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Passive immunotherapy and probiotic agents in enteric infections in children

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ABSTRACT

Diarrhoeal diseases remain one of the leading causes of global childhood morbidity and deaths. Rotavirus and pathogenic *Escherichia coli* are the most common causes of acute diarrhoeal illness in children. Oral rehydration therapy (ORT), although effective in management or prevention of dehydration, does not reduce the duration or severity of illness. Hence, there is a need for the development of new interventions to further improve therapy for gastrointestinal (GI) tract infections. We hypothesized that neutralizing antibodies from an immunized animal can be used to modulate infections due to diarrhoeal pathogens. The growing understanding of the host-microbe interactions and the role of indigenous flora on competence of the immune system allowed us to also hypothesize that probiotic agents (health promoting bacteria) could be useful adjuncts in the management of infectious diarrhoea. *Helicobacter pylori* infection is another common infection that affects 50% of world population, which is associated with peptic ulcer disease, gastritis and gastric cancer. Current antibiotic therapy is complex, expensive, associated with adverse events, and often induces emergence of resistance. Therefore, alternative approaches for treating such infections are highly desirable. The objectives of this thesis were to evaluate the potential of oral administration of pathogen-specific antibodies derived from different animal sources, notably immunoglobulin from immunized bovine colostrum (HBC) in rotavirus-induced diarrhoea (paper I), from milk concentrate (BIC) in *E. coli*-induced diarrhoea (paper II) and *H. pylori* infections (paper III), and hyperimmune chicken egg yolk (HEY) in rotavirus (RV) induced diarrhoea (paper IV) in children. We also evaluated the therapeutic efficacy of a probiotic agent, *Lactobacillus paracasei* (ST11) in children with diarrhoea (paper V). In order to find a stable form of antibodies and determine potentials for cost-effective, large-scale production needed for use in the developing countries, we evaluated if fragments derived from llama, produced in yeast, are capable of reducing diarrhoea morbidity in mice (paper VI). Finally, to see the therapeutic potentials and to optimize the dose a separate study with IgY was conducted in an infant mouse model of rotavirus-induced diarrhoea (paper VII).

We found impressive results with HBC in ameliorating rotavirus diarrhoea (Paper I), but did not see any effect on the course of *E. coli*-induced diarrhoea or *H. pylori* infection (Paper II and III) in children. We observed a modest beneficial effect of IgY treatment in reducing morbidity in children with rotavirus diarrhoea (Paper IV). In the study with probiotics, we failed to observe any beneficial effect of ST11 treatment in the management of rotavirus diarrhoea; however, the probiotic treatment significantly ameliorated morbidity in children with non-rotavirus diarrhoea (Paper V). In paper VI, for the first time we demonstrated that monovalent llama-derived antibody fragments produced in yeast was able to bind to rotavirus G3 strains in vitro. In vivo, this fragment reduced morbidity from rotavirus-induced diarrhoea in mice (Paper VI) demonstrating potentials for its therapeutic use in humans. The use of an animal model of rotavirus diarrhoea allowed us to evaluate therapy with higher doses of IgY, and the results suggested that an improved therapeutic effect could be achieved by increasing the dose (paper VII). Conclusion: Administration of specific antibodies showed an impressive clinical efficiency against rotavirus diarrhoea but no impact on *E. coli*–induced diarrhoea or *H. pylori* infection. Feeding of *Lactobacillus paracasei* ST11 did not produce any clinical benefit in rotavirus diarrhoea; however, produced clinical benefits to patients with diarrhoea not due to rotavirus (possibly induced by *E. coli* or other un-identified bacteria). These divergent effects observed is interesting, which suggests efficacy of orally administered antibodies against viral pathogens, and a possible effect of orally administered probiotic bacterium on bacterial diarrhoea. From these findings, it seems likely that a combination therapy using antibodies and probiotic agents could elicit therapeutic benefit in settings where diarrhoea in a majority of children are caused by rotavirus or *E. coli*.

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PASSIVE IMMUNOTHERPAY AND PROBIOTIC
AGENTS IN ENTERIC INFECTIONS IN
CHILDREN

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Stockholm 2006
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To my family
8.1  STUDY SITES ........................................................................................................38
8.2  BANGLADESH ................................................................................................38
8.3  ICDDR, B: ........................................................................................................40
  8.3.1  The Clinical Sciences Division (CSD) ..........................................................42
  8.3.2  Nandipara ..................................................................................................43
  8.3.3  Participants of the clinical studies ...............................................................44
  8.3.3.1  Diarrhoeal disease (papers I, II, IV and V) ..............................................44
  8.3.3.2  Helicobacter pylori..................................................................................45
  8.3.3.3  Animals ..................................................................................................45

9.  ETHICS ..................................................................................................................46

10.  METHODS ..........................................................................................................46
  10.1  FLUID AND ELECTROLYTE BALANCE (PAPER I, II, IV, V) .......................46
  10.2  ELISA ............................................................................................................47
  10.3  EIA FOR ANTI-LPS ANTIBODIES (PAPER II) ............................................47
  10.4  $^{13}$C-UREA BREATH TEST (PAPER III) ..........................................................47
  10.5  VHH1 FRAGMENT PREPARATION ..................................................................48
  10.6  SAMPLE SIZE ESTIMATION .........................................................................49
  10.6.1  Randomization ..........................................................................................49
  10.7  STATISTICAL ANALYSIS ............................................................................50
  10.8  END POINTS ..................................................................................................51
  10.9  OUTCOME MEASURES ..................................................................................51

RESULTS ...............................................................................................................52
  11.1  HYPERIMMUNE BOVINE COLOSTRUM (HBC) IN ROTAVIRUS DIARRHOEA
        (PAPER I) .......................................................................................................53
  11.2  BOVINE IMMUNOGLOBULIN MILK CONCENTRATE (BIC) IN ETEC AND EPEC
        INDUCED DIARRHOEA IN CHILDREN (PAPER II) ..........................................53
  11.3  HYPERIMMUNE BOVINE COLOSTRUM IN Helicobacter pylori INFECTION
        (PAPER III) .....................................................................................................54
  11.4  HYPERIMMUNIZED CHICKEN EGG YOLK (HEY) IN CHILDREN WITH ROTAVIRUS
        DIARRHOEA (PAPER IV) ................................................................................55
  11.5  Lactobacillus paracasei ST11 STUDY (PAPER V) ............................................55
  11.6  LLAMA-DERIVED ANTIBODY FRAGMENTS (VHH) IN ROTAVIRUS INFECTION
        (PAPER VI) .....................................................................................................56
        11.6.1  In vitro neutralization of rotavirus ..........................................................56
        11.6.2  In vivo efficacy in mouse model .............................................................57
  11.7  EGG YOLK IMMUNOGLOBULIN (IGY) IN A MOUSE MODEL OF ROTAVIRUS
        GASTROENTERITIS (PAPER VII) .......................................................................58

DISCUSSION ...........................................................................................................60

CONCLUSIONS AND FUTURE PERSPECTIVES .........................................................69

ACKNOWLEDGEMENTS .........................................................................................71

REFERENCES .........................................................................................................74
ABSTRACT

Diarrhoeal disease remains one of the leading causes of global childhood morbidity and mortality. Rotavirus and pathogenic Escherichia coli are the most common causes of acute diarrhoeal illness in children. Oral rehydration therapy (ORT), although effective in management or prevention of dehydration, does not reduce the duration or severity of illness. Hence, there is a need for new interventions to further improve therapy for gastrointestinal (GI) tract infections. We hypothesized that neutralizing antibodies from immunized animal can be used to modulate infections due to diarrhoeal pathogens. The growing understanding of the host-microbe interactions and the role of the indigenous flora on competence of the immune system allowed us to also hypothesize that probiotic agents (health promoting bacteria) could be useful adjunct in the management of infectious diarrhoea. Helicobacter pylori infection is another common infection and affects 50% of world population, and is associated with peptic ulcer disease, gastritis and gastric cancer. Current antibiotic therapy is complex, expensive, is associated with adverse events and often induces resistance. Therefore, alternative approaches for treating such infections are highly desirable. The objectives of this thesis were to evaluate the potential of oral administration of pathogen-specific antibodies derived from different animal sources, notably immunoglobulin from immunized bovine colostrum (HBC) in rotavirus-induced diarrhoea (paper I), from milk concentrate (BIC) in E. coli-induced diarrhoea (paper II) and H. pylori infections (paper III), and hyperimmune chicken egg yolk (HEY) in rotavirus (RV) induced diarrhoea (paper IV) in children. We also evaluated the therapeutic efficacy of a probiotic agent, Lactobacillus paracasei (ST11) in children with diarrhoea (paper V). In order to find a stable form of antibodies and determine potential for cost-effective, large-scale production needed for use in the developing countries, we evaluated if fragments derived from llama, produced in yeast, are capable of reducing diarrhoea morbidity in mice (paper VI). Finally, to see the therapeutic potentials and to optimize the dose a separate study with IgY was conducted in an infant mouse model of rotavirus-induced diarrhoea (paper VII).

We found impressive results with HBC in ameliorating rotavirus diarrhoea in children (Paper I), but did not see any effect on the course of E. coli-induced diarrhoea or H. pylori infection (Paper II and III) in children. We observed a modest beneficial effect of IgY treatment in reducing morbidity in children with rotavirus diarrhoea (Paper IV). In the probiotics study, we failed to observe any beneficial effect of ST11 treatment in the management of rotavirus diarrhoea; however, the probiotic treatment significantly ameliorated morbidity in children with non-rotavirus diarrhoea (Paper V). In paper VI, we demonstrated that monovalent llama-derived antibody fragments, produced in yeast, were able to bind to rotavirus G3 strains in vitro. In vivo, these fragments reduced morbidity from rotavirus-induced diarrhoea in mice (Paper VI) demonstrating a potential for its therapeutic use in humans. The use of a mouse model of rotavirus diarrhoea allowed us to evaluate therapy with higher doses of IgY, and the results suggested that an improved therapeutic effect could be achieved (paper VII). Conclusion: Administration of specific antibodies showed an impressive clinical efficiency against rotavirus diarrhoea but had no impact on E. coli–induced diarrhoea or H. pylori infection. Feeding of Lactobacillus paracasei ST11 did not produce any clinical benefit in rotavirus diarrhoea; however, the bacteria produced clinical benefits to patients with diarrhoea not due to rotavirus (possibly induced by E. coli or other un-identified bacteria). The divergent effects observed are interesting; our results suggest efficacy of orally administered antibodies against viral pathogens, and a possible effect of orally administered probiotic bacteria on bacterially induced diarrhoea. From these findings, it seems likely that a combination therapy using antibodies and probiotic agents could elicit therapeutic benefit in settings where diarrhoea in a majority of children are caused by rotavirus or E. coli.
LIST OF PUBLICATIONS

This thesis is based on 7 original papers, which are referred to in the text by their roman numerals (I-VII)


VII Sarker SA, Pant N, Juneja LR, Hammarström L. Gastroenteritis in suckling mice caused by rhesus rotavirus can be successfully treated with egg yolk immunoglobulin (IgY) Submitted.
## TABLE OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>BIC</td>
<td>bovine immunoglobulin concentrate</td>
</tr>
<tr>
<td>CRSC</td>
<td>Clinical Research and Service Centre</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ETEC</td>
<td>enterotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EPEC</td>
<td>enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>GALT</td>
<td>gut associated lymphoid tissue</td>
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<tr>
<td>HBC</td>
<td>hyperimmune bovine colostrum</td>
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<td>HEY</td>
<td>hyperimmunized egg yolk</td>
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<td>H. pylori</td>
<td><em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>ICDDR, B</td>
<td>International Centre for Diarrhoeal Diseases Research, Bangladesh</td>
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<tr>
<td>IgY</td>
<td>egg yolk immunoglobulin</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>L. paracasei</td>
<td><em>Lactobacillus paracasei</em></td>
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<tr>
<td>LT</td>
<td>heat labile toxin</td>
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<tr>
<td>MALT</td>
<td>mucosa associated lymphoid tissue</td>
</tr>
<tr>
<td>ORS</td>
<td>oral rehydration solution</td>
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<tr>
<td>RRV</td>
<td>rhesus rotavirus</td>
</tr>
<tr>
<td>RV</td>
<td>rotavirus</td>
</tr>
<tr>
<td>ST</td>
<td>heat stable toxin</td>
</tr>
<tr>
<td>ST11</td>
<td><em>Lactobacillus paracasei</em></td>
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<tr>
<td>UBT</td>
<td>urea breath test</td>
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<td>VHH</td>
<td>heavy chain antibody fragment</td>
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INTRODUCTION

Diarrhoeal disease remains a major threat to human health globally. In the early eighties, diarrhoeal diseases accounted for about 4.6 million deaths from around 1 billion episodes of illness in children younger than 5 years each year [Snyder and Merson, 1982]. A decade later, even without significant change in incidence, the number of deaths attributable to diarrhoeal diseases dropped to 3.3 million per year [Bern et al., 1992]. This reduction in death is attributed to implementation of Oral Rehydration Therapy (ORT), coordinated by the WHO [Victora et al., 2000]. Most recent estimates indicate that the number of deaths had been further reduced to 2.5 million [Kosek et al., 2003]. However, diarrhoal disease remains a leading killer of young children. Some data suggest it accounts for 15% of cause-specific mortality among under-five children, which is exceeded only by acute lower respiratory infections (18%) [Anonymous, 2004]. The major burden of diarrhoal illness is currently experienced by the developing world, where children suffer from 6-7 episodes per year compared to only one episode in the developed countries [Santosham et al., 1997]. Factors such as poor water supply and sanitation, lack of education and personal hygiene, malnutrition and HIV associated immunodeficiency underlie the high incidence of diarrhoeal diseases in the developing countries. In developed countries, deaths due to diarrhoeal illness are rare, and the effects of these illnesses are often measured in financial terms. In the United States, 25 million episodes of diarrhoeal illness occur among under-five children, leading to 200 000 hospital admissions each year [Santosham et al., 1997]. This account for 2% of the outpatient visits, costing US$50/visit, and 4% of all admissions, costing US$ 2307/admission [Zimmerman et al., 2001]. Despite the successes in the control of diarrhoeal diseases, the developed countries remain under the threat of enteric pathogens emerging and re-emerging in the developing countries due to increasing travel globally.

Current management of diarrhoeal illness involves prevention and management of dehydration using oral or intravenous rehydration, as appropriate, and continued feeding, including breastfeeding for young infants. Additionally, therapy with effective antimicrobial agents is required for management of shigellosis and severe cholera. Recently, the WHO and UNICEF has recommended routine use of zinc for 10-14 days in the management of diarrhoea in young children, irrespective of aetiology [Fontaine, 2006]. However, its successful implementation in the developing countries remains to be seen. Recently, an orally administrable cholera vaccine has been marketed in the United States mostly for travellers’
use; however, its prohibitive current cost and the need for more than one dose for better protective efficacy, particularly among young children, will not allow its routine use in the public health programmes of developing countries. An oral rotavirus vaccine, determined to be effective and safe in controlled trials, was withdrawn soon after its marketing due to intussusceptions among the vaccinees [Bines, 2005; Chen and DeStefano, 1998]. Another oral rotavirus vaccine has just been approved for marketing in the United States [Clark et al., 2006] and Europe [Vesikari et al., 2006] however; this vaccine would also be unlikely to be used by the developing countries in their national programmes because of the high cost. Antimicrobial therapy, while useful, requires continuous monitoring of susceptibility of the pathogens and dissemination of such information to health care providers-not an easy task and hence lacking in the developing countries. More importantly, emergence of resistant strains is common, which leads to therapy with ineffective agents and its associated problems. Even in cases where measures are effective, quite often the treatment regime is economically and logistically impossible to administer.

In the 1890s Paul Ehrlich proposed colostrum as a vehicle for transporting immune factors, “Antikörper”, from the mother to her offspring [Ehrlich, 1890]. Primarily via transfer of immunoglobulin and associated factors, breast milk offers passive protection to newborn babies and infants against enteric pathogens. The historical concept of “immune milk”, i.e. the transfer of passive immunity via lacteal antibodies, dates back to the 1950s [Campbell and Petersen, 1959; Campbell and Petersen, 1963]. The mechanisms underlying passive immunity, however, were recognised only in the early 1960s, when the chemical structure of immunoglobulin (Ig) was elucidated. Particularly, the identification of the mucosal or secretory immune system in the 1970s provided new insights into the role of secretory antibodies in the prevention and treatment of enteric infections in mammals [Lamm et al., 1978]. Since the 1980s, an increasing number of studies have shown that immune milk preparations, based on bovine antibodies derived from milk or colostrums from immunized cows, may be useful in the prevention and treatment of human and animal diseases caused by enteropathogenic microbes (for review see [Davidson, 1996; Hammarstrom et al., 1994; Weiner et al., 1999]). However, the practical application of milk-derived antibodies is limited due to the difficulties in their large-scale production. In the 1990s, laying hens attracted considerable attention as an alternative, inexpensive source of antibodies for large-scale production. In animals, IgY has proved to be useful in the treatment of a variety of GI infections including rotavirus, C. jejuni, and E. coli (for review see [Hammarstrom et al.,
However, there is still controversy regarding the stability of IgY during its passage through the GI tract. An alternative to using conventional immunoglobulin is the use of monovalent fragments derived from heavy chain antibodies found in Camelidae [Hamers-Casterman et al., 1993]. These antibodies are devoid of light chains, have a broad antigen-binding repertoire [Arbabi Ghahroudi et al., 1997] and are extremely thermo-stable [van der Linden et al., 1999].

For over a century, researchers have suggested that live bacterial cultures, such as those found in yoghurt, might be useful in the prevention and treatment of gastrointestinal disorders [Metchnikoff, 1907]. Lactobacilli belong to the normal commensal bacterial flora of the human intestine and are currently being investigated extensively as probiotics (health-promoting bacteria), and their antidiarrheal properties have been investigated since the 1960s [Beck and Necheles, 1961]. Several recent controlled clinical trials, conducted mostly in the developed countries, have shown that selected strains of lactobacilli such as Lactobacillus casei (strain GG), Lactobacillus reuteri and Lactobacillus acidophilus exhibit both therapeutic and prophylactic effects in children with viral, but not bacterially induced diarrhea. With few exceptions, the studies did not quantitatively evaluate stool volume, a WHO recommended criterion for assessing severity of diarrhoeal illnesses. As a majority of diarrhoea episodes are severe in the populations in poor communities, it is essential to define the role of probiotic agents in such populations.

This thesis will address the historical perspectives, and the present and the future applications of immune therapy for infectious diseases, including the current challenges in their applications for prevention and management of gastrointestinal infections. A short description of the mucosal immune system, the role of the indigenous flora in host defence, the current and future prospects for applications of passive immune therapy for infectious diseases, characteristics of different IgGs used for passive immunotherapy, their mechanisms of actions and the usefulness of probiotics in the treatment of gastrointestinal infections will be presented in the following sections.
1. THE IMMUNE SYSTEM

The physiologic function of the immune system is defence against infectious microbes. However, non-infectious foreign substances can also elicit immune responses. Defence against microbes is mediated by the early reactions of **innate immunity** (also called natural or naive immunity) and the later responses of **adaptive immunity**. The innate immunity consists of cellular and biochemical defence mechanisms that are in place even before infection poised to rapidly respond to infections. The principle components of innate immunity are: (i) physical and chemical barriers such as epithelia and anti-microbial substances produced at epithelial surfaces (ii) phagocytic cells (neutrophils, macrophages) and natural killer cells (NK cells); (iii) serum proteins, including the complement system and other mediators of inflammation; and (iv) cytokines, that regulate and coordinate many of the activities of the cells involved in innate immunity.

In contrast to innate immunity, there are other immune responses that are stimulated by exposure to infectious agents, which increase in magnitude and in defense capabilities with each successive exposure to a particular microbe. Because this form of immunity develops as a response to infections and adapts to the infection, it is called **adaptive immunity**. The defining characteristics of adaptive immunity are their exquisite specificity for distinct molecules and the ability to “remember” and respond more vigorously to repeated exposures to the same microbe. The adaptive immune system is able to recognize and react to a large number of microbial and non-microbial substances. In addition, it has an extraordinary capacity to distinguish among different, even closely related, microbes and molecules and for this reason it is called **specific immunity**. The components of adaptive immunity are **lymphocytes** and their products. Foreign substances that induce specific immune response or are the targets of such responses are called **antigens**. There are two major types of lymphocytes: **B lymphocytes** or **B cells**, which, when activated, differentiate into plasma cells that secrete antibodies; and **T lymphocytes** or **T cells**, of which there are two main classes. One class differentiates into cytotoxic T cells, which kill cells infected with viruses, whereas the second class of T cells differentiates into cells that activate other cells such as B cell and macrophages. The two classes are distinguished by the expression of the cell-surface protein CD4 and CD8 respectively. CD8 cells are also called **cytotoxic T cells** and CD4 T cells are called **helper T cells**. Among the T helper cells there are three subsets, Th1, Th2, 7
and Th3. Th1 is proinflammatory and produces cytokines such as interferon γ, tumor necrosis factor alpha (TNFα), and IL-2, whereas Th2 cells produce antiinflammatory cytokines such as transforming growth factor β (TGF β), IL-4, IL-5, IL-6 and IL-10.

1.1 THE MUCOSAL IMMUNE SYSTEM

One of the most important compartments of adaptive immune system is the mucosal immune system (commonly described by the acronym MALT), which is increasingly gaining attention as an area of great potential for the development of vaccines and immunotherapy. Besides its gastrointestinal locations, MALT includes lymphoid tissues in respiratory tract mucosa (trachea and bronchi, TALT and BALT) [Brandtzaeg et al., 1999]. They are thin and permeable barriers to the interior of the body because of their physiological activities in food absorption (the gut), gas exchange (the lungs), sensory activities (eyes, nose, mouth, and throat), and reproduction (uterus and vagina). The gut mucosal surface is particularly vulnerable to infection. The permeability of the surface lining facilitates vulnerability to infection, and is not surprising that a vast majority of infectious agents invade the human body by these routes.

The mucosa associated peripheral lymphoid tissues that lines the gut are called gut-associated lymphoid tissue or GALT. In the mucosa of the gastrointestinal tract, lymphocytes are found in large numbers within the epithelial layer, scattered throughout the lamina propria, and in organised collections in the lamina propria such as Peyer's patches. In addition to the organized lymphoid tissue, the mucosal immune system contains a distinctive repertoire of lymphocytes and plasma cells, which are scattered throughout the lamina propria of the gut wall [Hayday, 2000]. This represents the effector cells of the gut mucosal immune system.

The intestinal lamina propria, beneath the epithelial layer, contains a large number of mixed populations of cells in clusters including T lymphocytes, most of which are CD4+ and have a phenotype of activated cells. The lamina propria also contains a large number of activated B lymphocytes and plasma cells, macrophages, dendritic cells, eosinophils and mast cells. In histological sections, over 15,000 lymphoid follicles can be demonstrated within the intestinal lamina propria of a healthy child [Ghia et al., 1998].
The sub mucosal layer beneath the lamina propria contains organized lymphoid tissues, and the Peyer’s patches, nodules of 30-40 lymphoid follicles, are prominent amongst them [Ghia et al., 1998], which is an extremely important site for induction of immune responses in the small intestine. The Peyer’s patches in the intestine have a distinctive appearance, forming a dome-like structure extending into the lumen of the intestine. Like lymphoid follicles in the spleen and lymph nodes, the central region of the mucosal follicles is rich in B-cells, which often contains germinal centres. Peyer’s patches also contains small numbers of CD4+ T cells, mainly in the interfollicular regions. The overlying layer of the follicle-associated epithelium of the Peyer’s patches contain specialized epithelial cells—multifenestrated or microfold cells or membranous (M cells). M cells lack microvilli, which takes up molecules and particles from the gut lumen by endocytosis or phagocytosis. They are then transported through the interior of the cells, in vesicles, to the basal membrane and then released into the extra-cellular space. This process is known as transcytosis. At their basal surface, cell membrane of M cells is extensively folded around underlying lymphocytes and antigen presenting cells, which take up the transported material, released from the M cells and process the antigen for subsequent presentation.

The T cells of the gut can be divided into two types. One type bears the conventional α: β T-cell receptor in conjunction with either CD4 or CD8, and participates in the conventional MHC mediated T-cell responses to foreign antigens [Porcelli and Modlin, 1999]. The second type is made up of T cells with unusual surface phenotypes such as TCR γ: δ and CD8 αα TCRα β. T cell bearing TCR γ:δ are particularly abundant in the gut mucosa compared to other lymphoid tissues [Park et al., 1999]. The receptors of these T cells do not bind the normal MHC:peptide ligands but instead binds a number of other ligands including MHC class 1B molecules. In humans, only about 10% of the intraepithelial lymphocytes are γδ cells [Hayday, 2000; Nagler-Anderson, 2001], and about 80% of them are CD8+ cells [Scott et al., 1993].

Antigen from pathogenic microorganisms is presented to T cells by antigen presenting cells such as macrophages and B lymphocytes. In the follicle, the antigen is presented to B cells by follicular dendritic cells. Upon encountering the foreign antigen in the Peyer’s patches, naïve lymphocytes (both T and B cells) are activated to become effectors cells, which include a reprogramming step that alters the migratory behaviour of the cells and directs them to extra lymphoid sites. Leaving the Peyer’s patches, the activated lymphocytes are carried via the
lymphatic draining, and they pass through the mesenteric lymph nodes to eventually end up in the thoracic duct, from where they circulate in the blood throughout the entire body. They re-enter (homing) the mucosal tissues from the small blood vessels lining the gut wall [Brandtzaeg, 1996], thereby disseminating a mucosal immune response. T cells of the mucosal immune system bearing γδ T cell receptors then recognize and kill infected epithelial cells showing atypical class I molecules, MIC A and MIC B [Leishman et al., 2001]. B cells on the other hand differentiate into plasma cells and secrete immunoglobulin (mainly IgA) into the mucosa.

1.2 IgA is the major class of antibody in gut

IgA is the major class of antibody produced in the mucosal immune system. The gastrointestinal and respiratory tracts are the two most common portals of entry for microbes. Defense against microbes that enter by these routes is provided by antibodies, largely IgA produced by the mucosal lymphoid tissues and secreted through the mucosal epithelium into the lumen of these organs. In the mucosal secretions, IgA binds to luminal microbes and toxins and neutralize them by blocking their entry into the host. Patients with selective IgA deficiency have an increased risk of recurrent gastrointestinal infections (for review see [Rosen, 2000] illustrating the important role of sIgA in the gut. Secretory immunity is the mechanism of protective immunity induced by oral vaccines such as polio.

IgA antibodies are found in two isotypic forms in humans, IgA1 and IgA2. In the blood, IgA is found mainly as a monomer, and the ratio of IgA1 and IgA2 is about 4:1. In mucosal secretions, IgA is almost exclusively produced as a dimer, with a ratio of IgA1 to IgA2 of about 3:2. IgA is produced in larger amounts than any other antibody isotype, mainly because of the size of the intestinal surface. It is estimated that a normal 70-kg adult secretes about 2 gram of IgA per day, which accounts for 60-70% of the total output of antibodies [Parren and Burton, 2001]. Switching to the IgA isotype is stimulated by transforming growth factor β (TGF β), produced by T cells and non lymphoid stromal cells, and the Th 2 cytokine IL-5. The probable reason that larger amounts of IgA are produced in the mucosal immune system than in other tissues is that isotype switching to IgA occurs most efficiently in mucosal lymphoid tissue, in part because IL-5 producing helper T cells are more numerous in mucosa than in other lymphoid tissues. IgA-producing B cells also have a special propensity to home to the mucosal tissue.
1.2.1 Transport of secreted IgA

IgA produced by B cells in the lamina propria and secreted in the form of a dimer, which is held together by co-ordinately produced J chain. From the lamina propria, the IgA must be transported across the epithelium into the lumen, a function that is mediated by a Fc receptor called poly Ig receptor [Brandtzaeg, 1995]. This receptor binds polymeric IgA or IgM, and transports them to the luminal surface of the gut by transcytosis. Upon reaching the luminal surface of the enterocytes, the antibody is released into the secretion by proteolytic cleavage of the intracellular domain of the Ig receptor. The cleaved extracellular part of the polymeric Ig receptor is known as the Secretory Component, frequently abbreviated as SC, which remains associated with polymeric IgA or IgM. The SC serves several physiological roles, including acting as a “glue” to bind secreted IgA and IgM to the mucus layer on the luminal surface of the epithelial layer of the gut, where they can bind to, or neutralize, gut pathogens and their toxic products. SC also protects these antibodies against proteolytic cleavage.

Secretory IgA (SIgA) is essential in protecting mucosal surfaces by ensuring immune exclusion. In addition, SIgA binds selectively to M cells in Peyer's patches (PP), resulting in its transport across the epithelium for targeting the dendritic cells (DC) in the dome region. The immunological consequences of such an interaction are unknown. However, in mice, SIgA has also been observed to trigger migration of DC to the T cell-rich regions of PP, and regulate expression of CD80 and CD86 on DC in PP, mesenteric lymph nodes and spleen, illustrating that mucosal SIgA exerts an Ag delivery function [Favre et al., 2005], contributing to the effector and/or regulatory pathways characteristic of the intestinal mucosal compartment.

1.3 Effector mechanisms of gut antibodies

IgA antibodies engage a distinct set of effector mechanisms for disposing of antigens. The simplest and most direct way in which antibodies can protect the host from pathogens or their toxic products is through binding and thereby blocking their access to the cells that they might infect and destroy. This mechanism is important for protection against bacterial toxins and pathogens such as viruses, preventing their entry and replication in the cells.
Binding by antibodies alone is however not sufficient to arrest the replication of bacteria that reside outside the cells. In this case, the role of IgA is to coat the bacterium and thus enable phagocytic cells to ingest and destroy them. The coating of pathogens and foreign particles in this way is known as opsonization. The Fc portions of antibodies (IgG or IgA) are recognized by Fc receptors present on the surface of phagocytic cells such as macrophages and neutrophils, which can thereby bind and engulf pathogens coated with antibodies of these isotypes [Ravetch and Clynes, 1998].

The third function of antibodies is to activate a system of plasma proteins known as complement that react with one another to opsonize pathogen, and induce a series of inflammatory responses that help to fight infections. Complement are heat labile component of normal plasma that augments the opsonization and killing of bacteria by antibodies. This activity “complements” the antibacterial activity of antibodies. Complement activation is mainly initiated by IgG or IgM. However IgM is much more efficient in activating complement than IgG [Cooper, 1985]. Complement activation can also occur directly by pathogens without the help of antibodies. The complement activated opsonized product is then engulfed by phagocytes [Frank, 1991].

1.4 The mucosal immune system and the normal bacterial flora

The normal microflora of the digestive tract plays an important role in maintaining competence of the immune system. Humans harbour over 400 species of commensal bacteria, which are present in large numbers in the colon and the ileum. These bacteria collectively weigh about 1 kg and outnumber human cells by approximately 10 times ($10^{14}$ as compared $10^{13}$ cells). Most of the time, we cohabit with our intestinal flora in a symbiotic relationship [Berg, 1996, 1999]. Although most basic research in bacterial-epithelial cross-talk involves pathogenic organisms, we now know that indigenous organisms can also communicate with the intestinal surface to enhance intestinal defence against bacteria-induced clinical disease. In most cases, the precise cellular mechanisms behind these responses have not been elucidated. However, competing with pathogenic bacteria for space and nutrients, preventing their colonization in the gut, is one protective activity of the normal gut flora of humans. This activity is illustrated by one of the adverse effects of antibiotics. Administration of an antibiotic kills a large number of commensal gut bacteria and thereby offers an ecological niche for pathogenic bacteria that would not otherwise be able to compete successfully with the normal flora. One example of a bacterium that grows in the antibiotic treated gut is
Clostridium difficile, which produces a toxin that can cause severe bloody diarrhoea [Kelly and LaMont, 1998].

The scale of the normal immune response to gut bacteria is illustrated by a study of animals delivered by caesarean section into a sterile environment, a situation in which the gut is not colonized by microorganisms. These are known as germ-free or gnotobiotic animals; they have marked reduction in the size of all peripheral lymphoid organs and reduced levels of antibodies of all isotypes. In neonates, the attachment of luminal bacteria to the epithelium actively stimulates epithelial and lymphocyte functions [Dai and Walker, 1999; Insoft et al., 1996], illustrating that bacterial colonization of the lumen is essential to mount a mucosal immune response.

There are circumstances where normal bacterial inhabitants of the gut cause disease, e.g. when there is a breach of integrity of the mucosa lining the gut. This can occur in association with poor blood flow in the gut, or after endotoxemia. In these circumstances, normally innocuous gut bacteria, such as non pathogenic Escherichia coli, can cross the mucosa, invade the blood stream and cause a fatal systemic infection. This illustrates the vital role of the mucosal surface as a barrier against infection. The normal gut flora may also become a cause of systemic infection in patients with immunodeficiency. This illustrates the role of the adaptive immune system in defending the body against gut bacteria, and also demonstrates that this response does not eliminate them from the lumen of the gut but creates a state resembling symbiosis.

Initial bacterial colonization of the gut of newborns has been studied in breastfed and bottle-fed infants. Exclusively breast-fed neonates develop a specific flora within a week of their birth that becomes dominant by one month [Balmer and Wharton, 1989; Langhendries et al., 1995]. Bacteria initially colonizing the gut of newborns are derived from the mother’s birth canal and large intestine [Langhendries et al., 1995]. Several luminal factors in breast-fed infants e.g. production of lactic acid creating an acid milieu, and the presence of oligosaccharides that compete for bacterial receptors on the mucosal surface preventing colonization by pathogens, and specific nutrients in breast milk (bifidus factor, lactoferrin, casein, and nucleotides) contribute to a luminal milieu that favours proliferation of these indigenous bacteria [Bernt and Walker, 1999]. In contrast, newborns on formula feeds at birth develop an intestinal flora that is rich in enterobacteria and gram-negative organisms due to
the lack of prebiotic modulatory factors present in breast milk. Due to the importance of the indigenous flora on gastrointestinal functions, studies have been conducted to assess the probiotic effect of this flora. The principal probiotics studied includes lactobacilli and bifidobacteria.

2. PASSIVE IMMUNIZATION

Antimicrobials are important tools in the management of infectious diseases. However, the control and treatment of infectious diseases is increasingly complicated by emergence of resistant organisms, the emergence and rapid dissemination of new pathogens, and a growing incidence of opportunistic infections. Identification and commercialisation of new antimicrobial agents has always been an expensive and time consuming process. Defining alternative strategies is therefore important for control of infectious diseases, including enteric infections. Over the last two decades, oral administration of specific antibodies prepared against a variety of enteric pathogens, has been tested with various degrees of success, both in animal models and in humans with diarrhoea, (see [Hammarstrom et al., 1994; Korhonen et al., 2000] for review). The fact that secretory immunoglobulins in breast milk protects neonates and young infants against diarrhoea due to a variety of infectious agents, such as enterotoxigenic Escherichia coli (ETEC), Vibrio cholerae, Shigella flexneri, Campylobacter jejuni and Helicobacter pylori [Thomas et al., 1993] has been the impetus for considering and testing passive immunotherapy in the management of such infections. The observation that patients with IgA deficiency are more vulnerable to respiratory and gastrointestinal tract infections also indicates an important role of antibodies in the mucosal defence, and suggests a useful role of passive immunity in the control and management of enteric infections. This review concentrates on studies in which different immunoglobulin preparations have been used along with their observed efficacies to prevent or treat enteric infections.

2.1 Human IgG

For a number of years, the use of intravenous Ig has been strongly endorsed as the treatment of choice in patients with primary and secondary immunodeficiencies [Buckley, 1994; Hammarstrom et al., 1994] and hematological, inflammatory, and neurological disorders, such as idiopathic thrombocytopenic purpura [Kuhne et al., 1998], Kawasaki’s disease
[Newburger et al., 1986], and Gullian-Barré syndrome [Bril et al., 1999]. Oral administration of purified human immunoglobulin also exhibited a prophylactic effect against the development of necrotizing enterocolitis in prematurely born infants [Eibl et al., 1988], and shows a therapeutic effect in diarrhoea associated with Campylobacter jejuni [Hammarstrom et al., 1993] Clostridium difficile [Tjellstrom et al., 1993], and chronic diarrhoea of undetermined etiology [Casswall et al., 1996] in normal infants. In hospitalized low birth weight babies, oral administration of human gammaglobulin containing rotavirus-neutralizing antibodies has been shown to modify the course and severity of rotavirus diarrhoea in children [Barnes et al., 1982; Guarino et al., 1991]. Oral administration of high doses of human IgG to healthy volunteers [Janson et al., 1995], and to patients with a variety of gastrointestinal infections (for references see [Hammarstrom et al., 1994]) was not associated with any apparent adverse events.

However, from a theoretical point of view, human IgG is expected to be less effective than human IgA due to its comparatively inferior proteolytic stability, and its possible interaction with complement and phagocytic cells might provoke an inflammatory reaction. Moreover, the use of human gammaglobulin is likely to be limited due to the risk of transmission of HIV, hepatitis B and C or prions. Additionally, such therapy is prohibitively expensive for routine use, particularly in developing countries where enteric infections are more common. There is thus a need to identify alternative sources of effective antibodies of non-human origin.

2.2 Bovine antibodies

In cows, antibodies are transported from serum to the milk and are present in high concentrations in colostrum, making it a suitable source for antibodies for therapy in humans. In fact, such antibodies have previously been found to be beneficial in the treatment of diarrhoea due to Cryptosporidium [Shield et al., 1993] and undetermined etiology in patients with AIDS [Plettenberg et al., 1993]. Bovine antibodies consist of three IgG subclasses – IgG1, IgG2 [Butler, 1983] and IgG3 [Rabbani et al., 1997], in addition to IgM, IgD, IgA and IgE. In contrast to human breast milk, in which sIgA is the dominant immunoglobulin class; IgG1 is the dominant immunoglobulin in bovine colostrum [Butler, 1983]. Passive immunization by colostral IgG1 antibodies protects the calves from neonatal infections. A cow produces about 1.5 kg of antibodies in the first few days after calving, making it an attractive source for large-scale production of IgG. The antibody titres in the colostrum of un-
immunized cows are rather low [Brussow et al., 1987; Hilpert et al., 1987], and thus, a large amount of material from non-immunized cows, possibly in the order of 100 g/day, would be required for effective therapy, making it unsuitable for pharmaceutical use. It is possible to produce high titres of specific antibodies in hyperimmunised bovine colostrum (HBC) following vaccination of the pregnant cows with antigens from specific infectious agents. In fact, HBC has been effectively used both for prophylactic [Davidson et al., 1989] and therapeutic purposes [Mitra et al., 1995]. The prophylactic effect has been demonstrated in adults challenged with enterotoxigenic *Escherichia coli* [Tacket et al., 1988], and a therapeutic effect has been described in the management of rotavirus diarrhoea in hospitalized children [Mitra et al., 1995]. Bacteriocidal activity against *Helicobacter pylori* has also been shown in bovine colostrum, indicating its potential role in passive immunization for this infection [Casswall et al., 2002; Korhonen et al., 1995]. Although such therapy is possible, it would be prohibitively expensive since a large dose is often required to produce a beneficial effect. Therefore, a source with a better potential for large-scale production of antibodies with high titers needs to be identified.

### 2.3 Chicken antibodies

Immunization of chickens for production of specific antibodies has several advantages including a non-invasive way of collecting antibodies. Over 100 years ago, it was shown that passive immunity is transferred from serum to egg yolk [Klemperer, 1893]. This mechanism is used in the production of antibodies from egg yolk, and hence the term egg yolk immunoglobulin (IgY) [Leslie and Clem, 1969]. Egg yolk is considered to be an interesting source of specific antibodies for oral immunotherapy. Another advantage is the enhanced immunogenity of mammalian proteins in birds due to their phylogenetic distance [Gassmann et al., 1990]. This makes it possible to produce antibodies against highly conserved mammalian proteins, and much less antigen is required to produce an efficient immune response [Larsson and Sjoquist, 1988]. Moreover, chicken antibodies tend to recognise the same protein present in a number of mammalian species, making them more widely applicable, and due to the evolutionary difference, chicken IgY reacts with more epitopes on mammalian antigens [Larsson et al., 1993].

Avian IgY has several attractive advantages over conventional IgG antibodies. The concentration of IgY in the egg yolk is approximately 25 mg/mL [Rose et al., 1974]. Since a
hen can lay up to 250 eggs in a year, the yield of hyperimmunised IgY could be very large. As 160 mg of IgY could be obtained from a single egg, one immunized hen could produce 40 grams of IgY in a year. Hatta et al. [Hatta et al., 1997] compared the productivity of IgY from the eggs laid by a hen over a year with that of IgG from the serum of a rabbit. Both animals were immunized with the same antigen and the quantity of IgY obtained from the eggs was 18 times higher than that of IgG from the serum of the rabbit.

Compared to the production and processing of other antibodies, the IgY technology offers several advantages such as: (i) no bleeding; only egg collection is required, (ii) IgY isolation is fast and simple, and (iii) only a small amount of antigen is required to obtain high and long lasting IgY titres in the egg yolk [Gassmann et al., 1990]. Considering these advantages, the European Centre for the Validation of Alternative Methods (ECVAM) has recommend the use of yolk antibodies instead of mammalian antibodies for animal welfare reasons [Schade et al., 1994].

2.3.1 Structural characteristics of IgY

IgY is the homologue of IgG, the major serum antibody found in mammals; however, it is structurally different. IgY contains two heavy (H) and two light (L) chains with a molecular mass of 180 kDa, larger than that of mammalian IgG (159 kDa). The heavy chain constant region of IgY has five domains - the variable domain (VH) and four constant domains (Cv1, Cv2, Cv3 and Cv4). Sequence comparison between IgY and IgG have shown that the Cγ2 and Cγ3 domains of mammalian IgG are closely related to the Cv3 and Cv4 domains of IgY, while the Cv2 domain is absent in γ chain, which is condensed to form the hinge region of IgG [Warr et al., 1995] (Figure 1).

![Figure 1: Structure of IgG and IgY (adapted from Warr et al., 1995)](image)
2.4 Llama antibodies

Passive immunization for treatment of enteric infections generally requires relatively large amounts of antibodies, particularly at mucosal sites where antibodies are rapidly cleared. Therefore, the above therapeutic modalities, although proven to be effective, become expensive due to the need for large doses of antibodies. The other limitations of the conventional IgG or IgY antibodies include their poor stability and complex nature that limits their large-scale production. Camels and llamas possess an unusual type of IgG antibodies (IgG2 and IgG3) that are devoid of light chains - referred to as "heavy chain" antibodies. The binding domains of these antibodies (VHH domains) show a high antigen binding affinity and are extremely stable [van der Linden et al., 1999]. The binding domain of the heavy chain antibodies consists only of the variable domain of the heavy chain (VHH) in contrast to the binding domain of conventional antibodies, which consists of a variable domain of the heavy chain (VH) and of the light chain (VL) (Figure 2).

In 1992, it was noted that camel milk contains high titres of antibodies against rotavirus [el Agamy et al., 1992]. Many people in African/Arabic countries regularly consume camel milk and therefore the VHH fragments are likely to be culturally acceptable as food products. The VHH fragments are well expressed and secreted by the baker’s yeast Saccharomyces cerevisiae, [van der Vaart] allowing a large-scale and cost effective production.
2.5 Mechanism of action of antibodies

Pathogens most commonly enter the body through the epithelial barriers of the mucosal lining of the respiratory, digestive, and urogenital tracts. Antibodies protect the mucosal surfaces, tissues, and blood from infections as the antibodies neutralize the pathogens or promote their elimination before they can establish significant infection.

High affinity antibodies can neutralize bacterial toxins [Robbins and Robbins, 1986] and inhibit infectivity of viruses [Mandel, 1976]. Many bacteria cause disease by secreting proteins, which damage or disrupt the function of the host cells. To have an effect, a toxin must interact specifically with a molecule that serves as a receptor on the surface of the target cells. In many toxins, the receptor-binding domain is on one polypeptide chain, whereas a second chain carries out the toxic function. Antibodies that bind to the receptor-binding site on the toxin molecule can prevent the toxin from binding to the cell and thus protect the cells from attack. Antibodies that act in this way to neutralize toxins are referred to as **neutralizing antibodies**. Antibodies that directly block binding of virus to surface receptor, neutralize viruses, and inhibit their infectivity.
Many bacteria have cell-surface molecules, called adhesins, which enable them to bind to the surface of the host cells. The adhesion of bacteria to cells within tissues can also contribute to the pathogenesis of infection. Antibodies can block attachment or adherence of pathogens to the cell surface [Roost et al., 1995]. IgA antibodies secreted onto the mucosal surfaces of the intestinal, respiratory, and reproductive tracts are particularly important in preventing infection by blocking the adhesion of bacteria, viruses, and other pathogens to the epithelial cells lining these surfaces. IgG antibodies against adhesins have been shown to be equally efficient as IgA antibodies in preventing damage at mucosal surfaces [Bessen and Fischetti, 1990]).

Another way by which antibodies can protect against infection is by initiation of complement activation, after binding to pathogens or after forming antibody:antigen complexes. One of the important effects of complement activation is the assembly of the terminal components of complement to form a membrane attack complex that results in a pore and which destroys the membrane integrity of the pathogen [Esser, 1991].

3. PROBIOTICS

Probiotics are defined as “live microorganisms that when administered in adequate amounts confer a health benefit on the host” [FAO-/WHO, 2002]. For over a century, researchers have suggested that live bacterial cultures, such as those found in yogurt, might be useful in the prevention and treatment of gastrointestinal disorders [Metchnikoff, 1907]. Most probiotic strains belong to the Bifidobacterium or Lactobacillus species and are part of the normal commensal bacterial flora of the human intestine [Reid et al., 2003; Servin, 2004]. Humans have been ingesting probiotics in fermented milk for thousands of years in the belief that they produce health benefits. For example, Persian tradition has ascribed Abraham’s fertility and longevity to his regular ingestion of yogurt [Boyle et al., 2006]. The proposed health benefits of probiotics have undergone increasingly rigorous scientific evaluation in recent years, and there is now strong evidence for their potential for treating and preventing human diseases. Their anti-diarrhoeal properties have been investigated since the 1960s [Beck and Necheles, 1961]. Several recent, controlled clinical trials have shown that selected strains of lactobacilli such as L. casei strain GG, [Guandalini et al., 2000; Isolauri et al., 1991; Isolauri et al., 1994; Majamaa et al., 1995] L. reuteri [Shornikova et al., 1997] and L. acidophilus [Simakachorn et al., 2000] exhibit both therapeutic and prophylactic effects in children with diarrhoea due to viral, but not due to bacterial, pathogens [Oberhelman et al., 1999; Szajewska et al., 2001].
3.1 Bacterial colonization and “probiotic-host cross talk”

Interaction between microorganism in the gut lumen and those attached to the mucosal surface and the host GI tract has been termed “host-microbiota–cross talk”. This communication is very complex and diverse and includes: competition/cooperation for nutrients, intra–and inter species communication; direct contact between components of the bacterium, e.g. peptidoglycan, and lipopolysaccharides, with host cell surfaces, secretion of bacterial compounds that can interact with the underlying epithelium, and “modulin”, which can directly affect host cell function and response (e.g. immune response, glycosylation changes etc).

Initial bacterial colonization of the intestine with an indigenous (non pathogenic) flora is an important component of the mucosal host defense in the human newborn. The pattern of development of the bowel flora results from a complex interplay between nutritional, immunological and environmental factors. It is generally accepted that the predominance of some bacteria, such as bifidobacteria, are beneficial and others, such as enterobacteria, Pseudomonas aeruginosa, and clostridia, are detrimental. Preterm infants in intensive care develop a very abnormal pattern of bowel colonization that contributes to the pathogenesis of neonatal necrotizing enterocolitis (NEC) [Hoy et al., 2000]. Healthy infants develop a colonizing microflora, which is dominated in the bowel by non-pathogenic species such as bifidobacteria [Harmsen et al., 2000]. The early preterm microbial colonization probably contributes to normal development through a number of different pathways. These include enhancement of the mucosal protective barrier [Orrhage and Nord, 1999; Panigrahi et al., 1994], modification of systemic immune responses [Sudo et al., 1997], competitive exclusion of less desirable microbes [Reid et al., 2001], protein and carbohydrate degradation, vitamin and butyrate production, and perhaps also mucosal cell differentiation [Hamzaoui and Pringault, 1998].

Very little is currently known about the specific molecular mechanisms by which the indigenous flora activates the intestinal host defence, and this will be an important area for investigations in the years to come. Using gnotobiotic mice, functional genomic studies have shown that the probiotic or indigenous organisms modulate the expression of genes involved in a broad range of important intestinal functions including nutrient absorption, angiogenesis,
xenobiotic metabolism and strengthening of the innate immune system [Hooper et al., 2001].
To understand this “cross talk”, studies that identify both the bacteria and host contributions require further investigation. For example, understanding of the molecular basis for nutrient sharing among members of the normal gut flora is essential for us to appreciate how the intestinal microbiota community is established and maintained and how it may be modified by probiotics to benefit the host.

One of the difficulties in assessing the role of probiotics in clinical practice is the limited understanding of their mechanisms of action. However, some of the biological effects of probiotics have recently been characterized.

3.2 Microbiological mechanisms of action

The human intestinal microbiota contains hundreds of different species of bacteria. The neonates are rapidly and extensively colonized in their passage through the birth canal, and the bacterial density rapidly rises to $10^{11}$ CFU/g, reaching $10^{14}$ microorganisms during adulthood [Dai and Walker, 1999]. Studies indicate that probiotic bacteria can significantly influence the composition of the healthy intestinal microbiota in newborns [Vendt et al., 2006] and also in adults [Benno, 1996]. It has been shown that the intestinal microbiota of infants is more amenable to manipulation by probiotic supplementation than that of adults [Vendt et al., 2006].

In disease states, probiotics can also affect the intestinal microbiota. Some of the protective mechanisms by which they inhibit the actions of pathogenic microbes have been elucidated. For example, in disease states associated with increased intestinal mucosal permeability, it has been shown that administration of Lactobacillus probiotics can decrease the intestinal mucosal permeability [Tannock et al., 2000]. Probiotics produce bacteriocins, hydrogen peroxide, and biosurfactant to aid their survival in the gastrointestinal tract and can competitively inhibit the adherence of more pathogenic bacteria to the intestinal epithelium. Many probiotic species induce mucin production by intestinal epithelial cells in vitro and some also induce the production of defensin-β2, an anti bacterial peptide [Facchini et al., 1992]. The most important mechanisms of probiotic actions, however, relates to the development, maturation, and regulation of mucosa-associated immune defences [Elmer and
McFarland, 2001; Hooper et al., 2002; Isolauri, 2001] and maintenance of normal crypt and epithelial cell architecture [Alam et al., 1994; Darmoul et al., 1997].

3.3 Immunological mechanisms of action

An important function of probiotics is its effect on the gut immune system. Most of the immunological effects of probiotics are likely to take place in the gut-associated lymphoid tissue, including Peyer’s patches [Walker et al., 2006]. A range of probiotic immune effects have been described, but direct evidence for the mechanism by which they achieve their beneficial effects is limited. Murine studies have shown that they enhance the function of the intestinal epithelial barrier. Hooper et al., discovered that intestinal commesals up-regulate the expression of mucin-encoding genes in the host intestinal epithelium, which stimulates the production of mucus to form a protective barrier [Hooper et al., 2001]. Other investigators have shown that Toll-like receptor (TLR) signaling by the commensal intestinal microbiota is essential for homeostasis of the intestinal epithelium and protection from epithelial injury [Rakoff-Nahoum et al., 2004]. Through pattern-recognition molecules on the commensal microorganism, TLRs stimulate the production of epithelial repair factors. This is likely to be an important mechanism of action of probiotics [Rakoff-Nahoum et al., 2004]. Activation of TLR by molecules, such as lipopolysaccharides flagellin, and lipoteichoic acid induce the production of cytokines through intracellular signaling pathways, which activate transcription factors like nuclear factor κB (NF-κB). Some non-pathogenic enteric bacteria exert an effect on intestinal epithelia cells by directly inhibiting the NF-κB pathway [Neish et al., 2000]. Others inhibit the same pathway by promoting the nuclear export of a NF-κB subunit, thus limiting the duration of NF-κB activation [Kelly et al., 2004]. These inhibitory effects on the pro-inflammatory NF-κB pathway may be an important mechanism by which probiotics regulate intestinal inflammation.

Clinical studies have shown specific immunologic actions of particular probiotics, e.g. increases in concentrations of the anti-inflammatory cytokine IL-10 in association with administration of Lactobacillus GG to infants [Pessi et al., 2000]. The enhanced in vivo generation of IL-10 may increase the anti-inflammatory properties of specific strains of probiotic bacteria, which is an additional consideration for their use in the treatment of patients with intestinal inflammation. Lactobacillus GG was also observed to up-regulate markers of phagocyte activation in healthy persons, but to down-regulate the same markers in
persons allergic to cow milk undergoing cow milk challenge [Pelto et al., 1998]. *Lactobacillus GG* has also been shown to promote local antigen-specific immune responses (particularly of the IgA class), and to prevent permeability defects, thus conferring controlled antigen absorption in allergic disorders [Majamaa and Isolauri, 1997]. It has also been suggested that specific probiotics have a potential to preferentially stimulate different subsets of T helper cells (Th1 or Th2) and thus modify intestinal inflammatory or allergic responses [Sutas et al., 1996].

### 4. ROTAVIRUS

Rotavirus is the leading cause of infantile gastroenteritis worldwide accounting for approximately 20% of diarrhoea-associated deaths in children less than 5 years of age [de Zoysa and Feachem, 1985]. Rotavirus was first identified in humans in 1973 when characteristic particles were observed in the cytoplasm of the duodenal epithelial cells obtained from young children admitted to the hospital with acute diarrhoea [Bishop et al., 1973]. While rotavirus infections are universal and occur regardless of socio-economic status or environmental conditions, the outcome and consequences differ significantly between developed and developing countries.

Rotaviruses belong to the Reoviridae family and are characterized by their segmented (11 segments) double-stranded RNA genome. Each of the 11 genes codes for a single product. Six of the proteins are found in the virus particles (vP1, vP2, vP3, vP4, vP6, vP7), whereas the remaining five proteins are non-structural (NSP1-NSP5). Rotavirus is classified into serogroups A-E based on antigenic properties. Only groups A-C have been shown to infect humans, however, most human disease is caused by group A. The group A Rotavirus is further classified into G (serotypes) and P types based on the identification of antigens on the outer capsid protein. Of the 14 serotypes (G types) that have been identified in various species; nine, particularly G1, G2, G3, and G4 and G9 are responsible for most infections [Cunliffe et al., 2002].

Rotavirus most commonly causes diarrhoea among infants and children aged 6-24 months; however, severe infections at a younger age occur more frequently in developing compared to developed countries [Ballal and Shivananda, 2002; Jain et al., 2001; Waters et al., 2000]. Children develop natural immunity after repeated exposure. Rotavirus epidemics peak in the winter in countries with a temperate climate. Rotavirus infections account for up to 60% and
40% of all diarrhoeal episodes in developing and developed countries respectively, resulting in an estimated 870 000 deaths each year [Perez-Schael et al., 1997].

4.1 Pathogenesis and pathophysiology of rotavirus gastroenteritis

A large number of investigators have studied the histological changes that take place in the intestine following rotavirus infection. In most cases these studies have involved naturally and experimentally infected animals and rotavirus infections have been studied more extensively in mice than in any other species. Rotavirus infects the mature enterocytes in the mid and upper part of the villi of the small intestine. Within 24 hours after infection with murine rotavirus, intestinal enterocytes appear swollen and vacuolated. Infection and histological changes are concentrated to the upper small intestine. Vacuolation of enterocytes is most prominent on the villous tips, but can occur in enterocytes throughout the villus. The observation that many enterocytes appear vacuolated led Osborne and co-workers to propose that fluid loss in diarrhoea is secondary to local villous ischemia, at least in mice [Osborne et al., 1991]. In contrast to animal studies, there are few pathology studies of jejunal mucosa in infants infected with rotavirus. Studies of biopsies have revealed shortening and atrophy of villi, distended endoplasmic reticulum, mononuclear cell infiltration, mitochondrial swelling and denudation of microvilli [Davidson and Barnes, 1979].

It is likely that the fluid and electrolyte secretion caused by rotavirus is not explained by one single mechanism. Experimental evidence supports a decreased rate of absorption of electrolytes and glucose/amino acids that may reflect both an attenuated absorptive area and a decreased epithelial cell transport capacity. Furthermore, the enzymatic activity in the brush border region is markedly decreased, indirectly lowering the rate of transport of glucose and amino acids. The magnitude of rotavirus-evoked fluid secretion is such that it is probably not explained solely by a decreased fluid and electrolyte absorption. Secretory mechanism(s) are also at work and its enterotoxin NSP4 [Estes et al., 2001] inhibits fluid and electrolyte transport of the villous epithelium by attenuating the Na-glucose symport SGLT1. Concomitantly, disaccharidase activity is also inhibited. It is possible that the Na-K pump in the basolateral membrane is also attenuated. Taken together, these events will lower the rates of fluid, electrolyte, and glucose absorption. The paracellular epithelial permeability is also increased by rotavirus and NSP4. Intracellular Ca\(^+\) concentration is increased in the intestinal epithelium in response to virus/NSP4 [Lundgren and Svensson, 2001]. This may evoke the
release of amines/peptides from the intestinal endocrine cells. Furthermore, cytokines, prostaglandin and NO are released from the enterocytes in response to rotavirus infection. All these biologically active compounds may alone, or together, activate neuronal dendrites located just underneath the intestinal epithelium and stimulate secretory reflexes in the enteric nervous system (ENS) [Lundgren and Svensson, 2001].

4.2 Current management of rotavirus gastroenteritis

Current management of rotavirus diarrhoea includes prevention and/or management of dehydration using oral or intravenous rehydration as appropriate, and continued feeding. Early resumption of normal feeding is encouraged to enhance mucosal repair and to minimize nutritional consequences of infection and diarrhoea. The children in developing countries might benefit from zinc supplementation, but its mode of delivery and cost effectiveness are yet to be determined in rotavirus diarrhoea. No specific therapy to either reduce the duration or the severity of illness is currently available. Therefore, rotavirus diarrhoea, which is considered to be a self-limiting disease, continues to contribute significantly to childhood deaths in the developing countries [Glass et al., 1996].

4.3 Active immunization against rotavirus infection

The development, testing and potential wider use of an effective, safe and inexpensive rotavirus vaccine would be the most efficient method for preventing severe disease and deaths. Rapid progress has recently been made, and several candidate rotavirus vaccines have been developed and tested. The rhesus-human reassortant, tetravalent vaccine (RRV-TV) has been reported to provide a high rate of protection against rotavirus diarrhoea in some trials [Joensuu et al., 1997; Perez-Schael et al., 1997] while others have observed only a low rate of protection [Lanata et al., 1996]. A live, attenuated rotavirus vaccine (Rotashield, Wyeth Laboratories, Marietta, PA), licensed and recommended for routine immunization of US infants, was quickly withdrawn from the market due to reported higher rates of intussusceptions among the vaccinees [Gay et al., 1999; Murphy et al., 2001]. Two rotavirus vaccines, Rotarix (GlaxoSmithKline Biologicals, Belgium) and RotaTeq (Merck & Co., USA) have recently completed Phase III clinical trials [Desselberger, 2005; Petersen et al., 2006]. Both vaccines appear to be safe with respect to intussusceptions, and are highly efficacious in preventing severe gastroenteritis due to rotavirus strains carrying predominantly serotype G1 [Cunliffe and Nakagomi, 2005]. Confirmation of the safety of
both vaccines will require extensive post marketing surveys. Moreover, assessment of the
ability of each vaccine to provide protection against an increasingly diverse population of
rotavirus strains will depend on continuous surveillance of circulating strains and
development of newer vaccines with changes in the predominate serotypes in different
geographical locations - not easy to accomplish in developing countries. There is thus a clear
need to define other, cost-effective and affordable interventions for prevention and
management of rotavirus diarrhoea.

4.4 Passive immuno-therapy for rotavirus-induced diarrhoea

There are good reasons to hypothesize that passive immunotherapy could be useful in the
management of human rotavirus diarrhoea. For example, oral administration of rotavirus-
neutralizing antibodies has been found to modulate the rotavirus-induced diarrhoea in
children. Beneficial prophylactic [Barnes et al., 1982] and therapeutic effects [Guarino et al.,
1991], in terms of reduction of duration and severity of rotavirus diarrhoea, have also been
observed in association with orally administered human IgG preparations. The use of human
gammaglobulin, however, is limited due to the risk of viral contamination and high
production and storage costs.

An alternative safe and easy to produce source of antibodies, was later developed by
vaccinating pregnant cows against human strains of rotavirus, generating hyperimmunized
bovine colostrum. This mode of therapy has also been effectively used for prophylactic
[Davidson et al., 1989] and therapeutic benefits [Mitra et al., 1995] in rotavirus diarrhoea in
hospitalized children.
5. **ESCHERICHIA COLI (E. COLI)**

*E. coli* is the most abundant facultative anaerobe in the colon and is considered as the workhorse of molecular biology; however, some strains are public health threats. Within *E. coli*, enterotoxigenic and enteropathogenic *E. coli* strains are the major pathotypes [Albert et al., 1995; Qadri et al., 2005]. This is supported by previous results in a hospital-and a community-based study in rural Bangladesh [Black et al., 1980]. The prominent role of ETEC and EPEC infections has also been observed in many other developing countries [Echeverria et al., 1989; Guerrant et al., 1983; Kim et al., 1989]. In most of the studies in the developing world, ETEC is known to be the most common enteric pathogen, accounting for approximately 20% of diarrhoea episodes. ETEC is the common cause of diarrhoea in children up to 2 years of age [Bhan et al., 1994], and a leading cause of traveler’s diarrhoea [Lima, 2001]. The incidence of ETEC in developing countries decreases between 5-15 years of age, and increases again after the age of 15; 25% of all ETEC diarrhoea occurs in adults [Qadri et al., 2000].

5.1 **Pathogenesis**

ETEC causes diarrhea by producing heat labile (LT) and/or heat stable (ST) enterotoxins, and or more of at least 22 different colonization factors, which contribute to their virulence [Gaastra and Svennerholm, 1996]. These bacteria possess adhesins, known as colonization factor antigens (CFA), which enable them to bind to small intestinal mucosa, a region where *E. coli* does not normally reside in substantial numbers. These colonization factors generally take the form of surface fimbriae that are 2-9 nm in diameter, which bind to specific receptors on the host cells [Gaastra et al., 2002]. Although the nature of the receptors is not known, many are likely to be oligosaccharides on glycoprotein or glycolipids. This conclusion is based on the observation that bacterial adhesion to susceptible cells *in vitro* can often be blocked by pre-incubating cells with simple sugars [Wolf, 1997]. The interaction between ETEC adhesins and receptors appears to be host specific, and thus, the adhesins of ETEC that infect domestic animals differ from those of human strains.

In contrast to their diverse colonization factors, ETEC secret only two varieties of enterotoxins, known as heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) [Sears and Kaper, 1996]. Heat-labile enterotoxin is closely related to cholera toxin, both biologically and antigenically. Both toxins have a similar subunit structure with a single A subunit and five identical B subunits, which, in their pentameric form, allow the toxin to bind to a
ganglioside receptor, known as GM1, on host cells. The bound toxin is internalized and
processed, freeing up the A1 subunit from the holotoxin and releases into the host cell
cytoplasm, it catalyzes the modification of a regulatory subunit of the membrane-associated
adenylase cyclase, leading to irreversible activation of the latter. The resultant accumulation
of intracytoplasmic cyclic-AMP (cAMP) ultimately leads to an altered electrolyte transport
by the enterocytes, most notably increased secretion of chloride ions by the crypt cells and
reduced absorption of sodium and chloride ions by the villous cells. Both of these actions
lead to luminal accumulation of electrolytes, which drags water into the intestine along the
osmotic gradient. If the volume of the accumulated intraluminal fluid exceeds the normal
absorptive capacity of the large intestine, the excess is evacuated as watery diarrhea, which
characterizes infection with ETEC and *Vibrio cholerae*. Apart from their action on the
activity of the adnyl cyclase, cholera toxin (CT) and, presumably LT, also produce diarrhea
by influencing the metabolism of prostaglandins and stimulation of the neurotransmitters of
the enteric nervous system [Mourad and Nassar, 2000]. ETEC–induced diarrhea is usually
mild and self-limiting; however, it can be severe in some patients and devastating in
malnourished and weaning infants in developing countries because of high purging rates
similar to that observed in cholera.

5.2 Current treatment

Current management of ETEC diarrhoea includes prevention or management of dehydration
using oral or intravenous rehydration as appropriate, and continued feeding - fluid and
electrolyte balance is a therapeutic priority. Early resumption of normal feeding is
encouraged to enhance mucosal repair and to minimize nutritional consequences of infection.
Administration of antibiotics, although potentially useful, is of limited use in the management
of ETEC diarrhoea in developing countries where the infections are common and the
pathogens are usually resistant to commonly used agents. Importantly, patients suspected of
being infected with these bacteria should not be treated with antibiotics because they may
enhance synthesis and release of the toxins from the bacteria, and increased the risk for
hemorrhagic colitis or HUS [Wong et al., 2000].
5.3 Active immunization against *E. coli* diarrhoea

In the early seventies, Felsenfeld and his co-workers conducted an oral vaccination trial in monkeys, which revealed that a polyvalent, vaccine provides a short-term protection against *E. coli* [Felsenfeld et al., 1972]. Some trials have also observed significant protective effects of an orally administered, colicin E2-killed whole cell vaccine [Evans et al., 1988]. Experimental animal studies and indirect evidence from clinical studies suggest that immunity against ETEC is mediated by secretory immunoglobulin A antibodies [Svennerholm and Holmgren, 1995]. The difficulties in the development of a vaccine are related to the large number of already identified antigenically distinct CFA of human ETEC strains, and presence of potentially more, as yet unidentified factors. Taking these into account, several vaccine candidates for oral immunization have been proposed in the last few years. A recent phase-II safety and immunogenicity trial of an oral, formalin-inactivated ETEC vaccine containing six colonization factors (CFA/I, CS1, CS2, CS3, CS4, CS5) along with one milligram of recombinant cholera toxin B subunit, the CF-BS-ETEC vaccine, was shown to be well tolerated by Bangladeshi children and to produce significant systemic IgA antibody responses [Qadri et al., 2003]. The development, testing and eventual use of an effective, safe and inexpensive *E. coli* vaccine would be the most efficient method for preventing severe disease and deaths. There is thus a clear need to define improved, cost effective intervention in the management of ETEC diarrhoea, until an effective, safe and inexpensive vaccine, affordable to the developing countries for routine use, becomes available.

6. **HELCOBACTER PYLORI INFECTION**

Gastric colonization by *Helicobacter pylori* was first demonstrated in 1984 [Marshall and Warren, 1984]. *Helicobacter pylori* is a gram negative, spiral, microaerophilic bacterium that infects the stomach of more than 50% of the human population worldwide [Mitchell, 1999]. The prevalence is much more higher developing countries where 90% or more of population usually acquire the infection early in life [Sarker et al., 1995; Thomas et al., 1999]. The prevalence of infection increases with age [Sarker et al., 1997], which is attributed to a birth cohort effect [Veldhuyzen van Zanten et al., 1994], the vast majority of which are acquired in
childhood in both developing and developed countries [Rothenbacher et al., 2000], possibly due to mother-to-child transmission [Malaty et al., 2000]. In the absence of eradication, most infections run a chronic course and persists for life [Miehlke et al., 1999], and causing chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and B cell lymphoma in some individuals [Howden, 1996; Kuipers et al., 1995]. Based on overwhelming epidemiological evidence, individual infected with *Helicobacter pylori* have been shown to have an increased risk of non-cardiac cancer compared to an uninfected control population [Huang et al., 1998; Xue et al., 2001] and in 1984, the World Health Organization declared *H. pylori* as a class I carcinogen [Anonymous].

6.1 Transmission

The exact means of transmission of *H. pylori* remains a puzzle. The involvement of pets in the transmission is extremely unlikely [Bode et al., 1998]. *H. pylori* has been demonstrated to contaminate water supplies [Hulten et al., 1996] and sheep milk [Dore et al., 1999]; however, they may represent a consequence of bacterial shedding from infected people rather than being an actual source of infection particularly in the population in the developing world with poor sanitation and hygiene. Fecal-oral transmission is certainly a possibility; however, it is not clear if *H. pylori* would actually be able to survive transit through the normal intestine. Oral-oral transmission has also been proposed [Mitchell, 1999] as it was possible to culture *H. pylori* from pharmacologically induced vomiting and/or diarrhoea in infected adults [Parsonnet et al., 1999]. Similar results have been obtained in children with spontaneous vomiting [Leung et al., 1999].

There is clear histological evidence for lifelong gastritis in almost all infected individuals, yet a large proportion of those colonized with *H. pylori* remain asymptomatic. Around 15-20% of all infected people develop serious gastroduodenal diseases such as chronic gastritis, gastric and duodenal ulcers, and gastric adenocarcinoma and lymphoma. It is estimated that at least 7 million cases of *H. pylori*-induced diseases occur worldwide each year [Parsonnet et al., 1999], and most of them develop several years after the initial colonization.
6.2 Virulence factors

The observed variations in disease outcome are likely to be mediated by an intricate interplay between bacterial, host, and environmental factors. There are substantial phenotypic and genotypic diversities between *H. pylori* isolates, both at the genomic level and in individual virulence factors. Strain-to-strain genetic variability in bacterial virulence factors e.g. vacA and cagA affects not only their ability to colonize and to cause disease but they also influence inflammation and gastric acid output. There is a clear association between colonization with cag A$^+$ strains with an increased risk of developing a symptomatic outcome such as peptic ulcer or gastric cancer in Western populations [Blaser and Crabtree, 1996; Kuipers et al., 1995]. Variations in the host immune response, on the other hand, determine the chronicity of *H. pylori* infection, reflected directly on gastric disease including gastric acid output, but also in determining density and location of colonization by *H. pylori*. For example, in patients with predominant antral gastritis, acid secretion is increased, thus predisposing to duodenal ulcer [McColl et al., 1998]. On the other hand, impaired acid production in corpus predominant gastritis is associated with gastric ulcers and gastric carcinoma [McCull and El-Omar, 2002].

*H. pylori* isolates are classified as cytotoxin-associated gene (*cag A*) positive *cag*$^+$ or negative *cag*$^-$, depending on the presence of a pathogenicity island (PAI) of 40 kb DNA in the chromosome, termed *cag PAI*, which encodes a type IV secretion system. The majority (70%–80%) of the clinical isolates of *H. pylori* from individuals living in developed countries express CagA. In some countries, such as Japan and Korea, the frequency of CagA positive strains may even reach 100% [Maeda et al., 1998; Park et al., 1998; Weel et al., 1996]. Strains carrying the *cag* PAI are referred to as type I strains and those lacking *cag*-PAI are referred to as type 2 strains. The Type I strains are associated with more severe gastric pathologies (i.e. peptic ulcer, gastric cancer) in adults [Blaser and Crabtree, 1996]. Type 2 strains are less virulent and associated with milder gastric pathologies (i.e. chronic gastritis). However, it is now known whether both type I and type II strains express the *vacA* gene.

6.3 Pathogenesis

The successful life-long colonization of the human stomach by *H. pylori* is achieved through a combination of factors, which address the different challenges presented by the harsh
gastric environment. Its helicoidal shape and the action of flagella allow it to cross the thick layer of mucus lining the stomach. *H. pylori* adheres strongly to gastric cells, and binding is probably mediated by several proteins and glycolipids. BabA [Boren et al., 1993] and SabA [Mahdavi et al., 2002], outer membrane proteins of *H. pylori*, bind to the Lewis B blood group antigen and sialyl Lewis x respectively of human cells.

*H. pylori* is capable to synthesize urease to buffer the pH of its immediate surroundings in the stomach. The urease might cause damage to the host cells through the production of ammonia, an agent known to be toxic. Urease might also help to recruit neutrophils [Megraud et al., 1992; Suzuki et al., 1992] and monocytes in the inflamed mucosa and to activate production of proinflammatory cytokines [Harris et al., 1996]. Membrane-permeant weak bases cause various cell alterations, including swelling of intracellular acidic compartments, alterations of vesicular membrane transport, depression of protein synthesis, and ATP production and cell-cycle arrest. The 88 kDa VacA toxin, an important virulence factor in the pathogenesis of peptic ulceration and gastric cancer, can induce multiple cellular activities, including cell vacuolization, membrane channel formation, disruption of endosomal/lysosomal function, apoptosis, and immunomodulation [Cover and Blanke, 2005].

6.4 Clinical features of *Helicobacter pylori* infection

*H. pylori* colonization of the human stomach is virtually always accompanied by chronic gastritis. Persistent *H. pylori* gastritis may lead to destruction of gastric glands and fibrosis, a condition termed atrophic gastritis [Dixon et al., 1996]. The presence of atrophic gastritis facilitates the development of intestinal metaplasia, dysplasia and gastric adenocarcinoma. There is a 2.7-12 times increased risk of distal adenocarcinoma in *H. pylori* infected individuals [Huang et al., 1998]. *H. pylori* infection is clearly linked to peptic ulcer disease both in adults and in children [Cover and Blaser, 1996]. In one study, *H. pylori* were found in 92% of children with duodenal ulcer and in 25% of patients with gastric ulcers [Macarthur et al., 1995]. As in adults, most children with *H. pylori* infection remain asymptomatic, and they do not report abdominal pain more often than age-matched non-infected children [Bode et al., 2003]. Whether uncomplicated, *H. pylori*-induced gastritis is responsible for abdominal symptoms in subgroups of children has not been determined, and prospective placebo-controlled therapy trials in symptomatic children are still missing.
There is increasing interest in the extra-intestinal manifestations of chronic *H. pylori* infections in the development of children. Acute infections, known to cause temporary low or no gastric acid production (achlorhydria), could lead to health disadvantages in children as reduced or no gastric acid production results in impairment of the “gastric barrier” leading to bacterial overgrowth in the stomach and increased susceptibility to enteric infections, a major public health concern linked to diarrhoea, malnutrition and growth failure in children living in the developing countries [Gilman et al., 1988; Sullivan et al., 1990]. As gastric acid is also crucial for iron (Fe) absorption, low gastric acid production may cause suboptimal absorption of certain iron salts commonly used in fortification, and children with achlorhydria may thus have reduced the absorption of all non-heme Fe. Several recent reports have indicated an association between *H. pylori* infection and anemia, iron deficiency, and iron deficiency anemia [Kostaki et al., 2003; Marignani et al., 1997; Seo et al., 2002; Yip et al., 1997], although the underlying mechanism has not been well described. There are also studies that show a significant relationship between *H. pylori* infection and delayed growth of young children [Goodman et al., 1997; Perri et al., 1997]. Other investigators have observed a much higher prevalence of *H. pylori* infections in type I diabetic patients who tested positive for gastric parietal cell autoantibodies compared to age-matched diabetic patients without those autoantibodies [Arslan et al., 2000].

It is well known that an increased intestinal permeability can lead to an increased exposure of bacterial and dietary antigens to the immune system, which precipitates or perpetuates inflammatory reactions. Several studies performed on cell monolayers have shown that *H. pylori* decrease transepithelial resistance, causing significant increase in horseradish peroxidase passage, an indicator of increased intestinal permeability. During *H. pylori* infection, moderate to severe gastritis develops, which is accompanied by increased epithelial permeability. To prove the hypothesis, a clinical study investigating the relationship between *H. pylori* infection and food allergy suggested that Cag positivity is more common in patients with food allergy. Furthermore, serum IgE against the most common dietary antigens was significantly higher in *H. pylori*-Cag A positive individuals compared to those who were Cag A negative [Figura et al., 1999]. By causing a low gastric acid output, the infection could lead to further health disadvantages in children. For example, by abolishing the gastric acid barrier, *H. pylori* infection might increase the risk for acquiring other infections [Howden and Hunt, 1987] via the gastrointestinal tract. Moreover, chronic infection, with its accompanying...
inflammation, may also be deleterious, and it has been suggested that this may stunt growth and lead to chronic diarrhoea [Sullivan et al., 1990].

6.5 Therapy

Following the exponential rise in *H. pylori*-related research over the past decade, and varying worldwide prevalence and characteristics of *H. pylori* related diseases, guidelines for management of *H. pylori* infection have been established locally, regionally, and internationally. However, among these guidelines the Maastricht 1 and Maastricht 2 consensus report (European *Helicobacter pylori* study Group, 1997) [Anonymous, 1997], the NIH consensus development conference [Anonymous, 1994], and the Asia Pacific consensus conference reports [Lam and Talley, 1998] are usually followed by practicing clinicians. There is a general consensus that *H. pylori*-infected children are considerably less susceptible to peptic ulcer disease than adults, and that there is no compelling evidence demonstrating a relationship between *H. pylori* infection and recurrent abdominal pain in children, except those with gastric and duodenal ulcer. It is, therefore, recommended that children should be investigated for *H. pylori* infection only when they present with symptoms or signs suggestive of organic disease that are serious enough to justify the risk/adverse events of therapy. All the consensus groups recommend triple therapy using a proton pump inhibitor (PPI) plus two antibiotics for 7-14 days as the treatment of choice for *H. pylori* infection in children. However, due to the concern over the increasing rate of *H. pylori* resistance to macrolide and metronidazole in children [Kalach et al., 2001], testing for susceptibility and reliable non-invasive tests to confirm eradication are recommended. Few studies have reported unacceptably low (<80%) eradication rates with triple regimes (omeprazole, clarithromycin and amoxicillin or metronidazole) [Fradkin et al., 1997; Oderda, 1997]. Poor compliance as a consequence of long treatment regimes, adverse effects, and the complexity of the treatment protocols were considered responsible for the failures [Dohil et al., 1997]. Compared to the adults and older children, the risk of reinfection is higher in children younger than 5 years. It is possible that the duration of the infection, dose and bioavailability of the drugs, or primary resistance to the first-line antimicrobials used in the study population might play a role. There is a general consensus that screening and treatment of *H. pylori* infection in asymptomatic populations is not required. Till date, there is no scientific evidence in favour of treatment for *H. pylori* in the absence of an established diagnosis of peptic ulcer disease in children [Robinson et al., 1997]. However,
most pediatric gastroenterologists tend to treat children with significant abdominal symptoms, even when no ulcer is demonstrated in endoscopic examinations [Blecker and Gold, 1997; Oderda, 1997; Robinson et al., 1997].
7. AIMS OF THE PRESENT THESIS

The aims of the thesis were to:

1. Evaluate the efficacy of oral administration of immunoglobulins (IgG) from immunized bovine colostrums (HBC) in diarrhoea due to rotavirus, ETEC, and in Helicobacter pylori infections in infants and young children.

2. Evaluate the efficacy of oral administration of egg yolk immunoglobulins (IgY) from immunized chicken in rotavirus-induced diarrhoea in children and in vivo in mice.

3. Evaluate the efficacy of monovalent llama-derived rotavirus specific antibody fragments produced in yeasts in reducing the morbidity of rotavirus-induced diarrhoea in mice.

4. Evaluate the therapeutic efficacy of a probiotic agent, Lactobacillus paracasei in ameliorating diarrhoea in children.

The efficacy trials in the clinics and community were randomized, double blind and placebo-controlled. The animal experiments were randomized, and placebo-controlled.
SUBJECTS AND METHODS

8. SUBJECTS

The children enrolled in the clinical studies were younger than 2 years of age since this age group is most susceptible to both rotavirus, *E. coli* (Anonymous, 2002) and *H. pylori* infection. The animal experiments on rotavirus infection were conducted in 4-days old Balb/c mice.

8.1 Study sites

The clinical studies were conducted at the Dhaka Hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The community-based study of hyperimmune bovine colostrum (HBC) in the management of *Helicobacter pylori* infections was conducted at Nandipare, a peri-urban community of Dhaka, where ICDDR,B operates a clinic 12 kilometer from the main campus. The animal experiments were conducted at the animal facility at Huddinge hospital, Stockholm, Sweden.

8.2 Bangladesh

The People’s Republic of Bangladesh has a long history spanning thousands of years. A land replete with history, that finally achieved independence in 1971 after a protracted 9-month long independence war against Pakistan. With an area of about 144,000 sq km, Bangladesh is situated between latitudes 20~34' and 26~38' north and latitudes 88~01' and 92~41' east. The major portion of Bangladesh lies within the broad delta formed by the rivers Ganga, Brahmaputra and Meghna, and regularly suffers from floods. The only significant area of hilly terrain, constituting less than one-tenth of the country's territory, is the Chittagong Hill Tracts District in the narrow southeastern part of the country. The country is bordered by India on the east, west and north and by the Bay of Bengal on the south. There is also a small strip of frontier with Burma on the southeastern edge. The land is a deltaic plain with a network of numerous rivers and canals (Photograph 1).
The estimated population of Bangladesh (2001) was 133,376,684, making it one of the ten most populous countries. The overall density, 890 persons per sq km (2,304 persons per sq mi) in 2001, is much higher than that of other countries except for microstates such as Singapore [Anonymous, 2001]. Population growth rate is around 1.6%. The vast majority of people live in rural areas and although the urban population is increasing, only about 21% of the total population lived in the urban areas in 1999. The distribution of the population is relatively even, except the Chittagong Hill Tracts District which is sparsely populated and the mangrove forest Sundarbans that is almost devoid of any population. Most of the people are relatively young: about 60% under the age of 25, and only 3% are 65 or older. Life expectancy at birth is increasing and is currently 61 years. Islam, the state religion, is the faith of 88% of the population, and nearly all belong to the Sunni sect. Hindus make up most of the remainder, but the country also has small communities of Buddhists, Christians and animists.
The official language is Bengali or “Bangla” but English is spoken in the administration and at the universities. Bangladesh is one of the most fertile areas on Earth, yet it is often plagued by natural disasters on a scale beyond imagination. Bangladesh is an agricultural country. Jute and tea are principal sources of foreign exchange. Being an agricultural country, with some three-fifths of the population engaged in farming, a vast majority of the population depends directly upon their own farm production for survival. The agriculture sector is complex, and labour-intensive, with a low technological and resource base. Although small, the industrial sector contributes significantly to export receipts, and provides employment and a market for cash crops. Jute products, mainly burlap sacking and carpet backing for export, and cotton textiles for domestic consumption predominate. Since the early 1980s the production of ready-made garments for the US market has grown rapidly. Bangladesh is the fifth largest supplier of cotton apparel to the United States, and it has begun exporting to West European markets. Breaking up ships for scrap, using methods that are highly labor intensive, now meets most of Bangladesh's domestic steel needs. Other industries include sugar, tea, leather goods, newsprint, pharmaceuticals, and fertilizer production. The GDP real growth rate is approximately 4%.

Bangladesh has a tropical monsoon climate with significant variations in rainfall and temperature throughout the country. There are four main seasons: (i) the pre-monsoon (March-May) has the highest temperatures and experiences the maximum intensity of cyclonic storms, especially in May; (ii) the monsoon (June-September) when the bulk of rainfall occurs; (iii) the post-monsoon (October-November) which, like the pre-monsoon season, is marked by tropical cyclones on the coast; and (iv) the cooler and sunny dry season (December-February). The mean annual temperature is about 25°C, with extremes of 4 and 43°C. Ground frosts can occur in the hills. Humidity ranges between 60 percent in the dry season and 98 percent during the monsoon. About 80 percent of the total rainfall occurs during the monsoon, and the average annual rainfall for the entire country is 2320 mm. Precipitation varies from 1110 mm in the west to 5690 mm in the northeast. The country is regularly subjected to drought, floods and cyclones.

8.3 ICDDR, B:

The International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), popularly known as “The Cholera Hospital” locally, is an international health research
institution located in Dhaka, the capital of Bangladesh (Photograph 2). The Centre is an independent, international non-profit organization. In collaboration with partners from academic and research institutions throughout the world, the Centre conducts research, training and extension activities as well as programme-based activities through four scientific divisions: Clinical Sciences Division, Laboratory Sciences Division, Public Health Sciences Division (PHSD) also Health Systems and Infectious Diseases Division (HSID). The Centre’s vision is that all people, especially the poor, can become healthier and can reach their full potential through the application of new knowledge. Oral Rehydration Solution (ORS) that has saved 40 million lives worldwide since its implementation, underwent the first successful clinical trial at this Centre in 1968.

Photograph 2: International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B)

With the changing trend in the world scenario in health and population over the years, ICDDR, B (Centre) has expanded its activities to address some of the most critical global health needs. The Centre’s mission is to develop and promote realistic solutions to the major health, population and nutrition problems facing the poor people of Bangladesh and other settings. The Centre was established in 1960 as the Pak-SEATO Cholera Research
Laboratory to find ways of controlling cholera and improving its management. Following the independence of Bangladesh, the Centre was renamed as the Cholera Research Laboratory. In 1963, the Centre implemented a health research programme in rural Matlab. Matlab is located about 55 km southeast of Dhaka. The Health and Demographic Surveillance System (HDSS), formerly known as the Demographic Surveillance System (DSS), is one of the major components of this field programme. Since 1966, the HDSS has maintained registration of births, deaths, and migrations, in addition to carrying out periodical censuses.

8.3.1 The Clinical Sciences Division (CSD)

The Clinical Sciences Division (CSD) conducts hospital- and community-based clinical research in the fields of diarrhoeal diseases, respiratory infections, nutrition, and child development. The division also operates the Dhaka Hospital of ICDDR, B that provides care and treatment to around 110,000 patients each year, of whom about 60% are under five children, with uncomplicated and complicated diarrhoeal diseases, and associated health problems including malnutrition and pneumonia. Prevention strategies such as immunization of children and women, education of mothers on prevention and home management of diarrhoeal diseases and malnutrition, counseling to lactating mothers on exclusive breastfeeding, and promotion of birth spacing and immunization are also undertaken at the Dhaka hospital. Provision of theoretical and hands-on training on case management of diarrhoeal diseases and associated health problems, and clinical research methodology are also important activities of the division. According to the severity of illness and presence/absence of complications and/or other health problems, patients are treated in either of the following units: Short Stay Unit, Longer Stay Unit, and Special Care Unit. CSD also has a research ward where controlled clinical trials and nutritional and metabolic studies are conducted.

Of the total population visiting the hospital, approximately 35% with mild diarrhoea are referred to another clinic established within the ICDDR, B campus. Operated by an independent NGO, Progoti Somaj Kolliyan Prostisthan (PSKP), the clinic franchises the patient care services of ICDDR, B. Around 58% of patients with clinical dehydration but no complications or other health problems are managed and treated at the Short Stay Unit with an average stay of 24 hours. The remaining 7% patients require admission to the Longer Stay Unit or Special Care Unit or to Research Wards (Annual Report ICDDR, B 2003). The overall case fatality is 0.3%. The rush of patients at Dhaka Hospital is illustrated in Photograph 3.
Since 1979, a Diarrhoeal Disease Surveillance system is functioning at the Dhaka Hospital of ICDDR,B. The system enrolls 2% of all patients attending the hospital to determine the aetiology of diarrhoea and antimicrobial resistance of enteric bacterial pathogens, and their changes over time, emergence of new pathogens, detection of outbreaks and their locations, and to monitor sociodemographic information. In about 80% patients a diarrhoeal pathogen can be isolated. From about 14,000 fecal specimen tested under the system from 1996 through 2001, rotavirus was isolated in 23.7%, *Vibrio cholerae* in 22.9%, and different types of diarrhoeagenic *E. coli* in 34.9%. Among under-five children (>8,000 stools tested), rotavirus was isolated from 37.9% [Anonymous, 2002]. Multiple pathogens were isolated from about a third of the specimens.

### 8.3.2 Nandipara

*Nandipara* is a peri-urban area of Dhaka city, about 12 kilometers from the ICDDR, B campus. In 1985, a weekly clinic was established to provide outpatient services to the
children and their mothers with common medical problems. About 3,000 people live in an area of only 2.5 square miles, and of them about 500 are under-five children. The average family size is 4.5 members. Municipal water is used for drinking and cooking; however, ditch water is used for bathing and washing. The environmental sanitation of the area is very poor with a majority of the inhabitants using hanging latrines, which contaminate the ditch water. Most houses are mud-walled with thatched dry-bamboo roofs.

8.3.3 Participants of the clinical studies

8.3.3.1 Diarrhoeal disease (papers I, II, IV and V)

Papers: I, II, IV & V: These clinical studies were conducted at the Research Ward of the Dhaka Hospital, ICDDR,B. Male infants and children between 4 months to 24 months of age, admitted to the Short Stay Unit of the Dhaka Hospital of ICDDR, B with history of watery diarrhoea of less than 24 hours duration were initially screened. Stool volume was one of the important measures and, therefore, only male children were enrolled to facilitate reliable separation of urine from stools using paediatric urine collection bags. The children who fulfilled all of the inclusion and exclusion criteria were observed for 4 hours while their fecal specimen were tested for presence of *V. cholerae* using dark field microscopy and for rotavirus using ELISA. Children with a positive ELISA for rotavirus, a negative dark field test for *V. cholerae* (specimen is illuminated obliquely by a special light microscopy condenser, that does not hit the object, resulting in a light phenomenon in which bacteria are illuminated against a dark background), and who passed >20 ml/kg of stool during the observation period (signifying severe diarrhoea), were included in the rotavirus studies (Paper I and IV). Children with a negative ELISA for rotavirus and negative dark field test for *V. cholerae* were included in the *E. coli* study (paper II). Children with a negative dark field test in spot samples were included in the probiotic study (paper V). Children with severe protein energy malnutrition (65% weight for age by the standard of the National Centre for Health Statistics), with systemic infection requiring antimicrobial therapy, or history of bloody diarrhoea, were excluded in all the studies. All the studies were randomized, double blind and placebo-controlled and the interventions with active or placebo products were given for 4 days in the passive immunotherapy studies with immunoglobulin (paper I, II and IV) and for 5 days in the probiotic study (paper V). The children in the HBC rotavirus study (paper I) were enrolled between March 1995 and December 1996; in the HBC-*E. coli* study (paper II) were enrolled between March 1995 and October 1997; in the IgY-rotavirus were enrolled
between January 1997 and June 1999; and in the probiotic study, they were enrolled between December 2002 to November 2004.

8.3.3.2 *Helicobacter pylori*

**Paper III**

This was a community-based study conducted at the Nandipara community clinic of ICDDR,B as described above. All asymptomatic infants and children aged 4-29 months in the community were initially listed according to a pre-assigned family number. They were then screened for *H. pylori* infection using $^{13}$C urea breath test (UBT), and those positive in the test were enrolled sequentially for the intervention study to receive HBC or placebo according to randomization between June 1995 and December 1996. However, there was a delay by 2-6 months in initiating the intervention following the screening, which was due to the time taken for transportation of breath samples, sent in batches, from ICDDR,B to the Karolinska Institute (KI), Sweden for analysis, time taken for the tests, and time taken for receiving the results. Children with severe PEM (<60% weight for age according to the National Centre for Health Statistics reference values), history of taking antibiotics preceding one month, or with other systemic infections were not included in the study.

8.3.3.3 **Animals**

**Paper VI and VII:**

*Animal model of rotavirus diarrhoea*

The study with the llama-derived, monovalent, anti-rotavirus fragment (paper VI) and chicken IgY antibodies (paper VII) in a murine model of rotavirus infection was performed in the animal facilities of the Karolinska University Hospital at Huddinge, Sweden. Before the experiments, rotavirus negative, pregnant Balb/c mice were obtained from Möllegård, Denmark. The mice were housed individually in the animal facility. Pups were born on day 19-20 of gestation. Four-day old pups were used for the experiments. A dose of 10 µl RRV ($2 \times 10^7$ pfu), fed orally, elicited typical diarrhoea in pups. Following rotavirus challenge, the respective antibody fragments or placebo were administered and daily diarrhoea prevalence was monitored. Both of the studies continued for 6 days. Suckling mice remained with their dams for the entire duration of the protection trial after birth. This murine model of rotavirus diarrhoea has been described previously [Ruggeri et al., 1998].
9. ETHICS

Both of the Research Review Committee (RRC) and the Ethical Review Committee (ERC) of ICDDR, B, and the Ethical Committee of the Karoliska Institute approved all the clinical research protocols. The animal Ethics Committee of the Karolinska Institute approved both the study protocols for animal experiments.

10. METHODS

The protocols, with regards to the outcome measurements, in different clinical studies were similar and are described in each of the original articles. The laboratory tests, including rotavirus ELISA, stool dark-field for *Vibrio cholerae*, stool culture for *Salmonella*, *Shigella*, and vibrios, and assessment of *E. coli* isolates for ETEC (ST and LT) and EPEC (paper I, II, IV, V) were done in the laboratories under the Laboratory Sciences Division (LSD) of the ICDDR, B. Thomas Casswall of Karolinska University Hospital at Huddinge, Sweden and I myself performed the urea breath tests (UBT) for screening for *Helicobacter pylori* infection at the Nandipara clinic, with the help and assistance of staff recruited for the study. Breath samples for UBT were stored in refrigerators before being sent for analyses to the Department of Clinical Chemistry at the Huddinge Hospital by airfreight. Complete blood count and serum electrolyte assays (paper I, II, IV, V) were performed at the diagnostic laboratories under the LSD of ICDDR,B.

10.1 Fluid and electrolyte balance (paper I, II, IV, V)

The children who participated in the clinical therapeutic studies were kept on a “cholera cot”. Nurses assigned to the research ward and health workers employed by the study supervised, measured and recorded intakes of food, ORS solution, and medicines; the study staff recorded the frequency of breast-feeding and also assisted the nurses in their work.

**Clinical assessments:** Study nurses measured and recorded body temperatures, and respiratory and radial pulse rates every 6 hours during the entire period of hospitalisation. Body weight was measured once daily using an electronic scale with a sensitivity of 10 g. Stool and urine output, and intake of fluids including ORS were measured every eight hours using a balance with a sensitivity of one gram and were expressed as grams per kilogram of
body weight per day. Both of these scales were calibrated daily in the morning. Study health workers recorded frequency of stools (number/day) under the supervision of the nurse and/or study research assistants. One of the investigators or the study medical officer performed clinical assessments of all enrolled patients at least once daily, preferably in the morning. ORS solution was administered in amounts equivalent to stool losses of individual children. The magnitude of dehydration was assessed following the WHO guidelines. All data were recorded on pre-designed and pre-tested case report forms (CRFs), and one of the investigators reviewed the forms for appropriateness of entries and their accuracy.

10.2 ELISA

The presence of rotavirus antigen (group A rotavirus-specific VP6 proteins) in stool (paper I, II, IV, V) was detected by Enzyme-linked Immunosorbent Assay (ELISA) in the Immunology Laboratory under the LSD, using a solid phase sandwich type enzyme immunoassay incorporating rabbit hyperimmune antisera produced at ICDDR,B and a anti-human RV-horseradish peroxidase conjugate (Dakopatt, Copenhagen, Denmark) using the same criteria for determination of positivity as suggested by the manufacturers of the Dakopatts kit [Unicomb et al., 1993].

10.3 EIA for anti-LPS antibodies (paper II)

The anti-\textit{E. coli} preparation was made in the middle of the nineteen hundred eighties. Although antibodies are very stable and retain their immunological properties for decades, the antibody titres of these products were determined by enzyme immunoassay for anti-LPS antibodies as described by Karlsson, et al. [Karlsson et al.].

10.4 $^{13}$C-urea breath test (paper III)

The $^{13}$C-urea breath test (UBT) is a non-invasive test of choice for detecting \textit{H. pylori} infections with the greatest accuracy. In this method, the labeled carbon ($^{13}$C) is administered orally to the subjects. In \textit{H. pylori} infected individuals, the bacteria produce urease to increase the pH that enables their survival in acidic milieu of the stomach. Urease converts urea, when fed orally, into ammonia and $^{13}$CO$_2$, which is transported to the lungs via the blood stream and exhaled when breathing.

The natural abundance of $^{13}$C in the atmosphere is very low. Moreover, $^{13}$C is also ingested in foods. Hence a small amount of $^{13}$C is normally expired in the breath. In the $^{13}$C-urea breath test, the ratio between $^{13}$C/$^{12}$C (relative enrichment) is measured by gas-
 chromatography/mass spectrometry. The difference between the relative enrichment at baseline (i.e. before urea is given orally) and the relative enrichment after 30 minutes is calculated. In older children, the breath samples (expired air) were collected by allowing them to blow into a test tube (vacutainer) via a straw. In infants and young children, the breath sample was collected via a locally constructed facemask. As food reduces the sensitivity [Rowland et al., 1999], the tests were performed after a fasting period of 4 hours. To slow down the gastric emptying to allow urea to remain in the stomach for longer time for reaction with urease, a test meal (milk) was provided.

In our study (paper III), children were studied after a 2-hour fast. Breath samplings were performed using a Laerdal ® mask connected with a one-way valve to an anesthesia gas tube. After approximately 10 breath cycles, samples of expired air were drawn from the bag by a syringe and transferred to a screw-capped vacutainer tube for storage and subsequent analysis. Basal samples were obtained and the children were given a test meal consisting of 100 mL of a whole milk formula. Ten minutes later, 50 mg of $^{13}$C urea, dissolved in 10 mL of water, was administered, and breath samples were collected 30 minutes later (post dose samples).

All samples were collected in duplicate, and the ratio of $^{13}$C/$^{12}$C was determined by gas-chromatography/mass spectrometry (Breath Mat, Flinigan Mat, GMBH, Bremen, Germany). Results were expressed as the relative enrichment of $^{13}$C in basal samples and post-dose samples, and were recorded as the change in relative enrichment. Values of $\geq 5.5^{\%}$ were considered positive [Logan et al., 1991]. UBT was performed at the time of screening, before enrollment in the study, immediately after completion of anti-\textit{H. pylori} treatment, and 1 month following completion of treatment.

10.5 VHH1 fragment preparation

A llama was immunised with a G3 rotavirus strain (RRV). cDNA fragments encoding the binding domains of heavy-chain-only antibodies (VHH fragments) were obtained from the B-cells of the immunized animal. These gene fragments were then introduced into the yeast strain, \textit{Saccharomyces cerevisiae} (baker’s yeast). The yeast was subsequently able to express the VHH fragments, and secrete the proteins into the growth medium from which they were purified.

ELISA tests were used to identify VHH fragments, which recognised RRV (G3, P [3]) as well as CK5 (G). Rotavirus neutralisation activity of the rotavirus-specific VHH fragments
was tested by plaque neutralisation assays, and infection inhibition was assessed using an indirect immunofluorescence assay (IFA). Immunoelectron microscopy (IEM) was used to determine the specificity towards human rotavirus isolates from faecal samples.

10.6 Sample size estimation

The samples size of paper 1 and 4 were estimated based on three outcome variables: (i) the duration of diarrhoea, (ii) stool output, and (iii) the duration of fecal excretion of rotavirus. To ensure 30% or greater reduction in all outcome variables in association with HBC or HEY therapy, the required sample size in each group (active or placebo) were estimated to be 37 for duration, 22 for stool output, and 25 for duration of fecal excretion of rotavirus at 5% significance and 80% power. Using the highest required sample size (n=37 in each group) and allowing for a 10% dropout, the total sample size became 80. In paper 2, the sample size was estimated based on the median duration of diarrhoea in a group of children with ETEC reported earlier [Black et al., 1980]. A reduction in the duration by 35% in association with BIC was anticipated. To detect this difference at 5% significance level and 80% power, a sample size of 33 children was required in each of the two groups.

In paper 5, the sample size was estimated based on the three outcome measures: duration of diarrhoea, stool volume, and stool frequency, which were earlier reported to be 74 hours [Azim et al., 2003], 217 g/kg per 48h [Alam et al., 2000] and 13.75/day [Faruque et al., 1996] in acute watery diarrhoea, respectively. Assuming that the intervention would result in at least a 25% reduction of all of these outcome measures, the sample size was determined to be 78, 102, and 100 respectively at 5% significance and 80% power. The largest sample size of 102 patients in each group was, therefore, chosen as a desired sample size. To adjust for 10% dropouts due to any reason, the final sample size was determined to be 115 patients in each group.

10.6.1 Randomization

A random permuted block design (to equalize the number of patients in the treatment groups after short intervals) was employed for allocation of patients to one of the treatment groups: active (hyperimmune bovine colostrums powder for paper I, II, III; hyperimmune egg yolk (HEY) powder for paper IV; and Lactobacillus paracasei ST11 for paper V) or placebo (for
all papers). All patients qualifying for enrolment were assigned a sequential numbers that had earlier been allocated in accordance with the random list.

The master randomization codes and two sets of code envelopes (for each study), which detailed the specific treatment allocation per patient, were generated by independent statisticians not associated with the respective studies. The master randomization code and one set of code envelopes were kept at the Karolinska Institute, Sweden, and the other set of code envelopes were kept at the Pharmacy of the Dhaka Hospital of ICDDR,B for dispensing the study drugs.

HBC or HEY and placebo powders were dispensed in identical bottles (paper I, II, III, IV) or indistinguishable sachets (paper V), sequentially numbered to correspond to the randomization for the respective studies. The codes of HBC or HEY and placebo were known to the hospital pharmacists, and identification of study and control groups were performed after acquisition and cleaning of the data, and performing analyses of unidentified groups.

The animal experiments (paper VI and VII) were also placebo-controlled. The mouse pups allocated for different doses of VHH (paper 6) or IgY (paper 7) were also chosen at random, as for the clinical studies.

10.7 Statistical analysis

All relevant data were entered into a personal computer and analyzed using the Statistical Package for Social Science (SPSS; Windows version). The major outcome variables were compared between the intervention and the placebo groups after evaluating their descriptive statistics for distribution. The quantitative outcome measures (stool output, ORS intake, frequency of stool) were compared using Student's 't' tests on primary data or after their appropriate transformation and also by an equivalent nonparametric test e.g. Mann Whitney U Test when data were not normally distributed. Categorical outcome measures (e.g., proportion of children or mouse pups with resolution of diarrhoea within study period or proportion of children cleared *H. pylori* infection following therapy) were compared by \( \chi^2 \) test or Fisher's exact test, as appropriate. For the duration of diarrhoea and the duration of viral excretion a Kaplan Meier survival analysis was performed.
10.8 End points

The children remained in the research ward for 6 days or until cessation of diarrhoea, which was defined as the time to passage of the last watery or loose stool before passage of two consecutive soft or formed stools, or no stool during at least two consecutive 8-hour periods.

10.9 Outcome measures

The outcome measures of the studies (paper I, II, IV, and V) were daily and cumulative stool output (expressed as g/kg of body weight), number of stools, ORS intake (mL/kg of body weight), duration of diarrhoea (hours) from the time of randomisation to study interventions until diarrhoea resolution, time to faecal clearance of rotavirus (by ELISA) in days, and number of stools. The outcome measure of *H. pylori*-bovine colostrum study (paper 3) was the rate of eradication of *H. pylori*, as indicated by a negative UBT. The outcome measures of the animal studies (paper 5 and 6) were prevalence of diarrhoea, as indicated by loose stools on gentle palpation of the abdomen or a smeared tail during the six days of the experiment.
RESULTS

In total, 902 children were screened for the four clinical studies (paper I, II, IV, V). Of them 240 were screened for the HBC–rotavirus study, 160 for the BIC-E. coli study, 230 for the HEY-rotavirus study, and 272 for the Lactobacillus paracasei study. Because of stringent enrolment criteria and a high rate of isolation of co-pathogens, the number of evaluable children in the HBC or HEY or BIC studies were rather low—if the 240 enrolled, only 80 children (30%) were eligible for enrolment in the HBC-rotavirus study, and only 63 out of the 160 children (39%) enrolled in the BIC-E. coli study were finally evaluable. In the HEY-rotavirus study (Paper IV) only 79/230 children (34%) fulfilled the enrolment criteria. As we wanted to evaluate the efficacy of L. paracaeli in diarrhoea due to rotavirus as well as non-rotavirus pathogens, particularly E. coli, the rate of enrolment was higher in this study (paper V) than the others studies; 230/272 (85%) evaluable children were finally enrolled. The trial profiles of the studies are shown in figure 3. For the paper III, 43/81 (53%) of the screened children had a positive UBT, indicating a high prevalence of H. pylori infections in that community.

In the animal experiment, a total of 120 pups were evaluated -57 in the VHH study and 63 in the IgY study.
11.1 Hyperimmune bovine colostrum (HBC) in rotavirus diarrhoea (paper I)

In this double blind, placebo-controlled clinical trial, 240 children were initially screened. Of them 85 (35%) with a positive stool ELISA for rotavirus were enrolled and randomised to receive the study interventions. Five children were later excluded due to presence of co-pathogens (n=2), presence of severe oral candidiasis (n=2) or parental withdrawal of consent (n=1). Of the 80 children, 40 received HBC and 40 received placebo. Both the groups of children were comparable with regards to age, nutritional status, pre-hospitalisation diarrhoea duration, and other demographic characteristics. The children who received HBC had significantly less daily and cumulative stool output compared to those who received the placebo; cumulative stool output over the 4-day study period was 31% lower in children who had received HBC. The frequency of stool (number/day) and the requirements of ORS (ml/kg/day) were also reduced in the HBC-treated children. The duration of diarrhoea from the time of initiation of therapy was significantly shorter in the HBC-treated group compared to the placebo group (72 hour vs. 96.4 hours, p=0.016), and the proportion of children who had their diarrhoea resolved within the 4 days of the study period was also significantly higher in children who received HBC (30 vs. 21, p=0.001). Fecal clearance of rotavirus occurred in a significantly higher proportion of children receiving HBC (95% vs. 50%, p=0.001).

11.2 Bovine immunoglobulin milk concentrate (BIC) in ETEC and EPEC induced diarrhoea in children (paper II)

One hundred and sixty children were consecutively randomised to BIC or placebo, and only 63 (39%) of them were positive for ETEC or EPEC as determined by DNA probing. Twenty-three children were infected with other E. coli (EAEC, or DAEC) and were later analysed as a second control group. Of the 63 children infected with ETEC or EPEC, 32 received BIC and 31 received the placebo product. The second control group (children infected with EAEC or DAEC) received non-relevant antibodies (BIC or ImulinR). Admission characteristics with regard to age, pre-hospitalisation diarrhoea duration, anthropometrics, and laboratory values were comparable between the three groups of children. There were no differences among the three groups in terms of daily and cumulative stool outputs or ORS intakes, and the stool frequencies were also not significantly different.
However, a marginal, statistically non-significant difference between the children in the BIC versus those in the non-relevant control group (EAEC or DAEC) was observed on day 3. During the 5-day study period, fecal clearance of *E. coli* occurred in 56% of the children in the BIC group, compared to 45% in the placebo and 35% in the non-relevant control group; however, the rates were not significantly different between groups. The mean ± SD duration of diarrhoea following intervention was 93.3 ± 69.9 hours for the BIC group, 99.5 ± 63.5 hours for the placebo group, and 125 ± 92 hours for the non-relevant groups and the differences were not significant.

11.3 Hyperimmune bovine colostrum in *Helicobacter pylori* infection

(paper III)

At the Nandipara community, 81 children were screened for *Helicobacter pylori* infection using UBT, and the test was positive in 43 (53%) children. Of the 43 UBT-positive children, 24 were randomly selected during the 54 weeks of the enrolment period. UBT was repeated after a mean of 5 months following the 1-month period of the interventions (HBC or placebo); 5 children positive at the initial screening became negative before the intervention (3 in the placebo group and 2 in the HBC group).

None of the *H. pylori* positive children became negative on completion of 1 month’s treatment with HBC. Only one child in the placebo group became negative after the 1-month treatment period but became positive again after 1 month. In this group, 2 positive children became negative one more month later (3 months following the start interventions). Of the 2 children in the HBC group who were negative before the study interventions, one remained UBT-negative while the other became UBT-positive after the treatment period, and the results remained the same one-month after the interventions were over. Of the 3 children in the placebo group who were negative at the beginning of the intervention, 2 became UBT-positive and the other remained UBT-negative at the end of the one-month’s treatment and also at follow up one month later.
11.4 Hyperimmunized chicken egg yolk (HEY) in children with rotavirus diarrhoea (Paper IV)

Two hundred and thirty infants and children were initially screened for presence of rotavirus antigen in stool by ELISA, and of them 71 (31%) in the intervention and 69 (30%) in the control group positive for rotavirus, met the other enrolment criteria, and were thus finally enrolled in the study. However, a total of 61 children (34 in the intervention and 27 in the control group) were subsequently excluded because of presence of one or more co-pathogens in their stool culture. Data for the remaining 79 children (37 in the HEY and 42 in the control groups) were analysed. Demographic and clinical characteristics of the children in the two groups were similar, with the mean ± SD of diarrhoea duration of diarrhoea before enrolment of 34.7 ± 10.4 and 31.5 ± 11.9 hours for children in the intervention and control groups respectively (P=NS). Compared to the placebo group, there was a trend for a lower daily (ml/kg) and cumulative stool output (ml/kg) among children in the HEY group; however, the difference was statistically significant only for day 1 (p=0.03). A similar trend of a lower requirements of ORS intake (ml/kg) and the frequency of stool (number/day) was observed among children in the HEY group, and again the differences were statistically significant only for day 1 (p=0.008 for ORS intake and 0.003 for stool frequency). The difference in duration of diarrhoea between the HEY (94.5 ± 40.5 hours) and the placebo group (93.3 ± 44.6 hours) was not statistically significant. However, the rates of clearance of rotavirus from stool were significantly different - over 50% and only 26% of the control and HEY-treated children continued to shed rotavirus in their stool on day 4 (p=0.002).

11.5 Lactobacillus paracasei ST11 study (paper V)

In total, 272 children were screened; however, 42 children were not enrolled due to a positive dark field microscopy for \textit{V. cholerae}, suspected systemic infections, electrolyte imbalance, or parental refusal to enroll their children in the study. Finally, 230 children who fulfilled the study criteria were enrolled and randomized to the treatment regimens. Of the 115 children treated with \textit{Lactobacillus}, 78 (68%) were ELISA-positive and 34 (30%) were ELISA-negative for rotavirus antigen. In the placebo group, 73 children (63%) were ELISA-positive and 42 (37%) were ELISA-negative for rotavirus antigen. In the \textit{Lactobacillus} group 10 (3 rotavirus positive and 7 rotavirus negative); and in the placebo group 14 (8 rotavirus positive...
and 6 rotavirus negative) children had a positive stool culture for vibrios and thus subsequently excluded from the analysis in accordance with the study protocol.

The admission characteristics of the 115 eligible children in each arm of the trial were comparable, and their clinical features, breast-feeding status, and biochemical, haematological and microbiological data were not different. The characteristics of the non-rotavirus children in the two treatment groups were also not different. On the basis of undifferentiated diarrhoea, *L. paracasei* ST11 treatment did not result in a significant reduction of daily or cumulative (days 1 through 6) stool output, ORS intake, or stool frequency as compared to the placebo treatment.

In rotavirus infected children, after excluding those with vibrios (n=75 in the Lactobacillus group and n=65 in the placebo group), the clinical outcomes, such as the duration of diarrhoea, stool frequency and daily or cumulative (days 1 through 6) volume of abnormal stools, time to recovery from diarrhoea, and the amount of ORS intakes, were similar to those in the placebo-group children.

In a subgroup analysis of 53 children (27 in the treatment and 36 in the placebo group) not infected with rotavirus (non-rotavirus children), the stool frequency (number/6 days) and cumulative stool output (g/kg/6 days), and total ORS intake (ml/kg/6 days) were all significantly lower in Lactobacillus-treated children than the children in the placebo group, and the differences were both statistically (p<0.05) and clinically significant. The mean duration of diarrhoea was also shorter in the children treated with Lactobacillus (77 ± 48 hours) than in the placebo-group (99 ± 51 hours) children, but the difference was not statistically significant; however, resolution of diarrhoea by the 6th day of the study occurred in a significantly higher proportion of children in the Lactobacillus group.

11.6 Llama-derived antibody fragments (VHH) in rotavirus infection

(paper VI)

11.6.1 *In vitro* neutralization of rotavirus

The 23 unique, rotavirus-specific antibody fragments produced in *E. coli* were tested for *in vitro* neutralization activity with the rotavirus. Nine of these 23 clones produced antibody
fragments capable of neutralizing the strain of rotavirus tested (CK5). Two of the clones originated from selection round 1, four from round 2 and three from round 3. Subsequently, the yeast-produced antibody fragments were re-tested in the in vitro neutralization assay. Fragments 2B10 and 1D3 were identified as the most effective in neutralizing rotavirus in the plaque assays. On the basis of above observations, 4 of the 8 neutralizing antibody fragments were selected for further studies in an in vivo mouse infection model (Fragment 1F1 selected from round 1, 2E4 from round 2, and fragments 2B10 and 1D3 from round 3).

11.6.2 In vivo efficacy in mouse model

In the following experiments, we studied the efficacy of the antibody fragments in preventing and treating rotavirus-induced diarrhoea in mice; this model system is frequently used for study of rotavirus infections (Edina et al., 1992). There were altogether 57 mouse pups enrolled in the in vivo experiment. The number of the pups in each of the groups were: six in 2B10; ten in 1D3; nine in 2E4; eleven in 1F1; eight in VHH; eight in unrelated VHH; and five in VHH/no RRV5.

To examine if the fragments could inhibit infection when bound to rotavirus (RV), selected VHH’s were premixed with (2×10⁷ ffu) RRV before challenge on day 1. Four-day-old mice pups were treated daily including day 0 (day before infection) up to and including day 4, with VHH’s and incidence of diarrhoea among the pups was assessed. Antibody fragment 2B10-treated pups experienced a marked reduction in the diarrhoea incidence; compared to the untreated group the proportion of pups with diarrhoea was significantly reduced on day 2. Moreover, on days 3, 4 and 5 none of the pups in 2B10-treated group displayed signs of diarrhoea compared to the majority of the pups in the other RRV infected groups. No statistically significant effect of the unrelated RR6-specific VHH (directed against an azo dye) was observed when compared to the untreated group. The mean numbers of diarrhoea days per mouse pup were calculated for each treatment group as the total number of diarrhoea days observed divided by the total number of pups in any treatment group, and this was observed to be significantly (p <0.05) less (0.33±0.21 days) in the fragment 2B10-treated group compared to the untreated group (2.87±0.29 days).

Based on these results, 2B10 was selected for further studies. To investigate the effect of 2B10 treatment for rotavirus infections, 2B10 was administered in different amounts to pups
2 hours after infection on 57 pups. In the 2B10-only groups nine received 100 µg, 10 received 50µg, eight received 20 µg, and nine received 5µg; and four received 2B10/no RRV and seven received VHH alone.

Low amounts of 2B10 (5 and 20µg) did not significantly influence the course of diarrhoea and could not prevent the infections from occurring. However, the higher doses, 50 and 100 µg, significantly reduced the mean number of days with diarrhoea per pup to 1.20±0.29 and 0.67±0.23 days respectively, compared to 14±0.26 days for the untreated group. As for the previous experiments, the effects on diarrhoea reductions were particularly noticeable on days 3 and 4 (\(p < 0.01\) for the 100 µg 2B10 dose).

11.7 Egg Yolk immunoglobulin (IgY) in a mouse model of rotavirus gastroenteritis (paper VII)

Oral challenge with RRV typically induced diarrhoea 24 hours post-inoculation, and diarrhoea peaked around days 3 to 4. A positive outcome of therapeutic feeding with IgY and a clear dose-response were observed. Four different doses of IgY, ranging between 0.01 mg/ml and 10 mg/ml were tested. The therapeutic effects of IgY was noticed starting at the very low concentration of 0.01 mg/ml, which produced a slight effect on diarrhoea incidence and diarrhoea duration by 0.9 days compared to the control mice or a 31% reduction. Administration of higher doses of IgY produced a stronger effect in reducing diarrhoea incidence (37 % reduction with 0.1 mg/ml dose, 53 % with 1 mg/ml dose, and 67 % reduction with 10 mg/ml dose). The statistical analysis revealed that the significance in reducing the incidence was observed starting at 1 mg/ml (in comparison to control mice, day 2 \(\chi^2 2.2, p=0.02\), day 3 \(\chi^2 5.0, p=0.025\) and day 4 \(\chi^2 3.3, p=0.06\). The beneficial effect was even more pronounced in pups receiving the 10-mg/ml doses on day 1 \(p=0.06\), day 2 \(p=0.003\), day 3 \(p=0.003\) and on day 4 \(p=0.0006\) compared to the control mice. IgY therapy also reduced diarrhoea duration, which too demonstrated a dose-response relationship. Treatment with the 10-mg/ml doses achieved the strongest reduction compared to the control mice and also compared to the mice receiving the lower amounts of IgY \(p=<0.0001\). A reduction in the proportion of days with diarrhoea was observed in pups treated with IgY, which started at the 0.01 mg/ml doses. Here again, the strongest effect was observed among mice receiving 10
mg/ml dose the proportion of days with diarrhoea in these mice was only 22 % compared to the 70% in control mice (p<0.00001).
DISCUSSION

Although passive immunization by oral administration of specific antibodies is a recent concept in human medicine, this has been extensively studied in animals, and it represents an attractive approach to establish passive immunity against gastrointestinal (GI) pathogens both in humans and animals [Carlander et al., 2000]. In the past, immunotherapy was carried out via systemic administration of specific antibodies for applications such as targeting agents for cancer diagnosis and therapy, inactivation of toxic substances including drugs, and passive immunotherapy for neoplastic or infectious diseases. There has been an increasing interest in the oral administration of specific antibodies that offers several advantages, e.g. reduced cost, ease of administration and potential for prevention and treatment of localized infections such as GI infections caused by enteric pathogens [Reilly et al., 1997]. The available anti-infective armamentarium has proven to be alarmingly insufficient to combat many of the microbes that cause diseases, such as drug-resistant microbes [Crabb, 1998], microbes for which therapy is not available, microbes that only cause disease in the setting of impaired immunity but are not pathogens in normal individuals, and microbes that do not respond to antibiotics. This has prompted research to explore if the oral administration of antibodies could serve as an acceptable alternative to antibiotics to treat enteric infections.

Traditionally, commercially available polyclonal antibodies have been produced in mammals such as mice, rats, rabbits, sheep, goats and horses and have been obtained from sera after immunization of these animals with antigens of interest. These antibodies cannot be prepared in large-scales because of the difficulty in obtaining large quantities of blood and also because of concerns about animal welfare. Since the hybridoma technology has been widely adapted as a method of choice for the preparations of monoclonal antibodies, various hybridomas have been cloned and grown in large quantities for indefinite periods. However, successful commercialization of any of the therapeutic monoclonal antibodies for infectious diseases is still far away due to the high cost of production of such antibodies. In medicine, human antibodies have been used both as intravenous therapy and as oral therapy. However, there are obvious advantages using antibodies from non-human sources, such as safety, efficacy, costs and logistics. Some of the real potential immunotherapeutic applications of antibodies for animal or human infectious diseases will require kilogram quantities of highly purified antibodies that is not feasible using human sources.
Several alternative approaches have been explored, including bovine colostrum. Bovine colostrum naturally contains high concentrations of antibodies. IgG1 accounts for 90% of the bovine immunoglobulins, and confers some, albeit limited therapeutic benefit against enteric pathogens. However, the major limitation of using bovine colostrum is the low titres of antibodies against the pathogens of interest. Hyperimmunization of cows with repeated, systemic inoculations of specific antigens provides a method for inducing increased titres of specific antibodies in their colostrum [Korhonen et al., 1977; Saif et al., 1984]. The efficacy of orally administered bovine colostrum has been documented in numerous studies in experimental animal models and also in clinical trials in humans [Bogstedt et al., 1996; Davidson, 1996; Hammarström et al., 1994; Weiner et al., 1999].

In most of the studies, the efficacy of the preparation has been tested against infections due to enteropathogenic \textit{E. coli}, rotavirus and Cryptosporidium. However, proof of the therapeutic value of such antibodies against these infections in a well-controlled design and particularly with subjects with severe diarrhoea is however lacking. We therefore performed a randomized, double blind, placebo-controlled clinical trials to evaluate the therapeutic efficacy of hyperimmune bovine colostrum containing specific antibodies against \textit{E. coli} and rotavirus in children with diarrhoea due to those pathogens. It may be mentioned that together, rotavirus and \textit{E. coli} accounts for nearly 80% of diarrhoea in under-five children [Anonymous, 2002]. In addition to our paper (paper I), two other double blind studies have evaluated the effect of bovine antibodies against rotavirus-induced diarrhoea [Mitra et al., 1995; Ylitalo et al., 1998]. These were only 2 studies conducted before our paper (paper 2) that evaluated the therapeutic efficacy of bovine hyperimmune immunoglobulin concentrate in the treatment of diarrhoea caused by \textit{E. coli} [Lodinova-Zadnikova et al., 1987; Mietens et al., 1979]. Those studies however, were not well designed with regard to the power of the study. Another study, conducted in children aged 3 to 18 months, reported amelioration of diarrhoea due to diarrhoeagenic \textit{Escherichia coli} in terms of stool frequency in association with the use of nonimmunised bovine colostrum [Huppertz et al., 1999].

In our first randomized, double-blind, controlled clinical study, a group of proven rotavirus-infected Bangladeshi children aged 4-24 months received 10 g of lyophilized hyperimmune bovine colostrum containing 3.6 g of anti-rotavirus antibodies orally in 4 divided doses for 4 days. Compared to the control group children who received non-immune colostrum, the children treated with HBC experienced a significant reduction in the duration and severity of
diarrhoea along with shortened duration of excretion of the virus. During the 4-day study period, HBC-therapy was associated with 31% reductions in stool frequency and output, and ORS intake and a 25% shortening of the diarrhoea duration. These findings are similar to those observed in an earlier trial conducted in the same settings to assess the impact of oral administration of a 100 mL, thrice daily dose of crude hyperimmune colostrum [Mitra et al., 1995]. For diarrhoeal children with vomiting and anorexia, feeding of this large volume (100 ml) of crude colostrum three times in a day may not be an easy task, and administering lyophilized powder may be easier as well as produce better compliance to therapy. Ylitalo et al [Ylitalo et al., 1998] used a similar mode of administration (100 mL of hyperimmune colostrum 4 times/day for 4 days for treating rotavirus-infected children) and observed trends but statistically non-significant improvements in all the variables examined: weight gain, duration of diarrhoea and numbers of stool. Hilpert et al, had observed a shortening in the duration of rotavirus excretion but no effect on clinical symptoms [Hilpert et al., 1987]. Possible explanations for these differences in the outcomes of these studies include illness severity and other related host factors. Using hyperimmune bovine colostrum obtained from cows immunized with human rotavirus (Wa strain semian type I), Ebina et al., were able to protect infants during a rotavirus outbreak [Ebina et al., 1985]. Other studies have provided evidence for the role of hyperimmune bovine colostrum in protecting rotavirus cross-infection [Davidson et al., 1989; Davidsson, 1994]. Based on the above findings, it may be concluded that hyperimmune colostral products have potentials for use in prophylaxis as well as treatment of rotavirus infection.

In contrast to the trials that provided evidence for usefulness of hyperimmune bovine colostrum in the treatment of rotavirus diarrhoea, no such benefits were observed with their use in the treatment of diarrhoea due to *E. coli*. In our study (paper II) we enrolled children aged 4-24 months with a history of diarrhoea of less than 48 hours duration. In the absence of a rapid test for the diagnosis of ETEC or EPEC infections, we initially enrolled and randomized 160 children, negative both for rotavirus by ELISA and cholera by stool dark field microscopy, to bovine immunoglobulin milk concentrate (BIC) or a placebo. Of these 160 children, only 63 (39.3%) were positive for ETEC or EPEC, as determined later by DNA probing. We based our statistical power calculations on an expected 30% difference in the outcome variables between the intervention and the control groups. Because of the high (61%) exclusion rate, the obtained sample size could detect a difference of at least 35% to make statistically significant. The modest differences of 15-20% as observed in our study did
not achieve a 5% level of significance due to the smaller-than-required sample size in our study, reducing statistical power, although the clinical usefulness of the observed difference could be argued.

Reduced potency of the product, used in a powder form, could have been a possible contributor to the inferior outcome benefits observed in our study, since the product used was produced in the mid eighties (almost 10 years before the trial), at the same time as the rotavirus HBC product, which was used successfully in our first trial with rotavirus diarrhoea. However, reanalysis of the powder disclosed its high antibody titre. Therefore, loss of potency is unlikely reason for the observed effects. It is also possible that the most common and clinically relevant strains of *E. coli* used in immunizing cows during the production period were markedly different from current strains with diverse virulence factors such as CFAs (ETEC) and EAF plasmids (EPEC) [Nataro and Kaper, 1998], resulting in inefficient neutralization.

The pathogenic mechanisms involved in diarrhoea due ETEC and EPEC may also explain the suboptimal effects of BIC. ETEC produces toxins, similar to cholera toxin, and in earlier studies hyperimmunized milk failed to produce a therapeutic effect in patients with cholera [McClead et al., 1988]. It is possible that the antibodies failed to reach and neutralize bacteria closely adhering to the mucosal surface and delivering toxin to the cells. EPEC possess an intricate pathogenic mechanism for attachment and effacement of small intestinal enterocytes, a process that may not be affected by the antibodies. Thus, a possible beneficial effect of bovine antibodies in *E. coli* diarrhoea is arguable.

It has been shown that chicken antibodies against enterotoxigenic *E. coli* could prevent the bacteria from adhering to the pig enterocytes. However, this blocking of adherence could only be achieved if the bacteria had been pre-incubated with the chicken antibodies [Jungling et al., 1991] before the challenge. This implies that there is potential for a prophylactic rather than a therapeutic effect of BIC. In fact, a lyophilized Ig concentrate (prepared from colostrum of cows immunized with several enterotoxigenic *E. coli* subtypes, fimbria types, and *E. coli* heat labile enterotoxin) was shown to provide complete protection against enterotoxigenic *E. coli* infection in volunteers [Tacket et al., 1988]. Protection of human volunteers against *E. coli* infection was also observed in studies performed by Freedman and coworkers using hyperimmunized milk against CFA [Freedman et al., 1998]. Based on the
findings till date, we believe that the currently available bovine antibodies may not provide clinical benefit in the management of diarrhoea due to ETEC and EPEC. However, production of high titre antibodies, encompassing those against important and common virulence factors for all diarrhoeagenic *E. coli*, may overcome the limitations of bovine antibodies.

Our third study relates to the use of oral antibodies from hyperimmunized cows against *H. pylori* infections in children. We failed to observe any beneficial effect of bovine anti-*H. pylori* antibodies, and none of the children receiving 1 gram (710 mg antibodies) of the hyperimmune bovine colostrum daily for 30 days could eradicate the bacteria. Limited data are currently available on the prophylactic and therapeutic efficacy of orally administered antibodies in the treatment of *H. pylori* infections. Preliminary clinical trials in patients with chronic gastritis and children with *H. pylori* infections have shown that daily administration of 20 g of an immune anti-*H. pylori* bovine Ig concentrate for adults and 12 g for children for 3–4 weeks could reduce both the colonization rate and the severity of the symptoms in most subjects; however, total eradication of the *Helicobacter* was observed in only 1 of the 9 adults and in none of the 20 treated children [Oona et al., 1994], a finding consistent with our results. Similarly, Opekun et al., [Opekun et al., 1999] reported that immune bovine colostrums derived immunoglobulin were not effective in decreasing the number of *H. pylori* present in the gastric mucosa of infected volunteers [Casswall et al., 2002].

There remain several limitations in performing therapeutic trials in *H. pylori* infected children in developing countries, the infection rates are high and up to 80% of the populations are infected with *H. pylori* [Rowland et al., 1999; Rowland et al., 1997]. Inaccurate diagnostic test makes it even more difficult to evaluate if the occurrences of reinfections are true or not. The study was planned in the mid nineties when the knowledge of transmission of *H. pylori* and reliability of UBT in identifying infections in infants was incomplete. However, reinfections remain a concern even with conventional anti-*Helicobacter pylori* therapy.

The colostrum product used in our study was made only one year before the trial, and was derived by immunizing Swedish cows 10 times using whole bacteria. Its antibody concentration was 100 times higher than the control preparations, and thus, its potency was expected to be high. Despite this, none of the children in our study could clear the *H. pylori* infection. Denaturation of the immunoglobulin during the gastrointestinal passage, resulting
in failure to clearing the infection cannot be ruled out. Placebo controlled clinical studies will be required to test the efficacy of immune colostral preparations as a potential adjunct to the chemotherapy currently practiced in the treatment of *H. pylori*–associated gastritis.

The prophylactic effect of egg yolk immunoglobulin (IgY) has previously been demonstrated in suckling mice with diarrhoea caused by human rotavirus [Ebina et al., 1990]. Recently, the beneficial role of immunoglobulin prepared from the egg yolk of hens immunoglobulin against *Helicobacter pylori* has been evaluated in a Mongolian gerbil model [Shin et al., 2002] and human volunteers [Shimamoto et al., 2002]. Before our study (paper 4), there was only one study in humans showing that chicken antibodies could influence *S. mutans* infections in dental caries. However, no clinical study was conducted that evaluated the therapeutic efficacy of specific chicken egg antibodies in children. To our knowledge, ours’ is the first study that evaluated the therapeutic efficacy of IgY in children with diarrhoea due to proven rotavirus infections (paper 4).

Compared to our HBC-rotavirus study, we observed a modest reduction of stool volume and earlier clearance of rotavirus from stools in children indicating a potential role of chicken IgY in the management of rotavirus diarrhoea. We can think of several explanations for the reduced clinical benefit of the IgY our study. The neutralization titre of the product used in this study was lower than that of the bovine IgG used in trials that showed better effects in the management of children with rotavirus diarrhoea (paper I). The hens were immunized only twice, and the IgY product was 10-15 times less potent than the bovine product. It is important to note that, the immunization of hens was performed using inoculation of human rotavirus serotypes Wa, RV5, RV3, and ST3 (representing serotypes 1, 2, 3 and 4 respectively). During the period of the study, there was emergence of an untypeable rotavirus strains. It is possible that some of the children may have been infected by serotypes other than those currently recognized, against which the antibodies were developed.

Chicken antibodies are more vulnerable to proteolysis *in vitro* than bovine antibodies [Shimuzu, 1992], although the *in vivo* stability of chicken antibodies is not yet known. An improved delivery system designed to protect the product from inactivation by gastric acid and the digestive enzymes could improve the effects of such antibodies. The effect of passive immunotherapy is dose dependent, and it is thus also possible that a better effect could have been obtained using preparations with higher antibody titres or using a higher dose.
By the time diarrhea is established in rotavirus infection or ETEC/EPEC infection, immunotherapy might not have a chance to act as there is already involvement of substantial number of enterocytes, affecting net absorption or secretion. Prophylactic therapy would therefore have a better chance to exert a positive influence. However, in our study, the HBC and IgY therapy did ameliorate the course at the peak of disease indicating that the therapy possibly acted by neutralizing the toxins. On the other hand, a prophylactic field trial conducted by Brunser and colleagues showed no positive effect of an infant formula containing anti-rotavirus and anti-\textit{E. coli} milk antibodies from hyperimmunized cows in a cohort of Chilean infants and children [Brunser et al., 1992].

Several recent, controlled clinical trials have shown that selected probiotic strains of lactobacilli such as \textit{L. casei} strain GG, [Isolauri et al., 1991; Isolauri et al., 1994; Majamaa et al., 1995] \textit{L. reuteri} [Shornikova et al., 1997], and \textit{L. acidophilus} [Simakachorn et al., 2000] exhibit both therapeutic and prophylactic [Oberhelman et al., 1999; Szajewska et al., 2001] effects in children with viral, but not bacterial diarrhoea. Duration of diarrhoea has been used as the primary measure of outcome in all previous studies. However, this measure alone is not considered optimal, and with few exceptions, the studies did not quantitatively evaluate stool output. Using quantitative diarrhoea criteria, as recommended by the World Health Organization, we evaluated a new probiotic strain, \textit{Lactobacillus paracasei} ST11, in the management of acute diarrhoea (paper 5) in children.

In the present trial we did not observe any beneficial effect of \textit{Lactobacillus} ST11 in the treatment of rotavirus diarrhoea. We had enrolled adequate numbers of children with proven rotavirus diarrhoea, and we matched the control groups before initiation of interventions that allows us to draw a firm conclusion on the lack of efficiency of the probiotic. The dose of the orally administered lactobacilli was high enough to exclude the possibility of a dose effect in comparison with previous studies. In fact, we used a daily dose $10^{10}$ CFU, and administered it dissolved in ORS, as has also been used in other studies. We also excluded the possibility of another reason for a false negative outcome, decay of the lactobacilli during the two-year storage period in Dhaka. At the end of the study, analysis of the leftover sachets showed the same viable bacterial count as that at the beginning of the study.
What factors could explain the discrepancy between our findings and those of previous studies in rotavirus-induced diarrhoea? One might relate to the severity of the illness. In the previously published studies, the children had less severe diarrhoea than our study children. In the five previous studies, the duration of illness after initiation of intervention ranged between 1.6 to 3.8 days, compared to 4.0 days as we observed in our control children. The only other study investigating the affect of probiotics in severe acute dehydrating diarrhoea, conducted in Brazil [Costa-Ribeiro et al., 2003], also observed similar lack of efficiency of Lactobacillus GG in hospitalized children with rotavirus gastroenteritis, as in our study. It is, therefore likely that neither Lactobacillus GG nor ST11 would be able to produce a beneficial effect in the management of rotavirus diarrhoea severe enough to hospitalize children in developing countries. It is possible that lactobacilli might produce a beneficial effect in the treatment of only the mild forms of rotavirus gastroenteritis in developed countries.

On the other hand, Lactobacillus ST11 feeding produced a positive impact on quantitative diarrhoea outcome parameters. It may be noted that our intention to study the impact of the intervention on rotavirus and non-rotavirus diarrhoea was predetermined in our clinical protocol, and not a post hoc analysis. The difference in the cumulative stool frequency and output, ORS intake, and diarrhoea duration were all statistically significant despite the small number of non-rotavirus patients in our study. The results of this study have important implications for scientific research on probiotic bacteria. To our knowledge, our study is the first that convincingly demonstrated both clinically relevant and statistically significant effect of a probiotic in the management of severe non-rotavirus diarrhoea applying the objective quantitative evaluation criteria recommended by the WHO. The results suggests that appropriately selected probiotics could have a place in clinical practice if their costs (influenced by production costs and the determination of the lowest efficient dose) are affordable to developing countries where the burden of bacterial childhood diarrhoea is still high.

Passive immunization for treatment of enteric infections generally requires relatively large amounts of antibody. Therefore, these therapeutic modalities, even if proven to be effective, would be expensive. The other limitations of the conventional IgG antibodies include their complex nature and poor stability in the gastrointestinal tract, limiting their large-scale production. Our sixth paper relates to a llama derived antibody fragment, VHH that was produced in yeast by the Unilever, the Netherlands. In our study (Paper 6), we evaluated, for
the first time, if antibody fragments produced in yeast would be capable of modulating the natural course of rotavirus diarrhoea.

VHH is a llama-derived, heavy chain anti-rotavirus antibody fragment. The protein has been produced using genetic modifying technology, transferring the genetic sequence for the antibody fragment from peripheral blood lymphocytes of llama to *Saccharomyces cerevisiae* (bakers yeast) via *Escherichia coli*. Although the VHH antibody fragments have a simple antigen binding structure with only one domain, it possesses a high affinity, and is extremely thermostable [van der Linden et al., 1999]. Furthermore, they are well expressed and secreted by the bakers yeast *Saccharomyces cerevisiae*, allowing for a cost-effective, large-scale production. We assessed the efficacy of this novel antibody fragment *in vitro* and *in vivo* by a proof of principle study in mice. In this way, we demonstrated, for the first time, that llama-derived antibody fragments are capable of influencing the course of rotavirus diarrhoea in mammals. Given these findings, it appears that the selected VHH fragment has all the properties to ameliorate severity of illness due to rotavirus infection in children. A clinical trial using VHH anti rotavirus antibody fragment would, therefore, be of potential interest and is currently underway.

We observed a modest therapeutic efficacy of IgY in children with rotavirus diarrhoea (paper 4), and thus we also investigated, in a small study in an animal model of rotavirus diarrhoea, if better therapeutic effect could be achieved using higher doses of IgY (paper 7). Oral administration of IgY or egg yolk has previously been found to be effective in the prevention of rotavirus diarrhoea in mice [Bartz et al., 1980; Ebina et al., 1990; Kuroki et al., 1993], calves [Kuroki et al., 1994] and cats [Hiraga et al., 1990]. However, a therapeutic study of IgY in animals is lacking. The use of an animal model has allowed us to evaluate therapy with higher doses of immunoglobulin in achieving an improved therapeutic effect. In the last paper (paper 7), we have evaluated a therapeutic potential of anti–rotavirus IgY as well as defined the dose needed for achieving a therapeutic benefit in mice. We observed that feeding higher doses of IgY had a stronger effect (37 % reduction with 0.1 mg/ml dose, 53 % with 1 mg/ml dose, and 67% with 10 mg/ml dose) in reducing diarrhoea prevalence. IgY therapy also reduced diarrhoea duration. Treatment with the 10 mg/ml dose was associated with the highest reduction in comparison to not only the control mice but also the mice receiving lower doses of IgY (F =6.7, p=<0.0001), confirming that therapeutic response of passive immunization is dose dependent [Ebina et al., 1990].
CONCLUSIONS AND FUTURE PERSPECTIVES

Antirotavirus antibodies from colostrum of immunized cows were effective in ameliorating rotavirus gastroenteritis in children. There is a need to carry out further well-controlled studies to define its role in different settings. The potential of these antibody preparations when incorporated into existing oral rehydration solution packets for case management of diarrhoea needs to be defined.

Colostral antibodies from cows immunized with whole bacteria and the heat-labile toxin of E. coli seem to ineffective for treatment of established E. coli diarrhoea. As virulence factors of EPEC/ETEC other than the toxin are associated with the pathogenesis of diarrhoea, future studies should focus on therapy containing antibodies against such virulence factor.

Immune bovine colostral immunoglobulin was not effective in eradicating H. pylori infection in asymptomatic children. Placebo-controlled clinical studies to test the efficacy of immune colostral preparations in symptomatic subjects and to evaluate such preparations as a potential adjunct to antibiotics in the treatment of Helicobacter pylori associated illness, needs to be elucidated.

The IgY treatment resulted modest beneficial effect in rotavirus diarrhoea in children. The finding is encouraging, although not a good as those with HBC and poses great attention for future investigation to define the dose, and to find out the effective way to protect the antibodies from inactivation by gastric acid and the digestive enzymes. The findings in mouse experiments support that higher dose of IgY is needed in achieving clinical effects and therefore helps to design future studies in humans.

Llama derived antibody fragments directed against rotavirus can reduce the morbidity of rotavirus induced diarrhoea in mice. This antibody fragment has a potential to significantly impact on the course rotavirus gastroenteritis and a potential for large-scale production affordable to developing countries. Future studies should therefore, determine the effectiveness in preventing or treating RV induced diarrhoea in humans.

The probiotic bacteria, Lactobacillus paracasei ST11 has a clinically significant benefit in the management of children with nonrotavirus possibly E. coli-induced diarrhoea, but it is
ineffective in those with rotavirus diarrhoea. Confirmation of this result with a larger number of children and a better definition of the etiology is clearly warranted.

As the studies with passive immunotherapy studies (HBC, IgY) and ST11 study were conducted with a similar protocol and patients, the divergent effects are interesting and suggest an antiviral effect of oral antibodies and a possible antibacterial effect of probiotic bacterium. It seems likely that a combination therapy using antibodies and probiotic agents could elicit therapeutic benefit in settings where diarrhoea in a majority of children are caused by rotavirus or *E. coli*. 
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