【参考資料】カンボジア王国シェムリアップ州 1,565 人の妊婦を対象にした In-house 二重抗原サンドイッチ ELISA 法を用いた E 型肝炎ウイルス感染の疫学的評価

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※ 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

【背景】E 型肝炎は世界で、毎年推定で 2,000 万人が感染し、推定 330 万人が E 型肝炎を発症し、そして、56,600 人が E 型肝炎の関連で死亡している。その病原体である E 型肝炎ウイルス（HEV）の有病率は、途上国を中心に高く、カンボジアは HEV 高侵淫地域として知られているが、途上国で使用可能な安価・簡便な検査方法がなかったため、HEV 感染状況の実態は明らかにされていなかった。また、HEV 感染の致死率は通常 1〜2%であるが、妊婦は 20%と高いことが知られている。以上の背景から、本研究は、安価・簡便な ELISA 法による HEV 抗体測定法を開発し、カンボジア妊婦集団の HEV 抗体陽性率を評価することを目的とした。

【方法】In-house 二重抗原サンドイッチ ELISA 法による HEV 抗体検査法を開発した。主要なコーティング抗原としては、C 末端にマウス Fc タグが付加されたリコンビナント HEV カプシドタンパク質（ORF2）を使用した。二次抗原には、His タグ付きのリコンビナント HEV 抗原タンパク質を使用し、これをビオチンで標識して、多価ストレプトアビジン HRP を用いた化学発光検出システムを構築した。
HEV 抗体測定法を評価するため、カンボジアのシェムリアップ州で 2020 年に田中 純子研究室が実施した B 型肝炎ウイルス母子感染実態調査※で得られた保存血清検体 1,565 人のうち検体 ID に基づき無作為に選択された 262 検体を使用し In-house ELISA 法の評価を行った。
※2020 年 2 〜 9 月に同州の 3 医療機関で妊婦健診を受けた全妊婦のうち同意の得られた 1,565 人が調査の対象である。この調査では、5 項目の社会人口学的項目(出生年、民族、最終学歴、児の数、世帯主の職業)および 2 項目の既往歴情報項目(輸血歴、手術歴)のアンケート調査を同時に実施している。

2 種類の HEV IgG 測定商用キット（特殊免疫研究所の IgG anti-HEV EIA、および Mikrogen GmbH の recomLine HEV IgG/IgM Line immunoassay）を基準として、感度、特異性、ROC-AUC、一致率、コーエンの κ 係数を算出し、また費用を比較した。
次に、全 1,565 検体の HEV 抗体を本手法で測定し、HEV 抗体陽性とそれに関連する因子の多変量解析を実施した。

【結果】262 例を対象に In-house ELISA 法を評価した結果、本手法は、特殊免疫研究所のキットを基準とした時、感度 76%、特異度 94.1%、一致度 92.4%、κ =0.61 であった。Mikrogen の recomLine LIA を基準と

した時、感度 71.4%、特異度 98.6%、一致度 94.3%、κ =0.76 であった。ROC-AUC はいずれも 0.85 で、良好な識別力を示した。一方、1 回あたりの検査費用は本手法 133 円、Mikrogen 社製キット 4,333 円、特殊免疫研究所製キット 1,680 円と本手法は最も安価であった。次に、本手法を用いて全保存検体の HEV 抗体を評価したところ、全 1,565 人の妊婦における抗 HEV 抗体陽性率は 11.6%（181/1,565）であり、年齢が高い集団で高値を示す傾向を示した。
HEV 抗体陽性者の中で 22.7%（41 人）が IgM 抗体陽性であり、主に若年女性で最近または進行中の HEV 感染を示していた。一方、IgM 抗体陽性の検体からは HEV RNA は検出されなかった。多変量解析の結果から、高年齢、妊婦の世帯主職業が公務員である、が HEV 抗体陽性と関連があることが示され、生涯にわたる暴露によるリスク増加が示唆されている。
【結論】カンボジア王国シェムリアップ州の妊婦集団では、HEV 抗体陽性率が 11.6%であり、南アジアの妊婦集団の HEV 陽性率（Mirzaev and Tanaka et al. BMC Infect Dis. 24:525, 2024）よりは低いものの、HEV 抗体陽性者のうちの 22.7%が IgM 陽性であり、妊娠中の急性肝不全のリスクがあることを示した。本研究で開発した In-house ELISA 法は、既存の商用キットと同程度の診断精度を示し、安価であることから、途上国等の資源が限られた状況での HEV 検出や HEV 流行状況の把握に役立つと考えられた。

A. 研究目的

According to recently accepted meta-analysis from our department (Mirzaev U, Tanaka J et al., BMC Inf.Dis., accepted, 2024) the seroprevalence of Hepatitis E in Cambodia is 19.6%, which means that Cambodia is endemic for HEV. However, there is no information about the HEV seroprevalence among pregnant women, which are at risk of development of severe clinical course with serious outcome [1].
The principal goal of this study was to develop a new in-house ELISA method that is user-friendly, cost-effective, and less prone to errors by laboratory personnel. Because pregnant women are at an increased risk of experiencing severe HEV infections, especially in highly endemic areas including Cambodia, we then estimated the prevalence of HEV among this specific population

B. 研究方法

The in-house ELISA was designed for large-scale screening in resource-limited settings. Its performance was benchmarked against two commercial tests: the Anti-HEV IgG EIA (Institute of Immunology, Co. Ltd) and the Anti-HEV IgG RecomLine LIA (Mikrogen). This study builds upon a previous research project on the investigation of mother-to-child transmission of hepatitis B virus infection undertaken in Cambodia in 2020, which involved 1565 pregnant women from three hospitals in the Siem Reap region using a convenient sampling strategy [2]. The blood samples were collected from all participants and stored at −25°C for later analysis, and a well-structured questionnaire in the local Khmer language was used to gather sociodemographic information.
After accuracy assessment, we estimated the prevalence of total anti-HEV using the in-house ELISA across all 1565 participants. Next, we conducted the detecting IgM among those who tested positive for total anti-HEV immunoglobulins, using an anti-HEV IgM RecomLine LIA, Mikrogen GmbH, Germany. For the final phase, the positive anti-HEV IgM cases were tested by RT-PCR to confirm HEV RNA presence.
Additionally, in the study, we estimated an epidemiological trend of HEV transmission and association between HEV infection prevalence and various socio-demographic factors based on previously developed questionnaire (Figure 1).

C. 研究結果

The newly developed In-house ELISA showed a sensitivity of 76% and specificity of 94.1% against the Institute of Immunology kit, with a Cohen's kappa of 0.61. Against the RecomLine LIA by Mikrogen, it demonstrated a sensitivity of 71.4% and specificity of 98.6%, with a Cohen's kappa of 0.76 (Table 1). Both tests had an area under the curve (AUC) of 0.85, indicating good diagnostic accuracy (Figures 2 and 3).
The prevalence of total anti-HEV among 1565 pregnant women was found to be 11.6% (181/1565). The prevalence of anti-HEV IgM among 181 total anti-HEV was 22.7% (41 cases), indicating recent or ongoing infection.
The prevalence of total anti-HEV varied significantly across age groups, with higher rates observed in older women. Multivariate analysis revealed no significant association between total anti-HEV immunoglobulins positivity and socio-demographic factors such as education level, occupation, family size, or history of blood transfusion and surgical operations, except age and occupation of head of household as public officer.
The anti-HEV IgM presence was not associated with any of those factors.

HEV RNA was not detected in any of the 41 anti-HEV IgM positive samples, suggesting the absence of active viral replication among the participants.

D. 考察

The new in-house ELISA assay showed strong agreement with established commercial tests, achieving agreement scores of 0.76 and 0.61 with RecomLine, Mikrogen, Germany, and the Institute of Immunology, respectively. Despite variations in sensitivity and specificity among commercial systems and the absence of a universally accepted standard for HEV antibody detection, this assay is a promising tool for widespread screening in HEV-endemic, resource-limited settings [3, 4].

Research on stored sera from pregnant women indicated an 11.6% positivity for anti-HEV antibodies, with seroprevalence increasing with age, suggesting lifelong HEV exposure. Additionally, 22.7% of these positive cases had IgM antibodies, predominantly in younger women, indicating recent or ongoing infections.

Given the severe risks HEV poses during pregnancy, especially in the third trimester, our study underscores the urgency of HEV screening and preventive measures, such as maintaining hygiene and avoiding undercooked meat. The strengths of this study include its large sample size, the use of a novel ELISA method, and comprehensive analysis of HEV immunoglobulin prevalence.

E. 結論

Our study on the prevalence of Hepatitis E Virus (HEV) among pregnant women in Siem Reap, Cambodia, found that tested positive for total anti-HEV antibodies 11.6%, and 22.7% among last were IgM positives , highlighting the significant risk of severe outcomes such as acute liver failure during pregnancy. The newly developed in-house double antigen sandwich ELISA method proved to be an effective diagnostic tool, particularly suitable for use in areas with limited resources, and it aids in better understanding the epidemiological trends of HEV.

The absence of HEV RNA in samples positive for anti-HEV IgM could indicate either false positive results or transient viremia. The study also identified factors such as older age and occupation as a public officer as being linked to higher rates of HEV seropositivity, suggesting increased risk due to prolonged exposure.

These findings underscore the importance of implementing stronger preventive strategies, including enhanced hygiene practices and food safety measures.

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む。）

なし

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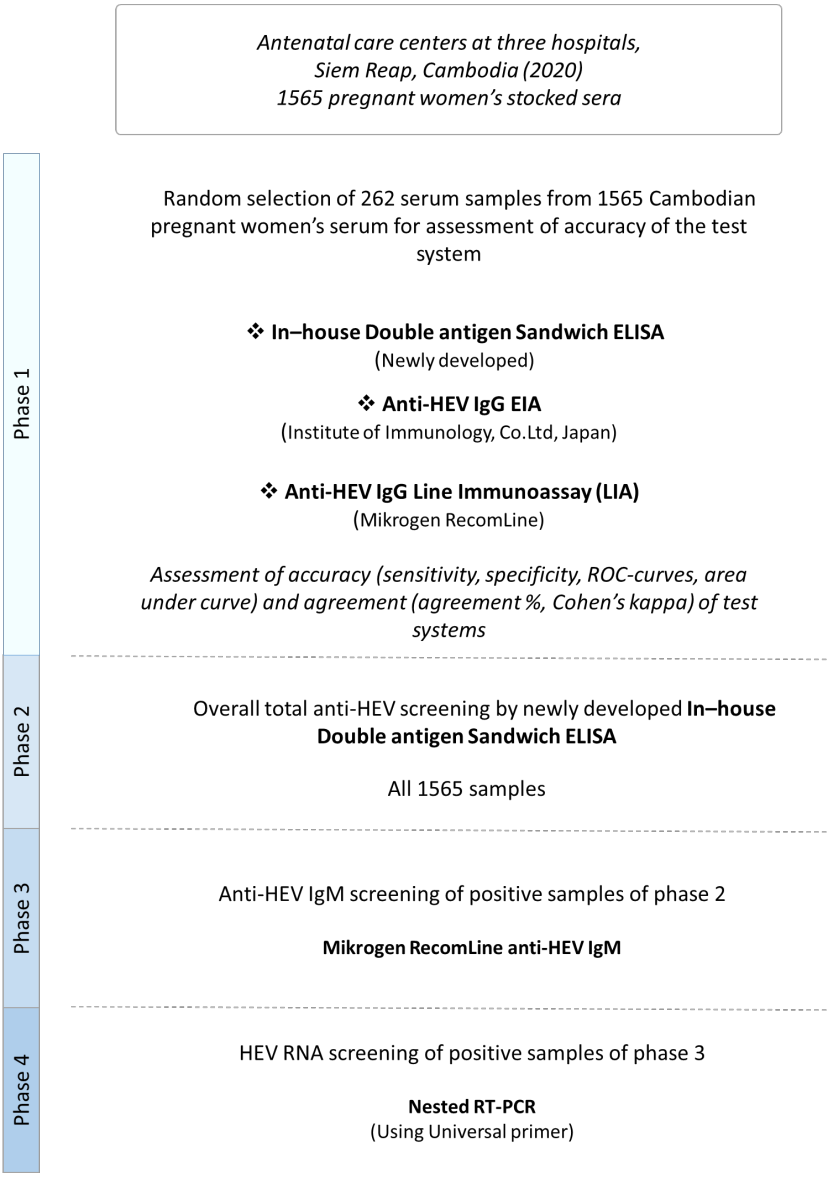

Figure 1. The outline and the phases of the study

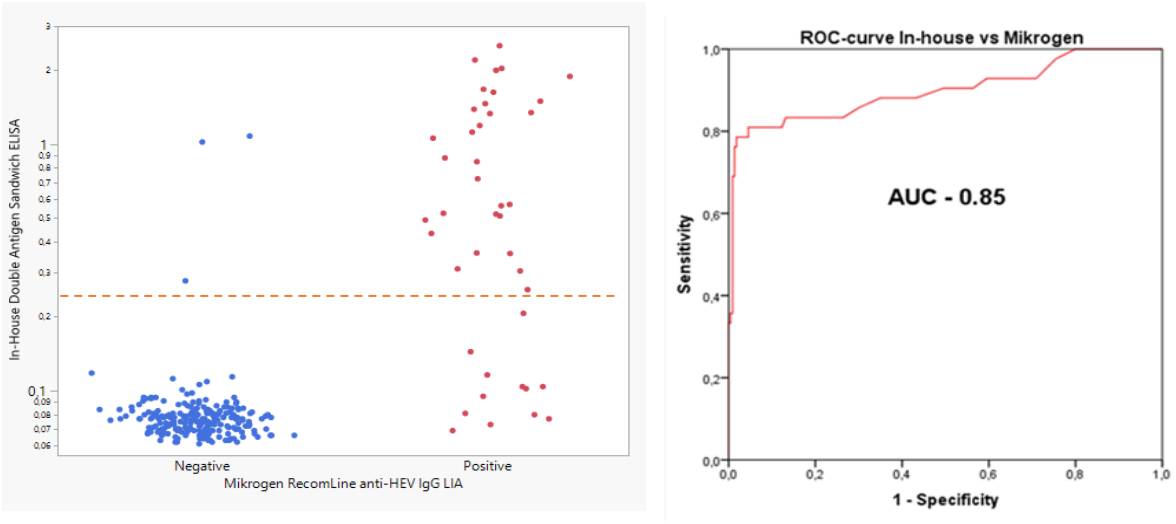

Figure 2. Comparison of commercial test system "RecomLine anti-HEV IgG", Mikrogen, Germany, and newly developed In-house Sandwich ELISA method

(Horizontal interrupted line – 0.24, OD cut-off value of In-house double antigen Sandwich ELISA; RecomLine anti-HEV IgM/IgG is line immunoassay (strips) is qualitative method, the positivity of the assay is measured by the number of lines appearance on the strip following the manufacturer's instructions).

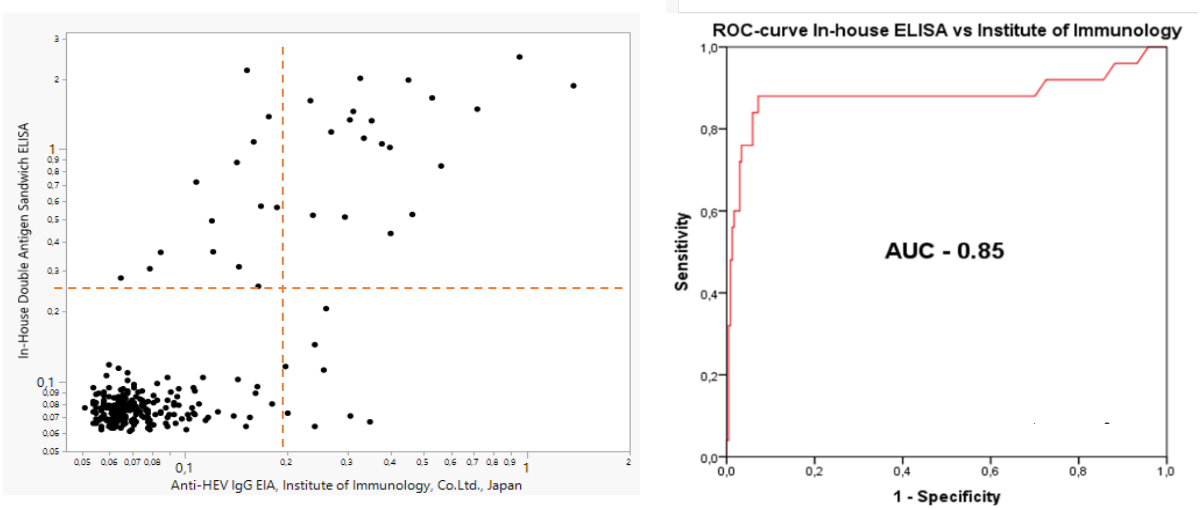

Figure 3. Comparison of commercial test system "anti-HEV IgG EIA", Institute of Immunology, Co. Ltd, Japan, and our newly developed In-house Sandwich ELISA method.
(Vertical red interrupted line – 0.198, OD cut-off value of Anti-HEV IgG EIA, Institute of Immunology, Co. Ltd, Japan; Horizontal interrupted line – 0.24, OD cut-off value of In-house double antigen Sandwich ELISA).

Table 1. Accuracy assessment of newly developed in-house double antigen sandwich enzyme-linked immunosorbent assay (ELISA) against two commercial test systems.

	Anti-HEV IgG EIA (Institute of Immunology)[a]		Anti-HEV IgG LIA (RecomLine; Mikrogen)[a]	
Test system	**Positive**	**Negative**	**Positive**	**Negative**
In-house double-antigen sandwich ELISA				
Positive	19	14	30	3
Negative	6	223	12	217
Total	25	237	42	220
Accuracy and agreement levels of the newly developed in-house double sandwich ELISA with each commercial test system as a reference method				
Sensitivity (%)	76.00		71.40	
Specificity (%)	94.10		98.60	
Agreement (%)	92.40		94.30	
Cohen's kappa	0.61		0.76	

Abbreviations: EIA, enzyme immunoassay; HEV, hepatitis E virus; IgG, immunoglobulin G; LIA, line immunoassay.

[a]The method was set as a reference ("gold standard") for the assessment of sensitivity and specificity.

【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

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.

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※ 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)

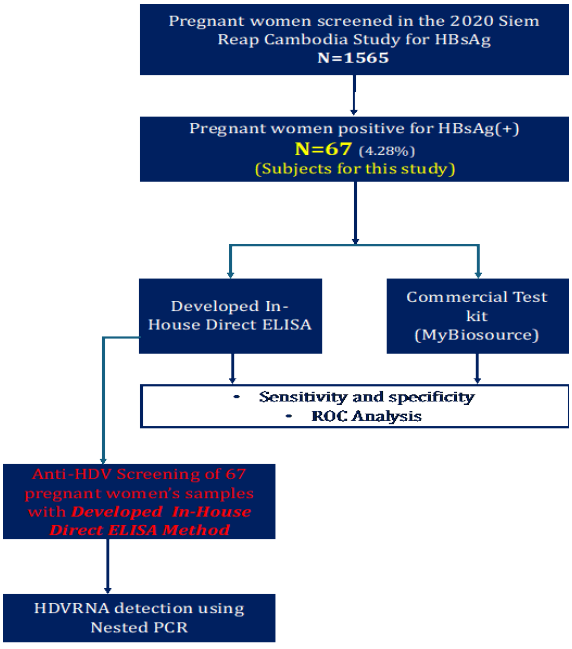

Figure 1: *Flow of study.*

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.

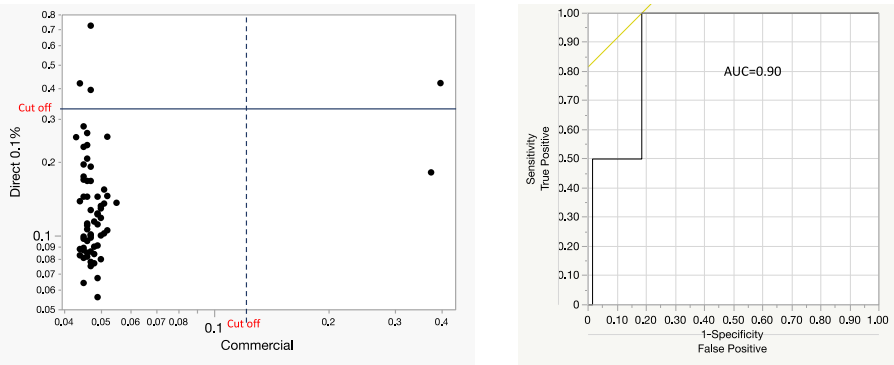

Figure 2: *ROC curve showing results of comparison of Developed in-house direct ELISA method and Commercial test.*

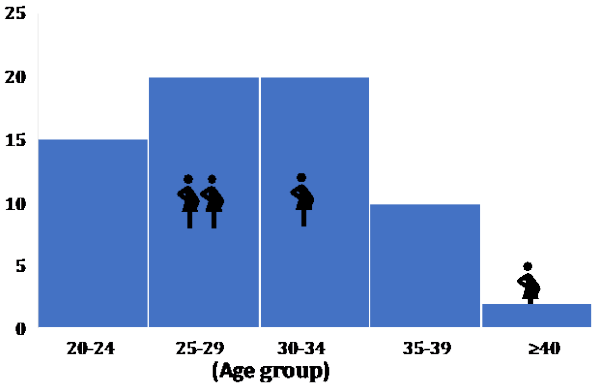

Figure 3: *Age distribution of 67 HBsAg (+) pregnant women showing distribution of four Anti-HDV positive pregnant women; Two in the 25–29-year group and one each in the 30 - 34 and over forty-year groups.*

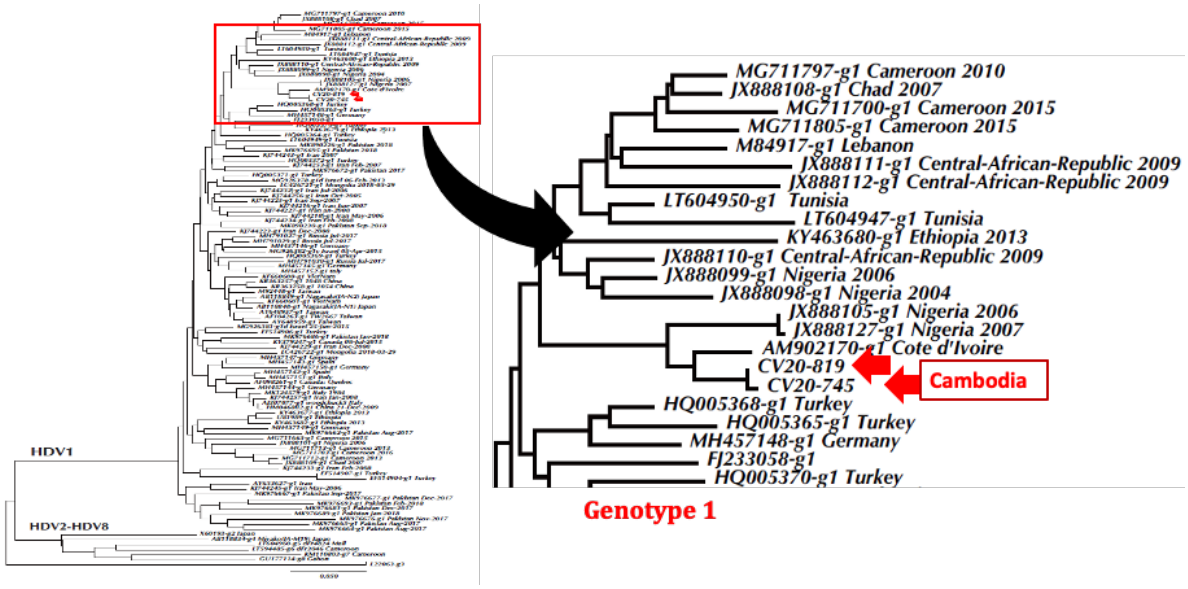

Figure 4: *Phylogenetic tree showing Genotype of HDVRNA positive pregnant women.*

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

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H. 知的財産権の出願・登録状況（予定を含む。）

なし

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