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Research Article

Epidemiology of Swine Hepatitis E Virus in Guangxi, China and Construction of Eukaryotic Expression Plasmid with ORF2 Gene for possible Vaccine

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Abstract

Hepatitis E (HE) is a zoonotic disease that can be transmitted through food or water contaminated from swine with HE virus (HEV) as an important natural reservoir host. This paper presents emperical data of epidemiological testing conducted at 14 cities in Guangxi, China, where random samples of 1387 serum samples were collected from the swine population. ELISA test for total antibodies of HEV showed that 1107 were positive in swine serum samples, with an average positive rate of 79.81%. In order to develop swine hepatitis E DNA vaccine, specific primers of immunogenic specific ORF2 gene targets for LB1, LB2, LB3 were designed and amplified using PCR assays. DNA amplicons were then directly cloned into T-A plasmid and expressed using pEASY-M1 eukaryotic expression vector system. The expression plasmids of pEASY-LB1, pEASY-LB2, pEASY-LB3 were transfected into 293T cells. These three target genes were detected by real-time fluorescent quantitative RT-PCR, indicating expression of eukaryotic ORF2 gene targets for LB1, LB2, LB3 in 293T cells. Analysis by SDS-PAGE and Western-blot indicated that expressed target proteins from 293T cells were transfected by eukaryotic expression plasmids. Further, antigenicity studies indicated corresponding results of HEV responses (data not shown). Therefore, three recombinants DNA of HEV ORF2 can be used for vaccine candidates. Further studies will be followed in vivo challenge and protection experiments in swine against HEV infection.

Keywords: Swine Hepatitis E Virus; ORF2; Eukaryotic Expression Plasmid; Real-Time Fluorescent Quantitative RT-PCR

Introduction

Hepatitis E (HE) featured with sporadic and epidemic waterborne outbreaks, primarily transmitted through fecal-oral route, is a new and important zoonotic disease worldwide [1]. It is a threat to people's health and animal industry, also showing a rising incidence in the developed and developing countries [2]. HEV can be transmitted through food or water contaminated with HEV. Swine are the important natural host and infection source of HE in people, primarily due to the close contact between human and swine population. The infection rate of swine have been investigated and found to be the highest among all the tested animals [3]. Recently 1387 sera samples were tested for anti-HEV IgG of swine from 14 cities in Guangxi, and results showed that 1107 serum samples were positive, with a positive rate of 79.81% [4,5].

HEV is a non-enveloped single stranded positive-sense RNA virus, classified into genotypes I, II, III, and IV, with genotypes I and II only infectious to humans while genotypes III and IV infectious to humans and various animals [6]. Although there are different isolated HEV strains, HEV has only one serotype [7]. The genome of HEV is approximately 7.3kb in length, containing 3 discontinuous and partially overlapping open reading frames (ORF), including ORF1, ORF2 and ORF3 [8]. The capsid protein (pORF2) which is encoded by ORF2 is a glycosylated protein containing 660 amino ac-

ids with at least 7 linear epitopes, and has multiple important immunological epitopes at its C-terminal end [9]. Although recent advances in the culturing have occurred [10,11], HEV is still difficult to grow in cell cultures ,which hamper the development of inactivated vaccine and attenuated vaccine. Currently, the key direction of HEV vaccine research is the development of genetic engineering vaccine, with a major concentration on ORF2. Different regions of ORF2 have been studied to select antigenic fragments with high immunogenicity, which is of a great significance for the development of HEV genetic engineering vaccine. Three continuous gene fragments B1, B2 and B3 of HEV ORF2 had been amplified from fresh swine feces and constructed prokaryotic expression plasmids pET32a-B1, pET32a-B2 and pET32a-B3, respectively [12]. Tests showed that all of these three recombinant plasmids could effectively express fusion protein with a good HEV antigenicity. However, these plasmids expressed lower proteins. Therefore, we constructed three recombinant eukaryotic expression plasmids pEASY-LB1, pEASY-LB2 and pEASY-LB3 shown to be more efficient in expressing proteins. We were able to identify high cell expression and HEV antigenicity, and consequently were able to obtained nucleic acid vaccine candidates of Swine Hepatitis E.

Materials and Methods

Samples

We conducted a seroepidemiologic study of HEV infection from 2008 to 2012 in Guangxi, China. We collected 1387 serum samples randomly from14 cities in Guangxi. HEV used for the study were from swine feces, DNA fragments were amplified as described previously [12].

Detection of antibodies to HEV in serum

Serum samples were tested for total antibodies of HEV by using WANTAI HEV-Ab ELISA kit (Wan Tai Pharmaceutical Co.,Beijing, China).

Construction of Recombinant Eukaryotic Expression Plasmids

Primers and Conventional PCR Amplification of Target Fragments

Utilizing ORF2 gene of HEV genome sequences published on GenBank , three pairs of primers for amplifying B1, B2 and B3 fragments were designed. All the 5' ends of the forward primers[B1-F:5'-gccaagcttgccatggaatatattcatccaaccaatccct-3';B2-F:5'-gccaagcttgccatggaacctagtgaacgcctgcattaccg-3'; B3-F: 5'- gccaagcttgccatggtagtcatccaggattatgacaatc -3'] contained *Hind*III restriction enzyme cutting site (underlined parts), start codon atg and Kozak sequence (g/a)nnatgg which can enhance the expression of target protein in eukaryotic cells. All the 5' ends of the reverse primers [B1-R: 5'- gcggatccttaaatcaccagctcagaggctatag -3'; B2-R: 5'- gcggatccttatcggactcaccaaggtcaatat -3';B3-R: 5'- gcggatccttactcagggcagaaatcatcgaaag -3']contained *Bam*HI restriction enzyme cutting site (underlined parts) and the reverse complementary stop codon tta. PCR products of LB1, LB2 and LB3 were 605bp, 602bp and 602bp in length respectively. The 50μl PCR reaction system was used for amplification of fragments LB1, LB2 and LB3, their compositions are as follows: 10×Taq Buffer (includes 20 mmol/l $MgCl_2$) 5 μl, dNTPs (10 mmol/l) 1μl, forward primers (B1-F/B2-F/B3-F) (25 μmol/l) 1μl, reverse primers (B1-R/B2-R/B3-R) (25 μmol/l) 1μl, Taq DNA Polymerase (5 U/μl) 0.5μl, DNA template (plasmids pET32a-B1 / pET32a-B2 /pET32a-B3) 1μl, ddH2O 40.5μl. The parameters for the PCR included a denaturation step at 95°C for 7min, followed by 35 cycles of denaturation for 50 sec at 94°C, annealing for 50 sec at 55°C, extension for 50 sec at 72°C and a final incubation at 72°C for 10min.

TA Clone

The PCR products were analysed in a 12 g/l agarose gel. The DNA band specific for the swine HEV was cut from the gel, purified with Gel Extraction kit (Tiangen, China).The purified PCR products of LB1, LB2 and LB3 were cloned in TA connected with the eukaryotic pEASY-M1 expression vector, and then the ligated products were transferred into Trans1-T1 Phage resistant chemically competent cells using pEASY-M1 Expression Kit (TransGen Biotech, China). Using PCR forward primers and general reverse primers of pEASY-M1 vectors, PCR was performed to select positive plasmids with the correct orientation.

Three positive plasmids were extracted using UNIQ-10 plasmid extraction kit (Sangon, China) and were identifed using double digestion with *Hind*III and *Bam*HI. Three positive plasmids named pEASY-LB1, pEASY-LB2 and pEASY-LB3 respectively were sequenced in a DNA sequence analyzer (ABI-PRISM3730 DNA analyzer, Invitrogen, USA).

Transfection of Recombinant Eukaryotic Expression Plasmids into 293T Cells

Plasmids DNA of pEASY-LB1, pEASY-LB2 and pEASY-LB3 in 50ul of Opti-MEM in reduced serum medium, mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount in 50μl of Opti-MEM Medium(Gibco, USA). Incubated for 5 minutes at room temperature,and combined the diluted DNA with the diluted Lipofectamine™ 2000 (Invitrogen, USA), AdMixture containing 100μl of complexes with plasmid concentration of 4 ug/ml to Human Embryonic Kidney Epithelial 293T Cells which were grown in 24-well plates to a conflu-

ent of 90%. Solutions were mixed gently by rocking the plate back and forth. Mixture containg cells were incubatedat 37°C in a CO_2 incubator for 48 hours prior to testing for transgene expression. The medium was changed by cell growth medium (MEM medium with 10% FBS) after every 4 hours.

Extraction and Reverse Transcription of mRNA

Total RNA was extracted from 293T Cells by using Trizol reagent (Invitrogen,USA)according the manufacturer's protocol. DNase I (RNase-free, Promega, USA) was used to remove the genomic DNA in RNA extracts. Diluted 1μl of RNA samples in 50ul of RNase-free H_2O, and the OD values were detected for RNA purity analysis by BioPhotometer plus (Eppendorf ,German). RNA samples were analysed in 1% agarose gel. Total RNA 1.0μg and ddH_2O 12μl were added in Rnase-free PCR tubes for RNA degeneration at 85°C for 5min, after then immediately cooled by ice to prevent RNA re-naturation. Reagents were add immediately into the tube as follow; Oligo (dT)(25 mmol/l)0.5μl, Random primer (25 μmol/l)0.5 μl, dNTPs (10 mmol/l) 2μl, RNase inhibitor (40 U/μl) 0.5 μl, 5 × buffer 4μl, M-MLV (200 U/μl) 0.5μl. 20ul reaction mixture was incubated at 30°C for 10min for priming, then at 42°C for 60min for reverse transcription, and finally at 85°C for 10min for reverse transcriptase inactivation. The complementary DNA (cDNA) was stored at −20°C until further use.

Real-Time Fluorescent Quantitative PCR Detection of Target Gene

Primers

According to the highly conserved segment of nucleotide sequence of three target genes of LB1, LB2 and LB3 and reference gene β-actin, four set primers [LB1 forward primer:5'- acatccccgcttacgtctac -3', LB1 reverse primer: 5'- ggcctcagttgccataatat -3'; LB2 forward primer: 5'- gcatagcattgaccctgttt -3', LB2 reverse primer: 5'- gggcgagagtagaacaactg -3'; LB3 forward primer: 5'- ggtcatcgtgtctgcatttc -3', LB3 reverse primer: 5'- gctagaacacccacagcagaa -3'; β-actin forward primer: 5'- tggatcagcaagcaggagta -3', β-actin reverse primer:5'- tcggccacattgtgaacttt -3'] were designed and synthesized (Sangon, China). SYBR Green I method was used for real-time fluorescent quantitative PCR. These three set of primers were designed to amplify 120 bp, 100 bp and 80 bp of ORF2, respectively.

Standard curves

The reverse transcription cDNA was used as the standard original concentrate, and the 1, 0.2, 0.04, 0.008, and 0.0016 fold concentrations of standards were obtained by 5 fold multiple proportion. The above mentioned standards were used as template for real-time PCR to obtain a standard curve. The 20μl reaction system was used for real-time PCR, with its compositions as follows; cDNA5 μl, forward primer (L1-F/ L2-F/ L3-F/β-F) (10 μmol/l) 0.5μl, reverse primer (L1-R/ L2-R/ L3-R/β-R) (10 μmol/l) 0.5μl, 2× SYBR Green qPCR SuperMix 10μl, dH_2O 4μl. The parameters for the real-time PCR as follows: at 50°C for 2min and then at 95°C for 2min, followed by 40 cycles of denaturation for 15sec at 95°C, annealing for 15sec at 60°C, extension for 30sec at 72 °C and a final incubation at 72°C for 10min.

$2^{-\Delta\Delta CT}$ relative quantitative method

On the basis of the Ct values resulted from the real-time PCR reaction, $2^{-\Delta\Delta CT}$ relative quantitative method was used to calculate the relative expression levels of target gene. Wherein, ΔCt = (target gene Ct - reference Ct) ± standard deviation, ΔΔCt = (ΔCt of target gene in transfected cells - ΔCt of gene in control cells) ± standard deviation, relative expression level = ($2^{-\Delta\Delta CT}$) ± standard deviation. Each sample was repeated three times.

SDS-PAGE and Western blot analysis

The transfected cells were lysed in lysis buffer, then the cell debris and the lysate was centrifuged for 5min, and the supernatant was taken for quantification of protein using BCA Protein Assay Kit (Keygen, China). The protein sample solution and the 5 × loading buffer were mixed at the ratio of 4:1 and boiled for 5min. SDS-PAGE was performed in the protein electrophoresis system BG-Power600i (BAYGENE, China) . After SDS-PAGE, protein bands were transferred onto a PVDF membrane (MILLIPORE, USA) and the membrane was treated with 5% nonfat milk overnight at room temperature. After washing three times (5min each time) with TBST, the membrane was incubated with the human anti-HEV (Wantai, China) serum diluted 1:500 with gentle agitation overnight at room temperature.After washing three times (5min each time) with TBST and incubated for 1h with diluted 1:1000 horse radish peroxidase(HRP)-conjugated goat Anti-human IgG (ZSGB-BIO,China). After washing three times with TBST (5min per time), and rinsing three times with distilled water (2min per time), the membrane was dipped in immobilon western chemilum HRP substrate (Millipore, USA) for color developing.

Results

Table 1 showed the occurrence of total antibodies to HEV in swine samples collected from different cities of Guangxi. ELISA test for total antibodies of HEV showed that 1107 were positive in swine serum samples, with an average positive rate of 79.81%,positive rate of HEV in all farm ranging 59.57-95.83%.

Table 1. Identification of serum antibodies to HEV in swine samples collected from different cities of Guangxi.

Region	No.samples	No.positive samples	Positive rate(%)
Nanning	342	309	90.35
Liuzhou	95	70	73.68
Guilin	70	60	85.71
Wuzhou	48	44	91.67
Beihai	45	32	71.11
Fangchenggang	69	54	78.26
Qinzhou	57	50	87.72
Guigang	132	102	77.27
Yulin	181	136	75.14
Baise	108	70	64.81
Hezhou	48	46	95.83
Hechi	89	62	69.66
Laibin	47	28	59.57
Chongzuo	56	44	78.57
Total	1387	1107	79.81

Conventional PCR Amplification for ORF2 gene

Three expected target fragments of LB1, LB2 and LB3 for HEV ORF2 gene were 605bp, 602bp and 602bp in length respectively as shown in Figure 1.

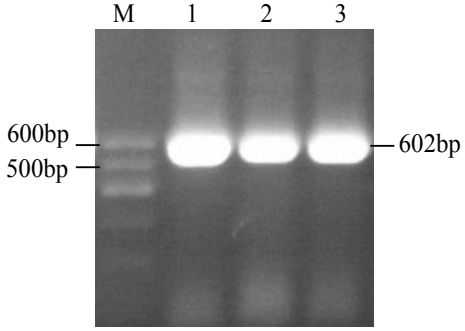


Figure 1. PCR amplification results of the HEV ORF2-LB1/LB2/LB3 fragment
M: 100 bp DNA Ladder Marker; 1: HEV ORF2-LB1; 2: HEV ORF2-LB2; 3: HEV ORF2-LB3

HEV ORF2 Recombinant Plasmids Digestion Identification

Double digestion with *Hind*III and *Bam*HI was performed on recombinant plasmids, and inserted target fragments respectively of 605 bp, 602 bp, and 602 bp could be seen, as well as fragments of the eukaryotic pEASY-M1 Expression Vector of 5437 bp. The sequencing results showed that all three continuous fragments were in line with expectations that the insertion direction was right and that there was no insertion or deletion among the sequences.

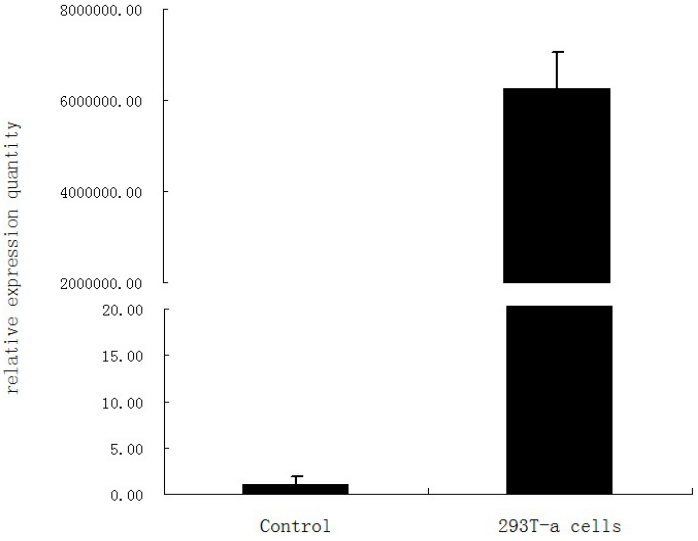
RNA purity analysis

Total RNA of the control 293T cells and those cells transfected by pEASY-LB1, pEASY-LB2 and pEASY-LB3 were respectively extracted, measured with OD values and calculated the ratio of OD260nm/OD280nm, results showed that four samples were all greater than 1.8, indicating that the extracted RNA was relatively pure with no protein contamination. Three complete rRNA bands of 28s rRNA, 18s rRNA 5s rRNA in each case, indicating that the extracted total RNA was relatively complete.

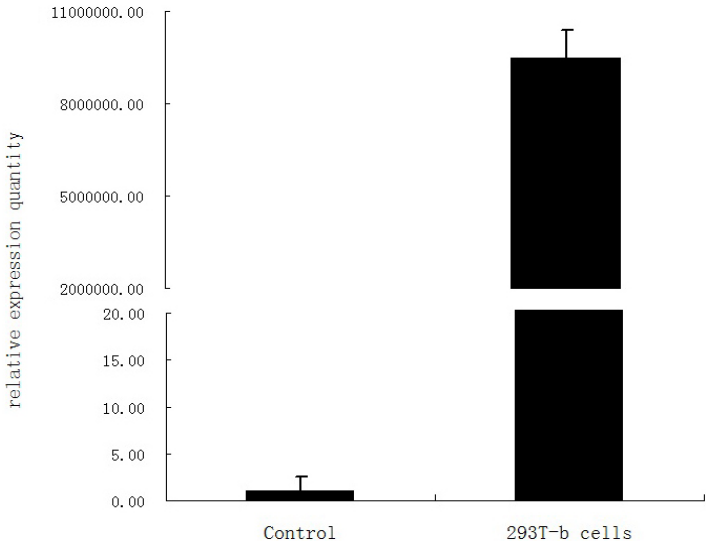
Expression level of target gene

The recombinant eukaryotic expression plasmids were respectively transfected into 293T, and a standard curve was established by real-time fluorescent quantitative PCR through detection of mRNA of target gene. Amplification efficiencies of target genes LB1, LB2 and LB3 and reference genes β-actin were 92.57%, 90.28%, 94.19% and 90.47%, respectively. Results of melting curve analysis showed that the melting curves of target and reference genes of the transfected cells and control cells were all unimodal with a single narrow and sharp peak, indicating good specificity of the amplified products. Gene expression profiles of target genes LB1, LB2 and LB3 in 293T-a, 293T-b and 293T-c and control cells were measured by $2^{-\Delta\Delta CT}$ relative quantitative method. The results (Figure 2) confirmed that those three recombinant eukaryotic expression plasmids had been successfully transfected in 293T cells.

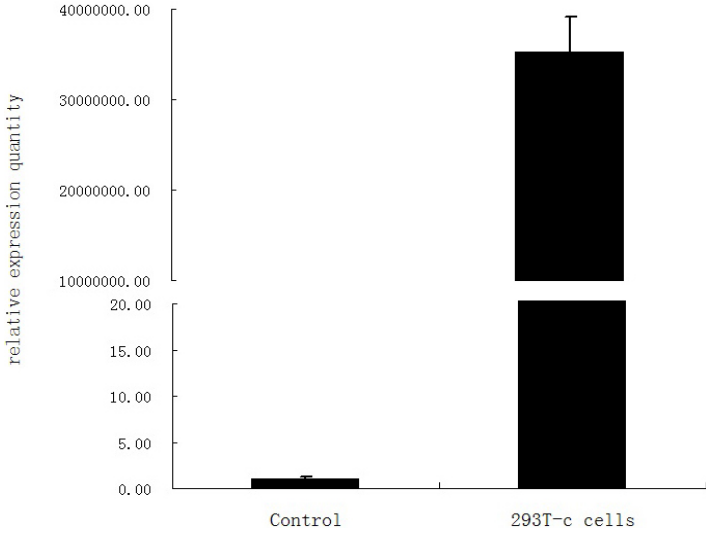
Figure 2. The expression level of target gene in recombinant eukaryotic expression plasmid transfected cells with the blank reference cells.



A: Target gene LB1 expression level map.



B: Target gene LB2 expression level map.



C: Target gene LB3 expression level map.

Protein identification

SDS-PAGE confirmed that target protein bands about 20.5 Ku were observed in all transfected 293T cells, while no such bands were observed in control 293T cells. Western-blot results (Figure 3) showed that there were target protein bands about 20.5 Ku in transfected 293T cells (293T-a, 293T-b and 293T-c), but not in control 293T cells, indicating that three candidate plasmids of pEASY-LB1, pEASY-LB2 and pEASY-LB3 all expressed HEV ORF2 proteins with biological activities.

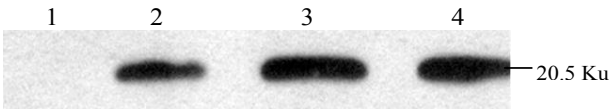


Figure 3. Western Blot identification results of transfected cells protein
1: 293T cells; 2: 293T-a cells; 3: 293T-b cells; 4: 293T-c cells

Discussion

[13-15] (1987;1990;1991) had researched on impacts on transcription and translation of site-directed mutagenesis of bases surrounding start codon atg and summarized the sequences at both ends of start codons in eukaryotes as: the efficiencies of transcription and translation were highest at (g/a) nnatgg, especially the g or a in positions -3 and g in positions +4 [16], which played key roles in enhancing the expression of the target sequence of recombinant eukaryotic expression plasmid in eukaryotic cells. This sequence was named Kozak sequence and used in the construction of expression vectors. In this study, in order to enhance the eukaryotic expression effects of target proteins in partial fragments of swine HEV ORF2, thereby enhancing the immune effect of the constructed swine HEV DNA vaccine, Kozak sequence gccatgg was designed at start codon atg of forward primer. Three constructed plasmids were detected by real-time fluorescent quantitative PCR and Western Blot test, result indicated that three recombinant eukaryotic expression plasmids containing Kozak sequence gccatgg were successfully transfected to cells and effectively expressed target proteins.

In this study, the eukaryotic expression vector pEASY-M1 and TA Fast Cloning (5mins) technique were utilized to clone the PCR products of three continuous fragments of native strain HEV ORF2. The eukaryotic expression vector pEASY-M1 has the following advantages: fast cloning with high cloning efficiency; direct cloning of the PCR products without digestion or purification; enhanced CMV promoter which can offer high efficient expression ability; V5 tag for easy detection of the target protein; Neomycin resistance genes facilitate the stable cell line selection. Therefore, operations of pEASY-M1 Expression Vector are more convenient and faster than the other conventional eukaryotic expression vectors, and it is an ideal eukaryotic expression vector for construction of recombinant plasmid of HEV nucleic acid vaccine.

The stable level of RNA transcription is one of the most convenient parameters in detecting gene expression activity of cells and tissues in gene expression analysis. Real-time fluorescent quantitative PCR detection of corresponding mRNA in cells transfected by recombinant plasmids can be applied for quantitative analysis of the gene expression lev-

el, superior to conventional methods of RNA detection and analysis such as northern blot and RNase protection test [17]. There are two quantification strategies in the templates of real-time fluorescent quantitative PCR: relative and absolute quantifications [18,19]. Absolute quantification is to calculate the amount of unknown sample using known standard curve. $2^{-\Delta\Delta CT}$ relative quantitative method is to achieve quantification through detection of changes in expression of target genes relative to reference genes, which is not only simpler and more economical than the absolute quantification, but also more reliable and accurate. But after all, there is a significant heterology between reference and target genes, hence this method requires that the amplification efficiencies of reference and target genes were basically similar [20]. In this study, PCR amplification efficiencies of target genes LB1, LB2 and LB3 and reference gene β-actin were 92.57%, 90.28%, 94.19% and 90.47%, respectively, basically similar to each other, hence $2^{-\Delta\Delta CT}$ relative quantification method could be applied for detection and analysis of target gene expression. Test results showed that the target gene was successfully expressed in transfected cells.

Nucleic acid vaccine, also known as gene vaccine or DNA vaccine, is cloning one or multiple antigen coding genes into the eukaryotic expression vector and directly inject the constructed recombinant plasmids into the animal and activate the immune system. The antigen protein is similar to recombinant protein vaccine ,and the only difference is that the antigen protein of nucleic acid vaccine is produced in the body of immune object [21]. The strong antigenicity of nucleic acid vaccine can induce long period of cellular immunity and humoral immune, which can ensure long presence of the antigen in the body, stimulating the body to produce a stronger and longer lasting immune response for protection. Nucleic acid vaccine is not only convenient for preparation and transformation, but also can fuse plasmids of different antigen genes to perform combined immunization. In recent years, many researchers [22-25] had conducted experimental studies on the development of nucleic acid vaccine of HEV ORF2 gene, and results showed that all of these nucleic acid vaccines had good antigenicity and immunogenicity. In this study, three recombinant eukaryotic expression plasmids which contained three continuous nucleic acid fragments of native HEV ORF2 were constructed respectively, and the results of expression in transfected cells and identification of HEV antigen confirmed that all these three eukaryotic expression plasmids could transfect cells and express target HEV proteins with antigenicity to HEV.

Conclusion

There is the high infection rate of swine HEV in Guangxi, China. It's the severe risk to human population in Guangxi. In order to develop swine HE vaccine candidates ,we have used more advanced and efficient eukaryotic expression plasmids of pEASY-LB1, pEASY-LB2, pEASY-LB3 which were transfect into 293T cells, and then three target genes were detected by real-time fluorescent quantitative RT-PCR. Gene expression profiles of target genes LB1, LB2 and LB3 in 293T-a, 293T-b and 293T-c and control cells were measured by $2^{-\Delta\Delta CT}$ relative quantitative method. SDS-PAGE and Western-blot confirmed that those three recombinant eukaryotic expression plasmids had been successfully transfected in 293T cells and expressed HEV ORF2 proteins with biological activities. The nucleic acid vaccine candidate of three continuous gene fragments of swine HEV ORF2 developed and validated in this study and will be used as a vaccine candidate in swine for protection against HEV infection.

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