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ARTICLE

Results of HDV RNA detection in male seminal fluid and female cervical swab samples using RT-PCR

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Abstract: Mongolia has an extremely high prevalence of hepatitis D virus (HDV), which leads to increased morbidity and mortality from hepatocellular carcinoma (HCC). HDV is the most severe form of chronic viral hepatitis (CVH). A recent study estimated that the prevalence of anti-HDV was 67.5% among hepatitis B surface antigen-positive cases in Mongolia. HDV infection is particularly prevalent in sexually active young people. The virus may be transmitted through sexual intercourse, and it is crucial to confirm this by detecting HDV RNA in semen and cervical swabs of patients with chronic hepatitis delta. This pilot study was conducted with 16 participants, including eight males and eight females. Viral RNA was isolated from the blood, male seminal fluid, and female cervical swab samples of all participants in the study. HDV RNA was quantified using RT-PCR in samples from blood, seminal fluid, and cervical swabs. This study found that the detection rate of HDV RNA differed between male and female samples, with 62.5% of seminal fluid samples in the male cohort being positive compared with 87.5% in the female cohort. The data shows a statistically significant difference between genders for the presence of HDVRNA in seminal fluid and cervical swabs (OR 0.238, 95% CI= 0.02-3.01). A significant correlation was found between blood HDV RNA quantity and HDV RNA presence status in seminal fluid and cervical swab samples, indicating a potential risk of sexual HDV transmission. The R^2 values for semen and cervical swab samples were 0.1095 and 0.9755, respectively.

Keywords: *HBV, HDV, Seminal fluid, Sexual transmission;*

INTRODUCTION

Hepatitis D, caused by HDV, represents a unique and often overlooked facet of viral hepatitis. HDV infects up to 70 million people worldwide, with 15–20 million developing chronic hepatitis delta (CHD), and the prevalence of CHD varies by region [1].

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There is a significant risk of morbidity and mortality due to end-stage liver disease and hepatocellular carcinoma (HCC) [2]. Mongolia has the highest prevalence of HDV among the world's population[3]. The viral genome consists of one single-stranded circular RNA of approximately 1.7 kb, which is the smallest genome known in animal virology [4]. Although hepatitis B (HBV) has long been recognized as a significant global health concern, HDV infection frequently occurs as a superinfection or co-infection with HBV, leading to more complicated liver disease outcomes [5]. Historically, HDV transmission has been predominantly associated with parenteral routes, such as contaminated needles and blood transfusions [5].

The first step in the diagnosis of HDV is an anti-HDV (anti-HDVIgM and IgG) antibody test in HBsAg-positive individuals. In both HDV co-infection and HDV superinfection, screening for HDV RNA using molecular techniques such as polymerase chain reaction (PCR) is necessary, as the goal is to quantify the circulating virus in the blood (RT-PCR) [6]. RT-PCR is a powerful molecular biology technique that has revolutionized the diagnosis of various diseases, including sexually transmitted infections (STIs). Traditional methods of STI diagnosis often lack the sensitivity and specificity required for early detection and precise identification of causative agents. RT-PCR, a molecular technique, offers numerous capabilities and features to address these shortcomings [7]. Its high sensitivity, specificity, and capacity for quantitative data make it indispensable for early detection and precise identification of STIs, patient management, epidemiological studies, and research. Understanding of how HDV spreads through sexual contact has evolved significantly in recent years, highlighting the need to examine this transmission mode. The sexual transmission of HDV primarily occurs through unprotected sexual contact with an infected partner, similar to the transmission of HBV [8]. It can be transmitted through vaginal, anal, or oral sex with an infected partner with active HDV and/or HBV infection. With the increasing prevalence of HDV infection in various parts of the world, sexual transmission of HDV has garnered increased attention from researchers and public health authorities. By delving into the dynamics of sexual transmission, we hope to contribute to a better understanding of this understudied aspect of HDV infection, ultimately facilitating more effective prevention and control strategies. In the era of advancing medical research and the quest to eliminate viral hepatitis as a global health threat, a thorough understanding of HDV transmission via sexual routes is crucial.

MATERIALS AND METHODS

Ethical approval. This study was conducted in accordance with established ethical principles and received approval from the ethics and research committees of the Mongolian National University of Medical Sciences under protocol number 2022/3–09. Informed consent was obtained from all participants, and the study adhered to the Declaration of Helsinki Guidelines for Human Research. The inclusion criteria were a diagnosis of both HBV and HDV, undergoing an HDV RNA viral load test, and a minimum age of 18 years.

Study design and participants. Patients from the Liver Center, Ulaanbaatar, Mongolia, were asked to participate in this study from October 2022 to December 2022. A total of 16 patients (8 male and 8 female) aged between 23 and 48 years participated in this study. Both male and female participants refrained from sexual activity for at least 48 hours before undergoing testing. Female participants abstained from using intravaginal products for the same duration. Seminal fluids were collected in sterile containers via masturbation, whereas vaginal/cervical

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specimens were collected using nasopharyngeal swabs dedicated to molecular biology tests.

Viral RNA preparation. Blood and sexual secretion samples were obtained from all participants for viral detection. Viral RNA was extracted from 150 µL of blood from each patient using the BioActGene® Virus Purification Kit (Bioactiva diagnostica GmbH, Germany) following the manufacturer's recommended protocols.

Detection of viral nucleic acids using Reverse Transcription Polymerase Chain Reaction. HDV detection was performed using the Bio-Rad "iTaq Universal Probes One-Step Amplification Kit" in a 25 µL total volume reaction. This reaction included 2x enzyme mix, reverse transcription, 400 nM of each primer targeting the HDV genotype 1 antigen region, 150 nM of the probe with a 6-FAM fluorescence dye at its 5' end and BHQ quencher at its 3' end, and 9 µL of the RNA extract.Amplification was conducted using an Agilent Technologies Stratagene Mx3005p thermal cycler for HDV detection and quantification under the following cycling conditions: 50°C for 20 min, 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. RT-PCR-based virus quantification was performed using HDV standards approved by the World Health Organization (WHO). The same RT-PCR method and conditions were applied to detect HDV RNA in the serum, cervical swabs, and semen plasma samples.

Statistical analysis. Odds ratio (OR) and correlation analyses (R^2) were used to assess the correlation between gender and HDV infection. A 95% confidence interval (CI) was used to estimate the precision of the OR. The correlation analysis was performed using Microsoft Excel (Microsoft 365 MSO).

Odds Ratio

$$OR = \frac{A/B}{C/D} = \frac{AD}{CB} [9]$$

A – the number of positive results in the male cohort

B – the number of negative results in the male cohort

C – the number of positive results in the female cohort

D – the number of negative results in the female cohort

95% confidence intervals (CI)

$$\text{Upper 95\% CI} = e\text{^}\ln(OR) + 1.96\sqrt{\frac{1}{A}+\frac{1}{B}+\frac{1}{C}+\frac{1}{D}} [9]$$

$$\text{Lower 95\% CI} = e\text{^}\ln(OR) - 1.96\sqrt{\frac{1}{A}+\frac{1}{B}+\frac{1}{C}+\frac{1}{D}} [9]$$

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RESULTS AND DISCUSSION

Analysis of the Viral Load in blood samples. Quantification of HDV viral load in the blood of both male and female participants was performed using RT-PCR, using the WHO-HDV international standard reference material (Table 1,2 and Figure 1). The threshold (dR) for all participants was consistent at 3362.115, indicating a standardized approach for determining cycle threshold (Ct). The lower the Ct value, the earlier the target nucleic acid was detected, indicating a higher initial amount in the sample.

Table 1. shows that male participants had Ct values ranging from 19.89 to 29.29 RT-PCR. The male participant with the highest viral load was M5 (6, 914, 000 IU/mL), and the lowest viral load was M6 (29, 500 IU/mL) in blood samples. As shown in Table 2, the Ct values among female participants varied, ranging from 16.06 to 26.22 for RT-PCR. The female participant with the highest viral load was F5 (60, 200, 000 IU/mL), and the lowest viral load was F4 (152, 000 IU/mL) in blood samples.

Table 1. HDV RNA was detected and quantified by RT-PCR in male participants' blood

№	Participants code	Threshold (dR)	Cycle threshold (dR)	Quantity (copies)
1.	M1	3362.115	27.00	63900
2.	M2	3362.115	25.77	78730
3.	M3	3362.115	21.98	875600
4.	M4	3362.115	20.63	2230000
5.	M5	3362.115	19.89	6914000
6.	M6	3362.115	29.29	29500
7.	M7	3362.115	22.08	4761000
8.	M8	3362.115	29.29	61510

Table 2. HDV RNA was detected and quantified by RT-PCR in female participants's blood

№	Participants code	Threshold (dR)	Cycle threshold (dR)	Quantity (copies)
1.	F1	3362.115	23.86	611000
2.	F2	3362.115	19.99	5970000
3.	F3	3362.115	16.32	51700000
4.	F4	3362.115	26.22	152000
5.	F5	3362.115	16.06	60200000
6.	F6	3362.115	23.24	881000
7.	F7	3362.115	25.19	1030000
8.	F8	3362.115	23.10	1209000

The x-axis represents the number of cycles, and the y-axis represents the fluorescence signal emitted by the fluorescent dye that binds to the amplified HDV RNA during each cycle. Ct is a crucial parameter obtained from the amplification plot. This is the number of cycles in which the fluorescence signal crossed a predetermined threshold. Figure 1 shows the amplification plots obtained by RT-PCR for HDV RNA in the blood samples of all the participants.

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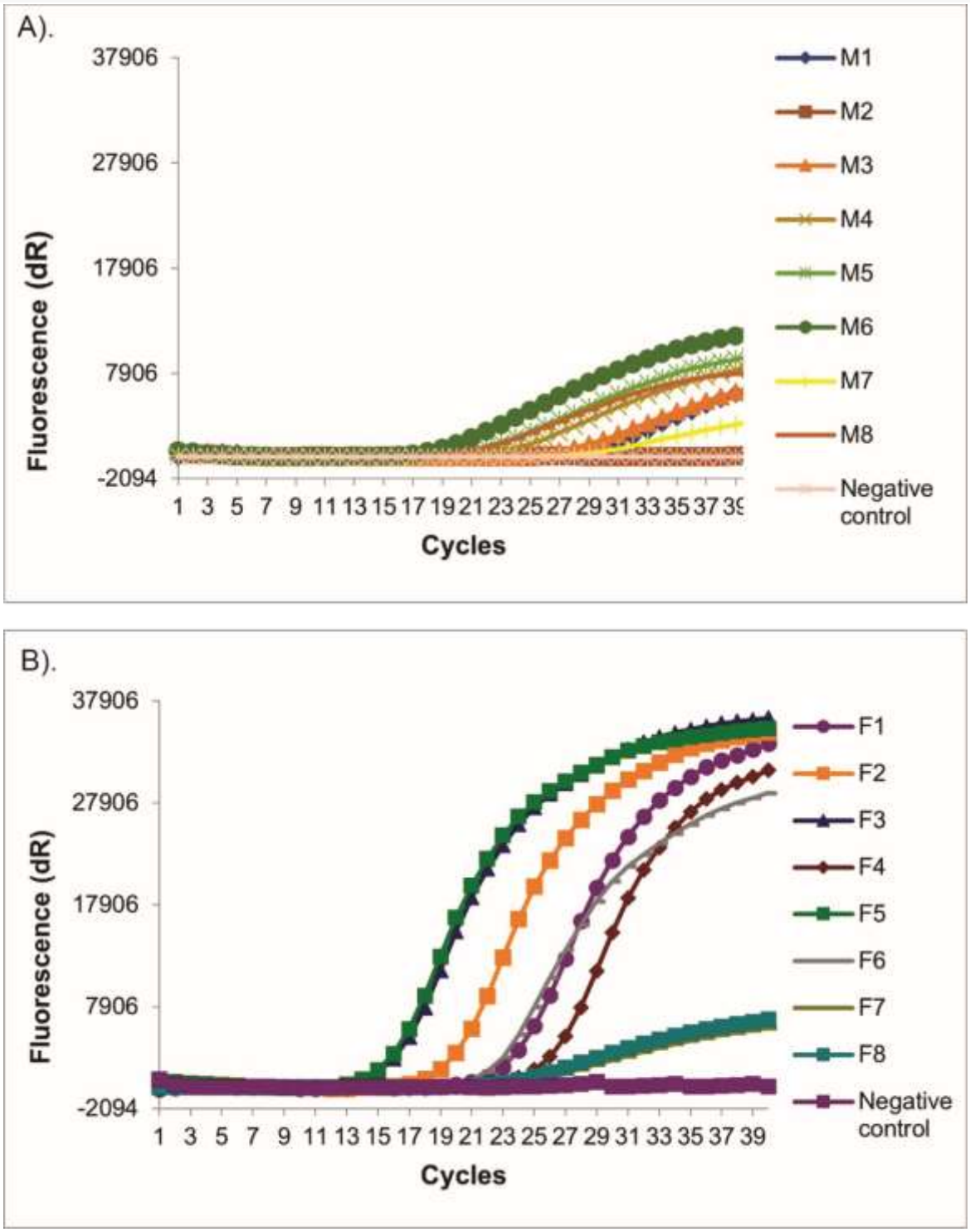

Figure 1. RT-PCR amplification plots for HDV RNA in the blood of participant **A). RT-PCR amplification plots for HDV RNA in the blood of male participants (B). RT-PCR amplification plots for HDV RNA in the blood of female participants**

Results of HDV RNA detection in male and female cervical swab samples. Viral infection was detected in the semen of HDV-infected male patients using RT-PCR and WHO-HDV international standard reference material.

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Table 3. Results from RT-PCR for the detection and quantification of HDV RNA in the semen of all male participants

№	Participants code	Threshold (dR)	Cycle threshold (dR)	Quantity (copies)
1	M1	1139.892	No Ct	0
2	M2	1139.892	No Ct	0
3	M3	1139.892	33.02	1860
4	M4	1139.892	No Ct	0
5	M5	1139.892	34.74	682
6	M6	1139.892	31.62	4230
7	M7	1139.892	35.11	549
8	M8	1139.892	29.17	17700

Table 3 shows that participants M1, M2, and M4 did not show detectable Ct values, indicating that HDV RNA was not detected in their seminal fluid samples during the study cycles. Participants M3, M5, M6, M7, and M8 showed Ct values ranging from 29.17 to 35.11, indicating the detection cycle of HDV RNA. They also had detectable quantities of HDV RNA in their seminal fluid samples, with copies ranging from 549 IU/mL to 17,700 IU/mL.

Female participants with detectable Ct values ranged from 29.01 to 36.46, whereas F4 did not. Participants with detectable Ct values showed varying amounts of HDV RNA in their cervical swab samples, ranging from 330 IU/mL to 25,300 IU/mL. Participant F3 had the highest viral load (25, 300 IU/mL), indicating a higher viral load compared to other female participants, as shown in Table 4.

Figure 2 shows the amplification plots obtained by RT-PCR for HDV RNA in male seminal fluid and female cervical swab samples.

Table 4. Results of RT-PCR to detect and determine the load of HDV in the cervical swabs of all female participants

№	Participants code	Threshold (dR)	Cycle threshold (dR)	Quantity (copies)
1	F1	2288.186	33.30	1760
2	F2	2288.186	31.66	4900
3	F3	2288.186	29.01	25300
4	F4	2288.186	No Ct	0
5	F5	2288.186	29.14	23500
6	F6	2288.186	36.00	330
7	F7	2288.186	36.46	248
8	F8	2288.186	33.93	1190

Results of statistical analysis. In the male cohort, 62.5 (5/8) of seminal fluid samples tested positive, whereas in the female cohort, 87.5% (7/8) of cervical swab samples tested positive. The OR for the association between gender and HDV infection status was 0.23809 (95% CI=0.02-3.01). An OR of less than 1 indicated a lower risk of HDV infection in males compared than in females. This study revealed a significant correlation between the quantity of blood HDV RNA and the presence of HDV RNA in the seminal fluid and cervical swab samples. The R^2 values,

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representing the proportion of variance explained by the correlation, were 0.0163 and 0.975 for seminal fluid and cervical swab samples, respectively.This study found no correlation between the presence of HDV RNA in seminal fluid and serum RNA load in the male cohort. In contrast, the female cohort showed a statistically significant and strong correlation between the load of HDV RNA in the blood and cervical swab samples (Figure 3).

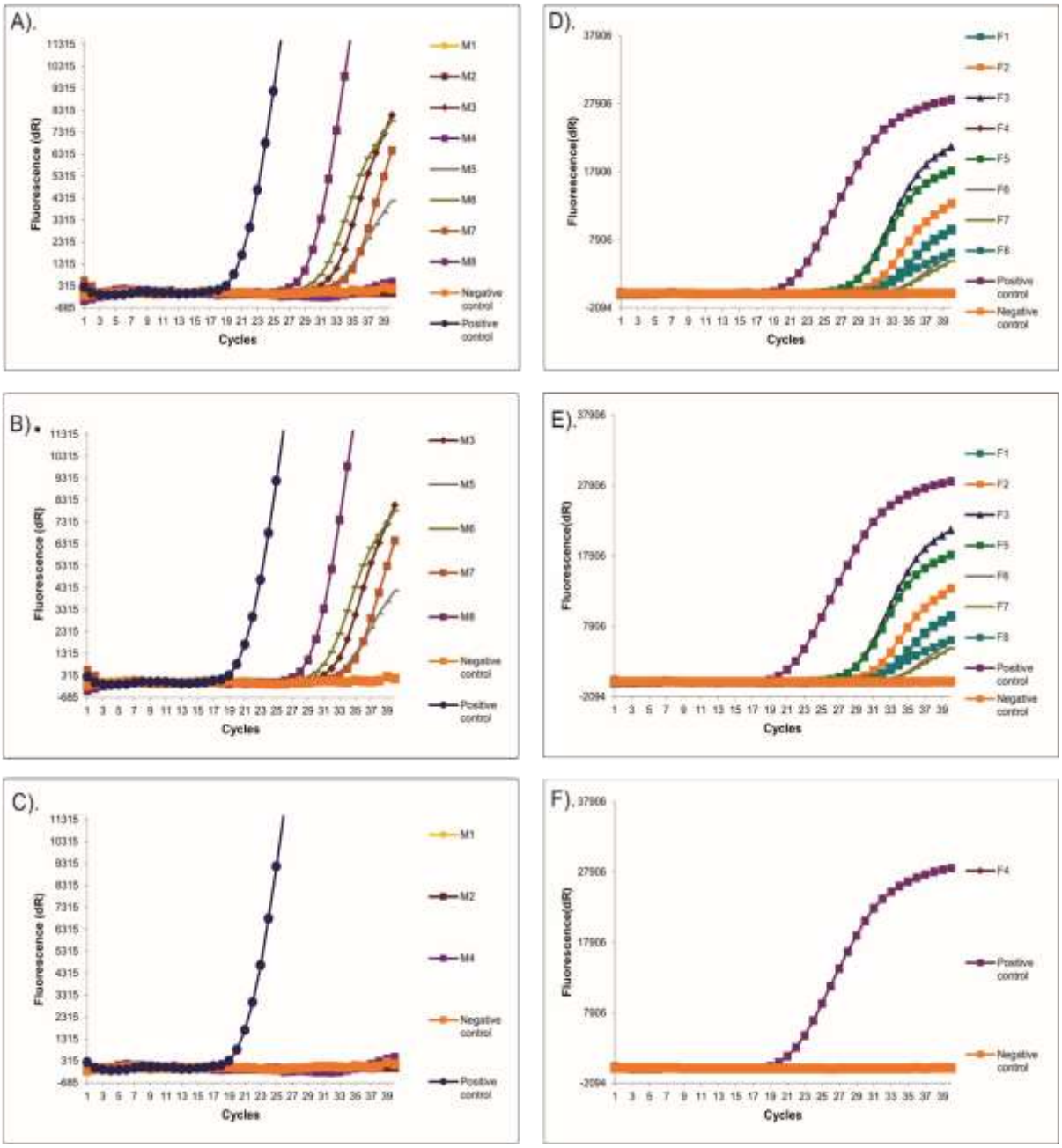

Figure 2. RT-PCR amplification plots for HDV RNA in male seminal fluid and female cervical swab samples. (A). HDV RNA was detected but not in the seminal fluid of all male participants (B). HDV RNA was detected in seminal fluid (C). HDV RNA was not detected in seminal fluid (D). HDV RNA was detected but not in the cervical swab samples (E). HDV RNA was detected in the cervical swab samples (F). HDV RNA was not detected in the cervical swab samples

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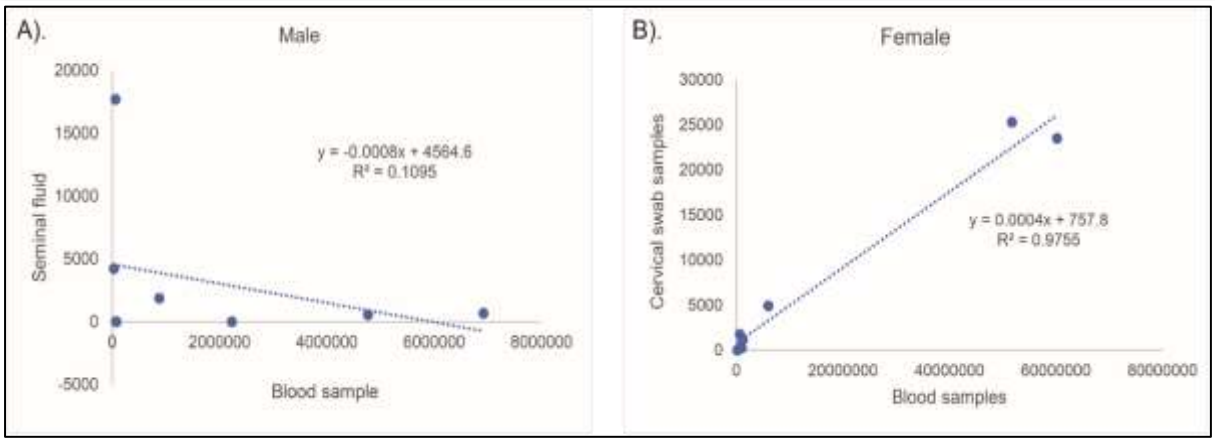

***Figure 3. Correlation analysis of blood HDV RNA levels and genital tract samples. (*A*).* Correlation between blood and seminal fluid in the male cohort (B).Correlation between the blood and cervical swab samples**

Mongolia has one of the world's highest rates of hepatitis B and delta coinfection. HDV is a significant public health issue in Mongolia. The prevalence of HDV infection in the Mongolian population is estimated to be 8.03% (5.26–12.08), meaning that a large proportion of the Mongolian population is infected with HDV, or 239 (157–360) thousand people are infected with HDV[10–12]. The WHO aims to eliminate viral hepatitis, human immunodeficiency virus, and sexually transmitted infections by 2030 [13]. Therefore, some countries, including Mongolia, have initiated mass screening campaigns for the detection of hepatitis viral infections in the entire population. This study is the first to report the detection of HDV RNA in male seminal fluid and female cervical swab samples. Currently, no assessment has been conducted in Mongolia to determine the risk of sexual transmission of HDV.

Having unprotected sex and multiple sexual partners can lead to sexually transmitted diseases. Many studies have confirmed that other viral hepatitis viruses, such as HBV and HCV, can be transmitted via sexual activity. However, few studies have shown that HDV can be transmitted through the sexual genital tract. In a 1990 study conducted by the Taiwanese researcher Jaw-Ching Wu et al. [14], the historical analysis of patients with acute HDV infection indicated a statistically plausible route of transmission through sexual contact with physical therapists. Furthermore, a study published in the same year by Liaw et al. [15] reported similar findings, demonstrating a high prevalence of sexual contact with prostitutes among patients with acute HBV infection, suggesting the potential for sexual transmission of the virus. Subsequently, in 1992, an American researcher, Lisa Rosenblum et al. [16], conducted a study involving prostitutes and drug addicts, revealing that the prevalence of HDV infection was comparable to that of other sexually transmitted infections and HBV. Additionally, a 1995 study by Jaw-Ching Wu et al [17] concluded that HDV transmission to spouses occurs during sexual intercourse with individuals with a history of involvement with prostitutes in Taiwan. In 2012, a French researcher Wael Mansour's [18] study demonstrated the presence of HDV markers in the semen of men, indicating a risk of HDV and HBV infection in the follicular fluid and semen of HDV-infected patients, regardless of the degree of macroscopic blood contamination. A 2017 study by Osiowy et al. [19] further confirmed the risk of HDV transmission among long-term cohabitants and spouses with HBV infections. Few of

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these studies are implicit and do not have direct evidence that used experiments for virus detection. To date, no study has successfully detected HDV RNA in genital tract samples in a sufficiently large cohort.

Therefore, our study is particularly unique and valuable, as it addresses this critical data gap and provides molecular biological evidence to support the assertion that HDV can indeed be sexually transmitted, which underscores the heightened susceptibility of women to HDV infection and emphasizes the importance of comprehensive investigation of these sex-specific differences. Furthermore, our research highlights the potential risks associated with high viral loads in the blood and semen, suggesting an increased likelihood of sexually transmitted HDV.This knowledge is pivotal for informing public health strategies and medical practices, especially for infection control and fertility management.

CONCLUSIONS

In conclusion, our study sheds light on the intricate dynamics of HDV transmission and the correlation between HDV RNA load, male seminal fluid, and female cervical swab samples and assesses the risk of sexually transmitted HDV.

Our pilot study showed that HDV RNA was detected in 75% of participants, and 25% were not detected. HDV RNA was detected in seminal fluid samples of 62.5% of male participants and cervical swab samples of 87.5% of female cervical swab samples. It is already known that the genetic material (DNA or RNA) of viral pathogens is the absolute indication of active infection. This concept has been widely utilized in the last two decades, and molecular biological diagnostic methods (PCR and RT-PCR) have become the gold standard for the detection and control of viral infections. In particular, the current outbreak of the global pandemic caused by SARS-Cov2 has led to the widespread introduction of polymerase chain reaction (PCR) technology, which has shown powerful characteristics, including high sensitivity and robustness. This study, though limited due to its small sample size, offers molecular biological evidence supporting the idea that HDV can be sexually transmitted, highlighting the need for future validation.

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