Defence Research and Development Canada | Recherche et développement pour la défense Canada

Expression of the envelope proteins of western equine encephalitis virus by adenovirus vectors

J.Q.H. Wu, D. Chau, C. Wong, W.-G. Hu, and C. McCullough

Defence R&D Canada

Technical Report

DRDC Suffield TR 2007-082

August 2007

Canada

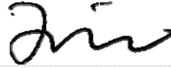
Expression of the envelope proteins of western equine encephalitis virus by adenovirus vectors

Josh Q.H. Wu, Damon Chau, Christina Wong, Wei-Gang, Hu, and Chelsea McCullough
Defence R&D Canada – Suffield

Defence R&D Canada – Suffield

Technical Report
DRDC Suffield TR 2007-082
August 2007

Author



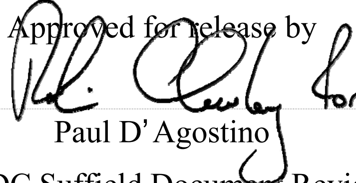
Josh Q.H. Wu

Approved by

Les P. Nagata

Head, Biotechnology Section

Approved for release by



Paul D'Agostino

Chair, DRDC Suffield Document Review Panel

© Her Majesty the Queen as represented by the Minister of National Defence, 2007

© Sa majesté la reine, représentée par le ministre de la Défense nationale, 2007

Abstract

Western equine encephalitis (WEE) virus is a potential biological warfare agent. The virus is mainly endemic in the western regions of Canada and the United States as well as Central and South America. Medical countermeasures against WEE virus are currently lacking. There are no commercial vaccines or anti-viral drugs available to protect Canadian Forces (CF) personnel at risk. We propose to develop WEE vaccine based on human-adenovirus-serotype-5 (HAd5) vectors expressing the structural proteins of WEE virus. To this end, this report describes the construction and characterization of a HAd5 vector expressing the envelope proteins of the 71V-1658 strain of WEE virus. The vector designated as Ad5-WEEV was generated by the transfection of 293 cells with an infectious HAd5 clone that contains the gene encoding the E3-E2-6K-E1 polyprotein of 71V-1658. The presence of the E3-E2-6K-E1 gene in Ad5-WEEV was confirmed by polymerase chain reaction (PCR). The expression of E1 and E2 envelope proteins from Ad5-WEEV was detected by Western blot using monoclonal antibody (MAb) 11D2 specific for E1 and MAb 3F3 specific for E2. The results from this report demonstrate the feasibility of HAd5 vectors as a platform for the expression of viral proteins of WEE virus and lay a foundation for further testing HAd5-vectored WEE vaccines in animals.

Résumé

Le virus de l'encéphalite équine de l'Ouest (EEO) est un agent de guerre biologique potentiel. Le virus est endémique surtout dans les régions occidentales du Canada et des États-Unis ainsi qu'en Amérique centrale et du Sud. On manque actuellement de contremesures médicales contre le virus EEC. Il n'existe pas de vaccins commerciaux ni de médicaments antiviraux qui soient disponibles pour protéger le personnel des Forces canadiennes (FC) exposé à ce risque. On propose de mettre au point un vaccin contre l'EEO basé sur les vecteurs d'adénovirus humains de sérotype 5 (HAd5) exprimant les protéines de structure du virus EEO. Ce rapport décrit, à cette fin, la construction et caractérisation d'un vecteur HAd5 exprimant les protéines d'enveloppe du virus EEO de souche 71V-1658. Le vecteur désigné Ad 5-VEEO a été généré par le transfert génétique de 293 cellules ayant un clone infectieux HAd5 contenant le gène encodant la poly protéine E3-E2-6K-E1 de souche 71V-1658. La présence du gène E3-E2-6K-E1 dans l'Ad5-VEEO a été confirmé par la réaction en cha'ne de la polymérase (RCP). L'expression des protéines d'enveloppe E1et E2 à partir de l'Ad5-VEEO a été détectée par transfert Western utilisant l'anticorps monoclonal (MAb) 11D2 spécifique pour E1 et MAb 3F3 spécifique pour E2. Les résultats de ce rapport démontrent la faisabilité des vecteurs HAd5 comme plateforme pour l'expression des protéines virales du virus EEO et établit les faits préalables à la recherche sur les animaux avec les vaccins EEO à vecteurs HAd5.

This page intentionally left blank.

Executive Summary

Background: Western equine encephalitis (WEE) virus is mainly endemic in the western regions of Canada and the United States as well as Central and South America. The virus is also a potential biological warfare agent. In nature, WEE virus is maintained through a cycle involving wild birds as reservoir hosts and *Culex tarsalis* mosquitoes as vectors. Humans and equines are infected by the virus through mosquito bites. WEE virus causes the infection of the central nervous system with signs and symptoms ranging from a mild flu-like illness to delirium, disorientation and coma. The overall case fatality rate is 3%, but increases to 8% in older adults. WEE virus can also be transmitted to humans through the aerosol route, resulting in a significantly higher mortality rate than that in mosquito-transmitted infections.

Neither human vaccine nor antiviral drugs are currently available for the prevention and treatment of WEE. While several vaccine candidates have been developed, they all have characteristics which make them unsuitable for the protection of Canadian Forces (CF) personnel. An inactivated vaccine available as an Investigational New Drug status is safe but not very effective, requiring multiple doses. Live attenuated vaccines are fast-acting and effective, but the vaccines could potentially revert to the virulent form.

Results: A new approach, using a viral vector genetically engineered to express proteins of the shell of WEE virus, is being pursued. The vector selected, human-adenovirus-serotype-5 (HAd5) vector, has a number of advantages. It has low pathogenicity and is safe for human use. Vaccines delivered by HAd5 vector induce swift and long-lasting immune protection, and induce immunity on mucosal surfaces which is the entry site for aerosolized WEE viruses.

The gene encoding the proteins of WEE virus was isolated from WEE virus and inserted into HAd5 vector. Cells inoculated with the resultant recombinant WEE vaccine (“Ad5-WEEV”) produced large amounts of WEE proteins.

Significance: This proof-of-concept study marks a significant milestone in the development of a potentially safe and effective vaccine against WEE virus.

Future plans: As a next step in the development of the HAd5-vectored WEE vaccine, experiments will be conducted to test its immunogenicity and efficacy in a mouse lethal challenge model of WEE virus.

Wu, J.Q.H. 2007. Expression of the envelope proteins of western equine encephalitis virus by adenovirus vectors. DRDC Suffield TR 2007-082. Defence R&D Canada – Suffield.

Sommaire

Contexte: Le virus de l'encéphalite équine de l'Ouest est endémique principalement dans les régions occidentales du Canada et des États-Unis ainsi qu'en Amérique centrale et du Sud. Le virus est aussi un agent de guerre biologique potentiel. Le virus EEO survit dans la nature en un cycle comprenant les oiseaux sauvages comme hôtes réservoirs et les moustiques *Culex tarsalis* comme vecteurs. Les humains et équins sont infectés par le virus par les morsures de moustiques. Le virus EEO cause l'infection du système nerveux central et des signes et symptômes allant d'une maladie ressemblant à une grippe bénigne au délire, la désorientation et le coma.

Le taux de mortalité est de 3% mais augmente à 8% chez les adultes plus âgés. Le virus EEO peut aussi se transmettre aux humains par la voie aérosol, résultant en un taux de mortalité plus haut que celui des infections transmises par les moustiques.

Il n'existe pas de vaccin humain ni de médicaments antiviraux qui soient actuellement disponibles pour la prévention et le traitement du VEEO. Plusieurs vaccins candidats ont été mis au point mais ils possèdent tous des caractéristiques qui les rendent inaptes à protéger le personnel des Forces canadiennes (FC). Un vaccin mort, disponible ayant le statut de drogue nouvelle de recherche, est sécuritaire mais n'est pas très efficace et requiert des doses multiples. Les vaccins à virus vivants modifiés agissent rapidement et sont efficaces mais ces vaccins ont le potentiel de reprendre leur forme virulente.

Résultats: On examine actuellement une nouvelle méthode qui utilise un vecteur viral génétiquement modifié pour exprimer les protéines de l'enveloppe du virus EEO. Le vecteur sélectionné, le vecteur d'adénovirus humain de sérotype-5 (HAd5) possède un certain nombre d'avantages. Il a une pathogénie faible et n'est pas dangereux pour les humains. Les vaccins livrés par le vecteur (HAd5) produisent une protection immunitaire prompte et durable et produit une immunité des muqueuses qui sont les orifices où pénètrent les aérosols de virus EEO.

Le gène encodant les protéines du virus EEO a été isolé du virus EEO et inséré dans le vecteur HAd5. Les cellules inoculées avec le vaccin recombinant EEO ("Ad5-VEEO") ont produit une grande quantité de protéines de VEEO.

Portée des résultats: L'étude de validation de principe marque une étape importante de la mise au point d'un vaccin contre le virus EEO qui ne comporterait potentiellement aucun risque.

Perspectives d'avenir: La prochaine étape de la mise au point d'un vaccin contre l'EEO de vecteur HAd5 consistera d'expériences conduites pour tester l'immunogénicité et l'efficacité d'un modèle de test de provocation létal du virus EEO d'une souris.

Wu, J.Q.H. 2007. Expression of the envelope proteins of western equine encephalitis virus by adenovirus vectors. DRDC Suffield TR 2007-082. R & D pour la défense Canada – Suffield.

Table of Contents

Abstract i

Executive Summary iii

Sommaire iv

Table of Contents v

List of Figures vi

Acknowledgements vii

Introduction 1

Materials and Methods 3

- Virus and cell 3
- Monoclonal antibodies 3
- Assembly of the gene encoding envelope proteins of 71V-1658 strain of WEE virus . 3
- Insertion of gene encoding the E3-E2-6K-E1 polyprotein into full-length HAd5 genome 5
- Generation of recombinant adenovirus Ad5-WEEV expressing envelope proteins of 71V-1658 strain of WEE virus 5
- Amplification, purification, and titration of recombinant adenovirus Ad5-WEEV 6
- PCR detection of the gene encoding the E3-E2-6K-E1 in viral DNA of Ad5-WEEV.. 6
- Western blot detection of the E1 and E2 proteins expressed from Ad5-WEEV 6

Results 8

- Generation of recombinant adenovirus Ad5-WEEV 8
- Recombinant adenovirus Ad5-WEEV contained the E3-E2-6K-E1 gene 9
- Ad5-WEEV expressed the E1 and E2 proteins of WEE virus 9

Discussion 11

Conclusion 12

References 13

List of Symbols/Abbreviations/Acronyms/Initialisms 16

List of Figures

Figure 1. WEE virus genome organization and the synthesis of structural proteins. 1

Figure 2. Assembly of the gene encoding envelope proteins of 71V-1658 strain of WEE virus. 4

Figure 3. Schematic representation of recombinant adenovirus Ad5-WEEV. 8

Figure 4. PCR Detection of the gene encoding the E3-E2-6K-E1 in recombinant adenovirus Ad5-WEEV 9

Figure 5. Recombinant HAd5 expressing E1 and E2 proteins of WEE virus 10

Acknowledgements

We thank Dr Les P. Nagata for providing plasmid pVHX-6.

This page intentionally left blank.

Introduction

Western equine encephalitis (WEE) virus is one of many biological agents which adversaries could use against the Canadian Forces (CF). Additionally, CF could be at risk to WEE virus infection when deployed in WEE endemic regions in the world. WEE virus is endemic mainly in western North America as well as Central and South America. In North America, the virus is maintained in a cycle involving the mosquito vector *Culex Tarsalis* and wild birds as amplification hosts. Natural outbreaks of WEE may occur when humans or horses are bitten by infected mosquitoes. WEE virus can also be transmitted to humans and animals by aerosol exposure with a significant higher mortality rate than that in mosquito-transmitted infections [1, 2].

The genome of WEE virus contains a positive single-stranded RNA [3]. The envelope proteins are encoded by a subgenomic mRNA and are produced by proteolytic cleavage of the E3-E2-6K-E1 polypeptide [4] (Fig. 1). E2 is initially synthesized as a precursor protein, PE2, which consists of E3 and E2, followed by cleavage of E3 from PE2 by furin-like protease activity. Subsequently, the E2 forms heterodimer with E1 and the complex is transported to the cell surface to become the envelope of the virus. Studies from VEE virus, which belongs to the same family as WEE virus, have demonstrated that the envelope proteins are the major determinants for the induction of immune protection against infection [5-7].

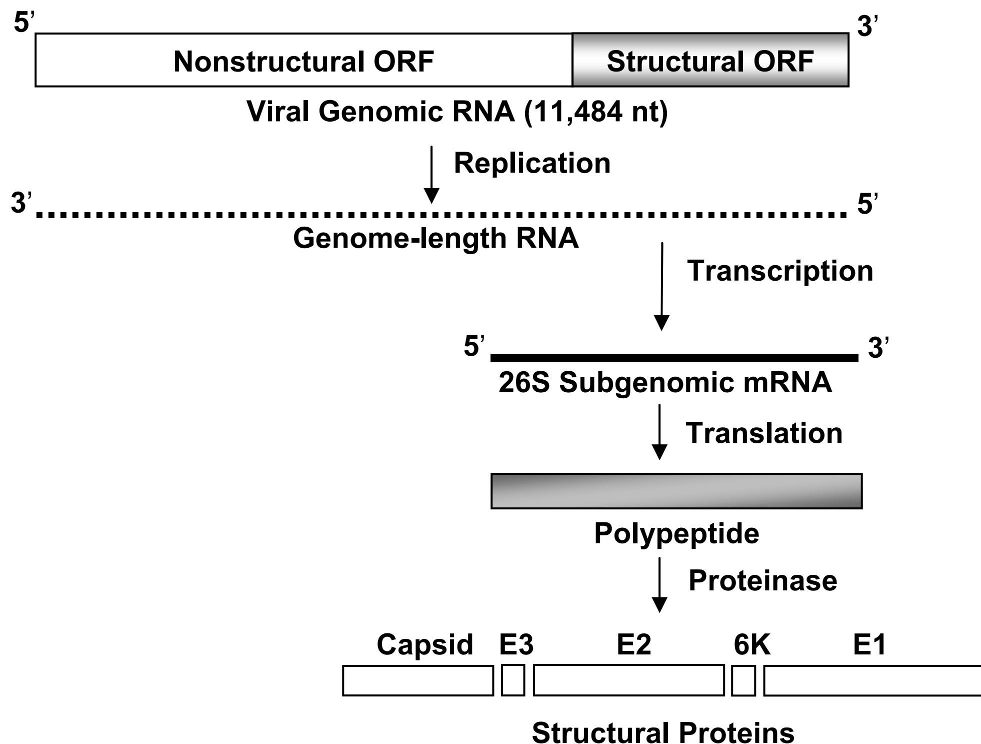


Figure 1. *WEE virus genome organization and the synthesis of structural proteins. ORF: open reading frame.*

The potential risk of WEE virus infections to the CF means that future vaccination against WEE virus is needed. However, there are no commercial vaccines available. Several vaccine candidates for WEE either require multiple injections to be effective or have safety concerns [8-11].

Replication-defective viruses which have deletions of the genes essential for viral growth have served as vectors for vaccine development [12]. The gene encoding a heterologous antigen can be delivered by these viral vectors into cells and the heterologous antigen is expressed and presented to MHC class I and class II molecules to induce immune responses. A viral vector derived from HAd5 has been used for the development of vaccines against varieties of viral infections such as Ebola, avian influenza, and foot-and-mouth disease [13-16]. HAd5 vectored vaccines expressing the E3-E2-6K envelop protein of VEE virus protect mice from aerosol challenge of VEE virus when used alone [17] or used as a booster for a DNA vaccine [18]. We have set out to investigate if HAd5 vector could be used for WEE vaccine development. As the first step of this project, we report the construction and characterization of a recombinant HAd5 expressing the envelope proteins of WEE strain 71V-1658.

Materials and Methods

Virus and cell

Recombinant adenovirus HAd5-EGFP, expressing enhanced green fluorescent protein (EGFP), was used as a control virus. This virus was constructed, purified, and titrated as previously described [19]. Human embryonic kidney 293 cells were purchased from the American Type Culture Collection (ATTC CRL-1573) [20]. These cells were used to generate, propagate, and titrate the recombinant adenoviruses. Propagation of 293 cells was previously described [19]. All the experiments were done with 293 cells within 30 passages.

Monoclonal antibodies

MAbs 3F3 and 11D2 were previously produced in DRDC Suffield laboratories [21]. MAb 3F3 was raised against the E2 of B11 strain of WEE virus and 11D2 was raised against the E1 of B11 strain. These MAbs also cross-react with the E2 and E1 of WEE virus 71V-1658 strain (unpublished data).

Assembly of the gene encoding envelope proteins of 71V-1658 strain of WEE virus

The gene encoding envelope proteins of the 71V-1658 strain of WEE virus was assembled through three steps (Figure 2). First, the gene encoding the envelope protein E3-E2 was isolated by PCR. To do this, plasmid pVHX-6 [8] was used as a template. Primers for PCR were the forward primer JQW3 (5'-**CAC C**AT GTC ACT AGT TAC AGC GCT ATG CGT GC- 3') and the reverse primer JQW5 (5' -TCA CTA AGC GTT GGT TGG CCG AAT GC-3'). These two primers were designed based on the GenBank sequence of WEE virus 71V-1658 strain (accession number NC_003908, nt 8250-9698; [22]). A start codon ATG (underlined) was incorporated into the forward primer (JQW3) and a stop codon TCACAT (underlined) was built in reverse primer (JQW5). In addition, four nucleotides CACC (bold) were added at the 5' end of forward primer JQW3 before the ATG start codon to facilitate directional cloning of PCR fragment into TOPO vector. PCR amplification was done using *Pfu*Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) with two minutes of initial denaturation at 95 °C, 25 cycles of 30 seconds of denaturation at 94 °C, 30 seconds of annealing at 55 °C, as well as 1 min of extension at 70 °C. Extension for 7 min of at 72 °C was added as a final step. The 1.4-kb PCR fragment was separated by 0.8% agarose gel and purified by QIAquick Gel Extraction kit (QIAGEN, Mississauga, ON, Canada). The purified PCR fragment was cloned into a linearized plasmid vector pcDNA3.1D/V5-His-TOPO (Invitrogen, Burlington, ON, Canada) to produce plasmid pcD3-WEE-E3-E2. The DNA sequence encoding E3-E2 was verified by DNA sequencing with CEQ8000 Genetic Analysis System (Beckman Coulter INC., Fullerton, CA, USA).

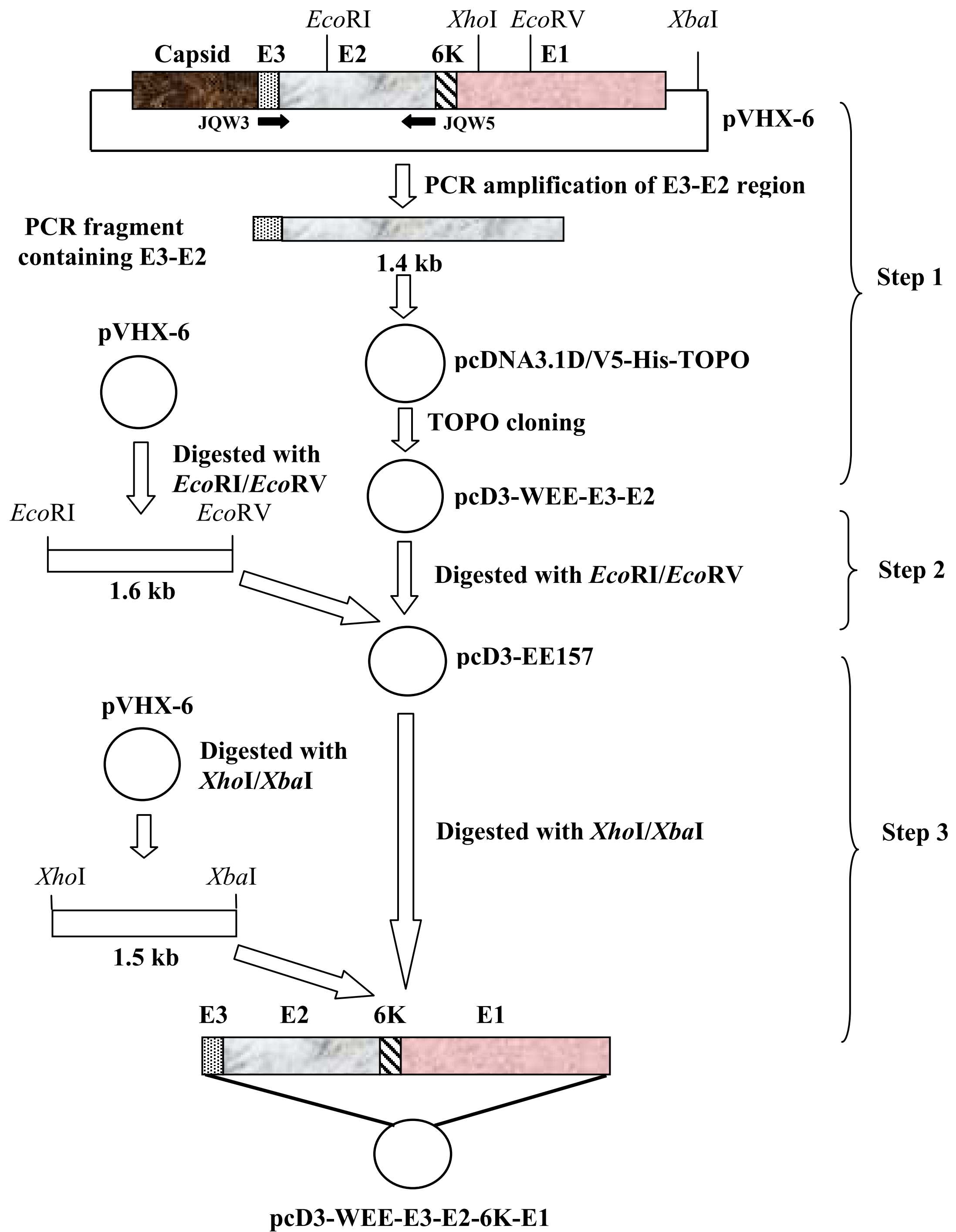


Figure 2. *Assembly of the gene encoding envelope proteins of 71V-1658 strain of WEE virus.*

In the second step of assembly, a 1.6-kb *Eco*RI-*Eco*RV fragment, containing DNA sequence encoding roughly the N-terminal half of 6K-E1protein, was isolated from plasmid pVHX-6. The DNA fragment was cloned into the plasmid pcD3-WEE-E3-E2 using the compatible restriction sites. The resultant plasmid was designated as pcD3-EE157.

In the final step of assembly of the gene encoding envelope proteins of the 71V-1658 strain, plasmid pVHX-6 was digested with restriction enzymes *Xho*I and *Xba*I and a 1.5-kb DNA fragment encoding the C-terminal half of 6K-E1 protein was isolated. This DNA fragment was then cloned into the *Xho*I-*Xba*I sites of the plasmid pcD3-EE157 to generate a plasmid pcD3-WEE-E3-E2-6K-E1 that contains the gene encoding the entire E3-E2-6K-E1 polyprotein of 71V-1658 strain.

Insertion of gene encoding the E3-E2-6K-E1 polyprotein into full-length HAd5 genome

To insert the gene encoding the E3-E2-6K-E1 of WEE strain 71V-1658 into full-length HAd5 genome, a transfer plasmid was made by ligating 3.3-kb *Hind*III-*Xba*I fragment isolated from pcD3-WEE-E3-E2-6K-E1 into the *Hind*III-*Xba*I sites of a plasmid vector pShuttle-CMV (Qbiogene, Carlsbad, CA, USA). The resultant plasmid, pSCMV-WEE-E3-E2-6K-E1, was linearized with a restriction enzyme *Pme*I and co-transformed with a plasmid pAdEasy-1 (Qbiogene) into *Escherichia coli (E. coli)* strain BJ5183 (Qbiogene). The pAdEasy-1 plasmid contains full-length HAd5 genome with deletions of genes encoding E1 and E3 proteins [23]. Through homologous recombination in *E. coli* BJ5183 strain [24], the gene encoding the E3-E2-6K-E1 was inserted into HAd5 genome, resulting in an infectious plasmid pAd5-WEE-E3-E2-6K-E1.

Generation of recombinant adenovirus Ad5-WEEV expressing envelope proteins of 71V-1658 strain of WEE virus

Recombinant adenovirus Ad5-WEEV was made by transfection of 293 cells with the plasmid pAd5-WEE-E3-E2-6K-E1. The plasmid DNA (40 μg total), purified by QIAGEN Plasmid Maxi kit (QIAGEN), was digested with restriction enzyme *Pac*I and purified by ethanol precipitation. Then, 8 μg of *Pac*I-digested DNA was incubated with 60 μl of Lipofectamine 2000 (Invitrogen) for 20 minutes at room temperature. The DNA-Lipofectamine 2000 complexes were added dropwise onto 293 cells seeded in a T25 flask. The transfected cells were incubated at 37 °C in a CO_2 incubator and monitored every day for the cytopathic effect (CPE). After cells showed CPE, they were harvested and recombinant HAd5 was released from the cells by three cycles of freeze and thaw in a total of 1 ml of Dulbecco's modified Eagle medium (D-MEM) supplemented with 2% defined fetal bovine serum (FBS), 1 mM sodium pyruvate, and antibiotics-antimycotics. The cell lysates were stored at -70 °C.

Amplification, purification, and titration of recombinant adenovirus Ad5-WEEV

Recombinant adenovirus Ad5-WEEV was amplified by infection of 293 cells grown in five T150 flasks. Each T150 flask was given 100 μL of Ad5-WEEV-infected cell lysate in a total of 5 ml of D-MEM supplemented with 2% defined FBS, 1 mM sodium pyruvate, and antibiotics-antimycotics. After 2-4 days of infection, the virus was harvested by collecting both supernatant and cell pellet. The virus was purified by BD Adeno-X virus purification kit (BD Biosciences, Mississauga, ON, Canada), aliquoted as 500 μl per vial, and stored at -70 °. The virus was titrated by tissue culture infectious dose 50 ($TCID_{50}$) assay [25]. The final titer of the virus stock was converted to plaque forming units (pfu)/ml.

PCR detection of the gene encoding the E3-E2-6K-E1 in viral DNA of Ad5-WEEV

Viral DNA from purified Ad5-WEEV was extracted by DNeasy Tissue Kits (QIAGEN) according to manufacture's instruction. Briefly, a total of 200 μl purified virus, containing about 10^7 pfu viruses, was mixed with 20 μl proteinase K and 200 μl Buffer AL. After incubation at 56 °C for 10 min, the sample was mixed with 200 μl pure ethanol and loaded onto DNeasy spin column. The column was washed sequentially with Buffer AW1 and AW2 and viral DNA was eluted from the column with 100 μl H_2O.

The purified Ad5-WEEV DNA was used as a DNA template for PCR detection of the gene encoding E3-E2-6K-E1. Primers used for PCR were forward primer S1 (5'-ACC ACG ACC ATG ACA TCA AG-3') and reverse primer JQW4 (5'-CCG CGC TCA GTC ATC TAC GTG TG-3'). The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). PCR was done by HotStarTaq DNA polymerase (Qiagene, Mississauga, Ontario, Canada) with an initial activation step of 15 min at 95 °C, 30 cycles of 1 minute of denaturation at 94 °C, 1 minute of annealing at 55 °C, and 2 minutes of extension at 72 °C. A final 10 min of extension at 72 °C was carried out after 30 cycles amplification. The DNA fragment obtained by PCR was visualized by ethidium bromide staining after electrophoresis in 0.8% agarose gel.

Western blot detection of the E1 and E2 proteins expressed from Ad5-WEEV

The expression of E1 and E2 envelope proteins by Ad5-WEEV was detected by western blot using NuPAGE Gel System and Western Breeze kit from Invitrogen. Confluent 293 cells grown in T25 flasks were infected with purified Ad5-WEEV at a multiplicity of infection (moi) of 1 in a total of 1 ml of D-MEM supplemented with 2% defined FBS, 1 mM sodium pyruvate, and antibiotics-antimycotics. Mock- and Ad5-EGFP-infected 293 cells were included as negative controls. At 24 h after infection, the cell pellet from each infected flask was collected and resuspended in 400 μl of 1X NuPAGE LDS sample buffer containing 5% (V/V) β-mercaptoethanol. The samples were boiled for 5 min and loaded onto 10% Bis-Tris NuPAGE Novex gel along with MultiMark Multi-Colored protein standard (Invitrogen). The

protein sample was electrophoresed at 200 V for 1 h in NuPAGE MOPS SDS running buffer. The eletrophoresed proteins were transferred to a nitrocellulose membrane [26] at 30 V for 2 h in NuPAGE transfer buffer supplemented with 10% (V/V) methanol using an XCell II Blot Module (Invitrogen). The nitrocellulose was blocked with blocking solution and then incubated with MAb 3F3 or 11D2 for 1.5 h at room temperature. The nitrocellulose membrane was rinsed four times with antibody wash buffer and incubated for 30 min with goat anti-mouse, alkaline phosphatase-conjugated antibody (Invitrogen). Protein bands were visualized using alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) from Invitrogen.

Results

Generation of recombinant adenovirus Ad5-WEEV

To express the E1 and E2 envelope proteins of the 71V-1658 strain of WEE virus, a recombinant adenovirus, Ad5-WEEV, was made based on a published method [23, 24]. We obtained Ad5-WEEV at 1 week after transfection of 293 cells. As shown in Fig. 3, the E3-E2-6K-E1 gene was cloned between the immediate-early promoter of cytomegalovirus (CMV) and the polyadenylation signal of simian virus 40 (SV40). The expression cassette replaced the E1 coding region of HAd5. Because E1 is required for HAd5 replication, the replacement of E1 by the expression cassette renders Ad5-WEEV replication defective.

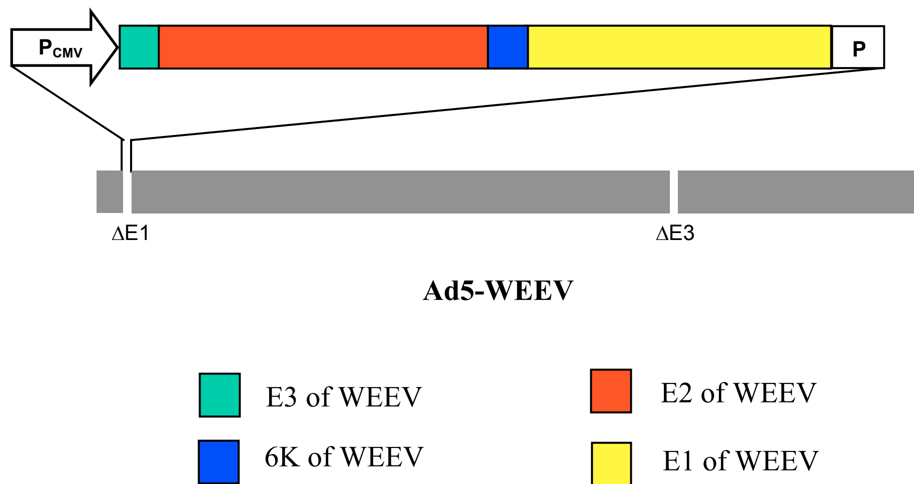


Figure 3. *Schematic representation of recombinant adenovirus Ad5-WEEV. P_{CMV}: the immediate-early promoter of human cytomegalovirus; P_A: the polyadenylation signal of simian virus 40; ΔE1 and ΔE3: deletions in E1 and E3 genes of adenovirus genome.*

A previous study has shown that a DNA vaccine candidate encoding structural proteins (capsid, E3-E2-6K-E1) of WEE virus completely protected mice against homologous challenge of the virus after three-dose injections [8]. Based on this result, we first attempted to generate recombinant HAd5 containing the gene encoding both capsid and E3-E2-6K-E1 but failed. We successfully cloned the gene into the HAd5 genome to generate an infectious DNA plasmid, but failed to obtain a recombinant HAd5 after transfection of 293 cells with the plasmid. At this point, we do not know if the failure to obtain recombinant adenovirus containing the gene encoding capsid and E3-E2-6K-E1 is due to the larger size of the gene or the detrimental effect of the capsid protein on the growth of recombinant HAd5.

Recombinant adenovirus Ad5-WEEV contained the E3-E2-6K-E1 gene

To determine whether the E3-E2-6K-E1 gene is present in the viral DNA genome of Ad5-WEEV, PCR was used to amplify the entire coding region of E3-E2-6K-E1. After amplification, the PCR product was separated on agarose gel and visualized with ethidium bromide staining. Figure 4 shows that a DNA band corresponding to the size (2.9 kb) of the entire coding region of E3-E2-6K-E1 gene appears in the PCR reaction of Ad5-WEEV (lane 2), indicating the presence of the E3-E2-6K-E1 gene in the Ad5-WEEV genome.

Figure 4. *PCR Detection of the gene encoding the E3-E2-6K-E1 in recombinant adenovirus Ad5-WEEV. Lane 1, Invitrogen high DNA mass ladder; Lane 2, PCR reaction.*

Ad5-WEEV expressed the E1 and E2 proteins of WEE virus

Western blot was used to confirm the expression of E1 and E2 envelope proteins from Ad5-WEEV. 293 cells were mock-infected, or infected with Ad5-EGFP or with Ad5-WEEV at multiplicity of infection (MOI) of 1. At 24 h post infection, cell lysates were collected and subjected to 10% SDS-PAGE. The eletrophoresed proteins were transferred to a nitrocellulose and probed, respectively, with MAb 11D2 specific for E1 envelope protein of WEE virus and MAb 3F3 specific for E2 [21]. MAb 11D2 reacted with a protein band with molecular mass of approximate 47 kDa in the cell lysates collected from Ad5-WEEV-infected cells (Fig. 5, lane 4). Similarly, MAb 3F3 reacted with a protein band with molecular mass of approximate 47 kDa in the cell lysates collected from Ad5-WEEV-infected cells (Fig. 5, lane 8). No such protein bands showed in mock-infected 293 cells (Fig. 5, lanes 2 and 6) or in Ad5-EGFP-infected 293 cells (Fig. 5, lanes 3 and 7). The 47-kDa protein bands detected by MAbs 11D2 or 3F3 are consistent with the molecular mass of the E1 and E2 envelope proteins of WEE virus strain 71V-1658 [7, 21].

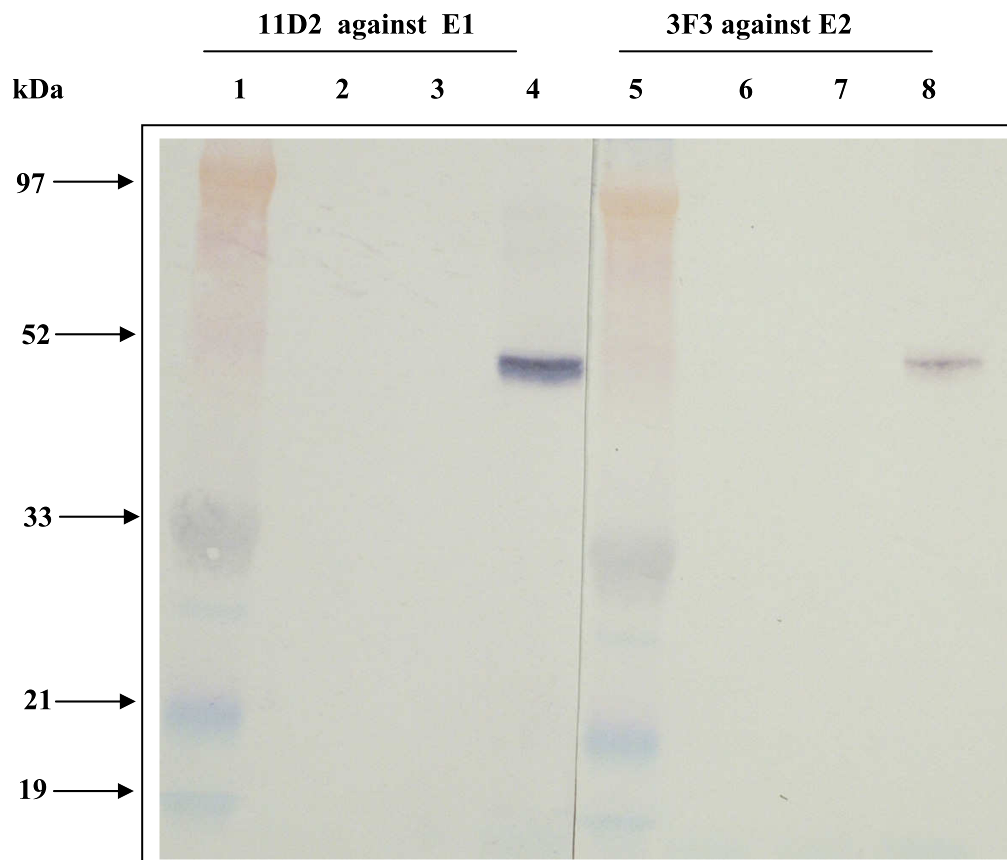


Figure 5. Recombinant HAd5 expressing E1 and E2 proteins of WEE virus. Proteins extracted from 293 cells mock-infected (lanes 2 and 6), or infected with Ad5-EGFP (lanes 3 and 7), or with Ad5-WEEV (lanes 4 and 8) were separated by 10% SDS-PAGE. The electrophoresed proteins were transferred to a nitrocellulose and probed with MAbs 11D2 specific for E1 and 3F3 specific for E2. Lane 1, MultiMark Multi-Colored standard (Invitrogen).

Discussion

This study demonstrates the feasibility of using the recombinant HAd5 as a platform to express the protective antigens of WEE virus. We constructed a recombinant HAd5, Ad5-WEEV, and confirmed that the gene encoding E3-E2-6K-E1 of WEE virus was inserted into the HAd5 genome and Ad5-WEEV expressed the E1 and E2 envelope proteins of the 71V-1658 strain of WEE virus. The study will facilitate the testing of potency and efficacy of an HAd5-vectored vaccine candidate for WEE.

Conventional approaches for developing WEE vaccines usually involve chemically inactivated whole viruses or live attenuated viruses. A formalin-inactivated WEE vaccine has been licensed to control the disease in horses [27]. A similar inactivated WEE vaccine under Investigational New Drug (IND) is also available for laboratory personnel at the risk of contracting WEE virus [10], but the immunogenicity of the inactivated vaccine is poor and short-lived, requiring multiple injections to be effective and frequent boosters to maintain the immunity. Compared to inactivated vaccines, live attenuated vaccines often give a quick and long-lasting protection because these vaccines induce an immune response by mimicking a natural infection without causing disease. Live attenuated vaccine candidates for WEE have been made by deletion of the PE2 furin cleavage site followed by selection of a second-site suppressor mutation in E1 or E2 gene [11]. Chickens vaccinated with a single dose of the live attenuated WEE vaccine were completely protected from the challenge of WEE virus [11]. Live attenuated vaccines, however, are vulnerable to reversion to virulent wild-type viruses and have the potential to spread the live-attenuated vaccine virus to susceptible populations.

An alternative approach for making the live attenuated vaccine for WEE is to use a viral vector to express the protective antigen(s) of WEE virus. A viral vector is typically a replication-deficient virus which is capable of efficiently delivering genes encoding viral subunit proteins. The viral proteins expressed from the viral vector will elicit an immune response against related viruses but without the disease associated with an actual infection. Therefore, viral vectored vaccines combine the safety of inactivated vaccines and the rapid induction of immunity of live attenuated vaccines.

We chose recombinant HAd5 as a vector for constructing WEE vaccine for the following reasons. Firstly, HAd5 is of low pathogenicity, causing a mild upper respiratory infection in humans. Secondly, HAd5 is extensively studied and methods for modification of the virus into a replication-defective viral vector and large-scale production of the vector are available. Thirdly, vaccines delivered by HAd5 vectors induce swift and durable humoral and cellular immune responses. For instance, rhesus monkeys immunized with a single-dose of HAd5 vectors encoding the gp140 envelope protein of human immunodeficiency virus generated both $CD8^+$ T lymphocytes and antibodies specific for the virus. The immune responses are readily detected as late as 151 weeks following initial vaccination [28]. In another study, a single-dose vaccination of non-human primates with an HAd5 vector encoding glycoprotein of Ebola virus protected those animals from a lethal challenge [15]. Finally, vaccines delivered by HAd5 vectors induce immunity on mucosal surfaces where the aerosolized viruses enter [29]. For example, intranasal vaccination of HAd5 expressing the envelope glycoprotein E2 of VEE virus protected mice against airborne challenge of VEE virus [17].

Conclusion

This report demonstrates the feasibility of using the recombinant HAd5 as a platform to express the protective antigens of WEE virus. The study is the first step toward the potency and efficacy studies of the HAd5-vectored WEE vaccine in animals. Several previous studies have shown that HAd5-based vaccines can be used as a single-dose vaccine against Ebola, avian influenza, and foot-and-mouth disease and that the protection is rapid, taking only 1 to 3 weeks to be effective. Therefore, the HAd5-vectored vaccine could be an ideal vaccine for biodefence because of its swift action. It is recommended that a safe, fast-acting WEE vaccine be built on a HAd5 platform.

References

1. Hanson, R.P., Sulkin, S.E., Beuscher, E.L., Hammon, W.M., McKinney, R.W., and Work, T.H. (1967). Arbovirus infections of laboratory workers. Extent of problem emphasizes the need for more effective measures to reduce hazards. *Science,* 158, 1283-1286.

2. Reed, D.S., Larsen, T., Sullivan, L.J., Lind, C.M., Lackemeyer, M.G., Pratt, W.D., and Parker, M.D. (2005). Aerosol exposure to western equine encephalitis virus causes fever and encephalitis in cynomolgus macaques. *J. Infect. Dis.,* 192, 1173-1182.

3. Schlesinger, S., and Schlesinger, M.J. (2001). Togaviridae: The viruses and their replication. In Fields Virology, Volume 2, 4th Edition Edition, D.M. Knipe and P.M. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 2265-2300.

4. Strauss, J.H., and Strauss, E.G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.,* 58, 491-562.

5. Mathews, J.H., and Roehrig, J.T. (1982). Determination of the protective epitopes on the glycoproteins of Venezuelan equine encephalomyelitis virus by passive transfer of monoclonal antibodies. *J. Immunol.,* 129, 2763-2767.

6. Hodgson, L.A., Ludwig, G.V., and Smith, J.F. (1999). Expression, processing, and immunogenicity of the structural proteins of Venezuelan equine encephalitis virus from recombinant baculovirus vectors. *Vaccine,* 17, 1151-1160.

7. Das, D., Gares, S.L., Nagata, L.P., and Suresh, M.R. (2004). Evaluation of a Western Equine Encephalitis recombinant E1 protein for protective immunity and diagnostics. *Antiviral Res.,* 64, 85-92.

8. Nagata, L.P., Hu, W.G., Masri, S.A., Rayner, G.A., Schmaltz, F.L., Das, D., Wu, J., Long, M.C., Chan, C., Proll, D., Jager, S., Jebailey, L., Suresh, M.R., and Wong, J.P. (2005). Efficacy of DNA vaccination against western equine encephalitis virus infection. Vaccine *23*, 2280-2283.

9. Schoepp, R.J., Smith, J.F., and Parker, M.D. (2002). Recombinant chimeric western and eastern equine encephalitis viruses as potential vaccine candidates. *Virology,* 302, 299-309.

10. Bartelloni, P.J., McKinney, R.W., Calia, F.M., Ramsburg, H.H., and Cole, F.E., Jr. (1971). Inactivated western equine encephalomyelitis vaccine propagated in chick embryo cell culture. Clinical and serological evaluation in man. *Am. J. Trop. Med. Hyg.*, 20, 146-149.

11. Turell, M.J., O'Guinn, M.L., and Parker, M.D. (2003). Limited potential for mosquito transmission of genetically engineered, live-attenuated western equine encephalitis virus vaccine candidates. *Am. J. Trop. Med. Hyg.,* 68, 218-221.

12. Dudek, T., and Knipe, D.M. (2006). Replication-defective viruses as vaccines and vaccine vectors. *Virology,* 344, 230-239.

13. Pacheco, J.M., Brum, M.C., Moraes, M.P., Golde, W.T., and Grubman, M.J. (2005). Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. *Virology,* 337, 205-209.

14. Gao, W., Soloff, A.C., Lu, X., Montecalvo, A., Nguyen, D.C., Matsuoka, Y., Robbins, P.D., Swayne, D.E., Donis, R.O., Katz, J.M., Barratt-Boyes, S.M., and Gambotto, A. (2006). Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *J. Virol.,* 80, 1959-1964.

15. Sullivan, N.J., Geisbert, T.W., Geisbert, J.B., Shedlock, D.J., Xu, L., Lamoreaux, L., Custers, J.H., Popernack, P.M., Yang, Z.Y., Pau, M.G., Roederer, M., Koup, R.A., Goudsmit, J., Jahrling, P.B., and Nabel, G.J. (2006). Immune protection of nonhuman primates against Ebola virus with single low-dose adenovirus vectors encoding modified GPs. *PLoS Med.,* 3, e177.

16. Sullivan, N.J., Geisbert, T.W., Geisbert, J.B., Xu, L., Yang, Z.Y., Roederer, M., Koup, R.A., Jahrling, P.B., and Nabel, G.J. (2003). Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature,* 424, 681-684.

17. Phillpotts, R.J., O'Brien, L., Appleton, R.E., Carr, S., and Bennett, A. (2005). Intranasal immunisation with defective adenovirus serotype 5 expressing the Venezuelan equine encephalitis virus E2 glycoprotein protects against airborne challenge with virulent virus. *Vaccine,* 23, 1615-1623.

18. Perkins, S.D., O'Brien, L.M., and Phillpotts, R.J. (2006). Boosting with an adenovirus-based vaccine improves protective efficacy against Venezuelan equine encephalitis virus following DNA vaccination. *Vaccine,* 24, 3440-3445.

19. Wu, Q. (2005). An improved method for gene delivery and expression in mammalian cells. Defence R&D Canada Suffield Technical Report 2005-015.

20. Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.,* 36, 59-74.

21. Long, M.C., Nagata, L.P., Ludwig, G.V., Alvi, A.Z., Conley, J.D., Bhatti, A.R., Suresh, M.R., and Fulton, R.E. (2000). Construction and characterization of monoclonal antibodies against western equine encephalitis virus. *Hybridoma,* 19, 121-127.

22. Netolitzky, D.J., Schmaltz, F.L., Parker, M.D., Rayner, G.A., Fisher, G.R., Trent, D.W., Bader, D.E., and Nagata, L.P. (2000). Complete genomic RNA sequence of western equine encephalitis virus and expression of the structural genes. *J. Gen. Virol.,* 81, 151-159.

23. He, T.C., Zhou, S., da Costa, L.T., Yu, J., Kinzler, K.W., and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA,* 95, 2509-2514.

24. Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., and Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. *J. Virol.,* 70, 4805-4810.

25. Mahy, B.W.J., and Kangro, H.O. (1996). *Virology Methods Manual,* Academic Press, San Diego, CA.

26. Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA,* 76, 4350-4354.

27. Barber, T.L., Walton, T.E., and Lewis, K.J. (1978). Efficacy of trivalent inactivated encephalomyelitis virus vaccine in horses. *Am. J. Vet. Res.,* 39, 621-625.

28. Santra, S., Seaman, M.S., Xu, L., Barouch, D.H., Lord, C.I., Lifton, M.A., Gorgone, D.A., Beaudry, K.R., Svehla, K., Welcher, B., Chakrabarti, B.K., Huang, Y., Yang, Z.Y., Mascola, J.R., Nabel, G.J., and Letvin, N.L. (2005). Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. *J. Virol.,* 79, 6516-6522.

29. Santosuosso, M., McCormick, S., and Xing, Z. (2005). Adenoviral vectors for mucosal vaccination against infectious diseases. *Viral Immunol.*, 18, 283-291.

List of Symbols/Abbreviations/Acronyms/Initialisms

ATCC	American Type Culture Collection
CF	Canadian Forces
CMV	cytomegalovirus
CPE	cytopathic effect
D-MEM	Dulbecco's modified Eagle medium
E. coli	Escherichia coli
EEE	eastern equine encephalitis
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
HAd5	human adenovirus serotype 5
IND	investigational new drug
kDa	kilodalton
MAb	monoclonal antibody
m.o.i.	Multiplicity of infection
nt	nucleotide
PCR	polymerase chain reaction
pfu	plaque forming unit
SDS-PAGE	sodium dodecyl sulfate polyacrylamide
SV40	simian virus 40
$TCID_{50}$	tissue culture infectious dose 50
VEE	Venezuelan equine encephalitis
WEE	Western equine encephalitis

DOCUMENT CONTROL DATA

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (the name and address of the organization preparing the document. Organizations for who the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in Section 8.)	2. SECURITY CLASSIFICATION (overall security classification of the document, including special warning terms if applicable)
Defence R&D Canada – Suffield PO Box 4000, Station Main Medicine Hat, AB T1A 8K6	Unclassified

3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S, C or U) in parentheses after the title).

Expression of the envelope proteins of western equine encephalitis virus by adenovirus vectors (U)

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

Wu, Josh Q.H.; Chau, Damon; Wong, Christina; Hu, Wei-Gang; McCullough, Chelsea

5. DATE OF PUBLICATION (month and year of publication of document)	6a. NO. OF PAGES (total containing information, include Annexes, Appendices, etc)	6b. NO. OF REFS (total cited in document)
August 2007	26	29

7. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

Technical Report

8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

Defence R&D Canada – Suffield

9a. PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant.)	9b. CONTRACT NO. (If appropriate, the applicable number under which the document was written.)
WBE 16qb32	

10a. ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.)	10b. OTHER DOCUMENT NOs. (Any other numbers which may be assigned this document either by the originator or by the sponsor.)
DRDC Suffield TR 2007-082	

11. DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification)

(x) Unlimited distribution
( ) Distribution limited to defence departments and defence contractors; further distribution only as approved
( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved
( ) Distribution limited to government departments and agencies; further distribution only as approved
( ) Distribution limited to defence departments; further distribution only as approved
( ) Other (please specify):

12. DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally corresponded to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected).

Unlimited

13. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C) or (U). It is not necessary to include here abstracts in both official languages unless the text is bilingual).

Western equine encephalitis (WEE) virus is a potential biological warfare agent. The virus is mainly endemic in the western regions of Canada and the United States as well as Central and South America. Medical countermeasures against WEE virus are currently lacking. There are no commercial vaccines or anti-viral drugs available to protect Canadian Forces (CF) personnel at risk. We propose to develop WEE vaccine based on human-adenovirus-serotype-5 (HAd5) vectors expressing the structural proteins of WEE virus. To this end, this report describes the construction and characterization of a HAd5 vector expressing the envelope proteins of the 71V-1658 strain of WEE virus. The vector designated as Ad5-WEEV was generated by the transfection of 293 cells with an infectious HAd5 clone that contains the gene encoding the E3-E2-6K-E1 polyprotein of 71V-1658. The presence of the E3-E2-6K-E1 gene in Ad5-WEEV was confirmed by polymerase chain reaction (PCR) The expression of E1 and E2 envelope proteins from Ad5-WEEV was detected by Western blot using monoclonal antibody (MAb) 11D2 specific for E1 and MAb 3F3 specific for E2. The results from this report demonstrate the feasibility of HAd5 vectors as a platform for the expression of viral proteins of WEE virus and lay a foundation for further testing HAd5-vectored WEE vaccines in animals.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifies, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

Adenovirus vector, alphavirus, vaccine

Defence R&D Canada

Canada's Leader in Defence
and National Security
Science and Technology

R & D pour la défense Canada

Chef de file au Canada en matière
de science et de technologie pour
la défense et la sécurité nationale

www.drdc-rddc.gc.ca