EXPRESSION OF E2 GENE OF CSFV AND ANALYSIS OF EPITOPE DIVERSITY

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SUMMARY

Infection of cells with classical swine fever virus (CSFV) is mediated by the interaction of envelope glycoproteins with receptor molecules on the cell surface. The E2 protein is one of the major envelope antigens for eliciting neutralising antibodies and conferring protective immunity. The study found a distinct substitution of two amino acids at positions 94 (R→G) and 97 (P→S) in the deduced E2 protein sequence of GPE strain of CSFV, which contributed to 0.5% difference from the original sequence. Nevertheless, successful E2 expression from the plasmid cassette was determined via *in vitro* analysis. Two different MAbs, WH303 and WH211, were used for E2 protein detection. The E2 detection was accomplished by using MAb WH211. However, the usage of the MAb WH303 for the E2 expression analyses was unsuccessful. It was observed that the alanine in minimal antigenic domain recognised by WH303 had been substituted with tyrosine in the critical epitope of E2 peptide of GPE$^-$ strain at position 163. Therefore, the mapping data for these neutralising epitopes will be useful for the development of diagnostic tests.

Keywords: Classical swine fever virus, envelope glycoprotein E2, E2 epitopes

INTRODUCTION

Classical Swine Fever (CSF) is a devastating contagious swine disease characterised by symptoms of hemorrhagic fever and immune depression. The disease usually leads to substantial economic losses (Van Oirschot, 2003). Because it is difficult to eliminate its reservoir and interrupt its transmission to susceptible hosts, it is important to increase the resistance of the hosts and develop a safer and more efficacious vaccine to reduce the incidence of CSF and mitigate its associated economic losses. In Malaysia, an attenuated GPE$^-$ Japanese strain of Classical Swine Fever Virus (CSFV) has been used for vaccine preparation to prevent pigs from being infected by this disease. Since the E2 gene of CSFV is the major envelope glycoprotein which is exposed to the outer surface of the virion, it represents an important target for induction of the immune response during infection. This protein can induce neutralising antibodies and confer protective immunity in pigs (Konig *et al.*, 1995).

In this study, an eukaryotic expression vector was constructed to express the CSFV E2 immunogen. The E2 gene sequence of GPE strain has an identical N-linked glycosylation site in terms of number and position, to other 10 CSFV strains as well as specified functional domains (Huang *et al.*, 2000). The E2 gene was cloned and studied for the *in vitro* expression efficiency in cell monolayers.

MATERIALS AND METHODS

Isolation of CSFV RNA and RT-PCR

CSFV RNA was extracted as described by Mittelholzer *et al.* (1997). Viral RNA was extracted using the TRIzol Reagent (Gibco, USA) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by biophotometer (Eppendorf, Germany). The first-strand cDNA spanning the entire E2 gene of CSFV was synthesised from the RNA template by the SUPERSCRIPT II (Life Technologies, U.S.A.) reverse transcriptase PCR (RT-PCR) procedure described by the manufacturer. The reverse primer synthesised for this purpose was END-E2 (5'-TGGGTAATAAGCTT CCCTATCAATAC-3') (Muyldermans *et al.*, 1993). Synthesis of cDNA was carried out in 20μl reaction volumes. One microgram of RNA was mixed with 1μl 10 mM dNTP and 2μl (150 ng/μl) of reverse primer before the mixture was heated to 65°C for 5 minutes and quickly chilled on ice for 2 minutes. The mixture was then spun before 4μl of 5x first strand buffer and 2μl of 0.1M DTT were added. The contents of the tube were mixed gently and incubated at 42°C for 2 minutes. Two hundred units of SUPERSCRIPT II was added into the tube, mixed properly by pipetting the content gently up and down, and incubated for 50 minutes at 42°C. Heating at 70°C for 15 minutes inactivated the reaction. The reaction mixture was then short spun and

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                    62        70         80         90        100        110        120
E2/GPE-  sequencedNDGTVKAICVAGSFKVTALNVVSRRYLASLHKGALSTSVTFELLFDGTNPSTEEMGDDFG
                          |||||||||||||||||||||||||||||||.||.|||||||||||||||||||||
E2/GPE-  GenBank  NDGTVKAICVAGSFKVTALNVVSRRYLASLHKRALPTSVTFELLFDGTNPSTEEMGDDFG
                   730        740        750        760        770        780
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Figure 1: Protein sequence homology between the cloned E2 gene of GPE⁻ strain and the GPE⁻ sequence deposited in GenBank (D49533). Differences in the deduced amino acids sequences are highlighted (at positions 94 and 97).

1μl (2 U) of RNAse H was added, incubated at 37°C for 20 minutes. This step was done to remove RNA complementary to the cDNA. The cDNA was used as a template for amplification in PCR.

Construction of E2-encoded expression plasmids

The primer sequence used to amplify the 1.2 kb E2 gene were: forward primer (SignE2) 5' –CG GGA TCC GCC ACC GTA CTA AGA GGA CAG ATC GTG C- 3' and reverse primer (TMR1-E2): 5'CG GAA TTC CTG TAA ACC AGC GGC GAG TTG TTC 3'. The primers contained *Bam*HI and *Eco*RI restriction sites (underlined), respectively. The appropriate initiation codon and stop codons are highlighted.

The cDNA template was added to a PCR reaction mixture containing 200 μM each deoxynucleotide triphosphate, 2.5 μM each primer, 1 unit Supertherm DNA polymerase (LPI, London, United Kingdom), and 5μl of 10x PCR buffer supplied by the manufacturer (containing 1.5mM magnesium chloride) in a total of 50 μl of reaction volume. The PCR was allowed to go at 94°C for 1 minute, then followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes. A final extension phase at 72°C for 5 minutes was also included.

Cloning and sequencing of cDNA of the E2 gene

The amplified E2 gene fragment was digested with *Bam*HI and *Eco*RI and then cloned into the same-enzyme digested plasmid pcDNA/Zeo+ (Invitrogen) which was an eukaryotic expression vector pcDNA/Zeo+. The in-frame sequence of the E2 gene fragment in the recombinant plasmid was verified by auto-sequencing using an ABI PRISM™ 377 DNA sequencer. Transformation of E. *coli* and preparation of DNA plasmids were carried out according to established procedures. Large-scale preparation of plasmid DNA was performed by QIAGEN Maxiprep kit.

Screening for E2 gene in vitro expression

The expression efficiency of the E2 gene was analysed by *in vitro* transfection of swine testicle (STE) and Vero monolayer cells in 24-well plate. Transient transfection was performed with Lipofectin reagent (Life Technologies) according to the manufacturer's instruction with some modifications. Briefly, the mixture of DNA and Lipofectin reagent was added to a 70% confluent monolayer cells for 6 hours before replenishment with serum media. Supernatant samples were harvested after 48 hours and kept at -20°C for ammonium sulfate precipitation (Ito, 2000) and SDS-PAGE analysis. The fractionated proteins were either stained with Coomassie blue or analysed by Western blots. The PBS-washed transfected cells were proceeded for immunoperoxidase assay. In *vitro* E2 expression was probed with E2-specific monoclonal antibodies (WH303 and WH211; Veterinary Laboratories Agency-Weybridge). Bound antibodies were detected by using HRP-conjugated goat anti-mouse (Kirkegaard & Perry Laboratories, USA), and DAB substrate for colour development.

RESULTS

Protein sequence analysis of the E2 dene

The recombinant pcDNA+E2 was sequenced using the universal M13 and END-E2 (reverse) primers. There were differences between the amplified E2 gene of GPE⁻ strain and the GenBank GPE⁻ sequence (D40533). These differences resulted in two amino acids substitutions at positions 94 (R→G) and 97 (P→S) in the deduced protein sequence (Figure 1). The overall protein sequences homologies of the sequenced E2 gene and documented sequence were 99.5%, whereby the 404 amino acids (aa) residues was predicted to be 45 kDA based from the calculated molecular weight output (WorkBench: SAPS).

Construction of the CSFV E2 encoded plasmid

Since the E2 gene is a part of a large ORF and does not contain its own initiation codon, the sequence CCACCATG, which contains an initiation codon and is compatible with Kozak's rule (1989), was engineered to the 5' end of E2 during PCR. The CSFV E2 gene cDNA was amplified by means of RT-PCR. An E2 PCR product

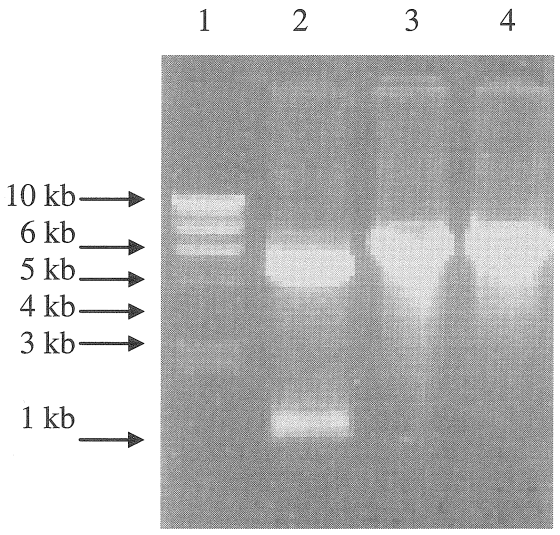

Figure 2: Restriction enzyme analyses of recombinant pcDNA+E2 (large scale plasmid extraction). Double digestions with *Bam*HI and *Eco*RI (lane 2) dropped the 1.2 kb E2 insert. Digestions with either *Eco*RI or *Bam*HI (lane 3 and 4) linearised the plasmid. Lane 1:1 kb DNA ladder.

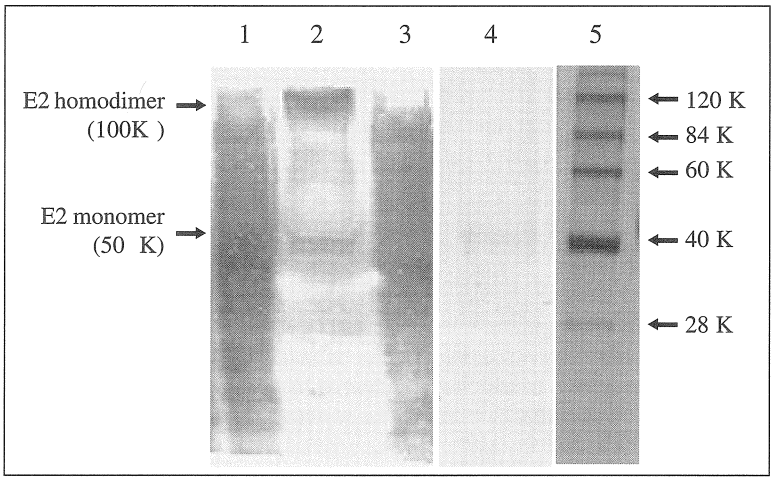

Figure 3: Western Blot analysis of the E2 protein using MabWH211. Prior to the Blot, the proteins were fractionated by 10% SDS-PAGE under non-reducing condition and protein size confirmed using BlueRanger™ Protein Marker. Lane 2: The different forms (monomer-50K, homodimer-100K) of E2 protein in GPE⁻ virions. Lane 4: E2 transient expression in a positive transfectants (50 K). Lane 5: BlueRanger™ Protein Marker. K=kDA.

of 1.2 kb was recovered and cloned (Figure 2). The recombinant pcDNA+E2 harboured the E2 gene, downstream to the hCMV promoter. A large scale preparation of the purified plasmid was performed for the transfection analysis.

In vitro expression analysis

With MAb WH211, it is possible to verify the homodimeric (110 kDa) and the monomeric forms (50 kDA) of the GPE⁻ virion. On the other hand, a protein approximately 50 kDA in size was detected from transfected cell lysate (Figure3). The observed increment of E2 predicted molecular weight from 45kDA®50kDA, could be attributed to glycosylation. Strikingly, the E2 protein was not detected by Western Blot when electrophoresis was performed in a reducing condition, suggesting the instability of the epitope in these conditions. Usage of the WH303 for the E2 expression analyses was unsuccessful. Even the E2 peptide of the positive control (GPE⁻ vaccine virus) could not be detected by Western Blot analysis. However, the E2 detection problem was solved by using WH211.

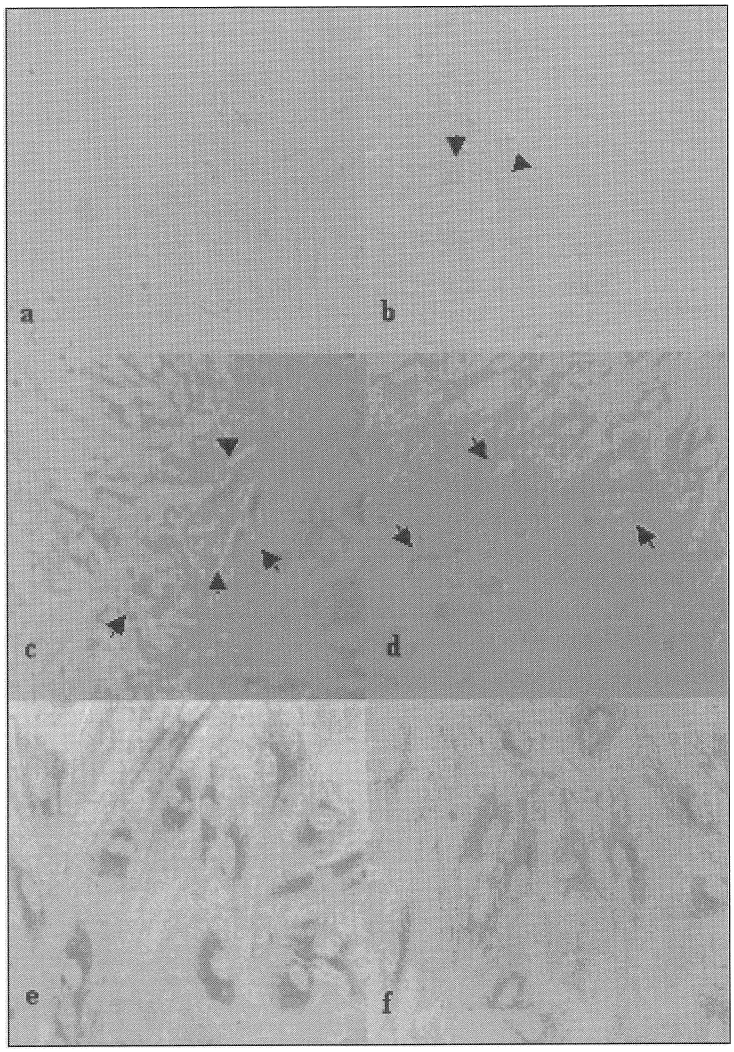

Figure 4: Immunoperoxidase detection of E2 protein expression in transfected cells culture. The distinct cytoplasmic expressions of the E2 protein (arrow heads) were indicated in Vero cells (b) and STE cells (c and d). Original magnifications of a (control Vero cells), b, c and d, were 400X. Higher magnification (1000X) for the expression in STE cells are shown in e and f.

The expression efficiency of the E2 gene was also analysed by *in vitro* transfection of STE and Vero cells in 24-well plates. STE cells showed a higher transfection efficiency than Vero cells and therefore were selected for most of the experiments. The cells were fixed at 48 hours post-transfection for an immunoperoxidase assay. Localisation of the E2 expression under light microscopy revealed that most of the E2 protein was distributed throughout the cytoplasm. The distinct cytoplasmic expression could only be demonstrated using MAb WH211 (Figure 4). No E2 protein was detected from the supernatant samples of the positive wells. It meant that the E2 protein was solely retained in the cell cytoplasm, without leakage in the medium.

DISCUSSION

This study successfully cloned the E2 gene of CSFV into a plasmid vector, pCDNA and analysed it. The featured *in vitro* E2 expressions indicated a membrane tropism, which could probably be associated with the presence of the transmembrane domain (TMR) at the C-terminus (Ahn, B.Y., Korea University: pers. comm.). The TMR of E2 not only serves to anchor the protein into the cellular membranes but also functions as a signal which targets E2 to the ER or the *cis*-Golgi region (Hulst *et al.*, 1993). The detection of the E2 protein in cells transfected with pCDNA+E2 via Western Blot analysis showed that the signal peptide was necessary for proper processing

of E2 protein. This observation is similar to that of Yu *et al.* (2001) who showed evidence that CSFV E2 could not be processed into the matured form of glycoprotein if there is a deletion of the signal peptide at the N'terminal. Mutagenesis study had shown the cystein residues in the N terminal of E2 are essential for antigenicity while those on the C-terminal portion presumably participate in disulphide bonding in dimer formation (Van Rijn *et al.*, 1994). However, the deletion on the C'-terminal half could be performed without effecting the binding capacity of MAbs (Van Rijn *et al.*, 1993).

The use of two E2-MAbs unraveled the underlying failure of E2 protein detection by WH303. As a matter of fact, both of these murine derived E2 specific MAb are directed against the existing epitopes at domain A (N'-terminal) of E2 envelope glycoprotein as defined by Wensvoort (1989). Although WH303 was claimed to be efficient in binding with all strains of CSFV including 3 vaccine strains, it was not reported whether it had been tested on the GPE$^-$ vaccine strain (Edwards *et al.*, 1991). The minimal antigenic domain recognised by WH303 had been defined to be a 9-mer peptide TAVSPTTLR (Lin *et al.*, 2000). Therefore, the single substitution of amino acid alanine to tyrosine (A→T) in the critical 9-mer peptide in the E2 of GPE$^-$, had possibly abolished the binding ability to WH303, and corroborated with the present findings. Negative Western Blot results were obtained constantly when the E2 peptides of GPE$^-$ virus were reacted with WH303. Deletion analyses performed by Lin *et al.*, (2000) suggested that a 3-mer peptide TAV of TAVSPTTLR contained residues critical for binding WH303. Previously, a panel of MAbs had been specifically classified for detecting all field strains of CSFV, vaccine strains of CSFV and BVDV/BDV, respectively, allowing an unambiguous differentiation between field and vaccine strains of CSFV on the one hand, and between CSFV and other pesti-viruses on the other (Edwards *et al.*, 1991). If this is the case, CSF outbreaks in areas that are using GPE$^-$ vaccine or any vector incorporating E2-expression cassette derived from a GPE$^-$ strain, could use WH303 MAb for specific diagnosis of CSFV infection.

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