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Design, Generation and Protein Expression of the Epitope-Based PEDV (Porcine Epidemic Diarrhea Virus) Vaccine

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ABSTRACT

Porcine Epidemic Diarrhea (PED) continues to cause huge economic losses in swine industry worldwide. Porcine Epidemic Diarrhea Virus (PEDV), the causative agent of PED, exhibits highly genetic variation; thus, a universal vaccine is desirable. Our main goal is to develop a universal vaccine using multi-epitope strategy. In this study, we designed, generated and studied the expression of the PEDV multi-epitope vaccines. Two new vaccine antigens, epiS(MHC) and epiS(CTL), were designed to contain CD8 T cell epitopes obtained from *in silico* prediction of the PEDV spike protein (S). The vaccines were then developed using 3 different vaccine systems. In HEK293A, the antigens produced by DNA vaccines and adenovirus-based vaccines exhibited only small amount of the protein. When expressed in *E. coli* expression system, only the epiS(MHC) protein displayed full-length and degraded forms of the protein when it is detected by Western blot. The results of protein expression by 3 different vaccine platforms suggest that epitope-based vaccine can be very unstable and susceptible to degradation and further modification is important to help improve stability and functionality of the vaccines.

Keywords: PEDV, Epitope-based vaccine, DNA vaccine, Adenovirus, *E. coli*

INTRODUCTION

A swine disease named Porcine Epidemic Diarrhea (PED) causes a huge trouble globally. Epidemic in Europe and Asia for a long time and in United States since 2013 leads to economic loss over $900 million (Paarlberg, 2014; Song & Park, 2012). PED shows diarrhea, dehydration, vomiting, severe enteritis, weight loss and anorexia, resulting in high morbidity and mortality (Song & Park, 2012). PED is caused by Porcine Epidemic Diarrhea Virus (PEDV). This virus can infect all ages of pigs; however, old pigs had survival chance more than 80% while sucking piglets showed 80-100% death rate after infection of PEDV (R.-Q. Sun, 2012).

PEDV belongs to the genus *Alphacoronavirus*, and it is an enveloped, positive-sense, single-stranded RNA virus (Song & Park, 2012). PEDV genome is approximately 28 kb long and encodes four structural proteins which are spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N). PEDV is one of the viruses that exhibit highly genetic variation. Based on S gene containing hypervariable regions, PEDV can be separated into two groups which are G1 as classical group found in Europe, Asia and America and G2 as epidemic or pandemic group reported in e.g. North America, South Korea and China (Lee, 2015). To protect pigs from PEDV infection, various vaccines had been developed from several strains of PEDV. However, these vaccines could not protect global strains of PEDV (Lawrence & Bey, 2015). Therefore, a universal vaccine that can provide protection against global strains of PEDV is desirable.

Universal vaccine is novel vaccine technology to protect host from broadly strains of particular pathogen (Rajão & Pérez, 2018). A universal vaccine can be developed based on (i) highly conserved region and (ii) multiple epitopes derived from various strains of the pathogen, called as epitope-based vaccine or multi-epitope vaccine. S protein have been reported to contain neutralizing epitope region, which is important for development of an effective vaccine capable of inhibiting PEDV entry into the host cell (D. Sun et al., 2008). However, cell-mediated immunity is also important in controlling virus infection. Cytotoxic T cells (CTLs) play a role in killing infected cells. Thus, T cell-based PEDV vaccine is also needed to provide protection against PEDV infection.

To date, none of CD8 T cell epitopes in the PEDV structural proteins is identified. With the aim to develop a universal vaccine, we have previously predicted putative CD8 T cell epitopes in various strains of PEDV using

immuno-informatic approach. In this study, we continued our work by designing new vaccine antigens composed of multiple CD8 T cell epitopes predicted from multiple strains of PEDV. The vaccines were then developed using 3 different vaccine systems: (i) DNA vaccine, (ii) adenoviral vector and (iii) prokaryotic expression system. Expression of the vaccine antigens by 3 different vaccine platforms was then studied using Western blot and immunofluorescence staining.

MATERIAL AND METHODS

Synthesis of gene containing strings of multi-epitopes

The codons of the epiS(MHC) and epiS(CTL) genes were designed based on pig's codon usage and optimized using Codon Optimization Tool (IDT) to avoid complex and restriction site in sequence. These antigen genes were synthesized by IDT, Singapore.

Generation of DNA vaccines

The epiS(MHC) and epiS(CTL) gene fragments were excised from the pUCIDT plasmid using *Pst*I and *Not*I restriction enzymes. Gel-purified DNA fragments were then ligated into plasmid pTH at *Pst*I and *Not*I sites. Recombinant plasmids were then generated by transforming *E. coli* DH5α with ligation mixture using heat shock method. The transformed bacteria were plated onto LB agar plate containing 100 µg/ml ampicillin and incubated at 37 °C overnight. Recombinant clones were screened and selected using rapid size screening method. Chosen recombinant clones were cultured and subjected to plasmid extraction. Plasmids from recombinant clones were then digested with restriction enzyme *Pst*I and *Not*I to confirm the presence of the antigen gene in the plasmid. Nucleotide sequence of the antigen genes in the plasmids were then confirmed by automate DNA sequencing.

Generation of recombinant adenovirus (rAd)

Recombinant adenovirus harboring antigen genes, epiS(MHC) and epiS(CTL), were generated using ViraPower Adenoviral Gateway Expression system (Invitrogen), following the manufacturer's instruction. The antigen gene fragments were cleaved from the pUCIDT plasmids using restriction enzymes *Xmn*I and *Not*I and then gel-purified. In parallel, the shuttle plasmid pENTR11 was also digested with *Xmn*I and *Not*I and then gel-purified. Ligation was performed and *E. coli* DH5α was then transformed with the

ligation mixture. Recombinant pENTR11 plasmids were screened using rapid size screening and then confirmed using restriction enzyme digestion and automate DNA sequencing.

The plasmids pENTR11.epiS(MHC) and pENTR11.epiS(CTL) were incubated with adenoviral plasmid pAd/CMV/V5-DEST in the presence of LR Clonase II for homologous recombination. The mixture then was used in transforming *E. coli* DH5α to generate recombinant clones bearing recombinant pAd. Recombinant plasmids, pAd.epiS(MHC) and pAd.epiS(CTL), were confirmed by rapid size screening and PCR amplification. Prior to cell transfection, recombinant pAd plasmids were digested with the *Pac*I restriction enzyme, followed by cleaning up using chloroform extraction. HEK293A cells grown in a 6-well plate were then transfected with the *Pac*I-cut plasmids using polyethylenimine (PEI) as a transfection agent. Transfected cells were incubated at 37 °C with 5% CO2 until cytopathic effect (CPE) developed. When 70-80% of the cells exhibited CPE (10-14 days), cells were harvested and lysed using freeze-thaw technique for harvesting crude viruses. The viruses were amplified in larger culture flasks and crude viral stock was prepared to be used in protein expression study.

Cell transfection and infection

HEK293A cell was cultured in DMEM supplemented with 10% FBS and antibiotics penicillin/streptomycin in a 37°C and 5% CO_2 incubator. To study protein expression by DNA vaccine, HEK293A cell grown in a 6-well plate were transfected with pTH.epiS(MHC) and pTH.epiS(CTL). Two microgram DNA was mixed with 4 µg of PEI and transfection was performed following the standard protocol. The mixture plasmid DNA and PEI was replaced with completed medium and then incubated at 37 °C with 5% CO_2. After a 48-h incubation, transfected cells were then collected and subjected to Western blot analysis and immunofluorescence staining assay.

To study the protein expressed by rAd, HEK293A cells grown to 80% confluence in 12-well plate were infected with crude stock of Ad.epiS(MHC) and Ad.epiS(CTL) diluted to 10^{-1} (1 ml/well). Additionally, one well was maintained as un-infected cells to be used as a negative control. Following a 48-h incubation, the cells were collected for Western blot analysis and immunofluorescence staining assay.

Generation of *E. coli* expression system and IPTG induction

The antigen gene fragments, epiS(MHC) or epiS(CTL), were obtained from the pUCIDT plasmids by cutting with *Hind*III and *Not*I, followed by gel purification. The antigen genes were then ligated into plasmid pET28b between the *Hind*III and *Not*I sites. *E. coli* DH5α was transformed with the ligation mixture. Recombinant clones bearing the plasmids pET28b.epiS(MHC) and pET28b.epiS(CTL) were screened using rapid size screening, followed by confirmation by cutting with *Hind*III and *Not*I. Nucleotide sequences of the antigen genes were confirmed by automate DNA sequencing.

E. coli BL21(DE3)pLysS, a protein expression host, was next transformed with the plasmids pET28b.epiS(MHC) and pET28b.epiS(CTL). In parallel, transformation with empty pET28b was also performed to generate a negative control clone. The transformed bacteria were plated onto LB agar plate containing 50 µg/ml kanamycin and incubated at 37 °C overnight. Recombinant *E. coli* BL21(DE3)pLysS clones bearing antigen genes were then subjected to protein expression study.

To induce an expression of the antigen genes in *E. coli* BL21(DE3)pLysS, the recombinant and negative control clones were inoculated into 4 ml of Luria-Bertani (LB) medium with 50 µg/ml kanamycin. The cultures were incubated at 37 °C with 200 rpm until OD_{600} reached 0.6 per milliliter (ml). Prior to IPTG induction, 1 ml of the culture was taken and used as non-induced sample. Isopropyl β-D-thiogalactopyranoside (IPTG) was added into each culture tube to a final concentration of 1 mM. The cultures were further incubated at 37 °C with shaking at 200 rpm. After a 4-h incubation, cells were harvested and lysed with lysis buffer. After centrifugation, protein samples were separated into supernatant (soluble protein) and pellet (inclusion body) fractions. The protein samples were then subjected to Western blot analysis.

Western blot analysis

HEK293A cells in 6-well plate were harvested using 150 µl of cold lysis buffer, (20 mM Tris, [pH 8.0], 137 mM NaCl, 10% Glycerol, 1% Nonidet P-40), containing 1X protease inhibitor cocktail (Sigma) and then transferred to a new 1.5 ml tube. *E. coli* BL21(DE3)pLysS, was collected by centrifugation. The cell pellets were then lysed homogenously in 200 µl

CelLytic B plus (Sigma-Aldrich), following the manufacturer's instruction. The mixtures were centrifuged at 12,000 rpm at room temperature for 5 min. Supernatant and pellet are referred to soluble proteins and inclusion body, respectively. Supernatant was transferred into a new 1.5 ml tube.

Prior to gel loading, the protein samples were mixed with sample buffer (2% SDS, 120% glycerol, 100mM DTT, 0.005% bromphenol blue and 0.05 M Tris HCl, pH 6.8) and heated at 95^0C for 5 minutes. The samples were then analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins from the gel were transferred to nitrocellulose membrane using semi-dry blot transfer equipment (Bio-Rad). The membranes were blocked with 5% skim milk in PBS buffer with 0.1% Tween (PBST) with agitation for 1 h. Next, mouse anti-V5 antibody diluted in 5% skim milk/PBST was added to the membrane. After a 3-h incubation at room temperature, the membranes were washed 3 times with PBST and then incubated with goat anti-mouse IgG HRP diluted in 5% skim milk/PBST, for 1 h 30 min at room temperature. The membranes were washed 3 times with PBST and then incubated with chemiluminescence substrate (Thermo scientific) for 5 min. The membranes were imaged using Odyssey® Fc Immaging System.

Immunofluorescence staining assay

HEK293A cells in a 12-well plate were fixed with 1.0% formaldehyde at room temperature for 10 min and then washed with PBS. Cells were permeabilized with cold 90% methanol and then incubated at 4 °C for 5 min. Cells were then washed once with PBS and blocked with 2% FBS/PBS for 1 h at room temperature. The blocking solution was replaced by mouse anti-V5 antibody diluted in PBS (350 µl/well). After incubation for 2 h at room temperature, the cells were washed twice with PBS. Donkey anti-mouse IgG conjugated with Alexa Fluor® 594 diluted in PBS was added to the cells and further incubated for 1 h at room temperature. The cells were washed twice with PBS and then stained the nucleus with DAPI diluted to 1:1,000 in PBS. After a 15-min incubation, the cells were washed once with PBS. Finally, fluorescence images were directly examined under an inverted fluorescence microscope at 40X objective lens (Olympus).

RESULTS AND DISCUSSION

Design of the multi-epitope antigens

In this study, we designed 2 new antigens, epiS(MHC) and epiS(CTL), which are composed of multiple predicted CD8 T epitopes. Predicted CD8 T cell epitopes of PEDV Spike protein obtained from *in silico* prediction using NetMHC 4.0 and NetCTLpan 1.1 tools were put together as a string of epitopes and named epiS(MHC) and epiS(CTL), respectively. As these 2 antigens are designed as CD8 T cell-based vaccines, we thus included extra amino acids to enhance their performance. Leader amino acids MN were added to the N-terminus to promote transcription and CTL response (Toes et al., 1997). The AAY spacer was added between epitopes as it has been suggested to help support cleavage by proteasome, which is important for antigen presenting process (Velders et al., 2001). V5 tag was added to the C-terminus to facilitate protein detection using anti-V5 antibody. The genes were codon-optimized with pig's codon usage to promote a high protein expression in pig (Nakamura, Gojobori, & Ikemura, 2000).

The epiS(MHC) antigen contains 25 putative epitopes predicted by NetMHC 4.0 tool and is composed of 316 amino acids (Figure 1A). The epiS(CTL) antigen contains 19 putative epitopes predicted by NetCTLpan 1.1 and consists of 248 amino acid residues (Figure 1B).

Figure 1. Details of the antigens. (A) Amino acid sequence of the epiS(MHC) antigen. (B) Amino acid sequence of the epiS(CTL). Epitopes are presented in light blue and green. V5 tag (IPNPLLGLD) at the C-terminus and AAY spacer are indicated.

Generation of DNA vaccines and protein expression study

DNA vaccines were generated by ligating the antigen genes with plasmid pTH, a mammalian expression vector. Recombinant plasmids were generated and nucleotide sequences of the antigen genes were confirmed by automate DNA sequencing. Expression of the antigens by two DNA vaccines, pTH.epiS(MHC) and pTH.epiS(CTL), was then investigated in human cell line, HEK 293A cell. The cells were transiently transfected with the DNA vaccines and then subjected to Western blot analysis and immunofluorescence staining.

Tested by Western blot analysis, both epiS(MHC) and epiS(CTL) proteins could not be detected at the expected size of 35 and 29 kDa, respectively (data not shown). Immunofluorescence staining was then used to directly visualize the antigen proteins in transfected cells. Both pTH.epiS(MHC)- and epiS(CTL)-transfected cells show only weak positive staining (Figure 2). This result indicates that although the epiS(MHC) and epiS(CTL) proteins were successfully expressed by DNA vaccine, the amount of the proteins in transfected cell is low.

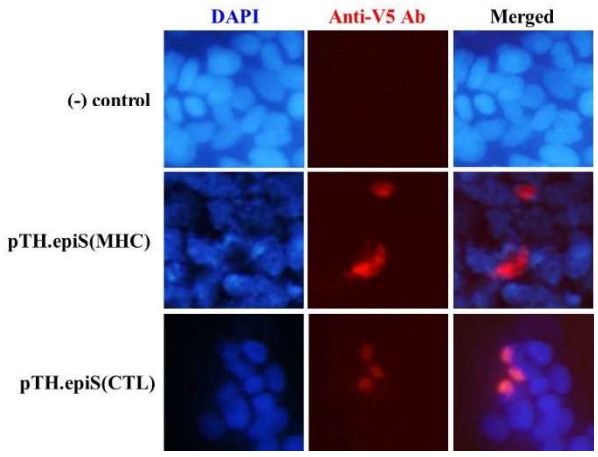

Figure 2. Antigen expression by DNA vaccines. Antigens were detected using ant-v5 antibody and immunofluorescence staining. DAPI was used to visualize the nucleus (blue). (-) indicted HEK 293A cells transfected with parental pTH. DNA vaccine-transfected cells are indicated.

Generation of adenovirus-based vaccines and protein expression study

Recombinant adenovirus (rAd) was generated to harbor the antigen genes. Recombinant viruses were rescued by transfecting HEK 293A cell with pAd.epiS(MHC) and pAd.epiS(CTL). CPE formation was clearly observed at day 9 post-transfection, suggesting a successful rescue of recombinant adenovirus. Interestingly, when we tried to amplify the virus, both rAd could not grow further and thus large viral stock preparation was not achieved and it is still unclear what the cause is. Expression of the epiS(MHC) and epiS(CTL) proteins by adenovirus were then investigated by infecting HEK293 cells with rAd and the infected cells were collected 48 h post-infection and then subjected to protein detection. When detected by Western blot analysis, both antigen proteins could not be detected (data not shown). Using anti-V5 antibody for detection, immunofluorescence staining showed only weak positive fluorescence in the cells infected with Ad.epiS(MHC) and Ad.epiS(CTL) (Figure 3), indicating low amount of the proteins within the infected cells. Possible explanation of this finding is that small peptide-based vaccines may be easily cleaved by proteases in host cells as AAY linker within the antigens promotes proteasome cleavage, making the proteins highly degradable and unstable (Velders et al., 2001).

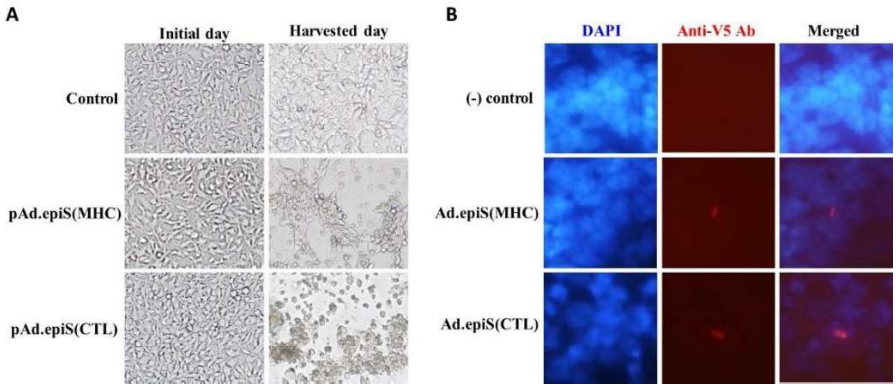

Figure 3. Generation of recombinant adenovirus and protein expression study. (A) Generation of adenovirus. HEK293A cell was transfected with plasmids pAd.epiS(MHC) and pAd.epiS(CTL). CPE formation was observed at day 9 post transfection. (B) Detection of the proteins using anti-V5 antibody and immunofluorescence staining. (-) control represents uninfected cells. DAPI was used to visualize the nucleus (blue).

Expression of the antigen proteins in *E. coli* expression system

The antigen gene fragments were inserted into pET28b, a prokaryotic expression plasmid. Expression of the antigens was then studied in *E. coli* BL21 (DE3)pLysS. After a 3-h IPTG induction, the cells were then collected and prepared as supernatant and inclusion body fractions. Western Blot analysis was employed to detect the proteins. The expected sizes of epiS(MHC) and epiS(CTL) are approximately 40 and 34, respectively.

Detected by anti-V5 antibody, while the epiS(CTL) sample showed no positive band in both soluble and inclusion body fractions, epiS(MHC) sample exhibited the band at the expected size of 40 kDa (Figure 4). However, the protein was found mainly in supernatant fraction and with only small amount as inclusion body. Besides the intact form, the cleaved fragments of approximately 30 and 25 kDa were also observed. These result suggests that the epiS(MHC) protein could be successfully produced in *E. coli* cell but it can be easily cleaved by the enzymes present in *E. coli* cell, thus making it unstable and unable to accumulate in large amount.

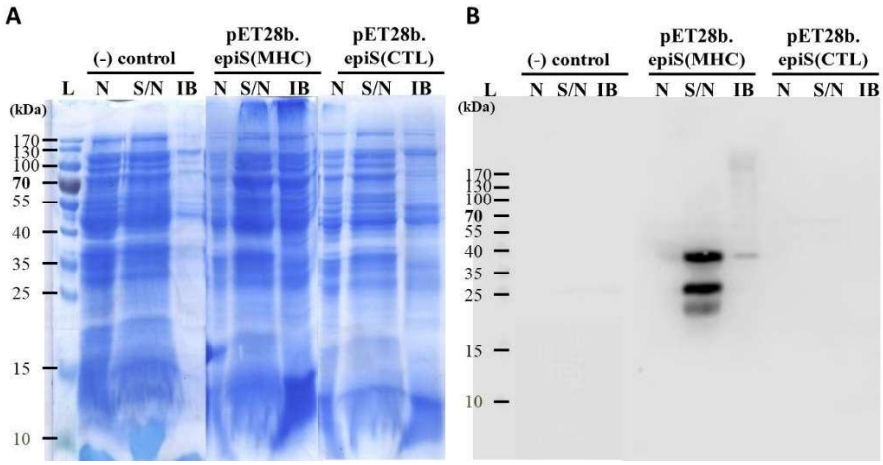

Figure 4 Western Blot analysis of the antigen proteins produced by *E. coli* BL21(DE3)pLysS. After a 4-h IPTG induction, cells were lysed and separated into soluble proteins (S/N) and inclusion bodies (IB). N indicates non-induced cell. L represents molecular-weight size marker. (-) control represents *E.coli* harboring empty pET28b.

CONCLUSION

Here, we designed and generated a universal vaccine by combining multiple predicted epitopes from various strains of PEDV into a string of epitopes. The vaccines were then developed using 3 different vaccine systems. Protein expression study revealed that the 2 new antigens were detected in a very low amount in mammalian expression systems regardless of vaccine types. However, expression in *E. coli* host system displayed a full-length form as well as cleaved fragments of the epiS(MHC) but not epiS(MHC). Altogether, these results suggest that an antigen designed based on a string of epitopes could be highly unstable and susceptible to enzymatic cleavage. Further modification is necessary to improve stability and functionality of the epitope-based vaccine.

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REFERENCES

Lawrence, P., & Bey, R. (2015). Recombinant Spike Protein Subunit Based Vaccine for Porcine Epidemic Diarrhea Virus (PEDV). In: US Patent 20,150,328,307.

Lee, C. (2015). Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. *Virology journal, 12*(1), 193.

Nakamura, Y., Gojobori, T., & Ikemura, T. (2000). Codon usage tabulated from international DNA sequence databases: status for the year 2000. *Nucleic acids research, 28*(1), 292-292.

Paarlberg, P. L. (2014). Updated estimated economic welfare impacts of porcine epidemic diarrhea virus (PEDV). *Purdue University, Department of Agricultural Economics, Working Papers, 14*(4), 1-38.

Rajão, D. S., & Pérez, D. R. (2018). Universal vaccines and vaccine platforms to protect against influenza viruses in humans and agriculture. *Frontiers in microbiology, 9*, 123.

Song, D., & Park, B. (2012). Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus genes, 44*(2), 167-175.

Sun, D., Feng, L., Shi, H., Chen, J., Cui, X., Chen, H., . . . Tong, G. (2008). Identification of two novel B cell epitopes on porcine epidemic diarrhea virus spike protein. *Veterinary microbiology, 131*(1), 73-81.

Sun, R.-Q. (2012). Outbreak of Porcine Epidemic Diarrhea in Suckling Piglets, China-Volume 18, Number 1—January 2012-Emerging Infectious Disease journal-CDC.

Toes, R. E., Hoeben, R. C., Van Der Voort, E. I., Ressing, M. E., Van Der Eb, A. J., Melief, C. J., & Offringa, R. (1997). Protective anti-tumor immunity induced by vaccination with recombinant adenoviruses encoding multiple tumor-associated cytotoxic T lymphocyte epitopes in a string-of-beads fashion. *Proceedings of the National Academy of Sciences, 94*(26), 14660-14665.

Velders, M. P., Weijzen, S., Eiben, G. L., Elmishad, A. G., Kloetzel, P.-M., Higgins, T., . . . Smith, L. (2001). Defined flanking spacers and enhanced proteolysis is essential for eradication of established tumors by an epitope string DNA vaccine. *The Journal of Immunology, 166*(9), 5366-5373.

Salleh, N.A., Rusul, G., Hassan, Z., Reezal, A., Isa, S.H., Nishibuchi, M., Radu, S. 2003. Incidence of *Salmonella* spp. in raw vegetables in Selangor, Malaysia. Food Control. 14: 475–479.

Sengun, I. Y., and Karapinar, M. 2004. Effectiveness of lemon juice, vinegar and their mixture in elimination of *Salmonella typhimurium* on carrots. Food Microbiology. 96: 301-305.

Sholberg, P. L., Haag, P., Hocking, R., and Bedford, K. 2000. The use of vinegar vapor to reduce postharvest decay of harvested fruit. Horticultural Science. 35: 898-903.

Tzortzakis, N. G. 2010. Ethanol, vinegar and origanum vulgare oil vapor suppress the development of anthracnose rot in tomato fruit. Food Microbiology. 142: 14-18.