An oral Vibrio vaccine for salmonids was developed. The vaccine was produced by spray coating lyophilized formalin-killed whole cells of *Vibrio anguillarum* (VA LS 1-74) onto non-pareil sugar beads. Then methacrylic acrylic acid copolymer (Eudragit L-30D) was applied as an enteric protective coating.

Using x-ray radiographic techniques, it was found that large particles (> 1.1 mm) remain in the fish stomach for more than 2 hours before they would enter the pyloric caeca. The pyloric sphincter which has an opening of 0.94 mm, acts as barrier to prevent the passage of large food particles in the stomach to the pyloric caeca. Based on this information non-pareil sugar beads of 18-20 mesh or smaller should be used as the vaccine carriers. A 15% (w/w) Eudragit L-30D coating is needed to provide enteric protection of the vaccine loaded sugar beads of 18-20 mesh size. Lower levels of coating resulted in the bead breaking down in the stomach and releasing contents prior to entering the pyloric caeca.
Since the lymphoid tissues are diffuse throughout the whole GI tract, it may not be necessary to target a vaccine to deliver antigens to a specific area of the intestinal tract, but only protect the antigens from gastric fluids.

In vitro dissolution studies indicate that 10% VA LS 1-74 loading was sufficient for rapid vaccine release (42% released in 30 minutes) and a 15% Eudragit L-30D coating was suitable for providing protection against stomach acid. The vaccine product used in vivo studies contained 10% VA LS 1-74 and 15% Eudragit L-30D on non-pareil sugar seeds of 18-20 mesh size.

Coho salmon were given the vaccine orally, and 30 days afterward a live challenge test was performed. There was no significant difference in the survival rates in a live bacteria challenge test with the positive control (83.3%) and test (80.3%) groups. Both had higher survival rates than the no vaccine fed control group. The serum and mucosal antibody levels to Vibrio were significantly higher (p<0.01) in the test group (19700 units/ml) than the other two groups (2530 units/ml in the positive control group and 617 units/ml in the negative control group). The antibody titer appears to be a better indicator for vaccine efficacy than survival rate of live bacteria challenge tests.

The oral Vibrio vaccine developed is effective, and the technique to protect the antigen can be applied to other antigens or proteins for oral delivery producing an economical pathway for mass vaccination of fish.
Development of Novel Oral Enteric-Coated Aquaculture Vibrio Vaccines

by

George Kaon Wong

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\[
\begin{align*}
1 &= \text{pH 4.5} \\
2 &= \text{pH 5.5} \\
3 &= \text{pH 6.0} \\
4 &= \text{pH 7.2}
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1 = 2 hours
2 = 4 hours
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DEVELOPMENT OF NOVEL ORAL ENTERIC-COATED AQUACULTURE VIBRIO VACCINES

INTRODUCTION

Vaccination has always been chosen as the best way to control an infectious disease in humans and fish. Most vaccination methods involve injections while oral vaccinations are not effective (Johnson et al. 1983). *Vibrio anguillarum* was used as the model bacterial pathogen and developed into an orally active vaccine against vibriosis in salmonids. Chapter I of the thesis focuses on the physiological aspects of the fish gut and the requirements needed for the antigen to reach the site of GI tract where it can interact with the immune system. Chapter II discusses the formulation procedure and results of dissolution of vaccine products. Chapter III includes an *in vivo* challenge of live bacteria and measurement of antibody levels after administering the vaccine to conclude the effectiveness of the oral Vibrio vaccine.
CHAPTER I

GI TRANSIT TIMES AND PHYSIOLOGY OF SALMONID
GASTROINTESTINAL TRACT
ABSTRACT

Histological staining of the coho salmon (*Oncorynchus kisutch*) GI tract revealed diffuse lymphoid tissues throughout the whole length of the GI tract. The pH of the stomach was found to be very acidic (pH 3.7 ± 1.2). Barium sulfate suspension stayed in the stomach for up to 2 hours and traveled throughout the length of the GI tract. After eight hours, most barium sulfate resided in the lower intestine. The opening of the pyloric sphincter of Coho salmon (mass 50-80 g) was measured to be 0.94 mm ± 0.10 mm. Vaccine beads coated with enteric coating of 15% w/w (methacrylic acrylic acid copolymer) and larger than 1.1 mm did not pass through the pyloric sphincter but stayed in the stomach for seven hours. Sugar beads of size 18-20 mesh (1.00 ± 0.06 mm) or smaller passed through the pyloric sphincter and are suitable carriers of the oral *Vibrio* vaccine. A 15% (w/w) methacrylic acrylic acid copolymer (Eudragit L-30D) enteric coating should be sprayed on top for enteric protection against the acidity of the fish stomach. Lower levels of coating with methacrylic acrylic acid copolymer break down in the stomach and release their contents there.
INTRODUCTION

To develop a successful oral vaccine, familiarity with the physiology, immunology and current vaccination practices is necessary. In fish there are two types of specific or adaptive immune responses characterized by antibody production (humoral immunity) and involvement of lymphocytes (cell mediated immunity, CMI). The sites of protective immunity can be found either systemically within the tissues of the body, (especially the organs of phagocytic filtration like kidney or spleen) or in integumentary organs like gut, gills and skin. It is very important that a vaccine stimulates both systemic and integumentary immune systems to offer maximum protection (1).

Intraperitoneal (i.p.) injection can stimulate the systemic immune response and was the first reported effective immunization method against vibriosis (2). However, disadvantages of i.p. injection of vaccines include intensive labor to administer, stress caused by handling and anesthesia, and a size limitation of the fish as it is only feasible to inject fish of 15 g and larger.

Although most of the current vaccines are administered by the method of immersion, researchers began with hyperosmotic immersion (3,4). Hyperosmotic immersion involves the immersion of fish in a hypertonic saline solution for a short time followed by a dip in an aqueous solution of the vaccine. The concept was that fish would
lose water when placed in the hypertonic salt solution and would take the water back up together with extensive amounts of vaccine when the fish was later placed in the vaccine solution. This vaccination method induces considerable stress on fish and it was quickly realized that direct immersion in the vaccine was equally efficacious (5).

Direct immersion involves dilution of the vaccine (usually 1 to 10 of most commercial vaccines), removal of the fish from the tanks, immersion of the fish in the vaccine solution for up to 30 seconds, draining of excess vaccine and returning of the fish to their tanks. This type of handling procedure can still be stressful to the fish and suppress their immune response to the vaccine (6).

The idea of simply feeding the vaccine together with the feed during routine husbandry is very attractive. It is a suitable method of vaccination for fish of any size and saves a lot of labor, time, and costs. It is also non-stressful to the fish. Oral vaccination was the first mass immunization method used with fish but it was not very effective (7). Theoretically, with oral vaccination both the integumentary (skin, gill and intestine) and the systemic immune response can be stimulated when the vaccine passes through the gastrointestinal tract and is absorbed into the blood stream (1). Oral administration of vaccine stimulating both integumentary and systemic immunity is especially important because experimentally induced infection by water-borne exposure demonstrated *Vibrio*
anguillarum (as well as Aeromonas salmonicida) enter the fish by penetrating the descending intestine and rectum while Vibrio ordalii can enter by penetrating the skin (8).

To fully understand how an immune response is elicited during oral vaccination, a basic knowledge of fish immunology and physiology of the gastrointestinal tract must be known. Important factors like GI transit times, the relationship between particle size and GI transit, pH's of the various segments of gut and the relationship of pH to antigen uptake during the digestive process should also be considered.

In mammals the gut-associated lymphoid tissue, in particular the Peyers' patches, plays an important role in the immunological defense against pathogens entering the gastrointestinal tract (9). Antigens enter from the intestinal lumen via specialized epithelium M cells across conventional absorptive cells and stimulate lymphocytes within Peyer's patches, which then migrate to mesenteric nodes for further maturation. After maturation these lymphocytes enter the systemic circulation as plasmablasts to redistribute along intestinal mucosal surfaces and produce secretory IgA antibodies.

In other vertebrates like reptiles and urodeles, lymphoid aggregates are also present in the gut (10,11). In carp, many scattered lymphoid-like cells are present in the epithelium and lamina propria although no aggregates are found (12). The hindgut of most teleosts contains a gut
segment that seems to specialize in the uptake and processing of antigens (13-20). At least a part of intubated antigens finally appear in intraepithelial macrophages of the second gut segment of carp (21,22). Enterocytes in the second gut segment of carp may be functional analogues of mammalian M cells.

Using immunocytochemical and electron-microscopy techniques, uptake and transport of soluble (ferritin) and particulate (Vibrio anguillarum) antigens from intestinal lumen to mucosal macrophages was studied by Rombout et al. (23). Both ferritin and *Vibrio anguillarum* were shown to be taken up by epithelial cells of the second gut segment, entrapped in supranuclear vacuoles and finally transported to large intraepithelial macrophages (23). After anal intubation of ferritin and *Vibrio anguillarum*, many small macrophages penetrate the intestinal epithelium and take up the antigens (23). However, these small macrophages disappear from the intestinal mucosa after 24 hours and similar events can be observed after anal saline intubation. Larger and less mobile macrophages stay in the intestinal epithelium and finally expose antigenic determinants of both ferritin and *Vibrio anguillarum* at their outer surface. This suggests an antigen-presenting function. These larger macrophages stay in the epithelium and may induce a local or mucosal response while small mobile macrophages may induce a systemic response (23).

Although macrophages dominate the epithelium of the
second gut segment of carp, basophilic and eosinophilic granulocytes are mainly found in the connective tissue of the first gut segment (24). Applying monoclonal antibodies against serum immunoglobulin (Ig) in an immuno-gold technique, only a small number of lymphoid cells appear to be Ig-immunoreactive at their external membrane. This suggests the presence of many more T than B cells in the intestinal mucosa. Plasma cells with Ig-immunoreactive cytoplasm can frequently be found in the head portion of the carp kidney while plasma cells with an Ig-immunoreactive cytoplasm are scarce in the intestinal mucosa. As mucosa plasma cells can regularly be found with electron microscopy, they possibly contain another class of Ig type antibody. On the other hand, macrophages and monocyte-like cells are found to be Ig-immunoreactive, suggesting the presence of immune complexes at their external membrane (24). The presence of antigen-presenting and immune complex-binding macrophages together with the high number of lymphocytes in the second gut segment of fish implies the existence of a local or mucosal immune system (24).

Anal immunization with Vibrio anguillarum can elicit mucosal as well as serum responses (25). This suggests that efficacy of oral vaccination can be improved by targeting the delivery and release of the vaccine to the second gut segment bypassing the stomach. The possibility exists that the high acid concentration in the stomach may denature or inactivate orally administered vaccines before they reach
the second gut.

In 1907 the acidity of the elasmobranch stomach was found by Sullivan (26) to be equivalent to 0.6% and 0.4% HCl in the free and bound forms, respectively, and this acidity increased markedly after feeding. Gastric acidity was once thought to be due to an organic acid (27). Dobreff showed that free HCl was the form of acid present in the stomach (28). Bayliss (29) has obtained the pH of the plaice (Pleuronectes platessa) stomach by sampling either the gastric juice or drops of water on the surface of an empty stomach. The pH values range from 2.4 to 7.6 with a mean value of 5.65. In 1929, Vonk (30) found the pH of Esox stomach to be 4.5 to 4.7 and pointed out that the pH on the food surface should be lower. By use of a capillary glass electrode, the pH of the surface of a prey lying within the stomach of an Esox was measured to be between 2.4 and 3.6 (31,32). At a depth of only 3 mm beneath the surface of the prey, the pH increased rapidly to a value of 4.7.

Although the major antigen for protection against Vibrio anguillarum is a lipopolysaccharide (33,34), most active immunogens are proteins which degrade when subjected to high acidic environment. To develop a potent oral vaccine, the antigen must be protected from the high acid environment in the stomach.

The goals of this part of the study were the identification of any lymphoid aggregates along the whole
gastrointestinal tract where vaccine delivery was to be targeted, measurement of GI transit times using x-ray radiography and determining the pH's in different segments of the GI tract. The coating level of Eudragit L-30D for protection of the vaccine from dissolving in the fish stomach was identified. The size of pyloric sphincter was measured to determine a suitable size of non-pareil sugar seeds to be used as carriers of future oral vaccines.
MATERIALS AND METHODS

Materials

Coho salmon (Oncorhynchus kisutch)

Coho salmon were donated by the Oregon State Department of Fish and Wildlife (ODFW) and transferred to the Salmon Disease Laboratory at Oregon State University. Each fish weighed between 50 to 80 g.

Non-pareil sugar seeds¹

Four different sizes of sugar seeds with mesh sizes 14-16 (1.4 ± 0.2 mm), 18-20 (1.0 ± 0.06 mm), 20-25 (0.85 ± 0.06 mm) and 25-30 (0.74 ± 0.03 mm) were selected for this part of the study (table I.1).

X-ray apparatus and barium sulfate suspension for upper GI

The X-ray machine was a Transworld 325V Radiographic X-ray system². Its output can range from 50 to 300 mA (milliAmperes) with a 125 KVP (Kilovolt Peak) maximum at all mA ratings. Dupont Cronex HiPlus intensifying screens were used together with Kodak XTL 8 x 10 films.

Barium sulfate suspension (72% w/v)³ for the upper GI radiography was obtained commercially and contained preservatives, suspending agents, natural and artificial flavor and artificial sweeteners.
Table I.1  Measurements of the Diameter of Eudragit L-30D Coated Non-pareil Sugar Beads.

<table>
<thead>
<tr>
<th>Level of enteric coating (w/w %)</th>
<th>Mesh size</th>
<th>Diameter(^a) (mm)</th>
<th>Standard error (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14-16</td>
<td>1.412</td>
<td>0.234</td>
</tr>
<tr>
<td>0</td>
<td>18-20</td>
<td>1.004</td>
<td>0.059</td>
</tr>
<tr>
<td>0</td>
<td>20-25</td>
<td>0.848</td>
<td>0.057</td>
</tr>
<tr>
<td>0</td>
<td>25-30</td>
<td>0.744</td>
<td>0.029</td>
</tr>
<tr>
<td>10</td>
<td>14-16</td>
<td>1.395</td>
<td>0.184</td>
</tr>
<tr>
<td>10</td>
<td>18-20</td>
<td>0.984</td>
<td>0.058</td>
</tr>
<tr>
<td>10</td>
<td>20-25</td>
<td>0.875</td>
<td>0.075</td>
</tr>
<tr>
<td>10</td>
<td>25-30</td>
<td>0.743</td>
<td>0.048</td>
</tr>
<tr>
<td>15</td>
<td>14-16</td>
<td>1.685</td>
<td>0.202</td>
</tr>
<tr>
<td>15</td>
<td>18-20</td>
<td>1.035</td>
<td>0.081</td>
</tr>
<tr>
<td>15</td>
<td>20-25</td>
<td>0.861</td>
<td>0.049</td>
</tr>
<tr>
<td>15</td>
<td>25-30</td>
<td>0.731</td>
<td>0.041</td>
</tr>
</tbody>
</table>

\(^a\) average of 50 measurements
Barium sulfate and Eudragit L-30 D loaded beads for GI transit times study

Sugar beads of mesh size 18-20 (1.0 ± 0.06 mm) were coated with barium sulfate\(^4\) and bovine serum albumin\(^4\) to yield a 40% (w/w) barium sulfate and 5% (w/w) bovine serum albumin final sugar bead product. The spray coater was an Aerocoat Strea-1 spray coater with a modified Lab-line/P.R.L. High Speed Fluid Bed Dryer\(^5\). Temperature was set at 25°C (ambient temperature) and nozzle pressure was maintained at 10 - 15 psi. A peristaltic pump\(^6\) was used to deliver the coating solution 15% w/v that contained both barium sulfate and BSA (8 parts by weight of barium sulfate to 1 part by weight of BSA) at a rate of about 6 ml/minute.

Different amounts of methacrylic acrylic acid copolymer (Eudragit L-30D\(^7\)) (0%, 5%, 10% and 15 % w/w final bead weight) were then spray-coated to serve as enteric coatings. Plasticizers were 10% triethyl citrate (TEC) (w/w of dry Eudragit) and 10% dibutylsebacate (DBS) (w/w of dry Eudragit) (see table I.2). The final Eudragit L-30D coating solution had a concentration of about 20% w/v. Similar coating conditions were used except the flow rate of coating solution was reduced to about 2.8 ml/minute to avoid clumping. Figures I.1, I.2 and table I.3 show the size and weight calibrations of these formulations.

Eudragit L-30D loaded sugar beads for pyloric sphincter size determination
Table I.2  Composition of Solution for Enteric Coating.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eudragit L-30Da (30% lacquer)</td>
<td>48.19 g</td>
</tr>
<tr>
<td>Triethyl citrate(^b)</td>
<td>1.81 g</td>
</tr>
<tr>
<td>Dibutyl sebacate(^c)</td>
<td>1.81 g</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>48.19 g</td>
</tr>
<tr>
<td>Total</td>
<td>100 g</td>
</tr>
</tbody>
</table>

\(^a\) Rohm Pharma, Waitestadt, West Germany

\(^b\) Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, U.S.A.

\(^c\) Sigma Chemical Company, St. Louis, Missouri, U.S.A.
The size of barium loaded non-pareil sugar beads with various levels of Eudragit L-30D (0, 5, 10 and 15%). Each bead contains 40% (w/w) barium sulfate and 5% BSA.
The mass of barium loaded non-pareil sugar beads with various levels of Eudragit L-30D (0, 5, 10 and 15%). Each bead contains 40% (w/w) barium sulfate and 5% BSA.

Figure I.2
Table I.3  Measurements of the Diameter of 40% (w/w) Barium Sulfate and 5% (w/w) Bovine Serum Albumin Loaded Non-pareil 18-20 Mesh Sugar Beads.

<table>
<thead>
<tr>
<th>Level of enteric coating (w/w %)</th>
<th>Diameter ( ^a ) (mm)</th>
<th>Standard error (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.081</td>
<td>0.054</td>
</tr>
<tr>
<td>5</td>
<td>1.110</td>
<td>0.061</td>
</tr>
<tr>
<td>10</td>
<td>1.111</td>
<td>0.071</td>
</tr>
<tr>
<td>15</td>
<td>1.155</td>
<td>0.065</td>
</tr>
</tbody>
</table>

\( ^a \) average of 50 measurements
Non-pareil sugar beads of different mesh sizes (14-30 mesh) were coated with either 10% or 15% Eudragit L-30D under the same conditions as described above. The diameters (0.74, 0.88, 0.98, 1.40, 0.73, 0.86, 1.04, 1.69 mm) and weights (0.30, 0.46, 0.70, 1.44, 0.30, 0.45, 0.72, 1.50 mg) of each of these formulations were shown in figures 1.3 and I.4, respectively.

Methods

Identification of lymphoid aggregates in GI tract

The whole fish gastrointestinal tract was isolated and fixed in 10% neutral phosphate buffered formalin for 24-48 hours. The tissue was then embedded in paraffin and sectioned with a microtome at 4 μm thickness. After staining with hematoxylin and eosin (H & E), a slide was made and each slide was cover-slipped with permount.

Determination of the pH along the GI tract

Each fish was fasted for 48 hours and immediately dissected after euthanasia. The pH in each segment of the GI tract (stomach, pyloric caeca, upper intestine - 1 cm from the pyloric caeca, lower intestine - 2 cm from the end of anus) was determined by a surface-sensitive pH probe. Other physical parameters including weight, total length of fish, lengths of esophagus to end of stomach, pyloric caeca and intestine were also recorded. A total of 22 fish were used in this part of the study.
Figure 1.3

The size of non-pareil sugar beads (14-16 M, 18-20 M, 20-25 M, 25-30 M) coated with various levels of Eudragit L-30D (0, 10 and 15%) as an enteric coating.
The mass of non-pareil sugar beads (14-16 M, 18-20 M, 20-25 M, 25-30 M) coated with various levels of Eudragit L-30D (0, 10 and 15%) as an enteric coating.
X-ray radiography on GI transit times

The settings on the X-ray machine were 300 milliAmperes (mA) with a 0.1 second exposure time (or 30 milliAmperes-second, mAs). The KVP was set at 46 and the focal film distance kept constant at 36 inches. During the experiment approximately 1 ml of barium sulfate suspension was given to each fish by oral intubation using a micropipette (figure 1.5). X-ray film exposure was performed on each fish at 0 h, 2 h, 5 h, 6 h and 8 h after administration of barium sulfate suspension.

Determination of Eudragit L-30D thickness for enteric protection against fish stomach acid

Eight fish were divided into four groups. The two fish in each group were intubated with the same amount of 40% barium sulfate (and 5% BSA) loaded sugar beads that contain the same level of Eudragit L-30 D coating. The four Eudragit coating levels were 0%, 5%, 10% and 15%. The same micropipette method of administration as described above was used for the vaccine beads and each of the dry sugar bead formulations was mixed with minimal amount (about 1 ml) of pH 4.5 phosphate buffer to facilitate pipetting. X-ray films were taken at 30 min, 2 h and 6 hr to determine the position of the beads in the GI tract. The level of Eudragit coating that would give enteric protection of the beads in the stomach was observed by noting which bead stayed in the stomach and which did not.
Each subject is orally intubated with a specific formulation using a micro-pipette. The subject is then put into the restrained container (squeeze bottle) situated in the background before the x-ray is taken.
Determination of the size of pyloric sphincter

The pyloric sphincter was unfolded longitudinally by a surgical scissor (figure I.6). The width, \( w \), (perimeter of the sphincter) was then determined using a photomicroscope\(^{10} \). The diameter of the sphincter was calculated using the following formula:

\[
\text{Diameter} = \frac{\text{Perimeter}}{3.1415}
\]

A total of 22 fish were examined in this part of the study.

Optimum bead size for oral vaccine formulation

Four sizes of non-pareil sugar seeds (from 14 to 30 mesh or 0.73 mm to 1.69 mm in diameter) and two levels of enteric coatings (10% and 15%) were used. Fish were divided into 8 groups and fasted for 48 hours before each was fed with a particular formulation of beads of a specified bead diameter (0.74, 0.88, 0.98, 1.40, 0.73, 0.86, 1.04 and 1.69 mm) (table I.4). There were 2 to 4 fish in each group. During the experiment each fish was intubated with similar weight (212-236 mg) of formulated beads (figures I.7 and I.8) using the same technique to administer the beads as described above. The fish was then dissected after 2.5 hours and the number of beads in the stomach was counted.
The pyloric sphincter is slit open longitudinally by a pair of surgical scissors and the size (perimeter) of the sphincter is examined under a microscope.
Table I.4  
Amount of Eudragit L-30D Coated Sugar Beads 
Orally Intubated into Each Fish.

<table>
<thead>
<tr>
<th>Level of enteric coating (w/w %)</th>
<th>Mesh size</th>
<th>Diameter</th>
<th>Mass</th>
<th>No. of beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mm)</td>
<td>(mg)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>14-16</td>
<td>1.40 ± 0.18</td>
<td>211.7 ± 4.2</td>
<td>147 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>18-20</td>
<td>0.98 ± 0.06</td>
<td>228.3 ± 5.5</td>
<td>325 ± 6</td>
</tr>
<tr>
<td>10</td>
<td>20-25</td>
<td>0.88 ± 0.08</td>
<td>233.0 ± 5.4</td>
<td>512 ± 12</td>
</tr>
<tr>
<td>10</td>
<td>25-30</td>
<td>0.74 ± 0.05</td>
<td>235.7 ± 4.2</td>
<td>775 ± 14</td>
</tr>
<tr>
<td>15</td>
<td>14-16</td>
<td>1.69 ± 0.20</td>
<td>223.0 ± 3.3</td>
<td>149 ± 2</td>
</tr>
<tr>
<td>15</td>
<td>18-20</td>
<td>1.04 ± 0.08</td>
<td>226.7 ± 3.7</td>
<td>316 ± 5</td>
</tr>
<tr>
<td>15</td>
<td>20-25</td>
<td>0.86 ± 0.05</td>
<td>223.3 ± 6.8</td>
<td>497 ± 15</td>
</tr>
<tr>
<td>15</td>
<td>25-30</td>
<td>0.73 ± 0.04</td>
<td>236.0 ± 3.6</td>
<td>781 ± 12</td>
</tr>
</tbody>
</table>

a average ± standard error
Figure I.7  The mass of 10 or 15% (w/w) Eudragit L-30D coated nonpareil sugar beads (with sizes 14-16, 18-20, 20-25 and 25-30 meshes) that are orally intubated to a fish to determine a suitable size of sugar beads as vaccine carriers.
The number of 10 or 15% (w/w) Eudragit L-30D coated nonpareil sugar beads (with sizes 14-16, 18-20, 20-25 and 25-30 meshes) that are orally intubated to a fish to determine a suitable size of sugar beads as vaccine carriers.

Figure I.8
RESULTS AND DISCUSSIONS

Identification of lymphoid aggregates in the GI tract

Because lymphoid tissues are responsible for immune response, they are the sites to be targeted for the delivery of oral vaccines. Lymphoid tissues were found to be diffuse throughout the length of the gastrointestinal tract of Coho salmon (figures I.9 to I.11) and lymphocytes were scattered throughout the esophagus, stomach, pyloric caeca and intestine. This agrees with previous findings in carp (9).

The presence of any organized lymphoid aggregates along the gastrointestinal tract of Coho salmon was not observed. Therefore, it may not be necessary to develop a formulation that will deliver the vaccine to a specific area of the intestinal tract, but to only protect the vaccine from gastric fluids. Uptake ability of antigen by lymphocytes in different areas of the intestinal tract may be the focus of research in the future. If lymphocytes in a particular area have significantly higher antigen uptake capabilities, it might be advantageous to focus the vaccine delivery to the lymphocytes in this area.

Determination of the pH along the GI tract

The pH's of different segments of the gastrointestinal tract were measured (figure I.12). Although stomach pH has a lot of variations (3.7 s.e. = 1.2), most observations were under pH 4. It is very likely that protein antigens will be
Figure I.9 Distribution of lymphoid tissues in the esophagus and stomach of Coho salmon. No organized lymphoid tissues are found.
Figure I.10 Distribution of lymphoid tissues in the pyloric caeca of Coho salmon. No organized lymphoid tissues are found.
Figure I.11 Distribution of lymphoid tissues in the middle and distal intestine of Coho salmon. No organized lymphoid tissues are found.
Figure 1.12  The pH of different segments of the Coho Salmon gastrointestinal tract. The pH ranges from acidic in the stomach (pH 3.7) to nearly neutral in the pyloric caeca (pH 6.9).
destroyed/denatured in this acidic medium. The pH's of the pyloric caeca, upper and lower portions of the intestines are very similar and all have a pH value close to 7.

The relative lengths of (a) esophagus-stomach \((3.6 \pm 0.3 \text{ cm})\), (b) pyloric caeca \((3.1 \pm 0.4 \text{ cm})\) and (c) intestine \((8.5 \pm 0.7 \text{ cm})\) to the total lengths \((19.2 \pm 1.0 \text{ cm})\) of fish are shown in figure I.13. The variations are small (less than 3.4\%) from fish to fish. The relationship between the weight and total length of a fish is also noted in figure I.14. Fish weight and fish lengths are highly related \((r^2 \text{ equal to 0.869})\).

X-ray radiography on GI transit times

There are a number of theories on the gastric emptying rate of fish and two of the most popular theories are the exponential model and the square root model \((35)\). Both of them are valid only under certain conditions.

Figures I.15 and I.16 show the x-ray radiographic pictures after intubation of 1 ml of barium sulfate suspension. By 2 hours, some of the barium sulfate suspension has emptied into the pyloric caeca. The barium sulfate suspension took 6 to 8 hours before it reached the intestine and had totally emptied from the pyloric caeca. X-ray film exposure of the barium sulfate and BSA enteric coated sugar beads was performed at 30 minutes, 2 hours and 6 hours after intubation.
Figure 1.13  Relative gut length in different parts of the GI tract to the total length of a fish. The variations are smaller than 3.5% from one fish to another in each segment. Total length of fish is 19.2 ± 1.0 cm.
The weight of fish, g, plotted versus the total length of the fish, cm ($r^2 = 0.869$).
Figure I.15  X-ray radiographic exposure pictures of 1 ml of barium sulfate (72% w/v) suspension in a Coho salmon at 15 min and 2 h after oral intubation. The barium sulfate suspension started to enter into the pyloric caeca from the stomach 2 hours after intubation.
Figure I.16 X-ray radiographic exposure pictures of 1 ml of barium sulfate (72% w/v) suspension in a Coho salmon at 5, 6 and 8 h after oral intubation. The barium sulfate suspension started to empty from the intestine 6 hours after intubation.
Determination of Eudragit L-30D thickness for enteric protection against fish stomach acid

Non-pareil sugar seeds of 18-20 mesh (diameter 1.00 ± 0.06 mm) loaded with barium sulfate and BSA were coated with various thickness of Eudragit L-30D and orally intubated into Coho salmon. Comparing figures I.1 and I.2 with figures I.3 and I.4, the barium sulfate loaded 18-20 mesh beads (diameters 1.08 mm to 1.16 mm and average mass 1.1 mg to 1.21 mg) are significantly larger and heavier than the unloaded 18-20 mesh beads (diameter 1.00 ± 0.06 mm and mass 0.62 mg).

Figures I.17-I.20 are x-ray exposures of fish after sugar beads having different levels of enteric coating are orally intubated. Non-enteric coated (0% w/w Eudragit L-30D) beads start to dissolve readily in the stomach and pass readily to the pyloric caeca and to the intestine within 2 and 6 hours, respectively. As the level of enteric coating increases to 5% or 10% (w/w), a larger number of beads stay intact in the stomach and a longer time (> 2 hours) is needed for the beads to dissolve and pass through the stomach. When the Eudragit L-30D level reaches 15% (w/w), the enteric coating prevents dissolution of the formulated beads in the stomach. Also in figure I.20, an obvious physical barrier is seen in the x-ray film. This is a pyloric sphincter and appears to serve the same role as the human pyloric sphincter to prevent large particles or undigested food from entering the next part of the
X-ray radiographic exposure pictures of 40% (w/w) barium sulfate and 5% (w/w) BSA loaded non-enteric coated non-pareil sugar beads (1.08 ± 0.05 mm in diameter) in fish at 30 minutes, 2 hours and 7 hours after oral intubation.
Figure I.18  X-ray radiographic exposure pictures of 40% (w/w) barium sulfate and 5% (w/w) BSA loaded enteric-coated (5% w/w of Eudragit L-30D) non-pareil sugar beads (1.11 ± 0.06 mm in diameter) in fish at 30 minutes, 2 hours and 7 hours after oral intubation.
Figure I.19  X-ray radiographic exposure pictures of 40% (w/w) barium sulfate and 5% (w/w) BSA loaded enteric-coated (10% w/w of Eudragit L-30D) non-pareil sugar beads (1.11 ± 0.07 mm in diameter) in fish at 30 minutes, 2 hours and 7 hours after oral intubation.
Figure I.20 X-ray radiographic exposure pictures of 40% (w/w) barium sulfate and 5% (w/w) BSA loaded enteric-coated (15% w/w of Eudragit L-30D) non-pareil sugar beads (1.16 ± 0.07 mm in diameter) in fish at 30 minutes, 2 hours and 7 hours after oral intubation.
gastrointestinal tract, the pyloric caeca.

Determination of the size of pyloric sphincter

The average diameter of pyloric sphincter is $0.94 \pm 0.1$ mm. This result is consistent with the previous finding that undissolved barium sulfate beads coated with 15% Eudragit (average diameter $> 1$ mm) remained in the stomach and did not pass to the pyloric caeca.

When the size of the pyloric sphincter was plotted against the weight of fish, a strong direct relationship is observed ($r^2 = 0.854$) (figure I.21). A plot of the diameter of pyloric sphincter against the total length of a fish was performed. A similar direct relationship exists between the size of the pyloric sphincter and the length of a fish ($r^2$ of 0.731) (figure I.22). From the $r^2$ values, the weight of a fish is a better predictor of the size of the pyloric sphincter than the length of the fish.

Optimum bead size for oral vaccine formulation

Amount of beads remaining in the stomach 2.5 hours after oral intubation were measured (figure I.23). Results with both 10% and 15% enteric coated beads were similar; i.e. that the maximum diameter of sugar beads suitable to allow passage through the pyloric sphincter is about 1 mm (approximately 18-20 mesh). Number of coated beads remaining in the stomach increased when the diameter of the beads was from 18-20 mesh size, 1 mm ($26.4 \pm 2.5\%$ remaining)
Figure I.21  The diameter of the pyloric sphincter, mm, plotted versus weight of the fish, g, ($r^2 = 0.854$).
Figure I.22 The diameter of the pyloric sphincter, mm, plotted versus the total length of the fish, cm \((r^2 = 0.731)\).
The amount of Eudragit L-30D coated non-pareil sugar left in the stomach of fish measured 2.5 hours after oral intubation. The diameters of each of these 8 formulations are from left to right: 0.73, 0.74, 0.86, 0.88, 1.04, 0.98, 1.69, 1.40 mm. The Eudragit coatings are either 10% or 15%.
to 14-16 mesh size, 1.4 mm (65.3 ± 3.4% remaining). This is also consistent with the results on the size of pyloric sphincter, which has an average value of 0.94 mm.

The 10% enteric coated beads pass to the pyloric caeca faster than the 15% enteric coated beads. It is possible that some of the 10% enteric coated beads start to dissolve in stomach and pass through to the pyloric caeca, as visualized by previous x-ray pictures. All of the four different size beads show the same trend.
CONCLUSIONS

No aggregated lymphoid tissue exists along the Coho salmon (Oncorynchus kisutch) gastrointestinal tract. It may not be necessary to target the delivery of the vaccine to a specific site along the GI tract except to bypass the stomach, which has a low pH and could destroy labile antigenic protein portions of the vaccine.

The pH of the stomach is low 3.7 ± 1.2 compared to the pH of the pyloric caeca and intestine, pH 7.0. It is very likely that protein antigens can be denatured in this low pH environment within 2 hours.

X-ray radiography studies showed that it takes about 2 hours before a barium sulfate suspension enters the pyloric caeca from the stomach, and 6 to 8 hours to reach the intestine. A 15% (w/w) Eudragit L-30D enteric coating is required to protect the 18-20 mesh size sugar beads from dissolving in the stomach.

The pyloric sphincter diameter is 0.94 mm ± 0.10 mm. Sugar beads of mesh size 14-16 (diameter 1.41 mm ± 0.23 mm) are too big to pass through the pyloric sphincter. Non-pareil sugar seeds of sizes 18-20 mesh (1.00 mm ± 0.06 mm) and smaller should be used as carriers of future oral vaccines so that the vaccine can easily pass through the pyloric sphincter from the stomach.

The application of 15% Eudragit L-30D coating on 18-20 mesh non-pareil sugar beads can be used to formulate oral
vaccines. The enteric coating level needs to be adjusted if smaller sugar beads are used when smaller fish (i.e. smaller pyloric sphincter) are being vaccinated.
ENDNOTES

1 Paulaur Corp., Princeton, New Jersey, U.S.A.
2 Transworld X-Ray Corporation, Charlotte, North Carolina, U.S.A.
4 J T Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.
5 Lab-Line/PRL, Melrose Park, Illinois, U.S.A.
6 Miniplus II peristaltic pump, Gilson Medical Electronics, Middleton, Wisconsin, U.S.A.
7 Rohm Pharma, Weiterstadt, West Germany
8 This part of the study was done by the courtesy of Dr. Jerry Heidel, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, U.S.A.
10 OPTIPHOT-POL Polarizing Microscope, Nikon Corporation, Tokyo, Japan
REFERENCES


CHAPTER II

FORMULATION AND DISSOLUTION STUDIES OF ENTERIC-COATED VIBRIO VACCINES
ABSTRACT

An in vitro dissolution study was performed on enteric-coated *Vibrio anguillarum* (VA LS 1-74) vaccine to determine the optimum vaccine loading level and methacrylic acrylic acid copolymer (Eudragit L-30D) coating thickness for enteric protection and rapid vaccine release. Sugar beads of size 18-20 mesh (diameter 1.00 mm ± 0.06 mm) were chosen to be the vaccine carriers. The pH at which Eudragit L-30D coating starts to dissolve was found to be pH 5.5. When the Eudragit coating level was 15% (w/w of final product), less than 20% of vaccine was released in a pH 1.5 simulated gastric fluid after 9 hours. In simulated intestinal fluid 15% Eudragit coating resulted in more than 50% vaccine release at pH 7.2 at 75 minutes. A 10% VA LS 1-74 loading gave the fastest release rate in simulated intestinal fluid (pH 7.2) with 50% of the vaccine being released in 98.7 ± 5 minutes. A 15% (w/w) Eudragit L-30D coating was enough to provide protection against dissolution in simulated gastric fluid (pH 4.5). Extended acid pretreatment time of 6 hours delayed 50% vaccine release in intestinal fluid by 20 minutes compared to 2 hour pretreatment in simulated gastric acid.
INTRODUCTION

Vibriosis is a bacterial disease of salt-water and migratory fish. Recent data indicate that annual losses due to the disease exceed 11 million pounds in Japan alone (1) and in severe cases there may be up to 40% mortality. Oral vaccination was the first mass vaccination method in fish (2). Its advantages over other methods of vaccination include no stressful handling and no interference with routine husbandry practice. However, most trials of oral vaccination have resulted in low efficacy compared to injection or immersion (3,4).

Simple (direct) immersion or dipping method was extensively studied and found to be very successful (1). Commercial vaccines currently available as well as results of some previous research provide some insight on how to formulate an oral Vibrio vaccine.

In 1980, Kusuda et al. studied the efficacy of 5 different concentrations of vaccine for bath-immunization against vibriosis in cultured ayu (5). The final percentages of survival in vaccinated groups 10 days after challenge were 90%, 89%, 85%, 75% and 65% in 0.5%, 0.3%, stock, 0.1% and 0.05% vaccine concentrations (diluted in water), respectively. The survival ratio in unvaccinated control was 45%. From observations on feeding activities of vaccinated fish, immunization in stock vaccine resulted in a more stressful environment for fish than in diluted vaccines.
During 1981 and 1982, experiments were performed by Anders with the aim of bath-immunizing juvenile rainbow trout (*Salmo gairdneri*) weighing under or near 1.2 g in fish farming conditions (6). Three methods were tested. The first preparation consisted of a vaccine whose antigens had been liberated with trypsin. It was added to the water in which trout were being held for 2 hours. The second vaccine was mixed with bentonite and the pH adjusted to 3. The trout were immersed in the vaccine for 30 seconds. The third method was analogous to the second, but the antigen had first been released with trypsin. No promising results were reported.

Horne et al. (7) investigated direct immersion in 1982. While intraperitoneal (i.p.) injections of formalized strain of *Vibrio anguillarum* gave nearly complete protection, direct immersion in the vaccine only offered 47% protection. The authors concluded that while i.p. was not a realistic commercial practice for fry and small fish, direct immersion could confer worthwhile protection against natural infection in a farmed population. Vaccination early in the season and repeated annually was recommended.

In 1982, Johnson et al. (8) measured the duration of immunity in salmonids vaccinated by direct immersion. The level of protective immunity was determined using commercial *V. anguillarum* (two serotypes) and *Y. ruckeri* (Hagerman strain) bacterin. The duration of protective immunity varied with the bacterin concentration, size and species of
fish, but the duration between the two bacterins was comparable. In fish under 1 g, duration of protective immunity was longest when the most concentrated bacterin was used. Generally, immunity lasted longer in larger fish. Immunity lasted for about 120 days in 1-g fish, about 180 days in 2-g fish, but about a year or longer in 4-g or larger fish. Coho salmon (Oncorhynchus kisutch) and sockeye salmon (O. nerka) retained immunity for a longer time and pink salmon (O. gorbuscha) the shortest time. Chinook salmon (O. tshawytscha) and rainbow trout (Salmo gairdneri) were intermediate.

Onset of immunity in salmonid fry was also determined by Johnson et al in 1982 (9). Level of protective immunity was measured by survival rates after bath challenge with virulent bacteria. Immersion time for effective vaccination was obtained within 5 seconds and protective immunity was demonstrated within 5 days at 18°C and within 10 days at 10°C. Minimum size of salmonid fry at which maximum protective immunity occurred was between 0 g (birth) and 5 g or an age between 1 and 2 years. Immunity appeared to be a function of size and not age. In fish under 1 year-old, the level of protective immunity could be increased by using a more concentrated bacterin.

In 1984 Tatner and Horne (10) detected a period of unresponsiveness in the very earliest groups of fry during direct immersion. Using a radiolabelled vaccine preparation, this was found to be due to a complete lack of
antigen uptake at these stages. Once the fry started to respond, they responded well and at relatively low water temperatures.

Tatner and Horne (11) also studied factors influencing the uptake of a $^{14}$C-labelled *Vibrio anguillarum* vaccine in direct immersion experiments with rainbow trout (*Salmo gairdneri*) in 1983. Immersion times of longer than 10 seconds did not increase vaccine uptake. A pre-immersion dip in a hyperosmotic solution had no effect on uptake, even at lower vaccine bath concentrations. It appeared that the head of the fish was implicated in vaccine uptake. Vaccine uptake decreased at lower temperatures, whereas the use of an adjuvanted vaccine and a soluble vaccine preparation led to increased antigen uptake. Larger fish took up more vaccine.

Another successful immersion vaccination was reported in 1984 by Kawano et al. (12) Excellent protection against vibriosis was provided to ayu, *Plecoglossus altivelis* by immersion with 5.32% NaCl solution containing either 0.94 (wet) g/l or 9.4 (wet) g/l of lyophilized cells of *Vibrio anguillarum*. The duration of protective immunity in the fish was at least 113 days. However, the serum from fish which had been vaccinated by either the immersion or oral administration did not show detectable levels of agglutinating antibodies against *Vibrio anguillarum*.

Similar results were obtained by Aoki et al. (13) using a vaccine solution of a formalin-killed culture of *Vibrio*
anguillarum. A high level of protection against artificial challenge was achieved. The immunized fish were protected against vibriosis when challenged 1 month after immersion.

Tatner in 1987 (14) used a radiolabel to quantitate the relationship between vaccine dilution, length of immersion time and antigen uptake in direct immersion experiments with rainbow trout, *Salmo gairdneri*. Provided the antigen concentration was not limiting, lengthening the immersion time did not result in greater uptake. However, when the antigen concentration was low, proportionally greater periods of time were needed for antigen uptake to occur. The variable exerting the largest effect on uptake, however, was fish size, with larger fish sequestering more antigen. A constant proportion of the total antigen uptake was found in the gut.

Increased understanding of the immune system leads one to expect local and mucosal response to play a major role during the vaccination process, partly because of the enormous surface areas of the skin, gills and especially the intestine during oral vaccination (15).

In 1988 Fujina and Nagai (16) investigated the ingestion and transport of bovine serum albumin (BSA) into the serum of chum salmon (*Oncorhynchus keta*) from the mucosal epithelial cells in the intestine. 20% BSA solution was administered into the intestine from the anus with a teflon tube. Results of radial immunodiffusion revealed that 5.1 to 96.1 micrograms of BSA were detectable in 1.0 ml
of the fish serum. This suggests the possibility of immunization of fish by the oral route.

McLean et al. (17) confirmed that the fish gut is able to absorb orally administered, intact proteins. In an attempt to ascertain whether the salmonid GI tract is able to absorb and subsequently transfer such macromolecules to the bloodstream and tissues, the authors orally intubated yearling chinook salmon with vitellogenin. Plasma and tissue accumulation of a lipo-glyco-phospho-protein (MW > 600,000 daltons) were followed over 6 hours. Vitellogenin can be considered as a model antigen in oral vaccination.

Potential problems exist in formulating oral vaccines. First of all, antigens must be maintained intact during the manufacturing process. A method must be developed to allow incorporation of bacterial antigens into feed without destroying the antigenicity (1). Excessive temperatures and harsh processes can often denature the antigens and render the vaccine ineffective. Palatability can also be a problem. A palatable vaccine can ensure immunization of every fish in the field.

Another problem which exists with oral vaccines resides in the location of the immune areas of the fish gut. It appears that the immune areas are located in the lower intestine (18,19,20). To reach the second gut, and thus to induce an immune response, the antigens must first pass through the stomach of the fish. It is possible that the high acid concentrations in the stomach may destroy the
antigens. This hypothesis is supported by the increased efficacy of Vibrio vaccines administered by anal intubation rather than orally (21). So far, microencapsulated and slow-release oral vibrio vaccines have been developed in 1989 but their efficacies were reported to be even lower than unprotected vaccine (22).

Recently oral vaccines are actively being developed for a number of human and animal diseases such as cholera (23-26), typhoid fever (27-30), rabies (31), vulvovaginal candidiasis (32), hepatitis B (33), influenza (34) and many others (chlamydiasis, colibacillosis, Yersinia enterocolitica, feline leukemia).

Cholera is caused by the human pathogen Vibrio cholerae 01 and share many common characteristics with Vibrio anguillarum, a fish pathogen. Vibrio cholerae adheres to mucosal enterocytes and produces an enterotoxin which has a molecular weight of 83,000 (35). This enterotoxin consists of two distinct types of subunits, A and B. Subunit A (MW 28,000) is noncovalently attached to five B subunits (each of 11,000 MW). Purified B subunit of cholera toxin retains membrane-binding capacity and protective immunogenicity and yet has no toxic activity as tested in animals (36).

Several approaches to the development of a safe and effective vaccine against Vibrio cholerae include (a) purified enterotoxin B subunit plus killed whole cells of Vibrio cholerae, (b) attenuated live Vibrio cholerae strains and (c) cloned Vibrio cholerae antigens in a Salmonella
carrier strain (37).

A major uncertainty about the oral vaccine is whether it requires protection from gastric acid to retain full immunogenicity in the small intestine. During a ganglioside ELISA, the B subunits exhibited almost no activity below a pH level of 3 (38,39). This lack of activity was explained to be the result of reversible dissociation of the B subunits from pentameric to monomeric form below a pH level of 4. Diminished IgG antibody responses to B subunits were also observed when B subunits were administered without antacids. The simplicity and equivalent immunogenicity of mixing vaccine with antacid for simultaneous administration were suggested (39).

In 1987, Chaicumpa et al. (40) used albino rats aged 7 to 9 weeks as an animal model to study the immunogenicity of Vibrio cholerae antigens. To protect the antigens from stomach acid, 1 ml of 5% NaHCO₃ was given prior to oral immunization.

In 1988, Pierce et al. (41) used intravenous cimetidine and NaHCO₃ to neutralize the gastric acid to increase the immunogenicity of their Vibrio cholerae 01 vaccine in rabbits.

Beside cholera vaccine, other vaccines can also be subject to acid degradation in the stomach and warrant enteric protection to increase their efficacies. One of these examples is the typhoid vaccine.

In 1983 Ty21a, a stable attenuated mutant of Salmonella
typhi, was considered a safe and protective oral vaccine when 3 doses of $10^9$ cells in saline were taken after neutralization of gastric acidity by 1 g NaHCO$_3$ (42). To identify a more convenient method of administering the vaccine, 141 U.S. adults received vaccine formulated in either one of the two ways: (a) in gelatin capsules administered with two additional gelatin capsules containing a total of 0.8 gm NaHCO$_3$ or (b) in enteric coated capsules. Rates of seroconversion of antibody were similar in all groups. Based on these observations a large-scale field trial of efficacy has been initiated in 90,000 school children 6 to 20 years of age in Santiago, Chile. One third of these subjects received one dose of enteric coated vaccine; one-third received two doses and the remainder received placebo.

Vaccine in enteric coated capsules was found to be a safe and practical means for mass immunization and more effective than a formulation consisting of gelatin capsules containing vaccine and NaHCO$_3$ (43). One dose of enteric coated vaccine was not sufficiently immunogenic, but two and three doses provided 54 to 62% protection for a period of 33 months and 18 months, respectively.

Levine at al. (44) confirmed that three doses of Ty21a attenuated Salmonella typhi oral vaccine in enteric coated formulation provided 67% protection for at least 3 years in a randomized, placebo-controlled field trial. Ty21a provides the same level of protection as the heat or phenol-
inactivated whole cell parenteral vaccine but it causes less adverse reactions.

In April 1990, this live oral vaccine (Ty21a) in the form of enteric coated capsules was approved by the Food & Drug Administration (FDA). One enteric coated vaccine capsule is to be taken on alternate days for a total of four capsules. A booster dose is recommended every four years if exposure is continuous or repeated (45).

Enteric coated capsules have also been used in the formulation of other vaccines that are acid-labile. A killed influenza vaccine consisting of 98 micrograms of Bangkok A hemagglutinin was given in enteric coated capsules and led to significant salivary and nasal IgA antibody rises in a 4-week period (46,47). The orally administered vaccine was associated with no more side effects than placebo and had less side reactions than the intramuscular route. The intramuscular route also did not stimulate antibody production in saliva and nasal secretions while secretory IgA antibody produced by oral vaccination may be important in protection against respiratory viral infections.

In 1986 Ishihara et al. (48) administered BCG vaccine in enteric coated capsules to 27 patients of malignant melanoma in additional to chemotherapy after surgical treatment while another 27 patients of the same stage of melanoma were treated with chemotherapy alone after surgery. The BCG group showed a significantly higher survival rate and a slightly higher disease free time.
So far the development of *Vibrio anguillarum* immersion vaccines have been discussed and some insights were given on how formulated oral vaccines which were ineffective, may have been degraded by the high acidic environment in the stomach. Several ways to improve the efficacy of an oral vaccine were (a) anal intubation of the antigen, (b) adding NaHCO₃ to the vaccine, (c) neutralizing the stomach acid by cimetidine or NaHCO₃ prior to oral immunization and (d) forming an enteric coated capsule of the vaccine.

The last method of improving efficacy of an oral vaccine has been chosen in this study. An enteric coated oral *Vibrio anguillarum* (VA LS 1-74) vaccine was developed. VA LS 1-74 was coated on the surface of small sugar beads and then Eudragit L-30D was applied on top to serve as an enteric coating. Reported here are the dissolution studies performed to analyze the release patterns of VA LS 1-74 vaccine developed. Methacrylic acrylic acid copolymer (Eudragit L-30D) was chosen to be the enteric coating material because it is extensively used in pharmaceutical industry. The coating films are insoluble in pure water, in buffer solutions below pH 5.0 and also in natural and artificial gastric fluids. However, the films are soluble in the neutral to weakly alkaline region of the digestive tract and in buffer solutions above pH 5.5.

Specific objectives of this study include identification of the spray coating conditions for VA LS 1-
VA LS 1-74 and Eudragit L-30D to avoid antigen degradation. The pH at which Eudragit L-30D starts to dissolve was examined. The loading level of VA LS 1-74 in the sugar beads for rapid vaccine release and the optimum coating thickness of Eudragit L-30D for enteric protection were identified together with a formulation which is to be used to run an in-vivo challenge test. Lastly, the effects of gastric acid pretreatment on dissolution are presented.
MATERIALS AND METHODS

Materials

_Vibrio anguillarum_, VA LS 1-74

A pure stock of VA LS 1-74 was obtained from a lyophilized source stored in a -70°C freezer\(^1\). The lyophilized bacteria were thawed and cultured on a tryptic soy broth agar plate. During any transfer process the purity of each colony was monitored by streaking out an agar plate and examined under a microscope after Gram's stains were performed.

Culture media and agar plates

Tryptic soy broth powder\(^2\), 30 grams, was dissolved in water to make up 1 liter of culture. To prepare agar plates 1.5 grams of agar powder\(^3\) was added to every 100 ml culture media.

Sugar beads\(^4\)

Non-pareil sugar beads of 18 - 20 mesh size with a diameter of 1.00 ± 0.06 mm and an average mass of 62 mg/100 beads were used. The sugar bead was coated with VA LS 1-74 to yield a final weight of 10% of the bead weight. Approximately 50 beads will deliver a dose of 3 mg lyophilized bacteria, which was chosen as the dose of the vaccine per day for each fish.
Enteric coating material

Eudragit L-305 (methacrylic acrylic acid copolymer) was chosen to provide a colorless latex and transparent enteric coating, which is insoluble in pure water, in buffer solutions below pH 5.0 and also in gastric fluids. This coating is soluble in buffer solutions above pH 5.5 and neutral to weakly alkaline region of the digestive tract. This product comes as a 30% dry lacquer.

Plasticizer/Lubricant

Polyethylene glycol 6000, PEG 60006, was the plasticizer and talc7 was the lubricant.

Reagents for Lowry's protein assay

A 2% sodium tartrate, Na$_2$C$_4$H$_4$O$_6$ solution (named A$_1$) was made by dissolving 2.0 grams of sodium tartrate in 100 ml of distilled water. A 1% copper sulfate, CuSO$_4$.5H$_2$O$^9$ solution (named A$_2$) was prepared by dissolving 1.0 gram in 100 ml of distilled water. A 2% sodium carbonate, NaCO$_3$$^{10}$ solution (named A$_3$) was made by dissolving 20.0 grams in 1 liter of 0.1 N NaOH$^{11}$. Solution A was prepared by mixing A$_1$, A$_2$ and A$_3$ in a 1:1:100 ratio. Folin & Ciocalteu's Phenol Reagent, 2.0 Normal$^{12}$, was obtained commercially and a 1:1 dilution in distilled water (named B) was used during the assay.

Methods

Mass culture techniques for Vibrio Anguillarum, VA LS 1-74
The optimum growth conditions for bacteria were found to be at 18°C in a culture room. Aseptic techniques were observed. All culture media, containers and glassware were autoclaved before and after use. All culture flasks contained tryptic soy broth as the growth media, had a cotton plug to avoid air contamination and were secured firmly on a shaker to provide aeration. To start, a test tube containing 10 ml of tryptic soy broth was inoculated with stock VA LS 1-74 grown on an agar plate. After 24 hours this culture was transferred to another 100 ml sterile media in a 250 ml flask. Another 24 hours elapsed and 10 ml portions of this culture were removed and added to 2 liter flasks, each of which contained 1 liter tryptic soy broth. To replenish the carbon source and neutralize the media pH, 20 ml of 20% dextrose and 0.8 ml of 10 N NaOH were added after 12 hours. The culture was then left on the shaker for another 12 hours before it was harvested with 3 ml (0.3% of culture volume) of formalin. A tryptic soy broth agar plate was streaked every time the culture was transferred from one flask to another to assure purity or sterility. The cells were centrifuged at 10,000 x g for 20 minutes under 4°C and were rinsed three times with phosphate buffered saline (PBS) (see Table II.I) before lyophilization. The lyophilization process was kindly performed by Mrs. Ilsa Kaattari (Instructor of Microbiology, Microbiology Department, OSU).
Table II.1 Composition of Phosphate Buffered Saline, PBS.

1. 1 liter Phosphate buffered saline (PBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

For convenience, a 10X PBS solution was usually prepared and diluted to one-tenth of its volume immediately before use.

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*a* Sigma Chemical Company, St. Louis, Missouri
Preparation of VA LS 1-74 loaded sugar beads

Lyophilized VA LS 1-74 was resuspended in 70% methanol\(^{19}\) or ethanol\(^{20}\) to produce a final concentration of 5% suspension. To assure homogeneity, this suspension was mixed in a blender\(^{21}\) at low speed for 15 seconds. It was then spray coated on non-pareil sugar seeds\(^{4}\) of mesh size 18-20 (1.00 ± 0.06 mm) to produce a final VA LS 1-74 loading of 10%, 15%, 20% or 25% (w/w of lyophilized VA LS 1-74/final product) for test products. The spray-coater was an Aerocoat Strea-1 spray coater with a modified Lab-line/P.R.L. High Speed Fluid Bed Dryer\(^{22}\). A peristaltic pump\(^{23}\) was used to deliver the suspension to the spray coater at a rate of about 2.5 ml/minute with constant mixing using a magnetic stirring bar. Temperature was set at 40°C and nozzle pressure was maintained at 10 - 15 psi. The vaccine loaded beads were dried in an oven at 60°C for 2 days before they were spray coated with Eudragit L-30D.

Enteric coating of VA LS 1-74 loaded sugar beads vaccines

The Aerocoat spray coater was used to apply Eudragit L-30D on the surface of dried VA LS 1-74 loaded sugar beads. The coating solution was Eudragit L-30D together with PEG 6000 (25% w/w of Eudragit) as a plasticizer and talc (7 parts of talc to 30 parts of Eudragit in weight) as a lubricant (Table II.2). Temperature was set at 35°C and nozzle pressure maintained at 10 - 15 psi. Rate of coating was about 2.2 to 2.5 ml/minute. Four enteric-coating levels
Table II.2 Composition of Enteric Coating Solution.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eudragit L-30D&lt;sup&gt;a&lt;/sup&gt; (30% lacquer)</td>
<td>440 ml</td>
</tr>
<tr>
<td>Polyethylene glycol 6000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.0 g</td>
</tr>
<tr>
<td>(25% w/w of dry Eudragit)</td>
<td></td>
</tr>
<tr>
<td>Talc</td>
<td>30.8 g</td>
</tr>
<tr>
<td>(7 parts to 30 parts of dry Eudragit in weights)</td>
<td></td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>q.s. 979 ml</td>
</tr>
</tbody>
</table>

*to make a 20% w/v solution*

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<sup>a</sup> Rohm Pharma, Weiterstadt, West Germany

<sup>b</sup> Sigma Chemical Company, St. Louis, Missouri, U.S.A.
(5%, 15%, 25% and 35% by weight of dried vaccine loaded beads) were examined and table II.3 is a summary of various formulations developed.

Dissolution of enteric coated VA LS 1-74 loaded sugar beads

All formulations in this study utilized non-pareil sugar seeds of mesh size 18-20 (1.00 ± 0.06 mm). Vaccine beads of each formulation containing 30 mg of lyophilized bacteria (based on theoretical calculation) were placed in a U.S.P. dissolution basket (figure II.1). This dissolution basket was then put inside a 250 ml beaker. Unless otherwise specified, 30 ml of U.S.P. simulated gastric fluid without enzymes (phosphate buffered to pH 4.5) (table II.4) was used in the first hour of the dissolution and replaced by 30 ml of U.S.P. simulated intestinal fluid without enzymes (phosphate buffered to pH 7.2) starting from the second hour of the dissolution study. The surrounding solution was agitated with a magnetic stirring bar at about 160 ± 20 rpm to ensure a uniform concentration of dissolved VA LS 1-74. Temperature was maintained constant by a water bath at about 18°C, which is close to the temperature (12°C) at which fish are kept. The sampling volume was 100 ul and the same amount of GI fluid was added to the dissolution fluid after a sample was taken. Each sample was studied in triplicate and analyzed by Lowry's protein assay. Standard calibration curve for VA LS 1-74 was set up on each day for different dissolution media.
Table II.3 Composition of Oral Enteric Coated Vaccines.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>VA LS 1-74 loading (%)</th>
<th>Eudragit L-30D coating (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>5</td>
</tr>
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<td>3</td>
<td>10</td>
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<td>10</td>
<td>25</td>
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<tr>
<td>5</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>35</td>
</tr>
</tbody>
</table>
During dissolution study, *Vibrio anguillarum* loaded and Eudragit L-30D coated sugar bead vaccine was put in a U.S.P. dissolution basket and the surrounding medium was agitated by a magnetic stirring bar to enhance mixing.
Table II.4 Composition of U.S.P. Simulated Gastric or Intestinal Fluid without Enzymes.

<table>
<thead>
<tr>
<th>a. U.S.P. simulated gastric fluid without enzymes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 g</td>
</tr>
<tr>
<td>HCl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0 ml</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>q.s. 900 ml</td>
</tr>
</tbody>
</table>

Adjust to desired pH with 10% HCl.

<table>
<thead>
<tr>
<th>b. U.S.P. simulated intestinal fluid without enzymes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8 g</td>
</tr>
<tr>
<td>0.2 N NaOH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>190 ml</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>400 ml</td>
</tr>
</tbody>
</table>

Adjust to desired pH with 10% HCl.

<sup>a</sup> Sigma Chemical Company, St. Louis, Missouri, U.S.A.
Determination of the pH at which Eudragit L-30D starts to dissolve

Vaccine beads coated with 10% VA LS 1-74 and 15% Eudragit L-30D were dissolved in simulated GI fluids of pH's 4.5, 5.5, 6.0 and 7.2. The sampling times were 15 min., 30 min., 45 min., 1 h, 1.25 h, 1.5 h, 1.75 h, 2 h, 2.5 h, 3 h, 6 h and 9 h.

Preliminary study

Non-enteric coated and enteric coated (15% Eudragit L-30D) vaccine beads containing 10% VA LS 1-74 were used in this part of the dissolution tests, which consisted of four (i-iv) different conditions. (i) The non-enteric coated vaccine was dissolved in a pH 7.2 simulated intestinal fluid. To compare, (ii) the enteric-coated vaccine was put in the same type of intestinal fluid or (iii) pH 1.5 simulated gastric fluid. Also, (iv) the enteric-coated vaccine was pretreated in pH 1.5 simulated gastric fluid for 1 hour before the vaccine was dissolved in pH 7.2 simulated intestinal fluid. The sampling times were 15 min., 30 min., 45 min., 1 h, 1.25 h, 1.5 h, 1.75 h, 2 h, 2.5 h, 3 h, 6 h and 9 h.

Determination of the appropriate VA LS 1-74 loading level for rapid vaccine release

Enteric-coated (15% Eudragit L-30D) vaccine beads loaded with various levels (10%, 15%, 20% or 25%) VA LS 1-
74 were dissolved in pH 4.5 simulated gastric fluid for the first hour and in pH 7.2 simulated intestinal fluid thereafter. The sampling times were 15 min., 30 min., 45 min., 1.25 h, 1.5 h, 1.75 h, 2 h, 3 h, 4 h, 6 h and 12 h.

Determination of the optimum Eudragit L-30D coating as enteric protection

Vaccine beads loaded with either 10% or 25% VA LS 1-74 and enteric-coated with various levels of Eudragit L-30D were dissolved in pH 4.5 simulated gastric fluid for the first hour and in pH 7.2 simulated intestinal fluid thereafter. The sampling times were 15 min., 30 min., 45 min., 1.25 h, 1.5 h, 1.75 h, 2 h, 3 h, 4 h, 6 h and 12 h.

Gastric acid pretreatment effect

Vaccine beads coated with 10% VA LS 1-74 and 15% Eudragit L-30D were pretreated in simulated pH 4.5 gastric fluid for 2, 4 or 6 hours and were then dissolved in pH 7.2 simulated intestinal fluid. The sampling times were 0 min., 15 min., 30 min., 45 min., 1 h, 2 h, 3 h, 5 h and 11 h.

Lowry's protein assay to measure amount dissolved

To quantitate the amount of VA LS 1-74 dissolved from our formulation, Lowry's protein assay method was used as follows:

To each sample, 2 ml of solution A (see Materials section) was added. After 10 minutes, 200 ul of reagent B
(see Materials) was added. After an incubation time of 30 minutes, the absorbance was read at 500 nm. The samples were separated from each other in such a manner that the absorbance was read exactly at the end of the 30 minute incubation.
RESULTS AND DISCUSSION

Figure II.2 is a typical calibration curve of *Vibrio anguillarum* at pH 7.0 using Lowry's protein assay. From the summary data of this standard curve (table II.5), the concentration of VA LS 1-74 and absorbance are highly linearly related with a high correlation coefficient, $r^2$ of 0.994.

Effect of pH on dissolution

To identify the exact pH at which the enteric coating will begin to dissolve, dissolution experiments were done at different pH's. Figure II.3 is the calibration curve of VA LS 1-74 at different pH's. The pH 7.2 standard curve was performed one more time to ensure the consistency of the results. It was observed that absorbance slightly increased for similar concentrations of VA LS 1-74 when pH increased. Figure II.4 is the dissolution graph at various pH's (table II.10). Enteric coated vaccine began to dissolve at pH's above 5.5 and this agrees with the manufacturer's specification for Eudragit L-30D.

Preliminary study

As a preliminary study, enteric coated (15% w/w Eudragit L-30D) and non-enteric coated VA LS 1-74 loaded (10% w/w) vaccine beads were dissolved in simulated gastric (pH 1.5) and simulated intestine fluid (pH 7.2) without
Figure II.2  Standard curve for *Vibrio anguillarum* LS 1-74 in pH 7.0 U.S.P. simulated GI phosphate buffer by Lowry's protein assay, estimated using linear regression.
Table II.5  Typical Standard Curve Data for Vibrio Anguillarum Concentration at pH 7.0 Estimated Using Linear Regression$^a$.

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. (mg/ml)</th>
<th>% Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.000</td>
<td>-0.010</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.115</td>
<td>0.232</td>
<td>116.2</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.195</td>
<td>0.403</td>
<td>100.7</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.280</td>
<td>0.584</td>
<td>97.4</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.368</td>
<td>0.772</td>
<td>96.5</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.485</td>
<td>1.022</td>
<td>102.2</td>
</tr>
</tbody>
</table>

Mean 102.6

S.D. 8.0

% C.V.$^d$ 7.8

$^a$ $R^2 = 0.996$

$^b$ Inversely estimated concentration = $-0.013 + 2.133X$ (Absorbance)

$^c$ % Theory = (Inversely estimated concentration/known concentration) x 100

$^d$ % Coefficient of variation = (S.D./Mean) x 100
Figure II.3 Standard curve for *Vibrio anquillarum* LS 1-74 in pH 4.5, 5.5 and 6.0 U.S.P. simulated gastric fluid by Lowry's protein assay, estimated using linear regression.
Table II.6  Standard Curve Data for *Vibrio Anguillarum*

Concentration in Figures II.4 and II.5 at pH 4.5
Estimated Using Linear Regression\(^a\).

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. (mg/ml)</th>
<th>% Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.050</td>
<td>-0.016</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.121</td>
<td>0.199</td>
<td>99.6</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.196</td>
<td>0.425</td>
<td>106.2</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.254</td>
<td>0.601</td>
<td>100.1</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.325</td>
<td>0.818</td>
<td>102.3</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.376</td>
<td>0.973</td>
<td>97.3</td>
</tr>
</tbody>
</table>

Mean 101.1  
S.D. 3.4  
\(^d\) %C.V. 3.3

\(^a\) \(R^2 = 0.997\)

\(^b\) Inversely estimated concentration = \(-0.169 + 3.035X\) (Absorbance)

\(^c\) \% Theory = (Inversely estimated concentration/known concentration) x 100

\(^d\) \% Coefficient of variation = (S.D./Mean) x 100
Table II.7 Standard Curve Data for *Vibrio Anguillarum*

Concentration in Figures II.4 and II.5 at pH 5.5

Estimated Using Linear Regression\(^a\).

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc.(^b) (mg/ml)</th>
<th>% Theory(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.055</td>
<td>-0.027</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.138</td>
<td>0.228</td>
<td>113.9</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.195</td>
<td>0.402</td>
<td>100.5</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.063</td>
<td>0.613</td>
<td>102.1</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.324</td>
<td>0.800</td>
<td>100.0</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.384</td>
<td>0.984</td>
<td>98.4</td>
</tr>
</tbody>
</table>

Mean 103.0

S.D. 6.3

%C.V.\(^d\) 6.1

\(^a\) \(R^2 = 0.997\)

\(^b\) Inversely estimated concentration = \(-0.197 + 2.991X\) (Absorbance)

\(^c\) % Theory = (Inversely estimated concentration/known concentration) x 100

\(^d\) % Coefficient of variation = (S.D./Mean) x 100
Table II.8 Standard Curve Data for *Vibrio Anguillarum*

Concentration in Figures II.4 and II.5 at pH 6.0

Estimated Using Linear Regression\(^a\).

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. (^b) (mg/ml)</th>
<th>% Theory (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.056</td>
<td>-0.015</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.127</td>
<td>0.196</td>
<td>98.2</td>
</tr>
<tr>
<td>3</td>
<td>0.60</td>
<td>0.202</td>
<td>0.418</td>
<td>104.5</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.269</td>
<td>0.615</td>
<td>102.4</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.338</td>
<td>0.819</td>
<td>102.4</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.388</td>
<td>0.967</td>
<td>96.7</td>
</tr>
</tbody>
</table>

Mean 100.8
S.D. 3.3
\%C.V.\(^d\) 3.2

\(^a\) \(R^2 = 0.997\)

\(^b\) Inversely estimated concentration = \(-0.180 + 2.953X\) (Absorbance)

\(^c\) \% Theory = (Inversely estimated concentration/known concentration) \times 100

\(^d\) \% Coefficient of variation = (S.D./Mean) \times 100
Table II.9  Standard Curve Data for *Vibrio Anguillarum*

Concentration in Figures II.4 and II.5 at pH 7.2

Estimated Using Linear Regression$^a$.

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. (mg/ml)</th>
<th>% Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.061</td>
<td>-0.025</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.141</td>
<td>0.207</td>
<td>103.7</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.214</td>
<td>0.420</td>
<td>104.9</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.284</td>
<td>0.623</td>
<td>103.8</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.346</td>
<td>0.803</td>
<td>100.4</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.404</td>
<td>0.972</td>
<td>97.2</td>
</tr>
</tbody>
</table>

Mean 102.0

S.D. 3.2

% C.V.$^d$ 3.1

$^a$ $R^2 = 0.999$

$^b$ Inversely estimated concentration = $-0.204 + 2.939X$ (Absorbance)

$^c$ % Theory = (Inversely estimated concentration/known concentration) x 100

$^d$ % Coefficient of variation = (S.D./Mean) x 100
Dissolution of 10% *Vibrio anguillarum* loaded and 15% Eudragit L-30D coated sugar bead vaccine (% release vs. time profile) in U.S.P. simulated gastric fluids of various pH's, 4.5, 5.5 and 6.0.
Table II.10 In Vitro Dissolution of VA LS 1-74 from 10% Vibrio Loaded and 15% Eudragit L-30D Coated Vaccine Beads at Different pH's.

<table>
<thead>
<tr>
<th>Dissolution</th>
<th>Mean Percent of Released(^a) ± SD(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>1(^c)</td>
</tr>
<tr>
<td>15 min</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>30 min</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>45 min</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1 hr</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1.25 hr</td>
<td>0.9 ± 1.3</td>
</tr>
<tr>
<td>1.5 hr</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>1.75 hr</td>
<td>0.9 ± 0.8</td>
</tr>
<tr>
<td>2 hr</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>2.5 hr</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>3 hr</td>
<td>2.4 ± 2.0</td>
</tr>
<tr>
<td>6 hr</td>
<td>6.2 ± 2.7</td>
</tr>
<tr>
<td>9 hr</td>
<td>3.9 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\) Mean value of three replicates
\(^b\) Standard deviation values for each mean percent
\(^c\) pH 4.5
\(^d\) pH 5.5
\(^e\) pH 6.0
\(^f\) pH 7.2
Figure II.5  Times for 50% release of *Vibrio anguillarum* from 10% VA LS 1-74 loaded and 15% Eudragit L-30D coated sugar bead vaccine in U.S.P. simulated gastric fluids of various pH's.

1 = pH 4.5  
2 = pH 5.5  
3 = pH 6.0  
4 = pH 7.2
enzymes. Figure II.6 depicts the calibration curves of VA LS 1-74 in simulated gastric (pH 1.5) and intestinal (pH 7.2), fluids, respectively while tables II.11 to II.12 summarize the data of these standard curves. Again, linear relationships were observed with correlation coefficients $r^2$ of 0.994 and 0.996.

Enteric coated vaccines did not dissolve in the low pH of 1.5 simulated gastric fluid after 9 hours (figure II.7) while the non-enteric coated vaccines were readily released in simulated intestinal fluid (pH=7.2). Eudragit L-30D coating slowed the release of VA LS 1-74 at the beginning but more than 50% was dissolved in about 1 hour (less than 70 minutes) in both cases (figure II.8). Pretreatment of enteric coated vaccines in gastric fluid for 1 hour seemed to speed up the dissolution rate slightly probably due to water penetration. 50% of VA LS 1-74 was dissolved in about 40 minutes after one hour acid pretreatment.

This study confirmed that Eudragit L-30D serves as a protective coating against stomach acid and the vaccine releases from the beads readily at the intestinal pH. Because the enteric coated VA LS 1-74 loaded beads would be incorporated into the Oregon Test Diet (OTD), which usually has a higher pH than stomach fluid, a certain proportion of the vaccine beads might be expected to dissolve in OTD. To determine the dissolution conditions for later studies, pH paper was used to determine the pH of OTD. The pH of intestine of Coho salmon (Oncorhynchus kisutch) was
Figure II.6  Standard curve for Vibrio anguillarum LS 1-74 in pH 1.5 and 7.2 U.S.P. simulated GI phosphate buffers by Lowry's protein assay, estimated using linear regression.
Table II.11 Typical Standard Curve Data for *Vibrio Anguillarum*

Concentration at pH 1.5 Estimated Using Linear Regression\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. (mg/ml)</th>
<th>% Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.074</td>
<td>0.021</td>
<td>102.1</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.128</td>
<td>0.203</td>
<td>101.3</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.175</td>
<td>0.360</td>
<td>90.1</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.256</td>
<td>0.632</td>
<td>105.3</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.295</td>
<td>0.763</td>
<td>95.4</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.372</td>
<td>1.021</td>
<td>102.1</td>
</tr>
</tbody>
</table>

Mean: 98.8

S.D.: 6.1

% C.V.: 6.2

\textsuperscript{a} \( R^2 = 0.993 \)

\textsuperscript{b} Inversely estimated concentration = \(-0.227 + 3.355X \) (Absorbance)

\textsuperscript{c} % Theory = (Inversely estimated concentration/known concentration) \times 100

\textsuperscript{d} % Coefficient of variation = (S.D./Mean) \times 100
Table II.12 Typical Standard Curve Data for *Vibrio Anguillarum*

Concentration at pH 7.2 Estimated Using Linear Regression$^a$.

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc.$^b$ (mg/ml)</th>
<th>% Theory$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.058</td>
<td>-0.030</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.141</td>
<td>0.210</td>
<td>105.2</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.217</td>
<td>0.431</td>
<td>107.7</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.281</td>
<td>0.617</td>
<td>102.8</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.344</td>
<td>0.799</td>
<td>99.9</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.404</td>
<td>0.973</td>
<td>97.3</td>
</tr>
</tbody>
</table>

Mean 102.6

S.D. 4.1

%C.V.$^d$ 4.0

---

$^a$ $R^2 = 0.996$

$^b$ Inversely estimated concentration = $-0.199 + 2.901X$ (Absorbance)

$^c$ % Theory = (Inversely estimated concentration/known concentration) x 100

$^d$ % Coefficient of variation = (S.D./Mean) x 100
Figure II.7 Dissolution of 10% Vibrio anguillarum loaded and 15% Eudragit L-30D coated 18-20 mesh sugar bead vaccine (% release vs. time profile) in U.S.P. simulated gastric fluid, pH 1.5 and simulated intestinal fluid, pH 7.2.
Table II.13 In Vitro Dissolution of VA LS 1-74 from 10% Vibrio Loaded and 15% Eudragit L-30D Coated Vaccine Beads.

<table>
<thead>
<tr>
<th>Dissolution Time</th>
<th>Mean Percent of Released$^a$ ± SD$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1$^c$</td>
</tr>
<tr>
<td>15 min</td>
<td>38.3 ± 6.2</td>
</tr>
<tr>
<td>30 min</td>
<td>37.5 ± 7.4</td>
</tr>
<tr>
<td>45 min</td>
<td>61.5 ± 11.3</td>
</tr>
<tr>
<td>1 hr</td>
<td>95.1 ± 25.0</td>
</tr>
<tr>
<td>1.25 hr</td>
<td>98.5 ± 25.0</td>
</tr>
<tr>
<td>1.5 hr</td>
<td>109.0 ± 19.7</td>
</tr>
<tr>
<td>1.75 hr</td>
<td>97.5 ± 7.1</td>
</tr>
<tr>
<td>2 hr</td>
<td>95.2 ± 14.2</td>
</tr>
<tr>
<td>2.5 hr</td>
<td>100.0 ± 5.4</td>
</tr>
<tr>
<td>3 hr</td>
<td>112.0 ± 8.8</td>
</tr>
<tr>
<td>6 hr</td>
<td>100.0 ± 2.1</td>
</tr>
<tr>
<td>9 hr</td>
<td>100.0 ± 19.6</td>
</tr>
</tbody>
</table>

$^a$ Mean value of three replicates

$^b$ Standard deviation values for each mean percent

$^c$ Non-enteric coated VA LS 1-74 loaded vaccine beads at pH 1.5

$^d$ Enteric coated VA LS 1-74 loaded vaccine beads at pH 7.2

$^e$ Enteric coated and pretreated in the gastric fluid for the first hour

$^f$ Enteric coated VA LS 1-74 loaded vaccine beads at pH 1.5
Figure II.8  Times for 50% release of Vibrio anguillarum from 10% VA LS 1-74 loaded and 15% Eudragit L-30D coated sugar bead vaccine in dissolution study.

1 = in intestinal fluid, pH 7.2,
2 = pretreated in gastric fluid for 1 hour,
3 = in gastric fluid, pH 1.5,
4 = non-enteric coated vaccine in intestinal fluid, pH 7.2
determined in parts of this thesis. The pH's were found to be 3.5-4.5 and 7.2, respectively. Unless otherwise specified, pH 4.5 simulated gastric fluid would be used during the first hour in later dissolution experiments. After the first hour, pH 7.2 simulated intestinal fluid would be used.

Figure II.9 is a typical standard curve for VA LS 1-74 at pH 4.5 or 7.2. Both regression lines show a linear relationship between absorbance and vaccine concentration with an $r^2$ of 0.999.

VA LS 1-74 loading effect on dissolution

With the same Eudragit L-30D coating level (15%) and pretreatment time (1 hour) in simulated gastric fluid, 10% loading of VA LS 1-74 gave the fastest release in simulated intestinal fluid (figures II.10). It took 98.7 ± 5 minutes for 50% dissolution (figure II.11). 15% loading had the slowest dissolution rate. Size of the vaccine bead seemed to play an important role in determining the release rate. Because VA LS 1-74 is loaded on sugar beads that are of the same size (mesh 18-20), an increase in loading will increase the size of the final product beads. Therefore, high VA LS 1-74 loading may have a slower dissolution rate. On the other hand, high VA LS 1-74 loading could result in a larger concentration gradient around the vaccine beads increasing the dissolution rate and offset the size effect. This may explain why 20% and 25% loaded beads had shorter times for
Figure II.9  Standard curve for Vibrio anguillarum LS 1-74 in pH 4.5 and 7.2 U.S.P. simulated GI phosphate buffers by Lowry's protein assay, estimated using linear regression.
Table II.14 Standard Curve Data for *Vibrio Anguillarum*

Concentration in Figures II.10 to II.17 at pH 4.5

Estimated Using Linear Regression.<sup>a</sup>

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. &lt;sup&gt;b&lt;/sup&gt; (mg/ml)</th>
<th>% Theory&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.060</td>
<td>0.005</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.133</td>
<td>0.214</td>
<td>107.0</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.198</td>
<td>0.408</td>
<td>101.9</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.254</td>
<td>0.576</td>
<td>95.9</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.327</td>
<td>0.796</td>
<td>99.5</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.399</td>
<td>1.012</td>
<td>101.2</td>
</tr>
</tbody>
</table>

Mean: 101.1

S.D.: 4.0

%C.V.<sup>d</sup>: 4.0

<sup>a</sup> R² = 0.999

<sup>b</sup> Inversely estimated concentration = -0.184 + 2.996X (Absorbance)

<sup>c</sup> % Theory = (Inversely estimated concentration/known concentration) x 100

<sup>d</sup> % Coefficient of variation = (S.D./Mean) x 100
Table II.15 Standard Curve Data for *Vibrio Anguillarum*

Concentration in Figures II.10 to II.17 at pH 7.2

Estimated Using Linear Regression\(^a\).

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. (mg/ml)</th>
<th>% Theory (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.055</td>
<td>0.013</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.110</td>
<td>0.178</td>
<td>89.0</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.187</td>
<td>0.409</td>
<td>102.2</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.252</td>
<td>0.604</td>
<td>100.6</td>
</tr>
<tr>
<td>5</td>
<td>0.80</td>
<td>0.315</td>
<td>0.793</td>
<td>99.1</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.386</td>
<td>1.004</td>
<td>100.4</td>
</tr>
</tbody>
</table>

Mean 98.3

S.D. 5.3

%C.V. \(^d\) 5.4

\(^a\) \(R^2 = 0.999\)

\(^b\) Inversely estimated concentration = \(-0.152 + 2.998X\) (Absorbance)

\(^c\) % Theory = (Inversely estimated concentration/known concentration) \times 100

\(^d\) % Coefficient of variation = (S.D./Mean) \times 100
Dissolution of various levels of *Vibrio anguillarum* loaded and 15% Eudragit L-30D coated sugar bead vaccine (% release vs. time profile) in U.S.P. simulated gastric fluid of pH 4.5 for the first 1 hour and then in U.S.P. simulated intestinal fluid of pH 7.2.
Table II.16 *In Vitro* Dissolution of VA LS 1-74 from Various Levels of Vibrio Loaded and 15% Eudragit L-30D Coated Vaccine Beads.

<table>
<thead>
<tr>
<th>Time</th>
<th>Dissolution</th>
<th>Mean Percent of Released $^a$ ± SD $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 $^c$</td>
<td>2 $^d$</td>
</tr>
<tr>
<td>15 min</td>
<td>0.0 ± 0.0</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>30 min</td>
<td>1.2 ± 2.7</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>45 min</td>
<td>3.2 ± 3.3</td>
<td>3.1 ± 2.4</td>
</tr>
<tr>
<td>1.25 hr</td>
<td>17.7 ± 4.1</td>
<td>9.5 ± 0.5</td>
</tr>
<tr>
<td>1.5 hr</td>
<td>41.6 ± 9.7</td>
<td>17.0 ± 1.2</td>
</tr>
<tr>
<td>1.75 hr</td>
<td>56.1 ± 8.8</td>
<td>33.2 ± 3.9</td>
</tr>
<tr>
<td>2 hr</td>
<td>60.1 ± 5.4</td>
<td>42.5 ± 3.8</td>
</tr>
<tr>
<td>3 hr</td>
<td>78.2 ± 5.3</td>
<td>50.1 ± 8.5</td>
</tr>
<tr>
<td>4 hr</td>
<td>99.3 ± 11.7</td>
<td>56.3 ± 11.4</td>
</tr>
<tr>
<td>6 hr</td>
<td>102.3 ± 1.1</td>
<td>75.3 ± 4.4</td>
</tr>
<tr>
<td>12 hr</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
</tbody>
</table>

$^a$ Mean value of three replicates

$^b$ Standard deviation values for each mean percent

$^c$ 10% VA LS 1-74 loading

$^d$ 15% VA LS 1-74 loading

$^e$ 20% VA LS 1-74 loading

$^f$ 25% VA LS 1-74 loading
Figure II.11  Times for 50% release of *Vibrio anguillarum* from various levels of VA LS 1-74 loaded and 15% Eudragit L-30D coated sugar bead vaccine in dissolution study.

1 = 10% VA LS 1-74 loading,
2 = 15% VA LS 1-74 loading,
3 = 20% VA LS 1-74 loading.
4 = 25% VA LS 1-74 loading.
50% release.

Effect of enteric coating levels on dissolution

Loading of VA LS 1-74 was kept constant (either 10% or 25%) and the levels of Eudragit L-30D coatings were varied from 5% to 35%. 5% Eudragit L-30D coating did not provide enough protection against dissolution in gastric fluid and more than 10% of the vaccine was released during the first hour in pH 4.5 simulated gastric fluid (figures II.12 and II.13). A 15% of Eudragit coating gave sufficient protection against gastric fluid and the dissolution rate in intestinal fluid was rapid, releasing 75% during the first hour in simulated intestinal fluid. Times for 50% release were longer for 25% or 35% Eudragit L-30D coating (figures II.14 and II.15). Higher coating levels (25-35%) of Eudragit L-30 acted as an extra barrier and delayed the release of the vaccine during dissolution studies.

To produce enteric coatings, a 3-5 mg application of Eudragit L-30D per cm² surface area of VA LS 1-74 loaded sugar beads is suggested by the manufacturer's product prospectus (Rohm Pharma, Weiterstadt, West Germany).

This agrees with the theoretical calculation:-

mass of non-pareil sugar beads = 62 mg/100 beads

density (150 g/175 cm³) = 0.857 g/cm³
diameter (calculated) = 0.89 cm

surface area = 0.0253 cm²/0.62 mg

15% w/w of Eudragit L-30D coating gives 3.68 mg
Figure II.12 Dissolution of various levels of Eudragit L-30D coated and 10% Vibrio anguillarum loaded sugar bead vaccine (% release vs. time profile) in U.S.P. simulated gastric fluid of pH 4.5 for the first 1 hour and then in U.S.P. simulated intestinal fluid of pH 7.2.
Table II.17 *In Vitro* Dissolution of VA LS 1-74 from 10% Vibrio Loaded and 15% Eudragit L-30D Coated Vaccine Beads at Different Pretreatment Time in Simulated Gastric Fluid.

<table>
<thead>
<tr>
<th>Dissolution Time</th>
<th>Mean Percent of Released$^a$ ± SD$^b$ 1$^c$</th>
<th>Mean Percent of Released$^a$ ± SD$^b$ 2$^d$</th>
<th>Mean Percent of Released$^a$ ± SD$^b$ 3$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>8.6 ±2.2</td>
<td>9.2 ± 0.4</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>15 min</td>
<td>40.2 ±7.2</td>
<td>8.7 ± 3.1</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>30 min</td>
<td>83.2 ±11.0</td>
<td>66.4 ± 9.8</td>
<td>37.7 ± 4.0</td>
</tr>
<tr>
<td>45 min</td>
<td>88.6 ±6.8</td>
<td>83.7 ±10.8</td>
<td>62.8 ± 8.2</td>
</tr>
<tr>
<td>1 hr</td>
<td>94.3 ±6.9</td>
<td>96.1 ± 3.9</td>
<td>74.3 ±10.7</td>
</tr>
<tr>
<td>2 hr</td>
<td>98.6 ±6.2</td>
<td>92.6 ± 2.5</td>
<td>82.6 ±11.9</td>
</tr>
<tr>
<td>3 hr</td>
<td>108.9 ±9.4</td>
<td>94.9 ± 5.1</td>
<td>84.2 ± 6.4</td>
</tr>
<tr>
<td>5 hr</td>
<td>102.9 ±6.3</td>
<td>95.5 ± 6.8</td>
<td>88.9 ± 9.1</td>
</tr>
<tr>
<td>11 hr</td>
<td>100.0 ±0.0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
</tbody>
</table>

$^a$ Mean value of three replicates

$^b$ Standard deviation values for each mean percent

$^c$ Pretreated in simulated gastric fluid for 2 hours

$^d$ Pretreated in simulated gastric fluid for 4 hours

$^e$ Pretreated in simulated gastric fluid for 6 hours
Figure II.13 Dissolution of various levels of Eudragit L-30D coated and 25% *Vibrio anguillarum* loaded sugar bead vaccine (% release vs. time profile) in U.S.P. simulated gastric fluid of pH 4.5 for first 1 hour and then in U.S.P. simulated intestinal fluid of pH 7.2.
Table II.18 *In Vitro* Dissolution of VA LS 1-74 from 25% *Vibrio* Loaded and Various Levels of Eudragit L-30D Coated Vaccine Beads.

<table>
<thead>
<tr>
<th>Dissolution Time</th>
<th>Mean Percent of Released&lt;sup&gt;a&lt;/sup&gt; ± SD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>2&lt;sup&gt;d&lt;/sup&gt;</th>
<th>3&lt;sup&gt;e&lt;/sup&gt;</th>
<th>4&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td></td>
<td>3.0 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td>6.0 ± 0.7</td>
<td>1.2 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>1.7 ± 1.4</td>
</tr>
<tr>
<td>45 min</td>
<td></td>
<td>10.0 ± 1.0</td>
<td>1.9 ± 0.8</td>
<td>2.7 ± 0.6</td>
<td>5.0 ± 3.9</td>
</tr>
<tr>
<td>1.25 hr</td>
<td></td>
<td>20.5 ± 2.5</td>
<td>6.7 ± 0.9</td>
<td>11.3 ± 1.6</td>
<td>18.2 ± 11.2</td>
</tr>
<tr>
<td>1.5 hr</td>
<td></td>
<td>42.6 ± 3.0</td>
<td>36.2 ± 7.2</td>
<td>33.8 ± 2.6</td>
<td>24.6 ± 4.4</td>
</tr>
<tr>
<td>1.75 hr</td>
<td></td>
<td>66.6 ± 5.1</td>
<td>64.0 ± 17.1</td>
<td>48.4 ± 1.4</td>
<td>42.8 ± 6.5</td>
</tr>
<tr>
<td>2 hr</td>
<td></td>
<td>86.1 ± 1.5</td>
<td>73.1 ± 18.2</td>
<td>59.8 ± 7.9</td>
<td>59.5 ± 10.0</td>
</tr>
<tr>
<td>3 hr</td>
<td></td>
<td>89.4 ± 3.1</td>
<td>74.2 ± 12.5</td>
<td>73.1 ± 5.4</td>
<td>69.6 ± 8.3</td>
</tr>
<tr>
<td>4 hr</td>
<td></td>
<td>87.6 ± 1.6</td>
<td>102.2 ± 9.7</td>
<td>81.3 ± 2.8</td>
<td>89.6 ± 4.1</td>
</tr>
<tr>
<td>6 hr</td>
<td></td>
<td>89.9 ± 1.0</td>
<td>93.0 ± 9.0</td>
<td>90.1 ± 7.6</td>
<td>98.1 ± 7.0</td>
</tr>
<tr>
<td>12 hr</td>
<td></td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean value of three replicates

<sup>b</sup> Standard deviation values for each mean percent

<sup>c</sup> 5% Eudragit L-30D coating

<sup>d</sup> 15% Eudragit L-30D coating

<sup>e</sup> 25% Eudragit L-30D coating

<sup>f</sup> 35% Eudragit L-30D coating
Figure II.14  Times for 50% release of *Vibrio anguillarum* from various levels of Eudragit L-30D coated and 10% *Vibrio anguillarum* loaded sugar bead vaccine in dissolution study.

1 = 5% Eudragit L-30D coating,
2 = 15% Eudragit L-30D coating,
3 = 25% Eudragit L-30D coating,
4 = 35% Eudragit L-30D coating.
Figure II.15  Times for 50% release of *Vibrio anguillarum* from various levels of Eudragit L-30D coated and 25% *Vibrio anguillarum* loaded sugar bead vaccine in dissolution study.

1 = 5% Eudragit L-30D coating,
2 = 15% Eudragit L-30D coating,
3 = 25% Eudragit L-30D coating,
4 = 35% Eudragit L-30D coating.
Eudragit L-30D per cm² and was the optimum loading for formulating the vaccine.

Effects of prolonged gastric fluid pretreatment

Because delayed gastric emptying or prolonged gastric acid exposure may affect the release pattern of a drug, this effect was investigated by pretreating our vaccine beads in pH 4.5 simulated gastric fluid for different periods of time. It was found that the longer the pretreatment time, the slower was the dissolution rate was (figure II.16). It took an increasingly longer time (8 to 20 minutes) to release 50% of our vaccine when acid pretreatment time increased from 2 to 6 hours (figure II.17). The increased release time can be explained by the observation that vaccine beads would clump together to form a large mass in the acidic medium over an extended period of time. As a result, the surface area exposed to the surrounding dissolution media decreased and dissolution was slowed. However, it was found that this clump would re-dissolve in simulated intestinal fluid, at the higher pH of 7.2.
Dissolution of 10% *Vibrio anguillarum* and 15% Eudragit L-30D coated sugar bead vaccine (% release vs. time profile) pretreated in U.S.P. simulated gastric fluid of pH 4.5 for different amount of times and then in U.S.P. intestinal fluid of pH 7.2.
Table II.19 **In Vitro** Dissolution of VA LS 1-74 from 10% Vibrio Loaded and Various Levels of Eudragit L-30D Coated Vaccine Beads.

<table>
<thead>
<tr>
<th>Time</th>
<th>Dissolution</th>
<th>Mean Percent of Released$^a$ ± SD$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1$^c$</td>
<td>2$^d$</td>
</tr>
<tr>
<td>15 min</td>
<td>6.7 ± 0.5</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>30 min</td>
<td>12.1 ± 1.6</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>45 min</td>
<td>13.5 ± 1.6</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>1.25 hr</td>
<td>14.9 ± 4.8</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>1.5 hr</td>
<td>54.7 ± 13.6</td>
<td>41.1 ± 0.9</td>
</tr>
<tr>
<td>1.75 hr</td>
<td>80.1 ± 4.9</td>
<td>61.5 ± 7.4</td>
</tr>
<tr>
<td>2 hr</td>
<td>88.6 ± 6.8</td>
<td>79.1 ± 5.5</td>
</tr>
<tr>
<td>3 hr</td>
<td>85.8 ± 5.6</td>
<td>87.1 ± 6.7</td>
</tr>
<tr>
<td>4 hr</td>
<td>88.3 ± 6.3</td>
<td>85.0 ± 7.5</td>
</tr>
<tr>
<td>6 hr</td>
<td>95.7 ± 3.8</td>
<td>96.2 ± 6.6</td>
</tr>
<tr>
<td>12 hr</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
</tbody>
</table>

$^a$ Mean value of three replicates  
$^b$ Standard deviation values for each mean percent  
$^c$ 5% Eudragit L-30D coating  
$^d$ 15% Eudragit L-30D coating  
$^e$ 25% Eudragit L-30D coating  
$^f$ 35% Eudragit L-30D coating
Figure II.17  Times for 50% release of *Vibrio anguillarum* from 10% VA LS 1-74 loaded and 15% Eudragit L-30D coated sugar bead vaccine in simulated U.S.P. simulated intestinal fluid, pH 7.2 after different pretreatment times in U.S.P. simulated gastric fluid, pH 4.5.

1 = 2 hours  
2 = 4 hours  
3 = 6 hours
CONCLUSIONS

Eudragit L-30D provides a good enteric coating and protects the vaccine from being released until the surrounding pH rises above 5.5.

The amount of VA LS 1-74 loading will affect the dissolution rate and the optimum level of vaccine loading for rapid release is 10%.

Eudragit L-30D coating level also affects the dissolution rate. Optimum coating level found for enteric protection against stomach acid was about 15%.

Prolonged pretreatment of enteric coated vaccine beads in simulated gastric fluid for more than 2 hours causes aggregation of the vaccine beads and delays the release of vaccine.
ENDNOTES

1 the -70°C freezer was located in Room 78 in the basement of Department of Microbiology, Nash Hall, OSU, Corvallis
2 Difco Pharmaceuticals, Michigan, U.S.A.
3 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
4 Paulaur Corp., Princeton, New Jersey, U.S.A.
5 Rohm Pharma, Weiterstadt, West Germany
6 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
7 Matheson Coleman and Bell, Division of the Matheson Company Inc., Nerwood, Ohio, U.S.A.
8 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
9 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
10 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
11 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
12 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
13 Model #AS01T26G7R, AMSCO laboratory, American Sterilizer Co., Erie, Pennsylvania, U.S.A.
14 Shakers from various companies are located on fifth floor of Nash Hall, Department of Microbiology, Oregon State University, U.S.A.
15 prepared from dextrose purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.
16 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
17 Sigma Chemical Company, St. Louis, Missouri, U.S.A.
18 Sorvall® RC-5B Refrigerated Super Speed Centrifuge, DuPont Instruments

19 Sigma Chemical Company, St. Louis, Missouri, U.S.A.

20 Sigma Chemical Company, St. Louis, Missouri, U.S.A.

21 Galaxie Osterizer blender, UL® listed 564A, Mexico

22 Lab-Line/PRL, Melrose Park, Illinois, U.S.A.

23 Miniplus II peristaltic pump, Gilson Medical Electronics, Middleton, Wisconsin, U.S.A.
REFERENCES


CHAPTER III

**IN VIVO CHALLENGE TEST AND ANTIBODY LEVELS**

**AFTER VACCINATION**
ABSTRACT

An oral enteric-coated *Vibrio anguillarum* (LS 1-74) vaccine was formulated by initially coating lyophilized bacteria (10% w/w) onto sugar beads (18-20 mesh or 1.00 ± 0.06 mm). A methacrylic acrylic acid copolymer (Eudragit L-30D) coating (15% w/w) was applied to serve as an enteric coating. Vaccine efficacy was determined by both an *in vivo* challenge with live bacteria and measurements of serum and mucus antibody levels by ELISA. Survival rates among the test group, positive and negative controls were 80.3%, 83.3% and 70.3%, respectively. Serum and mucus antibody levels were found to be significantly greater (8-32 times greater in the serum and 1.4-1.8 times greater in the mucus) in the test group (p<0.001). Serum and mucosal antibody levels are better indicators for the evaluation of the efficacy of an oral vaccine than the survival rate. Using this formulation technique to develop other oral vaccines or to deliver acid-labile proteins is promising.
INTRODUCTION

Outbreak of bacterial disease has always been a threat to fish farming and most farmers are willing to pay extra costs to vaccinate their fish against potential infections (1). However, despite over 40 years of effort there are only a few commercially available bacterial fish vaccines (2) for the control of enteric redmouth, furunculosis and vibriosis. These vaccines are mostly administered by immersion or spray methods (3,4,5,6) while the oral route of administration does not provide good protection.

In a postal survey, the amount of money that experienced farmers were willing to pay for 100% vaccine efficacy was positively correlated with the average annual vibriosis mortality rate (1). New farmers who had experiences with vibriosis were prepared to pay substantially more for vaccination than were vibriosis-free farmers. If younger fish were guaranteed vibriosis-resistant, most farmers who indicated an initial preference for rearing older fish, would instead purchase the younger fish (fingerlings or yearlings). All farmers are prepared to pay at least the full cost of vaccination for this protection.

Oral vaccination at present is not as effective as injection or direct immersion. However, it has certain advantages over other methods of vaccination. These include no stressful handling and no break in routine because
vaccine can be incorporated into the fish feed and fed to
the fish during routine husbandry. Also, oral vaccination
was the first (7,8,9) and the preferred route if possible
for mass vaccination to protect fish against vibriosis
because there is no size limitation and because of reasons
mentioned above.

Since 1960 oral vaccines against vibriosis have been
studied for more than three decades by Japanese (Endo, 1961
and Hayashi, 1964) and American investigators (Nelson,
Fryer, Fletcher and White, 1973; Rohovec, 1975) using either
formalin-killed and/or lyophilized whole cells of Vibrio
anguillarum (10). In 1972 Fryer et al. (11) found
significant protection in chinook salmon from orally
administered, formalin-killed, lyophilized sonicates of V.
anguillarum. As little as 200 micrograms per fish per day
for 20 days was sufficient for immunization. Natural
challenges resulted in a 95% loss in control group versus
37% in test group but booster feedings did not provide
increased protection.

In 1975 Rohovec et al. (12) administered orally
formalin-killed wet-packed or formalin-killed lyophilized
whole cells of Vibrio anguillarum to salmon. Concentrations
ranging from 0.5 to 10 mg wet weight of vaccine per gm of
ration were effective in controlling vibriosis. Increasing
the number of consecutive days the oral vaccine was
administered from 10 to 45 days did not increase the degree
of protection. Agglutinating antibodies were detected in
parenterally vaccinated fish but not in orally immunized fish (12).

In 1983, lipopolysaccharide fraction extracted by Westphal's method from the formalin killed bacterin was used as an extracted vaccine (13). Lipopolysaccharide, a heat stable antigen in the cell wall was found to be an effective immunogen. Oral bacterin against vibriosis in cultured ayu showed that fish were effectively immunized with heated bacterin and that agglutinin secreted in the body surface mucus of the immunized fish prevented pathogenic bacterium *Vibrio anguillarum* from adhering to the skin of fish (13). These findings suggested that a heat stable antigen on the surface of the bacterium can be used to form a vaccine against vibriosis.

Although oral vaccination has been shown to provide a degree of protection, oral vaccines were significantly less effective than intraperitoneal injections (4). In 1983, Agius et al. (14) compared an extract antigen with whole cell bacterins administered to rainbow trout intraperitoneally or orally in food. Intraperitoneal vaccination resulted in virtually 100% protection within 2 weeks whereas oral vaccination resulted in maximum protection of 50% to 70% after 8 weeks. When administered orally, formalin killed cells were better than extracts. This result was supported by data from experiments on how antigen uptake in fish occurs during the vaccination process (15).
Smith (15) used various radiolabelled proteins and latex particles sensitized with radiolabelled proteins to compare hyperosmotic infiltration and bath methods for fish vaccination. While there was some uptake of soluble proteins using the bath method, uptake was greatly increased when the proteins were in the particulate form. Size of an antigen was concluded to be an important factor for immunization.

However, the major problem of oral vaccination resides on the delivery of antigens to certain positions of the fish gut where immune responses can be elicited. A number of studies were performed to help understand the basic physiology and immunology of fish. While antigen uptake through the gill is implicated for vaccination by immersion (16,17), pinocytosis (18) and processing of antigens by epithelial cells and macrophages (19,20,21) were observed in several teleosts and in particular, the second gut segment of carp when antigens were administered orally. McLean et al (22) confirmed that the fish gut is able to absorb and subsequently transfer macromolecules to the bloodstream and tissues. This implies if the fish can absorb orally administered intact proteins, systemic immune response can occur.

High acid concentration in the stomach may denature or inactivate the orally administered vaccine or antigens before the vaccine reaches the second gut. Vaccine degradation by stomach acid is supported by increased
protection against vibriosis after anal intubation of the vaccine (23). Systemic and mucosal immune response can also be observed when *Vibrio anguillarum* was deposited to the second gut by anal administration (24).

In 1989, Lillehaug (25) formulated two oral Vibrio vaccines that were protective against digestive degradation. One of these contained 30% lyophilized *Vibrio anguillarum* in a slow-release matrix and the other consisted of 20% lyophilized whole-cell granules coated with 10% Eudragit L for enteric protection against stomach acid. *In vivo* challenge of live bacteria showed higher mortality rates in the fish that were given these slow-release or enteric coated vaccines than unprotected vaccines. The author explained the lack of immune response by the slow release and enteric-coated vaccine by the reduction in absorption of lipopolysaccharide antigens by the fish. The lipopolysaccharide antigen is located on the surface of vibrio. Also, lipopolysaccharides are usually not deactivated by the stomach acid.

The ultimate goal of this study was to formulate an oral vibrio vaccine that can withstand the high acid environment of fish stomach and be released rapidly in the second segment of the fish gut for absorption to stimulate both systemic and local (mucosal) immune response. In the previous two chapters, the importance of fish gut physiology, immunology, and pH's along the gastrointestinal tract have been discussed in regards to formulation of a
vaccine. The most optimum antigen loading level and enteric coating thickness for protection were presented.

The objectives of this final chapter include an in vivo challenge of live bacteria in the test group, positive and negative controls to assess effectiveness of the vaccine. Using ELISA, both serum and mucus antibody levels were measured after vaccination and an in vivo challenge performed to evaluate the effectiveness of the vaccine. A better predictor of vaccine efficacy was found to be antibody levels after in vivo challenge rather than comparing mortality rates during in vivo challenge. Discussions of the prospects of an oral enteric coated vaccines and the potential application in human and animal diseases are presented.
MATERIALS AND METHODS

Materials

Vaccines for the test group

The vaccine used during the in vivo challenge test contained 10\% (w/w) of VA LS 1-74 and 15\% (w/w) of Eudragit L-30D\(^1\). The vaccine was incorporated into Oregon Test Diet (OTD)\(^2\) and fed to the fish directly for 30 days.

Unprotected vaccines for the positive control and negative control group

Unprotected or non-enteric-coated vaccine for the positive control group was formed by embedding lyophilized whole cells of VA LS 1-74 directly into OTD\(^2\). Fish in the negative control group were fed with plain OTD\(^2\).

Fish for in vivo challenge

Experimental fish were Coho salmon (Oncorhynchus kisutch) donated by Oregon hatcheries and the Oregon Department of Fish and Wildlife (ODFW). Each fish weighed about 80 grams.

Holding facility

Oral vaccination took place in the old Fish Disease Laboratory on Highway 20 while the new Salmon Disease Laboratory was being built. In January 1990, the new Salmon Disease Laboratory was open and the fish were transported to
the new facility where the in vivo bacteria challenge occurred and serum and mucus samples were taken for antibody assays. The Salmon Disease Laboratory is a 9,000 ft² facility. Pathogen-free water is provided from two wells each 48 feet deep. The water goes through a column where excess gas is eliminated and supplemental oxygen is added to a concentration of 10 ppm. Water temperature is maintained at 12.5°C and effluent from the wet laboratory is treated with chlorine to ensure that no infectious agents used in the laboratory exit via the water. The capacity of each tank used during the experiment was 100 L and 25 fish were put into each tank.

ELISA reagents

Vibrio antigens were prepared by research assistants in Dr. Stephen Kaattari's Laboratory (Nash Hall, Oregon State University, Corvallis). Three antigens were chosen with the following abbreviations throughout the study (table III.1).

(i) VAE.PT = Vibrio antigen extract prepared by Prasad Turaga
(ii) VAE.DC = Vibrio antigen extract prepared by Don Chen
(iii) LPS = lipopolysaccharide antigen

Bovine serum albumin³ (BSA)

Biotin-labelled anti-fish antibody (1-14 or WARR's)

1-14 is a mouse monoclonal antibody developed by Dr. G. Warr from University of South Carolina. Stock 1-14 solution was obtained from Dr. Kaattari's research assistant (Nash
Table III.1 Abbreviations for Various Antigens and Reagents Used During ELISA.

(i) VAE.PT = Vibrio antigen extract prepared by Prasad Turaga

(ii) VAE.DC = Vibrio antigen extract prepared by Don Chen

(iii) LPS = lipopolysaccharides antigen

(iv) BSA = bovine serum albumin

(v) WARR's = mouse monoclonal antibody 1-14 developed by Dr. G. Warr

(vi) SA.HRPO = strepavidin-horsradish peroxidase

(vii) ABTS = azino-bis(ethylbenzthiazoline-6-sulfonic acid)

(viii) TBS = tris buffer saline

(ix) T-TBS = tween-tris buffered saline

(x) SAS = saturated ammonium sulfate

(xi) PBS = phosphate buffered saline

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a Prasad Turaga and Don Chen were research assistants of Dr. Stephen L. Kaattari, Department of Microbiology, Nash Hall, Oregon State University, Corvallis.
Strepavidin-Horseradish Peroxidase (SA.HRPO)\textsuperscript{4}

Strepavidin-HRPO was purchased in the lyophilized form and 2.5 ml of sterile water was injected into the 0.25 mg vial, which has an activity of 130 units/mg.

Substrate solution

Ten ml of citric acid buffer (table III.2), 5 ml of $\text{H}_2\text{O}_2$\textsuperscript{5} and 75 ul of ABTS\textsuperscript{6} (10 mg/ml of 2,2 azino-bis(ethylbenzthiazoline-6-sulfonic acid) are mixed together in distilled water.

ELISA equipment

Equipment consisted of ELISA plates\textsuperscript{7}, tupperware containers as moist chambers for incubation, ELISA plate reader\textsuperscript{8}, parafilm for wrapping ELISA plates during incubation to avoid excessive loss of evaporation, and wet paper towels to maintain moisture content inside incubation chamber.

Methods

Vaccination procedures

A total of 225 Coho salmon (\textit{Oncorhynchus kisutch}) each weighing about 80 grams were put into nine tanks with 25 fish in each tank. Of the nine tanks, three tanks were chosen randomly to be the test group, three to be the positive and three to be the negative controls (see table III.3 for the assignment of each group and corresponding tank numbers). All the fish were fed with regular OTD and
Table III.2 Buffers Used in ELISA.

1. Coating buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Na}_2\text{CO}_3^a$</td>
<td>1.59 g</td>
</tr>
<tr>
<td>$\text{NaHCO}_3^a$</td>
<td>2.93 g</td>
</tr>
<tr>
<td>$\text{NaN}_3^a$</td>
<td>0.20 g</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}$</td>
<td>q.s. 1000 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 9.6 and store no longer than 2 weeks at 4°C.

2. Tris buffer saline (TBS)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma base$^a$</td>
<td>6.07 g</td>
</tr>
<tr>
<td>EDTA$^a$</td>
<td>0.37 g</td>
</tr>
<tr>
<td>NaCl$^a$</td>
<td>8.70 g</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}$</td>
<td>q.s. 1000 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 8.0.

3. Tween tris buffered saline (T-TBS)

Add 1% (v/v) of tween 20$^a$ to Tris buffer saline (TBS).

4. Citric acid buffer

Prepare a 0.2% w/v of citric acid$^a$ and adjust pH to 4.0.

---

$^a$ The Sigma Chemical Company, St. Louis, Missouri, U.S.A.
Table III.3 Allocation of Fish in Each Group During Oral Vaccination and *In Vivo* Challenge.

<table>
<thead>
<tr>
<th>Tank number</th>
<th>Assigned group</th>
<th>Number of fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(Not Used)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>positive control</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>positive control</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>positive control</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>negative control</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>negative control</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>negative control</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>test group</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>test group</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>test group</td>
<td>25</td>
</tr>
</tbody>
</table>

**Total** = 225
acclimated in their tanks for a month before vaccination trials started in order to avoid stress and palatability problems. During the vaccination period, each fish in the test and positive controls was given daily 3 mg of lyophilized VA LS 1-74 in the form of enteric coated beads or unprotected vaccine, respectively. Same amount of OTD was given to the negative control group. Vaccination period continued for a total of 30 days.

In vivo challenge

Live bacteria challenge took place 47 days after the last administration of oral vaccine. Water supply was turned off and water level brought down to one inch above the dorsal fin of most fish in the tank. One liter of VA LS 1-74 with an optical density (O.D.) of 1.5 was then added to each tank. After 20 minutes the water level was slowly returned to the original level by turning on the water supply again. Mortalities were collected daily after challenge and vibriosis was confirmed by culturing live bacteria from the dead fish kidney. The bacteria were examined alive or after Gram’s stain under the microscope.

Methods of collecting serum and mucus samples for ELISA

Serum and mucus samples were collected from surviving fish for antibody assays 34 days after the initiation of live bacteria challenge.

Mucus samples from fish in each tank were expressed and
collected in a clear plastic bag containing 20 ml of water. To do this, each fish from a particular tank was put into the same bag for 30 seconds before skin mucus was wiped off by the inside surface of the bag.

Serum samples were then immediately after mucus samples were obtained. After euthanasia, the caudal peduncle was severed diagonally to allow blood samples to be collected into small centrifuge tubes. After storage at 4°C overnight, the serum was removed from the clot and residual red blood cells were removed by 20 second centrifugation in a microfuge\(^9\). The serum was kept at -20°C until being assayed for antibodies by ELISA.

After centrifugation at 2500 rpm\(^{10}\) (750 x g) for 10 minutes, the mucus protein was concentrated by saturated ammonium sulfate (SAS) (see SAS precipitation protocol in the next section) and dialyzed against PBS\(^{11}\) (phosphate buffered saline) to become more concentrated. Protein contents were determined by Lowry's assay method (refer to chapter II) before antibody levels were measured by ELISA.

Standard serum and mucus samples were obtained from previously immunized Coho salmon while control serum and mucus samples were taken from Coho salmon that had never been exposed to *Vibrio anguillarum* antigens.

SAS precipitation protocol for concentrating mucus samples

An equal volume (5 ml) of saturated ammonium sulfate\(^{12}\) (approximately 70 g/100 ml at 25°C) was added slowly to the
mucus sample (5 ml) over 30 minutes. Precipitate was collected after centrifuging at 3,000 rpm\textsuperscript{13} (1100 x g) for 30 minutes, and resuspended in 5 ml of H\textsubscript{2}O. This procedure was repeated one more time and the precipitate was resuspended in a minimal amount of PBS\textsuperscript{11}. These samples were then dialyzed against 1 liter of PBS\textsuperscript{11} overnight in a 4\textdegree C cold room for a total of three times.

**ELISA procedure (table III.4)**

Because ELISA consisted of several steps involving different reagents, various concentrations of different antigens (VAE.PT, VAE.DC and LPS), WARR's (1-14) and SA.HRPO were tested to determine the optimum conditions for the ELISA assay. The first part of the "results and discussion" section in this chapter describes the steps in developing the protocol. A brief description of the final protocol (table III.d) is as follows: Each well of a Elisa plate was coated with 100 ul of VA extract that was previously prepared and diluted to one one-hundredth of its original concentration using a coating buffer (table III.2). The plate was covered with parafilm and incubated in a tupperware container overnight at 17\textdegree C. After removal of the coating solution, 200 ul of 1% bovine serum albumin in tween tris-buffered saline (T-TBS, see table III.2) were added and the plate was incubated in the same manner for 1.5 hours at 37\textdegree C. The plate was then washed three times with T-TBS. In a series of three steps, 100 ul of diluted serum
Table III.4  Procedure of ELISA.

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Dilution in T-TBS</th>
<th>Amount</th>
<th>Incubation time</th>
<th>Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>antigen(^b)</td>
<td>1/100(^c)</td>
<td>100 ul</td>
<td>overnight</td>
<td>17°C</td>
</tr>
<tr>
<td>2.</td>
<td>BSA</td>
<td>1%</td>
<td>200 ul</td>
<td>1.5 hr</td>
<td>37°C</td>
</tr>
<tr>
<td>3.</td>
<td>sera/mucus</td>
<td>varies</td>
<td>100 ul</td>
<td>1.5 hr</td>
<td>17°C</td>
</tr>
<tr>
<td>4.</td>
<td>WARR's (1-14)</td>
<td>1/1000</td>
<td>100 ul</td>
<td>1.5 hr</td>
<td>Room</td>
</tr>
<tr>
<td>5.</td>
<td>SA.HRPO</td>
<td>1/200</td>
<td>100 ul</td>
<td>1.5 hr</td>
<td>Room</td>
</tr>
<tr>
<td>6.</td>
<td>Substrate</td>
<td>undiluted</td>
<td>100 ul</td>
<td>none</td>
<td>---</td>
</tr>
<tr>
<td>7.</td>
<td>Read O.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

\(^a\) Steps 2 to 5 are followed by three washes of T-TBS using a squirt bottle.

\(^b\) VAE.PT was chosen to be our antigen instead of VAE.DC or LPS in our final assays.

\(^c\) Antigens were diluted in coating buffer (table III.b).
or mucus sample, 100 ul of 1/1000 dilution of WARR's solution and 100 ul of 1/200 dilution of Strepavidin-HRPO were added. The incubation time for each of these three steps was 1.5 hours, followed by three washes of T-TBS. The incubation temperature was 17°C for the serum or mucus samples and room temperature (20°C) for WARR's (1-14) solution and Strepavidin-HRPO. The final step was the addition of 100 ul of the substrate solution (containing citric acid buffer, H2O2 and ABTS, see Materials) and the O.D. was periodically measured and recorded by an ELISA reader at 405 nm.

Statistical analysis

To evaluate any significant difference of the serum and mucus antibody levels among the three groups of fish, an analysis of variance was performed in additional to multiple group comparison using Tukey's HSD method and confidence intervals.
RESULTS AND DISCUSSION

In vivo challenge of live bacteria

Fish began to die of vibriosis on day 5 and continued to die till day 18 after challenged with live bacteria (Fig. III.1). Although the test group had a higher survival rate than the negative control group (80.3% or 57/71 vs 70.3% or 52/74), slightly more fish died in the test group than the positive controls (19.7% or 14/71 vs 16.7% or 12/72 mortalities). There were less than 75 fish in each group during the live bacteria challenge because some fish were infected with cold water bacterial disease during the vaccination period and were removed from the study. The results of the survival challenge test do not provide evidence that enteric protected vaccine was better than the unprotected non-enteric coated vaccine. Measurement of the serum and mucus antibody levels to detect differences in the antibody levels among the fish in the test group, positive and negative controls was performed. However, there was no standard ELISA protocol for antibodies against Vibrio anguillarum.

The first task in determining serum or mucus antibodies in Coho salmon (Oncorhynchus kisutch) would be the optimization of reagent concentrations and incubation times in each step of ELISA. The following section can serve as a guideline during a need to develop a new antibody assay using ELISA method.
Figure III.1  Percent survival rate after in vivo challenge of live *Vibrio anguillarum* after oral vaccination. The vaccine (test) group has a survival rate of 80.3% or 10% higher than the survival rate in the control group.
Development of an ELISA protocol for antibodies against VA LS 1-74

As noted in table III.4, ELISA for antibodies consists of several steps involving an antigen, bovine serum albumin (BSA), serum or mucus samples, mouse monoclonal anti-fish antibody (WARR's) and strepavidin-horseradish peroxidase (SA.HRPO). The first step in developing the assay would be the selection of an appropriate antigen.

Selection of an antigen

Two Vibrio anguillarum extracts were chosen (VAE.PT and VAE.DC) and prepared by Prazad and Don Chen, research assistants of Dr. Stephen L Kaattari, Department of Microbiology, Oregon State University, Corvallis, Oregon) and a Vibrio anguillarum lipopolysaccharides (LPS) fraction.

To compare the effectiveness of an antigen, the addition of antibody to the ELISA plate in the second step can be skipped. Antigens that are of higher concentrations or higher binding affinity to the ELISA plate will bind in a larger quantity and more closely onto the ELISA plate. As a result, less WARR's will bind to the ELISA plate and subsequently less SA.HRPO and substrate will react together in the final step to give a smaller value of absorbance.

Figure III.2 shows the absorbance of different antigens at various dilutions. LPS did not show any binding abilities to the ELISA plate in any range of dilutions. On the other hand, VAE.PT and VAE.DC gave the best binding capacity at about 1% dilution. To choose between VAE.PT and
Figure III.2 O.D. vs. antigen concentration in phosphate buffered saline to compare the binding abilities of three different antigens (VAE.PT, VAE.DC and LPS) to ELISA plate. VAE.PT gave the best O.D.-concentration profile.
VAE.DC, the protein contents in each of these antigens were first measured using Lowry's protein assay. Figure III.3 is a calibration curve of *Vibrio anguillarum* antigens concentrations using bovine serum albumin as a standard.

Figure III.4 compares the binding abilities of VAE.PT and VAE.DC on the basis of same protein content. The absorbance increase was observed after 5 and 10 minutes and the results were very consistent. Because VAE.PT resulted in a smoother curve and slightly larger drop in absorbance in the dilution range of 1/10 to 1/100,000, VAE.PT was chosen to be the antigen for the ELISA assay. 1/10 and 1/100 dilution factors were used in later experiments to determine the optimum concentrations for other reagents.

**Optimization of antigen, WARR's and SA.HRPO concentrations**

To identify the best dilution factor for each reagent, two different concentrations were selected for each reagent (table III.6) and the ELISA assay was conducted with both standard immunized (STD) and normal non-immunized (NS) sera (figure III.5). Using the same amount of each reagent, VAE.PT gave a much better result than VAE.DC. All of samples 3 to 6 had a higher absorbance in the standard immunized sera (STD.VAE.PT) than normal non-immunized sera (NS.VAE.PT). On the other hand, only sample numbers 1, 5 and 7 gave slightly higher absorbance in the immunized sera samples when VAE.DC was used. This again suggested that VAE.PT should be used in the ELISA. Dilution factors as
Concentration curve for Vibrio anguillarum antigens in phosphate buffered saline using bovine serum albumin as standard. Absorbance vs. concentration with line estimated from linear regression.
Table III.5 Calibration Curve Data for *Vibrio Anguillarum* Antigen Concentration Estimated Using Linear Regression$^a$.

<table>
<thead>
<tr>
<th>Std. No.</th>
<th>Conc. (mg/ml)</th>
<th>Absorbance</th>
<th>Inv. Est. Conc. (mg/ml)</th>
<th>% Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.01</td>
<td>0.000</td>
<td>0.005</td>
<td>--------</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>0.016</td>
<td>0.045</td>
<td>89.3</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>0.040</td>
<td>0.102</td>
<td>101.9</td>
</tr>
<tr>
<td>4</td>
<td>0.50</td>
<td>0.512</td>
<td>0.517</td>
<td>103.3</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>0.409</td>
<td>0.991</td>
<td>99.1</td>
</tr>
</tbody>
</table>

Mean 98.4
S.D. 6.3
% C.V.$^d$ 6.3

$^a$ $R^2 = 0.9995$

$^b$ Inversely estimated concentration = 0.006 + 2.410X (Absorbance)

$^c$ % Theory = (Inversely estimated concentration/known concentration) x 100

$^d$ % Coefficient of variation = (S.D./Mean) x 100
Figure III.4 O.D. vs antigen concentration in phosphate buffered saline to compare the binding abilities of different antigens (VAE.PT and VAE.DC) to ELISA plate. VAE.PT has a better binding ability.
Table III.6  Dilution Factors of Various Reagents in Figure III.5 to Find the Optimum Concentrations for ELISA Assay.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Antigen(^a)</th>
<th>WARR'S(^b)</th>
<th>SA.HRPO(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/10</td>
<td>1/20</td>
<td>1/30</td>
</tr>
<tr>
<td>2</td>
<td>1/100</td>
<td>1/20</td>
<td>1/30</td>
</tr>
<tr>
<td>3</td>
<td>1/10</td>
<td>1/100</td>
<td>1/30</td>
</tr>
<tr>
<td>4</td>
<td>1/100</td>
<td>1/100</td>
<td>1/30</td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>1/20</td>
<td>1/100</td>
</tr>
<tr>
<td>6</td>
<td>1/100</td>
<td>1/20</td>
<td>1/100</td>
</tr>
<tr>
<td>7</td>
<td>1/10</td>
<td>1/100</td>
<td>1/100</td>
</tr>
<tr>
<td>8</td>
<td>1/100</td>
<td>1/100</td>
<td>1/100</td>
</tr>
</tbody>
</table>

\(^a\) 200 ul of diluted antigen was used to coat the ELISA plate.

\(^b\) 100 ul of diluted reagent was used in each specific step.
Various antigens and reagents concentrations

Figure III.5
ELISA on standard immunized sera (STD) and normal non-immunized sera (NS) using different antigens (VAE.PT or VAE.DC) and various amounts of SA.HRP, WARR's. Table III.6 lists the exact amount of each reagent used.

Table 111.6 lists the exact amount of each reagent used.
high as 1/100 for both WARR's and SA.HRPO were still effective in distinguishing immunized from non-immunized serum.

To minimize the amount of each reagent used, further dilution of the reagents and the WARR's in 1% bovine serum albumin were performed (table III.7 and III.8). Figures III.6 and III.7 are ELISA results for non-immunized and immunized sera, respectively. All the curves for the non-immunized sera did not show any significant increase in absorbance implying minimal amount of antibodies existed in non-immunized sera against the selected Vibrio antigen (VAE.PT). The amount of standard immunized sera gave higher absorbance values suggesting the presence of antibodies against the Vibrio antigen.

Conditions in sample #4 (1/100 dilutions of antigen, 1/1000 dilutions of WARR's, 1/200 dilutions of SA.HRPO) gave the best ELISA result with small and minimal increase in absorbance for non-immunized sera while a significant change of absorbance was obtained over a range of serum quantity (Figure III.8). A summary of the final ELISA protocol is listed in table III.4 in the Materials and Methods section of this chapter.

Serum antibody assay

Two typical ELISA curves of standard serum samples from previously immunized Coho salmon are shown in figure III.9. The 50% point of the O.D. range was consistently given by 1
Table III.7 Dilution Factors of Various Reagents in Figure III.6 to Find the Optimum Concentrations for ELISA.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WARR'S&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SA.HRPO&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/100</td>
<td>1/100</td>
<td>1/100</td>
</tr>
<tr>
<td>2</td>
<td>1/100</td>
<td>1/100</td>
<td>1/200</td>
</tr>
<tr>
<td>3</td>
<td>1/100</td>
<td>1/1000</td>
<td>1/100</td>
</tr>
<tr>
<td>4</td>
<td>1/100</td>
<td>1/1000</td>
<td>1/200</td>
</tr>
<tr>
<td>5</td>
<td>1/100</td>
<td>1/1000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1/100</td>
</tr>
</tbody>
</table>

<sup>a</sup> 200 ul of diluted antigen (*Vibrio anguillarum* extract) was used to coat the ELISA plate

<sup>b</sup> 100 ul of diluted reagent was used in each specific step.

<sup>c</sup> 1% BSA (bovine serum albumin) was used to dilute instead of TTBS (tween 20 in tris-buffered saline)
Table III.8 Dilution Factors of Various Reagent in Figure III.7 to Find the Optimum Concentrations for ELISA.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WARR'S&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SA.HRPO&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/100</td>
<td>1/100</td>
<td>1/100</td>
</tr>
<tr>
<td>2</td>
<td>1/100</td>
<td>1/100</td>
<td>1/200</td>
</tr>
<tr>
<td>3</td>
<td>1/100</td>
<td>1/1000</td>
<td>1/100</td>
</tr>
<tr>
<td>4</td>
<td>1/100</td>
<td>1/1000</td>
<td>1/200</td>
</tr>
<tr>
<td>5</td>
<td>1/100</td>
<td>1/1000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1/100</td>
</tr>
</tbody>
</table>

<sup>a</sup> 200 ul of diluted antigen (Vibrio anguillarum extract) was used to coat the ELISA plate

<sup>b</sup> 100 ul of diluted reagent was used in each specific step.

<sup>c</sup> 1% BSA (bovine serum albumin) was used to dilute instead of TTBS (tween 20 in tris-buffered saline)
Figure III.6  ELISA on normal non-immunized sera using different amounts of SA.HRPO and WARR's. Table III.7 lists the exact amount of each reagent used.
Figure III.7 ELISA on standard immunized sera using different amounts of SA, HRPO and WARR's. Table III.8 lists the exact amount of each reagent used.
Figure III.8 Typical ELISA from standard immunized and normal non-immunized sera.
Figure III.9  Typical ELISA curve obtained from standard immunized sera. 1 ul gives about 50% maximum response and is assigned to have 1 antibody unit.
ul of the standard serum, which was arbitrarily assigned to be 1 antibody unit. In other words, 1 ml of the standard serum is equivalent to 1,000 antibody units. An amount equivalent to 1 ul of standard serum was then added to each Elisa plate during assay to serve as a reference for the determination of antibody levels of various samples.

Figure III.10 showed a preliminary result of some immunized serum and mucus samples from a fish in tank #9 of the test group. The ELISA curve of the serum sample was similar to that of the standard immunized serum, indicating that a significant amount of antibodies existed in the serum. On the other hand, the mucus sample from immunized fish did not suggest the presence of any antibodies when compared to the result observed from non-immunized serum. However, it was later discovered that the antibodies in the mucus samples were too dilute to be detected.

Figures III.11 to III.19 are ELISA results of sera samples from fish in different tanks. The horizontal line represents the O.D. given by 1 ul of standard immunized serum of Coho salmon on the same ELISA plate. As has been mentioned before, standard immunized sera was arbitrarily assigned to have 1 antibody unit (equivalent to 1000 units per ml). For example, figure III.14 illustrates Elisa results of 5 fish in a negative control tank #5. The horizontal line represents the O.D. value of the standard serum and it required an average 3 ul of sample serum in each fish to attain the same O.D. value. As a result, the
Figure III.10  First ELISA on standard immunized sera, immunized sera and mucus from tank #9 (test group) and normal non-immunized sera.
Figure III.11  ELISA result on positive control sera from fish in tank #2 (878 ± 256 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.12  ELISA result on positive control sera from fish in tank #3 (3135 ± 1600 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.13  ELISA result on positive control sera from fish in tank #4 (3580 ± 2200 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.14  ELISA result on negative control sera from fish in tank #5 (412 ± 44 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.15  ELISA result on negative control sera from fish in tank #6 (454 \pm 179 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.16  ELISA result on negative control sera from fish in tank 
#7 (985 ± 164 units/ml). The horizontal line represents 
the O.D. increase of 1 ul standard immunized serum which 
was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.17 ELISA result on test group sera from fish in tank #8 (35740 ± 11180 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.18  ELISA result on test group sera from fish in tank #9 (7020 ± 148 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
Figure III.19 ELISA result on test group sera from fish in tank #10 (14060 ± 5580 units/ml). The horizontal line represents the O.D. increase of 1 ul standard immunized serum which was arbitrarily assigned to be 1 antibody unit/ul.
serum antibody level was 333 (1000/3) units per ml for these fish.

The bar chart in figure III.20 shows the antibody levels of 45 fish, (5 from each tank or 15 fish in each of the test group, positive and negative controls). Fish in the test group had significantly higher levels of antibody than both the positive and negative controls (p < 0.0001, table III.9). 95 percent confidence intervals and Tukey's HSD gave the same results (table III.10).

Mucus antibody levels

As pointed out in the previous section, the mucus antibody levels were too dilute to be detected. To make the sample more concentrated, saturated ammonium sulfate (SAS) was used to precipitate the protein contents in mucus samples. Because some mucus samples were contaminated with fecal materials and the final sample volumes of mucus collected were not exactly the same, antibody levels in each group were normalized on the basis of protein content. The protein content of mucus samples was determined by Lowry's protein assay method for the comparison. The calibration curve of protein content is shown on figure III.21 using bovine serum albumin as standard. Protein concentration of mucus sample from each tank varied from 30 to 536 mcg/ml. (figure III.22).

During the ELISA of mucus, 1 ul of standard immunized serum was again added to each ELISA plate to serve as a
Figure III.20 Serum antibody levels of fish from tanks in different groups (positive, negative controls and test).
Table III.9  Analysis of Variance\textsuperscript{a} on Serum Antibody Levels Following Oral Vaccination \{ ln(antibody) \}.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>S.S.</th>
<th>df</th>
<th>M.S.</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>17.85</td>
<td>2</td>
<td>8.92</td>
<td>24.10</td>
</tr>
<tr>
<td>Between tanks</td>
<td>2.22</td>
<td>6</td>
<td>0.37</td>
<td>1.78</td>
</tr>
<tr>
<td>Error</td>
<td>7.49</td>
<td>36</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27.55</td>
<td>44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} ANOVA results was generated by a statistics software called Statgraphics
Table III.10  Multiple Range Analysis for ln (serum antibody levels) by Group.

a. Method: 95 Percent Confidence Intervals

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Average</th>
<th>Homogeneous Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>15</td>
<td>2.686</td>
<td>*</td>
</tr>
<tr>
<td>Positive</td>
<td>15</td>
<td>3.078</td>
<td>*</td>
</tr>
<tr>
<td>Test</td>
<td>15</td>
<td>4.173</td>
<td>*</td>
</tr>
</tbody>
</table>

b. Method: 95 Percent Tukey HSD Intervals

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Average</th>
<th>Homogeneous Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>15</td>
<td>2.686</td>
<td>*</td>
</tr>
<tr>
<td>Positive</td>
<td>15</td>
<td>3.078</td>
<td>*</td>
</tr>
<tr>
<td>Test</td>
<td>15</td>
<td>4.173</td>
<td>*</td>
</tr>
</tbody>
</table>

's on the same vertical line represents homogeneous groups.
Calibration curve of protein concentration in fish mucus using bovine serum albumin as standard. Absorbance vs. concentration with line estimated from linear regression.
Figure III.22  Protein concentrations in fish mucus from tanks in different groups (positive, negative controls and test).
reference point. The whole standard curve was plotted in figure III.23 to ensure that 50% inhibition was given by about 1 ul of standard immunized sera.

Figure III.24 depicts the O.D. increase of mucus samples from various tanks while figure III.25 shows the same result but the O.D. was divided by the O.D. given by 1 ul of standard immunized sera, which served as an internal standard for each ELISA plate. Results in each group were pooled and the average calculated (figure III.26). The difference in antibody levels of each group are more easily seen. Mucus antibody levels represented by sample O.D./standard O.D. were from 0.85 to 1.35 in the test group, 0.75 to 0.95 in the positive control and 0.5 to 0.75 for the negative control group. Analysis of variance showed significantly higher mucus antibody levels in the test group (1.4 to 1.8 times greater) than both positive or negative controls (p < 0.001, table III.11). The same results were tested by multiple range analysis of 95 percent confidence intervals and Tukey's HSD intervals (table III.12).

The results suggest that the newly developed oral enteric coated vaccine can elicit good systemic and local (mucosal) immune response. A significantly better immunity, measured by higher serum and mucus antibody levels, was observed in the test group. It also confirmed previous findings that oral vaccine efficacy can be improved if the vaccine can be protected from stomach acid degradation (23).

Because in vivo challenge of live bacteria could not
Figure III.23  ELISA standard curve obtained from standard immunized sera for mucus samples. 1 ul gives about 50% inhibition and is assigned to have 1 antibody unit.
Figure III.24  ELISA result on mucus samples from fish in various tanks (positive, negative controls and test group).
Figure III.25  ELISA result on mucus samples from fish in various tanks (positive, negative controls and test group).
Figure III.26  Overall average ELISA result on mucus samples of fish from various groups (positive, negative controls and test).
Figure III.27  Mucus antibody levels of fish from tanks in different groups (positive, negative controls and test).
Table III.11  Analysis of Variance\textsuperscript{a} on O.D. Mucus Sample/O.D. Standard Following Oral Vaccination.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>S.S.</th>
<th>df</th>
<th>M.S.</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>0.776</td>
<td>2</td>
<td>0.388</td>
<td>24.59</td>
</tr>
<tr>
<td>Between tanks</td>
<td>0.023</td>
<td>6</td>
<td>0.004</td>
<td>0.24</td>
</tr>
<tr>
<td>\textit{ln} (protein content)</td>
<td>0.052</td>
<td>4</td>
<td>0.013</td>
<td>0.82</td>
</tr>
<tr>
<td>Error</td>
<td>0.331</td>
<td>21</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.280</td>
<td>33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} ANOVA results was generated by a statistics software called Statgraphics
Table III.12 Multiple Range Analysis for O.D. Mucus Sample/O.D. Standard by Group.

a. Method: 95 Percent Confidence Intervals

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Average</th>
<th>Homogeneous Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>10</td>
<td>0.687</td>
<td>*</td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
<td>0.875</td>
<td>*</td>
</tr>
<tr>
<td>Test</td>
<td>13</td>
<td>1.078</td>
<td>*</td>
</tr>
</tbody>
</table>

b. Method: 95 Percent Tukey HSD Intervals

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Average</th>
<th>Homogeneous Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>10</td>
<td>0.687</td>
<td>*</td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
<td>0.875</td>
<td>*</td>
</tr>
<tr>
<td>Test</td>
<td>13</td>
<td>1.078</td>
<td>*</td>
</tr>
</tbody>
</table>

*'s on the same vertical line represents homogeneous groups.
distinguish any difference between the test group and positive controls, the conclusions made from total mortalities or survival rates may not be a good end point to indicate vaccine efficacy. This may be the reason why opposite results were observed by Lillehaug in 1989 (25) and Johnson & Amend in 1983 (23).

Johnson & Amend (23) administered their suspension vaccine by anal intubation and found better survival rates among these vaccinated fish than fish that were given the vaccine orally. On the other hand, Lillehaug (25) concluded his slow-release pellet and enteric coated granule vaccines to be less efficacious than unprotected vaccines when they were administered orally. The size of Lillehaug's vaccines was in the range of 1 to 2 mm and each fish had an average weight of 50 g. As pointed out in chapter I of this thesis, the average diameter of the pyloric sphincter is 0.94 mm ± 0.12 mm. It is very likely that Lillehaug's vaccine beads took a long time to pass through to the pyloric sphincter into the pyloric caeca. If the vaccine time of residence in the stomach was too great, the vaccine may have been released in the stomach instead of the intestine as thought.

To develop a successful oral vaccine, protection of the antigen against possible degradation in the stomach must be achieved while later having the vaccine released rapidly into the intestine where absorption and interaction with the immune system can occur. In this thesis dissolution experiments were performed on all vaccines developed using
both USP simulated gastric (26) and intestinal (27) fluids without enzymes to determine the optimum levels of VA LS 1-74 loading and thickness of enteric coating for acid protection and rapid release in the second gut. This type of study is routinely done in the pharmaceutical industry and requested by the Food and Drug Administration (FDA) for drug approvals (28).

It is true that lipopolysaccharide from *Vibrio anguillarum* cell walls is the major antigen for protection against vibriosis (13,29) and may not be subject to acid degradation in the stomach. However, it is desirable for the vaccine to be deposited in the second gut where antigens are processed and interaction with the immune response occurs (19,20,21). Slow-release prills or enteric coated granules prepared by Lillehaug were less efficacious than unprotected vaccines because of decreased antigen absorption as explained by the author. On the other hand, the vaccine developed here has both the characteristics of enteric protection and rapid release in the intestine. Therefore, it generated better immune response than unprotected vaccine.

The prospect of an oral enteric protected vaccine can be very promising. First of all, similar vaccines can be made to control enteric red mouth or furunculosis, where commercially available vaccines are administered by immersion but are not effective when given orally. Secondly, this method of preparing oral vaccine is
especially applicable when the major antigen is protein and will denature in an acidic environment. Last but not least, it can be applied to formulate vaccines for human or other animal use. Up till the end of 1989, there was only one oral vaccine for human use, which is inactivated poliovirus vaccine. Early this year, the second oral human vaccine, live oral typhoid vaccine Ty21a in the form of enteric capsules, was approved by the FDA (30). Oral vaccines are actively being developed for many human and animal diseases such as influenza (31,32,33) or rabies (34).
CONCLUSIONS

In vivo studies have shown that our enteric coated oral Vibrio vaccine can provide protection against live bacteria challenge. Furthermore, both mucosal (integumentary) and systemic immune responses were elicited. Also, serum and mucosal antibody levels may be better indicators of the effectiveness of a vaccine during an in vivo challenge than survival rate.

Application of this technique of loading of the antigen on non-pareil sugar beads and enteric coating the antigen to formulate other oral vaccines for animal or human diseases is promising. Also, other protein drug moieties can be delivered in the same manner if they are susceptible to destruction in the acidic environment of the stomach. So far, no vaccine exists for the bacterial kidney disease caused by Renibacterium salmoninarum in salmonids. However, a soluble antigen has been identified (35) and it can be used as the next prototype antigen for this vaccine delivery system.

Future research should include:

Determination of the size of fish that would be used in vaccination trials. As has been shown, a major factor for the success of oral vaccine is the size of the pyloric sphincter. It prohibits the passage of large particles to the pyloric caeca.

Identification of a suitable carrier to load the
Select antigens onto. Non-pareil sugar beads of mesh size 18-20 were used in this project. When smaller fish are used, a smaller carrier bead will be needed for the surface to load the vaccine on.

Choosing a prototype antigen (BSA or other proteins) and development of an ELISA for antibodies against that antigen. Serum and mucosal antibodies levels should always be used to indicate the effectiveness of future oral vaccines.

Spray coat the antigen on carriers and apply an enteric coating on top to serve as a protection against stomach acid. Find out the optimum coating conditions.

Run in vitro dissolution studies on formulated vaccines. Determine whether antigens undergo any degradation or denaturation and determine the best combination of levels of enteric coating and antigen loading for enteric protection and rapid release.

Establish a dose-response curve to determine the optimum levels of antigen in carriers (non-pareil sugar beads?).

Choose the best formulation(s) and run in vivo challenge of live bacteria. Determine and compare serum, mucosal antibody levels (a) before the vaccination procedure, (b) after the vaccination procedure but before the in vivo challenge and (c) after the in vivo challenge.

Run statistical analysis on antibody levels and mortalities to conclude the effectiveness.
ENDNOTES

1 See chapter II for exact procedure.

2 Courtesy of Dr. Jerry D Hendricks, Professor of Food Science and Technology, Food Toxicology and Nutrition Laboratory, Oregon State University, Corvallis, OR

3 The Sigma Chemical Company, St. Louis, Missouri, U.S.A.

4 The Sigma Chemical Company, St. Louis, Missouri, U.S.A.

5 The Sigma Chemical Company, St. Louis, Missouri, U.S.A.

6 The Sigma Chemical Company, St. Louis, Missouri, U.S.A.

7 Costar ELISA plate, #3590, Cambridge, Massachusetts, U.S.A.

8 Autoreader Model EL 310, Bio-Tek Instruments, Inc., Burlington, Vermont, U.S.A.

9 Beckman Microfuge E™, Spinco Division, Palo Alto, California, U.S.A.

10 SorvallR RC-5B Refrigerated Super Speed Centrifuge, DuPont Instruments

11 see Table II.a

12 The Sigma Chemical Company, St. Louis, Missouri, U.S.A.

13 Beckman Model T J-6 Centrifuge, Palo Alto, California, U.S.A.
REFERENCES


BIBLIOGRAPHY


