A Thesis

For the Degree of Master of Science in Veterinary Medicine

Construction of a Recombinant *Salmonella enterica* serovar Typhimurium Vaccine Carrying *Rhodococcus equi*-VapA

Department of Veterinary Medicine

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

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(Supervised by professor DuSik Lee)

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Abstract

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Rhodococcus equi is a gram positive bacteria and important causative pathogen of purulent bronchopneumonia in young foals. It has specific antigen VapA which determines the pathogenicity of *R. equi* in horse. Even though various studies have been reported for the development of the vaccines using *Vap*A as an immunogen source, there is not commercialized vaccine available to control *R. equi* until now. In this study, we applied the attenuated *Salmonella enterica* serovar Typhimurium, H683 as a vehicle floating *Vap*A to immunize against *R. equi.* The *VapA* gene was inserted in pMAL-c2 vector, cloned and expressed in *E. coli* cell. Purified recombinant MBP-VapA was used for the production of rabbit antiserum and was used as the coating antigen of the ELISA. For the mucosal vaccine construction, the *Vap*A gene

was amplified by PCR and cloned into *asd* + pYA292 vector. The cloned plasmid was transformed into *E. coli* △*asd* strain, H681 and was retransformed into H683. The retransformation of pYA292-*Vap*A was confirmed by PCR. Expression of target protein in H683 was confirmed by SDS-PAGE and western blot analysis. Recombinant VapA was expressed at cell surface of H683. The attenuated *Salmonella enterica* serovar Typhimurium, H683 expressing VapA (SeTy-V vaccine) was orally administrated to 6 week-old mouse. Though the markedly highest humoral immune responses were not appeared, there were somewhat higher titre tendency than the control group.

KEYWORDS: *Rhodococcus equi*; VapA; *Salmonella* Typhimurium; Vaccine.

1. INTRODUCTION

Rhodococcus equi is a Gram-positive rod, facultative intracellular bacterial pathogen. It has been increasingly recognized as an important causative pathogen of purulent bronchopneumonia in young foals and even in immunodeficient human hosts like AIDS human patients [3, 8, 10, 31, 32].

R. equi will potentially multiply in horse manure and persist a long time on contaminated pastures [4, 39, 46]. Infections usually occur between 1 month and 6 months and clinical symptoms were comes out in 4-12 weeks aged foals [30]. This organism reaches at the lung by inhalation dusty manure- contaminated environments and usually causes pyogranulomatous bronchopneumonia in affected foals, occasionally with extensive abscesses [4]. And it causes rarely some cases of enteritis and osteomyelitis [4, 21, 47]. It may result in considerable losses through the costs for diagnosis and treatment, in some cases of death. So it can be considered significant economical problems to the horse breeding industry.

Previous studies have shown that the *R. equi* from the pathogenic equine contains a virulence plasmid of approximately 85 kb which encoded a 15-17 kDa surface protein [42]. From among these virulence associated protein, VapA was most widely studied and became an important marker for the isolation of virulence *R. equi* in equine [43]. The PCR method was used for the rapid identification of virulence plasmid of *R. equi* based on the DNA sequence obtained [40]. Some researches revealed that the pathogen with the virulence plasmid had an ability to survive within alveolar macrophages. Also,

it could replicate within the phagosome and prevent phago-lysosome fusion [19, 20]. But, the pathogen cured of the virulence plasmid was unable to survive and replicate in macrophages, and dramatically decrease lethality in foals and mice [17, 41, 42]. Many studies suggested that the VapA antigen was important key for development of vaccine [13, 25, 33]. However, no vaccines are developed for use as a commercial vaccine up to now. Therefore, this study focused on Salmonella vaccine vector expressing VapA proteins as a mucosal vaccine system.

At first finding of the vaccine, the facts that *Salmonella enterica* serovar Typhimurium (SeTy) establishes an infection by colonization and antigenic presentation at the gut associated lymphoid tissue (GALT) and leads immune response might be a key point to development of a new efficient mucosal immune vaccine [6]. Later, it was constructed a balanced-lethal system in which the *asd* gene of *Streptococcus mutans* is present in a plasmid complementing a △*asd* mutation in the chromosome of the SeTy vaccine strains [27]. The *asd* gene encodes aspartate β-semialdehyde dehydrogenase, an enzyme in the biosynthetic pathway for synthesis of Diaminopimeric acid (DAP). The DAP enzyme is an essential component of the peptidoglycan of the Gram negative bacteria cell wall [35]. In the condition of absence the DAP, *asd* mutated bacteria finally undergoes lysis. Consequently, *asd* + vector and △*asd* host system have been considered useful vaccine candidates.

Attenuated SeTy vaccine strains have been genetically modified to express another pathogen's antigens [44]. These orally administered vaccines are colonized at the GALT (Peyer's patches) and secondary lymphatic tissues, like liver and spleen [15]. It had been shown to induce protective immune responses about various diseases in host and mice models [2 ,5, 16, 29].

In this study, we expressed a *Vap*A gene in pMAL protein fusion and purification system and purified the fusion protein. Also, we constructed a recombinant live attenuated SeTy vaccine expressing VapA antigen (SeTy-V). The vaccine candidate, SeTy-V, was administrated orally to mice in an attempt to induce protective immune responses.

2. MATERIALS AND METHODS

1. Expression of VapA proteins in *E. coli*

Bacterial strains and medium

The isolation of virulent *R. equi* was carried out from lung of foals with bronchopneumonia which were submitted to Veterinary Bacteriology laboratory in Veterinary Medicine, Cheju National University, Korea. The pathogen was cultured on Brain Heart Infusion broth (BD, USA) at 37℃ for 48 h [41]. Some of colonies which were shown smooth, round and tend to coalesces were picked and the presence of gene virulent *Vap*A was confirmed by PCR [35]. The expression of VapA protein was confirmed by western blot analysis [41]. Strains were stocked in 20% glycerol and stored at -80℃ for further testing.

Construction of TOPO-VapA cloning

The TOPO TA Cloning Kits (Invitrogen, Co., California, USA) are so highly efficient, 5 minutes, one step cloning strategy. In this kits, the DNA ligase was not used for the construction, but specific enzyme called *Topoisomerase* I from the vaccinia virus was enhance the directly insertion of Taq polymerase amplified PCR products into a plasmid vector (Fig. 1).

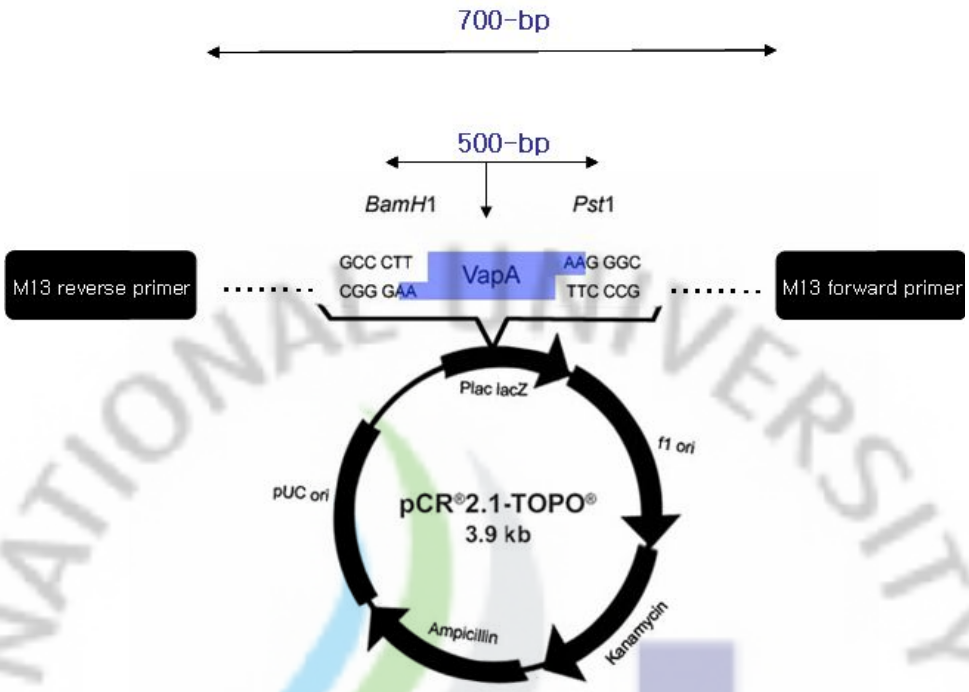


Figure 1. Genetic map of pCR-TOPO vector (Invitrogen, Co. California, USA).

The amplified gene of *Vap*A was inserted between (T) residues of multi cloning site. To verify of insertion of gene, PCR was conducted with M13 universal primer.

For the amplification of *Vap*A gene, PCR was performed using AccuPower ™PCR PreMix (Bioneer, DaeJeon, Korea). The *Vap*A specific primers were synthesized by Bioneer ;

*Vap*A-F1: 5'- GGA TCC ACC GTT CTT GAT TCC GGT AG-3'
(forward primer)
*Vap*A-R: 5'-GCG ACT GCA GTT AGG CGT TGT GCC AGC TAC-3'
(reverse primer)

The PCR premix containing application specific enzyme in an easy to resuspend, lyophilized premix of dNTPs, reaction buffer, a tracking dye and a stabilizer was suspended with 1 μl of template DNA, 1 μl of each 20 pmol primer and distilled water up to total 20 μl. The amplification was performed by TaKaRa Thermal Cycler (gradient model, Takara) by initial denaturation at 94℃ for 2 min, followed by 25 cycles of 94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min, and a final extension at 72℃ for 7 min. The PCR products were cloned into pCR®2.1-TOPO® vector and transformed into DH5α, *Escherichia(E.) coli* competent cell. The cloning and transformations were carried out by manufacturer's instructions strictly. Briefly, 2 μl of PCR products, 1 μl of salt solution, 2 μl of distilled water and 1 μl of contained pCR®2.1-TOPO® vector was reacted 5 min in room temperature. The 2 μl of products were reacted with 100 μl of DH5α for 10 min on ice and transformed by 42℃ heat shock for 30 sec. Then, these were added 250 μl of SOC medium (Invitrogen) on ice and incubated at 37℃ rotary shaking 200 rpm for 1 h and spread into Luria Bertani (LB) agar (composed with 1% Bacto™ Tryptone , 0.5% BBL™ yeast extract, 1% NaCl, 1.5% Bacto™ Agar,

adjusted pH with NaOH (Merck, Germany)) containing 100 ㎍/㎖ of ampicillin (Sigma-Aldrich Corp., St. Louis, MO, USA), 100 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma), 40 ㎎/㎖ of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma) and incubation 37℃, overnight. The Lac - colonies, white, were selected from LB-ampicillin plate. For verifying of *Vap*A gene's insertion, PCR was conducted by above mentioned program but using the universal primer M13 contained at the kit (Invitrogen). The confirmed colonies were multiplicated and stocked in 20% glycerol for the next. The successfully constructed plasmid, TOPO-*Vap*A was purified by DNA purification System (Promega Corp., Madison, Wi., USA) and stored at -20℃ for the next experiment.

Cloning of MBP-*Vap*A plasmid and purification

For the production of VapA protein, pMAL protein fusion and purification system which was developed by New England Biolab Inc. was used. The pMAL-c2 plasmid contains the *mal*E translation initiation signal and *tac* promoter (Fig. 2). The target gene is inserted in downstream of the *mal*E gene which encoding maltose binding protein (MBP) and resulting in the expression of large amounts of MBP fusion protein (Fig. 3). The 15 ㎕ of PCR products and pMAL-c2 (40 ㎍/㎖) vectors are separately digested with 1 ㎕ of endonuclease *Bam*H1 and *Pst*1 in 10K buffer (Takara Bio Inc, Japan), distilled water up to 30 ㎕ and incubation at 37℃ overnight. After confirmation of well digestion by gel electrophoresis, these were separately purified with the QIAquick Gel Extraction Kit (Qiagen, Velencia, CA, USA) and purified product was ligased with the T4 DNA ligase. The construction of pMAL-VapA was transformed into DH5α by rubidium chloride method [34]. The transformants were grown in LB agar plate containing 50 ㎍/㎖ of ampicillin. For verifying of *Vap*A's expressions, sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis was done with MBP monoclonal antibody (NEB, Beverly, MA, USA). The protein purification procedure was followed by manufacturer's instructions. Briefly, DH5α harbored pMAL-VapA plasmid were incubated in Rich broth (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose) that containing 50 ㎍/㎖ of ampicillin overnight. The 10 ㎖ of cultures were inoculated 1,000 ㎖ of Rich broth. After 4 h incubation, when the cell concentration was about ~10^8 cells/㎖, IPTG was added in the cultures at final concentration of 0.3 mM. After 2 h of induction, the cell were harvested by centrifugation

4,000 g x 20 min at 4℃ and resuspended in 50 ㎖ of column buffer (CB: 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, 10 mM -mercaptoethanol) and froze in -20℃ overnight. The other day, samples were slowly thawed in cold water, sonicated in short pulse of 15 seconds for 6 times. Then, centrifuge at 9,000 g x 30 min and the supernatant (soluble fraction) were diluted 1 : 5 with column buffer. An amylose lesin (NEB) was poured in the column and the diluted soluble fractions were loaded in the column at a flow rate of 1 ㎖/min. The fusion proteins in column were eluted with CB containing 10 mM maltose. The protein concentration was measured by bradford assay (Bio-Rad, Hercules, CA, USA). Approximately 2 g/㎖ of protein in a total volume 8 ㎖ were transferred to dialysis tubing (Sigma) and dialyzed at 4℃ in 2 L of sterilized PBS for 5 h initially, followed by two more changes of the same PBS over the next 48 h. The purified protein was confirmed with SDS-PAGE and western blot analysis as described below.

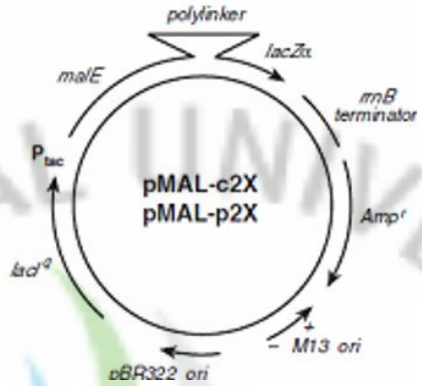


Figure 2. Genetic map of pMAL-c2 vector (NEB).

pMAL-$_C$2 vector contains a *tac* promotor and *mal*E gene. The target gene was inserted at the polylinker site, and the target protein was produced as a MBP fusion protein.

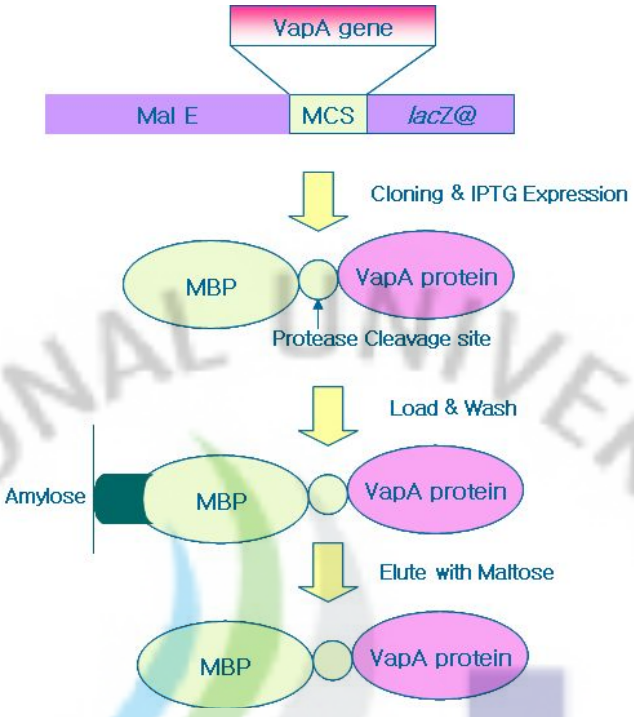


Figure 3. Illustration of protein fusion and purification system (NEB).

The target gene was inserted into multi cloning site and the *Mal*E gene was operated by IPTG system. The expressed MBP fusion protein was purified by affinity chromatography using amylose resin. The MBP fusion protein was attached with amylose resin and they were eluted with maltose buffer.

SDS-PAGE and western blot anaylsis

To obtain the Anti-VapA polyclonal antiserum from rabbit, 2 g/㎖ of purified MBP-VapA proteins were entrusted to the research institute, Peptron Inc (DaeJeon, KOREA). The obtained antiserums were used for immunoblotting of *Vap*A's expression.

Purified MBP fusion VapA samples were boiling at 95℃ for 5 min in 1x SDS sample buffer and then separated at 8% polyacrylamide gel by SDS-PAGE. The gel was stained with Coomassie briliant blue [22] and for the western blot analysis, the proteins on 8% gel were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) by electroporatically (Fast-blot, Biometra). Then, the membrane was blocked with Tris-buffered saline (TBS; 20 mM Tris (pH 7.4), 200 mM NaCl) that contained 5% skim milk. The membrane was proved first with 1:10,000 monoclonal anti-MBP antibody (NEB) and then with a 1:1,000 goat anti-rabbit IgG conjugated to HRP (Sigma). Detection of VapA was carried out with the substrate 4-chloro-1-naphthol (Bio-rad, Hercules, CA, USA) and H_2O_2.

2. Construction of Anti-VapA oral Vaccine

Materials

For the transformation of recombinant plasmid, *E. coli* H681, △*asd* mutant strain and SeTy H683, a △*aro* △*asd* mutant strain were used as the recipient strains. These were kindly provided by Dr. David Pascual in the Laboratory of Veterinary Molecular Biology, Montana State University [44].

These are obligate requirement of DAP and will undergo lysis in environmental without DAP [14]. *Asd* + plasmid pYA292 was chosen for carrying *Vap*A gene (Fig. 4). All strains were cultured in LB broth, in rotary shaker 180 rpm at 37℃. 100 μl/ml of Ampicillin was added for the maintenance and screening the transformants. 50 μl/ml of DAP was supplemented on media for the *asd* - strains growth.

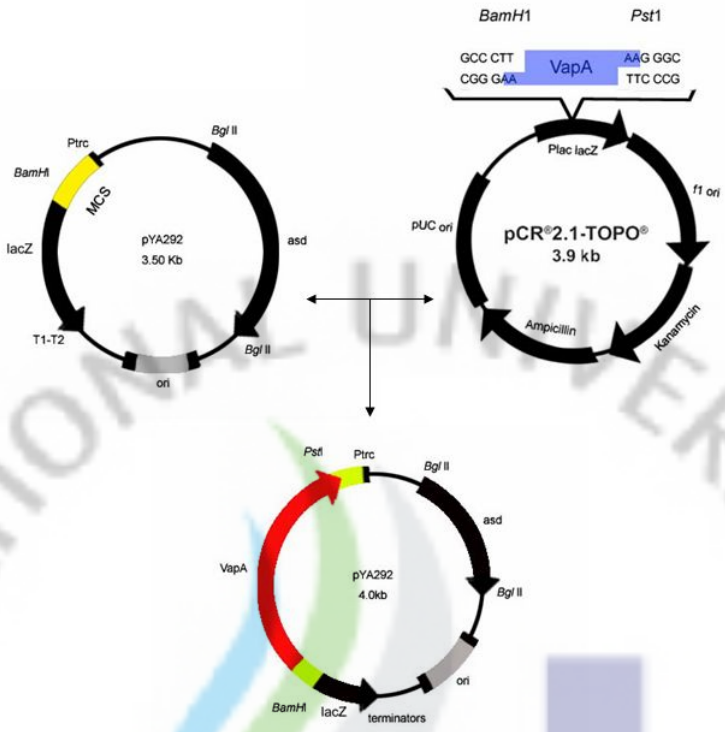


Figure 4. Construction of a recombinant plasmid for transformation to live oral vaccine.

The *asd* + plasmid, pYA292, and TOPO-*Vap*A was treated with *Bam*H1 and *Pst*I, separately. The cutting zone of insertion and vector was ligased with T4 DNA ligase.

Salmonella vaccine construction

For the construction of pYA292-*Vap*A plasmid, *Vap*A gene was amplified by PCR using the specific primer ;

*Vap*A F2: 5'-AGG CGG ATC CAC CAT GAA GAC TCT TCA CAA GAC-3' (the *Bam*H1 site is underlined)

*Vap*A R: 5'-GCG ACT GCA GTT AGG CGT TGT GCC AGC TAC-3' (the *Pst*I site is underlined).

First, amplified *Vap*A gene was inserted into pCR®2.1-TOPO® vector and constructed a TOPO-*Vap*A plasmid, as described above. The plasmid and pYA292 were digested with endonuclease *BamH*1 and *pst*I. The fragment of *Vap*A and pYA292 were purified separately using the QIAquick Gel Extraction Kit (Qiagen). The purified plasmids were ligased with T4 ligase in 16℃, overnight. The electroporation of ligated mixture to *E.coli* H681 and successfully transformants were grown into LB deprived of DAP. The purified plasmid obtained from strain H681 was then electroporated into H683 and grown into LB deprived of DAP. The presence of *Vap*A gene was confirmed by PCR each step and the expression of the VapA protein from H681 and H683 was identified by western blot analysis. For the identification of that the VapA protein expression was resulted from the SeTy-V vaccine cell surface or not, the strains were suspended in sterile distilled water and centrifuged at 20,000 g for 15min. The supernatants (inner part protein) and pellets (surface part protein) were separately collected and analyzed by western blot analysis. The SeTy-V vaccine was stocked with 20% glycerol in -80℃.

3. Local immunization of Anti-VapA oral Vaccine to mice

Immunization of mice

Six weeks old female BALB/c mice were immunized intra-gastrically and intra-nasally with recombinant strains of SeTy-V (vaccine), SeTy-P (vector) and PBS alone. For inoculum preparation, a single bacterial colony was picked and grown in 10 ml LB broth for 10-12 h at 37℃ with shaking at 180 rpm. The bacterial suspensions were collected by centrifugation at 6,000 g for 5 min and washed with three times and resuspended with 0.85% sterile saline. The bacterial cell concentration was adjusted to $1x10^9$ CFU (200 μl /mouse). Following starvation of food and water for 4 h, 6 weeks old mice (five mice per group) were pretreated with 200 μl of 50% saturated sodium bicarbonate solution and dosed orally with 200 μl of SeTy-V, SeTy-P (vector) and PBS using a feeding needle on days 0 and 14.

Serum and fecal Samples

Serum and fecal samples were collected on days 7, 14, 21, 28, 35, 42, 49 and 56 after 2'nd immunization. Sera was prepared from the blood samples of retro-orbital vein using capillary pipette and stored at -20℃. The fecal samples were prepared by placing 100 mg fecal material into 1 ml 1 x PBS supplemented 0.1% sodium azide and vortexing for 15min in room temperature. Samples were centrifuged and the supernatants were collected and stored at -20℃.

ELISA

For identifying of the titration of IgG and IgA, a standard ELISA has been described. ELISA was performed using 96 well microplates were coated with purified 2 μg/well of MBP-VapA. MBP only coated plates were used as control plates. Plates were blocked with 1% BSA in 1 x PBS for 1 h at 37℃. After blocking, plates were rinsed three times with 1 x PBS- Tween 20 (0.05%) solution. Samples were then added with sera dilution at 1:30 and fecal sample dilution at 1:10 for 2 h at 37℃. After rinse the plates, secondary antibodies were adjusted at 1:1,000 dilutions of goat anti-mouse IgG-HRP (SIGMA) for sera samples and goat anti-mouse IgA-HRP (SIGMA) for fecal samples 1 h at 37℃. After three times washing step, plates developed using ABTS (2.2'-azino bis(3-ethylbenzthiazoline-6-sulfonic acid), SIGMA) solutions and hydrogen peroxide (H_2O_2). Absorbance was read at 405/492nm using a Spectra III spectrophotometer (SLT-Labinstrument, Austria).

3. RESULTS

The confirmation of virulence gene of *R. equi* by PCR

R. equi from the pathogenic foals had a virulent plasmid showing surface protein VapA. The virulence plasmid was confirmed by PCR and showing 560 bp product (Fig. 5). The protein expression of VapA was identified by western blot analysis and it showed 15-17 kDa positive band [data not shown]. The VapA protein of live *R. equi* was especially well expressed in BHI agar than other medium including Blood agar base, Nutrient agar and LB agar medium [data not shown].

Sub-cloning of the VapA gene into pCR®2.1-TOPO®

The 560bp-*Vap*A gene amplified from the *R. equi* was cloned into pCR®2.1-TOPO®. The vector possesses M13 reverse priming sites at multi cloning site. If the cloning was successfully achieved, the PCR using M13 universal primer shows about ~700 bp product containing of 560 bp *Vap*A gene. The presence of VapA gene was confirmed by PCR. (data not shown) The transformation in DH5α was also successfully conducted.

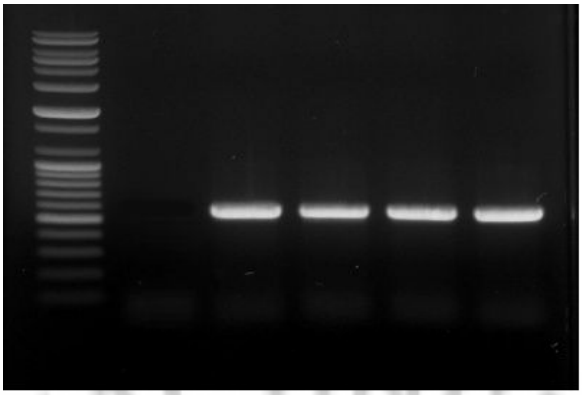


Figure 5. Identification of the 560 bp PCR products, *Vap*A of virulent *R. equi.*

Lane M, 100 bp plus DNA ladder (Bioneer, DaeJeon, Korea); lane 1, *Vap*A-negative *R. equi*, Re1, stocked in our laboratory; lane 2, *Vap*A-positive *R. equi,* Re73; lane 3, *Vap*A-positive *R. equi*, Re74; lane 4, *Vap*A-positive *R. equi*, Re75; lane 5, *Vap*A-positive *R. equi,* Re63.

Expression and purification of MBP-VapA fusion protein

The *Vap*A gene amplified by PCR from TOPO-*Vap*A was digested with endonuclease *Bam*H1 and *Pst*I. These were successfully ligased with pMAL-c2 vector. The pMAL-*Vap*A construction was transformed into DH5α, and the expression of fusion protein was induced for 2 h with IPTG at 37℃ and cell lysates were analysed by SDS-PAGE and Western blot analysis (Fig. 6). After MBP-VapA protein was induced fully by IPTG, these were purified using affinity chromatography with amylose resin column. The soluble fraction of sonicated cell was attached at amylose resin and purified with column buffer contained 10% maltose. The purified MBP-VapA was loaded after successfully dialyzed. Induced band of MBP and MBP-VapA were confirmed by SDS-PAGE and western blot analysis with monoconal MBP antibody. The assay showed 43 kDa MBP (Fig. 6. lane2) and ~60 kDa MBP fusion VapA protein (Fig. 6. lane 4) of lysated cells and purified ~60 kDa MBP-VapA protein (Fig. 6. lane 5).

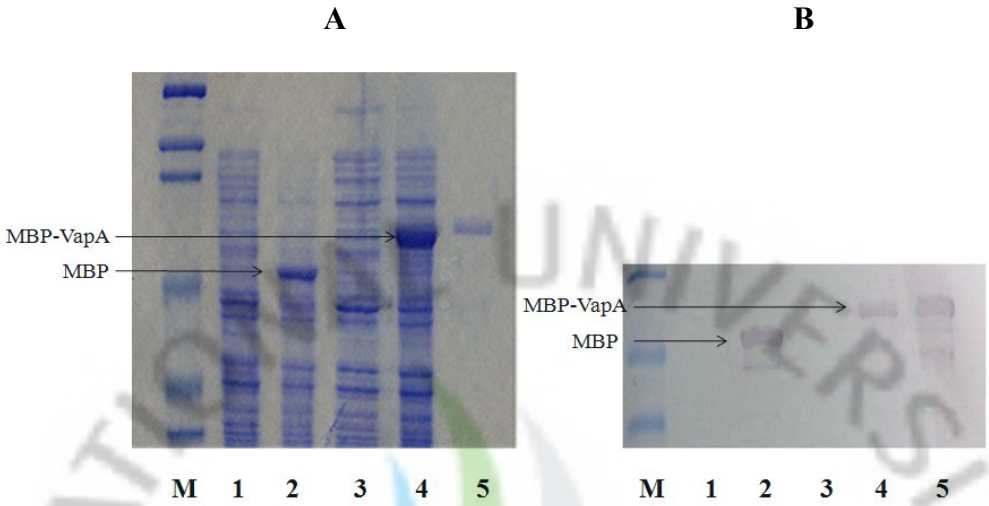


Figure 6. SDS-PAGE (A) and Western blot analysis (B) of pMAL-*Vap*A construct and purified MBP-VapA protein.

Lane M, Broad range marker (BioRad); lane 1, Uninduced pMAL ; lane 2, induced pMAL with IPTG for 2 h; lane 3, Uninduced pMAL-*Vap*A construct ; lane 4, Induced pMAL-*Vap*A construct with IPTG for 2 h; lane 5, Purified MBP-VapA. Western blot analysis (B) with MBP monoclonal antibody (NEB, Beverly, MA, USA) indicates the facts of induced MBP and MBP-VapA.

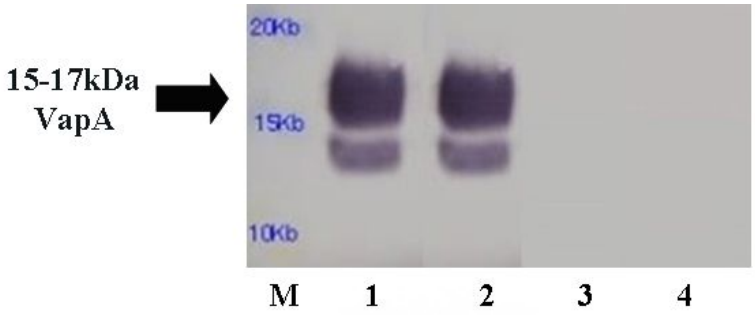


Figure 7. Western blot analysis with whole VapA positive (lane 1, lane 2) and negative (lane 3, lane 4) *R. equi* with polyclonal antiserum from MBP-VapA protein.

Lane M, Broad range marker (BioRad); lane 1, VapA positive strains Re74; lane 2, VapA positive strains Re63; lane 3, VapA negative strains Re1; lane 4, VapA negative strains Re2. Western blot analysis was carried out with immunized rabbit serum 1:1,000 as a 1'st antibody and goat anti rabbit IgG - HRP, 1:1,000 as a 2'nd antibody substrate 4-chloro-1-naphthol (Bio-Rad) and H_2O_2 was used for the color development.

Recombinant *S.* Typhimurium vaccine expressing VapA antigen

The *S.* Typhimurium vaccine expressing specific antigen was able to stimulate mucosal immune system. We tried to construct a Salmonella vaccine vector expressing VapA antigen. The vaccine candidate, H683, couldn't live without DAP. Therefore, the asd+ plasmid pYA292 was ligased with *Vap*A restricted with endonuclease *Bam*H1 and *Pst*1. The construct was transformed into *E. coli* △*aro* strain, H681. And purified plasmid was retransformed into *S.* Typhimurium △*aro*, △*asd* strain, H683. All strains of H681 and H683, H681-pYA292 and H683-pYA292, H681-p*Vap*A and H683-p*Vap*A were lysated to SDS-PAGE assay. This assay didn't give a strictly distinguish that the protein express was present or not (data not shown). But the western blot analysis using the H681-*Vap*A and H683-*Vap*A was predominantly provide the distinct expression result (Fig. 8. lane 3, 5) with the positive control VapA expression (Fig. 8. lane 1).

In the western blot analysis, identifying the fact that SeTy-V cell produce the VapA protein at the cell surface or not gave a positive responses in pellet sample on the contrary of supernatant sample (Fig. 9). This result indicated that the recombinant SeTy-V vaccine express the VapA protein at the surface part of vector system.

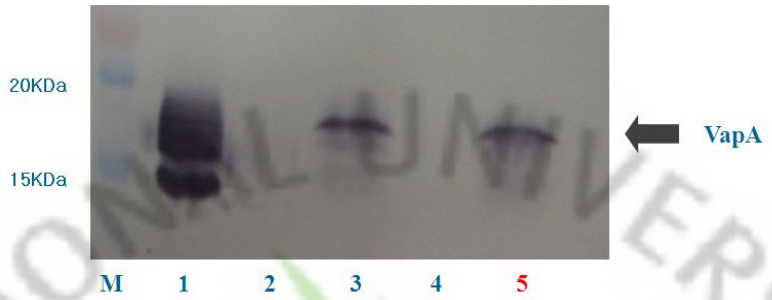


Figure 8. Identification of the expression of VapA in vaccine candidates.

Lane M, Broad range marker (BioRad); Lane 1, *Vap*A-positive *R. equi,* Re74; Lane 2, Attenuated strain, *E. coli* H681, carrying pYA292; Lane 3, attenuated strain, *E. coli* H681, carrying pYA292-*Vap*A; Lane 4, attenuated strain, SeTy H683, carrying pYA292; Lane 5, attenuated strain, SeTy H683, carrying pYA292-*Vap*A.

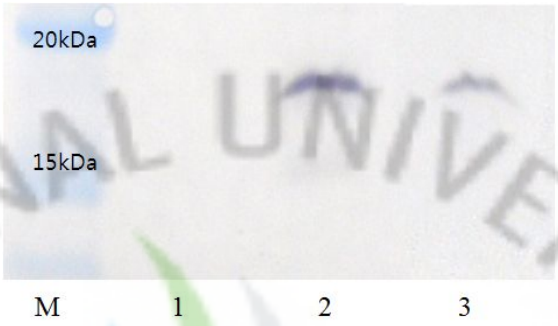


Figure 9. Western blot analysis, for the verifying of VapA expression region of vaccine cell

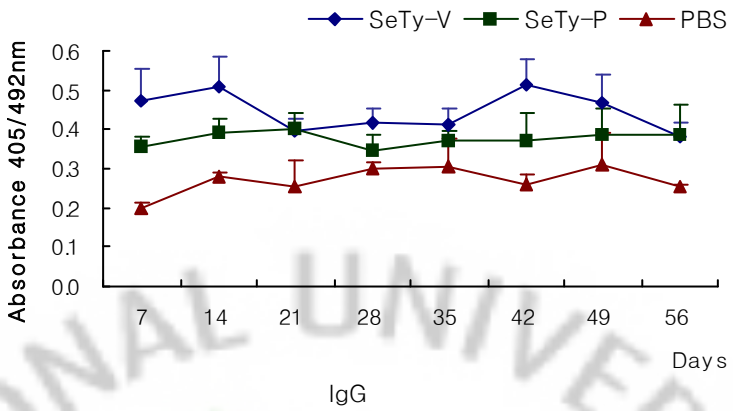
Lane M, Broad range marker (BioRad); Lane 1, supernatant sample of lysated cells; Lane 2, pellet sample of lysated cell; Lane 3, whole vaccine cell lysated as a positive control. In the western blot analysis using VapA specific antiserum, the positive immune response was identified in pellet sample of lysated cell. In the consideration of this result, the recombinant SeTy-V vaccine express the target protein at the cell surface.

Detection of VapA-specific antibody

VapA specific IgG in serum and the specific IgA in feces were identified by ELISA (Fig. 9). A specific IgG in vaccine administration group (Fig. 9A) had OD values of 0.38 ± 0.034 ~ 0.51 ± 0.08 for 56 days after 2'nd immunization. It was somewhat higher than SeTy-P control group, 0.35 ± 0.036 ~ 0.39 ± 0.038 OD values and PBS only administration group, 0.20 ± 0.015 ~ 0.31 ± 0.017 OD values. However, the markedly increasing tendency of IgG were not present throughout 7 to 56 days after immunization.

VapA specific IgA in vaccine administration group (Fig. 9B) had OD values of 0.04 ± 0.016 ~ 0.14 ± 0.058 for 56 days after 2'nd immunization. It was somewhat higher than SeTy-P control group, 0.06 ± 0.016 ~ 0.09 ± 0.012 OD values and PBS only administration group, 0.027 ± 0.008 ~ 0.055 ± 0.001 OD values. However the markedly high IgA production point were not present throughout 7 to 56 days.

A.



B.

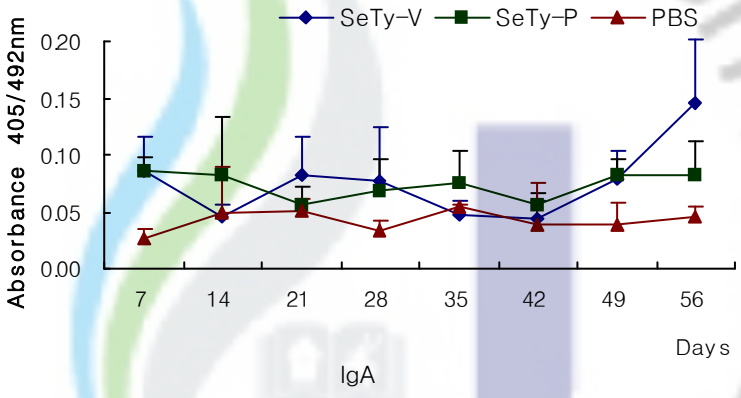


Fig. 9. Vaccination with SeTy-V (vaccine candidate), SeTy-P (plasmid control), PBS (control) for the identification of IgG from serum (A) and IgA from fecal (B).

VapA specific IgG in serum and the specific IgA in feces were higher OD values than SeTy-P, PBS administration group's. But the specific increasing antibody tendency or specific highest titre point for 56 days were not present.

4. DISCUSSION

Over the past twenty to thirty years, significant progress has been made towards understanding the mechanisms of protective immunity of *R. equi*. Previous vaccination attempts have not produced reliable protection. Direct oral immunization of foals with a modified bacterin offered partial protection, but did not result in induction of a lasting anamnestic responses [7]. After that, it is asserted that intracellular pathogen, *R. equi*, generally requires a Th1- type responses for protective immunity, the DNA vaccines which can be used for elicit a Th1 response are potentially useful for protection against the pathogens [23, 37]. So the *Vap*A-based DNA and protein vaccines were studied out for the immunity. They induced a significant Th1 responses, but did not enhance clearance of *R. equi* in the murine model [45].

To date, studies using various VapA vaccines have failed to produce immunity in mice [45]. So the goal of the present study was development of a multivalent vaccine against equine *R. equi* infection using mucosal immune system. A variety of different attenuated strains of *Salmonella* have been employed as vaccine vectors to orally deliver antigens of viral, bacterial and parasitic origin to the immune system [2, 5, 9, 12, 16]. For example, *Salmonella* strains expressing the *E. coli* fimbrial protein K99 were used to immunize adult mice, and the resulting anti-K99 T-cell responses were examined [5]. Also, an *aro*A attenuated strain of *Salmonella enterica* serovar Typhimurium (SL3261) has been used to deliver the *Yersinia pestis* V antigen as a candidate oral plague vaccine. Immunoblot analysis showed V antigen expression in SL3261 in vitro and intragastric immunization of mice with the

recombinant *Salmonella* resulted in the induction of V antigen-specific serum antibody responses. But the antibody responses induced by the recombinant Salmonella did not correlate with the protection afforded [16]. And the eukaryotic expression vector pCMV containing the gene of the glycoprotein D (gD) of the herpes simplex virus 2 was used to transform *Salmonella.* The oral immunization with the transformed Salmonella elicited a strong cellular immune response in both, the mucosal and systemic compartments [36]. As a parasite field, an *aro*A- and *aro*D- attenuated strain of *Salmonella* Typhimurium (BRD509) had been used to deliver the recombinant eukaryotic plasmid pSAG(1-2)/CTA2/B expressing a multi-antigenic gene encoding SAG1 and SAG2 of *Toxoplasma gondii* linked to A2/B subunits of cholera toxin as a candidate oral *T. gondii* vaccine [37].

The ability of live recombinant *Salmonella* vaccines to colonize at the GALT, Peyer's patches and antigen presenting system leads immune responses [6]. Antigenic exposure at mucosal sites activates mucosal B and T-lymphocytes to emigrate from the induction site and home to various mucosal effector sites. This pathway has almost been demonstrated for S-IgA antibody responses at mucosal surfaces mediated by B cells, but similar events are assumed to take place with T cell [24]. Different immunization routes, such as oral, rectal, and intranasal can influence for inducing the generalized mucosal immune responses. Specially, It was asserted that oral immunization induced a more restricted mucosal response, as reflected by a more restricted homing receptor profile than nasal immunization [24]. Also, the fact that nasal immunization induced antibodies in a broader range of tissues, such as saliva and the urogenital tract, than oral immunization reflected the more restricted nature of oral immunization [24]. This vaccine delivery approach had been

shown to be effective at inducing humoral and cellular immune responses [18, 36].

Also, we constructed a recombinant live attenuated SeTy vaccine expressing VapA antigen (SeTy-V). The vaccine candidate, SeTy-V, was administrated orally to mice in an attempt to induce protective immune responses.

In this study, we constructed a recombinant live attenuated SeTy vaccine expressing VapA antigen (SeTy-V). It expressed stably the VapA at the vaccine cell surface, in vitro. But the case of administration of the vaccine orally in mice, the statistical significance was not nearly enough for humoral immune responses although the vaccinated group gives a relatively higher IgG and IgA values than control groups. There are some possible explanation of why VapA-expressing SeTy-V lacks the ability to induce the antigen production. First, mucosal vaccine candidates couldn't enough produce the proteins in vivo conditions. Recently, there are studies that intragastric immunization with recombinant *Lactobacillus casei* expressing flagellar antigen gave a antibody - independent protective immunity against *Salmonella enterica* serovar enteritidis [1]. This mucosal vaccine candidates could not elicit the specific antibody production, but it was mainly attributable to cell mediated immune responses and protective immunity [1]. Also, this research revealed that large of purified protein administration had a power to induce the humoral immune responses. Then, lack of the ability of vaccine's antigen producing potent could be a main problem for induction of humoral immune responses in vivo test. Second, if the vaccine stability in gastric condition of mice were not enough, the vaccine couldn't give a antigen presenting to mucosal immune associated cells. Last, the use of BALB/c mice could be not

enough for the challenge model for development of *R. equi* vaccine. This murine strains was known to respond with a Th2 bias to infection by intracellular pathogens [48]. This strain of mice and the route of challenge may not be ideally suited for *R. equi* vaccine studies. Therefore, these vaccines need to be tested in the equine or another animal model that closely similar with the pulmonary infection in foals.

In the future, we have to check the cellular immune responses through IFN-γ production by splenic cells and challenge the pathogenic *R. equi* to mouse group of vaccinated or not for identifying the survival rates. For the enhance of the immune effect of mucosal vaccine, adjusted a adjuvant to live SeTy vaccine for giving a protective effects in gastro-intestinal track could be also considered.

The currently licensed oral vaccines, such as the live oral polio, adenovirus, cholera and typhoid vaccines have proven to be practical and reliable public health tools [10, 25, 27, 37]. Thus other strategies including stable state of vaccine, boost and therapeutic regimen for large antibodies production and giving a potent protective effect to foals could be employed to enhance the efficacy of this vaccine.

5. CONCLUSION

Previous studies have shown that the *R. equi* from the pathogenic equine, contained a virulence plasmid of approximately 85 kb which encoded a 15-17 kDa surface protein, virulence associated protein A (*Vap*A).

For identifying of the characteristics of *Vap*A from *R. equi*, the MBP-fusion protein expression system was used. Expression of the full-length *Vap*A in *R. equi* was 15-17 kDa. In this study, the 60- kDa MBP-VapA fusion protein was detected in *E. coli*.

The purified protein was immunized throughout the rabbit and plentiful immunized serum were acquired. It was confirmed that acquired serum was reacted with 15-17 kDa VapA protein from virulent *R. equi* by western blot analysis.

For the development of new vaccine candidates for *R. equi*, we constructed a recombinant live attenuated SeTy-V vaccine carrying *R. equi* VapA antigen. The vaccine candidate successfully expressed the surface protein of VapA.

The candidate SeTy-V vaccine was orally administrated to 6 weeks mouse group on days 0 and 14 days. Analysis of VapA-specific antibodies after the 2'nd immunization gave a generally higher titre than non-vaccinated group but didn't result in the presence of significance.

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초 록

Rhodococcus equi 의 VapA를 발현하는 재조합 *Salmonella enterica* serovar Typhimurium 백신의 설계

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*Rhodococcus equi*는 어린망아지의 화농성 기관지성폐렴을 일으키는 중요한 병원체이다. *R. equi*의 병원성의 결정은 VapA 항원에 의하며, 많은 연구에서 이를 이용한 새로운 백신 개발에 주력하고 있다. 그러나 이 병원균에 대한 상업화된 백신은 아직 개발되어있지 않다. 본 연구는 *Salmonella enterica* serovar Typhimurium, H683 백신주에 VapA를 발현시켜 *R. equi*에 대한 국소면역백신을 개발하고자 실시하였다. 이를 위하여 VapA 유전자를 pMAL-c2 vector에 삽입하여 *E. coli*에서의 발현과 특징을 살펴보고, MBP-VapA fusion protein을 분리하였다. 정제된 MBP-VapA는 VapA에 대한 항혈청 제조와 ELISA의 coating 항원으로 사용하였다. TOPO-TA vector에 sub-cloning된 *Vap*A 유전자를 *asd*+ pYA292 벡터에 클로닝한 후 SeTy, H683 백신주에 삽입하였다. 설계한 백신 후보주(SeTy-V)의 표면항원에는 VapA가 발현되었다. 개발된 재조합백신 후보주

를 6주령 마우스에 경구투여하여 8주간 혈청과 분변에서의 IgG와 IgA 역가를 ELISA로 분석하였다. 혈청과 분변 시료에서는 현저하지는 않았으나 VapA에 대한 특이 IgG 및 IgA가 존재함이 확인되어 국소면역백신으로서의 가능성이 확인되었다. 개발된 후보주는 발현의 조건과 면역보강제의 보완이 추가적으로 연구된다면 효과적인 *R. equi*에 대한 국소면역백신으로서 적용이 가능할 것으로 사료된다.

주요어: *Rhodococcus equi*; VapA; *Salmonella* Typhimurium; Vaccine

감사의글

바쁘게 달려온 시간을 뒤로하며 ... 대학 생활부터 시작된 제주에서의 8년은 제게 너무나 많은 것들을 주었습니다. 지난 모든 기억들이 영화필름처럼 머릿속을 스쳐지나갑니다.

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