A COMPARISON OF ALTERNATE MUCOSAL ROUTES OF PROPHYLACTIC IMMUNISATION USING A MOUSE MODEL OF HELICOBACTER INFECTION

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PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
Abstract

Throughout history a diversity of animal species have been used and studied extensively in the development of vaccines for the benefit of humans and animals alike. As mice are a relatively easy species to maintain, handle and manipulate and have the advantage of being cost effective, they are commonly employed as animal models in the investigation of immunisation strategies against mucosal associated pathogens. Vaccine research against the human gastric pathogen Helicobacter pylori is extensively conducted in a mouse model and typically uses intra-gastric administration for the testing of potential vaccine candidates. An inherent complication with this route however, is that the vaccine constituents may be inadequately delivered to sites of specific immunity and consequently may not be the optimal method for vaccine delivery. In the present study a mouse model of H. pylori infection was used to determine the efficacy of alternate mucosal routes of immunisation from examination of protective immunity, immune responses and the practical aspects of vaccine administration. Commencing with the optimisation of intra-intestinal immunisation, the direct injection of a H. pylori vaccine to initiator sites of the mucosal immune system established baseline data of dose rates for the comparative analysis of intra-gastric, intra-nasal and intra-rectal immunisation. Following the development of simple administration techniques whilst maintaining the welfare of the animals, intra-nasal immunisation was shown to elicit the highest levels of prophylaxis against H. pylori challenge. Effective prophylaxis was also shown to be dependent upon a specific ratio of the vaccine constituents. When using whole cell lysate of H. pylori and the mucosal adjuvant cholera toxin, the ratio of antigen:adjuvant for optimal protective immunity was 10:1. The outcomes of this study have proved conclusively the necessity for optimisation of all aspects of immunisation in an animal model of infection.
Statement of Originality

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgment is made in the text.

I also declare that the intellectual content of this thesis is the product of my own work, even though I may have received assistance from others on style, presentation and language expression.

(Signature)
Acknowledgments

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<tbody>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
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<tr>
<td>CSA</td>
<td>Campylobacter Selective Agar</td>
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<tr>
<td>CT</td>
<td>Cholera Toxin</td>
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<tr>
<td>d.H₂O</td>
<td>De-ionised Water</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbtant Assay</td>
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<tr>
<td>FAE</td>
<td>Follicle Associated Epithelium</td>
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<td>GALT</td>
<td>Gut Associated Lymphoid Tissue</td>
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<tr>
<td>GSSA</td>
<td>Glaxo Selective Supplement Agar</td>
</tr>
<tr>
<td>HBA</td>
<td>Horse Blood Agar</td>
</tr>
<tr>
<td>Hp</td>
<td><em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>HpL</td>
<td><em>Helicobacter pylori</em> Lysate</td>
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<tr>
<td>I/G</td>
<td>Intra-Gastric</td>
</tr>
<tr>
<td>I/I</td>
<td>Intra-Intestinal</td>
</tr>
<tr>
<td>I/N</td>
<td>Intra-Nasal</td>
</tr>
<tr>
<td>I/R</td>
<td>Intra-Rectal</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina Propria</td>
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<tr>
<td>LT</td>
<td>Heat-labile Toxin of <em>Escherichia coli</em></td>
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<tr>
<td>MALT</td>
<td>Mucosal Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>NALT</td>
<td>Nasal Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PP</td>
<td>Peyers’ Patch</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory Immunoglobulin A</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific Pathogen Free</td>
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<tr>
<td>SSI</td>
<td>Sydney Strain 1</td>
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Chapter 1
Introduction

1.1 GENERAL INTRODUCTION

Many pathological disorders of animals can be attributed to microorganisms entering the host through the mucosal surfaces, and it would be reasonable to assume that reduced productivity and subsequent economic loss are a direct consequence of such diseases. Indeed, conditions including listeriosis, a causative agent of abortion in sheep,\textsuperscript{[64]} paratuberculosis resulting in milk production losses in the dairy cattle industry,\textsuperscript{[159]} and tuberculosis causing weight loss and eventually death in a range of domesticated species,\textsuperscript{[60]} are examples of diseases that have challenged veterinary research to find effective preventative measures. Since the late 19\textsuperscript{th} century, significant achievements have been made to improve animal health and welfare through immunisation,\textsuperscript{[101]} and an animal health regime incorporating vaccination programs in conjunction with improved standards of animal husbandry and veterinary intervention has greatly enhanced animal wellbeing.\textsuperscript{[143]}

An important aspect to the development of successful immunisation strategies is the route by which a vaccine may be effectively delivered. In the instance of superficial and systemic pathogens, immunisation commonly utilises parenteral administration of a vaccine, that is, by routes alternate to that of the gastrointestinal tract, and typically in these cases is an appropriate and effective means of delivery.\textsuperscript{[45]} However, when dealing with organisms that infiltrate mucosal-associated lymphoid tissue, due consideration should be given to mucosal routes of delivery.\textsuperscript{[139]} Immunisation against the human pathogen poliomyelitis is a classic example of vaccine evolution with respect to immunising against an organism that enters and infects the host through the mucosal surfaces. Polio was first vaccinated against by parenteral administration with the
inactivated virus Salk vaccine, then later with live attenuated orally administered Sabin vaccine which demonstrated and underlined an improved mucosal immunity by using the most appropriate route of delivery.\[^{145}\] Aside from the comparison of efficacy of the two vaccines, possibly the most important aspect of the latter, Oral Sabin, was the simple nature of its administration.

Deciding upon the most suitable route of immunisation from a variety of possibilities is dependent upon a number of determinants. The site of infection, the nature of the pathogen to be immunised against and the means by which antigenic substances may be effectively delivered to sites of specific immunity are significant factors. Furthermore, particular emphasis should be given to the practical aspects of vaccine administration, such as the ease of operation, cost effectiveness and the impact upon the target species. Addressing these issues may prove to be important in the development of successful vaccines against a host of mucosal associated pathogens. For instance, recent research has shown that an effective vaccination against brucellosis may be achieved via intra-nasal immunisation whereas hitherto parenteral administration of vaccine candidates has been largely ineffectual in controlling the disease.\[^{160}\]

Undoubtedly, the advancement in animal immunisation practices is a direct result of the development of vaccines against a range of diseases that affect humans. Ironically it is an animal model that is typically used for the pathological diagnosis of such infections and the subsequent investigation into appropriate control tactics. In keeping with this precept, the following thesis has concentrated on the development of immunisation techniques using an animal model of infection against an important pathogen in humans, *Helicobacter pylori*.

A standardised mouse model for *H. pylori* infection has been established and lends itself to the further investigation and development of immunisation strategies against gastrointestinal disease.\[^{89}\] Importantly, early studies of *H. pylori* infection have shown that the mucosal route of intra-gastric immunisation induces higher levels of protection than the parenteral routes of intra-peritoneal or intra-venous immunisation,\[^{14}\] thus supporting the significance of the role of alternate routes of mucosal immunisation.
1.1.1 Animals & Immunisation: A Brief History

The earliest known medical work was the Corpus Hippocraticum, compiled by Hippocrates (circa 460-377 BC) the father of medicine. The work contained descriptions of pig experimentation providing an insight into human health, illness and death. Throughout the ages, animals were used primarily for advances in basic medical science such as anatomy and physiology, however the first recorded attempts at immunological therapies and prophylaxis were undertaken in humans in 6th century China.\[56\] The Chinese developed a technique in an attempt to deal with the threat of smallpox. As described in an early text “The Golden Mirror of Medicines” they had learnt that a healthy subject could be safeguarded against the disease by inoculation from a pustule taken from a person with only a mild form of the infection, thus providing protective immunity.\[132\] Obviously, the major drawback with variolation, as the practice was later termed, was that the recipient was at considerable peril of contracting smallpox in potentially its most extreme and lethal form. In spite of this, the value of variolation appears to have outweighed the risks and was adopted throughout Asia for centuries and finally introduced to England in 1721 by Lady Mary Wortley Montagu.\[125\]

According to folklore, it was the chance remark of a milkmaid “I cannot take the smallpox, since I have had the cowpox”, that inspired the British doctor, Edward Jenner to begin experiments with a much safer method of protection against smallpox. His initial investigations were conducted using animals, suggesting that microparticles taken from horsepox could be transferred to ovine udders then subsequently on to dairy maids.\[155\] This work climaxed in 1796 with the first successful immunisation, that of a young boy, James Phipps. Jenner inoculated Phipps with a sample of pus taken from a cowpox-infected milkmaid. Six weeks later the boy was challenged, unsuccessfully, with smallpox, thus demonstrating resistance.\[161\] The term vaccination, from the Latin vacca meaning cow, was born and the technique adopted worldwide despite total ignorance of the nature of infection and the immune response. Though the general acceptance of vaccination against smallpox is accredited to Jenner, some historians may argue that other earlier researchers were responsible.\[155\] Nevertheless, in further investigations, Jenner was the first to differentiate between distemper and rabies, and prove that the former was indeed infectious but not communicable to man.\[74\] Thus a significant breakthrough into the
understanding of disease using animals as experimental models had been made and furthered the cause of vaccination development.

The actual science of preventative medicine, that is, preventing disease rather than merely dealing with its effects began in earnest with the work of the 19th century French chemist Louis Pasteur. Based upon his germ theory, he reasoned that unseen airborne microbes were at the heart of spoiling wine. Extrapolating this principle he correctly proposed that disease was the result of microscopic organisms invading the body, thus dispelling the myth of disease being spontaneously generated.\cite{125, 132} Pasteur had the foresight to recognise Jenners approach that an avirulent strain could be used to protect against a virulent strain. He also understood the importance of attenuating an organism so as to reduce it’s virulence.\cite{128} These two essential observations made in animal models of disease were crucial for the development of his vaccines effective against fowl cholera (1880) and anthrax-causing bacteria (1881).\cite{127, 129} At a similar time-point in history, Dr. Burdon-Sanderson at the Brown Animal Sanitary Institution, University of London, made the remarkable discovery of microbial attenuation by passage through an alternate species.\cite{9} He used anthrax taken from infected cattle to inoculate guinea pigs. The result being that the rodents died, but the bovine animals inoculated with blood and the pulp of diseased spleens taken from these donors was used to successfully immunise against anthrax challenge.

Robert Koch, the German bacteriologist and Nobel laureate established the principle of proof of aetiology. Koch’s Postulate, published in 1884, provided for a greater understanding of the nature of disease and became a prerequisite for future immunisation strategies. This work established the necessity of quality control when using animal models to interpret disease outcomes.\cite{156, 125, 132}

Throughout history animals have been used and studied extensively in the development of vaccines and to gain insights into the immune response. Humans have benefited significantly from these studies, and the same principles for immunisation led to the successful development for vaccines such as polio (1954) and rubella (1962), culminating with humankind’s’ greatest achievement to date when the World Health Organisation formally announced in 1980 the worldwide eradication of smallpox.\cite{125, 132}
Despite the remarkable success of vaccination programs, the revelation and evolution of antibiotics in the latter half of the 20th century has perhaps overshadowed the significance of immunisation. When penicillin was discovered and developed for use in the treatment of a range of bacterial pathogens, it was heralded as the “miracle drug” for which the world was waiting. However, in the relatively short period of time since Fleming, Florey and Chain won the Nobel Prize in 1945, there has been an emergence of antibiotic resistant strains of bacteria.

The gram positive bacteria *Staphylococcus aureus* is well documented for its resistance to antibiotics, and is an increasing concern particularly in hospitals.\[^{68}\] Similarly, and with respect to the pathogenic organism used within this study, antibiotic resistant strains of *H. pylori* have been demonstrated by a number of independent investigations to be jeopardising the classical antimicrobial therapies.\[^{46, 69, 98, 113}\] Salmonellosis, a serious and widespread bacterial disease affecting both livestock and man is another instance where treatment with antibiotics has little or no effect, and indeed their use has been shown to possibly exacerbate the condition.\[^{148}\]

With the evolution of antibiotic resistance to some strains of bacteria, and as no treatment is currently available for viral infections, scientific thought has been prompted into a re-evaluation of the control measures used against disease. A multifaceted approach including continued and improved surveillance, reduction in the unnecessary use of antibiotics and the application of other strategies such as vaccination, will be required for effective disease control.\[^{68}\]

### 1.1.2 Animals & Immunisation: An Ethical Perspective

Advances in human and veterinary medicine, and in particular further vaccine development will, for the foreseeable future, be dependent upon experimentation and research requiring living animals. Even in light of the animal care and ethics ethos of refine, reduce and replace, animal models of experimentation will continue, however regrettable, for two very important reasons. First, it is at present impossible to replicate *in-vitro* the complexity of the interactions between different organs and systems that exist in
all living animals. And secondly, and no doubt re-enforced by the threat of litigation, there is a requirement to test the safety of any substance intended for commercial use in humans or animals. Without the aid of an animal model, the toxicity of any substance is reliant upon specific tests on simplified systems, however, there are numerous ways in which any such substance can be toxic, and this toxicity can only be fully evaluated in the living animal.

How then does modern science address the issue of the ethical use of animals for research and the necessity to use animal models? The overall welfare of the laboratory animal may be categorised into two areas. In the first instance there are causes of animal suffering such as inadequate veterinary attention, incompetent animal handling techniques and inappropriate housing and husbandry, which are avoidable through good management practices. Secondly, there are those experimental procedures to which the animal may be subjected that can be refined or replaced in order to reduce any distress. For example, experiments using the Draize test, whereby the toxicity of a substance is tested in the eye of the rabbit, is an instance of a draconian practice now prohibited in many countries because of its impact on animal welfare. Such legislation has required researchers to find and use alternatives to animal models and has led to procedures such as the increased use of tissue cultures to replace many in-vivo experiments. If live animals need to be used because alternative experimental techniques are not available, then simple considerations such as better experimental design and technical refinement, without compromising scientific or statistical validity, should be incumbent upon the investigator.

With respect to the development of immunisation strategies, the fundamental techniques of delivery used for testing vaccines in an animal model is an area open to such refinement. Administration of vaccines in animals commonly uses some form of chemical restraint, and as such use of anaesthesia requires a high degree of technical proficiency and may create additional stress to the animal, simplified methods need to be developed. Furthermore, improvement in the efficacy of immunisation may arise from the use of alternate routes of delivery, thereby producing a greater consistency in resultant data, and also most importantly ensuring the betterment of animal welfare through the limitation of unnecessary experimental replication.
1.2 THE MUCOSAL IMMUNE SYSTEM

The gastrointestinal tract extending from the buccal cavity to the rectum and the respiratory and urogenital tracts comprise some of the largest and most complex immunological organs in the body. During normal physiological function, such as the ingestion of food, the submucosal areas of the gastrointestinal tract may be confronted on a continual basis by potentially pathogenic organisms. The mucosal epithelial layer serves as a deterrent to such infection. Non-immunological mechanisms such as gastric acidity, enzymes, peristalsis, commensal microflora and mucus act as an obstruction at the mucosal surface to such pathogens.\textsuperscript{[12, 84, 162]}

However, specific immunological processes also exist to combat such organisms and comprise the mucosal immune system. The mucosal immune system is organised into several interconnecting compartments representing either inductive (initiation of immune response from antigen uptake) or effector sites (expression of immune response) and is generally referred to as the common mucosal immune system.\textsuperscript{[72]}

1.2.1 Mucosal-Associated Lymphoid Tissue

The protection afforded by the immunological barrier arises from widely separated lymphoid cells such as T cells, lamina propria lymphocytes (LPLs') and antibody secreting cells (ASCs') or dispersed aggregates of non-encapsulated lymphoid tissue and is referred to as the mucosal-associated lymphoid tissue (MALT). The lymphoid cells of MALT are either present as diffuse aggregates or are organised into nodules containing germinal centres.

The fundamental difference between lymphoid tissue associated with the spleen, thymus and lymph nodes and that of the mucosa-associated lymphoid tissue is that MALT is in immediate contact with foreign antigenic substances. A single layer of epithelial cells sealed by tight junctions, otherwise known as simple epithelia, lines the mucosal surfaces of the gastrointestinal, respiratory, and urogenital tracts.\textsuperscript{[117]}
In simple epithelia, antigen sampling and uptake is limited to sites where the mucosal surface is overlaid by specialised epithelium otherwise known as follicle-associated epithelium (FAE) which is in direct contact with organised lymphoid tissue. Such structures are found in the nasal cavity (nasal associated lymphoid tissue, NALT) and the alimentary canal (gut associated lymphoid tissue, GALT), and typically contain abundant antigen presenting cells and lymphocytes. Indeed, as lymphocytes are known to constitute up to 25% of all cells present in the mammalian small intestine, it should be regarded as a major lymphoid organ.\textsuperscript{[4, 149]}

1.2.2 \textit{Gut-Associated Lymphoid Tissue}

The gut-associated lymphoid tissue appears as aggregated lymphoid tissues or solitary lymphoid follicles throughout the mammalian gastrointestinal tract and has been described by O'Hagan into four distinguishable anatomical regions.\textsuperscript{[122]}

a) The lamina propria (LP): The layer of thin connective tissue underlying and supporting the epithelium of the mucous membrane, containing large numbers of plasma cells, macrophages, neutrophils, eosinophils and mast cells.

b) The intra-epithelial lymphocytes: Dispersed between the epithelial cells of the mucous membrane.

c) Isolated lymphoid follicles: Present throughout the small and large intestine, such as the vermiciform appendix of rabbits and the chicken bursa of Fabricius.

d) The Peyers' patches (PP): Clusters of lymphoid follicles found along the wall of the small intestine and are similar in function to the isolated lymphoid follicles but are structurally seen as aggregates of lymphoid follicles. The structure of the Peyers' patches includes a dome region overlying the follicle. The follicle contains an interfollicular area, the T cell zone and toward the base, germinal centres, the B cell zone. The epithelium covering the dome comprises cuboidal epithelial cells.
Plate 1.1 Schematic Representation of Peyers’ Patch
Plate 1.2 Exteriorised Ileum with Peyers’ Patch

Exteriorised ileum of mouse small intestine showing location of Peyers’ patch. 
Peyers’ patch inset (x 5 original magnification)
The lamina propria and the intra-epithelial lymphocytes are defined as diffuse lymphoid tissue and express an immune response, whilst the GALT structures responsible for initiating an immune response are the isolated lymphoid follicles and the Peyers’ patches.

Macromolecules with antigenic potential are generally excluded from the intestine by the mucosal barrier.\[^{97}\] Whilst absorption of such antigenic material can occur in neonates, it typically ceases in the adult of the species as the epithelial cell membrane matures.\[^{161}\] Therefore in order to establish infection or initiate an immune response, invasive enteric bacteria, viruses and antigens must somehow pass through this barrier of intestinal epithelium. It is becoming clear that a common target for intestinal mucosa penetration is the specialised epithelial cell of Peyers’ patches, the microfold or membranous M cell.\[^{67}\]

M cells act as antigen-sampling cells, transporting luminal antigens across the thin apical rim of cytoplasm to the underlying lymphatic cells of the mucosal immune system.\[^{18}\] This is accomplished by the M cells basolateral surface being deeply invaginated to form a pocket thus creating a relatively short distance between the apical and basolateral surface thereby allowing rapid transfer.\[^{117}\] The antigen presenting cells of the Peyers’ patch (dendritic cells, interlocking cells in T cell areas and follicular dendritic cells in B cell areas) achieve the processing and presentation of antigens, which have crossed the epithelial barrier through the M cells.

Following contact with antigenic material via M cells the germinal centres of the Peyers’ patches develop and initiate a mucosal immune response. In studies using animals deficient of such initiator sites, a significant reduction in an antigen specific immune response is observed.\[^{157}\] Therefore the Peyers’ patches have been identified as important inductive sites of the mucosal immune response.\[^{45,133}\]

Other areas of mucosal-associated lymphoid tissue have functionally similar lymphoid follicles containing M cells, such as in the nasal cavity and particularly in the rectal and colonic mucosa.\[^{84,123}\]
Plate 1.3 Schematic Representation of M Cell
1.2.3 **Mucosal Immune Response**

The previously described Peyers’ patches are considered important inductive sites, and it is generally accepted that the majority of mucosal immune responses are elicited from MALT, which lack a lymphatic supply but instead can sample foreign material from the epithelial surfaces.\(^5\) As discussed, the digestive mucosa has the ability to sample antigens and microorganisms through M cells in order to trigger the development of either tolerance, that is, an active state of unresponsiveness by lymphoid cells to a particular antigen (tolerogen), or the initiation of an immune response.\(^5\) A local immune response is established when antigens gain access to lymphoid tissue within these sites and stimulate antigen-specific lymphocytes. After activation these cells enter the lymphatics, pass into circulation and back to the lamina propria of the gut for example, where they may secrete immunoglobulin A (IgA) that can be transported into the gut to prevent colonisation of microorganisms.

The role of CD4+ T cells in this process cannot be overlooked when discussing the immune response. Commonly referred as T helper cells (Th), these lymphocytes are regarded as the orchestrators of most immune reactions. When T cell receptors on the surfaces of activated Th cells encounter antigens from antigen presenting cells (APC’s) the Th cells trigger the two parallel immune responses of Th1 (cell-mediated immune response) and Th2 (humoral immune response) simultaneously. The two responses are initiated and defined by the secretion of cytokines, small proteins that have a biological effect on other cells in the area of production.

The cell-mediated immune response involves cytotoxic T cells recognising and destroying infected somatic cells. This is accomplished by APC’s producing the cytokine interleukin 1 (IL-1) and thus activating Th1 cells to produce, amongst others, IL-2 and IL-12. Interleukin-2, commonly known as T cell growth factor causes proliferation of more T cells specific to the target antigen.

The humoral immune response involves the ability of B lymphocytes to identify foreign antigens. Following the production of an alternative set of cytokines by Th2 cells, such as IL-4, IL-5, IL-6 and IL-13, immunoglobulins are produced in large numbers and are
directed against the antigen. The immunoglobulins, or antibodies, bind to the identified antigens and label them for destruction by one of a number of mechanisms. One such mechanism is that provided by IgM. Produced in a primary immune response, IgM activates complement proteins causing osmosis and ultimately destruction of the foreign cell. Produced at the latter stages of a primary response and in a secondary response another immunoglobulin, IgG, has a similar function to that described above for IgM. Additionally IgG labels cells thus allowing their identification and destruction by macrophages. Yet another mechanism is that performed by IgA. A determinant of humoral mucosal immunity is secretory immunoglobulin A (sIgA) and studies have shown that specific IgA levels detected in one mucosal region serve as reliable indicators for specific IgA at distal mucosal sites.\(^\text{1,5, 10, 4, 10, 6}\) Found in intestinal and vaginal secretions in addition to tears and saliva, sIgA has important functions. Instances of inhibition of bacterial adhesion, agglutination of pathogenic organisms and the neutralisation of some viruses by binding to surface proteins are attributable to sIgA.\(^\text{92}\)

As previously discussed, M cells are believed to be the site responsible for generation of a mucosal immune response. In vivo studies have shown that the peroral administration of the mucosal adjuvant cholera toxin to subject animals clearly leads to a strong mucosal immune response being generated at the intestinal level.\(^\text{19, 6, 15, 3}\) Additionally, antibody secreting cells (ASC) are found sequentially in the lamina propria, mesenteric lymph nodes and spleen following immunisation with cholera toxin.\(^\text{96}\) Both of these observations suggest that there is a migration of committed cells from a single source rather than systemic antigen uptake. Pierce et al.\(^\text{131}\) surmised that ASC’s arise in the Peyers’ patches, thus supporting the importance of the intestinal route of immunisation. To further emphasise this point, studies using Interleukin 4 “knock-out” mice, in which the germinal centres of the Peyers’ patches fail to develop, show a marked deficiency in antigen specific immune response.\(^\text{157}\) Mucosal-associated lymphoid tissue is therefore important when considering the local immune response at mucosal surfaces.\(^\text{10, 3, 16}\)

The induction of a mucosal antibody response at distant sites throughout the mucosal immune system is well documented.\(^\text{1, 8, 20, 36, 62, 71, 75, 76, 78, 108, 142}\) The concept of a common mucosal system, whereby immunisation at various inductive sites induces an immune response at other, remote mucosal surfaces has been illustrated, amongst others, by
Kantele et al.\textsuperscript{[7]} In this particular investigation, rectal immunisation elicited a potent immune response in nasal secretions, tears and in the rectum, suggesting that there is an interconnection between different mucosal surfaces via circulating lymphocytes.\textsuperscript{[130]} However, as previously described, strong evidence exists to propose that specific sIgA is preferentially induced at the site of administration.\textsuperscript{[53]} Even though high titres of sIgA are observed at local and distal sites, a significant prophylactic immune response is not necessarily evoked at the site of infection.\textsuperscript{[165]}

These findings would support the concept of compartmentalisation, whereby the application of immunogens at one inductive site evokes an immune response at adjacent or physiologically related sites, with the response generated being proportionately reduced at more distal and/or less related effector sites.\textsuperscript{[115, 94, 114, 134, 138]}

\textbf{1.2.4 Mucosal Adjuvants}

The sole use of purified antigens as vehicles for immunisation when delivered orally has proved to be largely ineffective due to the immunogens being readily digested in the gastrointestinal tract and because they usually only give short lived, low-titred responses even when administered in large and repeated doses.\textsuperscript{[12, 107, 137]} Therefore there exists a necessity for substances to act as adjuvants on local immune responses to perorally administered immunogens.\textsuperscript{[93]} To impress this point, the comparison of two related studies, that of Michetti et al.\textsuperscript{[110]} and Kreiss et al.\textsuperscript{[80]}, showed that an antigen, in this instance recombinant \textit{H. pylori} urease, when used in conjunction with the adjuvant, heat-labile toxin of \textit{Escherichia coli} (LT), was immunogenic, yielding higher antibody titres than the administration of antigen alone.

Immunologically, an adjuvant is any substance that non-specifically enhances the immune response to antigen. With respect to the mucosal immune response, the mechanism of adjuvants is not entirely understood. Cholera toxin (CT), another mucosal adjuvant, markedly potentiates the immune response suggesting that it has the ability to increase gut permeability, thus facilitating luminal antigen access to gut mucosae.\textsuperscript{[34, 95]} Stimulation of a significant mucosal immune response is observed when CT is administered orally,
inducing an immune response to unrelated proteins and in the process nullifying oral
tolerance to these proteins.\textsuperscript{[35, 63]} Unfortunately, many of the mucosal adjuvants, including
cholera toxin have inherent complications. Diarrhoea and rapid fluid loss in many species,
particularly humans, represents a major drawback with the use of LT and CT despite their
attributes as adjuvants.\textsuperscript{[32, 61, 110]} However, while the diarrheagenic dose of CT in humans is
1 – 5 \( \mu g \) per 70 kg of body weight, some animal species can tolerate relatively high
concentrations without the associated toxic effects.\textsuperscript{[137]} For example, the laboratory mouse
with an approximate body weight of 25 g is not adversely affected with a CT dose rate of
10 \( \mu g \).\textsuperscript{[137]}

Much work has been done to explore the potential of eliminating the adverse side effects
of CT by removing the toxic component of the molecule, however it is these very sub-units
that are responsible for its remarkable immune stimulation properties.\textsuperscript{[34, 76, 90, 96, 138, 164]}
Therefore it would appear that one aspect of future immunisation progress lies in the
development of new generation adjuvants without gastrointestinal toxicity. Furthermore, it
may be necessary to utilise alternate routes of administration whereby the toxicity of
adjuvants may be bypassed altogether. Indeed, these two approaches have been
successfully illustrated in a recent study. Guy \textit{et al}\textsuperscript{[50]} demonstrated marked prophylaxis
against \textit{H. pylori} challenge using a \textit{Helicobacter} antigen, urease, with four entirely
different adjuvants and administered subcutaneously. The efficacy of such prophylaxis
was comparable to that exhibited by the antigen in combination with labile toxin when
administered orally.
1.3 ROUTES OF IMMUNISATION

Parenteral administration of vaccines has frequently been used against many mucosal associated pathogens, however, recent studies comparing the efficacy of parenteral and mucosal immunisation against such organisms have provided strong evidence for the preferential use of mucosal routes of vaccine delivery.\textsuperscript{[12, 19, 59, 134]} Furthermore, the realisation that many infectious diseases arise or are acquired through mucosal surfaces has focused attention on immunological protection via the mucosae.\textsuperscript{[17, 137]} For example, \textit{Brucella abortus} is a mucosal pathogen which has previously been treated by the parenteral administration of a vaccine.\textsuperscript{[160]} Primarily affecting cattle as a causative agent of contagious abortion, immunisation by this route, whilst limiting incident rate of abortion, does not induce 100\% immunity in the host. However, trials conducted in both guinea pig and mouse models of \textit{B. abortus} infection have demonstrated the potential for a successful vaccine against the organism when administered via the mucosal surfaces.\textsuperscript{[160]}

Developing immunisation strategies against other mucosal associated pathogens may see the delivery of vaccines through a variety of routes, and as a recent approach in vaccine development is to utilise the natural route of infection for vaccine delivery, mucosal immunisation is emerging as an important procedure.\textsuperscript{[139]} By way of illustration, when targeting pathogens of the gastrointestinal tract, enteric immunisation via orally administered vaccines is an obvious choice, with the significant advantages of being a relatively simple route of delivery, inexpensive and imparting minimal stress to the subject.\textsuperscript{[17, 45, 145]} Of course mucosal immunisation is not exclusively limited to oral delivery only, but may see vaccines delivered via alternate mucosal sites. However, with particular reference to human medicine, the idiosyncrasies of human nature may deter the use of certain routes. For instance, intra-rectal immunisation may prove a significant and efficacious route for vaccine administration against mucosal pathogens, particularly those associated with the lower bowel.\textsuperscript{[20]} Whilst any such aversion may apply to humans, in veterinary terms its application should not be prejudiced against.

In the present study, four mucosal routes of immunisation, intra-intestinal, intra-gastric, intra-nasal and intra-rectal, are examined in a mouse model of infection and evaluated for prophylactic efficacy against the gastric pathogen \textit{Helicobacter pylori}.  

Plate 1.4 Schematic Representation of Sites of Immunisation
1.3.1 **Intra-Intestinal Immunisation**

The main site for antigen uptake within the gastrointestinal tract is known to be via the Peyers’ patches of the ileum, presumably through M cells. The typical method of delivery to these inductive sites whereby vaccine administration presents immunogens to the Peyers’ patches is via the intra-gastric route. However, accurate presentation cannot be guaranteed owing to a number of deleterious factors. Degradation by the principle constituents in the gut, absorption at non-specific sites and whole or partial regurgitation contribute to lack of dose rate precision. Furthermore, soluble proteins are not usually immunogenic when delivered directly to the gut lumen as, unlike particulate antigens, they are not readily taken up by M cells. Appropriate administration techniques that can circumvent such barriers and which are independent of the efficacy of various oral delivery systems are necessary to investigate the full potential of possible vaccine candidates. Intra-intestinal immunisation, in other words the administration of a vaccine by direct injection of the Peyers’ patches, is emerging as a suitable technology for such inquiry. Recent studies have shown that both local and systemic immune responses can be elicited from direct administration to the intestinal and gastric mucosae. An investigation by Muir et al. showed that priming with adjuvant by direct Peyers’ patch injection vastly enhanced the immune response with comparison to priming orally.

In contrast to the relatively simple nature of the methodologies associated with more conventional routes of immunisation, the actual technique of direct injection of the Peyers’ patches requires manipulative surgery performed under general anaesthesia. Given that intra-intestinal immunisation demands a high level of technical competency, the routine use of such a procedure would be limited in veterinary practices, and certainly would not be applicable to human medicine. However, despite any such paucity in everyday employment, intra-intestinal immunisation may prove to be a significant technique for research purposes in an animal model of investigation. For example, a novel approach for the delivery of antigen to sites of uptake is by the utilisation of polymers or micro-particles, whereby an enteric-coated capsule, administered orally, effectively protects vaccines against inactivation by gastric factors and releases antigen to the Peyers’ patches. Preliminary investigation of the efficacy of vaccines delivered in this fashion may be initially screened by intra-intestinal immunisation. Furthermore, direct injection of the
Peyers’ patches can serve as a precursor for analysis of alternate routes of delivery by providing base line data from maximal intestinal immunisation.\textsuperscript{[19, 28]}

Intra-intestinal immunisation has been described by Dunkley et al.\textsuperscript{[27]} as possible immunisation strategy in a mouse model of \emph{H. pylori} infection. Using a \emph{H. pylori} citrate synthase homologue protein as the vaccine antigen combined with Freund’s incomplete adjuvant, this study demonstrated that the direct injection of a vaccine into the sub-serosa of the Peyers’ patches of mice induced a prophylactic immune response against \emph{H. pylori} challenge. However, the level of colonisation attained in the infected control groups was relatively low as the bacterial burden, assayed by viable plate counts, was in the order of $10^4$ colony-forming units per gram (CFU/g) of stomach tissue, whilst contemporary studies using \emph{H. pylori} infected mice typically show colonisation levels of $10^5$ - $10^6$ CFU/g.\textsuperscript{[152]}

Furthermore, the possibility of a placebo effect arising from the methodology alone was not evaluated. Notwithstanding these apparent shortfalls, intra-intestinal immunisation once fully validated in a mouse model of \emph{H. pylori} infection may prove to be a valuable technique in the further development and screening of \emph{H. pylori} vaccines.

\subsection{Intra-Gastric Immunisation}

There are two main possibilities for vaccine delivery by way of oral immunisation. Administration of live attenuated organisms in the first instance, and secondly by antigens which have the capacity to bind and be absorbed at the intestinal level, generating a local immune response and possibly a systemic response against pathogens that colonise and invade the mucosa.\textsuperscript{[154]} This study explores the use of the latter vaccine design.

Parenteral immunisation has often been used preferentially over peroral immunisation because of unreliable uptake of antigen in perorally administered vaccines.\textsuperscript{[45]} Such a response may occur when vaccines are administered via this route if the antigens present in the vaccines are in the form of particles or large peptides, and as a consequence may be poorly delivered to sites of specific immunity.\textsuperscript{[45]}
Other reasons may be many but include acid degradation, spontaneous or enzymic breakdown and inadequate absorption of the vaccine. However, in light of the evolution of vaccines such as the development and use of mucosal adjuvants, oral immunisation is emerging as a prominent site for mucosal delivery. The main advantage of orally administered vaccines is the convenience of being a relatively simple procedure to carry out, and when performed competently there is no detriment to the animal regardless of the number of administrations performed or duration of inoculation schedules.

Oral immunisation is a generalised term, and simply implies that the administration of vaccines is via the mouth. In the present study, oral immunisation is further defined as intra-gastric immunisation, and is the method used for the peroral delivery of vaccines. In theory, the technique of intra-gastric administration of vaccines bypasses the lymphoid tissue associated with the buccal and nasal cavities and delivers the aliquot directly to the stomach where the normal physiological function of gut transit presents the antigen to the suggested intestinal inductive sites of the Peyers’ patches. However, the methodology of intra-gastric administration, whereby the intent of delivery is directly to the stomach, needs to be validated as immunisation by way of oral administration may succeed in antigen uptake through a variety of mucosal surfaces other than the specified target initiator sites. Inaccuracy of vaccine presentation may be attributed to poor design of delivery or inadequate technique, and it could be reasoned that such technical unreliability would necessarily impact upon experimental replication.

With respect to gastric *Helicobacter* infection in animals models, intra-gastric immunisation elicits significantly higher levels of protective immunity against bacterial challenge than the parenteral routes of intra-peritoneal, intra-dermal and subcutaneous administration. Given that intra-gastric administration is the technique used for the experimental infection of the stomach in animal models, may further explain why this route of delivery is so popular with the vast majority of investigators involved with *H. pylori* vaccine research.
1.3.3 Intra-Nasal Immunisation

The nasal mucosae is known to be the first site of contact with inhaled antigens, however, the nature of immune responses and the role of nasal-associated lymphoid tissue (NALT) in those responses have been rarely studied.\(^{58}\) NALT consists of unencapsulated lymphoid cell aggregates with an overlying epithelium containing M-like cells. In rodents the lymphoid tissue is presented as a bilateral strip beneath the posterior nasal passage and is analogous to the Waldeyer’s ring of tonsils and adenoids in humans and non-human primates.\(^{82}\) NALT cells typically contain a higher proportion of T lymphocytes but the total B lymphocyte frequency is lower by approximately 20\% than that of Peyers’ patches.\(^{58}\) However, Wu et al.\(^ {170}\) demonstrated by histological examination, that NALT cells taken from the same naïve animals reveal morphology more similar to the Peyers’ patch with respect to lymphocyte size and granularity (indicative of maturity), than cells taken from the lamina propria and other mucosal effector sites. This observation is indicative that NALT, like the Peyers’ patch, is another mucosal inductive site rather than an effector site. The strategic location of NALT in the upper respiratory tract and its ability to distribute immunocompetent cells to mucosal effector sites indicate its important role in respiratory and oral immunity and in intra-nasal immunisation.\(^{169}\)

Immunisation against smallpox via intra-nasal administration was practised in 6\(^{th}\) century China by either blowing powdered scabs of smallpox lesions into the nose or inserting cotton wads smeared with the contents of a smallpox vesicle.\(^ {56, 132}\) The efficacy of such seemingly dramatic methods must have recorded some success for the practice to continue down the ages.\(^ {132}\) It is now known that intra-nasal immunisation is a very effective route for inducing mucosal immunity, however, the cellular mechanism responsible for regulating and disseminating these responses is not fully understood, due in part by the lack of attention intra-nasal administration has received in the past.\(^ {170}\) Nevertheless, this alternate mucosal route is once again gaining prominence, as recent experimental data has shown that intra-nasal immunisation is in many instances more effective in producing a mucosal immune response than intra-gastric immunisation.\(^ {59, 70, 171}\) Furthermore, recent research with a variety of animal species has shown that intra-nasal immunisation with mucosal adjuvants and/or antigens elicited detectable antibody responses in plasma, saliva and nasal and vaginal secretions.\(^ {51, 62, 119, 137}\)
Three unrelated investigations have come to similar conclusions as to the effectiveness of intra-nasal immunisation to produce significant mucosal immune responses at distal effector sites.\textsuperscript{1, 62, 146} Each study, using different animal models and experimental protocols, reported significantly higher IgA levels in vaginal fluids and faeces when immunised intra-nasally than the response generated locally by intra-rectal administration. Whether this data translates to effective prophylactic immunisation against \textit{H. pylori} remains to be validated, but certainly the findings would be suggestive that this route is potentially an effective mode of vaccine administration to distal target sites as well as locally.

1.3.4 Intra-Rectal Immunisation

The rectal mucosa is known to be abundant with lymphoepithelial structures analogous to Peyers’ patches of the small intestine.\textsuperscript{123} However, whilst there has been much research of the intestinal M cells that overlie the Peyers’ patches, comparatively little is understood concerning those in the large intestine. What is known is that repeated rectal immunisation with cholera toxin has been shown to induce strong mucosal and systemic antibody-secreting-cell (ASC) responses.\textsuperscript{36} Local administration of antigen to rectal mucosa results in higher ASC responses than systemic or distal mucosal delivery. Furthermore, rectal mucosae can serve as inductive sites for systemic ASC responses and the rectal route of administration an effective method for the induction of immune protection.\textsuperscript{20, 36}

In a recent study, a number of different mucosal routes were trialed in BALB/c mice by Lagranderie \textit{et al} \textsuperscript{83} investigating potential vaccine candidates for the HIV virus. Whilst all routes induced significant IgA and IgG levels with strong cytotoxic responses in splenocytes the study indicated that oral and in particular the rectal routes of administration were the most effective means of delivery to this line of enquiry. Similarly, and in spite of possible human disinclination of utilising the rectal route, Crowley \textit{et al} \textsuperscript{20} reported that IgA and IgG antibody levels were appreciably raised in women following rectal immunisation with an anti-influenza vaccine. Eriksson \textit{et al} \textsuperscript{36} investigated the local administration of antigen to the rectal mucosa in monkeys, resulting in higher ASC
responses than systemic or distal delivery, thus further suggesting that the rectum is an important target site for the induction of a significant immune response.

Kleanthous and co-workers have shown that rectal immunisation with the recombinant *Helicobacter* antigen urease can elicit high levels of anti-urease IgA in a mouse model of *H. pylori* infection.\(^{176}\) IgA was originally considered important for protective immunity against *H. pylori* infection.\(^{21, 90, 124}\) Recent studies however have produced evidence that whilst high titres of IgA are detectable in animals immunised with *Helicobacter*-antigen, in the absence of an adjuvant they are not protected from *H. pylori* challenge.\(^{159, 165}\) However, this study found that a significant level of prophylaxis against *H. pylori* was obtainable when the antigen was combined with the mucosal adjuvant heat-labile toxin of *Escherichia coli* (LT) and administered via the rectal route.

These results are important when reflecting upon some of the pitfalls encountered with oral administration of vaccines such as degradation and denaturation of antigens by enzymes and gastric fluids. Intra-rectal immunisation may possibly circumvent such factors and deliver immunogens intact to sites of specific immunity.\(^{71}\)
1.4 **THE HELICOBACTER PYLORI MOUSE MODEL**

In principle, the development of the *Helicobacter pylori* mouse model and the resultant immunological therapies derived from the initial and continuing research were, and still are, for the benefit of human medicine. However, as there are numerous mucosal associated pathogens, and with the continuing discovery of other *Helicobacter* organisms in a wide range of animal species, the utilisation of this model and the techniques developed within this study have potential within the animal husbandry industries.

1.4.1 **Helicobacter pylori**

Microbiologists in the later stages of the 19th century and early 20th century had observed spiral-like bacteria in the mammalian stomach, though very little attention was afforded them at the time.\textsuperscript{[3, 25, 73, 140]} For over a century, the assumption was that the hostile environment of the stomach was considered sterile despite these early observations.\textsuperscript{[85]} A revelation in microbiology occurred when Barry Marshall and Robin Warren first established the presence of a spiral bacterium isolated from the stomach of human patients with associated chronic gastritis in 1982.\textsuperscript{[100]} The organism is now known as *Helicobacter pylori*, and its implication with gastroduodenal disease has destroyed the dogma that stomach ulcers were the result of conditions such as stress and poor diet.\textsuperscript{[7]} *H. pylori* is now considered an important pathogen in humans, being a leading causative agent in chronic gastritis and contributing to the development of peptic ulcers and gastric cancer.\textsuperscript{[86]} Consequently, *H. pylori* has been designated a Group 1 carcinogen, as decreed by a working party of the World Health Organisation International Agency for Research on Cancer.\textsuperscript{[168]}

Naturally only infecting humans and some primates, *H. pylori* is a gram negative, spiral-shaped bacilli being $\approx 2.5 \mu m - 3.5 \mu m$ in length with a diameter of $\approx 0.5 \mu m - 1 \mu m$.\textsuperscript{[86, 144]} Possessing up to 5 sheathed flagella, the organism is microaerophilic and characterised by the production of large quantities of the enzyme urease, which converts urea to ammonia and carbon dioxide.\textsuperscript{[29, 48]}
a. Scanning electron micrograph depicting spiral morphology (original magnification x 35,000).
b. Transmission electron micrograph of negative stain preparation showing multiple polar flagella (original magnification x 15,000).
c. Phase contrast microscopy (original magnification x 1,000).

Plate 1.5 Helicobacter pylori
(Collage courtesy of Ms. Jani O’Rourke & Ms. Lucinda Thompson, UNSW)
1.4.2 Other Helicobacters’

A number of *Helicobacter* species have now been identified in the gastric mucosa of a diverse spread of mammals and are associated with gastritis.\(^6,^{30, 39, 41, 57, 88}\) Additionally, the ongoing discoveries of new *Helicobacter* organisms that colonise mucosal tissue other than the stomach are being connected with a range of conditions such as inflammatory bowel disease of sheep.\(^{147}\)

*Helicobacter bilis*, isolated from the liver of aged inbred mice is associated with chronic hepatitis and hepatomas,\(^{42}\) whilst *Helicobacter hepaticus*, which colonises the liver, colon and caecum is linked to multifocal necrotic hepatitis and colitis.\(^{10, 43, 81}\)

These animal models of experimental infection will be fundamental in contributing to the understanding of not only their associated pathogenesis but in the development of control strategies in animals as well as humans.

1.4.3 Antimicrobial Therapies

For many years gastroduodenal disease was treated by acid-suppressant therapy under the supposition that poor diet and stress were the prime contributing factors related to stomach ulcers. The discovery of *H. pylori,\(^{1001}\) and its association with peptic ulcers initiated the development of numerous antimicrobial treatments.\(^{105, 136, 156}\) All early treatments proved to be unsuccessful at permanently eradicating the infection until the combination of bismuth subcitrate, tetracycline and metronidazole was shown to prevent *H. pylori* infection and duodenal ulcer relapse.\(^{44, 49}\)

“Triple therapy” as it came to be known is the standard management for the infection, however, drug resistance strains of *H. pylori* particularly to metronidazole are beginning to emerge.\(^{69, 118}\) Furthermore, antibiotics do not protect against reinfection or indeed initial infection of the organism thereby necessitating the requirement for an alternative to antimicrobial therapies.
1.4.4 *Development of the Helicobacter Model in Mice*

Rodents, particularly mice and rats, make excellent research models due largely to cost viability, extensive knowledge of background data and ease of handling and husbandry. As an individual species, the laboratory mouse has been utilised in many instances as models for mucosal infection,[102, 120, 121, 126, 167] and indeed a mouse model of *Helicobacter* infection has been developed.[77, 89]

The early models of *Helicobacter* infection involved large animals. Although these models provided important data, they were cumbersome to work with and expensive to maintain. In 1990 a closely related *Helicobacter* organism, *H. felis* was found to readily colonise mice with colonisation being restricted to the gastric mucosa.[23, 87] At that time, the only species *H. pylori* colonised were gnotobiotic piglets and primates.[38, 79] Attempts to establish a valid *H. pylori* mouse model by numerous independent research groups around the world produced only partial success with wide variability and poor colonisation levels.[11, 33, 40]

A *Helicobacter* workshop held in Lausanne, Switzerland in 1995 discussed the necessity of a mouse model that fulfilled certain criteria.[111] The requirements were for a suitable animal model that would be able to successfully colonise and maintain colonisation regardless of the number of *in-vitro* passages. Furthermore the model should supply reliable data on the grading of colonisation and pathology. In that same year Marchetti *et al* [99] reported successful colonisation of mice by clinical isolates of *H. pylori*.

Two years later, in 1997, Lee *et al* [89] isolated a strain of *H. pylori* taken from a human biopsy, which had been cultured *in-vitro* then passaged, using the technique described by Marchetti *et al* [99], through various specific pathogen free mouse strains. The mouse strains BALB/c, DBA/2 and C3H/He were all successfully colonised with C57BL/6 producing the highest levels of 10^6-10^7 colony-forming units per gram of stomach. The isolate met the Lausanne criteria and gained the nomenclature of Sydney Strain 1 (SS1).
1.4.5 Current Immunisation Practices

Enduring for the life span of the host, *H. pylori* infection is not self-limiting despite the presence of a specific immune response against it, thereby prompting early investigators to suggest that immunisation against the organism would be ineffectual.\[^{14}\] However, in the final decade of the 20\textsuperscript{th} century, significant progress in *Helicobacter* research demonstrated the potential for future vaccine development. Using peroral immunisation in a mouse model of infection, several groups produced evidence proposing prophylactic and therapeutic immunisation strategies that fully protected against *Helicobacter* challenge or eradicated existing infection respectively.\[^{14, 21, 26, 109}\][135]

These early investigations of *Helicobacter* immunisation often used gastric urease assay or histological examination of prepared stomach sections to assess prophylaxis or therapeutic clearance.\[^{54, 76}\] Both forms of analysis are now known not to guarantee detection of low to moderate levels of infection, and gastric urease analysis fails to be quantitative in any conclusive manner.\[^{152}\] Sutton et al\[^{152}\] demonstrated that bacterial levels of $10^4$ organisms per gram of mouse stomach tissue were undetectable not only by gastric urease analysis but also by histology. A more sensitive analysis of *Helicobacter* colonisation assesses colony-forming units, whereby gastric bacterial load is ascertained from serial dilutions of homogenised stomach tissue and subsequent viable plate counts on a selective culture media. This latter technique has indicated that the current immunisation protocols for *Helicobacter* infection neither totally eradicate nor provide 100\% protection against the organism.\[^{152}\]

The initial immunisation studies, whilst encouraging, were limited in their findings with respect to determining the optimal strategies for of all components of vaccine administration, in other words, optimisation of the animal model. The concept of optimisation of immunisation needs to evaluate a number of variables such as route of administration, dose rates of antigen and adjuvant and time points of immunisation.

The latter variable, time points of immunisation, has been investigated by Sutton et al\[^{152}\]. The data indicated that two time points only, prime at day 0 and booster at day 21, produced a marked prophylactic improvement to that of immunising with the commonly used protocol of four time points at days 0, 7, 14 and 21. Three separate experiments using
different strains of mice for each (inbred C57BL/6, inbred BALB/c and outbred Quachenbush Swiss) revealed that the efficacy was consistent throughout. The authors suggested the possible explanation that when immunising at time points too close together, effector T cells are perpetually generated rather than allowing the full production of *Helicobacter*-specific memory T cells. Whilst effector T cells are responsible for the initiation of an immune response, the memory T cells intercept a later bacterial challenge.

Accurate dose rates must also be essential criteria for optimisation. Too little antigen may be ineffectual whilst overdosing may induce or exacerbate oral tolerance. The majority of current experimental immunisation programs against *H. pylori* are reliant upon the administration of antigen in combination with a mucosal adjuvant such as cholera toxin (CT) or heat-labile toxin of *Escherichia coli* (LT). The adjuvant properties of CT on gut mucosal immune responses in mice were investigated by Lycke *et al.*[^3] This study produced evidence that incremental dose rates of CT when administered perorally produced corresponding increments in antibody producing cells. A plateau was reached at 10 µg per dose. This dose rate has become the basis when incorporating CT as a mucosal adjuvant in the development of immunisation strategies against *Helicobacter*.

Whilst many researchers use 10µg of cholera toxin per mouse per dose as the standard for a mucosal adjuvant, the dose rate of antigen has been more arbitrary. Typically, antigen is either whole cell lysate of *H. pylori* or a purified single antigen.[^13, 26, 75, 76, 80, 90, 109, 110, 135, 139] Using a customary antigen dose rate of 1 mg of *Helicobacter* whole cell lysate, the *Helicobacter* vaccine research group at the University of New South Wales led by Professor Adrian Lee, explored the prophylactic potential of an incremental reduction of antigen dose rates.[^152] Marked prophylaxis was found in the group of animals administered with the lowest dose rate of 200 µg of lysate when delivered with CT. More significantly however, this dose rate produced a higher level of protection than in those mice immunised with the nominal 1 mg of antigen + 10 µg of CT. As an explanation for this result, the authors have discussed data from other studies suggesting that that low doses of antigen are responsible for a Th2-type immune response whereas higher doses induce the production of a Th1-type response. Because immunisation against *Helicobacter* possibly induces a Th2-type response, effective prophylaxis may be dose rate dependent.
As previously reported the administration of the vaccine for both priming and subsequent booster doses has typically been via the oral-gastric route. *H. pylori* colonisation is localised to the stomach, leading to much investigation being focused on peroral administration of vaccines. Indeed, early vaccination studies have suggested that parenteral routes of administration do not protect against challenge, whereas, peroral administration provides for a prophylactic response.\(^{13, 31, 47, 76}\) Furthermore, the route of delivery despite the target organ appears to have a major bearing on efficacy. To wit, Lee *et al.*\(^{90}\) demonstrated that oral immunisation was superior to direct intra-gastric immunisation even though the stomach was the specific site of delivery for both methods of administration. This may suggest that antigen uptake was occurring in the oro-buccal cavity via the presence of M-like cells in the minor salivary glands, and that the hostile environment of the stomach was potentially degrading the constituents and preventing efficient immunisation.

Finally, in a recent study by Saldinger *et al.*,\(^{1139}\) emphasis was placed on the lack of understanding of the effector mechanisms that prevent or cure *Helicobacter* infection. The likely scenario of future immunisation strategies discussed included the use of new mucosal adjuvants without gastrointestinal toxicity, combinations of two or three antigens used in synergy and the use of alternate routes of vaccine delivery such as intra-nasal and intra-rectal immunisation.
1.5 OVERALL OBJECTIVES

Because *H. pylori* colonises the gastric mucosa of the stomach in humans and experimentally infected animal models, it affords the opportunity to target various mucosal surfaces as suitable routes for immunisation investigation.\(^{86,89,90}\) The specific objectives and intended outcomes of this study were primarily to investigate the viability of utilising a number of mucosal routes of prophylactic immunisation against *H. pylori*. The actual development and application of such delivery methods was pivotal to the study with the intention that subsequent conclusions drawn would be discussed as potential precursors for future immunisation strategies against the organism as well as other *Helicobacter* species.

The initial studies were founded upon the development and optimisation of intra-intestinal immunisation, the focus being on ascertaining that direct injection of the Peyers’ patches of the ileum could provide important base line data for further examination of mucosal routes of administration. Such experimentation was underpinned by the technical refinement of surgical manipulation, associated anaesthesia, micro-injection of the site of specific immunity and tissue analysis.

The second phase of the study was to evaluate the efficacy of alternate routes of gastric, nasal and rectal immunisation by examination of the immune response and prophylaxis against bacterial challenge. Comparative studies between these routes were based upon the optimised route of direct intra-intestinal immunisation. A principle aim in this component of the work was to devise and evolve practical methodology in the development of vaccine administration techniques via mucosal-associated lymphoid tissue.

The practical aspects of the methodology was not solely confined to the development of delivery systems but also examined the means by which the study was initiated, conducted and the data obtained. Described in detail within the materials & methods section are the fundamental techniques of *in-vivo* and *in-vitro* investigation. Often overlooked in complete definition in many scientific papers, this study refined and concisely illustrated all components of the research, a factor fundamental to the concept of experimental reproduction.
1.5.1 Specific Aims

- To ascertain by intra-intestinal immunisation that the Peyers’ patches of the ileum are important inductive sites of the mucosal immune response and serve as a significant focal point for the further evaluation of immunisation strategies against *H. pylori*.

- To develop, refine and detail three fundamental techniques of vaccine administration, intra-gastric, intra-nasal and intra-rectal, in an animal model of scientific investigation.

- To determine whether alternate mucosal routes of vaccine delivery are efficacious in eliciting a significant immune response and/or prophylaxis against *H. pylori* challenge.
Chapter 2
Materials & Methods

2.1 INTRODUCTION

The laboratory methods used for this study and described within this chapter were performed not only for the experimental work contained herein, but also potentially for commercial purposes. All protocols and their execution conformed to the requirements of the code for Good Laboratory Practices (GLP). All brands, lot numbers and specific quantities were recorded in addition to rigorous compliance to standard operating procedures and manufacturers recommendations where applicable.

All methodologies and handling of materials were performed in a safe working environment and in compliance with the Australian & New Zealand Standards for Safety in Laboratories (AS/NZ S2243.1-10).

The basic maintenance of all laboratory animals was in accordance with the standard operating procedures of the School of Microbiology and Immunology Animal Facility, University of New South Wales, and conducted by appropriately qualified laboratory animal technicians. Animal husbandry and subsequent experimental procedures were appropriate to the guidelines as defined by the Code of Practice for the Use of Animals for Scientific Purposes and within the scope of the Animal Research Act. All animal experimental procedures performed herein were approved by the University of New South Wales’ Animal Care and Ethics Committee (approval number 99/116). Complete and concise records were maintained of all animal movements in addition to recording all aspects of health monitoring and experimental procedures.
2.2 **BUFFERS & SOLUTIONS**

Standard buffers and solutions were prepared for a diversity of purposes. All preparations were quality controlled for contamination by incubating a sample at 37° C for 48 hours. Assessment of sterility was made by light microscopy. Preparations were discarded if they failed to meet specifications or exceeded their prescribed shelf life and replaced with fresh stocks.

2.2.1 **0.1 M Phosphate Buffered Saline**

**Constituents:**

Sodium dihydrogen orthophosphate (NaH$_2$PO$_4$·2H$_2$O) \(4.37 \text{ g/L}\)

di-Sodium Hydrogen orthophosphate, anhydrous (Na$_2$HPO$_4$) \(10.22 \text{ g/L}\)

Sodium Chloride (NaCl) \(8.5 \text{ g/L}\)

**Formulation:**

Salts were weighed and dissolved in d.H$_2$O to \(\approx 90\%\) of total volume. The pH was adjusted to 7.2 and volume brought to specification in volumetric glassware. Solution was dispensed into appropriate glass containers and sterilised by autoclave at 121° C and 100 Kpa for 15 minutes. The sterilised aliquots were stored at room temperature.

2.2.2 **Physiological Saline**

**Constituents:**

Sodium Chloride (NaCl) \(8.5 \text{ g/L}\)

**Formulation:**

Sodium Chloride was weighed and dissolved in d.H$_2$O to \(\approx 90\%\) of total volume. The pH was adjusted to 7.0 and volume brought to specification in volumetric glassware. Solution was dispensed into appropriate glass containers and sterilised by autoclave at 121° C and 100 Kpa for 15 minutes. The sterilised aliquots were stored at room temperature.
2.2.3  

**10% Neutralised Buffered Formalin**

**Constituents:**

- Formaldehyde solution (40%)  
  100 mL/L
- Sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O)  
  4.37 g/L
- di-Sodium Hydrogen orthophosphate, anhydrous (Na₂HPO₄)  
  10.22 g/L

**Formulation:**

Salts were weighed and dissolved in d.H₂O to = 80% of total volume. In the confines of a fume hood, a precise aliquot of 40% formaldehyde solution was added and the pH was adjusted to 7.0. The volume was brought to specification in volumetric glassware. Solution was dispensed into an appropriately sealed glass vessel and stored at room temperature.

2.2.4  

**Urease Reagent**

**Constituents:**

- Sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O)  
  0.44 g/L
- di-Sodium Hydrogen orthophosphate, anhydrous (Na₂HPO₄)  
  1.02 g/L
- Urea (2%)  
  20.0 g/L
- Phenol Red (0.05%)  
  0.5 g/L
- Sodium Azide (NaN₃)(0.02%)  
  0.2 g/L

**Formulation:**

All salts were weighed and dissolved in sterile d.H₂O to = 90% of total volume. The urea, phenol red and sodium azide were separately weighed out and individually dissolved in the salt solution. The pH was adjusted to 5.7 and volume brought to specification in volumetric glassware. Solution was dispensed into a glass vessel and stored at room temperature. Immediately prior to use, the pH of the reagent was measured and adjusted accordingly.
2.2.5  *Brain Heart Infusion Broth*

**Constituents:**

Brain Heart Infusion (CM225, Oxoid, Bassingstoke UK) 37.0 g/L

**Formulation:**

Brain Heart Infusion (BHI) powder was weighed and dissolved in d.H₂O and adjusted to volume. The solution was dispensed into appropriate glass containers then sterilised by autoclave at 121° C and 100 Kpa for 15 minutes. Aliquots were stored at room temperature.

2.2.6  *Brain Heart Infusion Broth with Glycerol*

**Constituents:**

Brain Heart Infusion (Oxoid) 37.0 g/L

Glycerol 310.0 g/L

**Formulation:**

Brain Heart Infusion was prepared as previously described. An appropriate volume of glycerol was sterilised by autoclave at 121° C and 100 Kpa for 15 minutes. 90 mL of BHI and 10 mL of glycerol were aseptically transferred into sterile 100 mL glass bottles and stored at 4° C.
2.3 **CULTURE MEDIA**

Four types of culture medium were used for the *in-vitro* growth of *H. pylori*. The choice of media was reliant upon the purpose for which the bacteria were to be utilised. In the instance of the preparation of a selective media, the addition of specific antibiotic supplements, prepared in advance, was required. All prepared media was quality controlled and discarded if contaminated or failed to meet specifications. Media was discarded when the prescribed shelf life had been exceeded.

2.3.1 **Horse Blood Agar**

Horse Blood Agar (HBA) was used for the resuscitation of *H. pylori* from frozen stock suspensions. Due to the non-addition of antibiotic supplements, HBA was a reliable indicator for the early detection of contaminants from stock cultures.

**Constituents:**

- Blood Agar Base No.2 (Oxoid, Basingstoke, UK) 38.0 g/L
- Defibrinated Whole Horse Blood (Oxoid, Melbourne, Australia) 50.0 mL/L
- Amphotericin B (refer 2.3.6) 0.5 mL/L

**Formulation:**

Blood agar base powder was weighed and thoroughly mixed with distilled water and adjusted to volume. The solution was dispensed into appropriate glass vessels and sterilised by autoclave at 121° C and 100 Kpa for 15 minutes. The sterilised solution was allowed to slowly cool to 46° C in a thermostatically controlled water bath. Horse blood and Fungizone® were added aseptically to the media and gently mixed. Aliquots of ≈ 20 mL were poured aseptically into sterile plastic 90 mm Petrie dishes (Techno-plas, SA, Australia). The plates were allowed to cool for a minimum of 1 hour then packaged tightly in polyethylene wrap to retain moisture and stored “face up” at 4° C. HBA plates were used within 1 week of manufacture.
2.3.2  *Campylobacter Selective Agar*

Campylobacter Selective Agar (CSA) was used for the culture of *H. pylori* for the preparation of sonicates.

**Constituents:**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Agar Base No.2 (Oxoid)</td>
<td>38.0 g/L</td>
</tr>
<tr>
<td>Defibrinated Whole Horse Blood (Oxoid)</td>
<td>50.0 mL/L</td>
</tr>
<tr>
<td>Amphotericin B (refer 2.3.6)</td>
<td>0.5 mL/L</td>
</tr>
<tr>
<td>Skirrow’s Selective Supplement (refer 2.3.5)</td>
<td>2.0 mL/L</td>
</tr>
</tbody>
</table>

**Formulation:**

Blood agar base powder was weighed and thoroughly mixed with distilled water and adjusted to volume. The solution was dispensed into appropriate glass vessels and sterilised by autoclave at 121°C and 100 Kpa for 15 minutes. The sterilised solution was allowed to cool to 46°C in a thermostatically controlled water bath. Horse blood, Skirrow’s selective supplement and Fungizone® were added aseptically to the media and gently mixed. Aliquot’s of ≈ 20 mL were poured aseptically into sterile plastic 90mm Petrie dishes (Techno-plas, SA, Australia). The plates were allowed to set then packaged tightly in polyethylene wrap to retain moisture and stored “face up” at 4°C. CSA plates were used within 1 week of manufacture.

2.3.3  *Selective Brain Heart Infusion Broth*

Selective Brain Heart Infusion Broth was the medium for growth of *H. pylori* and was used for the *in-vivo* infection and bacterial challenge of mice.

**Constituents:**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Agar Base No.2 (Oxoid)</td>
<td>38.0 g/L</td>
</tr>
<tr>
<td>Horse Serum (Oxoid, Melbourne, Australia)</td>
<td>50.0 mL/L</td>
</tr>
<tr>
<td>Amphotericin B (refer 2.3.6)</td>
<td>0.5 mL/L</td>
</tr>
<tr>
<td>Skirrow’s Selective Supplement (refer 2.3.5)</td>
<td>2.0 mL/L</td>
</tr>
</tbody>
</table>
Formulation:
Brain heart infusion powder was weighed and thoroughly mixed with distilled water and adjusted to volume. 300 mL aliquot's of dissolved BHI were dispensed into 1 litre glass conical flasks and sealed with a non-absorbent cotton wool bung. The tops were covered with aluminium foil and secured by autoclave tape, then sterilised by autoclave at 121°C and 100 Kpa for 15 minutes. Prepared flasks (excluding serum and antibiotics) were stored at room temperature with a shelf life of four weeks maximum.

2.3.4 Glaxo Selective Supplement Agar

Glaxo Selective Supplement Agar (GSSA) is a highly selective media, and was used for the culture and recognition of colony forming units of *H. pylori* as an assay of in-vivo bacterial load.

Constituents:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Agar Base No.2 (Oxoid)</td>
<td>38.0 g/L</td>
</tr>
<tr>
<td>Defibrinated Whole Horse Blood (Oxoid)</td>
<td>50.0 mL/L</td>
</tr>
<tr>
<td>Amphotericin B (refer 2.3.6)</td>
<td>0.5 mL/L</td>
</tr>
<tr>
<td>Glaxo Selective Supplement A (refer 2.3.7)</td>
<td>1.5 mL/L</td>
</tr>
<tr>
<td>Glaxo Selective Supplement B (refer 2.3.7)</td>
<td>500 μL/L</td>
</tr>
</tbody>
</table>

Formulation:
Blood agar base powder was weighed and thoroughly mixed with distilled water and adjusted to volume. The solution was dispensed into appropriate glass vessels and sterilised by autoclave at 121°C and 100 Kpa for 15 minutes. The sterilised solution was allowed to cool to 46°C in a thermostatically controlled water bath. Horse blood, selective antibiotics and Fungizone® were added aseptically to the media and gently mixed. Aliquots of ≈ 20 mL were poured aseptically into sterile plastic 90 mm Petrie dishes (Techno-plas, SA, Australia). The plates were allowed to set then packaged tightly in polyethylene wrap to retain moisture and stored “face up” at 4°C. GSSA plates were used within 1 week of manufacture.
2.3.5 **Skirrow's Selective Supplement**

Antibiotic supplement required for the preparation of Campylobacter Selective Agar and Selective Brain Heart Infusion Broth.

**Constituents:**
Vancomycin Hydrochloride (Eli Lilly & Co, West Ryde, Australia) 5 mg/mL
Polymyxin B Sulfate (Sigma, St Louis, MO, USA) 0.16 mg/mL
Trimethoprim (Sigma) 2.5 mg/mL

**Formulation:**
Trimethoprim was dissolved in minimal 95% ethanol. Vancomycin and Polymyxin B were added and adjusted to volume in sterile distilled water. Desired aliquot’s of the solution were aseptically transferred into pre-sterilised glass vessels via a 0.22 μm micro-filter (Minisart®, Sartorius, VIC, Australia), and sealed with a screw cap. Prepared antibiotics were stored at −20º C for up to six months. Immediately prior to use, antibiotics were removed from cold storage and allowed to thaw at room temperature.

2.3.6 **Amphotericin B**

Fungicide supplement required for the preparation of Campylobacter Selective Agar, Glaxo Selective Supplement Agar and Selective Brain Heart Infusion Broth.

**Constituents:**
Amphotericin B (Fungizone®, Squibb, Princetown, NJ, USA) 5 g/L

**Formulation:**
Amphotericin B was dissolved in sterile distilled water and adjusted to volume. Desired aliquot’s of the solution were aseptically transferred into pre-sterilised glass vessels via a 0.22 μm micro-filter (Minisart®, Sartorius, VIC, Australia), and sealed with a screw cap. Prepared antibiotic was stored at −20º C for up to six months. Immediately prior to use, antibiotic was removed from cold storage and allowed to thaw at room temperature.
2.3.7 **Glaxo Selective Supplements**

Antibiotic supplements were required for the preparation of Glaxo Selective Supplement Agar. The antibiotics were prepared as two independent components (GSSA-A & GSSA-B) so as to avoid chemical interaction and precipitation. The two formulations were added separately upon manufacture of the media.

**GSSA-A**

**Constituents:**
- Vancomycin Hydrochloride (Eli Lilly & Co) 6.67 mg/mL
- Polymyxin B Sulfate (Sigma) 0.22 mg/mL
- Bacitracin (Sigma) 13.33 mg/mL

**Formulation:**
Vancomycin, Polymyxin B and Bacitracin and were dissolved in sterile distilled water and adjusted to volume. Desired aliquot’s of the solution were aseptically transferred into pre-sterilised glass vessels via a 0.22 μm micro-filter (Sartorius), and sealed with a screw cap. Prepared antibiotics were stored at −20°C for up to six months. Immediately prior to use, antibiotics were removed from cold storage and allowed to thaw at room temperature.

**GSSA-B**

**Constituents:**
- Nalidixic Acid (Sigma) 2.14 mg/mL

**Formulation:**
Nalidixic Acid was dissolved in sterile distilled water and adjusted to volume. Desired aliquot’s of the solution were aseptically transferred into pre-sterilised glass vessels via a 0.22 μm micro-filter (Sartorius), and sealed with a screw cap. Prepared antibiotic was stored at −20°C for up to six months. Immediately prior to use, antibiotic was removed from cold storage and allowed to thaw at room temperature.
2.4 **BACTERIOLOGICAL CULTURE**

*Helicobacter pylori* is notable for being a fastidious organism when cultured *in vitro*. Rigorous conformity to media preparation protocol and attention to detail was essential for the maintenance of a contamination free viable culture. The strain of *H. pylori* used was the mouse adapted ‘Sydney Strain 1’ (SS1) and obtained from Ms Jani O’Rourke (School of Microbiology & Immunology, University of New South Wales).

2.4.1 **Bacterial Maintenance**

Stock *H. pylori* was maintained by harvesting fresh cultures from CSA plates with BHI plus glycerol. The suspensions at a bacterial count of \( \approx 10^8 \) organisms per mL were aliquoted into 1 mL Nunc Cryo Tubes™ (Nunc, Roskilde, Denmark) and stored at \(-80^\circ C\).

2.4.2 **Resuscitation of Bacteria**

*H. pylori* was resuscitated by removal of stock from deep freeze and allowing to fully thaw at \(4^\circ C\). When completely liquefied, approximately 200 \( \mu \)L of suspension was aseptically dispensed onto a HBA plate and distributed over the entire surface of the media using a sterilised glass spreader. Plates were incubated “face up” at \(37^\circ C\) for 48 hours in a Steri-Cult 200 incubator (Selby Scientific Ltd, NSW, Australia) with 10% CO\(_2\) and 95% relative humidity.

*Figure 2.1 Bacterial Spreader*
2.4.3 Solid Culture

Following resuscitation of *H. pylori* on HBA plates, the bacteria were sub-cultured onto CSA plates. A platinum loop was sterilised by flaming through a Bunsen burner. A sample of bacteria was picked up from the original culture by the sterilised loop and streaked upon a fresh plate. Plates were incubated “face up” at 37°C for 48 hours in a Steri-Cult 200 incubator (Selby Scientific Ltd, NSW, Australia) with 10% CO₂ and 95% relative humidity.

![Figure 2.2 Bacterial Loop](image)

2.4.4 Liquid Culture

Sterile 1 litre conical flasks containing 300 mL of BHI and sealed with a non-absorbent cotton wool plug were used for liquid culture of *H. pylori*. The organisms were harvested from CSA plates with BHI that had been cultured for 48 hours as previously described. The bacterial suspension from two plates was aseptically transferred to a flask with a sterile Pasteur pipette. Skirrow antibiotic supplement (600 μL) and Amphotericin B (150 μL) were added aseptically and the flask loosely resealed with the plug. Each prepared flask was placed into an anaerobe jar with an anaerobic gas generating kit (Anaerobic System BR38, Oxoid) and incubated at 37°C for 48 hours with continuous gentle shaking.

Following the incubation period, the culture was examined for contamination by phase contrast light microscopy and dispensed into sterile 50 ml centrifuge tubes (Sarstedt, Germany). Suspensions were centrifuged at 2500 RPM for 10 minutes and supernatant removed. The pellet was re-suspended in minimal BHI then re-examined.
Plate 2.1 Pictorial Representation of Bacterial Culture

a. Resuscitation of bacteria on solid media
b. Bacterial culture on solid media
c. Bacterial culture in liquid media
2.5  

**PREPARATION OF BACTERIA FOR INFECTION**

Live *H. pylori* was prepared from liquid culture as previously described for the infection of naïve mice and the bacterial challenge of immunised mice. Liquid culture was the medium of choice, providing greater bacterial motility than that obtained from solid cultures. The bacterial count was adjusted to approximately $10^8$ viable organisms per millilitre, thereby providing a dose rate of $10^7$ organisms per mouse in a 100 μl suspension. In all instances *H. pylori* was administered directly to the stomach of recipient mice via oro-gastric gavage.

2.5.1  

**Quantification of Bacterial Suspension**

Following preparation of live *H. pylori* from liquid culture, the bacterial count was quantified by haemocytometer under light microscopy (400x). Either diluting with BHI or centrifugation to concentrate adjusted the suspension accordingly.

Bacterial numbers of the final suspension were confirmed by retrospective plate counts of colony forming units. The sample was diluted 1:10$^1$, 1:10$^2$, 1:10$^3$, 1:10$^4$, 1:10$^5$, 1:10$^6$, 1:10$^7$, 1:10$^8$, 1:10$^9$ and 1:10$^{10}$ by successively dispensing 100 μl of each diluted suspension into a sterilised test tube containing 900 μl of BHI.

A 100 μl sample of each serial dilution was aseptically transferred onto GSSA plates and evenly distributed across the entire surface with a sterilised glass spreader. Plates were incubated “face up” at 37°C for 5 days in a Steri-Cult 200 incubator (Selby Scientific Ltd, NSW, Australia) with 10% CO$_2$ and 95% relative humidity. Colony forming units were read following the incubation period and specific bacterial load ascertained and recorded.
2.6 **ANTIGEN PREPARATION FOR IMMUNISATION**

The antigen used to immunise mice for all of the experimental work of this study was whole cell lysate of *H. pylori*. The lysate was derived from solid culture medium and produced in-house by the proceeding protocols.

2.6.1 **Bacterial Preparation**

*H. pylori* was cultured from stock supply onto HBA plates and incubated for 48 hours as previously described. The culture was assessed to be free of contaminates by phase contrast microscopy (oil immersion, 1000x) and subcultured onto CSA plates. Following a further 48 hour incubation period, the culture was harvested aseptically with minimal 0.1 M phosphate buffered saline (PBS) and placed into sterile 10 mL centrifuge tubes. Suspensions were centrifuged at 9000 RPM for 10 minutes and supernatant removed. The pellet was re-suspended in minimal 0.1 M PBS and re-centrifuged as above to remove all traces of residual media. The suspensions were stored at −80°C until required for sonication.

2.6.2 **Whole Cell Lysate Preparation**

Whole bacterial cell lysate of *H. pylori* was prepared using a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT). Bacterial suspensions were sonicated at a rate of 1 minute/mL with a duty cycle of 50% (0.5 pulses/second). A power output of 60 watts was initiated and sequentially reduced in the event of frothing. To avoid potential protein degradation from excessive heating, suspensions were kept on ice whilst sonicating. Phase contrast light microscopy (oil immersion, 1000x) was used to ensure no intact organisms remained.
2.6.3 **Protein Estimation**

Protein estimations for the bacterial sonicates were made using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Absorbancess were read using a Bio-Rad Microplate Reader and protein estimations calculated with the Bio-Rad Microplate Manager® program (version 2.2 for Macintosh). Bovine Serum Albumin (BSA; Sigma) was used as the standard and 0.1 M phosphate buffered saline (0.1 M PBS) as a blank.

BSA was diluted in 0.1 M PBS from 0.2 mg/mL to 1.2 mg/mL in increments of 0.2 mg. The whole cell sonicate was diluted 1:50, 1:100, 1:200 in 0.1 M PBS. The diluted samples were placed in duplicate into 96 well, flat bottom, microtitre plates (Linbro®/Titertek®, ICN Biomedicals, NSW, Australia). Bio-Rad reagent A (25 μL) and Bio-Rad reagent B (200 μL) were added to each well and mixed by gentle tapping. The solutions were allowed to incubate for 15 minutes at room temperature. Absorbancess were read at 655 nm and protein estimations calculated. Sample dilutions, which best fit the standard curve were used to estimate the quantity of protein contained in the sample.

The sonicate, otherwise called lysate, was diluted in 0.1 M PBS to a protein concentration of 1mg/mL and dispensed as 1 mL aliquot's into sterile 2 mL Nunc Cryo Tubes™ (Nunc, Roskilde, Denmark). Lysates were stored at –80°C until required for use.
2.7 **ANIMALS**

All *in-vivo* work in this study used the *H. pylori* mouse model as developed by the School of Microbiology & Immunology, UNSW.\(^{89}\) Original source of supply of mice was from the Biological Resource Centre, Little Bay, NSW.

2.7.1 **Specific Pathogen Free Status**

All mice sourced and used were advised by the supplier to be free from the pathogens described in *Table 2.1*.

*Table 2.1 Specific Pathogen Free Status of Animal Model*

<table>
<thead>
<tr>
<th>VIRAL</th>
<th>BACTERIAL</th>
<th>PARASITIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Minute Virus of Mice</em></td>
<td><em>Corynebacterium kutscheri</em></td>
<td><em>Nematodes</em></td>
</tr>
<tr>
<td><em>Mouse Hepatitis Virus</em></td>
<td><em>Pasteurella pneumotropica</em></td>
<td><em>Intestinal Protozoa</em></td>
</tr>
<tr>
<td><em>Pneumonia Virus of Mice</em></td>
<td><em>Pasteurella multocida</em></td>
<td><em>Cestodes</em></td>
</tr>
<tr>
<td><em>Reovirus – 3</em></td>
<td><em>Streptococcus pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td><em>Lymphocytic Choriomenigitis</em></td>
<td><em>Streptococcus moniliformis</em></td>
<td></td>
</tr>
<tr>
<td><em>Theiler’s Encephalomyelitis</em></td>
<td><em>Staphylococcus aureus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Mycoplasma pulmonis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>CAR bacillus</em></td>
<td></td>
</tr>
</tbody>
</table>

Additional to these organisms, all animals were verified free of detectable *H. pylori* and other gastric *Helicobacters' from assessment by viable plate counts of colony forming units. However, freedom from other lower bowel *Helicobacter* species was not tested.
2.7.2  Housing & Husbandry

Mice were housed under barrier conditions in the School of Microbiology & Immunology, University of New South Wales, Animal Facility, located at Kensington campus. Animal maintenance was performed by qualified animal technicians and in accordance with in-house standard operating procedures. All materials and equipment required for daily routine husbandry was sterilised by autoclave and assessed for freedom of contamination prior to use. Environmental monitoring was conducted routinely for the early detection of any compromise to the barrier status.

All mice were housed separately from any other species and maintained in a controlled environment appropriate to species specific requirements as defined in Table 2.2.

<table>
<thead>
<tr>
<th>Table 2.2 Environmental Parameters for Maintenance of Mouse Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Temperature 21 – 23°C</td>
</tr>
<tr>
<td>• Relative Humidity 55 – 65%</td>
</tr>
<tr>
<td>• Air Supply High Efficiency Particulate Air</td>
</tr>
<tr>
<td>• Air exchange 18 per hour</td>
</tr>
<tr>
<td>• Light Source Tubular fluorescent (350 – 400 lux)</td>
</tr>
<tr>
<td>• Photo Period 12 hours light : 12 hours dark</td>
</tr>
<tr>
<td>• Noise Levels Background &lt; 50 dBA</td>
</tr>
</tbody>
</table>

Animals were maintained in polypropylene “shoe-box” style cages fitted with flat-top stainless steel lids with recessed feed hopper and water bottle cradle attached. Stocking density did not exceed more than ten animals per cage. Direct bedding of sterilised extruded paper pellet (Fibre Cycle, QLD.) was used in all cages at a quantity sufficient to absorb all bodily excretions and potential water bottle spills. Environmental enrichment in the form of sterilised cardboard rolls and wooden blocks was supplied to all cages.

A controlled diet of autoclavable rat and mouse ration (Gordons Specialty Stockfeeds, Yanderra, NSW) in pelleted form was supplied ad-libitum in feed hopper. Glass bottles with stainless steel sipper tubes provisioned for water (sterilised by autoclave) and was also provided ad-libitum.
Glass 600 mL water bottle

Rubber bung

One-way sipper tube

Feed hopper

Stainless steel wire mesh lid

Polypropylene rodent box

150mm

250mm

400mm

Plate 2.2 Pictorial Representation of Mouse Cage
2.8 FUNDAMENTAL ANIMAL TECHNIQUES

Throughout the course of this research, a variety of fundamental animal techniques were used which are described in this chapter. Procedures of an advanced technical nature, such as the surgical manipulation of intra-intestinal immunisation and the methodologies of alternative vaccine administration are detailed in the relevant chapters.

2.8.1 Physical Restraint

For the majority of experimental procedures performed upon the animals, such as intra-gastric gavage, intra-nasal and intra-rectal immunisations, there existed a requirement to restrain the mouse by physical means. Restraint needed to simply and effectively immobilise the subject whilst not causing any undue stress or physical harm.

Basic physical restraint was achieved by capturing animal at base of tail then grasping the scruff at base of neck firmly between thumb and forefinger (Figure 2.3a). The body of the mouse was then immobilised by restraining the tail between palm and little finger (Figure 2.3b). From this position a variety of moderately invasive procedures could be performed without the necessity to use chemical restraint, that is anaesthesia or sedation.
2.8.2 Animal Identification

Monitoring individual experimental animals over the course of the investigation required an adequate identification system. A permanent method of identification was necessary, as up to ten mice were housed per cage. Ear notching was the technique of choice, being relatively simple to perform, enduring and cost effective.

Each mouse was physically restrained as previously described and the selected ear swabbed with 70% ethanol. The notch was made with an ear punch (Becton Dickinson, USA) on the specified ear at a predetermined location corresponding to the numerical designation.

![Diagram of ear notches for identification]

Figure 2.4 Mouse Identification Ear Notch Code
2.8.3 Intra-Gastric Gavage

Oral administration of a substance, whether that is a drug, infective agent or experimental feeding, is an often-performed procedure on small laboratory animals. Where there exists a definite requirement for such a substance to be delivered directly to the stomach, intra-gastric gavage is commonly practiced. Bacterial infection of mice with *H. pylori* was achieved by direct administration of a bolus into the mouse stomach via intra-gastric gavage. This technique involved the delivery of specified aliquot by the insertion of a gavage into the buccal cavity, down the oesophagus and into the stomach.

Individual mice were physically restrained by hand and immobilised by application of a firm grip about the scuff and tail. The gavage was inserted into the space between the left incisors and molars and guided in a caudal direction to the right ramus of the mandible. Passage was generally facilitated by the onset of a swallowing reflex as the gavage approached the pharynx allowing progression into the oesophagus. The neck of the mouse was gently extended to provide a straight line between the oesophageal orifice and the cardiac sphincter. The gavage was inserted down the oesophagus into the stomach and specific aliquot delivered.

*Figure 2.5 Restraint for Intra-Gastric Gavage*
2.8.4  Surgical Anaesthesia

Surgical anaesthesia of mice was achieved by the combination of the dissociative anaesthetic Ketamine (Parnell Laboratories, NSW), the sedative/analgesic Xylazine (Parnell Laboratories, NSW) and sterile distilled H₂O. This anaesthetic cocktail when administered via the intra-peritoneal (I/P) route produced a sleep time of approximately sixty minutes.

The correct volume of drug to be administered for the corresponding prescribed dose rate was calculated by:

\[ \text{Administration volume} = \frac{\text{dose rate} \times \text{body weight}}{\text{concentration}} \]

Where:
- Prescribed dose rate of 50 mg/kg of Ketamine at a concentration of 100 mg/mL.
- Prescribed dose rate of 50 mg/kg of Xylazine at a concentration of 20 mg/mL.
- Distilled H₂O added at ratio of x2 volume of combined anaesthetic agents.

Calculated volumes of both anaesthetic agents and d.H₂O were aseptically dispensed into an appropriate sterile glass vessel. The addition of d.H₂O allowed for a manageable volume for administration thereby limiting the possibility of accidental overdose. Short-term storage (maximum of 2 days) of the anaesthetic was at 4°C until required.

The subject was physically restrained and inclined cranially downwards so as to allow the abdominal mass to drop forward thus avoiding penetration of the viscera. The site of injection was swabbed with 70% ethanol (Figure 2.6a). A 27G needle attached to a 1 mL tuberculin syringe was inserted at an angle approximately 30° from the horizontal through the skin medial to the right flank and cranial to the inguinal canal. Avoiding mammary glands, the needle was advanced through the abdominal muscle and the syringe aspirated prior to administration of anaesthetic (Figure 2.6b). Aspiration of the syringe allowed for visual indication of penetration of the viscera and was to be avoided.
When inserting the needle, caution was required to avoid shallow injection, as Ketamine may cause tissue necrosis if administered subcutaneously. Similarly, deep injection was avoided so as to prevent retro-peritoneal or intra-renal administration.

For all procedures where anaesthesia was used, mice were maintained on a thermostatically controlled heating pad to prevent hypothermia and food and water withheld until fully recovered.
2.8.5 **Blood & Serum Collection**

A number of anatomical sites may be used for collection of blood from mice. Cardiac puncture, performed under general anaesthesia, was the method of choice for all experimental work in this study.

Serial blood sampling, with no detriment to the animal, was accomplished when the sample size of each collection was limited to a volume ≤ 6% of body weight and time points restricted to no more than once every two weeks. Terminal blood sampling was also performed whereby the maximum obtainable volume of blood was collected. In this instance, exsanguination of the subject was followed by precautionary cervical dislocation so as to ensure death.

Subject mice were anaesthetised to a surgical plane as previously described and placed in dorsal recumbency on a thermostatically heated pad. The upper abdominal and lower thoracic region was swabbed liberally with 70% ethanol. A 27G needle attached to a tuberculin syringe was elevated approximately 15° from the horizontal axis of the sternum and inserted beneath the xyphoid cartilage slightly left of midline. The needle was aspirated and slowly moved cranially until blood was visually indicated in the barrel of the syringe. The plunger was gently withdrawn allowing blood to flow into the syringe by virtue of cardiac pressure alone.

Blood was transferred from the syringe to collection tubes (Microvette® CB 1000 S tubes, Sarstedt, Germany) and stored at 4°C for a minimum of 1 hour and no more than 24 hours. Blood samples were then centrifuged at 10000 RPM for approximately 10 seconds and the supernatant (serum) removed. The serum was aseptically transferred to 1.5 mL micro-centrifuge tubes (Greiner, Labortechnik, Germany) and stored at −20°C until required for analysis.
Plate 2.3 Blood Collection Flow Diagram

- **a.** Anaesthesia by I/P injection
- **b.** Dorsal recumbancy
- **c.** Cardiac puncture
- **d.** Blood transfer
2.8.6 Stomach Collection

Mice were euthanased and the abdominal cavity opened by incising with sharp/sharp iris scissors. The stomach was removed by severing at the pyloric and cardiac sphincters. The non-glandular region was removed in addition to any attached mesentery. The remaining portion consisting of the antrum and body was opened along the lesser curvature. Residual ingesta was removed by gently scraping with the reverse side of a scalpel blade. The stomach sample was washed in physiological saline and blotted dry on absorbent paper towelling. Bisecting along the greater curvature resulted in two identical halves, one of which was placed into fixative for histological purposes, the remaining half placed into a sterilised aliquot of 0.85% physiological saline and utilised for the culture of viable plate counts.

Figure 2.7 Pictorial Representation of Stomach Depicting Regions
a. Euthanasia of subject and abdominal cavity opened
b. Stomach exteriorised (magnification x 2)
c. Stomach removed (magnification x 8)
d. Stomach opened along lesser curvature. Non-squamous epithelium removed. Ingesta removed (magnification x 8)

Plate 2.4 Stomach Collection Flow Diagram
2.8.7 Euthanasia

The humane killing of mice, euthanasia, was performed for two reasons. In the first instance it was necessary to euthanase animals at the conclusion or end point of each experiment for tissue collection and analysis. Secondly, any animal displaying signs of abnormal behaviour, ill health or any other condition to the detriment of the wellbeing of the animal was euthanased as a requirement of the Animal Care & Ethics Committee approval. In either instance for this work, euthanasia of any animal was always performed in the isolation of all other animals.

Asphyxiation by inhalation of carbon dioxide was used for euthanasia of animals that exhibited signs of ill health. A lethal chamber was partially filled (≈ 50%) with CO₂. The animal was placed into the chamber, the lid sealed and additional CO₂ added to 100% capacity. The animal remained in the chamber for a minimum of 5 minutes. Animals to be terminated for tissue collection as a requirement of the experimental procedure were initially surgically anaesthetised. Maximum blood was collected for every harvest and death typically resulted from exsanguination. To ensure death, all animals were submitted to cervical dislocated at completion of tissue harvest. In this instance the mouse was restrained on a flat surface and the base of the tail was grasped between the thumb and forefinger. A solid flat object such as a scalpel blade handle held in reverse was positioned at the base of the skull. The scalpel handle was pushed forward whilst the hand holding the tail was pulled backward. When the spinal cord was severed, a 2-4 mm space was palpable between the occipital condyles and the first cervical vertebra.

![Figure 2.8 Cervical Dislocation](image-url)
2.9 DETECTION OF HELICOBACTER PYLORI INFECTION

The detection of *H. pylori* infection in mice was assessed by two independent methodologies. A rapid test for detection of *Helicobacter* infection was based on a gastric biopsy urease assay developed by Hazell *et al.*\textsuperscript{[54]} and was used as an initial indication of efficacy of immunisation. A quantitative analysis of *Helicobacter* gastric bacterial colonisation was assessed from viable plate counts of serial dilutions taken from homogenised stomach tissue. This latter assay, being the most sensitive of the two detection methods, was used for all statistical analysis of colonisation levels.

2.9.1 Gastric Urease Assay

Urease reagent was added to flat-bottomed micro-titre plates (Linbro\textsuperscript{®} / Titerpak\textsuperscript{®}, ICN Biomedicals) at 150 μL per well and sealed with clear adhesive tape. The stomach of a euthanased mouse was removed and sectioned as previously described and a portion of antrum (= 5mm ∅) placed into the reagent of an individual well then re-sealed with the tape and allowed to incubate at room temperature.

Alternatively, a 100 μL sample was taken from the initial suspension of the homogenised stomach tissue (as described in section 2.9.2 Viable Plate Counts) and aseptically transferred into flat-bottomed micro-titre plates to which 100 μL of urease reagent was added. The wells were sealed with tape and incubated at room temperature as above.

In either method, the procedure was performed for each individual animal and reagent examined by ‘eye’ for colour change at 1 hour and 24 hours post harvest. The initial reagent colour was yellow, changing to deep red when high levels of *Helicobacter* were present. Scores from 0-3 were recorded for each animal corresponding to the colour change. A score of 0 was appointed to no colour change whilst a score of 1 and 2 indicated a slight and moderate change respectively with a complete colour change scoring 3. A score of 2 or 3 was indicative of *Helicobacter* infection, whilst a score of 0 or 1 corresponded to reduced or possibly zero infection.
2.9.2 Viable Plate Counts

The bacterial load of *H. pylori* infected mice was ascertained by harvesting the stomach from each euthanased mouse (as previously described), homogenising the tissue sample and culturing on a selective media from subsequent serial dilutions.

The saline suspended stomach was thoroughly homogenised using an Ultra Turrax (Janke & Kunkel. IKA, Laboratechnik). Preceding the processing of each individual sample the rotor of the Ultra Turrax was rinsed with sterile distilled water, immersed in 95% ethanol, flamed over a Bunsen burner and rinsed again in dH₂O. Samples were maintained on ice prior to plating. Using a Gilson P1000 calibrated automatic pipette (Gilson Medical Electronics, France), each sample was diluted 1:10¹, 1:10², 1:10³ and 1:10⁴ by successively dispensing 200 μL of each diluted suspension into a sterilised test tube containing 1800 μL of physiological saline. A 200 μL sample of original suspension and each serial dilution were aseptically transferred onto GSSA plates. The deposit was evenly distributed across the entire surface with a glass spreader previously sterilised by immersing in 95% ethanol and flaming over a Bunsen burner.

Plates were incubated “face up” at 37° C for 5 days in a Steri-Cult 200 incubator (Selby Scientific Ltd, NSW, Australia) with 10% CO₂ and 95% relative humidity. Following incubation period, viable organisms were read by counting single colony forming units (CFU’s) from the dilution with a bacterial count between 30 and 300. Colonies typically appeared symmetrical and approximately 0.5-1 mm in diameter. Where there was reason to suspect that the incubation period was insufficient to produce readable colony forming units, plates were returned to the incubator for a further 24-48 hours or until such time that satisfactory growth had occurred. To confirm that colonies were *Helicobacter* species, a small strip of blotting paper (approximately 5 mm x 30 mm) was dipped in urease reagent then gently pressed against a colony. A small red dot was presented if *Helicobacter* positive. The number of bacteria per gram of stomach was calculated as follows:

\[
CFU = \frac{\text{Number of colonies} \times 10^{\text{dilution factor}}}{\text{Weight of stomach in grams}}
\]

The bacterial load was thus calculated and recorded for each individual animal.
Plate 2.5 Viable Plate Counts
2.10 **ENZYME-LINKED IMMUNOSORBENT ASSAY**

Enzyme-Linked Immunosorbent assays (ELISA) were used to detect anti-*H. pylori* IgG antibody titres in the sera of subject animals. The assays were based on the methodology developed by Mitchell *et al.*\textsuperscript{112} initially designed for the detection of anti-*H. pylori* IgG antibody in human serum.

2.10.1 **ELISA Blocking Buffer**

**Constituents:**

0.1M Phosphate Buffered Saline (pH 7.2)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Azide (Na$_N_3$)</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>Gelatin</td>
<td>1 g/L</td>
</tr>
</tbody>
</table>

**Formulation:**

Sodium azide and gelatin were weighed and suspended in PBS. Solution was gently heated to dissolve then dispensed into glass vessels and stored at room temperature.

2.10.2 **ELISA Wash Buffer**

**Constituents:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>85.0 g/L</td>
</tr>
<tr>
<td>Sodium Azide (Na$_N_3$)</td>
<td>2.0 g/L</td>
</tr>
<tr>
<td>Tween 20 0.5%</td>
<td>5.0 mL/L</td>
</tr>
</tbody>
</table>

**Formulation:**

Sodium chloride and sodium azide were weighed and dissolved in specified volume of sterile d$_{2}H_2$O. Specified volume of Tween 20 was added and solution well mixed. Wash Buffer was stored at room temperature and diluted 1:10 in sterile d$_{2}H_2$O when required.
2.10.3 **Diethanolamine Buffer**

**Constituents:**
- Diethanolamine \(96 \text{ mL/L}\)
- Magnesium chloride (MgCl\(_2\)-6H\(_2\)O) \(50.0 \text{ mg/L}\)
- Distilled H\(_2\)O

**Formulation:**
Diethanolamine Buffer (DEA) was dissolved in approximately 80% of total volume of distilled water. Magnesium chloride was weighed out and added to the solution. The pH was adjusted to 9.8 and distilled water added to the required level. The solution was dispensed into an appropriate glass bottle that was totally wrapped in aluminium foil to exclude light. The prepared DEA buffer was stored in the dark at 4\(^\circ\) C and brought to room temperature prior to use.

2.10.4 **Detection of Anti-*H. pylori* IgG Antibody**

Ninety-six well flat-bottomed micro-titre plates (Linbro\(^\circledR\) / Titertek\(^\circledR\), ICN Biomedicals) were coated with 100 \(\mu\)L of *H. pylori* sonicate at 100 \(\mu\)g/mL and sealed with cling wrap. Thus coated, the plates were stored at 4\(^\circ\)C for a minimum of 48 hours and maximum of 30 days prior to use.

When required, the plates were removed from cold storage and allowed to reach room temperature, nominally = 22\(^\circ\)C, then washed 3 times with 150 \(\mu\)L of ELISA wash buffer. 200 \(\mu\)L of ELISA blocking buffer was added to each well and incubated for 2 hours at room temperature.

Following blocking, the plates were washed once with 150 \(\mu\)L of wash buffer and 100 \(\mu\)L aliquots of the standard and samples diluted in blocking buffer were added to each well in duplicate. Standard positive sera taken from mice immunised with *H. pylori* whole cell lysate + cholera toxin was diluted 1/10 in blocking buffer.
The plates were incubated for 1 hour at room temperature then washed three times in wash buffer to remove unbound antibody. 100 µL of alkaline-phosphatase labelled anti-mouse IgG diluted 1/2000 with blocking buffer was added to each well and incubated for 1 hour at room temperature.

The plates were then washed three times in wash buffer and three times in distilled H₂O then incubated in the dark for approximately thirty minutes at 30° C. Following incubation a 100 µL aliquot of substrate was added to all wells. The substrate contained DEA plus one disodium p-nitrophenyl phosphate tablet for every 8.3 mL of solution (Sigma, 104® phosphate substrate tablets).

The reaction was stopped by adding 100 µL of 3M NaOH to each well, and absorbances read at 405 nm using a microplate reader (Model 3550, Bio-Rad). *H. pylori* IgG antibody levels were standardised by expressing the sample absorbance as a ratio of the positive control. All samples which fell below the absorbency score of the negative control were recorded as a zero value.
2.11  STATISTICAL ANALYSIS

Tables of animal body weights and graphs of bacterial colonisation were depicted as arithmetical means of the sample group. One standard deviation (STDEV) was calculated independently for each group by the following formula:

\[
\text{STDEV} = \sqrt{\frac{n \sum x^2 - (\sum x)^2}{n (n-1)}}
\]

The significant difference in animal body weights between control and manipulated animals was compared using the Chi-square \((x^2)\) test.

Comparisons of infection levels as determined by colony forming units and serum IgG responses as determined by ELISA were performed using the non-parametric ANOVA Tukey-HSD multiple range test, using the Statistical Package for the Social Sciences (SPSS) computer software. Significance was accepted at 95\% (significance level 0.05)
2.12 PRESENTATION OF FIGURES

Unless otherwise stated and acknowledged, all plates and figures presented herein were the original product of the author.

Original line drawings were hand-drawn by the author. Schematic representations were created using Adobe Photoshop™ (version 5.0, Adobe Systems Inc). Original photographs for plates were taken by the author using a Pentax® K1000 camera.

All images were produced using a Color One scanner (Macintosh) connected to a Power Macintosh (model 7100/800av) and scanned in by Ofoto® (version 2, Light Source Computer Images Inc). The scanned images were imported to Adobe Photoshop™ in order to make alterations to the background, reproduce and sharpen.
Chapter 3
Intra-Intestinal Immunisation

3.1 INTRODUCTION

Many independent studies in a mouse model of Helicobacter infection have typically used peroral immunisation to investigate and demonstrate the possibility for a successful vaccine against the human pathogen H. pylori.\textsuperscript{14, 21, 26, 47, 90, 109, 124, 135, 150, 152} However, peroral administration of vaccines using non-replicating antigens may see degradation in the gut, a reduction of the immunogenic properties of soluble proteins, and characteristically poor immune responses with short duration.\textsuperscript{16, 116, 122} Therefore an alternative method of administration is necessary to initially test the potential of an oral vaccine without the inherent complications associated with oral delivery systems. Any such technique would be required to deliver immunogens intact and with precision to sites responsible for the initiation of an immune response.

As has already been mentioned, the principal site for antigen uptake within the gastrointestinal tract following enteric immunisation is known to be via the Peyers’ patches of the ileum,\textsuperscript{45, 161} and recent studies have shown that intra-intestinal immunisation is more efficacious in enhancing an immune response compared to that of oral immunisation.\textsuperscript{28, 116} The administration of immunogens by the direct injection of the Peyers’ patches has been used by Dunkley \textit{et al.}\textsuperscript{27} to evaluate the vaccine potential of the citrate synthase homologue protein in a mouse model of \textit{H. pylori} infection. However, whilst providing further evidence that intra-intestinal immunisation may be an important experimental technique for the preliminary screening of \textit{H. pylori} vaccines, the study was inconclusive in evaluating the impact of procedure upon scientific data and animal welfare.
The data contained within this chapter aims in the first instance to provide proof of principle that the direct injection of the Peyers’ patches with a vaccine consisting of whole cell lysate of *H. pylori* and cholera toxin can stimulate a significant protective immune response against subsequent *H. pylori* challenge. Furthermore, by physically bypassing the mechanisms associated with M cells, antigen uptake by the Peyers’ patches, and hence prophylaxis, may possibly be accomplished without the requirement for a mucosal adjuvant. Most importantly however, any prophylactic effect either with or without the inclusion of an adjuvant was to be solely attributable to the vaccine and not in any part as a consequence of the methodology.

Secondly, the procedures associated with intra-intestinal immunisation in the present study were developed with the welfare of the animal model at the forefront of experimental design. Any detrimental impact upon animal health as a consequence of the applied techniques would be ethically unacceptable. Furthermore, any effect upon the normal physiological and behavioural function of the animals may possibly influence data and therefore jeopardise scientific replication.

Finally, the fundamental purpose of intra-intestinal administration of the *H. pylori* vaccine used in the present study was to obtain maximal intestinal immunisation by circumventing the previously described deleterious factors associated with peroral administration. As ultimately the principle direction of this investigation was to compare the efficacy of alternate mucosal routes of immunisation in a mouse model of *H. pylori* infection, the resultant data from intra-intestinal immunisation was intended to establish a baseline of dose rates from which to commence such evaluation.

The outcome of these aspects of the study may provide evidence that intra-intestinal immunisation is an important route for the examination and development of potential *H. pylori* vaccine candidates.
3.2 **METHODODOLOGY OF INTRA-INTESTINAL IMMUNISATION**

The procedure of intra-intestinal immunisation necessitated invasive surgery requiring suitable surgical anaesthesia. The chosen anaesthesia was selected on the basis of sleep time required, ease of administration, species specificity, minimisation of risk of anaesthetic complications and post operative analgesia.

Fundamental to the development of the techniques described here was the selection of the appropriate equipment. Largely the species requirements and the specific nature of the procedure to be performed govern the choice of surgical instruments. For instance, surgical manipulation of rodents would necessitate the use of fine instruments, whereas procedures of a similar nature performed on large animals would use instruments appropriately scaled to the task at hand. Additionally, the investigator’s personal preference will dictate those items to be included in the surgical pack.

The actual methodology of intra-intestinal immunisation involved the micro-injection of a test substance into the sub-serosa of the Peyers’ patch of mice. These sites are often only 1-2mm in diameter, particularly in those mice that are naïve to any infection where the Peyers’ patches will not have fully developed germinal centres. Bearing this in mind it is often difficult to locate the Peyers’ patches with the naked eye let alone manipulate them. Therefore, the use of some form of magnification lens was essential for successful manipulation and injection of such a relatively small site.

3.2.1 **Surgical Preparation**

The logistics of all aspects of the surgical procedure, including pre- and post-operative care, were considered and implemented in advance to day of surgery. Instruments were selected, cleaned, packed and sterilised. Animal body weights were recorded 1 day in advance in preparation for calculation and administration of anaesthetic. The toenails of all subjects were trimmed to avoid entanglement with suture material. Clean dry cages were set up in readiness for recovering animals. Surgical procedure was performed early in the AM to enable observation of recovering animals throughout the course of the day.
As with any operation where the gastrointestinal tract is exteriorised and manipulated, critical attention to aseptic technique for all aspects of preparation and procedure was required to avoid possible infection. All surgical instruments were pre-packed and sterilised by autoclave at 121°C and 100 Kpa for 15 minutes. Added to the kit post-sterilisation was a micro-syringe (sterilised by 70% ethanol followed by ultra-violet irradiation) and suture material (sterilised by gamma irradiation).

Anecdotal evidence indicates that most surgical procedures in the mouse do not require the operator to wear a cap, mask, gloves or gown. However, it is generally advised that due to the nature of gastrointestinal surgery, barehanded contact with the viscera must be avoided and all material in the periphery of the operating area sterile. Therefore it was considered prudent that prior to surgical procedure, each operator performed a full surgical scrub of hands and forearms with an antiseptic hand-wash preparation and donned a sterile mask, gown and gloves. Attendants assisting operator performed surgical scrub and donned sterile gown only.

The subject mice were individually anaesthetised to a surgical plane with a combination of Ketamine® (50 mg/kg) and Xylazine® (50 mg/kg), (Parnell Laboratories, NSW, Australia) as described in section 2.8.4, providing a sleep time of approximately 60 minutes. All fur about the abdominal area was removed with fur clippers (Oster Golden A5, Sunbeam Corporation, USA). The subject was then placed in dorsal recumbency onto a sterile drape and transferred to a thermostatically controlled heating pad.

Approximately 100 μL of sterile physiological saline, administered with a 1 mL tuberculin syringe, was used to irrigate each eye to prevent corneal drying. A sterile drape, with a central section removed to reveal the surgical site, was placed over the subject. The entire site was swabbed with 70% ethanol followed by a liberal application of 10% w/v iodine solution.
Plate 3.1 Sterile Surgical Pack

a. Dissecting Forceps
b. Forceps (sharp)
c. Scissors (sharp/sharp)
d. Scissors (blunt/blunt)
e. Autoclip® applicator
f. Wound clips
g. Needle holders
h. Probe
i. Suture (6-0 Dexon™)
j. Micro-syringe
3.2.2 **Surgical Technique**

Prior to commencement of surgical procedure, anaesthesia of the subject animal was assessed by three independent methods as described in *Table 3.1*.

<table>
<thead>
<tr>
<th>Test</th>
<th>Clinical Signs</th>
<th>Level of Anaesthesia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puff test</td>
<td>Movement of whiskers and ear to a puff of air</td>
<td>Minimal sedation</td>
</tr>
<tr>
<td>Pupillary</td>
<td>Dilation of pupils and failure to contract to light stimulus</td>
<td>Surgical anaesthesia</td>
</tr>
<tr>
<td>response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pedal reflex</td>
<td>Failure to withdraw hind paw in response to pinch</td>
<td>Surgical anaesthesia</td>
</tr>
</tbody>
</table>

The Peyers’ patches are located in the small intestine increasing in size and frequency distal to the stomach, becoming conspicuous in the ileum, where they infiltrate the submucosal layer. Exposure of the ileum was accomplished by two incisions. The initial incision was made through the skin followed by a second incision through the muscle fascia. Using sharp/sharp iris scissors, a 1cm longitudinal incision was made through the skin, right of the mid-ventral line and cranial to the transverse plane, thus exposing the sheath of the rectus abdominus.

*Figure 3.1 Site of Surgical Incision*
Iris scissors, blunt/blunt, were carefully inserted between the skin and sheath of each subject and “blunt” dissection performed to separate these two layers of tissue. An incision with sharp/sharp iris scissors was made through the rectus abdominus and parietal peritoneum to expose the abdominal cavity.

The greater omentum was gently moved aside with a probe to reveal the small intestine. Avoiding the ascending colon, transverse colon and caecum, the small intestine was located and exteriorised by gently grasping with forceps and withdrawn from the abdominal cavity with the aid of a probe. Extreme care was taken to prevent damage to mesentery (omental bursa) and associated blood vessels. The small intestine was splayed out and Peyers’ patches located.

A single Peyers’ patch was raised with forceps and 30 gauge needle attached to a 50 μL Gastight® micro-syringe (Hamilton Company, USA) was inserted into the sub-serosa. Deep insertion into the lumen of the small intestine was avoided by holding the syringe parallel to the ileum. A 5 μL aliquot of test substance was administered to a Peyers’ patch. Successful delivery was recognised by a discernible swelling or “bleb” of the Peyers’ patch. Four such Peyers’ patches were manipulated in this fashion thereby giving a total dose volume of 20 μL per animal.

At completion of administration the small intestine and mesentery were carefully replaced into the abdominal cavity. To prevent adhesion, the cavity was irrigated with = 250 μL of sterile physiological saline prior to re-positioning of the greater omentum, and closure. The rectus abdominus was closed by suturing with 6-0 Dexon™ (Davis-Geck, USA) using a single interrupted pattern. The skin was closed with sterile stainless steel wound clips applied with an Autoclip® applicator (Becton Dickinson, USA).

All surgically manipulated subjects were maintained on the heating pad and eye drops of sterile physiological saline continued until fully recovered. Similarly, food and water was withheld until animals regained total consciousness and full physiological and behavioural function. Wound clips were removed at 1-week post-surgery with an Autoclip® remover (Becton Dickinson, USA) under simple restraint and without the necessity for anaesthesia.
Plate 3.2 Intra-Intestinal Immunisation Flow Diagram

a. Anaesthetised subject
b. Abdominal area shaved
c. Sterile surgical site
d. Incision of skin & muscle
e. Ileum exteriorised
f. Micro-injection of PP
g. Ileum replaced & cavity irrigated
h. Abdominal muscle closed
i. Skin closure & recovery
Plate 3.3 Peyers' Patch Injection

Micro-injection of a Peyers’ patch. (Inset x 5 original magnification)
3.3 OPTIMISATION OF INTRA-INTESTINAL IMMUNISATION

Prior to any conduct of procedure and subsequent investigation of optimisation, the choice of a suitable animal model must first be made. The criteria for selection of an appropriate strain of mouse were twofold. In the first instance, and as previously discussed, the size of the Peyers’ patches in mice is relatively small. Therefore, in order to give the investigator every possible chance of a successful injection, a large strain would be desirable, with the anticipation that the animals’ size would correspond directly to the size of the Peyers’ patches. Secondly, the use of an outbred strain would partially encompass the genetic diversity of the laboratory mouse, and the data obtained used as a general indicator across the species. Outbred Quackenbush Swiss were selected for these attributes, and because it is renowned for its robust physiology.

As previously discussed, the optimisation of an animal model of immunisation needs to ascertain the optimal value of a number of factors. One such variable is that of the time-course of immunisation. Using intra-gastric administration, two time points of immunisation only, prime at day 0 and booster at day 21, was shown by Sutton et al\textsuperscript{152} to produce a marked prophylactic improvement against \emph{H. pylori} challenge to that of immunising with four time points at days 0, 7, 14 and 21. This result is not only important for improved efficacy of immunisation, but also equally significant for methodology that may impact on animal health such as the invasive nature of direct injection of the Peyers’ patches and its requirement to use anaesthetic agents. Therefore, the minimal possible number of administrations performed to the individual animal, to obtain maximal immunisation, was deemed to be essential criteria for successful intra-intestinal immunisation.

Of similar importance in the pursuit for optimisation is the determination of appropriate dose rates. Comparative to the more commonly used routes of mucosal immunisation there is very little published material on intra-intestinal immunisation. As a consequence, the lack of substantial data for optimal dose rates of antigen and adjuvant prior to this study provided a fundamental purpose for the investigation. Typically a 10 \( \mu \)g dose rate of cholera toxin per immunisation has become the basis when incorporating CT as the mucosal adjuvant in the development of orally administered vaccine strategies against
Helicobacter infection.[126, 93, 135, 151] Similarly, previous investigations have generally used 1 mg of H. pylori whole cell lysate (HpL) as the antigenic component for such peroral immunisation.[26, 134, 151] However, Sutton et al. [122] demonstrated in a contemporary study that a reduced antigen concentration of 200 μg per immunisation produced significantly improved prophylaxis against bacterial challenge. Even more recently, the same group has successfully used as little as 100 μg of whole cell lysate for a number of immunisation strategies [unpublished data, Helicobacter research group, UNSW]. To determine a suitable concentration and volume for H. pylori whole cell lysate and cholera toxin to be delivered by direct Peyers’ patch injection, an assessment of doses rates from similar studies was made.[27, 116] In a pilot investigation preceding the present complete study it was found that 10% of the nominal amount administered orally (100 μg HpL + 10 μg CT) was sufficient to stimulate a protective immune response when delivered via Peyers’ patch injection.[151]

The philosophy of “less is best”, though an oversimplification of the immune processes at work, may apply to a successful immunisation strategy against H. pylori. Certainly for the intention of optimising intra-intestinal immunisation in a mouse model, reduced numbers of immunisations and dose rates may prove the most efficacious.

3.3.1 Experimental Plan

To ascertain the efficacy and optimal dose rate for direct intra-intestinal immunisation, specific pathogen free, female Quackenbush-Swiss (Q/S) mice aged = 8 weeks were selected as the animal model. The subject mice were prophylactically immunised with whole cell lysate of H. pylori (HpL) + cholera toxin (CT). As described in Table 3.2, test groups (n=10) were administered with a placebo, antigen only, adjuvant only or ratios of antigen plus adjuvant complexes. Phosphate buffered saline (PBS) was used as the placebo.

As a general indicator of animal health, all animal body weights were measured at 1 day prior to priming immunisation, 1 week post booster immunisation and at completion of study.
Table 3.2 Intra-Intestinal Immunisation Experimental Regime

<table>
<thead>
<tr>
<th>Group Identification</th>
<th>Dose Rate</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Non-infected</td>
<td>Nil</td>
<td>N/A</td>
</tr>
<tr>
<td>B Intra-gastric placebo</td>
<td>0.1 M PBS</td>
<td>100 μL</td>
</tr>
<tr>
<td>C Intra-gastric antigen + adjuvant</td>
<td>100 μg HpL + 10 μg CT</td>
<td>100 μL</td>
</tr>
<tr>
<td>D Intra-intestinal placebo</td>
<td>0.1 M PBS</td>
<td>4 x 5 μL</td>
</tr>
<tr>
<td>E Intra-intestinal adjuvant only</td>
<td>1 μg CT</td>
<td>4 x 5 μL</td>
</tr>
<tr>
<td>F Intra-intestinal antigen only</td>
<td>10 μg HpL</td>
<td>4 x 5 μL</td>
</tr>
<tr>
<td>G Intra-intestinal antigen + adjuvant</td>
<td>0.1 μg HpL + 0.01 μg CT</td>
<td>4 x 5 μL</td>
</tr>
<tr>
<td>H Intra-intestinal antigen + adjuvant</td>
<td>1 μg HpL + 0.1 μg CT</td>
<td>4 x 5 μL</td>
</tr>
<tr>
<td>I Intra-intestinal antigen + adjuvant</td>
<td>10 μg HpL + 0.1 μg CT</td>
<td>4 x 5 μL</td>
</tr>
<tr>
<td>J Intra-intestinal antigen + adjuvant</td>
<td>1 μg HpL + 1 μg CT</td>
<td>4 x 5 μL</td>
</tr>
<tr>
<td>K Intra-intestinal antigen + adjuvant</td>
<td>10 μg HpL + 1 μg CT</td>
<td>4 x 5 μL</td>
</tr>
</tbody>
</table>

Mice were immunised twice (priming dose at day 0 and booster dose at day 21) via the intra-intestinal route with chequer-board dose rates of antigen and adjuvant. An intra-gastric immunisation group was incorporated as a control, also primed at day 0 and boosted at day 21.

At day 50 all groups were challenged (except uninfected controls) with live *H. pylori*. A bacterial count of approximately $10^7$ organisms in a 100 μL suspension of BHI was delivered perorally to each animal by intra-gastric gavage (refer to method 2.8.3).

At four weeks post challenge, mice were euthanased by CO₂ asphyxiation and stomachs harvested for bacterial culture. Analysis of viable colony forming units of serial dilutions from homogenised stomach tissue (refer to method 2.9.2) was used to determine levels of *H. pylori* infection. An initial assessment of infection levels was made using the gastric urease assay (refer to method 2.9.1).
3.3.2 Results

Animal body weights, pre- and post-experimental procedure were monitored and recorded over the course of the study. No significant difference was observed in body weights between the control and manipulated animals, that is, there was no statistical difference between the non-immunised non-infected animals (Group A) and any other group in the study.

The group average of animal weights increased between 8 and 20 weeks at a gain typical for the strain and age (Table 3.3 & Figure 3.2).

Table 3.3 Animal Body Weights for Intra-Intestinal Immunisation
Body weights (in grams) are expressed as the arithmetical mean (± standard deviation) for each group of 10 animals at designated time points.

<table>
<thead>
<tr>
<th>ID</th>
<th>Pre Immunisation (= 8 weeks old)</th>
<th>Post Immunisation (= 12 weeks old)</th>
<th>At Harvest (= 20 weeks old)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>34.6 ± 2.4</td>
<td>36.7 ± 2.2</td>
<td>38.1 ± 2.1</td>
</tr>
<tr>
<td>B</td>
<td>36.6 ± 2.3</td>
<td>36.9 ± 2.8</td>
<td>38.1 ± 2.5</td>
</tr>
<tr>
<td>C</td>
<td>35.3 ± 2.6</td>
<td>36.4 ± 3.9</td>
<td>37.4 ± 3.9</td>
</tr>
<tr>
<td>D</td>
<td>36.1 ± 2.7</td>
<td>35.7 ± 2.7</td>
<td>37.8 ± 2.1</td>
</tr>
<tr>
<td>E</td>
<td>35.0 ± 2.9</td>
<td>34.3 ± 2.8</td>
<td>36.2 ± 2.6</td>
</tr>
<tr>
<td>F</td>
<td>35.1 ± 3.5</td>
<td>35.0 ± 3.2</td>
<td>36.2 ± 3.1</td>
</tr>
<tr>
<td>G</td>
<td>36.0 ± 2.4</td>
<td>35.3 ± 2.9</td>
<td>37.0 ± 2.5</td>
</tr>
<tr>
<td>H</td>
<td>35.7 ± 2.7</td>
<td>35.4 ± 2.6</td>
<td>37.3 ± 2.5</td>
</tr>
<tr>
<td>I</td>
<td>35.1 ± 2.9</td>
<td>34.8 ± 2.9</td>
<td>36.3 ± 2.6</td>
</tr>
<tr>
<td>J</td>
<td>35.3 ± 3.3</td>
<td>35.3 ± 3.2</td>
<td>36.6 ± 2.7</td>
</tr>
<tr>
<td>K</td>
<td>36.0 ± 2.3</td>
<td>35.5 ± 2.5</td>
<td>36.1 ± 2.1</td>
</tr>
</tbody>
</table>

Additionally, no discernible difference in behavioural function from continual daily observation of all animals was perceived between any of the groups prior to, during and at completion of the study.
Figure 3.2 Comparison of Body Weights for Intra-Intestinal Immunisation

Mean group body weights recorded during course of experimental procedure. Only one intra-intestinal antigen + adjuvant group (10\(\mu\)g HpL + 1\(\mu\)g CT) included which is indicative of all other intra-intestinal dose rates.
An initial indication of colonisation levels was made by gastric urease assessment (refer to section 2.9.1). Results by reagent colour change were visually indicated approximately 1 hour post harvest and processing.

<table>
<thead>
<tr>
<th>Group Description</th>
<th>Individual Animal Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A (Non-Infected)</td>
<td>○</td>
</tr>
<tr>
<td>B (I/G 0.1M PBS)</td>
<td>●</td>
</tr>
<tr>
<td>C (I/G 100µg HpL + 10µg CT)</td>
<td>●</td>
</tr>
<tr>
<td>D (I/I 0.1M PBS)</td>
<td>●</td>
</tr>
<tr>
<td>E (I/I 1µg CT)</td>
<td>●</td>
</tr>
<tr>
<td>F (I/I 10µg HpL)</td>
<td>●</td>
</tr>
<tr>
<td>G (I/I 0.1µg HpL + 0.01µg CT)</td>
<td>○</td>
</tr>
<tr>
<td>H (I/I 1µg HpL + 0.1µg CT)</td>
<td>○</td>
</tr>
<tr>
<td>I (I/I 10µg HpL + 0.1µg CT)</td>
<td>●</td>
</tr>
<tr>
<td>J (I/I 1µg HpL + 1µg CT)</td>
<td>○</td>
</tr>
<tr>
<td>K (I/I 10µg HpL + 1µg CT)</td>
<td>○</td>
</tr>
</tbody>
</table>

**COLOUR CODE**

○ = 0  
● = 1  
●● = 2  
●●● = 3

*Figure 3.3 Gastric Urease Analysis following Intra-Intestinal Immunisation*

Scores from 0-3 were recorded for each animal corresponding to the colour change. A score of 2 or 3 was indicative of *Helicobacter* infection, whilst a score of 0 or 1 corresponded to reduced or possibly zero infection.
The colour change of urease reagent was translated and recorded as a numerical score from 0-3 as described in Figure 3.3. The percentage of animals in each group considered by gastric urease assay to be protected from *H. pylori* challenge is indicated in Table 3.4.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Non-infected</td>
<td>N/A</td>
</tr>
<tr>
<td>B Intra-Gastric 0.1 M PBS</td>
<td>0%</td>
</tr>
<tr>
<td>C Intra-Gastric 100 μg HpL + 10μg CT</td>
<td>90%</td>
</tr>
<tr>
<td>D Intra-Intestinal 0.1 M PBS</td>
<td>0%</td>
</tr>
<tr>
<td>E Intra-Intestinal 1 μg CT</td>
<td>0%</td>
</tr>
<tr>
<td>F Intra-Intestinal 10μg HpL</td>
<td>0%</td>
</tr>
<tr>
<td>G Intra-Intestinal 0.1 μg HpL + 0.01 μg CT</td>
<td>60%</td>
</tr>
<tr>
<td>H Intra-Intestinal 1 μg HpL + 0.1 μg CT</td>
<td>80%</td>
</tr>
<tr>
<td>I Intra-Intestinal 10 μg HpL + 0.1 μg CT</td>
<td>40%</td>
</tr>
<tr>
<td>J Intra-Intestinal 1 μg HpL + 1 μg CT</td>
<td>40%</td>
</tr>
<tr>
<td>K Intra-Intestinal 10 μg HpL + 1 μg CT</td>
<td>100%</td>
</tr>
</tbody>
</table>

The non-infected control animals were all negative to urease analysis (Figure 3.3). The placebo in both routes of administration provided zero evidence of protection, as did the intra-intestinally delivered antigen only (10μg HpL) and adjuvant only (1 μg CT). The positive immunisation control of 100 μg HpL + 10μg CT administered via the intra-gastric route provided protection in 90% of the animals.

All antigen + adjuvant dose rates administered via the intra-intestinal route prevented *H. pylori* colonisation in a proportion of the mice. The highest level of prophylaxis from intra-intestinal immunisation, with 100% of animals protected, was observed in the mice immunised with 10 μg HpL + 1 μg CT. 80% and 60% of mice were protected in the groups administered 1 μg HpL + 0.1 μg CT and 0.1 μg HpL + 0.01 μg CT respectively. Only 40% of mice were protected from bacterial challenge from both groups intra-intestinally immunised with 10 μg HpL + 0.1 μg CT and 1 μg HpL + 1 μg CT.
Figure 3.4 Bacterial Colonisation following Intra-Intestinal Immunisation

Level of \textit{H. pylori} colonisation following immunisation and challenge in Q/S mice. Data expressed as arithmetical means of colony forming units per gram of stomach tissue (error bars indicate standard deviation) in 10 animals.
Following analysis of gastric urease assay, prophylactic efficacy was further evaluated by assessment of viable plate counts from homogenised stomach tissue. Incorporated in the study as a ‘sentinel’ for the detection of contamination, the uninfected control group, that is the group of animal’s naïve to any procedure, were free from any detectable incidence of *H. pylori* colonisation at the completion of the study. This data were observed from the gastric urease assay (*Figure 3.3*) and viable plate counts (*Figure 3.4*).

The intra-gastric administered placebo (I/G PBS) animals were used as an infection control. The data presented in *Figure 3.4* indicated that this group recorded a mean bacterial load of 2.99 x 10^6 colony forming units per gram of stomach tissue (CFU/gram). This level of colonisation was consistent for the strain of mouse used and established a base line for comparison of colonisation levels for all other groups. Similarly, the group administered the placebo via direct injection of the Peyers’ patches (I/I 0.1 M PBS) recorded a mean colonisation of 3.78 x 10^6 CFU/gram, and was also consistent for the strain of mouse. There was no significant difference observed between these two routes of immunisation when administered the placebo alone (refer *Table 3.5*).

Further examination of *Figure 3.4* indicated that all groups administered with an antigen/adjuvant (HpL + CT) complex via intra-intestinal immunisation elicited varying degrees of a prophylactic immune response against *H. pylori* challenge. Furthermore, the intra-gastric immunisation group administered 100 μg HpL + 10μg CT also provided a level of prophylaxis at 4.89 x 10^5 CFU/gram.

The group intra-intestinally immunised with whole cell lysate only (10 μg HpL) provided a prophylactic response with a colonisation level of 1.62 x 10^6 CFU/gram. However, the group of animals administered the mucosal adjuvant cholera toxin (CT) only via the intra-intestinal route, exhibited a mean bacterial level of colonisation of 3.13 x 10^6 CFU/gram and did not produce a prophylactic response.

Whilst all animals immunised with an antigen/adjuvant complex elicited a prophylactic response, there was evidence of clearly different levels of efficacy. Statistical analysis of differences in bacterial colonisation levels was performed on all groups with the significant differences between those groups illustrated in *Table 3.5*. 
**Table 3.5 Statistical Analysis for Intra-Intestinal Immunisation**

Groups of animals that have significantly different levels of bacterial colonisation from any other group are designated by ● with a significance level of p < 0.05. As an example, group B is significantly different from groups A, C, F, G, H, I, J & K, but not significantly different from groups D & E.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Nil)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>B (i/g PBS)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>C (i/g 100 μg HpL + 10 μg CT)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>D (i/i PBS)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>E (i/i 1 μg CT)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>F (i/i 10 μg HpL)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>G (i/i 0.1 μg HpL + 0.01 μg CT)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>H (i/i 1 μg HpL + 0.1 μg CT)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>I (i/i 10 μg HpL + 0.1 μg CT)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>J (i/i 1 μg HpL + 1 μg CT)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>K (i/i 10 μg HpL + 1 μg CT)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
</tbody>
</table>

The data presented in Table 3.5 indicates that all antigen/adjuvant complexes of intra-intestinal immunisation when delivered at a ratio of 10:1 (10 μg HpL + 1 μg CT), (1 μg HpL + 0.1 μg CT) and (0.1 μg HpL + 0.01 μg CT) were significantly different from all other groups with the exception of the intra-gastrically immunised mice. The latter were also immunised with a 10:1 antigen/adjuvant ratio (100 μg HpL + 10 μg CT). No significant difference was observed between any of the groups immunised with an antigen/adjuvant ratio of 10:1.

The groups of animals intra-intestinally immunised with and antigen/adjuvant ratio of 100:1 (10 μg HpL + 0.1 μg CT) and 1:1 (1 μg HpL + 1 μg CT) were significantly different from the infection control animals and the groups with an antigen/adjuvant ratio of 10:1.
3.4 DISCUSSION

The major results obtained from this component of the study were viewed from two perspectives. In the first instance, and of the upmost importance, was that the actual methodology was expected to be accomplished with precision and with no detrimental effects to the animals. Secondly the demonstration of prophylactic responses being elicited by direct injection of the Peyers’ patches and establishing base line data of dose rates would be essential in the further examination of alternate mucosal routes of vaccine administration. These two outcomes, though closely linked, have been discussed on their own merits.

3.4.1 Validation of Methodology

An important outcome of investigating intra-intestinal immunisation was to ascertain that the direct administration of immunogens to a specific site of immunity was a valid scientific procedure for the further investigation of mucosal immunisation strategies. Any quantitative effect associated with the methodology alone, either to the physiology of the animals or influence on the resultant data, would be considered unacceptable.

As described in section 3.3.2, a group of animals naïve to any procedure and undisturbed for the entire program were included in this investigation. As recorded, there were no detectable levels of *H. pylori* colonisation in this group. This was predictable as a random sample of animals was initially screened for *H. pylori* infection upon arrival. Assessment of colony forming units from homogenised stomach tissue (refer section 2.9.2) taken from these animals indicated that the colony was free *H. pylori* prior to commencement of the study. Furthermore, as all animals were maintained to a strict specific pathogen free protocol, and all procedures performed aseptically, the potential for any contamination was effectively eliminated. The inclusion of a group of uninfected and non-immunised animals were used in the first instance as monitor for contamination. Most importantly however, as this experiment involved surgical intervention, it was necessary to incorporate a group of animals that could serve as a base line for the detection of any influence on data resulting from the procedure.
Comparisons of body weights were used as an indicator for the monitoring of health status over the course of experimental procedure. The groups that underwent Peyers’ patch injection experienced a marginal weight loss following surgical manipulation. The reduction however, was negligible (no statistical difference as determined by chi-square test) between all manipulated animals and those of the uninfected controls, and any such weight loss was regained approximately 1 week post-surgery, with all animals adding body weight typical for the age and strain. Furthermore, there was no detectable evidence of impact on behavioural function. The outcome of no discernible detrimental effects upon the manipulated animals when compared to the uninfected controls, and indeed no losses, satisfied the ethical obligation toward the welfare of the animals. Similarly, statistical analysis of the data was unaffected through maintained animal numbers.

Since the techniques applied were invasive, one would hypothesise that they could lead to physiological stress. Such stress may have positive or negative bearing upon prophylaxis. As there was no significant difference between the colonisation levels observed in the group administered a placebo (0.1 M PBS) intra-intestinally to the corresponding group given the placebo orally, the result could indicate that the procedure did not exacerbate infection. Conversely, it can also be reasoned that no prophylactic response was obtained from the procedure alone.

3.4.2 Efficacy of Intra-Intestinal Immunisation

The second major objective of intra-intestinal immunisation was to establish and analyse base line data for consideration of the projected investigations of alternate mucosal routes of immunisation. Additionally, the prospect of a prophylactic response arising from intra-intestinal immunisation would present the technique as an important methodology for the evaluation of future strategies.[27, 65, 91, 120, 141]

From analysis of the data, protective immunity from bacterial challenge was observed from the direct injection of immunogens to the Peyers’ patches, thus satisfying one of the principal objectives of this study. As determined from gastric urease assay (Figure 3.3 and Table 3.4), all animals immunised via the intra-intestinal route with an antigen/adjuvant
complex elicited a prophylactic response in a percentage of the animals. The range of protection varied considerably, and was dependent upon the dose rate concentration and the proportion of antigen as to adjuvant. Gastric urease assay clearly indicated that a ratio of 10:1 between whole cell lysate of *H. pylori* and cholera toxin produced the highest levels of prophylaxis and that neither antigen only or adjuvant only was effective.

Whilst the initial indication of efficacy of intra-intestinal immunisation was obtained from gastric urease assay, the findings were confirmed and further defined by the more sensitive assay of viable plate counts. As anticipated, one of the groups to exhibit the highest levels of colonisation were the animals administered with the placebo (0.1 M PBS) via the intra-intestinal route, presenting a mean bacterial load of $3.78 \times 10^6$ colony forming units per gram of stomach tissue (CFU/gram). This data correlated well with the infected control group (intra-gastric placebo of 0.1 M PBS), which produced a mean bacterial load of $2.99 \times 10^6$ CFU/gram and with no significant difference between the two groups. Similarly, the intra-intestinal administration of adjuvant only (1 μg CT) was not significantly different from the placebo groups, with an infection level of $3.13 \times 10^6$ CFU/gram. In reiteration of the discussion of validation of methodology, these data are important when considering any impact on colonisation from the experimental procedure. As no significant difference was observed between the intra-gastrically administered placebo and the intra-intestinally administered placebo, it may be suggested that the surgical procedure alone had no experimental effect.

From examination of Figure 3.4 and Table 3.5, all groups administered with an antigen/adjuvant complex demonstrated varying degrees of prophylaxis with a high proportion of those groups immunised via the intra-intestinal route comparing favourably with those animals vaccinated by the intra-gastric route. Furthermore, there was no statistical difference observed between the animals immunised with as little as 0.1% of the dose rate used for intra-gastric immunisation. Notably, all groups intra-intestinally immunised with an antigen/adjuvant ratio of 10:1, regardless of the dose rate, produced the lowest levels of colonisation, with no significant difference observed between these animals and that of the intra-gastric immunisation (I/G 100 μg HpL + 10 μg CT) group. This result provides evidence of a wide degree of immunisation tolerance capable of providing protection from bacterial challenge.
The two groups intra-intestinally immunised with an antigen/adjuvant ratio of 1:1 and 1:100 produced mid-level colonisation with $1.15 \times 10^6$ and $2.45 \times 10^6$ CFU/gram respectively. Clearly from these data, a 10:1 antigen/adjuvant complex elicited a significantly better prophylactic response than any other combination. This phenomenon of apparent ratio dependence was to be explored in greater detail later in the study.

Of particular interest, was the infection level observed in the group of animals administered exclusively antigen (10 μg HpL) via the intra-intestinal route. Whilst significantly different from the 10:1 antigen/adjuvant groups, the observed bacterial load of $1.62 \times 10^6$ CFU/gram showed a prophylactic response similar to, and with no significant difference to that of the intra-intestinally immunised groups with an antigen/adjuvant ratio of 1:1 and 1:100. Therefore, in the context of similarity between the colonisation levels of these three regimes, it should be considered that a prophylactic effect was in evidence in the absence of a mucosal adjuvant. The necessity of using a mucosal adjuvant to assist facilitation of luminal antigen access to gut mucosae when perorally administering vaccines has been previously discussed.\cite{34, 95} However, by bypassing the mechanisms associated with antigen uptake by M cells of the Peyers’ patches, immunogens may possibly be presented directly to site of specific immunity.\cite{18, 67, 116} Furthermore, gastrointestinal degradation of proteins may be avoided by direct administration to inductive sites of the mucosal immune system.\cite{16, 45, 122, 133}

It is well documented that peroral immunisation protects against \textit{H. pylori} challenge in a mouse model of infection.\cite{21, 47, 89, 90, 135} The comparative data for prophylaxis between the two routes of intra-intestinal and intra-gastric immunisation provides strong evidence that direct administration to the actual sites of antigen uptake provides efficient prophylactic immunisation. This finding could be important for future investigations of vaccine candidates where initial screening can be accomplished in the absence of the deleterious factors associated with peroral administration.\cite{16, 122}
Chapter 4
Alternate Routes of Immunisation

4.1 INTRODUCTION

The selection of an appropriate route of immunisation against a mucosal associated pathogenic organism requires evaluation of a number of determinants. As reviewed, the site and nature of bacterial infiltration and the means by which antigenic substances may be effectively delivered to sites of specific immunity are significant factors. Equally important to vaccination success is the determination of the practical aspects of vaccine administration. Consideration should be given to the technical ease by which a subject may be immunised, including time and cost effectiveness, without compromising precision or indeed efficacy.

It would be implausible to assume that any one particular route of administration would elicit the best possible effect without the trial and examination of other alternate routes. However, many previous Helicobacter investigations have only tested one model of immunisation, with oral immunisation typically taking precedence over other potentially efficacious routes. The technique of peroral administration does have fundamental attributes that may account for its popularity. Antimicrobial therapy, administered perorally, has proven to be an effectual treatment against existing Helicobacter infection. The route is also the obvious choice for the administration of live attenuated vaccines. Furthermore, experimental infection is typically, and effectively, induced by intra-gastric delivery. In view of these traits, and the fact that the target site for vaccination is the stomach, the likelihood of peroral administration being generally accepted and adopted for a majority of immunisation strategies against H. pylori is high. However, the data from the preceding chapter has clearly indicated that the direct injection of an immunogen to the Peyers'
patches elicits significant prophylaxis against *H. pylori* challenge comparative to that of intra-gastric immunisation. This result is suggestive that routes alternate to that of peroral administration may be equally or more efficacious. Whilst the intra-intestinal delivery of vaccines provides valid scientific data and accuracy in presentation, as illustrated by maximal intestinal immunisation, it is cumbersome in operation. Requiring an adept technical proficiency and incurring the costs associated with surgical manipulation, intra-intestinal immunisation would be inappropriate for large-scale vaccine trials, commercial applications or as an everyday methodology for use in the field. Therefore consideration of other mucosal routes of immunisation with an inherently simple methodology should be evaluated.

Prior to evaluation, the selection of any route would be required to fulfil criteria appropriate to the condition such as the site and nature of the pathogenic organism, the species to be immunised or gender specificity. For example, the efficacy of intra-vaginal immunisation has been demonstrated in clinical trials as an important methodology for treatment of specific mucosal associated pathogens.\(^8\), \(^{36}\), \(^{78}\) However, the procedure is limited to use only in females, and whilst applicable to communicable diseases of the urogenital tract, may possibly have limitations with reference to gastro-intestinal infection. Two mucosal routes that have the potential for a greater diversity of application are intra-nasal and intra-rectal. These two routes have been used with success against a variety of mucosal infections, \(^{[1, 20, 36, 59, 62, 71, 75, 119, 134, 137]}\) and recently, their application has been used in vaccine trials against *H. pylori* with encouraging results.\(^{[76, 165]}\)

Whilst the aforementioned routes have been examined independently, very little comparative analysis has been performed. One investigation by Kleanthous *et al.*\(^{[76]}\) did compare intra-rectal and intra-nasal. Though whilst the investigation was pertinent to its intended aims, the study used differing vaccination regimes for the two routes of immunisation, thereby comprising, in terms of comparisons, the statistical validity of scientific data and possible impact on the subject animals. Therefore, the purpose of this chapter was to investigate the efficacy and draw comparisons in a controlled study of the alternate routes of intra-gastric, intra-nasal and intra-rectal, with the intention of discussing the outcomes as precursors for vaccination against other mucosal associated pathogens.
4.2 METHODOLOGY OF IMMUNISATION

The comparative analysis of the three routes of intra-gastric, intra-nasal and intra-rectal immunisation was dependent in part upon the development of each of the methodologies under examination. All of the procedures presented in this section are adaptations of universally accepted and utilised protocols in the experimental mouse model with the welfare of the animal paramount.

In the course of immunisation practices on small laboratory animals many experimental investigators commonly utilise some form of chemical restraint. The methodology of intra-gastric, intra-nasal and intra-rectal administration have been described elsewhere whereby anaesthesia of the subject was required.\(^{[23, 90, 93, 109, 137, 139]}\) A key element of this work was to develop and establish simple methodology for administering substances in the absence of such regimes. The use of anaesthetic agents could well impact upon the experimental protocol and in the very least create additional stress to the subject.

As described in the preceding chapter, the selection of all devices used for the administration of substances must be appropriate to the species. Similar to the selection of surgical equipment, the choice of delivery apparatus was determined by not only that befitting the subject but influenced by personal choice and in particular the volume of test substances to be administered. For instance, when designing the device for intra-gastric immunisation, the selection criteria for the gauge and length of the gavage tubing was based on the animals size, with the flexible nature of such tubing suiting its intended purpose.

Fundamental to the overall success of the techniques is the ability to perform the procedures without the assistance of a second person. Bearing in mind that the animal must first be captured then restrained for the duration of administration allows only one hand to operate the device. Furthermore, the added necessity to perform multiple doses was also an essential requirement when treating a number of subjects. Therefore all devices were designed using items of equipment commonly used with one hand in everyday laboratory and medical procedures.
a. Automatic pipette used for intra-nasal immunisation
b. Stock polyethylene tubing (ID $\varnothing$ 0.58 mm, OD $\varnothing$ 0.98 mm)
c. Tubing attached to a 23 gauge needle on a 1 mL syringe used for intra-gastric immunisation
d. Stainless steel probe attached to a 1 mL syringe used for intra-rectal immunisation

Plate 4.1 Devices for Alternate Routes of Immunisation
4.2.1 Intra-Gastric Immunisation

Peroral, or per os, administration of a drug is by definition through the mouth (L. per, through, + os, mouth). In most circumstances, peroral administration of a drug may be given to mean oral or intra-gastric, as this is typically the route for either the buccal cavity or stomach respectively. Confusion often surrounds what actually constitutes oral and intra-gastric immunisation and the differences between the two routes. In many instances, the term oral administration may be taken to imply intra-gastric administration. This however, in the strictest sense, is an inaccurate use of terminology. Intra-gastric immunisation (L. intra, within, + G. gastér, stomach) requires the vaccine to be delivered directly to the stomach, bypassing all mucosal associated lymphoid tissue allied with the buccal cavity, oesophagus and respiratory tract. On the contrary, oral immunisation should be defined as the route by which vaccine is delivered into the buccal cavity, where the target may be one or many of the aforementioned MALT structures in addition to the stomach.

For all experimental work in this study, intra-gastric gavage was used for the direct administration of a specified aliquot of vaccine into the mouse stomach. Delivery was achieved by the insertion of a gavage needle into the buccal cavity, down the oesophagus then ultimately into the stomach.

Purpose manufactured gavage needles are available from a range of distributors. These are typically made from stainless steel or platinum and come in a variety of sizes. They may be straight or curved and have a small bulb attached to the end to prevent perforation of the gut. The novice often encounters difficulty inserting this type of gavage due to the size, shape and inflexible nature of the apparatus. A more simple and cost effective system was developed and refined for, and used extensively throughout the experimental component of this thesis. The delivery device consisted of single lumen polyethylene tubing (internal Ø 0.58 mm, external Ø 0.96 mm; Critchley Electrical Products Pty Ltd, Auburn, NSW) connected to a 23 gauge hypodermic needle (Plate 4.1c). The tubing was slipped over the shaft of the needle, such as a sleeve, with a portion extending from the point. The length of the extension was based on a measure from the nose to the last rib of the mouse. The prepared gavage needle was in turn attached to a 1 mL tuberculin syringe.
Attention to detail was essential in the preparation of the gavage needle, the uptake of vaccine into the syringe and the subsequent technique of insertion. As previously described, the target for intra-gastric immunisation was the stomach, and any contact with the vaccine with any other MALT structure would be considered a compromise to the experimental protocol.

Prior to capture and restraint of the subject animal, an aliquot was drawn-up into the syringe in the absence of the needle (Figure 4.1a). The needle was attached and the air gap expunged by gentle flicking of the syringe in conjunction with depressing the plunger. Any residual vaccine external to the tubing was removed by wiping on a sterile paper tissue then the apparatus set aside in readiness (Figure 4.1b).

Individual mice were captured by grasping at the base of the tail between thumb and forefinger of one hand and about the scuff of the neck with thumb, forefinger and index finger of the other hand (Figure 4.1c). The subject was rotated so that the body of the mouse was at rest in the palm of the hand and physically restrained by the application of a firm grip about the scuff and tail (Figure 4.1d). With the mouse thus totally immobilised, the gavage was inserted between the left incisors and molars and guided to the right of the ramus mandibulae (Figure 4.1e). Typically the subject attempted to gnaw on the tubing thus impeding insertion. Such behaviour was alleviated by a small puff of air about the face.

As the gavage approached the oesophagus, the neck of the subject was gently extended thus providing an imaginary straight line that existed with the syringe, the oesophageal orifice and the cardiac sphincter. The syringe was brought in line and the gavage gently moved in a caudal direction (Figure 4.1f). Passage of the device was generally facilitated by the onset of a swallowing reflex as the gavage approached the pharynx allowing advancement into the oesophagus. Any resistance experienced to the gavage was generally indicative of insertion into the trachea. In these instances, the gavage was entirely withdrawn and the procedure re-attempted.

Progression of the gavage was continued down the oesophagus and into the stomach where a specific aliquot was delivered (Figure 4.1f).
Figure 4.1 Intra-Gastric Administration Flow Diagram

There was no physical indication that the gavage had indeed entered the stomach, however, the estimation of tubing length as previously described should correlate to insertion. Additionally, as rodents do not exhibit oesophageal reflex, that is they do not regurgitate, delivery into the oesophagus virtually guaranteed successful delivery to the stomach.
Plate 4.2 Intra-Gastric Immunisation
4.2.2 Intra-Nasal Immunisation

By definition intra-nasal immunisation (\textit{L. intra}, within, \textit{+ L. nasus}, nose) that is, within the nasal cavity, delivers vaccine to the nasal sinus. In mice, antigen is presented to the bilateral strip of lymphoid tissue beneath the posterior nasal passage thereby inducing specific antibody-secreting cells in NALT and the draining cervical lymph nodes.\textsuperscript{51,158}

The means by which a vaccine may be efficiently and effectively delivered nasally required a technique that was simple to perform and comparatively non-invasive to the animal. Anecdotal evidence has indicated that many previous investigations have administered vaccines by inserting a gavage-like needle into the nasal sinus, with the subject often encountering some degree of physical damage to the soft tissue. Additionally, such a procedure is typically performed under anaesthesia thereby exacerbating an already stressful condition. The procedure described here circumvents such drawbacks by depositing the test substance at the base of the nares and allowing the subject to inhale the aliquot by normal physiological means.

The delivery device consisted of a 1-100 \( \mu \text{L} \) pipette tip (Sarstedt, SA, Australia) attached to a Gilson P100 calibrated automatic pipette (Gilson Medical Electronics, France) (\textit{Plate 4.1a}). Smaller pipettes and tips are available but their use was discouraged, as visual indication of delivery was unsatisfactory.

Individual mice were physically restrained by hand and immobilised by the application of a firm grip about the scuff and tail (\textit{Figure 4.2a}). Total immobilisation of the head was essential for successful administration (\textit{Figure 4.2b}). Using the automatic pipette, a 20 \( \mu \text{L} \) aliquot of test substance was taken up into the pipette tip (\textit{Figure 4.2c}). Excess vaccine external to the pipette tip was removed by carefully wiping with a sterile paper tissue.

The tip of the pipette was placed proximal to the nares and specific aliquot deposited (\textit{Figure 4.2d}). The normal inhalation by the subject delivered the test substance into the nasal sinus. The subject remained restrained for a short period (\( \approx 2-3 \) seconds) until visually confirming that administration was successful (\textit{Figure 4.2e}).
Whilst the technique described here conformed to the criteria of being simple and relatively non-invasive, no guarantee could be made as to the exact volume being delivered to NALT. It would be possible that a partial amount might be in fact ingested or a component of the vaccine may remain external to the sinus.
Plate 4.3 Intra-Nasal Immunisation
Intra-Rectal Immunisation

Intra-rectal immunisation (L. *intra*, within, + L. *rectus*, straight) that is, within the rectum, delivers vaccine to the terminal portion of the digestive tract, extending from the sigmoid colon to the anal canal. The target immune sites for administration were the colonic patches analogous to the Peyer’s patches of the small intestine.

The delivery device consisted of a straight stainless steel probe with a small bulb attached to the end to prevent perforation of the gut. The probe was connected to a 1 mL tuberculin syringe (*Plate 2.1d*). Prior to capture and restraint of the subject animal, an aliquot was drawn-up into the syringe (*Figure 4.3a*). The air gap was expunged by gentle flicking of the syringe in conjunction with depressing the plunger, and any residual vaccine external to the probe was removed by wiping with a sterile paper tissue. To facilitate insertion of the needle into the rectum, a minimal amount of a water-based lubricant was applied to the bulbous tip of the probe with a sterile cotton bud (*Figure 4.3b*) and set aside in readiness.

Individual mice were physically restrained by hand and immobilised by the application of a firm grip about the scuff and tail (*Figure 4.3c*). With the subject thus immobilised the anus was gently stroked with another sterile cotton bud so as to encourage defecation prior to insertion (*Figure 4.3d*). When satisfied that the bowel had been at least partially evacuated, the probe was gently inserted into the anus. A slight twisting action applied to the needle in conjunction with the lubricant facilitated insertion into the rectum. The probe was advanced slowly in a cranial direction along the colon for approximately 2 cm and the specific aliquot delivered (*Figure 4.3e*).

The subject remained restrained for a short period of approximately 2 to 3 seconds until operator was satisfied that administration was successful. Any portion of the aliquot being excreted visually indicated unsuccessful delivery.
As for intra-nasal immunisation, intra-rectal was a relatively elementary procedure, however as was the case with the former, there were minor complications associated with such a simple technique. The greatest obstacle was the potential for the vaccine to be excreted with faeces. Performing the technique at a time following the animals’ normal sleep period, whereby the colonic content of faeces is decreased, would possibly alleviate this potential.
Plate 4.4 Intra-Rectal Immunisation
4.3 Efficacy of Alternate Routes of Immunisation

As explained in the preceding chapter, an appropriate strain of the mouse model needed to be selected prior to commencement of the study. Whereas, an outbred strain was used for the development of intra-intestinal immunisation, for the reasons previously described, an inbred strain of mouse was considered applicable to this line of enquiry due to the comparative nature of the study. An additional characteristic would be one that tolerates and adapts well to repeated handling. The inbred BALB/c was chosen as a suitable animal model as the strain is renowned for its placid temperament, ease of handling, and has been widely used for numerous investigative purposes.

The reliability of a comparison of alternate routes of delivery depends upon the elimination of as many variables as possible. The aforementioned use of an inbred strain of mouse effectively removes genetic diversity. To exclude the potential of sex-linked factors (unless desired), the animal model should also be of the same gender across the entire investigation. Correlates of prophylaxis between routes can be directly assessed by the use of identical dose rates, and fundamentally, though often overlooked, the same operator should perform all methodologies. The logistics of the latter criteria may often be impossible to fulfil entirely due to the volume of animals used in the research or the nature of the procedures performed. However, anecdotal evidence from previous studies has clearly indicated subtle differences in resultant data when the experimental techniques applied have been performed by a number of investigators. [unpublished data, Helicobacter vaccine research group, UNSW]

As previously indicated, the principle aim of method development was in the refinement of procedure. Underpinning such refinement were ethical and fiscal considerations. The ethical obligation of the investigator towards the welfare of the animals was paramount. Methods were developed so as to have minimal physical impact and zero mortality. Additionally, the volume of animal numbers used was restricted in accordance with the ethos of reduction as per the guidelines for animals used for scientific purposes. However, all group sizes, and the number of controls incorporated, were designed to allow for valid statistical analysis whilst still operating within budgetary constraints.
4.3.1 Experimental Plan

To ascertain the efficacy of alternate routes of immunisation, specific pathogen free, female BALB/c mice aged ≈ 8 weeks were used as the animal model. The subject mice were prophylactically immunised with whole cell lysate of *H. pylori* (HpL) and/or cholera toxin (CT) via the three routes of intra-gastric, intra-nasal and intra-rectal. Test groups (n=10).

<table>
<thead>
<tr>
<th>Group Identification</th>
<th>Dose Rate</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Inoculated control</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B Intra-gastric adjuvant only</td>
<td>10 µg CT</td>
<td>100 µL</td>
</tr>
<tr>
<td>C Intra-gastric antigen + adjuvant</td>
<td>100 µg HpL + 10 µg CT</td>
<td>100 µL</td>
</tr>
<tr>
<td>D Intra-gastric antigen + adjuvant</td>
<td>10 µg HpL + 10 µg CT</td>
<td>100 µL</td>
</tr>
<tr>
<td>E Intra-nasal adjuvant only</td>
<td>10 µg CT</td>
<td>10 µL</td>
</tr>
<tr>
<td>F Intra-nasal antigen + adjuvant</td>
<td>100 µg HpL + 10 µg CT</td>
<td>10 µL</td>
</tr>
<tr>
<td>G Intra-nasal antigen + adjuvant</td>
<td>10 µg HpL + 10 µg CT</td>
<td>10 µL</td>
</tr>
<tr>
<td>H Intra-rectal adjuvant only</td>
<td>10 µg CT</td>
<td>100 µL</td>
</tr>
<tr>
<td>I Intra-rectal antigen + adjuvant</td>
<td>100 µg HpL + 10 µg CT</td>
<td>100 µL</td>
</tr>
<tr>
<td>J Intra-rectal antigen + adjuvant</td>
<td>10 µg HpL + 10 µg CT</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Mice were immunised twice via the three described routes at day 0 (priming dose) and day 21 (booster dose). An infected control group was incorporated as a control, administered a placebo (PBS) at day 0 and at day 21.

At four weeks post immunisation all groups were challenged with live *H. pylori*. A bacterial count of approximately $10^7$ organisms in a 100 µL suspension of BHI was delivered perorally to each animal by intra-gastric gavage.

At four weeks post challenge, mice were euthanased by CO$_2$ asphyxiation and stomachs harvested for bacterial culture. The level of *H. pylori* colonisation was determined by assessment of colony forming units as previously described.
4.3.2 Results

Animal body weights, pre- and post-experimental procedure were monitored and recorded over the course of the study as a general indicator of animal health. No significant difference was observed in animal body weights between the control and manipulated animals. Consistent weight gain was observed in all groups appropriate to weight for age as described in Table 4.2 and Figure 4.4.

Table 4.2 Animal Body Weights for Alternate Routes of Immunisation

Body weights (in grams) are expressed as the arithmetical mean (± standard deviation) for each group of 10 animals at designated time points.

<table>
<thead>
<tr>
<th>ID</th>
<th>Pre Immunisation (≈ 8 weeks old)</th>
<th>Post Immunisation (≈ 12 weeks old)</th>
<th>Post Challenge (≈ 16 weeks old)</th>
<th>At Harvest (≈ 20 weeks old)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>23.6 ± 1.3</td>
<td>24.7 ± 1.3</td>
<td>25.5 ± 1.2</td>
<td>26.3 ± 1.4</td>
</tr>
<tr>
<td>B</td>
<td>24.0 ± 1.6</td>
<td>25.2 ± 1.3</td>
<td>25.7 ± 2.0</td>
<td>26.0 ± 1.7</td>
</tr>
<tr>
<td>C</td>
<td>24.6 ± 1.2</td>
<td>25.3 ± 1.4</td>
<td>26.0 ± 1.2</td>
<td>26.4 ± 1.5</td>
</tr>
<tr>
<td>D</td>
<td>23.9 ± 1.7</td>
<td>25.2 ± 1.4</td>
<td>25.8 ± 1.5</td>
<td>25.9 ± 1.4</td>
</tr>
<tr>
<td>E</td>
<td>24.0 ± 2.1</td>
<td>24.6 ± 1.9</td>
<td>25.5 ± 1.7</td>
<td>25.9 ± 1.6</td>
</tr>
<tr>
<td>F</td>
<td>24.2 ± 1.5</td>
<td>24.9 ± 1.7</td>
<td>25.4 ± 2.1</td>
<td>26.0 ± 1.9</td>
</tr>
<tr>
<td>G</td>
<td>23.9 ± 2.1</td>
<td>24.5 ± 1.7</td>
<td>25.8 ± 1.6</td>
<td>26.8 ± 1.2</td>
</tr>
<tr>
<td>H</td>
<td>24.8 ± 1.7</td>
<td>25.5 ± 1.0</td>
<td>26.2 ± 1.9</td>
<td>26.8 ± 1.8</td>
</tr>
<tr>
<td>I</td>
<td>23.9 ± 2.1</td>
<td>24.4 ± 1.8</td>
<td>25.2 ± 1.8</td>
<td>25.7 ± 1.6</td>
</tr>
<tr>
<td>J</td>
<td>24.4 ± 1.9</td>
<td>25.3 ± 1.6</td>
<td>26.0 ± 1.5</td>
<td>26.4 ± 1.7</td>
</tr>
</tbody>
</table>

Additionally, no discernible difference in behavioural function from continual daily observation of all animals was perceived between any of the groups prior to, during and at completion of procedure.
Figure 4.4 Comparison of Body Weights for Alternate Routes of Immunisation

Mean group body weights recorded during course of experimental procedure.
LEGEND

A  I/G 0.1 M PBS
B  I/G 10 μg CT
C  I/G 100 μg HpL + 10 μg CT
D  I/G 10 μg HpL + 10 μg CT
E  I/N 10 μg CT
F  I/N 100 μg HpL + 10 μg CT
G  I/N 10 μg HpL + 10 μg CT
H  I/R 10 μg CT
I  I/R 100 μg HpL + 10 μg CT
J  I/R 10 μg HpL + 10 μg CT

Figure 4.5 Bacterial Colonisation following Alternate Routes of Immunisation (1)
Level of *H. pylori* colonisation following immunisation and challenge in BALB/c mice. Data expressed as arithmetical means of colony forming units per gram of stomach tissue (error bars indicate standard deviation) in 10 animals.
Colonisation levels of the infected control group were in the order of $10^5$ organisms per gram of stomach tissue, thus corresponding well with the nominal infection levels for the strain of mouse used. The significant differences between all groups are illustrated in Table 4.3

### Table 4.3 Statistical Analysis for Alternate Routes of Immunisation

Groups of animals that have significantly different levels of bacterial colonisation from any other group are designated by ○ with a significance level of $p < 0.05$. As an example, group B is significantly different from groups A, C, D, F & G, but not significantly different from groups E, H, I & J

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (I/G PBS)</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>B (I/G 10 µg CT)</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>C (I/G 100 µg HpL + 10 µg CT)</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>D (I/G 10 µg HpL + 10 µg CT)</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>E (I/N 10 µg CT)</td>
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<td>I (I/R 100 µg HpL + 10 µg CT)</td>
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<td>J (I/R 10 µg HpL + 10 µg CT)</td>
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All groups were significantly different from the infected control group. The intra-gastrically administered 100 µg HpL + 10 µg CT and the corresponding intra-nasally administered 100 µg HpL + 10 µg CT were significantly different from all other groups. These two immunisation regimes were not significantly different from each other. All rectally administered groups (adjuvant only and both antigen/adjuvant complexes) were not significantly different from each other. All three rectally immunised groups were not statistically different from either the intra-gastrically or intra-nasally administered adjuvant only or antigen/adjuvant complex of 10 µg HpL + 10 µg CT.
4.4 IMMUNE RESPONSE OF ALTERNATE ROUTES

As previously discussed, other investigators have demonstrated significant immune responses to *H. pylori* immunisation via a diversity of mucosal routes of administration. The purpose of the following experiment was to verify these findings in the mouse model of infection used within this study. Additionally, it was anticipated that a correlation could be made between any immune responses generated and the prophylaxis observed from subsequent bacterial challenge.

4.4.1 Experimental Plan

To compare the immune response of intra-gastric, intra-nasal and intra-rectal immunisation, specific pathogen free, female BALB/c mice aged = 8 weeks were used as the animal model. The subject mice were prophylactically immunised with whole cell lysate of *H. pylori* (HpL) + cholera toxin (CT). Test groups (n=10).

<table>
<thead>
<tr>
<th>Group Identification</th>
<th>Dose Rate</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>A  Infected control</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B  Intra-Gastric immunisation</td>
<td>100 μg HpL + 10 μg CT</td>
<td>100μL</td>
</tr>
<tr>
<td>C  Intra-Nasal immunisation</td>
<td>100 μg HpL + 10 μg CT</td>
<td>10μL</td>
</tr>
<tr>
<td>D  Intra-Rectal immunisation</td>
<td>100 μg HpL + 10 μg CT</td>
<td>100μL</td>
</tr>
</tbody>
</table>

At commencement of the study, all groups were anaesthetised by intra-peritoneal administration of Ketamine and Xylazine (50 mg/kg each) and 100μL of blood collected by cardiac puncture.

At one week post bleed, following a recovery period, mice were immunised twice via the three prescribed routes at day 0 (priming dose) and day 21 (booster dose). Blood samples were again taken from all groups as previously described at three weeks post immunisation.
At four weeks post immunisation all groups were challenged with live *H. pylori*. A bacterial count of approximately $10^7$ organisms in a 100 µL suspension of BHI was delivered perorally to each animal. At four weeks post challenge, mice were euthanased by CO$_2$ asphyxiation and stomachs harvested. The level of *H. pylori* colonisation was determined by assessment of colony forming units.

### 4.4.2 Results

No mortalities were recorded and no discernible difference in behavioural function from continual daily observation of all animals was perceived between any of the groups following blood collection from cardiac puncture.

The detection of an immune response indicated no significant difference between the three routes of intra-gastric, intra-nasal and intra-rectal immunisation. All immunised animals produced statistically significant elevated levels of circulating IgG antibody levels compared to the non-immunised group.

Comparative analysis by viable plate counts (refer Figure 4.7) indicates that intra-gastric and intra-nasal immunisation each produced a significant prophylactic response exhibiting a bacterial load of $2.28 \times 10^4$ CFU/gram and $1.39 \times 10^4$ CFU/gram respectively. The two routes were not statistically different from each other. The intra-rectal immunisation group elicited a mid-level reduction in colonisation, with a bacterial load of $1.33 \times 10^5$ CFU/gram, and was significantly different from both intra-gastric and intra-nasal immunisation. Intra-rectal, though whilst indicating a reduced level of infection, was not significantly different from the uninfected control group. This result was consistent with the data obtained from the previous experiment of examination of efficacy of alternate routes of immunisation (refer section 4.3.2).
LEGEND

A  Infected control (Nil)
B  Intra-gastric immunisation (100 μg HpL + 10 μg CT)
C  Intra-nasal immunisation (100 μg HpL + 10 μg CT)
D  Intra-rectal immunisation (100 μg HpL + 10 μg CT)

Figure 4.6 Immune Response of Alternate Routes of Immunisation

Serum IgG antibody response to *H. pylori* whole cell lysate in immunised BALB/c mice (10 animals per group). Data expressed as arithmetical means (error bars indicate standard deviation).
Figure 4.7 Bacterial Colonisation following Alternate Routes of Immunisation (2)

Level of *H. pylori* colonisation following immunisation and challenge in BALB/c mice. Data expressed as arithmetical means of colony forming units per gram of stomach tissue (error bars indicate standard deviation) in 10 animals.
4.5 DISCUSSION

There were three major areas of interest resulting from the investigation of alternate mucosal routes of immunisation. First, the practical aspects of vaccine administration via the various routes involving the technical procedure and impact upon the animal subject were evaluated. Secondly, the comparison of efficacy of prophylactic immunisation against bacterial challenge for each route was assessed. Finally, the immune response generated from each route of immunisation was examined and any correlation between such a response and prophylaxis was discussed.

4.5.1 Validation of Methodologies

An important aspect of this work was to develop and refine alternate mucosal routes of immunisation with relevance to the practical aspects of vaccine administration. There were two key areas of interest to this component of the investigation. First, the procedures had to have minimal impact upon the animal subject. Secondly, the procedures were evaluated on the basis of operator ease and efficiency.

Similar to the monitoring of those animals that underwent Peyers' patch injection, comparisons of body weights were used as an indicator of health status. All groups, without exception, gained weight applicable to age and strain throughout the course of experimental procedure. Additionally, no mortalities were experienced and all subjects appeared normal in physiological and behavioural function. As discussed in Chapter 3, the outcome of no detrimental effects was important from not only an ethical standpoint but from the scientific aspect as well.

Further to the aspect of procedure impacting on data and animal health, any inclusion of an unnecessary variable was considered unacceptable for competent comparative analysis. Consequently, the major experiment used to determine prophylactic efficacy (section 4.3) was conducted independently from the experiment used to ascertain the immune response (section 4.4), whereby mice were anaesthetised and blood collected from cardiac puncture. However, as the animals used for the latter experiment were immunised, they afforded the
opportunity to repeat previous observations following bacterial challenge and subsequent assessment of prophylaxis. From examination of the prophylactic responses illustrated in Figure 4.5 and the corresponding regimes depicted in Figure 4.7, it is obvious that the data in the first experiment was replicated in the second. Therefore it is reasonable to theorise, without stating conclusively, that in this instance, the technique of cardiac puncture with associated surgical anaesthesia produces no discernible effect to the investigation.

As detailed in section 4.2, all three methodologies of intra-gastric, intra-nasal and intra-rectal immunisation are simple in the nature of equipment required and performance of the procedure. Without consideration for targeting specific immune initiator sites, the personal preference of the operator would no doubt dictate the choice of methodology. Anecdotal evidence would suggest a predisposition by investigators to use intra-gastric gavage, primarily in light of its employment to routinely experimentally infect small laboratory animals. However, there is marginally more skill required to gavage an animal or use a rectal probe than the simple use of a pipette to apply an aliquot external to the nares. Additionally, of the three procedures, intra-nasal administration, is the only delivery method where an implement is not physically inserted into an orifice. It would not be unreasonable to presume that the less invasive the technique, the less stress the animal would experience. Not discounting the issue of ethics, the addition of any stress related factors could influence scientific data.

4.5.2 Efficacy of Alternate Routes of Immunisation

From examination of the bacterial burden of all groups challenged, the data would indicate that there was a prophylactic effect that corresponds directly to the proximity of vaccine administration to that of specific sites of immunity. Intra-gastric and intra-nasal immunisation, when delivered at the dose rate ascertained as optimal by intra-intestinal immunisation, produced significant prophylactic responses. The same dose rate of 100 μg HpL + 10 μg CT when delivered via the rectal route was statistically less efficacious. Apparently, immunisation at the initiator sites of the nasal associated lymphoid tissue and that of the intestinal Peyers' patches have stimulated a significantly greater prophylactic response in the closely related effector site of the stomach than that exhibited by distal
initiator sites associated with the colon. Such an outcome would possibly be anticipated if comparatively low antibody titres had been detected in those animals immunised intrarectally. However, in view of the significant systemic immune responses generated by all three routes of immunisation, the data is consistent with other investigations of compartmentalisation, where immunogens introduced at one inductive site may evoke an immune response at adjacent or physiologically related sites, with the response generated being proportionately reduced at more distal and/or less related effector sites.\textsuperscript{[53, 94, 114, 134, 138]}

Comparing the arithmetical means between intra-nasal and intra-gastric when the antigen/adjuvant complex was administered at a ratio of 10:1, indicates that while there was no statistical significant difference in prophylaxis, intra-nasal was arguably superior in eliciting a prophylactic response. Upon critical assessment of these two routes, the degree of error, as indicated by standard deviation, was greater in those groups immunised intra-gastrically. Even more revealing however was the resultant data from the antigen/adjuvant ratio of 1:1 (10 µg HpL + 10 µg CT) delivered by intra-nasal administration, which produced statistically greater reduction of infection than the corresponding regime for intra-gastric. While this reduction was less than that recorded for the 10:1 relationships, it is clearly evident that over a range of dose rates, intra-nasal immunisation is the most efficacious against \textit{H.pylori} challenge.

An interesting outcome from intra-rectal immunisation was that regardless of the dose rate, either antigen/adjuvant complexes or adjuvant alone, a prophylactic response against \textit{H.pylori} challenge was elicited from this route when analysed against the infection control group. All intra-rectal regimes were statistically similar, which suggests that a level of prophylaxis against \textit{H.pylori} was independent of an antigen/adjuvant combination when delivered by this route. In other words, apparently the mucosal adjuvant cholera toxin was solely responsible for the prophylactic effect observed in all three intra-rectal regimes. In counterpoint to the possible argument that the technical procedure itself was responsible for the prophylactic effect, the correlation between all three routes administered adjuvant only is suggestive that cholera toxin is indeed generating an immune response. Needless to say, a comparative analysis between the administration of cholera toxin and a placebo, via the routes in question, would be required to confirm or refute this observation. However, in studies such as the work of Kleanthous \textit{et al}\textsuperscript{[76]} examining intra-rectal and intra-nasal
immunisation strategies, there has been no suggestion of a placebo effect arising purely from the method of administration.

Of particular interest was the observation that by all three routes, adjuvant only elicited a protective response significantly different from the infected only control animals. Why this is so may be open to conjecture. Possibly, a humoral immune response is being elicited from the generation of antibody producing cells, thus bearing upon colonisation. In conflict with this speculation, is that it is widely acknowledged that whilst high titres of IgA are detectable following immunisation with cholera toxin, protective immunity against *Helicobacter* is inconsequential.\(^{[76, 150, 165]}\) Most importantly however was that there was no indication of any such response from those animals administered cholera toxin by direct injection of the Peyers’ patches as examined in Chapter 3. A possible hypothesis may be that the introduction of such a potent mucosal adjuvant is somehow integrating with unidentified antigenic material when administered via the mucosal surfaces. The animals used throughout this study were known to be free of all gastric *Helicobacters’,* however the inclusion of intestinal and lower bowel *Helicobacters’* was undefined. In further explanation, it may be reasoned that the administration of cholera toxin via a number of mucosal routes is not entirely absorbed at the sites of specific immunity in the first instance. Partial amounts may in fact encounter antigenic material analogous to *H. pylori* thus forming an antigen/adjuvant complex sufficient to initiate a degree of protective immunity. Such a possibility could be circumvented when cholera toxin is delivered directly to sites of specific immunity, bypassing any such antigenic material, as is the case for intra-intestinal immunisation.

### 4.5.3 Immune Response of Alternate Routes of Immunisation

Due to the recent discovery of *H. pylori* and its ability to colonise a hitherto supposed sterile organ, the stomach has become a relatively new site of interest to immunologists. Comparatively little is known about the immunology of the stomach, but what is appreciated is that there does exist a full compliment of immune cells. Immunoglobulins IgG, IgM and IgA are found in the mucosa and gastric juices.\(^{[158, 172]}\) CD4+ and CD8+ T cells in similar proportion to B cells are found in the gastric mucosa.\(^{[157]}\) Additionally,
specialised antigen presenting cells such as dendritic cells are apparent in the gastric tissue,\cite{22} which led Ernst et al\cite{137} to surmise that antigen sampling and consequently the induction of an immune response could be taking place within the stomach. Accepting this premise and with respect to the findings of the present study, it could be reasoned that certainly through the administration of vaccines via the intra-gastric and intra-nasal routes, the stomach could well indeed perform antigen sampling. As previously described in the section detailing methodologies, intra-gastric delivers test substances directly to the stomach, and intra-nasal, whilst depositing an aliquot to nasal associated lymphoid tissue, may in fact see a portion arrive at the stomach through normal physiological processes.

The immune response elicited from those animals immunised produced statistically similar levels of circulating antibody, with all three routes of intra-gastric, intra-nasal and intra-rectal administration exhibiting an anti-\textit{H. pylori} IgG response significantly higher than the non-immunised animals. However, upon closer examination of Figure 4.6, intra-rectal immunisation produced a noticeably higher antibody response to that of intra-gastric and intra-nasal immunisation. This data did not correlate directly with that observed for the prophylactic response. Prior to examination of the immune response, it was anticipated from the results of prophylactic efficacy, that intra-nasal and intra-gastric immunisation may elicit higher levels of IgG than that of intra-rectal immunisation. This assumption was also based on \textit{in vivo} studies investigating alternate routes of immunisation against other mucosal associated pathogens. For instance, Staats et al\cite{146} described a superior vaginal anti-HIV antibody response in mice immunised nasally compared to those animals immunised rectally or vaginally. However, with relation of the immune response to \textit{H. pylori} examined here, those animals immunised rectally exhibited appreciably higher antibody levels, and it may be reasoned that whilst inductive sites distal to the effector site of the stomach were indeed generating a distinct systemic immune response, a significant immune against a gastric pathogen such as \textit{H. pylori} was unattainable due the previously described concept of compartmentalisation.\cite{194,154,138}

In a study of a similar nature, Kleanthous et al\cite{176} reported considerable levels of anti-urease immunoglobulin A (IgA) in mice immunised against \textit{H. pylori} with recombinant urease and heat-labile toxin of \textit{Escherichia coli} (LT) via the oral, nasal and rectal routes. This outcome was suggestive that significant prophylaxis against \textit{H. pylori} was possible
via all three routes. However, the importance of IgA with respect to immunisation against *Helicobacter* has been drawn into question. It was originally suggested that secretory IgA was a major contributing constituent for prophylaxis against *H. pylori* infection.\(^{21, 90, 124}\) Later studies have since contradicted this assumption, and it is now evident that whilst high titres of IgA are detectable in animals immunised with *Helicobacter*-antigen in the absence of adjuvant, they are not protected from subsequent bacterial challenge.\(^{76, 150, 165}\)

Recalling the earlier discussion of the mucosal immune system, M cells of the intestinal Peyers’ patches, colonic mucosae and nasal associated lymphoid tissue are believed to be responsible for generation of a mucosal immune response. *In vivo* studies of peroral administration of cholera toxin by Svennerholm *et al.*\(^{153}\) and Lycke *et al.*\(^{96}\) have shown that such a response is generated at the intestinal level with antibody secreting cells found in mucosal effector sites. Yet again, many assumptions made about immunological processes can be contradictory. To wit, Sutton *et al.*\(^{150}\) used mice lacking B lymphocytes (\(\mu\)MT mice) in a successful therapeutic immunisation regime against *H. pylori* infection. This would suggest that whilst an immune response was generated, resulting in reduction of bacterial colonisation, the mechanism for such a response was antibody independent. How therapy was achieved in this instance remains to be determined with the authors indicating that some novel immune mechanism may be involved. This opinion would appear to be reinforced from the evidence of intra-rectal immunisation presented herein of high antibody levels being contradictory to the corresponding level of prophylactic efficacy. Clearly, a comprehensive understanding of the mucosal immune system associated with *Helicobacter* infection may require many more years of investigation.

Whilst intra-rectal immunisation proved to elicit markedly lower levels of prophylaxis comparative to that of intra-gastric and intra-nasal immunisation, the fact that a significant humoral immune response was generated from this route is encouraging for future studies. The data from the Kleanthous investigation,\(^{76}\) in addition to results obtained from the present study would suggest that whilst all three routes are capable of stimulating a significant immune response, the intra-rectal route of immunisation may have particular advantages. For example, an important issue when considering the oral use of potent mucosal adjuvants such as cholera toxin is the unpleasant side effect of diarrhoea. If effective mucosal delivery could bypass the stomach and small intestine, then such
complications may be reduced. A further factor in favour of intra-rectal immunisation over that of the intra-gastric immunisation is that it may circumvent degradation and denaturation of antigens by enzymes and gastric fluids and deliver immunogens intact to sites of specific immunity.[71]

Finally, although the effector mechanisms of immunity against *H. pylori* are not entirely understood, the fact that a significant humoral immune response is unquestionably generated by intra-rectal immunisation gives strong indication that this route warrants continued investigation. Furthermore, as discussed in the present study the importance of the concept of compartmentalisation suggests that the use of the intra-rectal administration of vaccines may prove to be an invaluable strategy against lower bowel *Helicobacters’* and possibly other colonic/caecal associated pathogens.
Chapter 5
Relationship between Antigen & Adjuvant

5.1 INTRODUCTION

The immunogenicity of foreign proteins administered in the absence of an adjuvant is low, with studies such as Michetti et al and Kreiss et al confirming that significantly higher antibody levels are produced when an adjuvant is included in vaccine preparations. Furthermore, the murine work of Lycke et al demonstrated that the sole administration of incremental amounts of the mucosal adjuvant cholera toxin reached a plateau at 10 μg per dose for the production of an immune response. These findings have influenced numerous investigations of immunisation in a mouse model of Helicobacter infection. The vaccines used in these studies typically incorporated relatively large concentrations of antigenic component in comparison to only a moderate proportion of adjuvant, however, little attention has been placed on the relationship between antigen and adjuvant with respect to the importance of the ratio between the two fundamental vaccine constituents.

Data from the previous two chapters of this study have suggested that a specific ratio between the dose rate of antigen and the dose rate of adjuvant may be required to elicit a protective immune response. The first recognisable instance of some such phenomena was noticed in the data obtained from the optimisation of intra-intestinal immunisation. As discussed in section 3.4.2, the animals immunised with antigen plus adjuvant complexes at a ratio of 10:1 produced the greatest levels of prophylaxis. A similar trend was observed from the data of Chapter 4 (refer section 4.5.2). Using a vaccine of H. pylori whole cell lysate and cholera toxin, the data contained within this chapter aims to define any specific ratio between antigen and adjuvant which correlates to significant prophylaxis in a mouse model of H. pylori infection.
5.2 IMMUNISATION WITH RATIOS OF ANTIGEN & ADJUVANT

To investigate the possibility that a ratio effect between vaccine constituents is of considerable importance and is consistent throughout a range of dose rates, intra-nasal administration was chosen as the most appropriate route of delivery for a number of reasons. In the first instance, targeting the nasal associated lymphoid tissue circumvents the possibility of acid degradation of the vaccine and other previously described deleterious factors associated with intra-gastric administration. Secondly, previous data from Chapter 4 has indicated that intra-nasal immunisation provides reduced statistical errors over the range of dose rates trialed. The third point for consideration was that intra-nasal immunisation was demonstrated to be a superior route compared to that of intra-gastric and intra-rectal in eliciting a prophylactic response over a limited range of antigen/adjuvant complexes. Finally, the simplistic nature of the technique, both in operator ease and cost effectiveness, recommended intra-nasal immunisation as the most suitable methodology for further investigative work.

In the present study, a vaccine preparation consisting of H. pylori whole cell lysate and cholera toxin has already been used to examine the prophylactic efficacy of intra-intestinal immunisation and alternate mucosal routes of vaccine administration. Although it is improbable that any potential H. pylori vaccine intended for commercial use would use such a crude vaccine, its employment in the laboratory, whilst economically producing relatively large volumes of antigen, adequately allows for the investigation of specific theories. However, due to issues such as the nature of antigen preparation, the possible risk of contamination, bacterial cell characteristics and variances between strains of H. pylori, whole cell vaccines are difficult to produce in a standardised form. Therefore, in an attempt to partially eliminate these variables, a single batch of antigen was prepared from the H. pylori mouse-adapted Sydney Strain 1 (SS1) and used solely for the entire course of the study including the examination of the relationship between antigen and adjuvant.

The concentrations and dose rates of antigen and adjuvant investigated in this chapter were based upon data obtained from Chapters 3 and 4, and structured about the hypothesis that an antigen:adjuvant ratio of 10:1 elicit the highest levels of protective immunity.
5.2.1 Experimental Plan

To ascertain the significance of an antigen/adjuvant relationship, specific pathogen free, female BALB/c mice aged = 8 weeks were selected as the animal model based upon their use and findings from previous work of this study.

As described in Table 5.1, test groups (n=10) were prophylactically immunised intranasally with either antigen only, adjuvant only or combinations of antigen plus adjuvant. An infected control group was administered a placebo of 0.1 M phosphate buffered saline (PBS) at a time schedule corresponding to all other test groups. Whole cell lysate of H. pylori (HpL) was used as the antigen and cholera toxin (CT) as the mucosal adjuvant. All test substances were administered at a volume of 10 µL per mouse per dose.

Table 5.1 Relationship between Antigen & Adjuvant Experimental Regime

<table>
<thead>
<tr>
<th>Group Identification</th>
<th>Dose Rate</th>
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<tbody>
<tr>
<td>A Infection control (placebo)</td>
<td>0.1 M PBS</td>
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<tr>
<td>B Antigen (HpL) only</td>
<td>100 µg HpL</td>
</tr>
<tr>
<td>C Adjuvant (CT) only</td>
<td>20 µg CT</td>
</tr>
<tr>
<td>D High dose antigen + adjuvant (ratio = 5:1)</td>
<td>100 µg HpL + 20 µg CT</td>
</tr>
<tr>
<td>E High dose antigen + adjuvant (ratio = 10:1)</td>
<td>100 µg HpL + 10 µg CT</td>
</tr>
<tr>
<td>F High dose antigen + adjuvant (ratio = 20:1)</td>
<td>100 µg HpL + 5 µg CT</td>
</tr>
<tr>
<td>G Mid dose antigen + adjuvant (ratio = 5:1)</td>
<td>50 µg HpL + 10 µg CT</td>
</tr>
<tr>
<td>H Mid dose antigen + adjuvant (ratio = 10:1)</td>
<td>50 µg HpL + 5 µg CT</td>
</tr>
<tr>
<td>I Mid dose antigen + adjuvant (ratio = 20:1)</td>
<td>50 µg HpL + 2.5 µg CT</td>
</tr>
<tr>
<td>J Low dose antigen + adjuvant (ratio = 2:1)</td>
<td>20 µg HpL + 10 µg CT</td>
</tr>
<tr>
<td>K Low dose antigen + adjuvant (ratio = 5:1)</td>
<td>20 µg HpL + 4 µg CT</td>
</tr>
<tr>
<td>L Low dose antigen + adjuvant (ratio = 10:1)</td>
<td>20 µg HpL + 2 µg CT</td>
</tr>
<tr>
<td>M Low dose antigen + adjuvant (ratio = 20:1)</td>
<td>20 µg HpL + 1 µg CT</td>
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</table>

The antigen/adjuvant complexes were defined as either high dose (100 µg HpL), mid dose (50 µg HpL) or low dose (20 µg HpL) with each HpL dose rate being further subdivided into antigen/adjuvant ratios of 5:1, 10:1 and 20:1.
Figure 5.1 Bacterial Colonisation following Ratios of HpL:CT Immunisation (1)
Level of *H. pylori* colonisation following intra-nasal immunisation with ratios of antigen and adjuvant and challenge in BALB/c mice. Data expressed as arithmetical means of colony forming units per gram of stomach tissue (error bars indicate standard deviation) in 10 animals.
The significant differences between all groups are illustrated in Table 5.2.

Table 5.2 Statistical Analysis for Ratios of HpL:CT Immunisation (1)

Groups of animals that have significantly different levels of bacterial colonisation from any other group are designated by ● with a significance level of $p < 0.05$. As an example, group B is significantly different from groups D, E, F, G, H, I, J, K, L & M but not significantly different from groups A & C.

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<tr>
<td>A (0.1 M PBS)</td>
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<td>B (100 µg HpL)</td>
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<td>C (20 µg CT)</td>
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<td>D (100 µg HpL + 20 µg CT)</td>
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All groups that were immunised with an antigen plus adjuvant complex elicited a prophylactic response exhibiting a statistical significant difference from that of the infected control group and in addition to those animals administered with antigen only and adjuvant only. A significant difference was observed between the adjuvant only group (20 µg CT) and the infection control group.

To provide a clearer indication of variance between the antigen/adjuvant immunised animals only, statistical analysis was performed in the absence of all control groups, that is, the infection control, antigen only and adjuvant only groups were excluded. These groups had high infection levels ($10^5$ CFU/gram of stomach) compared to low level infection ($10^4$ CFU/gram of stomach) all other immunised groups.
Figure 5.2 Bacterial Colonisation following Ratios of HpL:CT Immunisation (2)
Level of *H. pylori* colonisation (excluding control groups of placebo, antigen only and adjuvant only) following intra-nasal immunisation with ratios of antigen and adjuvant and challenge in BALB/c mice. Data expressed as arithmetical means of colony forming units per gram of stomach tissue (error bars indicate standard deviation) in 10 animals.
The significant differences between all groups immunised with an antigen/adjuvant complex are illustrated in *Table 5.3*.

*Table 5.3 Statistical Analysis for Ratios of HpL:CT Immunisation (2)*

Statistics performed in the absence of all control groups. Groups of animals that have significantly different levels of bacterial colonisation from any other group are designated by • with a significance level of p < 0.05. As an example, group D is significantly different from groups E, J & K, but not significantly different from groups F, G, H, I, L & M.

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<th>D (100 µg HpL + 20 µg CT)</th>
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The data presented in *Table 5.3* indicates that the high dose at 10:1 antigen/adjuvant ratio was significantly different from:

- High dose: Ratio 5:1
- Mid dose: Ratio 5:1
- Mid dose: Ratio 20:1
- Low dose: Ratio 2:1
- Low dose: Ratio 5:1
- Low dose: Ratio 20:1

There was no recorded significant difference between any of the groups at an antigen/adjuvant ratio of 10:1.
5.3 DISCUSSION

The administration of the adjuvant alone (CT only) produced a level of protection that was significantly different compared to that of the infected control animals and was consistent with that observed and discussed in the preceding chapter.

As presented in Figure 5.1 and Table 5.2, the mice immunised with ratios of antigen and adjuvant, ranging from the high to low concentration of the constituents, were protected from bacterial challenge. All groups exhibited a statistically significant reduction in colonisation from that of the three control groups of infected only, antigen only and adjuvant only, and provides further evidence of a wide degree of immunisation tolerance capable of providing protection from *H. pylori* challenge. However, whilst all groups immunised with antigen and adjuvant produced significant prophylaxis, there were subtle differences between those groups. Statistical examination of the data in the absence of all control groups (Figure 5.2 & Table 5.3) clearly indicated a specific relationship between the dose rate of antigen in conjunction with the dose rate of adjuvant to provision for optimal protective immunity. An antigen/adjuvant ratio of 10:1 proved to elicit the highest levels of prophylaxis against bacterial challenge. This result for a ratio of 10:1 was consistent across the range of the three dose rates trialed.

The reduction of cholera toxin proportional to the amount of whole cell lysate (20:1 ratio) produced a reduction in prophylaxis comparable to that of the 10:1 ratio. It may be reasoned that the reduction in cholera toxin equates to the original study of Lycke et al.[93] whereby incremental amounts of cholera toxin when administered via mucosal routes reached a plateau at 10 µg in producing an immune response. In argument to this explanation is that when the dose rate of whole cell lysate was reduced so that a 10:1 antigen/adjuvant existed, significant prophylaxis was attained.

Even more interesting however is that an increase in cholera toxin proportional to the amount of whole cell lysate (5:1 ratios) resulted in a similar reduction in prophylactic efficacy to that of the 20:1 antigen/adjuvant groups. This trend was exacerbated with a reduced ratio (2:1). A possible explanation may lie at the biochemical level where it is
An additional antigen/adjuvant ratio of 2:1 was added to the low dose group for further evaluation of any trend. This ratio was not included in the mid and high range dosages as it would have required administration of 25 µg CT and 50 µg CT respectively. Such concentrations were excluded from the study for three reasons. In the first instance, cholera toxin is an expensive product, therefore, it was required to be used judiciously. Secondly, the gastrointestinal toxicity of such concentrations upon mice was undetermined, therefore the physiological impact and ethical obligations could possibly be compromised. Finally, ethics approval for this study was granted on the basis of previously approved investigations with reference to the maximum permissible dose rate of cholera toxin being 20 µg per mouse.

All mice were immunised twice via the intra-nasal route at day 0 (priming dose) and day 21 (booster dose). At four weeks post immunisation all animals from all groups were challenged with live *H. pylori*. A bacterial count of approximately $10^7$ organisms in a 100 µL suspension of BHI was delivered perorally to each animal.

At four weeks post challenge, mice were euthanased by CO₂ asphyxiation and their stomachs harvested. The level of *H. pylori* colonisation was ascertained by assessment of colony forming units as previously described (refer method 2.9.2).

### 5.2.2 Results

Animal health was monitored for the duration of the experiment, and physiological and behavioural function was unaltered in any of the groups. No mortalities were recorded over the course of the investigation.

Colonisation levels of the infected control group were in the order of $10^5$ colony forming units per gram of stomach tissue, thus corresponding well with the nominal infection levels for the strain of mouse used. Similarly, animals administered antigen only exhibited were not significantly different from the infected controls.
feasible that there exists a requirement for specific concentrations of the vaccine constituents, and their interaction, for an effective immunogen to be established.

Demonstrated by the data contained herein and previously by independent investigations,\textsuperscript{[14, 135]} it is accepted that a whole cell lysate of \emph{H. pylori} can stimulate protective immunity of varying degrees when immunising mice in conjunction with the mucosal adjuvant cholera toxin. It was evident from this investigation that whole cell lysate and cholera toxin combined at a ratio in the order of 10:1 between antigen and adjuvant respectively, suggested optimal prophylactic immunisation against \emph{H. pylori} challenge regardless of the dose rates trialed.

\emph{H. pylori}, like any organism, is composed of many antigens, therefore, it is also possible that the relationship between antigen and adjuvant is proportional to the antigenic component of whole cell lysate. Additionally, whole cell lysate of \emph{H. pylori} contains material with adjuvant properties such as lipopolysaccharides.\textsuperscript{[115]} Nevertheless, if the instance of reproducible ratio effects is recognisable, this reinforces the established theory of ratio importance. To further explore this phenomenon, a single purified \emph{H. pylori} antigen, such as Catalase, could be used in conjunction with cholera toxin. Because Catalase constitutes approximately 3\% of total cell protein for \emph{H. pylori},\textsuperscript{[55]} it could be reasoned that a ratio proportional to this percentage of the constituent might exist between catalase and cholera toxin for optimal prophylaxis. Similarly Urease, another \emph{H. pylori} antigen approximating 6\% of whole cell lysate could be singly or even in combination with another protein(s).\textsuperscript{[29]} A beneficiary of such an investigation would be that although a number of investigations have shown the potential for a successful vaccine by using crude antigen preparations, it would be unlikely that this would be used as standard practice in a commercially viable product. A single antigen or multiple purified antigens in synergy would be the most likely candidates.
Chapter 6
Conclusions & Future Studies

6.1 CONCLUSIONS

The development of immunisation strategies for diseases linked to mucosal associated pathogens in both humans and animals is reliant upon efficient, effective and ethical experimentation using living animals. The studies described herein have provided valuable scientific information regarding the further development of immunisation strategies against the human pathogen Helicobacter pylori. These studies conducted in a mouse model of H. pylori infection were underpinned by the obligation of the investigator to ascertain that the welfare of the animals involved was maintained throughout the duration of the investigation. Furthermore, and underlining the significance of this work, was that the outcomes of this study have clearly shown the necessity for optimisation of many of the variables associated with immunisation in an animal model.

Whilst providing important data, previous studies examining the efficacy of potential vaccines against H. pylori infection in an animal model have drawn their conclusions from immunisation strategies that have never been fully optimised.\textsuperscript{[26, 27, 50, 76, 77, 90, 135]} The assumptions prior to the present study relating to dose rates, the most efficacious route of administration and the importance of the relationship between the vaccine constituents of antigen and adjuvant are likely to have been less than ideal.

In the current study, prophylactic immunisation against H. pylori infection in a mouse model was further optimised by a comparison of the efficacy of alternate routes of vaccine administration and the determination of the optimal vaccine constituents and dose rates.
The initial investigation of this study was to optimise the route of presentation of antigen to inductive sites of the mucosal immune system by the administration of a *H. pylori* vaccine to the Peyers’ patches in the mouse model. The direct injection of whole cell lysate of *H. pylori* and cholera toxin into the Peyers’ patches showed these sites to be responsible for the generation of a significant prophylactic response against subsequent bacterial challenge, and provided proof of principle that intra-intestinal immunisation was an important technique for future evaluation of potential *H. pylori* vaccine candidates. Furthermore, this route of administration established baseline data for vaccine dose rates from maximal intestinal immunisation by circumventing gastric acidity, poor absorption and breakdown by enzymes associated with peroral administration,\(^{16, 122}\). Immunisation via the intra-intestinal route revealed that only 0.1% of the dose typically used for peroral immunisation was required for a significant prophylactic response and provided the first evidence that a specific ratio between the antigen and adjuvant components of the vaccine may be essential for successful prophylaxis.

Notwithstanding the importance of these findings, the data would have been compromised if the technique of intra-intestinal immunisation affected animal wellbeing. Any bias upon the data as a direct result of the procedure would not only call into question the technique as a reliable investigative device but more importantly, the ethical consideration for the welfare of the animals would have been breached. However, despite the invasive nature of the methodology, no detectable evidence of any such influence was observed on the animals’ health, thus recommending this to be a suitable technique for further investigative purposes with the potential to provide valuable information for vaccine development against other gastro-intestinal pathogens. For example, given that the rectal mucosa is known to contain lymphoepithelial structures comparable to the Peyer’s patches of the small intestine, the same principles of methodology could be applied to the development of immunisation strategies against pathogens that infect the large bowel.\(^{110, 81, 122, 147}\)

The results obtained from intra-intestinal immunisation provided the background data to commence evaluation of alternate routes of immunisation at other sites of mucosal associated lymphoid tissue. Although previous investigations such as that by Kleanthous *et al* \(^{75}\) have compared efficacy of alternate routes of immunisation in a mouse model of *H. pylori* infection, these studies used different dose rates and in addition no direct correlation
was made between immune response and protection against bacterial challenge. The studies presented here have investigated and directly compared the prophylactic efficacy, systemic immune response and the practical aspects of vaccine delivery between intra-gastric, intra-nasal and intra-rectal immunisation in the *H. pylori* mouse model. Following administration of identical concentrations of vaccine to each of these three routes, the degree of protective immunity and humoral immune response were found to differ.

The present study found intra-nasal administration of a whole cell lysate of *H. pylori* in combination with cholera toxin to be the most efficacious route of administration for establishing a significant prophylactic response against *H. pylori* challenge. Intra-nasal immunisation proved not only to elicit the best prophylactic response over a range of dose rates, but also by its very nature, the procedure is the simplest. Compared to intra-gastric or intra-rectal immunisation in terms of the time involved to immunise an individual mouse, the equipment required and the subsequent method of administration, intra-nasal immunisation was the most efficient. An obvious advantage of this finding is that the level of competency required to perform intra-nasal immunisation is less than that for intra-gastric or intra-rectal immunisation, thereby allowing an investigator with limited animal handling skills to conduct research without the aid of specialised equipment or training. Furthermore, vaccine trials using large numbers of animals would be accomplished more expediently and with fewer costs incurred than with the commonly performed route of intra-gastric immunisation.

The finding that intra-nasal immunisation is the most effective means of delivery of the *H. pylori* vaccine in a mouse model may have significant application elsewhere. Over recent years a number of *Helicobacter* species have been isolated from the gastric mucosa of a variety of animal species.[^6] In a number of these animals these *Helicobacters* have also been associated with gastritis[^31]. Whilst this study used only small laboratory animals which are easily restrained by hand, any attempt at immunisation in large and potentially dangerous species would most likely require the use of chemical restraints necessary for peroral administration of the vaccine. If the equally efficacious route of intra-nasal administration could be applied in these instances without the aid of sedation, then the potential veterinary complications associated with anaesthesia may be avoided.
Although intra-nasal administration proved to be the most efficacious route of immunisation against *H. pylori* challenge, intra-gastric administration produced significant prophylaxis and was shown to be a reliable means of delivering substances to the small laboratory animal. Intra-gastric gavage is an effective technique for the experimental infection of the gastro-intestinal tract, and many investigators will no doubt continue with its use for such purposes. However, as many previous studies have used some form of anaesthesia to sedate the mouse prior to intra-gastric gavage, the simple method described here has shown that not only is any such chemical restraint unnecessary, the procedure presented no detectable evidence of detriment to the animals.

Given that *H. pylori* colonises the gastric mucosa, the target effector site for immunisation by all routes of administration was the stomach. Although immunisation at the distal inductive site of the rectal mucosa evoked a protective immune response, the level of protective immunity was proportionately reduced compared to that of immunisation from intra-gastric and intra-nasal administration. This finding was consistent with previous investigations that have suggested the theory of compartmentalisation whereby the administration of immunogens to a specific inductive site produces an immune response proportional to the proximity and physiological relationship of the immune effector sites. However, even though the prophylactic effect against *H. pylori* challenge was reduced in mice immunised by the rectal route, a significant systemic anti-*H. pylori* immune response was generated by intra-rectal immunisation and would indicate that the administration of immunogens to the rectal mucosa might have application against pathogens that infect the large bowel. By extrapolating the concept of compartmentalisation to the large intestine and disease associated with pathogens that inhabit the rectum, colon and caecum, it could be reasoned that the colonic patches, analogous to Peyers’ patches of the ileum might indeed be the optimal sites for antigen uptake. With the discovery of new *Helicobacter* species that colonise the gastrointestinal tract other than the stomach, intra-rectal immunisation may possibly be a successful alternative to that of intra-gastric immunisation as a mucosal route for vaccine administration. *Helicobacter hepaticus* for example is known to colonise the caecum and large bowel and has been linked with inflammatory bowel disease. The use of intra-rectal immunisation may be essential in the successful development of vaccines against such a pathogen.
Throughout this study it became clear that the ratio of antigen to adjuvant was critical to the level of protective immunity. The hypothesis that effective prophylaxis was ratio dependent was examined by intra-nasal immunisation across a spread of dose rate concentrations. An antigen/adjuvant ratio of 10:1 proved to elicit the highest levels of prophylaxis against bacterial challenge and was consistent across the range of the three dose rates trialed. In previous studies, Sutton et al.\textsuperscript{[132]} demonstrated by using a standard antigen/adjuvant dose rate of 1 mg of whole cell lysate of \textit{H. pylori} (HpL) and 10 μg of cholera toxin (CT) that lower concentrations of antigen produced greater levels of protective immunity in mice. Whilst maintaining the 10 μg concentration of CT, the group proved that an 80% reduction of HpL from 1 mg to 200 μg was more efficacious against \textit{H. pylori} challenge. The authors suggested that the lower dose rate of antigen was responsible for the stimulation of a greater Th2-type response, therefore leading to a greater reduction in bacterial burden. Whilst this hypothesis may be valid, it must also be reasoned from the data presented here that the most efficacious dose rates presented by Sutton were indeed approaching the optimal antigen/adjuvant ratio of 10:1.

In conclusion, as there are inherently many variables associated with research using living animals, it would be prudent to eliminate those that are unnecessary. The studies presented here have shown conclusively that there is a fundamental requirement for the investigator to optimise, with a humane approach, any animal model intended for scientific investigation. As other \textit{Helicobacter} species are being connected to gastrointestinal diseases in a range of animal species, control strategies are still in developmental stages.\textsuperscript{[10, 40, 41, 42, 43]} The search for potential vaccines against these pathogens will be a key issue and appropriate mucosal routes of vaccine administration will play an important role in the progress of therapeutic and prophylactic measures.\textsuperscript{[138]}
6.1.1 Summary of Major Results

1. The direct administration of a vaccine to the Peyers’ patches of the ileum provided effective intestinal immunisation, and was an important baseline for the further examination of alternate mucosal delivery systems.

2. In the absence of chemical restraint and detriment to the animals, intra-gastric, intra-nasal and intra-rectal administration of vaccine proved to be successful routes of delivery.

3. Intra-nasal immunisation was shown to be the most efficacious route of immunisation against *H. pylori* challenge. This route elicited the highest levels of protective immunity with the lowest recorded margins of error across a spread of dose rates. In addition, intra-nasal immunisation was the easiest procedure to perform and had the least requirement for specific purpose equipment and specialised training.

4. A significant systemic immune response to *H. pylori* was shown using all three routes of intra-gastric, intra-nasal and intra-rectal immunisation.

5. Intra-rectal immunisation, whilst generating the highest levels of circulating Immunoglobulin G (IgG), showed the least efficacious prophylactic response to *H. pylori* challenge, thus supporting the concept of compartmentalisation and providing further evidence to support the view that prophylaxis against *H. pylori* is not antibody driven.

6. Effective prophylaxis was dependent upon a specific ratio of vaccine constituents. When using a whole cell lysate of *H. pylori* and cholera toxin, the ratio of antigen to adjuvant required for optimal prophylaxis was 10:1.
6.2 **FUTURE STUDIES**

Further investigation of immunisation strategies against *H. pylori* will address a number of areas of importance before a viable vaccine will be realised, if at all. Research to find alternative adjuvants without the gastrointestinal toxicity associated with cholera toxin and labile toxin will be one such area, as will the continued exploration of the processes involved with *Helicobacter* infection and immunity. Future studies emanating directly from the data obtained from this study should focus on a complete understanding of the effector mechanisms of the immune response, and in particular on the further development and optimisation of the animal model.

The disparity between prophylaxis and systemic immune response observed from immunisation via intra-rectal immunisation further implies that effector mechanisms are other than antibody dependent. The fact that the generation of a potent circulating IgG response did not correlate to significant prophylaxis should be of interest to immunologists. Another immunological aspect worthy of attention was the observation of a prophylactic response in those animals administered exclusively antigen via the intra-intestinal route in the absence of cholera toxin. Furthermore, the use of cholera toxin as mucosal adjuvant raises yet more questions by its ability to generate a level of protection without the inclusion of known antigenic material.

In many investigations of *Helicobacter* infection, prophylaxis or therapeutic clearance has primarily been assessed by using either gastric urease assay or histology.\(^{154,76}\) Sutton *et al*\(^{152}\) demonstrated that bacterial levels of \(10^4\text{-}10^5\) organisms per gram of mouse stomach tissue were undetectable by either of these two assays, therefore, previous conclusions of an immunisation regime producing a ‘sterilising’ effect may have been largely misguided. The data presented here confirmed that whilst the direct administration of vaccine to the Peyers' patches provided highly effective intestinal immunisation, total protection may not be possible. However, as the link between level of infection and disease status has yet to be fully evaluated an effective vaccine may not be required to totally eliminate 100% of organisms.
Whilst the animal model has been further refined through this investigation, other variables such as time points of immunisation may be further explored. An effective prophylactic immunisation regime against *H. pylori* using two time points only, prime at day 0 and booster at day 21, has been demonstrated by Sutton *et al.*\(^{[152]}\). In a follow-up experiment, this group produced a marked prophylactic improvement to the aforementioned strategy when mice were immunised with three time points at days 0, 21 and 70 (unpublished data).

Using the mouse as an experimental animal model has many advantages. They are a cost-effective proposition when considering the volume of animals necessary for large-scale experimentation where statistical analysis is not to be compromised. They are also a relatively easy species to maintain, handle and manipulate. However, within the context of evaluating the most efficacious route of administering vaccines, the mouse model has some drawbacks when extrapolating to other species. For instance, the mouse does not exhibit oesophageal reflex; that is, they do not vomit, which could possibly present problems when using intra-gastric gavage in a species that does regurgitate. Therefore, examination of efficacy of alternate routes of immunisation and the development of appropriate delivery techniques should be evaluated in other species.

Finally, even though the objective of this study was to further define a vaccination strategy against *H. pylori*, this study may propose the viability of using alternate routes of administration against a variety of mucosal associated pathogens.
References


68. Johnson AP. 1998. Antibiotic resistance among clinically important gram-positive bacteria in the UK. J Hospital Infect. **40**: 17-26


