



UNIVERSITI PUTRA MALAYSIA

***MOLECULAR CHARACTERIZATION AND METHYLATION STATUS OF
TORQUE TENO VIRUS ISOLATION AMONG HEPATITIS PATIENTS IN A
PUBLIC HOSPITAL IN PAHANG, MALAYSIA***

SITI NURZULAIKHA BINTI HAZANUDDIN

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By

SITI NURZULAIKHA BINTI HAZANUDDIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of Master of
Science**

September 2020

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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September 2020

Chair : Zulkefley Othman, PhD
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Hepatitis diseases also known as asymptomatic at the early stage of infection. There are viruses classified as commensal yet opportunistic pathogens with a high infection rate, including anellovirus. Torque teno virus (TTV) is a small, non-enveloped and circular single-stranded DNA anellovirus that infects the human population worldwide, especially hepatitis patients. A high prevalence of TTV was reported from various countries to include within the Asian continent. However, there is no information regarding the TTV prevalence among hepatitis patients in Malaysia. Hence, this study decided to determine the genotype of TTV as well as to construct the phylogeny tree of TTV isolated among the hepatitis community in Hospital Tengku Ampuan Afzan (HTAA) Kuantan. Other than that, this study aims to determine the mutation by DNA methylation in the CpG distribution of TTV isolates. The sample subject selected is hepatitis patients who attended HTAA for a medical appointment. A total of 137 hepatitis patients have been recruited and the plasma DNA was tested with three sets of primers by using the standard PCR method to amplify the coding and noncoding region of TTV. It was discovered that 87.5% and 73.7% of the subjects showed the presence of the noncoding region of TTV when two different sets of primers were used (UTR(a) and UTR(b)). Meanwhile, for the presence of the N22 region which is the selected coding region located in ORF1 specifically among Group 1 TTV, 20% of positivity were shown. The other 80% of positively detected TTV isolates without N22 region could not be classified into any groups as it is require further sequence analysis. Out of 101 positive UTR(b) samples which have been subjected for sequencing, only 41 samples produce complete readable sequences which belong to TTV genome. Low concentration amplicon and samples presented with multiple bands which have the possibility of co-infection were excluded from the analysis. By the construction of the phylogeny tree, conserved TTV isolates in hepatitis patients were identified in Clade A with the majority of numbers. Despite that, TTVMY02 is the first TTV Malaysian isolate from hepatitis patients with partial whole genome sequence of 3,265 kb and was determined by phylogeny relation to have a high

identity percentage with KAV isolate, which grouped in Group 2. The protein translated region for all ORFs in TTVMY02 isolates were well described on its physiochemical properties; and ORF1 and ORF2 appeared to be more hydrophobic while ORF3 more hydrophilic. Lastly, the selected TTV Malaysian isolate was further investigated for the methylation properties in the region consist of the highest CG percentage among a few CpG island by bisulfite sequencing and methylation-specific PCR. The result showed the CpG methylation in the TTV genome with 80% proved to be methylated in the selected island. Although the methylation of CpGs which is an addition of methyl group at cytosine base is considered as mutation, the alteration could be the way for the TTV virus to escape from the immune cells. Besides, there is no further investigation on the effect of TTV DNA methylation towards a cell line, but the postulation could be made based on PPV genome structure due to the similarity shared between both viruses. In conclusion, the characteristics of the TTV genome described in the study could be suggested that the virus is a non-pathogenic virus yet potentially leading to latent infection without any outbreak or disease outcome.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**MOLEKUL PENCIRIAN DAN METILASI KAJIAN ISOLASI TORQUE TENO
VIRUS YANG DIEKSTRAK DARIPADA DARAH PESAKIT HEPATITIS DI
HOSPITAL AWAM DI NEGERI PAHANG, MALAYSIA**

Oleh

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Penyakit hepatitis juga dikenali sebagai penyakit yang tiada gejala pada peringkat awal jangkitan. Terdapat virus yang diklasifikasikan sebagai patogen komensal namun oportunistik dengan kadar jangkitan yang tinggi, termasuk anellovirus. Virus Torque teno (TTV) adalah anellovirus yang mempunyai ciri-ciri seperti bersaiz kecil, berstruktur bulat, dan juga tidak berkantung telah menjangkiti ramai orang, terutamanya pesakit hepatitis. Penyebaran TTV yang tinggi dilaporkan daripada pelbagai negara termasuk negara dalam kalangan benua Asia. Namun, tidak ada informasi mengenai data kelaziman (prevalence) TTV dalam kalangan pesakit hepatitis di Malaysia. Oleh itu, kajian ini memutuskan untuk menentukan data kelaziman jangkitan TTV dalam kalangan pesakit hepatitis di Hospital Tengku Ampuan Afzan (HTAA) Kuantan. Subjek sampel yang telah dipilih adalah pesakit hepatitis yang menghadiri HTAA untuk temujanji perubatan. Sebanyak 137 pesakit hepatitis telah direkrut dan DNA plasma diuji dengan tiga set primer dengan menggunakan kaedah PCR standard. Telah ditemui bahawa 87.5% dan 73.7% subjek positif jangkitan TTV dengan wujudnya kawasan tanpa kod atau "noncoding" apabila menggunakan dua set primer yang berbeza iaitu primer UTR(a) dan UTR(b). Sementara itu, untuk kehadiran kawasan N22 yang merupakan kawasan pengekodan yang dipilih yang terletak di Kawasan ORF1 khususnya di dalam TTV Kumpulan 1, 20% subjek menunjukkan keputusan positif. 80% sampel pesakit hepatitis yang dikesan positif tanpa kawasan N22 tidak dapat diklasifikasikan ke dalam kumpulan mana pun kerana memerlukan analisis urutan yang lebih lanjut. 101 sampel UTR (b) positif telah menjalani urutan dan hanya 41 sampel menghasilkan urutan yang menghasilkan urutan yang sempurna dan dikenalpasti termasuk sebagai urutan TTV. Amplikon yang mempunyai kepekatan rendah dan sampel yang dipercayai mempunyai beberapa jenis

jangkitan TT telah dikeluarkan daripada analisis. TTVMY02 adalah isolat TTV Malaysia yang pertama daripada pesakit hepatitis dengan urutan keseluruhan genom keseluruhan. 3,265 kb menunjukkan mempunyai peratusan identiti yang tinggi dengan isolat KAV, yang dikumpulkan dalam Kumpulan 2. Selain itu, kawasan terjemahan protein untuk semua ORF dalam isolat TTVMY02 telah dijelaskan dengan baik mengenai sifat fisiokimia; dan ORF1 dan ORF2 menunjukkan ciri-ciri yang lebih hidrofobik manakala ORF3 lebih hidrofilik. Isolat TTV Malaysia yang dipilih terus disiasat untuk sifat metilasi di rantau ini yang terdiri daripada peratusan CG tertinggi di beberapa pulau CpG dalam urutan TTV oleh "bisulfite" penjujukan. Hasil kajian menunjukkan terdapat penentuan CPG metilasi dalam TTV genom dengan 80% terbukti di pulau yang dipilih. Walaupun metilasi CpGs yang merupakan penambahan kumpulan metil pada pangkalan sitosin dianggap sebagai mutasi, tetapi perubahan tersebut dapat menjadi jalan keluarnya virus TTV dari sel-sel imun. Di samping itu, tidak ada penyelidikan lebih lanjut mengenai pengaruh metilasi DNA TTV terhadap garis sel, tetapi postulasi dapat dibuat berdasarkan struktur genom PPV kerana kesamaan yang dikongsi antara kedua virus. Kesimpulannya, ciri-ciri genom TTV yang dijelaskan dalam penelitian ini dapat didalilkan bahawa virus TTV adalah virus bukan patogen namun berpotensi menyebabkan jangkitan lewat tanpa gejala yang berbahaya.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

UPM	Universiti Putra Malaysia
aa	amino acid
ALT	Alanine aminotransferase
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAV	Chicken Anemia Virus
CpG	5'—C—phosphate—G—3'
dsDNA	double-stranded DNA
EDTA	Ethylene diamine tetra acetic acid
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HIV	Human Immunodeficiency virus
HSC	hematopoietic stem cell
HTAA	Hospital Tengku Ampuan Afzan
HVR	Hypervariable region
ICTV	International Committee on Taxonomy of Viruses
mRNA	messenger RNA
MOPD	Medical Outpatient Department
MSP	Methylation specific PCR
nt	nucleotide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction

QV	Quality value
RDA	Representational difference analysis
ssDNA	single-stranded DNA
TTV	Torque teno virus
TTMV	Torque teno mini virus
TTMDV	Torque teno midi virus
TAIP	TTV-derived apoptosis-inducing protein
UTR	Untranslated region
VP	Viral protein

CHAPTER 1

INTRODUCTION

1.1 Background of study

Hepatitis is one of the upsetting global health issues which had infected over 325 million people globally, specifically 49 million people in the South East Asia region, not excluding Malaysia (WHO., 2019). It is a slow yet progressive disease that involved a hepatotropic virus-induced inflammatory reaction in the liver. Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D and Hepatitis E are the five infectious hepatitis viruses known. The most virulence viruses which could lead to deleterious effect such as liver cancer are Hepatitis B and C (Ruksana., 2016). Despite of the ability of both virus to cause chronic hepatitis, it does not cause any symptoms to the infected host at the early stage of infection and known as asymptomatic infection (WHO, 2020)

Asymptomatic infection is defined as an outbreak that has been investigated in real-time among the exposed people who are not manifesting any symptoms, yet demonstrates seroconversion (Potasman., 2017). There are few viruses considered to be commensal and opportunistic pathogens which include members of the herpesvirus, adenovirus, papillomavirus, anellovirus, polyomavirus and circovirus families (Wylie et al., 2014). Back in 1997, a post-transfusion Japanese hepatitis patient was detected with a novel DNA virus sequence while in searching of the hepatitis etiology and later categorized as Alphatorquevirus genus in the family of Anelloviridae and named as Torque teno virus (TTV) (Nishizawa et al., 1997).

More than 39 genotypes of TTV have been discovered under five major phylogeny group (Group 1, 2, 3, 4 and 5), and two additional minor groups was recently found (Group 6 and 7) (Huebei., 2017). The divergence between the phylogeny group was calculated to be more than 50% and each genotype shared a similarity of less than 70% (Irshad, Singh, Agarwal, & Joshi, 2008 & Mi et al., 2014). The circular structure of single-stranded DNA non-enveloped TTV is divided into two significant regions; potential coding and noncoding region (Spandole et al., 2015). TTV which sizes in the range of 30 – 50 nm in diameter and 3.8 kb in length contains 3739 base pairs (bp) which expressing 3 messenger RNAs (mRNAs) (Rezahosseini et al., 2019). These mRNAs use alternative start codons (AUGs) for translation and encode at least 6 open reading frame (ORF) proteins.

TTV-hepatitis infected patients were shown to have a correlation of TTV viraemia load with the immune status of infected individuals (Omid et al., 2019). For

example, Tokita et al (2002) had evaluated that a high percentage of HCV patients co-infected with TTV is significantly associated with higher age, history of blood transfusion and complication of cirrhosis. Meanwhile, for HBV, patients who co-infected with TTV has significant associations in plasma samples of liver transplanted patients, with determined cirrhosis (Kazemi et al 2015). Also, multiple speculations suggested that TTV can induce liver damage by the association of TTV viremia with liver enzyme elevation (Chen et al., 1999). Other than that, Omid et al (2019) reported the estimated detectable viral load for TTV remains about 102 to 108 copies/ml and >90% of these are cleared by the immune system. The pathogenesis of virus clearance by the immune system without eliciting any symptoms seems to have a relation with the DNA methylation mechanism.

The epigenetic mechanism has a role in the regulation of the viral replicative cycle. One of the interrelated epigenetic mechanisms which actively replicated in eukaryotic cells is DNA methylation (Francessa 2013). DNA methylation is one of the prime forms of epigenetic modifications in the eukaryotic genome and occurs on cytosines that are located prior to guanines to yield 5-methylcytosine (Herman and Havlin., 2003). CpG dinucleotides which are underrepresented in most of the small DNA viruses (Toth 2012) is believed to associate with gene silencing which can inhibit gene expression by various mechanisms (Portela & Esteller, 2010; Kacem & Feil, 2009). Therefore, the silencing gene and inhibition of gene expression of TTV might help TTV to escape from the immune system of the infected individuals without causing any illnesses symptoms. On top of that, a new and emerging concept includes the view that viruses may have deleterious or beneficial immunomodulatory effects other than affecting the adaptive and innate immune response (Gentile & Micozzi., 2016).

1.2 Problem Statement

For almost 20 years since the discovery of TTV, many studies were conducted with multilevel health background for sample subject, yet the information regarding TTV infection among countries in South East Asia continent, especially Malaysia, is still limited. Although TTV infection is categorised as asymptomatic infection due to no particular disease is related to the infection, yet the investigation has to be conducted as critical as other symptomatic infection. The reason is to prevent any epidemic caused by these asymptomatic viruses as the virus could be actively transmitted during the pre-symptomatic phase of infections (e.g. influenza, hepatitis A). Hence, timely diagnosis and perhaps treatment of asymptomatic infections is of prime importance. In addition, the postulation about TTV co-infection with the hepatitis virus is hype among TTV studies. Therefore, this study decided to explore more on the prevalence of TTV infection among hepatitis patients with the addition of the identification of TTV genotype isolated among the Malaysian population in terms of its molecular characterization and CpG methylation status. Other than that, the determination

of CpG distribution and methylation pattern in TTV could give a new theory for in-depth understanding of the association of viral expression with viral latency.

1.3 Study Significance

The prevalence studies on the TTV have shown a much wider range of virus localization including people with acute and chronic hepatitis, blood donors, drug users, dialysis patients and healthy individuals as well. However, different geographical locations studied will vary the prevalence of TTV and the genotypes discovered. From the complete genome analysis of TTV, the epigenetic side of TTV on the viral DNA methylation in CpG island which occurs at cytosine bases will be observed.

Furthermore, there is little or no information especially in Malaysia's population regarding the epigenetic changes of CpG methylation in TTV. Hence, in understanding TTV, this present study may give an idea on the prevalence and genotype of TTV isolated in Malaysia's populations with additional information on the epigenetic changes of CpG methylation in TTV.

1.4 Objectives

1.4.1 General objective

This study is to investigate the molecular characterization and methylation status of Torque Teno Virus (TTV) isolated from Hospital Tengku Ampuan Afzan (HTAA) hepatitis patients.

1.4.2 Specific objectives

- i. To determine the genotype of Torque Teno Virus (TTV) isolated from hepatitis patients
- ii. To construct a phylogeny tree of TTV isolated from hepatitis patients.
- iii. To determine the CpG distribution and methylation pattern in Torque Teno Virus isolated from hepatitis patients

1.4.3 Research question

- 1) Is there any information regarding TTV studies in Malaysia?

Yes, there are two studies has been conducted in 2017 and 2019 to observe the infection rate of TTV. However, the study population selected for these studies are only among healthy individuals and pneumonia patients.

- 2) Why hepatitis patients' population in Malaysia was selected to be the sample study population in this study?

Hepatitis patients were selected based on the previous studies which proposed observation of high TTV infection rate among hepatitis patients due to the replication sites of TTV which has been found in the hepatocytes. Other than that, the first TTV study conducted among hepatitis patients in Malaysia will propose a new isolate which has its uniqueness parallel to our genetic pattern inheritance.

- 3) Why CpG methylation pattern was selected for epigenetic study in TTV genome?

It is because the CpG methylation pattern is one of the common event of mutation which occur in viruses. However, there are more studies conducted in observing the methylation pattern in large size viruses compared to small size viruses, including TTV. Besides, the methylation pattern is postulated to have a connection with viral latency; the ability of a virus to remain dormant within the host cell

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