【参考資料】シェムリアップ（カンボジア）の HBsAg 陽性妊婦における HDV 陽性率と遺伝子分布：In-House Direct ELISA 法の開発と評価（中間報告）

Sero-prevalence and Genotype Distribution of HDV Among HBsAg-Positive Pregnant Women in Siem Reap, Cambodia: Development and Evaluation of an In-house Direct ELISA method.

田中 純子

Golda A. Akuffo, Ko Ko, 杉山 文, 秋田 智之, 高橋 和明
広島大学 大学院医系科学研究科 疫学・疾病制御学

※ 本稿では、厚生労働科学研究費補助金（肝炎等克服政策研究事業）
以外で実施したウイルス肝炎の疫学研究を参考資料として記載する。

研究要旨

B 型肝炎ウイルス（HBV）持続感染者のうち約 5%が D 型肝炎ウイルス（HDV）に感染しているとされるが、その疫学的実態は十分明らかになっていない。WHO guidelines for the prevention, diagnosis, care and treatment for people with chronic Hepatitis infection, (WHO 2024[1]) states that におい すべての HBV 持続感染者に HDV スクリーニング検査を行うことを推奨しており、HBV 高浸淫地区であるアジア・アフリカ地域での検査普及には、より安価で高精度の検査法の確立が求められる。

本研究では、HDV 抗体を検出するための In-House Direct ELISA 法の開発を試みた。

シェムリアップの HBsAg 陽性妊婦 67 人の保存血清を用いて HDV 抗体を測定し、市販の測定試薬による測定結果と比較することで有効性を評価した。

その結果、市販の測定試薬を基準とした場合の In-House Direct ELISA 法の感度および特異度は、それぞれ 50.0%および 95.4%であった。In-House Direct ELISA 法によって 4 検体（4/67、5.97%、95%CI：1.65-14.59）で HDV 抗体が検出された。そのうち 2 検体では HDV RNA 陽性であり、いずれも HDV 遺伝子型 1 であることが同定された。

本研究において開発した In-House Direct ELISA 法は、市販の ELISA キットと比較して安価かつ入手しやすい試薬を用いており、使用する検体量も少なく済むことから、HBV 高浸淫地区であるアジア・アフリカ地域における HDV 抗体検査の普及に貢献できる可能性がある。

なお、本研究では用いた検体の HDV ウイルス量が少なかったことが HDV 抗体検出感度に影響した可能性があり、高ウイルス量の検体による追試験を予定している。

A. 研究目的

Hepatitis Delta infection previously known as the Orphan Disease because it was widely neglected due to its rarity and little awareness is caused by the Hepatitis delta virus (HDV); a 1.7kb virus which is a defective virus of the Hepatitis B virus (HBV)[2]. The coexistence of Hepatitis D Virus (HDV) infection among individuals already infected with Hepatitis B Virus surface antigen (HBsAg) has significant clinical implications, particularly during pregnancy[3]. Cambodia, like many low resourced settings lacks information on the prevalence of anti-HDV in spite of its high HBV prevalence. This is due to the inaccessibility of cheap

accurate testing[4]. This study presents a unique context where 67 Cambodian pregnant women positive for HBsAg were assessed for the prevalence of anti-HDV. The research not only outlines the epidemiological landscape but also introduces a novel method for anti-HDV detection. In recognition of resource limitations often encountered in settings like Siem Reap. , An In-house Enzyme-Linked Immunosorbent Assay (ELISA) was developed and meticulously evaluated for its efficacy. This paper explores the intersection of these critical elements—high Anti-HDV prevalence, pregnancy, and the introduction of a tailored diagnostic tool—offering insights that hold relevance for both local healthcare contexts and broader discussions on infectious diseases in resource-limited settings.

B．研究方法

This study is a continuum of a previous study on mother-to-child transmission of HBV among 1565 pregnant women in Siem Reap, Cambodia in 2020. Among the 1565 women, 67 (4.28%) tested positive for HBsAg. This study involved the development of an In-house direct Elisa method.

This was compared with a Commercial ELISA(My BioSource, Inc, San Diego, USA) kit to assess its accuracy. A 96-well microtiter plate was coated overnight with 50 µL of 500ng Recombinant HDVAg in 0.02M Tris-HCL at 4°C. The next day, the coating antigen was removed, and the wells blocked with 200 µL of 2% Human Serum Albumin (HSA) in 0.02M Tris-HCL. After 1 hour of incubation at room temperature, the plate was washed three times with a washing buffer (8.9g NaCl, 0.05% polysorbate 20 in 0.02M Tris-HCL) using an automatic microplate washer.

Next, 50µL of patient samples and positive/negative controls, diluted ten times with 5% HSA in 0.1% Tween 20 in 0.02M Tris-HCL, were added to the wells and incubated at 37°C for 1 hour. The plate was washed again, and 50 µL of peroxidase labelled anti-HDV IgG horseradish peroxidase (HRP), diluted 2000 times, was added and incubated at 37°C for 1 hour.

After further washing, 50 µL of TMB substrate was added to each well and the plate was kept in the dark at room temperature for 30 minutes. Readings were taken at 450 nm after adding 50 µL of stop solution. The cut-off for the ELISA test was determined as the mean absorbance of the negative controls multiplied by four.

To assess the new method, all 67 samples from HBsAg pregnant women were analyzed with both the developed direct ELISA method test and the commercial test. Sensitivity and specificity of the new test was calculated by ROC curve analysis the commercial test as the gold standard. To ascertain the prevalence of anti-HDV we detected Anti-HDV in all 67 HBsAg-positive sera using a newly developed in-house ELISA methods.

HDV RNA was tested among all anti-HDV positive samples using nested polymerase chain reaction (nested PCR). Relevant information related to HDV was extracted from questionnaires used for the HBV study. Refer to (Figure 1)

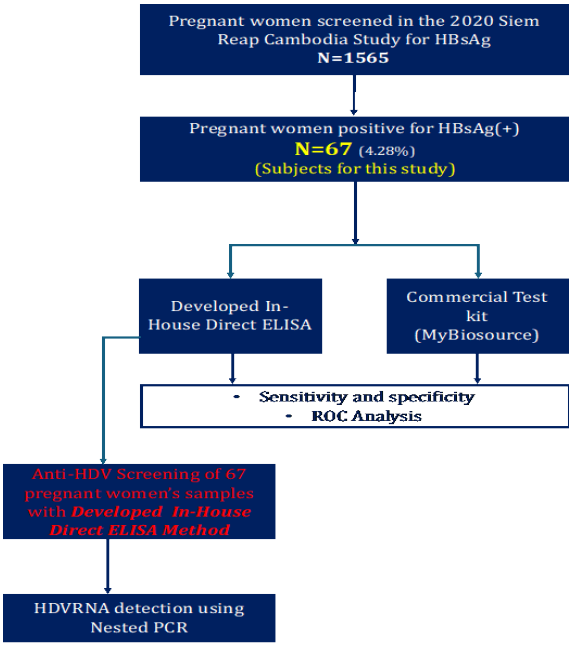

Figure 1: *Flow of study.*

C. 研究結果

In this study the sensitivity and specificity of the Direct In-house ELISA compared with the commercial test were 50.0% and 95.4%, respectively (figure 2). Anti-HDV was detected in 4(4/67) samples by the in–house Direct ELISA method, giving a prevalence of 5.97% (95% CI:1.65-14.59). Of the anti-HDV positive samples, 2(2/4) tested HDV RNA positive, with both samples belonging to the HDV1 genotype with close similarities to the west African types (figure 4).

Prevalence of HDVRNA was 2.99% (95% CI: 0.36-10.4) among the 67 HBsAg positive pregnant women while the Anti-HDV prevalence among all pregnant women was 0.26% (95% CI: 0.07-0.65). The ages pregnant women who were anti-HDV positives ranged between 21 and 49 years old, with majority of the pregnant women in the 25 to 29 age range (Figure 3). Among the anti HDV pregnant women none of them had been given blood transfusion, had surgery, or been HB vaccinated. None of them tested positive for HCV, syphilis, or HIV. On the other hand, both pregnant women who tested HDVRNA positive had highest HBV viral titers of 6.67 x 10^7 copies/ml and 2.94 x 10^4 compared to the rest who were also AntiHDV positive. Two of these anti HDV positive them were infected with HBV genotype C whilst the rest were unknown.

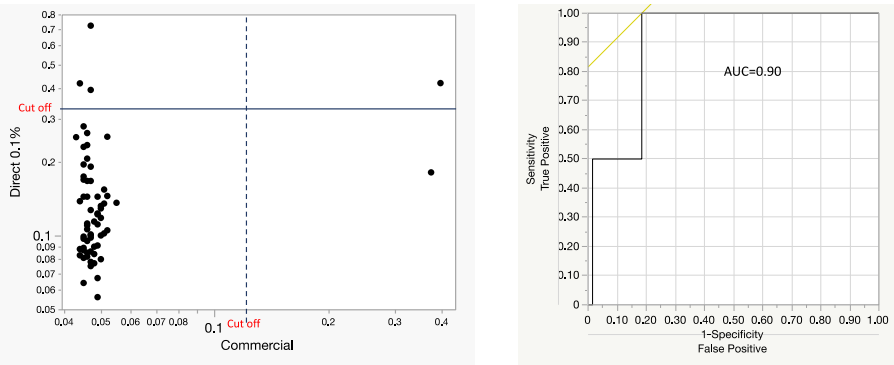

Figure 2: *ROC curve showing results of comparison of Developed in-house direct ELISA method and Commercial test.*

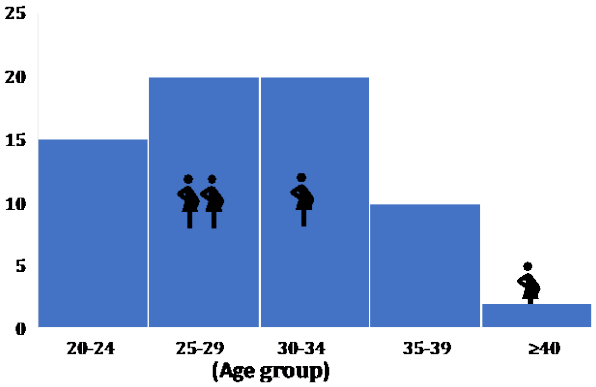

Figure 3: *Age distribution of 67 HBsAg (+) pregnant women showing distribution of four Anti-HDV positive pregnant women; Two in the 25–29-year group and one each in the 30 - 34 and over forty-year groups.*

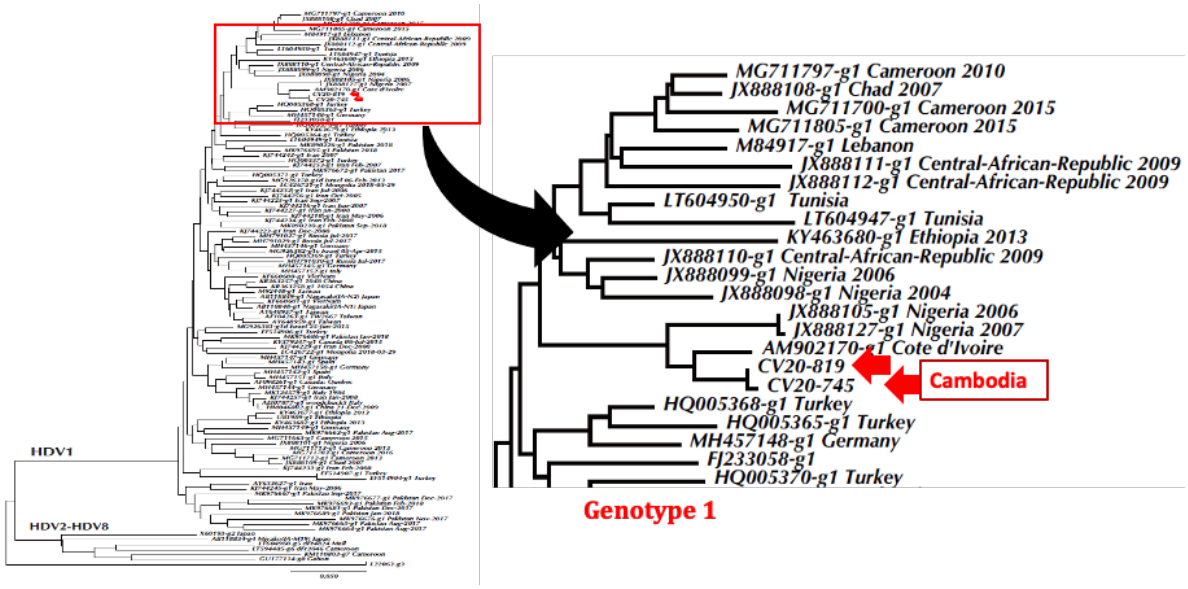

Figure 4: *Phylogenetic tree showing Genotype of HDVRNA positive pregnant women.*

D. 考察

Our in house developed ELISA method with a sensitivity and specificity of 50.0% and 95.4%, respectively was effective in detecting anti-HDV among 67 pregnant women who were HBSAg positive from among 1565 pregnant women. With our method we estimated the prevalence of HDV in Cambodia as 5.97% in line with the achievement of the WHO goal of elimination of viral hepatitis, it is imperative that more testing should be done, in order to ascertain actual prevalence of HDV in especially resource limited countries[5]. Mass testing is only possible with the availability of testing methods which currently are in assemble in some low-income countries[6]. In house methods like ours which are affordable can be utilized for this, as they have been proven to be efficacious.

However, it is worth noting that the sensitivity of our test method was low because of the limited sample size used in evaluating its performance and the lower HDV viral titers among the pregnant women included in our study resulting in low AntiHDV titres.

This study was able to establish anti-HDV prevalence for Cambodia and especially for the precious group of pregnant women. which was nonexistence in the past. The global prevalence of HDV is currently pegged at 5% among HBsAg positive patients[7]. Thus, the prevalence for Cambodian Pregnant women recorded in our study was high. Which is because in comparing the prevalence of HBV in Cambodia which is 4.28% to the global prevalence which was 3.2%, Cambodia is deemed to have a high prevalence of HBV[8]. This explains the high HDV prevalence. The Genotype distribution of HDV found in Cambodia confirms that genotype 1 is the most common genotype found worldwide even though it was expected that as a Southeast Asian Country the genotype could be II and IV[9]. Our ever precaution should

E. 結論

Our newly developed in-house ELISA method is effective in accurately detecting anti-HDV Antibodies among HBsAg positive pregnant women. The prevalence of Anti HDV was 5.97% and the RNA prevalence 2.99% among pregnant women. Additionally, the genotype 1 was genotype detected in Cambodia. As recommended by WHO, more tests like ours should be developed to make testing assessable to all to reduce the burden of viral hepatitis.

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

1. Akuffo Golda, Ko Ko, Bunthen E, Serge Ouoba, Aya Sugiyama, Tomoyuki Akita, Kazuaki Takashi, Junko Tanaka. Prevalence of Anti-HDV Among 67 HBsAg positive Pregnant Women in Siem Reap, Cambodia, Japanese Society of Hepatology conference, 7.9.2023, Tokyo Japan.
2. Akuffo Golda, Ko Ko, Bunthen E, Serge Ouoba,

Aya Sugiyama, Tomoyuki Akita, Kazuaki Takashi, Junko Tanaka, Prevalence of Anti-HDV Among 67 HBsAg positive Pregnant Women in Siem Reap, Cambodia, 28.3.2024, APASL Kyoto, Japan.

H. 知的財産権の出願・登録状況（予定を含む。）

なし

References

1. World Health Organization, *Guidelines for the prevention, diagnosis, care and treatment for people with chronic hepatitis B infection*. (2024).
2. Stockdale, A. J. *et al. The Global Prevalence of Hepatitis D Virus Infection: Systematic Review and Meta-Analysis Prevalence of Anti-HDV Among 67 HBsAg Positive Pregnant Women in Siem Reap, Cambodia*. *Journal of hepatology* vol. 73 (2020).
3. Stockdale, A. J. *et al.* Prevalence of hepatitis D virus infection in sub-Saharan Africa: a systematic review and meta-analysis. *Lancet Glob Health* 5, e992–e1003 (2017).
4. Hayashi, T. *et al.* The global hepatitis delta virus (HDV) epidemic: what gaps to address in order to mount a public health response? *Archives of Public Health* 79, (2021).
5. Razavi, H. A. *et al.* Hepatitis D double reflex testing of all hepatitis B carriers in low-HBV- and high-HBV/HDV-prevalence countries. *J Hepatol* 79, 576–580 (2023).
6. Palom, A. *et al.* Implementation of anti-HDV reflex testing among HBsAg-positive individuals increases testing for hepatitis D. *JHEP Rep* 4, 100547 (2022).
7. Stockdale, A. J. *et al.* The global prevalence of hepatitis D virus infection: Systematic review and meta-analysis. *J Hepatol* 73, 523–532 (2020).
8. Bunthen, E. *et al.* Residual risk of mother-to-child transmission of HBV despite timely Hepatitis B vaccination: a major challenge to eliminate hepatitis B infection in Cambodia. *BMC Infect Dis* 23, 1–12 (2023).
9. Sharafi, H., Rezaee-Zavareh, M. S., Miri, S. M. & Alavian, S. M. Global distribution of hepatitis d virus genotypes: A systematic review. *Hepat Mon* 20, e102268 (2020).