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Rescue of Duck Hepatitis Virus-1 from Cloned cDNA by Using T7 RNA Polymerase

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Abstract: In the present study, researchers describe a rescue system for duck hepatitis virus-1 using T7 RNA polymerase. This strain can not induce typical cytopathic effect in BHK-21 cells. First, there is used the vitro transcripts to infect BHK-21 cells. Then the infected cell supernatant were inoculated into chicken embryos to rescue the virus and the cDNA clone was engineered to contain one silent nucleotide change to create a BssHII site to distinguish from the parental virus. The rescued virus was able to result in embryonic death and have similar infection with parental virus in chicken embryos indicating that the cDNA clones were replication competent. It is proposed that this rescue system may benefit the investigation on viral virulence determinants and molecular virology of DHV-1 virus.

Key words: Duck hepatitis virus type 1, infectious cDNA, rescue system, competent, rescaed viras, China

INTRODUCTION

Duck virus hepatitis which caused by duck hepatitis virus is an acute and fatal disease of young ducklings characterized by its high mortality rate and rapid transmission. Since the 20th century, duck hepatitis became one of the most serious infectious diseases which brought huge economic losses.

Duck hepatitis virus have been described to three serotypes (DHV-I to DHV-III) and no antigenic relationships have been found between these three serotypes (Levine and Fabricant, 1950; McNulty, 2001; Sandhu *et al*., 1992).

Duck hepatitis virus belongs to the family of picornaviruses and more close to the member of Parechovirus (Kim *et al*., 2006). Due to the different characteristics of the genome, duck hepatitis virus has been suggested that it should be assigned to a new genus in the picornaviruses family (Ding and Zhang, 2007; Tseng *et al*., 2007).

The genome of DHV-1 is a single-stranded, polyadenylated, positive-sense RNA of approximately 7690 nucleotide (Kim *et al*., 2006; Tseng *et al*., 2007). The genome of DHV-1 contains one single long Open Reading Frame (ORF), the ORF of 2250 codons which codes for a polyprotein containing structural and non-structural proteins. The P1 region encodes the capsid proteins VP0, VP3 and VP1. The P2 and P3 regions encode non-structural proteins with P2 encoding 2A-2C and P3 encoding 3A-3D. The production of cDNA clone has been crucial for the production and characterization of viral proteins. However, duck hepatitis virus can not propagate well in cell cultures and produce Cytopathogenic Effect (CPE) which makes it difficult to recover this kind of virus. It is only reported that egg-attenuated strains of DHV could passage through some kinds of primary cells for example the Chicken Embryomic Fibroblast (CEF) (Davis and Woolcock, 1986). A highly infectious cDNA clone of the ZJ-V isolate of DHV-1 was generated and has a typical CPE on BHK-21 cells (Yun *et al*., 2010). In this study, researchers aimed at rescuing the DHV-1 virus which have no CPE in BHK-21 cell cultures from the infectious cDNA clone.

MATERIALS AND METHODS

Cells, virus and chicken embryos: The SS isolate which isolated from a duck suffering duck hepatitis virus-1 in Guangdong province was chosen as the parental virus. The baby hamster kidney cell line (BHK-21) was cultivated in Dulbecco's Modified Eagle's Medium (DMEM) that was supplemented with 10% Fetal Bovine Serum (FBS) at 37°C. The chicken embryos were specific pathogen-free and inoculated at 37°C.

RNA extraction and RT-PCR: Viral RNA was extracted from the egg embryo allantoic fluid using TRIzol (invitrogen) according to the manufacture's instructions. First-stand cDNA was synthesized using the primer Oligod $(T)_{14}$ with M-MLV reverse Transcriptase (Takara). Three fragments covering the complete genome of the virus were amplified with PfuUltra II Fusion HS DNA Polymerase (Stratagene). PCR was done using specific primers at several position along the template RNA.

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Construction of the full-length cDNA: The cDNA fragments were assembled into a single clone from three overlapping cDNA fragments by use of the restriction sites SalI, BamHI, PstI, MluI. First, the fragment of A-C were amplified by primers. The A fragment was cloned into transformed pOK12 at their respective sites named pOKA. Fragment B was released from pGEM-T Easy (Promega) vector with BamHI and PstI and ligated to plasmid pOKA to produce recombinant plasmid pOKAB. At last, fragment C was digested both with PstI and MluI and ligated to pOKAB. The recombinant vector containing the full-length cDNA was checked by sequencing and restriction endonuclease analysis and named pOKDHV. In order to distinguish the recombinant virus from the parental virus, an BssHII site was designed in primer BF containing silent mutation of nucleotide A-C, at position 1061. T7 RNA polymerase at the 5'end and a 20 base poly (A) tail at the 3'end were introduced by primers.

***In vitro* transcription:** To linearize the plasmid, pOKDHV was digested by AclI and treated with proteinase K. The linearized plasmid DNA was used for *in vitro* transcription with T7 RNA Polymerase (Takara) according to the manufacture's instructions. The RNA was purified by RNeasy mini kit (Qiagen) according to the manufacture's instructions.

Transfection of BHK-21 cells and Infection of chicken embryos: In order to obtain the rescued virus, 5 μg of *in vitro* transcribed RNA was mixed with 10 μL DMRIE-C (invitrogen) and added into Opti-medium (invitrogen). After exposure to the mixture at 37°C for 4 h, the infected cells were washed and overlaid with DMEM that contained 10% FBS. Then the cells were cultivated at 37°C for 48 h. Then the infected cells were injected into three 9 days old SPF chicken embryos after 3 times of freeze-thawing. Each of them was inoculated with 200 μL cell supernatant. As a negative control, three chicken embryos were injected with 200 μL PBS.

Indirect Immunofluorescence Assays (IFA): BHK-21 cells grown in six-well plates were washed twice with PBS for 15 min. Cells were fixed in acetone; ethanol (3:2) at room temperature for 5 min. The fixed cells were washed with PBS for three times. DHV-1 antiserum (1:50 dilution of an antiserum raised in chicken) prepared in 3% BSA in PBS were incubated for 30 min at 37°C. Cells were washed with PBS and stained with the goat anti-chicken immunoglobulin G fluorescent secondary antibody (1:100 dilution) for 30 min at 37°C. Then cells were washed with PBS. Samples were observed under a fluorescence microscope.

RT-PCR for virus detection and genetic marker analysis: Allantoic fluid of infected chicken embryos and negative control were harvested after 48 h inoculation and were extracted by Trizol (invitrogen). The 691 bp fragment was amplified by RT-PCR from RNA extraction which contains the genetic marker. The products were digested with BssHII and analyzed on a 1% agarose gel.

Sequence analysis: Three fragment were amplified by RT-PCR with amplified primer from the rescued virus. Samples were ligated to pGEM-T easy vector for sequencing. The sequences of the rescued virus and the parental virus were compared by using MegAlign (DNASTAR).

Embryo median lethal dose (ELD50): Seventy SPF chicken embryos were divided into seven groups. Each group has ten chicken embryos. The 5th-passaged rescued virus were diluted (1:10) into seven dilutions (10^{-1} to 10^{-7}) and each dilution was injected into one group. Each chicken embryo was injected with 0.2 mL dilutions. Ten chicken embryos were kept as the PBS-inoculation control.

RESULTS

Construction and sequencing of the full-length DHV cDNA clone: The full-length cDNA clone was constructed in three steps. The A fragment was 1st cloned into transformed pOK12 at their respective sites, named pOKA. Then, the fragment B and C were ligated into the pOKA to obtain the full-length cDNA clone. In order to distinguish the recombinant virus from the parental virus, an BssHII site was designed in primer BF containing silent mutation of nucleotide A-C at position 1061.

Compared with the parental virus, the full-length cDNA clone had no nucleotide changes and the silent mutation was exsited. The pOKDHV has T7 RNA polymerase at the 5'end and a 20 base poly (A) tail at the 3'end.

Infectivity of *in vitro* transcripts: RNA was *in vitro* transcribed from the AclI-linearized full-length cDNA clone with T7 RNA polymerase. Then the RNA were cleared by the RNeasy mini kit (Qiagen). First, the *in vitro* transcripts were infected the BHK-21 cells but there was no CPE. Then the infected cell supernatant were injected into SPF chicken embryos to obtain the infectivity.

Total 48 h later, all embryonated chicken eggs inoculated with infected cell supernatant were killed. None of the chicken embryos inoculated with PBS died in the experiment.

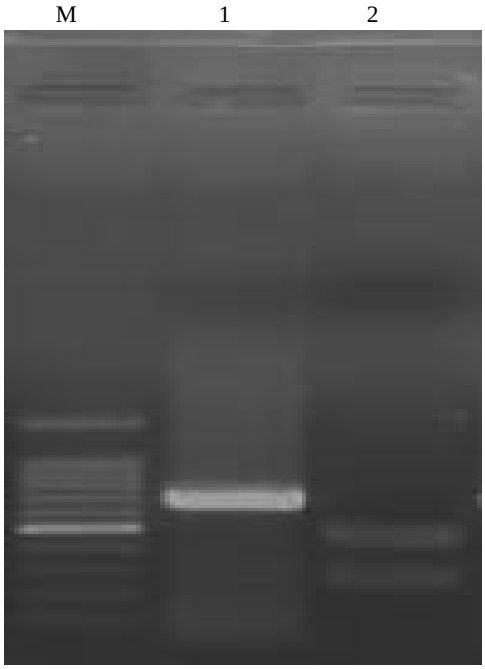

Fig. 1: Introduction of a genetic marker into the resured virus. BssHII restriction site which is present only in resuced virus was introduced in the full-length cDNA clone of SS strains to distinguish from the parental virus. After RNA was extracted from the egg embryo allantoic fluid, RT-PCR was performed with primer MF/MR to amplify the 691 bp fragment. The amplicons were digested with BssHII and analyzed on a 1% agarose gel. The presence of a BssHII restriction site resulted in fragments of 265 and 426 bp. M: 100 bp DNA Marker: 1) Parental virus digested with BssHII and 2) Cloned virus digested with BssHII

Discrimination between the cloned virus and the parental SS virus: In order to distinguish recombinant virus from the parental virus, a BssHII restriction site was engineered into the cDNA clone. To detect the rescued virus, allantoic fluid of infected chicken embryos were extracted by TRizol (invitrogen). A 691 bp fragment was amplified by RT-PCR from RNA extraction which contains the genetic marker. The RT-PCR fragment derived from the cloned virus was cleaved by BssHII, generating two fragments, one of 426 bp and one of 265 bp (Fig. 1). In contrast, the PCR fragment derived from the parental isolate was not cleaved by BssHII.

IFA: To determine if viral protein was expressed in DHV RNA-transfected BHK-21 cells, an DHV-positive BHK-21 cell line was analyzed by IFA. The results showed that there was viral protein expression in the transfected cells (Fig. 2).

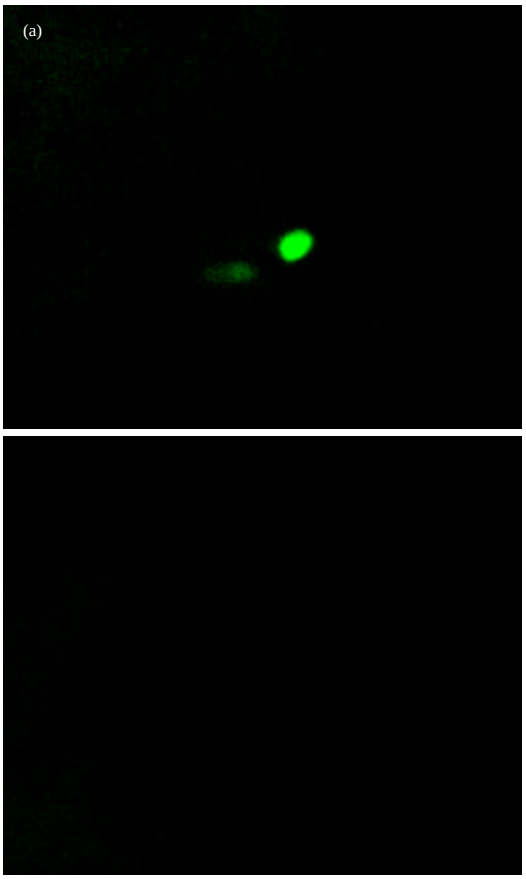

Fig. 2: a) Fluorescence microscopy of BHK-21 cells 48 h after transfection with the full-length cDNA clone of DHV-1 and b) normal BHK-21 cells

Results of ELD50: A quatitative method was adopted to detect the embryo median lethal dose (ELD50) of rescued virus and parental virus to test whether the rescued virus displayed the same infection as the parental virus in chicken embryos. After observing for 7 days, the ELD50 result of rescued virus and parental virus was $10^{-4.83}$ and $10^{-4.68}$ according to Reed-Muench method.

DISCUSSION

Reverse-genetic systems are often used to study the determination of virulence factors and also for the elucidation of mechanisms in viral pathogenesis. An infectious cDNA clone of highly virulent ZJ-V isolate of DHV-1 was generated for the 1st time by using SP6 promoter in 2009. The rescued virus has apparent CPE and has a similar infectivity to that of the parent virus (Yun *et al*., 2010). In the present study, researchers report the construction of the full-length cDNA clone of the strain SS of DHV which has no CPE in BHK-21 cell. Some picorna viruses were obtained infectivity from

clone cDNA by *in vitro* rescue for further research (Cohen *et al*., 1987; Kandolf and Hofschneider, 1985; Westrop *et al*., 1989). Researchers chose T7 RNA polymerase as promoter. The cole sequence of T7 promoter was fused directly to the 5'end of the viral genome by PCR. In other research of picornaviruses, the poly (A) tail may effect on the infectivity of the RNA (Bergamini *et al*., 2000; Grubman *et al*., 1979). In the research, a ploy (A) tail containing 20 A residues was added into the 3'end of the genome by Oligo d (T) primer (Nam *et al*., 2002).

In order to linearized the full-length cDNA clone, a AclI recognition site was added into the end of the ploy (A) tract which can not introduce other residues into the cDNA clone. Few of reports show the DHV-1 can grow well in BHK-21 (Yun *et al*., 2010). So, the *in vitro* transcripts were infectious toward BHK-21 cell 1st then the cell supernatant were injected in embryonated chicken eggs to rescue the virus.

The 50% embryo lethal dose (ELD50) indicated the RNA transcripts transcribed from the cDNA clone were highly infectious by direct injection into chicken embryos. Sequence analysis demonstrated that the recombinant virus shared the highest homology in nucleotide sequence with its parental virus. In addition, the rescued virus from the full-length cDNA clone can be growth stably in chicken embryos.

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