Epidemiological assessment of hepatitis E virus Infection among 1565 pregnant women in Siem Reap, Cambodia using an In-house double antigen sandwich ELISA.

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Abstract

Background

This study investigated Hepatitis E Virus (HEV) prevalence among pregnant women in Siem Reap, Cambodia, by developing a cost-effective, user-friendly in-house ELISA for detecting total anti-HEV immunoglobulins.

Materials and Methods

The in-house ELISA was designed for large-scale screening in resource-limited settings. Its performance was benchmarked against two commercial tests: the Institute of Immunology's anti-HEV IgG EIA and Mikrogen's anti-HEV IgG RecomLine LIA. The in-house ELISA demonstrated a sensitivity of 76% and 71.4%, and a specificity of 94.1% and 98.6% against the two commercial tests, respectively, with overall agreement rates of 92.4% and 94.3%.

Results

Among 1565 tested pregnant women, 11.6% were anti-HEV positive. Prevalence increased with age, particularly in women aged 35-40 and over 40. No significant associations were found with education, number of children, family size, or history of blood transfusion and surgery, except for the occupation of the family head as a public officer. Of the total anti-HEV positive women, 22.7% had anti-HEV IgM, indicating recent or ongoing infection.

Conclusion

The study concluded that the in-house ELISA is a viable option for HEV screening in regions with limited resources due to its high accuracy and cost-effectiveness. It is particularly suitable for large-scale studies and public health interventions in areas where HEV is endemic and poses a significant risk to pregnant women.

1. Introduction

HEV's global presence is substantial, affecting approximately one-third of the world's population [1]. The epidemiology of HEV can be categorized into four distinct types of prevalence by geographical zones: hyperendemic, endemic, with distinctive epidemiologic pattern and countries with autochthonous cases. Notably, Hepatitis E is hyperendemic in many countries across southern Asia (such as India, Bangladesh, Bhutan, Nepal, Pakistan, and Sri Lanka), southeast Asia (including Burma, Cambodia, Indonesia, Thailand, Vietnam, and Laos), central Asia (like Kazakhstan, Tajikistan, and Uzbekistan). In these regions, Hepatitis E infections can occur as both widespread, ongoing health concerns which could cost high economic burden. The most common cause of the disease in hyperendemic areas is HEV-1 [1–3].

Over the years, research has been conducted to understand the prevalence of hepatitis in Cambodia. A study conducted between 1996 and 2017 in Phnom Penh, Cambodia, found that the overall prevalence of anti-HEV IgG and IgM in Phnom Penh, Cambodia, were 41.1% and 2.7%, respectively, with a decreasing trend of anti-HEV IgG over the years [4]. Another study mentioned the prevalence of anti-HEV IgG from 7.2% to 12.7% [5]. A study conducted from 2010 to 2014 in the Siem Reap province found the prevalence of anti-HEV IgG to be 18.4% among the general population [6]. These studies have indicated that the prevalence of anti-HEV IgG antibodies is notably high among the population. While these surveys offer valuable insights, they are limited to specific timeframes or had been done several years ago, a comprehensive trend analysis of HEV infection in Cambodia remains a challenge.

The diagnosis of HEV is primarily based on the detection of anti-HEV antibodies, including IgM, IgG, and IgA, targeting ORF-2 and ORF-3 encoded proteins [7]. However, the performance of commercial anti-HEV ELISA test systems can vary significantly, with differences in sensitivity and specificity. A study evaluating four commercial HEV ELISA kits for IgM and IgG found that the sensitivities of different kits for anti-HEV IgM ranged from 82.6% to 86%. Each kit for anti-HEV

IgM was highly specific (97.8–100%). The sensitivities of all kits to detect anti-HEV IgG had a substantial agreement (87.2–91.9%), but some tests were more specific than the others [8]. Another study evaluated eight commercially available HEV serum antibody IgM- and IgG-specific ELISAs for genotype 1 and 3 HEV infections. The results of the study demonstrated different sensitivities and specificities of the test systems. The study found that low anti-HEV IgM concentrations were better detected by DSI, Mikrogen, and All Diag, making these tests the most sensitive in the study. On the other hand, Euroimmun, MP, and Dia.pro showed lower sensitivity than the other tests. Regarding anti-HEV IgG, the results revealed similar sensitivities among the tests. However, there was a striking overall lack of concordance among the results [9]. A comparison of five commercial assays for the detection of anti-HEV IgM and IgG in a clinical setting found that with the two most sensitive assays, anti-HEV IgG was identified in 16% of the blood donor samples and in 66% of patients with suspected HEV infection [10, 11]. There are some other studies which are also reporting about discordance of the results of different commercial test systems [12–14]. This variation can impact the interpretation of results and the understanding of HEV prevalence in different populations.

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. Such an ELISA system could be financially viable for use in regions with limited resources, where highly skilled laboratory personnel may be scarce. Additionally, an in-house ELISA system with strong specificity could be employed in large-scale screening efforts in hyperendemic areas. Because pregnant women are at an increased risk of experiencing severe HEV infections [15], especially in highly endemic areas including Cambodia, we then estimated the prevalence of HEV among this specific population.

2. Materials and Methods

2.1. Study Design and Site

This study builds upon a previous research project on investigation of mother-to-child transmission of hepatitis B virus (HBV) infection conducted in Cambodia, which involved 1565 pregnant women from three hospitals in Siem Reap region using convenient sampling strategy in 2020 [16]. 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 socio-demographic information.

2.2 Structure of the research

The present study utilized preserved serum samples from pregnant women and was divided into four distinct phases. The initial phase encompassed the creation of a new In-house Sandwich ELISA technique and its comparison with two commercially available kits: the anti-HEV IgG EIA from the Institute of Immunology, Co. Ltd, Tokyo, Japan, and the anti-HEV IgG RecomLine LIA, from Mikrogen GmbH, Germany. For this stage, we adopted a random sampling approach and selected 262 samples for analysis from a total of 1565 pregnant women.

The second phase of the study focused on estimating the prevalence of total anti-HEV immunoglobulins using the newly developed In-house ELISA method across the entire sample set of 1565 pregnant women's serum. This phase also examined the epidemiological patterns of HEV transmission based on data from a previously conducted questionnaire. The results of the questionnaire were broken down by age cohorts, education level, occupation of the household, number of children, number of family members the pregnant woman is living with, and history of blood transfusion and surgical operations.

The third phase determined the prevalence of IgM among the positive samples for total anti-HEV immunoglobulins using RecomLine anti-HEV IgM kit, Mikrogen.

The final phase, the fourth step, involved molecular analysis of the samples that tested positive for anti-HEV IgM (Figure 1).

Figure 1. The outline and the steps of the study. This figure shows a sequential testing protocol for HEV infection in pregnant women, from initial test evaluation through to the final confirmation of viral RNA presence.

2.3 Development of In-house Double Antigen Sandwich ELISA method for detection of total anti-HEV immunoglobulin in serum samples

The In-house double antigen Sandwich ELISA procedure involved the use of specific antigens. The primary coating antigen is a recombinant Hepatitis E (HEV) virus capsid protein (ORF2) with a C-terminal mouse Fc-Tag (The Native Antigen Company, UK), produced in HEK293 cells. The secondary antigen is also a recombinant Hepatitis E virus antigen protein with a His Tag (ABCAM, UK), likewise, produced in HEK293 cells. These proteins each consisted of 1 - 660 amino acids.

To enhance the chemiluminescent signal, the secondary antigen was biotin-labeled and employed in conjunction with polyclonal Streptavidin HRP (BD Bioscience, U.S.), during the reaction. All the steps of the ELISA test were carried out using 96 wells Corning ELISA plates (Corning Inc., U.S.).

Each well of the Corning plate was first coated with 50µL of 500 ng/mL HEV ORF2 Fc-Tag protein prepared in a 0.02 M Tris-HCl buffer, and the plate was incubated overnight at 4°C. The coating antigen was manually removed and then blocked with 2w/v% human albumin diluted in 0.02 M Tris-HCl, along with 0.01v/v% Polysorbate 20 (Tween-20) for one hour at room temperature. The wells were washed three times using a washing buffer consisting of 0.9w/v% sodium chloride, and 0.01v/v% Polysorbate 20 in 1000 mL of 0.02M Tris-HCl with automated microplate washer (Thermo Scientific™ Wellwash™, Thermo Fisher Scientific Inc., U.S.)).

Next, 17 µL of each serum sample was dilution with 34µL of dilution buffer containing 5w/v% human albumin and 0.01% Polysorbate 20 in 0.02M Tris-HCl to get final threefold dilution. Total 50µL of threefold diluted serum samples were added to each assigned well and incubated at

37°C for 60 minutes.

Then in-house Biotin-labelled HEV Ag His-Tag at concentration of 400ng/mL was prepared with the abovementioned dilution buffer and later mixed with polyclonal Streptavidin HRP, which was further diluted a thousandfold. Then, 50 µL of mixture containing both the diluted antigen and polyclonal Streptavidin HRP were added to the wells in equal proportions. The plate was incubated again at 37°C for 60 minutes and then washed three times with same washing buffer using automated washer and one time manually followed by inverting the microplate and tapping firmly onto absorbent paper to ensure all wash buffer were clearly blotted dry.

The plate was then revealed with 50µL of TMB solution - KPL "Sure Blue", microwell peroxidase substrate (SeraCare Life Sciences, USA) and incubated in the dark at room temperature for 30 minutes and the reaction was stopped with 50µL of a KPL TMB stop solution (SeraCare Life Sciences, USA). The plate was read on microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific Inc., U.S.) at 450 nm.

2.4 The determination of the cut-off value for the newly developed In-house ELISA

The cut-off value for the newly developed In-house ELISA was determined by multiplying three times the mean optical density (OD) values obtained from the negative control samples and it was 0.24 [17].

2.5 Assessment on performance of In-house Double Antigen Sandwich ELISA against two commercially available anti-HEV ELISA kits

To evaluate the diagnostic accuracy of the In-house Double Antigen Sandwich ELISA, two commercially available anti-HEV ELISA kits were employed: the anti-HEV IgG EIA from the Institute of Immunology, Co. Ltd., Tokyo, Japan (quantitative ELISA method), and anti-HEV IgG RecomLine LIA, from Mikrogen GmbH, Germany (qualitative line ELISA method). Both test

179 systems were strictly followed according to the manufacturers' protocols.

180 The sample size for this phase of the study was calculated based on the alternative hypothesis
181 that the In-house test system would have a sensitivity and specificity of around 70%, while the
182 null hypothesis accepted a sensitivity and specificity of 50%. Given the prevalence of anti-HEV
183 IgG in the general population was close to 20% [6], this number was used as the level of
184 prevalence. The calculation resulted in a requirement of 245 serum samples for the assessment
185 of the accuracy of the newly developed In-house Double Antigen Sandwich ELISA method [18].
186 A total of 262 serum samples were randomly selected from among 1565 pregnant women for
187 the assessment of the In-house Double Antigen Sandwich ELISA method. These 262 samples
188 were subsequently tested using both the two commercial test systems and the newly developed
189 In-house Double Antigen Sandwich ELISA. ROC-curves, agreement percentages, and Cohen
190 kappa were used to demonstrate the test accuracy.

191

192 ***2.6. Detection of anti-HEV IgM among total anti-HEV positives***

193 All total anti-HEV positive specimens were investigated for anti-HEV IgM using the Mikrogen anti-
194 HEV IgM RecomLine LIA strictly following the manufacturer's instruction and the qualitative
195 results were interpreted accordingly.

196

197 ***2.7. Detection of HEV RNA among total anti-HEV positives***

198 All anti-HEV IgM positive samples were then screened for HEV RNA. The nucleic acid was
199 extracted from 50 µL of sample using SMI-TEST Ex R&D and the final pellet was dissolved in 10
200 µL of RNase free water. HEV RNA was screened by two rounds of nested reverse transcriptase
201 polymerase chain reaction (nested RT-PCR) using the universal primer sets targeting HEV Open
202 Reading Frame 1 (ORF 1). The first round of nested RT-PCR was performed using Prime Script
203 One Step Enzyme Mix (TAKARA Bio CO. Ltd, Japan) using outer sense primers (HE7-1: 5'-

GCAGACCACRTATGTGKTCG-3', HE7-2: 5'-CCACRTATGTGGTCGAYGCC-3') and outer antisense primers (HE7-3: 5'-ACMARCTGSCGRGGYTGCAT-3', HE7-4: 5'-CGYTGRATWGGRTGRTTCCA-3'). The thermal cycle was as follows: 45°C for 10 seconds, 94°C for 2 minutes followed by 35 cycles of 98°C for 10 seconds, 55°C for 15 seconds, 68°C for 30 seconds, and then a final cycle at 68°C for 2 minutes. The second round of nested RT-PCR was performed using Ex Taq Hot Start (TAKARA Bio. Ltd, Japan) using inner sense primers (HE7-5: 5'-TGKTCGAYGCCATGGAGGC-3', HE7-6: 5'-TCGAYGCCATGGAGGCCCA-3') and antisense primers (HE7-7: 5'AYGCCATGGAGGCCCAYCAG-3', HE7-8: 5'-CKRACYACCACAGCATTCGC-3', HE7-9: 5'-GGCCKRACYACCACAGCATT-3'). The thermal cycle included 30 cycles of 98°C for 10 seconds, 55°C for 30 seconds and 72°C for 1 minute. The amplicon was then visualized by Gel electrophoresis.

2.8. Statistical Analysis

The statistical analysis involved various methods to assess and compare data using SPSS Ver.29 (IBM SPSS Statistics, U.S.). Descriptive statistics were used to present the baseline characteristics of both the anti-HEV seropositive and seronegative groups. Normality was evaluated using the Shapiro–Wilk test. When the assumptions of normality were met, an independent t-test was employed to compare the two groups. Otherwise, the Mann–Whitney U test was used. For comparisons between the groups in terms of categorical variables, the Pearson chi-square test was used when there were enough observations in each cell of the cross table. Otherwise, Fisher's exact test was utilized. Odds ratios were calculated to compare the two groups regarding the investigated outcomes. Univariate and multivariate regression analysis was conducted to identify factors associated with HEV seropositivity. The results are broken down by age cohorts, education level, occupation of the household, number of children, number of family members the pregnant woman is living with, and history of blood transfusion and surgical operations.

To evaluate the accuracy of the laboratory technique, we employed a variety of statistical

measures including Receiver Operating Characteristic (ROC) curves, Area Under the Curve (AUC), as well as sensitivity and specificity. Additionally, we quantified the level of concordance using percentage agreement and Cohen's kappa coefficient. A significance level of 0.05 (two-sided) was considered as the threshold for statistical significance.

2.9. Ethical consideration

This study was approved by the Epidemiological research Ethic Committee of Hiroshima University (No. E-1693) and the Cambodian National Ethic Committee for Health Research (No. 223-NECHR). Before each study procedure, all subjects gave their informed consent. For participants younger than 18 years of age, informed consent was obtained from their legal guardians and the informed assent was obtained from the participants accordingly. All research activities were carried out in conformity with the Declaration of Helsinki.

3. Results

3.1 Sensitivity and specificity of In-house Double Antigen Sandwich against commercial kits

The assessment of inhouse double antigen sandwich ELISA was conducted in 262 randomly selected serum samples among total 1565 pregnant women collected in Siem Reap, Cambodia in 2020. The accuracy of the newly developed method was evaluated against two commercial test systems. Against Institute of Immunology, the in-house double sandwich ELISA provided sensitivity of 76% (19/25) and specificity of 94.1% (223/237) with overall agreement at 92.4% and Cohen’s kappa 0.61. Nevertheless, against RecomLine LIA, Mikrogen, the sensitivity was 71.4% (30/42) and specificity was 98.6% (217/220) with overall agreement of 94.3% at Cohen’s kappa 0.76. (Table 1)

The evaluation involved the use of ROC curves and a comparison of the OD values, as depicted in Figures 2 and 3. While both tests exhibited an area under the curve (AUC) of 0.85, there were

variations in the agreement percentages and Cohen's kappa values between the two methods (Table 1).

Figure 2. Comparison of commercial test system "anti-HEV IgG RecomLine LIA", 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).

3.2 Prevalence of HEV seromarkers among 1565 pregnant women in Siem Reap

Using in-house double antigen sandwich method, total anti-HEV was detected in 181 out of total 1565 pregnant women providing the prevalence at 11.6 % (95% CI 10 – 13.2%). Furthermore, among 181 total anti-HEV positives, 41 samples tested positive for anti-HEV IgM by RecomLine LIA, Mikrogen resulting in the prevalence of 22.7% (95% CI 17.2 – 29.4%). The prevalence of total anti-HEV among 181 positive cases showed that, the distribution by age group was as follows: 2.8% for those up to 19 years old, 14.9% for the 20-24 age group, 30.9% for those aged 25-29, 27.6% for the 30-34 age group, 18.8% for those aged 35-40, and 5% for those aged 40 and above (Table 2). However, when the data was adjusted for age groups, the prevalence rates changed significantly. The adjusted prevalence rates were 7.1% (5/70) for those under 20 years old, 9.5% (83/878) for the 20-29 age group, 14.7% (84/571) for those aged 30-39, and 19.6% (9/46) for individuals aged 40 and above, indicating significant differences across age groups with upward trend the age with total anti-HEV Ig prevalence.

3.3 Risk factors associated with HEV seromarkers positivity among pregnant women in Siem Reap

The overall sample size was 1565 pregnant women, out of which 181 (11.6%) tested positive for total anti-HEV. Among these 181 women, 41 (22.7%) were also positive for anti-HEV IgM, indicating a recent or ongoing HEV infection.

In terms of age cohorts, the prevalence of total anti-HEV increased with age, with the highest prevalence observed in the 35-40 and ≥40 age groups. The multi1111111variate analysis showed that the odds of total anti-HEV positivity were significantly higher in the 35-40 (AOR=2.90; 95% CI 1.06-7.92; p=0.03) and ≥40 (AOR=3.54; 95% CI 1.07-11.7, p=0.03) age groups compared to the 15-19 age group. However, the prevalence of anti-HEV IgM was highest in the 30-34 age group, but the association was not statistically significant in the multivariate analysis.

In the multivariate analysis, there was no significant association between educational level and the detection of any anti-HEV antibodies, similar to the findings for occupation in relation to anti-HEV IgM antibodies. The number of children and family members the pregnant woman is living with, as well as history of blood transfusion and surgical operations, did not show a significant association with total anti-HEV or anti-HEV IgM positivity in the univariate analysis. (Table 3).

3.4 Detection of HEV RNA among anti-HEV IgM positive pregnant women in Siem Reap

The nested RT-PCR based HEV RNA screening revealed no presence of HEV RNA among 41 anti-HEV IgM positive pregnant women.

4. Discussion

Our investigation identified a prevalence of 11.6% for total anti-HEV immunoglobulins among a sample of 1565 pregnant women from Siem Reap, Cambodia. This finding is consistent with outcomes from other research conducted across diverse geographical regions and nations. For instance, a Chinese study, which included 32,678 pregnant women, reported a seroprevalence

of anti-HEV IgG of 13.17% (95% CI 11.19–15.28) [19]. A comprehensive systematic review and meta-analysis, which incorporated 52 studies (11,663 pregnant women), discovered a seroprevalence of HEV of 3.5% in asymptomatic women, who were predominantly from high endemic areas, and 49.6% in symptomatic women [20]. In the African context, the overall pooled seroprevalence of HEV among pregnant women was 29.13%, with the highest seroprevalence reported from Egypt (84.3%) and the lowest prevalence reported in Central Africa (6.6%) [21]. The high incidence of Hepatitis E virus (HEV) infection among pregnant women is a significant health issue, given the severe health implications it can have, including acute liver failure, loss of the fetus, and heightened maternal mortality [20, 21]. According to the World Health Organization, if pregnant women contract Hepatitis E in their third trimester, the mortality rate can be as high as 20–25% [22]. In some parts of South-East Asia, such as India, this mortality rate can escalate to 30% [23–25].

The prevalence of Hepatitis E (HEV) in Cambodia varies across different regions and populations. A cross-sectional study conducted in the Siem Reap province found an anti-HEV IgG prevalence of 18.4% among the general population [6]. Another study conducted in Phnom Penh between 1996 and 2017 reported an overall prevalence of 41.1% for anti-HEV IgG, with a significant decrease in prevalence over the past two decades. Several factors have been identified as risk factors for HEV infection in Cambodia. These include male gender, age above 30 years, and Phnom Penh residency [4]. The decline in HEV prevalence over time may be attributed to improvements in sanitation conditions, food safety, and access to clean water in the country. The high prevalence of HEV in Cambodia, including frequent cases of early HEV infection, suggests that measures to prevent the spread of the virus are urgently needed [26]. The country's population remains exposed to HEV, and the infection is considered highly endemic. The occurrence of HEV in Cambodia surpasses that in certain other areas, underscoring the need for collaborative efforts at both national and regional levels to address this emerging disease,

329 particularly given its heightened impact on pregnant women.

330 Prior to its implementation, the newly developed in-house Double Antigen Sandwich ELISA
331 technique was evaluated against two established commercial assays for its accuracy in detecting
332 anti-HEV IgG antibodies. These commercial assays were the anti-HEV IgG EIA by the Institute of
333 Immunology, Co. Ltd., based in Tokyo, Japan, and the anti-HEV IgG recomLine LIA test by
334 Mikrogen GmbH from Germany. In a comparative analysis using a random selection of 262 cases,
335 the Japanese Institute of Immunology's test identified 25 positive instances, whereas the
336 German Mikrogen RecomLine test detected 42 positive cases. The in-house developed ELISA
337 method ascertained 33 cases as positive within the same cohort. The variation in diagnostic
338 sensitivity, particularly noted in the Institute of Immunology's assay, suggests that assays with
339 lower sensitivity may be more adept at identifying higher concentrations of antibodies during
340 the acute phase of HEV infection, but may not be as effective in detecting antibodies during the
341 later stages of the infection [27]. Many studies have found significant differences in sensitivity
342 and specificity among commercial test systems, further complicating the task of comparison.
343 Additionally, the in-house developed test system is intended for total immunoglobulins, which
344 may also contribute to the complexity of the comparison. Comparison studies often reveal
345 disparities in the prevalence of immunoglobulins when using the same serum samples [28–30].
346 It's important to note that there is not a universally accepted "gold standard" method for
347 detecting HEV antibodies [14, 31, 32]. The studies from Mansuy et al. from France demonstrate
348 the difference in same test systems in the same area (52.5% versus 16.6%) [33, 34]. Al-Absi et
349 al.'s study employed a "silver standard" to assess the accuracy of commercial or in-house ELISA
350 test systems. The underlying concept of the silver standard was to enhance the likelihood of true
351 positives and true negatives while reducing the probabilities of false positives and false negatives.
352 In this proposed silver standard test, only samples that tested positive in three or more different
353 assay sets were considered as positive [28]. This approach would be more justified if there were

an additional commercial test system available or if there were more consistent results between two commercial test systems (with 20 cases showing concordance in two commercial test systems). Comparison of test systems using ROC-curves analysis revealed the same levels of area under curve (AUC) as 0.85. At the same time, it did not demonstrate the real accuracy of our newly developed test system. Instead, for this issue, we used agreement percentages and Cohen's kappa values. Our developed In-house method demonstrated a high level of agreement and Cohen's kappa with Mikrogen RecomLine LIA.

The serological analysis of 181 cases with positive total anti-HEV using the Mikrogen test system for IgM prevalence revealed 41 cases as positive. However, HEV RNA was not detected in any of the 41 anti-HEV IgM positive cases. Certain researchers have documented the reduction in anti-HEV IgM levels within a period of four to six months following acute infection [35, 36]. As a result, employing assays with lower sensitivity might result in an earlier inability to detect anti-HEV IgM after acute infection. The considerable variability in sensitivity among different assays, up to 19-fold, could impact the recorded duration of anti-HEV IgM persistence, spanning from a few weeks to three months [37]. While this absence of HEV RNA could be attributed to the brief viremia period in the blood of HEV-infected pregnant women, it remains challenging to entirely rule out the possibility of false positive results. Notably, the Anti HEV IgM test systems from the same producer, Mikrogen (RecomWell EIA and RecomLine LIA), yielded non-concordant results. This discrepancy suggests that determining the prevalence of false positive results compared to PCR test results is not a straightforward task. [38].

Exploring factors associated with the prevalence of hepatitis E immunoglobulins was facilitated through questionnaire data collected in a previous study [16]. Notably, the analysis, as presented in Table 3, identified a significant association of age and the occupation of the head of the household with the prevalence of total anti-HEV Ig, particularly among those employed as public officers. We found that the prevalence of total anti-HEV antibodies was observed to increase

with age, showing statistically significant differences across various age groups. Our results align with several other studies that have reported a similar age-dependent increase in anti-HEV IgG prevalence. For instance, a study conducted in Bulgaria observed a stepwise increase in anti-HEV IgG prevalence with advancing age in several sub-populations [39]. Another study from Europe found a significant increase in prevalence of anti-HEV IgG in older people in comparison with more younger ones [40]. Similarly, a studies from South Korea and Japan reported an increase in anti-HEV IgG prevalence corresponding to age [41, 42].

However, it's worth noting that not all studies have found a significant relationship between age and anti-HEV IgG prevalence. A study from Tehran, Iran, found the highest rate of anti-HEV IgG in the age group over 60 years and the lowest rate in the age group under 29 years, but no significant relationship was found between positive IgG antibody against HEV and different age groups [43]. Additionally, a large multi-ethnic youth cohort in China found no significant differences in anti-HEV IgG prevalence among different age groups [44].

In our study, we evaluated the occupational roles of the head of households to determine their socioeconomic status and the prevalence of anti-HEV antibodies. Initially, we hypothesized a significant link between farmers and a higher prevalence of total anti-HEV antibodies. However, the outcomes of our multivariable analysis highlighted an association between the “Public officer" and a total anti-HEV positivity.

The association between the "Public officer" occupation and anti-HEV positivity in our study suggests that additional factors, potentially including environmental exposure, lifestyle choices, or other unrecognized risk factors, might influence HEV transmission within this group. Further research is necessary to delve into these associations and to better understand the factors that contribute to the higher prevalence of anti-HEV antibodies among public officers.The variation in findings across different studies could be attributed to several factors, including differences in study populations, geographical location, and exposure to HEV. Despite these variations, the

general trend observed in our study and others suggests that the likelihood of having anti-HEV IgG antibodies increases with age, possibly reflecting cumulative exposure to the virus over a person's lifetime.

Interestingly, our univariable and multivariable analysis of anti-HEV IgM positivity a high prevalence of anti-HEV IgM among 30-34 age group, but the association was not statistically significant. This finding is noteworthy as it suggests that younger pregnant women may be at a higher risk of recent HEV infection. This may align with some studies that have reported higher rates of HEV infection among younger individuals [43, 45].

However, it's important to note that the interpretation of anti-HEV IgM results can be complex. Anti-HEV IgM can persist for several months after the acute phase of the infection, and cross-reactivity with other infections can sometimes lead to false-positive results [14]. Therefore, while our findings suggest a higher prevalence of recent HEV infection among younger pregnant women, further studies are needed to confirm this trend and to understand the underlying reasons.

HEV infection during pregnancy, especially in the third trimester, can lead to severe outcomes, including fulminant hepatitis and increased maternal and fetal mortality and morbidity. Therefore, our findings underscore the importance of HEV screening and preventive measures among pregnant women, particularly those in the younger age groups. These measures could include maintaining hygienic practices, avoiding consumption of undercooked meat, and potentially vaccination once a safe and effective vaccine becomes widely available.

The study's strengths include its use of a large and well-characterized group of pregnant women, the evaluation of the newly developed in-house ELISA method, and a comprehensive approach to assess the prevalence of anti-HEV immunoglobulins. However, it's essential to acknowledge certain limitations, such as potential recall bias in questionnaire-based data collection and the study's cross-sectional nature, which restricts the ability to establish causal relationships.

5. Conclusion

In conclusion, this study contributes valuable insights into the seroprevalence of anti-HEV immunoglobulins among pregnant women in Cambodia. The accuracy assessment of the newly developed in-house ELISA method highlights its potential as a reliable diagnostic tool. The findings regarding factors associated with HEV seropositivity, as well as the absence of active HEV infection among the cohort, provide essential information for public health initiatives and future research in the field of hepatitis E. Further longitudinal studies are warranted to investigate the dynamics and long-term consequences of HEV infection among pregnant women in this region.

Availability of Data

The dataset used and analyzed in the current study is available from the corresponding author on reasonable request.

Competing Interest

The authors declare no competing interest.

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Authors Contribution

JT and KT conceived and designed the study and the data analysis concept. TA, JT supervised and monitored the survey. UM, KK, and KT performed laboratory measurement. UM, EB, ZP, GA, CC, AS managed the data. UM, KK, TA analyzed the data. UM, KT, JT interpreted the data. UM, KK drafted the manuscript. All authors read and approved the final version of the manuscript.

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Abbreviations:

ELISA - Enzyme-Linked Immunosorbent Assay
IgM - Immunoglobulin M
IgG - Immunoglobulin G
IgA - Immunoglobulin A
HRP - Horseradish Peroxidase
OD: Optical Density
EIA - Enzyme Immunoassay
LIA - Line Immunoassay
RNA - Ribonucleic Acid
ROC - Receiver Operating Characteristic
AUC - Area Under the Curve

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Table 1. The accuracy assessment of the newly developed In-house double antigen Sandwich ELISA against two commercial test systems.

Test systems		**Anti-HEV IgG EIA (Institute of Immunology) ***		**Anti-HEV IgG (RecomLine LIA, Mikrogen) ***	
		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
The accuracy and agreement levels of the newly developed In-house double Sandwich ELISA with each of commercial test systems as a reference method.					
Sensitivity (%)		76		71.4	
Specificity (%)		94.1		98.6	
Agreement (%)		92.4		94.3	
Cohen's kappa		0.61		0.76	

*The method was set as reference ("gold standard") for assessment of sensitivity and specificity

Table 2. Sociodemographic and anamnestic characteristics of 1565 pregnant women in Siem Reap, Cambodia

Variables	***Total (N = 1565)***		***Total anti-HEV negative (n = 1384)***		***Total anti-HEV positive (n = 181)***		***p-value***	***Anti-HEV IgM negative (n=140)***		***Anti-HEV IgM positive (n=41)***		***p-value***
	Frequency	**(%)**	**Frequency**	**(%)**	**Frequency**	**(%)**		**Frequency**	**(%)**	**Frequency**	**(%)**	
Age (mean±SD)	28.3 ± 5.7		28.1 ± 5.5		29.8 ± 5.8		<0.001	30 ± 5.6		29 ± 6.4		0.30
15–19	70	4.47	65	4.7	5	2.8	<0.01	2	1.4	3	7.3	0.26
20–24	338	21.60	311	22.5	27	14.9		20	14.3	7	17.1	
25–29	540	34.50	484	35.0	56	30.9		47	33.6	9	22.0	
30–34	391	24.98	341	24.6	50	27.6		38	27.1	12	29.3	
35–39	180	11.50	146	10.5	34	18.8		25	17.9	9	22.0	
≥ 40	46	2.94	37	2.7	9	5.0		8	5.7	1	2.4	
Education level												
≤ Primary School	324	20.7	291	21.0	33	18.2	0.41	26	18.6	7	17.1	0.59
High School	857	54.76	752	54.3	105	58.0		84	60	21	51.2	
University	384	24.54	341	24.6	43	23.8		30	21.4	13	31.7	
Occupation of household												
Farmer/Fisherman/Laborer	255	16.29	230	16.6	25	13.8	0.03	20	14.3	5	12.2	0.78
Public Officer	217	13.87	178	12.9	39	21.5		29	20.7	10	24.4	
Private Company Employee	495	31.63	432	31.2	63	34.8		47	33.6	16	39	

Self-Employed	598	38.21	544	39.3	54	29.8		44	31.4	10	24.4	
Number of children (median (IQR))	1 (1;2)		1 (1;2)		1 (1;3)		0.01	2 (1;3)		1 (1;2)		0.03
Blood transfusion history												
No	1527	97.57	1348	97.4	179	98.9	0.22	138	98.6	41	100	0.44
Yes	38	2.43	36	2.6	2	1.1		2	1.4	0	0	
Surgical history												
No	1361	86.96	1205	87.1	156	86.2	0.74	120	85.7	36	87.8	0.73
Yes	204	13.04	179	12.9	25	13.8		20	14.3	5	12.2	

Table 3. Factors associated with total anti-HEV and anti-HEV IgM positivity among pregnant women in Cambodia.

Variables		**Overall n=1565**	**Total anti-HEV (+) n (%)**	**Total anti-HEV IgG positivity**						**Overall n=181**	**anti-HEV IgM (+) n (%)**	**anti-HEV IgM positivity**					
				Univariate analysis			**Multivariate analysis**					**Univariate analysis**			**Multivariate analysis**		
				OR	[95% CI]	p-value	AOR	[95% CI]	p-value			OR	[95% CI]	p-value	AOR	[95% CI]	p-value
Age cohorts	*15-19*	70	5 (2.8)	1	[Ref.]	-	1	[Ref.]	-	5	3 (7.3)	1	[Ref.]	-	1	[Ref.]	-
	20-24	338	27 (14.9)	0.24	[0.42-3.04]	0.81	1.03	[0.38-2.79]	0.95	27	7 (17.1)	0.23	[0.03-1.7]	0.15	0.19	[0.02-1.57]	0.12
	25-29	540	56 (30.9)	0.84	[0.58-3.89]	0.4	1.34	[0.51-3.53]	0.54	56	9 (22)	0.13	[0.02-0.9]	0.04	0.11	[0.01-0.85]	0.04
	30-34	391	50 (27.6)	1.32	[0.73-4.96]	0.18	1.76	[0.67-4.66]	0.25	50	12 (29.3)	0.21	[0.03-1.41]	0.11	0.17	[0.02-1.38]	0.10
	35-40	180	34 (18.8)	2.21	[1.13-8.09]	0.02	2.90	[1.06-7.92]	0.03	34	9 (22)	0.24	[0.03-1.68]	0.15	0.28	[0.03-2.5]	0.25
	≥40	46	9 (5.0)	1.94	[0.98-10.14]	0.053	3.54	[1.07-11.7]	0.03	9	1 (2.4)	0.08	[0.01-1.29]	0.08	0.10	[0.01-2.02]	0.13
Education level	*No education/*	324	33 (18.2)	1	[Ref.]	-	1	[Ref.]	-	33	7 (17.1)	1	[Ref.]	-	1	[Ref.]	-

	Primary School																
	Junior High School/ High School	857	105 (58.0)	0.99	[0.81-1.86]	0.325	1.67	[0.93-2.3]	0.09	105	21 (51.2)	1.59	[0.54-4.66]	0.90	1.2	[0.4-3.6]	0.73
	College or University	384	43 (23.8)	0.43	[0.69-1.79]	0.665	0.5	[0.67-1.96]	0.61	43	13 (31.7)	1.86	[0.15-23.58]	0.40	1.88	[0.13-26.59]	0.64
Occupation of household	*Farmer/ Fisherman/ Laborer*	255	25 (9.8)	1	[Ref.]	-	1	[Ref.]	-	25	5 (12.2)	1	[Ref.]	-	1	[Ref.]	-
	Public officer	217	39 (18)	2.55	[1.17-3.45]	0.011	[1.14-3.64]		0.016	39	10 (24.4)	1.43	[0.42-4.83]	0.57	1.11	[0.27-4.64]	0.88
	Private Company Employee	495	63 (12.7)	1.18	[0.82-2.19]	0.24	[0.79-2.26]		0.272	63	16 (39)	1.36	[0.44-4.23]	0.59	1.09	[0.31-3.87]	0.89
	Self-Employed	598	54 (9)	0.36	[0.55-1.5]	0.721	[0.53-1.51]		0.69	54	10 (24.4)	0.91	[0.27-3.01]	0.88	0.90	[0.24-3.34]	0.88
Number of children	*1-3*	1469	166 (11.3)	1	[Ref.]	-				166	41 (100)	1	[Ref.]	-			
	≥4	96	15 (15.6)	1.56	[0.86-2.81]	0.143				15	0 (0)	939	-	0.99			
Number of family members whom pregnant women is living with	*1-4*	794	108 (12)	1	[Ref.]	-				108	26 (63.4)	1	[Ref.]	-			
	≥5	590	73 (11)	0.87	[0.63-1.21]	0.431				73	15 (36.6)	0.74	[0.32-1.7]	0.48			
Blood transfusion history	*No*	1527	179 (11.7)	1	[Ref.]	-				179	41 (100)	1	[Ref.]	-			
	Yes	38	2 (5.3)	0.383	[0.9-1.63]	0.194				2	0 (0)	0.01	0 -	0.99			
Surgical operations	*No*	1361	156 (11.5)	1	[Ref.]	-				156	36 (87.8)	1	[Ref.]	-			

history	*Yes*	204	25 (12.2)	1.14	[0.72-1.8]	0.579	25	5 (12.2)	0.83	[0.25-2.69]	0.76
					R^2 (Cox and Snell's) – 0.138; p<0.001					R2 (Cox and Snell's) – 0.053; p=0.621	

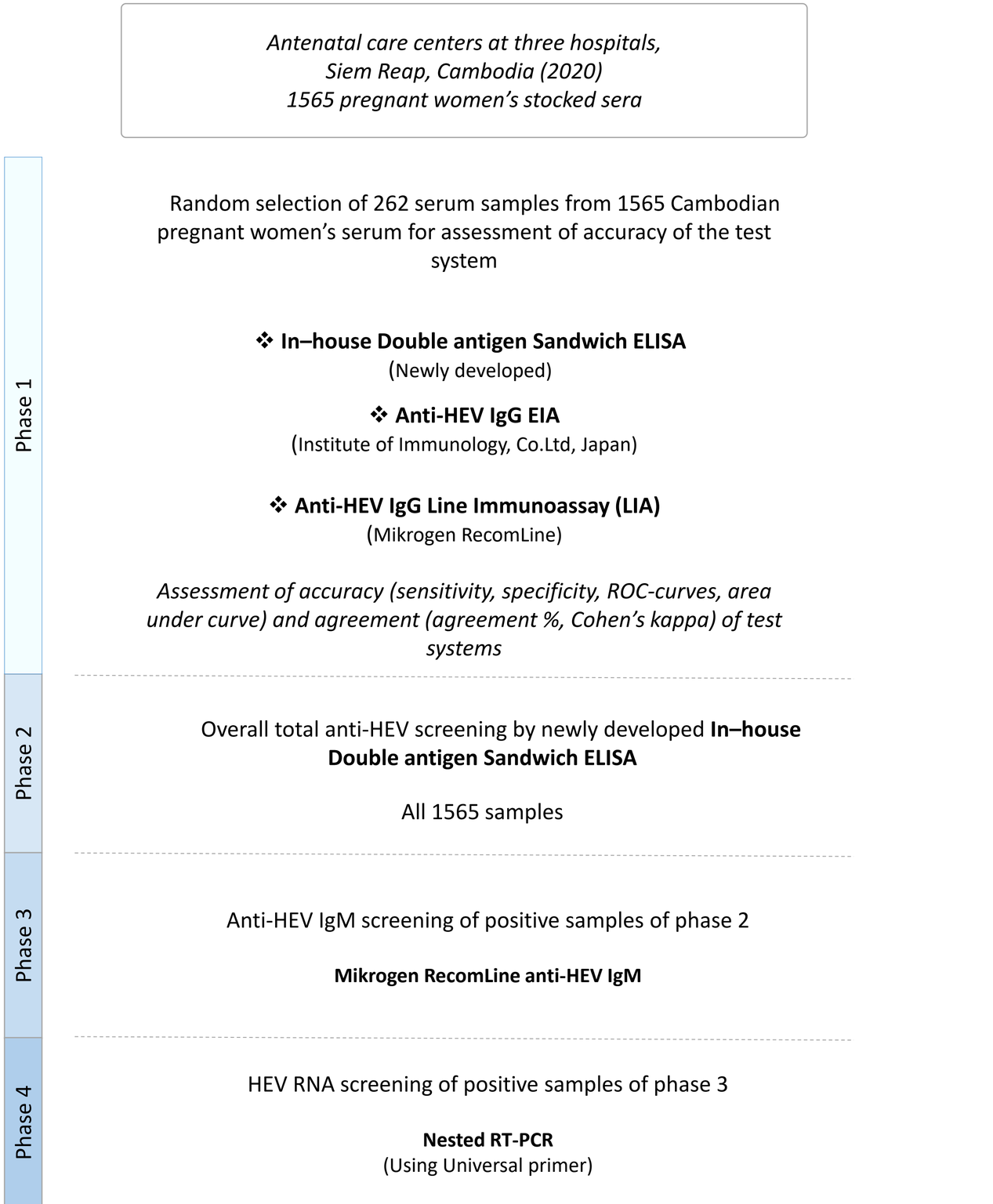

Figure 1. The outline and the steps of the study.

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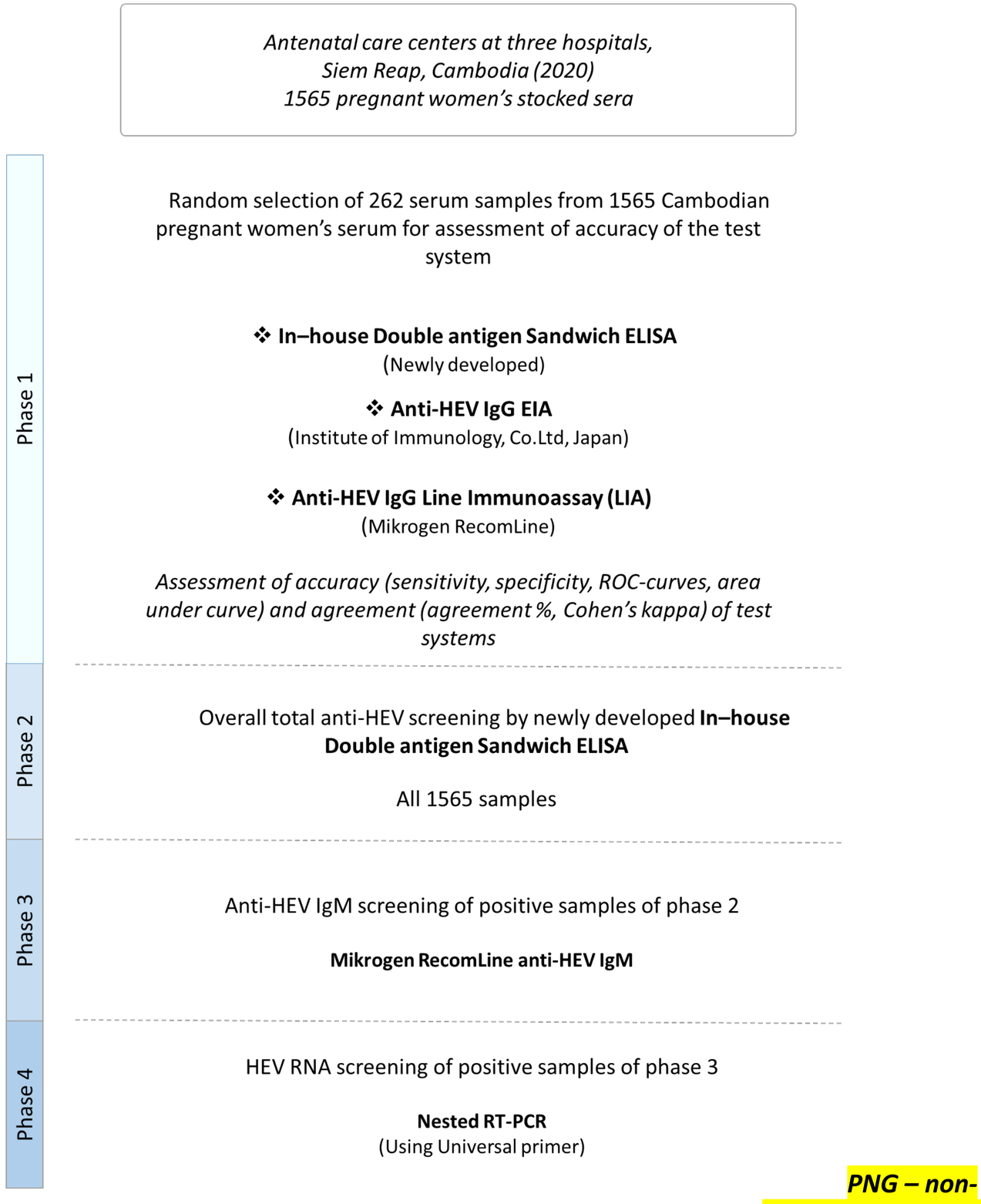
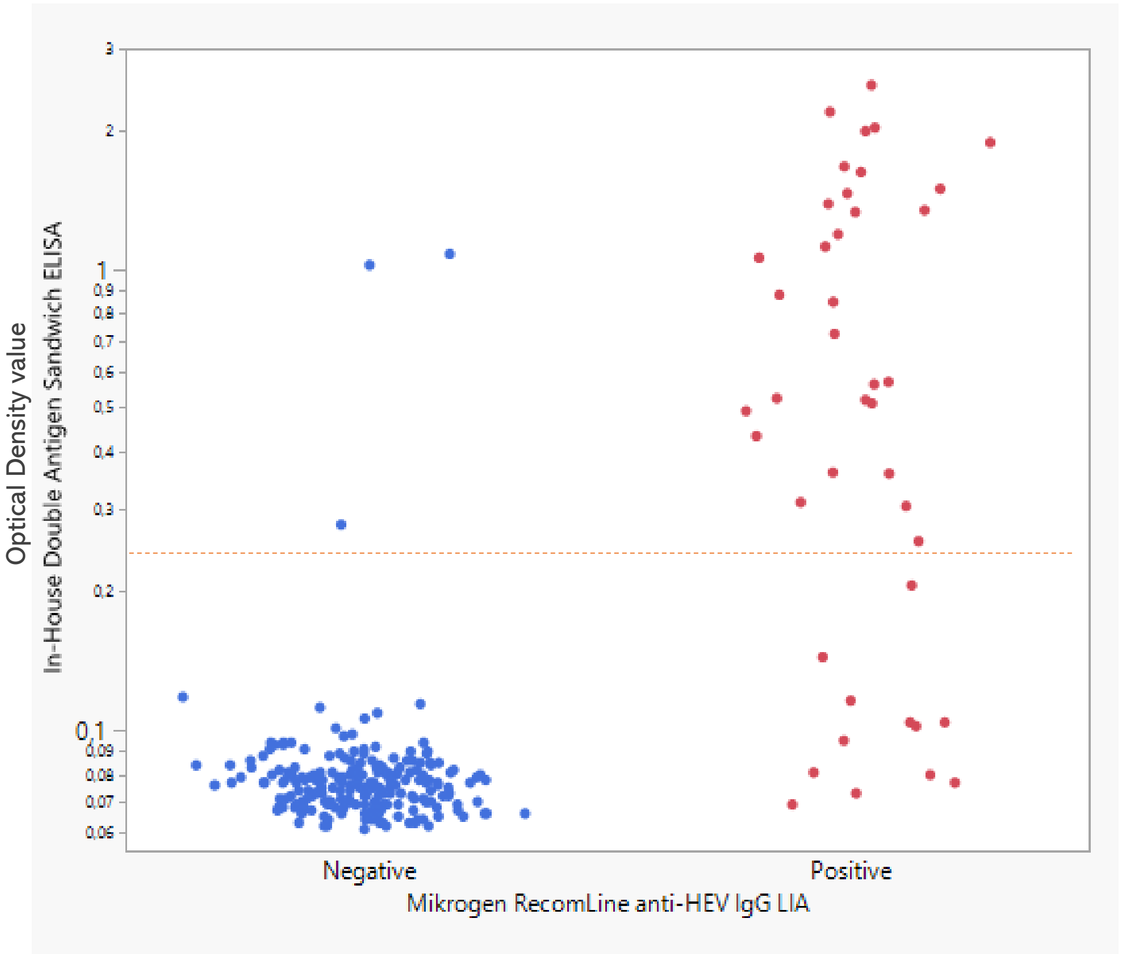


Figure 1. The outline and the steps of the study.



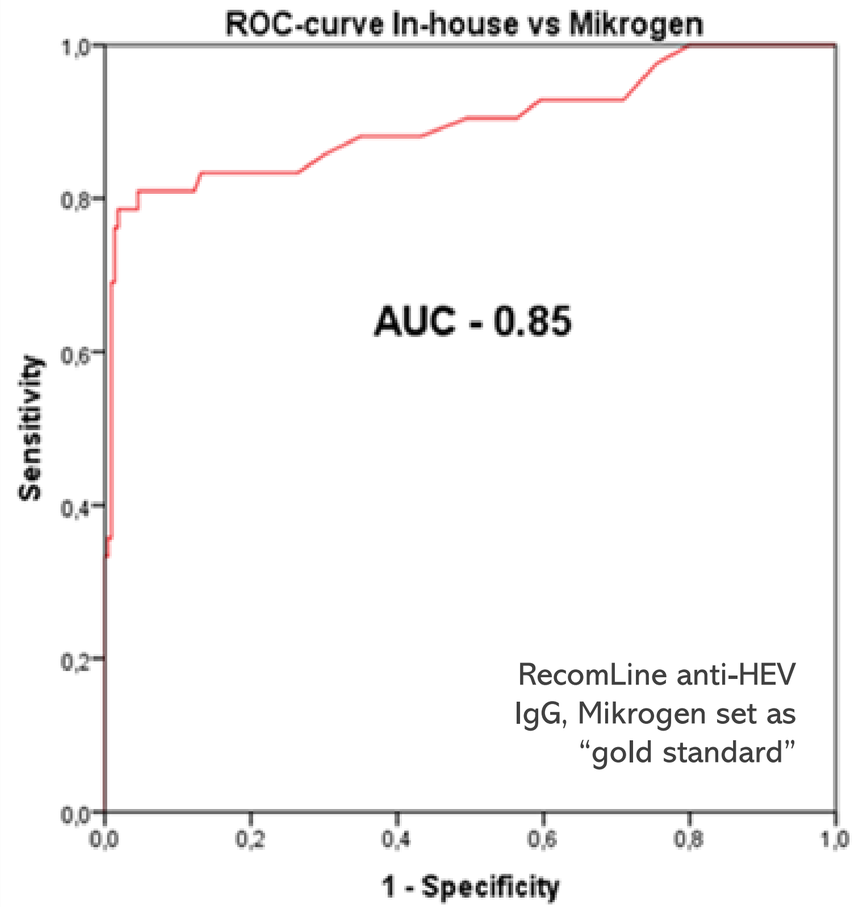
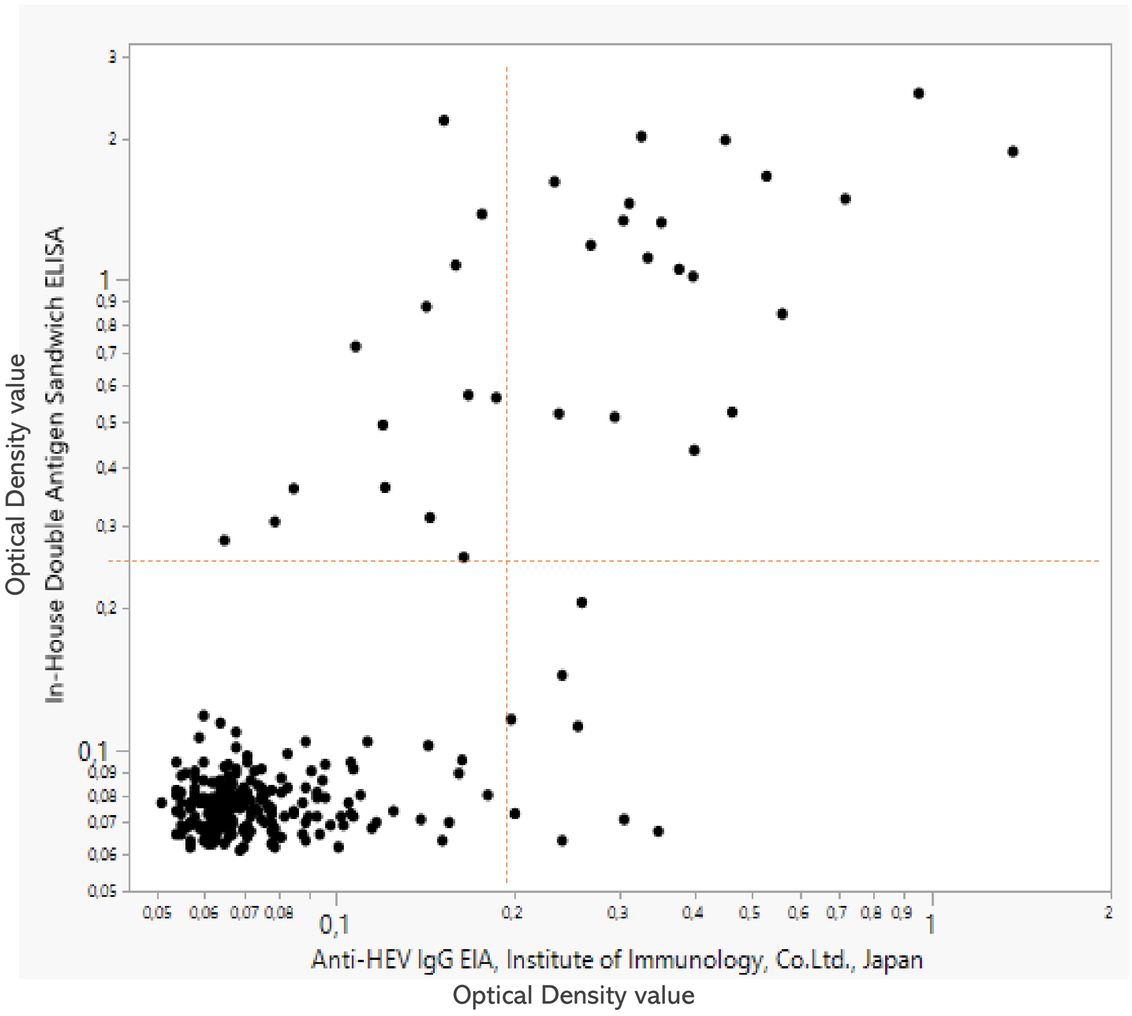


Figure 2. Comparison of commercial test system "RecomLine anti-HEV IgG", Mikrogen, Germany, and newly developed In-house Sandwich ELISA method

(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).



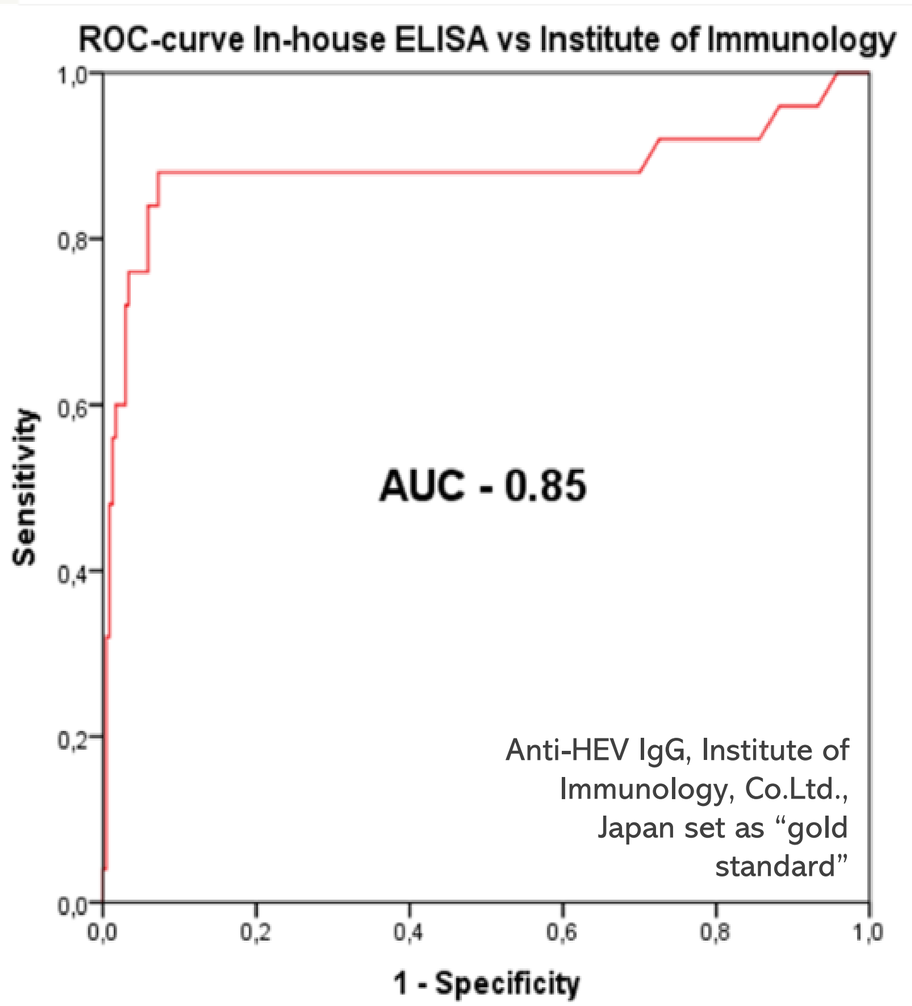


Figure 3. Comparison of commercial test system "anti-HEV IgG EIA", Institute of Immunology, Co. Ltd, Japan, and our newly developed In-house Sandwich ELISA method.
(Vertical red interrupted line – 0.198, OD cut-off value of Anti-HEV IgG EIA, Institute of Immunology, Co. Ltd, Japan; Horizontal interrupted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