jayapura belonged to the subtype *adr*. Mulyanto *et al.* (1997) also reported that 76.9% of the 13 isolates of adult population of Papuan in Jayapura belonged to the subtype *adr*. Lusida *et al.* (2008) found that 85.2% of the 23 isolates from blood donors (mostly Papuans) in Papua belonged to the subtype *adr*, too. Our results confirmed the difference of the most predominant HBV subtype from Papuans with non-Papuans even though they lived in the same places.

All HBV isolates with subtype *adw2* and *ayw1* belonged to genotype B, *adrq+* to genotype C and *ayw2* to genotype D. No HBV C/*adrq-* isolates were found here. This was consistent with the HBV genotypes and subtypes relationship. In comparison to our results, the subtype *adr* is rarely found in genotype B, subtype *adw2* can be found in genotypes A, C, F, G and subtype *ayw2* can be found in genotypes A and C (Kramvis *et al.* 2005).

By analyzing the phylogenetic relatedness on part of the S gene, it was found that many HBV isolates (40%) from non-Papuans (Javanese and Moluccans) belonged to the Papuan clusters, HBV/C6 (6NP, 27NP and 49NP) and HBV/D6 (7NP, 25NP, 26NP). Surprisingly, it seemed that there could be a horizontal transmission of HBV from Papuans to non-Papuans living in Papua, in an adequate number. Two isolates from Javanese and one isolate from Sulawesi people (30NP, 37NP and 57NP) were classified into HBV/B3 (Java and Papua). The reported HBV/B3 isolates from Papua by Lusida *et al.* (2008), were possibly obtained from Javanese living in Papua. Two isolates from Javanese (9NP and 18NP) were classified into HBV/B7 (Alor, East Nusa Tenggara). The possible reason was the fact that there were many East Nusa Tenggara people living in Papua, especially in transmigrating areas. Besides, one isolate (12NP) from East Nusa Tenggara people was classified into HBV/C1 (South East Asia). Again, it might be caused by horizontal transmission, because there were many Thailand seamen who came to Papua. Interestingly, some HBV/B isolates from Javanese and Sulawesi people (14NP, 39NP and 55NP) were not definitely classifiable into the previous subgenotype B (B1-B7). They had possibly distinct clusters which reflected the specificity of the ethnicity in the HBV distributions, but further investigation about other regions, such as the pre-S or even the whole region of HBV DNA is needed. Finally, all HBV/C isolates from non-Papuans are separated from Far East Asia (HBV/C2), New Caledonia-Polynesia (HBV/C3), Australia (HBV/C4) and the Philippines (HBV/C5) clusters (Fig 2). This confirmed that the existence of HBV genotypes-subtypes in Papua was unique.

Overall, it could be concluded that many HBV isolates from non-Papuans in Jayapura belonged to the Papuan clusters, but others had different genotype-subtype patterns which frequencies were dependent on ethnicity. There could be a domination of HBV from Papuans, but the existence of HBV from non-Papuans must be considered as another important role to determine the circulation of the HBV genotypes-subtypes in Papua.

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