Identification And Evaluation Of Patient Serum Isolates As A Source Of Inoculum For 3D In Vitro Cell Culture: A Tool For Drug Discovery

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Introduction

Hepatitis B Virus (HBV) is currently a leading healthcare problem affecting more than 240 million people worldwide, despite the availability of a highly effective vaccine [1, 2]. Drug therapies are available on the market for the treatment of HBV, but these rarely provide a cure. The development of novel compounds is hampered by the lack of easily accessible animal models and sophisticated in vitro tools that recapitulate the full viral life cycle [3]. Patient isolates of HBV are infrequently used in in vitro HBV cultures, but could prove useful for identifying novel therapies for a cure of HBV.

Here we aimed to identify HBV positive patient isolates capable of infecting primary human hepatocytes in a 3D microfluidic device, and compared this with an infection launched from HepG2.2.15 derived virus. The same cell lots were infected with infectious inoculum and cultured for 14 days.

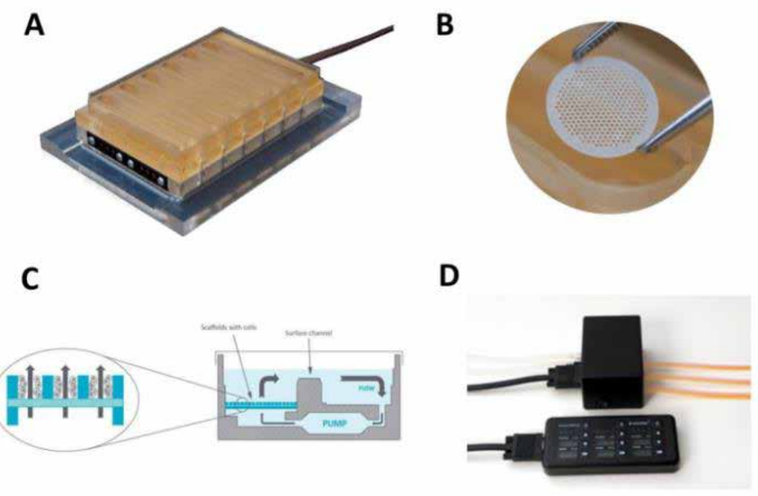


Figure 1: LiverChip® Hardware

A. LiverChip® is a 12 well perfused cell culture system.
B. Hepatocytes form three dimensional tissue structures in an array of channels through a collagen I-coated scaffold.
C. Media flows through the channels due to the action of a pneumatically operated pumping mechanism.
D. The speed and direction of flow can be adjusted using an electronic controller.

Methods

Cryopreserved primary human hepatocytes (PHH) were obtained from Life Technologies (USA) or QPS Hepatic Biosciences (USA). 0.6 x 10^6 hepatocytes were seeded into each LiverChip® well in Williams' E medium containing supplements. Infection was launched using commercially available HBV positive serum (SeraLab and Quest Biomedical) or HBV genotype D derived from HepG2.2.15 cell culture supernatant concentrated via PEG precipitation. Whole HBV genome sequences were obtained by ultra-deep sequencing. Cultures were infected on Day 0 in the presence of 4% polyethylene glycol 8000 (PEG) at various multiplicity of infection (MOI) expressed as genome equivalents per cell. Complete media changes were performed every 2 – 3 days, unless the experimental protocol dictated otherwise.

Assays

HBV surface antigen (HBsAg) was measured using a CLIA according to the manufacturer's instructions (AutoBio Diagnostics, China). For HBV DNA, total DNA was extracted from cell culture medium using QIAamp MinElute Virus Spin Kit along with a standard curve generated with Acrometrix HBV panel. qPCR was run with TaqMan reagent and specific probes and primers.

Total RNA was extracted using the RNeasy mini kit (Qiagen). RNA was converted to cDNA using the high capacity cDNA synthesis kit (Life Technologies). Gene expression profiles were quantified in equivalent cDNA samples using either TaqMan specific assays or SYBR reagents and specific probes and primers for total HBV RNA and pre-genomic (pg) RNA on an ABI QuantStudio 6 real time PCR system. Cells were stained with 50 µg/ml Phalloidin-TRITC, counterstained with Hoechst and imaged on an Upright Zeiss LSM 510 confocal microscope.

Results 1 – Hepatocyte Microtissues in LiverChip®

PHH cultured in 3D (in the LiverChip® platform) for 14 days form microtissues, demonstrating polarity of cells and the presence of bile canaliculi (Fig. 2). This has been identified as essential for effective HBV infection and particularly the presence of the hepatic basolateral sodium-dependent-bile transporter, sodium taurocholate co-transporting polypeptide (NTCP) [4]. PHH cultured in LiverChip® have been shown to express higher levels of NTCP than those cultured in 2D (data previously presented).

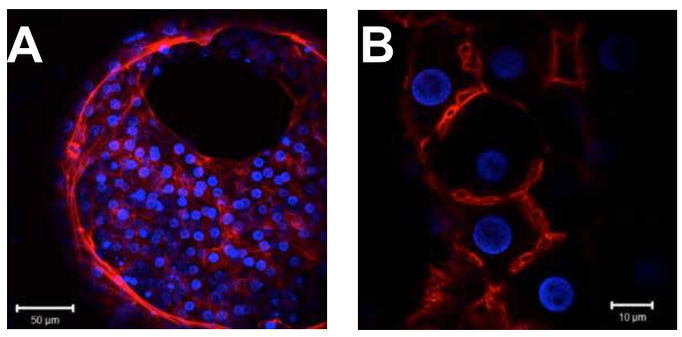


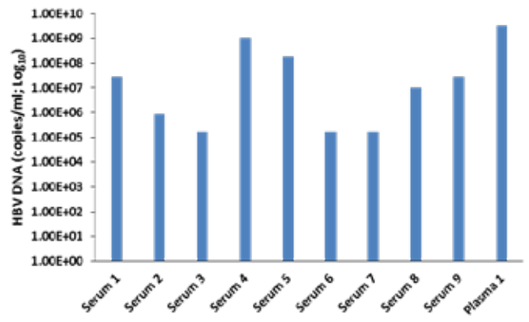
Figure 2: Hepatocytes cultured in 3D form liver microarchitecture

PHH were cultured in 3D and imaged by confocal microscopy; (A, B) stained for actin (red) and nuclei (blue) to show polarised cells..

Results 2 – HBV Infection in LiverChip®

HBV DNA levels were assessed from 10 human HBV positive serum and plasma samples identifying varying levels of HBV DNA (Fig. 3A). Using these 10 patient samples, infections were launched with an addition of 10 % serum or plasma resulting in a range of HBsAg levels at Day 8 or 9 in culture (Fig. 3B). Data from further experiments with Serum 5, demonstrates greater cumulative HBsAg over 7 days than that measured in the inoculum indicating the presence of a productive infection (Fig. 3C). Interestingly, a positive infection with patient inoculum was not correlated with HBV DNA levels in patient serum. Both Serum 4 and Plasma 1 had higher HBV DNA levels than Serum 5, but did not yield a positive infection.

A



B

Sample ID	HBV DNA (copies/ml)	HBsAg (IU/ml)
Serum 1	2.72E+07	0.00
Serum 2	8.57E+05	0.00
Serum 3	1.67E+05	0.00
Serum 4	1.08E+09	0.97
Serum 5	1.71E+08	1.90
Serum 6	1.58E+05	0.00
Serum 7	1.69E+05	0.00
Serum 8	9.79E+06	8.10
Serum 9	2.81E+07	2.34
Plasma 1	3.24E+09	1.59

C

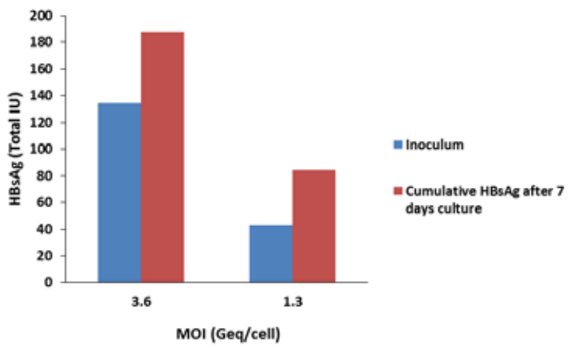


Figure 3: Hepatocytes infected with HBV positive human serum in LiverChip® produce a productive infection (A) HBV DNA was measured in multiple patient serum and plasma samples; (B) HBsAg measured at Day 8 or 9 in culture from infections launched with 10 % patient serum or plasma (C) Cumulative production of HBsAg launched with MOI 1.3 and 3.6, over 7 days were measured in cell culture medium at varying time points to determine HBV infection.

Results 3 – Human Serum

PHH were infected with HBV positive patient serum (Fig. 3A, Serum 5) at MOI 1.3 and 3.6 in LiverChip®. HBV DNA and HBsAg in the cell culture supernatant was measured over a 14 day period post infection (Fig. 4). Despite the presence of HBV DNA, replication intermediates (Fig. 5) and HBsAg, no HBeAg was measureable. No HBeAg mutations were found to be present, to account for the lack of antigen production.

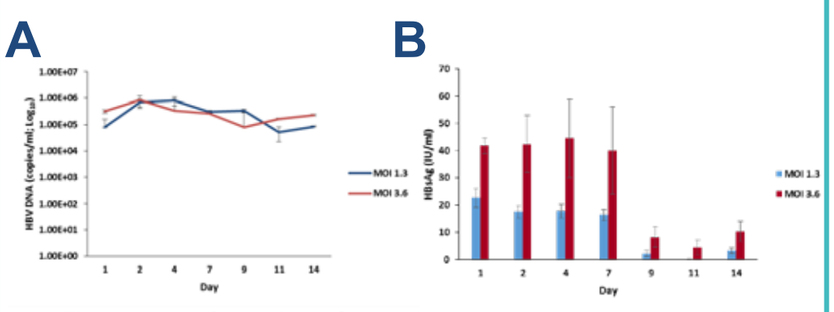


Figure 4: Infectivity of hepatocytes with patient derived virus

PHH were cultured in LiverChip® for 14 days and were infected with HBV (MOI: 1.3 and 3.6) (A) HBV DNA production in culture medium was measured by qPCR; (B) HBsAg production was measure in culture medium by ELISA.

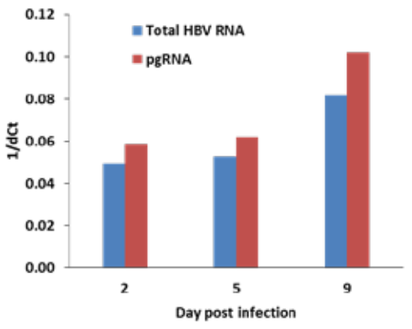


Figure 5: The expression of HBV replication intermediates in LiverChip®

Total RNA was extracted from PHH infected with patient inoculum at MOI 3.6. Gene expression analysis was performed using SYBR reagents and specific primers for pgRNA and total HBV RNA.

Results 4 – HepG2.2.15 derived virus

PHH were infected with concentrated HepG2.2.15 derived virus at an MOI of 80 in LiverChip®. HBV DNA, HBeAg and HBsAg in the cell culture supernatant was measured over a 14 day period post infection (Fig. 6).

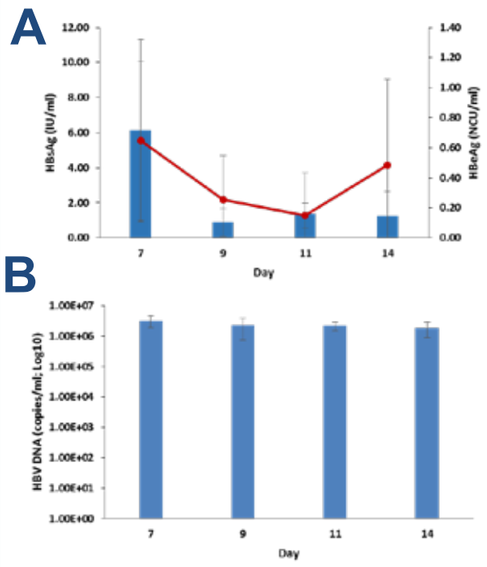


Figure 6: Infectivity of HepG2.2.15 derived virus in LiverChip®

PHH were cultured in LiverChip® for 14 days and were infected with HBV (MOI: 80) (A) HBsAg (Bar) and HBeAg (Line) production was measured in culture medium by ELISA. (B) HBV DNA production was measure in culture medium by qPCR.

Conclusions

- Phenotypic analysis of PHH cultured in LiverChip® indicate that microtissues should be susceptible to HBV infection.
- One patient inoculum was identified from 10 samples, as capable of producing a positive HBV infection in PHH cultured in LiverChip® platform.
- PHH infected with patient inoculum produced HBV DNA for up to 14 days as well as HBsAg. There was an absence of HBeAg production, despite the lack of relevant genetic mutations.
- HepG2.2.15 derived virus also produced a productive infection in LiverChip®, but at a lower level than demonstrated with patient inoculum. HBV DNA, HBsAg and HBeAg were present.
- Both patient inoculum and HepG2.2.15 derived virus produce positive HBV infection in LiverChip® microtissues, useful for preclinical testing of novel treatments for the disease.

References

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