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# Assembly of pseudorabies virus genome-based transfer vehicle carrying major antigen sites of S gene of transmissible gastroenteritis virus: Potential perspective for developing live vector vaccines

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## Abstract

Two severe porcine infectious diseases, pseudorabies (PR) and transmissible gastroenteritis (TGE) caused by pseudorabies virus (PRV) and transmissible gastroenteritis virus (TGEV) respectively often result in serious economic loss in animal husbandry worldwide. Vaccination is the important prevention means against both infections. To achieve a PRV genome-based virus live vector, aiming at further TGEV/PRV bivalent vaccine development, a recombinant plasmid pUG was constructed via inserting partial PK and full-length gG genes of PRV strain Bartha K-61 amplified into pUC119 vector. In parallel, another recombinant pHS was generated by introducing a fragment designated S1 encoding the major antigen sites of S gene from TGEV strain TH-98 into a prokaryotic expression vector pPROEX HTc. The SV40 polyA sequence was then inserted into the downstream of S1 fragment of pHS. The continuous region containing S1 fragment, SV40 polyA and four single restriction enzyme sites digested from pHS was subcloned into the downstream of gG promoter of pUG. In addition, a LacZ reporter gene was introduced into the universal transfer vector named pUGS-LacZ. Subsequently, a PRV genome-based virus live vector was generated via homologous recombination. The functionally effective vector was purified and partially characterized. Moreover, the potential advantages of this system are discussed. © 2006 The International Association for Biologicals. Published by Elsevier Ltd. All rights reserved.

**Keywords:** Pseudorabies virus; Transmissible gastroenteritis; Genome-based transfer; Live vector vaccine

## 1. Introduction

Transmissible gastroenteritis virus of swine (TGEV) is a coronavirus, one of the most important causative agents of enteric infection. The infection is associated with high morbidity in animals of all ages and with high mortality in suckling piglets and may cause devastating economic problems [1–4]. TGEV is composed of three major structural proteins, the spike (S), the integral membrane (M) glycoprotein, and the nucleocapsid (N) protein, containing 1447, 262 and 382 amino acids, respectively

[5–7]. A positive single-strand RNA approximately 4.3 kb in size, regulates and encodes the synthesis of S protein [8,9]. Virus neutralization in the absence of complement is directed against the S protein [10–12]. The amino-terminal half of glycoprotein S encodes four major antigenic epitopes primarily responsible for inducing neutralizing antibodies [13,14].

It was reported that TGEV cause typically local infection, and the secretory immunoglobulin A (S-IgA) induced by the mucosal (i.e., respiratory or digestive tract) inoculation may exert effective anti-infection immunity [15–17]. Pregnant sows exposed orally to TGEV develop such immunity and confer passive protection to the suckling piglets via colostrum and milk containing high neutralizing antibody titers [18,19].

Like TGE, PRV infection (Aujeszky's disease, AD) is another serious, worldwide problem in swine that cause

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significant economic losses. In young piglets as well as in the other susceptible species, PRV infection is often fatal, and animals die from central nervous system disorders [20]. Taxonomically, PRV belongs to the herpesvirus group of enveloped animal viruses, which constitute a large family of DNA viruses whose genome consists of linear, double-stranded, noncircularly permuted DNA molecules [21]. The genome of the herpesviruses has been divided into three or five classes (depending on different nomenclatures); correspondingly, the genome of PRV is a class 2 or class D [22,23]. PRV genome of approximately 150 kb encodes at least seven glycoproteins [24,25]. gE (gI), gC (gIII), gI (gp63), and gG (gX) are not required for viral growth in tissue culture and are therefore designated non-essential [26–29]. In contrast, gB (gII), gD (gp50), gL and gH (gL and gH are a complex) are thought to be essential for PRV replication [30–34]. The multiple nonessential regions within the large size genome of PRV allows the insertion of large and/or multiple DNA fragments encoding foreign antigens to create vectored vaccine. Furthermore, PRV has a broad host range, infecting most mammals and some avian species. However, higher primates including humans are not susceptible to infection [20]. These characteristics, together with the verified immunogenicity [35] and the nononcogenic properties, make it suitable to exploit PRV and its genome for vaccine development.

Vaccination is a very effective prophylactic measures to protect animals against numerous viral diseases including TGE. However, current traditional inactivated and attenuated vaccines are less effective than desired due to failure of vaccination to prevent viral shedding or reversion of attenuated vaccine strain to virulence strain. In addition, high cost to vaccination reduces the use of current vaccine [36]. With the development of biotechnology and molecular virology, use of pseudorabies virus (PRV) as a vector for delivery of vaccine antigens is potential for development of improved vaccines.

In this paper, we report construction of a PRV genome-based universal transfer plasmid by assembling the essential gene expression and regulation components. The amino-terminal half of glycoprotein S encoding four major antigenic sites of TGEV was inserted into the plasmid. Via homologous recombination, the S1 fragment was integrated into the PRV vaccine strain Bartha K-61 genome and a recombinant PRV strain expressing the S1 fragment of TGEV was selected by plaque purification and partially characterized. The recombinant strain may be used for further expression of proteins of interest, and for preparation of effective bivalent recombinant TGEV/PRV vaccine with potentially commercial value.

## 2. Materials and methods

### 2.1. Cells and viruses

Vero cells (Africa green monkey kidney cell line) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 8% fetal bovine serum (Gibco) at 37 °C in a CO<sub>2</sub> incubator. The pseudorabies virus (PRV) vaccine strain Bartha K-61 used for construction of transfer plasmid and

co-transfection was propagated in Vero cells monolayer containing 5% fetal bovine serum. The plaque-titration of Bartha K-61 and the PRV genome-based recombinant were determined on the same Vero cells.

### 2.2. Construction of transfer plasmid pUGS-LacZ

Vero cells infected with PRV strain Bartha K-61 were collected and resuspended in phosphate-buffered saline (PBS) and treated with 1% sodium dodecyl sulfate (SDS) and proteinase K (100 µg/ml) for 2 h. Two fragments named Ga and Gb containing partial PK and full-length gG genes of PRV genome were amplified respectively by PCR using the extracted genome as template. Ga fragment was amplified with sense primer 5'-TTTTGATGGAGGG**CATGCTGAAGCG**-3', whose position is on *SphI* site of PK gene, and anti-sense primer 5'-ATTTAGCACGACGATGTACAGGCCGG-3', whose position is downstream of *PstI* site of gG gene. Gb fragment was amplified with sense primer 5'-TTTACGTGAACGCCAGCCTCGTGCTGCAG-3', whose position is on *PstI* site of gG gene, and anti-sense primer 5'-TTTGAATTCGCCACGTGGCGGTAGCCGTCG-3', whose position is nearby the most 3' end of gG gene. A recombinant plasmid pUG was constructed via inserting Ga and Gb into *SphI* and *EcoRI* site of cloning vector pUC119 (TaKaRa). In parallel, another recombinant plasmid pHS was generated by introducing the amino-terminal half (about 2.2 kb) of glycoprotein S of TGEV designated S1 subunit encoding four major antigenic sites into *BamHI* and *EcoRI* sites of a prokaryotic expression vector pP<sub>RO</sub>EX HTc (GIBICO). SV40 polyA sequence derived from plasmid pEGFP-N1 (CLONTECH) was amplified by specific sense primer 5'-TCGGCATGGACGAGCTGTACAAGTA-3' and anti-sense primer 5'-AAACTGCAGGGCACCTCGACCCCAAAA-3'. The restriction enzyme sites of primers are in bold. The polyA was then inserted into *XbaI* and *PstI* sites, locating the downstream of S1 fragment of pHS. The continuous fragment containing S1, multiple cloning sites (MCS) from pP<sub>RO</sub>EX HTc and SV40 polyA gene was digested from pHS, and was subsequently subcloned into *BamHI* and *PstI* sites of gG promoter downstream of pUG. The new recombinant was termed pUGS. In addition, a report gene LacZ controlled by the immediate early promoter of CMV was inserted into the *PstI* site of pUGS. The construction flowchart of the PRV transfer plasmid designated as pUGS-LacZ containing major antigen sites of S gene of TGEV is briefly illustrated in Fig. 1.

### 2.3. Verification of the recombinant

The recombinant pUGS-LacZ was identified by restriction enzyme digestion and PCR. Using pUGS-LacZ as template, the S1 fragment of TGEV and polyA sequence amplified by specific primers were subjected to sequencing. Four single restriction enzyme sites (*StuI*, *SstI*, *SpeI* and *NspV*) derived from the MCS of pP<sub>RO</sub>EX HTc in pUGS-LacZ were available for foreign gene insertion directly.

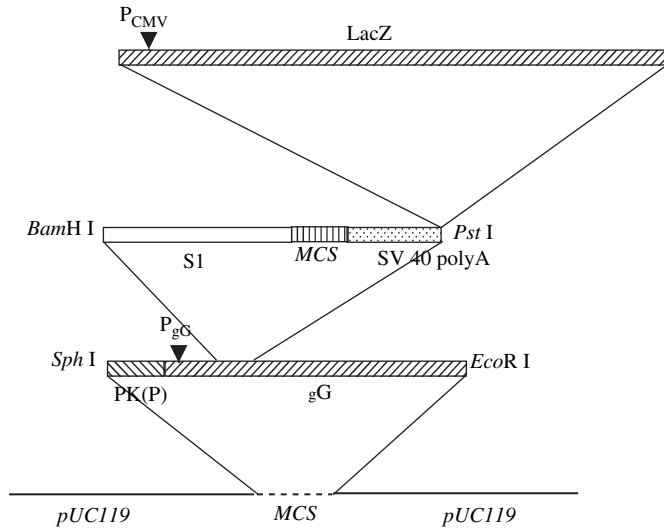


Fig. 1. Construction of transfer vector pUGS-LacZ. After construction of a recombinant pUG via inserting Ga and Gb of PRV into vector pUC119, another continuous fragment containing S1 of TGEV, MCS of vector pPROEX HTc as well as SV 40 polyA sequences was introduced into gG promoter downstream of pUG. Finally, a reporter gene LacZ controlled by the immediate early promoter of CMV was located at the *Pst*I site of pUGS-LacZ. For details, see Section 2.

#### 2.4. Recovery of recombinant virus

The linear transfer plasmid (pUGS-LacZ) was digested with *Kpn*I, and co-transfected with PRV genome into Vero cells cultured on 35-mm-diameter dishes at the density of  $200 \times 10^3$ /ml. Transfection was performed using Lipofectamin 2000 reagent (Invitrogen) according to the manufacturer's instructions. The virus-contained supernatant was harvested, clarified and used to inoculate fresh Vero cells for plaque purification after appearance of cytopathic effect (CPE). Screening for recombinants for the LacZ reporter gene utilized selection of blue plaques on Vero cell layers in the presence of X-gal. After three cycles of purification, a stock of positive recombinant virus designated rBartha/S1 was obtained.

#### 2.5. Identification of recombinant virus by PCR

To confirm if the S1 region (containing S1 fragment, SV40 polyA and LacZ sequences) was still incorporated in the genome of the recombinant virus, the genomic DNA of rBartha/S1 was extracted as described above, and a pair of site-specific primers was designed according to physical map analysis of pUGS-LacZ for PCR identification. In brief, the sense primer 5'-GTCACCAAGAGGTCGCGGCT-3' was located upstream of *Sph*I site of PK gene on PRV genome. The anti-sense primer 5'-AGGTTTCAATGAGATTCCAC-3' was located at nucleotides 97 to 116 of the S1 fragment. Subsequently, the PCR product was visualized by agarose gel electrophoresis (1% agarose, 100 V for 40 min, 0.8  $\mu$ g/ml ethidium bromide included in gel) and subsequent UV transillumination.

#### 2.6. Growth characteristics of the recombinant virus

To explore the effect of foreign gene insertion on growth of recombinant virus, we analyzed the kinetics curve of Bartha K-61 and rBartha/S1 under multiple growth conditions on Vero cell monolayer. Triplicate monolayers of Vero cells were infected with either rBartha/S1 or Bartha K-61 virus at a multiplicity of infection (MOI) of 0.001, and the supernatants were harvested at 6-h intervals to determine the virus titer by plaque assay.

### 3. Results

#### 3.1. Construction and identification of transfer plasmid pUGS-LacZ

By taking advantage of DNA *in vitro* recombination techniques, we had constructed a PRV genome-based universal transfer plasmid. Simultaneously, the S1 fragment of TGEV was inserted into downstream of gG gene controlled by gG promoter. In order to enhance the expression of S1 fragment, SV40 polyA sequence was introduced into the downstream of foreign gene. LacZ expression cassette was inserted into the most 3' end of SV40 polyA for rapid screening and purification of recombinant virus (see Section 2). S1 of TGEV and SV40 polyA regions of recombinant pUGS-LacZ were identified respectively by primer-specific PCR (Fig. 2A,B). All PCR products were identical with the theoretical calculation. In addition, the restriction enzymes analysis of pUGS-LacZ demonstrated the integration of four single restriction sites (*Stu*I, *Sst*I, *Spe*I and *Nsp*V) derived from MCS of pPROEX HTc with the recombinant (Fig. 2C,D). pUGS-LacZ therefore should be a universal transfer vector, which facilitates the insertion of foreign genes. The sequencing results of these PCR products indicated there are no mutations, insertion or deletion of genetic material during the subcloning process (data not shown).

#### 3.2. Recovery of recombinant virus rBartha/S1

We co-transfected the linear transfer plasmid pUGS-LacZ and genome of PRV strain BarthaK-61 into Vero cells. The S1 fragment, SV40 polyA and Lac Z expression cassette were integrated into the downstream of promoter of gG gene of the parental virus genome (Fig. 3). Thus the blue plaques appeared on the cell monolayer when adding X-gal (Fig. 4), and in this study, it was evident that the linearization of transfer plasmid has enhanced the recombination frequency (data not shown).

#### 3.3. PCR analysis of recombinant virus rBartha/S1

To confirm the presence of TGEV S1 fragment in the genome of rBartha/S1, viral DNA was extracted from purified virus and analyzed by PCR. The sense primer corresponds to the upstream PK gene out of the homologous arm. The anti-sense primer is complementary to nucleotide positions 97 to 116 in

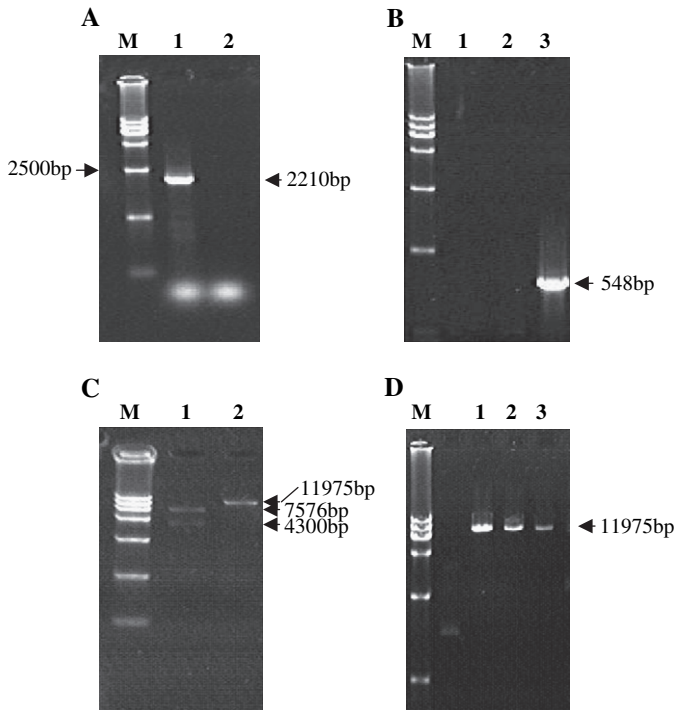


Fig. 2. Identification of recombinant pUGS-LacZ. (A) Identification of S1 fragment of TGEV by PCR: lane 1, the expected PCR product, about 2210 bp; lane 2, negative control. (B) Identification SV40 polyA fragment by PCR: lanes 1–2, negative controls; lane 3, the expected PCR product, about 548 bp. (C) Identification of pUGS-LacZ with restriction enzyme digestion: lane 1, pUGS-LacZ digested with *Pst*I produced two bands, about 7675 bp (recombinant pUGS) and 4300 bp (LacZ expression cassette) respectively; lane 2, pUGS-LacZ digested with *Nsp*V, about 11,975 bp. (D) Validation of the MCS in pUGS-LacZ: lanes 1–3, pUGS-LacZ digested with *Stu*I, *Sst*I, *Spe*I respectively produce the linear pUGS-LacZ, about 11,975 bp as calculated. The DNA marker used here was designated M.

S1 fragment. Two control templates were amplified with the specific primers respectively. One is parental virus Bartha K-61, the other is transfer plasmid pUGS-LacZ. The results of agarose gel electrophoresis demonstrated that rBartha/S1 yielded an expected fragment of about 1.3 kb, whereas BarthaK-61 and pUGS-LacZ showed no positive band (Fig. 5).

### 3.4. Growth characteristics of the recombinant virus

To examine the effect of foreign gene insertion on growth of recombinant virus, we analyzed the kinetics curve of Bartha K-61 and rBartha/S1 under multistep growth conditions on

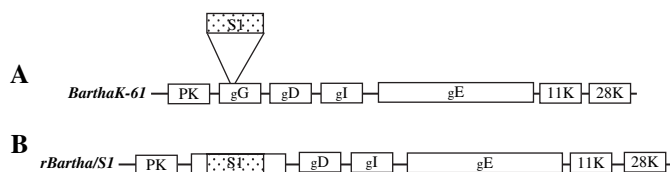


Fig. 3. Recovery of recombinant virus rBartha/S1. (A) S1 region (containing S1 fragment, SV40 PolyA, LacZ and MCS sequences) was integrated into parental virus genome. (B) The recombinant virus carrying S1 region. Therefore, gE gene is not intact due to the insertion.

infected Vero cell monolayer. The virus titration curve determined by plaque assay indicated that the kinetics of replication for both rBartha/S1 and Bartha K-61 were very similar, though there was a slight delay of replication of recombinant virus (Fig. 6).

## 4. Discussion

TGEV and PRV are important pathogens of pigs of all age groups, causing high mortality in piglets. In both cases, vaccination is an indispensable prevention means.

In the case of traditional TGEV vaccines, although the S-IgA in colostrum of sows vaccinated by virulent or attenuated vaccine can provide effective protection to suckling piglets, the potential dissemination and prevalence of infectious agent to piglet remains. Moreover, current conventional virus vaccines may lead to the recovery of virulence and the emergence of antigenic variant viruses. In contrast, the inactivated vaccine is relatively safe, but it produces weaker lactogenic protection to the piglets. It is supposed that immunogenicity of the latter is too low to induce adequate S-IgA. Currently, some genetic engineering subunit vaccines are also being under investigated [37–39]. Although this kind of vaccines may prevent the infection to piglet from authentic virus, the low immunogenicity, difficulty of inoculation and production cost, etc. are still bottlenecks for their actual application. However, this kind of vaccines lack the complete viral architecture, stimulating worse immune response compared with traditional vaccines.

There is a need for the development of alternative vaccine strategies to overcome the shortcomings of the current conventional vaccines. Some recombinant viral-vectored vaccines derived from adenovirus, baculovirus, vaccinia virus, herpesvirus or retrovirus have been studied for that purpose [40–48]. Besides vaccinia virus and herpesvirus including PRV, other above-mentioned viruses share some inherent deficiencies for vaccine exploration, such as smaller nonessential region size, need for helper virus or specific host cell lines and poor safety. The application of vaccinia virus vector is also partially limited due to its relatively narrow host range. In contrast, PRV, as a member of herpesvirus demonstrates some significant advantages: firstly, there are multiple non-essential regions within the large genome of PRV, allowing up to 40 kb insertion of foreign genes; secondly, this virus can result in efficient mucosal infection capable of inducing a stable specific immune response; furthermore, PRV indicates no evidence for oncogenesis [49]. More recently, a bivalent vaccine prepared by integrating NS1 protein gene of Japanese encephalitis (SA14-14-2) virus into PRV genome illustrated the enormous potential and exploited prospects for future application of PRV genome-based vector [50].

It is well known that S protein of TGEV is the major antigenic determinant. Particularly major antigenic sites located in the N terminal half of S gene are mainly responsible for eliciting corresponding humoral and cellular immune responses. [17,39,43]. We designated this fragment



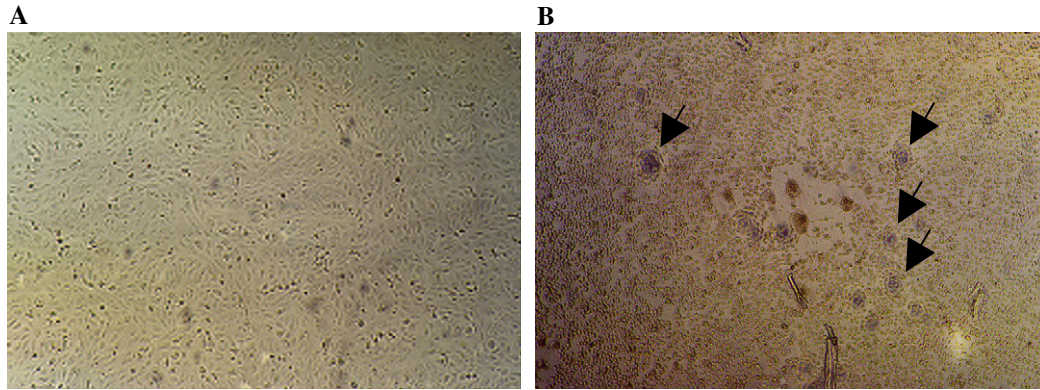


Fig. 4. Blue plaques on Vero cells in presence of X-gal ( $\times 150$ ). (A) Uninfected Vero cell monolayer. (B) Blue plaques appeared after adding X-gal on Infected Vero cell monolayer with rBartha/S1, several of which have been indicated with black arrows.

as S1 subunit of TGEV. Five recombinants containing either the full-length or the S1 subunit of TGEV S gene have been constructed via inserting the foreign genes into the E3 region of porcine adenoviruses of serotype 5. Three of them all induced TGEV- and PAdV-5-specific virus-neutralizing antibodies by oral immunization of pigs [17]. This suggests that generation of TGEV/PRV live vector vaccine has potential for use as an improved vaccine, because adenoviruses and PRV are similar in terms of inducing mucosal immunity. Our previous experiment also indicated that the DNA vaccine encoding the S1 subunit of TGEV can elicit more potent humoral and cellular immune responses in immunized mice than that induced by DNA vaccine encoding full-length S gene of TGEV (unpublished data).

The aim of our study is to construct a PRV genome-based expression vector carrying S1 subunit of TGEV in order to develop a novel bivalent vaccine candidate for PRV and TGEV or other related immunogens in the future.

The constructed PRV genome-based high-efficiency transfer vector was composed of a strong promoter, polyA and length-optimal homologous arm sequences, etc. In order to facilitate gene expression, the S1 fragment was located at the downstream of gG glycoprotein promoter regulated by immediate early and early gene products. Some foreign genes, such as human tissue plasminogen activator (tPA), *Escherichia coli* LacZ or glycoprotein E1 of hog cholera, have been expressed by the same way [51–53]. It was reported that the heterologous fragment insertion in gG gene gave rise to no changes in cell tropism and virulence [54]. Interestingly, however, we found that the cytopathic effect (CPE) caused by the recombinant was morphologically different with that infected by parental virus (Bartha K-61), in which the former demonstrated vacuolization, and the latter showed syncytia formation (data not shown). Further experiments are needed to determine if the dissimilarity was due to the deletion of gG gene or the insertion of foreign gene. The aim for insertion of SV40 polyA sequence in the construct is to improve the stability of mRNA, facilitating mature mRNA through nuclear membrane into cytoplasm and enhancing the foreign gene expression. Using the parental vaccine virus as template to amplify homologous arms of enough length will guarantee both effective recombination frequency and safety. Theoretically, the recombination probability between PRV genome and transfer vector in cellular nucleus is about one in a thousand; the integration of Lac Z reporter gene greatly contributes to the screening of recombinant virus. Meanwhile, PCR performed with site-specific primers has demonstrated the incorporation of S1 fragment of TGEV into the PRV genome. The recombinant virus displayed the similar replicative kinetics as the parental virus, which allows us to hypothesize that the expression of S1 subunit of TGEV can be detected in the future. The transfer vector possesses significant universality due to the introduction of multiple cloning sites; therefore, it can also be utilized for generation of multifold and/or multivalent vaccines.

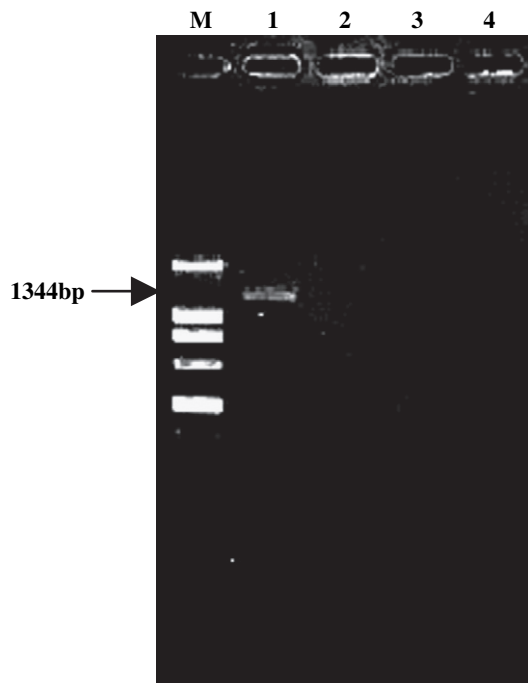


Fig. 5. PCR analysis of recombinant virus rBartha/S1. Lane 1, the PCR product from genome of rBartha/S1, about 1344 bp as expected. Lane 2, the PCR result from genome of BarthaK-61. Lane 3, The PCR result from pUGS-LacZ. Lane 4, negative control for PCR reaction.

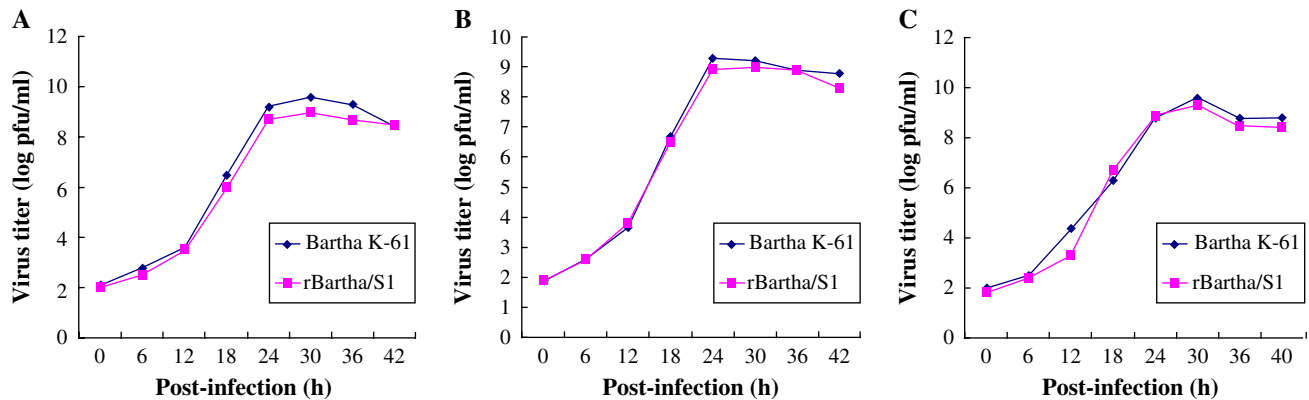


Fig. 6. Multistep growth curve of Bartha K-61 and rBartha/S1. Vero cells monolayer in 35-mm-diameter dishes was infected with virus at a MOI of 0.001 and incubated at 37 °C in DMEM containing 5% fetal bovine serum. Supernatants were harvested at 6-h interval for virus titration. In this panel, A, B and C are the values of triplicate experiments, respectively.

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