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Research Article

A Sensitive and Rapid Assay for Investigating Vertical Transmission of Hepatitis B Virus via Male Germ Line Using EGFP Vector as Reporter

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Hepatitis B virus (HBV) constitutes a serious menace to man. DNA recombination and sequencing, interspecific in vitro fertilization, single-embryo PCR and RT-PCR were employed to establish a sensitive and rapid assay for exploring the vertical transmission of viruses via male germ line. Plasmid pIRES2-EGFP-HBs which expressed enhanced green fluorescent protein as reporter for the expression of hepatitis B virus *S* gene was successfully constructed and confirmed by PCR, EcoR I and Sal I digestion, and DNA sequencing. After exposure to the plasmid, human spermatozoa were used to fertilize with zona-free hamster ova. Two-cell embryos were collected and classified into group A with green fluorescence and group B without green fluorescence under fluorescence microscope. The results showed that HBs DNA positive bands were detected in the embryos with green fluorescence (PCR and RT-PCR) and positive control (PCR) indicating expression of pIRES2-EGFP-HBs, and not observed in the embryos without green fluorescence and negative controls (PCR and RT-PCR) indicating no pIRES2-EGFP-HBs in the cells. The advantages and application foreground of this assay for study on vertical transmission of viruses such as HCV, HIV, HPV, and SARS via germ line were discussed.

1. INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus, replicating almost exclusively in the liver; see [1]. Although effective recombinant vaccines are available, HBV infection is still a major global health problem and each year, acute and chronic HBV infection causes about 1 million deaths [2]. Hepatitis B is a worldwide public health problem. WHO estimates that approximately 350 million people are chronically infected with hepatitis B virus (HBV), and that the prevalence is more than 20% in certain highly endemic area of Africa and Asia (EPI NEWS, No. 25, 2002). Sequelae of HBV infection are severe. Up to 15% of carriers eventually develop primary hepatic carcinoma; the younger a patient is when they acquire chronic infection, the higher the risk of primary hepatocellular carcinoma. Premature mortality from chronic liver disease occurs in 15–25%. Therefore, studies on transmission of HBV are of substantial importance in virology as well as in public health.

The transmission routes of HBV through blood transfusion [3, 4]; body fluids including serum, saliva, vaginal secretions, breast milk, and semen [5, 6]; intrauterine infection [7, 8]; cell, tissue, and organ transplantation [9, 10] and others including hemodialysis units and intravenous drug injection [11, 12] have been documented. The known transmission of HBV does not constitute a menace to man because we are able to identify effective preventive measures. The unknown transmissions have greater risk potential because we do not know where they are when HBV attacks us. In 1985, Hadchouel et al. [13] confirmed the presence of integrated HBV DNA sequences in spermatozoa from two of three patients with HBV infection using the molecular hybridization. They assumed that the presence of integrated sequences in spermatozoa suggested the possibility of true vertical transmission of HBV via germ line. Nobody confirmed their assumption in more than ten years, because neither experimental animals nor cell culture systems had been available. Study on vertical transmission of HBV genes

using embryos produced from human oocytes fertilized with human spermatozoa carrying HBV genes would be an ideal model, but such a system presents major logistical, moral, ethical, and methodological problems. Thus, it is crucial to establish a proper assay for such study.

Spermatozoa of a wide variety of species such as mouse, guinea pig, and human can fuse with zona-free hamster oocytes. Interspecific in vitro fertilization has been widely used by investigators for studying on morphological and molecular details of sperm-egg interactions [14, 15]. Huang et al. employed this method for investigating transmission of HBV via male germ line and avoided the mentioned moral and ethical problems. They firstly provided the direct evidence that HBV DNA integrated into human sperm chromosomes and showed that human sperm carrying HBV genes can pass through oolemma to enter into the oocyte and complete fertilization normally [16]. Other investigators demonstrated that after fertilization the sperm-mediated HBV genes are able to replicate themselves and express their functions at mRNA and protein level in early embryonic cells [17–19]. But it was the sample-consuming, time-consuming, and energy-consuming to utilize the mentioned experimental model because not all sperm either from the semen of patients with HBV chronic infection or from the human spermatozoa exposed in vitro to the recombinant plasmid pBR322-HBV were integrated by HBV DNA. After fertilization it was difficult to see which embryo contained HBV genes delivered by human sperm. To obtain the mentioned results, many embryos as the test materials were needed and the experiment had to be repeated many times in the previous studies.

Enhanced green fluorescent protein (EGFP) as a good reporter has been widely used for biological and medical research. In the present study we constructed a recombinant plasmid pIRES2-EGFP-HBs containing HBs and EGFP genes, which enable us to distinguish between the embryos with andwithout HBs gene through observation of the presence of green fluorescence. The aim of this study was to provide a rapid and sensitive assay for exploring feasibility of vertical transmission of HBV and other viruses via the germ line.

2. MATERIALS AND METHODS

All experiments of the present work were performed under the approval of the Internal Review Board of Shantou University Medical College.

2.1. Materials

Biggers- *Whitten* -Whittingham (BWW) medium supplementedwith 0.3% bovine serum albumen (BSA, Sigma Chemicals Co., ST. Louis , MO) was prepared for human sperm preparation, oocyte collection, insemination and subsequent handling. Ovum culture medium (OCM, from Flow Laboratories, Germany) containing 10% heat-inactivated fetal bovine serum was used in the post-insemination culture.

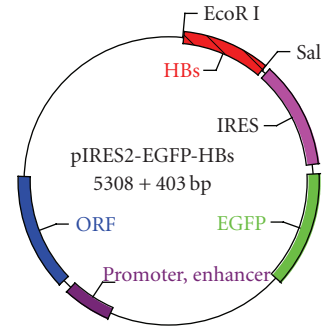


Figure 1: Diagram of constructed recombinant plasmid pIRES2-EGFP-HBs.

2.2. Methods

2.2.1. Construction of recombinant plasmid pIRES2-EGFP-HBs

The expression vector for HBs and EGFP was constructed as Figure 1.

The HBs gene was amplified (403 bp) from plasmid pBR322-HBV by PCR and subcloned to plasmid pIRES2-EGFP to construct recombinant plasmid pIRES2-EGFP-HBs.The PCR mixture consisted of 5 μL 10X Taq buffer, 4 μL of 2.5 mM of MgCL2, 200 μmol/L of each deoxynucleoside-triphosphate (Invitrogen, Guangzhou, China), 20 pmol of each primer with EcoR I and Sal I restriction sites (Takara Biotech, Japan) including forward: 5′-CGGAATTCTATC-GCTGGATGTGTCTGC-3′ and reverse: 5′-GGTCGACAG-ACTTGGCCCCCAATAC-3′, 2.5 U Taq polymerase and sterile ddH_2O in a final volume of 50 μL. The amplification program was as follows: denaturing at 94°C for 5 minutes, and 35 cycles each at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 45 seconds, followed by a final extension at 72°C for 10 minutes. The amplification products were routinely analyzed by staining with ethidium bromide, after electrophoresis on 1% agarose gel and then purified using DNA fragment purification kit (Takara, Beijing, China). The purified DNA was recombined into plasmid pIRES2-EGFP using T4 ligase kit (Takara) and subjected transformation into *Ecoli* DH5α according to manufacture instruction. The successful construction was confirmed by PCR, EcoR I, and Sal I digestion and DNA sequencing.

2.2.2. Preparations of human spermatozoa

Semen samples collected from the normal man were kept in a CO_2 incubator (37°C/50 mL/L CO_2 in air) for 30 minutes in order to be liquefied. Highly motile spermatozoa were recovered from the semen with swim-up method. The sperm suspension thereby obtained was centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in BWW with 0.3% BSA and centrifuged again. The washed spermatozoa were suspended in 5 mL of 10 μM ionophore solution for 10 minutes to facilitate the capacitation and washed twice,

and then suspended in BWW with 3.5% BSA to allow capacitation.

2.2.3. Exposure of spermatozoa to plasmid pIRES2-EGFP-HBs

Two and half hours after the beginning of capacitation, the spermatozoa was exposed to plasmid pIRES2-EGFP-HBs. Briefly, a total of 100 μL mixture containing 1 μL plasmid pIRES2-EGFP-HBs (1.5 μg/ml), 6 μL liposome, and 93 μL HEPES buffered saline was incubated at room temperature for 15 minutes, and then was added to sperm sample and kept in the incubator for another 1.5 hours. After that, the sperm sample was washed five times in 5 mL fresh BWW.

2.2.4. Preparation of golden hamster oocytes

The mature hamsters were induced to superovulate by intraperitoneal injection of 40 IU of pregnant mare serum gonadotropin (PMSG, Ningbo Hormone Product Co., Ltd., China) on day 1 of oestrous cycle followed by administration of 40 IU human chorionic gonadotrophin (hCG, Ningbo Hormone product Co., Ltd.) 72 hours later. Superovulated oocytes were collected from the ampullar region of oviducts 17 hours after hCG injection and freed from cumulus cells in 0.1% hyaluronidase (Sigma). Cumulus-free oocytes were washed twice in BWW andtreated with 0.1% trypsin (Sigma)to remove the zona pellucida, then washed twice immediately in BWW.

2.2.5. Insemination and postinsemination culture

Insemination was performed with the sperm suspensions at a concentration of 10^6/mL. The oocytes were kept in the sperm suspension for 20–30 minutes and then transferred and incubated in the sperm-free BWW under mineral oil (Sigma) for another 1 hour to ensure sperm penetration. After washing twice in OCM, the groups, containing five oocytes per group, were cultured in a droplet (60 μL each) of OCM under oil in a plastic Petri dish kept in a CO_2 incubator (37°C , 50 mL/L CO_2 in air) for 24 hours.

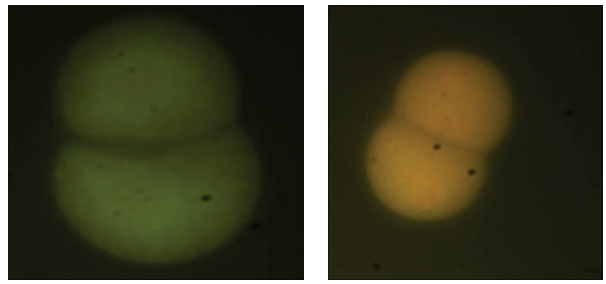
2.2.6. Preparations of embryos and grouping

Twenty four hours after insemination, two-cell embryos were collected and classified into two groups under fluorescence microscope (Axiovert 40 CFL, Zeizz , Germany) including group A with green fluorescence (Figure 2(a)) and group B without green fluorescence (Figure 2(b)).

The two-cell embryos from group A and B were washed three times in cold 1X PBS to remove serum from the medium, and then each embryo was transferred singly into a PCR tube for PCR and RT-PCR, respectively.

2.2.7. Single-embryo PCR

Approximately, 4.5 μL of cell lysis buffer were added to each single-embryo of group A and B, mixed thenincubated at 70°C for 10 minutes. The cell lysate of embryo of group A and B was used as a DNA template, *and* pIRES2-EGFP-HBs and sterile ddH_2O as the positive and negative controls, respectively. All procedures of single-embryo PCR were the same as mentioned in Section 2.2.1.



(a) (b)

Figure 2: Two-cell embryo with green fluorescence (a) and two-cell embryo without green fluorescence (b) 24 hours after insemination (arrows). 400x.

2.2.8. Single-embryo RT-PCR

RNAwas extracted from each single-embryo of groups A and B. Single-embryo RT-PCR was performed using the cells-cDNA II kit. Briefly, 49.5 μL ice-cold cell lysis II buffer was added to each single-embryo sample and the mixture was incubated at 75°C for 10 minutes. Approximately, 0.12 U DNase I was added to the mixture which then incubated at 37°C for 30 minutes. To deactivate DNase, the samples were incubated at 75°C for 5 minutes. Reverse transcription was preformed according to the kit protocol. PCR amplification was carried out with 5 μL cDNA of each sample from reverse transcription reaction as a template, HBs specific primer pair mentioned above, and two negative controls (minus template and minus reverse transcription). The amplification step was carried out using the same conditions of normal PCR to amplify HBs gene as mentioned above. About 20 μL of each RT-PCR product were made visible by staining with EB after electrophoresis on 1.2% agarose gel. This experiment was repeated three times under the same condition.

3. RESULTS

3.1. Construction of recombinant plasmid pIRES2-EGFP-HBs

Identification of recombinant plasmid pIRES2-EGFP-HBs was shown as Figure 3. The successful construction was confirmed by PCR, EcoR I, and Sal I digestion, DNA sequencing, and BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

3.2. The rate of two-cell embryos

A total of 350 zona-free golden hamster oocytes were fertilized with human spermatozoa in this study. 201 embryos were obtained including 105 one-cell and 96 two-cell embryos (Figures 4(a) and 4(b)). The fertilization rate was 57.42% (201/350). About 47.76% (96/201) fertilized eggs

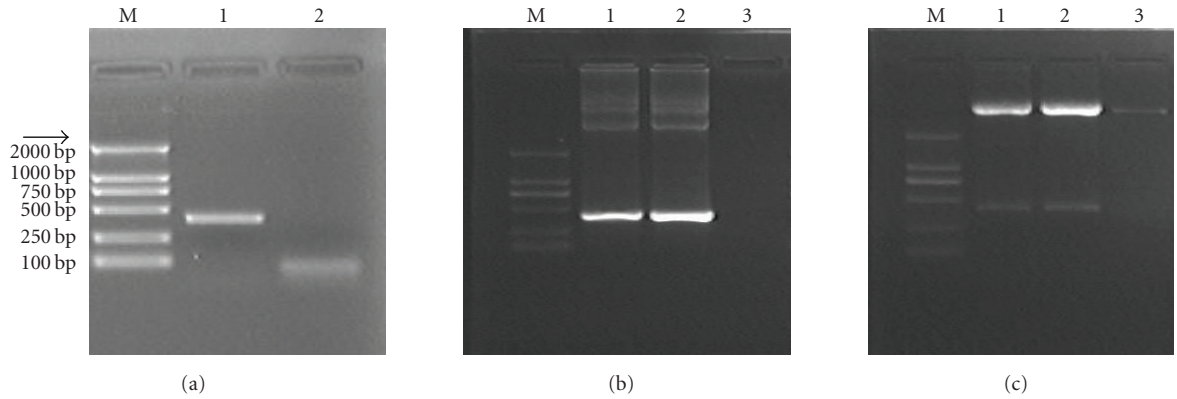


Figure 3: Identification of the recombinant plasmid pIRES2-EGFP-HBs. (a) PCR result of HBs gene before transformation. M: Markers; 1: HBs gene; 2: negative control. (b) PCR result of HBs gene after transformation. M: Markers; 1 and 2: HBs gene; 3: negative control after transformation. (c) Result of enzyme digestion. M: Markers; 1 and 2: pRIS2-EGFP-HBs digested using EcoiR I and Sal I after transformation; 3: pRIS2-EGFP empty as native control.

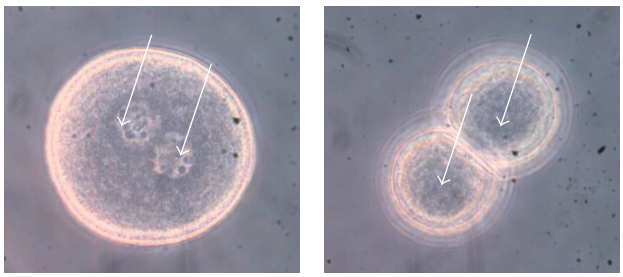


Figure 4: One-cell embryo showing a male and a female pronuclei (a), and two-cell embryo one nucleus in each, (b) 24 hours after insemination (arrows). 400 X, under phase contrast microscope.

could develop into two-cell stage of embryonic development. 33 of them (34.37%) showed green fluorescence.

3.3. Single-embryo PCR results

Single-embryo PCR results are shown in Figure 5. The HBs DNA positive bands (403 bp) were detected in three samples from single embryos with green fluorescence and the positive controls, and not detected in three samples from single embryos without green fluorescence and the negative control. All amplification products were found to be reproducible when reactions repeated three times using the same reaction conditions.

3.4. Single-embryo RT-PCR results

Single-embryo RT-PCR results are shown in Figure 6. The HBs cDNA positive bands (403 bp) were detected in three samples from single embryos with green fluorescence, and not detected in three samples from single embryos without green fluorescence and the negative controls (−RT and −T). All amplification products were found to be reproducible when reactions repeated three times using the same reaction conditions.

4. DISCUSSION

In the present study, the sensitive and rapid assay was established to explore the feasibility of virus vertical transmission via male germ line based on the work of many scientists. It consisted of the following procedures: the construction of recombinant plasmid pIRES2-EGFP-HBs, the exposure of human sperm to virus DNA, interspecific in vitro fertilization between human sperm and zona-free golden hamster ova, identifiable embryos with sperm-mediated virus genes, single-embryo PCR, and single-embryo RT-PCR.

We successfully constructed the recombinant plasmid pIRES2-EGFP-HBs (Figure 1), which was confirmed by PCR, EcoR I and Sal I digestion, DNA sequencing, and BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) (Figure 3). This plasmid contains HBs and EGFP genes, which expressed EGFP as reporter for HBs gene expression. It enables us to distinguish between the embryos with and without HBs gene through observation of the presence of green fluorescence (Figure 2), and to demonstrate the existence and expression of human sperm-mediated HBs genes in host embryonic cells (Figures 5 and 6). Interspecific in vitro fertilization between human sperm and zona-free golden hamster ova established by Yanagimachi et al. [20] was widely used in the research on reproductive biology. We extended its application to the area of virus transmitting research and introduced it into the present assay.

In theory, the existence and expression of HBs gene should be detected in two-cell embryos with green fluorescence and not detected in those without green fluorescence. The results of single-embryo PCR and single-embryo RT-PCR confirmed our assumption. HBs DNA positive bands were detected in the embryos with green fluorescence (PCR and RT-PCR) and positive control (PCR) and not observed in the embryos without green fluorescence and the negative controls (PCR and RT-PCR) (Figures 5 and 6). The same

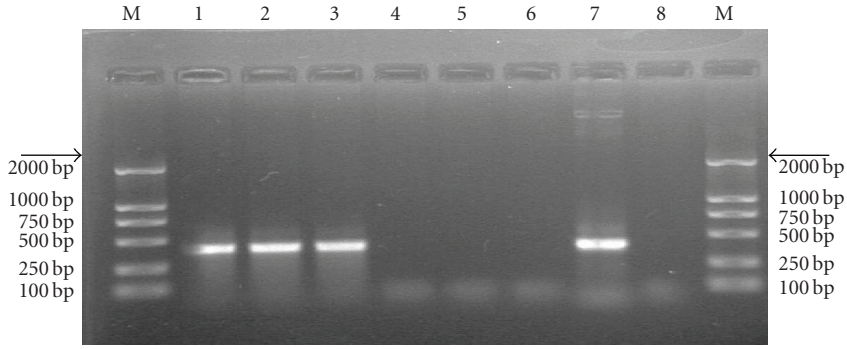


Figure 5: PCR product of HBs gene: M: Markers; 1, 2, and 3: the samples from three single embryos with green fluorescence showing HBs DNA positive bands, respectively; 4, 5, and 6: the samples from three single embryos without green fluorescence showing negative results for HBs DNA; 7: the positive control; 8: the negative control.

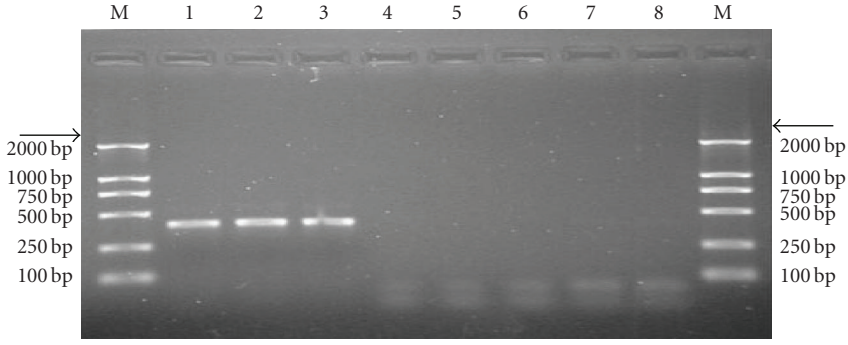


Figure 6: RT-PCR product of HBs gene: M: Markers; 1, 2, and 3: the samples from three single embryos with green fluorescence showing HBs cDNA positive bands, respectively; 4, 5, and 6: the samples from three single embryos without green fluorescence showing negative results for HBs DNA; 7: the negative control (−RT); 8: the negative control (−T).

results were obtained in the repeated experiments which demonstrated the exactness and efficiency of our assay as well as the reliability of our previous research results, that is, the sperm-mediated HBV genes are able to express their functions in host early embryonic cells [16–19, 21].

Zona pellucida is a glycoprotein coat surrounding the egg proper. It protects the fragile eggs and embryos from physical damage [22]. We use zona-free oocytes in the current experimental assay because our previous studies have confirmed that the zona pellucida could not become a barrier to the entry of virus DNA sequences into the oocytes and virus DNA fragments were able to enter into oocytes and integrate into their chromosomes either in vitro or in vivo experiments [16–19].

The present assay has the following advantages. (1) The researchers can exactly know which embryo contains human sperm-mediated virus genes through observation of green fluorescence and avoid the blindness occurred in the previous experiments. The results which were obtained by performing many experiments in the previous studies could be gained just in one experiment now. Thus, it saves much time and energy. (2) The test sample needed a batch of embryos in the previous studies and now a single embryo already meets the needs of experiments in the present study. It not only economized the experimental materials but also made experimental results more exact and reliable. (3) The present assay would make it easy to explore vertical transmission of other viruses (X) via human spermatozoa if recombinant plasmid pIRES2-EGFP-HBs was replaced by pIRES2-EGFP-X. (4) The present assay also avoided the moral, ethical, and methodological problems which would present when the vertical transmission of sperm-mediated virus genes were studied using embryos from fertilization between human oocytes and spermatozoa.

With progresses of virology, epidemiology, molecular biology, and genetics, more and more viruses have been found to be associated with human health. The studies on the transmission of viruses are of substantial importance in virology as well as in public health. Our study provided a very useful assay for study on the exact vertical transmission of viruses via the germ line. It might open a wide area for basic and clinical research because severe threats to humanity are posed by many viruses, such as HCV, HIV, SARS, and the like.

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