www.bti.org.in
ISSN 0974-1453
Research article

AMPLIFICATION AND CLONING OF FOWL ADENOVIRUS 4 HEXON GENE IN ALPHAVIRUS REPLICON BASED VECTOR

Upasna Sharma[1] and Anant Rai[2*]
[1]IFTM University, Moradabad, Uttar Pradesh, India.
[2]Institute of Biotechnology & IT, Bareilly-243122, Uttar Pradesh, India.
***Corresponding author: raia48@gmail.com**

ABSTRACT

The 2916 bp FAV4 hexon gene was amplified using Pfu DNA polymerase and cloned in *Stu*I site of pAlpha vector. The recombinant plasmid was characterized by RE analysis, colony PCR and sequencing.

Key words: PCR, gene, cloning, alpha vector, amplification, fowl adenovirus 4.

INTRODUCTION

Fowl adenoviruses are prevalent in India and cause disease in chickens. FAV 4 is a pathogen mainly isolated from hydropericardium syndrome (HPS) in chickens. The heart and liver are mainly affected causing up to 75% mortality in young chickens. Hence this *Stu*dy will help towards developing a DNA vaccine against this disease. In this context, cloning of the hexon gene can be a promising tool, as it could open avenues for further characterization of the hexon gene, in relation to the development of serotype specific diagnostic tests, sequencing studies and development of recombinant vaccines in the near future. The hexon is the major surface protein of the virus, on which type, group and subgroup specific antigenic determinants are located; hence characterization of the hexon at the molecular level is of utmost importance.

The usual DNA vaccines do not confer sufficient immune responses required for protection which could be due to inadequate expression of antigen (Leitner *et al*., 2006). This could be overcome by expressing the target antigen under the control of an alpha viral replicase (Zhou *et al*., 1994; Herweijer *et al*., 1995; Kanojia and Rai, 2016) with the ability of alpha viruses to produce large amounts of viral mRNA (Schlesinger, 2001; Leitner *et al* 2006) and subsequently protein. Incorporation of alphavirus replicons into plasmid DNA vectors for amplification of RNA expressing the gene of interest has been used to improve DNA vaccine (Dubensky *et al*., 1996). Plasmid vectors encoding the alphavirus-RNA replicase have shown highly immunogenic in murine models at doses up to thousand-

fold lower than those used for conventional plasmid vectors (Hariharan *et al*., 1998; Leitner *et al*., 2000a; Leitner *et al*., 2000b) and are effective when used as vaccines against cancer or viral infections (Anderson *et al*., 2000). The present work was undertaken to construct a recombinant plasmid DNA with FAV4 hexon gene.

MATERIALS AND METHODS

Virus

The Fowl adenovirus serotype 4 adapted to BGM70 cell line at passage 14 (P14) and the purified FAV4 viral DNA was available in the Biotechnology Lab of the Institute.

Primers

The PCR primers in the hexon gene region were used for amplification of 2916 bp hexon gene.

F 5'GGAATTCCAAATGTCAGCAGTAGGCGATTGTGTTACTA 3' 38-mer 354-383
R5'CGGGATCCCGGTGGTCCCCGTCATGCCGTCGCTCTAA 3' 37-mer 3269-3243

Primer pair F &R was designed by Barua and Rai (2003) from FAV 10 hexon gene sequence and the primer positions and were calculated from FAV1 hexon gene reported by Raue and Hess (1998).

BGH reverse primer: **5'TAG AAG GCA CAG TCG AGG3'**

Plasmid Vector

The pAlpha vector available in the Biotechnology lab of IBIT Bareilly was used for cloning. It contains replicase gene of Alphavirus Sindbis virus to amplify the foreign gene at very high copy number, CMV promoter and BGH polyadenylation signal sequence. The BGH reverse primer sequences are used in sequencing (Figure.1).The size of vector is 10799 bp.

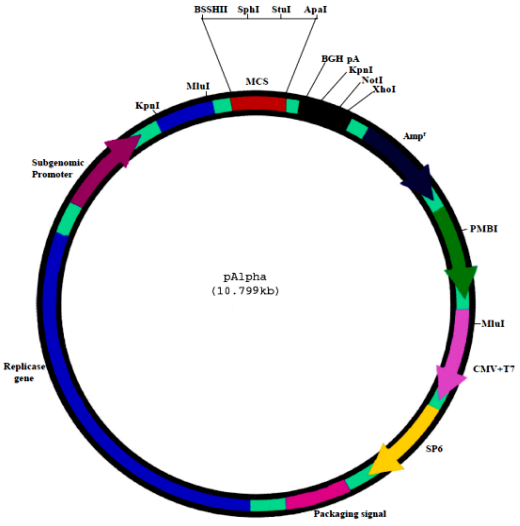


Figure1. pAlpha vector

Bacterial strain

Escherichia coli strain DH5α (Promega, Madison) available in Biotechnology lab of IBIT was used for transformation of recombinant plasmids.

Amplification of hexon gene

For PCR, the 50 µl reaction mix in a thin walled microfuge tube contained 10 x PCR buffer 5 µl, 25 mM $MgCl_2$ 3 µl, Q-solution10 µl, 10 mM dNTP mix 1 µl, F

primer (25pmol/µl) 2µl, R Primer (25pmol/µl) 2 µl, fav4 DNA (100 ng) 4 µl, Pfu DNA polymerase 2 µl, DEPC water 21µl. The tubes were placed in the thermocycler (QuantaBiotec, UK) attached with a laptop installed with Quanta Biotec PCR software. The PCR cyclic conditions were denaturation at 94ºC for 4 min followed by 40 cycles of denaturaion at 94ºC for 60 sec, annealing at 55ºC for 60 sec and extension at 72ºC for 90 sec and then the final extension at 72ºC for 5 min, then hold at 4ºC. These were fed into the program and PCR was started.

Analysis of amplified products

The PCR products were electrophoresed on a 0.7% agarose gel containing 0.5 µg/ml ethidium bromide in 1X TAE buffer. Five µl of each PCR product was mixed with 1 µl of gel loading buffer (Promega). Samples were loaded alongside known molecular weight markers and electrophoresed at 50V for 5 min and thereafter at 100 V for appropriate time. The bands were viewed in transilluminator and the product size was estimated by comparing with the markers.

Plasmid PCR

The plasmid DNA was isolated using TELT method (He *et al*., 1990) and was subjected to PCR amplification for using reaction mix shown above. Five µl of the PCR product was run on a 1% agarose gel.

DNA extraction from agarose gel

The gel extraction of DNA fragments was done using MinElute Gel extraction kit (Qiagen, Germany) following manufacturer's instruction.

Vector preparation

Blunt site was created by *Stu*I enzyme. A 50 µl reaction mixture was prepared containing pAlpha vector DNA (100 ng/µl) 20 µl, NE Buffer 4 (10X) NE Biolab 5µl, nuclease free water 22 µl, *Stu*I enzyme (NE Biolab, 10, 000U/ml 3 µl. The reaction mixture was incubated at 37°C overnight. The linearized plasmid was checked on 0.7% agarose gel electrophoresis and then gel extracted and quantified. The calf intestinal alkaline phosphatase (CIAP) was used to dephosphorylate the linearized plasmid. The 50 µl reaction mixture contained nuclease free water 34µl, 10X buffer 5 µl, linearized vector pAlpha (100ng/µl) 10µl, CIAP enzyme (20 U/µl, Promega) 1µl. It was incubated at 37°C for 30 min. But to be on safer side, 1µl CIAP was added again and re-incubated at 37°C for 30 min. Enzyme was then inactivated by heating the reaction mixture at 75°C for 10 min. The phenol-chloroform precipitation was used following the protocol of (Sambrook and Russell, 2001).

Blunt end ligation of pAlpha vector and FAV4 hexon gene

A 10 µl reaction mixture was prepared containing DW 1 µl, 10X ligation buffer 1 µl, pAlpha vector (100 ng/µl) 0.5 µl, fav4 hexon gene (150 ng/µl) 4 µl, 30% PEG 8000 (Amresco) 1.5 µl, T4 DNA ligase (Fermentas 20U/µl) 2 µl.

One step competent cell preparation and transformation

It was done as per Chung *et al*., (1989).

Characterization of recombinant plasmid

The recombinant plasmid was characterized by restriction endonuclease digestion, colony PCR, sequencing and gene expression in cell culture. Fifteen transformant colonies were selected randomly; half was amplified in LB broth containing 100 µg/ml ampicillin and plasmid DNA was isolated by TELT

method. Another half was used for colony PCR.

Restriction Enzyme Analysis

The presence of gene in right orientation in the recombinant plasmid was confirmed by RE analysis. The fav4 hexon gene sequence, pAlpha vector sequence and pAlpha-fav4hex recombinant plasmid DNA sequence were analysed by DNAstar (Lasegene) Mapdraw software so as to select appropriate restriction enzymes for determining the right gene orientation. The *Apa*I, *Xho*I and *Mlu*I restriction enzymes were found to be useful in determining the right orientation of the gene. The analysis done is shown in Table 1 and Figure 2. The recombinant plasmid was digested with *Apa*I, *Xho*I and *Mlu*I to confirm the right orientation of fav4 hexon gene. The digested products of plasmid were analysed on 0.7/1% agarose gel.

Table 1. RE sites on fav4hex, pAlpha and pAlpha-fav4hex DNA.

DNA	RE	Cut sites	DNA fragments expected
Fav4 hexon	*Apa*I	150	
	*Xho*I	1868	
	*Mlu*I	914	
pAlpha vector	*Apa*I	7760	
	*Xho*I		
	*Mlu*I	7760, 9964	
	*Stu*I	7784	
pAlpha-fav4hex	*Apa*I	7925, 10730	10985, 2805
	*Xho*I	9633, 10852	12500, 1219
	*Mlu*I	7743, 8688, 12894	8574, 4200, 945

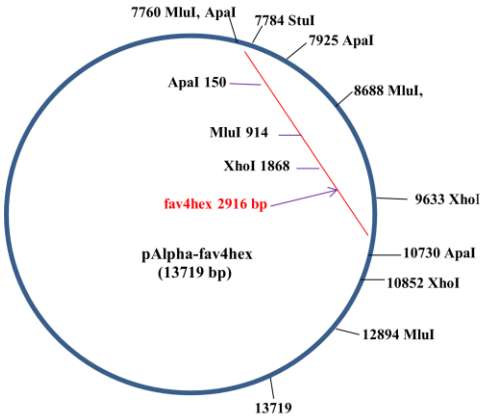


Figure 2. Analysis of pAlpha-fav4hex recombinant plasmid containing fav4hex gene in right orientation showing the RE sites.

The 6 µl recombinant plasmid DNA was mixed with 1µl restriction enzyme and 3 µl nuclease free water, incubated at 37°C overnight and then electrophoresed in 0.7% agarose (Low EEO HiMedia) gel using 1X TAE. The released fragments were compared against 1 kb DNA ladder. The recombinant plasmid DNA sequence was analyzed by DNAstar Map Draw software and these restriction enzymes were found to be appropriate to detect the right orientation of gene. The concentration and purity of plasmid DNA was done spectrophotometrically by taking absorbance at 260 nm and 280 nm in Eppendorf Biophotometer D30 using micro cuvette which shows reading of DNA concentration in µg/ml as well as its purity.

After confirming the right orientation of the hexon gene in recombinant plasmid by RE analysis and colony PCR, the sequencing of the gene insert was got done using BGH reverse primer to further confirm the gene insert as well as its orientation.

RESULTS

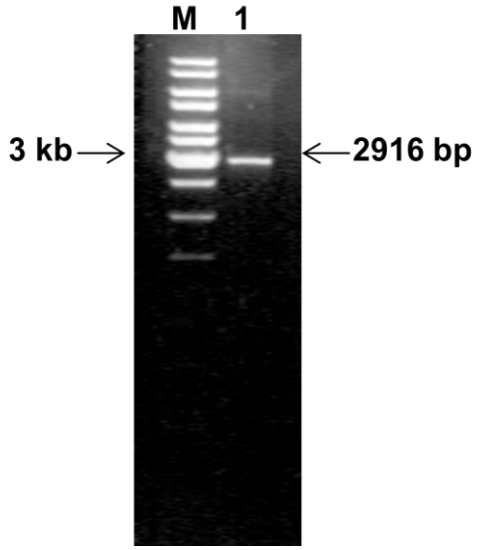
Amplification and Cloning of FAV4 hexon gene

FAV4 hexon gene (2916 bp) was successfully amplified using FAV4 DNA

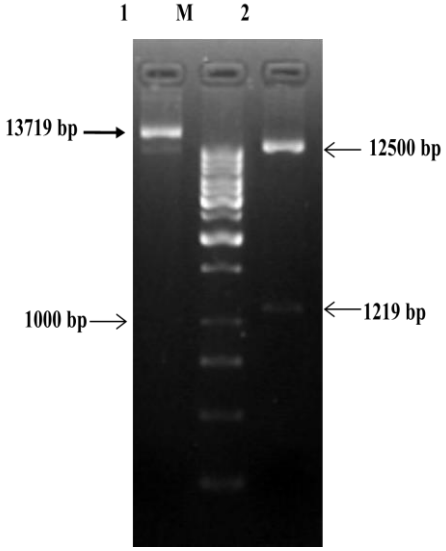
as a template, gene specific F and R primers and Pfu DNA polymerase in presence of required components using the cyclic conditions (Figure 1). It was ligated to linear pAlpha vector and the constructed plasmid was then transformed into *E.coli* DH5α competent cells and plated on ampicillin containing agar plate. After 16 h of incubation at 37^0C, discrete colonies were observed on plate whereas no colonies were observed on control plates. Fifteen discrete colonies were selected randomly and grown in LB broth containing ampicillin. The recombinant plasmid DNA was isolated and characterized by restriction enzyme analysis. Digestion with *Xho*I, *Apa*I and *Mlu*I confirmed the right orientation of the hexon gene (Table 2, Figure 4-7). The fragment sizes obtained were similar to expected sizes. Sequencing of the insert using BGH reverse primer showed that G gene was in right orientation. The confirmed recombinant plasmid DNA of 13719 bp size was designated as pAlpha-fav4hex plasmid DNA.

Table 2. Fragments of pAlpha-fav4hex plasmid DNA by RE analysis.

Restriction enzyme	Expected fragment sizes
*Apa*I	10985 bp, 2805 bp
*Xho*I	12500 bp, 1219 bp
*Mlu*I	8574 bp, 4200 bp, 945 bp



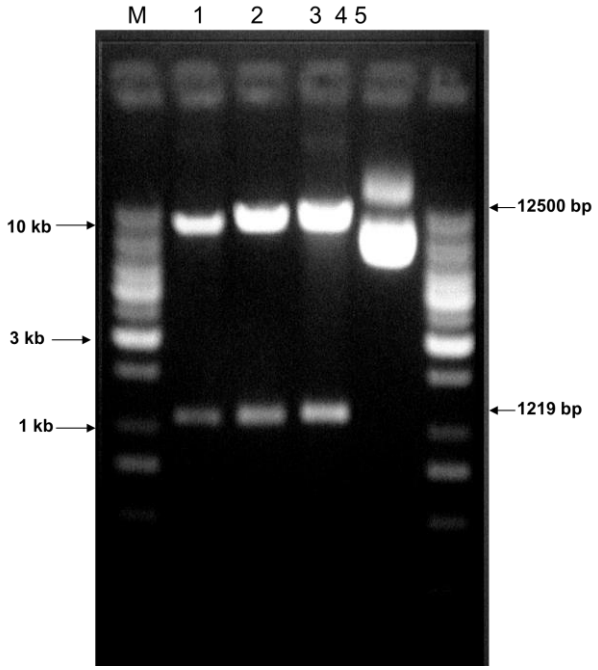
(Figure 3)



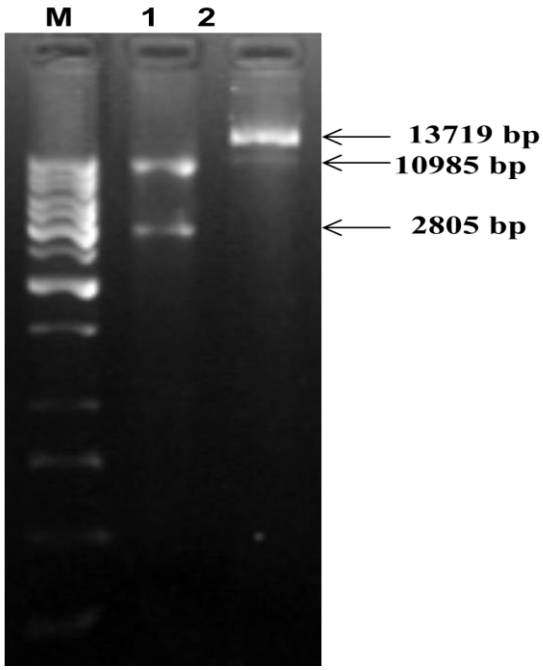
(Figure 4)

Figure 3. PCR amplification of FAV4 hexon gene. Lane M: 1 kb DNA ladder, 1: FAV4 hexon gene amplified.

Figure 4. RE analysis of pAlpha-fav4hex. Lane M: 1 kb DNA ladder, 1: Uncut pAlpha-fav4hex, 2:Digestion of pAlpha-fav4hex with *Xho*I yielding 12500 bp and 1219 bp fragments.



(Figure 5)



(Figure 6)

Figure 5. RE analysis of pAlpha-fav4hex by *Xho*I. Lane M:1 kb ladder, 1-3: Digestion of pAlpha-fav4hex rplasmid with *Xho*I yielding 1219 bp and 12500 bp fragments, Lane 4: Uncut pAlpha-fav4hex 13719 bp.

Figure 6. RE analysis of pAlpha-fav4hex by *Apa*I. Lane M: 1 kb DNA ladder, 1: Digestion of pAlpha-fav4hex with *Apa*I yielding 2805 bp and 10985 bp fragments, 2: Uncut pAlpha-fav4hex 13719 bp.

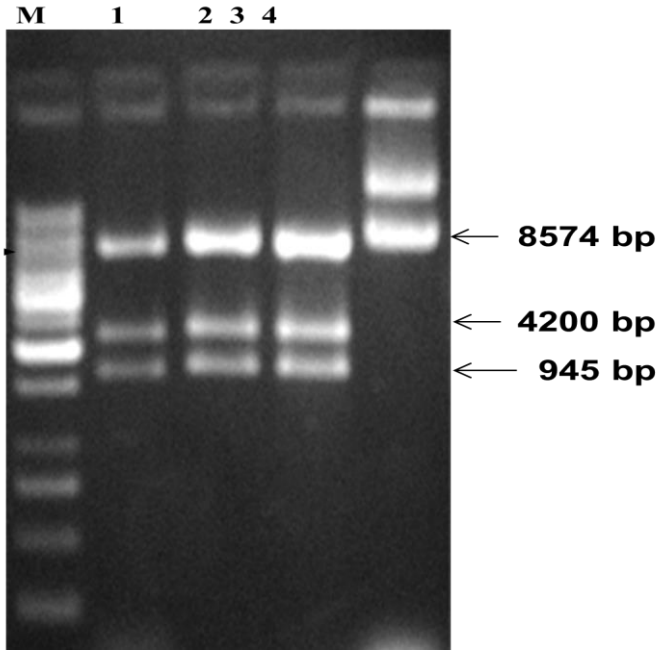


Figure 7. RE analysis of pAlpha-fav4hex by *Mlu*I. Lane M: 1 kb DNA ladder, 1-3: Digestion of pAlpha-fav4hex with *Mlu*I yielding fragments of 8574 bp, 4200 bp and 945 bp, 4:Uncut pAlpha-fav4hex.

DISCUSSION

Cloning of the hexon gene can be a promising tool, as it could open avenues for further characterization of the hexon gene, in relation to the development of serotype specific diagnostic tests, sequencing studies and development of recombinant vaccines in the near future. In the present study, the hexon gene of fowl adenovirus 4 was successfully amplified using Pfu DNA polymerase which produces blunt ended PCR product. It was helpful to clone in the pAlpha vector since there was a *Stu*I site in the MCS of this vector which when cut by *Stu*I enzyme, the blunt ends are created. Similarly one step competent cell preparation and transformation using TSS solution (Chung *et al*., 1989) was a very simple, easy to perform method which readily completes the experiment. The plasmid DNA isolation using TELT method (He *et al*., 1990) is very simple method, having very few steps and easy to perform requiring few chemicals and yielding plasmid DNA of good quality which is suitable for use in molecular biology experiments. The cloning of the hexon gene in right orientation was successfully done. It also produced high level of SN antibody response on immunization of chicken and also produced high cell mediated immune response. Since, no satisfactory effective vaccine against FAV4 infection is available, the cloned recombinant plasmid DNA could be used as DNA vaccine for protection against the disease after conducting field trials of the vaccine.

Sheppard *et al*., (1995) identified the gene for the major capsid protein (hexon) of FAV10. Sandey *et al*. (2008) reported cloning of hexon gene in a replicase based vector and its immunological response in chicken when given along with an IBD VP2 containing recombinant plasmid. The cloning of hexon gene of FAV4 of Indian origin has been reported by Barua and Rai (2003) who cloned the 2916 bp hexon gene amplified by self-designed primers in a mammalian expression vector pcDNA3.1/V5-his-Topo vector (5523 bp, Invitrogen) in India. The field trial of the vaccine showed that it was safe and potent and sera collected from 25 vaccinated birds revealed SN titre of sera 128-256 against FAV4.

REFERENCES

Anderson C, Liljestrom P, Stahl S, Power UF (2000). Protection against respiratory syncytial virus (RSV) elicited in mice by plasmid DNA immunization encoding a secreted RSV G protein-derived antigen. FEMS Immunol Med Microbiol. 29: 247–253.

Barua S and Rai A (2003). Cloning of hexon gene of fowl adenovirus 4 in mammalian expression vector. Indian J Comp Microbiol Immunol Infect Dis. 24: 33-38.

Chung CT, Niemela SL, Miller RH (1989). One step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci USA. 86: 2172-2175**.**

Dubensky TW Jr, Driver DA, Polo JM, Belli BA, Latham EM, Ibanez CE, Chada S, Brumm D, Banks TA, Mento SJ, Jolly DJ, Chang SMW (1996). Sindbis virus DNA-based expression vectors: utility for in vitro and in vivo gene transfer. J Virol. 70:508–519.

Hariharan MJ, Driver DA, Townsend K, Brumm D, Polo JM, Belli BA, Catton DJ, Hsu D, Mittelstaedt D, McCormack JE, Karavodin L, Dubensky Jr. TW, Chang SMW, Banks TA (1998). DNA immunization against herpes simplex virus: efficacy using a Sindbis virus-based vector. J Virol. 72:950–958.

He M, Wilde A and Kaderbhai MA (1990). A single step procedure for small scale preparation of *Escherichia coli* plasmid. Nucleic Acids Res. 18: 1660.

Herweijer H, Latendresse JS, Williams P, Zhang G, Danko I, Schlesinger S, Wolff JA (2008). A Plasmid-Based Self-Amplifying Sindbis Virus Vector. Human Gene Therapy. 6 (9): 1161-1167.

Kanojia A and Rai A (2016). Amplification and cloning of rabies virus virulent glycoprotein gene in pAlpha vector. Biotech Int. 9: 1-10.

Leitner, WW, Ying H, Driver DA, Dubensky TW, Restifo NP (2000a). Enhancement of tumor-specific immune response with plasmid DNA replicon vectors. Cancer Res. 60:51–55.

Leitner WW, Ying H, Restifo NP (2000b). DNA and RNA-based vaccines: principles progress and prospects. Vaccine. 18:765–777.

Leitner WW, Bergmann-Leitner, Hwanga LN, Restifoa NP (2006). ES Type I interferons are essential for the efficacy of replicase-based DNA vaccines. Vaccine. 12: 5110-5118.

Raue R and Hess M (1998). Hexon based PCRs combined with restriction Enzyme analysis for rapid detection and differentiation of fowl adenoviruses and egg drop syndrome virus. J Virol Methods. 73: 211-217.

Sambrook J and Russel DW (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandey M, Rai A, Saxena A (2008). Development of replicase based DNA vaccine containing fowl adenovirus 4 hexongene. Biotech Int. 1: 98-153

Schlesinger S (2001). Alphavirus vectors: Development and potential therapeutic applications. Exp Opin Biol Ther. 1:177–191.

Sheppard M, McCoy RJ, Werner W (1995). Genomic mapping and sequence analysis of the fowl adenovirus serotype 10 hexongene. J Gen Virol. 76: 2595-2600.

Zhou X, Berglund, P, Rhodes G, Parker SE, Jondal M, Liljeström P (1994). Self-replicating Semliki forest virus RNA as recombinant vaccine. Vaccine. 12:1510-1514.