Investigations into the Use of the M-protein for the Production of a Vaccine Against *Streptococcus equi* Subspecies *equi* Using Chicken IgY

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Abstract

Strangles is a destructive disease in horses caused by an infection of the bacteria *Streptococcus equi* subspecies *equi*. Current vaccines designed to protect horse populations for this infection are only 50% effective at preventing the disease. The aim of this study was to develop a vaccine against *Streptococcus equi* by targeting the antiphagocytic virulence factor of the bacteria, the M-Protein (SeM18). SeM18 was expressed using the ampicillin resistant pET vector system and purified by metal affinity column using Cu(II). Purified protein was used to vaccinate chickens, and the anti-SeM18 IgY present in the yolk of the eggs produced by these chickens was purified. IgY was purified by SDS PAGE and Western Blot analysis. SDS PAGE of the induced protein showed a distinct band at 18 kDa, indicating the presence of SeM18 in the fraction. Analysis of the purified egg yolk detected the presence of anti-SeM18 IgY. This IgY would then be used to vaccinate horses against *Strep. equi* infection.

Acknowledgements

Dr. Sergey Artiushin with the Gluck Equine Research Center Department of Veterinary Science University of Kentucky for the stock SeM18 protein and the recipe for Lysozyme Buffer used for the breakup of inclusion bodies in purification of expressed SeM18 from the pET vector. Dr. Mark Hemric for extraordinary support and the use of his lab. Dr. Randall Hubbard for allowing his chickens to be inoculated with *Streptococcus equi* subspecies *equi*.

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Introduction

Etiology

Streptococcus equi subspecies *equi* is a Gram-positive bacterium of the Lancefield's group C. *In vitro*, it grows in long chains of irregularly shaped cocci, and its colonies are an amber color and have the appearance of mucus. When isolated, it is encapsulated and is highly virulent. *Strep. equi* causes hemolysis, which is the rupture of red blood cells.

Pathogenesis

When this organism is inhaled or ingested by members of the family Equidae, the cells attach to the tonsil and adjacent lymphoid nodules (Wilson, 1988). Enzymes and toxins are then released by the bacteria that damage nearby cells and cause inflammation of the affected area. The success of the bacterial infection is significantly aided by the presence of the M-protein (SeM18) within the bacterial capsule of *Strep. equi*. Immune cells, neutrophils and macrophages, in the host animal generally serve to protect it from infection by phagocytizing foreign material such as bacteria. The M-protein released by *Strep. equi* inhibits such phagocytosis. Opsonization is a process by which foreign particles in the body are marked by the complement system that attracts phagocytes to this particle to destroy it. The M-protein released by *Strep. equi* inhibits complement deposition, thereby preventing opsonization (Boschwitz & Timoney, 1994).

Other factors released by *Strep. equi* that contribute to its virulence include capsular hyaluronic acid, hemolysins, hyaluronidase, leukocytotoxins, and mitogens

(Chanter, Collin, Mumford, 1994). The hyaluronic acid capsule and SeM18 also increase the strength of this bacterial infection by helping the bacteria attach to host cells. The bacteria move to the submandibular and retropharyngeal lymph nodes, and, even though SeM18 has decreased the effectiveness of immune response in the host organism, many neutrophils still migrate to these lymph nodes (Timoney, 2004). However, due to the decreased ability of these neutrophils to phagocytize the bacteria as a result of SeM18's inhibition and the fact that the bacteria grow in chains, these neutrophils are ineffective in clearing the infection and instead become a source of accumulation of dead cells in the lymph nodes. The other sources of accumulation in the lymph nodes are necrotic tissue and extracellular organisms. Usually, abscessing of lymphoid tissue only occurs in the lymph nodes in the head and neck, but rarely this process will spread to other lymph nodes throughout the body. Often the rupture of the retropharyngeal abscesses will leak into the guttural pouch causing guttural pouch empyema, which is simply the accumulation of puss and septic exudates in this area. This can cause swelling and pain in the neck area and can even affect the horse's breathing. Because *Strep. equi* infection leads to constriction of the upper airway due to a number of different factors, it has also been called "strangles" (Taylor & Wilson, 2006).

Epidemiology

Strangles was first described in 1888, though this condition that affects horses had been referenced for centuries (Todd, 1910). Horses normally have bacteria growing in the nasopharyngeal areas that are harmless to the animal and yet help to keep other more dangerous infections from taking the horse by competition. These bacteria are called normal flora, and while *Streptococcus equi* subspecies *zooepidemicus* is part of a horse's

normal flora, *Strep. equi* subspecies *equi* are not. Yearlings and young adult horses have been found to be the most susceptible, with yearlings being most severely affected by the bacterial infection (Sweeney, Whitlock, Meirs, 1987).

Overcrowding, mixing of horses from different areas, weaning, traveling, severe weather, concurrent illness, and improper nutrition can lead to increased transmission of the disease between horses (Reed, Bayly, Selon, 2004). This is because, after two to three days of the infection, horses shed *Strep. equi* in nasal secretions. Therefore, any factor that keeps horses in close contact with each other lends itself to increasing transmission of bacterial infections. If the bacteria in the nasal secretions lands on feeders, water bucks, tack, handlers, etc., and these items are then used for a different horse, this new horse is likely to come into contact with this virulent bacterial infection. Once outside of the horse's upper respiratory tract, heat, sunlight, desiccation, and the many disinfectants used on most well-managed farms, the organism does not survive for more than a few weeks (Wilson, 1988).

Immunology

Because the innate immune response is so hindered by SeM18 and other antiphagocytic factors released by the bacteria to increase the strength of the infection, the most important protective immune response to *Strep. equi* is the specific immune response.

Specific immune response. The specific immune response includes the production of antibodies specifically designed by B cells to bind to specific antigens on the bacteria and lead to their degradation. Undifferentiated B cells in the horse present many different antibodies on their surfaces, and when one of these antibodies comes into

contact with a protein on the bacteria that it precisely fits, this antibody/antigen complex is taken up by the B cell and proteolyzed into peptides. These peptides, which have the capability to produce an immune response, are presented on the surface of the B cell by its MHC II molecules. The matching helper T cell is then attracted to the combination of MHC and antigen, and it releases lymphokines and thereby activates the B cell. The B cell then produces millions of copies of the antibody, which circulate in the blood and lymph fluid. These antibodies bind to the antigens of the bacterial infection throughout the body and mark them for destruction. Antibodies can be produced that are designed to bind to the many different antigens on the surface of the bacteria. The most effective antigen to target with production of specific antibodies has been shown to be the M-protein (Timoney & Eggers, 1985).

Memory cells and the secondary response to infection. When B cells and T cells are activated during the specific immune response, some of the proliferating cells are selected to become memory cells. These memory cells contain antibodies for the specific antigen in question, and when that antigen is subsequently encountered, the memory cells mount a secondary immune response, which can give a stronger response much more quickly. Following a natural infection of *Strep. equi* subspecies *equi*, 75% of horses are able to mount an effective secondary response against future infection for four years (Hamlen, Timoney, Bell, 1994). One special form of a secondary response occurs when a foal is born to an immune mare. *Strep. equi* specific antibodies can be passed to the newborn horse from a mother who has built up these antibodies. This results in temporary immunity for the foals, but this phenomenon disappears after approximately three months (Galan & Timoney, 1987).

Clinical Signs

Clinical signs of a Strangles infection can be seen after three to eight days of having come into contact with the bacteria. These symptoms include a fever of 103°-106°F, anorexia, depression, and mucopurulent nasal discharge (Yelle, 1987). In the effort to fight off the infection, the number of leukocytes in the blood of the horse increases significantly, and this effect can be visualized when a sample of blood is taken and observed under microscope. In addition, the lymph nodes swell with the increase in production of lymph fluid to fight this bacterial infection. These lymph nodes abscess and rupture, and the horse generally recovers in one to two weeks (Reed et al., 2004).

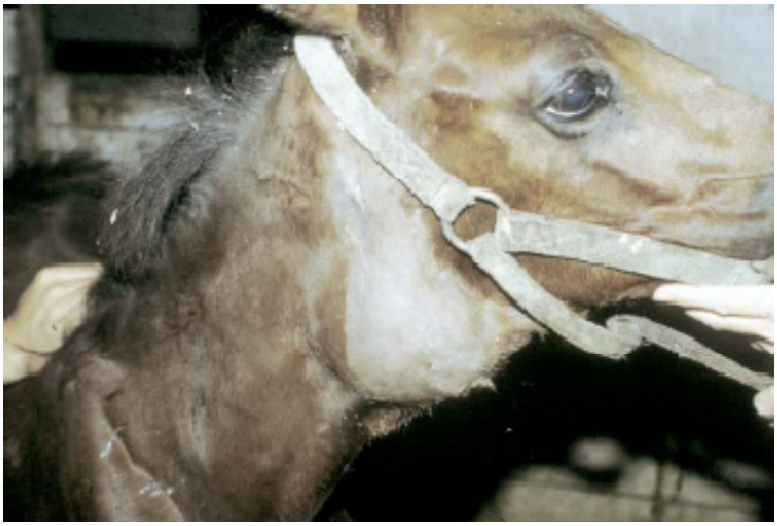

Figure 1 Medial retropharyngeal lymph node abscess visible in Viborg's triangle (Taylor & Wilson, 2006).

The major concern with Strangles infection is that the lymph nodes may become large enough to press in against the walls of the upper respiratory tract and keep the horse from breathing. If this is happening, the horse will often make a distinctive straining noise when trying to breathe. A tracheotomy, which is a surgical procedure in which an

incision is made in the front of the animal's neck that connects directly to the trachea, may need to be performed so that the horse does not asphyxiate.

Diagnosis

A definitive diagnosis can be made by a culture of nasal washes or fluid aspirated from the abscessed lymph nodes.

Polymerase chain reaction. Polymerase Chain Reaction (PCR) is a molecular biology technique that generates many copies of a particular DNA sequence for gene sequencing, DNA-based phylogeny, or functional analysis of genes. PCR can accomplish in a few hours what previously took weeks by inserting the target DNA into bacteria vectors for amplification. PCR utilizes three steps: denaturation, annealing, and elongation. Denaturation, which is the melting of the DNA sequence thereby taking away its characteristic conformation and separating the two strands of the double helix, is performed by heating the sequence to approximately 94 degrees Celsius. Annealing occurs when a complementary primer strand binds by hydrogen bonds to the denatured DNA strand as it cools to about 54 degrees. These primers are short segments of single-stranded nucleic acids that initiate synthesis. Because primers are highly specific and initiate the production of DNA copies, the choice of appropriate primer is an extremely important aspect of implementing the PCR technique.

The elongation step occurs when DNA polymerase attaches at the primers and copies the DNA template in both directions using the bases adenosine, guanine, cytosine, and thymine present in the PCR reaction mixture. PCR is performed in multiple cycles, and each cycle doubles the number of DNA copies, resulting in exponential amplification of the target DNA sequence. PCR has been used to identify the DNA sequence of the

Strep. equi SeM18 gene, and is three times more sensitive in detecting *Strep. equi* than culture (Timoney & Artiushin, 1997).

Enzyme-linked immunosorbent assay. Another important means of diagnosis involves taking a sample of the horse's blood and measuring serum levels of SeM18 antibody with an enzyme-linked immunosorbent assay (ELISA) test. An ELISA test involves coating a surface with an unknown amount of antigen. The antibody for the specific antigen in question is applied to the surface, and this antibody is linked to an enzyme. When a substrate is added to the surface containing both antigen and antibody, this will cause a visually noticeable change in the material, most often a color change. A color change therefore indicates the presence of the antigen, in this case, SeM18 (Sheoran, Sponseller, Holmes, 1997). It is possible for a horse to come into contact with Strep. equi and remain asymptomatic yet serve as carrier for the bacterial infection to spread to other horses in its environment. For this reason, diagnostic testing of animals that appear to be well is important to prevent the spread of this disease (Collins & Wernery, 1999).

Treatment

Treatment for horses that have developed a *Strep. equi* infection has been a matter of controversy. Most horses require only rest in a warm, dry setting with access to soft, moist feed and fresh water, with nonsteroidal antiinflammatory drugs such as pheylbutazone and flunixin meglumine to reduce fever and pain. However, antimicrobial drugs can be used to treat the infection more effectively. With use of antimicrobial drugs at the onset of fever and nasal discharge and the length of time to recover is reduced, lymph node abscesses do not form. Procaine penicillin G at 22,00IU/kg twice per day for

ten to fourteen days is the standard prescription (Wilson, 1988). If lymph abscesses develop, they should be lanced and drained daily until they return to normal size. To prevent *Strep. equi* infection from occurring, three vaccines are available. Unfortunately, vaccines only effectively protect 50% of Strangles cases from occurring.

Vaccines and the secondary response. Vaccines are an important immunological tool for the prevention of disease. The idea behind a vaccine is the secondary response of the animal immune system. When an animal has been exposed to a bacteria or virus in the past, its immune system contains memory cells that "remember" the specific biochemistry of that foreign particle. These memory B cells and T cells can more quickly respond to a second infection and eliminate it before substantial symptoms are perceived. A vaccine contains a virus or bacteria and exposes the animal to it, so it can have an appropriate secondary response when it comes into contact with the virus or bacteria in nature. A vaccine can be an attenuated, which means that it is a weakened form of the bacteria or virus. A vaccine may contain a dead virus or bacteria. A vaccine may contain the most antigenic parts of the virus or bacteria but not the whole organism. If the animal were injected with a simple culture of the *Strep. equi* bacteria, it would succumb to infection, and a problem would have been created rather than prevented. By exposing the animal's immune cells to a non-dangerous form of the bacteria, it is able to mount a response when they encounter that antigen again in its virulent form.

The strangles vaccine. The most effective Strangles vaccine involves injecting the animal with a low concentration of the SeM18 protein so that when it encounters the complete *Strep. equi* bacteria, it is able to recognize the SeM18 protein and respond much more strongly and quickly to prevent infection (Hoffman, Staempfli, & Prescott, 1991).

These vaccines are administered intramuscularly. Administration of vaccines with bacterial extracts has been shown to cause purpura hemorrhagica, an immune-mediated, type III hypersensitivity, and high concentrations of SeM18 increase the risk for this reaction. (Pusterla, 2003)

Developing an effective vaccine for *Strep. equi* is currently a matter of much research, as the most effective vaccine is only 50% effective. Because *Strep. equi* shares greater than 95% DNA homology with *Streptococcus zooepidemicus*, another less virulent beta-hemolytic Streptococcus, many of the proteins produced by the two bacteria are identical. Interestingly, however, the immune response to *Strep. zooepidemicus* does not protect the animal from *Strep. equi* infection (Bazeley, 1942). For this reason, in order to develop an effective vaccine against *Strep. equi*, those proteins that are immunoreactive and are not shared by these two bacterial strains should be investigated as to their use in a vaccine. The proteins that have been shown to be expressed by *Strep. equi* but not *Strep. zooepidemicus* include Se18.9, SeM18, SePE-H, and SePE-I (Tiwari, Quin, Artiushin, Timoney, 2007). Interestingly, there is an allele for the SeM18 protein in Strep. zooepidimicus, but it is not expressed (Timoney, Artiushin, Boschwitz, 1997).

In the process of developing a vaccine, inactivated whole bacteria and several recombinant proteins have been used, but with little success. Vaccines using attenuated whole bacteria have conferred immunity to an insignificant number of horses, and more often induced adverse reactions (Jorm, 1990). Several mouse studies indicated that vaccines targeting the SeM18 protein of Strep. equi effectively cause generation of SeM18 reactive antibodies in the animal. However, vaccines specifically targeting SeM18 have had little success in protecting horses. One study found that “lack of

efficacy of vaccines is probably not due to failure to stimulate serum bactericidal antibodies but failure to stimulate pharyngeal antibodies to the *Strep. equi* M protein" (Galan & Timoney, 1987). Several mouse studies also indicated that a recombinant hyaluronate associated protein (HAP) caused the generation of protective antibodies against this protein but likewise did not protect vaccinated horses from developing strangles (Chanter, et al., 1999). These mysterious results imply that a vaccine with multiple recombinant proteins may be necessary so that multiple immune responses can be mounted to clear the bacteria.

One intranasal vaccine, the "Pinnacle I.N.", developed by Timoney, is of particular interest. A non-specifically attenuated strain of Strep. equi was developed by chemical mutagenesis that resulted in non-defined point mutations throughout the genome. After being injected into the animal, back mutations can occur and bring the bacteria up to full virulence. This vaccine has not been licensed due to safety concerns, despite the fact that it may protect up to 100% of horses (Timoney, 1993). In an attempt to improve the safety of this vaccine, deletions of the 3' end of *hasA* gene and the 5' end of the *hasB* gene were performed, making this bacterial mutant unable to reverse to full virulence. Studies have been inconclusive as to whether these deletions make the vaccine safer for horses.

Another important landmark in the development of an effective Strangles vaccine is the live attenuated strain TW 928 developed by Intervet. The *aroA* gene was deleted from this bacterial strain, resulting in a 10^4-fold decrease in virulence in mouse studies. When horses were intramuscularly vaccinated, they had 100% protection from future *Strep. equi* infection, but severe adverse reactions occurred, which made further use of

this vaccine impossible. Lower concentrations of the TW 928 strain were used, and while the decreased concentration decreased adverse reactions, it also decreased the protection conferred by the vaccine. Small collections of pus developed in the top layers of the skin at the site of injection, and these pustules contained the TW 928 strain. With decreased concentrations of TW 928 in the vaccine, less pustules formed and less protection was offered. With increased concentrations of TW 928 in the vaccine, more pustules formed and more protection was offered. These results suggest that the pustules play a significant role in the generation of an immune response against *Strep. equi* (Jacobs, Goonerts, Nuijten, Hartford, & Foster, 2000).

Though live attenuated vaccines may provide the best protection, multi-component subunit vaccines based on several immunogenic Strep. equi surface proteins remain the vaccines of choice in current veterinary practice. This is because of the severe adverse effects experienced by a significant number of horses vaccinated with the live attenuated bacterial strains. By combining the protective effects of inoculating with recombinant proteins, increased protection can be conferred to the animal without the level of adverse effects experienced with live attenuated bacterial strains (Flock, Karlstrom, Lannergard, & Guss, 2006).

IgY for the Development of Effective Vaccines

On the frontier of research in vaccine development is the use of Immunoglobin Y from egg yolk to confer immunity. When an egg is produced in the ovary, immunoglobins from the hen are transferred to the yolk at a concentration of 6-13 mg/ml, comparable to the concentration of IgY in the hen's serum. IgY is phylogentically similar to mammalian IgG and IgE, but its structure is significantly different (Wart, Magor,

Higgins, 1995). In this case, the M protein from *Strep. equi* could be injected into a hen, and this hen's immune system would produce antibodies against this bacteria. When this hen lays eggs, it will passively immunize each egg by transferring anti-*Strep. equi* IgY antibodies to the yolk of each egg. IgY from collected eggs can be purified and administered orally or otherwise to a horse to protect it against *Strep. equi*.

Advantages of IgY. Chicken antibodies are preferable to mammalian antibodies for research and immunotherapeutic purposes for several reasons. First, eggs are a much more efficient way of collecting large amounts of antibodies. It is much easier to make a chicken lay an egg than it is to bleed a mouse, goat, or rabbit. Hens produce five to six eggs per week, which results in a concentration of antibodies equivalent to 75 to 90 ml of serum. It would take an immunized rabbit three to five weeks to produce that same amount and would also involve pain for the animal. In addition, the cost of caring for a chicken is much less than that of caring for a rabbit (Rollier, Charollois, Jamard, Trepo, Cova, 2000). A second benefit of the use of chicken antibodies is the phylogenetic distance between avian antibodies and mammalian antigens. Because the proteins that a chicken contains in its body and that it comes into contact with in its environment are significantly different than the proteins that a mammal contains in its body and that it comes to into contact with in its environment, chickens recognize many more of the antigens on the *Strep. equi* as foreign and can therefore mount a stronger response to eliminate the infection. More antibodies bind to the molecule, amplifying the immune response.

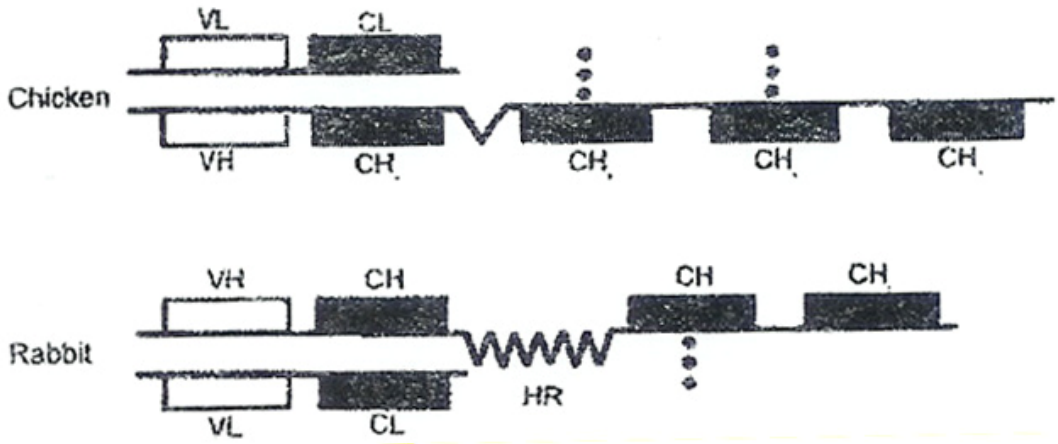

Figure 2 Comparison of chicken IgY with mammalian IgG, showing the significant structural differences between the two which make chicken IgY preferable to IgG for use in vaccine development (Tini, Jewell, Camenisch, Chilov, Gassmann, 2002).

A third benefit of the use of chicken antibodies is that it reduces intereference. Chicken IgY do not cross react with mammalian IgG and therefore avoid interference due to rheumatoid factors (Tini et al., 2002). In addition, chicken antibodies to not activate the human complement system. A fourth benefit of the use of chicken antibodies is that they are incredibly stable. They can be stored for over ten years at four degrees Celcius without loss of antibody activity or for over six months at room temperature. IgY can withstand pH 4-9 (Lee, Chang, Lee, Lee, Koo, 2002). IgY has been administered orally to prevent and treat a variety of diseases, including rotavirus in mice, human rotavirus, bovine coronavirus, entertotoxigenic *E. coli* in pigs, Salmonella species in mice, *Helobacter pylori*, *Streptococcus mutans*, and *Pseudomonas aeruginosa* for treatment of Cystic fibrosis in humans (Zhang, 2003).

Premise for Study

The aim of this study was to develop a vaccine against *Streptococcus equi*. This particular vaccine targeted the antiphagocytic protein SeM18, which makes substantial contributions to the virulence of the *Streptococcus equi* bacterial infection and is unique

to this bacteria. I describe in this paper the characterization of SeM18 and the cloning and expression of its structural gene in *Escherichia coli*. For this procedure, the pET system was used, which is a powerful system for the cloning and expression of recombinant proteins in *E. coli*. I describe the purification of SeM18 from the induced culture and its inoculation into a hen for the production of antibodies. I describe the purification of the resulting IgY and its potential use as vaccine against *Streptococcus equi*.

Methods and Materials

Expression of the M-protein

In order to express the SeM18 protein, it was necessary to grow a starter culture of the pET recombinant in a λDE3 lysogen. The pET recombinant protein was provided by Sergey Artiushin from the Gluck Equine Research Center at the Department of Veterinary Science of the University of Kentucky. To prepare the pET system, the target gene that programs the SeM18 protein was cloned in pET plasmids using bacteriophage T7 transcription signals. The target protein was established in a non-expression host, then the plasmid was transferred into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV*5 control. The plasmids contained a T7*lac* promoter, which is a lac operator sequence just downstream of the T7 promoter. This provided safety for the *E.coli* because the target gene product may have interfered with normal cell function and therefore inhibit its ability to produce mass quantities of the protein. The source used in this experiment to induce was Isopropyl β-D-1-thiogalactopyranoside (IPTG).

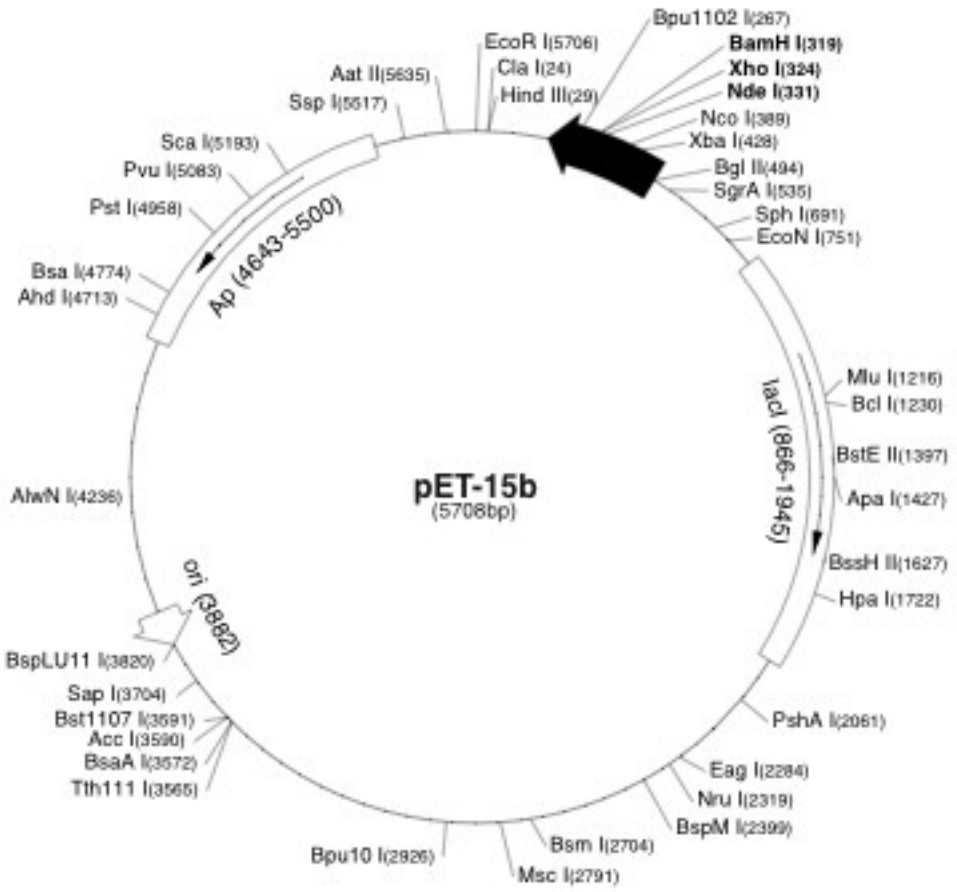

Figure 3 The pET-15b vector used to culture and express SeM18.

From a glycerol stock of the *E. coli* with the SeM18 protein gene already established, 20µL was transferred to an agar plate, having been prepared with Luria Agar from Sigma-Aldrich and a 1M concentration of ampicillin. Ampicllin was used because the pET vector system was designed to be amp-resistant. Therefore, only the *E. coli* containing the recombinant protein should have grown because virtually no other bacteria present in the lab environment could be amp-resistant. The Luria agar plate was incubated for 24 hours at 37°C to allow ample time for the bacteria to grow. Three colonies were selected and transferred to three 3mL culture tubes containing Lurai Broth from Sigma-Aldrich containing 1M ampicillin. The cultures were incubated in the

shaking incubator until the bacterial growth reached an OD_{600} of 0.6, a process which took approximately five hours. The entire contents of these culture tubes were each then transferred to a flask containing 100mL Luria Broth with ampicillin. These flasks were then incubated at 37°C with shaking until the bacterial growth reached an OD_{600} of 0.6, which took approximately three hours. To these cultures, 100µL of 1M IPTG was added and continued to incubate at 37°C with shaking overnight. The IPTG induced culture was centrifuged at 14,000 x g for thirty minutes. A sample of the supernatant was kept for comparison on SDS PAGE, and the rest discarded.

Purification of M-protein with Metal Affinity Column

The pellet was resuspended in 1/20 the volume of Buffer A (8M urea, 20mM TRIS, 100mM NaCl, pH 8.0) of the initial culture, which was about 4.8mL. The Buffer contained 8M urea in order to break up the inclusion bodies. To disrupt the cells, the solution was frozen in the -80°C freezer for thirty minutes and then allowed to thaw. Viscosity was reduced by passing the solution through a syringe needle several times. In order to pellet cell debris, the mixture was then centrifuged at 14,000 x g for thirty minutes. The pellet was saved for comparison on SDS PAGE. The supernatant from this centrifugation was loaded onto a metal affinity column.

The column was prepared using a His-binding resin because the pET vector system contained a His-tag. Using a 1x8cm column, the slurry was poured and allowed to settle. The column was filled with buffer A several times to wash the column thoroughly. The buffer A was drained to a level just slightly above the gel top, at which point 0.5mL of 0.1 M $CuSO_4$ solution was added to the column and allowed to enter. Buffer A was then added to the column to wash the column thoroughly until all excess Cu(II) had been

eluted, which required about 25mL. After buffer A had drained to a level just at the top of the gel, approximately 4mL of the supernatant containing the recombinant protein was added to the top of the gel and allowed to enter. 5mL fractions were collected from this point. The column was filled with buffer A to elute all unwanted proteins and nucleotides. A_{280} measurements were taken of each fraction by transferring the contents of each fraction into a quartz cuvette and measured by Genesys spectrophotometer. When the measurements neared 0.000 absorbance, the column was washed with buffer B (8M urea, 20mM TRIS, 100mM NaCl, 100mM Imidazole, pH 8.0). 1mL fractions were collected and A_{280} measurements taken with the spectrophotometer in a quartz cuvette.

SDS PAGE Analysis

The most concentrated of both the wash fractions and the elution fractions were prepared to be applied to an SDS PAGE gel. The original induced culture in LB broth, the original supernatant from the first centrifugation, the second pellet from the second centrifugation, the wash fraction that gave the highest absorbance, the elution fraction that gave the highest absorbance, and a molecular weight standard were prepared for SDS PAGE. 15µL of each were transferred to seven different 1.5mL eppendorf tubes. To each tube excluding the molecular weight standard, 15µL Sample Buffer (0.5 M Tris-HCl pH6.7, 20% SDS, 20mL glycerol, a pinch of Bromphenol Blue, and 0.5mL Beta-mercaptoethanol) were added. The tubes were boiled for five minutes, microcentrifuged for thirty seconds, and transferred to the gel. The gel used was 4-20% poly-acrylamide from Bio-Rad. The SDS PAGE apparatus was from Bio-Rad. The Electrophoresis Buffer (3M Tris Base, 14.4M glycine, 1M sodium dodecyl sulfate) filled the inner and outer chambers and was used to wash each lane of the gel. The gel was run at 200V until the

lanes reached the end of the gel, which was approximately forty minutes. The gel was then stained for one hour with coomassie blue stain, following which it was destained with a methanol and 10% acetic acid destain overnight. Pictures of the gel were then taken with a gel camera.

Production of Chicken Antibodies

A 500 µg/mL concentration of stock SeM18 protein was prepared and used to immunize one chicken. The eggs collected from this hen were used to isolate IgY designed for the SeM18 protein.

Isolation of IgY from Egg Yolk

Purification of IgY. The yolk was separated from the egg without breaking the yolk and was mixed with three parts 0.1M Phosphate Buffered Saline (PBS) pH 7.5 and one part17.5% polyethylene glycol (6000 MW), giving a final concentration of 3.5% PEG. This mixture was stirred for twenty minutes at room temperature. The sample was centrifuged at 3000 rpm for twenty-five minutes. The supernatant was removed and poured through a cheesecloth to remove any residual yolk lipids and granules. Two parts 17% PEG was added to the one part supernatant and stirred for one hour in an ice bath to precipitate the IgY. The cold was used to keep proteins from being denatured while precipitating. The solution was then centrifuged for twenty-five minutes at 3000 rpm. The supernatant was kept for later testing, and the IgY in the pellet was dissolved in an equal volume of PBS.

SDS PAGE of IgY. An SDS PAGE gel was run using the same procedure as that run with fractions from the metal affinity column. The culture supernatant, Triton-X 100

extract, glass bead treated cells, and cell pellet were run alongside a molecular weight standard.

Western blot of IgY. The proteins were transferred to a nitrocellulose membrane where they were detected using antibodies specific to the target SeM18 protein. The gel from the SDS PAGE was placed in Transfer Buffer (3.03 g Tris, 14.4 g Glycine, 20% v/v methanol, 1L deionized water, pH 8.3) to equilibrate for fifteen minutes. The fiber pads and filter paper were soaked in transfer buffer. A fiber pad was placed on the black side of the Western Blot cassette, followed by a piece of filter paper and then the gel. The wetted nitrocellulose membrane was laid over the gel, making sure that no air bubbles were present between the gel and the membrane. Another sheet of wetted filter paper was placed on the membrane followed by another fiber pad. The sandwich was closed and placed in the electrophoresis module. This entire apparatus was placed in the tank, filled with transfer buffer, and the unit was run overnight at 30V, constant 90mA. Anti-IgY antibodies was applied to the filter paper to detect the presence of anti-SeM18 antibody from the egg yolk.

Results

Expression of the M-protein

The bacterial colonies of the pET vector grew at 37°C. The colonies were an off-white color and a circular shape, characteristic of *Streptococcus* growth on Luria Agar. The agar was infused with ampicillin, a powerful antibiotic. Therefore, only the amp-resistant *E. coli* used as the vector for the SeM18 protein should have grown. As there was only one kind of colony, it was determined that they were, in fact, the *E. coli* of interest. Approximately five hours was required to grow the 3mL culture tube to an

OD_{600} of 0.6; approximately two and a half hours was required to grow the 100mL culture flasks to an OD_{600} of 0.6.

Purification of M-protein with Metal Affinity Column

The His-Tag in the resin effectively bound SeM18, as shown by the fact that switching to the elution buffer caused the release of a significant amount of protein. The initial washing of the column gave very high concentration of material. Some of this may include nucleotides as well as unwanted protein. (Figure 4) The elution with buffer B showed significant concentration of protein that had not been collected using buffer A, which indicates that it had bound to the metal affinity column and was likely SeM18. High concentration of SeM18 in the early fractions tapered off to essentially zero absorbance. (Figure 5)

A

Fractions	OD_{600}
1	4
2	3.95
3	0.451
4	0.09
5	0.035

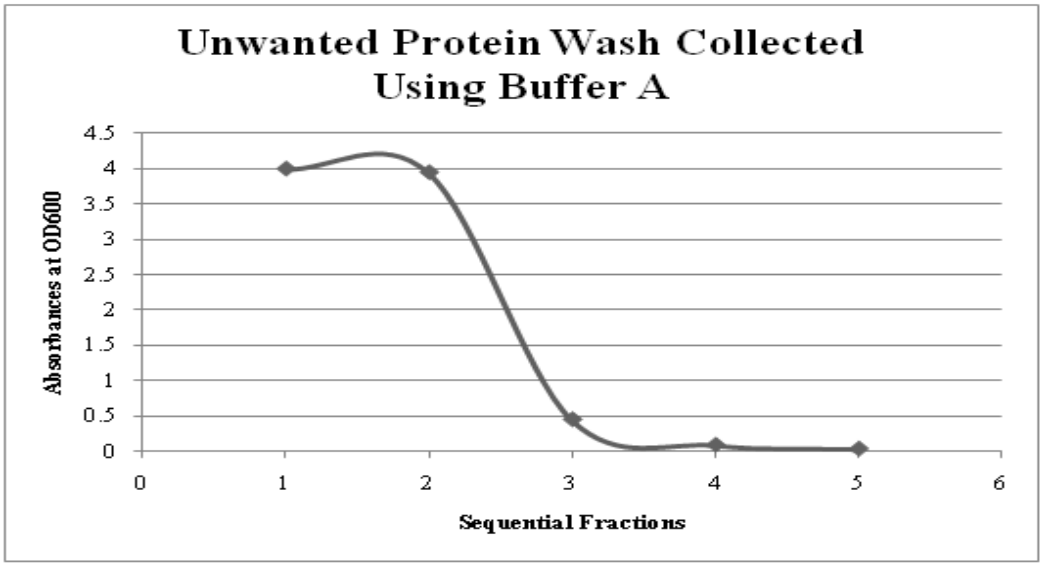

Figure 4 (a) Absorption values obtained from the wash fractions using buffer A. (b) Graphical representation of absorption values at OD_{280}. High concentrations tapered off to essentially zero absorption before switching to the elution buffer.

B

Fractions	OD_{600}
1	0.587
2	1.637
3	1.396
4	1.08
5	0.744
6	0.633
7	0.579
8	0.514
9	0.463
10	0.397
11	0.369

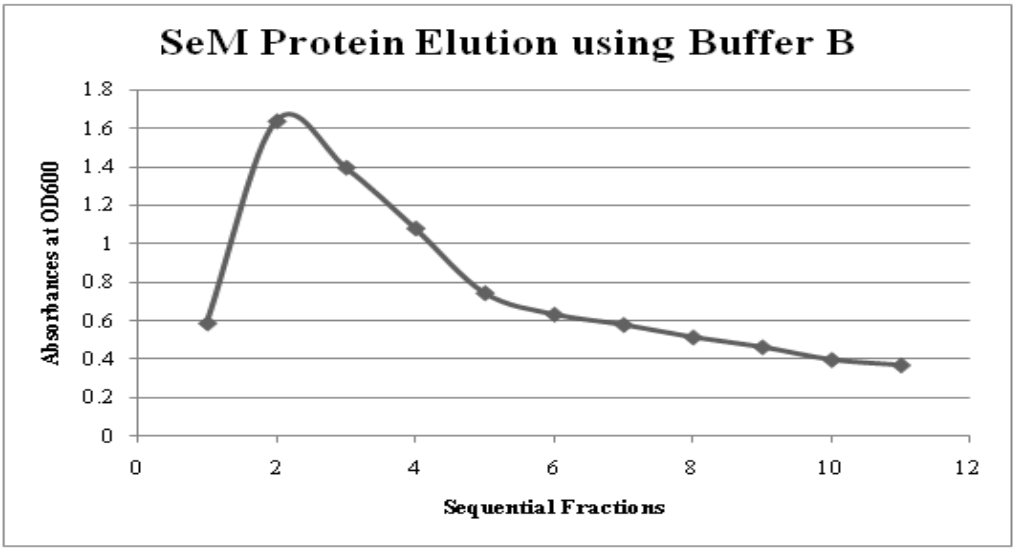

Figure 5 (a) Absorption values obtained from the elution fractions using buffer B. (b) Graphical representation of absorption values at OD_{280}. High concentrations tapered off to essentially zero absorption.

SDS PAGE Analysis

SDS Page analysis resulted in the presence of a band at the 18 kDa molecular weight standard measuring point. This was the expected molecular weight for the fragment of SeM18 being used in this study. The appearance of this distinct band was taken as proof of the presence of this SeM18 fragment in the purified protein sample (Figure 6).

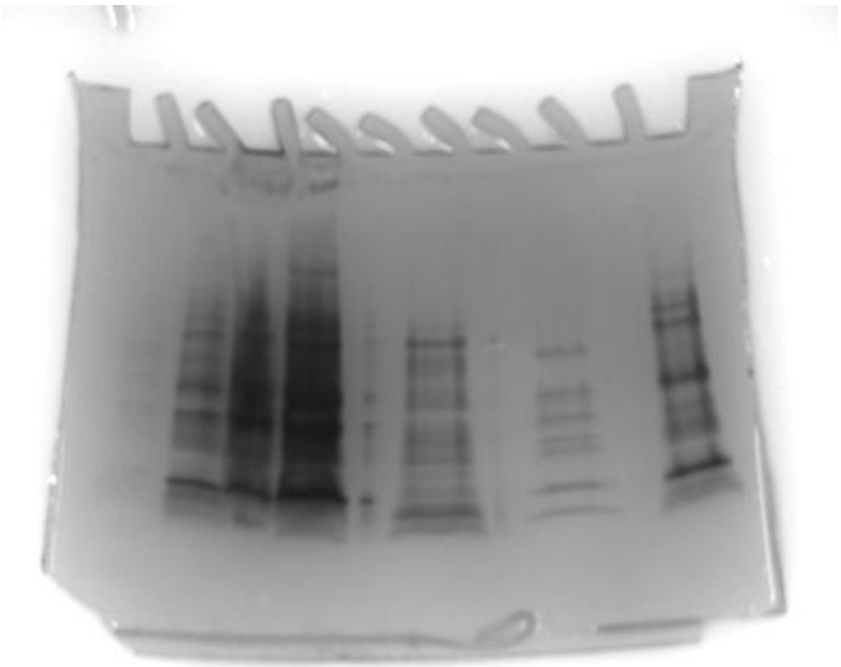

Figure 6 Digital image of the SDS PAGE gel after staining. From left to right: Lane 1 contained the original supernatant from the centrifugation of the induced culture, Lane 2 contained the induced culture in LB broth, Lane 3 contained the second pellet from the centrifugation of the lysed cells, Lane 4 contained the second supernatant before being applied to the column, Lane 5 was blank, Lane 6 contained the highest concentration of the unwanted protein fractions, Lane 7 was blank, Lane 8 contained the molecular weight standard, Lane 9 was blank, and Lane 10 contained the highest concentration of the eluted SeM18.

Production and Isolation of Anti-SeM18 IgY

The resulting Western Blot showed bands corresponding to the 18kDa molecular weight standard, which indicates the presence of the SeM18 protein. It was expected to see a band in the lane that contained the stock protein as well as the lane that contained the eluted protein. In the lane that contained the stock protein, there is a band at the 18kDa mark, but there is also a lot of protein at higher molecular weight marks. This may mean that the stock had degraded due to experimental conditions. It may also mean that the stock had been contaminated with other proteins due to improper storage. However, it can be accepted that when the stock was used in previous experiments it did contain the SeM18 protein in adequate amounts because it was successfully eluted from the metal

affinity column and resulted in the production of anti-SeM18 IgY. In the lane that contained the eluted protein, the darkest band corresponded to the 18kDa molecular marker. This indicates that SeM18 was successfully produced by the pET vector and isolated from the bacteria. It was expected that the lane containing the molecular weight standard would not show up on the Western Blot because IgY should not have bound to it. This was, in fact, the case. It was expected that the lane containing the IgY would show up on the Western Blot, since the secondary antibody was anti-IgY. The molecular weights were not expected to be at 18kDa, because the light and heavy chains of IgY are not 18kDa, but of greater molecular weight. The fact that a band was visible in the IgY lane that corresponds to 18kDa is therefore puzzling. This may mean that the IgY had somehow already bound SeM18 during the experiment (Figure 7). Overall, the Western Blot indicated that anti-SeM18 IgY was successfully produced.

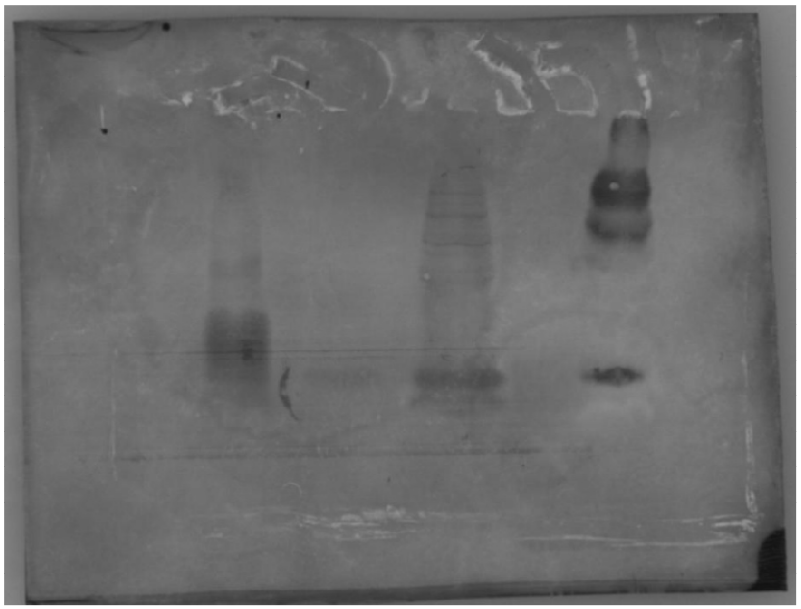

Figure 7 Digital image of Western Blot after development. From Left to Right: Lane 2 contained the stock SeM18 protein, Lane 4 contained the molecular weight standard, Lane 6 contained the highest fraction of the eluted SeM18 protein, and Lane 9 contained the IgY produced by inoculated chickens against SeM18.

Discussion

These results indicate that SeM18 was successfully expressed and is present in the final protein fraction to be used to vaccinate horses for the prevention of *Streptococcus equi* subspecies *equi*. The next step for the continuation of this study would be to inoculate horses and test for the efficacy of this vaccine to prevent *Strep. equi* infection. It would be advantageous to add protein fragments of several of the other immunogenic proteins secreted by *Strep. equi* to increase the strength of the secondary response when the horse encounters this bacteria in its environment. These include Se18.9, SePE-H, and SePE-I. This procedure for producing anti-SeM18 IgY will be adopted by the Liberty University Immunology class curriculum.

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