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RESEARCH ARTICLE

Hepatitis B Virus Surface Antigen Subtype in Positive HBsAg Subjects in Mengwi District, Badung, Bali-Indonesia

Made Agus Hendrayana[1,2]*, I Putu Astawa[3]

1. *Microbiology Department, Faculty of Medicine, Udayana University, Bali-Indonesia.*
2. *Biomedicine Doctoral Program, Post Graduate Program, Udayana University, Bali-Indonesia.*
3. *Orthopedic and Traumatology Department, Faculty of Medicine, Udayana University-Sanglah General Hospital, Bali-Indonesia.*

***Corresponding Email Author:** *agus_hendrayana@unud.ac.id*

Abstract

Indonesia is a country with a high prevalence of hepatitis B virus infection and can develop into chronic hepatitis in the community. Indonesia is a multiethnic country with a vast territory, so it is estimated that the prevalence of each hepatitis B virus subtype varies greatly in different regions. Sequences in the S region gene encoding amino acids can show hepatitis b virus subtypes through exclusive variations in subtype determinants of HBsAg. This hepatitis B virus subtype is epidemiologically important, because it can show differences in geography and ethnicity in its spread. This study aims to analyze the hepatitis B virus subtype in HBsAg positive subjects in Mengwi, Badung, and Bali-Indonesia. The subjects of the study were 75 patients who came to the Mengwi I Badung Primary Health Center. All serum samples were examined for HBsAg by the ELISA method. In HBsAg positive serum samples, hepatitis B virus subtypes are determined. The DNA is extracted from serum samples that are HBsAg positive. Amplification of portions of DNA S gene from serum HBsAg positive was performed by polymerase chain reaction first-round and second-round. The nucleotide sequence of the hepatitis B virus is converted into amino acid sequences and multiple alignments are performed. The hepatitis B virus subtype was determined by amino acid substitution analysis at positions 122, 127, 134, 159, 160 and 177 in the S gene assisted by using a computer program. The subjects with HBsAg positive were obtained from 11 out of 75 samples (14.7%). The results of hepatitis B virus subtype analysis found that almost all samples, 10 of 11 samples (90.9%) had *adw2* subtypes, while one sample (9.1%) had *adrq* + subtype.

Keywords: *HBsAg, Subtype, HBV, Mengwi.*

Introduction

Hepatitis due to viral infection is still a problem in Indonesia where some regions in Indonesia have a high prevalence so that it can be said that Indonesia has high hepatitis B endemicity levels and can develop into chronic [1-3]. People with hepatitis B Virus (HBV) carriers in a healthy-look population in Indonesia are reported to be in the range of 4-20.3%, so Indonesia is a country with a high prevalence of HBV infection.

Another report, the Core Working Party for Asia-Pacific Consensus on Hepatitis B and C also states that Indonesia has a moderate to high hepatitis B endemicity level [2, 4]. HBV gene in region S (surface) encodes HBV envelope containing HBsAg. Based on the sequence of nucleotide sequences in the S region genes that encode amino acids can show HBV subtypes through a variable determinant of HBsAg subtypes that are exclusively paired [5, 6]. The HBV subtype is the identification of a determinant and two pairs of alela variations *d/y* and *w/r,* which can divide HBsAg according to the immunological characteristics of the protein into four major subtypes, namely: *adw, adr, ayw*, and *ayr*.

Other classification systems have divided HBV into nine subtypes, namely *adw2, adw4, ayw1, ayw2, ayw3, ayw4, adrq +, adrq-* and *ayr* [7]. Sequences in the S region gene encoding amino acids can show HBV subtypes through exclusive variations in subtype determinants of HBsAg.

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In the S region gene products, variations of amino acids in certain positions are related to the subtypes of HBsAg subtypes that are exclusively paired. The molecular basis of variations in *d/y* and *w/r* both depends on the substitution of Lys / Arg in number codons 122 and 160 amino acid sequence regions S. From sequence codons number 101 to 180 of 44 small genes HBV region S, codon number 127 is a codon which plays a role in the variation *w1-w4*, where *w1/w2*, *w3* and *w4* are each encoded by Pro, Thr and Leu in that position. Expression *w1* also depends on Arg at position 122, Phe at 134 and or Ala on 159. Other determinants are known as *q* expressed from most strains of *adw4* and some from *adr*.

Codons that play a role in the determinant expressions *q* are codons no 177 and 178 [6, 8]. Indonesia is a multiethnic country with a vast territory, so it is estimated that the prevalence of each HBV subtype varies greatly in different regions. All the main subtypes of HBV are found in Indonesia. *adw, ayw* and *adr* subtypes are dominant in western Indonesia, Maluku and Papua. *adr* subtypes are very predominant in Papua and parts of Sumatra, while the subtype's *ayw* (*ayw1* to *ayw4*) most commonly found in southern Sulawesi, Lombok, East Nusa Tenggara (Flores, Sumba, Sumbawa, Timor) and Maluku. The *ayr* subtype is very rarely found in Indonesia [9].

Also reported in Yogyakarta, *adw* was the most commonly found HBV subtype (74%), followed by *adr* subtype (11%) and other subtypes (Hadiwandowo et al., 1994). Other studies report that the *adw* subtypes (*adw2* and *adw4*) are most often found in Sumatra, Java, southern Kalimantan, Bali, Lombok, Ternate and Morotai [1]. In a study by Mulyanto et al. in Bali, in the city of Denpasar, it was found that HBV subtypes that predominantly infected patients were *adw* subtypes (63%), then followed by *adr* subtypes (24%), *ayw* (13%) and *ayr* (0%) [9].

This HBV subtype is epidemiologically essential, because it can show differences in geography and ethnicity in its spread. By studying the pattern of distribution of subtypes, we can obtain a description of the pattern of population migration in the past, because HBV infection from a subtype that is transmitted to other individuals will show the same subtype.

The subtype turns out to be related to ethnic and genetic factors, this is especially true for chronic sufferers [9]. Bali is a world tourism destination so that if there are health problems in Bali, especially infectious diseases, it becomes a serious concern, especially by countries with many people visiting Bali. From several studies in the last 20 years, the percentage of HBV infection in Bali is quite high, although there is not much research on HBV in Bali. This study aims to analyze the HBV subtype in HBsAg positive subjects in Mengwi, Badung, Bali.

Material and Method

This study is a descriptive observational study with an exploratory study design. In this study 75 serum samples were collected from subjects who came to the Mengwi I Primary Health Center and all samples resided or lived around the Mengwi District area.

Measurement of HBsAg Titers

All samples collected were then serologically tested for HBsAg using the ELISA method using the HBsAg commercial kit, Axiom Diagnostic, Cat.no.88 03 18 which was done according to the procedures contained in the kit.

HBV DNA Extraction

Serum from samples that were found to be HBsAg positive was then extracted to isolate HBV DNA uses commercial kit QIAamp DNA Mini kit (Qiagen, Inc. Cat. No.51304) which is done according to the procedures contained in the kit.

DNA Amplification by PCR (First Round)

Reaction mixture prepared with a total volume of 25 μl was made in a sterile microcentrifuge tube with a composition: Master mix (Fermentas) 12.5 μl, Primer P7 (5'-GTG GTG GAC TTC TCT CAA TTT TC-3 ') 25 pmol / μ1 1 μ1, Primer P8 (5'-CGG TAW [A/T] ACT ACT AAA GGG [A/C] GAT-3 ') 25 pmol / μ1 1 μl, DW 1 μl, and DNA 9 , 5 μl. The mixture was inserted into the thermal cycler machine and the program file on the machine was run. The first round PCR was carried out in 40 cycles where each cycle consisted of stages: denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C and extension for 2 minutes at 72°C.

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DNA Amplification by PCR (Second Round)

If the first round PCR amplification is negative, then a second round PCR is performed using the HBS1 primer (5'-CAA GGT ATG TTT TG-3 'CCC GTG) and HBS2 primer (5'-AAA GCC CTG ACTG A-3 CGA ACC') with the same conditions as first-round PCR.

Detection of PCR Products by Electrophoresis

The 2% agarose gel was prepared which contained ethidium bromide and then placed in the apparatus gel electrophoresis and added 1x buffer TBE until the gel was completely submerged. A marker of 10 μl was mixed gently pipetting with 10x loading buffers as much as 2 μl which had been dropped over the parafilm. The first round PCR product of 7 μl was taken and mixed gently pipetting with 10x loading buffers as much as 2 μl which had been dropped over the parafilm, then put into the slot gel. Gel electrophoresis apparatus is closed and run with 100 volts for approximately 30 minutes, then seen under ultraviolet light short wave. Then the results appear to be documented with Polaroid cameras.

Isolate DNA with Low Melting Agarose

The DNA seen on electrophoresis with ordinary agarose gel was then repeated using agarose low melting gel. The agarose gel using agarose L (low melting agarose) 2% was prepared, and then a mixture of DNA 18 μl and 10x loading buffer 2 μl were prepared. This mixture is applied to the slot gel at intervals without using a marker and then the electrophoresis machine is run. Electrophoresis results were seen with ultraviolet light long wave. A gel containing DNA is cut with a cutter that is washed every time it cuts. The gel piece is inserted with tweezers into a sterile 1.5 μl Eppendorf tube. Then DNA isolation of the PCR results from low melting agarose was done using a QIAamp DNA Mini kit (Qiagen, Inc.) commercial kit with work procedures according to the procedures contained in the kit.

Labeling with PCR for Sequencing

Labeling with PCR for sequencing using the Ready Reaction Cycle Sequencing Kit (ABI Prism Big Dye Terminator vl. L, Applied Biosystems).

Sequencing reaction with a total volume of 20 μl was made in a sterile micro centrifuge tube with a composition: DNA PCR 5 μl, primary sense or antisense 4 pmol / μl for HBV: 1.5 μl (primer P7), Ready reaction mixture Big Dye Terminator 2 μl, Big Dye Buffer 7 μl, DW 4.5 μl mixed with gently pipetting. Then put in a regulated thermal cycler machine to a temperature of 94°C for 3 minutes. PCR was carried out in 25 cycles. Each cycle consists of stages: denaturation: for 10 seconds at 96°C, annealing for 5 seconds at 50°C, extension for 4 minutes at 60°C. After the cycle is complete, it is cooled to 4°C.

Purification of the Results of DNA Labeling with Ethanol Precipitation

The tube containing 20 μl sequencing reaction was removed from the thermal cycler and rotated briefly, then aspirated and transferred into a sterile 1.5 ml Eppendorf tube. Then sodium acetate as much as 2.5 μl and absolute ethanol as much as 50 μl were added to the tube and then mixed using the flicked tube. The mixture was incubated at room temperature for 5 minutes. Then centrifuged at 15,000 rpm for 15 minutes at 6°C.Existing supernatants are aspirated carefully and thrown into containers.

Then 70% Ethanol as much as 100 μl is added to the tube. Centrifuged at 15,000 rpm for 7 minutes at 6°C.The existing supernatants are aspirated carefully and thrown into the container of the container containing DNA pellets in an open state wrapped in plastic wrap and dried using a vacuum pump for 15 minutes. After that dry DNA pellets can be stored at 4°C after being tightly closed with aluminum paper.

DNA Sequencing

DNA sequencing uses an ABI Prism 310 Genetic Analyzer (Perkin Elmer) machine. Previously a DNA mixture was prepared before sequencing. 25 formal of HiDi formaldehyde was added to a tube containing dry DNA pellets. Centrifuged and then incubated at 9°C for 2 minutes. Then incubated in ice for about 3 minutes and then spin down. The tubes are kept in ice until they are ready to be analyzed, then aspirated and transferred into a special sterile microtube for sequencing.

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Then the tube is inserted into the machine and run on the ABI Prism 310 Genetic Analyzer engine.

HBV Subtype Analysis

The results of the nucleotide sequences of the specimens were translated into amino acid sequences and then multiple alignments were carried out, and analyzed for certain codon sequences. Specimen HBV subtypes were determined based on the results of amino acid analysis of specimens at positions 122, 127, 134, 159, 160 and 177 in the S HBV gene, using the Genetyx Version 9.0 for Windows.

Result

Based on the results of examination of HBsAg levels obtained only 11 of 75 samples (14.7%) with positive HBsAg levels. Of the 11 samples, one was female (9.1%), and the majority of HBsAg positive from the sample were found in men, 10 of 11 samples (90.9%).

From 11 HBsAg positive samples after PCR examination, it was found that 11 samples (100%) could be identified by PCR, which consisted of eight samples that were positive with PCR using P7-P8 primary pair with 541 bp amplification and three positive samples using PCR HBS1-HBS2 primer with 259 bp amplification results from S. gene. Most of the 8 samples from 11 (72.7%) can be detected by PCR using P7-P8 primary pair (first round), while the remaining 3 from 11 samples (27.3%) which can be detected with the primary pair of HBS1-HBS2 (second round).

Multiple alignment of amino acid sequence S region number 116-183 which is encoded by nucleotides 500-703 in HBV S region genes which refers to HBV isolates with accession number D00329, which is 11 sequences of patients with hepatitis symptoms in Mengwi, Badung and 38 sequences from the international DNA data bank (DDBJ / GenBank) shown in the Figure 1.

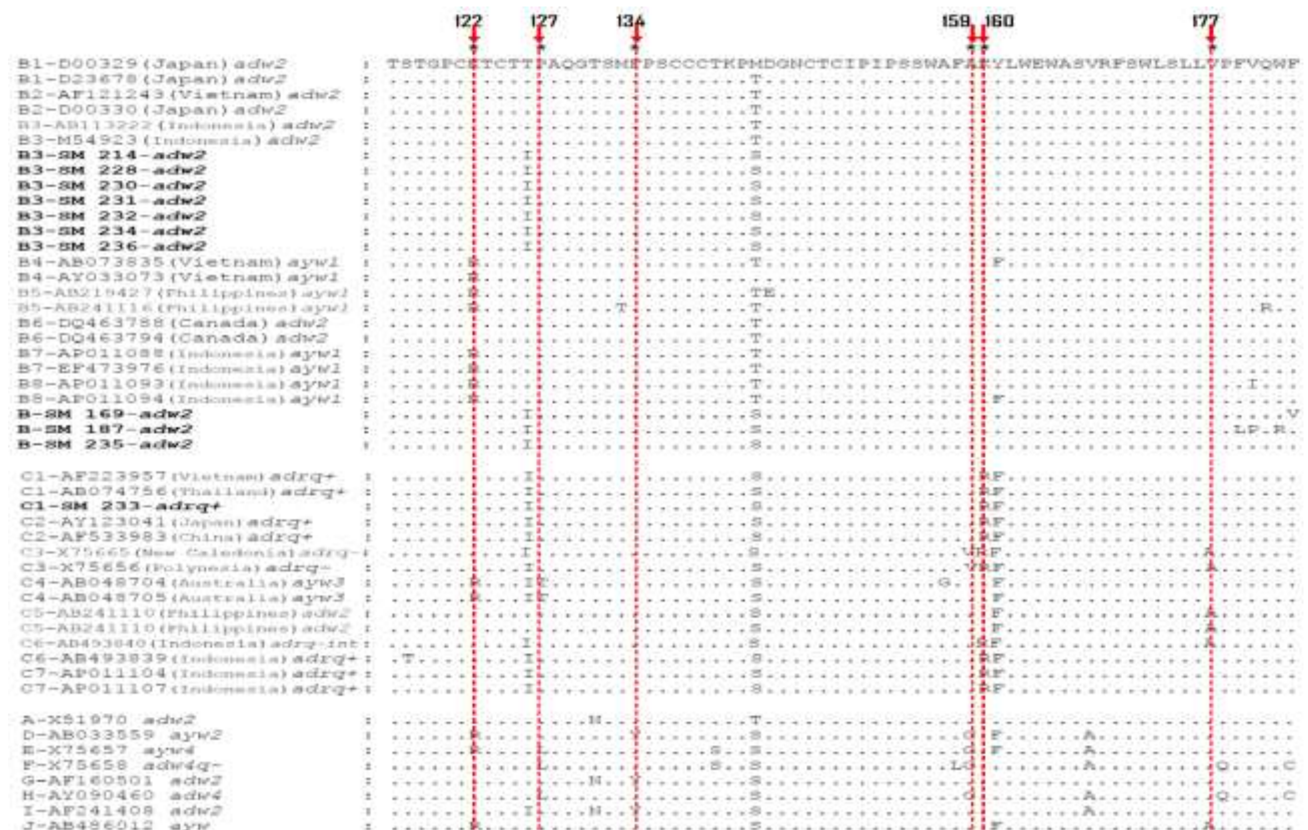

Figure 1: Multiple alignments of amino acid sequences numbered 116-183 in S region genes of various subgenotypes and HBV subtypes of sample isolates and isolates from international DNA databanks (DDBJ/GenBank); Note: Isolates with bold print indicate sample isolates

In the multiple alignments in figure 1, it can be shown the amino acid substitution analysis in sequence numbers 122, 127, 134 and 160 so that the HBV subtype can be known from the sample isolates. Amino acid substitution analysis Ala with Val at position 159 (A159V) and amino acid Val with Ala at position 159 (V159A) in HBV subtype *adr* can be known whether or not there is a determinant of *q*. Isolates from sample number SM 233 which had the *adr* subtype did not show substitution of amino acids

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A159V and V159A, so that isolates of sample number SM 233 could have a determinant of *q* (*q* +). The HBV subtype of the sample isolates can be seen in the following Table 1.

Table 1: HBV subtype of isolates from the study sample

Subject No.	HBV subtype
SM 169	*Adw2*
SM 187	*Adw2*
SM 214	*Adw2*
SM 228	*Adw2*
SM 230	*Adw2*
SM 231	*Adw2*
SM 232	*Adw2*
SM 233	*Adrq+*
SM 234	*Adw2*
SM 235	*Adw2*
SM 236	*Adw2*

Table 1 shows that analysis of HBV subtypes of all sample isolates, almost all samples, 10 out of 11 samples (90.9%) had *adw2* HBV subtypes, while one sample (9.1%) had *adrq* + HBV subtype. It can be said that the results of the HBV subtype analysis of all samples showed that the *adw2* subtype was the dominant subtype of all isolates in the study sample

Discussion

From the results of HBV subtype analysis of all sample isolates, almost all samples (90.9%) had *adw2* HBV subtypes, while one sample had HBV *adrq* + subtype. *adw2* subtype is the dominant subtype of all isolates in the study sample. The *adw* subtype is found in a wide area ranging from Africa, the Eastern Mediterranean region, West Asia to North India, Western Europe, North and South America. In Asia and Oceania the subtype *adr* is mostly found in North China, Korea, large islands in Japan, Malaysia, Burma and Thailand, while the subtype adw is mainly found in the southern parts of South China, Taiwan, Okinawa, and Amami, the Philippines and Indonesia [10].

Utama et al. obtained HBV *adw2* subtypes and *adrq* + subtypes found to be dominant in infection by HBV genotypes B and C in Indonesia [11]. This HBV subtype can epidemiologically show differences in geography and ethnicity in its spread. The pattern of distribution of subtypes can describe the pattern of population migration in the past, because HBV infection from a subtype that is transmitted to other individuals will show the same subtype. The subtype turns out to be related to ethnic and genetic factors, this is especially true for chronic sufferers [9].

Mulyanto *et al*. study show that the ancestors of Indonesia's easternmost population infected with the HBV subtype *adr* appear to have come from Melanesia where the subtype *adr* is found. Analysis of amino acid substitution in positions 159 and 177 of the S gene classifies HBV isolates from New Caledonia (Melanesia) and French Polynesia (Polynesia) into the *adrq*-subtype [5, 9].

All the main subtypes of HBV are found in Indonesia. *adw, ayw* and *adr* subtypes are dominant in western Indonesia, Maluku and Papua. *adr* subtypes are very dominant in the Papua region and part of Sumatra [9]. Also reported in Yogyakarta, *adw* is the most commonly found HBV subtype, followed by *adr* subtypes and other subtypes [12]. Other studies report that the *adw* subtypes (*adw2* and *adw4*) are most often found in Sumatra, Java, southern Kalimantan, Bali, Lombok, Ternate and Morotai [1].

There are several theories about the native population of the Indonesian population, but the most accepted theory is the theory by Brandes. The theory by Brandes states that native Indonesians are of Austronesian descent. Austronesian descendants can be divided into two, namely Western and Eastern Austronesian.

Populations of western Austronesian descendants include Kalimantan, Sumatra, Bali and islands in West Nusa Tenggara where in this zone HBV *adw2* subtypes are dominant. Eastern Austronesians include Sulawesi, Nusa Tenggara and South Maluku where *ayw* subtypes are dominant [9, 13].

From increasing population mobility and changing demographic composition in Mengwi, Badung, Bali in the past few years it has not been known whether there has been a shift in the HBV subtype pattern in Mengwi because there has never been any data or research describing the HBV genotype pattern in Mengwi.

Some limitation in this study includes the number of samples and the distribution of

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sampling that is less extensive and less scattered from all regions in Mengwi and Badung Regency so that data have not been represented that represent the actual population conditions in Mengwi District and Badung Regency.

Conclusion

The results of HBV subtype analysis of all HBsAg positive samples showed that almost all samples, 10 out of 11 samples (90.9%) had *adw2* HBV subtypes, while one sample (9.1%) had *adrq* + HBV subtype.

Conflict of Interest

The author declares there is no conflict of interest regarding all elements in this study.

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