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# Milk Antibodies for the Treatment of Gastroenteritis in Developing Countries

**By**

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**DECLARATION**

I declare that the research presented in this thesis, is my own work. The results presented have not been submitted for assessment, in full or part, at any other tertiary institution.

Mohammad Ahmad

/ /2010

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**ABSTRACT**

Gastroenteritis caused by bacterial and viral pathogens has been the cause of death to more than 1.7 million children less than five years of age in developing countries. Rotavirus and Enterotoxigenic *Escherichia.coli* (ETEC) are among the main causes of gastroenteritis and diarrhoea that causes dehydration, and even death.

Using immunotherapy against gastroenteritis is not novel. Previous works succeeded to treat gastroenteritis and diarrhoea using immunotherapy. The idea that dams produce antibodies against different types of antigens, and these antibodies are transferred to milk as passive immunity to offspring, has been implemented in this project to produce polyclonal antibodies against ETEC O78:h11, NL053 (O6:h16) and rotavirus SA11 in the milk of sheep. The levels of these polyclonal antibodies were measured in milk and serum using enzyme-linked Immunosorbent assay.

Fifty Balb/c mice were used in this study. The mice were inoculated with ETEC O78:h11 and then challenged using milk enriched with antibodies against ETEC O78:h11 or against enterotoxigenic *Escherichia.coli* strain O6:H16 and rotavirus strain SA11 to measure the effectiveness of antibodies in neutralising the pathogens of interest. Heat stable enterotoxin was measured using the commercial kit COLIST (Denka Seiken, Japan) to assess the effectiveness of milk in neutralising ETEC O78:h11.

Survival of the antibodies in the intestine of Balb/c mice was studied through measuring the level of antibodies against ETEC strain O78:h11, O6:H16 and rotavirus SA11 in the faecal samples during milk treatment period and after weaning.

Results showed that antibody level in serum and milk increased after three boosts of each vaccine. The antibody level in the intestine of mice was high during milk treatment and sharply decreased after weaning. Heat stable enterotoxin test was negative for the treated groups 1 and 3 whereas it was positive for group 2. This group was given the milk without antibodies

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**ABBREVIATIONS**

| | |
|---|---|
| ACIP | United States Advisory Committee on Immunisation Practice |
| ADP | Adenosine diphosphate |
| BIC | Bovine immunoglobulin milk concentrate |
| Ca+ | Calcium Ion |
| cAMP | Cyclic adenosine monophosphate |
| CDC | Disease Control and Prevention |
| CFA | Colonisation factor antigens |
| CFTR | Cystic fibrosis transferase receptor |
| CFU | Colonisation factor unit |
| CT | Cholera toxin |
| DAEC | Diffusely adherent *E. coli* |
| dsRNA | Double-stranded ribonucleic acid |
| EAEC | Enteroaggregative *E. coli* |
| ECP | *E. coli* common pili protein |
| EHEC | Enterohaemorrhagic *E. coli* |
| EIEC | Enteroinvasive *E. coli* |
| ELISA | Enzyme linked immunosorbent assay |
| ENS | Enteric Nervous System |
| EPEC | Enteropathogenic *E. coli* |
| ETEC | Enterotoxigenic *Escherichia.coli* |
| GC-C | Guanylate cyclase C |
| GD1 | Disialoganglioside |
| GM1 | Ganglioside |
| GTP | Guanosinetriphosphate |
| Ig | Immunoglobulin |

| | |
|---|---|
| K+ | Potassium ion |
| LT | Heat labile enterotoxin |
| MIC | Milk immunoglobulin concentrate |
| Na+ | Sodium ion |
| NaCl | Sodium chloride |
| NAD | Nicotinamide adenine dinucleotide |
| NSP | Non structural proteins |
| ORS | Oral rehydration salt solution |
| ORT | Oral rehydration therapy |
| PBS | Phosphate-buffered saline |
| PGE1/2 | Prostaglandin1/2 |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PLC-IP3 | Phospholipase C-inositol 1,3,5 triphosphate |
| rCTB | Recombinant cholera toxin B |
| RER | Rough endoplasmic reticulum |
| RNA | Ribonucleic acid |
| Rpm | Round per minute |
| SGLT1 | sodium-glucose co-transporter 1 |
| ST | Heat stable enterotoxin |
| Tia | Toxigenic invasion loci A |
| TibA | Toxigenic invasion loci bA |
| UNICEF | United Nations Children's Fund |
| USD | United State Dollar |
| VLP | Virus Like Particles |
| VP | Viral proteins |
| WHO | World Health Organisation |

# CHAPTER 1: INTRODUCTION AND REVIEW OF THE LITERATURE

## 1.1 Gastroenteritis

Gastroenteritis, a major health problem in developing countries, is the infection of the digestive system. In 2009, the World Health Organisation (WHO) estimated that diarrhoeal diseases caused by gastroenteritis was ranked third in causes of death in children less than five years old with about 1.7 million deaths every year (Figure 1). Gastroenteritis is also considered the major health risk for travellers to developing countries (WHO, 2009). The common feature between these two groups is the lack of access to clean water and food due to poor sanitation.

Diarrhoea is a major symptom of gastroenteritis and usually indicates infection in the gastrointestinal tract. The clinical definition of diarrhoea is the passage of three or more loose or liquid stools within 24 hours (Clemens et al., 2004).

Gastroenteritis diseases cause diarrhoea, which may led to dehydration and death in children less than five years of age in developing countries. According to World Bank, developing countries included all low-income, middle-income countries and countries in transition(Ross and Harmsen, 2001). Table 1 shows list of developing countries according to World Bank classification, 2010.

Gastroenteritis results from bacterial, viral, parasitic and protozoal infection. Variety of microorganisms such as, rotavirus, enterotoxigenic *Escherichia coli*, *Vibrio cholera* and *Shigella* spp cause gastroenteritis and diarrhoea form a serious life threatening, especially, in developing countries. These areas tend to lack hygiene measurements, sanitation and medical supplies (Cukor and Blacklow, 1984). For example, in Bangladesh a study showed that children who live in urban areas had suffered from diarrhoea caused by enterotoxigenic *E.coli* transmitted through the faecal-oral route (Qadri et al., 2007b). Children less than five years old are the most susceptible to gastroenteritis in these countries. Surveillance and diagnosis of gastroenteritis are less organised in developing countries. This is due to the limited access to modern laboratory procedures (Holtz, 2007). Accurate documentation depends largely on laboratory facilities that demand high cost materials and equipment. For example, a Taiwanese study estimated that the mean cost for treatment of a child with rotavirus gastroenteritis sent to a hospital to be about USD 558.69

(Mast et al., 2010). Moreover, the specific disease burden is particularly complex due to a multiplicity of the causing agents and their serotypes. This demands more laboratory tests and consequently the associated cost escalates.

Minimising the effects of gastroenteritis and diarrhoeal diseases can be achieved through maintenance of clean water, improvement of hygiene and sanitary measures and availability of nutrition. Public education programs that disseminate information about the causes and the consequences of gastroenteritis and diarrhoea will help to increase the personal awareness and encourage disease prevention. For example, teaching children at schools about personal hygiene and waste disposal will help to minimise the surface water pollution that will minimise diarrhoeal diseases. A one to one survey held in five developing countries measured the knowledge of rotavirus(Simpson et al., 2007). The results from this study showed that knowledge of rotavirus was extremely low. Good nutrition and availability of clean food and water helped patients who had suffered from diarrhoea to recover in shorter time (Alam and Ashraf, 2003).

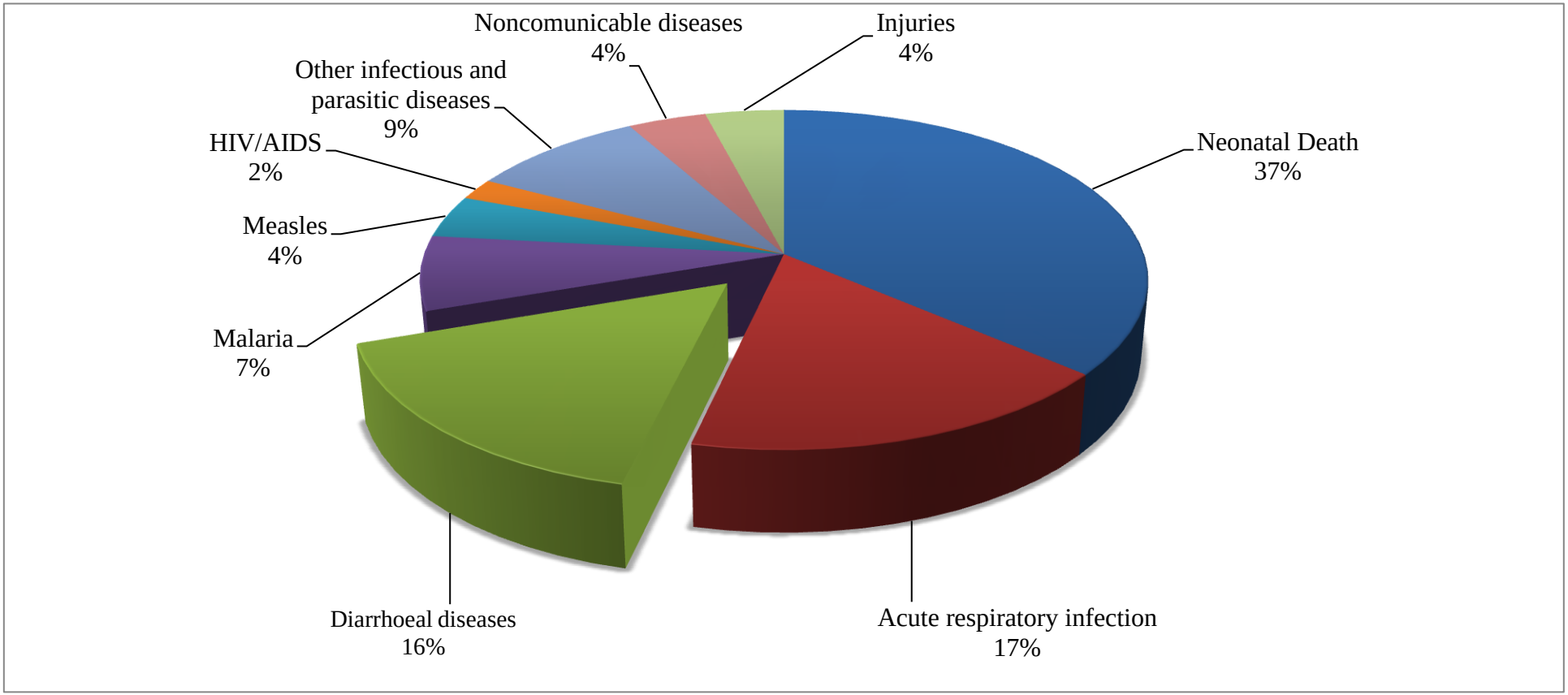



Figure 1 Global burden of diseases in children less than five years old. According to data complied by the World Health Organisation. An estimate 10.4 million succumb on an annual basis (WHO, 2008) updated February, 2009. Diarrhoeal disease caused by gastroenteritis accounts for 16% of the total deaths; third behind neonatal deaths and mortalitiy caused by acute respiratory infection.

Table 1 List of developing countries according to World Bank 2010.

| | | | | | | |
|---|---|---|---|---|---|---|
| Afghanistan | Cape Verde | Fiji | Kiribati | Mayotte | Poland | Swaziland |
| Albania | Central African Republic | Gabon | Korea, Dem. Rep. | Mexico | Romania | Syrian Arab Republic |
| Algeria | Chad | Gambia, The | Kosovo | Micronesia, Fed. Sts. | Russian Federation | Tajikistan |
| American Samoa | Chile | Georgia | Kyrgyz Republic | Moldova | Rwanda | Tanzania |
| Angola | China | Ghana | Lao PDR | Mongolia | Samoa | Thailand |
| Argentina | Colombia | Grenada | Latvia | Montenegro | São Tomé and Principe | Timor-Leste |
| Armenia | Comoros | Guatemala | Lebanon | Morocco | Senegal | Togo |
| Azerbaijan | Congo, Dem. Rep | Guinea | Lesotho | Mozambique | Serbia | Tonga |
| Bangladesh | Congo, Rep. | Guinea-Bissau | Liberia | Myanmar | Seychelles | Tunisia |
| Belarus | Costa Rica | Guyana | Libya | Namibia | Sierra Leone | Turkey |
| Belize | Côte d'Ivoire | Haiti | Lithuania | Nepal | Solomon Islands | Turkmenistan |

| | | | | | | |
|---|---|---|---|---|---|---|
| Benin | Cuba | Honduras | Macedonia, FYR | Nicaragua | Somalia | Uganda |
| Bhutan | Djibouti | India | Madagascar | Niger | South Africa | Ukraine |
| Bolivia | Dominica | Indonesia | Malawi | Nigeria Pakistan | Sri Lanka | Uruguay |
| Bosnia and Herzegovina | Dominican Republic | Iran, Islamic Rep. | Malaysia | Palau | St. Kitts and Nevis | Uzbekistan |
| Botswana | Ecuador | Iraq | Maldives | Panama | St. Lucia | Vanuatu |
| Brazil | Egypt, Arab Rep. | Jamaica | Mali | Papua New Guinea | St. Vincent and the | Venezuela, RB |
| Bulgaria | El Salvador | Jordan | Marshall Islands | Paraguay | Grenadines | Vietnam |
| Burkina Faso Burundi | Eritrea | Kazakhstan | Mauritania | Peru | Sudan | West Bank and Gaza |
| Cameroon | Ethiopia | Kenya | Mauritius | Philippines | Suriname | Yemen, Rep. |
| Zambia | | | | | | |
| Zimbabwe | | | | | | |

Immunisation against specific diseases is a promising approach that may minimise the effect of some infectious pathogens and thereby avoiding serious illness and death. For example, vaccination against cholera decreased the numbers of deaths dramatically in Africa and Latin America (Parashar, 2009). Vaccine development against enteric pathogens represents a serious challenge. This is due to the diversity of organisms causing gastroenteritis and the large number of pathogens that require mucosal immunity induction in the gut. Another challenge arises due to mixed gastroenteritis infection, when more than one pathogen is involved in the disease process. For example, rotavirus and ETEC are a common mixed infection. In this case, vaccination against rotavirus will solve half the problem (Grimprel et al., 2008).

Previous studies investigated and tracked the causes of gastroenteritis and diarrhoea in children in developing countries. Table 2 shows the previous studies of the causes of diarrhoea in children less than five years old from different countries. In 1956, Bhattacharya *et.al* studied the causes of diarrhoea in children in Calcutta in India. The study revealed that enterotoxigenic *E.coli* was the main cause of diarrhoea (De, 1956).

In 1976, a study was undertaken to monitor the clinical characteristics of disease due to enterotoxigenic *E.coli* in 176 adult males that had been admitted to a hospital at Dacca, Bangladesh. The patients showed moderate to severe dehydration, diarrhoea and abdominal pain. The study confirmed that ETEC was the cause acute, severe cholera-like diarrhoea in the studied cases (Merson et al., 1980).

Enterotoxigenic *E.coli* was the most frequently isolated and identified pathogen from patients of all ages in two years study held in Bangladesh from 1977 to 1979. The total number of cases was 14,491. In children less than two years old enterotoxigenic *E.coli* was isolated from 25% of the cases (Black et al., 1980).

In a study between 1978 and 1989, a total number of 1,183 rotavirus-positive specimens from eight countries of four continents were studied to determine the most common global serotype in circulation and which vaccine might provide good protection. The study showed the importance of rotavirus as a cause of diarrhoea in those countries (Woods et al., 1992).

Table 2 Studies that have linked specific pathogens as causes of diarrhoea in children less than 5 years of age in different countries

| **Cause of diarrhoea** | **Total cases** | **Countries** | **Year** | **Reference** |
|---|---|---|---|---|
| Enterotoxigenic *E.coli* | Not Available | India | 1956 | (De, 1956) |
| Enterotoxigenic *E.coli* | 176 | Bangladesh | 1976 | (Merson et al., 1980) |
| Enterotoxigenic *E.coli* | 14,491 | Bangladesh | 1977-1979 | (Black et al., 1980) |
| Rotavirus | 1,183 | USA, Israel, Peru ,S.Korea, S.Africa and China | 1978-1989 | (Woods et al., 1992) |
| Rotavirus, *E. coli* and *Shigella* spp. | 265 | Jordan | 1993-1994 | (Youssef et al., 2000) |
| Enterotoxigenic *E.coli* | 489 | Indonesia | 2000-2001 | (Subekti et al., 2003) |
| Rotavirus and Enterotoxigenic *E.coli* | 1,275 | Egypt | 2000-2002 | (Wierzba, 2006) |
| Enteroaggregative *E.coli* | 836 | Vietnam | 2001-2002 | (Nguyen et al., 2005) |
| Enterotoxigenic *E.coli* | 321 | Bangladesh | 2002-2004 | (Qadri et al., 2007b) |

Another two-year study performed in Jordan (1993 to 1994) to determine the cause of diarrhoea in children less than 5 years old and showed that rotavirus was the main cause of diarrhoea followed by *E.coli* and *Shigella* in 256 children (Youssef et al., 2000).

Nguyen *et.al.* (2001 to 2002) studied a total number of 836 children aged between 1 day and 60 months of age in Hanoi, Vietnam. The study showed that enteroaggregative *E.coli*, enteroinvasive *Ecoli*, enteropathogenic and ETEC were the main causes of diarrhoea in the studied cases (Nguyen et al., 2005).

In Egypt, a two-year clinic-based surveillance study of childhood diarrhoea was carried out. A total of 1,275 stool samples were collected. The samples were examined to determine the cause of gastroenteritis and diarrhoea in children. Rotavirus followed by ETEC were the main causes of diarrhoea (Wierzba, 2006).

There have been many previous studies investigating the causes of gastroenteritis and diarrhoea in developing countries (Table 2). These studies confirmed that rotavirus and ETEC cases were the most prevalent and serious among all patients less than five years old. Both pathogens are the main causes of death in developing countries.

In this study, rotavirus and enterotoxigenic *E.coli* were studied as the main causes of gastroenteritis.

## 1.2 Rotavirus

Based on previous studies, rotavirus is the first in rank among all other microbial causes of diarrhoea in children less than five years old. The importance of studying rotavirus came after the isolation of rotavirus in 1969 (Mebus et al., 1969) and described by Ruth Bishop as a cause of children gastroenteritis (Bishop et al., 1973). It was described as the etiological agent of neonatal calf diarrhoea (Babiuk, 1977). Rotavirus has since been identified as one of the major causes of diarrhoea in humans and animals (Babiuk, 1977). Rotavirus is 70nm in diameter and icosahedra in shape (Figure 2). It belongs to the family *Reoviridae*. Seven rotavirus serogroups (A to G) have been described and studied. Most human pathogens belong to groups A, B or C. Group A rotaviruses are the most important from a public health standpoint (Dennehy, 2008). However all groups (A to G) infect animals (Ramig, 2004).

### 1.2.1 Antigenic variability of rotavirus

The main morphological feature of rotavirus is the wheel-like structure shape. This characteristic feature of rotavirus under electron microscope gave the virus its name (*rota = wheel*). The genome of rotavirus consists of 11 segments of double-stranded RNA encoding six structural proteins (VP1-VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6) (Desselberger et al., 2009). Each protein has a specific role in the life cycle and the pathogenesis of the virus. Figure 2 and Table 3 show the RNA segments and the encoded antigens linked with their specific role in the pathogenesis and life cycle of rotavirus.

Rotaviruses are characterised by the presence of 11 segments of double-stranded RNA (dsRNA) enclosed in a triple-shelled protein capsid. Two outer proteins define serotypes of rotavirus: the protease sensitive protein (VP4) and the major glycoprotein (VP7). The two outer proteins induce neutralising antibodies and designate G (VP7) and P (VP4) viral serotypes (Araujo et al., 2001). A binary system of rotavirus classification to designate the neutralisation specificity of both VP7 and VP4 proteins was established (Santos, 2005).

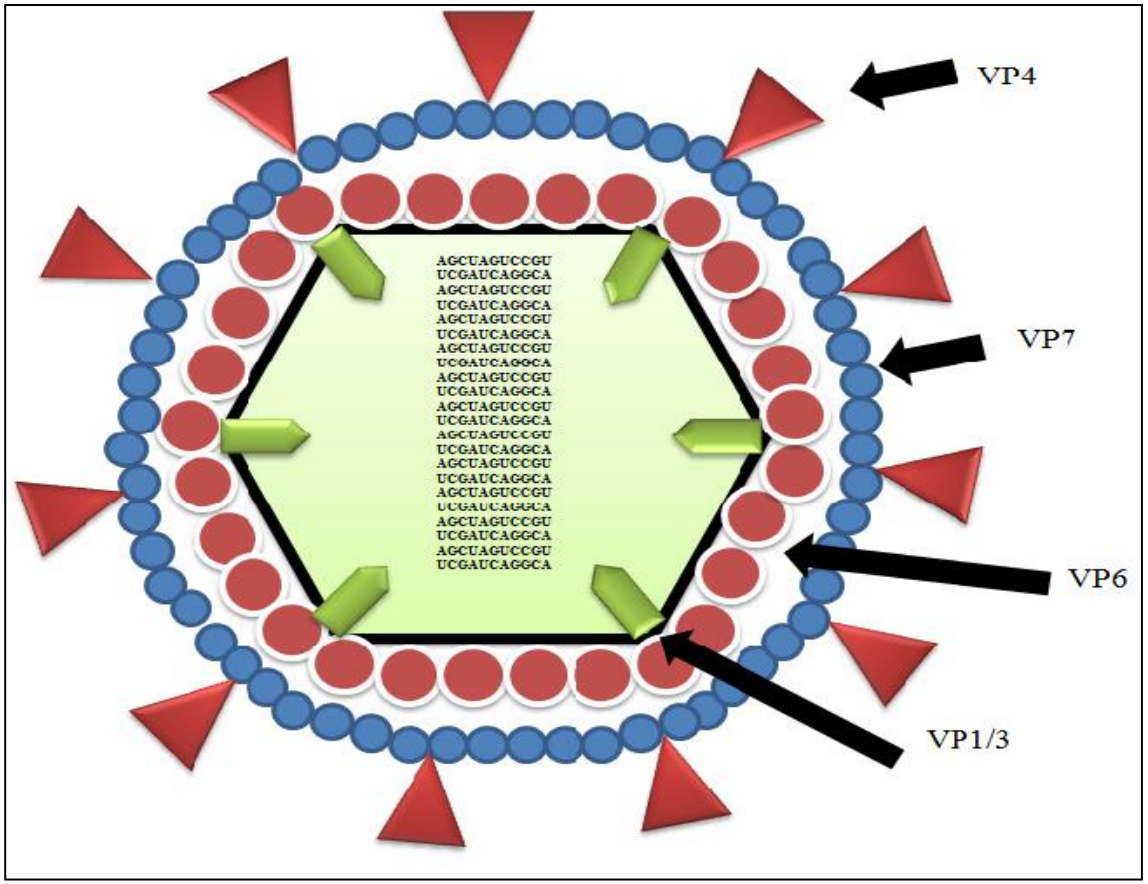



Figure 2 Schematic representation of rotavirus antigens (VP1/3, VP4, VP6 and VP7).

Table 3 Different rotavirus proteins and their role in the virus pathogenesis.

| Segment of dsRNA | Protein | Role in Pathogenesis | Reference |
|---|---|---|---|
| 1 | VP1 | Required for replicase activity of VLPs. | (Zeng et al., 1996) |
| 2 | VP2 | Functions in RNA binding and virus assembly. | (Boyle and Holmes, 1986) |
| 3 | VP3 | Required for guanylyl transferase and capping enzyme activity. | (Liu et al., 1992) |
| 4 | VP4 | Required for heamagglutination, antigen neutralisation, protease-enhanced infectivity virulence and cell attachment. | (Ball et al., 2005) |
| 6 | VP6 | Required for trimerisation and formation of tubules. | (Estes, 1989) |
| 9 | VP7 | Target rough endoplasmic reticulum (RER) and exploit it as maturation site for rotavirus. | (Estes, 1989) |
| 5 | NSP1 | Has affinity for rotavirus mRNA binding. | (Hua et al., 1994) |
| 7 | NSP2 | Drives with NSP5 the formation of viroplasm-like structures (VLS). | (Fabbretti et al., 1999) |
| 8 | NSP3 | Required for 3'-specific viral mRNA binding. | (Poncet et al., 1993) |
| 10 | NSP4 | Functions as an intracellular receptor for addition of the outer coat and spike. | (Ball et al., 2005) |
| 11 | NSP5/6 | Is essential for the assembly of viroplasms and virus replication. | (Campagna et al., 2005) |

VP = viral protein, VLP = virus like particles, NSP = non structural protein

### 1.2.2 Epidemiology

A study summarised all the literature that had been published from 1986 to 2000 describing deaths caused by childhood diarrhoea (Parashar, 2003). Based on this study, 1.4 billion diarrhoea episodes in children less than 5 years of age occur every year. About 25 million clinic visits, 2 million hospitalisations, and more than 440,000 deaths were caused by rotavirus gastroenteritis every year. Most of the death cases occurred in developing countries (Steele et al., 2009). Figure 3 shows the global percentage of viral episodes that required clinical visits, hospitalisation and those that resulted in death in 2003.

Another study (1989 to 2004) performed to analyse 45,571 strains collected globally from 124 studies reported from 52 countries on five continents. The study revealed that four common G types (G1, G2, G3, and G4) in conjunction with P[8] or P[4] represented over 88% of the rotavirus strains worldwide (Santos, 2005). The G1P[8] represents about 65% of the worldwide prevalence of rotavirus, and 34%, 23%, 34% in South America, Africa and Asia respectively (Figure 4). Specifically, G9P[8] has become highly prevalent in many countries in Europe and Australia, with somewhat lower incidence rates in South America, Africa and Asia (O'Ryan, 2009).

A total of 345 children less than 5 years old who had acute gastroenteritis were enrolled in a surveillance study in Morocco (Benhafid et al., 2009). The study revealed that 30.6% of samples were G1[P8], 26% were G9[P8], 7.5% were G2[P6], 3.7% were G1[P6] and 0.7% were G2[P8].

Rotavirus studies also refer to the economical importance and the loss due to outbreaks of rotavirus infections and immunisation. Zimmerman in 2001 discussed the median cost of rotavirus hospitalisation in United States and the results showed that the median cost of diarrhoea-associated hospitalisation was USD 2,307 and that of rotavirus-associated hospitalisation was USD 2,303. Median cost of diarrhoea and rotavirus-associated outpatient visits were USD 47 and USD 57, respectively (Zimmerman, 2001).

Another study in the USA in 2006 assessed the health and economical impacts of a national rotavirus immunisation program (Widdowson et al., 2007). The study revealed

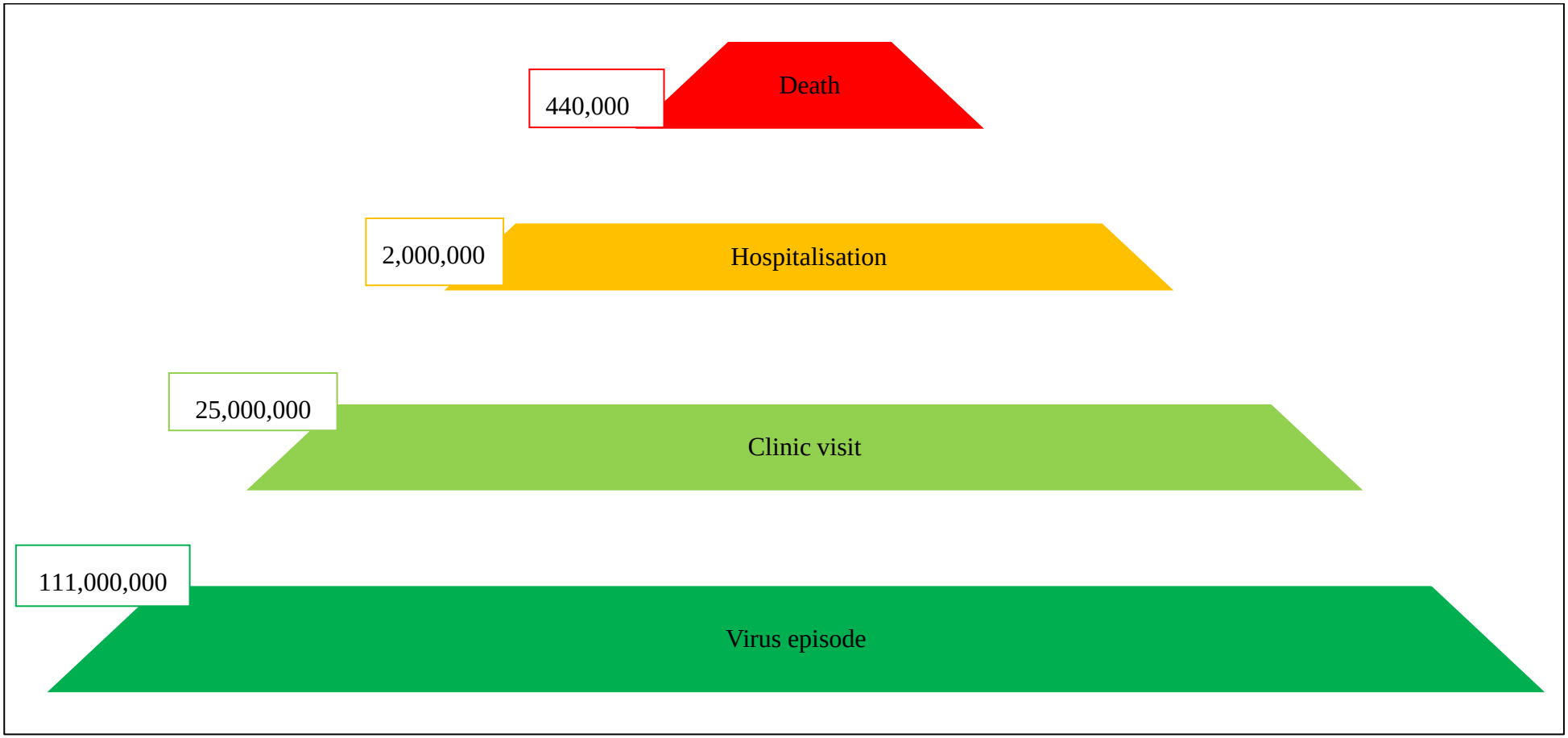



Figure 3 Estimated global prevelance of rotavirus infection in 2003. The numbers are adapted from Parshar (2003). Rotavirus caused approximatly 440,000 deaths per annum, average 50 deaths per hour.

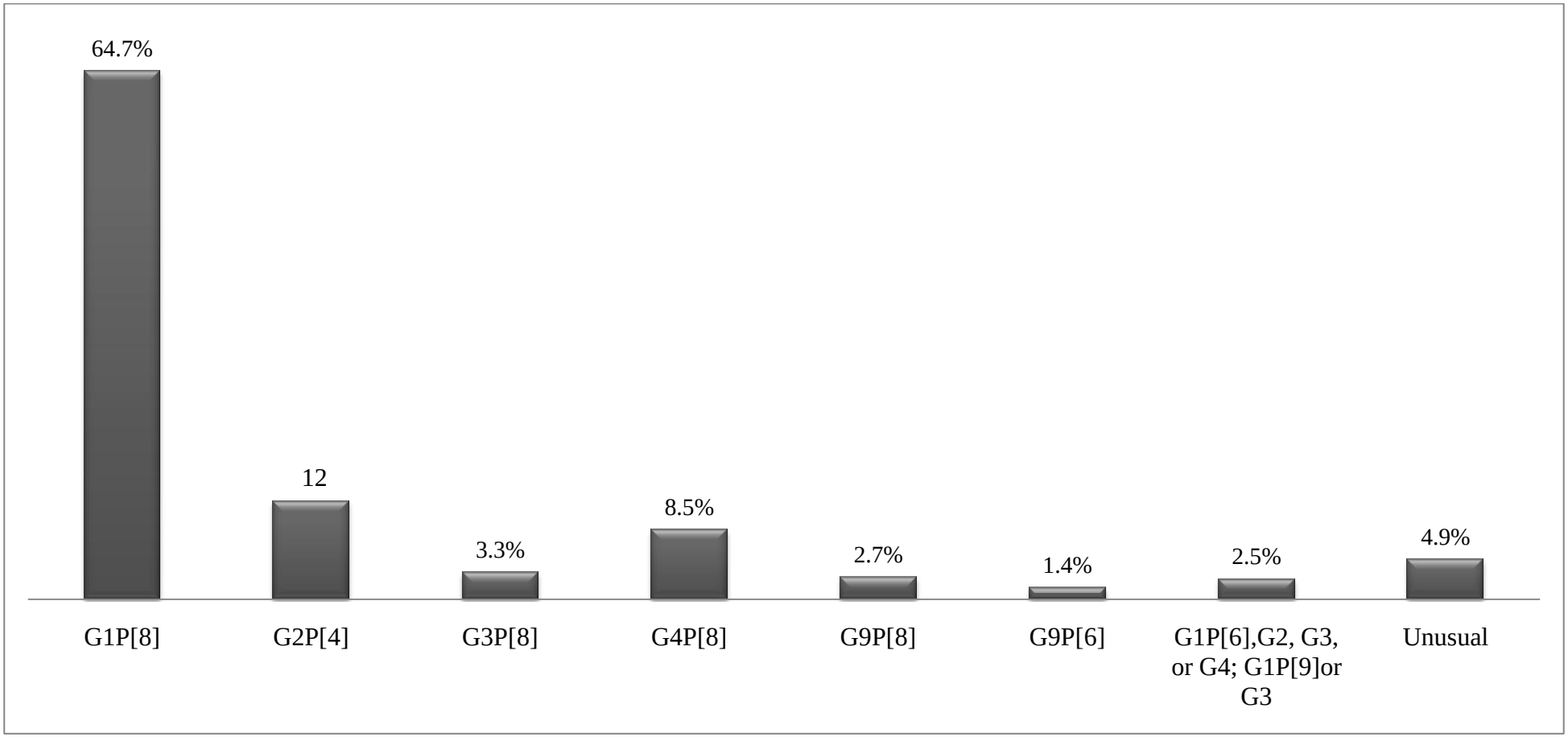



Figure 4 The relative distrbution of various strains of human rotavirus A (P&G) from 124 studies reviewed by Santos, 2005. The predominant strain G1P[8] was the most prevalent strain in Asia, Africa and South America.

that vaccination cost USD 138 per case averted, USD 3,024 per serious case averted and USD 197,190 per life-year saved. The total cost was USD 515 million to the health care system and USD 216 million to the society.

In conclusion, rotavirus has been and remained a serious cause of death in children less than five years of age. Specific strains are endemic in specific geographical areas. Moreover, from an economical aspect, rotavirus infection has cost health care systems such as the USA health care system millions of dollars.

### 1.2.3 Pathogenesis

Different animal models to study rotavirus pathogenesis have been developed. These include the rabbit, mouse (Ward, 1990), guinea pig (Zijlstra et al., 1999), rat (Salim et al., 1995) and calf (Carpio M., 1981) (Table 4). Further details using a variety of inbred mouse strains are summarised in Table 5. The small intestine consists of villi and crypts. Each villus is lined with mature enterocytes that are specifically developed for absorption and secretion by the digestive system. In general, illnesses caused by rotavirus infection are classified into four clinical settings; mild cases require home care alone, moderate cases requiring clinical visits, severe cases require hospitalisation and cases resulting in death. There are four scenarios for the mechanisms of rotavirus-induced diarrhoea: malabsorption, secretion, villus ischemia and intestinal motility.

#### 1.2.3.1 Malabsorption

As rotavirus start to synthesis its proteins, this process leads to a disruption in $Ca^{+}$ ion homeostasis which leads to increase in $Ca^{+}$ permeability through the intestinal epithelium and increase $Ca^{+}$ concentration in the plasma membrane and the endoplasmic reticulum. This triggers a chain of events that lead to cell lysis (Pérez et al., 1998).

The enterotoxin non structural protein 4 (NSP4) is a multifunctional protein encoded by segment 10 of the dsRNA rotavirus (Ball et al., 2005). NSP4 triggers phospholipase C-inositol 1,3,5 triphosphate (PLC-IP3) cascade resulting in the release of $Ca^{+}$ from intracellular stores (Dong et al., 1997). The increase of $Ca^{+}$ concentration caused by NSP4 is not related to the increase due to phospholipase C (PLC) stimulation (Tian et al., 1995). The effects of NSP4 in the process of infection may also play a role in the

Table 4 Human and animal models for studying pathogenesis of rotavirus infection

| **Model** | **Strain** | **Age** | **Pathogenesis** | **Histology** | **Reference** |
|---|---|---|---|---|---|
| Mice | Balb/C | < 2 weeks | Infect villous cell | Changes in the villous but not in crypt cells | (Little and Shadduck, 1982) |
| Rat | Not available | 5 days | Infects jejunal and ileum villi | Reduction in total mucosal thickness resulted in a true flat mucosa | (Salim et al., 1995) |
| Rabbit | Pathogen free New Zeland | Not available | Impairs intestinal brush- border membrane $Na^+$ solute co-transport activities | Not available | (Halaihel et al., 2000a) |
| Guinea Pig | Not available | 3-5 days | Infects ileum villi | Villous atrophy | (Yuan et al., 1996) |
| Calves | Not available | Neonatal | Infects jejunal and illeum villi | Reduction of jujenal villus length due to stunting of villi and replacement of columnar with cuboidal epithelium at the tips of villi | (Carpio M., 1981) |
| Human | Not applicable | <5 years | Infects villus epithelium of the small intestine | Destruction of the microvilli in the small intestine, | (Jourdan et al., 1997, Ramig, 2004) |

Table 5 Previous studies that have used mouse models to study rotavirus infection

| Mice strain | Virus strain | Year | Type of study | Reference |
|---|---|---|---|---|
| Balb/C | EDIM[1] | 1982 | Pathogenesis of rotavirus infection | (Little and Shadduck, 1982) |
| CD-1 | SA11[2], NCDV[3], UK[4] | 1986 | Oral immunisation | (Offit et al., 1986) |
| Balb/C | EDIM | 1988 | Histological changes in the intestine | (Osborne et al., 1988) |
| Balb/C | EDIM, WC3[5] | 1990 | Immunisation | (Ward, 1990) |
| Balb/C | EDIM | 1991 | Pathology and histology of rotavirus infection | (Osborne et al., 1991) |
| Balb/C, CBA/H | SA11 | 1992 | Passive immunity against rotavirus | (Both et al., 1993) |
| NA | BRV[6], BRV-UK[7], SA11, | 1995 | Antibodies against VP7 and VP4 | (Ijaz et al., 1995) |
| Balb/C | EDIM | 1997 | Vaccination against VP6 | (Choi et al., 1997) |
| ddy | PO-13[11], Ty-3[12], SA-11 | 2001 | Avian to mammal rotavirus transmission | (Mori et al., 2001) |
| $CB17_{Scid}$ | RRV, WC3 | 2004 | Rotavirus vaccine (WC3-PV) | (Qiao, 2004) |
| Balb/C | RRV | 2007 | Egg yolk immunoglobulin against rotavirus | (Sarker, 2007) |
| ICR | ECwt[13] | 2010 | Rotavirus infection in the intestinal villi | (Guerrero et al., 2010) |

[1]Epizootic diarrhoea of infant mice, [2]Simian rotavirus, [3]Nebraska calf diarrhoea virus, [4,5,6,7]Bovine rotavirus, [8,13]Murine rotavirus, [9.10] Human rotavirus, [11]Pigeon rotavirus. [12]Turkey rotavirus.

dysregulation leading to diarrhoea. This indicates the ability of inactivated rotavirus particles to induce diarrhoea (Shaw et al., 1995).

Loss of fluids could happen due to the failure of $Na^+$-linked nutrients absorption process. The process happens due to a disturbance of $Na^+$ and $K^+$ concentration. The disturbance leads to a change in the intracellular concentration of $Na^+$ and $K^+$. This in turn could impair the electronuteral NaCl absorption (Michelangeli, 2003). The segment NSP4 may also be involved, as its peptide is a specific and non-competitive inhibitor of sodium-glucose co-transporter 1( SGLT1) (Halaihel et al., 2000b).

Infection also reduces the expression of the digestive enzymes at the apical surface of the infected enterocytes. Rotavirus can cause paracellular leakage. It can alter the structure of polarised enterocytes by affecting $Ca^+$ sensitive proteins (F-actin, villin and tubulin) that damage the microvilli and lead to a drop in transepithelial resistance. This results in losing the tight junctions between enterocytes (Tafazoli et al., 2001).

#### 1.2.3.2 Secretion

Secretion of fluids and loss of water is a consequence of the activation of chloride secretion caused by NSP4. This leads to increase in Ca+ concentration in crypt epithelium (Morris and Estes, 2001). The Enteric nervous system (ENS) is activated during rotavirus infection stimulating the crypt cell to secret electrolytes and water (Lundgren, 2003).

#### 1.2.3.2 Villus ischemia

Villus ischemia was only observed in mice. It was a result of the release of vasoactive agents from rotavirus and caused local villus ischemia and damage to the enterocytes (Osborne et al., 1991).

#### 1.2.3.4 Intestinal motility

Enteric nervous system controls intestinal motility. Rotavirus affect ENS which increases the peristaltic motility of the small intestine and decreases their transit time leading to diarrhoea (Michelangeli, 2003).

### 1.2.4 Prevention and treatment

Prevention is better than cure. In order to prevent rotavirus infection; previous attempts were carried out to develop an effective vaccine. The first attempt was in 1980 (Vesikari et al., 1984). Later, four different approaches were used; the Jennerian approach, modified Jennerian approach, non-Jennerian approach and virus like particle approach. The least successful effort was the RotaShield® vaccine developed by Wyeth Ayerst. RotaShield® was withdrawn on October 1999 after production of 11million doses of the vaccine. This action was based on the recommendations of the United States Centre for Disease Control and Prevention (CDC) and the United States Advisory Committee on Immunisation Practice (ACIP). They found a link between vaccination and intussception, especially during the first 2 weeks after administration of the first dose (Kapikian et al., 2005). On the other hand, two licensed vaccines are now available over the counter with no history of side effects, Rotateq® 2006 and Rotatrix® 2008 (Ciarlet and Schodel, 2009). Table 6 shows different approaches to develop rotavirus vaccine and the most recent status for each approach.

Despite preventative measures, treatment protocols are required for children who do not have the benefit of vaccination program. Rotavirus infection causes diarrhoea and loss of body fluids that lead to dehydration and possibly death. The principle treatment model of rotavirus infection is oral rehydration therapy (ORT). This therapy is to compensate fluid loss and prevent body dehydration. Oral rehydration therapy may be administered either by oral or intravenous route depending on the degree of dehydration (Alam and Ashraf, 2003). Oral rehydration salt solution (ORS) is recommended for ORT as a formula from World Health organisation and the United Nations Children's Fund (UNICEF). Intravenous route is recommended for initial management of severe dehydration due to diarrhoea followed by ORT with ORS solution. The formulation is sodium 75 mmol/L, potassium 20 mmol/L, chlorine 65 mmol/L, citrate 10 mmol/L, glucose 75 mmol/L, osmolarity 245 mosmol (WHO, 2001).

Table 6 Different approaches used for the development of rotavirus vaccines

| Vaccine | Formulation | Serotype [Genotype] | Company | Status |
|---|---|---|---|---|
| Jennerian approach | | | | |
| RIT 4237 | Monovalent live oral bovine NCDV | P[1]G6 | Smith Kline Beecham (Belgium) | Discontinued |
| WC3 | Monovalent live oral bovine WC3 strain | P[5]G6 | Merck (USA) | Discontinued |
| RRV | Monovalent live oral rhesus MMU 18006 strain | P[3]G3 | DynCorp (USA) | Discontinued |
| LLR | Monovalent live oral lamb LLR strain | P[12]G10 | Lanzhou Institute of Biological Products (China) | Active Licensed (China 2000) |
| Modified Jennerian approach | | | | |
| WaXUK | Monovalent live oral human (Wa) bovine (UK) reassortant | P[8]G6 | DynCorp (USA) | Active Phase I |
| WaXDS-1XUK | Monovalent live oral human (Wa and DS-1)-bovine (UK) reassortant | P[8]G2 | DynCorp (USA) | Active Phase I |
| RotaShield® | Quadrivalent live oral human rhesus (RRv) reassortants | P[3]G1,G2,G3 and G4 | Wyeth Ayerst (USA) | Licensed (USA 1998) withdrawn 1999 |
| RotaTeq® | Pentavalent live oral human-bovine (WC3) reassortants | P[5]G1,G2,G3,G4 and P[8]G6 | Merck (USA) | Active Licensed (2006 USA) |

| | | | | |
|---|---|---|---|---|
| UK-based reassortants | Quadrivalent live oral human-bovine (UK) | P[5]G1,G2,G3 and G4 | NIH (USA) | Licensed USA |
| Non-Jennerian approach | | | | |
| M37 | Monovalent live oral human neonatal M37 strain | p[6]G1 | DynCorp (USA) | Discontinued |
| Rotatrix | Monovalent live oral human 89-12 strain | P[8]G1 | GlaxoSmithKline (Belgium) | Active licensed (2008 USA) |
| RV3 | Monovalent live oral human neonatal RV3 strain | P[6]G3 | DynCorp (USA) | Active phase I-II |
| 116E | Monovalent live oral human neonatal 116E strain | P[11]G9 | Bharat Biotech (India) | Active phase III |
| 1321 | Monovalent live oral human neonatal 1321 strain | P[11]G10 | Bharat Biotech (India) | Active phase III |
| BIRVI | Monovalent inactivated human AU64 strain | P[4]G1 | Biken (Japan) | Under development |
| Virus like particles approach | | | | |
| VLPs | Simian (SA11) virus-like particle | VP2/6 | Lederle/Green Cross (Korea) | Under development |

### 1.3 *Escherichia.coli*

*E.coli* is a member of the family *Enterobacteriacea*. The family is characterised as rod-shape, gram negative and a facultative anaerobe. *E.coli* is a commensal organism of the human colonic flora. Typically *E.coli* colonises the infant gastrointestinal tract and plays a role in maintaining the intestinal physiology (Myron, 1987). *E.coli* has the ability to cause different infections to humans through their highly adapted clones (Drasar and Hill, 1974).

*E.coli* was serotyped according to its O (somatic), H (flagellar) and K (capsular) surface antigen profiles (Kenny et al., 1997, Prager et al., 2003). A total of 170 different O antigens, each defining a serogroup, were recognised. Bacterial agglutination test was used to determine the presence of K antigen. This antigen is for an *E. coli* strain that was inagglutinable by O antiserum but became agglutinable when the culture heated.

The diversity of molecular structures, including fimbriae, conferred the K phenotype This led microbiologists to suggest restructuring the K antigen designation to include only acidic polysaccharides (Lior, 1996). Therefore, proteinaceous fimbrial antigens were excluded from the K series and given F designations (Orskov, 1982). Using the combined designation of O and H antigens to define the serotype of isolated *E.coli* can be associated with certain clinical syndromes (Table 7).

In general, it is not the serologic antigens themselves that confer virulence; the serotypes and serogroups serve as identifiable chromosomal markers that correlate with specific virulent clones (Whittam et al., 1993). There are six diarrhoeagenic pathovars *E.coli*: enterotoxigenic *E.coli* (ETEC), enteropathogenic *E.coli* (EPEC), enterohaemorrhagic *E.coli* (EHEC), enteroinvasive *E.coli* (EIEC); which includes Shigella), enteroaggregative *E.coli* (EAEC) and diffusely adherent *E.coli* (DAEC) (Croxen and Finlay, 2009).

### 1.4 Enterotoxigenic *E.coli* (ETEC)

Previous studies used animal models to understand the ETEC infections and the complexity of antigens structure. These studies aimed to explain the immune response for ETEC infection and develop an effective vaccine. Table 8 shows the different studies that used ETEC on animal models.

Table 7 Different *E.coli* pathovars and clinical diseases

| ***E.coli* Pathovars** | **Strain examples** | **Disease** | **Transmission** | **Mode of action** | **Reference** |
|---|---|---|---|---|---|
| Enteropathogenic *E. coli* (EPEC) | O55:H6,NM | Watery diarrhoea | Faecal and oral | Attach to intestine and produce lesions called localised adherence | (Donnenberg and Kaper, 1992, Seifert and DiRita, 2006) |
| Enterohaemorrhagic *E. Coli* (EHEC) | O26:H11,H32, | Bloody diarrhoea (Haemolytic Uremic Syndrome) | Faecal and oral | Attach the intestine through common pillus, (haemorrhagic diarrhoea) | (Croxen and Finlay, 2009, Seifert and DiRita, 2006) |
| Enterotoxigenic *E. coli* (ETEC) | O6:H16<br>O78:H11,H12 | Watery diarrhoea (traveller's) | Faecal and oral | Colonises the intestine and produces toxins | (Roy et al., 2009b, Seifert and DiRita, 2006) |
| Enteroinvasive *E. coli* (EIEC) | O159:H2,NM | Dysentery and bloody diarrhoea | Faecal and oral | Invades the submucosa by microfold cells | (Croxen and Finlay, 2009, Seifert and DiRita, 2006) |
| Enteroaggregative *E. coli* (EAEC) | O3:H2<br>O15:H18 | Watery diarrhoea | Faecal and oral | Produces an aggregative adherence pattern | (Weintraub, 2007, Seifert and DiRita, 2006) |
| Diffusely adherent *E. coli* (DAEC) | O2:H6<br>O6:H27 | Acute diarrhoea and urinary tract infections in adults | Faecal and oral | Produce a diffuse adherence pattern that covers the entire surface | (Scaletsky et al., 2002, Lopes et al., 2005) |

Table 8 The mouse models used in different studies of enterotoxigenic *E.coli*

| **Mouse strain** | **Year** | **Study type** | **Results** | **Reference** |
|---|---|---|---|---|
| CF1 | 1980 | Pathogenesis | Young mice susceptible to ETEC K99+ adhesion | (Runnels et al., 1980) |
| Balb/C | 1990 | Pathogenesis | Adult mice could be used to study rotavirus infection | (Ward, 1990) |
| Balb/C | 2003 | Immune response | Mice could be used to study immunogenicity and pathogenicity of ETEC | (Byrd et al., 2003) |
| Balb/C | 2009 | Vaccine development | More than 80% of the mice were protected against ETEC challenge | (Liu et al., 2009) |

Enterotoxigenic *E.coli* elaborates two defined groups of enterotoxin: heat-stable (ST) and heat-labile (LT) enterotoxin (Myron, 1987). Enterotoxigenic *E.coli* was first recognised as a cause of diarrhoeal disease in piglets. Studying ETEC in piglets explained the mechanism of the disease and highlighted the role of enterotoxins. These studies paved the way for the researchers to study ETEC in humans. The first study identified that certain *E.coli* strains isolated from the stools of children with diarrhoea caused fluid secretion in ligated rabbit intestinal loops (Taylor, 1961).

At present, ETEC is diagnosed as the most common cause of traveller's diarrhoea and the fatal pathogen for children less than five years old (Nataro and Kaper, 1998). Traveller's diarrhoea refers to an enteric disease acquired when a person travels from a developed country to a developing area of the world. The incubation period of most enteropathogens is 7 to 10 days after they return to a developed area (De Las et al., 1999). Enterotoxigenic *E.coli* strains, *Shigella*, *Campylobacter* and *Salmonella* spp. are responsible for 80% of diarrhoeal episodes in travellers. The most recognised and common cause of traveller's diarrhoea is ETEC. It accounts for about 40% of the cases of traveller's diarrhoea. In children who live in developing countries, ETEC results in 20% of the hospitalised diarrhoeal episodes (Qadri et al., 2005).

### 1.4.1 Pathogenesis of ETEC

The first step of ETEC pathogenesis is the colonisation of the mucosal surface of the small intestine using the colonisation factors (McConnell et al., 1991). The second step is the elaboration of enterotoxins to trigger reactions and cause diarrhoea. Both LT and ST play major roles in the process of diarrhoea (Nataro and Kaper, 1998, Croxen and Finlay, 2009). Table 9 summarises ETEC antigens and their role in the pathogenesis of diarrhoea.

#### 1.4.1.1 Heat-labile toxins (LT)

The LTs of *E.coli* in general share some similarities with cholera toxin (CT) elaborated by *Vibrio cholera*, especially, in structure and function (Fan et al., 2004, Sixma et al., 1993). The LT consists of two main serogroups, LT-I and LT-II. Both show no immunological cross-reaction. Serogroup LT-I is expressed by *E.coli* strains that are pathogenic for both humans and animals. LT-II is found primarily in animal hosts (Brandal et al., 2007).

**LT-I**

The toxin LT-I is composed of one A subunit and five identical B subunits (Brandal et al., 2007, Lasaro et al., 2008). The B subunits are arranged in a ring shape and bind strongly to the ganglioside GM1 (Lasaro et al., 2008). The A subunit is split into two peptides A1 and A2. These peptides are responsible for the enzymatic activity of the toxin. The genes encoding LT (*elt* or *etx*) reside on plasmids that also may contain genes encoding ST and/or colonisation factor antigens (CFAs).

The peptide A1 targets the ADP-ribosyl moiety and transfers it from NAD to alpha subunit of the guanosinetriphosphate GTP-binding (GS). The GS stimulates adenylate cyclase activity that leads to increase the level of intracellular cyclic AMP. The increasing level of cAMP activates cAMP dependent protein kinase (A kinase). The kinase leads to activation of chloride channel CFTR (Sears and Kaper, 1996, Croxen and Finlay, 2009). The previous process results in chloride secretion and inhibition of NaCl absorption. The inhibition draws water passively resulting in osmotic diarrhoea.

An alternative way for enterotoxins to cause diarrhoea is through stimulating the secretion of fluids from intestine (Sears and Kaper, 1996). Previous studies considered PGE1 and PGE2 as mediators in the mechanism of diarrhoea. Synthesis and release of arachidonic acid metabolites such as prostaglandins and leukotrienes can stimulate electrolyte transport and intestinal motility.

Enteric nervous system (ENS) as intestinal motility regulator plays role in the process of diarrhoea. It controls the peristaltic movement of the small intestine. In addition, CT and LT decrease the absorption of fluids from the intestinal lumen(Michael Field et al., 1972).

Table 9 Different ETEC antigens and their specific modes of action

| **Antigens** | | | **Role in pathogenesis** | **Reference** |
|---|---|---|---|---|
| Heat-labile toxins | LT-I[1] | Subunit A (A1, A2) | Stimulate AC[2] which increases the level of cAMP[3] This in turn increases P. kinase A that leads to osmotic diarrhoea and increases chloride secretion which acts on CFTR[4] chloride channels | (Lasaro et al., 2008) |
| | | Subunit B | Binds to ganglioside GM1 | |
| | LT-II | LT-IIa | Increases cAMP which increases chloride secretion leading to osmotic diarrhoea | (Spangler, 1992) |
| | | LT-IIb | Increases cAMP which increases chloride secretion leading to osmotic diarrhoea | |
| Heat-stable toxins | ST | STa (STh and STp) | Bind to GC-C[5] that increases GC. This increases the level of cAMP leading to osmotic diarrhoea | (Hasegawa and Shimonishi, 2005), |
| | | STb | Causes villus atrophy and increase of $HCO3^{+}$, $Ca^{+}$, PGE2[6], serotonin and ENS[7] concentration | (Daniel Dubreuil, 2010) |

| | | | |
|---|---|---|---|
| Colonisation factors | CFA (25 CFs) | Enhance adhesion and colonisation to the intestinal mucosa | (Fleckenstein et al., 2009) |
| Other fimbrial operons | Type I fimbriae | Induce intestinal colonisation | (Sokurenko et al., 1998) |
| | *E. coli* common pilus | Cause pilus assembly | (Blackburn et al., 2009) |
| Non-fimbrial adhesins/ invasions | Tia and TibA invasions | Induce adherence and epithelial cell invasion | (Fleckenstein et al., 2009) |
| | EtpA | Forms bridge binding between the host cell receptors and the tips of ETEC flagella | (Roy et al., 2009b) |

[1]Labile toxin, [2]Adenylate cyclise, [3]Cyclic adenylate monophosphate, [4]Cystic fibrosis transferase receptor, [5]Guanylylcyclase-C, [6]Prostaglandin E2, [7]Entirc nervous system.

**LT-II**

The LT-II has two antigenic variants, LT-IIa and LT-IIb. The LT-II causes diarrhoea by increasing the intracellular cAMP levels with similar mechanisms to those involved in LT-I toxicity. One step is different, using GD1 as a receptor instead of GM1 (Spangler, 1992) (Table 9).

### 1.4.1.2 Heat-stable toxins (ST)

There are two serogoups of ST, STa and STb. These are different in structure and mode of action (Croxen and Finlay, 2009). Genes of both classes are found predominantly on plasmids, and some ST-encoding genes were found on transposons (Nataro and Kaper, 1998).

Two variants, STp (ST porcine or STIa) and STh (ST human or STIb) were isolated from pigs or humans, respectively. Both variants are human ETEC strains. The major receptor for STa is a membrane-spanning enzyme called guanylate cyclase C (GC-C) (Basu et al., 2010). The GC-C is located in the apical membrane of the intestinal epithelial cells. Binding of ligands to the extracellular domain stimulates the intracellular enzymatic activity. Binding of STa to GC-C stimulates GC activity. This increases intracellular cGMP levels (Guo et al., 2007). This activity stimulates chloride secretion and/or inhibition of sodium chloride absorption. The stimulation results in net intestinal fluid secretion. Ultimately, the CFTR chloride channel is activated, leading to secretion of $Cl^-$ ions into the intestinal lumen (Huang et al., 2009). In contrast to the 15- to 60-min lag time needed for LT to translocate and activate the basolateral adenylate cyclise complex, STa acts much faster due to the apical location of its cyclase receptor.

Alternative mechanisms of action were proposed for STa and involves prostaglandins, calcium, and ENS (Foster and Geof, 2009). The secretory response to STa may also involve phosphatidylinositol and diacylglycerol release, activation of PKC, elevation of intracellular calcium levels and microfilament (F-actin) rearrangement (Nataro and Kaper, 1998).

**STb**

The STb is described mainly in pigs, although, there have been some reported cases in human with ETEC elaborating STb (Dubreuil, 2008). The STb induces diarrhoea by the

effect of histological damage that caused in the intestinal epithelium. The damage is expressed as loss of villus epithelial cells and partial villus atrophy (Dubreuil, 2008). The STb stimulates the secretion of bicarbonate ions from intestinal cells (Daniel Dubreuil, 2010). On the other hand, STb does not show an effect on increasing the intracellular cAMP or cGMP concentrations. However, STb increases the intracellular calcium levels from extracellular sources (Susan et al., 2006). In addition, STb has a stimulatory effect on PGE2 and serotonin. This suggests a role for ENS in the secretory response to this toxin (Susan et al., 2006, Daniel Dubreuil, 2010).

#### 1.4.1.3 Colonisation factors (CF)

Previous researches investigated the mechanisms of adherence and colonisation of the intestinal epithelium used by ETEC strains (Fleckenstein et al., 2009, Navaneethan and Giannella, 2008, Freedman et al., 1998, Rodas et al., 2009, Vidal et al., 2009, Xia et al., 2010). Adherence to the enterocytes is the first step in the pathogenesis of ETEC infection, an event mediated by surface fimbriae (also called pili) (Mu et al., 2008, Navaneethan and Giannella, 2008, Croxen and Finlay, 2009). Fimbrial antigens form the basis for species-specific differentiation of ETEC and as a problem for vaccination development. For example, ETEC strains expressing K99 are pathogenic for calves (Foster and Geof, 2009), lambs and pigs (Kwon et al., 1999). Whereas K88-carrying organisms are able to cause disease only in pigs (Cassels and Wolf, 1995).

From the aspect of vaccine development, colonisation factors (CFs) are very important targets (Vidal et al., 2009). Twenty-five different proteinacious structures of CFs have been described to date. Most structures are plasmid-encoded (Fleckenstein et al., 2009). The structural morphology and antigenicity of CFs are diverse. Three different structures were described: fimbrial, fibrillar and helical. The structures had variable lengths up to more than 20 mm (Xia et al., 2010). The CFA/I fimbriae were studied intensively. No definite receptor has been identified for CFA. However, the proposed mechanism of action was through binding the glycoprotein conjugates on the cell surface (Fleckenstein et al., 2009).

#### 1.4.1.4 Other fimbrial operons

**Type I fimbriae**

ETEC produces a common type I fimbriae. There is no clear role for type I fimbriae in ETEC infection process. However, some studies showed that they could be suited for intestinal colonisation (Sokurenko et al., 1998).

**Common pilus**

The role of *E.coli* common pili protein (ECP) in the process of ETEC infection is not fully understood. Some studies have discussed its role in pillus assembly (Blackburn et al., 2009).

#### 1.4.1.5 Non-fimbrial adhesins/invasins of ETEC

**Tia and TibA invasions**

The two invasion loci tia and tib encode distinct proteins on ETEC. Tia is an outer membrane protein encoded on a large pathogenicity island and encoded by tia loci (Elsinghorst and Kopecko, 1992, Mammarappallil and Elsinghorst, 2000). The role of Tia and TibA in the pathogenesis of ETEC are exemplified in the adherence and invasion of the epithelial cells of the small intestine (Mammarappallil and Elsinghorst, 2000, Fleckenstein et al., 2009).

**EtpA**

The EtpA, EtpB and EtpC are members of the family TPS exoprotein. The main function of EtpA is promoting the adhesion of the small intestine. Previous studies showed that the role of EtpA is to form a bridge binding the host cell receptor and the ETEC flagella (Roy et al., 2009b). The EtpA has been the subject to many vaccine development attempts. These attempts revealed that vaccination of mice with either a truncated recombinant EtpA fragment (Roy, 2008) or the full length EtpA glycoprotein (Roy et al., 2009a) protect mice by preventing the colonisation of the intestin (Fleckenstein et al., 2009).

## 1.4.2 Epidemiology of ETEC

### 1.4.2.1 Relationship between Age and Infection

Previous studies stated that ETEC was a common cause of gastroenteritis and diarrhoea in children less than two years old (Subekti et al., 2003, Nguyen et al., 2005, Youssef et al., 2000, Merson et al., 1980, Wierzba, 2006, Qadri et al., 2007b). In Bangladesh, a study showed that 19.5% of 321 children less than 2 years old suffered from diarrhoea due to ETEC (Qadri et al., 2007b). Another study in Egypt confirmed ETEC as a common cause of diarrhoea in infants (Wierzba, 2006).

Al-Gallas *et al.* and Qadri *et al.* suggested that infections due to ETEC declined between the age of 5 and 15 and increased again at adolescence because of different environmental and immunological factors (Nataro and Kaper, 1998, Al-Gallas et al., 2007, Qadri et al., 2007a, Qadri et al., 2005). Based on animal studies, the age related infection was explained by the presence or disappearance of the K99 receptor in the intestine (Runnels et al., 1980). However, factors such as individual variability and repeated infection during childhood affected the infection progress and disease development (Clemens et al., 2004).

### 1.4.2.2 Mixed Infection (co-infection)

Mixed infection usually occurs in the case of ETEC infection, and other enteropathogens (Grimprel et al., 2008). Co-infection may disturb and mislead the diagnosis of the actual cause of diarrhoea. This has led to the lack of understanding of the actual pathogenesis of the infection (Grimprel et al., 2008). Mixed infection is frequent and can occur in up to 40% of cases (Peltola and Siitonen, 1991, Black, 1993, Albert et al., 1999, Qadri et al., 2000). Poor sanitation and contaminated food and water are among the main reasons that have increase the opportunity of mixed infection in some areas.

In cases of children infected with ETEC, rotavirus is the most common pathogen that interferes and causes mixed infection. This is followed by other bacterial enteropathogens (e.g., *V. cholerae*, Shigella spp., *Cryptosporidium*, *Campylobacter jejuni* and Salmonella spp.) (Qadri et al., 2007b). In case of traveller's diarrhoea, enteroaggregative *E.coli* and *Campylobacter* spp are the common pathogens that occur together with ETEC (Shah et al., 2009).

Previous studies discussed the importance of mixed infection with ETEC and showed the different mixed pathogens. Albert *et al.* studied 814 children with diarrhoea in Bangladesh. The study showed that rotavirus was the major pathogen in case of mixed infection that formed about 20% of the studied population (Albert et al., 1999).

Another study by Youssef *et al.* in Jordan showed that 15.5% of the samples from 265 children less than five years old suffered from diarrhoea were due to mixed infection. In this population, rotavirus, EPEC and ETEC were the main causes of the mixed infection cases (Youssef et al., 2000).

Other pathogens were studied in Kenya, India and Jamaica revealed that *Shigella*, *Vibrio*, *Campylobacter* and *Salmonella* were the causes of the mixed infection (Zhi-Dong et al., 2002). *Ascaris Lumbricoides* was the prevalent pathogen in Mozambique (Mandomando et al., 2007). Table 10 summarises the pathogens found in mixed infections with ETEC.

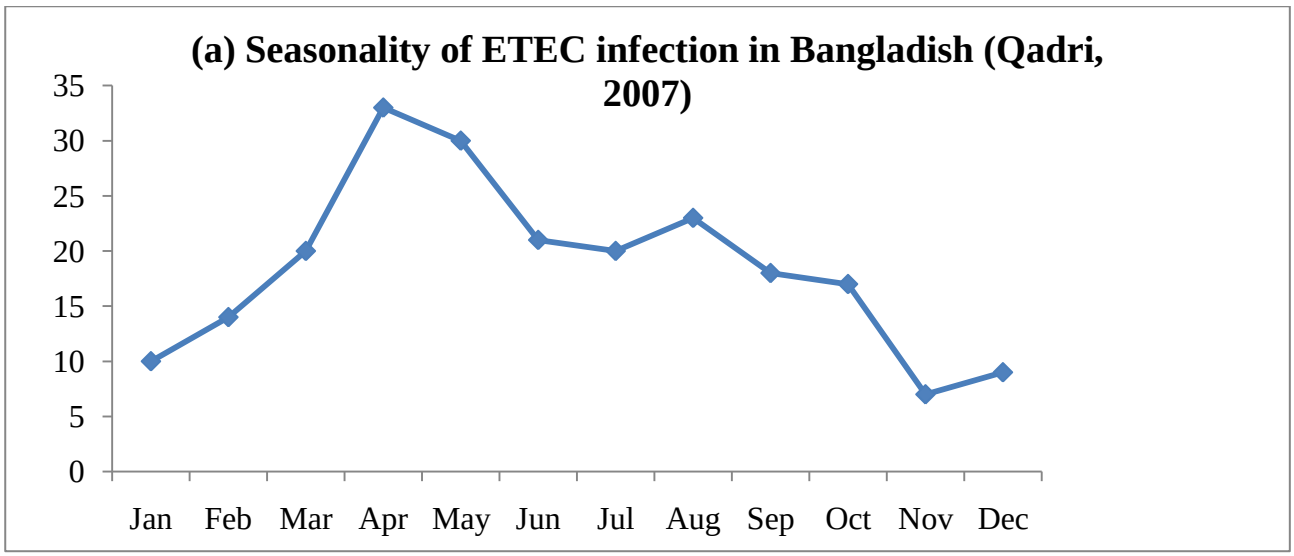
#### 1.4.2.3 Frequency of ETEC outbreak

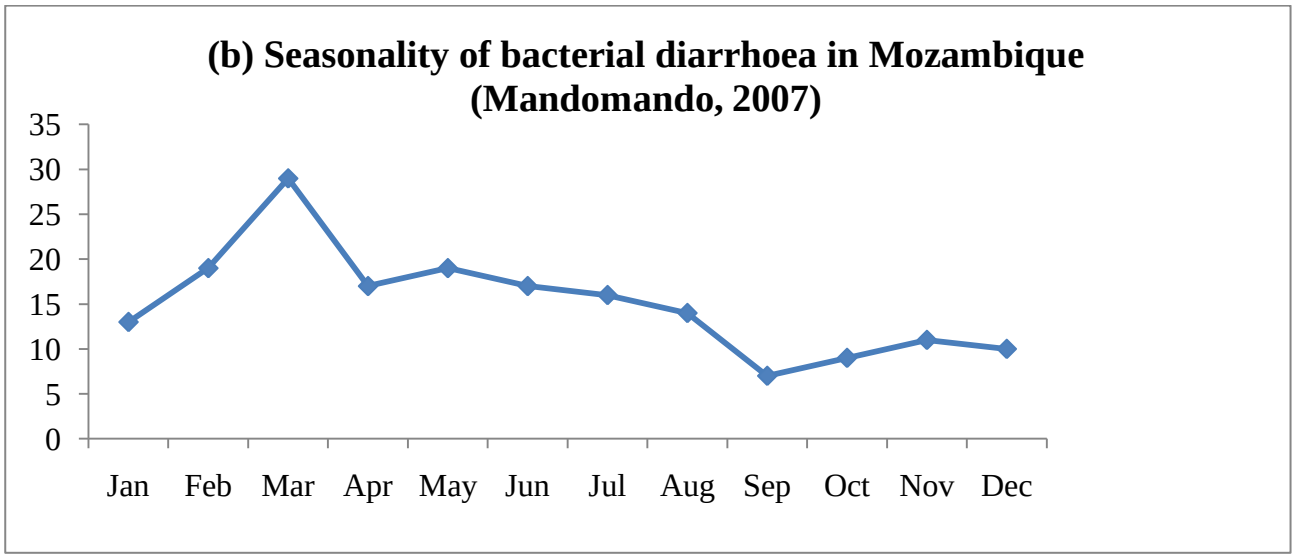
Infection due to ETEC is more frequent during warm seasons (Mandomando et al., 2007). A study in Bangladesh showed that although ETEC was endemic in Bangladesh, outbreaks and high incidence of ETEC infection was mostly at the beginning of spring (Albert et al., 1995). High temperature in warm seasons afforded a good environment and the best conditions for optimal growth of ETEC (Qadri et al., 2005).

Figure 5 shows ETEC incidence in three different countries, Bangladesh (Asia), Mozambique (Africa) and United Mexican States (South America). In Bangladesh and Mozambique the high incidence and outbreaks tended to occur during the warm seasons March, April and May. In The United Mexican states, high incidence and outbreaks were more in June, July and august(Estrada-Garcia et al., 2009). In conclusions, outbreaks in developing countries increased in warm seasons, which give the pathogens the best environment to grow and spread.

Table 10 Pathogens found in mixed infections with ETEC in different studies.

| **Pathogens mixed with ETEC** | **Period** | **Country** | **Total cases** | **Cases of mixed infection (%)** | **Reference** |
|---|---|---|---|---|---|
| Rotavirus | 1993-1994 | Bangladesh | 814 | 20% | (Albert et al., 1999) |
| Rotavirus, EPEC | 1993-1994 | Jordan | 265 | 15.5% | (Youssef et al., 2000) |
| Rotavirus | 1996-1998 | Bangladesh | 4662 | 15.6% | (Qadri et al., 2000) |
| Shigella, Vibrio, Campylobacter and Salmonella | 1996-1998 | Kenya, India and Jamaica | Not available | 7% | (Zhi-Dong et al., 2002) |
| *A. lumbricoides* | 2000-2001 | Mozambique | 529 | 10.6% | (Mandomando et al., 2007) |



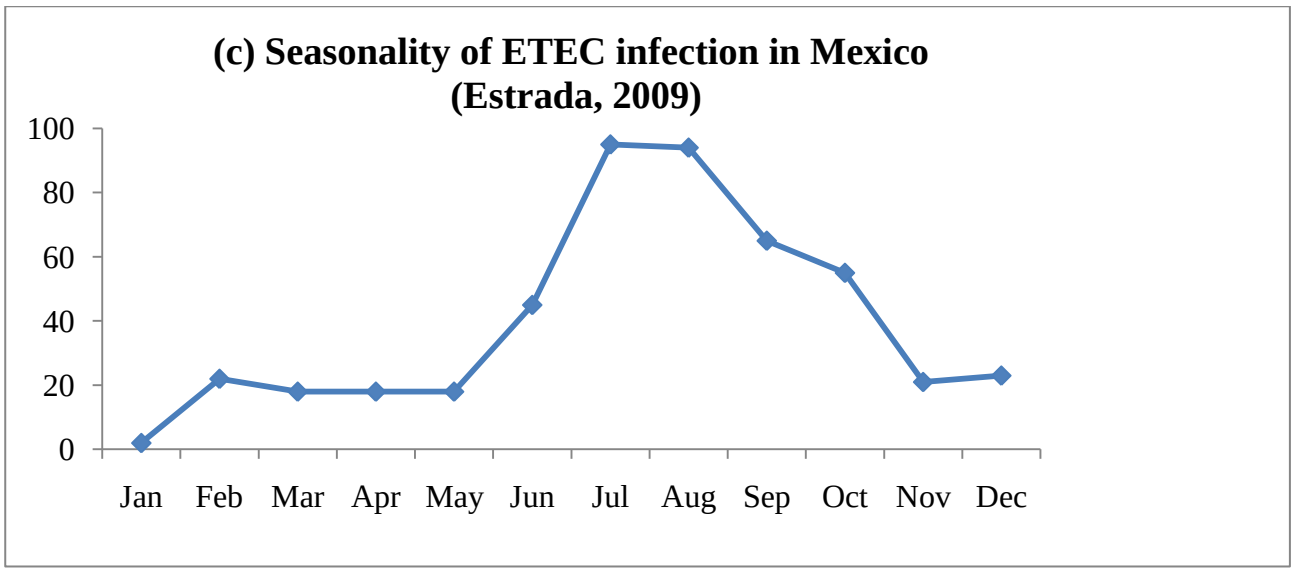




Figure 5 Seasonality of ETEC prevalence in Bangladesh (Asia), Mozambique (Africa) and United States of Mexican (South America). Outbreaks occurred in hot seasons in the mentioned countries.

#### 1.4.2.4 Contaminated Food and Water as Predisposing Factor for ETEC infection

Contaminated food and water harbor ETEC and spread the infection as a result (Begum et al., 2007). In developing countries, where ETEC is endemic, studies showed that surface water contaminated with ETEC is considered a predisposing factor in developing diarrhoea and gastroenteritis (Ohno et al., 1997, Begum et al., 2007).

Transmission of ETEC by processed food products outside the developing world is less commonly seen but well documented. In 1977, Sack *et al.* found that of 240 isolates of *E.coli* from animal food originated in the United States, 8% were found to contain ETEC. The strain produced either LT and ST or both (Sack et al., 1977).

Danielsson *et al.* reported a food born diarrhoea outbreak in Sweden in 1970 (Danielsson, 1979). A study in 2009 evaluated desserts prepared in popular restaurants in the tourist cities of Guadalajara, Mexico, Houston and Texas. The study aimed to determine the importance of coliform and *E.coli* as causes of traveller's diarrhoea (Vigil et al., 2009). Another study found that ETEC was the main cause of diarrhoea in contaminated infant's weaning food (Clemens et al., 1997).

#### 1.4.2.5 Mortality from ETEC Diarrhoea

According to the World Health Organisation, more than 380,000 deaths in children less than five years old are caused by ETEC every year (WHO, 2003). Most cases occur because of a lack of medical support, rehydration therapy and adequate diet management. In contrary, death numbers could have been reduced if patients with ETEC received adequate treatment and health support.

### 1.4.3 Potential treatment of ETEC

Treatment of ETEC infection falls into three main aspects; prevention and treatment of dehydration, the sustainability of patient's diet and antibiotic therapy. Prevention can be achieved by proper cleaning and adapting hygiene measures in food and beverage preparation. Vaccination against ETEC has been a subject of much research. Attempts for vaccine development are presented below (section 1.4.4). The management of dehydration is through using the ORT solution and preventing further fluid loss. Using antibiotics against ETEC infection prevents or reduces the complications, shortens the duration of the illness and reduces mortality. On the other hand, the cost of using

antibiotics, and the possible resistance development are concerns that have been raised and has provided the impetus to develop more effective and inexpensive therapeutic methods (Alam and Ashraf, 2003).

### 1.4.4 Vaccine against ETEC

Developing an effective vaccine against ETEC has been the goal of many studies (Taxt et al., Mietens, 1979, Freedman et al., 1998, Walker et al., 2007). Immunisation against ETEC has been an effective prophylactic strategy. Vaccine design was based on the pathogenesis of ETEC. These studies highlighted the importance of fimbrial antigens CFA, CS1-CS6 and LT/ST in the mechanism of action and disease progress (Byrd et al., 2003, Navaneethan and Giannella, 2008, Fleckenstein et al., 2009).

Different approaches were used to develop an effective vaccine against ETEC; killed oral vaccine, live attenuated oral vaccine and other approaches. Dukoral™ by Sweden BIO (SBL) was the first vaccine licensed and available over the counter. Using recombinant cholera toxin B (rCTB) SBL made the first killed oral vaccine. An attempt by Acambis in the United Kingdom using two non-toxigenic ETEC strains to express CFA/I as live attenuated oral vaccine is in phase II. Other approaches include using transcutanous immunising patches or using edible transgenic plants to express the antigens and develop an effective vaccine. Table 11 summarises different attempts for ETEC vaccine development and the recent status information.

Table 11 Different vaccine approaches including the serotype, the company and the status towards developing a vaccine against ETEC infection

| Vaccine | Serotype | Company | Status |
|---|---|---|---|
| **Killed Oral Vaccine** | | | |
| Dukoral™ | rCTB Cholera | SBL (Sweden) | licensed |
| Killed, oral, whole-cell ETEC and CTB | Whole-cell five strains of ETEC &CTB | University of Göteborg (Sweden) | Phase II |
| Oral killed CF-ETEC vaccine | Engineered *E coli* bacteria K12 | University of Göteborg (Sweden) | Not available |
| **Live attenuated oral vaccines** | | | |
| PTL002 and PTL003 | CFAII | Acambis (UK) | Phase I |

| | | | |
|---|---|---|---|
| Two nontoxinogenic ETEC strains that express CFA/I | CFAI | Acambis (UK) | Phase II |
| Attenuated *Shigella* vectors that express ETEC fimbrial and LT antigens | CFA/1, CS2, CS3 and CS4 | University of Maryland (USA) | Phase I |
| spi-VEC oral live attenuated typhoid vector that deliver ETEC antigens | *E.coli* LT-B subunit | Microscience (UK) | Not available |
| Engineered attenuated *S flexneri* 2a that express the ETEC fimbriae subunit CfaB | CFAI and LT-B | Walter Reed Army Institute of Research | Phase I |
| Peru15pCTB live attenuated oral cholera | Cholera and ETEC | AVANT | Phase I |
| **Other ETEC vaccines approaches** | | | |
| Transcutaneous immunisation patch | A mixture of fimbrial antigens (CS6 and LT) | IOMAI Corp (USA) | Phase II |
| Edible transgenic plants | Express the cholera toxin B subunit (CTB) or the LT B subunit. | University of Delhi South Campus (India) | Not available |

## 1.5 Passive immunisation

B-cells produce five types of immunoglobulin these are, IgA, IgM, IgE, IgG and IgD, also called antibodies. Immunoglobulins (Ig) have been used as a treatment tool against infectious diseases (Hammarstrom et al., 1993, Casadevall et al., 2004). The theory was based on the ability of antibodies to neutralise infectious agents and prevent the progress of a disease. Antibodies could be administered via intravenous injection or oral administration. The fact that antibodies can transfer to a dam's milk and form a very important formula for their offspring especially during the first days of life has paved the way to use these animals as models for different pathogens and to use milk therapy against different diseases.

Polyclonal antibodies are produced from different clones of B-cell. On the other hand, monoclonal antibodies produced from on clone of B-cell. The advantage of polyclonal antibodies is the production of large insoluble immune complexes with polyvalent antigens (Schalkhammer, 2002).

Neutralising antibodies bind to the specific epitope and resulted in shape deformity and inactivation of the antigen. For example, attached antibodies to picornaviruses will result in aggregation, virion stabilisation, inhibition of virus attachment and capsid deformity of the virus. This will prevent the infection and stop the disease process (Burton, 2001).

### 1.5.1 ETEC immunotherapy approaches

Development of ETEC vaccine is based on two strategies: blocking adherence and/or toxin activity. Colonisation factors are necessary for ETEC to adhere to the intestinal mucosal lining. After adherence, LT or ST or both are expressed resulting in watery diarrhoea (Lapa et al., 2008).

### 1.5.2 Using immunotherapy against Gastroenteritis

Immunotherapy defined as a treatment designed to initiate immunity to a disease or enhance the resistance of the immune system to an active disease process (Dictionary, 2010). Using immunotherapy to target infectious agent and microorganisms is not novel. The proposed theory in this study suggested that injecting animals with the inactivated enteropathogens of interest, *E.coli* and rotavirus would enhance the immune

system of the immunised animal. This in turn would result in the production of antibodies. These antibodies would transfer through milk as a passive immunity to their offspring.

This study targeted ETEC and mixed infection of rotavirus and *E.coli* as a major cause of gastroenteritis in developing countries. Both vaccines against ETEC and mixed vaccine of rotavirus and *E.coli* were used and were injected to ewes as an animal model to produce polyclonal antibodies.

Previous studies were carried out to treat infectious diseases in general and gastroenteritis in specific using antibodies produced in animals. Table 12 shows previous studies used antibodies to treat gastroenteritis caused by variety of enteropathogens. The antibodies were derived from bovine's milk after immunisation. except for Sarkar (2009) who used egg yolk to produce IgY and treat rotavirus infection.

Table 12 Previous studies on immunotherapy including the cause of diarrhoea, source of antibodies and their efficacy

| **Cause of diarrhoea** | **Source of antibodies** | **Efficacy** | **Year** | **Reference** |
|---|---|---|---|---|
| *E.coli* | Bovine milk | + | 1979 | (Mietens, 1979) |
| Rotavirus | Bovine milk | Prevent outbreak | 1985 | (Ebina, 1985) |
| Rotavirus | Bovine milk | + | 1987 | (Brussow, 1987) |
| ETEC | Bovine milk | + | 1998 | (Freedman et al., 1998) |
| ETEC | Bovine milk | + Prophylactic / - Treatment | 2000 | (Casswall, 2000) |
| Rotavirus | Bovine milk | + | 2004 | (Kvistgaard, 2004) |
| Rotavirus | Bovine milk | + | 2007 | (Bojsen et al., 2007) |
| Rotavirus | Egg yolk | + | 2007 | (Sarker, 2007) |

Mietens *et al.* (1979) used milk immunoglobulin concentrate (MIC) containing antibodies against enteropathogenic *E. coli* strains. The resulted MIC was given to 60 patients suffering from diarrhoea due to enteropathogenic *E.coli*. Forty eight percent of the patients showed positive results after the treatment (Mietens, 1979).

In 1985, Ebina *et al.* used bovine to produce milk with antibodies against rotavirus. The resulted colostrum (Rota colostrums) was used as a prophylactic treatment against rotavirus infection and during the period of rotavirus outbreak. Six infants were given the milk as a prophylactic treatment. The result showed that the colostrums protected children from rotavirus infection without any side effects. In the control group, six infants were given 20ml of the market milk and develop rotavirus infection after outbreak (Ebina, 1985).

Two years later, Brussow *et al.* did a study that showed milk immunoglobulin concentrate had powerful antiviral activity, even against very high doses of infectious rotaviruses. The study proposed that milk immunoglobulin concentrate was used to induce passive immunity to infantile rotavirus gastroenteritis (Brussow, 1987).

A study held in 1998 by Freedman targeted ETEC and produced a hyperimmune bovine milk antibody product with a specific activity against purified colonisation factor antigens (CFAs). In the study, 25 volunteers were given hyperimmune milk and then challenged with ETEC. The resulted antibodies against CFAs alone were sufficient for protection (Freedman et al., 1998).

The therapeutic efficacy of an oral bovine immunoglobulin milk concentrate (BIC) from cows hyperimmunised with ETEC and EPEC strains were tested by Casswall *et.al*. Results showed a positive prophylactic effect but negative treatment effect (Casswall, 2000).

Kvistgaard *et al*. concluded that bovine macromolecular whey protein fraction had an efficient and versatile inhibitory activity against rotavirus (Kvistgaard, 2004). Bojsen.*et al*. studied the whey product of bovine milk as an alternative to colostral-derived products for rotavirus treatment (Bojsen et al., 2007).

Another interesting study used egg yolk as a source of IgY for the treatment of rotavirus. Results showed that therapy with IgY was dose dependent (Sarker, 2007).

In conclusion, using immunotherapy against gastroenteritis and diarrhoeal diseases is effective as both a prophylactic and therapeutic tool.

### 1.6 Diversification of sheep in developing countries

In this study, sheep was the animal model to produce antibodies against the enteropathogens of interest. Sheep have been domesticated since 9000 B.C. Sheep are multi-purpose animals that produce wool, meat and milk. They are distributed all over the world with the majority in Asia, Africa and Oceania (Zygoyiannis, 2006). Sheep's milk contains higher levels of total solids and major nutrients than goat's and cow's milk (Park et al., 2007).

Using sheep as an animal model for producing antibodies against ETEC and rotavirus has many advantages in treating gastroenteritis. Sheep are easily domesticated and widely available, especially in developing countries. Figure 6 shows the distribution of sheep population in the world. About 65% of sheep are in Asia and Africa, where most developing countries lie.

Sheep milk is a human consumable. Producing milk enriched with antibodies against enteropathogens like rotavirus and *E.coli* gives milk an additional value beside its nutritional value. In the very remote areas of developing countries, it is difficult to maintain the good conditions and achieve the sustainability in providing vaccines. This is due to the lack of transportation and suitable storing conditions to preserve vaccines. Using sheep to produce antibodies enriched milk will provide one solution toward affording good and efficient treatment in these areas.

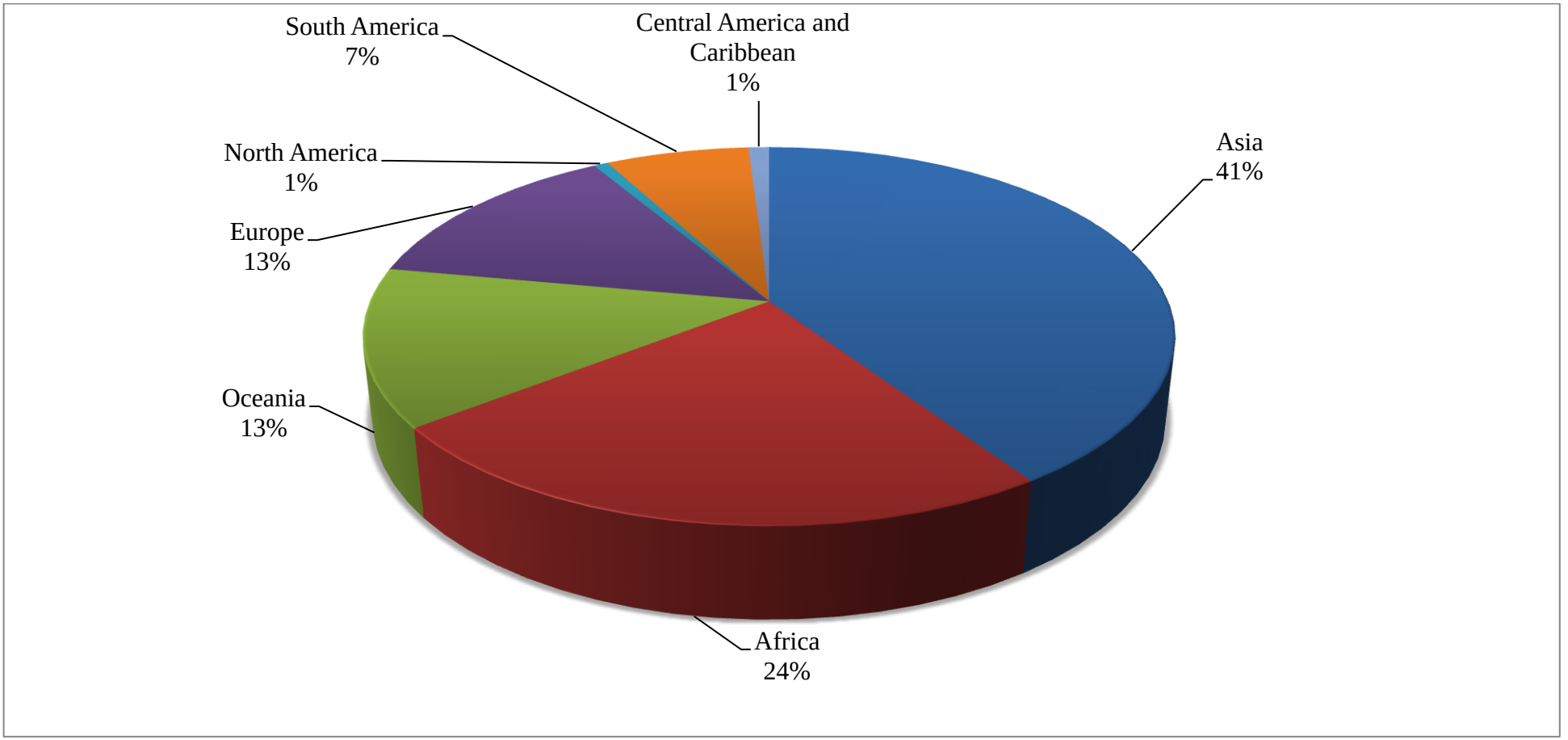



Figure 6 Sheep population distribution all over the world. Where, most of the developing countries lie, 65% of sheep were in Asia and Africa. Number of sheep = $10^6$X percent in each area (Zygoyiannis, 2006).

## 1.7 Antibody Applications in Forensic Science

Forensic science is a branch of science used in the resolution of legal disputes (White, 2004). Antibodies are utilised in forensic investigations through different assays such as, enzyme immunoassay, radioimmunoassay and affinity chromatography. The use of antibodies in forensic investigations is defined as forensic serology.

Antibodies are used to investigate biological samples such as blood, mucous, urine and semen in forensic cases. In general, the antibody-antigen complex is the main indicator for positive and negative samples. In forensic toxicology, antibodies were raised against some types of toxins for immunotherapeutic and diagnostic approaches (Nusair and Tay, 2010). In conclusion using antibodies in forensic science has many applications and is considered a very useful tool in the diagnostic process.

## 1.8 AIMS OF THE STUDY

The aim of this research was to develop polyclonal antibodies against two enteropathogens, rotavirus and *E.coli*. These two enteropathogens are the main cause of gastroenteritis which leads to severe dehydration in susceptible patients particularly children. Diarrhoeal disease in children is the third most common cause of death of children under the age of 5 years in developing countries(WHO, 2009). Effective treatment will result in the possibility of relieving pain and preventing death caused by dehydration occurring because of vomiting, diarrhoea and abdominal pain. In order to achieve this, the following were completed:

1. Polyclonal antibodies were produced in the milk of sheep. Sheep were chosen to be the animal model for immunisation against gastroenteritis; the milk enriched with specific antibodies capable of neutralising the pathogen of interest. These pathogens commonly affect children less than 5 years of age in developing countries and, sheep are the majority of livestock population in these countries.

2. Milk and serum samples were used to measure the specific antibodies using *in vitro* methods such as, enzyme linked immunosorbent assay (ELISA) and electrophoresis.

3. Mice were challenged with the ETEC followed by milk treatment with the specific antibodies enriched milk derived from sheep vaccinated against ETEC and antibodies enriched milk derived from sheep vaccinated with mixed vaccine contains ETEC and rotavirus.

4. The effectiveness of antibodies in the gastrointestinal tract was assessed through measuring the antigen and the antibody levels in the faecal samples of mice during milk treatment and after weaning.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 VACCINE PREPARATION

### 2.1.1 Preparation of the *Escherichia coli* O78:h11 vaccine

*Escherichia coli* O78:h11 used for the vaccine preparation was Heat Stable (ST) and Heat Labile (LT) enterotoxin positive and had Colonisation Factor Antigen/I (CFA/I). The bacterial culture was stored at -80°C at the Forensic Biological Laboratory of Centre for Forensic Science, University of Western Australia prior to use. The method used for this vaccine preparation was modified from procedure developed by Allen *et al.*, (2006). Firstly, the frozen bacterial culture was left in the vial at room temperature for at least half an hour to allow culture to completely thaw. A 1ml aliquot was used to spike 2ml of Luria broth (LB), a medium that contained tryptone pancreatic digest of casein (10g/L) yeast extract (5g/L) and NaCl (10g/L) (Sigma-Aldrich, USA). The culture was incubated at 37°C overnight. After successful growth the bacteria was further purified. The original culture diluted 1:100 in LB and grown at 37$^{o}$C overnight. The turbidity from the resultant growth had an optical density of 0.13 at 600nm as determined using a NanoDrop®ND1000 spectrophotometer (ThermoScientific, USA). Subsequently a 40ml sample of the culture was centrifuged at 4,000rpm for 20 minutes at 4°C using Eppendorf® bench centrifuge (Eppendorf AG, Germany). The resultant pellet was resuspended in 1.6ml of sterile Phosphate-Buffered Saline (PBS) (pH 7.4); yielding a bacterial solution of approximately, 1x10$^{8}$CFU/ml. Serial dilutions were prepared from the previous culture ranging from 1x10$^{-1}$CFU/ml to 1x10$^{-9}$CFU/ml. A 0.1ml aliquot of each dilution was streaked over a MacConkey agar plate (Oxoid, Australia). The ingredients of the agar were peptone (20gm/L), lactose (10gm/L), bile salts (1.5gm/L), NaCl (5.0gm/L), neutral red (0.05gm/L), crystal violet (0.001gm/L) and agar (15.0gm/L). The plates were incubated overnight at 37°C. On the next day, the number of colonies on each plate was counted to determine the actual dose of the vaccine.

The bacteria used in the vaccine was inactivated using formalin treatment (Sigma-Aldrich, USA). Formalin was added to the bacterial solution to form 1M suspension. The suspension was incubated for 2 hours at 37°C with continuous gentle agitation. Subsequently, the suspension was incubated for 3 days at 4°C. After that, suspension

was washed twice with sterile PBS (pH 7.4). A streak from the suspension was plated onto LB agar which composed of the same ingredients as LB broth but with addition of agar (12g/L). The plates were incubated at 37$^{o}$C for two weeks. The plates were checked on a regular basis and lack of the growth on the plate was used as an indicator of complete inactivation of *E.coli*. The suspension was stored in aliquots at 4°C. On the vaccination day, 0.5ml of the suspension was completely emulsified in 0.5ml TiterMax Gold® (Sigma-Aldrich, USA) using one syringe, blunt needle method to form 1ml of the vaccine.

### 2.1.2 Preparation of the mixed vaccine (Rotavirus SA11 and *E.coli* O6:H16)

The vaccine containing a mixture of Rotavirus SA11 and *E.coli* O6:H16 was kindly provided by Immuron Limited (Melbourne, Australia). The mixed vaccine was in an inactivated form of a dose of1.5x10$^{8}$virions/ml rotavirus SA11 mixed with 5x10$^{8}$CFU/ml *E.coli* O6:H16emulsified in the adjuvant Montanide ISA740® (SEPPIC, France).

## 2.2 SHEEP IMMUNISATION PROTOCOL

Two female Merino sheep (designated no.1 and no.2) were separated into individual pens. They were housed alongside their lambs to maintain lactation and for their general welfare. The animals were allowed to adapt to the surroundings of the large animal facilities (LAF) at University of Western Australia, for two weeks. After adaptation, 20ml milk and 5ml blood samples were taken from each ewe as reference controls. Milk was collected by hand. Prior to collection, the lambs were put aside, to allow milk to pool in, the udder Oxytocins was not required to facilitate milk let down. Blood samples were collected using 18-gauge needles from the jugular vein. The site of injection was wool-clipped prior to sample collection and disinfectant was used after. No complications were encountered.

Sheep no.1 was immunised with the prepared *E.coli* O78:h11 vaccine. Sheep no.2 was immunised with the mixed vaccine formula. The vaccines were injected into the muscle in the hind quadriceps on Day 0, 15 and 29. Milk and blood samples were collected from each group on Day 0, 8, 22 and 36. The samples collected at Day 0 before immunisation were used as controls. All samples were centrifuged at 4,000rpm for 20 minutes at 4°C using Eppendorf® bench centrifuge (Eppendorf AG, Germany). The

resultant serum samples and reduced fat milk were labelled and stored at -20°C for further tests. All animal studies were conducted according to protocols approved by Animal Ethics Committee of the University of Western Australia (reference No. RA/3/100/921).

## 2.3 MOUSE CHALLENGE STUDY USING MILK

Fifty 21-day-old female Balb/c mice, Animal Resource Centre (Perth, Australia), were used for mice studies. Upon arrival, the animals were allowed to acclimate to their new environment in the Biomedical Research Facility (BRF) at the University of Western Australia for 1 week prior to any experiment. Mice were housed in autoclaved micro-isolator cages that were lined with autoclaved bedding. The mice were separated into five groups (10 mice per group). Each group was subject to a different treatment regime as outlined below (Table 13), faecal samples were collected from animals and specific indicators were measured. Mice had free access to autoclaved water and food.

Prior to commencement of the experiment, all mice were weighed and faecal samples were collected and pooled from each group. All mice were gavaged orally with 40µl 5% bicarbonate buffer to neutralise their stomach acidity directly before the bacterial inocula. A description of the protocol carried out on each group of animals is summarised in Table 13.

Groups 1, 2 and 3 were challenged with the *E.coli* pathogen. Specifically, 0.2ml of $1x10^7$CFU/ml *E.coli* O78:h11 was introduced into the animals using a 20-gauge gavage needle (Popper and Sons, Inc, USA). The animals in Group 4 and 5 received 0.2ml normal saline instead of bacterial suspension. Faecal samples were pooled and collected every 24 hours from each group. After 24 hours of the inoculation, each mouse in Group 1 was treated with 0.2ml milk enriched with polyclonal antibodies against *E.coli* O78:h11. All mice in Group 2 were treated with 0.2ml milk without antibodies. Group 3 and 5, mice were treated with 0.2ml milk enriched with polyclonal antibodies against Rotavirus SA11 and *E.coli* O6:H16. Finally, Mice in Group 4 were gavaged with 0.2ml milk without antibodies. The indicator of infection to as well as the measure of successful neutralisation was assessed by measuring relevant antibodies and antigen levels in the faeces of the mice (see section 2.4.2). All mice were euthanised at the end of the experiment using a single intra peritoneal injection of 160mg/kg pentobarbitone per mouse.

Table 13 Summary of the mice experiments showing the three test groups challenge with pathogenic *E.coli* O78:h11 (group 1, 2 and 3) and the two control groups (4 and 5), that received saline in place of *E.coli*

| Study Group (*n=10 in each*) | Pre-treatment regimen | Infection protocol at time = 0 hour | Treatment Protocol | Time in hours when faecal samples were collected for testing. Treatment amounts shown in ml of milk administered * | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 24 hours | 48 hours | 72 hours | 96 hours | 120 hours | 144 hours | 162 hours | 180 hours |
| 1 | Collection of faecal samples for testing as pre-treatment control ( at time prior to treatment) | Infection of subjects by gavage with *E.coli* O78:h11 | Milk enriched with specific polyclonal antibodies against *E.coli* O78:h11 | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.0ml | 0.0ml | 0.0ml |
| 2 | | | Treatment with milk with no specific antibodies | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.0ml | 0.0ml | 0.0ml |
| 3 | | | Milk containing anti-Rotavirus SA11 and anti-*E.coli* O6:H16 antibodies | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.0ml | 0.0ml | 0.0ml |
| 4 | | Control animals receiving saline as gavage | Milk with no specific antibodies | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.0ml | 0.0ml | 0.0ml |
| 5 | | | Milk containing anti-Rotavirus SA11 and anti-*E.coli* O6:H16 antibodies | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.2ml | 0.0ml | 0.0ml | 0.0ml |

* 0.2ml of milk used for treatment. 0.0ml = animals were weaned of milk treat regime

## 2.4 ANTIBODY PRODUCTION IN SHEEP

### 2.4.1 Measurements of anti *E.coli* O78:h11 polyclonal antibodies in serum and milk samples from sheep

Polyclonal antibodies against *E.coli* O78:h11 in the serum and milk samples harvested from sheep no.1 were measured using an indirect Enzyme Linked Immunosorbent Assay (ELISA). Firstly, an overnight culture of *E.coli* O78:h11 was prepared on LB agar plates at 37$^{o}$C. The bacteria were collected in a tube to which 50ml 0.1M carbonate/bicarbonate solution (pH 9.6) was added. The bacterial suspension was inactivated by immersing the tube into a hot water bath at 60$^{o}$C for 30 minutes.

The inactivated bacterial suspension was centrifuged at 4,000rpm for 20 minutes at 4$^{o}$C. The resultant pellet was suspended in 1ml carbonate/bicarbonate solution and the turbidity reading of 0.3 at 660nm was measured. Subsequently, 0.1ml of the suspension was added to each well of the 96-well flat-bottom plates (Bio-Rad, USA). The plate was covered with a biofilm and incubated overnight at 4°C. On the next day, the wells were washed with 1M PBS containing 0.05% Tween-20. A blocking buffer was prepared by mixing 2% bovine serum albumin (BSA) in 1M PBS. A 0.1ml aliquot of this blocking buffer was added to each well. The plate was incubated overnight at 4°C. Wells were washed twice using 1M PBS containing 0.05%. Tween-20.

The amount of the antibody in the serum and milk samples were measured at four different dilutions (undiluted, 1/10, 1/100, 1/1000). The samples were processed in duplicates. The plates were kept overnight at 4$^{o}$C. After washing, a 50µl aliquot of the secondary antibody, peroxidase conjugate anti-sheep IgG produced in donkey (Sigma Aldrich, USA) at a dilution of 1/5000 was added to each well. Subsequently, the plates were washed and a 50µl of liquid substrate, 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich, USA) was added to each well. The plates were incubated in the dark for 30 minutes at room temperature. Finally, 50µl of 2M HCl was added to each well to stop the reaction. The plate was read at 450nm using Enzyme Linked Immunosorbent Assay reader (Bio-Rad Laboratories, USA).

### 2.4.2 Measurements of anti rotavirus SA11 and *E.coli* O6:H16 polyclonal antibodies in serum and milk samples from sheep

The method described above (section 2.4.1) was also used to measure the levels of polyclonal antibodies against rotavirus SA11 and *E.coli* O6:H16 produced in sheep no.2. A commercial Rotavirus SA11 antigen (Fitzgerald Industries International, USA) was prepared at a concentration of 0.39mg/ml. The *E.coli* O78:H11 antigens were prepared as described above. The antigens were diluted in the carbonate /bicarbonate buffer to a concentration of 20µg/ml.

## 2.5 DETECTION OF THE ANTIBODIES AND ANTIGENS IN THE MOUSE CHALLENGE STUDIES

Faecal samples were collected from animals in all five groups. The levels of antibodies and enterotoxin antigens in the faeces were quantified. The levels in the five groups were compared to assess the efficacy of antibody therapy. Samples were collected and stored at -80$^{o}$C prior to testing.

### 2.5.1 Measurements of anti *E.coli* O78:h11, anti rotavirus SA11 and *E.coli* O6:H16 polyclonal antibodies in faecal samples from mice

The faecal samples were thawed, weighed and placed in 1ml PBS (pH 7.4). The pooled faecal samples were measured at four different dilutions (undiluted, 1/10, 1/100, 1/1000). The ELISA for quantification of polyclonal antibodies is as summarised above (section 2.4.1).

### 2.5.2 Detection of Enterotoxigenic *E.coli* O78:h11 heat stable ST enterotoxin in the faeces of mice after culturing on MacConkey agar.

Serial dilutions of the faeces were prepared in 1%peptone water (Oxoid Pty Ltd, Australia). A 100µl aliquot of each dilution was streaked over a MacConkey agar plate and incubated overnight at 37$^{o}$C. Five different colonies that morphologically resembled *E.coli* were picked from each plate and confirmed using COLIST EIA (Denka Seiken CO., Ltd, Japan). The monoclonal antibodies used in this kit are capable of detecting both STh (ST produced by *E.coli* of human origin) and STp (ST produced by *E.coli* of animal origin). The 96-wells plate was coated with synthesised enterotoxin and stabiliser. Culture filtrate was added to the wells, followed by anti-ST monoclonal

antibodies conjugated with peroxidase. Bound antigen antibody was detected with the substrate. The reaction was stopped after 15 minutes with the stopping solution (0.75mol/L sulphuric acid). The colour changes were observed visually and quantified using spectrophotometer (Bio-Rad Laboratories, USA) at 450nm.

### 2.5.3 Measurements of *E.coli* O78:h11 heat stable enterotoxin antigen in faecal samples filtrate from mice

Measurements of *E.coli* O78:h11 heat stable enterotoxin (ST) antigen was performed using a commercial kit, COLIST EIA (Denka Seiken CO., Ltd, Japan). The method used was based on the manufacturer's instructions and as described above (see section 2.5.2).

The faecal samples collected from the animals for the enterotoxin measurements were immediately stored at -80$^{o}$C to preserve enterotoxin antigens. Prior to the test, 0.1g of the faecal sample was diluted in 1ml PBS (pH7.4). The pellets were crushed using metal spatula. They were mixed thoroughly on a vortex and let sit on ice for 45 minutes. Subsequently, the suspensions were centrifuged 5 minutes at 4°C.

Heat stable enterotoxin was measured using the faecal filtrate from the previous step. 100µl of the faecal filtrate were added to each microwell of the kit as a sample. After the reaction stopped, the plate was read at 450nm using spectrophotometer (Bio-Rad Laboratories, USA).

# CHAPTER. 3 RESULTS

## 3.1 VACCINE FORMULA

Two vaccine preparations were administered one with a single antigen and second containing antigens against two microorganisms. The *Escherichia coli* O78:h11 vaccine dose used was at a concentration of $1x10^8$CFU/ml. The mixed vaccine dose was $1.5x10^8$virions/ml rotavirus SA11 mixed with $5x10^8$CFU/ml *Escherichia coli* NL053. The mice inoculums dose of bacteria was $1x10^7$CFU/ml *E.coli* O78:h11.

## 3.2 ANTIBODY PRODUCTION IN SHEEP

### 3.2.1 Quantification of polyclonal antibodies against *E.coli* O78:h11 in serum and milk of sheep no1.

Sheep no.1 was immunised with a $1x10^8$CFU/ml dose of *E.coli* O78:h11. Figure 7 shows the antibody levels in serum and milk samples against enterotoxigenic *E.coli* O78:h11. The vaccine was given to sheep no.1 on Day 0, 15 and 29. The blood and milk samples were collected at Day 0, 8, 22 and 36. Serum and milk samples were prepared and antibody levels were assessed by ELISA. Absorbance of the samples was measured at wavelength 450nm and at 1/1000 and 1/100 dilution for serum and milk respectively. Figure 7 shows an increase in the polyclonal antibodies against *E.coli* O78:h11 in serum. The amount of antibodies increased as shown by the increase in absorbance from 0.10 to 0.79. The absorbance decreased slightly after the second injection to 0.73. After the third injection, the absorbance reading of antibodies in serum increased slightly to 0.76.

Milk samples were processed to remove fat and diluted to a 1/100 dilution prior to ELISA. The levels of polyclonal antibodies against *E.coli* O78:h11 show an increase after first injection from 0.06 to 0.60, and then a slightly increase after the third injection to peak at 0.63 as shown in Figure 7.

### 3.2.2 Quantification of polyclonal antibodies against Rotavirus SA11 and *E.coli* O6:H16 in serum and milk of sheep no2.

Mixed vaccine stimulated the immune system of sheep no.2 to produce antibodies against both rotavirus SA11 and *E.coli* O6:H16. The antibody levels were measured and interpreted in Figure 8. At a 1/1000 dilution, antibodies in serum showed an

increase in the levels against rotavirus SA11 (0.05 to 0.7) after the first injection. This increase observed in milk at a 1/100 dilution, indicated by the increase in the level from 0.04 to 0.52. It was shown that the levels of polyclonal antibodies against rotavirus SA11 in serum were higher when compared to the levels found in milk. There was a slight decrease in the absorbance reading after the second injection to 0.68, 0.50 in serum and milk respectively. Subsequently after the third injection, the level of polyclonal antibodies against rotavirus SA11 increased to an absorbance reading of 0.74, followed by increase in the level in milk to 0.59.

In similar comparison, Figure 9 was prepared to show the antibody levels against *E.coli* O6:H16 in serum and milk of sheep no.2. In serum samples at a 1/1000 dilution, polyclonal antibodies against *E.coli* O6:H16 showed an increase in the absorbance reading from 0.04 to 0.58 after first injection. The level showed a slight decrease at day 22, then another increase after the last injection (0.60). Polyclonal antibodies in milk samples showed same trend as serum. The level showed an increase after first injection (0.40) and another increase after the last injection (0.42) at day 36.

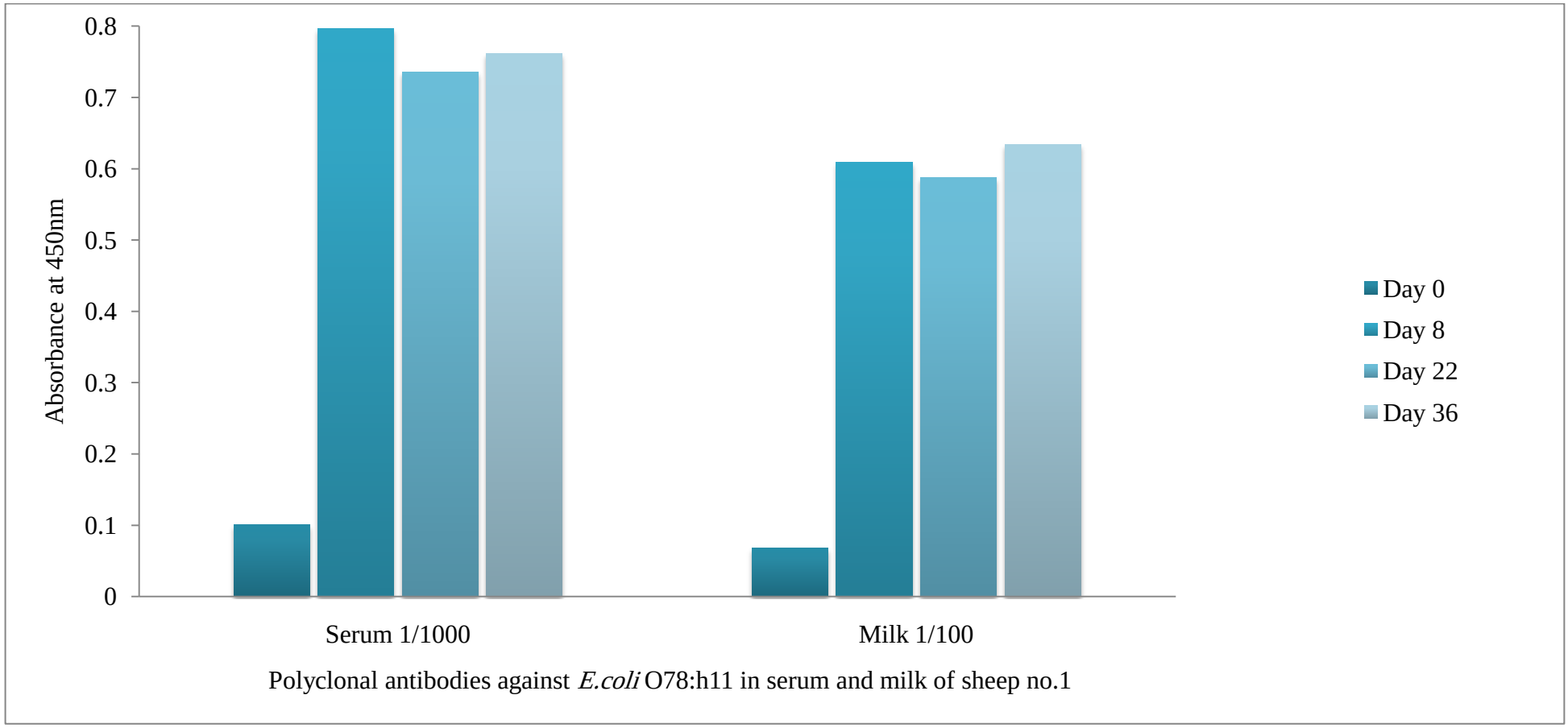



**Figure 7** The absorbance of polyclonal antibodies against *E.coli* O78:h11 in serum samples at a 1/1000 dilution and milk samples at a 1/100 dilution from sheep no.1 using ELISA. The sheep was immunised at Day 0, 15 and 29. Sample collection days were 0, 8, 22 and 36.

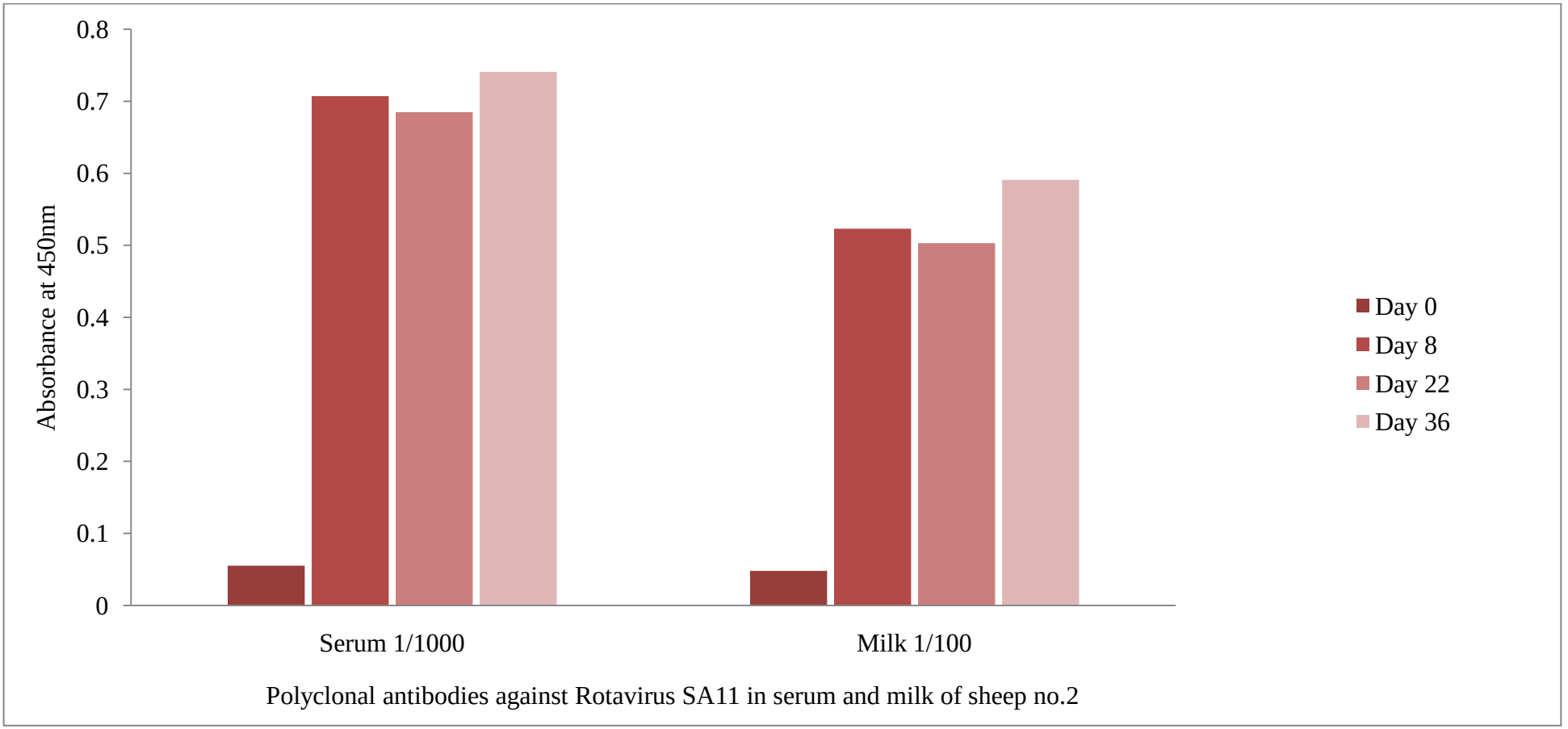



**Figure 8** The absorbance of polyclonal antibodies against rotavirus SA11 in serum samples at a 1/1000 dilution and milk samples at a 1/100 dilution from sheep no 2 using ELISA. The sheep was immunised at day 0, 15 and 29. The sample were collected at day, 0, 8, 22 and 36.

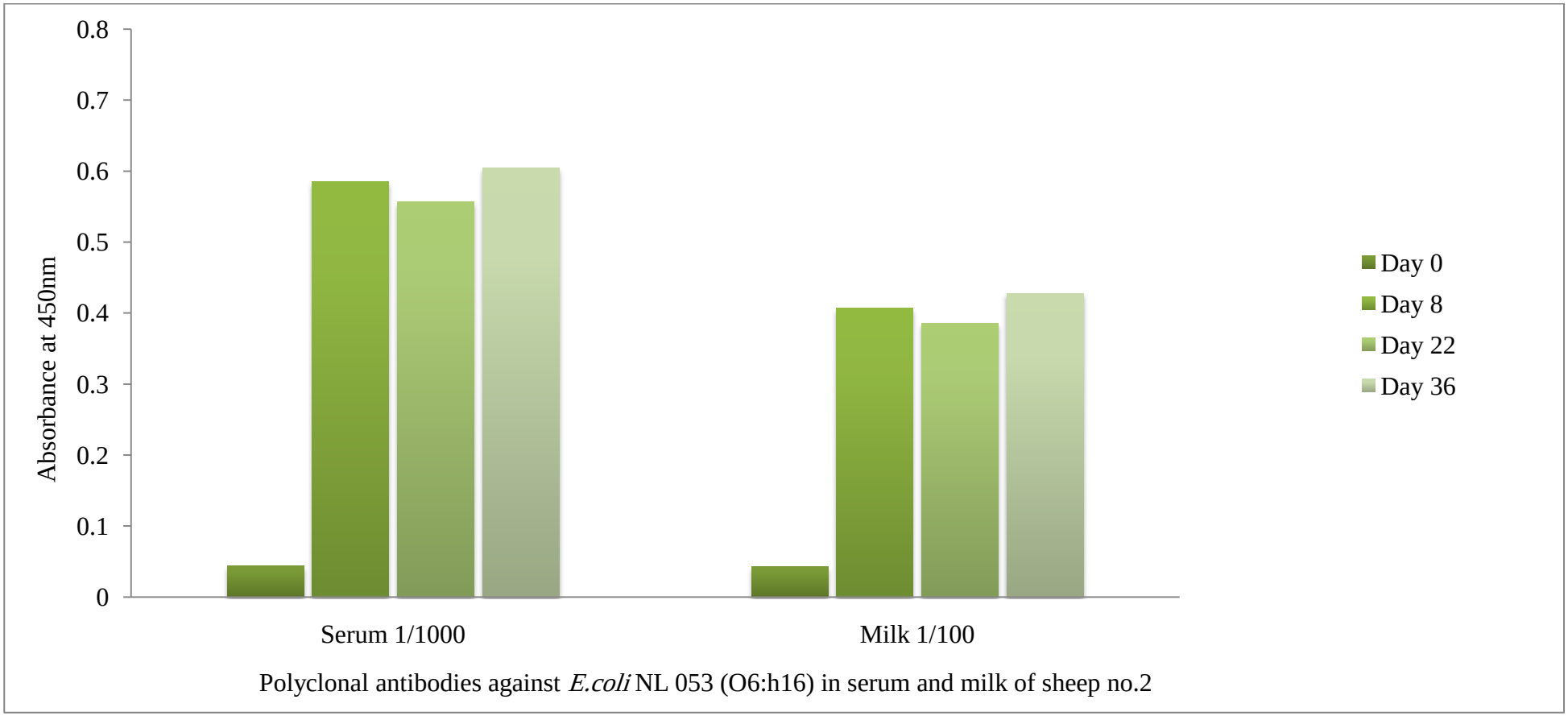



**Figure 9** The absorbance of polyclonal antibodies against *E.coli* NL 053 (O6:H16) in serum samples at a 1/1000 dilution and milk samples at a 1/100 dilution from sheep no.2 using ELISA. The sheep was immunised at day 0, 15 and 29. The samples were collected at day 0, 8, 22 and 36.

## 3.3 ANTIBODIES AND ANTIGENS IN THE MOUSE CHALLENGE STUDIES.

### 3.3.1 Quantification of polyclonal antibodies against *E.coli* O78:h11, Rotavirus SA11 and *E.coli* O6:H16 in the mice faecal samples

Survival of polyclonal antibodies against *E.coli* O78:h11, Rotavirus SA11 and *E.coli* O6:H16 in gastrointestinal tract of mice was studied during and after the milk treatment period. Two groups 1 and 5 were studied to quantify polyclonal antibodies against *E.coli* O78:h11, rotavirus SA11 and *E.coli* O6:H16 in undiluted faecal samples. Milk was administered at Hours 24, 48, 72, 96 and 120. Faecal samples were collected every 24 hours during milk treatment and every 18 hours after milk treatment of mice study. ELISA was used to quantify the polyclonal antibodies in undiluted faecal samples.

Antibodies survival in the gastrointestinal tract during milk treatment and after weaning were measured for group 1 mice. Figure 10 shows the levels of polyclonal antibodies against *E.coli* O78:h11 in faecal samples of mice in group 1. During time 0 to 24 hours, the level of polyclonal antibodies was almost zero because no milk was administered during this period. First milk administration was at Hour 24. The level increased at Hour 48 (0.39), and then the level showed a slight decrease during the period from Hour 72 (0.38) to Hour 144 (0.32). Sharp decrease observed at Hour 162 (0.122) and at Hour 180 (0.02).

Figure 11 shows the levels of polyclonal antibodies against rotavirus SA11 and *E.coli* O6:H16 in faecal samples of mice in group 5. As per group 1, no increase was observed during the time 0 to 24 hours. The level of Polyclonal antibodies against rotavirus and *E.coli* O6:H16 increased at Hour 48 to peak 0.31 for rotavirus and 0.18 for *E.coli* O6:H16. The levels for both rotavirus SA11 and *E.coli* O6:H16 showed a slight decrease during the time 72 to 144. During the last 36 hours, the level of antibodies dropped to the zero level.

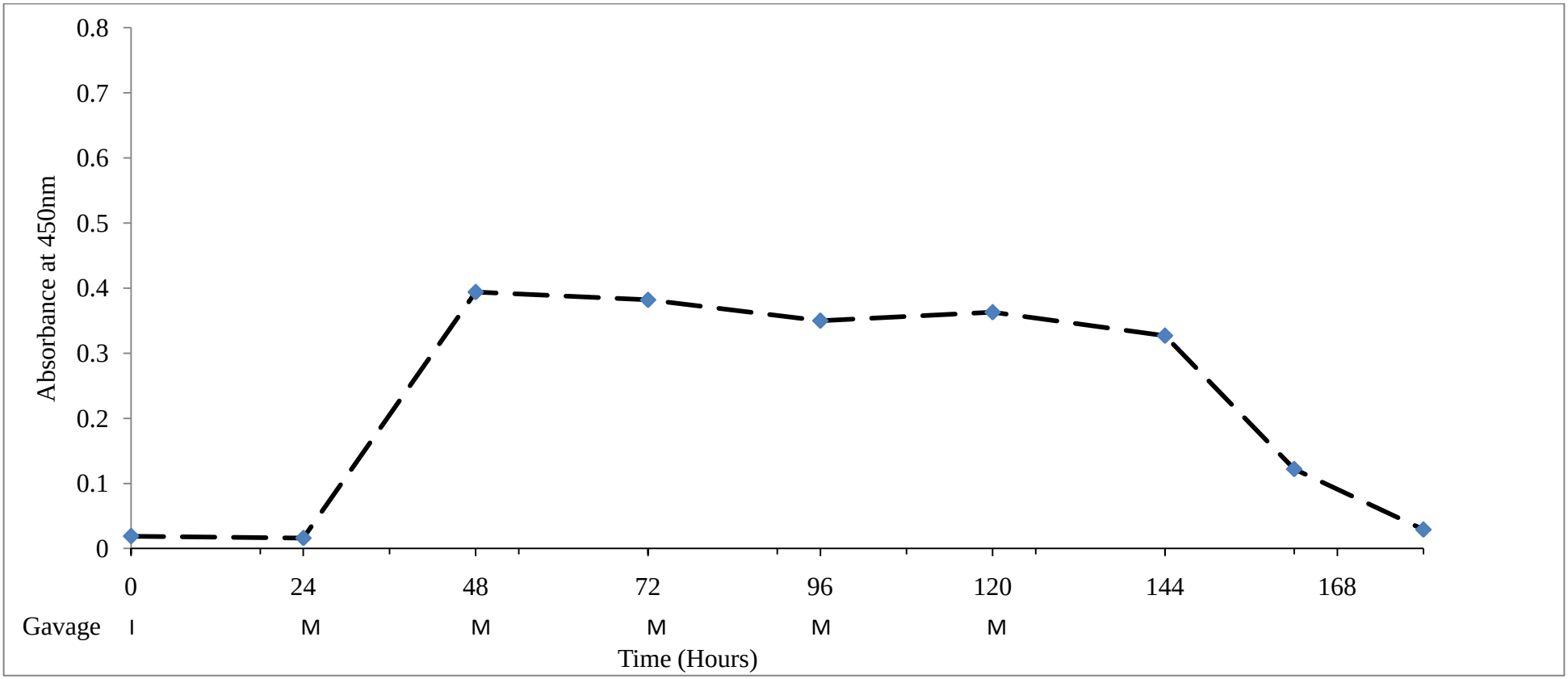



**Figure 10** The absorbance of polyclonal antibodies against *E.coli* O78:h11 in undiluted pooled faecal samples using ELISA. The samples were collected from Balb/c mice challenged with *E.coli* O78:h11. Subsequently the *E.coli* infection was treated with milk containing polyclonal antibodies raised specifically against *E.coli* O78:h11 in sheep milk. "I" denotes the time that the Infection was established and "M" denotes when milk samples were administered.

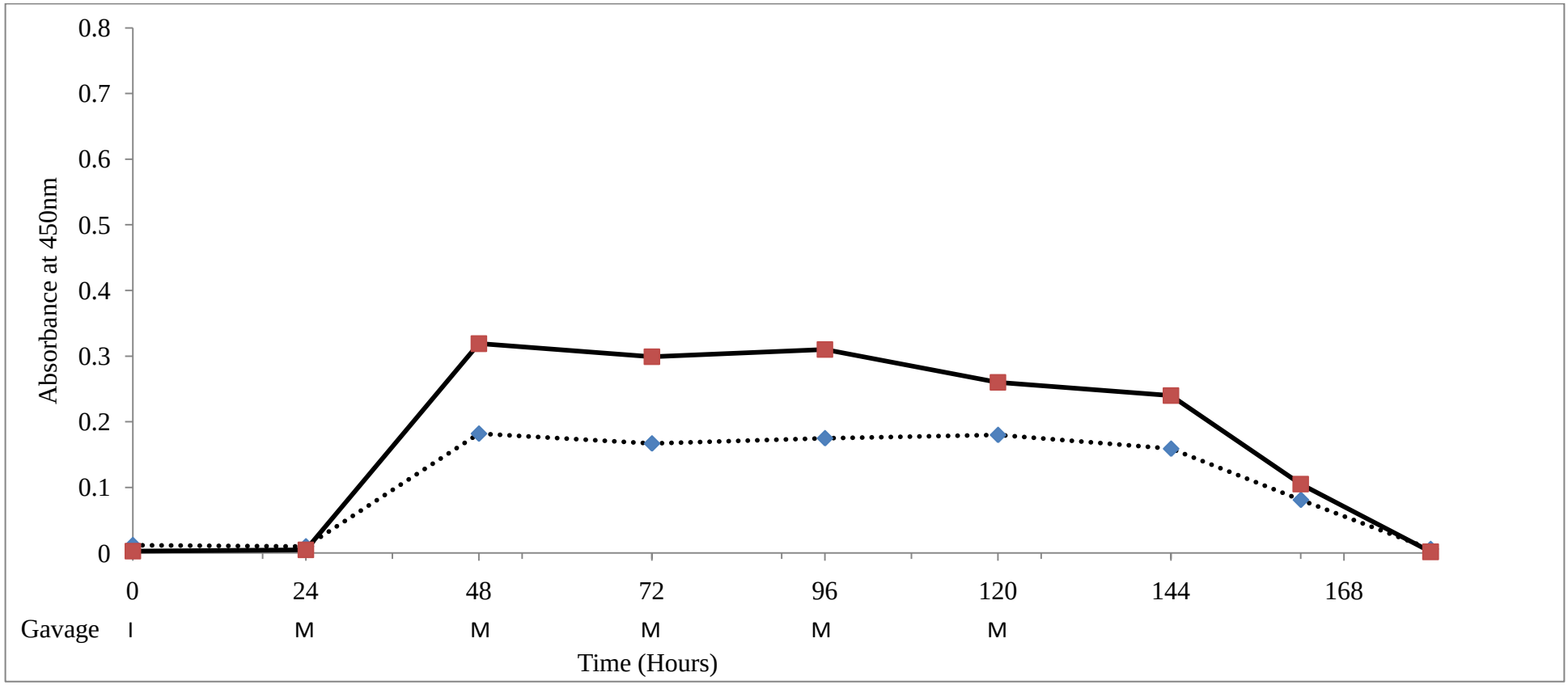



**Figure 11** The absorbance of polyclonal antibodies against rotavirus SA11 (—) and *E.coli* O6:H16 (····) in undiluted pooled faecal samples using ELISA. The samples were from Balb/c mice treated with milk containing polyclonal antibodies raised specifically against rotavirus SA11 and *E.coli* O6:H16 as a mixed vaccine in sheep milk. "I" denotes the time that the Infection was established and "M" denotes when milk samples were administered.

### 3.3.2 *E.coli* O78:h11 heat stable enterotoxin (ST) antigen in faecal samples from mice

Faecal samples were collected from each group of mice. They were pooled and stored at -80$^{o}$C prior to analysis. The samples from each group were mixed thoroughly and a 0.1g subsample processed as a representation of the infection status of each group. Table 14 shows the response of Group 1, 2, 3, 4 and 5 to protection with milk. The response was assessed based on the quantification of *E.coli* ST measured by COLIST. Briefly, three test groups, namely 1, 2 and 3 were challenged with *E.coli* O78:h11. In the animals of all three groups milk enriched with specific antibodies (group 1and 3) and a placebo (group 2) were introduced at time 24, 48, 72, 96 and 120. The animals in groups 1, 2 and 3 were *E.coli* ST positive 24 hours after challenge. Moreover, they remained positive at 48, 72 hours. After 96 hours, the COLIST results from Group 1 were negative, whilst the COLIST results from Group 2 and 3 remained positive. After 120 hours, only the COLIST results from Group 2 were positive and all groups showed negative results at 144 hours.

Figure 12 shows *E.coli* O78:h11 enterotoxin quantification for group 1, 2 and 3. According to the manufacturer's procedure, the kit reading less than or equal to 0.5 were considered *E.coli* ST positive and reading more than 0.5 were considered *E.coli* ST negative. The figure shows negative result for ST at time 0. At time, 24, 48 and 72 results were positive for all three groups. After 96 hours, results for group 1 were negative and remained positive for Group 2 and 3. After 120 hours, results were positive just for Group 2 and become negative after 144 hours for all groups.

Group 4 and 5 were *E.coli* or infection negative controls. Instead of receiving a gavage with inoculums of the pathogen, animals were infected with saline. In group 4, no side effects were observed because of milk administration on mice. Both Group 4 and 5 showed negative results for *E.coli* ST.

Table 14 Five groups of mice were infected with *E.coli* (1, 2 and 3) or received a saline placebo (4 and 5). Each group received the treatment regimen shown. The COLIST Kit used to detect the presence of *E.coli* O78:h11 ST at time 24, 48, 72, 96, 120 and 144 hours.

| Study Group (*n=10 in each*) | Pre-treatment regimen | Infection protocol at time = 0 hour | Treatment Protocol administered at 24, 48, 72, 96 and 120 hours | Result for heat stable ST detection on each day of mice experiment | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 24 hours | 48 hours | 72 hours | 96 hours | 120 hours | 144 hours |
| 1 | Collection of faecal samples for testing as pre-treatment controls | Infection of subjects by gavage with *E.coli* O78;h11 | Milk enriched with specific polyclonal antibodies against *E.coli* O78:h11 | + | + | + | - | - | - |
| 2 | | | Treatment with milk with no specific antibodies | + | + | + | + | + | - |
| 3 | | | Milk containing anti-Rotavirus SA11 and anti-*E.coli* O6:H16 antibodies | + | + | + | + | - | - |
| 4 | | Control animals receiving saline as gavage | Milk with no specific antibodies | - | - | - | - | - | - |
| 5 | | | Milk containing anti-Rotavirus SA11 and anti *E.coli* O6:H16 antibodies | - | - | - | - | - | - |

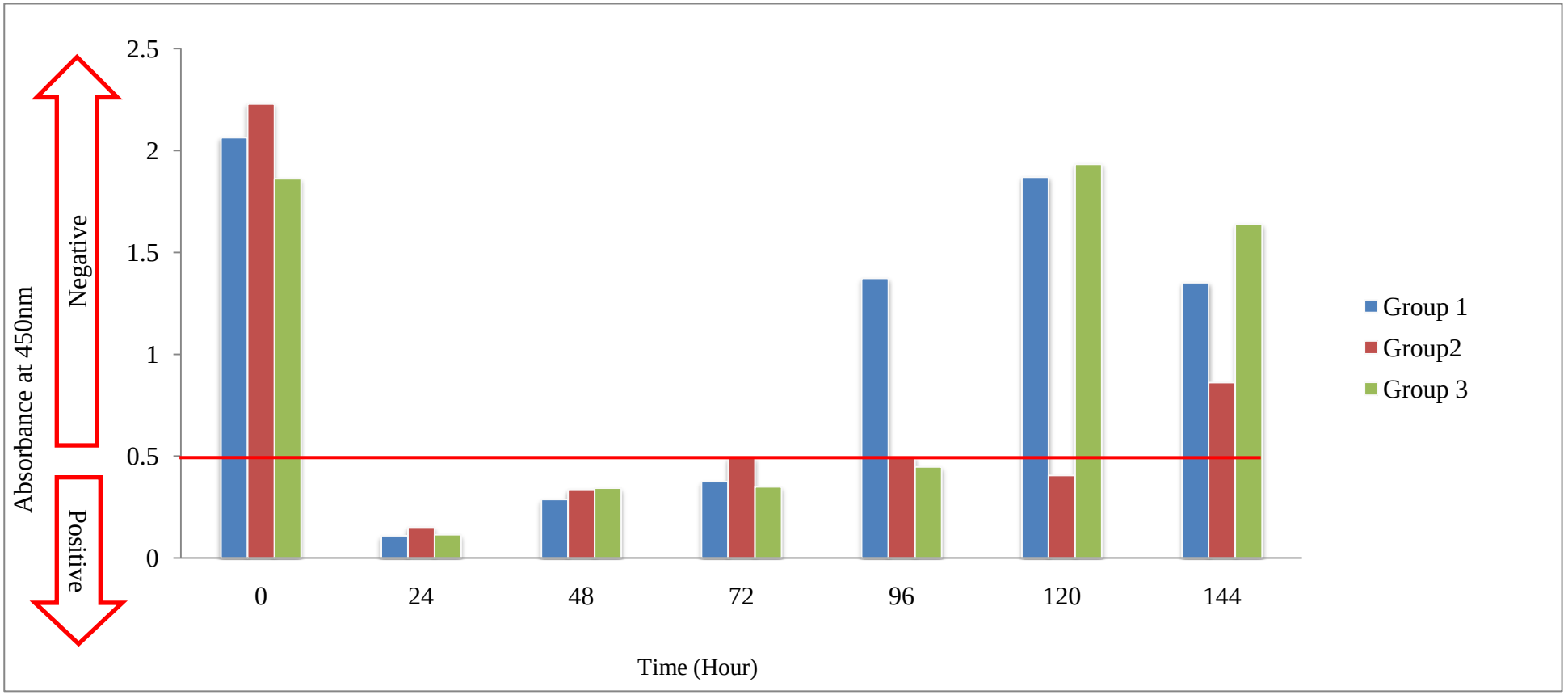



**Figure 12** The absorbance values used as the measure of the presence of *E.coli* ST in faecal samples from animals in groups 1, 2 and 3.According to the guideline from the manufacturer of the kit readings less than or equal to 0.5 were considered *E.coli* ST positive and readings more than 0.5 were considered *E.coli* ST negative.

# CHAPTER 4: DISCUSSION AND CONCLUSION

## 4.1 DISCUSSION

Gastroenteritis caused by rotavirus and enterotoxigenic *E.coli* (ETEC) causes more than 1.7 million deaths every year. Most of deaths occurred in children less than five years of age in developing countries. The availability and sustainability of treatment for gastroenteritis will help to decrease number of death cases of the affected populations. Treatment regimens should be designed to ensure maximum possible compliance by the parents of affected children.

Drug delivery to human in many developing countries is problematic. This is due to lack of basic infrastructure in these areas including electricity, roads and transportation which will affect the shelf life of the drugs or vaccines that required special storing conditions, usually at $4^{o}$C. For example, outreach territories in Pohnpei Island of Micronesia posed logistical problem for the delivery of cholera vaccine due to the remoteness of the communities (Calain et al., 2004). Hence, this work was conceived to access the once popular antibody based therapy (Alexander, 1943).

Treatment of gastroenteritis caused by rotavirus and ETEC using polyclonal antibodies in the milk of ruminants offers a pragmatic solution in developing countries. Access to milk is linked to the availability of high numbers of ruminants in these countries. More than 65% of the global sheep population is distributed over Asia and Africa where most of developing countries lie (see section 1.6). Most dairy herds raised in countries where incidence of diarrheal diseases is high. According to Food and Agriculture Organisation (FAO), the number of dairy animals is the largest in developing countries. For example, in India the number of dairy animals is about 100,000,000. In India, the number of deaths resulting from diarrheal diseases is very high (see Table 15).

In this study, sheep were used to produce antibodies in milk against three different strains of enteropathogens that cause gastroenteritis. These enteropathogens are ETEC strains O78:h11, O6:H16 and rotavirus strain SA11. The resulted antibodies in milk were measured using ELISA.

Table 15 Regions with significant deaths arising from Diarrheal Disease (DD) are located in countries with the world's largest dairy herds where there is easy access to milk

| Rank | Countries and numbers of deaths | | Dairy Animals | |
|---|---|---|---|---|
| | Top 20 DD | Under 5 Death | Number | Rank |
| 1 | India | 549,663 | 99,459,000 | 1 |
| 17 | Sudan | 15,893 | 67,095,000 | 2 |
| 2 | China | 206,016 | 58,342,186 | 3 |
| 8 | Brazil | 44,376 | 25,000,000 | 5 |
| 6 | Pakistan | 63,210 | 24,487,000 | 6 |
| 5 | Bangladesh | 79,440 | 21,985,000 | 7 |
| 16 | Turkey | 16,934 | 16,629,386 | 9 |
| 7 | Ethiopia | 55,741 | 10,386,000 | 13 |
| 4 | Indonesia | 79,788 | 9,068,970 | 16 |
| 12 | Kenya | 24,684 | 9,042,000 | 17 |
| 10 | Afghanistan | 25,269 | 8,468,000 | 20 |
| 13 | Egypt | 23,616 | 6,400,000 | 24 |
| 20 | Iraq | 12,289 | 4,550,150 | 29 |
| 19 | Yemen | 14,039 | 3,165,000 | 38 |
| 14 | Myanmar | 18,821 | 2,290,002 | 44 |
| 11 | Uganda | 25,232 | 2,100,000 | 47 |
| 3 | Nigeria | 93,808 | 18,257,000 | 54 |
| 18 | Thailand | 15,098 | 300,000 | 115 |
| 15 | Viet Nam | 17,160 | 135,120 | 131 |
| 9 | Philippines | 27,504 | 5,700 | 165 |

Source: FAOSTAT (2006), WHO (2000), UN (2005)

Balb/C mice used to study the efficacy of antibodies in milk against the ETEC induced infection in mice groups. Mice were challenged with the pathogen and then gavaged with antibodies enriched milk to measure the effectiveness of the resulted milk.

Survival of polyclonal antibodies in the gastrointestinal tract of mice was studied during the milk treatment period and after weaning. Faecal samples were collected and antibody levels were measured using ELISA.

The first sheep (no.1) immunised against ETEC showed a high level of antibodies in serum and milk after the first immunisation against *E.coli* O78:h11. This increase was an indication of the stimulation of immune response against the antigens in the vaccine. The data showed an increase in the antibody levels in serum higher than those in milk. This is expected as the first encounter of the antigens would be in the blood then the antibodies would leak into the milk. This forms the passive immunity passed from mother to offspring. Colostrum is the first milk formed in the mammary gland of dams. Colostrum protect offspring of mammalian species through the high level of immunoglobulin that concentrated in milk during late pregnancy and first few days after parturition (Bitzan et al., 1998). Newborn animals that do not receive the "first milk" or colostrums during first 24 hours of birth will be susceptible to pathogens and diseases that might resulted in death (Uruakpa et al., 2002)

The slight increase in the antibody level after the second and the last immunisation was due to production of large numbers of B-cells. The B-cells were bearing antigen-specific cell-surface antibodies (Stanley A. Plotkin et al., 2008).

In serum and milk, there were certain levels of antibodies at Day 0 (absorbance reading is 0.101). This was due to possible previous exposure of the sheep to the same antigens. In general, *E.coli* is a very common pathogen. This proposed a high possibility of previous exposure.

Sheep no.2 responded to the mixed vaccine and the levels of antibodies increased after first injection. In general, the response of sheep no.2 to the mixed vaccine was lower than that of sheep no.1 to *E.coli* O87:h11 prepared vaccine. This can be explained by the difference between the nature of the involved antigens, the individual variability between the sheep and due to the difference of the adjuvant. The adjuvant of the mixed vaccine was Mountanide®, while that for the *E.coli* O87:h11 was TiterMax®. In

addition, the *E.coli* O78:h11 vaccine prepared in-house was composed of whole-cell antigens. The larger the antigen's size the higher the immune response. Adjuvants have been used for sixty years to increases the response of immune system to the vaccinated antigen (Gupta et al., 1993). Different types of adjuvant stimulate the immune system to different levels of response (Geerligs et al., 1989). For example, different types of adjuvant have been used in developing a vaccine to *leishmania*. These adjuvants show variability in response and side effect (Mutiso et al., 2010).

Vaccine could be manufactured as a single antigen or as multiple antigens. Using a single antigen vaccine will elicit a higher immune response and produce more antibodies (Roy et al., 1990).

Figure 9 shows the same trend of antibody increase in serum and milk against *E.coli* O6:H16. The dilution was different though. It was 1/1000 for serum samples compared to 1/100 for milk samples. This indicated the lower concentration of antibody levels in milk compared to levels in serum of both sheep against both vaccines. The explanation of this might be due to the fact, that the antigens were injected. Thus, the immune response would have started at blood then antibodies excreted into milk.

Survival of antibodies against *E.coli* O78:h11 in the gastrointestinal tract of mice measured in undiluted pooled faecal samples is shown in Figure 10. The level studied indicated the passage of the milk enriched with antibodies in the gastrointestinal system of mice. The resulting levels showed absence of the antibodies at hour 0 and then a sudden increase after 24 hours following the milk antibody administration. The levels then showed a slight decrease during the treatment hours up to 144 hours. After 162 hours, the level dropped sharply. A second reduction was observed after 180 hours. In general, the antibody levels were lower in undiluted pooled faecal samples than the levels in milk. This was due to loss of antibodies in the digestion and absorption process of the mice's gastrointestinal system.

Figure 11 shows the same trend of antibodies against rotavirus SA11 and *E.coli* O6:H16 that increased after 24 hours of the milk challenge and then decreased towards very low level after 162 and 180 hours of the same challenge. In general, the levels of faecal antibody against *E.coli* O87:h11 was higher than those against the mixed vaccine. This was due to higher antibody levels against *E.coli* O87:h11 in milk compared to those from sheep no.2 immunised against the mixed vaccine.

Figure 12 and Table 13 show Group 1, 2 and 3 mice response to milk challenge. Group1, 2 and 3 were challenged with *E.coli* O78:h11. Group 1 was gavaged with milk enriched with polyclonal antibodies raised specifically against *E.coli* O78:h11. Group 2 was gavaged with milk without antibodies and group 3 was gavaged with milk enriched with polyclonal antibodies raised specifically against *E.coli* O6:H16 and rotavirus SA11. The three groups were negative to ST at time 0 before milk administration. This was for the control samples. After 72 hours, they were ST positive due to the course of the infection. The infection usually takes 1 to 2 days for incubation and 3 to 4 days for recovery in case of diarrhea the course last 3 to 4 days and is self limited (Qadri et al., 2005). The first three groups of mice were ST positive at 72 hours (3 days) then Group 1 and 2 were negative afterwards. This recovery indicated that the challenged milk enriched with polyclonal antibodies was effective in enhancing the recovery process. In another word, the polyclonal antibodies neutralised the inoculated antigens in mice.

On the other hand, Group 2 mice showed positive results even after the milk challenge. This was due to using regular milk (milk without the specific polyclonal antibodies) to treat the group. Group 3 showed positive result for 96 hours then showed negative results at Hour 120 and 144. This means that polyclonal antibodies against *E.coli* O6:H16 (CFAII, CS1, CS3, ST+, LT+) were effective against *E.coli* O78:h11 (CFAI, ST+, LT+) and succeed to neutralise the antigens. ETEC O6:h16 Group 2 showed negative result at last day after 144 hours, which could be due to self-recovery because of the low dose used for inoculation.

The overlap in between the high incidence of diarrhoeal diseases countries and the large number of dairy herds in these countries serves the idea of using these dairy herds as rescuer. This could be achieved through the production of polyclonal antibodies in the ruminant's milk against diarrhoea causing enteropathogens and using this milk as a treatment tool in addition to the nutritional value of the milk (see Table 16).

At present the World Health organisation and the United Nations Children's Fund (UNICEF), recommend and prescribe Oral Rehydration Solution (ORS) for dehydration treatment. ORS is delivered as a sachet containing about 40g sugar, 3.5g salt. Parents should dissolve this sachet in one litre of clean water to be ready to address diarrheal disease. Comparing ORS to a one litre of milk, which composed of 880g of water, 46g

of sugars and 1.6g of salts, shows the extra ingredients in milk. The electrolyte properties of milk are similar to the oral rehydration solution (ORS).

The water in the milk has been filtered through the mammary glands where no pathogens can pass (see Table 16). Moreover, using ORS will help to reduce the symptoms of dehydration, but it did not eliminate the causative agents. On the other hand, using milk enriched with antibodies against enteropathogens will serve as a rehydration solution and as a treatment tool.

Table 16 Milk contains electrolytes, but has the added benefit of nutrition and germ-free water

| Components | ORS | Milk | |
|---|---|---|---|
| Sugar (g) | 40.0 | 46.0 | Electrolyte |
| Salt (g) | 3.5 | 1.6 | |
| Protein (g) | 0.0 | 39.0 | Nutrition |
| Fat (g) | 0.0 | 33.0 | |
| Vitamins (g) | 0.0 | 0.4 | |
| Volume (ml) | 1,000 | 1,000 | Water |

## 4.2 CONCLUSION AND RECOMMENDATIONS

In conclusion, developing polyclonal antibodies in the milk of ruminants is an effective way; at least to combat the variety of infectious diseases. This has been supported by previous works and studies (see Table 12). For example, Mietens,(1979) succeeded in treating 84% of patients infected with *E.coli*.using milk immunoglobulin concentrate (Mietens, 1979). In another study, milk immunoglobulin with a specific activity against colonisation factor protected 14 volunteer out of 15 form *E.coli* infection (Freedman et al., 1998). In addition, the flexibility of milk manufacturing into powder or pellets gives the suggested method of producing polyclonal antibodies enriched milk competitive characteristics over using other vaccines. There are processes to dry milk at relatively low temperatures (i.e. $70^{o}$C). Water is removed from milk using a process called spray drying which used hot air to dry milk. Another way is to use freeze-drying method, which is convenient for preserving the nutrients of milk. This will minimise protein denaturation and as a result maintain the antibody therapeutic activity in milk (Singh and Creamer, 1991). Processed milk will facilitate the transportation and storing to implement at developing countries.

Moreover, using mixed vaccine of rotavirus and enterotoxigenic *E.coli* to produce milk enriched with polyclonal antibodies was effective against ETEC infection. This could be a good indicator of the possibility that the produced milk against both would be effective also against rotavirus. Yet, this possibility has not been tested in this research. Further investigation is needed. If the outcome was promising then producing such milk would be more valuable as mixed infection is very common in developing countries. Immunoglobulins in ruminants' milk provide antimicrobial protection against the specific vaccinated pathogen (Korhonen, 2000).

Further research is recommended in the scope of increasing the efficacy of the vaccine, antibodies purification and characterisation. This might help in harvesting more antibodies. In addition, exploring the possibility of using other animals to produce the polyclonal antibodies enriched milk such as camels. Camel has a unique type of antibodies that lack the light chain and made it the smallest antigen binding fragment (Muyldermans, 2001). It is important to investigate the best species to produce the milk as their response might be higher to the vaccines and their antibodies might be more efficient in neutralising the pathogens of interest. The production animals should be

considered after assessment of nature of agriculture specific to the region. In addition, the selection of the most antigenic prevalent vaccine will result in maximum protection. For example, human-bovine rotavirus reassortant vaccine contains wide common variety of antigens. Using such, vaccine might end up in developing universal therapeutic milk that would help treating a wide range of gastroenteritis. This in turn, would save many children all around the world. Developing such therapy would add nutritional value to the treatment characteristics; this value essentially needed in developing countries.

# CHAPTER 5: REFERENCES


AL-GALLAS, N., BAHRI, O., BOURATBEEN, A., BEN HAASEN, A. & BEN AISSA, R. 2007. Etiology of Acute Diarrhea in Children and Adults in Tunis, Tunisia, with Emphasis on Diarrheagenic Escherichia coli: Prevalence, Phenotyping, and Molecular Epidemiology. *Am J Trop Med Hyg,* 77**,** 571-582.

ALAM, N. H. & ASHRAF, H. 2003. Treatment of Infectious Diarrhea in Children. *Pediatric Drugs,* 5**,** 151-165.

ALBERT, M. J., FARUQUE, A. S. G., FARUQUE, S. M., SACK, R. B. & MAHALANABIS, D. 1999. Case-Control Study of Enteropathogens Associated with Childhood Diarrhea in Dhaka, Bangladesh. *J. Clin. Microbiol.,* 37**,** 3458-3464.

ALBERT, M. J., FARUQUE, S. M., FARUQUE, A. S., NEOGI, P. K., ANSARUZZAMAN, M., BHUIYAN, N. A., ALAM, K. & AKBAR, M. S. 1995. Controlled study of Escherichia coli diarrheal infections in Bangladeshi children. *J. Clin. Microbiol.,* 33**,** 973-977.

ALEXANDER, H. E. 1943. Experimental Basis for Treatment of Haemphilus Influenzae Infections. *Am J Dis Child,* 66**,** 160-171.

ARAUJO, I. T., FERREIRA, M. S. R., FIALHO, A. M., ASSIS, R. M., CRUZ, C. M., ROCHA, M. & LEITE, J. P. G. 2001. Rotavirus Genotypes P[4]G9, P[6]G9, and P[8]G9 in Hospitalized Children with Acute Gastroenteritis in Rio de Janeiro, Brazil. *J. Clin. Microbiol.,* 39**,** 1999-2001.

BABIUK, L. A. 1977. Rotavirus isolation and cultivation in the presence of trypsin. *Journal of clinical microbiology,* 6**,** 610.

BALL, J. M., MITCHELL, D. M., GIBBONS, T. F. & PARR, R. D. 2005. Rotavirus NSP4: A Multifunctional Viral Enterotoxin. *Viral Immunology,* 18**,** 27-40.

BASU, N., ARSHAD, N. & VISWESWARIAH, S. 2010. Receptor guanylyl cyclase C (GC-C): regulation and signal transduction. *Molecular and Cellular Biochemistry,* 334**,** 67-80.

BEGUM, Y. A., TALUKDER, K. A., NAIR, G. B., KHAN, S. I., SVENNERHOLM, A. M., SACK, R. B. & QADRI, F. 2007. Comparison of enterotoxigenic Escherichia coli isolated from surface water and diarrhoeal stool samples in Bangladesh. *Canadian Journal of Microbiology,* 53**,** 19-26.

BENHAFID, M., YOUBI, M., KLENA, JOHN D., GENTSCH, JON R., TELEB, N., WIDDOWSON, M. A. & ELAOUAD, R. 2009. Epidemiology of Rotavirus Gastroenteritis among Children <5 Years of Age in Morocco during 1 Year of Sentinel Hospital Surveillance, June 2006–May 2007. *The journal of infectious diseases,* 200**,** S70-S75.

BISHOP, R., DAVIDSON, G. P., HOLMES, I. H. & RUCK, B. J. 1973. Virus Particles in Epithelial Cells of Doudenal Mucosa from Children with Acute Non-Bacterial Gastroenteritis. *The Lancet,* 302**,** 1281-1283.

BITZAN, M. M., GOLD, B. D., PHILPOTT, D. J., HUESCA, M., SHERMAN, P. M., KARCH, H., LISSNER, R., LINGWOOD, C. A. & KARMALI, M. A. 1998. Inhibition of Helicobacter pylori and Helicobacter mustelae Binding to Lipid Receptors by Bovine Colostrum. *The journal of infectious diseases,* 177**,** 955-961.

BLACK, R. E. 1993. Epidemiology of diarrhoeal disease: implications for control by vaccines. *Vaccine,* 11**,** 100-106.

BLACK, R. E., MERSON, M. H., RAHMAN, A. S. M. M., YUNUS, M., ALIM, A. R. M. A., HUQ, I., YOLKEN, R. H. & CURLIN, G. T. 1980. A Two-Year Study of Bacterial, Viral, and Parasitic Agents Associated with Diarrhea in Rural Bangladesh. *The journal of infectious diseases,* 142**,** 660-664.

BLACKBURN, D., HUSBAND, A., SALDANA, Z., NADA, R. A., KLENA, J., QADRI, F. & GIRON, J. A. 2009. Distribution of the Escherichia coli Common Pilus among Diverse Strains of Human Enterotoxigenic E. coli. *J. Clin. Microbiol.,* 47**,** 1781-1784.

BOJSEN, A., BUESA, J., MONTAVA, R., KVISTGAARD, A. S., KONGSBAK, M. B., PETERSEN, T. E., HEEGAARD, C. W. & RASMUSSEN, J. T. 2007. Inhibitory Activities of Bovine Macromolecular Whey Proteins on Rotavirus Infections In Vitro and In Vivo. *J. Dairy Sci.,* 90**,** 66-74.

BOTH, G. W., LOCKETT, L. J., JANARDHANA, V., EDWARDS, S. J., BELLAMY, A. R., GRAHAM, F. L., PREVEC, L. & ANDREW, M. E. 1993. Protective Immunity to Rotavirus-Induced Diarrhoea Is Passively Transferred to Newborn Mice from Naive Dams Vaccinated with a Single Dose of a Recombinant Adenovirus Expressing Rotavirus VP7sc. *Virology,* 193**,** 940-950.

BOYLE, J. F. & HOLMES, K. V. 1986. RNA-binding proteins of bovine rotavirus. *J. Virol.,* 58**,** 561-568.

BRANDAL, L. T., LINDSTEDT, B. R.-A., AAS, L., STAVNES, T.-L., LASSEN, J. R. & KAPPERUD, G. 2007. Octaplex PCR and fluorescence-based capillary electrophoresis for identification of human diarrheagenic Escherichia coli and Shigella spp. *Journal of Microbiological Methods,* 68**,** 331-341.

BRUSSOW, H. 1987. Bovine milk immunoglobulins for passive immunity to infantile rotavirus gastroenteritis. *Journal of clinical microbiology,* 25**,** 982.

BURTON, D. R. 2001. *Antibodies in viral infection* Springer.

BYRD, W., MOG, S. R. & CASSELS, F. J. 2003. Pathogenicity and Immune Response Measured in Mice following Intranasal Challenge with Enterotoxigenic Escherichia coli Strains H10407 and B7A. *Infect. Immun.,* 71**,** 13-21.

CALAIN, P., CHAINE, J.-P., JOHNSON, E., HAWLEY, M.-L., O'LEARY, M. J., OSHITANI, H. & CHAIGNAT, C.-L. 2004. Can oral cholera vaccination play a role in controlling a cholera outbreak? *Vaccine,* 22**,** 2444-2451.

CAMPAGNA, M., EICHWALD, C., VASCOTTO, F. & BURRONE, O. R. 2005. RNA interference of rotavirus segment 11 mRNA reveals the essential role of NSP5 in the virus replicative cycle. *J Gen Virol,* 86**,** 1481-1487.

CARPIO M., B. J., BABIUK L. 1981. Comparative virulence of different bovine rotavirus isolates. *Can. J. Comp. Med,* 45**,** 38-42.

CASADEVALL, A., DADACHOVA, E. & PIROFSKI, L.-A. 2004. Passive antibody therapy for infectious diseases. *Nature Reviews Microbiology,* 2**,** 695-703.

CASSELS, F. J. & WOLF, M. K. 1995. Colonization factors of diarrheagenicE. coli and their intestinal receptors. *Journal of Industrial Microbiology and Biotechnology,* 15**,** 214-226.

CASSWALL, T. H. 2000. Treatment of enterotoxigenic and enteropathogenic Escherichia coli-induced diarrhoea in children with bovine immunoglobulin milk concentrate from hyperimmunized cows: a double-blind, placebo-controlled, clinical trial. *Scandinavian journal of gastroenterology,* 35**,** 711.

CHOI, A. H. C., KNOWLTON, D. R., MCNEAL, M. M. & WARD, R. L. 1997. Particle Bombardment-Mediated DNA Vaccination with Rotavirus VP6 Induces High Levels of Serum Rotavirus IgG but Fails to Protect Mice against Challenge. *Virology,* 232**,** 129-138.

CIARLET, M. & SCHODEL, F. 2009. Development of a rotavirus vaccine: Clinical safety, immunogenicity, and efficacy of the pentavalent rotavirus vaccine, RotaTeq®. *Vaccine,* 27**,** G72-G81.

CLEMENS, J., SAVARINO, S., ABU-ELYAZEED, R., SAFWAT, M., RAO, M., WIERZBA, T., SVENNERHOLM, A.-M., HOLMGREN, J., FRENCK, R., PARK, E. & NAFICY, A. 2004. Development of Pathogenicity-Driven Definitions of Outcomes for a Field Trial of a Killed Oral Vaccine against Enterotoxigenic Escherichia coli in Egypt: Application of an Evidence-Based Method. *Journal of Infectious Diseases,* 189**,** 2299-2307.

CLEMENS, J. D., RAO, M. R., CHAKRABORTY, J., YUNUS, M., ALI, M., KAY, B., VAN LOON, F. P. L., NAFICY, A. & SACK, D. A. 1997. Breastfeeding and the Risk of Life-threatening Enterotoxigenic Escherichia coli Diarrhea in Bangladeshi Infants and Children. *Pediatrics,* 100**,** e2-.

CROXEN, M. A. & FINLAY, B. B. 2009. Molecular mechanisms of Escherichia coli pathogenicity. *Nat Rev Micro,* 8**,** 26-38.

CUKOR, G. & BLACKLOW, N. R. 1984. Human viral gastroenteritis. *Microbiol. Mol. Biol. Rev.,* 48**,** 157-179.

DANIEL DUBREUIL, J. 2010. STb and AIDA-I: The missing link? *Critical Reviews in Microbiology,* 0**,** 1-9.

DANIELSSON, M.-L. 1979. Enterotoxigenic enteric bacteria in foods and outbreaks of food-borne diseases in Sweden. *J. Hyg., Camb.,* 83.

DE LAS, C., ADACHI & DUPONT 1999. Review article: travellers' diarrhoea. *Alimentary Pharmacology & Therapeutics,* 13**,** 1373-1378.

DE, S. N., K. BHATTACHARYA, AND J. K. SARKAR 1956. A study of the pathogenicity of strains of Bacterium coli from acute and chronic enteritis *J. Pathol. Bacteriol.*, 71**,** 201-209.

DENNEHY, P. H. 2008. Rotavirus vaccines: an overview. *Clinical microbiology reviews,* 21**,** 198.

DESSELBERGER, U., MANKTELOW, E., LI, W., CHEUNG, W., ITURRIZA-GOMARA, M. & GRAY, J. 2009. Rotaviruses and rotavirus vaccines. *Br Med Bull,* 90**,** 37-51.

DICTIONARY 2010. Immunotherapy definition.

DONG, Y., ZENG, C. Q.-Y., BALL, J. M., ESTES, M. K. & MORRIS, A. P. 1997. The rotavirus enterotoxin NSP4 mobilizes intracellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1,4,5-trisphosphate production. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 3960-3965.

DONNENBERG, M. S. & KAPER, J. B. 1992. Enteropathogenic Escherichia coli. *Infect. Immun.,* 60**,** 3953-3961.

DRASAR, B. S. & HILL, M. J. 1974. *Human intestinal flora,* London, United Kingdom., Academic Press.

DUBREUIL, J. D. 2008. Escherichia coli STb toxin and colibacillosis: knowing is half the battle. *FEMS Microbiology Letters,* 278**,** 137-145.

EBINA, T. 1985. Prevention of rotavirus infection by oral administration of cow colostrum containing antihumanrotavirus antibody. *Medical Microbiology and Immunology,* 174**,** 177.

ELSINGHORST, E. A. & KOPECKO, D. J. 1992. Molecular cloning of epithelial cell invasion determinants from enterotoxigenic Escherichia coli. *Infect. Immun.,* 60**,** 2409-2417.

ESTES, M. K. 1989. Rotavirus gene structure and function. *Microbiology and molecular biology reviews,* 53**,** 410.

ESTRADA-GARCIA, T., LOPEZ-SAUCEDO, C., THOMPSON-BONILLA, R., ABONCE, M., LOPEZ-HERNANDEZ, D., SANTOS, J. I., ROSADO, J. L., DUPONT, H. L. & LONG, K. Z. 2009. Association of Diarrheagenic

Escherichia coli Pathotypes with Infection and Diarrhea among Mexican Children and Association of Atypical Enteropathogenic E. coli with Acute Diarrhea. *J. Clin. Microbiol.,* 47**,** 93-98.

FABBRETTI, E., AFRIKANOVA, I., VASCOTTO, F. & BURRONE, O. R. 1999. Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm- like structures in vivo. *J Gen Virol,* 80**,** 333-339.

FAN, E., O'NEAL, C. J., MITCHELL, D. D., ROBIEN, M. A., ZHANG, Z., PICKENS, J. C., TAN, X.-J., KOROTKOV, K., ROACH, C., KRUMM, B., VERLINDE, C. L. M. J., MERRITT, E. A. & HOL, W. G. J. 2004. Structural biology and structure-based inhibitor design of cholera toxin and heat-labile enterotoxin. *International Journal of Medical Microbiology,* 294**,** 217-223.

FLECKENSTEIN, J. M., HARDWIDGE, P. R., MUNSON, G. P., RASKO, D. A., SOMMERFELT, H. & STEINSLAND, H. 2009. Molecular mechanisms of enterotoxigenic Escherichia coli infection. *Microbes and Infection,* 12**,** 89-98.

FOSTER, D. M. & GEOF, W. S. 2009. Pathophysiology of Diarrhea in Calves. *The Veterinary clinics of North America. Food animal practice,* 25**,** 13-36.

FREEDMAN, DANIELÂ J., TACKET, CAROLÂ O., DELEHANTY, A., MANEVAL, DAVIDÂ R., NATARO, J. & CRABB, JOSEPHÂ H. 1998. Milk Immunoglobulin with Specific Activity against Purified Colonization Factor Antigens Can Protect against Oral Challenge with Enterotoxigenic Escherichia coli. *The journal of infectious diseases,* 177**,** 662-667.

GEERLIGS, H. J., WEIJER, W. J., WELLING, G. W. & WELLING-WESTER, S. 1989. The influence of different adjuvants on the immune response to a synthetic peptide comprising amino acid residues 9-21 of herpes simplex virus type 1 glycoprotein D. *Journal of Immunological Methods,* 124**,** 95-102.

GRIMPREL, E. M. D., RODRIGO, C. M. D. P. & DESSELBERGER, U. M. D. 2008. Rotavirus Disease: Impact of Coinfections. *Pediatric Infectious Disease Journal Rotavirus: Evidence for Vaccination,* 27**,** S3-S10.

GUERRERO, C. A., SANTANA, A. Y. & ACOSTA, O. 2010. Mouse intestinal villi as a model system for studies of rotavirus infection. *Journal of virological methods,* In Press, Corrected Proof.

GUO, D., TAN, Y.-C., WANG, D., MADHUSOODANAN, K. S., ZHENG, Y., MAACK, T., ZHANG, J. J. & HUANG, X.-Y. 2007. A Rac-cGMP Signaling Pathway. *Cell,* 128**,** 341-355.

GUPTA, R. K., RELYVELD, E. H., LINDBLAD, E. B., BIZZINI, B., BEN-EFRAIM, S. & GUPTA, C. K. 1993. Adjuvants -- a balance between toxicity and adjuvanticity. *Vaccine,* 11**,** 293-306.

HALAIHEL, N., LIEVIN, V., ALVARADO, F. & VASSEUR, M. 2000a. Rotavirus infection impairs intestinal brush-border membrane Na+-solute cotransport activities in young rabbits. *Am J Physiol Gastrointest Liver Physiol,* 279**,** G587-596.

HALAIHEL, N., LIEVIN, V., BALL, J. M., ESTES, M. K., ALVARADO, F. & VASSEUR, M. 2000b. Direct Inhibitory Effect of Rotavirus NSP4(114-135) Peptide on the Na+-D-Glucose Symporter of Rabbit Intestinal Brush Border Membrane. *J. Virol.,* 74**,** 9464-9470.

HAMMARSTROM, V., C. I. EDVARD SMITH & HAMMARSTROM, L. 1993. Oral immunoglobulin treatment in Campylobacter jejuni enteri.

HASEGAWA, M. & SHIMONISHI, Y. 2005. Recognition and signal transduction mechanism of Escherichia coli heat-stable enterotoxin and its receptor, guanylate cyclase C. *The Journal of Peptide Research,* 65**,** 261-271.

HOLTZ, C. 2007. *Global health care: issues and policies,* Sudbury, Jones and Bartletr.

HUA, J., CHEN, X. & PATTON, J. T. 1994. Deletion mapping of the rotavirus metalloprotein NS53 (NSP1): the conserved cysteine-rich region is essential for virus-specific RNA binding. *J. Virol.,* 68**,** 3990-4000.

HUANG, F., ROCK, J. R., HARFE, B. D., CHENG, T., HUANG, X., JAN, Y. N. & JAN, L. Y. 2009. Studies on expression and function of the TMEM16A calcium-activated chloride channel. *Proceedings of the National Academy of Sciences,* 106**,** 21413-21418.

IJAZ, M. K., ALKARMI, T. O., EL-MEKKI, A. W., GALADARI, S. H. I., DAR, F. K. & BABIUK, L. A. 1995. Priming and induction of anti-rotavirus antibody response by synthetic peptides derived from VP7 and VP4. *Vaccine,* 13**,** 331-338.

JOURDAN, N., MAURICE, M., DELAUTIER, D., QUERO, A. M., SERVIN, A. L. & TRUGNAN, G. 1997. Rotavirus is released from the apical surface of cultured human intestinal cells through nonconventional vesicular transport that bypasses the Golgi apparatus. *J. Virol.,* 71**,** 8268-8278.

KAPIKIAN, ALBERT Z., SIMONSEN, L., VESIKARI, T., HOSHINO, Y., MORENS, DAVID M., CHANOCK, ROBERT M., LA MONTAGNE, JOHN R. & MURPHY, BRIAN R. 2005. A Hexavalent Human Rotavirus–Bovine Rotavirus (UK) Reassortant Vaccine Designed for Use in Developing Countries and Delivered in a Schedule with the Potential to Eliminate the Risk of Intussusception. *The journal of infectious diseases,* 192**,** S22-S29.

KENNY, B., ABE, A., STEIN, M. & FINLAY, B. B. 1997. Enteropathogenic Escherichia coli protein secretion is induced in response to conditions similar to those in the gastrointestinal tract. *Infect. Immun.,* 65**,** 2606-2612.

KORHONEN, H. 2000. Milk immunoglobulins and complement factors. *The British Journal of Nutrition,* 84**,** 75.

KVISTGAARD, A. S. 2004. Inhibitory effects of human and bovine milk constituents on rotavirus infections. *Journal of Dairy Science,* 87**,** 4088.

KWON, D., KIM, O. & CHAE, C. 1999. Prevalence of genotypes for fimbriae and enterotoxins and of O serogroups in Escherichia coli isolated from diarrheic piglets in Korea. *J Vet Diagn Invest,* 11**,** 146-151.

LAPA, J. A., SINCOCK, S. A., ANANTHAKRISHNAN, M., PORTER, C. K., CASSELS, F. J., BRINKLEY, C., HALL, E. R., VAN HAMONT, J., GRAMLING, J. D., CARPENTER, C. M., BAQAR, S. & TRIBBLE, D. R. 2008. Randomized Clinical Trial Assessing the Safety and Immunogenicity of Oral Microencapsulated Enterotoxigenic Escherichia coli Surface Antigen 6 with or without Heat-Labile Enterotoxin with Mutation R192G. *Clin. Vaccine Immunol.,* 15**,** 1222-1228.

LASARO, M. A., RODRIGUES, J. F., MATHIAS-SANTOS, C., GUTH, B. E. C., BALAN, A., SBROGIO-ALMEIDA, M. E. & FERREIRA, L. C. S. 2008. Genetic Diversity of Heat-Labile Toxin Expressed by Enterotoxigenic Escherichia coli Strains Isolated from Humans. *J. Bacteriol.,* 190**,** 2400-2410.

LIOR, H. 1996. *Classification of Escherichia coli,* Wallingford, United Kingdom, CAB International.

LITTLE, L. M. & SHADDUCK, J. A. 1982. Pathogenesis of rotavirus infection in mice. *Infect. Immun.,* 38**,** 755-763.

LIU, J.-K., HOU, X.-L., WEI, C.-H., YU, L.-Y., HE, X.-J., WANG, G.-H., LEE, J.-S. & KIM, C.-J. 2009. Induction of Immune Responses in Mice after Oral Immunization with Recombinant Lactobacillus casei Strains Expressing Enterotoxigenic Escherichia coli F41 Fimbrial Protein. *Appl. Environ. Microbiol.,* 75**,** 4491-4497.

LIU, M., MATTION, N. M. & ESTES, M. K. 1992. Rotavirus VP3 expressed in insect cells possesses guanylyltransferase activity. *Virology,* 188**,** 77-84.

LOPES, L. M., FABBRICOTTI, S. H., FERREIRA, A. J. P., KATO, M. A. M. F., MICHALSKI, J. & SCALETSKY, I. C. A. 2005. Heterogeneity among Strains of Diffusely Adherent Escherichia coli Isolated in Brazil. *J. Clin. Microbiol.,* 43**,** 1968-1972.

LUNDGREN, O., AND L. SVENSSON (ed.) 2003. *The enteric nervous system and infectious diarrhea,* Amsterdam, The Netherlands.: Elsevier Science BV.

MAMMARAPPALLIL, J. G. & ELSINGHORST, E. A. 2000. Epithelial Cell Adherence Mediated by the Enterotoxigenic Escherichia coli Tia Protein. *Infect. Immun.,* 68**,** 6595-6601.

MANDOMANDO, I. M., MACETE, E. V., RUIZ, J., SANZ, S., ABACASSAMO, F., VALLES, X., SACARLAL, J., NAVIA, M. M., VILA, J., ALONSO, P. L. & GASCON, J. 2007. ETIOLOGY OF DIARRHEA IN CHILDREN YOUNGER THAN 5 YEARS OF AGE ADMITTED IN A RURAL HOSPITAL OF SOUTHERN MOZAMBIQUE. *Am J Trop Med Hyg,* 76**,** 522-527.

MAST, T. C., CHEN, P.-Y., LU, K.-C., HSU, C.-M., LIN, H. C., LIAO, W.-C., LIN, D.-P., CHEN, H.-C. & LAC, C. 2010. Epidemiology and economic burden of rotavirus gastroenteritis in hospitals and paediatric clinics in Taiwan, 2005-2006. *Vaccine,* 28**,** 3008-3013.

MCCONNELL, M. M., HIBBERD, M. L., PENNY, M. E., SCOTLAND, S. M., CHEASTY, T. & ROWE, B. 1991. Surveys of Human Enterotoxigenic

Escherichia coli from Three Different Geographical Areas for Possible Colonization Factors. *Epidemiology and Infection,* 106**,** 477-484.

MEBUS, C. A., N. R. UNDERDAHL, M. B. RHODES, A. & TWIEHAUS, M. J. 1969. Calf diarrhea (Scours): reproduced with a virus from a field outbreak. Nebraska Res Bull.

MERSON, M. H., SACK, R. B., ISLAM, S., SAKLAYEN, G., HUDA, N., HUQ, I., ZULICH, A. W., YOLKEN, R. H. & KAPIKIAN, A. Z. 1980. Disease Due to Enterotoxigenic Escherichia coli in Bangladeshi Adults: Clinical Aspects and a Controlled Trial of Tetracycline. *The journal of infectious diseases,* 141**,** 702-711.

MICHAEL FIELD, DAVID FROMM, QAIS AL-AWQATI & WILLIAM B. GREENOUGH, I. 1972. Effect of Cholera Enterotoxin on Ion Transport across Isolated Ileal Mucosa. *J Clin Invest,* 4**,** 796-804.

MICHELANGELI, F., AND M. C. RUIZ (ed.) 2003. *Physiology and pathophysiology of the gut in relation to viral diarrhea,* Amsterdam,: Elsevier Science BV.

MIETENS, C. 1979. Treatment of infantile E. coli gastroenteritis with specific bovine anti-E. coli milk immunoglobulins. *European Journal of Pediatrics,* 132**,** 239.

MORI, Y., SUGIYAMA, M., TAKAYAMA, M., ATOJI, Y., MASEGI, T. & MINAMOTO, N. 2001. Avian-to-Mammal Transmission of an Avian Rotavirus: Analysis of Its Pathogenicity in a Heterologous Mouse Model. *Virology,* 288**,** 63-70.

MORRIS, A. P. & ESTES, M. K. 2001. Microbes and Microbial Toxins: Paradigms for Microbial-Mucosal Interactions: VIII. Pathological consequences of rotavirus infection and its enterotoxin. *Am J Physiol Gastrointest Liver Physiol,* 281**,** G303-310.

MU, X.-Q., SAVARINO, S. J. & BULLITT, E. 2008. The Three-Dimensional Structure of CFA/I Adhesion Pili: Traveler's Diarrhea Bacteria Hang on by a Spring. *Journal of Molecular Biology,* 376**,** 614-620.

MUTISO, J. M., MACHARIA, J. C. & GICHERU, M. M. 2010. A review of adjuvants for Leishmania vaccine candidates. *Journal of Biomedical Research,* 24**,** 16-25.

MUYLDERMANS, S. 2001. Single domain camel antibodies: current status. *Reviews in Molecular Biotechnology*, 74**,** 277-302.

MYRON, M. L. 1987. Escherichia coli That Cause Diarrhea: Enterotoxigenic, Enteropathogenic, Enteroinvasive, Enterohemorrhagic, and Enteroadherent. *The journal of infectious diseases,* 155**,** 377-389.

NATARO, J. P. & KAPER, J. B. 1998. Diarrheagenic Escherichia coli. *Clin. Microbiol. Rev.,* 11**,** 142-201.

NAVANEETHAN, U. & GIANNELLA, R. A. 2008. Mechanisms of infectious diarrhea. *Nat Clin Pract Gastroenterol Hepatol,* 5**,** 637-647.

NGUYEN, T. V., LE VAN, P., LE HUY, C., GIA, K. N. & WEINTRAUB, A. 2005. Detection and Characterization of Diarrheagenic Escherichia coli from Young Children in Hanoi, Vietnam. *J. Clin. Microbiol.,* 43**,** 755-760.

NUSAIR, S. & TAY, G. 2010. Milk Could Save You By Neutralizing Poison. *The Internet Journal of Laboratory Medicine,* 4.

O'RYAN, M. 2009. The Ever-Changing Landscape of Rotavirus Serotypes. *The Pediatric Infectious Disease Journal,* 28**,** S60-S62 10.1097/INF.0b013e3181967c29.

OFFIT, P. A., CLARK, H. F., BLAVAT, G. & GREENBERG, H. B. 1986. Reassortant rotaviruses containing structural proteins vp3 and vp7 from different parents induce antibodies protective against each parental serotype. *J. Virol.,* 60**,** 491-496.

OHNO, A., MARUI, A., CASTRO, E. S., REYES, A. A. B., ELIO-CALVO, D., KASITANI, H., ISHII, Y. & YAMAGUCHI, K. 1997. Enteropathogenic Bacteria in the La Paz River of Bolivia. *Am J Trop Med Hyg,* 57**,** 438-444.

ORSKOV, I., F. ORSKOV, A. BIRTH-ANDERSON, M. KANAMORE, AND C. SVANBORG-EDEN 1982. O, K, H and fimbrial antigens in E. coli serotypes associated with pyelonephritis and cystitis. *Scand. J. Infect. Dis,* 33**,** 18-25.

OSBORNE, M. P., HADDON, S. J., SPENCER, A. J., COLLINS, J., STARKEY, W. G., WALLIS, T. S., CLARKE, G. J., WORTON, K. J., CANDY, D. C. A. & STEPHEN, J. 1988. An Electron Microscopic Investigation of Time-Related

Changes in the Intestine of Neonatal Mice Infected with Murine Rotavirus. *Journal of pediatric gastroenterology and nutrition,* 7**,** 236-248.

OSBORNE, M. P., HADDON, S. J., WORTON, K. J., SPENCER, A. J., STARKEY, W. G., THORNBER, D. & STEPHEN, J. 1991. Rotavirus-Induced Changes in the Microcirculation of Intestinal Villi of Neonatal Mice in Relation to the Induction and Persistence of Diarrhea. *Journal of pediatric gastroenterology and nutrition,* 12**,** 111-120.

PARASHAR, U. D. 2003. Global illness and deaths caused by rotavirus disease in children. *Emerging infectious diseases,* 9**,** 565.

PARASHAR, U. D. 2009. Rotavirus Vaccines--Early Success, Remaining Questions. *New England Journal of Medicine, The,* 360**,** 1063.

PARK, Y. W., JUAREZ, M., RAMOS, M. & HAENLEIN, G. F. W. 2007. Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Research,* 68**,** 88-113.

PELTOLA, H. & SIITONEN, A. 1991. Prevention of traveller's diarrhoea by oral B-subunit/whole cell cholera vaccine. *Lancet,* 338**,** 1285.

PÉREZ, J. F., CHEMELLO, M. E., LIPRANDI, F., RUIZ, M.-C. & MICHELANGELI, F. 1998. Oncosis in MA104 Cells Is Induced by Rotavirus Infection through an Increase in Intracellular Ca2+Concentration. *Virology,* 252**,** 17-27.

PONCET, D., APONTE, C. & COHEN, J. 1993. Rotavirus protein NSP3 (NS34) is bound to the 3' end consensus sequence of viral mRNAs in infected cells. *J. Virol.,* 67**,** 3159-3165.

PRAGER, R., STRUTZ, U., FRUTH, A. & TSCHAPE, H. 2003. Subtyping of pathogenic Escherichia coli strains using flagellar (H)-antigens: serotyping versus fliC polymorphisms. *International Journal of Medical Microbiology,* 292**,** 477-486.

QADRI, F., AHMED, T., AHMED, F., BHUIYAN, M. S., MOSTOFA, M. G., CASSELS, F. J., HELANDER, A. & SVENNERHOLM, A.-M. 2007a. Mucosal and Systemic Immune Responses in Patients with Diarrhea Due to CS6-Expressing Enterotoxigenic Escherichia coli. *Infect. Immun.,* 75**,** 2269-2274.

QADRI, F., SAHA, A., AHMED, T., AL TARIQUE, A., BEGUM, Y. A. & SVENNERHOLM, A.-M. 2007b. Disease Burden Due to Enterotoxigenic Escherichia coli in the First 2 Years of Life in an Urban Community in Bangladesh. *Infect. Immun.,* 75**,** 3961-3968.

QADRI, F., SVENNERHOLM, A.-M., FARUQUE, A. S. G. & SACK, R. B. 2005. Enterotoxigenic Escherichia coli in Developing Countries: Epidemiology, Microbiology, Clinical Features, Treatment, and Prevention. *Clin. Microbiol. Rev.,* 18**,** 465-483.

QADRI, F. D., SWADESH KUMAR FARUQUE, A. S. G. FUCHS, G. J., ALBERT, M. J., SACK, R. B. & SVENNERHOLM, A.-M. 2000. Prevalence of Toxin Types and Colonization Factors in Enterotoxigenic Escherichia coli Isolated during a 2-Year Period from Diarrheal Patients in Bangladesh. *J. Clin. Microbiol.,* 38**,** 27-31.

QIAO, H. 2004. A comparison of the effects of oral inoculation with Rotashield and pentavalent reassortant rotavirus vaccine (WC3-PV) on suckling CB17scid mice. *The Journal of general virology,* 85**,** 2245.

RAMIG, R. F. 2004. Pathogenesis of Intestinal and Systemic Rotavirus Infection. *J. Virol.,* 78**,** 10213-10220.

RODAS, C., INIGUEZ, V., QADRI, F., WIKLUND, G., SVENNERHOLM, A.-M. & SJOLING, A. 2009. Development of Multiplex PCR Assays for Detection of Enterotoxigenic Escherichia coli Colonization Factors and Toxins. *J. Clin. Microbiol.,* 47**,** 1218-1220.

ROSS, D. C. & HARMSEN, R. T. 2001. Official Financing for Developing Countries. Washington, D.C.

ROY, K. 2008. The EtpA exoprotein of enterotoxigenic Escherichia coli promotes intestinal colonization and is a protective antigen in an experimental model of murine infection. *Infection and immunity,* 76**,** 2106.

ROY, K., HAMILTON, D., OSTMANN, M. M. & FLECKENSTEIN, J. M. 2009a. Vaccination with EtpA glycoprotein or flagellin protects against colonization with enterotoxigenic Escherichia coli in a murine model. *Vaccine,* 27**,** 4601-4608.

ROY, K., HILLIARD, G. M., HAMILTON, D. J., LUO, J., OSTMANN, M. M. & FLECKENSTEIN, J. M. 2009b. Enterotoxigenic Escherichia coli EtpA mediates adhesion between flagella and host cells. *Nature,* 457**,** 594-598.

ROY, P., URAKAWA, T., VAN DIJK, A. A. & ERASMUS, B. J. 1990. Recombinant virus vaccine for bluetongue disease in sheep. *J. Virol.,* 64**,** 1998-2003.

RUNNELS, P. L., MOON, H. W. & SCHNEIDER, R. A. 1980. Development of resistance with host age to adhesion of K99+ Escherichia coli to isolated intestinal epithelial cells. *Infect. Immun.,* 28**,** 298-300.

SACK, R. B., SACK, D. A., MEHLMAN, I. J., ØRSKOV, F. & ØRSKOV, I. 1977. Enterotoxigenic Escherichia coli Isolated from Food. *The journal of infectious diseases,* 135**,** 313-317.

SALIM, A. F., PHILLIPS, A. D., WALKER-SMITH, J. A. & FARTHING, M. J. 1995. Sequential changes in small intestinal structure and function during rotavirus infection in neonatal rats. *Gut,* 36**,** 231-238.

SANTOS, N. 2005. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. *Reviews in Medical Virology,* 15**,** 29.

SARKER, S. A. 2007. Successful Treatment of Rotavirus-induced Diarrhoea in Suckling Mice with Egg Yolk Immunoglobulin. *Journal of health, population, and nutrition,* 25**,** 465.

SCALETSKY, I. C. A., FABBRICOTTI, S. H., CARVALHO, R. L. B., NUNES, C. R., MARANHAO, H. S., MORAIS, M. B. & FAGUNDES-NETO, U. 2002. Diffusely Adherent Escherichia coli as a Cause of Acute Diarrhea in Young Children in Northeast Brazil: a Case-Control Study. *J. Clin. Microbiol.,* 40**,** 645-648.

SCHALKHAMMER, T. G. M. 2002. *Analytical Biotechnology,* Berlin, Germany.

SEARS, C. L. & KAPER, J. B. 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol. Rev.,* 60**,** 167-215.

SEIFERT, H. S. & DIRITA, V. J. 2006. *Evolution of microbial pathogens* Washington, DC, ASM.

SHAH, N., DUPONT, H. L. & RAMSEY, D. J. 2009. Global Etiology of Travelers' Diarrhea: Systematic Review from 1973 to the Present. *Am J Trop Med Hyg,* 80**,** 609-614.

SHAW, R. D., HEMPSON, S. J. & MACKOW, E. R. 1995. Rotavirus diarrhea is caused by nonreplicating viral particles [published erratum appears in J Virol 1996 Aug;70(8):5740]. *J. Virol.,* 69**,** 5946-5950.

SIMPSON, E., WITTET, S., BONILLA, J., GAMAZINA, K., COOLEY, L. & WINKLER, J. L. 2007. Use of formative research in developing a knowledge translation approach to rotavirus vaccine introduction in developing countries. *BMC Public Health,* 7**,** 281-11.

SINGH, H. & CREAMER, L. K. 1991. Denaturation, aggregation and heat stability of milk protein during the manufacture of skim milk powder. *Journal of Dairy Research,* 58**,** 269-283.

SIXMA, T. K., KALK, K. H., VAN ZANTEN, B. A. M., DAUTER, Z., KINGMA, J., WITHOLT, B. & HOL, W. G. J. 1993. Refined Structure of Escherichia coli Heat-labile Enterotoxin, a Close Relative of Cholera Toxin. *Journal of Molecular Biology,* 230**,** 890-918.

SOKURENKO, E. V., CHESNOKOVA, V., DYKHUIZEN, D. E., OFEK, I., WU, X.-R., KROGFELT, K. A., STRUVE, C., SCHEMBRI, M. A. & HASTY, D. L. 1998. Pathogenic adaptation of Escherichia coli by natural variation of the FimH adhesin. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 8922-8926.

SPANGLER, B. D. 1992. Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. *Microbiol. Mol. Biol. Rev.,* 56**,** 622-647.

STANLEY A. PLOTKIN, WALTER A. ORENSTEIN & PAUL A. OFFIT 2008. *Vaccines,* Elsevier Inc.

STEELE, A. D., CUNLIFFE, N., TUMBO, J., MADHI, SHABIR A., DE VOS, B. & BOUCKENOOGHE, A. 2009. A Review of Rotavirus Infection in and Vaccination of Human Immunodeficiency Virus-Infected Children. *The journal of infectious diseases,* 200**,** S57-S62.

SUBEKTI, D. S., LESMANA, M., TJANIADI, P., MACHPUD, N., SRIWATI, SUKARMA, DANIEL, J. C., ALEXANDER, W. K., CAMPBELL, J. R.,

CORWIN, A. L., BEECHAM, H. J., SIMANJUNTAK, C. & OYOFO, B. A. 2003. Prevalence of enterotoxigenic Escherichia coli (ETEC) in hospitalized acute diarrhea patients in Denpasar, Bali, Indonesia. *Diagnostic Microbiology and Infectious Disease,* 47**,** 399-405.

SUSAN, M. T., ANTHONY, S.-T., LISA, M. C. & IAN, R. H. 2006. Weapons of mass destruction: virulence factors of the global killer Enterotoxigenic Escherichia coli. *FEMS Microbiology Letters,* 263**,** 10-20.

TAFAZOLI, F., ZENG, C. Q., ESTES, M. K., MAGNUSSON, K.-E. & SVENSSON, L. 2001. NSP4 Enterotoxin of Rotavirus Induces Paracellular Leakage in Polarized Epithelial Cells. *J. Virol.,* 75**,** 1540-1546.

TAXT, A., AASLAND, R., SOMMERFELT, H., NATARO, J. & PUNTERVOLL, P. Heat-Stable Enterotoxin of Enterotoxigenic Escherichia coli as Vaccine Target. *Infect. Immun.***,** IAI.01397-09.

TAYLOR, J., M. P. WILKINS, AND J. M. PAYNE. 1961. Relation of rabbit gut reaction to enteropathogenic Escherichia coli. *Br. J. Exp. Pathol.,* 42**,** 43-52.

TIAN, P., ESTES, M. K., HU, Y., BALL, J. M., ZENG, C. Q. & SCHILLING, W. P. 1995. The rotavirus nonstructural glycoprotein NSP4 mobilizes Ca2+ from the endoplasmic reticulum. *J. Virol.,* 69**,** 5763-5772.

URUAKPA, F. O., ISMOND, M. A. H. & AKOBUNDU, E. N. T. 2002. Colostrum and its benefits: a review. *Nutrition Research,* 22**,** 755-767.

VESIKARI, T., ISOLAURI, E., D'HONDT, E., DELEM, A., ANDRÉ, F. & ZISSIS, G. 1984. PROTECTION OF INFANTS AGAINST ROTAVIRUS DIARRHOEA BY RIT 4237 ATTENUATED BOVINE ROTAVIRUS STRAIN VACCINE. *The Lancet,* 323**,** 977-981.

VIDAL, R. M., VALENZUELA, P., BAKER, K., LAGOS, R., ESPARZA, M., LIVIO, S., FARFJN, M., NATARO, J. P., LEVINE, M. M. & PRADO, V. 2009. Characterization of the most prevalent colonization factor antigens present in Chilean clinical enterotoxigenic Escherichia coli strains using a new multiplex polymerase chain reaction. *Diagnostic Microbiology and Infectious Disease,* 65**,** 217-223.

VIGIL, K. J., JIANG, Z.-D., CHEN, J. J., PALUMBO, K. L., GALBADAGE, T., BROWN, E. L., YIANG, J., KOO, H., DUPONT, M. W., ERICSSON, C.,

ADACHI, J. A. & DUPONT, H. L. 2009. Coliform and Escherichia coli Contamination of Desserts Served in Public Restaurants from Guadalajara, Mexico, and Houston, Texas. *Am J Trop Med Hyg,* 80**,** 606-608.

WALKER, R. I., STEELE, D. & AGUADO, T. 2007. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic E. coli (ETEC) disease. *Vaccine,* 25**,** 2545-2566.

WARD, R. L. 1990. Development of an adult mouse model for studies on protection against rotavirus. *Journal of virology,* 64**,** 5070.

WEINTRAUB, A. 2007. Enteroaggregative Escherichia coli: epidemiology, virulence and detection. *J Med Microbiol,* 56**,** 4-8.

WHITE, P. 2004. *Crime scene to court: the essentials of forensic science,* Cambridge, UK, Royal Society of Chemistry.

WHITTAM, T. S., WOLFE, M. L., WACHSMUTH, I. K., ORSKOV, F., ORSKOV, I. & WILSON, R. A. 1993. Clonal relationships among Escherichia coli strains that cause hemorrhagic colitis and infantile diarrhea. *Infect. Immun.,* 61**,** 1619-1629.

WHO. 2003. *Initiative for Vaccine Research (IVR)* [Online]. Available: http://www.who.int/vaccine_research/diseases/e_e_coli/en/ [Accessed 2010].

WHO. 2009. *Diarrhoeal Diseases (Updated February 2009)* [Online]. Available: http://www.who.int/vaccine_research/diseases/diarrhoeal/en/index.html [Accessed 2009].

WHO, U. A. 2001. Reduced osmolarity oral rehydration salts (ORS) formulation: a report from a meeting of experts jointly organized by UNICEF and WHO, Unicef House, New York, USA,. New York: UNICEF and WHO.

WIDDOWSON, M.-A., MELTZER, M. I., ZHANG, X., BRESEE, J. S., PARASHAR, U. D. & GLASS, R. I. 2007. Cost-effectiveness and Potential Impact of Rotavirus Vaccination in the United States. *Pediatrics,* 119**,** 684-697.

WIERZBA, T. F. 2006. Clinic-based surveillance for bacterial-and rotavirus-associated diarrhea in Egyptian children. *The American journal of tropical medicine and hygiene,* 74**,** 148.

WOODS, P. A., GENTSCH, J., GOUVEA, V., MATA, L., SANTOSHAM, M., BAI, Z. S., URASAWA, S. & GLASS, R. I. 1992. Distribution of serotypes of human rotavirus in different populations. *J. Clin. Microbiol.,* 30**,** 781-785.

XIA, D., SAVARINO, S. J. & BULLITT, E. 2010. Structure and Assembly of CFA/I pili from Enterotoxigenic Escherichia Coli That Cause Traveler's Diarrhea. *Biophysical Journal,* 98**,** 417a-417a.

YOUSSEF, M., SHURMAN, A., BOUGNOUX, M.-E., RAWASHDEH, M., BRETAGNE, S. & STROCKBINE, N. 2000. Bacterial, viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children from northern Jordan. *FEMS immunology and medical microbiology,* 28**,** 257.

YUAN, L., WARD, L. A., ROSEN, B. I., TO, T. L. & SAIF, L. J. 1996. Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J. Virol.,* 70**,** 3075-3083.

ZENG, C. Q., WENTZ, M. J., COHEN, J., ESTES, M. K. & RAMIG, R. F. 1996. Characterization and replicase activity of double-layered and single- layered rotavirus-like particles expressed from baculovirus recombinants. *J. Virol.,* 70**,** 2736-2742.

ZHI-DONG, J., LOWE, B., VERENKAR, M. P., ASHLEY, D., STEFFEN, R., TORNIEPORTH, N., VON SONNENBURG, F., WAIYAKI, P. & DUPONT, H. L. 2002. Prevalence of Enteric Pathogens among International Travelers with Diarrhea Acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay). *Journal of Infectious Diseases,* 185**,** 497.

ZIJLSTRA, R. T., MCCRACKEN, B. A., ODLE, J., DONOVAN, S. M., GELBERG, H. B., PETSCHOW, B. W., ZUCKERMANN, F. A. & GASKINS, H. R. 1999. Malnutrition Modifies Pig Small Intestinal Inflammatory Responses to Rotavirus. *J. Nutr.,* 129**,** 838-843.

ZIMMERMAN 2001. Cost of diarrhea-associated hospitalizations and outpatient visits in an insured population of young children in the United States. *The Pediatric Infectious Disease Journal,* 20(1)**,** 14-19.

ZYGOYIANNIS, D. 2006. Sheep production in the world and in Greece. *Small Ruminant Research,* 62**,** 143-147.

# APPENDICES

## Appendix A. Sheep descriptions

Sheep descriptions and sample collection days with milk and serum absorbance

| **Sheep No.** | | **1**<br>**Vaccinated against rotavirus SA11 and ETEC O6:H16** | | | | | | | | **2**<br>**Vaccinated against ETEC O78:h11** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Sample for Rotavirus measurements** | | | | **Samples for ETEC O6:H16 measurements** | | | | | | | |
| Sample collection Days | | D 0 | D8 | D22 | D36 | D 0 | D 8 | D 22 | D 36 | D 0 | D 8 | D 22 | D 36 |
| **Serum** | Absorbance at 450nm | 0.055 | 0.707 | 0.685 | 0.741 | 0.044 | 0.585 | 0.557 | 0.604 | 0.101 | 0.797 | 0.736 | 0.762 |
| **Milk** | Absorbance at 450nm | 0.048 | 0.523 | 0.503 | 0.591 | 0.043 | 0.407 | 0.386 | 0.428 | 0.068 | 0.609 | 0.588 | 0.634 |

**Appendix B. Faecal samples absorbance**

Faecal samples collection days and absorbance in each day

| **Time after milk immunotherapy (Hour)** | **Status** | **0** | **24** | **48** | **72** | **96** | **120** | **144** | **162** | **180** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Against rotavirus SA11** | Absorbance at 450nm | 0.003 | 0.005 | 0.319 | 0.299 | 0.31 | 0.26 | 0.24 | 0.105 | 0.002 |
| **Against *E.coli* NL055** | Absorbance at 450nm | 0.012 | 0.01 | 0.182 | 0.167 | 0.175 | 0.18 | 0.159 | 0.081 | 0.006 |
| **Against ETEC O78:h11** | Absorbance at 450nm | 0.019 | 0.016 | 0.394 | 0.382 | 0.35 | 0.363 | 0.327 | 0.122 | 0.029 |

## Appendix C. Monitoring sheet for mice experiment

Monitoring sheet for the mice experiment

| **After each inoculation or booster/sheep** | Day 0 | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 |
|---|---|---|---|---|---|---|---|
| **TIME** | | | | | | | |
| Answer (Yes or No) | | | | | | | |
| Bodyweight | | | | | | | |
| Active? | | | | | | | |
| Rough hair coat? | | | | | | | |
| Eating? | | | | | | | |
| Drinking? | | | | | | | |
| Normal gait/posture? | | | | | | | |
| Diarrhoea | | | | | | | |
| Vocalisation? | | | | | | | |
| Type of breathing | | | | | | | |

| **After each inoculation or booster/sheep** | Day 0 | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 |
|---|---|---|---|---|---|---|---|
| **TIME** | | | | | | | |
| Answer (Yes or No) | | | | | | | |
| Pain score (1-4) | | | | | | | |
| Observers initials | | | | | | | |

PAIN ASSESSMENT SCORING

0 = normal behaviour and faeces

1 = mild behaviour and physiological changes (decreased food/water consumption) and pasty faeces

2 = moderate pain (includes observation in group 1, mild diarrhoea or semi liquid faeces, reluctance to move)

3 = severe pain/distress (includes all observation in groups 1 and 2, profound diarrhoea or liquid faeces)

4 = moribund

Please contact a member of the veterinary staff for group 2 or higher animals.

* N= normal, L= laboured, R = rapid, S = Shallow

**In case of score 2, 3 fluid administration will be considered**? Sterile saline (0.9% NaCl) is recommended for most injections. Do not administer fluids if kidney function is impaired without first consulting the veterinary staff. Only conscious animals may be given oral fluids. Fluids should be warmed by running the syringe under warm water to avoid hypothermia or shock, especially in the smaller rodents. The preferred routes are (i/v 0.5ml max total, s/c 0.3 per site, 1 ml per animal) , monitoring the site of injction for any reaction during the administration of fluid and after.