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Short communication

Induction of protective immune responses against EV71 in mice by baculovirus encoding a novel expression cassette for capsid protein VP1

Balraj Premanand [a], Tanja K. Kiener [a], Tao Meng [a], Yun Rui Tan [a], Qiang Jia [a], Vincent T.K. Chow [b], Jimmy Kwang [a,b,*]

[a] Animal Health Biotechnology, Temasek Life Sciences Laboratory, National University of Singapore, 117604 Singapore, Republic of Singapore
[b] Department of Microbiology, Faculty of Medicine, National University of Singapore, 1 Research Link, Singapore, Republic of Singapore

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ABSTRACT

EV71 is a major causative agent of hand, foot and mouth disease (HFMD) and is responsible for large outbreaks in various Asian Pacific countries. In the present study, we generated the recombinant baculovirus (Bac-VP1) encoding VP1 in a novel expression cassette. The transmembrane domain of hemagglutinin of the H3N2 influenza virus was included in the cassette as a minimal membrane anchor for VP1. The protective immunity of Bac-VP1 was investigated in a mouse model. The results showed that mice vaccinated with live Bac-VP1 had strong VP1 specific antibody responses. In an *in vitro* neutralization assay Bac-VP1 sera exhibited cross-neutralization against homologous and heterologous EV71 strains with a maximum titer of 1:512. Passive immunization studies confirmed that these sera were able to provide 100% protection against 5 MLD_{50} of mouse adapted EV71 (B4 strain). This study revealed that baculovirus displaying VP1 with a HA transmembrane domain efficiently induced cross-neutralizing antibody responses in mice.

1. Article outline

Enterovirus 71 (EV71) is a single stranded RNA virus of the genus Enterovirus in the family *Picornaviridae* which is responsible for large outbreaks of HFMD in various parts of Asia Pacific (Wu et al., 1999; Shih-Min Wang et al., 1999; Hiroshi Komatsu et al., 1999). Currently, there is no commercial antiviral therapy or vaccine against EV71 infection. To address this, the baculovirus system has been developed as a novel tool for vaccine production, serving as a viral vector for the surface display of antigens (Miller, 1998; Kost and Condreay, 2002; Madhan et al., 2010). Traditionally, surface display of foreign proteins on the baculovirus envelope was achieved by using its native membrane protein gp64 as a fusion partner (Boublik et al., 1995; Madhan et al., 2010). In our previous study, we used the full-length gp64 membrane protein for the baculovirus surface display of VP1 as an EV71 subunit vaccine (Meng et al., 2011). In this approach however, VP1 is incorporated into the baculovirus envelope along with the extracellular domain of gp64 (485 amino acids) (Fig. 1a) which might interfere with the budding process. In the present study, we generated the recombinant baculovirus (Bac-VP1) using a novel expression cassette containing the 27 amino acid long transmembrane domain of the influenza virus H3N2 hemagglutinin (HA) as a fusion partner for VP1 (Fig. 1b). We hypothesized that the presence of this minimal transmembrane region might facilitate the budding process and could achieve higher baculovirus titers than fusion to full length gp64. Briefly, Bac-VP1 encoding the novel expression cassette was constructed by amplifying the full length VP1 from EV71 (C4) virus and inserting it into the pFastBac1 vector containing the immediate early promoter of White Spot Syndrome virus (WSSV ie1). HA transmembrane domain and gp64 cytoplasmic domain (ctd) were introduced in frame to the C-terminus of VP1 using the primers listed in Table 1. Finally, the construct was integrated into the baculovirus genome and the recombinant bacmid was transfected into Sf9 cells (Bac to Bac system, Invitrogen). Expression of VP1 in insect cells was visualized by confocal microscopy (Fig. 2a). Further, the budded virus particles were purified by sucrose gradient centrifugation (O'Reilly et al., 1992). VP1 localization on the baculovirus envelope was confirmed by Western blot and TEM analysis (Fig. 2b and c). As a positive control vaccine, inactivated EV71-C4 (GenBank accession #EU703813) was used. Equal titers (10^7 PFU/ml) of EV71-C4 and Bac-VP1 were inactivated with binary ethylenimide (BEI) (Rueda et al., 2000) or formaldehyde (Ong et al., 2010). Before immunization, the amount of VP1 in each vaccine candidate was quantified using the Odyssey Application Software Version 1.2 (Li-COR). Quantification was based on the comparison between the fluorescent intensity of incorporated VP1 to a standard curve of purified VP1

* Corresponding author at: Animal Health Biotechnology, Temasek Life Sciences Laboratory, National University of Singapore, 117604 Singapore, Republic of Singapore. Tel.: +65 68727473; fax: +65 68727007.
E-mail address: kwang@tll.org.sg (J. Kwang).

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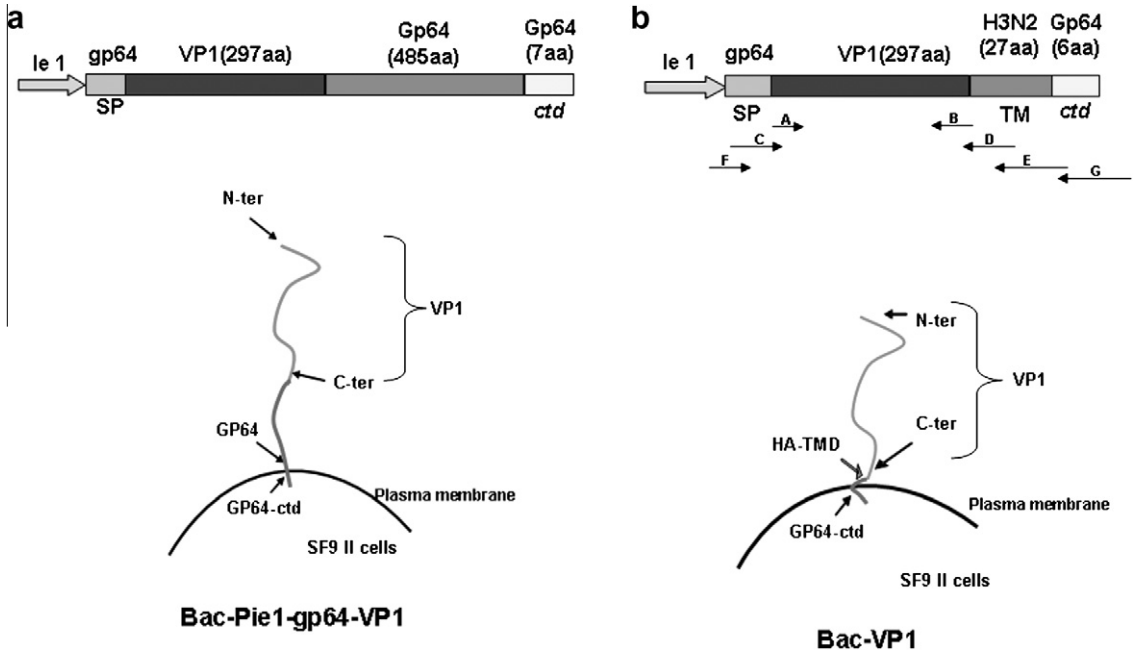

Fig. 1. Schematic illustration of the fusion gene and its anchoring pattern on SF9-II cells. The expression cassettes and the anchoring patterns of VP1 expressed on the Sf9-II plasma membrane. (a) Bac-Pie 1-gp64-VP1 and (b) Bac-VP1 (arrows indicates the primers used for cloning). SP-signal peptide, TM-transmembrane domain, ctd-cytoplasmic domain.

Table 1
Primers used in the cloning experiments.

Primer	Orientation	Primer sequence
A	Forward	GCATTCTGCCTTTGCGGATCTGCAGGGAGATAGGGTGGCAGATG
B	Reverse	CATTTGCGCGTTGCAGTGCTCAAGAGTGGTGATCGCTGTG
C	Forward	TTTGGCGGCGGCGGCGCATTCTGCCTTTGCGGGAGATAGGGTGGCAGATGTAATTG
D	Reverse	CATGATATGGCAAAGGAAATCCATAGGATCCAAAGAGTGGTGATCGCTGTGCGAC
E	Reverse	TTGGCAGGCCCACATGATGAACCCCAACAAAGCAACACAAAGCAAAAAACATGATATGGCAAAGGAAATCCA
F	Forward	AATCGGTCCGATGGTAAGCGCTATTGTTTTATATGTGCTTTTGGCGGCGGCGGCGCATTCTG
G	Reverse	AATAAGCTTTTAATATTGTCTATTACGGTTTTGGCAGGCCCACATGATGAACC

protein. The formalin and BEI inactivated whole virus contained between 118–120 ng/μg of total VP1 protein while Bac-VP1 contained approximately 39 ng/μg of VP1. Interestingly, this VP1 content was higher than the 13 ng/ug of total VP1 protein of the previously published Bac-Pie1-gp64-VP1 construct (data not shown).

Nine female Balb/c mice (6 wks old) were immunized intramuscularly with 200 μl of either 10^7 PFU/ml of live Bac-VP1 or inactivated Bac-VP1 containing 0.78 μg of VP1, and a subsequent booster was given on day 21. As negative controls, 9 mice were injected with either purified wild type baculovirus (Bac-wt) or PBS. As positive controls, 100 μl of 10^7 PFU/ml of formalin inactivated EV71-C4 virus or 10^7 PFU/ml of BEI inactivated EV71-C4 virus, which contained approximately 1.18 and 1.20 μg of VP1 respectively, were injected intramuscularly with complete Freund's adjuvant and the subsequent booster dose was given with incomplete Freund's adjuvant. All the animal procedures were conducted under institutionally approved protocols. To determine the *in vivo* transduction ability of Bac-VP1, three mice each from the Bac-VP1, inactivated Bac-VP1 and PBS immunized groups were euthanized on day 6. Muscle tissue was collected in 10% (w/v) buffered formalin, embedded in paraffin and immunohistochemical analysis was done by using anti-VP1 monoclonal antibody (mAb). The results revealed that live baculovirus was able to transduce the muscle cells and express VP1 (Fig. 3).

Serum samples from the immunized mice were collected on days 14, 28, 42 and 56. VP1 specific antibodies in the sera were measured by indirect enzyme-linked immunosorbent assay (ELISA). The results showed that mice vaccinated with BEI inactivated EV71 elicited the highest antibody levels against VP1. In contrast, the antibody levels of mice vaccinated with formalin inactivated EV71 were comparatively lower (data not shown). Interestingly, mice vaccinated with Bac-VP1 had enhanced specific antibody responses compared to the Bac-Pie1-gp64-VP1 construct of our previous study (Meng et al., 2011). Serum microneutralization tests against 100 $TCID_{50}$ of both homologous and heterologous EV71 genotypes were performed on day 56 according to the previously described protocol (Meng et al., 2011). The results showed that mice immunized with BEI inactivated EV71 had significantly higher neutralizing antibody titers (up to 2^{10}) compared with mice vaccinated with formalin inactivated EV71 (up to 2^6, $p < 0.001$; Fig. 4). Interestingly, sera from Bac-VP1 immunized mice were nearly as efficient in neutralizing different EV71 genotypes (2^8–2^9) as sera from BEI inactivated EV71 controls. Additionally, these neutralization titers were significantly higher compared with those elicited by Bac-Pie1-gp64-VP1 (2^6) in our previous study (Meng et al., 2011).

The level of protective immunity induced by our vaccine constructs was determined in a suckling mouse model. First, mouse-adapted EV71-B4 strain was produced by inoculating 1-day-old Balb/c mice intraperitoneally (i.p.) with 10^7 $TCID_{50}$ of parental EV71-B4 strain (Genbank accession #AF316321.2). Five days later, the mice were euthanized and their brain tissues were dissected. After homogenization, the tissue lysates were collected, sterile fil-

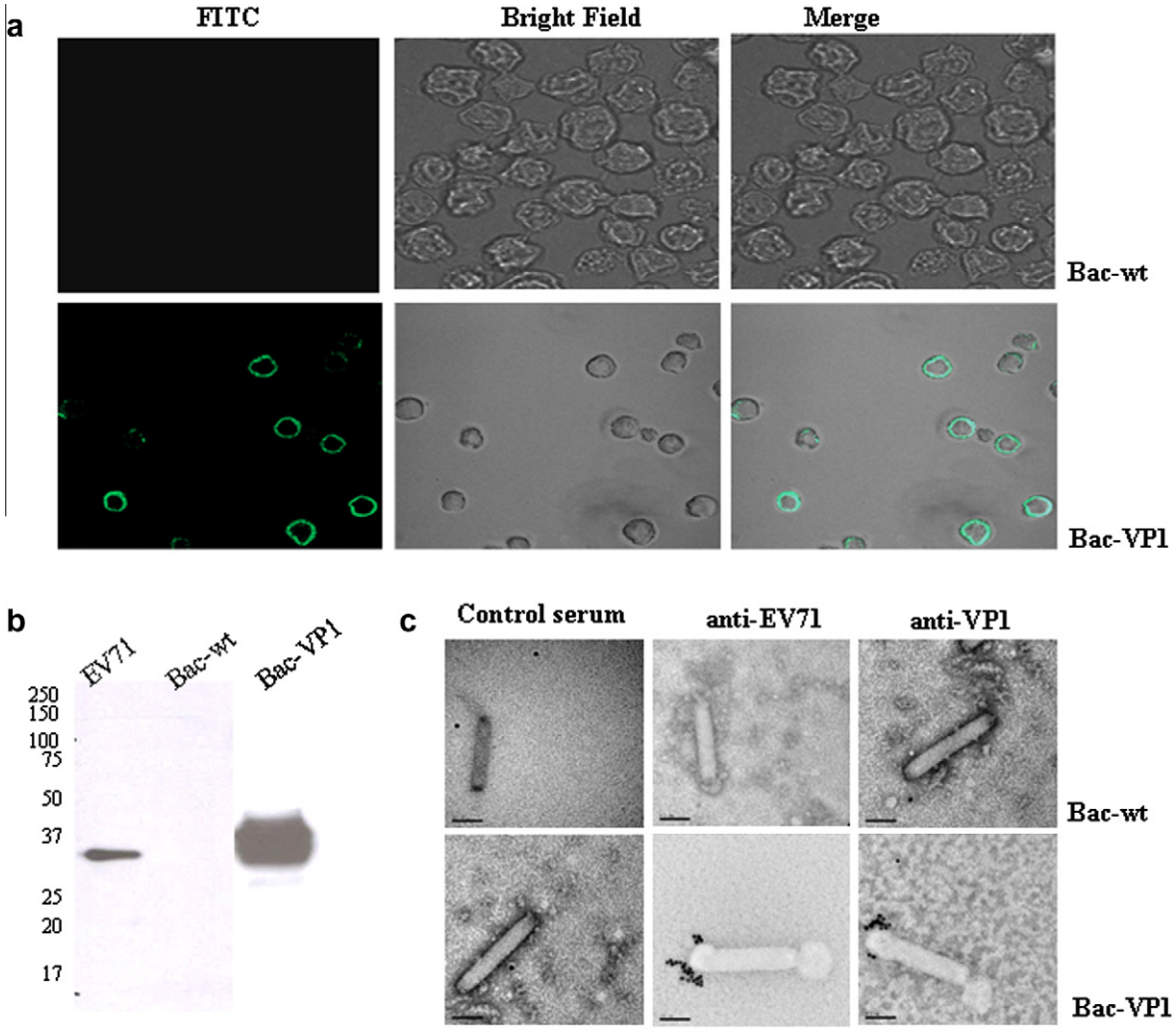

Fig. 2. Anchoring of VP1 on SF9-II cells and VP1 incorporation into the baculovirus envelope. (a) Confirmation of the anchoring of VP1 on the plasma membrane of Sf9-II cells infected with Bac-VP1. No anchoring of VP1 was observed on the plasma membrane of Sf9-II cells infected with Bac-wt. The cells were cultured on sterile cover slips and infected at an MOI of 0.1. Cells were fixed with 4% PFA and blocked with 2% bovine serum albumin for 30 min at 37 °C. VP1 was detected by a polyclonal primary antibody against bacterially expressed VP1 raised in guinea pig (1:300 dilution; in house production) followed by a secondary FITC-conjugated rabbit anti-guinea pig mAb (1:100 dilution; Dako). (b) Western blot analysis of inactivated EV71, Bac-wt and Bac-VP1 using anti-VP1 mAb and respective secondary antibody. (c) Immunogold electron micrographs of purified Bac-VP1 and Bac-wt detected by negative control serum, anti-EV71, and anti-VP1 mouse polyclonal sera as the primary antibody (1:500; in house production) and anti-mouse IgG conjugated to 10 nm gold particles as the secondary antibody (1:25; Sigma).

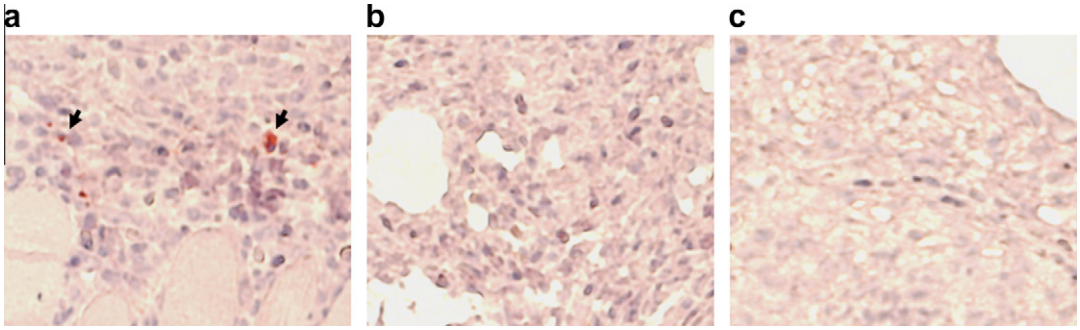

Fig. 3. Bac-VP1 transduction and expression of VP1 *in vivo*. (a) Live Bac-VP1, (b) inactivated Bac-VP1, and (c) PBS immunized mice muscle tissues were embedded in paraffin and sectioned. Immunohistochemical staining was carried out using anti-VP1 mAb and HRP-conjugated rabbit anti-mouse antibody. Arrows indicate the successful transduction and expression of VP1.

tered and used for further inoculation (i.p.) into 1-day-old Balb/c mice. Six mouse brain passages later, the adapted virus caused 100% mortality in 6 days old mice. A passive immunization study was conducted using 6 days old Balb/c mice inoculated (i.p.) with

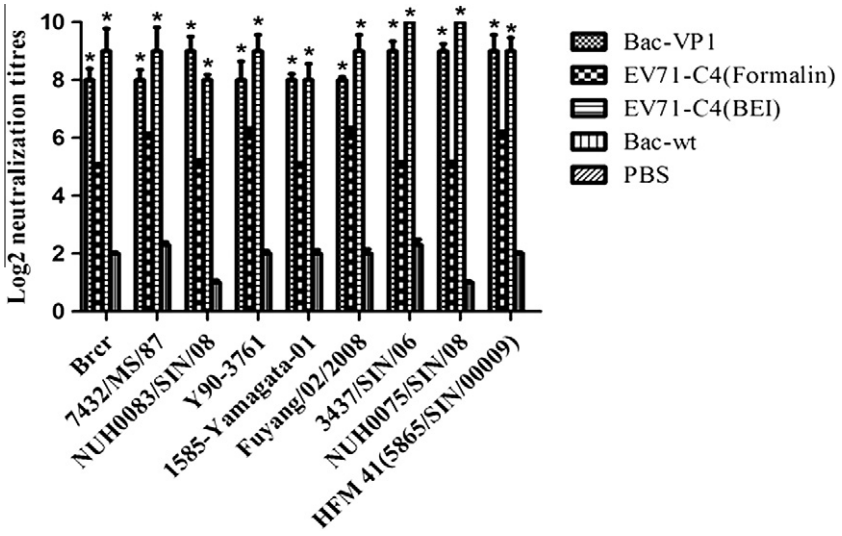

Fig. 4. Microneutralization assay. Sera, collected from mice 56 days post vaccination, were first incubated at 56 °C for 30 min to inactivate the complement. Twenty-five microlitre of serial twofold dilutions of sera were then mixed with 25-μl of 100 $TCID_{50}$ of virus, and incubated at 37 °C for 2 h to neutralize infectious virus. The mixtures were then transferred to 96-well plates with more than 90% confluent monolayers of RD cells grown in DMEM containing 5% FBS. After incubation for 5 days at 37 °C, the neutralizing antibody titers were read as the highest dilution of sera that completely inhibited virus growth. The data represent the mean ± SD of three independent assays. *Denotes data points of statistical significance ($p < 0.001$) compared to EV71 (Formalin) vaccinated mice.

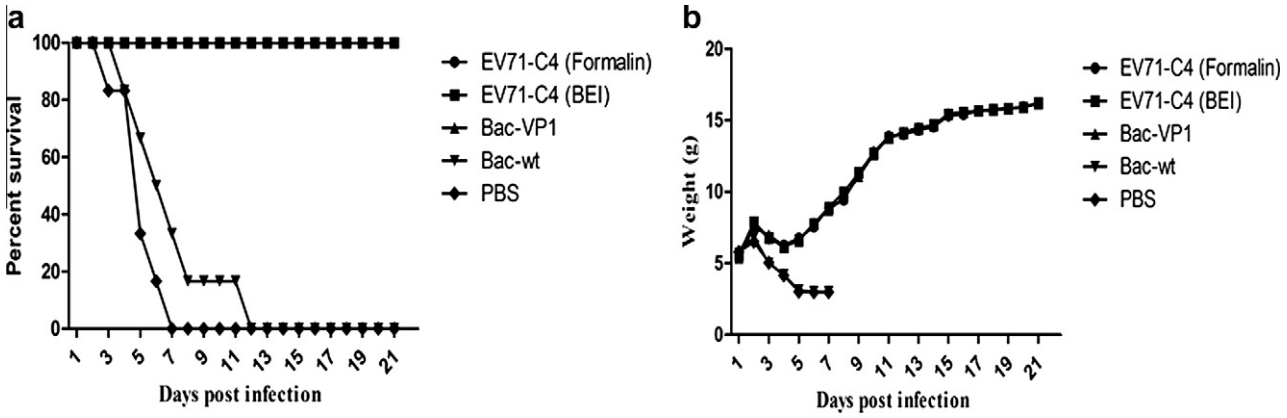

Fig. 5. *In vivo* passive immunization studies. Six days old Balb/c mice were inoculated i.p. with mouse adapted EV71-B4 strain at a dose of 5 MLD_{50} per mouse. Twenty-four hours later, each mouse was passively immunized with sera obtained from the vaccinated adult mice. The control groups received sera from Bac-wt and PBS immunized mice. Mortality and body weight loss were monitored until 21 days post infection. (a) Percent survival. (b) Body weight.

5 MLD_{50} of the mouse adapted EV71-B4. Twenty four hours later, each mouse was passively immunized with serum obtained from a previously vaccinated mouse. Mice that received sera from negative control groups (Bac-wt and PBS) presented with the typical symptoms of EV71 infection 3 days post-inoculation. Seven days post-inoculation, all the mice developed muscle limb paralysis and subsequently died. In contrast, mice that were treated with sera from the Bac-VP1, EV71 (Formalin) or EV71 (BEI) vaccinated mice remained healthy and survived throughout the 21-day observation period (Fig. 5). Immunohistochemical analysis was conducted on the brain tissues from unprotected and protected mice. EV71 antigens were detected in the brains of unprotected mice passively immunized with Bac-wt or PBS derived sera but not in the brains of survivors protected by Bac-VP1 or EV71 derived sera (Fig. 6). Furthermore, real time quantitative analysis of the brain tissues put the total viral RNA copies of unprotected mice at approximately 1×10^4 copies/ml, whereas no viral copies could be detected in the protected mice (data not shown).

Several strategies have been applied to develop EV71 vaccines with the most advanced being inactivated whole virus vaccines. The inactivated vaccine showed protective activity against lethal EV71 infection in mouse models (Bek et al., 2011; Dong et al., 2011; Ong et al., 2010). A commercially produced formalin-inactivated vaccine (Sinovac Biotech Ltd., Beijing, Peoples' Republic of China) showed protection against lethal challenge with the mouse-adapted subgenogroup B3 strain MP-26M (Chua et al., 2008). This vaccine candidate has recently completed a phase 1 clinical trial (ClinicalTrails.gov Identifier NCT01267903). One of the major disadvantages of inactivated vaccines is the possible degradation of epitopes due to the inactivation process which can reduce the efficiency of a vaccine. In addition, these vaccines need proper bio-containment facilities for their production which are not readily available in developing countries. Hence, it is important to develop a safe, cost effective and easily produced EV71 vaccine. Many viruses, which are currently being employed for gene therapy, utilize humans as a host for their replication. In contrast, baculovirus is incapable of replication in mammalian cells (Tjia et al., 1983) and more importantly, there is no preexisting immunity against baculovirus in humans (Strauss et al., 2007). Although these observations might make baculovirus one of the

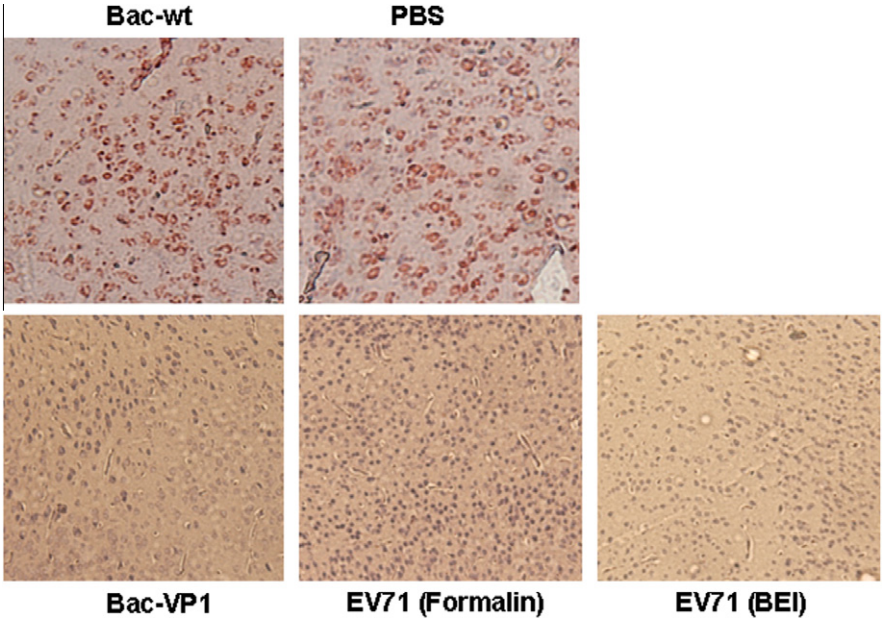

Fig. 6. Immunohistochemical analysis of EV71 in brain tissue. Brain tissue of unprotected and protected mice was collected, embedded in paraffin and sectioned. Immunohistochemical staining was carried out by using anti-VP1 mAb (1:100) followed by HRP-conjugated rabbit anti-mouse antibody (DAKO).

safest vaccine vectors, its application in humans remains to be verified and toxicity studies must be performed in animals before its use in clinical trials.

In conclusion, we have shown that Bac-VP1 is efficient in inducing high neutralization antibody titers that can cross-neutralize all subgenogroups. Bac-VP1 does not require sophisticated bio-containment infrastructure. Further, the VP1 protein of any new subgenogroup could be converted into an efficient vaccine in a short period of time. Currently we are evaluating the oral efficacy of the vaccine and the safety of the baculovirus system, after which we intend to advance to the clinical stage if our findings are favorable.

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