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Construction, *in Silico* Analysis, and *in Vitro* expression of DNA vaccine candidate encoding human Rotavirus capsid protein VP6

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Abstract
Rotavirus is common cause of severe diarrhea which responsible for 200,000 deaths of children per year. Several live attenuated rotavirus vaccines have been developed and licensed, but it is important to develop new generation of rotavirus vaccine with better safety. In this research, we developed rotavirus DNA vaccine candidate encoding immunogenic VP6 protein from human rotavirus RV4 (HRV-RV4 VP6). VP6 gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) and inserted into pVAX1 vector. The resultant recombinant plasmid, pVAX1-VP6, was analyzed and transfected into mammalian cells. The results revealed that HRV-RV4 VP6 gene, encoding a protein of 397 amino acids, has high homology to VP6 protein of human rotavirus G1P[8]. Three point mutations were found (T246I, H316Y, and I385V) and predicted did not affect the three-dimensional (3D) structure of the HRV-RV4 VP6 protein. However, epitope analysis showed that these mutations might increase the antigenicity of HRV-RV4 VP6. Expression results confirmed that VP6 was expressed since 24 hours after transfection in Vero cells. This study suggested that pVAX1-VP6 can be further developed as rotavirus DNA vaccine candidate because it contained potential epitopes and could be expressed in mammalian cells.

Keywords: Rotavirus; DNA vaccine; VP6; Epitope.

INTRODUCTION

Rotavirus infection is the leading cause of severe diarrhea, responsible for approximately 200,000 deaths per year in children under five years of age worldwide (Tate *et al.*, 2016a). A tetravalent rhesus-based live attenuated rotavirus vaccine (RotaShield) has been developed and introduced to the market in 1998, but one year later it was withdrawn because of association with increased risk of intussusception (CDC, 1999a; CDC, 1999b). Several years later, the new rotavirus vaccines (RotaTeq and Rotarix) have been developed and licensed. Both live attenuated vaccines were shown to be highly efficacious in preventing rotavirus gastroenteritis and safe in clinical trial (Vesikari *et al.*, 2006; Ruiz-Palacios *et al.*, 2006). However, recent studies identified that these vaccines were also associated with small increased risk of intussusception in children 8 to 11 weeks (Tate *et al.*, 2016b).

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DNA vaccine can be alternatives for rotavirus vaccine development to minimize the risk of intussusception. DNA vaccine is non-replicating vaccine that can induce both humoral and cellular immune responses (Kutzler *et al.*, 2008). These vaccines have shown success in most animal studies, had good safety and tolerability profile in human (Kibuka *et al.*, 2010; Liu, 2011). Several studies of rotavirus DNA vaccine development containing gene VP4, VP6, and VP7 from murine rotavirus have been done and shown promising (Herrmann *et al.*, 1996; Chen *et al.*, 1998; Herrmann *et al.*, 1999; Yang *et al.*, 2001).

Rotaviruses are non-enveloped icosahedral virions, composed of three protein layers (Estes *et al.*, 1989). Rotavirus genome made up of 11 segments of double stranded (ds) RNA, encoding 6 structural (VP) and 6 non-structural (NSP) protein (Pesavento *et al.*, 2006). VP6 is the major structural protein in rotavirus particles which plays a key role in the morphology of the virion by interacting with the outer layer and inner layer proteins (Pesavento *et al.*, 2006). Interestingly, VP6 is antigenically conserved protein among group A

rotaviruses, so thus VP6-based vaccines could potentially provide heterotypic protection (Chen *et al.*, 1998).

In this study, we developed recombinant plasmid expressing VP6 protein derived from the human rotavirus strain RV4 (HRV-RV4) as rotavirus DNA vaccine candidate for human use. In silico analysis was performed to predict the structure and potential epitopes of VP6. Finally, in vitro study was conducted to analyze the capability of DNA vaccine candidate to be expressed in mammalian cells.

MATERIALS AND METHODS

Cell Culture and Virus Strai

MA104 and Vero cells were grown at 37^0C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, Gibco). HRV-RV4 (provided by Bio Farma) was propagated in MA104 cell culture using serum free medium. The virus was pretreated with 10 µg/mL trypsin (Sigma) before added to the cells. The culture was incubated for 2 d and harvested by freezing and thawing, followed by centrifugation. Rotavirus RNA was isolated from culture supernatant using SV total RNA isolation system (Promega).

Amplification of HRV-RV4 VP6

A pair of PCR primer was designed according to the published sequence of the human rotavirus VP6 in GenBank (no. X57943.1). cDNA was generated by RT-PCR that performed in a 50 µl mixture containing 1X AccessQuick master mix, 100 ng of each primer, 3 µl of extracted RNA, and 1 µl of AMV-reverse transcriptase (Promega). The reaction was run in a thermocycler with the following program: incubation at 45^0C for 45 min, 30 cycles composed of denaturation at 94^0C for 1 min, annealing at 45^0C for 2 min, and extension at 72^0C for 1 min. cDNA sequence was used for designing a new pair of PCR primer that will be used to clone the VP6 gene into pVAX1 plasmid.

The re-amplification was performed in a 50 µl mixture containing 1X AccuPrime PCR buffer I, 50 pmol of each primer, 1 µl of DNA, and 0.5 µl of AccuPrime Taq high fideality (Invitrogen). The reaction was run in a thermocycler with the following program: denaturation at 94^0C for 2 min, 30 cycles composed of denaturation at 94^0C for 1 min, annealing at 56^0C for 1 min, and extension at 72^0C for 1 min, and was ended with a final extension step of 7 min at 72^0C. The amplified product was analyzed on 1.2% agarose gels stained with SYBR Safe DNA gel stain (Invitrogen) and purified by QIAquick gel extraction kit (Qiagen).

Construction of Recombinant Plasmid pVAX1-VP6

The cDNA encoding human rotavirus VP6 protein was inserted by cohesive-end ligation into *EcoR*V and *Xho*I sites of pVAX1 expression vector (Invitrogen). The recombinant plasmid, pVAX1-VP6, was transformed into *Escherichia coli* TOP10 by heat shock at 42^0C for 2 min. The transformed bacteria were plated onto LB plate with 50 µg/ml kanamycin for selection. Plasmids candidates were identified by PCR and sequencing.

in Silico Analysis of HRV-RV4 VP6

The HRV-RV4 VP6 gene and protein sequences were compared with VP6 gene and protein sequences available in the GenBank database using BLAST software. The three-dimensional structure of the HRV-RV4 VP6 protein was predicted by SWISS-MODEL (Arnold *et al.*, 2006; Guex *et al.*, 2009; Kiefer *et al.*, 2009; Biasini *et al.*, 2014). Visualization of the molecules were done using PyMOL. Epitope predictions (MHC-I and MHC-II binding predictions) were made by IEDB analysis resource (Nielsen *et al.*, 2003; Peters *et al.*, 2005; Lundegaard *et al.*, 2008; Sidney *et al.*, 2008; Wang *et al.*, 2008; Wang *et al.*, 2010; Kim *et al.*, 2012; Andreatta *et al.*, 2016) using allele from Austronesian ethnic (HLA-A*11:01, HLA-B*15:02, HLA-DPA1*02:01/DPB*05:01, HLA-DQA1*05:01/DQB*03:01, and HLA-DRB1*15:02). Protein docking prediction was created using ClusPro server (Comeau *et al.*, 2004a; Comeau *et al.*, 2004b; Kozakov *et al.*, 2006; Kozakov *et al.*, 2013).

in Vitro Transfection and Expression of HRV-RV4 VP6

pVAX1-VP6 plasmid was transfected into Vero cells in ordered to detect the mRNA and protein expression of VP6. Vero cells were cultured in a 96-well tissue culture plate until the cells reached approximately 70-90% confluence. Plasmid DNA transfection was performed with Lipofectamine 3000 (Invitrogen) as specified by the manufacturer. Briefly, 0.1 µg of plasmid DNA was diluted with 5 µl OptiMEM containing 0.2 µl P3000 reagent. This master mix of DNA was added to 5 µl of OptiMEM medium containing 0.3 µl of Lipofectamine 3000 and incubated for 10 min at room temperature. DNA-liposome complexes were added to each well containing of Vero cell with 100 µl of OptiMEM. Transfected cells were incubated at 37^0C, 5% CO2, for 24-120 h. For negative control, the same preparation was made but without addition of plasmid DNA.

HRV-RV4 VP6	0	MEVLYSLSKTLKDARDKIVEGTLYSNVSDLIQQFNQMIVTMNGNDFQTGG	50
HRV-G1P[8] VP6	0	MEVLYSLSKTLKDARDKIVEGTLYSNVSDLIQQFNQMIVTMNGNDFQTGG	50
HRV-RV4 VP6	51	IGNLPVRNWTFDFGLLGTTLLNLDANYVENARTTIEYFIDFIDNVCMDEM	100
HRV-G1P[8] VP6	51	IGNLPVRNWTFDFGLLGTTLLNLDANYVENARTTIEYFIDFIDNVCMDEM	100
HRV-RV4 VP6	101	ARESQRNGVAPQSEALRKLSGIKFKRINFDNSSEYIENWNLQNRRQRTGF	150
HRV-G1P[8] VP6	101	ARESQRNGVAPQSEALRKLSGIKFKRINFDNSSEYIENWNLQNRRQRTGF	150
HRV-RV4 VP6	151	VFHKPNIFPYSASFTLNRSQPMHDNLMGTMWLNAGSEIQVAGFDYSCAIN	200
HRV-G1P[8] VP6	151	VFHKPNIFPYSASFTLNRSQPMHDNLMGTMWLNAGSEIQVAGFDYSCAIN	200
HRV-RV4 VP6	201	APANIQQFEHIVQLRRALTTATITLLPDAERFSFPRVINSADGATIWFFN	250
HRV-G1P[8] VP6	201	APANIQQFEHIVQLRRALTTATITLLPDAERFSFPRVINSADGATTWFFN	250
HRV-RV4 VP6	251	PVILRPNNVEVEFLLNGQIINTYQARFGTIIARNFDTIRLSFQLMRPPNM	300
HRV-G1P[8] VP6	251	PVILRPNNVEVEFLLNGQIINTYQARFGTIIARNFDTIRLSFQLMRPPNM	300
HRV-RV4 VP6	301	TPAVNALFPQAQPFQYHATVGLTLRIESAVCESVLADANETLLANVTAVR	350
HRV-G1P[8] VP6	301	TPAVNALFPQAQPFQHHATVGLTLRIESAVCESVLADANETLLANVTAVR	350
HRV-RV4 VP6	351	QEYAIPVGPVFPPGMNWTELITNYSPSREDNLQRVFTVASIRSMLIK	397
HRV-G1P[8] VP6	351	QEYAIPVGPVFPPGMNWTELITNYSPSREDNLQRIFTVASIRSMLIK	397

Figure 1: Amino acid alignment of HRV-RV4 VP6 and HRV-G1P[8] VP6 (GenBank no. AEK69541.1). Conserved histidine residue (His 153) was indicated by yellow color. Mutations were indicated by magenta color (T246I, H316Y, and I385V).

Transcription analysis of VP6 gene was performed using RT-PCR. mRNA of Vero cells were isolated using SV total RNA isolation system (Promega). Amplification was performed in a 50 µl mixture containing 1X Super Script buffer, 50 pmol of each primer, 3 µl of RNA, and 1 µl of RT/Platinum Taq mix (Invitrogen). The reaction was run in a thermocycler with the following program: incubation at 45^0C for 45 min, 30 cycles composed of denaturation at 94^0C for 1 min, annealing at 56^0C for 2 min, and extension at 72^0C for 1 min. The amplified product was analyzed on 1.2% agarose gels stained with SYBR Safe DNA gel stain (Invitrogen).

Expression of VP6 protein was identified by immunofluorescence. Transfected cells were washed with washing buffer (PBS containing 0.05% Tween 20). Cell fixation was performed by fixation solution (methanol-acetone) for 10-15 min at room temperature. Blocking solution (washing buffer containing 1% BSA) was added to each well and incubated for 2h at room temperature. First antibody (provided by Bio Farma) was added to each well and incubated for 1 h at room temperature. Cells were washed with washing buffer before addition of second antibody (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen) followed by incubation for 1h at room temperature. Cells were washed with washing buffer before observed by fluorescence microscope.

RESULTS

Construction of Recombinant Plasmid pVAX1-VP6

HRV-RV4 VP6 gene was amplified by PCR with the length of 1219 bp. VP6 gene was successfully inserted into pVAX1 (3000 bp) plasmid generated recombinant plasmid pVAX1-VP6 (4219 bp), indicated by PCR results (data not shown). The nucleotide sequence of the VP6 showed that the gene maintained in correct open reading frame encoding a protein of 397 amino acid.

in Silico Analysis of HRV-RV4 VP6

The nucleotide sequence of HRV-RV4 VP6 gene had 98% homology to VP6 gene of human rotavirus (HRV) G1P[8] (GenBank no. JN258858.1) (data not shown). The amino acid sequence of HRV-RV4 VP6 protein showed 99% homology to VP6 protein of HRV-G1P[8] (GenBank no. AEK69541.1) (data not shown). The amino acid alignment result between HRV-RV4 VP6

and HRV-G1P[8] VP6 showed that both protein had histidine residue at position 153 which strictly conserved among group A rotaviruses but not in other serogroups (Fig. 1) (Mathieu *et al.*, 2001). This residue responsible for Zn^{+} binding, facilitated by a hydrogen bond between the Nδ of His 153 and the main chain carbonyl of residue 339 from adjacent subunit (Mathieu *et al.*, 2009). The protein alignment results also showed that three point mutation was found in HRV-RV4 VP6 protein (T246I, H316Y, and I385V) (Fig. 1).

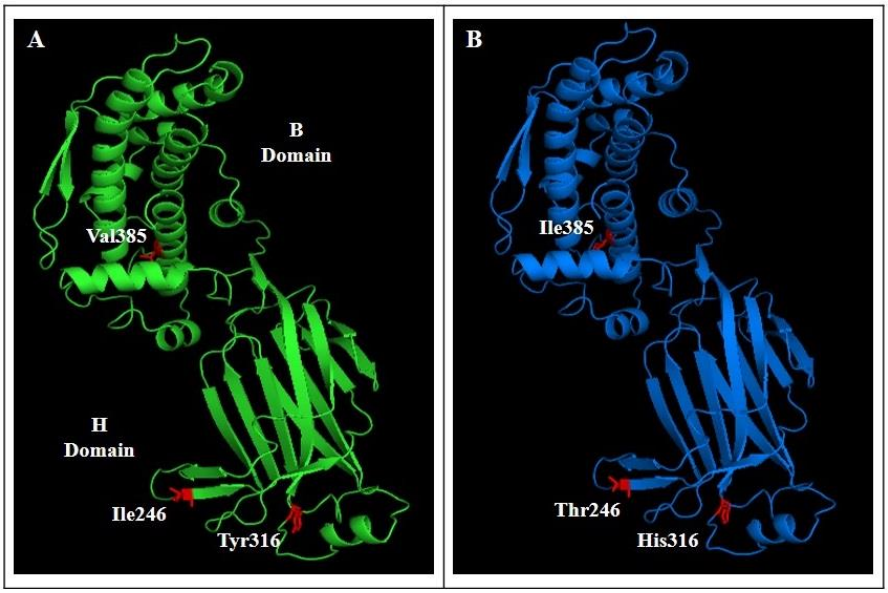

Figure 2. 3D structure prediction of HRV VP6. (A) Structure of HRV-RV4 VP6. (B) Structure of HRV-G1P[8] VP6 (GenBank no. AEK69541.1 VP6).

Three-dimensional (3D) structure of the HRV-RV4 VP6 protein was predicted based on corresponding crystal structure of rotavirus bovine intermediated capsid protein VP6 (SMTL id. 4v7q.1) (Fig. 2). The VP6 protein has two domains, B domain consist of eight α-helixes and H domain consist of β-sheet (Mathieu *et al.*, 2001; Pesavento *et al.*, 2006). Based on 3D structure of HRV-RV4 VP6 protein, the mutations were located at B domain (I385V) and H domain (T246I and H316Y) (Fig. 2). These mutations were predicted did not give any changes on the structure of the VP6 protein (Fig. 2).

Epitope prediction was conducted using IEDB software to identify the HRV-RV4 VP6 fragments which can bind to MHC-I and MHC-II molecules. The data showed that this protein has many potential epitopes (data not shown). The data also showed that all mutations which occurred at HRV-RV4 VP6 protein were located at epitope regions. This is silico result was in line with the previous in vivo analysis conducted by Choi *et al.* (2000), which showed T246I and I385V mutations were located at epitope regions (Choi *et al.*, 2000). However, different result was showed for H316Y mutation. This might caused by different allele which used on both research.

Effect of mutations on VP6 protein antigenicity were predicted based on the percentile rank (PR) value. A small numbered PR indicates high affinity of the candidate epitopes to the allele. PR value showed that the mutation at position 246 gave the highest effect to the VP6 antigenicity (Table 1). This might caused by the different properties of threonine and isoluecine.

Further study to identify the effect of the mutation to VP6 antigenicity was done by docking analysis (Fig. 3). Docking prediction of HLA-A*11:01 (receptor) with $VP6_{244-252}$ epitope (ligand) showed that the third residue (P3) of the ligand was crucial for epitope binding. P3 is one of the anchors that will bind to HLA-A*11:01. Binding probability of the epitopes will increase if P3 is the hidrophobic residue (Leu, Phe, Try, Ile, or Ala) (Chujoh *et al.*, 1998). So that, $VP6_{244-252}$ HRV-RV4 which has Ile at P3 position has better binding probability to HLA-A*11:01, compared with $VP6_{244-252}$ HRV-G1P[8] which has Thr (polar residue) at P3 position. Ile was predicted will interact with hidrophobic residue Val67 that located at binding region of HLA-A*11:01 (Fig. 3). These results suggested that the mutations might increase the antigenicity of HRV-RV4 VP6.

Expression of HRV-RV4 VP6 in Vero Cells

pVAX1-VP6 expression analysis performed by seeing the presence of VP6 mRNA and VP6 protein for 120 h. The results showed that the transcription of VP6 gene was detected since 24 h post-transfection (Fig. 4), indicated by single band with 1219 bp in size, which corresponds to the size of VP6 gene.

VP6 transcription will occurred if pVAX1-VP6 plasmid can pass through the nuclear membrane and enter the nucleus of transfected Vero cells. The plasmid entry into the nucleus of dividing cells was facilitated by the reorganization of the nuclear membrane when mitosis occured (Dean *et al.*, 2005). Another researchs showed that plasmid entry into the cell nucleus could be facilitated by lipoplex. Fusion of lipopex with nuclear membrane will initiates release of the plasmid into the nucleus (Kamiya *et al.*, 2002; Khalil *et al.*, 2006).

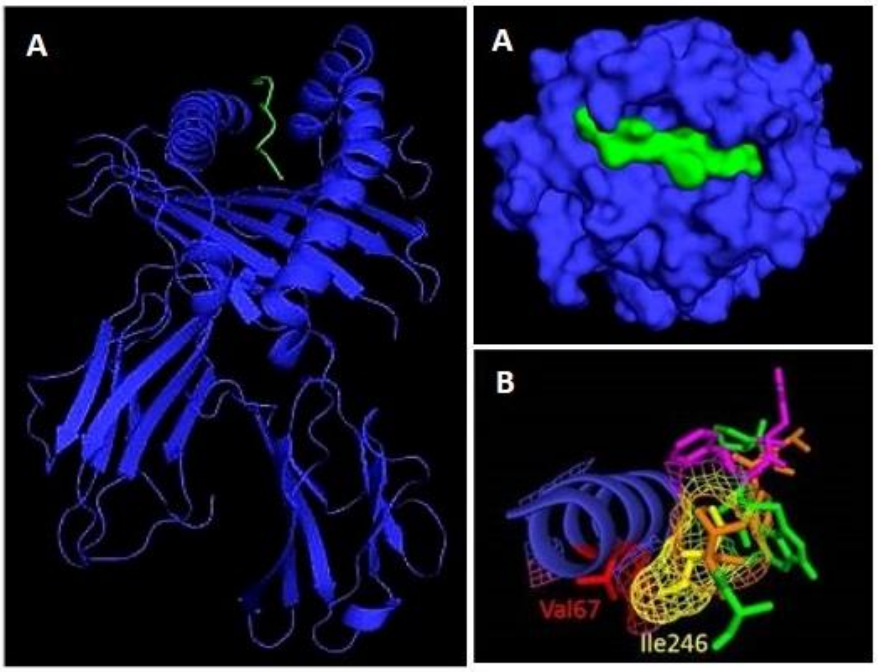

Figure 3. Docking prediction of HLA-A*11:01 with $VP6_{244-258}$. (A) Complex structure of $VP6_{244-258}$ and HLA-A*11:01. (B) Interaction of $VP6_{244-258}$ Ile246 residue (yellow) with HLA-A*11:01 Val67 residue (red) at receptor binding site.

The results also showed that VP6 protein production had been occurred since 24 h post-transfection, indicated by fluorescence cells (Fig. 4). The previous research conducted by Ljungberg *et al.* (2007), which using the same expression vector (pVAX1-GFP), also showed that GFP protein expression was detected since 18-24 h post-transfection. Protein expression reached the peak after 48 h post-transfection, but then decreased gradually (Ljungberg *et al.*, 2007).

The immunofluorescence assay results showed that production of VP6 protein decreased gradually and only small number of fluorescence cells were detected after 120 h post-transfection (Fig. 4). VP6 gene expression is determined by the number of pVAX1-VP6 which internalized into the cell, because pVAX1 is not designed to replicate in mammalian cells and do not integrate with host cell chromosome. Protein production reduction can also caused by the death of transfectant and the half-life of the plasmid (Weiss *et al.*, 2012). The half-life of DNA plasmid in the cytoplasm is about 90 minutes (Weiss *et al.*, 2012). DNA in the cytoplasm can not survive for a long time because it can be degraded by cytosolic nuclease (Weiss *et al.*, 2012).

CONCLUSION

Recombinant plasmid expressing VP6 protein derived from HRV-RV4 (pVAX1-VP6) has been successfully constructed. This protein shared high homology with VP6 protein of HRV-G1P[8] (GenBank no. AEK69541.1), but three point mutation was identified (T246I, H316Y, and I385V). These mutations were predicted did not effect the 3D structure of the VP6 protein, but it might increased the antigenicity of VP6. Expression results confirmed that pVAX1-VP6 could be expressed in mamalian cells. All these data suggested that pVAX1-VP6 can be further developed as a DNA vaccine candidate against rotavirus.

Conflict of interest

There is no conflict of interest associated with the present manuscript.

References

Andreatta M and Nielsen M (2016) Gapped sequence alignment using artificial neural networks: application to the MHC class I system. *Bioinformatics*, **32**: 511-7.

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environtment for protein structure homology modelling. *Bioinformatics*, **22**: 195-201.

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucl. Acid Res*., **42**(W1): W252-W258.

CDC (1999a) Intussusception among recipients of rotavirus vaccine-United States, 1998–1999. *MMWR*, **48**(27): 577–581.

CDC (1999b) Withdrawal of rotavirus vaccine recommendation. *MMWR*, **48**(43): 1007.

Chen SC, Jones DH, Fynan EF, Farrar GH, Clegg JCS, Greenberg HB, Herrmann JE (1998) Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. *J. Virol*., **72**(7): 5757-5761.

Choi AHC, Basu M, McNeal MM, Flint J, VanCott JL, Clements JD, Ward RL (2000) Functional mapping of protective domains and epitopes in the rotavirus VP6 protein. *J. Virol*., **74**(24): 11574-11580.

Chujoh Y, Sobao Y, Miwa K, Kaneko Y, Takiguchi M (1998): The role of anchor residues in the binding of peptides to HLA-A*1101 molecules. *Tissue Antigens*, **52**(6): 501-509.

Comeau SR, Gatchell DW, Vajda S, Camacho CJ (2004a) ClusPro: an automated docking and discrimination method for the prediction of protein complexes. *Bioinformatics*, **20**(1): 45-50.

Comeau SR, Gatchell DW, Vajda S, Camacho CJ (2004b) ClusPro: a fully automated algorithm for protein-protein docking. *Nucl. Acid Res*., **32**: W96-W99.

Dean DA, Strong DD, Zimmer WE (2005): Nuclear entry of nonviral vectors. *Gene Therapy*, **12**(11): 881-890.

Estes MK and Cohen J (1989) Rotavirus gene structure and fuction. *Microbiol. Rev*., **53**(4): 410-449.

Guex N, Peitsch MC, Schwede T (2009) Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. *Electrophoresis*, **30**(S1): S162-S173.

Herrmann JE, Chen SC, Fynan EF, Santoro JC, Greenberg HB, Wang S, Robinson HL (1996) Protection against rotavirus infections by DNA vaccination. *J. Infect. Dis*., **174**(Suppl 1): S93-S97.

Herrmann JE, Chen SC, Jones DH., Tinsley-Bown A, Fynan EF, Greenberg HB, Farrar GH (1999) Immune responses and protection obtained by oral immunization with rotavirus VP4 and VP7 DNA vaccines encapsulated in microparticles. *Virology*, **259**(1): 148-153.

Kamiya H, Fujimura Y, Matsuoka I, Harashima H (2002) Visualization of intracellular trafficking of exogenous DNA delivered by cationic liposomes. *Biochem. Biophys. Res. Comm*., **298:** 591–597.

Khalil IA, Kogure K, Akita H, Harashima H (2006) Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol. Rev*., **58(1)**: 32-45.

Kiefer F, Arnold K, Kunzli M, Bordoli L, Schwede T (2009) The SWISS-MODEL Repository and associated resources. *Nucl. Acid Res*., **37**: D387-D392.

Kibuuka H, Kimutai R, Maboko L, Sawe F, Schunk MS, Kroidl A, Shaffer D, Eller LA, Kibaya R, Eller MA, Schindler KB, Schuetz A, Milliard M, Kroll J, Dally L, Hoelscher M, Bailer R, Cox JH, Marovich M, Birx DL, Graham BS, Michael NL, de Souza MS, Robb ML (2010) A Phase ½ study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus serotype 5 boost vaccine in HIV-uninfected East Africans (RV 172). *J. Infect. Dis*., **201**(4): 600-607.

Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, Lundegaard C, Sette A, Lund O, Bourne PE, Nielsen M, Peters B (2012) Immune epitope database analysis resource. *Nucl. Acid Res.*, **40**: W525-30.

Kozakov D, Brenke R, Comeau SR, Vajda S (2006) PIPER: an FFT-based protein docking program with pairwise potentials. *Proteins*, **65**: 392-406.

Kozakov D, Beglov D, Bohnuud T, Mottarella S, Xia B, Hall DR, Vajda, S (2013) How good is automated protein docking? *Proteins: Struc. Funct. Bioinf.*, **81**(12): 2159-2166.

Kutzler MA and Weiner DB (2008) DNA vaccines: ready for prime time? *Nat. Rev. Genet*., **9**(10): 776-788.

Liu MA (2011) DNA vaccines: an historical perspective and view to the future. *Immunol. Rev*., **239**(1):62-84.

Ljungberg K, Whitmore AC, Fluet ME, Moran TP, Shabman RS, Collier ML, Kraus AA, Thompson JM, Montefiori DC, Beard C, Johnston RE (2007) Increased immunogenicity of a DNA-launched venezuelan equine encephalitis virus-based replicon DNA vaccine. *J. Virol*., **81**(24): 13412-13423.

Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, and Nielsen M (2008) NetMHC-3.0: accurate web accessible predictions of human, mouse, and monkey MHC class I affinities for peptides of length 8-11. *Nucl. Acid Res.*, **36**: W509-512.

Mathieu M, Petitpas I, Navaza J, Lepault J, Kohli E, Pothier P, Prasad BVV, Cohen J, Rey FA (2001) Atomic structure of the major capsid protein of rotavirus: implications for the architecture of the virion. *EMBO*, **20**(7): 1485-1497.

Nielsen M, Lundegaard C, Worning P, Lauemøller SL, Lamberth K, Buus S, Brunak S, Lund O (2003) Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. *Protein Sci.*, **12**: 1007-1017.

Pesavento JB, Crawford SE, Estes MK, Prasad BVV (2006) Rotavirus proteins: structure and assembly. *CTMI*, **309**: 189-219.

Peters B and Sette A (2005) Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. *BMC Bioinformatics*, **6**: 132.

Ruiz-Palacios GM, Perez-Schael I, Velazquez FR, Abate H, Breuer T, Clemens SC, Cheuvart B, Espinoza F, Gillard P, Innis BL, Cervantes Y, Linhares AC, Lopez P, Marcia-Parra M, Ortega-Barria E, Richardson V, Rivera-Medina DM, Rivera L, Salinas B, Pavia-Ruz N, Salmeron J, Ruttimann R, Tinoco JC, Rubio P, Nunez E, Guerrero ML, Yarzabal JP, Damaso S, Tornieporth N, Saez-Llorens X, Vergara RF, Vesikasri T, Bouckenooghe A, Clemens R, De Vos B, O'Ryan M (2006) Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *N. Engl. J. Med*., **354**: 11-22.

Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, Sette A, Peters B (2008) Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries. *Immunome Res*., **4**: 2.

Tate JE, Burton AH, Pinto CB, Parashar UD (2016a) Global, regional, and national estimates of rotavirus mortality in children <5 years of age, 2000-2013. *Clin. Infect. Dis*., **62**(S2): S96-105.

Tate JE, Yen C, Steiner CA, Cortese MM, Parashar UD (2016b). Intussusception rates before and after the introduction of rotavirus vaccine. *Pediatrics*, **138**(3): e20161082.

Vesikari T, Matson, DO, Dennehy P, Damme PV, Santosham M, Rodriguez Z, Dallas MJ, Heyse JF, Goveia MG, Black SB, Shinefield HR, Christie CDC, Ylitalo S, Itzler RF, Coia ML, Onorato MT, Adeyi BA, Marshall GS, Gothefors L, Campens D, Karvonen A, Watt JP, O'Brien KL, Dinubile MJ, Clark HF, Boslego JW, Offit PA, Haeton PM (2006) Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *N. Engl. J. Med*., **354**: 23-33.

Wang P, Sidney J, Dow C, Mothé B, Sette A, Peters B (2008) A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. *PLoS Comput. Biol*., **4**(4): e1000048.

Wang P, Sidney J, Kim Y, Sette A, Lund O, Nielsen M, Peters B (2010) Peptide binding predictions for HLA DR, DP and DQ molecules. *BMC Bioinformatics*, **11**:568.

Weiss R, Tendeloo VV, Scheiblhofer S, Thalhamer J (2012) General mechanisms of gene vaccines. *Gene Vaccines*. Springer, ISBN: 9783709104385, 1-35.

Yang K, Wang S, Chang KO, Lu S, Saif LJ, Greenberg HB, Brinker JP, Herrmann (2001) Immune responses and protection obtained with rotavirus VP6 DNA vaccines given by intramuscular injection. *Vaccine*, **19**(23-24): 3285-3291.