ORIGINAL ARTICLE

Hepatitis B virus: a new platform for *in vitro* studies of infection, replication cycle and pathogenesis

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Abstract

Hepatitis B virus (HBV) research has been hampered by the lack of suitable and reproducible cell culture systems that reliably mimic the viral life cycle. Several infection stages and metabolic aspects related to HBV cycle still need to be elucidated. The aim of this research was the study of techniques aiming at developing a sustainable in vitro platform of hepatitis B virus infection, in a simplified and low maintenance approach, evaluating the continuity of infection in cell culture throughout many passages, using a positive serum pool to the virus in human hepatocellular carcinoma. The viral load was quantified by real-time polymerase chain reaction. The cells underwent a freeze and thaw cycle, followed by seeding, and the new culture was analyzed to quantify the viral load. An aliquot was used to detect the surface antigen (HBsAg), by chemiluminescence. The detection of the core antigen (HBcAg) was performed by flow cytometry and by immunofluorescence microscopy. Viral load remained detectable throughout the studied period, 50 days after initial infection. The process of freezing and seeding produced detectable viral load for a 7-day period. HBsAg was reagent in the infected cells, confirming the maintenance of infection. The flow cytometry result indicated 11.85% of HBcAg positive cells, which demonstrates that new viral particles were at the assembly stage. Indirect immunofluorescence using epiluminescence microscopy allowed the detection of viral HBcAg in the interior of infected cells, confirming the results obtained by flow cytometry. The platform for infection in cell culture was successfully obtained during the studied period, which represents the possibility to apply this model in a continuous practice, to support several biotechnological purposes.

Keywords: Hepatitis B. Cell Culture. *in vitro* Infection. Hepatocellular Carcinoma Cells.

How to cite

INTRODUCTION

The World Health Organization (WHO) estimates that, in 2015, chronic hepatitis B infection reached 257 million people worldwide, and death toll related to this disease reached 887,000 of the patients in the same year, mostly attributed to hepatocellular carcinoma and cirrhosis. In 2016, 27 million people were informed of their infection, while only 16.7% of these diagnosed people were on treatment (World Health Organization, 2020).

Throughout the years, hepatitis B virus (HBV) research has been hampered by the lack of suitable and reproducible cell culture systems that reliably mimic the viral life cycle. Several infection stages and metabolic aspects related to HBV cycle still need to be elucidated, especially concerning the biogenesis, homoeostasis and turnover of the cccDNA reservoir, whereas HBV infection is characterized by a narrow species and tissue tropism (Revill et al., 2019; Sai et al., 2016). The extended maintenance of these systems presents numerous challenges, such as technical difficulties to keep the virus in the replication phase and with the detectable viral load during various passages and the costs associated with complicated methodologies to preserve the virus in lytic phase and to prepare cells in order to make them susceptible to infection. Therefore, until now, the HBV replication mechanisms described in the literature were obtained from genetic and biochemical methodologies, of systems basically limited to primary human hepatocytes (Nishitsuji et al., 2017; Witt-Kehati et al., 2016).

In vitro methods include varied uses of animal and human tissues, isolated cells, cells lines, and cellular components (Castel & Gómez-Lechón, 1997). For instance, a good example of an *in vitro* replication system was the one successfully developed by Wakita et al. (2005) for the hepatitis C virus (HCV). This system is based on the transfection of human hepatocarcinoma cells, of the Huh lineage, highly permissive to this virus, using a subgenomic replicon (SGR) of the genotype 2a, named JFH1 (*Japanese Fulminant Hepatitis 1*), which is maintained active and replicating in a straightforward routine, with no additional steps to treat the infected cell culture. Furthermore, because it presents the main viral replication cycle stages, releasing infective particles to the extracellular medium, this can be considered an ideal model to study viral behavior when parasitizing the host cell (Wakita et al., 2005; Park & Rehermann, 2014).

A variety of sublines of Huh-7 cells, such as Huh-7.5, have been shown to support HCV efficiently; thus, these lines have been used widely for screening and characterizing antiviral medicines. The robust replication of HCV in Huh-7.5 cells is explained by a defect in innate immune signaling. A key molecule of innate interferon signaling, RIG-I, carries a mutation that results in low innate antiviral IFN responses upon HCV infection (Omura et al., 2019).

The existing *in vitro* HBV replication systems are not as efficient as the one available for HCV since they solely provide low levels of infection or they need immunomodulation to maintain the HBV replication stable. Additionally, these systems are not susceptible to HBV isolates derived from patients, only supporting low levels of infection or require immunomodulation to replicate HBV (Ortega-Prieto et al., 2018). The lack of an adequate replication system for the HBV directly affects the persistence of hepatitis B in the population, forasmuch as it complicates the development of more effective therapies, through research of new medications, of pharmacological interactions and of the cellular signaling pathways employed by the virus. Hence, the therapy against HBV is limited to reducing the evolution of the hepatic disease and its primary consequences, specifically cirrhosis, hepatocellular carcinoma and, ultimately, death (Nishitsuji et al., 2015, 2017; Ortega-Prieto et al., 2018; World Health Organization, 2015). For that reason, an efficient experimental system, that is still lacking, supporting hepatitis B *in vitro* infection would assist several research areas, including the identification of new biomarkers and the study of the hosts genetics, allowing even the analysis of the response to certain compounds or medications to HBV infection (Sai et al., 2016; Witt-Kehati et al., 2016; Nishitsuji et al., 2015).

OBJECTIVE

Study techniques attempting to develop a sustainable *in vitro* platform of HBV infection, in a simplified and low maintenance method, and to evaluate the continuity of the cell culture infection throughout many passages and storage conditions, using hepatocellular carcinoma Huh-7.5 cells.

METHODS

Cell Culture

Hepatocellular carcinoma Huh-7.5 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium), containing 10% fetal bovine serum (FBS), 1% non-essential amino acids solution, 10,000 IU/mL penicillin and 10 mg/mL streptomycin, maintained in a 5% CO_2 incubator at 37 °C. After > 90% cell confluence was reached at a 25 cm² flask, cells were detached employing trypsin 0.25% and EDTA solution (Sigma-Aldrich, UK reagents).

HBV Infection

The viral isolate was obtained by HBV positive pool serum previously screened, with viral load quantified by real-time Polymerase Chain Reaction (PCR). In order to infect cells, 25% of the original volume was maintained in the culture flask after detachment and, when ∼ 30% confluence was reached, HBV positive pooled serum (1:4) in DMEM was added. In the 19th day of infection, an aliquot was separated, centrifuged at 300 ×g , for 10 min, at 4 °C, and the pellet was resuspended at 10% dimethyl sulfoxide in FBS and stored in cryogenic tube at − 80 °C. Subsequently, the cells were thawed and seeded in another cell culture flask. The supernatant was collected and quantified by real-time PCR.

Quantitative Real-Time PCR

Real-time PCR was performed using the Abbott m2000 Real-Time system (Abbott Laboratories, IL, USA). For measurement of viral load, 500 µL of HBV positive human serum pool, or the supernatant of cell culture, were used for extraction using an automated system based on magnetic microparticles, followed by the purification of DNA samples. HBV DNA and internal control were amplified by the Abbott m2000rt equipment, and the amplification cycle at which fluorescence is initially detected is proportional to the log of the HBV DNA concentration present in the original sample, therefore allowing the quantitation by a calibration curve.

HBsAg Detection by Qualitative Chemiluminescent Immunoassay

After cellular detachment by trypsin, an aliquot (100 µL) was centrifuged and resuspended in lysis buffer (Tris 10 mmol/L pH 7.4; NaCl 150 mmol/L; 1 mmol/L EDTA), with protease inhibitors phenylmethanesulfonyl fluoride 100 mmol/L and leupeptin 1 mg/mL. Cells were lysed with a sonicator and the volume was adjusted to 300 µL in 3% FBS in PBS. Afterward, the samples were analyzed by the automated chemiluminescence system Abbott Architect (Abbott Laboratories, IL, USA), and the result calculated by the ratio of the sample (S) response to the cutoff (CO), established from the systems' calibrators, both measured in relative light unit (RLU). The assay is considered reagent when the calculated ratio is > 1.1.

HBcAg Detection by Flow Cytometry

The first stage consisted of Huh-7.5 cells fixation. 35 days after infection, frozen cells were thawed with PBS, centrifuged; the supernatant was discarded and resuspended in 3% FBS in PBS. Cells were incubated for 10 min in cytometry buffer (REF 349202, BD Biosciences), for cell fixation. The supernatant was removed and incubation succeeded, now with saponin 0.5% in PBS, followed by two washing steps with PBS. Cell concentration was adjusted to 1×10^6 cells/mL. The assay was divided into A) permeabilized and fixed cells and human anti-IgG conjugated with fluorescein isothiocyanate (FITC); B) permeabilized and fixed cells, incubated with human serum not reactive with anti-HBc, and anti-IgG-FITC; C) permeabilized and fixed cells, incubated with human serum reactive with anti-HBc, and anti-IgG-FITC. Tubes B and C were incubated in serum for 30 min, washed with PBS, centrifuged and the

supernatant discarded. Ensuing, 100 μL of anti-IgG-FITC was added to all tubes and incubated overnight, in the dark. Next day the tubes were centrifuged, supernatant discarded and 350 μL of cytometry buffer was added.

HBcAg Detection by Indirect Immunofluorescence Microscopy

After thawing of infected cells, they were centrifuged and the supernatant discarded, later resuspended in 500 μL of PBS. Then, 5 μL were pipetted into 2 marked areas of an immunofluorescence slide, and it was added to A) human serum reactive with anti-HBc (positive control); B) human serum not reactive with anti-HBc (negative control); in a 1:5 dilution in PBS, for 30 min. The slide was washed with ethanol and 1 drop of anti-IgG-FITC was added for 1 h. Finally, the slide was washed with water, then PBS, and 1 drop of Evans blue dye. The slide was visualized in an epiluminescence microscope.

STATISTICAL ANALYSIS

The statistical analysis was performed using Statistica 10 (StatSoft, Inc.) software. The Kolmogorov-Smirnov test (K-S d = 0.463; $p < 0.01$) was used to verify the assumption of normality, and it demonstrated that the viral load was not normally distributed throughout time. For this reason, Spearman correlation matrix was applied. Probability levels of <.05 were considered to be significant.

RESULTS

The HBV positive serum pool employed for the cell culture infection of Huh-7.5 cells presented viral load of 7.7 log (2.02×10^8 copies/mL).

The evaluation of the viral load results on the supernatant was performed throughout the study, during 50 days, indicated in Figure 1. The moderate negative linear correlation obtained ($r = -0.4030$) indicated that time has a reasonable influence over the quantity of HBV DNA copies, which remained detectable for the whole studied period. The *p-value* (.07) indicates that the viral load variation is not statistically significant. This is due to the viral load remaining relatively stable, despite the sudden decrease on DNA copies in the first days of incubation, probably related to the passages, washes and medium changes that removed the virus off the supernatant.
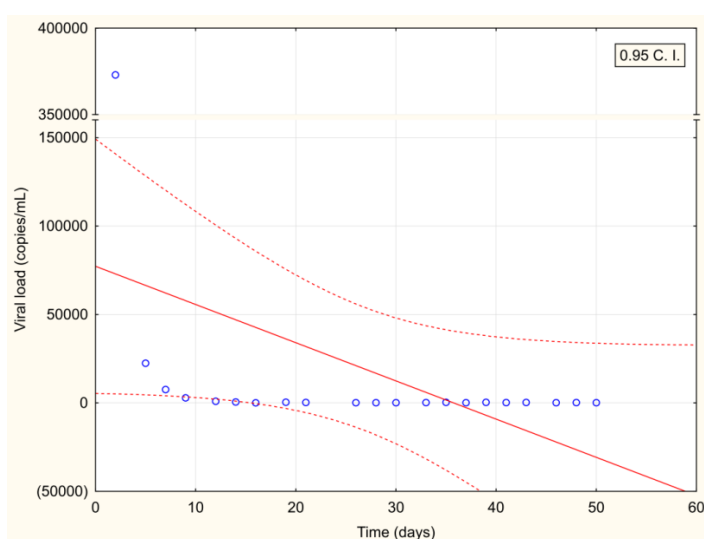

Figure 1. Viral load quantitation by real-time Polymerase Chain Reaction. Legend: Viral load in copies/mL. Results analyzed by statistic correlation ($p = 0.07$).

After thawing, the cells were reseeded in a cell culture flask and the infection was maintained for another 7 days, with detectable viral load by quantitative PCR.

To determine the virus surface antigen presence (HBsAg), the qualitative chemiluminescent immunoassay was performed. The result indicated a ratio S/CO = 3.74, confirming the viral infection on the cell culture supernatant after passages and washes. This antigen may be the only indicator of asymptomatic carriers in persons with chronic hepatitis B.

The result obtained by flow cytometry, showed in Figure 2, indicated 11.85% of positive cells to intracellular HBcAg (with anti-HBc and anti-IgG-FITC+), calculated from the subtraction of the cells that presented unspecific reaction, in the group of cells exclusively incubated in anti-IgG-FITC (Figure 2A), together with the group that was incubated in serum not reactive with anti-HBc (Figure 2B), from the cells anti-HBc positive, incubated in serum reactive with this antibody (Figure 2C).
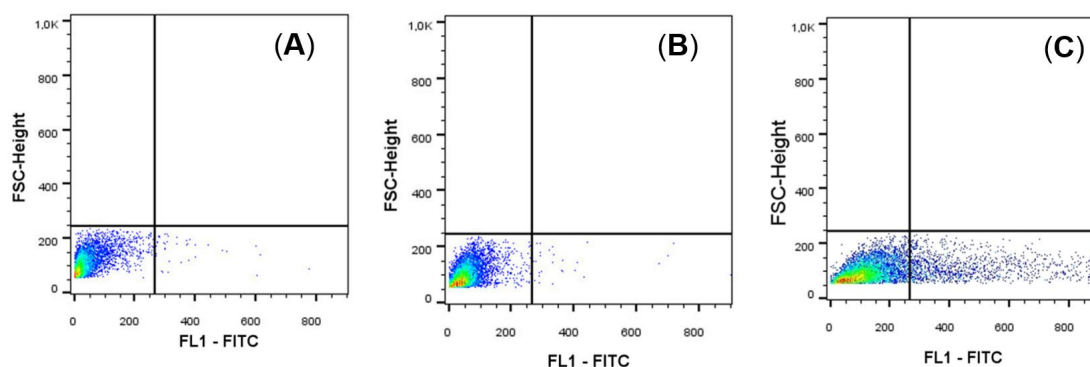
Figure 2. 10,000 events analyzed by flow cytometry.

Legend: (**A**) cells were incubated in anti-IgG-FITC (**B**) cells were incubated in serum not reactive with anti-HBc, and anti-IgG-FITC; (**C**) cells were incubated in serum reactive with anti-HBc, and anti-IgG-FITC.

The indirect immunofluorescence by epiluminescence microscopy (Figure 3) allowed the detection of the viral antigen inside the infected cells, using specific antibodies to the intracytoplasmic core protein, confirming the results obtained by flow cytometry. The fluorescent marker FITC, when excited at 490 nm, produces the green dye observed in Figure 3B, composed by HBV infected cells incubated in serum reactive with anti-HBc; this antibody, in its turn, marked with anti-IgG-FITC. In the cells incubated in serum not reactive with anti-HBc (Figure 3A), the characteristic emission of green fluorescence cannot be observed.
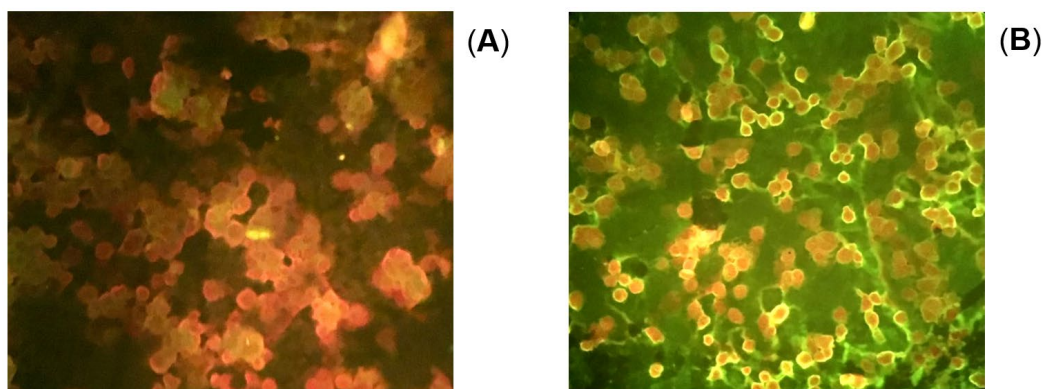
Figure 3. Indirect immunofluorescence by epiluminescence microscopy of the Huh-7.5 cells infected by hepatitis B virus. Legend: (A) cells were incubated in serum not reactive with anti-HBc, and anti-IgG-FITC; (B) cells were incubated in serum reactive with anti-HBc, and anti-IgG-FITC.

DISCUSSION

This research aimed at studying techniques to develop a cellular infection system with HBV, experimentally substantiated by different methods, using Huh-7.5 cells, which has already been shown to support HCV efficiently (Omura et al., 2019). Viral load remained detectable even after several passages, washes, freezing and reseeding procedures. Therefore, the proposed system remained stable regarding viral infection, in diverse conditions, *in vitro*. According to the literature (Shah & Singh, 2007), the use of HepG2 cells to study HBV could not be maintained through an extensive period (about 10 days), and viral DNA was detected for 7 days only. The cell lineage HB 611, of fetal hepatocytes, likewise, is not susceptible to HBV after the 10th day of infection (Ochiya et al., 1989; Bchini R, et al.,1990; Michailidis et al., 2017).

According to Tsukiyama-Kohara & Kohara (2014), there are many difficulties associated with the maintenance of an animal model, *in vivo*, like the *Tupaia belangeri,* which justifies the better applicability of immortalized cells, derived from hepatocellular carcinoma. In that case, it is also necessary to dialyze and dilute the serum of patients before the infection of *tupaias* hepatocytes (Walter et al., 1996).

Huh-7.5 cells are frequently used for *in vitro* assays with HCV (Dang SS, et al., 2019 ; Michailidis et al., 2017 ; Tsukiyama-Kohara & Kohara, 2014; Walter et al., 1996, Shirasago Y et al., 2015; Zhong J et al., 2006), presented susceptibility to HBV infection, for a 50-day period of analysis. Even with a dramatic decrease in the viral load, approximately after the 10th-day post culture infection, the viral DNA remained detectable during the whole observed period. The culture medium was changed every other day, the supernatant removed, not accumulating. Moreover, the number of cells suffered variations between measurements, due to the need to detach cells with trypsin to maintain the culture, and so, decrease the total population to allow more space in the flask, to stimulate cell growth. It is also important to highlight that not all the viral particles are infective, in other words, the virus cannot infect all the Huh-7.5 cells. That is the main reason for the percentage result of the flow cytometry assay, which evaluated the core protein HBcAg that constitutes the internal envelope, where viral DNA and polymerase enzyme, responsible for viral replication in already infected cells, are contained. This test detected the core protein in 11.85% of the infected cells, after several passages and washes, which means that approximately 10 to 20% of the cells at this stage were compatible to internalize the virus or to express viral genes.

The same method of marking HBcAg with anti-HBc and human anti-IgG conjugated to FITC was employed to observe this antigen in Huh-7.5 cells by indirect immunofluorescence using epiluminescence microscopy, with positive results observed on the cells incubated in serum reactive with anti-HBc. This antigen is solely detectable when the virus is replicating since it is needed for the assembly phase of new viral particles. The presence of anti-HBc antibodies in HBs-Ag negative patients can be suggestive of a resolved infection, or low-grade chronic infection (classified as occult infection), passive transference, the immune response of the co-infected one with HIV host, or non-specific antibody of cross-reactivity (Coppola et al., 2016; Gessoni et al., 2014; Lopes & Schinoni, 2011).

The study of the presence of the surface antigen HBsAg allowed the confirmation of the viral infection. HBsAg corresponds to the external viral coating. Situations where this antigen is positive and anti-HBc is negative in the blood may indicate the final phase of the incubation period, or it can be related to immunocompromised patients (Coppola et al., 2016; Gessoni et al., 2014; Lopes & Schinoni, 2011).

CONCLUSION

The proposed platform produced an expressive quantity of viral particles during the period of investigation, as demonstrated by different methodologies. Therefore, the *in vitro* infection system using Huh-7.5 cells has potential for a research to be carried out on the molecular mechanisms related to the disease and viral replication control studies.

Additional research must be carried out in order to improve both the knowledge regarding the virus genotype used in the infection and the details of *in vitro* replication, and also to refine and better understand the platform.

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