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Environmental and biological interactions of Hepatitis B virus in leeches – a molecular investigation

Alican Bilden[1,A-B,D,F] ✉, Merve Kahraman[1,B,E], İbrahim Halil Şahin[2,D-E], Nadia İbrahim Kamil Kamil[1,B-D], Ömer Karakamış[3,C], Elif Sevim[3,C,F], Muttalip Çiçek[1,A,E-F]

[1] *Medical Parasitology, Ahi Evran University, Kırşehir, Turkey*
[2] *Medical Services and Techniques, Eren University, Bitlis, Turkey*
[3] *Medical Biology, Ahi Evran University, Kırşehir, Turkey*
A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

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Abstract

Objective. The aim of the study is to investigate whether transovarial transmission of the Hepatitis B virus occurs in leeches, and whether the virus is released into the external environment through their secretions.

Materials and Method. 52 reproductive leeches (*Hirudo verbana*) were experimentally fed with hepatitis B infected human blood. From these reproductive leeches, juveniles were produced. Additionally, diverse leech-associated samples were collected, encompassing water and soil from the habitats of the reproductive leeches, faeces, body surface secretions, and cocoon shells. Each sample was analyzed for the presence of the hepatitis B virus using advanced molecular methods, specifically, real-time quantitative polymerase chain reaction.

Results. 90 juveniles, 15 leech-associated samples, and 13 cocoon shells were analyzed. Analyses did not reveal the presence of HBV in any of the 90 juveniles, or in the leech-associated samples. The results suggest that HBV is neither vertically transmitted to the juveniles through transovarial transmission, nor disseminated into the external environment through secretions or other biological materials linked to leeches.

Conclusions. The study concludes that leeches (*Hirudo verbana*) do not facilitate the transovarial transmission of HBV. Moreover, the absence of HBV in the environmental samples highlights the minimal risk of viral spread via leech secretions, or associated materials. These findings provide critical insights for the ecological management of leech populations, especially in minimizing viral transmission risks. In the literature, studies on transovarial transmission in leeches are quite limited, and it has been concluded that while the data from the presented study are valuable, they are insufficient and highlight the need for further research in this field.

Key words

ecological safety, molecular analysis, transovarial transmission, leeches, Hepatitis B virus, *Hirudo verbana*

INTRODUCTİON

Leeches are segmented worms belonging to the subclass Hirudinea within the phylum Annelida. They function primarily as temporary blood-feeding ectoparasites on mammals, and occasionally on amphibians, such as frogs and tadpoles, as well as on turtles and small fish [1, 2]. In addition to parasitic species, predatory leeches have also been documented [3]. To date, more than 600 leech species have been identified worldwide, inhabiting a wide range of ecosystems, including freshwater, marine, estuarine, and moist terrestrial environments [4, 5].

Leeches are hermaphroditic organisms, possessing both male and female reproductive structures. Their typical life cycle comprises three main stages: egg, which is enclosed within a protective cocoon; juveniles; and reproductively mature hermaphroditic adults [6]. Leeches have long held a place of significance in various ecological and biomedical contexts. As obligate haematophagous organisms, certain species such as *Hirudo verbana* are known for their intricate biological mechanisms, including their ability to feed on vertebrate blood while secreting a range of bioactive compounds [7–11].

Leeches hold a significant place in ecological systems, raising curiosity about the mechanisms through which pathogenic agents are transmitted within their environments, and to their juveniles. Transovarial transmission refers to the vertical transfer of pathogens from parent to juveniles and is a well-documented phenomenon in arthropod vectors, such as mosquitoes and ticks [12]. However, the mechanism of transovarial transmission in leeches remains poorly understood [13]. The biological adaptations of leeches in diverse ecosystems suggest that the presence or absence of this mechanism holds critical ecological and medical significance [14]. For example, studies have shown that mosquito vectors play a pivotal role in the dissemination and persistence of zoonotic pathogens through transovarial transmission, particularly under adverse environmental conditions [15]. Despite this, the relationship between the haematophagous nature of leeches, their gut microbiota, and immune systems, as well as how pathogens reach their reproductive organs, remains unclear [13].

The close contact of leeches with both human and animal blood presents a risk factor that necessitates investigation

✉ Address for correspondence: Alican Bilden, Medical Parasitology, Ahi Evran University, Kırşehir, Turkey
E-mail: alican.bilden@ahievran.edu.tr

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into the vertical transmission of potential pathogenic agents. Some haematophagous parasites have been reported to retain pathogens in their guts for extended periods, with these pathogens remaining biologically active [16]. One study demonstrated that leeches could ingest and temporarily retain bloodborne pathogens within their digestive systems. However, under controlled conditions, no detectable levels of these pathogens were observed after 4–6 months post-feeding, indicating effective microbial degradation over time [17]. In parallel, environmental parameters such as temperature have been shown to influence the efficiency of transovarial transmission in vector organisms, particularly mosquitoes. Lower temperatures, for instance, have been associated with increased vertical transmission rates for certain arboviruses [15].

Environmental and host-related factors, such as feeding status, time of collection, and habitat source, have been shown to influence the composition of gut microbial communities in medicinal leeches. These variations may indirectly affect host-microbe interactions and potential microbial transmission pathways [18]. Moreover, environmental cues, such as carbon dioxide, temperature, humidity, and host-derived chemical signals, are known to influence tick-host interactions. Understanding these sensory-driven behaviours may contribute to the development of novel vector control methods [19]. Given the ecological importance of these organisms, it is evident that further investigation is needed to elucidate their roles in pathogen dynamics, particularly in relation to viruses such as hepatitis B virus (HBV).

The aim of this study is to determine whether *Hirudo verbana* facilitates the transovarial transmission of HBV, and whether HBV can be detected in environmental samples associated with leeches. A controlled experimental study was conducted using real-time quantitative polymerase chain reaction (RT-qPCR) to test this hypothesis.

MATERIALS AND METHOD

Ethical Approval. The study was approved by the Ethics Committee of Ahi Evran University in Kırşehir, Turkey (Approval No. E-77504701-204.01.07-00000715179).

Acquisition of feeding blood. The feeding blood used in this study was obtained from a patient diagnosed with Hepatitis B using the RT-qPCR method at the Virology Laboratory of Kırşehir Training and Research Hospital. In accordance with ethical guidelines and after obtaining the necessary approvals, 450 mL (one unit) of blood was collected from the patient. The RT-qPCR analysis revealed a viral load of 6.5×10^5 IU/mL. The blood was utilized as feeding blood for the reproductive leeches used in the study.

Acquisition and feeding of leeches. A total of 52 reproductive leeches, weighing between 2–4.2 grams, were obtained from the Medical Leech Breeding and Research Laboratory of Kırşehir Ahi Evran University Faculty of Medicine. Prior to the experiment, the leeches were starved for a period of 3 months to prepare them for the study. Following the 3-month fasting period, feeding blood, preheated to 36–37 °C, was introduced into the dried intestine, which was then placed in the container housing the reproductive leeches. This setup allowed the leeches to attach themselves to the surface of the intestine and feed effectively (Fig. 1).

Figure 1. Reproductive leeches feeding on Hepatitis B-infected blood preheated to 36–37 °C within a dried intestine setup

After feeding, each leech was observed to have at least doubled its initial weight. All leeches were individually weighed, and their pre- and post-feeding weights were recorded (Fig. 2). Note: Pre-feeding and post-feeding weights (g) are compared for each individual leech (n=52)

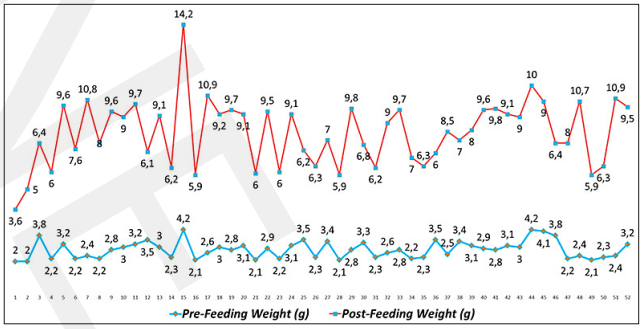


Figure 2. Changes in the weights of reproductive leeches before and after feeding

After the feeding process was completed, the leeches were transferred to water with identical parameters. Following the completion of an approximately one-month regurgitation period, the leeches were placed in soil tanks for cocoon production. During this process, cocoon monitoring was conducted bi-weekly, and the water in which the leeches were kept was changed twice a week. Additionally, the vitality and behaviour of all leeches were observed daily. This process continued for a total of 7 months.

Experimental setup and sample collection. A total of 52 *Hirudo verbana* leeches were used in the study. The experimental environment was arranged to provide 1 liter of water per leech, using a tank with a total capacity of 60 liters. To simulate the natural living conditions of the leeches, peat soil with high water retention capacity, free of fertilizers and chemicals, was placed in a perforated container positioned at the centre of the tank. Garden stones of various sizes were placed in the remaining areas of the tank to enhance habitat simulation. Before introducing the reproductive leeches into the tank, water parameters were carefully adjusted to maintain a pH of 6.8–7.2, a temperature of 24–25 °C, dissolved oxygen (D.O.) levels of 80–90%, and electrical conductivity of 20–50 µS/cm. Throughout the study, the water in the tank was changed twice a week to ensure proper hygiene and water quality.

During the 7-month study period, samples associated with the leeches were collected approximately every 40–45 days. These samples were categorized as 'The Habitats of Reproductive Leeches: Water and Soil Samples', 'Faeces of Reproductive Leeches', and 'Body Surface Secretions of Reproductive Leeches', and stored at -20 °C. Additionally, every 2 weeks, the soil in which the reproductive leeches were housed was inspected, and any cocoons laid by the leeches were collected and transferred to separate soil with optimal conditions. The cocoons were incubated for 35 days to allow for the emergence of juveniles. After this period, all cocoons were manually opened, and the juveniles were counted individually. Both the juveniles and the cocoon shells were numbered and stored at -20 °C.

No mortality was observed among the reproductive leeches during the study period. At the end of the study, 5 leeches were randomly selected from the 52 reproductive leeches, sacrificed, and stored at -20 °C. Additionally, to ensure test reliability and evaluate changes in viral load, one positive control leech fed with infected blood, and one negative control leech fed with healthy blood, were sacrificed at the sixth month of the study, and stored at -20 °C.

DNA extraction and quantitative real-time PCR (RT-qPCR). All samples were processed as independent replicates and analyzed in triplicate. Nucleic acid extraction was performed (Tab. 1), and the process was successfully completed for all specimens. Detection and quantification of HBV DNA in the extracted samples were carried out using a commercial RT-qPCR kit (Bosphore HBV Quantification Kit, Anatolia Geneworks, Turkey) on the Montania 4896 Real-Time PCR system, in accordance with the manufacturer's protocol. The kit targets the S gene region of HBV and is capable of detecting genotypes A through J. According to the manufacturer, the quantification range of the assay is 10 to 1×10^9 IU/mL, with an analytical sensitivity of 10 IU/mL. The results were calculated as the arithmetic mean of the triplicate reactions and are expressed in IU/mL (Tab. 2). The conversion factor used was 1 IU = 4.5 ± 0.2 copies/mL.

Table 1. Summary of DNA extraction procedures and sample details

Specimen type	No. of samples	DNA extraction kit used
Positive control leeches	1	DNeasy Blood & Tissue Kit (Qiagen, Germany)
Negative control leeches	1	DNeasy Blood & Tissue Kit (Qiagen, Germany)
Reproductive leeches	5	DNeasy Blood & Tissue Kit (Qiagen, Germany)
The habitats of reproductive leeches: water and soil samples	5	PowerSoil DNA Isolation Kit (MO BIO, USA)
Feces of Reproductive Leeches	5	DNeasy Blood & Tissue Kit (Qiagen, Germany)
Body surface secretions of reproductive leeches	5	DNeasy Blood & Tissue Kit (Qiagen, Germany)
Juvenil leeches	90	DNeasy Blood & Tissue Kit (Qiagen, Germany)
Cocoon shells	13	DNeasy Blood & Tissue Kit (Qiagen, Germany)

Note. A PowerSoil DNA Isolation Kit was used for soil-related samples, while the DNeasy Blood & Tissue Kit was applied to biological samples, such as leeches and their secretions.

RESULTS

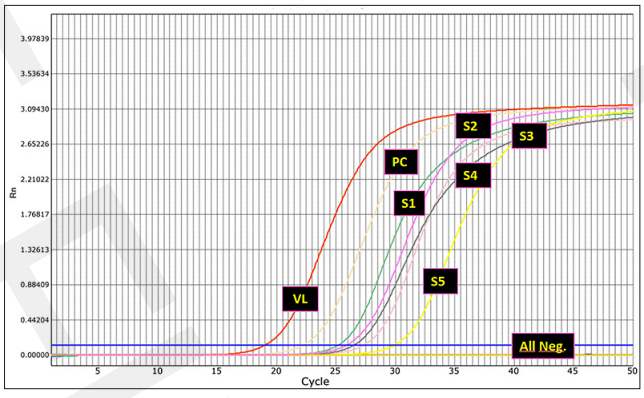
In this study, a total of 5 reproductive leeches, 13 cocoons (1st Cocoon: 7 juveniles; 2nd Cocoon: 11 juveniles; 3rd Cocoon: 14 juveniles; 4th Cocoon: 0 juveniles; 5th Cocoon: 10 juveniles; 6th Cocoon: 3 juveniles; 7th Cocoon: 3 juveniles; 8th Cocoon: 9 juveniles; 9th Cocoon: 6 juveniles; 10th Cocoon: 10 juveniles; 11th Cocoon: 4 juveniles; 12th Cocoon: 13 juveniles; 13th Cocoon: 0 juveniles), 90 juvenile leeches, 5 "Habitats of Reproductive Leeches: Water and Soil Samples" samples, 5 "Feces of Reproductive Leeches" samples, and 5 "Body Surface Secretions of Reproductive Leeches" samples were analyzed. Extraction and PCR processes were successfully applied to and completed for all samples.

The PCR results were reported using the arithmetic means of triplicate measurements for each sample. The feeding blood, reproductive leeches, leech-associated samples, and the positive and negative control leeches' average HBV viral loads and results are presented in tabular form (Table 2). Except for the reproductive leeches and the positive control leech, all samples tested negative for HBV in the RT-qPCR assays. Amplification curves and Ct values for all samples were also visualized (Figure 2).

Table 2. HBV RT-qPCR results, sample types, and viral loads

Specimen type	No. of samples	Average Viral Load (IU/mL)	Result
Feeding blood	1	6.5×10^5	**Positive**
Positive control leech	1	1.078×10^4	**Positive**
Reproductive leeches	5	3.3×10^3	**Positive**
Habitats of reproductive leeches: water and soil samples	5	<10	Negative
Faeces of reproductive leeches	5	<10	Negative
Body surface secretions of reproductive leeches	5	<10	Negative
Juvenil leeches	90	<10	Negative
Cocoon shells	13	<10	Negative
Negative control leeches	1	<10	Negative

Note: Viral loads below the detection limit (<10 IU/mL) are reported as negative. Positive results indicate detectable levels of HBV



*__VL (Viral Load of Leech-Feeding Blood):__ Amplification curve for HBV-infected blood used to feed the leeches.
**__PC (Positive Control Leech):__ Amplification curve showing HBV detection in the positive control leech.
***__S1, S2, S3, S4, S5 (Reproductive Leeches):__ Amplification curves for reproductive leeches tested for HBV.
****__All Neg (Negative Samples):__ Amplification curves for water, soil, feces, body secretions, juvenile leeches, cocoon shells, and the negative control leech.

Figure 2. Amplification Curves and Ct Values for All Samples Analyzed in the HBV RT-qPCR Assay

DİSCUSSİON

The roles of leeches in ecosystems and the preservation of sustainable ecological environments are critically important for both biodiversity and ecosystem balance. In aquatic ecosystems, leeches are integral components that support ecosystem services. However, the increasing medical and commercial use of these organisms has led to over-harvesting, posing a significant threat to their natural populations [20, 21]. The ecological and biological safety of medicinal leeches, such as *Hirudo verbana*, needs to be evaluated more comprehensively.

This study evaluated the potential for transovarial transmission of Hepatitis B Virus (HBV) and its environmental dissemination by *Hirudo verbana*. The results demonstrated that HBV was neither vertically transmitted through leeches nor detected in environmental samples (Tab. 2, Fig. 2). These findings suggest that the potential of leeches to carry and disseminate HBV is minimal, posing a low virological safety risk. Supporting evidence from the literature aligns with these results. For instance, one study investigated the survival of various pathogens in the intestines of medicinal leeches, and found that protozoan parasites such as *Toxoplasma gondii, Trypanosoma brucei brucei,* and *Plasmodium berghei,* along with bacteriophages and several bacterial species, remained viable for extended periods, particularly under low-temperature conditions. In experiments conducted at 3 °C, 22 °C, and 32 °C, pathogen viability in leech intestines persisted for up to 6 months at 3 °C. Although electron microscopy revealed no evidence of these pathogens penetrating the salivary glands or reproductive organs, the possibility of indirect transmission was acknowledged [16].

Similarly, another study demonstrated that *Hirudo verbana* leeches, when fed with bovine blood and kept at 20 °C under controlled laboratory conditions for 7 months, exhibited a progressive reduction in viral titers. Bovine Viral Diarrhea Virus (BVDV) titers dropped to undetectable levels within 3 months, while Reovirus and Murine Parvovirus titers fell below detection thresholds after 4 months. The study also highlighted the role of intestinal symbiotic bacteria, such as *Aeromonas hydrophila veronii,* in contributing to viral degradation and inactivation [17]. Furthermore, another study reported that leeches fed with mammalian viruses including Bovine Parvovirus (BPV), Feline Calicivirus (FCV), Equine Arteritis Virus (EAV), and Equine Herpesvirus Type 1 (EHV-1), showed undetectable levels of infectivity within 23–29 weeks when maintained at 10 °C. However, when the temperature was elevated to 30 °C after the sixth week, the inactivation process was significantly accelerated.

These findings collectively support the conclusion that maintaining leeches at approximately 20 °C provides optimal conditions for digestive inactivation of blood-borne pathogens, thereby minimizing their potential role in disease transmission [22]. The findings also suggest that leeches may act as potential vectors for human and animal pathogens, such as HIV, Hepatitis B, *Toxoplasma gondii*, and *Plasmodium berghei*, in regions where these pathogens are endemic [16]. Although these studies do not directly investigate transovarial transmission or environmental dissemination, their results demonstrate that the infectivity and persistence of pathogens can diminish under certain environmental conditions and over time. Notably, low temperatures and the presence of symbiotic microorganisms appear to reduce both the survival duration and infectivity of pathogens. These factors may contribute to a lower risk of environmental release and direct transmission, thereby enhancing the virological safety of leeches. Collectively, these findings help clarify how environmental parameters and the biological characteristics of hematophagous organisms influence pathogen dynamics and transmission potential.

In this context, the results of the presented study are indirectly supported by previous findings on pathogen inactivation and containment in leech systems. Moreover, one study emphasized the limited understanding of the impacts of human-derived pathogens on animal populations, and highlighted the need to improve knowledge about how zoonotic and reverse zoonotic agents affect ecosystem health [23]. The current study contributes to this discourse by demonstrating that HBV, a blood-borne pathogen, is not transmitted transovarially in leeches and does not pose a significant environmental dissemination risk. These findings underscore the importance of further research to address gaps in current knowledge regarding pathogen interactions with the immune systems and the gut microbiota of haematophagous organisms.

CONCLUSİONS

The study shows that *Hirudo verbana* presents a low risk of transovarial pathogen transmission and environmental dissemination. However, broader studies investigating different viral species are essential for a better understanding of the ecological and biological impacts of these mechanisms. Given the contributions of leeches to both ecosystem health and human health, it is critical to protect their natural populations and enhance biosecurity standards. The presented findings not only provide a foundation for future research, but also make a significant contribution to the existing literature.

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