Aetiology of acute diarrhoea in hospitalized children Tripoli, Libya

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TO MY FAMILY

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Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
BM	Basement Membrane
cAMP	cyclic Adenosine Monophosphate
CCDA	Charcoal Cefoperazone Desoxycholate Agar
CDD	Control diarrhoea Disease
CDT	Cytolethal Distending Toxin
CFA	Colonization Factor Antigen
cGMP	cyclic Guanosine Monophasphate
CLT	Cholera-Like Toxin
CNS	Central Nervous System
CT	Cholera Toxin
DNA	Deoxy ribonucleic Acid
EAST	Enteroaggregative Stable Toxin
ECM	Extra Cellular Matrix
EPI	Expending Programme Immunization
ER	Endoplasmic Reticulum
HB	Haemoglobin
HC	Heamorrhagic Colitis
HIV	Human Immunodefieciency Virus
HUS	Haemolytic Uraemic Syndrome
IVD	Intravenous Drip
IVF	Intravenous Fluid
LEE	Locus of Enterocytes Effacment
LT	Labile Toxin

LPA	Lymphocyte Proliferation Assay
NSP4	Non-Structural Protein 4
OR	Odds Ratio
ORS	Oral Rehydration Solution
ORT	Oral Rehydration Therapy
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SIg A	Secretory Immunoglobulin A
ST	Stable Toxin
TTSS	Type III Secretion System
VP	Viral Proteins
VT	Verocytotoxin
W/V	Weight/Volume

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Abstract

Two studies were performed to detect aetiology of acute diarrhoea in hospitalized children aged under five years in Tripoli, Libya. In the first study, two hundred and thirty-six stool samples were obtained. Of these, 118 were from patients admitted for acute diarrhoeal disease, and 118 age and sex-matched concurrent controls were admitted for other reasons. In the second study, eight hundred and ten stool specimens were collected 405 from patients and the same number from controls. All were admitted to the Aljala Children's Hospital, Tripoli, Libya. The first study was conducted between August 1st 1997 and 31st October 1997 and the second study was conducted between January 1st 2003 and 31st December 2003. One or more enteric pathogens were isolated from 55.9% of patients and 13.6% of controls in the first study and 68.1% of patients and 12.6% of controls in the second study.

In the first study, the organisms were identified from stool of patients; *Escherichia coli* 22.9%, rotavirus 15.3%, *Campylobacter* 7.6%, *Proteus* 7.6%, *Klebsiella* 4.2%, *Entamoeba histolytica* 4.2%, *Cryptosporidium* 2.5% and *Giardia lamblia 1.*7%, *Salmonella* was not isolated in both studies, because not growth on ordinary media were used (MacConkey agar and Blood agar) but usually growth on Salmonella-Shigella medium where not used.

In the second study, enteropathogens identified were: rotavirus 30.4%, Escherichia coli 16.5%, *Entamoeba histolytica* 12.5%, *Campylobacter* 6.7%, *Giardia lamblia* 3.7%, *Cryptosporidium* 3.2%, *Klebsiella* 2.9%, *Proteus* 2.2%.

Libyan children under six months of age were the most susceptible to acute diarrhoea requiring admission to the hospital. This was remarked in 46.6% of patients in the first study and 43.2% in the second. Exclusive breast feeding in Libyan children was of a very low percentage. In the first study it was 19.5% and in the second 29.1%.

Children use dummies were about 3 times more at risk of diarrhoea than the non users in the first study (OD: 5.95, 95%, CI: 1.120-2.37, $p<0.001$) and RR: 3.1 and (O: : 2.986, 95%. CI: 2.13-4.16, $P<0.001$) and RR: 2 3 in the second, this fell to children being twice as much at risk of contracting diarrhoea.

Watery stool was the most common stool consistency of patients in both studies. Fever was the most common clinical feature associated with acute diarrhoea in the two studies (72.9% and 71.6% respectively). Vomiting was present in 51.7% of patients among the first study and 58.3% among the second. Moderate and isotonic dehydration were the most common degree and type of dehydration across both studies. In the second study, Rotavirus peaked during the winter (cold season) while bacteria and parasites peaked during the summer and autumn seasons.

API 20E and API Campy were used to identify bacterial isolated by cultures and the number of confirmed cases decreased from 142 to 112 samples.

Polymerase Chain Reaction was adopted in the second study to confirm detection of *Campylobacter* spp. these were identified by API Campy technique, where all *Campylobacter* spp. were identified as the same as detected by API Campy and for 24 samples.

Chapter 1

INTRODUCTION

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1.1 General introduction

Diarrhoeal diseases are a major cause of childhood morbidity and mortality, especially in developing countries. According to World Health Organization (WHO) figures, acute diarrhoea due to gastroenteritis (GE) causes about 750 million episodes of diarrhoea a year. From this, there are 4-5 million deaths in children under the age of 5 years in developing countries (Anon. 1982). In 1992, it was estimated that 2.9 million deaths worldwide could be attributed to diarrhoeal disease, with most of the deaths occurring in developing countries (Murry and Lopez 1997). In 1998, it was estimated that 2.2 million deaths, most of whom were under 5 years of age were caused by diarrhoea (Victoria *et al*. 2000). The highest rates of illness are in children under the age of 2 years (Dupont 1995). The prospective, community-based studies reveal that acute gastrointestinal illnesses are extremely common ranging from one to three illnesses per year in the developed countries and five to 18 illnesses per person per year in impoverished areas of tropical, developing countries. In the latter areas, diarrhoeal diseases are major causes of morbidity as well as leading causes of death (Guerrant *et al*. 1990). In these low income countries, diarrhoea accounts for as many as 21 percent of all deaths in children under the age of 5 years (Parashar *et al*. 2003b & Kosek *et al*. 2003). These figures are striking when compared with the developed world, where diarrhoea is associated with fever in 1 percent of deaths in children (Parashar *et al*. 2003b). The highest age-mortality rate (8.5 per 1000/yr) occurred in children younger than 1 year of age (Kosek *et al*. 2003).

Latin American children under 5 years of age will have an average of 5 to 10 diarrhoeal episodes per year. The highest attack rate will occur during the first year of

life. Differences in diarrhoeal mortality are observed among individual countries; in Mexico, mortality rates due to diarrhoea are 6 per 1000 children, in Peru 7 in 1000, and in Chile 3 in 1000. Mortality associated with acute diarrhoea is highest among infants less than 1 year of age, where it averages the rate of 20 per 1000 children (Lanata *et al.* 1992). It is well known that diarrhoeal disease is one of the leading causes of illness and death in young children. But it is not clear how accurate the morbidity and mortality estimates are? (Parashar *et al.* 2003b). In 1976, Rohde and Northrup estimated that annually, diarrhoea killed up to 5 million children in developing countries (Rohde and Northrop 1976). Snyder and Merson (1982) analysed 24 longitudinal, prospective, community-based studies of diarrhoea published between 1954 and 1979. They estimated that children under 5 years old had 2.2 episodes of diarrhoea per year, resulting in 4.6 million deaths (Snyder and Merson 1982). In 1986, a committee of the US Institute of Medicine revised the diarrhoea mortality estimate to 3.5 million deaths per year, based on published data and field experience (Anon. 1986). Bern *et al.* (1992) then updated Snyder and Merson's is analysis by using studies published from 1980 to 1990. They documented diarrhoea morbidity of 2.6 episodes per child per year, a similar finding to that reported a decade earlier, but with lower mortality 3.3 million deaths per year (Bern *et al.* 1992). Kosek *et al.* (2003) have recently made a further update by reviewing 60 studies of diarrhoea morbidity and mortality published between 1990 and 2000. They concluded that diarrhoea accounts for 21% of all deaths in children under the age of 5 and causes 2.5 million deaths per year, although diarrhoea morbidity remains relatively unchanged.

In developed countries, diarrhoeal disease is an important cause of morbidity with significant health cost (Ferson 1996). In the United States, for example, where attack rates range from 1 to 5 episodes per child per year (Guerrant *et al.* 1990), it accounts for some 500 deaths annually and 10% of preventable post neonatal infant deaths, and

costs the health care system $ 264 million (Ho *et al.* 1988). Over 5% of consultations made by general practitioners for children under 5 years in England and Wales are for infectious disease (Anon. 1995a) and the age specific incidence of inpatient episodes for infectious intestinal disease in England is highest in children under 5 years at 6.3 in 100,000 per year (Anon. 1995b). In Southeast Asian counties such as Malaysia, the mortality resulting from diarrhoeal illness is low, but continues to be a major cause of hospital admission in children (Lee and Ooi 1999). Infectious intestinal disease in children is associated with mortality, although rarely in developed countries (Crowley *et al.* 1997). In the United States, an estimated 211 to 375 million episodes of acute diarrhoea occur each year (1.4 episodes per person per year); such episodes are responsible for more than 900,000 hospitalizations and 6000 deaths annually (Herikstand *et al.* 2002).

Despite the different methods and sources of information, each successive review of the diarrhoea burden over the past three decades has demonstrated declining mortality but relatively stable morbidity (Parashar *et al*. 2003b). The decline in global diarrhoea mortality is confirmed by detailed information from monitoring in certain countries over time (Victoria *et al*. 2000). Increased use of oral rehydration therapy, (ORT) improved nutrition, increased breast feeding, better supplement feeding, enhanced female education, measles immunization, and improvements in hygiene and sanitation are all believed to have contributed to this decline (Parashar *et al*. 2003).

1.2 Definition of diarrhoea

Diarrhoea can be defined as increased frequency and fluidity of the stool; that is, the passage of stool containing excess water and electrolytes losses. Acute diarrhoea is defined as the presence of three or more loose, watery stools within a 24-hour period (Anon. 1995c). Dysentery, often termed bloody diarrhoea, indicates visible blood and

mucous in diarrhoeal stools. Episodes of diarrhoea lasting more than 14 days are referred to as persistent diarrhoea.

1. 3 Clinical features

Acute diarrhoea usually manifests as an increase in the frequency or volume of stool. Fever is a common symptom and is usually associated with invasive pathogens. Bloody stools are also present in diarrhoea caused by invasive pathogens. Vomiting is observed more frequently in viral diarrhoea. Abdominal cramps are present in some cases. Dehydration is one of the most important clinical features. Assessment of dehydration requires a great deal of experience. In general an onset of excessive stools and vomiting leads to severe dehydration. Dehydration can be classified into mild, moderate and severe based on the severity of body fluid loss. Patients with mild dehydration appear almost clinically normal, yet they may have lost considerable body fluid (4-5% body weight loss i.e. mild dehydration). Fluid losses in moderate dehydration (6-9% body weight loss) and in severe dehydration (10% or more of body weight loss) are greater. Symptoms and signs particularly useful in assessment of dehydration and monitoring of rehydration are: thirst, radial pulse, blood pressure, skin elasticity, anterior fontanella in infants, appearance of tongue, mucous membranes, eyes, eyeball tension, tears, respiration, urinary output, estimation of body weight and central nervous system manifestations.

1.4 Diarrhoea and malnutrition

In developing countries diarrhoea and malnutrition are closely related: decreased food intake during diarrhoeal episodes leads to weight loss and malnutrition and diarrhoea is more severe and more prolonged in malnourished children. Intake of food is often voluntarily reduced by the child's mother during illness. Marasmus (very low body

weight) and, less commonly, Kwashiorkor (low body weight) results from deficiency of protein and energy as calories in the diet. Protein energy deficiency may develop during diarrhoea episodes leading to stunting of growth. In children with malnutrition, episodes of diarrhoea are prolonged and frequently associated with complications of illness (Guerrant 1994). In certain areas, micronutrient deficiencies are common. The micronutrients of potential critical to general infant health in these regions include iron, iodine, vitamin B12, folate, vitamin A and zinc. Of these, vitamin A and zinc deficiencies are particularly important to diarrhoeal disease. Vitamin A deficiency is associated with the development of both acute and persistent diarrhoea (Usha *et al.* 1991). Deficiency in tissue level of zinc is associated with diarrhoea and atrophy of intestinal mucosa (Hambidge 1992). However this is less consistent and generally does not support the use of vitamin A supplementation for reducing morbidity associated with acute diarrhoea (Grotto *et al*. 2003). Zinc deficiency, in particular, suppresses immune system function and is associated with an increased prevalence of persistent diarrhoea (Bitarakwate *et al*. 2003).

1.5 Breast feeding and diarrhoea

The neonatal period is particularly critical in this respect, because the newborn is immediately exposed to a large number of micro-organisms, foreign proteins and chemicals. Breast-feeding is therefore important, not only as a natural immunological 'substitution therapy' or 'passive vaccine', but also because immune - modulating factors in breast milk may exert an important impact on the suckling s developing immune system (Brandtzaeg 1998 and 2002). Epidemiological data indicate that the risk of dying from diarrhoea could be reduced 14-24 times in breast-fed children (Anonymous 1994). Lactating mammary glands are part of an integrated immune system with local production of antibodies, mainly consisting of secretory

immunoglobulin A (SIg A). The main purpose of the secretory antibody system is, in cooperation with innate mucosal defence mechanisms, to perform immune exclusion. Most important to this end, SIg A inhibits colonization and invasion by pathogens, and carries the pathogens and their products back to the lumen, thus avoiding cytolytic damage to the epithelium (Norderhaug *et al.* 1999). In addition to antibodies and free glycoproteins called membrane secretory components, other components are thought to protect the very young children. These include innate defence factors such as lysozyme, lactoferrin, peroxidase, complex oligosacharides, fatty acids and mucins. Moreover, a variety of leukocytes occur in colostrum and later milk together with macrophages, neutrophilic granulocytes and cytokines (Goldman 1993). Two studies published in Latin American and Asia at the end of the 1980s found that exclusively breastfed infants had considerably reduced risks of diarrhoea (and other infections) compared with infants who either received other foods or liquids along with breast milk or were fully weaned off breast feeding (Popkin *et al.* 1990). Similar results have been published more recently from a more industrialized setting (Kramer *et al.* 2001).

1.6 Diarrhoea in children and adults

Diarrhoea is the most common cause of death in children under 3 years of age because a young child's body has not yet developed acquired immunity to infection. During an episode of diarrhoea, some children do not obtain enough food and drink because many people believe that it is dangerous to feed a child who has diarrhoea. This may result in starvation leading to malnutrition. Children are also not able to state that they are thirsty and often are not supplied with enough drink. If malnourished children get diarrhoea this makes the malnutrition worse. Children are more likely than adults to suffer the ill-affects of other infections which are often present during diarrhoea. In the native adult population, although acute diarrhoea occurs, it is not a major problem and

is not considered a major public health issue. Gastroenteritis in adults is usually not severe and does not require medical intervention, the exceptions to this being acute diarrhoea caused by *Vibrio cholerae* (*V. cholerae*) (Prado and Ó Ryan 1994), Typhoid, Eenteropathogenic *Escherichia coli* (*EPEC*) and *Enterohaemorrhagic Escherichia coli* (*EHEC* e.g. 0157).

1.7 Traveller's diarrhoea

Traveller's diarrhoea remains the most frequent infectious illness among travellers from industrialized countries visiting destinations in developing countries. Groups at particularly high risk of symptoms include infants, people with impaired gastric acid barrier and travellers with co-existing medical problems. Traveller's diarrhoea may be particularly severe and long-lasting in small children (Pitzinger *et al.* 1991). Traveller's diarrhoea is usually caused by faecal contamination of food and drink. Bacterial agents predominate, especially Enterotoxigenic *Escherichia coli* (*ETEC*) and Enteroinvasive *Escherichia coli* (*EIEC*) which are responsible for up to 60% of cases. *Salmonella, Campylobacter* and other species of bacterial pathogens, as well as *Giardia lamblia* (*G. lamblia*) and *Entamoeba histolytica (Ent. histolytica)*, each cause fewer than 5% of the cases (Gomi *et al*. 2001). Rotavirus and *Cryptosporidium* (*Cr.*) may also be responsible for some cases.

Despite extensive microbiological assessment, approximately 20% of all cases remain of undetermined aetiology. (Behrens and Steffen 2003). Indeed, the importance of *Campylobacter jejuni* (*C. jejuni*) as a cause of traveller's diarrhoea (Taylor 1992) has led to the observation that the spectrum of illness in travellers is similar to that described in their country of origin. Bacterial agents probably cause most of these cases because they can be prevented by use of antimicrobial agents (Ericson 2000).

1.8 Persistent diarrhoea

The mortality rate from acute diarrhoea is being reduced significantly by the increased use of ORT. Prolonged diarrhoea (14-20 days) is emerging as an increasingly important cause of morbidity and mortality. Between 3% and 10% of acute diarrhoeal cases occurring in developing regions progress to a course of 14 days or longer (Guerrant 1994). Most enteropathogens that have been isolated in acute diarrhoea have also been isolated in persistent episodes (Bhan 1989). However, persistent diarrhoea is emerging as a serious public health problem. Causes of persistent diarrhoea are multiple and include certain enteropathogens such as Enteroadherent *E. coli* (*EAEC*), *Yersinia enterocolitica* (*Y. enterocolitica*), enteric adenovirus, *Isospora belli, Cyclospora cayelerensis*, *G. lamblia*), Microsporidium, *Cryptosporidium parvum* (*Cr. parvum*), transitory lactose intolerance, and moderate to severe malnutrition (Bhan *et al*. 1989 and Fang *et al.* 1995). The incidence of this diarrhoea is also high, suggesting that both conditions may have common risk factors. Virulence factors of individual infectious agents besides specific host factors may influence the occurrence of both acute and persistent diarrhoea (Lanata *et al*. 1992).

1.9 Pathophysiology

1.9.1 Bacterial enteropathogens

1.9.1.1 Adherence factors. These factors include pili, glycoproteins, or other surface proteins that allow bacteria to colonize the intestine. The presence of one or more of these factors that bind to specific surface receptors in the enterocyte is critical for adhesion in the first phase of infection. (Prado & Ó Ryan 1994).

1.9.1.2 Toxin production. The major toxins produced by enteropathogenic bacteria are enterotoxins and cytotoxins. These toxins can cause diarrhoea by activation of

secretory mechanisms in the intestinal mucosa. *ETEC* produces both heat-stable (ST) and heat-labile (LT) toxins. These produce secretion of sodium and water through stimulation of the cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) circuits, respectively. Cytotoxins produce direct cell damage, mainly through inhibition of protein synthesis. The best characterized enteric cytotoxins are the Shiga and Shiga-like toxins (Prado & Ó Ryan 1994). Some agents, such as *V. cholerae* and *ETEC*, produce enterotoxins that cause diarrhoea by activation of secretory mechanisms in the intestinal mucosa. *ETEC* produces both heat-stable (STa and STb) and heat-labile (LTI and LTII) toxins. Other pathogens such as *Shigella dysenteriae* (*Sh. dysenteriae*) and *V. paraheamolyticus*, produce cytotoxins that lead to destruction of the intestinal epithelium, hence resulting in inflammatory diarrhoea. Some agents, such as *Staphylococcus aureus* (*Staph. aureus*) and *Bacillus cereus,* produce neurotoxins that induce diarrhoea by acting directly on the nervous system. Dysentery results from either the production of toxins or from direct invasion of the intestinal mucosa by pathogens such as *Shigella* organisms and *EIEC*. Some enteric pathogen, such as *Salmonella typhi* (*S. typhi*) and *Y. enterocolitica*, can penetrate the intestinal mucosa and disseminate through the regional lymphatics to enter the bloodstream, resulting in systemic manifestations (Podewils *et al.* 2004).

1.9.1.3 Invasiveness. The capacity of Gram negative bacteria to invade and multiply in a cell, causing cell destruction, is determined by the lipopolysaccharide composition of the bacterial cell wall in combination with production and liberation of specific plasmids and chromosomal genes that vary from one enteropathogen to another. The presence of some of these factors is a prerequisite for disease (for example virulence plasmids in *Shigella* and *Yersinia* infections). Others factors add to pathogenesis, albeit not essential (enzymes and surface antigens in Shigella and *EPEC* infections). The genetic basis for these pathogenic characteristics is located in plasmids, the

chromosome and bacteriophages (Prado & Ó Ryan 1994). Sequencing the virulence plasmid of the *Shigella flexneri* (*Sh. flexneri*) 5a strain M90T has allowed investigators to identify the genes encoding approximately 25 proteins secreted by the type III secretion system (Buchrieser *et al.* 2000). This secretion system, like that in some other Gram-negative bacteria, translocates the *Shigella* effector molecules from the bacteria cytoplasm of the host cell (Galan & Collmer 1999). A 31-kb segment of the virulence plasmid, called the entry region or *ipa/mxi-spa* locus, comprises the pathogenicity island, which is actually sufficient for entry of Shigella into intestinal epithelial cells, for apoptotic death of macrophages and for activation of polymorpho-nuclear leukocytes (Ingersoll *et al.* 2002). The island is organized into two divergently transcribed operons (Fernandez & Sansonetti, 2003 and Ingersoll *et al*. 2002).

1.9.2 Viral entropathogens

Information on the pathophysiology of rotavirus as well as other enteric viral infections has been obtained mostly from animal studies and from a few biopsy specimens obtained from seriously infected children. Thus, the pathophysiological mechanisms proposed for viral gastroenteritis, although probably correct, cannot be assured at this time. During the recovery phase, the enteroblastic cells will mature and reconstruct the villous structure.

Diarrhoea that occurs may be a result of one or more of the following: (1) decrease in surface area, (2) disruption in epithelial integrity, (3) disaccharide deficiency, and/or (4) imbalance of counter current mechanisms with a net result of intestinal fluid secretion. It is not clear which of these mechanisms play a greater role in the pathogenesis of rotavirus or other enteric viral diarrhoea (Prado & Ó Rayan 1994). Enteric viruses cause diarrhoea through multiple mechanisms, including interfering with gastrointestinal motility or destroying the intestinal epithelium and reducing

brush border enzymes (Moon 1997). Studies in mice indicate that a non-structural protein of rotavirus 4 (NSP4) acts as an enterotoxin and causes secretory diarrhoea by altering epithelial cell function and permeability (Morris *et al.* 1999 and Estes 2001). Recent data indicate that a specific cleavage product of NSP4 (aa 112 to 175) is actively secreted from infected cells and that NSP4 is released from the apical side of infected epithelial cells via an atypical pathway that bypasses the Golgi apparatus and involves lipid microdomains called rafts (Sapin *et al.* 2002 and Zhang *et al.* 2000). Other studies indicate that rotavirus antigen and Ribonucleic acid (RNA) are present in serum of children with acute rotavirus infection, suggesting that rotavirus perhaps escapes the intestinal tract in children, resulting in antigenaemia and possible viremia (Blutt *et al.* 2003). However, since NSP4 was detected in vivo at the basement membrane (BM) with antibodies against aa 114 to 135 and 120 to 147, it can be concluded that the released peptide sequence contains at least the enterotoxic domain. After basal release, NSP4 would bind to extra cellular matrix (ECM) proteins laminin-β3 and fibronectin (Boshuizen *et al.* 2004). Laminin and fibronectin are multifunctional proteins with diverse biological activities. They can influence cell adhesion, growth, morphology, differentiation, migration and agglutination as well as the assembly of the ECM (Colognato and Yorchenco 2000).

1.9.3 Parasitic enteropathogens

Protozoa that produce diarrhoea have adherence mechanisms that allow the organism to attach to the intestinal epithelium. Once adhered, the organism produces inflammation at the submucosa, rapid cell turnover and atrophy of the intestinal villi (Prado & Ó Rayan 1994). Specific surface structures that facilitate parasite adhesion include surface glycoproteins such as glycophorin A and B, complement receptors, adsorbed components of the complement cascade fibronectin, and N-

acetylglucosamine conjugates. Numerous investigators have suggested that toxic products elaborated by parasitic protozoa are responsible for at least some aspects of pathology.

1.10 Aetiology of acute diarrhoea

Many recent advances have allowed a more clear understanding of the role of different viral and bacterial agents as a cause of diarrhoeal disease worldwide, rapid identification of new aetiologic agents of acute diarrhoea. The discovery of new pathogenic mechanisms of enteric infections and the elaboration of new molecular-based diagnostic techniques for diarrhoeal pathogens have taken place during the past 25-30 years. Most causes of diarrhoeal disease are due to the action of pathogenic organisms in the small or large bowel. Currently, the leading aetiological agents of diarrhoea in developed and developing countries are bacteria (Escherichia coli (E. coli) ; *ETEC*, *EPEC, EIEC*, *EHEC* and Enteroaggrigative *EC* (*EAggEC*), *Campylobacter* spp., *Shigella* spp., *Salmonella* spp., cytotoxigenic *Clostridium difficile* (*Cl. difficile*), *Clostridium perfringens* (*Cl. perfingens*), *V. cholerae*, *Y. enterocolitica, Staph. aureus* and *Bacillus* spp.*)*, viruses (rotaviruses, enteric adenoviruses, noroviruses, caliciviruses, astrovirus and coronavirus), and parasites (*Ent. histolytica*, *G. lamblia, Cryptosporidium* and *Cyclosporium),* which are the most common aetiological agents of acute diarrhoea.

1.10.1 Bacterial Agents

Diarrhoeal diseases caused by enteric bacterial pathogens have become less prevalent in industrialized countries (with the exception of *Campylobacters* and *Salmonellas*), but remain an important cause of morbidity and mortality in developing countries, especially in infants and children in tropical countries. Many bacteria can cause

outbreaks of diarrhoea. Identification of the pathogen causing disease in an individual or outbreak depends not only on a microbiology laboratory, but also on the history of the episode, clinical examination of patients, inspection of the stools and epidemiological knowledge. Children in developing countries are exposed to a wide range of bacterial enteric pathogens at a very early age and suffer numerous episodes of diarrhoeal illness as a result. The predominant bacterial pathogens vary with the age of the child and also with time, showing both seasonal and secular trends, as well as geographical variation (Podewils *et al*. 2004).

1.10.1.1 *Escherichia coli*

E. coli are motile Gram negative bacilli, some strains are capsulated, they are aerobic and facultative anaerobes and they grow on simple media.

These are normal inhabitants of the intestine of man and animal. However, some can cause disease in man. *E. coli* is responsible for a number of diseases, including septicaemia, urinary tract infections, neonatal meningitis, appendicitis, peritonitis and gastroenteritis. *E. coli* is the predominant facultative anaerobe of human colonic flora and colonizes the infant gastrointestinal tract within hours of birth (Nataro & Kaper 1998). Although most *E. coli* strains are harmless or even beneficial to their human host, several highly adapted clones are capable of causing diarrhoeal disease. From the 6 classes of diarrhoeagenic *E. coli*, 5 classes have been defined by the presence of specific virulent characteristics: *ETEC*, *EPEC*, *EIEC*, *EHEC* and *EAggEC*.

A sixth category is Diffuse adherent *EC* (*DAEC*). The role of this strain that attaches diffusely to Hela and Hep-2 cells in diarrhoeal disease has been controversial. The scant information currently available on pathogenic mechanisms associated with *DAEC* does not allow a clear differentiation among true pathogenic and non pathogenic *DAEC* strains (Prado & Ó Ryan 1994). All five diarrhoeagenic *E. coli* are a cause of disease in children in the developing world but, *EHEC*, including *E. coli*

0157:H7, are the agents of diarrhoea more commonly identified in developed countries (Podewils *et al.* 2004). As a group, diarrhoeagenic *E. coli* are the most common bacteria detected in studies from economically developing countries, causing 30 to 40 percent of acute diarrhoeal episodes in children (Albert *et al.* 1999 and Torres *et al.* 2001). *EAggEC*, *EPEC* and *ETEC* cause endemic watery diarrhoea and have been reported most frequently in children younger than 2 years of age (Albert *et al.* 1999). *ETEC*, *EAEC* and *EHEC* are toxin producers; *EPEC*, *EAEC* and *DAggEC* have characteristic adhesion patterns. *EIEC* is distinguished by capacity to invade epithelial cells and the inability to produce toxins (Rodrigues *et al.* 2004). *ETEC* is a common cause of traveller's diarrhoea in economically developing countries. *EIEC* generally causes diarrhoea with fever and blood indistinuishable from *Shigella* infections in all ages. Shigatoxin-producing *E. coli* (*STEC*) has been established as the main etiologic agent of Haemolytic Uraemic Syndrome (HUS) (Lopez *et al.* 2000) and reported as a common cause of bloody diarrhoea among some developing countries (mainly Chile and Argentina), but these findings have not been universal (Ó Ryan *et al.* 2005).

1.10.1.1.1 *Enterotoxigenic E. coli*

Epidemiology *ETEC* has a worldwide distribution and is a major health hazard in adults and children in developing countries and is spread is by the faecal-oral route either directly or indirectly via food or water. Infants are at particular risk during weaning. In community-based studies in developing countries *ETEC* are responsible for 15-20 % of cases of diarrhoea in developing countries. The infective dose is high in the normal host colonies forming unit (c.f.u; 10^6-10^{10}). *ETEC* is among the most frequently detected enteric pathogens in the stools of children under 5 years of age in developing countries and in travellers to those regions who developed acute watery diarrhoea. In Latin American, *ETEC* is a common cause of diarrhoeal disease in children (Prado & Ó Ryan 1994).

Pathogenesis This is the most clearly identifiable diarrhoegenic *E. coli* associated with production of acute diarrhoea. The first phase of *ETEC* infection is adherence to the enterocyte mediated by adherence fimbria, denominated colonization factors that attach to specific ganglioside M_1 ($GM_{1)}$ ganglioside receptors on the enterocyte surface. The bacteria then release their toxins. The genes for these adhesions are coded in plasmids, and six different antigen determinants have been identified for *ETEC* fimbria (CS1 to CS6). These antigens form combinations that are grouped into families denominated colonization factor antigen (CFAI, CFAΠ, and CFAIV). After attachment, *ETEC* produce and release an enterotoxin, LT and / or ST that is responsible for the secretion of sodium and water into the intestinal lumen. Both toxins bind to receptors in intestinal epithelial cells and insert an enzymatic subunit that modifies a G protein associated with the adenylete cyclase complex. The increase of cyclic AMP and increased synthesis of prostaglandins by toxin-stimulated cells initiates a metabolic cascade that results in a significant increase of secretion of fluid and electrolytes in to the intestinal lumen. There are no specific histo-pathological changes to be seen in the intestinal mucosa, and no evidence of inflammation (Hart and Shears 2003).

1.10.1.1.*2 Enteropathogenic E. coli*

Epidemiology The first infections with *EPEC* were described in the UK and the USA in the 1940s and 1950s, in epidemics of infantile diarrhoea (Bray & Beavan 1948 and Neter 1959). Nowadays they are a cause of sporadic disease. In developing countries *EPEC* is still a major cause of infantile diarrhoea. *EPEC* was initially identified from common-source outbreaks of diarrhoea among infants by their serological characters. A range of O serotypes of *E. coli* was then identified which became widely used to detect *EPEC* strains known to be previously associated with these outbreaks. *EPEC* has also been associated with Traveller's diarrhoea.

Transmission is by the faecal-oral route, either directly or in food or water. The infective dose appears to be low at less than 10^4 c.f.u. (Hart and Shears 2003). *EPEC* strains cause acute secretory diarrhoea, largely in children of 2 years of age and younger and especially in those who are 6 months or younger. *EPEC* strains are associated with chronic diarrhoea in children but rarely cause disease in adults (Podewils *et al*. 2004).

Pathogenesis *EPEC* strains were the first *E. coli* strains associated with diarrhoea. There are two factors of pathogenic mechanisms, one of these is a fimbrium of 60 mD called adherence factor (AF). The presence of this factor alone is not sufficient to cause illness. The second factor is a surface protein, coded by chromosomal gene eae, which seems responsible for the alterations in the cytoskeleton of the intestinal cell and the disruption of the micro villi structure (Jerse *et al*. 1990). Significant toxin production has not been shown for *EPEC* strains, although the clinical characteristics of *EPEC*-associated diarrhoea suggest that secretory mechanisms may be involved (Prado & Ó Ryan 1994). The ingested *EPEC* adhere to the mucous overlying the small intestinal enterocytes using fimbriae. On contact with enterocytes, pathogenicity island (PAI), (called the locus of enterocytes effacement; LEE) associated genes are activated. This induces the formation of a type III secretion system (TTSS) that delivers effector molecules across both bacterial membranes and through a pilus-like structure into the enterocyte (Vallance & Finlay 2000). Secreted effectors include Tir (translocatable intimin receptor), which becomes inserted in the enterocyte membrane. This has affinity for intimin, a surface protein on the *EPEC*. Thus *EPEC* is unique in synthesizing, secreting and inserting its own receptor into host cell membranes. This then mediates the intimate attachment of *EPEC* to the enterocyte surface. Subsequently the brush border is lost by a process of vesiculation (Embaye *et al*. 1989). PAI often encode both effector molecules responsible for disease and secretion

system that deliver these effectors to host cells. Diarrhoeageinc *EPEC* and *EHEC* are attaching/ effacing (A/E) bacterial pathogens that attach to host intestinal epithelium and efface brush border microvilli, forming A/E lesions (Frankel *et al.* 1998 and Clarke *et al.* 2003). The factors are often encoded by PAI present in the genomes of pathogenic, but absent in non-pathogenic strains (Hacker and Kaper 2000). Many key virulence factors shared by A/E pathogens reside in the LEE), a PAI essential for A/E lesion formation (Deng *et al* 2001 and Taauschek *et al.* 2002 and Deng *et al.* 2003).

The LEE contains 41 genes and encodes a TTSS, a common virulence mechanism for many human and plant pathogens (Hacker & Kaper 2000, Galan 2001 and Cornelis 2002). TTSSs are conserved organelles that deliver bacterial effectors proteins capable of modulating host functions for forming such organelle (Clarke *et al.* 2003). Recently a study showed a large repertoire of non-LEE- encoded effectors indicates that diseases mediated by A/E pathogens may require coordinated action of effectors encoded by the LEE and at least three other PAIs (Deng *et al.* 2003).

1.10.1.1.3 *Enteroinvasive E. coli*

Epidemiology *EIEC* are not a common cause of diarrhoea. A recent study performed in Santiago, Chile, found that these organisms were associated with 2% to 3% of diarrhoea cases, more commonly in children older than 2 years of age. Diarrhoea caused by these organisms is most commonly watery, although dysentery, similar to that observed for *Shigella* enterocolitis, also occurs in *EIEC* infections (Prado & Ó Ryan 1994). Infection is uncommon in children under 1 year of age but may be a cause of traveller's Diarrhoea (Hart and Shears 2003). *EIEC* strains cause bloody mucoid diarrhoea and share many biochemical and pathogenic properties with *Shigella* organisms. Watery diarrhoea is also observed and fever is a common finding (Podewils *et al.* 2004).

Pathogenicity These *E. coli* strains have the capacity to colonize, invade, and destroy colonic cells, properties that are genetically coded by chromosomal and plasmid Deoxy ribonucleic acid (DNA). Enteroinvasive *E. coli* (*EIEC)* harbor a 120 mD plasmid that shares homology with the virulence plasmid of Shigella. These organisms additionally elaborate an enterotoxin, which is more intensely expressed in low iron environments. This toxin stimulates secretion of water and electrolytes at the small intestine level (Fasano *et al.* 1990).

1.10.1.1.4 *Enterohemorrhagic E. coli*

Epidemiology Infection with *EHEC* was initially described in industrialized countries. Here they tend to cause outbreaks of infection, usually as result of the consumption of incompletely cooked beef (Karmali 1989). *EHEC* can be part of the normal enteric flora of cattle, sheep, goats, cats and dogs in which they cause no disease. It is transmitted to humans primarily through consumption of contaminated foods, such as raw or uncooked ground meat products and raw milk. The incidence of *EHEC* infections varies by age group, with the highest incidence of reported cases occurring in children aged under 15 years (0.7 cases per 100,000 in the United States). It is estimated that up to 10% of patients with *EHEC* infection may develop Haemolytic Uraemic Syndrome (HUS), with a case-fatality rate from 3% to 5%. *E. coli* O157 is the most important *EHEC* serotype in relation to public health; other serotypes have frequently been involved in sporadic cases and outbreaks (Anon. 2005). The infective dose is low (less than 10^2 c.f.u.), and person-to-person spread also occurs (Hart and Shears 2003). *EHEC* infections are more common occurrences in developed countries, although a large outbreak has been described in Swaziland (Effler *et al.* 2001).

Pathogenesis *EHEC* produce attaching effacement, limited to the terminal ileum and colon, and have most of the genes in the LEE pathogenicity island of *EPEC*.

In addition, they release one or both of the toxins, verocytotoxin (VT) 1 or 2. These

toxins are also called Shiga-like toxins (SLT)1 and 2; they inhibit protein synthesis and are cytotoxic (Roe & Gally 2000). There are three pathogenic characteristics attributable to *EHEC* strains: (1) adherence is mediated by plasmid coded fimbria, (2) attachment and effacement lesions identical to that observed for classic *EPEC* occurs and is mediated by a chromosomal gene similar to the *EPEC* eae, and (3) these strains produce one and / or two phase encoded toxins, similar to the *Shigella dysenteriae* I toxin, and called SLT1 and SLT Π (formerly called verotoxins because of their capacity to destroy vero cells). At least 10 *EHEC* serogroups and 55 serotypes have been described, of which the most commonly found are serogroups 0157, 026, and 0111 and serotypes 0157: H7, 026: H11, 026;H32, 0111;H8, and 0111: H30 (Levine *et al.* 1987). *EHEC*-associated diarrhoea can present with watery stools when infection is moderate or with gross bloody diarrhoea when infection is severe (Prado & Ó Ryan 1994). *STEC* produces either or both of two phage-encoded toxins, Stx_1 and Stx_2 (Popoff 1998) which are thought to cause the vascular endothelial damage observed in haemorhagic colitis (HC) and HUS patients (Podewils 2004).

1.10.1.1.5 *EnteroAggregative E. coli*

Epidemiology *EAggEC* are the most recently discovered pathogenic group (Nataro *et al.* 1998). Diagnosis is by culture of *E. coli* that produce a distinctive aggregative pattern on cultured cells and that contain *EAggEC* pathogenicity genes detectable by PCR (Steiner *et al.* 1998). *EAggEC* are being recognized increasingly as important diarrhoeal pathogens, although the mechanism by which they act remains poorly understood. They are associated with watery diarrhoea in young children and have been reported as a cause of persistent diarrhoea in children and Human Immunodeficiency Virus (HIV) in adults (Podewils *et al.* 2004).

Pathogenicity These *E. coli* strains tend to autoaggregative in Hep-2 or HeLa cell culture system in a stacked brick pattern, which is why they have been termed

autoaggregative *E. coli,* although enteroaggregative may be more appropriate. Autoaggregation is determined by the presence of a 55 to 65 mD plasmid that codes for adherence fimbria, a smooth lipopolysacharide, and a new heat-stable enterotoxin named enteroaggregative stable toxin (Nataro *et al.* 1992). A second thermolabile toxin antigenically related to *E. coli* hemolysin has also been detected for some of these strains (Savarino *et al.* 1991). These *E. coli* can cause necrosis of micro-villi, and mononuclear infiltration of the submucosa (Prado & O' Ryan 1994).

1.10.1.1.6 *Diffuse Adherent E.coli (DAEC)*

The role of these sixth- category *E. coli* strains that attach diffusely to HeLa and Hep-2 cells in diarrhoeal disease has been controversial. Some studies have reported isolation of these strains more frequently in children with diarrhoea than in controls and in children with persistent diarrhoea (Baqui *et al.* 1992). In Santiago, *DAEC* infection seems to be more common in older children and has a seasonal distribution similar to *ETEC*. Other studies, including those with volunteers, did not show an association of this organism with diarrhoea production (Gomes *et al.* 1989 and Tacket *et al.* 1990). The diffuse adhesion pattern of aggregation has been associated with the presence of fimbriae (Bilge *et al.* 1989). Enterotoxin production has not yet been shown for these *E. coli* strains. The scarce information currently available on pathogenic mechanisms associated with *DAEC* does not allow a clear differentiation among true pathogenic and non-pathogenic *DAEC* strains (Prado & Ó Ryan 1994).

1.10.1.2 *Campylobacter* spp.

Campylobacteriosis is a collective description for infectious diseases caused by members of the bacterial genus *Campylobacter.* In 1977, Skirrow confirmed the findings of a Belgian team and described a simple technique for culturing *Campaylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) from stool specimens, which allowed widespread isolation of these organisms (Skirrow 1977).

Although the species names of *C. jejuni* and *C. coli* were derived from an initial association with enteric disease in animals (Jones *et al.* 1931 and Doyle 1948), they are the most important human pathogens in this genus, with the former usually responsible for the majority of enteric *Campylobacter* infections (80-90%; Nachamkin and Blaser 2000). However, a variety of other species, including *C. upsaliensis*, *C. hyointestinalis* and *C. lari*, also infect humans (Mishu *et al.* 1992). Campylobacteriosis is a zoonosis and has in recent years been recognized as a primary cause of childhood diarrhoea which is often accompanied by abdominal pain with bloody stool. Infection in young adults may be related to the popularity among that age group for consuming fast food and takeaway meals (Skirrow 1990). *Campylobacter* is one of the most frequently isolated bacteria from the stools of infants and children in developing countries (Coker *et al*. 2002).

Campylobacters are slim (1.5-6.0 μm long and 0.2-0.5 μ m wide), Gram-negative rods, which are spirally curved with tapering ends. They are microaerophilic and grow best in the presence of 5% O_2, 10% CO_2 and 85% N_2. The cell usually possesses a polar flagellum at one or both ends. Motility is of the darting type with a cork screw like movement.

The clinical spectrum of *Campylobacter* enteritis ranges from a watery, non-inflammatory diarrhoea to a severe inflammatory diarrhoea with abdominal pain and fever. Disease is less severe in developing countries than in developed countries (Oberhelman and Taylor 2000). *C. jejuni* infection is the most frequently identified infection preceding Guillain-Barre syndrome (GBS), an autoimmune disorder of the peripheral nervous system, which is characterized by acute flaccid paralysis (Nachamkin *et al.* 1998).

Epidemiology *Campylobacter* enteritis is the most frequent form of acute bacterial diarrhoea in industrialised countries, where it is affects people of all ages but with a

distinctive bimodal distribution, particularly affecting children aged < 4 years and young adults aged 15-44 years (Tauxe 2001). In developing countries where *Campylobacter* are hyper-endemic, the disease is confined to young children who, through repeated exposure to infection, develop immunity early in life (Oberhelman & Taylor 2000 and Coker *et al*. 2002). Disease is normally restricted to children with no apparent peak in adults. *Campylobacter* is part of the natural intestinal flora of a wide range of domestic and wild birds and animals. Although identification of the origin of a particular infection is rarely made, transmission is probably most commonly *via* the surface of meat as a result of faecal contamination during slaughtering. Other sources include untreated water, untreated milk and sewage contamination. Pet contact, particularly with puppies with diarrhoea, is also a probable source (Ketley 1997). Most estimates of incidence in developing countries are from laboratory-based surveillance of pathogens responsible for diarrhoea. *Campylobacter* isolation rates in developing countries range from 5 to 20% (Oberhelman & Taylor 2000). The *Campylobacter* associated with diarrhoea appears to differ between developed and developing countries. In the developed world, both children and adults are at risk for *Campylobacter* infection, and transmission of the organism has been associated with consumption of un-pasteurized milk, undercooked meats, contaminated water, and travel to *Campylobacter* endemic areas (Andrews 1998). In contrast, *Campylobacter* is endemic in the developing world, and infection is usually limited to children, suggesting that a high level of exposure in early life leads to the development of protection (Calva *et al* 1988). In developing countries, *Campylobacter* enteritis has no seasonal preference; in contrast, in developed countries epidemics occur in the summer and autumn (Altekruse *et al.* 1999). The rate of *Campylobacter* infections worldwide has been increasing with the number of cases often exceeding those of salmonellosis and shigellosis (Altekruse *et al.* 1999). *Campylobacter* spp. are the

major cause of bacterial gastrointestinal disease in the United Kingdom: more than 60,000 cases of infection were reported in 1999. In June 1999, the dioxin crisis, caused by dioxin-contaminated food components, resulted in the withdrawal of chicken and eggs from the market in Belgium. Through the sentinel surveillance system, a corresponding decrease in *Campylobacter* infections during June 1999 was noticed (Vellinga and Van Loock 2002). Barbecues and similar activities appear to present special hazards for infection, as they permit easy transfer from raw meat to hands and other foods, and thence to the mouth. Raw or inadequately heat-treated milk and inadequately treated water have been incriminated as sources of massive outbreaks of infection (Frost *et al*. 2002). *Campylobacter* can be shed asymptomatically (Ali *et al*. 2003).

Pathogenesis: Infection by *Campylobacter* leads to enterocolitis involving intestinal tissue damage. This host cell invasion and perhaps cytotoxin production with subsequent tissue destruction are likely to be key elements in pathogenesis. Enterocyte damage (responsible for a loss of net fluid absorption) and eventual destruction of epithelia integrity (with a resultant leakage of serosal fluid) leads to diarrhoea. Epithelial disruption, however, may not be the only mechanism that results in net fluid loss. One of the notable pathological changes associated with clinical disease is intestinal inflammation (Ketley 1997). The virulence determinants are generally not well characterized and some are rather controversial. In association with food or water, *Campylobacter* enter the host intestine surviving the stomach acid barrier and colonize the distal ileum and colon. Following colonization of the mucus and adhesion to intestinal cell surfaces, *Campylobacters* perturb the normal absorptive capacity of the intestine by damaging epithelial cell function either directly, by cell invasion or the production of toxins, or indirectly, following the initiation of an inflammatory response. As these possible mechanisms are not mutually exclusive, any combination

may have a role depending on the host status and attributes of the infecting strain (Ketley 1997). Effective colonization requires chemotaxis. Thus, *Campylobacters* have mechanisms to detect chemical gradients and linked motility functions that enable the cell to move up or down the gradient (Takata *et al.* 1992). Motility of *Campylobacter* spp. necessitates the production of the flagellum, the best characterized virulence determinant of *Campylobacter*. A combination of the flagellum and cell shape is believed to give campylobacter an unusually high level of motility in viscous environments. Observations suggest that, at high viscosity, cell shape and flagellar conformation (or both) may change and this perhaps results in *Campylobacter* remaining motile with longer path lengths. This behaviour has relevance to the penetration of the mucous that overlays the intestinal epithelium. The adhesion to host cells is not actually necessary as the bacterium is able to remain in the intestine by successfully colonizing the mucus. *C. jejuni* and *C. coli* are certainly able to adhere to tissue culture cells without subsequent invasion (Everest *et al.*1992). Assume that adhesion to the epithelial cell surface is necessary for subsequent invasion of the cell, although adhesion and invasion may not be needed for intestinal colonization *per se* (Lee *at al.* 1986). In order to colonize the intestine, *Campylobacters* must be able to compete with the resident flora and to avoid non-specific host defences. Iron is an essential element for all living organisms and pathogenic bacteria obtain this throughout the infection process. *Campylobacters* have not been shown to produce siderophores, but they are able to use exogenous siderophores (Field *et al.* 1986). There is evidence supporting a role for host cell invasion in *Campylobacter*-mediated disease. Inflammation and bacteraemia strongly suggest that cellular invasion is an important pathogenic mechanism (Ketley 1997). *Campylobacter* may cross the intestinal epithelium by translocation or epithelial cell invasion followed by cell lysis. An additional epithelial translocation pathway would

be via M cells. Such a route has been proposed for several enteric pathogens including *Shigella* (Sansonetti *et al.* 1991). Intestinal infection with *Campylobacter* is often associated with an inflammatory response where polymorpho-nuclear leucocytes (PMNLs) and monocytes infiltrate the intestinal epithelium (Russell *et al.* 1989). Given this inflammatory response, the interaction between campylobacter and professional phagocytes is of potential importance (Ketley 1997). Although tissue invasion could be solely responsible for the clinical picture resulting from infection, toxins may also contribute to the disease process. The production of toxins is another unclear area. *Campylobacter* spp. reportedly produces a variety of toxins including a cholera-like toxin (CLT) and several cytotoxins (Ketley 1997). Enterotoxin production by *C. jejuni* was first described in 1983 (Ruzi-Palacios *et al.* 1983). The evidence for a role and even the production of the enterotoxin is not convincing. In support (reviewed by Wassenaar 1997), there is a cell product that, like cholera toxin, elongates CHO cells which is detected with a GM1-based ELISA and fluid cross reacts immunologically with *E. coli* labile toxin (LT) and *Vibrio cholera* toxin (CT), has been partially purified and specific antisera raised to it. CLT production was additionally reported to correlate with the watery diarrhoea observed in patients in developing countries (reviewed by Guerrant *et al.* 1992). A newly characterised heat-labile and trypsin-sensitive cytotoxin is known as cytolethal distending toxin (CDT). In the last decade, the gene encoding CDT, *cdtB*, has been found in the *Campylobacter* spp. (Pickett *et al.* 1996) and in several sero-types of *E. coli* (Elwell *et al.* 2001 and Tóth 2003). With the exception of *Salmonella enterica serovar typhi* in which only cdtB gene is found (Haghjoo and Galán 2004), in all other microbes CDT consists of a tripartite polypeptide complex of CdtA (30 kDa), CdtB (29 kDa) and CdtC (21 kDa, encoded by the corresponding genes *cdtA, cdtB and cdtC* (Lara-Tejero & Galán 2002). Although considerable divergences in the predicted amino acid sequences of each

CDT subunit exist among different microbes, the *cdtB* genes have conserved structural similarities to the mammalian DNase1 catalytic and Mg^2-binding sites (Lara-Tejero & Galan 2000). There is some evidence that CDT is a virulence determinant for naturally occurring and experimentally induced enteric diseases associated with *C. jejuni* (Fox *et al.* 2004), *E. coli* (Pandey *et al.* 2003) and *Shigella* (Russell *et al.* 1989). A recent study showed that cdtB gene and CDT activity in *C. jejuni* strains isolated from healthy macaques and from macaques with clinical signs of diarrhoea. Although the cdtB gene also present in *C. coli* strains isolated from symptomatic and asymptomatic macaques, only lysates obtained from the *C. jejuni* isolates caused cytoplasmic and nuclear enlargement to gather with cell cycle arrest of HeLa cells in the G2/M phase that is characteristic of CDT activity (Dassanayake *et al.* 2005).

1.10.1.3 *Salmonella* spp.

The genus *Salmonella* is part of the family of enterobacteriaceae. On the basis of host preference and disease manifestations in man, the salmonellae can be conveniently placed into two broad categories: First *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, and *S. paratyphi* C. These serotypes are primarily host-adapted to man and cause a bacteraemic illness also known as enteric fever in which diarrhoea rarely plays a major role. Second; *S. enteritidis, S. typhimurium* and other serotypes, the order of prevalence is variable according to geography and time (Hohmann 2001). They have a narrow host range and occasionally infect humans. This usually involves invasive infection (Chiu *et al.* 1999).

Salmonella are important pathogens in human and animals. They are classified according to toxins to two species in the genus salmonella: *S. enterica* (six subspecies) and *S. bongori* (one subspecies). Some serovars such as *S. typhi* and *S. paratyphi* are highly adapted to the human without other known natural hosts, while others such as *S. typhimurium* have a broad host range and infect a wide variety of animal hosts. A few

serovars such as *S. choleroesuis* have a narrow host range and occasionally infect humans (Chiu *et al.* 2005).

Morphology: *Salmonella* spp. are Gram-negative bacilli, motile and non-capsulated.

Epidemiology The organisms are widely distributed in the animal kingdom. Domestic animals, notably cattle, pigs and poultry are frequent excretors and many wild animals are further infected. Household pets such as dogs, cats, birds and turtles are all potential, albeit rare, sources of human infection. Human cases and convalescent carriers are also critical sources. Inadequately cooked meat or precooked foods are contaminated by raw meat in the kitchen and these represent important vehicles of transmission. *Salmonella* may survive deep freezing and adequate thawing is essential prior to cooking (Hart and Shears 2003). Transmission of infections by *Salmonellae* is facilitated by low standards of personal and communal hygiene which facilitate faecal-oral transfer of micro-organisms; animals can be vectors, and food-borne transmission is considerable. Poultry or its products are involved as vehicles of transmission in nearly 50% of outbreaks (Duguid and Northe 1991). In recent years, fresh shell hen eggs infected through vertical transmission have emerged as an important source of *S. enterditis* infection in both Europe and USA (Cowden 1990). In developing countries, the epidemiological pattern is different as large-scale rearing of food animals is not common and methods of cooking differ. *Salmonella* is uncommon in adults.

Pathogenesis These bacteria grow intracellularly, a condition that seems to be facilitated by inhibition of phagocytic respiratory burst and phagolysosome fusion. This capacity has been shown to be genetically regulated (Field *et al.* 1989). Enterotoxins similar to *E. coli* ST and LT have been described for some *Salmonella*.

1.10.1.4 *Shigella* spp.

Shigellosis, the acute enteric infection caused by bacteria of genus *Shigella,* has a world-wide distribution with an estimated annual incidence of 164.7 million cases, of

which 163.2 million occur in developing countries leading to 1.1 million deaths. Sixty-nine percent of all episodes and 61 percent of all *Shigella*-related deaths involve children younger than 5 years old (Kotloff *et al*. 1999).

Shigella spp. are aerobic, gram negative bacilli, non-motile and non-capsulated.

Epidemiology Shigellosis is common in many tropical countries and can cause large epidemics of diarrhoea. *Shigella* infection spreads by the faecal-oral route. Because of the low infectious dose of 10 to 100 organisms, person-to-person transmission is probably the most common (Qzuah 1998). However, transmission by contaminated food, drinking water, swimming pools, and flies has also been documented (Edwards 1999). The highest incidence of Shigellosis is in young children; usually those 1 to 5 years old (Kotloff *et al*. 1999 and Prado *et al.* 1999).

In developed countries, *Sh. sonnei* is the most common species; reports from several locations demonstrate an increase in its relative prevalence in the last several years (Edwards 1999 and Kotloff *et al*. 1999). In developing countries, *Sh. flexneri* is most common, with outbreaks that are often caused by *Sh. dysenteriae* (Qzuah 1998).

Pathogenesis A key element in the pathogenesis of shigellosis is the ability of the bacteria to invade colonic mucosa. The invasion process depends on a 210-to 220-Kb plasmid, which is necessary, but not sufficient, for full virulence (Edwards 1999, Sansonetti 2001 and Ashkenazi 2002). Sequencing the virulence plasmid of the *Sh. flexneri* 5a strain M90T has allowed investigators to identify the genes encoding approximately 25 proteins secreted by the TTSS (Buchrieser *et al*. 2000). A 31-kb segment of the virulence plasmid, called the entry region or *ipa*/*mxi-spa* locus, *comprises* the pathogenicity island, which is actually sufficient for entry of *Shigella* into intestinal epithelial cells, for apoptotic death of macrophages and activation of polymorphonuclear leukocytes (Ingersoll *et al*. 2002).

Shigella spp. produce two enterotoxins, ShET-1 and ShET-2. The former is produced mainly by *S. flexneri* serotype 2a whereas the latter is produced by all *Shigella* spp. (Vargas *et al.* 1999). Shiga toxin, encoded chromosomally and found mostly in *Sh. dysenteriae* serotype 1 is a potent protein-synthesis inhibitor that primarily targets vascular endothelium (Sandving 2001 and Bitzan & Loo 2003). This toxin mediates the Heaemolytic Uraemic Syndrome (Bitzan & Loo 2003). These enterotoxins may contribute to the profuse volume and watery diarrhoea often observed in initial stages (Ashkenazi 2004).

1.10.1.5 *Vibrio cholerae*

The genus *Vibrio* is composed of many species of curved bacilli, where 12 species have been implicated in human infections. *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are the most prominent. Vibrios are comma-shaped, aerobic, Gram-negative bacteria which have a characteristic darting movement.

Epidemiology: *V. cholerae* needs to be considered among the possible causes in children living in endemic areas (currently south Asia and Africa). *V. cholerae* O1 and *V. cholerae* O139 can cause epidemics and pandemics in areas of extreme poverty, and in areas with massive population migrations associated with natural disasters or long-lasting wars (Sack *et al.* 2004). Cholera is also endemic along the Gulf Coast of the USA. The disease is closely associated with poverty, overcrowding and low socio-economic status (Cook 2003). Humans are the only known natural hosts of *V. cholera*. Transmission is by ingestion, of contaminated water or food. The infective dose is high, up to 10^{11} c.f.u bacteria being required. The seventh pandemic, which was caused by *V. cholerae* O1 biotype El Tor, began in Asia in 1961 and spread to Africa, Europe, and Oceania in the 1970s and 80s. The largest epidemics of cholera since the mid 1990s have been in Africa. By June 1995, more than 1 million cases and 10000 deaths resulting from the disease had been reported in the Americas. In East Africa,

excessive rain and flooding in 1997 were followed by extensive outbreaks of cholera in Somalia and northern Kenya. In August 2000 there was an extensive outbreak in South Africa, with over 75,000 cases reported in March 2001 (Hart and Shears 2003).

Pathogenesis *Vibrio cholerae* is non invasive; Pathogenesis is due to an enterotoxin that causes excessive fluid and electrolytes loss. Cholera toxins (CT) consist of two subunits A and B. A subunit comprising two polypeptides, A1 and A2, linked by a disulfide bond. The B subunit binds with high affinity and specificity to GM1, a ganglioside present in the epical membranes of all intestinal epithelial. The A subunit is then able to migrate through the epithelium cell membrane. This subunit leads to a transfer of ADP ribose from necotinamide-adenosine dinucleotide (NAD) to GTP resulting increase in cAMP production which, by inhibiting sodium chloride absorption and stimulating chloride excretion, leading to loss of water and electrolytes. Accessory cholera exotoxin was discribed in 1993, and increases transmembrane iron transport (Hart and Shears 2003). Additional toxins and other factors are now known to be involved in cholera pathogenesis. Zonula occludens toxin (Zot) increases the permeability of small intestinal mucosa by affecting the structure of intrercellular tight junctions (Colombo *et al.* 1994).

1.10.1.6 *Yersinia enterocolitica*

Y. enterocolitica may cause illness ranging from a self-limited enterocolitis to a potentially life-threatening systemic infection (Cover and Aber 1989). Septicemia caused by *Y. enterocolitica* may occur in normal hosts as well as in those with underlying disorders (Bottone 1997). *Y. enterocolitica* invades ileal enterocytes and M cells in Peyer's patches, where it multiplies. This produces an inflammatory diarrhoea. Bacteria may pass to local lymph nodes, then produce systemic disease (Hart and Shears 2003).

Epidemiology All *Yersinia* infections are zoonotic, with humans being the accidental

hosts. These are worldwide in distribution, but are more frequently found in temperate zones than in the tropics. Even in temperate countries infection is more prevalent in colder climates and is more common in the winter (Cover and Aber 1989). In most surveys of acute diarrhoeal disease where *Y. enterocolitica* was sought, it was either absent or present in less than 1% of cases (Gomes *et al.* 1991). The reservoir for *Y. enterocolitica* is a variety of animal species including: birds, frogs, fish, snails, oysters and most mammals. The organism is excreted in faeces from pigs and cattle. *Y. enterocolitica* causes a range of gastrointestinal illnesses in humans and is primarily transmitted via the consumption of contaminated food or water (Bottone 1997).

Pathogenesis pathogenic *Yersinia* spp. Including: *Y. enterocolitica, Y. pseudo tuberculosis* and *Y. pestis,* carry a virulence plasmid (pYV) harboring genes for protein TTSS, a set of up to 6 effector proteins *Yersinia* outer proteins (Yops) and regulators for gene expression and Yop secretion. When contacted with target cells, Y*ersinia spp* employ their TTSS to translocate six effector Yops (YopH, YopO/YpkA, YopP/YopJ, YopE, YopM and YopT) in to the cytoplasm. These interfere with a variety of cell functions including adhesion, phagocytosis, superoxide anion release, and cytokine secretion and impair the balance of survival versus apoptosis (Cornelis *et al.* 1998). It has recently become evident that polypeptides secreted by the type III machinery may represent an exception to this widely held paradiagm (Cornelis 2003).

1.10.1.7 *Clostridium* spp.

The organisms are present in soil, water, and sewage and as part of the normal microbial flora in the gastrointestinal tracts of animals and humans. Two species *Clostridium difficile* and *Clostridium perfringens* are associated with diarrhoeal disease (Hart and Shears 2003).

1.10.7.1 *Clostridium difficile*

Clostridium difficile is a major cause of nosocomial colitis, generally occurring after

antibiotic-induced alterations of bowel flora (Bartlett 2002). *Cl. difficile* has been recognized as a cause of antibiotic-associated pseudomembranous colitis. The organism and toxin can be detected in asymptomatic infants but their presence in older individuals is related to disease (Hart and Shears 2003).

Epidemiology The organism is ubiquitous. *Cl. difficile* is part of the normal intestinal flora in a small number of healthy people and hospital patients. Although the bacterium can be found worldwide it is probably an unusual cause of diarrhoeal disease in developing countries (Griffin 1989). *Cl. difficile* is responsible for almost all cases of PBC and for 15 to 25% of cases of antibiotic-associated diarrhoea (Bartlett 2002).

Pathogenesis Two large toxin proteins: TcdA or toxin A and TcdB or toxin B are thought to be primary virulence of *Cl. difficile.* These toxins are encoded by two separate genes, named *tcdA* and *tcdB* together with three additional genes they form a 19.6-kb pathogenicity locus.

1.10.7.2 *Clostridium perfringens*

Two forms of diarrhoeal disease are associated with *Cl. perfringens*. The first is a food-poisoning illness due to ingestion of *Cl. perfringens* types A or the α-toxin (enterotoxin) it produces. Type A strains of *Cl. perfringens* cause food poisoning with abdominal cramps and diarrhoea, soft tissue infections and primary septicaemia. The second *Cl. perfringens* type C, in contrast, is common in certain areas of the tropics and produces a severe necrotic enteritis (Hart and Shears 2003).

Epidemiology: *Cl. perfringens* type C infection may occur sporadically but also in epidemics. It occurs at any age but is more likely to present as acute toxic or acute surgical problems in children under 10 years of age (Shann *et al* 1979).

Pathogenesis The pathogenicity of *Cl. perfringes* is potentially attributed primarily to toxins and enzymes produced by this organism. *Cl. perfringens* type C produces both

α and β-toxins which, it is presumed, are responsible for disease manifestations (Hart and Shears 2003). A-toxin, the most important toxin and the one produced by all types of the *Cl. perfringens*, is a lecithinase that lyses erythrocytes, platelets, leucocytes and epithelial cells. β-toxin is responsible for the necrotic lesions in necrotizing enteritis.

1.10.2 Enteric viruses

A variety of viruses can be found in the gastrointestinal tract. These include non-pathogenic bacteriophages and human viruses that use the gastrointestinal tract as a portal of entry such as enteroviruses, hepatoviruses and some adenoviruses (non-group F). The latter group rarely if ever cause diarrhoeal disease. (Hart *et al.* 2003).

Rotavirus causes from 25 to 70% of GE cases in children severe enough to require hospitalization (Parashar *et al.* 2003a). Astrovirus, human caliciviruses and enteric adenovirus together represent as many as 20 to 30% of cases of diarrhoea in areas where bacterial infections are found less commonly (Ó Ryan, *et al.* 2005).

1.10.2.1 Rotaviruses

Rotaviruses are the leading cause of severe, dehydrating gastroenteritis among children, accounting for one-third of all hospitalization for diarrhoea and an estimated 500,000 deaths worldwide each year (Glass *et al.* 2003). Rotavirus infection is found as frequently in developed as in developing countries. Rotaviruses are classified by group, subgroup, and serotypes. Seven groups (A–G) of human and animal rotaviruses have been identified. The majority of human infections are with this group A rotaviruses. Group B rotavirus infections appear to be uncommon but have been associated with large outbreaks of infection in China and India (Krishnan *et al.* 1999). Up to a third of adult humans have serological evidence of infection with group C. Rotavirus is a member of the family Reoviridae.

Epidemiology Human rotavirus is a major cause of gastroenteritis among infants and young children and is the most common cause of severe dehydrating diarrhoea in this age group. In hospital-based surveys it is found to be responsible for 25-65% of cases, while in community-based surveys rotavirus is detected in 5-40% of cases. The peak of infection in most parts of the world occurs from 4 months to 3 years. There is a difference in the peak ages of infection between developed and developing countries. The median age of children hospitalized with rotavirus gastroenteritis in Africa was 6 months and 81% were under 1 year, whereas in developed countries the median age is over 11 months. The total prevalence of rotavirus illness in children in developing and industrialised nations was estimated to be approximately 111 million episodes of gastroenteritis that require home care only, 25 million clinic visits, and 2 million hospitalizations in children <5 years of age worldwide (Parashar *et al*. 2003a). Rotavirus is assumed to be transmitted person to person by the faecal-oral route. Air-borne transmission may also be possible.

The infective dose is low (10^2-10^3 virus particles). The incubation period is usually 1-3 days (Hart *et al* 2003).

Pathogenesis Rotavirus infects the mature villous enterocytes of the upper small intestine. It is unable to infect the immature villous crypt cells or colonic enterocytes. Rotavirus enters the cell by endocytosis or by direct penetration if VP4 has been cleaved to VP5 and VP8. Rotavirus non-structural protein 4 (NSP4) is an enterotoxin that was identified as an important agent in symptomatic rotavirus infection. Three mechanisms have been described for the pathogenesis of rotavirus diarrhoea. In the first 12-24 hours post infection enterocytes are intact but levels of the brush border disaccharides (sucrose, maltose, lactose) are greatly decreased (Batt *et al*. 1995). This is apparently due to interference with transport of the enzymes to the brush border (Jourdan *et al*. 1998). The result of this is that disaccharides in the diet cannot be

hydrolysed to monosaccharides and thus cannot be absorbed, producing an osmotic diarrhoea. Secondly, NSP4, which is involved in rotavirus assembly, has a collateral effect in opening calcium channels in the enterocyte. This causes an efflux of sodium and water and thus a secretory diarrhoea (Ball *et al.* 1996). Finally, the raised intra-enterocyte calcium concentration causes them to die by necrosis (Perez *et al.* 1998). Recent studies indicated that a specific cleavage product of NSP4 (aa112 to 175) is actively secreted from infected cells and that NSP4 is released from the apical side of infected epithelial cells via an atypical pathway that bypasses the Golgi apparatus cells and involves lipids microdomains called rafts (Zhang *et al.* 2000 and Sapin *et al.* 2002).

1.10.2.2 Adenovirus 40/41

Human adenoviruses are subdivided into six subgenera (A-F), which contain a total of 49 serotypes. Only types 40 and 41 (subgenes F) have been clearly associated with diarrhoeal disease. In most surveys they are second in importance to rotaviruses in causing GE, being responsible for 0.9-11% of cases in hospitalized children. Similarly in community-based studies adenoviruses have been shown to be important causes of childhood diarrhoea (Cruz *et al.* 1990). Most infections occur in children under 2 years (Barners *et al.* 1998). Diagnosis: Electron microscopy is unable to distinguish adenovirus 40/41 from other adenoviruses but immuno-electron microscopy can. There are now a number of antigen detection kits including ELISA and LPA, which are highly sensitive and specific. Genomic detection is additionally by DNA hybridization or PCR (Hart *et al.* 2003).

Epidemiology Most infections occur in children under 2 years (Barners *et al.* 1998). Enteric adenoviruses belonging to serotypes 40 and 41 have been associated with persistent diarrhoea, generally in children under 4 years (Matson *et al.* 2000). They are

spread from person to person by the faeco-oral route and are food-borne. There is no apparent seasonality for infection (Hart *et al.* 2003).

1.10.2.3 Small round viruses

These viruses are a group of morphologically similar but antigenically unrelated or only partially related small round viruses. These latter are 25-35 nm in diameter. Astrovirus was first described in 1975. It is small (28 nm) round and unenveloped with a star shape. There are at least eight human astrovirus serotypes (Hast V 1-8) but serotype 1 is commonly detected (Shi *et al.* 1994). However, other serotypes can be responsible for outbreaks of food-borne infection (Belliot *et al.* 1997). Caliciviruses are 35-39 nm in diameter. Human caliciviruses (HuCVs) are a group of nonenveloped, small, round viruses that belong to two genera in the family caliciviridia; the noroviruses and sapoviruses (previously called "Norwalk- like viruses" and "saporo-like viruses," respectively). HuCVs, of which noroviruses are the prototype, cause more than 90 percent of outbreaks of acute gastrointestinal illness in the United States and account for approximately 23 million cases of diarrhoeal disease per year, according to the pathogen-associated method of calculation (Mead *et al.* 1999 and Fankhauser 2002). Attack rates have been as high as 41 percent, reflecting the propensity of infection with noroviruses to cause emesis and voluminous stool and vomits and the large number of organisms in stools and vomits, plus the low inoculums (fewer than 100 viral particles) required to produce infection (Musher and Benjamin 2004). Others viruses include: the coronavirus (Zhang *et al.* 1994), torovirus (Jamieson *et al.* 1998) picobirnaviruses (Ludert & Liprandi 1993) and pestiviruses (Yolken *et al.*1989). They have been found to be associated with diarrhoeal disease. The etiologic role of noroviruses in moderate to severe childhood GE in developing countries is being studied, but primarily data from developed countries indicate that they may be the second most common viral agent after rotavirus, accounting for 4 to

19% of episodes of severe GE in young children (Pang *et al.* 1999). Noroviruses are the most common cause of GE outbreaks and affect all age groups, whereas sapoviruses invariably affect children (Fankhauser *et al.* 2002 and Rockx *et al.* 2002).

1.10.3 Parasitic agents

Human intestinal protozoan infections are discovered worldwide in both developing countries and the industrialized world. Pathogenic intestinal protozoa produce disease by infecting the small or large intestine, or both. Intestinal protozoa are found in highest prevalence in developing countries, where they are responsible for a substantial burden of disease. The small intestinal protozoa *Giardia lamblia*, *Cryptosporidium parvum* (*Cr. Parvum*) and *Cyclospora* have their major impact in children, while the large bowel pathogen *Entamoeba histolytica* infects all age groups but has its most profound effects in adults.

1.10.3.1 *Entamoeba histolytica*

Clinical features: Diarrhoea is the major symptom and is watery initially but subsequently develops the features of steatorrhoea often associated with nausea, abdominal discomfort, bloating and weight loss. Although it is self-limiting in the majority of healthy immunocompetent people, possibly 30-50% proceed to suffer persistent diarrhoea, usually with features of steatorrhoea.

Epidemiology Amoebiasis, the infection caused by the parasitic protozoan, *Ent. histolytica,* is responsible for 70,000 deaths per annum (Anon. 1998). Spreading occurs *via* the faecal-oral route and food and drink contaminated through exposure to human faeces. Crowding and poor sanitation contribute to its prevalence in Asia, Africa and Latin American. Approximately 10% of the world's population is infected, yet 90% of infected persons are asymptomatic. Epidemics occur when raw sewage contaminates the water supply. High risk groups include travellers,

immunocompromised individuals and possibly children in day-care centres. Severe infections occur in very young children, pregnant women, the malnourished, and individuals taking corticosteroids (Farthing *et al.* 2003).

Pathogenesis the cyst of *Ent. histolytica* is resistant to gastric acid, and on ingestion it passes into the small intestine. The amoeba within the cyst becomes active in the neutral or alkaline environment of the small intestine. Trophozites use the galactose and N-acetyle-D-galactosamine (Gal/galNAc)-specific lectin to adhere to colonic mucins and colonize the large intestine (Petri *et al.* 2002). Several virulence factors have been identified, such as adhesion molecules, contact-dependent cytolysin, proteases, haemolysins and phagocytic activity. Trophozoites must subsequently penetrate the mucous layer and adhere to the host cells. *Ent. histolytica* enhance mucous secretion, alter its composition and deplete goblet cells of mucin, thereby rendering the epithelial surface more vulnerable to invasion. Once the mucous barrier has been broken down, *Ent. histolytica* reaches the luminal surface of enterocytes and initially produces a contact-dependent focal and superficial epithelial erosion. Invasion of the colonic and caecal mucosa by *Ent. histolytica* begins in the interglandular epithelium (Farthing *et al.* 2003).

1.10.3.2 *Giardia lamblia*

Epidemiology: Giardiasis is found worldwide but has a high prevalence in the developing world, where prevalence rates can reach 20-30%. In the industrialized world, prevalence varies between 2% and 5%. Age-specific prevalence rises throughout infancy and childhood declining only in adolescence. Giadiasis is well recognized as occuring in travellers, although overall it accounts for no more than 5% of cases of traveller's diarrhoea. The major reservoirs of *Giardia* cysts are the human host and contaminated surface water. A recent survey in the USA suggested that there may be as many as 2.5 million cases of Giardiasis per year (Furness *et al.* 2000).

Giardiasis is a zoonosis and cross-infection by beaver, cattle, dogs and rodents that they as a constant reservoir. Ingestion of at least 10 to 100 cysts can cause infection in humans. Water–borne transmission probably represents a relatively small proportion of the total infections worldwide. The disease is spread by faecal-oral transmission. Sexual transmission is also known to occur. (Farthing *et al.* 2003).

Pathogenesis when a new host consumes a cyst, the host's acidic stomach simulates cysts for liberate of trophozoites which migrate to the duodenum and proximal jejunum, where they attach to the mucosal wall by means of a ventral adhesive disk. *Giardia* growth in the small intestine is stimulated by bile, carbohydrates and low oxygen tension. However, recent work in tissue culture cell lines has revealed that Giardia trophozoites induce localized condensation of F-actin and loss of perijuctional α-actin (Teoh *et al.* 2000). There is increasing evidence to suggest that T-cell activation within the intestinal mucosa can produce villous atrophy (Scott *et al.* 2000). A recent theory suggests that the symptoms are the result of a brush border enzyme deficiency rather than invasion of the intestinal wall. There is some evidence to suggest that *Giardia* trophozoites produce cytopathic substances that might be responsible for this disruption of epithelial structure and function. *Giardia* is known to produce a variety of proteinases that could find cleavage sites in proteins of the microvillous membrane (Farthing *et al.* 2003).

1.10.3.3 *Cryptosporidium*

Characteristics; the infected form is the oocyst, which has probably already sporulated before shedding, excystation happens in the small intestine, with four sporozoites being released from each oocysts. The sporozoite attaches to the apical membrane of the host epithelial cell where that attachment induces reorganization of the host-cell actincytoskeleton and protrusion of the host-cell membrane around the sporozoite to form a vacuole in which the organism remains intracellular but extracytoplasmic

(Chen & La Russo 2000 and Tzipori & Widmer 2000). Restriction fragment length polymorphism (RFLP) analysis was the first technique to illustrate variation between isolates of *Cryptosporidium parvum* by PCR sequence analysis, especially of the rRNA gene loci. These techniques broadly confirm that the species *Cr. parvum* comprises at least two major genotypes, one of which infects humans exclusively and the other with a broader range of host specificity infecting animals besides humans (McLauchlin *et al.* 1999).

Epidemiology Data from the Public Health Laboratory Service in North Wales, based on diagnostic laboratory returns, show an age distribution of infection, with peak between 1 and 5 years, and a marked reduction over the age of 35 years (Casemore 1990). Cryptosporidial infection accounts for 2.2% of cases of diarrhoea in immuno-competent persons in developed countries and 6.1% of cases of diarrhoea in immuno-competent persons in developing countries. Cryptosporidial infection is more common in immunocompromised persons, especially those with Acquired immunodeficiency syndrome (AIDS). In developed countries, it occurs in 14 % of patients with AIDS and diarrhoea; in developing countries, it occurs in 24% of such patients (Guerrant 1997). In the developed world cryptosporidiosis is found in outbreaks clustered around day-care nurseries. Substantial evidence has accumulated implicating *Cr. parvum* in outbreaks of water–borne diarrhoea and in stable endemic childhood diarrhoea among the poor of the developing world (Smith & Rose 1990). Infection is frequently spread by person-to-person transmission, by animal and indirectly through the environment (mainly water). Zoonotic transmission from cattle and sheep to human is known, and these animals are the most significant reservoirs of human disease. As few as 10 to 100 oocysts can cause infection (Chen *et al.* 2002).

Pathogenicity *Cr. parvum* attaches to the microvillous membrane and causes loss of microvilli and results in malabsorption. An enterotoxin-like activity has been detected

in faecal extracts, which may cause abnormal absorption and impaired epithelial permeability, but no enterotoxin has been purified (Farthing 2000). The organism activates secondary signal pathways in the host cell, thereby altering cell function (McCole *et al.* 2000). *Cr. parvum* activates the nuclear factor-*k*B (NF-*k*B). Activation of NF-*k*B induces the production of cytokines and chemokines, such as interlukin-8, to trigger an inflammatory reaction and stimulates an anti-apoptonic survival signal in directly infected cells (McDoland *et al.* 2000). *Cr. parvum* induces secretion of 5-hydroxytryptamine and prostaglandin E_2 into the lumen. Enterotoxin activity, which produces chloride secretion *in vitro*, has been detected in faecal extracts from infected calves (Chen *et al.* 2002). *Cr. parvum* also induces apoptosis in epithelial cells, resulting in damage to epithelial barrier.

1.10.3.4 *Cyclospora*

Cyclospora is a coccidian parasites that is taxonomically related to *Isospora species*, C*ryptosporidium parvum*, and *Toxaplasma gondi*. A single species infecting humans, *Cyclospora cayetanensis*, has been identified. Each oocyst has two sporocysts. Excystment results in the liberation of two sporozoites which are crescent-shaped approximately 9 µm in length and 1.2 µm in width (Ortega *et al.* 1993).

Epidemiology *Cyclospora* spp. were first identified in individuals with a history of foreign travel and those infected with AIDS. The global prevalence of the infection is unknown, although a prevalence of 4-7% has been reported in foreign residents in Nepal, with peak prevalence rates occurring during the warmer months with higher rainfall (Hoge *et al.* 1993). *Cyclospora* caused diarrhoea has now been described in the Americas, the Caribbean, Africa, Bangladesh, South-East Asia, Australia, England and Eastern Europe. Transmission appears to be by the faecal-oral route, with water being the foremost vehicle. However studies conducted in Guatemala and Peru suggested that having certain animals (e.g., dogs, chickens and ducks) increased the

risk for human infection by *Cyclospora* (Bern *et al.* 1999 and 2002). The first waterborne outbreaks in the USA were reported in 1995 (Huang *et al.* 1995). However, in 1996 a major outbreak of Cyclosporiasis was investigated in the USA which was found to be due to the ingestion of Guatemalan raspberries (Herwaldt *et al.* 1997).

Pathogenesis The mechanism by which this organism produces diarrhoea has not been clearly established. Still, the organism occupies an intracellular location within enterocytes, and histological examination of small intestinal biopsies has demonstrated mild reduction in villous height, with associated mucosal inflammation and increased numbers of intraepithelial lymphocytes (Bendall *et al.* 1993). No specific virulence factors have yet been identified. Diarrhoea caused by *Cyclospora* infection has been associated with the development of GBS (Farthing *et al.* 2003).

1.11 Detection and diagnosis of enteric pathogens

There are over 40 different enteropathogens with the ability to cause gastroenteritis, although not all are sought even in the best-funded laboratories (Hart *et al.* 2003). Detection and diagnosis of aetiological causes of acute diarrhoea due to enteropathogens (bacterial, viral and parasitic) infections are the most common agents detected in the standard laboratory. Assessment of stool characteristics is a key feature in determining potential pathogens causing an acute diarrhoea episode. Liquid non-bloody stools are associated predominantly with a secretory/small intestinal dysfunction process, whereas dysenteric (blood and pus) or bloody stools are generally associated with an inflammatory/invasive process of the colon. Mucous can be present in both situations and should be differentiated from pus. History and clinical features assist in proper investigations that can lead to a swift and accurate diagnosis. Stool culture to isolate the pathogens is still the cornerstone of diagnosis (Edwards 1999). When a stool specimen is received by a laboratory, firstly inspection of colour,

consistency, odour and presence of mucous or blood occurs. The stool will be divided into parts, one for examination by light microscope to detect any ova, cyst and then to search for faecal leukocytes and spiral bacteria, whilst second part is for bacterial culture. The third part is for viral detection and the residual is placed into a refrigerator for further investigation.

1.11.1 Bacterial isolation

Specific diagnosis of aetiological bacterial cause depends on stool culture on different media and other identification and confirmation tests: serological typing, detection of toxin, biochemical tests (API) and Polymerase Chain Reaction (PCR). The latter is one of the molecular biology techniques now available; these have much greater sensitivity, provide results within a short time and may prove useful for organisms that are difficult or slow to culture.

Stool culture on media: MacConkey agar, *Salmonella-Shigella* agar and *Campylobacter* Blood-Free Selective agar medium. Blood agar for isolation of *Escherichia coli, Salmonella, Shigella* and *Campylobacter,* respectively, are the most common types of media used. Tetrathionate broth, peptone-sorbitol bile broth, and alkaline peptone water were used as enrichment media for *Salmonella, Yersinia* and *Vibrio* bacteria, in that order. The plates are incubated at 35-37 ˚C for 18-24 hours, except for Campylobacter media, which is incubated at 42˚C for 48-72 hours in anaerobic jar. After the incubation period, if there is growth, the morphology of colonies (size, colour, shape, haemolysis and odour) will be noted. If the types of colony vary, it will be necessary to perform a subculture to obtain pure colonies for bacterial type.

The second step is direct microscopy following staining (Gram stain) for rapid classification and identification of bacterial organism according to the morphological

feature of each organism.

Escherichia coli spp. are Gram negative bacilli, capsulated and motile: their size range from 1.1 to 1.5 μm in width and 2.0 to 6.0 μm in length and the colonies are pink in colour due to fermentation of lactose on MacConkey agar medium.

Identification is performed by using a conventional bacteriological technical method and confirmed by serology using appropriate antiserum (slide and tube agglutination tests). Two toxins of *E. coli* production are LT and ST toxin. A GM1 receptor ELIZA is performed to test for LT production. Commercial competitive STEIA (Oxoid) is used for detection of ST production. Non-fermenting *E. coli* colonies on sorbitol MacConkey palates will be tested by anti-O:157 serum to investigate (Shiga toxin-producing *E. coli*).

Other identification tests are phage typing and commercial biochemical-based identification system API 20E (bioMerieux, France) which are used for identification and confirmation tests.

PCR technique for diarrhoeaginic of *E. coli;* after preparation of Deoxy ribonucleic acid (DNA) templates for PCR. A smear of *E. coli* is inoculated into a tube with 5 ml of phosphate-buffered saline then boiled for 20 min followed by centrifugation at 2.500 xg for 10 min. The supernatant is placed in a PCR machine after preparation of programme (Multiplex PCR assay). The DNA templates are subject to multiplex PCR with specific primers for the detection of the virulence markers: *eaeA* (gene of *EHEC* and *EPEC*), *vt1* and *vt2* (*Shiga toxin* 1 and 2 of *EHEC*), *eltB* and/ or *estA* (gene of *ETEC)*, *ial* (gene of *EIEC*) and pCVD (gene of *EAEC*).

Campylobacter spp. are Gram negative rods, slim (1.5-6.0 μm long and 0.2-0.5 μm wide), which are spirally curved, with tapering ends. They are microaerophilic and grow best in the presence of 5% O_2, 10% CO_2, 85% N_2. The cell usually possesses a polar flagellum at one or both ends. Motility is of the darting type with a corkscrew

like movement. They are microaerophilic and grow best in the presence of 5% O_2, 10% CO_2, 85% N_2. The organisms grow well on selective media Modified Charcoal Cefoperazone Desoxycholate Agar blood free medium (CCDA), (Oxiod) placed in an anaerobic jar and incubated at 42° C for 48 hours. Colonies that arise are colourless or grey with a metallic sheen. They are oxidase and catalase positive but urease negative, hippurate test is positive in *C. jejuni* and negative in *C. coli*. Commercial biochemical technique test API Campy (bioMerieux, France) is adopted for identification of *Campylobacter.* Further biochemical tests are used for speciation. *C. jejuni* and *C. coli* are sensitive to Erythromycin and Nalidixic acid but *C. lari* is resistant. Commercial kits are available for the direct detection of *C. jejuni* and *C. coli* antigens in faeces, e.g., by latex agglutination (Hindiyeh *et al.* 2000). Serotyping of *Campylobacter* spp by Penner method is the most common method utilised to confirm *Campylobacter* spp (Penner *et al.* 1983). Other serotyping schemes for *C. jejuni* and *C. coli* are based on direct agglutination of heat-stable antigens (Frost *et al* 1998).

PCR-based identification methods can detect several *Campylobacter* spp., including some uncommon species that are difficult to culture (Kulkarni *et al.* 2002).

A novel PCR for the detection and speciation of thermophilic *Campylobacter* spp. (Jackson *et al* 1996). DNA is isolated from pure cultures of these strains (the control is used; the reference strain of *Campylobacter*) by use of lysozyme/sodium dodecyle sulphate lysis/proteinase K digestion. Redissolved samples were diluted 100-fold in pure water, and 50 µl used as the target for amplification. DNA primers and PCR amplification Oligonucleotides BO4263 and BO4264 are synthesized by Appligene (Illkirch, France). The amplification reaction is performed in a volume 100 µl. The PCR machine programme used (programmable thermal reactor) was for denaturation and primer annealing. After completing the reaction, PCR products are visualized by gel electrophoresis on 2% NuSieve 3:1 agrose. The stain then used was

ethidium bromide.

Salmonella spp is a genus, Gram-negative bacilli, motile and non-capsulated.

Cultural characters: aerobes and facultative anaerobes. They grow on nutrient agar, MacConkey's medium, *Salmonella-Shigella* agar medium and (DCA) medium after enrichment in selenite F broth. They produce pale non-lactose fermenting colonies on XLD. They produce pink colonies with or without a black centre. Biochemical activities: *S. typhi* ferment glucose, maltose and mannite with production of acid only. It is indole negative and H_2S positive. *S. paratyphi* ferments glucose, maltose and mannite with production of acid and gas. It is H_2S negative and indole negative. *S. paratyphi* B and C possess the same biochemical activities except that H_2S production is positive. Serological characters: they possess O (somatic) and H (Flagellar) antigens. Biochemical-based identification system API 20E (bioMerieux, France) is utilised as an identification and confirmation test.

Other bacteria can cause acute diarrhoea and need to use specific medium.

Shigella spp. Cultural characters: They produce pale non-lactose fermenting colonies on MacConkey's medium and Desoxycholate citrate agar (DCA) and pink colonies on XLD after enrichment in selenite F broth. Some strains ferment glucose alone with production of acid e.g. *Sh. dysenteriae*. Others ferment glucose and mannite with acid only e.g. *Sh. flexneri*, *Sh. boydii* and *Sh. sonnei*. The latter is a late lactose fermenter. They are indole variable. Four species (serogroups) are defined on the basis of serologic or biochemical reactions, namely, *Sh. dysenteriae*, serogroup A; *Sh. flexneri* serogroup B; *Sh. boydii*, serogroup C; and *Sh. sonnei*, serogroup D (Edwards 1999). Immune assay with monoclonal antibodies to the IpaC protein detected with both *Shigella* spp. and Enteroinvasive *E. coli* in 165 specimens, with high sensitivity and specificity (Szakal *et al.* 2003). Biochemical-based identification system API 20E (bioMerieux, France). Detection of *Shigella* is by a PCR assay targeting the ipaH gene.

Other bacteria can cause acute diarrhoea and need to use specific medium.

Vibrio cholerae is Gram-negative, comma-shaped and motile. Aerobic and facultatively anaerobic Vibrios possess both flagellar and somatic antigens. They are oxidase positive and ferment sucrose and glucose but not lactose. *V. cholerae* strains are divided according to O-antigens; *V. cholerae* 01 is the cause of cholera. Other non-O1 and O139 *V. cholerae* strains may occasionally cause diarrhoea. *V. cholerae* are characterized by an ability to grow in alkaline conditions (pH >8.0). Alkaline broth is used to grow the organism selectively from faecal samples. A special selective medium thiosulphate-citrate-bile salt-sucrose agar (TCBS) medium, *V. cholerae* form characteristic yellow colonies on this medium. Specific slide agglutination reactions for O antigens distinguish *V. cholerae* from non-cholera vibrios. Biochemical tests are further used for confirmation.

Yersinia enterocolitica is a Gram-negative bacillus isolated by culture of stool or blood culture or by serology.

Clostridia are anaerobic sporing Gram-positive rods. There are four species, but only two cause food poisoning; *Cl. perfringens* is a Gram-positive rod with sub-terminal oval-shaped spores which form irregular colonies, it is normally surrounded by a zone of haemolysis on blood agar and ferments a variety of sugars with production of gas. *Cl. perfringens* are recognised based on surface antigens and the major types of lethal toxins. *Cl. difficile* is large Gram-positive bacillus with terminal spores. The organism can be isolated from faeces by anaerobic culture on elective media. Toxin production is confirmed by exposure of tissue culture cells to culture filtrates of the organism. Diagnosis can additionally be made by direct detection of toxin in faeces; incubation of tissue culture cells with faecal filtrates; typical cytopathic effect can be recognized within 48 hours or by use of commercial assays (ELISA, latex agglutination) based on specific antitoxin is available (Elliott *et al.* 1997).

1.11.2 Enteric viruses detection

Four enteric viruses cause diarrhoea in humans: rotavirus, astrovirus, human caliciviruses divided into genera, norovirus and sapovirus and enteric adenoviruses (Ó Ryan *et al*. 2005).

Rotavirus

Many laboratories (especially in developing countries) are unable to detect rotavirus or other enteric viruses as a routine procedure or it may not form part of their protocol due to a scarcity of resources.

Morphology on electron microscopy. It is approximately 75 nm in diameter and has a genome of 11 double-stranded ribonucleic acid segments. It has a characteristic double -shelled capsid which gives it a wheel- shaped appearance, lacks an envelope and is composed of an internal core and an inner and outer capsid. Diagnosis is made by direct detection in faeces by electro microscopy, ELISA or latex agglutination test and PCR

Adenovirus 40/41

Adenoviruses are un-enveloped DNA viruses with an icosahedral capsid 70-90 nm in diameter. Diagnosis is made by detection of virus by electron microscope in faeces. Electron microscopy cannot distinguish adenovirus 40/41 from other adenoviruses whereas immuno-electron microscopy can. There are now a number of antigen detection kits including ELISA and LPA, which are highly sensitive and specific. Genomic detection is also possible by DNA hybridization or PCR (Hart *et al.* 2003).

1.11.3 Parasitic identification

Many parasites are human infections, some pathogenic and others non-pathogenic. The identification of parasites from stool specimens requires good personal experience and facilities in laboratories enable to detect parasites and differentiate between

pathogenic and non-pathogenic. A large number of pathogenic parasites cause diarrhoea, but the most common (especially in children) which cause acute diarrhoea are *Ent. histolytica*, *G. lamblia* and *Cryptosporidium.*

Entamoeba histolytica is distinguished from other intestinal amoeba by morphological characteristics of diagnostic importance. The classic stool ova and parasite examination whereby *Ent histolytica* is identified by its appearance in trichorome- or iron hematoxylin-stained stool specimens. This is insensitive and cannot differentiate *Entamoeba histolytica* from the non-pathogenic *Entamoeba dispar* (*Ent. dispar*) because, identical-appearing parasite (Haque *et al.* 2003a). The size of trophozoite (infective stage) is large and ranges from 20 to 40 μm in diameter. Under examination by light microscope, trophozoite appears to have two zones within cytoplasma; an outer zone and an inner zone in diameter. The cysts are round or oval and 10-16 μm in diameter, in immature cyst a single nuclei is present and mature infective cyst contain 4 small nucleui. The diagnosis should ideally be based on the detection in stool of *Ent. histolytica*-specific antigens or DNA and by the presence of antibodies in serum (Haque *et al.* 2003a). Several biological differences have been described between *Ent. histolytica* and *Ent. dispar*, but none fully explains why *Ent. dispar* is unable to produce invasive disease. *Ent. dispar* produces less protease (Bruchhaus *et al.* 1996), it does not bind to target cells as strongly and is less cytotoxic, has a thinner glycocalyx, higher surface charge and has less phagocytic activity than *Ent. histolytica* (Espinosa-Canntellano *et al.* 1998). Diagnosis of amoebic colitis by antigens capture ELISA demonstrate higher isolation rate of *Ent. histolytica* (Abd-Alla and Ravdin 2002).

Amplification of amoeba DNA fragments by PCR has been proven to constitute a sensitive and specific method to detect *Ent. histolytica* or *Ent. dispar* from human faeces (Verweij *et al.* 2000). Recently developed closed-tube, real-time PCR for detection and differentiation of *Ent. histolytica* and *Ent. dispar* directly from faecal

samples (Blessmann *et al.* 2002). This method reduces the time required and the risk of cross-contamination.

Giardia lamblia The trophozoite when fixed for light microscope is 12-15 μm long and 5-9 μm wide. It has two nuclei and four symmetrically placed flagella. The cyst is ovoid, approximately 7-10 μm. A single stool examination has a sensitivity of 50 to 70%; the sensitivity increases to 85 to 90% with three serial specimens (Furness *et al.* 2000). Sensitive and specific ELISAs for *Giardia* antigens have been developed and some of these assays are now marketed commercially. Sensitivity and specificity are reported to be 87-100% (Fedorko *et al.* 2000). However, microscopy-negative cases of giardiasis do exist and the more sensitive methods of detection such as ELISA or DNA-based diagnostic techniques will eventually prove the cases (Butcher and Farthing 1988). A PCR-based assay that amplifies the intergenic spacer region of multicopy rRNA gene followed by nested PCR appears to be both rapid and reliable in detecting *Giardia* in stool (Ghosh *et al.* 2000).

Cryptosporidium spp. oocyst, the shape is oval or spherical and the size 4-6 μm. Three staining methods are in common using: auramine staining modified Ziehl-Neelsen staining and immunofluorescence use monoclonal antibodies to oocysts. ELISAs that incorporate anti-oocyst antibody to detect cryptosporidial antigen in stool have been developed (Ungar 1990). Indirect fluorescent; immunofluorescent antibody test has been developed that will detect serum antibodies to oocyst antigens (Farthing *et al.* 2003). Several different assays using PCR have been published for detection of DNA in stool or biopsies (Webster 1993). Restriction fragment length polymorphism (RFLP) analysis was the first technique to demonstrate variation between isolates of (*Cr. parvum*) by PCR, sequence analysis especially of the rRNA gene loci. These techniques broadly confirm that the species *Cr. parvum* comprises at least two major genotypes, one of which infects humans exclusively and the other with a broader range

of host specificity infecting animals besides humans (McLauchlin *et al*. 1999).

1.12 Management of acute diarrhoea

1.12.1 Assessment of the Patient

Effective prevention of mortality and management of morbidity associated with acute diarrhoeal disease among children is dependent on making a timely and accurate assessment of the status of dehydration. The mainstay of management of diarrhoeal disease is the assessment of dehydration and the appropriate replacement of fluid and electrolytes. The current WHO integrated management of childhood illness as a set of guidelines (Anon. 1996a). There are additional practice guidelines for treatment of acute gastroenteritis illness in children (Anon. 1996b). Although diarrhoeal disease can produce dehydration at any age, its impact is greatest in those aged under 5. This is as a result of their relatively greater surface area and thus greater fluid loss through skin. Infants require 2.5 times more water per kg. of body weight than older individuals. Fluid and electrolyte loss is also greatly exacerbated by vomiting. Both the initial degree of dehydration and response to rehydration therapy should be monitored clinically. The assessment of dehydration depends on the body weight loss (%), clinical state and signs. Mild dehydration is when the child has 5% of body weight loss, clinical state is not unwell and there are signs of dehydration; thirsty and dry mucous membranes. Moderate dehydration is when the child has 5-10% of body weight loss, clinical state is apathetic and there are signs of dehydration; sunken eyes, sunken fontanelle, tachypnoea, oliguria and loss of skin turgor. Severe dehydration is when a child has 10- 15% of body weight loss, clinical state is shocked and there are signs of dehydration; hypotension and peripheral circulatory failure. The critical state is when the child has >15% of body weight loss, clinical state is moribund and there are signs of dehydration; severe shock and the child may be comatose.

1.12.2 Oral rehydration therapy

For children with mild to moderate dehydration, ORT should be initiated with ORS, administered at a rate of 75 ml/kg of body weight per hour over a period of 4 hours (Anon. 1995c). ORS contains a mixture of glucose, sodium, potassium, and chloride in a bicarbonate base. The optimal osmolarity of ORS, determined by the concentration of sodium and glucose, was re-evaluated recently (Hahn *et al.* 2001 and 2003) WHO and the United Nations Children's Fund (UNICEF) recently revised their guidelines to suggest use of ORSs that are hyposmotic to solutions previously promoted after several clinical trials documented distinct advantages (Anon. 2002 and Hahn *et al.* 2003). The new WHO, ORS contains 13.5 g/L glucose, 75 mmol/L sodium, 20 mmol/L potassium, 65 mmol/L chloride, and 30 mmol/L base bicarbonate solution, for a total osmolarity of 245 mOsm/L (Anon. 2002). For children who are unable to tolerate ORS via the oral route, nasogastric feeding can be used (Podewils 2004). Amylase-resistant starch added to standard World Health Organization glucose-ORS significantly shortened the duration of diarrhoea compared with G-ORS (Raghupathy *et al.* 2006).

1.12.3 Intravenous rehydration therapy

Approximately 90% of children will respond to oral rehydration therapy. The remainder are generally infants with severe dehydration or those with profuse vomiting or a high purging rate. These will require rehydration by the intravenous route. Suitable solutions include: Ringer's lactate (Hartman's), consisting of NaCl 6.2g, KCl 0.4g, sodium lactate 2.3g and 2 ml 50% glucose in 1 litre of solution; Dhaka solution (NaCl 5g, $NaHCO_3$ 4g, KCL 1g and 50% glucose per litre); or acetate solution (NaCl 5g, KCl 1g, sodium acetate 6.5g and 2ml 50% glucose per litre of solution). Severe dehydration and shock are a medical emergency, and intravenous rehydration therapy should be administered immediately. Lactated ringer solution,

normal saline, or similar solution should be administered at a rate of 30 ml/kg of body weight until pulse, perfusion, and mental status return to normal; in some instances, large doses may need to be administered more rapidly, which might require using multiple intravenous lines. Electrolyte levels should be assessed, although therapy can be begun safely in the absence of these data (Podewils *et al.* 2004). As soon as the child's condition is stable and mental status is normal, therapy can be altered to the oral route, as described previously.

1.12.4 Antimicrobial drugs

Anti-microbial agents are not usually recommended for the treatment of acute diarrhoea (Anon. 1990). In general, infants with acute watery diarrhoea are best managed without recourse to antibiotics. However, if there is evidence of systemic spread, *V. cholera*, dysentery, *Campylobacter* and *Shigella,* then antimicrobials will shorten the course of diarrhoea and ameliorate its effects. With the advent of Fluoroquinolones such as Ciprofloxacin and Ofloxacin, debate on the use of antimicrobials has been reopened: First, there is no doubt that the widespread indiscriminate use of antimicrobials, often in sub-therapeutic regimens, encourages resistance in both pathogens and normal enteric flora (Shears 1993). Second, for most individuals with acute gastroenteritis, diarrhoea will subside after a few days without the use of an antimicrobial agent. Third, indiscriminate use of antimicrobial agents will lead to increase in bacterial resistance with in the community, increase in antimicrobial-related side effects, in medical costs and to prolonged bacterial excretion in some individuals. In addition, for most cases of noncomplicated bacterial gastroenteritis, antimicrobials have not proven to be of significant benefit to the patient. Erythromycin and Azithromycin dehydrate are adopted for treatment of *Campylobacter* infections. Trimethoprin (TMP)-Sulphamethoxazole (SMX) may be used for treatment of *V. cholera* infection in younger children, whereas Doxcycline or

Tetracycline are used more frequently for older children and adults. Appropriate treatment for shigella dysentery includes TMP-SMX and Ampicillin (Anon. 1995c). Oral Nitazoxanide is recommended for the treatment of diarrhoeal disease associated with *G. lamblia* or *Cr. parvum* and TMP-SMX is effective treatment for cyclosporiasis (Anon. 2003). From recent data it emerges that Fluroquinolones should be the first line of therapy and Cephalosporins should be used as a second line (Alam and Bhatnagar 2006)

1.12.5 Antidiarrhoeal drugs

1.12.5.1 Anti-motility drugs e.g. Loperamide Hydrochloride, Diphenoxylate with Atropine, Tincture of opium and Codeine. These may reduce the frequency of stool passage in adults. However, they do not decrease the volume of stool in young children. Moreover, sedation may occur at usual therapeutic doses and central nervous system toxicity has been reported for some agents. None of those agents should be given to infants or children with diarrhoea (Anon. 1995c). Loperamide further does not decrease fluid and electrolytes secretion of stool of children with acute diarrhoea. Many reports demonstrated a relation between Loperamide and paralytic ileus in infants and young children. These drugs should cosequently be avoided (Anon. 1996a). Racecadotril could be preferable to Loperamide as it may induce less secondary ileus (Wang *et al.* 2005). There now seems to be a real possibility that anti-secretory therapy will become more widely available in the future (Farthing 2006).

1.12.5.2 Adsorbent drugs such as Kaolin, Pectin, Smectite, activated Charcoal and Cholestyramine. These are used for treatment of diarrhoea on the basis of their ability to bind and inactivate bacterial toxins. None of these agents has proven practical value in the routine treatment of acute diarrhoea in children (Anon. 1995c). In a study in critically ill tube-fed patients receiving antibiotics, Pectin tended to prevent diarrhoea more efficiently than placebo (Sohultz *et al.* 2000). In contrast, a recent meta-analysis

on the randomized trials of enteral solutions enriched with fibres discovered no benefit in the subset of critically ill patients (Yang *et al.* 2005). Clearly, larger studies are warranted to make recommendations for the use of fibres (Wiesen *et al.* 2006).

1.12.6 Nutritional supplements

Breast milk can be given to infants even during acute rehydration; otherwise, food should not be given during the acute rehydration phase but should be initiated as soon as possible after fluid levels are restored. Energy intake should continue to be increased as tolerated following the diarrhoeal episode to allow for catch-up growth and continued development (Anon. 1995c). Micronutrient deficiencies have been associated with increased incidence, severity and duration of diarrhoea and other diseases. During and after weaning, infants need to receive calories, proteins, and micronutrients. Increasing the energy density of weaning food is crucial in settings where malnutrition is common. A few randomized trials have demonstrated that vitamin A supplementation reduced the incidence and duration of diarrhoeal disease (Huttly *et al.* 1997), especially among children with measles (Anon. 2002) or with vitamin A deficiencies (Chowdhury *et al.* 2002). A trial of zinc supplementation in India also demonstrated a clinically significant decrease in the severity and duration of diarrhoea (Sazawal *et al.* 1995). In contrast, a recent study in infants in Pakistan, India and Ethiopia showed no differences (Fischer *et al.* 2006) This randomized, placebo controlled trial was designed to assess the safety and efficacy of 10-mg Zinc supplementation for the treatment of acute diarrhoea in infants. There are no differences zinc and placebo groups. Young infants do not appear to benefit from zinc supplementation for the treatment of diarrhoea (Fischer *et al.* 2006). Recently, clinical trials study suggest potential beneficial effects of probiotic therapy for preventing and treating antibiotic-associated diarrhoea, acute diarrhoea including rotavirus-including diarrhoea, traveller's diarrhoea and diarrhoea-predominant irritable bowel syndrome.

Probiotics may serve as a functional food in the treatment of diarrhoea (Yan 2006).

1.13 Control and prevention of diarrhoea

In industrialized countries it has been the separation of human and animal excreta from potable water and foodstuffs that has contributed to the great decline in the incidence of diarrhoeal disease. The relative importance of the various programs and the specific cost- effective interventions that should be pursued in developing regions where resources are scarce remains uncertain. Development of strong collaborative research programs are necessary to help identify the optimal approach to reducing the health consequences of diarrhoea (DuPont 1991). Specific areas known to be critical are outlined below. The two most cost-effective measures in the control of diarrhoea and its consequences are probably promotion of breast feeding and widespread implementation of oral rehydration treatment programmes. Measures of potential value in controlling infantile diarrhoea include: safe supply of water, sewage removal systems, personal and food hygiene, avoiding use of human excreta for fertilizing crops, breast feeding, food and nutrient supplementation, health care, family planning, controlling the fly population and measles immunization. The household characteristics relating to diarrhoea occurrence include: number of household children, homes with a poor state of repair, water service, sanitation system, food being exposed to room temperature between meals, poor rodent control and presence of animals in the home (Wright *et al.* 1991). Education concerning food hygiene principles resulting in the discontinuance of the practice of leaving prepared foods at ambient temperatures between meals should lead to a reduced rate of enteric disease. Individual measures include careful personal hygiene, especially hand washing, and limited use of antacids, anti-motility drugs and antimicrobial agents.

Despite the high-quality water and food supplies available in the United States and

other socioeconomically developed areas of the world, outbreaks of food-borne and waterborne disease continue to occur, generally due to improper handling and storage of food (Olsen *et al*. 2000). Promotion of hand washing has proven to be a highly effective measure in decreasing the incidence of diarrhoea among people living in high-risk areas, such as settlements in Pakistan (Luby 2004).

1.14 Vaccines

The number of vaccines available to prevent enteric infections is scarce but expected to increase in the future with the development of new technologies (Nataro and barry 2004).Two typhoid vaccines are currently approved for clinical use. The Vi capsular polysaccharides vaccine is approved for children older than 2 years. A single parenteral dose provides a high level of protection (Dizer *et al*. 2002).

The live attenuated vaccine, Ty21a, is similarly efficacious for a period of at least 5 years (Cryz *et al.* 1995). Live genetically attenuated vaccines for *Shigella* are currently in various stages of development. The vaccines are serotype-specific, meaning that multivalent vaccines will be required to protect against the most prevalent serotypes worldwide (e.g. *Sh. dysenterie* type 1, *Sh. sonnei*, and *Sh. flexneri* 2a, 3a and 6) (Hala and Venkatesan 1997). Two oral vaccines, one live-attenuated and one inactivated, have been licensed in industrialized countries. CVD 103 HgR was the first recombinant live-attenuated cholera vaccine that proved to be well-tolerated, immunogenic and protective and is the only such vaccine presently licensed for human use (Podewils *et al*. 2004). An oral cholera vaccine consisting of non-toxic, highly immunogenic cholera toxin B subunit protein in combination with heat-and formalin-killed *V. cholera* O1 classical and E1 Tor *Vibrios* has been developed in Sweden. This B subunit-whole cell (D-WC) vaccine has proved to be safe and protective against cholera.

The most advanced *ETEC v*accine is a killed whole cell formulation plus recombinant cholera toxin B subunit. This vaccine proved safe and immunogenic in adult volunteers as well as in children aged between 2 and 12 (Ahren *et al*. 1998 and Savarino *et al*. 1998). Efforts to develop rotavirus vaccines were pursued when improvements in hygiene and sanitation did not appear to reduce disease incidence because rates were similar in less developed and industrialized countries (Podewils *et al*. 2004). In 1998, a rotavirus vaccine was licensed in the United States and was recommended for routine immunization of infants. This vaccine, a tetravalent rhesus-human reassortment rotavirus tetravalent vaccine RRV-TV was developed to target the four most common G serotypes of rotavirus, G1-G4. However, RRV-TV was withdrawn in 1999 when it was causally linked with intussusception at an estimated rate of 1 case per 11,000 vaccinated infants and the manufacturer ceased its production (Podewils *et al*. 2004). Other rotavirus vaccines are now being developed. Two live oral rotavirus vaccines (Rotarix) and (RotaTeq) have been licensed in Europe and the USA respectively and in several other countries (Heaton *et al*. 2005). These vaccines are based on slightly different principles to achieve broad immunity against the diverse strains of rotavirus in circulation. Rotarix was prepared from an individual human strain that replicates well in the intestine and is shed in the stool. RotaTeq is a combination of five bovine-human reassortant that replicate poorly in the gut. Each vaccine has proven to be highly effective in preventing severe rotavirus diarrhoea in children and found safe from the possible complication of intussusception (Glass *et al.* 2006).

1.15 Brief history of acute diarrhoea in Libya

1.15.1 Introduction and background information

Libya is located in North Africa between Egypt to the east, Tunisia and Algeria to the

west and Sudan and Chad to the south (Fig. 1). It covers approximately 1,750,000 sq km and the population was approximately 4 million in the 1984 census and estimated to be 5.5 million in 2000. Most of the people inhabit the towns of Tripoli, Zawia, Misrata, Sert, Benghazi and Sabha. Tripoli is the capital city of Libya and is located in the northwest of the country besides the Mediterranean sea.

Health Programmes especially those related to childhood illness were developed at national level in Libya, e.g. Expanded Programme of Immunisation (EPI), Control of Diarrhoeal Diseases (CDD), Acute Respiratory Illness (ARI). Some were successful, but others not. EPI was the most successful programme where the uptake rate of the six main vaccines reached more than 95%.

Control of Diarrhoeal Diseases. This programme was established during 1986. A national plan was implemented and many activities were performed and the oral rehydration therapy (ORT) uptake rate reached above 80%. Libya was among the most successful countries to cease using anti-diarrhoeal drugs for management of diarrhoea in children aged under 5 years. A breast feeding programme was developed but more activities need to be carried out and an evaluation effort is required. Unfortunately, there have been no significant activities related to CDD since the mid-1990s. The programme was not evaluated although diarrhoea related admission to hospitals was reduced. Since the era of ORS recommended by WHO, the incidence of acute diarrhoea morbidity (668.4 per 1000 infant), and mortality (18.45 per 1000 infant) declined to a satisfactory level in only a few areas of the country e.g. Tripoli and Benghazi, Libya (Sehary 1999).

1.15.2 Review of previous studies carried out on acute diarrhoeal disease in children aged under 5 years in Libya

The few studies that have been carried out relating to acute diarrhoea in children aged

under 5 in Libya have been based upon reviewing hospital records. Analysis of a 2-year study of 1249 admissions with GE (1988-1989) revealed its tremendous impact on Tripoli Children's Hospital morbidity (31%) and mortality (45%) statistics (Ben Halim 1991). Infants comprised 92% of patients and 59% were aged 6 months or younger. More boys (55%) than girls were admitted. GE was equally common in both summer and autumn. Dehydration was isonatraemic in 94%, hyponatraemic in 4% and hypernatraemic in 2%; and 97% of patients were wholly artificially fed. Pathogenic bacteria were isolated from 32% of patients' stools. Incidence of *Salmonella* was 21.3%; of *E. coli* 6.7%; of *Staph. aureus*. 2.1%; and *Shigella* 1.9%. The mortality rate was 2.9% and case fatality rate was 9% for boys and 10.3% for girls. 94% were infants and 75% of patients were aged 6 months and under. Conditions detrimental to survival were identified as protein energy malnutrition (22%); shock (20%); and aspiration pneumonitis – bronchopneumonia (7.5%) (Ben Halim 1991).

Another study was conducted between September, 1992 and August, 1993 to determine the aetiology of diarrhoea in Libyan children within the Tripoli area (Ghenghesh 1997). It included 161 children with diarrhoea (cases) and 161 age and sex matched controls. The age of children ranged from a few days up to 3 years. Most (86%) of the children were less than one year of age. Standard methods were adopted to detect enteropathogens (bacterial agents and rotavirus but parasitic agents were not sought in this study). Rotavirus and *Campylobacter* spp. studied 147 and 100 cases and 90 and 100 controls respectively. Rotavirus was detected in 35% of cases and 2% of controls, *Salmonella spp*. in 11% and 4%, *Shigella* spp. in 6% and 1%, *Aeromonas* spp. in 15% and 17%, *Campylobacter* spp. in 6% and 3%, *Yersinia* enterocolitica in 0.6% and 0.0%, enteropathogenic *E. coli* in 15% and 9%, enterotoxigenic *E. coli* in 11% and 11%, enteroinvasive *E. coli* in 0% and enterohaemorrhagic *E. coli* (serogroup 0157:H7) in 7% and 4% respectively (Ghenghesh 1997).

1.16 The reasons for these studies

1. The lack of studies focusing upon acute diarrhoea in children under 5 years of age.
2. The studies that have been made are dated and limited. They are additionally too short, not exceeding one year.
3. The lack of cooperation between pediatricians and microbiologists in this field, meaning that previous studies concentrated on either the clinical or microbiological aspect, but not both.

1.17 The aims of the study include:

1. Establishing a relationship between clinical symptoms and the aetiology of acute diarrhoea. .
2. Finding the factors contributing to the cause of acute diarrhoea besides the age group (s) that are prone to infection.
3. Finding the relationship between the cause of acute diarrhoea and time of year it occurs (seasonal variation).
4. Using new methods to diagnose the aetiology of acute diarrhoea not used in Libya e.g. selective media, API and PCR.
5. Increasing the detection rate of the aetiology of acute diarrhoea.

Figure. 1 Map of Libya

Chapter 2

Patients Sampling Methods

CHAPTER 2

PATIENTS AND SAMPLING METHODS

2.1 Selection of patients and case controls

Two studies were performed to detect the aetiology of acute diarrhoea in hospitalized children in Tripoli, Libya. There are variations between the two studies according to the study protocol.

The first study was conducted over a short period of time (3 months), with a small number of cases (118 patients) and routine laboratory techniques (light microscopic examination, Gram stain, oxidase test, blood and MacConkey agar media and selective medium for *Campylobacter* isolation were used). In the light of results from the first study, the second study was performed over a whole year. A large number of cases (405 patients) were enrolled and advanced laboratory techniques were completed (API 20E, API Campy and PCR) besides as routine laboratory techniques. Other data collected was along the same lines.

In the first study, stool samples were collected from children (patients and controls) between the age of 1 to 60 months who were admitted to Aljala Children's Hospital in Tripoli, Libya, during the period between August and October 1997. The second study ran from January 1st, 2003 to December 31st, 2003. The age of children here was between 1 to 60 months who were admitted to the same hospital aforementioned.

Faecal specimens were obtained from children who had acute diarrhoea and from a control group. The criterion for diagnosis of acute diarrhoea was that the children passed three or more loose or watery stools per day for a minimum of one day and for no longer than two weeks prior to admission. Controls were selected from children who were admitted to hospital for a reason other than acute diarrhoea, such as upper and lower respiratory infection, asthma, uriary tract infection and heart diseases, and

they were matched for age and gender. An additional criterion was that neither patients nor controls had received antibiotics in the preceding two weeks. A questionnaire (case sheet) used to collect information related to the protocol of this study from mothers of patients who were interviewed in detail upon first contact. This questionnaire was ompleted for each patient where details of clinical history, feeding practice, household environment and demographic information (age, sex, etc.) were recorded. Other additioned information about the patient's health was also taken from their files. The questionnaire was prepared by clinician and microbiologist, with the remit that information sought might be relevant to the aims of the study. All information was analysed by statistical programmes. A copy of the questionnaire used is displayed in appendix 1 and laboratory results are shown in appendix 2.

2.2 Definition of diarrhoea

Diarrhoea can be defined as increased frequency and fluidity of stool, that is, the passage of stool containing excess water and electrolytes losses. For this study, acute diarrhoea was defined as the presence of three or more loose watery stools within a 24 hours period (Anon. 1995c).

2.3 Data collection

The collecting information included:

2.3.1 Clinical history, examination and investigation on admission

Acute diarrhoea usually manifests as an increase in the frequency or volume of stool. Fever is a regular occurrence; vomiting and dehydration are also common clinical features which need careful observation. Assessment of dehydration requires a great deal of experience. Symptoms and signs used in assessment of dehydration (were assessment by Junior doctors at admission) and monitoring of rehydration include:

thirst, radial pulse, blood pressure, skin elasticity, anterior fontanella in infants, and appearance of the tongue, mucous membranes, eyes, eyeball tension, tears, respiration, urinary output and central nervous system manifestations. The classification of the type and degree of dehydration was according to WHO guidelines for clinical assessment of signs of dehydration to detect the degree of dehydration (mild, moderate and severe) and serum sodium of patient to detect the type of dehydration (isotonic; 130 to 150, hypotonic <130 and hypertonic dehydration >150 mmol/l). The degree of dehydration at the time of admission was calculated by subtracting the naked weight at admission from the naked weight following rehydration using the same weight scale (Angela *et al.* 1989).

$$\text{Degree of true dehydration (\%)} = \frac{\text{post rehydration weight} - \text{admission weight}}{\text{post rehydration weight}} \times 100$$

In general when stool and vomiting are extremely profuse then dehydration is severe. Patients with mild dehydration appear almost clinically normal yet they may have lost considerable body fluid (4-5% body weight loss, in mild dehydration, moderate dehydration: 6-9% body weight loss, severe dehydration: 10% or more body weight loss).

2.3.2 Body weight

The weight of patient was monitored on admission and daily after admission (by Nurses) until resolution of diarrhoea and biochemical results returned to normal.

Body weight is classified into four groups according to the standard charter percentile of Libyan children (Tajouri 1978, personal connection, see Appendix 3) as used in Libyan paediatric hospitals; <10%, 10 to <50%, 50% and >50% of age for weight. The weight for age of patients after rehydration and weight for age controls offer a good indication for the nutritional status of Libyan children.

2.3.3 Haematology and biochemistry

The cut-off point where a child is considered to have anaemia when haemoglobin (HB) is concentrated in the blood is ≤ 9 gdl^{-1}.

Haemoglobin concentration, serum sodium, potassium and urea were analyzed in Aljala hospital's laboratory; Hb% as part of complete blood counts (CBC) analysis by machine (Sysmex-1000), serum sodium and potassium by machine (AVL 988-3 Electrolyets analyzer) and blood urea by machine (Beehman-BUN analyzer). All investigation of haematology and biochemistry was done by the laboratory of Aljala Children's Hospital in Tripoli, Libya.

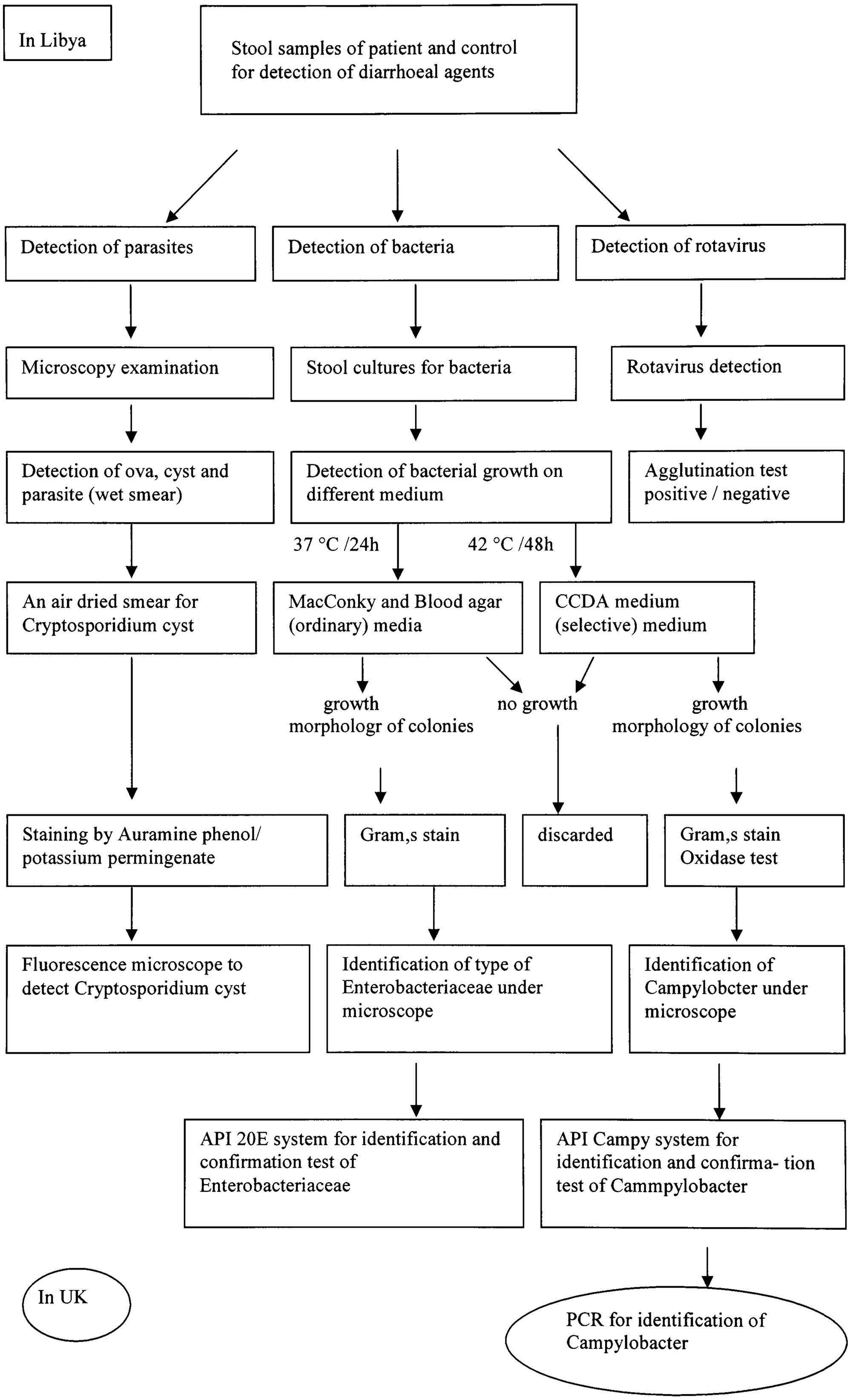
2.4 Specimen collection

Faecal specimens were obtained from all patients and controls as soon as possible after the patient was admitted to the hospital. For each stool specimen, the consistency and presence of blood or mucous (or both) were noted. Two stool specimens were collected from each patient and case control. The first fresh stool sample was examined under light microscope within 1 hour for the presence of ova, cysts and parasites at the hospital's laboratory in the hospital. The second stool specimen was transported in an ice bag to the central laboratory in Tripoli within 2 hours of collection for detection of micro-organisms; stool culture for bacteria, identification of rotavirus by agglutination latex test and detection of *Cryptosporidium* cysts by staining. Residual stool specimens were refrigerated for a few days and then stored in a deep freezer.

2.5 Storage of bacteria

The colonies of any bacteria was isolated (after subculture to obtain pure culture), was picked up with a sterilised cotton swab and placed into small tubes containing storage medium and stored in deep a freezer (-20 °C) for other confirmation tests.

Figure 2. Protocol of stoolsamples for detect of diarrhoeal agents



medium and stored in deep a freezer (-20 °C) for other confirmation tests.

The protocol of detection and identification of micro-organisms in both studies will be summarized in (Figure 2).

2.6 Identification of micro-organisms

2.6.1 Identification of parasites

Two methods were used for detection of parasites:

2.6.1.1 Wet preparation for ova, cysts, and parasites. A drop of normal saline and iodine was placed on the slide and a small amount of stool was mixed in using a wooded stick. This was then examined under a light microscope.

2.6.1.2 An air dried smear for *Cryptosporidium*

A small amount of stool was picked up on a cotton swab and this was then spread onto a slide which was dried in air at room temperature. A positive control slide was used with each batch.

2.6.1.3 Method of staining of *Cryptosporidium* (Nicols and Thom 1984).

Auramine phenol/potassium permanganate was used for staining for *Cryptosporidium.* The film was fixed in methanol for 3 minutes, the slide was stained with Auramine-phenol (1% solution) for 10-15 minutes, then was washed with tap water. Subsequently it was decolourised with 0.5 % acid alcohol for 2 minutes, then washed again with tap water, the slide was then finely counter-stained with 0.01 % (w/v aqueous) potassium permanganate for 1- 3 minutes. The slide was rinsed in tap water and air-dried. After drying, a drop of oil was placed on the slide and was examined under a fluorescent microscope to detect the cysts of *Cryptosporidium*.

2.6.2 Isolation of bacteria

All faecal specimens (patients and controls) were cultured for pathogens: *Escherichia*

coli and *Campylobacter,* because both bacteria were the most common bacterial cause of acute diarrhoea in Libyan children according to the last studies performed in Libya. The following media were used: Blood agar, MacConky agar and *Campylobacter* selective media.

2.6.2.1 Blood agar base (oxoid blood agar base):

Typical formula (in g l^{-1}): Protease peptone 15.0; Liver digest 2.5; Yeast extract 5.0; sodium chloride 5.0; Agar 12.0; Ph 7.4 ± 0.2 (CM271).

Medium was prepared according to manufacturer's instruction (Oxoid Ltd, Wade Road, Basingstoke, Hampshire, RG 24 8PW, UK).

Method: The powder was suspended 40 g in 1 litre of distilled water. The suspension was brought to the boil so all solids were dissolved evenly. The medium was sterilised by autoclaving at 121°C for 15 minutes for blood agar, the base was cooled to 50°C and then 7 % (v/v) of defibrinated horse blood SR was added. This was mixed with gentle rotation and poured into Petri dishes.

2.6.2.2 MacConkey agar:

Formula: (in g l^{-1}): Peptone 20.0; Lactose 10.0; Bile salts No. 3 (Oxoid L56) 1.5; sodium chloride 5.0; Neutral red 0.03; Crystal violet 0.001; Agar No. 3 (Oxoid L13) 15.0; Ph 7.1 ± 0.2 (CM115).

Method: The powder was suspended 51.5 g in 1 litre of distilled water. The suspension was brought to the boil so all the solids were dissolved evenly. It was then sterilised by autoclaving at 121°C for 15 minutes, cooled to 50 °C and poured into Petri dishes. The surface was dried of the gel before inoculation.

Faecal samples of patients and controls were inoculated directly on MacConkey and Blood media were incubated aerobically at 37 °C for 24 h.

The result was read after 24 h for MacConkey and Blood agar media. The detection of bacteria growth was confirmed by tests according to the protocol of the Central Tripoli

Laboratory in Libya (colonal morphology, lactose fermentation, non-lactose ferment, Gram stain, biochemical tests, oxidase test and sensitivity to antibiotics).

2.6.2.3 Preparation of Modified Charcoal Cefoperazone Desoxycholate Agar blood free medium (CCDA)

Campylobacter Blood-Free Selective Agar Base modified (CCDA-Preston). It was prepared according to the manufacturer's instruction (Oxoid CM 739). 22.75gm of *Campylobacter* blood free selective agar base was suspended in 500 ml of distilled water and brought to boiling point to dissolve the solids. It was then sterilised by autoclaving at 121°C for 15 minutes. The medium was cooled to 50°C. One vial of antibiotic supplement was added aseptically. This step inhibits the growth of bacteria except *Campylobacter.* CCDA selective supplement (SR 155) was reconstituted with 2 ml of sterilized water. This was mixed well and poured into sterile Petri dishes. (Batch No: CM 239) 500 Gm. (Unipath Ltd, Basingstoke, Hampshire, UK).

Faecal samples of patients and controls were inoculated directly on CCDA medium which was incubated in an anaerobic jar after adding 10 ml of water to the *Campylobacter* gas generating kits (BBL pack plus Becton and Dickinson, UK), which was placed in the jar and incubated at 42°C for 48 h.

2.6.2.4 Maximum recovery deluent

Maximum recovery deluent media was prepared according to the manufacturer's instruction (Oxoid CM 733). 9.5 gm of powder were added to 1 litre of distilled water mixed well and distributed to a final container 9 ml sterilised by autoclaving at 121°C for 15 minutes. The medium was then cooled and placed in the fridge prior to use.

Faecal suspension was prepared to inoculate on to CCDA medium by putting 1g of stool in 9 ml of maximum recovery diluent and mixing well on a vortex mixer.

A sample was removed with a sterile loop and spread on the Petri dishes containing CCDA medium, which together with a positive control were incubated in an anaerobic

jar.

The result was read after 48 h for CCDA medium. The detection of *Campylobacter* was confirmed by tests according to the protocol of the Public Health Laboratory (now Health Protection Agency), Royal Preston Hospital in the UK (colonal morphology of *Campylobacter*, Gram stain, oxidase test, biochemical tests and biotyping).

If growth occurred and the morphological features of colonies were typical of *Campylobacter* an oxidase test was performed, otherwise plates were discarded. If the cultures were oxidase positive a Gram stain was performed to confirm the morphology of campylobacter.

2.6.2.5 Oxidase test

Method A few crystals of NNN′N′-tetramethyle-p-phenylene-diamine dihydrochloride (Sigma-Aldrich Co. Fancy Road, Poole, Dorset, BH 12 4QH, England) were dissolved in 5 ml sterile deionized water. A sterile cotton swab was soaked into prepared oxidase reagent and was used to select a single colony of the test organism. The appearance of a pink /violet colour within 10 seconds was taken as indicative of a positive reaction.

Cultures were then streaked to form single colonies from CCDA medium onto Blood agar and CCDA media to obtain pure cultures and incubated microaerophilically at 47°C for 48 h in an anaerobic jar. The colonies of campylobacter from Blood agar medium were picked up with a sterilised cotton swab and put into small tubes containing storage medium (Brain- heart- infusion broth medium).

2.6.2.6 Brain heart infusion

Brain heart infusion media was prepared according to the manufacturer's instruction (Oxoid CM 225). 37 gm of media were added to 1 litre of distilled water and mixed well and distributed into 2 ml screwed tubes and sterilized by autoclaving at 121°C for 15 minutes. Stored cultures were removed from the freezer, subcultured and prepared for API 20E (for bacterial isolation) and API Campy for *Campylobacter* was isolated.

Campylobacter was also stored onto the FTA-Card and transported to the UK for PCR.

2.6.2.7 Identification of bacteria

2.6.2.7.1 API 20E and API Campy

Identification for enterobacteiaceae and other non-festidious Gram-negative rods: API 20E and API campy (Biomeireux, UK) were prepared according to the manufacturer's instruction. These tests were performed in Libya while *Campylobacter* identification by PCR was performed in the UK.

2.6.2.7.2 Polymerase Chain Reaction

Methods for the microbiological isolation of *Campylobacter* using selective enrichment culture lack sensitivity and are unreliable for detecting low numbers of these organisms.

Recovery may be particularly poor if large numbers of indigenous micro flora are present, or overgrowth with co-contaminating species occurs. In addition, relatively fastidious pathogens such as *Campylobacter* spp. may lose viability before samples can be processed in the laboratory (Jackson *et al.* 1996). Recent modifications to enrichment culture methods have increased selectivity and decreased the time to necessary detect positive samples (Raben and Slavik 1994). These improvements do not substantially improve the recovery of damaged organisms, and still require a prolonged period of incubation (Jackson *et al*. 1996).

The polymerase chain reaction offers a highly sensitive and specific alternative to selective enrichment for detection and identification of bacterial pathogens (Lampel *et al*. 1990).

2.6.2.7.2.1 Materials and methods of PCR

The reference strain of *Campylobacter jejuni* NCTC 11168 was provided by Dr. D.

Wareing (Dynal Biotech Ltd, Microbiology R & D, Department of Biological Science, University of Central Lancashire, Preston PR1 2HE, UK).

Campylobacter isolated from patients and controls were removed from storage (deep freezer) and prepared for storage on FTA-Cards according to the manufacturer's instruction (Whatman BioScience Ltd. Granta Park, Abington, Cambridge, CB1 6GR, United Kingdom) and transported to the UK.

2.6.2.7.2.2 FTA-Card

FTA products are composed of a sophisticated filtration matrix impregnated with a patented formulation of powerful protein denaturants, a chelating agent, and a free-radical trap designed to protect and capture nucleic acids, It is non-toxic to humans yet prevents the growth of bacteria and other microorganisms. It additionally rapidly inactivates organisms such as blood-borne pathogens, offering user safety. Such protective qualities permit nucleic acids collected on FTA matrices to be stored at room temperature without degradation for many years.

A colony in 5 to 10 µl was resuspended of phosphate buffer solution (PBS) in a micro centrifuge tube. A single spot (5 µl) was applied onto the FTA matrix card immediately after spotting on the card and a pencil was used to outline each sample spot. This was useful before the FTA matrix had dried for locating samples on the matrix. The samples were allowed to dry one h or overnight at room temperature. The cards were then stored in an environmentally-controlled dark location at room temperature.

2.6.2.7.2.3 DNA primers and PCR amplification

Oligonucleotides BO4263 (5′-AGAACACGCGGACCTA-TATA-3′) and BO4264 (5′-CGATGCATCCAGGTAAT-GTAT-3′) were synthesized by Appligene (IIIKirch, France). These primers amplify a 250 base pair product, and are designed from the sequence of an open reading frame adjacent to and downstream of a novel *C. jejuni*

two component regulator gene (Jackson 1995).

The amplification reaction was performed in a volume of 100 µl containing: 50 µl of test DNA sample; 50 mmol l^{-1} Tris-HCL (ph 9.0); 16 mmol l^{-1} $(NH_4)_2SO_4$; 50 mmol l^{-1} KCL; 7 mmol l^{-1} $MgCL_2$; 0.2 mg ml^{-1} BSA; 200 µmol l^{-1} each dATP, dCTP, dTTP and dGTP; mol l^{-1} of each primer, and 2.5 units of *Tag* DNA polymerase (Promega). After overlying samples with 50 ml mineral of oil, the PCR was carried out on a Hybaid HB-TRI programmable thermal reactor (Hybaid Ltd, Teddington, Middlesex, UK). The amplification parameters were an initial 4 minutes' denaturation at 95°C, followed by 40 cycles of primer annealing (60°C, 45 seconds), chain extension (72°C, 45 s) and denaturation (95°C, 45 s). A terminal extension step (72°C, 5 minutes) completed the reaction. PCR products were visualized by gel electrophoresis on 2% NuSieve 3:1 agarose (FMC Bioproducts, Rockland, ME, USA), stained with ethidium bromide (50 µg ml^{-1}).

2.6.3 Rotavirus screen

The Rota screen, a rapid and simple latex agglutination test for detection of rotavirus in faecal specimen (Rota screen M 80 Microgen Biopredaclis Ltd England) was utilised. The test was performed according to the manufacturer's literature. Faecal specimens were tested within 2 hours of collection or stored overnight at 2-8 °C.

The standard method was as follows: a 10 % suspension of faeces was prepared by transferring 0.1g of sample into 1.0ml of extraction buffer in a screw capped tube and mixing the contents well. The tubes were then centrifuged at 1000 g for 10 minutes. Supernatant (5 ml) was pipetted onto each of two wells on the test slide. One drop of well-mixed test latex reagent was added to one well and one drop of well-mixed control latex reagent was added to the other well. The contents of each well were mixed by using a separate mixing stick for each sample covering the entire area of the well. The slide was gently rocked and left for 2 minutes before reading the results. A

positive result was indicated by agglutination of the test latex reagent with no agglutination of the control latex reagent. Results were negative where no agglutination of either the test latex reagent or the control latex reagent appeared within 2 minutes.

2.7 Statistical analysis

Analyses were generated using statistical software package SPSS version 11.0 (SPSS, Inc., Chicago, IL). The correlation between diarrhoea and potential risk factors as registered in the questionnaire was assessed. McNemar,s chi sequare test p-values were calculated using the two-tailed test and significance was measured at the $P < 0.05$ level. The strength of the correlation of risk factors was estimated by odds ratio (OR) and their corresponding 95% confidence intervals (CI). The most of clinical variables will be analysed by univariate and multivariate. Averages, ranges and percentages of positive samples were calculated.

For seasonal distribution, Spring, Summer, Autumn and Winter (March to May, June to August, September to November and December to February) were used.

Chapter 3

Results of Demographical Findings

CHAPTER 3

Demography, Clinical Findings and Aetiological Agents Detection

Completed questionnaires were received from two studies and were analyzed by using the statistical package SPSS version 11.0 (SPSS, Inc., Chicago, IL).

In the first study, two hundred and thirty-six stool samples from children were examined. Of these, 118 were from patients admitted for acute diarrhoea (called patients) and 118 were from children who were admitted to hospital for a reason other than acute diarrhoea (called controls). All were admitted to the Aljala Children's Hospital in Tripoli, Libya, between 1st August and 31st October 1997.

The second study ran from 1st January to 31st December 2003. Eight hundred and ten stool samples from children were examined here. Of these, 405 were from patients admitted for acute diarrhoea and 405 were from controls as before. All were admitted to the same hospital.

3.1 Demographical findings

3.1.1 Age

The age distribution of patients is shown in tables 1a and 1b.

The age distribution ranged from 1 to 60 months in both studies. Children were divided into six groups. In the first study, the largest number of patients was in the 1 to 6 months age group. This represented 55 children (46.6%). The smallest group was among children aged from twenty-five to thirty-six months and included only three children (2.5%). The second largest number of patients was in the 7 to 12 months age group and represented 38 children (32.2%). The age group below one year was represented by 93 (78.8), while the age group from above one year to two years

Table 1. Age and gender characters of patients (a) First study

Age of children /months	Gender Male	(%)	Female	(%)	Total of children number (№)	(%)
1-6	31	26.3	24	20.3	55	46.6
7-12	22	18.6	16	13.6	38	33.2
13-18	7	5.9	5	4.2	12	10.1
19-24	3	2.6	3	2.5	6	5.2
25-36	2	1.7	1	0.8	3	2.5
37-60	2	1.7	2	1.7	4	2.6
Total	67	56.8	51	43.2	118	100

Table 1b. Second study

Age of children /months	Gender Male	(%)	Female	(%)	Total of children (№)	(%)
1 - 6	96	23.7	79	19.5	175	43.2
7 - 12	57	14.1	52	12.8	109	26.9
13 -18	21	5.2	18	4.4	39	9.6
19 - 24	18	4.4	14	3.5	32	7.9
25 - 36	15	3.7	9	2.2	24	5.9
37 - 60	16	4.0	10	2.5	26	6.5
Total	223	55.1	182	44.9	405	100

represented 18 patients (15.3%).

In the second study, the largest number of patients was again in the 1 to 6 months' age group and represented 175 children (43.2%). The smallest age group was aged from 25 to 36 months and included 24 children (5.9%). Again, the second largest number of patients was in the 7 to 12 months' age group and represented 109 (26.9%). The age group below one year represented 284 (70.1), while the age group from above one year to two years represented 71 patients (17.5%). Acute diarrhoea was most frequently encountered in children aged below one year and was observed in 93 cases (78.8%) in the first study and 284 (70.1%) in the second.

3.1.2 Gender

The prevalence of acute diarrhoea was slightly greater in males than in females in both studies (Table 1a and 1b). The number of males was 67 (56.8%) and the number of females 51 (43.2%) in the first study. In the second study, the number of males was 223 (55.1%) and that of females 182 (44.9%). The ratio of male: female was (1.3:1) in the first study and (1.2:1) in the second. There were no significant differences between males and females in either study ($p > 0.05$).

3.1.3 Maternal educational level

The maternal educational level of mothers of patients and mothers of controls is displayed in table 2a and 2b. The type of maternal education was classified into five groups. For convenience we use the first group (illiterate) as baseline. Every other group is compared to the baseline. The first group contained mothers of patients without formal education (illiterate); the second group, primary school education, the third group, secondary, the fourth group, teacher or college. The fifth group had the highest education level (graduated from university or high college).

Table 2. Maternal educational level (a) First study

Level of education	Mother of patients № of mothers	(%)	Mother of controls № of mothers	(%)	OR	p-value	95% CI
Illiterate	24	20.3	21	17.8	1		1 to 1(by default)
Primary school	36	30.5	34	28.8	0.915	0.820	0.425 to 1.968
Secondary school	22	18.6	27	22.9	0.719	0.419	0.323 to 1.601
Teacher or college	31	26.3	28	23.7	0.909	0.804	0.430 to 1.922
Higher education	5	4.2	8	6.8	0.553	0.361	0.155 to 1.974
Total	118	100	118	100			

Table 2b. Second study

Level of education	Mothers of patients № of mothers	(%)	Mothers of controls № of mothers	(%)	OR	P-value	95% CI
Illiterate	63	15.6	57	14.1	1		1 to 1(by default)
Primary school	102	25.2	96	23.7	0.979	0.929	0.619 to 1.549
Secondary school	75	18.5	79	19.5	0.872	0.559	0.552 to 1.378
Teacher or college	124	30.6	119	29.4	0.954	0.835	0.613 to 1.484
Higher education	41	10.1	54	13.3	0.690	0.177	0.403 to 1.183
Total	405	100	405	100			

In the first study, the first group contained twenty-four mothers of patients (20.3%) and 21 mothers of controls (17.8%). The second group had thirty-six mothers of patients (30.5%), compared to thirty-four mothers of controls (28.8%). The third group comprised twenty-tow mothers of patients (18.6%) and twenty-seven mothers of controls (22.9%). The fourth group had thirty one mothers of patients (25.3%) and twinty-eight mothers of controls (23.7%) and the fifth group consisted of five mothers of patients (4.2%) and eight mothers of controls (6.8%). The run of the McNemar,s test has shown that children from educated mothers at any level are at lesser risk than (odds ratio <1) illiterate ones but the evidence is not statistically significantly ($p>$ 0.05). See table 2a.

In the second study, the first group contained sixty-three mothers of patients (15.6%) and fifty-seven mothers of controls (14.1%). The second group was one hundred and two mothers of patients (25.2%) and ninety-sixe mothers of controls (23.7%). The third group was seventy-five mothers of patients (18.5%) and seventy-nine mothers of controls (19.5%). The fourth group consisted of one hundred and twenty-four mothers of patients (30.6%) and one hundred and ninten mothers of controls (29.4%). Forty-one mothers of patients (10.1%) and fifty-four mothers of controls (13.3%) were represented within the fifth group. The run of the McNemar,s test has shown that the level of education of mothers of patients in both studies again did not statistically differ from the control mothers ($p>$ 0.05). See table 2b.

3.1.4 Family size

Family size was divided to four groups: in the first group, the number of families with a single child, in the second, families with 2 to 4 children, the third, families with 5 to 9 children; and the fourth group, families with 10 to 12 children (Table 3a and 3b).

In the first study, the number of children ranged from 1 to 12 with a median of 4

children mean 4.6. For the first group, the number was 7 families of patients (5.9%) and 19 families of controls (16.1%). For the second group, the number was 57 families of patients (48.3%) and 63 families of controls (53.4%). The third group contained 46 families of patients (39.0%) and 34 families of controls (28.8%). The number was 8 families of patients (6.7%) and 2 families of controls (1.7%) in the fourth. The largest group was where the number of children was from 2 to 4 (57; 48.3%) families of patients and (63; 53.4%) of families of controls. The smallest group was families where the number of children was from 10 to 12 (8; 6.7%) families of patients and (2; 1.7%) families of controls. The McNemar's chi sequare test is showing that children from family with more than one child are significantly more at risk of illness than children from single child family (baseline), but the confidence interval is too wide becauuse of the small unmber of cases in the category (10-11). See table 3a.

In the second study, the numbers of children in the families examined in this study, the range was from 1 to 11 children with a median of 4 children mean 4.5. For the first group, the number was twenty-eight families of patients (6.9%) and 56 families of controls (13.8%). The second group was 200 families of patients (49.4%) and 208 families of controls (51.4%). The third group was 156 families of patients (38.5%) and 130 families of controls (32.1%). The fourth group was 21 families of patients (5.2%) and 11 families of controls (2.7%). The largest group contained families where the number of children was from 2 to 4 (200; 49.4%) families of patients and (208; 51.4%) of families of controls, and the smallest group contained families where the number of children was from 10 to 11 (21; 5.2%) families of patients and (11; 2.7%) of families of controls. The McNemar's chi sequare test is showing that children from family with more than one child are significantly more at risk of illness than children from single child family (baseline). See table 3b.

Table 3. Family size of patients and controls (a) First study

Number of childrn/family	Families of patients № of family	(%)	Families of controls № of family	(%)	OR	p-value	95% CI
1	7	6.0	19	16.1	1		1 to 1 (by default)
2 – 4	57	47.5	63	53.4	2.409	0.071	0.926 to 6.268
5 – 9	46	39.8	34	28.8	3.966	0.008	1.426 to 11.029
10 - 12	8	6.7	2	1.7	11.294	0.008	1.862 to 68.492
Total	118	100	118	100			

Table 3b. Second study

Number of children/family	Families of patients № of family	(%)	Families of controls № of family	(%)	OR	p-value	95% CI
1	28	6.9	56	13.8	1		1 to 1 (by default)
2-4	200	49.4	208	51.4	2.205	0.004	1.279 to 3.800
5-9	156	38.5	130	32.1	2.795	0.000	1.571 to 4.977
10-11	21	5.2	11	2.7	3.999	0.002	1.672 to 9.568
Total	405	100	405	100			

3.1.5 Number of children who lived in Tripoli and other areas

In the first study, the number of children who lived in Tripoli was 142/236 (60%) and those who lived outside the capital was 94/236 (40%). In the second study, the number of children who lived in Tripoli was 453/810 (56%) whilst 352/810 (44%) lived outside Tripoli. No specefic area with more cases could be identified but children lived from outside Tripoli or those referred from primary hospitals were usually suffering from sever diarrhoea.

3.2 Clinical findings

3.2.1 Stool nature of patients

3.2.1.1 Number of motions per day

The number of motions per day are shown in table 4a and 4b.

In the first study, the number of stools per day for each patient was reported. Children were divided to three groups: the first group comprised patients with 4 to 6 motions per day and had 60 patients (50.8%); the second group had from 7 to 9 motions per day and had 43 patients (36.5%); the third group ≥10 motions per day contained 15 patients (12.7%). The mean was 5.4 and the median was 6. The second study yielded similar results. Patients with from 4 to 6 motions per day numbered 198 (48.9%), the second group with 7 to 9 motions per day had 147 (36.3%), and the third group from ≥ 10 motions per day had 60 (14.8%). The mean was again 5.4 and the median 6.

3.2.1.2 Consistency of stool

The stool consistency was noted for every patient and the results are illustrated in table 4. Both studies gave similar results. In the first study, the number of patients who passed loose mucous stool was thirty-nine (33.1%), the number of patients who passed watery stool was sixty-nine (58.5%) and the number who passed mixed stool with blood was ten (8.4%). Watery stool was the most common stool consistency of stool

patients and proved to be statistically different from loose, mucous or bloody stool (χ^2 = 15.42, $p < 0.05$).

In the second study, the number of patients who passed loose mucous stool was 133 (32.8%), the number of patients who passed watery stools were 229 (56.6%) and the number of patients who passed stool mixed with blood 43 (10.6%). Watery stool was once more the most common stool consistency of stool patients and statistically different from loose, mucous or bloody stool (χ^2 = 20.57, $p < 0.05$). Both studies provided similar results for the stool nature of patients.

3.2.1.3 Duration of diarrhoea

Duration of diarrhoea was reported before admission of patients until it ceased and the results are shown in table 4. The results were again similar in both studies.

In the first study, diarrhoea started prior to admission and ceased after one day in 32 (27.1%) patients, from two to three days in 56 (47.5%) patients, from four to six days in 23 (19.5%) patients and from ≥ 7 days in 7 patients (5.9%). The mean was 3.6 and the median was 3 days.

In the second study, diarrhoea started one day before admission in 101 (24.9%) patients, from two to three days in 197 (48.6%) patients, from four to six days in 86 (21.3%) patients and from ≥ 7 days in 21 patients (5.2%). The mean was also 3.9 and the median 3 days. Both studies gave similar results for the duration of diarrhoea.

Duration of diarrhoea of 2-3 days was found to be the most common in the first and second stidies with 56 (47.5%) and 197 (48.6%) respectively.

3.2.2 Abdominal cramps

The number of patients who had abdominal cramps was reported before or during admission. In the first study, the number of patients who had these was 38 (32.2%) and the number of patients who did notwas 80 (67.8%). For the second study, the number

Table 4. Stool nature of patients (a) First study

Number of motion Motion/day	(№)patients	(%)	Stool appearance Consistency	(№)patients	(%)	Duration Duration/day	(№)patients	(%)
4-6	60	50.8	loose + mucous	39	33.1	1	32	27.1
7-9	43	36.5	watery	69	58.5	2-3	56	47.5
≥10	15	12.7	mixed with blood	10	8.4	4-6	23	19.5
						≥7	7	5.9
Total	118	100		118	100		118	100

Table 4b. Second study

Number of motion Motion/day	(№)patients	(%)	Stool appearance Consistency	(№)patients	(%)	Duration Duration/day	(№)patients	(%)
4-6	198	48.9	loose + mucous	133	32.8	1	101	24.9
7-9	147	36.3	watery	229	56.6	2-3	197	48.6
≥10	60	14.8	mixed with blood	43	10.6	4-6	86	21.3
						≥7	2	5.2
Total	118	100		118	100		118	100

of patients who had abdominal cramps was 96 (23.7%) and the number who did not have them was 309 (76.3%).

3.2.3 Fever

The number of patients who had fever reported before and during admission is displayed in table 8. The cut-off point for definition of fever was ≥ 38.0 °C. If the patients had temperatures < 38 °C the patient was not recorded as having fever.

3.2.3.1 Fever grade

Fever grade was classified in five groups: from 38.0 to 38.4, 38.5 to 38.9, 39.0 to 39.4, 39.5 to 39.9 and ≥ 40 °C (Table 5a and 5b).

In the first study, the number of patients who had fever was 86 (72.9%), while the number of patients not reporting fever was 32 (27.1%). The number of patients in the first group was 37 (31.4%), the second was 11 (9.3%), the third was 25 (21.2%), the fourth was 8 (6.8%) and the fifth group was 5 (4.2%). Fever was a clinical feature associated with acute diarrhoea.

In the second study, the number of patients who had fever was 290 (71.6%), while the number of patients not reporting fever was 115 (28.4%). The number of patients in the first group was 95 (23.4), in the second 49 (12.1%), the third 78 19.3%), the fourth 54 (13.3%) and the fifth 14 (3.5%). Fever was again a clinical feature associated with acute diarrhoea.

There were some dissimilarities between the two studies. The group of patients who had low grade fever (38.0 to 38.4) was higher in the first study (37; 31.4%) than in the second (95; 23.4%) while high grade fever (39.5 to ≥ 40) was higher in the second study (68; 16.8%) when compared to the first (13; 11%).

3.2.3.2 Duration of fever

The duration of patients' fever before and during admission was varied (Table 5a and

Table 5. Fever* of patients (a) First study

Fever grade °C	№ of patients	(%)	duration/days	№ of patients	(%)
38.0-38.4	37	31.4	1	20	16.9
38.5-38.9	11	9.3	2-3	41	34.8
39.0-39.4	25	21.2	4-6	18	15.3
39.5-39.9	8	6.8	≥ 7	7	5.9
≥ 40	5	4.2			
Total	86	72.9		86	72.9

* Only patients with temperatures ≥ 38 °C were recorded

Table 5b. Second study

Fever grade °C	№ of patients	(%)	duration/days	№ of patients	(%)
38.0-38.4	95	23.4	1	49	12.1
38.5-38.9	49	12.1	2-3	167	41.2
39.0-39.4	78	19.3	4-6	52	12.9
39.5-39.9	54	13.3	≥ 7	22	5.4
≥ 40	14	3.5			
Total	290	71.6		290	71.6

5b). The minimum was one d and the maximum seven d. In the first study, the number of patients who had fever for one d was 20 (16.9%), from two to three d was 41 (34.8%), from four to six d was 18 (15.3%) and seven d or more was 7 (5.9%). The mean was 3.06 and the median was 2 d.

In the second study, the number of patients who had fever for one d was 49 (12.1%), from two to three d was 167 (41.2%), from four to six d was 52 (12.9%) and seven or more d was 22 (5.2%). The mean was 3.42 and the median 2 d.

There was some disparity between the two studies. The group of patients who had fever for one day was higher in the first study (20; 16.9%) while duration of fever from 2-3 days was higher in the second study (167; 41.2% table 5b).

3.2.4 Vomiting of patients

The vomiting results of patients are displayed in table 6. The number of vomits per d (frequency) and for how long (duration/days) per patient were recorded.

In the first study, the number of patients who had vomiting before and during admission was 61 (51.7%), while the number of patients who had no vomiting at admission was 57 (48.3%). Vomiting as a clinical feature was not associated with acute diarrhoea in the first study ($p > 0.05$). In the second study, the number of patients who had vomiting before and during admission was 236 (58.3%), while the number of patients who had no vomiting was 169 (41.7%). Vomiting as a clinical feature was associated with acute diarrhoea ($p < 0.05$).

4.2.4.1 Frequency of vomiting

The frequency of vomiting per day was classified into three groups: the first group From 1 to 3 times per day; the second 4 to 6 times per d; the third 7 times or more per day.

Table 6. Vomiting patients (a) First study

Frequency/day	№ of patients	(%)	Duration/days	№ of patients	(%)
1-3	15	12.7	1	16	13.6
4-6	42	35.6	2-3	32	27.1
≥ 7	4	3.4	4-6	9	7.6
			≥ 7	4	3.4
Total	61	51.7		61	51.7

Table 6b Second study

Frequency/day	№ of patients	(%)	Duration/days	№ of patients	(%)
1-3	55	13.6	1	47	11.6
4-6	164	40.5	2-3	151	37.3
≥ 7	17	4.2	4-6	23	5.7
			≥ 7	15	3.7
Total	236	58.3		236	58.3

In the first study, the number of patients in the first group was 15 (12.7%), the second group was 42 (35.6%) and the third group was 4 (3.4%). The mean was 4.6 and the median was 5.

In the second study, the number of patients in the first group was fifty-five (13.6%), the second group was one hundred and sixty-four (40.5%) and the third was seventeen (4.2%). The mean was 4.9 and the median 5. The minimum frequency was one and the maximum ten. The frequency of vomiting was higher in the second study (44.7%) than in the first (39%).

3.2.4.2 Duration of vomiting The frequency of vomiting per day was classified into four groups: the first 1 time per day; the second 2 to 3 times per d; the third 4 to 6 times per d; the fourth 7 times or more per d.

In the first study, the number of patients in the first group was 16 (13.6%), the second group 32 (27.1%), the third 9 (7.6%) and the fourth 4 (3.4%). The mean was 3.25 and the median 3.

In the second study, the number of patients in the first group was forty-seven (11.6%), the second group was one hundred and fifty-one (37.3%), the third twenty-three patients (5.7%) and the fourth group fifteen patients (3.7%). The mean was 3.45 and the median 3. The minimum duration was one day and the maximum was eight days. The duration of vomiting was longer in the second study (46.7%) than in the first (38.1% ; table 6).

3.2.5 Dehydration

Dehydration is a clinical feature of acute diarrohea. The estimation of the type and degree of dehydration at admission is crucial in order to commence the proper treatment by correction of dehydration. Fig. 3.

3.2.5.1 Type of dehydration

The type of dehydration was classified into three types (according to the serum sodium concentration). Fig. 3.

3.2.5.1.1 Isotonic dehydration

In the first study, the number of patients who had isotonic dehydration was 90 (76.3%). In the second, the number of patients who had isotonic dehydration was 307 (75.8%).

3.2.5.1.2 Hypotonic dehydration

In the first study, the number of patients who had hypotonic dehydration was 16 (13.5%). In the second study, the number of patients who had hypotonic dehydration was 42 (10.4%).

3.2.5.1.3 Hypertonic dehydration

In the first study, the number of patients who had hypertonic dehydration was 12 (10.2%). In the second study, the number of patients who had hypertonic dehydration was 56 (13.8%). The type of dehydration did not differ between the two studies.

3.2.5. 2 Degree of dehydration

The degree of dehydration was classified into three types; (according to the percentage of body weight loss): mild dehydration is when the patient has 4-5% of body weight loss, moderate dehydration when the patient has 6-9% of body weight loss, and severe dehydration when the patient has 10% or more of body weight loss.

3.2.5.2.1 Mild dehydration

In the first study, the number of patients who had mild dehydration at admission was 38 (32.2%). In the second, the number of patients who had mild dehydration at admission was 134 (33.1%).

3.2.5.2.2 Moderate dehydration

In the first study, the number of patients who had moderate dehydration at admission

Figure 3. Type and degree of dehydration of patients (a) First study

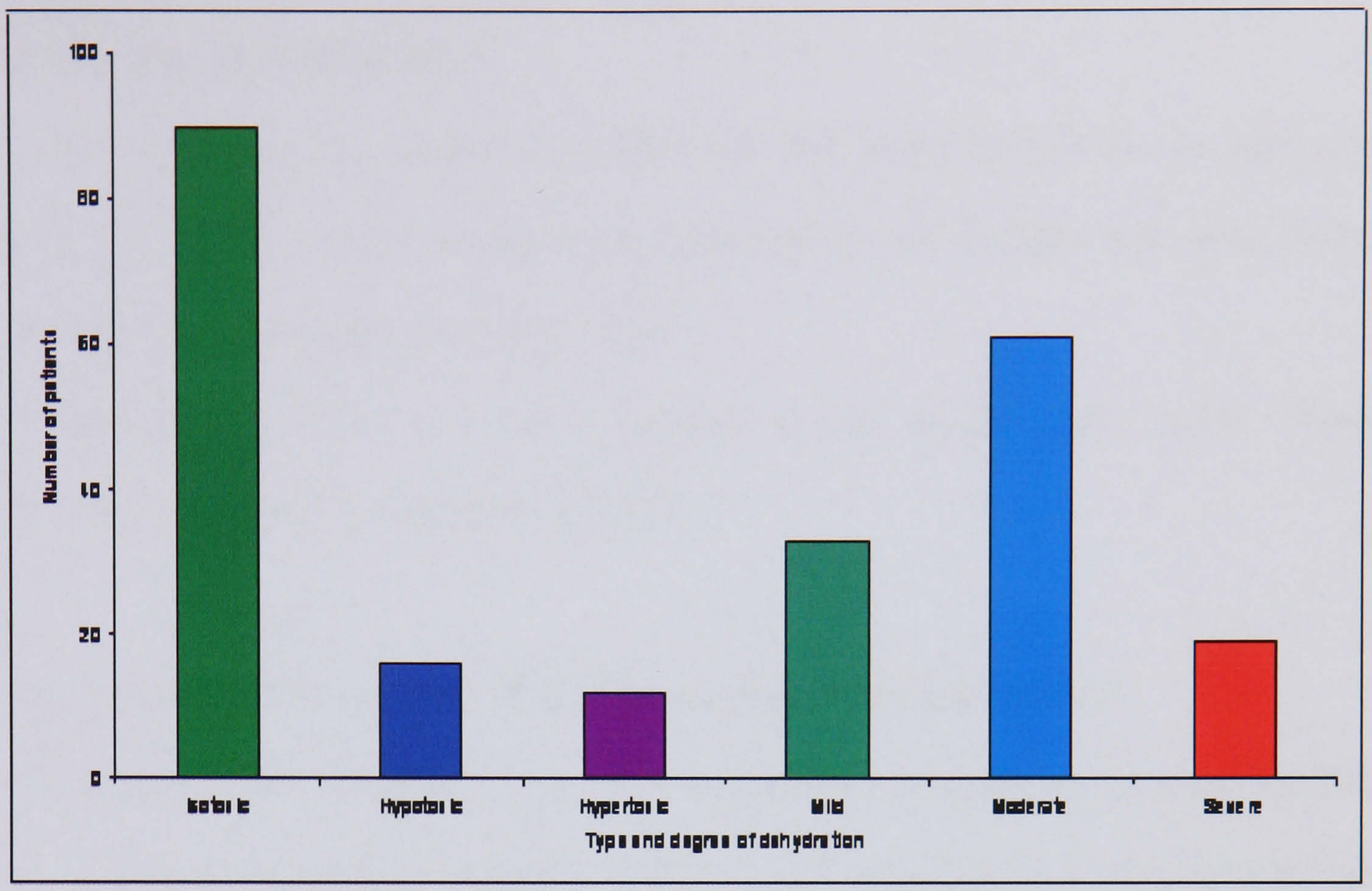
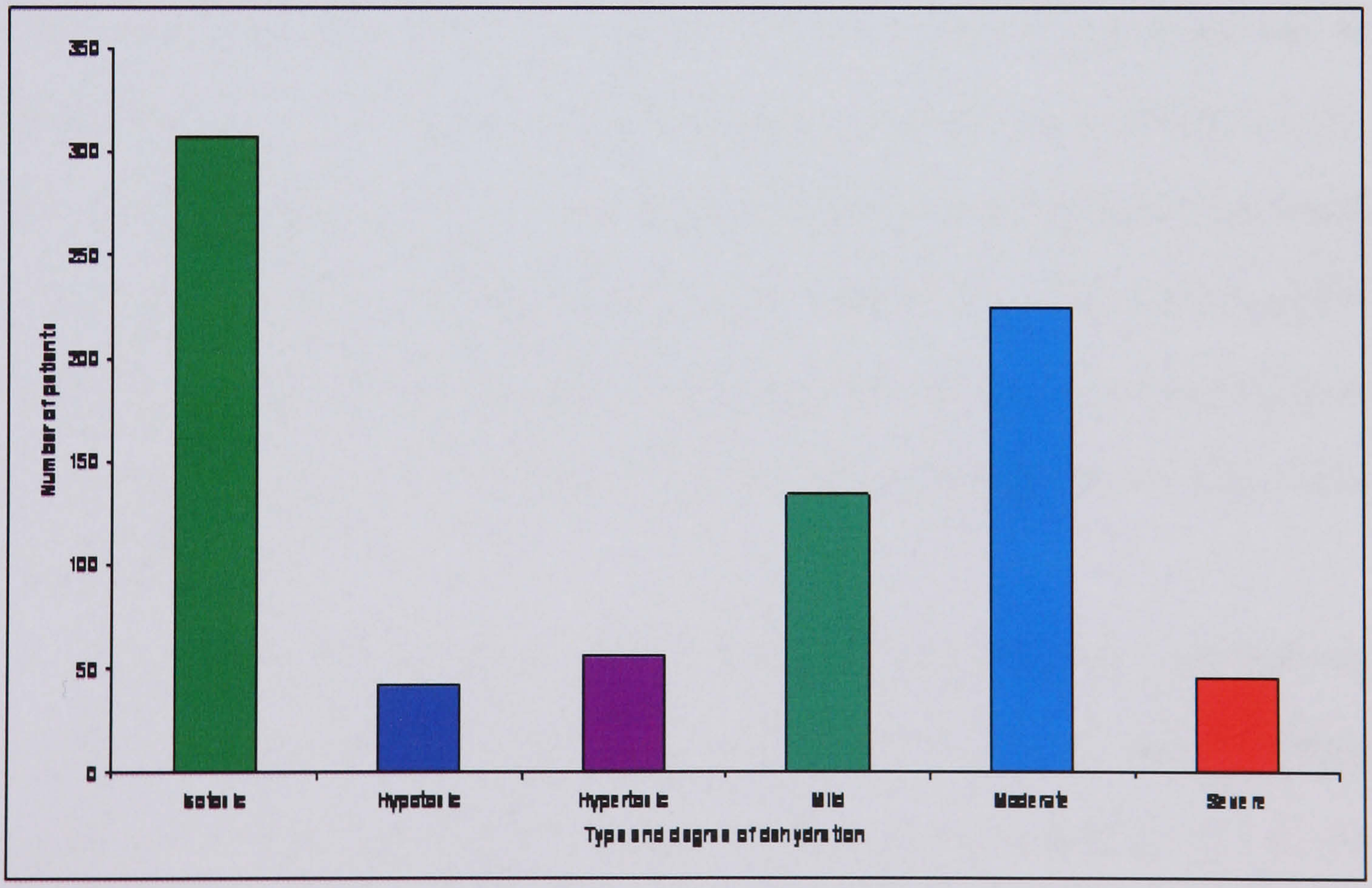


Figure 3b. Second study



was 61 (51.7%). In the second study, the number of patients who had moderate dehydration at admission was 225 (55.5%).

3.2.5.2.3 Severe dehydration

In the first study, the number of patients who had severe dehydration at admission was 19 (16.1%), whilst in the second the number of children who had severe dehydration at admission was 46 (11.4%).

Moderate dehydration was more frequent in the second study, while severe dehydration was more frequent in the first.

3.2.6 Haemoglobin concentration in blood of patients and controls

The haemoglobin concentration in blood of patients and controls was classified into four groups: first group, haemoglobin (Hb) ≤ 9 gdl^{-1} (anaemia), second group Hb from 9.1 to 10.9, third group Hb from 11 to 11.9, and the fourth group Hb ≥ 12. See table 7a and 7b. In the first study, the number of patients of the first group was 4 (3.4%) and was 1 only (0.8%) of control, the number of patients of the second group was 58 (49.2%) and was 49 (41.5%) of controls, the number of patients of the third group was 24 (20.3%) and was 35 (29.7%) of controls and the number of patients of the fourth group was 32 (27.1%) and was 33 (28.0%) of controls. By using a haemoglobin concentration level ≤ 9 as a baseline, the test is showing that higher haemoglobin concentration is associated with lower risk but this association is not statistically significant.

In the second study, the number of patients in the first group was 24 (5.9%) and was 17 (4.2%) of the controls, the number of patients in the second group was 162 (40.0%) and was 154 (38.0%) of controls, the number of patients in the third group was 118 (29.1%) and was 129 (31.9%) of controls, and the number of patients in the fourth group was 101 (24.9%) and was 105 (25.9%) of controls. By using a haemoglobin

Table 7. Haemoglobin concentration of patients and controls (a) First study

Haemoglobin gdl^{-1}	№ of patients (%)	№ of control (%)	OR	p-value	95% CI
≤ 9	4	3.4	1	0.8	1	1 to 1 (by default)
9.1-10.9	58	49.2	49	41.5	0.253	0.217	0.029 to 2.241
11.0-11.9	24	20.3	35	29.7	0.146	0.087	0.016 to 1320
≥ 12	32	27.1	33	28.0	0.205	0.156	0.023 to 1.826
Total	118	100	118	100			

Table 7b. Second study

Haemoglobin gdl^{-1}	№ of patients (%)	№ of controls (%)	OR	p-value	95% CI
≤ 9	24	6.0	17	4.2	1	1 to 1 (by default)
9.1-10.9	162	40.0	154	38.0	0.485	0.390	0.387 to 1.449
11.0-11.9	118	29.1	129	31.9	0.652	0.205	0.336 to 1.264
≥ 12	101	24.9	105	25.9	0.674	0.266	0.337 to 1.349
Total	405	100	405	100			

concentration level ≤ 9 as a baselne, the test is showing that higher haemoglobin concentration is associated with lower risk but this association is not statistically significant. The variances were not significant between the two studies, in the first study where Hb ≤ 9 (mild anaemia) represented 4 (3.4%) patients and 1 (0.8%) of the controls and was represented 24 (5.9%) patients and 17 (4.2%) of the controls in the second study.

3.2.7 Blood urea and serum electrolytes of patients

The blood urea, serum potassium and sodium of patients were classified in three groups; first group, urea ≤ 19 mg/dl, potassium ≤ 3.3 mmol/l and sodium < 130 mmol second group, urea 20 – 59 mg/dl, potessium 3.4 – 5.3 mmol/l and sodium 130 -150 mmol and third group urea ≥ 60 mg/dl, potassium ≥ 5.4 and sodium > 150 mmol/l. (See table 8).

3.2.7.1 Blood urea

In the first study, the number of patients of the first group was 44 (37.3%), the number of patients of the second was 63 (53.4%) and the number of the third group was 11 (9.3%). The mean was 27.68 and the median 23.90. In the second study, the number of patients in the first group was 155 (38.3%), the number in the second group was 209 (51.6%) and that in the third group was 41 (10.1%). The mean was 27.24 and the median 23.48.

3.2.7.2 Serum potassium

In the first study, the number of patients in the first group was 20 (17.0%), the number of patients in the second was 93 (78.8%) and the number in the third group was 5 (4.2%). The mean was 3.94 and the median 3.90. In the second study, the number of patients in the first group was 67 (16.5%), the number of patients in the second was 318 (78.5%) and the number in the third group was 20 (5.0%). The mean was 3.95 and

Table 8. Blood urea and electrlytes of patients (a) First study

Urea mg/dl	№ of Patients	(%)	Potassium mmol	№ of patients	(%)	Sodium mmol	№ of patients	(%)
≤ 19	44	37.3	≤ 3.3	20	17.0	< 130	16	13.5
20 – 59	63	53.4	3.4- 5.3	93	78.8	130-150	90	76.3
≥ 60	11	9.3	≥ 5.4	5	4.2	> 150	12	10.2
Total	118	100%		118	100%		118	100%

Table 8b. Second study

Urea mg/dl	№ of Patients	(%)	Potassium mmol	№ of patients	(%)	Sodium mmol	№ of patients	(%)
≤ 19	155	38.3	≤ 3.3	67	16.5	< 130	42	10.4
20-59	209	51.6	3.4-5.3	318	78.5	130-150	307	75.8
≥ 60	41	10.1	≥ 5.4	20	5.0	> 150	56	13.8
Total	405	100%		405	100%		405	100%

the median 3.92.

3.2.7.3 Serum sodium

In the first study, the number of patients in the first group was 16 (13.5%), the number of patients in the second was 90 (76.3%) and that of patients in the third group was 12 (10.2%). The mean was 137.8 and the median 135.5. In the second study, the number of patients in the first group was 42 (10.4%), the number in the second group was 307 (75.8%) and the number of patients in the third group was 56 (13.8%). The mean was 137.6 and the median 137.3.

3.2.8 Treatment of dehydration

Three types of treatment were used for correction of dehydration among patients; firstly ORT, secondly IVT and thirdly mixed (ORF and IVD; Table 9a). In the first study, the number of patients given ORT was 10 (8.5%), the number of patients given IVT was 31 (26.3%) and the number of patients administered both ORT and IVT was 77 (65.2%). In the second study, the number of patients given ORT was 56 (13.8%), the number of patients given IVT was 102 (25.2%) and the number of patients administered both ORT and IVT was 247 (61.0%). ORT was used more frequently in the second study (Table 9b).

3.2.9 Stay of patients in hospital

The duration of stay of patients in the hospital was classified in three groups; the first group spent from one to three d, the second from four to seven d and the third from eight to eleven d. In the first study, the number of patients in the first group was 17 (14.4%), the number of patients in the second was 89 (75.4%) and the number in the third was 12 (10.2%). The mean was 5.4 and the median 5.0 d. In the second study, the number of patients in the first group was 90 (22.2%), in the second 284 (70.1%) and in

Table 9. Type of rehydration therapy and duration of stay in hospital (a) First study

Type of fluid	№ of patients	(%)	Stay in hospital/days	№ of patients	(%)
Oral	10	8.5	1-3	17	14.4
IVF	31	26.2	4-7	89	75.4
Oral + IVF	77	65.3	8-11	12	10.2
Total	118	100%		118	100%

Table 9b. Second study

Type of fluid	№ of patients	(%)	Stay in hospital/days	№ of patients	(%)
Oral	56	13.8	1-3	90	22.2
IVF	102	25.2	4-7	284	70.1
Oral + IVF	247	61.0	8-11	31	7.7
Total	405	100%		405	100%

the third 31 (7.7%). The mean was 5.03 and the median 5.00 d. More patients required hospitalisation for 4-7 d in the second study (Table 9a and 9b).

3.2.10 Body weight of patients and controls

The body weight of children was classified in four groups; first group < 10% of age for weight, second group from 10 to < 50%, third group 50% and fourth group > 50%. In the first study, the number of first group was 30 (25.4%) patients and 8 controls (6.8%; table 10a). The number of patients of second group was 48 (41.7%) and 29 (24.6%) in the controls. The number of patients in the third group was 13 (11.0%) and 24 (20.3%) in the controls. The number of patients in the fourth group was 27 (22.9%) and 57 (48.3%) in the controls. The weight range of all patients and controls was 3.2 - 18.1 kg. The mean was 7.2 and the median 6.8. The patient group was significantly different from the control group in their weight for age of first group under nutrition < 10% of patients (χ^2= 15.18, p <0.05). In the second study, the number of patients in the first group was 115 (28.4%) and the controls 32 (7.9%; table 10b). The number of patients in the second group was 150 (37.0%) and the controls 91 (22.5%). The number of the patients in the third group was 57 (14.1%) and the controls 98 (24.2%). Finally the number of patients in the fourth group was 83 (20.5%) and the controls 184 (45.4%). The weight range of all patients and controls was 3.3 - 17.8 kg. The mean was 7.1 and the median 6.7 kg. None of the patients with acute gastroenteritis demonstrated signs of marasmus or kwashiorkor in both studies. The number of patients with undernutrition was 115 (28.4%) and 32 (7.9%) in the controls in the second study. The patient group was significantly different from the control group in their weight for age of the first group under nutrition < 10% ($p < 0.05$) of the second.

Table 10. Body weight of patients and controls (a) First study

Age for weight (%)	№ of patients	(%)	№ of controls	(%)
< 10%	30	25.4	8	6.8
10 - < 50%	48	40.7	29	24.6
50%	13	11.0	24	20.3
> 50%	27	22.9	57	48.3
Total	118	100%	118	100%

Table 10b. Second study

Age for weight (%)	№ of patients	(%)	№ of controls	(%)
< 10%	115	28.4	32	7.9
10 - < 50%	150	37.0	91	22.5
50%	57	14.1	98	24.2
> 50%	83	20.5	184	45.4
Total	405	100%	405	100%

3.2.11 Extraintestinal manifestations associated in some patients

In the first study, certain other diseases were found in 19/118 (16.1%) of patients with diarrhoea. Chest infection was the most common disease associated with diarrhoea (9/19; 47.4%), followed by febrile convulsion (4/19; 21%), meningitis (3/19; 15.8%) and heart disease (3/19; 15.8%). In the second study, other diseases were discovered in 87 (21.5%) patients with diarrhoea. Chest infection was again the most common disease associated with diarrhoea 45/87 (51.7%), followed by febrile convulsion 18/87 (20.7%), meningitis 13/87 (14.9%), heart disease 6/87 (6.9%) and renal disease 5/87 (5.8%).

3.3 Aetiological detection agents isolated from patients and controls

Bacteria, viruses and parasites were detected in stool specimens obtained from patients and controls as described in sampling and methods. The results are displayed in table 11 and figure 4. Bacteria were responsible for most cases in the first study, but rotaviruses applied to the second.

In the first study, the microbiological aetiology of 118 stool specimens from children with acute diarrhoea was identified as either single or mixed infection in 66/118 (55.9%) of patients. The aetiology of the remaining 52/118 (44.1%) patients was un-determined.

Bacterial agents were isolated from 50/118 patients (43.3%), rotaviruses were identified in 18/118 patients (15.3%) and parasites were identified in 10/118 (8.4%) patients (Table 11a). Infection of patients by a single pathogen was present in (46.6%) and infection by mixed pathogens in 11/118 patients (9.3%) where more than one pathogen was detected in the same patient (table 11a). Mixed infections were either two 10/118 (8.4%) or three pathogens 1/118 (0.8%). Bacteria + rotavirus were present in 5/118 (4.2%) patients, mixed two types of bacteria were present in 2/118 (1.7%) patients, bacteria + parasite were present in 2/118 (1.7%) of patients and rotavirus + parasite were present in 1/118 (0.8%) patients. A mixed infection by three agents, two bacterial agents + rotavirus was present in 1/118 (0.8%) patients. No mixed infections by more than one parasitic agent were detected in this study. Stool specimens from the 118 children in the control group were examined for enteric pathogens which were identified in 16/118 (13.6%) children. Bacterial agents were isolated from 9/118 (7.6%), rotaviruses from 3/118 (2.6%) and parasites from 4/118 (3.4%) controls. No mixed infections were observed in the control group. Pathogens detected in the patients' group were significantly different from the control group $p<0.05$).

Table 11. Identification of bacteria, rotavirus and parasites in faecal specimens of patients and control with single or mixed infections (a) First study

Type of infection	Patients 66/118		Control 16/118	
	№ of patients	(%)	№ of controls	(%)
1 agent	55	46.6	16	13.6
2 age	10	8.5		
3 agents	1	0.8		
No agents detected	52	44.1	102	86.4
Total	118	100%	118	100%
Single agent				
E. coli	21	17.8	5	4.3
Campylobacter	6	5.1	1	0.8
Proteus	6	5.1	2	1.7
Klebsiella	4	3.4	1	0.8
Rotavirus	11	9.3	3	2.6
Ent. histolytica	2	1.7	2	1.7
G. lamblia	2	1.7	1	0.8
Cryptosporidium	3	2	1	0.8
2 agents				
Ent.histolytica + *Campylobacter*	1	0.8		
Ent. histlytica + *E. coli*	1	0.8		
Ent. histolytica + rotavirus	1	0.8		
E. coli + *Proteus*	2	1.7		
E. coli + rotavirus	2	1.7		
Rotavirus + *Campylobacter*	2	1.7		
Rotavirus + *Proteus*	1	0.8		
3 agents				
Rotavirus + *E. coli* + *klebsiella*	1	0.8		
Total	66	55.9	16	13.6%

* No mixed pathogens.

Table 11. Identification of bacteria, rotavirus and parasites in faecal specimens of patients and control with single or mixed infections (b) Second study

Type of infection	Patients 276/405		Control 51/405	
	№ of patients	(%)	№ of controls	(%)
1 agent	239	59.0	51	12.6
2 agents	36	8.9		
3 agents	1	0.2		
No agents detected	129	31.9	354	87.4
Total	405	100%	405	100%
Single agent				
E. coli	52	12.8	11	2.7
Campylobacter	16	4.0	4	1.0
Proteus	6	1.5	3	0.7
Klebsiella	5	1.2	2	0.5
Rotavirus	98	24.2	12	3.0
Ent. histolytica	37	9.1	11	2.7
G. lamblia	12	3.0	5	1.2
Cryptosporidium	13	3.2	3	0.7
2 agents				
E. coli + *Proteus*	3	0.7		
E. coli + rotavirus	6	1.5		
E. col + *Ent. histolytica*	2	0.5		
E. coli + *G. lamblia*	3	0.7		
Campylobacter + rotavirus	4	1.0		
Campylobacter + *Ent. histolytica*	4	1.0		
Rotavirus + *Klebsiella*	7	1.7		
Rotavirus + *Ent. histolytica*	7	1.7		
3 agents				
Rotavirus + *E. coli* + *klebsiella*	1	0.2		
Total	276	68.1	51	12.6%

* No mixed pathogens.

In the second study, the microbiological aetiology of 405 stool specimens from children with acute diarrhoea were identified as either single or mixed infections in 276/405 (68.1%) patients. The aetiology of the remaining 129/ 405 (31.9%) was not detected. Bacterial agents were isolated from 113/405 (27.9%) patients, rotaviruses were identified in 123/405 (30.4%) patients and parasites were identified in 79/405 (19.3%) patients. Infection of patients by a single agent was present in 239/405 (59.0%) and infection of patients by mixed agents was present in 37/405 (11.4%; table 11b). Mixed infections were with either two pathogens in 36/405 (8.9%) patients or three pathogens in 1/405 (0.2%) patients. Of the mixed agents, two bacteria were in 3/405 (0.7%), bacteria + rotavirus in 17/405 (4.2%) patients, bacteria + parasite in 9/405 (2.2%) and rotavirus + parasite were detected in 7/405 (2%) patients and mixed infection by three agents, bacteria + rotavirus + parasite was present in 1/405 (0.2%) patients. No mixed infections with greater than one parasitic agent were detected.

Stool specimens from 405 children as a control group were investigated for detection of bacteria, rotavirus and parasites were in 51/405 (12.6%) controls.

Bacterial agents were isolated from 20/405 (4.9%) controls, rotavirus was detected in 12/405 (3%) controls and parasites were discovered in 19/405 (4.7%). Again, no mixed agents were observed in the control group. Bacterial, rotavirus and parasites detected in the patient group were again significally different from the control group (χ^2= 3.99, p<0.02).

3.3.1 Bacteriological findings

3.3.1.1 Isolation of bacteria

The type of bacteria isolated from patients and controls in the first study is shown in table 11a. The most common bacterial pathogens was *Escherichia coli* found in 27/118 (22.9%) patients and lowest isolation of bacterial pathogens was *Klebsiella*

Figure 4. The number of each aetiological agen was identification from patients

(a) first study

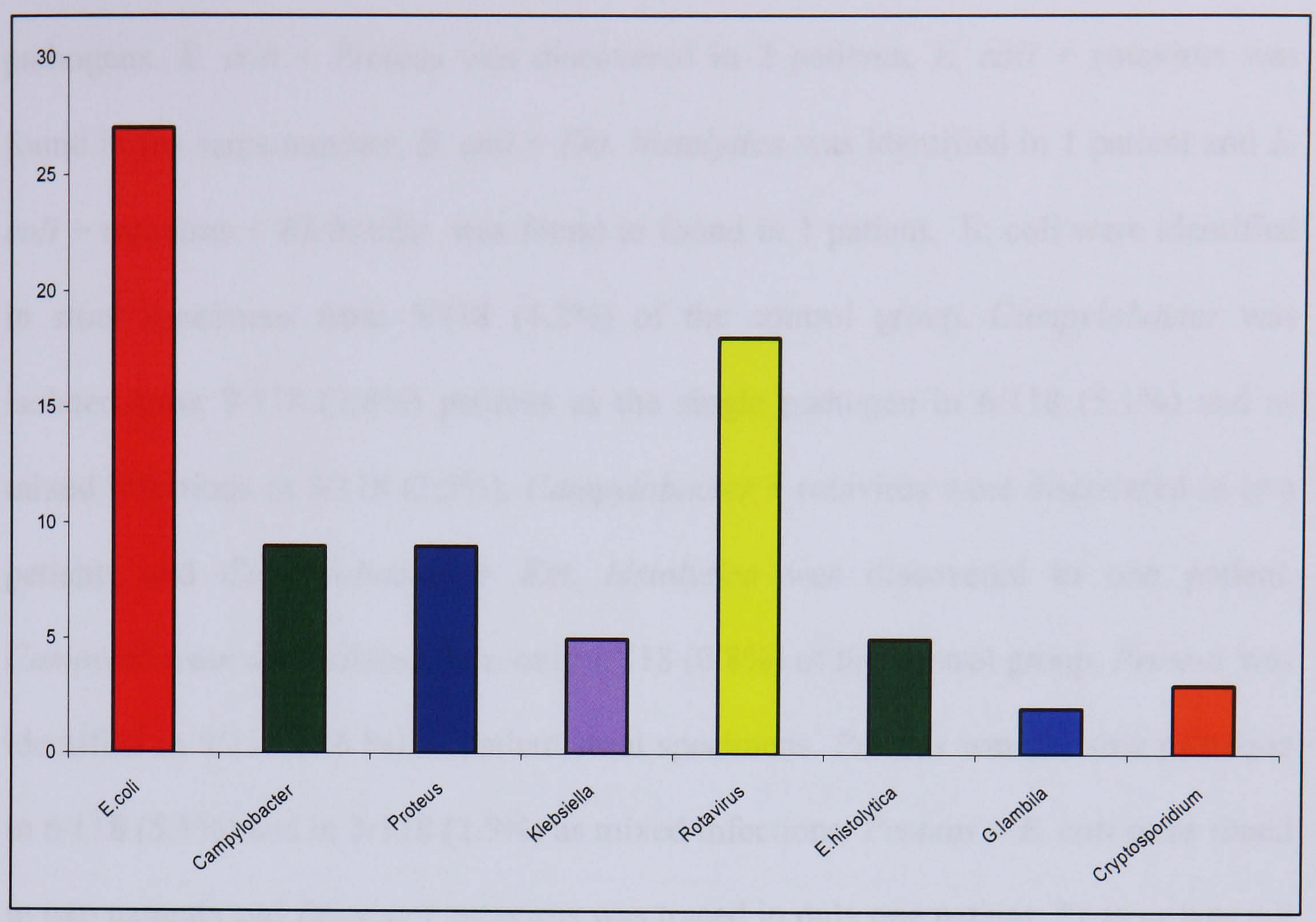
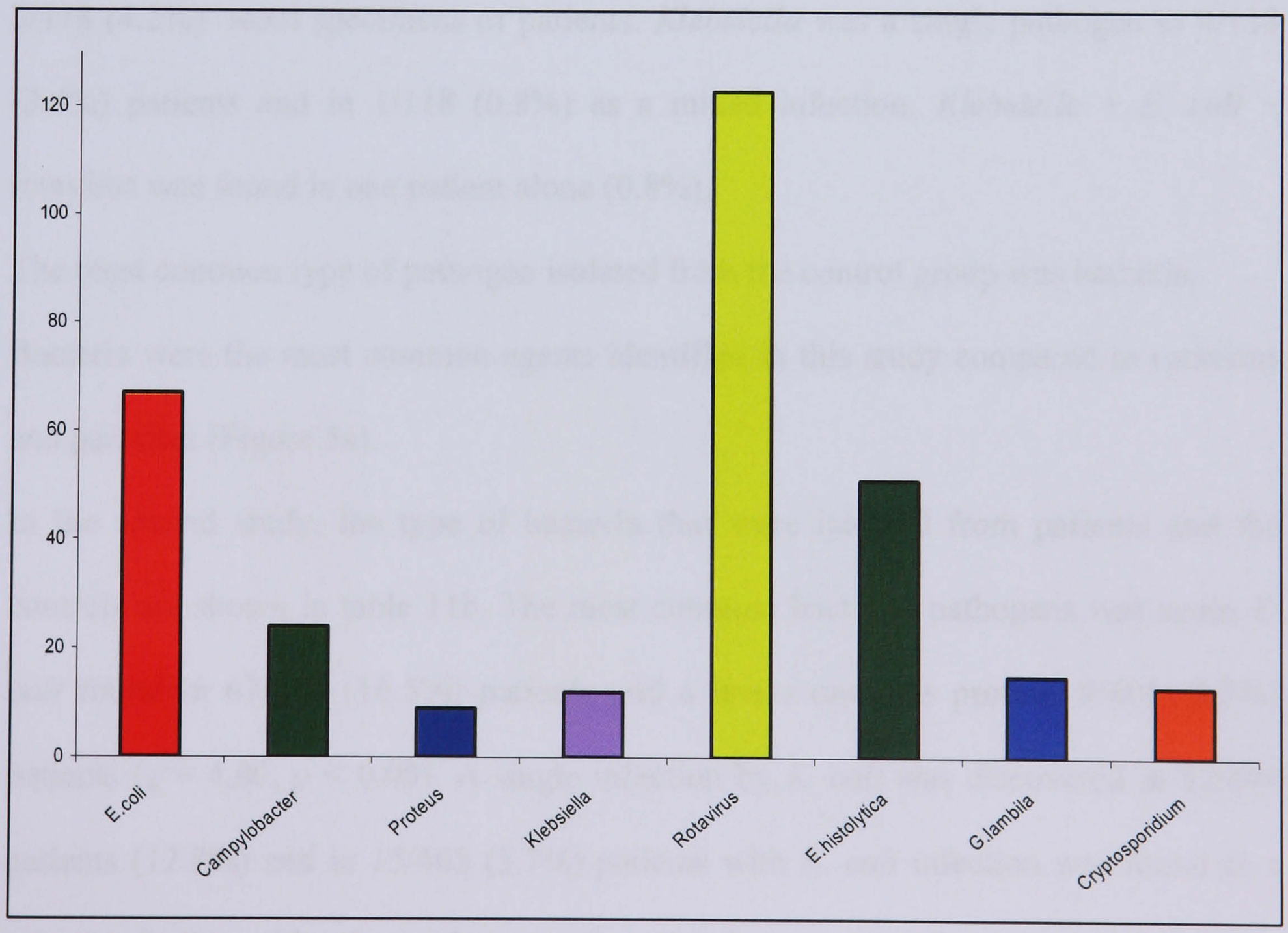


Figure 4b. Second study



found in 5/118 (4.2%) of patients (χ^2= 19.65, $p < 0.05$). A single infection by *E. coli* was discovered in 21/118 (17.8%) and in 6/118 (5.1%) as mixed infections with other pathogens. *E. coli* + Proteus was discovered in 2 patients, *E. coli* + rotavirus was found in the same number, *E. coli* + *Ent. histolytica* was identified in 1 patient and *E. coli* + rotavirus + *Klebsiella* was found in found in 1 patient. E. coli were identified in stool specimens from 5/118 (4.2%) of the control group. *Campylobacter* was isolated from 9/118 (7.6%) patients as the single pathogen in 6/118 (5.1%) and as mixed infections in 3/118 (2.5%). *Campylobacter* + rotavirus were discovered in two patients and *Campylobacter* + *Ent. histolytica* was discovered in one patient. *Campylobacter* was isolated from only 1/118 (0.8%) of the control group. *Proteus* was identified in 9/118 (7.6 %) of patient stool specimens. *Proteus* was the sole pathogen in 6/118 (5.1%) and in 3/118 (2.5%) as mixed infections. *Proteus* + *E. coli* were found in two patients and *Proteus* + rotavirus was traced in only one patient. *Proteus* was not isolated from any stool specimens of the control group. *Klebsiella* was discovered in 5/118 (4.2%) stool specimens of patients. *Klebsiella* was a single pathogen in 4/118 (3.4%) patients and in 1/118 (0.8%) as a mixed infection. *Klebsiella* + *E. coli* + rotavirus was found in one patient alone (0.8%).

The most common type of pathogen isolated from the control group was bacteria.

Bacteria were the most common agents identified in this study compared to rotavirus and parasites (Figure 5a).

In the second study, the type of bacteria that were isolated from patients and the controls are shown in table 11b. The most common bacterial pathogens was again *E. coli* found in 67/405 (16.5%) patients and a lesser one was proteus 9/405 (2.2%) patients (χ^2= 4.00, $p < 0.05$). A single infection by *E. coli* was discovered in 52/405 patients (12.8%) and in 15/405 (3.7%) patients with *E. coli* infection was found as a mixed infection with other pathogens; *E. coli* + *Proteus* were discovered in 3 patients,

Figure 5. The number of bacterial, viral and parasitic were identification from patients (a) first study

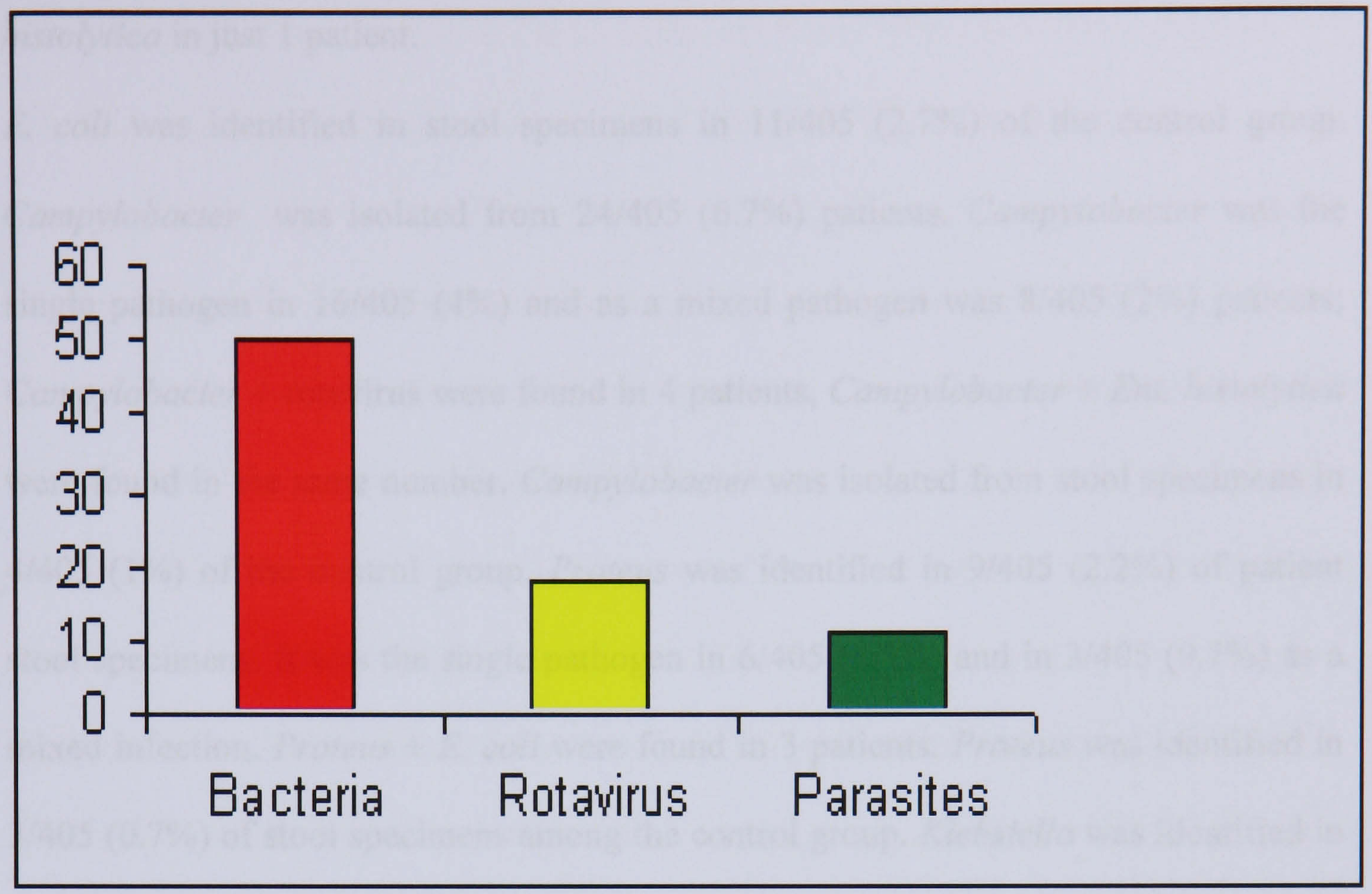
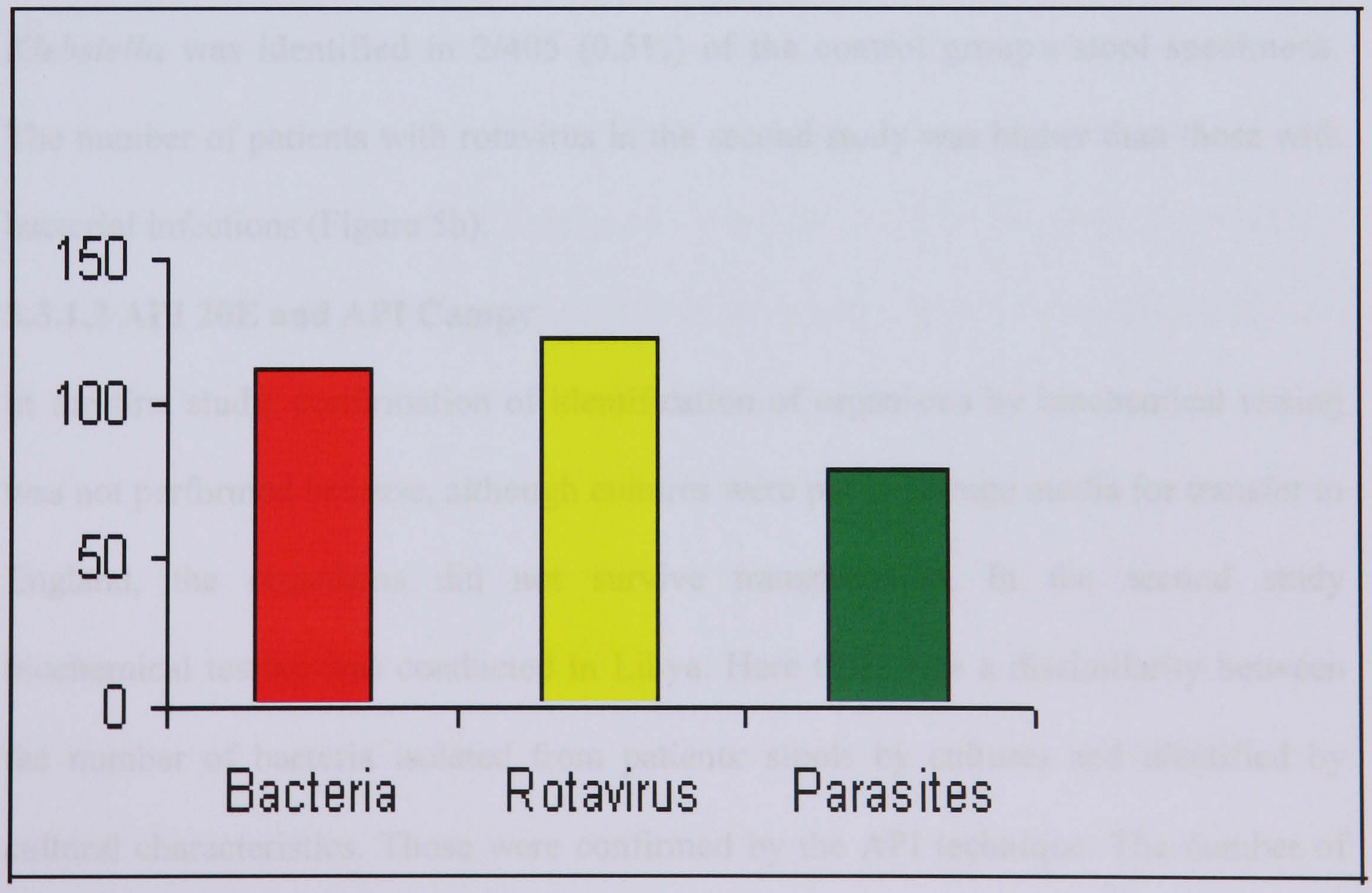


Figure 5b. Second study



E. coli + rotavirus was found in 6 patients, *E. coli* + *Ent. histolytica* was found in 2 patients *E. coli* + *G. lamblia* were found in 3 patients and *E. coli* + rotavirus + *Ent. histolytica* in just 1 patient.

E. coli was identified in stool specimens in 11/405 (2.7%) of the control group. *Campylobacter* was isolated from 24/405 (6.7%) patients. *Campylobacter* was the single pathogen in 16/405 (4%) and as a mixed pathogen was 8/405 (2%) patients; *Campylobacter* + rotavirus were found in 4 patients, *Campylobacter* + *Ent. histolytica* were found in the same number. *Campylobacter* was isolated from stool specimens in 4/405 (1%) of the control group. *Proteus* was identified in 9/405 (2.2%) of patient stool specimens. It was the single pathogen in 6/405 (1.5%) and in 3/405 (0.7%) as a mixed infection. *Proteus* + *E. coli* were found in 3 patients. *Proteus* was identified in 3/405 (0.7%) of stool specimens among the control group. *Klebsiella* was identified in 13/405 (3.2%) patients. *Klebsiella* was the single pathogen in 5/405 (1.2%) and in 8/405 (2%) as a mixed infection. *Klebsiella* + rotavirus were found in 7 patients and 1 /405 (0.2%) in combination with other pathogens *Klebsiella* + *E. coli* + rotavirus.

Klebsiella was identified in 2/405 (0.5%) of the control group's stool specimens. The number of patients with rotavirus in the second study was higher than those with bacterial infections (Figure 5b).

3.3.1.2 API 20E and API Campy

In the first study, confirmation of identification of organisms by biochemical testing was not performed because, although cultures were put in storage media for transfer to England, the organisms did not survive transportation. In the second study biochemical testing was conducted in Libya. Here there was a dissimilarity between the number of bacteria isolated from patients' stools by cultures and identified by cultural characteristics. Those were confirmed by the API technique. The number of bacteria identified by culture was 142/405 (35.1%) from stool of patients and the

number of bacteria confirmed by API was 112/405 (27.7%). The difference was 30 (7.4%) of bacterial agents. The number of bacterial organisms unconfirmed by API 20 E was 27; *Escherichia coli* 19 (12 as a single and 7 as a mixed infection), *Proteus* 4 (2 as a single and 2 as a mixed infection) and *Klebsiella* 4 (2 as a single and 2 as a mixed infection; Table 12).

The number of *Campylobacter* isolated by culture was 27 and the number unconfirmed by API Campy was 3 (2 as a single and 1 as a mixed infection; Table 13). The number of bacterial organisms isolated from stools of the control group by cultures was 27/405 (6.7%) and the number of bacteria confirmed by API technique was 20/405 (4.9%). The difirence was 7 (1.7%) of bacterial agents. The number of bacterial agents not confirmed by API 20E 6; *Escherichia coli* was 3, *Proteus* 2, *Klebsiella* 1 (Table 14). The number of *Campylobacter* unconfirmed by API Campy was 1 (Table 15).

API 20E results

Diarrhoeagenic *Escherichia coli* of a number of biotypes were isolated by cultures from stool specimens of patients and confirmed by identification with the API 20E. The number of *E. coli* confirmed was 76; 32 *E. coli* had very good identification (ID 79.9-99.8%, T 0.57-0.96) and 35 had good identification (ID 78.5-98.1%, T 0.35-0.8). The number of *Proteus* confirmed was 9; 2 *Proteus* had v. good identification (ID 72 – 99.9%, T 0.72), 3 *Proteus* with acceptable identification of the genus (ID 66.9%, T 0.72) and 4 proteus doubtful profiles (99.8-99.9%, T 38-39). The number of *klebsiella* confirmed was 12; 2 v. good identification (ID 99.1%, T 0.77), 4 *Klebsiella* good identification to the genus (ID 76.3%, T 0.44), 3 *Klebsiella* good identification (ID 97.4%, T 1.0) and 3 *Klebsiella* doubtful profiles (ID 82.8%, T 0.42; Table 12).

Escherichia coli were isolated by culture from stool specimens of the control group and confirmed with API 20E. The number was 11 *E. coli*; 4 *E. coli* was identified as

very good (ID 99.2% to 99.4%, T 0.57 to 0.96), one v. good identification to the genus (ID 79.9%, T 0.73) and 6 good identification (ID 92.2% to 98.1%, T 0.6-0.63). The number of *Proteus* confirmed was 3; 1 v. good identification (ID 99.9%, T 0.72) and 2 acceptable identification to the genus (ID 66.9%, T 0.72). The number of *Klebsiella* confirmed was 2; 1 good identification (ID 97.4%, T 1.0) and 1 good identification to the genus (ID 76.3%, T 0.44; Table 13).

API Campy result

Campylobacter spp. were isolated by selective medium (CCDA) from stool specimens of patients and confirmed by identifiying with the API Campy. The number was 24 *Campylobacter* spp; 19 *Campylobacter jejuni*1, 7 v. good identification (ID 99.0-99.8%, T 0.86-0.92), and 12 good identification (ID 97.6-98.9%, T 0.87-0.99). 5 *Campylobacter jejuni* were identified as *Campylobacter jejuni* spp *jejuni*2 and 14 as *Campylobacter jejuni* spp. *jejuni*1. The number of *Campylobacter coli* was 5; 1 v. good identification (ID 99.6%, T 0.91) and 4 good identification (ID 98.2 to 98.9%, T 0.9 to 0.93; table 14).

Campylobacter spp. were isolated by selective medium from stool specimens of the control group and confirmed by identifiying with API Campy. The number was 4 *Campylobacter* spp; 3 *Campylobacter jejuni,* 1 very good identification (ID 99.8%, T 0.86) and 2 good identification (ID 97.9-98.4%, T 0.87-0.9). One *Campylobacter jejuni* was identified as *Campylobacter jejuni spp jejuni*2 and 2 as *Campylobacter jejuni spp jejuni*1. The number *of Campylobacter coli* was one, good identification (ID 98.6%, T 0.9; table 15).

Table 12. Comparison between bacterial isolation by cultures and API 20E from stool of patients

Type of bacteria	№ of bacterial agents isolated by cultures	(%)	№ of profiles of API 20E	№ of bacterial identified	(%)	ID (%)	T
1. *Escherichia coli*	86	74.1		67	77.9		
			7 144 572 x (2)			99.8	0.63
			7 044 142 x (4)			78.5	0.35
			5 544 572 x (3)			99.2	0.63
			5 144 772 x (5)			97.2	0.63
			5 144 752 x (3)			99.7	0.67
			5 144 562 x (3)			99.8	0.85
			5 144 712 x (5)			98.1	0.6
			5 144 560 x (4)			99.5	0.57
			4 044 532 x (2)			81.5	0.67
			5 144 452 x (3)			99.8	0.75
			5 104 570 x (5)			98.0	0.53
			5 144 572 x (3)			99.5	0.96
			5 144 550 x (2)			99.5	0.72
			5 144 150 x (4)			92.8	0.56
			5 144 002 x (3)			79.5	0.66
			5 144 500 x (2)			79.9	0.73
			5 044 442 x (3)			93.0	0.58
			4 044 553 x (4)			94.8	0.54
			1 044 542 x (2)			93.4	0.76
			1 044 402 x (3)			93.0	0.75
			1 044 110 x (2)			96.7	0.8
2. *Proteus*	13	11.2		9	69.2		
			0 456 021 x (2)			99.9	0.72
			0 474 060 x (3)			99.8	0.38
			0 436 000 x (3)			66.9	0.72
			2 734 000			99.9	0.39
3. *Klebsiella*	17	14.7		13	76.5		
			5 014 573 x (5)			76.3	0.44
			5 255 773 x (3)			97.4	1.0
			5 204 543 x (2)			99.1	0.77
			5 204 777 x (3)			82.8	0.42
Total	116	100.0		89	77.4		

Table 13. Comparison between bacterial isolated by cultures and API 20E from stool of control group

Type of bacterial	№ of bacterial isolated by cultures	(%)	profiles of API 20E	№ of bacterial identified	(%)	ID (%)	T
1. *Escherichia coli*	14	63.7		11	78.6		
			5 544 572			99.2	0.63
			5 144 712 x (2)			98.1	0.6
			5 144 570			99.5	0.68
			5 144 572			99.5	0.96
			5 144 500			99.9	0.73
			5 044 513 x (3)			92.2	0.61
			5 144 772			97.2	0.63
			5 144 560			99.5	0.57
2. *Proteus*	5	22.7		3	60		
			0 436 000 x (2)			66.9	0.72
			0 456 021			99.9	0.72
3. *Klebsiella*	3	16.6		2	66.7		
			5 014 573			97.4	1.0
			5 255 773			99.1	0.77
Total	22	100.0		16	72.7		

Table 14. Comparison between campylobacter isolated by culture and API Campy from stool of patients

Type of *Campylobacter*	№ of *Campylobacter* isolated by culture	(%)	profiles of API Campy	№ of *Campylobacter* identified	(%)	ID (%)	T
1. *Campylobacter jejuni*	21	77.8		19	90.5		
			6 100 554 x (3)			97.6	0.89
			6 501 555 x (2)			99.0	0.92
			6 100 544 x (2)			98.5	0.9
			6 701 555 x (3)			99.8	0.89
			6 101 514 x (2)			98.4	0.87
			6 101 554 x (2)			98.4	0.99
			6 101 555			98.9	0.94
			6 301 504 x (2)			99.8	0.86
			6 500 554 x (2)			97.7	0.87
2. *Campylobacter coli*	6	22.2		5	83.3		
			6 401 524 x (2)			98.9	0.9
			6 420 534			99.8	0.94
			6 421 514 x (2)			98.2	0.93
Total	27	100.0		24	88.9		

Table 15. Comparison betwwen campylobacter isolated by culture and API Campy from stool of control group

Type of *Campylobacter*	№ of *Campylobacter* isolated by culture	(%)	profiles of API Campy	№ of *Campylobacter* identified	(%)	ID (%)	T
Campylobacter jejuni	5	71.4		3	60		
			6 301 504			99.3	0.95
			6 101 514			99.8	0.86
			6 500 554			99.4	0.93
Campylobacter coli	2	28.6		1	50		
			6 421 504			98.9	0.9
Total	7	100.0		4	57.1		

3.3.1.3 Polymerase Chain Reaction

The polymerase chain reaction gives a highly sensitive and specific alternative to selective enrichment for the detection and identification of bacterial pathogens (Hill et *al.* 1990). A novel PCR assay that will detect and identify the thermophilic, enteropathogenic *Campylobacter* spp. commonly associated with human infection was utilised. Restriction analysis was not employed.

In this study the method was adopted as described in chapter 2 to confirm detection and identification of 28 *Campylobacter* spp. isolated from stool specimens of Libyan children; 24 from children with acute diarrhoea and 4 from children without diarrhoea by use of selective medium (CCDA) and identified by API Campy technique in Tripoli central laboratory, Libya. All *Campylobacter* were subcultured and pure *Campylobacter* colonies were placed on FTA card sample collection and transferred to England for the PCR technique.

Results

Amplification of *Campylobacter* from children of the second study and control (*Campylobacter jejuni* NCTC 11168 was provided by Dr. D. Wareing (Dynal Biotech Ltd, Microbiology R & D, Department of Biological Sciences, University of Central Lancashire, Preston PR1 2HE, UK). The profiles of which on gel electrophoresis commencing were characteristic for the species amplified (Figures 6 and 7).

When commencing work, the amount of hyperLadder V (marker) 25 µl was taken and the marker spaced bands were joined together, especially lower bands due to the large amount used (25 µl) instead of the proper amount (5 µl). HyprLader V produces 12 regularly spaced bands, ranging from 25 to 500 bp. After correction the amount of HyperLader V by (5 µl), the reaction was more distinct and identification of the site of restriction was detected.

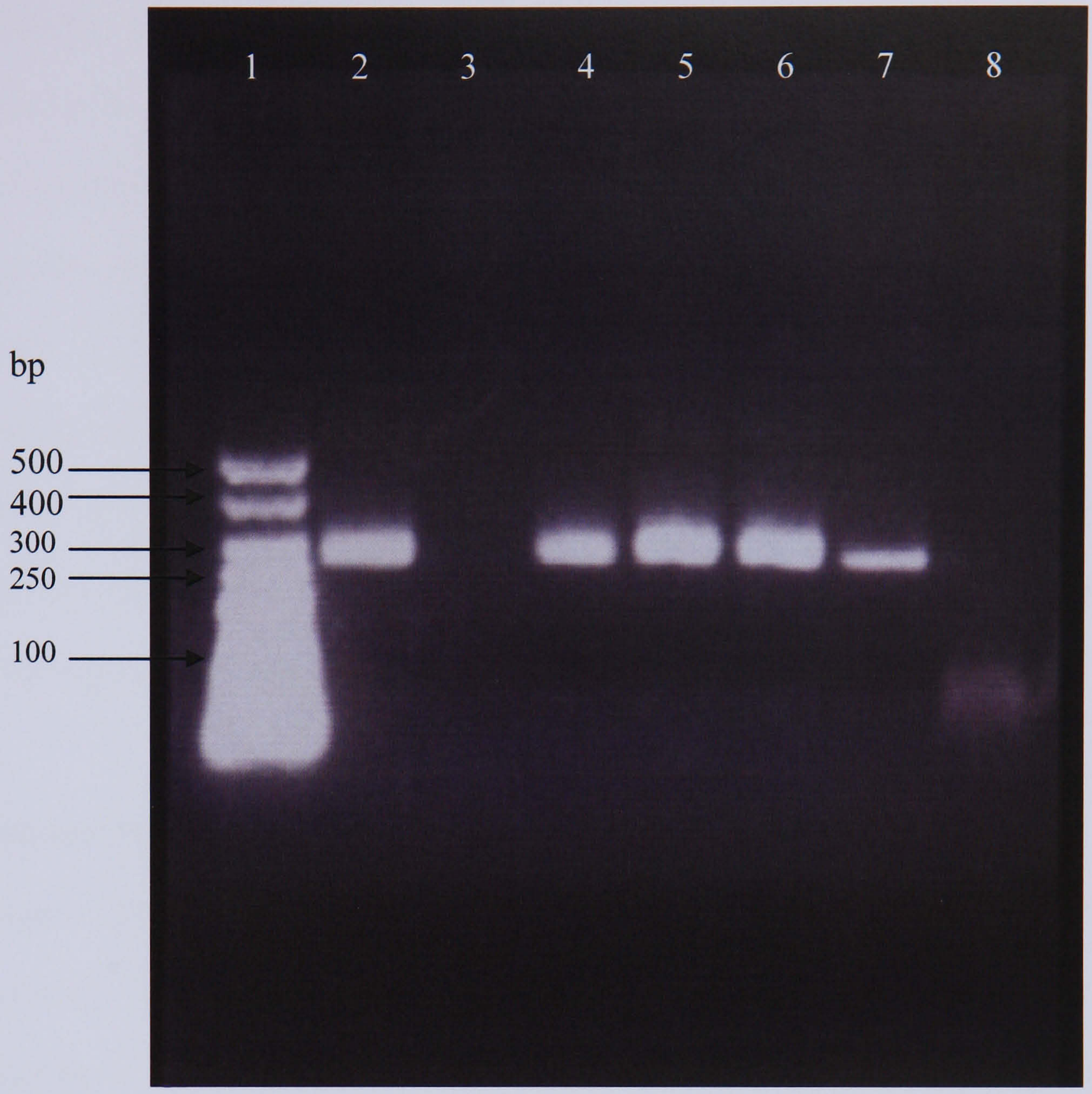


Figure 6. PCR amplification products from the two *Campylobacter* spp.

(lane 1) molecular weight marker, (lane 2) *C. jejuni* positive control, (lane 3) Negative control, (lanes 4-6) *C. jejuni* isolated from stool specimens of patients and control, (lane 7) *C. coli.*

In the first samples the site of restriction showed between 200 and 300 bp. Moreover the upper bands were more clear than the lower bands and reaction was satisfied. This indicated that the *Campylobacter jejuni* and *Campylobacter coli* samples and positive control were on the same line at approximately 250 bp (Figure 6).

When the second sample of hyperLader V was reduced to 5 µml the reaction was clearer and the site of restriction of *Campylobacter jejuni* was the same band at approximately 250 bp.

24 of *Camylobacter* spp. (19 *Campylobacter jejuni* and 5 *Campylobacter coli*) were isolated from stools of patients and 4 (3 *Camplobacter jejuni* and 1 *Campylobacter coli*) from the control group were identified by PCR to compare the results of API Campy. The detection of *Campylobacter* spp. was 100%.

3.3.2 Virological findings

Rotavirus test results are displayed in (Table 11a and 11b).

In the first study, rotavirus was detected in 18/118 (15.3%) of patient stool specimens. The single pathogen was identified in 11/118 (9.3%) and in 7/118 (5.9%) patients as a mixed infection with other pathogens. Rotavirus + *E. coli* were also detected in 2 patients, rotavirus + *Campylobacter* were detected in 2 patients, rotavirus + *Proteus* were discovered in 1 patient too, rotavirus + *Entamoeba histolytica* were detected in 1 patient and rotavirus + *E. coli* + *Klebsiella* were detected in a sole case. Rotavirus was present in 3/118 (2.6%) stool specimens from the control group.

Rotavirus infection of patients was significantly different from the control group ($p < 0.05$).

In the second study, rotavirus was detected in 123/405 (30.4%) patient stool specimens, the single pathogen in 98/405 (24.2%) and in 25/405 (6.2%) patients as a mixed infection with other pathogens. Rotavirus + *E. coli* were identified in 6 patients,

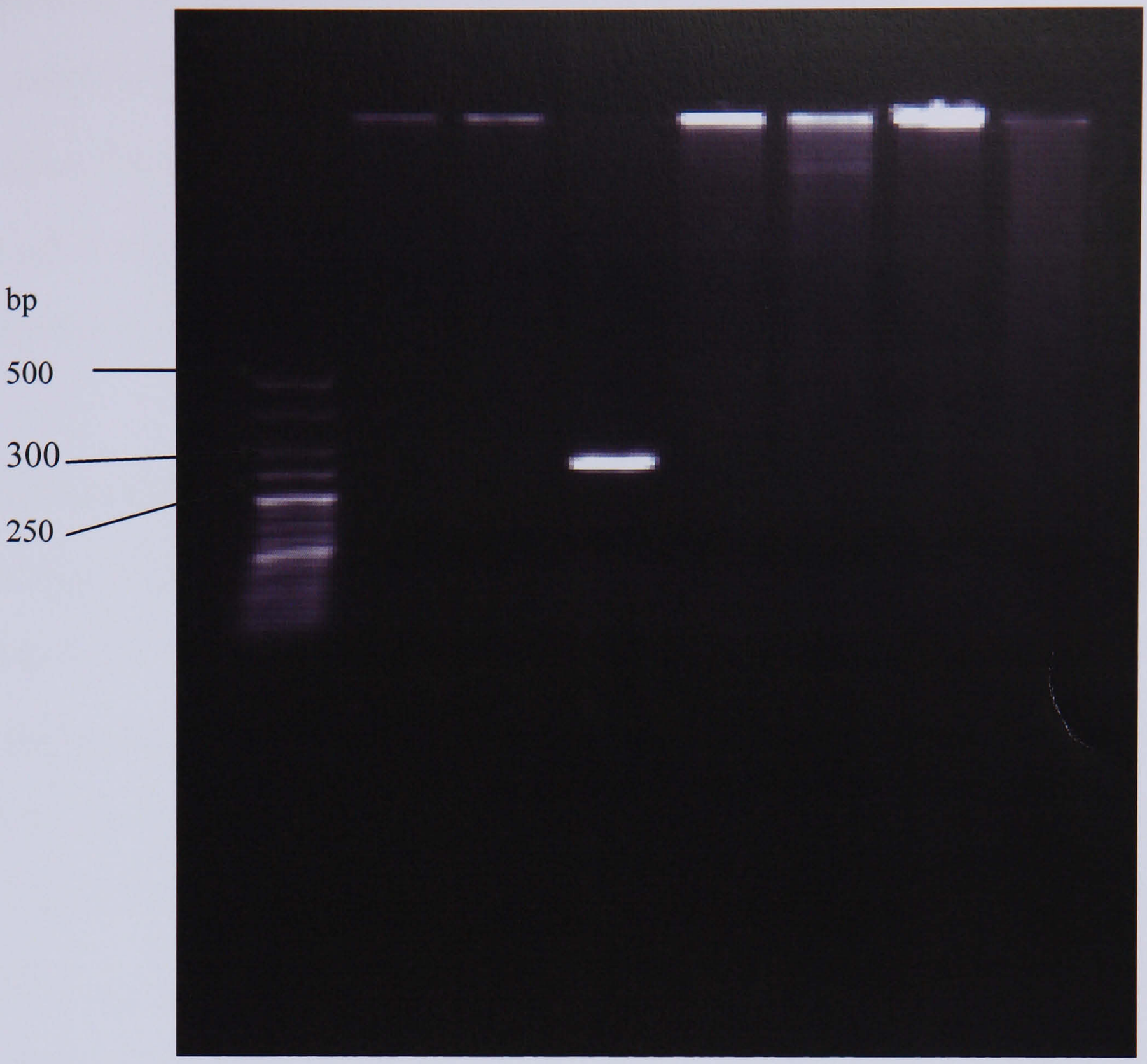


Figure 7. PCR Amplification products showing one *Campylobacter* spp. restriction site of enzyme. (lane 1) marker, (lane 2,3) negative, (lane 4) *Campylobacter jejuni*, (lane 5-7) and (lane 8) negative control.

rotavirus + *Camyplobacter* were identified in 4 patients, rotavirus + *Klebsiella* were identified in 7 patients, rotavirus + *Entamoeba histolytica* were identified in 7 patients and rotavirus + *E. coli* + *Ent. histolytica* in 1 patient. Rotavirus was discovered in 11/405 (2.7%) stool specimens of the control group. Patients' infection with rotavirus was again significantly different from the control group ($p < 0.05$) and was much higher than in the first study.

3.3.3 Parasite findings

The results of examination of stool specimens for parasites are displayed in table 11. In the first study, parasites were discovered in 10/118 (8.5%) stool specimens of patients and as a single pathogen was in 7/118 (6.0%) patients besides being a mixed pathogen in 3/118 (2.5%) patients. *Entamoeba histolytica* was the most common cause of parasitic infection. *Ent. histolytica* was detected in 5/118 (4.2%) stool specimens of patients, as a single pathogen in 2/118 (1.7%) and in 3/118 (2.5%) as a mixed infection. *Ent. histolytica* + *E. coli* were discovered in one patient, *Ent. histolytica* + *Campylobacter* in one patient and *Ent. histolytica* + rotavirus also in one patient. *Ent. histolytica* was detected in 2/118 (1.7%) stool specimens from the control group. *Giardia lamblia* was identified in 2/118 (1.7%) stool specimens of patients and in 1/118 (0.8%) stool specimens from the control group. No mixed infections with *G. lamblia* were detected. *Cryptosporidium* was discovered in 3/118 (2.5%) patient stool specimens and in 1/118 (0.8%) control group specimens. In first study, *Cryptosporidium* was not detected as mixed infection with other pathogens. Parasite infection of patients was not statistically significantly different from the control group ($p < 0.05$). In the second study, parasites were detected in a higher number of stool specimens of patients (79/405; 19.5%). A single parasite was detected in 62 (15.3%) patients and as mixed infections in 17/405 (4.2%). *Entamoeba histolytica* was the most

common cause of parasitic infection and was detected in 51/405 (12.6%) stool specimens of patients, as a single pathogen in 37/405 (9.1%) and in 14/405 (3.5%) as a mixed infection. *Ent. histolytica* + *E. coli* were detected in 2 patients, *Ent. histolytica* + *Campylobacter* were detected in 4 patients, *Ent. histolytica* + rotavirus were detected in 7 patients and *Ent. histolytica* + *E. coli* + rotavirus were detected in 1 case. *Ent. histolytica* was detected in 11/405 (2.7%) stool specimens of the control group. *G. lamblia* was detected in 15/405 (3.7%) stool specimens of patients, as a single pathogen in 12/405 (3%) and in 3/405 (1.2%) patients as a mixed pathogen. *G. lamblia* + *E. coli* were detected in 3 patients. *G. lamblia* was further detected in 5/405 (1.2%) stool specimens of the control group. *Cryptosporidium* was detected as a single pathogen in 13/405 (3.2%) patient stool samples. No mixed infections with *Cryptosporidium* were detected in the second study also. *Cryptosporidium* was detected as a single pathogen in 3/405 (0.7%) of stool specimens of the control group. Parasitic infection of patients varied significantly from the control group in the second study ($p < 0.05$).

3.3.4 Summary

Libyan children of below 1 year were the most susceptible to acute diarrhoea requiring hospitalisation in both studies (78.8% and 70.1% respectively). There was no difference between males and females in either study. In addition, there was no statistically significant difference between patients and controls vis ā vis educational level of mothers. Family size differed in both studies, except the group family size ≥ 5 child ($P < 0.05$). Watery stool was the most common stool consistency in both studies ($P < 0.05$). Bloody diarrhoea was approximately 5 to 6% and there was slight variance between the two studies for duration of diarrhoea. The mean was 3.6 and 3.9 and the median was 3 days in both studies. Fever was present in 72.9% of patients in the first

study and in 71.6% in the second. Fever was the most prevalent clinical feature associated with acute diarrhoea in both studies ($P < 0.05$). The group of patients who had fever for one day was higher in the first study while the group from 2-3 days was higher among the second.

Vomiting was present in 51.7% of patients in the first study and 58.3% among the second study. Vomiting was not associated with acute diarrhoea in the first study ($P > 0.05$) but was in the second ($P < 0.05$). Type and degree of dehydration was most commonly isotonic and moderate at more than 70% and 50% in both studies. Very few children had low haemoglobin ≤ 9 (mild anaemia; 3.4% and 3.7%) of patients. %) respectively. Serum potassium and sodium were within their normal limits in more than 75% of cases in both studies. Urea ≥ 60 (abnormal) was present at 9% in first study and 10% of the second. ORT proved to be of little use for treatment of dehydration, representing only 8.5% in the first study and 13.8% in the second. Regarding the duration for patient stay in hospital, the mean was 5.39 and the medium was 5 in the first study, and the mean was 5.03 and median 5 in the second. No patients with acute diarrhoea demonstrated signs of marasmus or kwashiorkor. a patient group was significantly different from the control group in their weight for age for the group of under-nutrition in both studies ($P<0.05$). No complications were recorded in either study (HUS or GBS). Chest infection was the most prevalent disease combined or associated with diarrhoea.

One or more enteric pathogens were detected from stool of patients (55.9%) and (13.6%) of controls in the first study and from 68.1% of patients and 12.6% of controls in the second study.

Bacteria were most common (43.3%) in the first study while rotavirus was most common in the second. *Campylobacter* was the second most frequently isolated bacteria in both studies. *Entamoeba histolytica* was the common parasite identification

in both studies. *Cryptosporidium* was more frequently detected in the second study. Watery stool, fever, and vomiting were common in patients with rotavirus infection in both studies.

API 20E and API Campy were used to identifiy bacterial isolated by cultures and the number of confirmed cases decreased from 142 to 112 samples in the second study.

Polymerase chain reaction was adopted in the second study to confirm detection of *Campylobacter* spp. These were identified by APA Campy technique, where all *Campylobacter* spp. were identified as the same as detected by API Campy and for 24 samples.

Chapter 4

Epidemiology & Risk Factors

CHAPTER 4

Epidemiology and Risk Factors for Childhood Diarrhoea

4.1 Seasonal distribution

The first study was conducted over a relatively short period (3 months) at the end of summer and beginning of autumn and no seasonal variation could be determined.

During the second study, the seasonality of detection of variation enteropathogens was determined and the results are shown in (Fig. 8). The highest frequency of isolation of enterobacteria was in the summer season (June, July and August). Parasites were detected more frequently in summer and autumn whereas infection with rotavirus was higher in winter (December, January and February). Enteropathogen detection rates in summer, winter, spring and autumn were 32.6% (112/344), 25.3% (87/344), 18.9% (65/344 and 23.2% (80/344) respectively (Fig. 8). The highest incidence was 42/314 (13.4%) in July and the lowest in April (16/314; 5.1%). The peak in July was due to the high prevalence of bacterial and parasite infections during that month (Fig. 8).

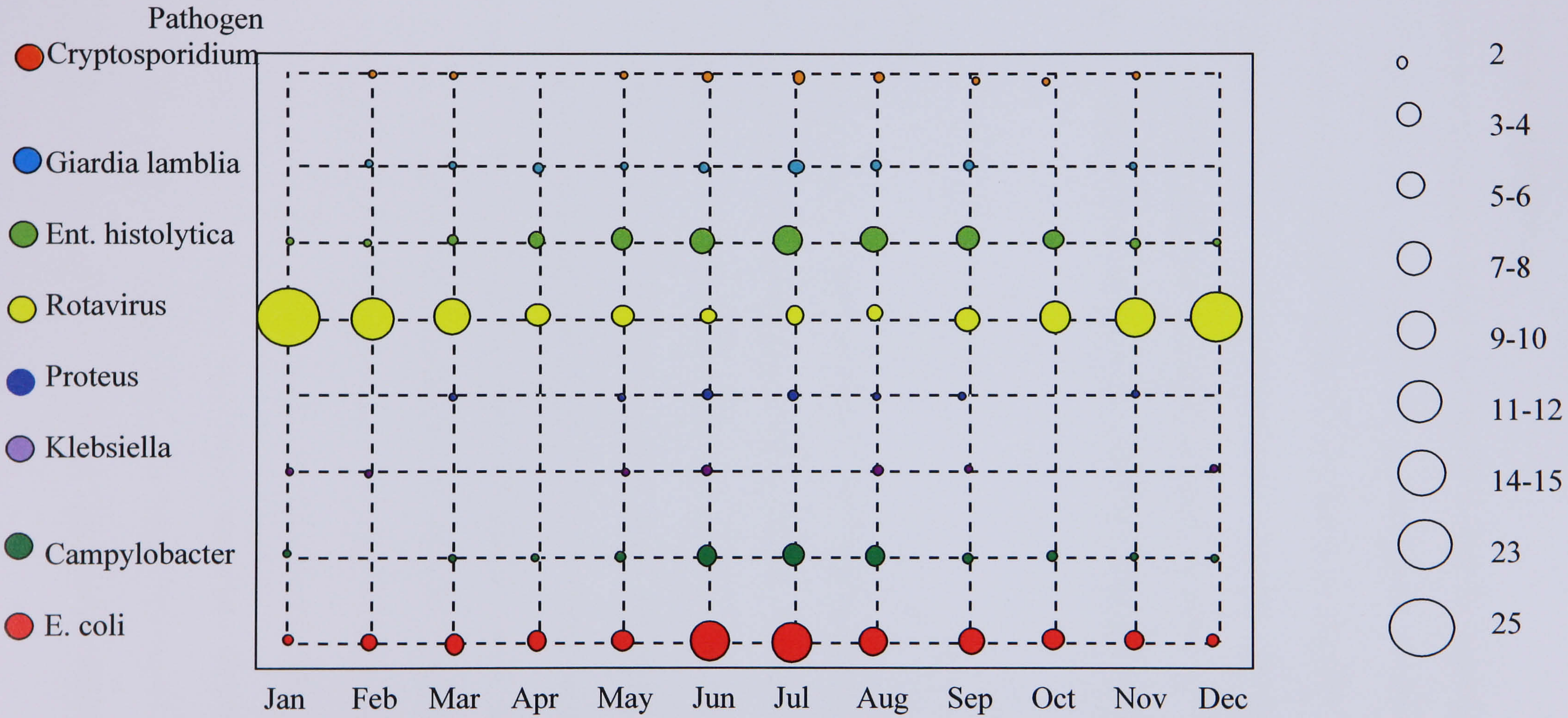
4.1.1 Seasonal variation of bacteria

Bacteria were more frequently isolated in the hot months from June through to October (74/112; 66.1%; Figure 8). The peak of bacterial isolation was July (22/112; 19.6%). Bacteria were most frequently isolated in the summer season (56/112; 50%). Bacteria were less frequently isolated in winter (12/112; 10.7%). The prevalence of bacterial infections in autumn and spring was 20.5% (23/112) and 18.8% (21/112) respectively.

4.1.2 Seasonal variation of rotavirus

The seasonality variation in the frequency of rotavirus infections differed markedly from that of bacterial and parasite infections (Fig. 8). The highest frequencies of rotavirus infection were found in the relatively cool months of November through to March (87/123 (70.7%). Rotavirus was most frequently detected in the winter season

Figure 7. The monthly distribution of each pathogen*detected of second study



(62/123; 50.4%). The peak month of prevalence of rotavirus identification was January with 25/123 (20.3%). Rotavirus infection was less prevalent in summer with 10/123 (8.1%). The frequencies of rotavirus infection in autumn and spring seasons were 29/123 (23.6 %) and 22/123 (17.8%) respectively.

4.1.3 Seasonal variation of parasites

The seasonal variation of parasite infection was similar to that for bacteria where the highest number of parasites were discovered in the hot months 54/79 (68.4%; Figure 8). The summer was the most common season for prevalence of parasites detected in this study 40/79 (50.6%). The peak month of prevalence of parasite infection was July with 16/79 (20.3%). Parasite infection was less common in winter (5/79; 12.7%). Parasite infections in autumn and spring were 18/79 (22.8%) and 16/79 (20.3%) respectively.

4.2 Type of feeding:

The type of feeding patients and controls received is displayed in tables 16a and 16b. In the first study, the number of patients who were breast fed was only five (4.2%) whilst this rose to ninteen in the controls (16.1%). The number of patients that were bottle fed was 73 (61.9%) and 25 (21.2%) in the control group. The number of patients with mixed feeding (breast and bottle) was 28 (23.7%) in the patient group and 50 in the control group (42.4%). Finally, the number of patients with solid feeding (normal food) was 12 (10.2%) and 24 in the controls (20.3%). For feeding type, bottle feeding is higher risk than breast feeding (baseline), whereas mixed feeding shows a protective effect and it marginally significant at 10% (p-value=0.108). The odds of having the diarrhoea for bottle feeding is 5 times higher than those who are breast fed. In the second study, the number of patients who were breast fed was 47 (11.6%) and 98 (24.2%) in the controls. The patients were again significantly different from the

Table 16. Type of feeding of patients and controls (a) First study

Type of Feeding	№ of patients	(%)	№ of controls	(%)	OR	p-value	95% CI
Breast feeding	5	4.2	19	16.1	1		1 to 1 (by default)
Bottle feeding	73	61.9	25	21.2	4.483	0.005	1.590 to 12.644
Mixed feeding	28	23.7	50	42.4	0.244	0.108	0.044 to 1.360
Solid feeding	12	10.2	24	20.3	Unstable solution	0.988	0 to ∞
Total	118	100	118	100			

Table 16b. Second study

Type of feeding	№ of patients	(%)	№ of Controls	(%)	OR	p-value	95% CI
Breast feeding	47	11.6	98	24.2	1		1 to 1 (by default)
Bottle feeding	189	46.7	104	25.7	4.927	0.000	2.827 to 8.587
Mixed feeding	86	21.2	167	41.2	1.348	0.390	0.682 to 2.664
Solid feeding	83	20.5	36	8.9	Unstable solution	0.985	0 to ∞
Total	405	100	405	100			

control group in their breast feeding patterns (x^2 = 18.19, p<0.05). The number of patients who were bottle fed was 189 (46.7%) and 104 (25.7%) in the controls. The number of patients with mixed feeding was 86 (21.2%) and 167 (41.2%) among controls. The number of patients with solid feeding was 83 (20.5%) and 36 (8.9%) in the controls. For feeding type, bottle and mixed feeding are higher risk than breast feeding (baseline). However the McNemar's chi sequere test shows that only bottle feeding is significantly associated with diarrhoa and the odds of having the diarrhoea is 5 times higher than those who are breast fed.

4.3 Type of water supply

The water supplies used by patients' families and controls in these studies were classified into four types (Table 17a and 17b): (1) tap water (chlorinated and therefore safe water supply), (2) bottled water, (also likely to be safe), (3) well water, (usually unsafe because it is a private source and not chlorinated), (4) storage water, (not safe and easily contaminated because the water is stagnant and there is no chlorination). In the first study, tap water was drunk by 65 (55.1%) patients and 55 (46.6%) controls. Bottled water was drunk by 8 (6.8%) in the patient group and by 9 (7.6%) in the control group. Well water was drunk by 35 (29.7) patients and by 32 (27.1%) of the controls. Storage water was drunk by 5 (4.2) patients and by 3 (2.5%) of the controls. Using the McNemar,s chi square test and using water tap as the baseline, there is no statistical evidence of an association between water type and diarrhoea.

In the second study, tap water was drunk by 237 (58.5%) in the patient group and by 212 (52.3%) in the control group. Bottled water was drunk by 75 (18.5%) of the patients and by 72 (17.8%) of the controls. Well water was drunk by 20 (4.9%) of the patients and 14 (3.5%) of the controls. Storage water was drunk by 26 (6.4%) in the patients and by 9 (2.2%) of controls.

Table 17. Type of water supply of patients and controls (a) First study

Type of water	№ of patients	(%)	№ of controls	(%)	OR	p-value	95% CI
Tap water	65	55.1	55	46.6	1		1 to 1 (by default)
Bottle water	8	6.8	9	7.6	0.696	0.151	0.234 to 2.069
Well water	35	29.7	32	27.1	0.806	0.532	0.410 to 1.584
Storage water	5	4.2	3	2.5	0.845	0.842	0.161 to 4.431
Total	113*	95.8	99**	83.8			

* This excludes (5) of patients which were exclusively breast fed.

** This excludes (19) of controls which were exclusively breast fed.

Table 17b. Second study

Type of water	№ of patients	(%)	№ of controls	(%)	OR	p-value	95% CI
Tap water	237	58.5	212	52.3	1		1 to 1 (by default)
Bottle water	75	18.5	73	18.0	0.957	0.848	0.612 to 1.497
Well water	20	4.9	14	3.5	1.361	0.410	0.633 to 2.836
Storage water	26	6.4	9	2.2	1.854	0.145	0.809 to 4.251
Total	358*	88.4	308**	76.0			

* This excludes (47) of patients which were exclusively breast fed.

** This excludes (97) of controls which were exclusively breast fed.

Using the McNemar,s chi square test and using water tap as the baseline, there is no statistical evidence of an association between water type and diarrhoea.

4.4 Animal contact of patients and controls

The number of patients and controls who had contact with animals was reported in patients as thirty-seven (31.4%) and among controls as thirty-five (29.7%) in the first study. Animal contact was not significantly statistically different between patient and control groups ($x^2 = 0.08$, $p > 0.05$). In the second study, the number of patients who had contact with animals was one hundred and seventeen (28.9%) and the number of controls was one hundred and four (25.7%). Animal contact was again not significantly statistically different between the patient and control groups ($p > 0.05$).

4.5 Use of dummies

In the first study, the number of patients who used dummies was sixty-eight (57.6%) and twenty-two (18.6%) of the controls and the number of not used dummies was fifty (42.4%) of the patients and ninty six (81.4%) of the controls (Table 18a). Those patients who used a dummy were substantially more likely to contract diarrhoea when compared with the control, by using McNemar's chi sequere test shows that dummy usage is highly associated with diarrhoea, that is the odds of being diarrhoea for dummy users are 2.5 times the odds of non dummy users.

The second study provided similar results. The number of patients who used dummies was 147 (36.3) and 65 (16%) of controls and the number of not used dummies was two hundred and fifty eight (63.7%) of the patients and three hundredand fourty (84%) of the controls (Table 18b). By using McNemar's chi sequare test shows that dummy usage is highly associated with diarrhoea, that is the odds of being diarrhoea for dummy users are 3 times the odds of non dummy users.

Table 18. Dummies used by patients and controls (a) First study

Use of dummies or non-use	Patients № of patients	(%)	Controls № of controls	(%)	OR	p-value	95% CI
Dummies used	68	57.6	22	18.6	2.5	11.57 $p<0.0007$	1.42 to 4.59
Dummies not used	50	42.4	96	81.4			
Total	118	100	118	100%			

Table 18b. Second study

Use of dummies or non-use	Patients № of patients	(%)	Controls № of controls	(%)	OR	p-value	95% CI
Dummies used	147	36.3	65	16.0	3.28	43.7 $p<0.001$	2.24 to 4.90
Dummies not used	258	63.7	340	84.0			
Total	405	100	405	100			

4.6 Age distribution of patients with different enteric pathogens

In the first study, the distribution of age of patients according to the aetiological agent was determined (Table 19a).

Bacteria were more prevalent in stools from 18/37 (48.6%) patients aged under 6 months and in 12/37 (32.4%) patients aged between 6-12 months. The lowest prevalence of bacterial infections was observed in patients aged over 12 months 7/37; 19%). The most common age range of patients with rotavirus infection was 6 to 12 m in 5/11 (45.4%) patients and < 6 m in 3/11 (27.3%). The prevalence of rotavirus infection was observed in patients aged > 12 months in 3/11 (27.3%). The most common age range of patients with parasite infection was > 12 months in 4/7 (57.1%) and in 2/7 (28.6%) patients aged from 6 to 12 m. The lowest prevalence of parasite infections was observed in patients aged under 6 m (1/7; 14.3%).

The patients' age group < 6 months was most common. A single infection was detected in 22/55 (40.0%), of the age group 6 to 12 months in 19/55 (34.6%) and the age group > 12 months in 14/55 (23.6%). Libyan children younger than 1 year were the most susceptible to infection with acute diarrhoea (76.4% of patients).

In the second study, bacterial infection was again more prevalent in patients aged < 6 m (36/79; 45.6%) and in 25/79 (31.6%) patients aged 6-12 m. The lowest prevalence of bacterial infections was observed in patients aged > 12 m (18/79; 22.8%).

Rotavirus was detected in stool specimens in 52/98 (53.1%) patients aged between 6-12 m and in 28/98 (30.6%) > 12 m. The lowest prevalence of rotavirus infection was observed in patients aged < 6 m (18/98, 16.3%). Parasites were detected in 30/62 (48.4%) of stool specimens of patients aged > 12 m and in 21/62 (33.9%) patients aged 6- 2 m. The lowest prevalence of parasitic infections was observed among patients aged < 6 m (11/62, 17.7%). Libyan children younger than 1 year were, again, the most likely to be found with acute diarrhoea (68.2%).

Table 19. Age distribution of patients with different enteric pathogens* (a) First study

Type of pathogen	Patients aged <6 m № of patients	(%)	Patients aged 6-12 m № of patients	(%)	Patients aged >12 m № of patients	(%)	Total (%)
Bacteria	18/37	48.6	12/37	32.4	7/37	19.0	100
Rotavirus	3/11	27.3	5/11	45.4	3/11	27.3	100
Parasite	1/7	14.3	2/7	28.6	4/7	57.1	100
Total	22/55		19/55		14/55		

* age distribution of patients for single infection only

Table 19b. Second study

Type of pathogen	Patients aged <6 m № of patients	(%)	Patients aged 6-12 m № of patients	(%)	Patients aged >12 m № of patients	(%)	Total (%)
Bacteria	36/79	45.6	25/79	31.6	18/79	22.8	100
Rotavirus	18/98	16.3	52/98	53.1	28/98	30.6	100
Parasite	11/62	17.7	21/62	33.9	30/62	48.4	100
Total	65/239		98/239		76/239		

* age distribution of patients for single infection only

There were slight variances between the two studies. In the first study, the prevalence of bacterial infections was higher than in the second study for infection of patients aged < 6 months 18/37 (48.6%) while the prevalence of rotavirous infection in the second study was higher than the first for infection of patients aged from 6 to 12 m Bacteria were more prevalent in stools from 18/37 (48.6%) patients aged under 6 52/98 (53.1%). The prevalence of parasite infections were higher for infection of patients aged > 12 m 4/7 (57.1%) in the first study and 30/62 (48.4%) in the second.

However, statistically is not significant because the number of child for age groups is not enough and p values were ($P > 0.05$).

4.7 Clinical features associated with each enteric pathogen

Clinical features associated with the pathogens are shown in (Table 20).

4.7.1 Fever

In the first study, fever was a presenting feature in significantly more patients with rotavirus infection 9/11 (81.8%) than with *Crypytosporidium* (reported in 1/3; 33.3%). Fever was the most clinical feature of acute diarrhoea associated with rotavirus infection of patients. Parasites and bacteria were intermediate and ranged between 50% and 70.3% manifesting fever.

In the second study, rotavirus infection was once more highly associated with fever and was reported in 76/98 (77.7%) while *Cryptosporidium* was only reported with fever in 5/13 (38.5%). Fever was the most clinical feature of acute diarrhoea associated with rotavirus infection of patients. Parasites and bacteria were intermediate and ranged between 48.7% and 67.3% of patients with fever.

4.7.2 Vomiting

In the first study, vomiting occurred more commonly in patients with rotavirus infection 8/11 (72.7%) and less commonly in patients with *Klebsiella* infection 1/4

(25%). Parasites and bacteria were intermediate and ranged between 42.8% and 51.5%. Vomiting was the second most clinical feature of acute diarrhoea associated with rotavirus infection ($p < 0.05$).

In the second study, rotavirus infection was again associated with vomiting which was observed in 74/98 (75.5%) while proteus was weakly associated with vomiting and observed in 2/6 (33.3%). Other bacteria and parasites were intermediate at 63.5% and 41.7%. Vomiting was the second most clinical feature of acute diarrhoea associated with rotavirus infection of patients ($p < 0.05$

4.7.3 Dehydration

In the first study, mild dehydration was noted in 38/118 patients and was most common in patients with parasite infection; *Cryptosporidium* and *G. lamblia* (3/3 and 2/2; 100%) and less common in patients with rotavirus (2/11;18.2%). Bacteria and *Ent. histolytica* were intermediate and ranged between 27% and 50%. Mild dehydration was the most clinical feature of acute diarrhoea associated with parasite infections ($p <$ 0.05).

Moderate dehydration was reported in 61/118 patients and was more common in patients with infection by *Campylobacter* 4/6 (66.7%) and less so in patients with *Proteus* 2/6 (33.3%). Rotavirus, bacteria, and *Ent. histolytica* were intermediate and ranged between 27.3% and 50% .

Severe dehydration was reported in 19/118 patients and was most prevalent in patients with rotavirus infection 6/11 (54.5%) and least common in those with *Proteus* infection 1/6 (16.7%). Severe dehydration was the most common clinical feature of acute diarrhoea associated with rotavirus infection of patients. Ther bacteria were intermediate and ranged from 19% to 25%. No severe dehydration was reported in patients who had parasitic infections or *Campylobacter.*

Table 20. Clinical features associated with different enteric pathogens (a) First study

Type of Pathogen*	№ of Patients	Fever	Vomiting	Abdominal Cramp	Type of dehydration Mild	Moderate	Severe
Escherichia coli	21	16	10	7	4	13	4
Campylobacter	6	4	3	3	2	4	
Proteus	6	3	4	2	3	2	1
Klebsiella	4	3	1	2	1	2	1
Rotavirus	11	9	8	3	2	3	6
Ent. histolytica	2	1	1	1	1	1	
G. lamblia	2	1	1	1	2		
Cryptosporidium	3	1	1	2	3		

* single infection only

Table 20b. Second study

Type of Pathogen*	№ of Patients	Fever	Vomiting	Abdominal Cramp	Type of dehydration Mild	Moderate	Severe
Escherichia coli	52	35	33	16	17	26	9
Campylobacter	16	9	10	9	7	9	
Proteus	6	3	2	3	2	4	
Klebsiella	5	3	2	3	2	3	
Rotavirus	98	76	74	18	19	47	32
Ent. histolytica	37	18	16	15	23	12	2
G. lamblia	12	6	5	4	9	3	
Cryptosporidium	13	5	6	8	13		

* single infection only

In the second study, mild dehydration was noted in 134/405 patients and once more was common in patients with parasite infection; *Cryptosporidium* was detected in 13/13 (100%), and *G. lamblia* 9/12 (75%) and proved less common in patients with rotavirus infection in 19/98 (19.4%). Mild dehydration was the most clinical feature of acute diarrhoea associated with parasite infections ($p < 0.05$). Mild dehydration in *Ent. histolytica* infection was 23/37 (62.2%) and bacteria were intermedite and ranged between 32.7% and 40%.

Moderate dehydration was noted in 225/405 (55.5%) patients and was most common in patients infected by *Proteus* (4/6; 66.7%) and was less common with infection by *G.lamblia* (3/12; 25%). Other bacteria were intermediate and ranged from 50% to 60%, whereas *Ent. histolytica* in 12/37 (32.4%) and rotavirus was in 47/98 (48%).

Severe dehydration was reported in 46/405 (11.4%) and was most common in patients with rotavirus infection (32/98 ;32.7%) and less common in *Ent. histolytica* infection (2/37; 5.4%). Severe dehydration was the most clinical feature of acute diarrhoea associated with rotavirus infection ($p < 0.05$). Severe dehydration was intermediate (9/52; 17.3% of patients with *E. coli* infection).

No severe dehydration was reported in patients who had other parasitic or other bacterial (*Proteus, Klebsiella* and *Campylobacter*) infections.

4.7.4 Abdominal cramps

In the first study, abdominal cramps were reported as a more common feature in patients with *Cryptosporidium* infection in 2/3 (66.7%) than rotavirus infection in 3/11 (27.3%; Table 21). Abdominal cramp was the most clinical feature of acute diarrhoea with *Cryptosporidium* infection ($p < 05$). Rotavirus, parasites, and bacteria were intermediate and ranged between 33.3% and 50% of patients.

In the second study, abdominal cramps were again reported as a more common feature in patients with *Cryptosporidium* infection in 8/13 (61.5%) and in this study with

Campylobacter 9 /16 (56.3%). Abdominal cramps in rotavirus infection were again lowest at 18/98 (18.4%). They were the most clinical feature of acute diarrhoea associated with *Cryptosporidium* infection ($p < 0.05$). Other parasites, and other bacteria were intermediate and ranged from 30.8% to 60%.

4.8 Stool appearance associated with aetiological pathogens in patients

The results are displayed in (Table 21).

In the first study, watery stools were common in 8/11 (72.7%) of rotavirus. Watery stool occurred in significantly more of the patients with rotavirus infection ($x^2 = 8.28$, $p < 0.05$) than in those with *E. coli* infection (6/21; 28.6%). Watery stools in other bacterial and parasite infections were intermediate and ranged between 50% and 66.7%. No watery stools occurred in infections with *G. lamblia* or in *Campylobacter* infections.

Mucous in the stool was a presenting feature in patients with *Campylobacter* infection (5/6; 83.3%) and was less common in patients with rotavirus infection 3/11 (27.3%). Other bacterial and parasite infections were intermediate and ranged between 33.3% and 66.7%. Mucous in the stool was significantly greater among patients with *Campylobacter* infection. Stool mixed with blood was common in patients with *G. lamblia* infection in 1/2 (50%) and less common in patients with *Campylobacter* infection 1/6 (16.7%). *E. coli* infection, bloody stool was detected in 5/21 (23.8%) patients. No bloody diarrhoea was observed in patients with rotavirus, *Cryptosporidium* or other bacterial infections.

In the second study, watery stools were further common in patients with rotavirus infection (74/98; 75.5%). Watery stool occurred in significantly more patients with rotavirus infection than patients with *Ent. histolytica* 7/37 (18.9%). For bacterial infections, watery stool was intermediate and ranged between 32.8% and 62.5%.

Table 21. Type of stool appearance associated with aetiological pathogens* (a)

First study

Type of Pathogen	№ of patients	Stool appearance Watery	Mucous	Bloody
Escherichia coli	21	6	10	5
Campylobacter	6		5	1
Proteus	6	4	2	
Klebsiella	4	2	2	
Rotavirus	11	8	3	
Ent. histolytica	2	1	1	
G. lamblia	2		1	1
Cryptosopridium	3	1	2	

* Single infection only

Table 21b. Second study

Type of Pathogen	№ of patients	Stool appearance Watery	Mucous	Bloody
Escherichia coli	52	9	27	16
Campyylobacter	16		13	3
Proteus	6	3	3	
Klebsiella	5	3	2	
Rotavirus	98	74	24	
Ent. histolytica	37	7	28	2
G. lamblia	12		9	3
Cryptosporidium	13	3	10	

* Single infection only

*Cryptosporidium i*nfection, watery stool was noted in 3/13 (23%) of patients. No watery stools occurred in infection with *G. lamblia* or *Campylobacter.*

Mucous in the stool was most prevalent in patients with *Campylobacter* infection (13/16; 81.3%) and also in patients with *Ent. histolytica* infection (28/37;75.7%). It proved less common in patients with rotavirus infection (24/98; 25.5%). Other parasites and bacteria were intermediate and ranged from 37.5% to 77%. Mucous with the stool was significantly greater in patients with *Campylobacter* infection.

Stool mixed with blood was common in patients with *E. coli* infection in 16/52 (30.8%) and less common in patients with *Ent. histolytica* 2/37 (5 %), *G. lamblia* in 3/12 (25%) and *Campylobacter* in 3/16 18.8%). No bloody diarrhoea was determined in patients with rotavirus, *Cryptosporidium* or other bacterial infections.

4.9 Frequencies of other parameters associated with different etiological agents

The results are shown in table 22.

In the first study, the mean frequency of motion per day was highest in patients with rotavirus infection (7.3 motions) than those with *G. lamblia* infection (4.6 motions). Other parasites and bacteria were intermediate and ranged between 5.4 and 6.9 motions. The mean duration of diarrhoea was shortest in the rotavirus infection at 3.2 days and longest in *Cryptosporidium i*nfection at 7.2 days. Other parasites and bacteria were intermediate and ranged between 4.6 and 6.8 days.

The mean stay in hospital was longest in patients with *Cryptosporidium* infection (7.3 days) and lowest in those with rotavirus infection (4 days). Other parasites and bacteria were intermediate and ranged from 4.5 to 6.4 days.

In the second study, the mean frequency of motion per day was greatest in patients with rotavirus infection (8.9 motions) and contrasted with those with *G. lamblia* infection (4-6 motions).

Table 22. Mean frequency of motions, mean duration and mean stay in hospital associated with aetiological pathogens (a) First study

Type of Pathogen*	№ of patients	mean frequency motions/day	mean duration diarrhoea/days	mean stay in hospital/days
Escherichia coli	21	6.9	4.7	5.6
Campylobacter	6	5.2	6.8	6.8
Proteus	6	5.4	4.6	4.3
Klebsiella	4	5.6	5.2	4.5
Rotavirus	11	9.3	3.2	4.0
Ent. histolytica	2	5.0	6.7	6.2
G. lamblia	2	4.6	6.2	6.4
Cryptosporidium	3	7.3	7.2	7.3

* Single infection only

Table 22b. Second study

Type of Pathogen*	№ of patients	mean frequency motions/day	mean duration diarrhoea/days	mean stay in hospital/days
Escherichia coli	52	6.7	4.3	5.8
Campylobacter	16	6.5	6.3	6.8
Proteus	6	5.2	4.6	4.6
Klebsiella	5	5.4	5.2	4.5
Rotavirus	98	8.9	3.4	4.3
Ent. histolytica	37	5.0	6.7	6.6
G. lamblia	12	5.3	6.5	6.5
Cryptosporidium	13	4.4	7.4	7.2

*Single infection only

Bacteria and other parasites were intermediate and ranged between 5.2 and 6.7 motions. The mean duration of diarrhoea was shortest in the rotavirus infection (3.4 days) and longest in the *Cryptosporidium* (7.4 days). Other parasites and bacteria were intermediate and ranged between 4.6 and 6.7 days.

The mean stay in hospital was longest in patients with *Cryptosporidium* infection (7.3 days) and lowest in patients with rotavirus infection at (4.3 days). Other parasites and bacteria were intermediate and ranged between 4.5 and 6.8 days.

4.10 Univariate and multivariate analysis

4.10.1 First study

Multivariate analysis is a natural extension of the univariate analysis. It enables us to see the effect of each prognostic factor but allowing for other confounding factors. Adjusted odds ratio will be obtained rather than crude odds ratio from univariate analysis. As mentioned in section (1.1), the design is a 1:1 matched case-control, therefore the appropriate method for multivariate analysis is the conditional logistic regression (or fixed-effects logit). The dependent variable is the variable describing whether a child is a "control" or a "patient". The dependent variable is binary that is The reason why we use logistic regration.

For the multivariate analysis, we used the feeding type, dummies, family size, and haemoglobin concentration, the other variables were making the model diverge and unstable. The results of the multivariate analysis is shown in the table 23. We can observe that only dummy use and bottle feeding are showing statistical significance and the effect of using dummy is 18-fold that of not using dummies, while bottle feeding increases the risk by 89. The effect of dummies and bottles on diarrhoea can be explained as these two items are exposed to all sorts of germs and often are picked up by children from a dirty floor without the parent noticing.

Table 23. The results of multivariate analysis (feeding type, dummies, family size and haemoglobin concentration) for first study

Predictors	Odds ratio	p-value	95% confidence interval for OR
Feeding type			
Breast feeding (baseline)	1		1 to 1
Bottle feeding	89.04	0.000	11.373 to 697.056 (too wide)
Mixed feeding	2.54	0.424	0.257 to 25.142
Solid feeding	Unstable		
Dummies			
No dummy use (baseline)	1		1 to 1
Dummy use	18.26	0.000	4.151 to 80.332
Family size			
1 child (baseline)	1		1 to 1
2 – 4	0.73	0.692	0.151 to 3.505
5 – 9	4.22	0.098	0.768 to 23.213
10 – 11	5.35	0.200	0.412 to 69.636
Haemoglobin concentration			
≤ 9 (Baseline)	1		1 to 1
9.1 – 10.9	7.663	0.145	0.495 to 118.392
11.0 – 11.9	3.007	0.424	0.202 to 44.690
≥ 12	8.269	0.151	0.463 to 147.611

4.10.2 Second study

The same procedure as the first study will be used for the second study, namely the conditional logistic regression. Feeding type has been removed because of non stability of the solution. Storage water, dummy usage and family size are showing the most significant effect for the presence of the diarrhoea. The results of the multivariate analysis is shown in the table 24.

4.10.3 The correlation between fevef and vomiting with each other clinical feaures

In this section we are investigating association of presence of fever with other factors such as presence of pathogens, stool duration, feeding type, vomiting, age and use of dummies in both study for univariate and multivariate analysis. See appendix 4.

4.10.3.1 Univariate analysis from first study

For this we created a binary variable "Fever" which has a value 0 if there is a body temperature below 38 °C, and a value 1 if presence of temperature of 38°C and above. We must bear in mind that this procedure enables us to test the factor affecting fever and not the cause of the fever. The following table shows a crosstabulation of fever with stool duration. In order to test for association we used a chi square test which shows a little evidence of association as the p-value slightly higher than the cut–off value of 0.05 ($\chi^2 = 7.3$, p-value=0.064), a larger sample show give significance.

We also tested the association of fever with the presence of pathogens as shown in the following table. A chi square test shows clearly no evidence of association ($\chi^2 = 0.51$, p-value=0.773). Association between Fever and feeding type has also shown no evidence ($\chi^2 = 1.02$, p-value=0.797). We also tested vomiting and fever association, which also was not statistically significant ($\chi^2 = 1.15$, p-value=0.283).

The association of fever with age is also not significant ($\chi^2 = 0.093$, p-value=0.954).

Table 24. The results of multivariate analysis (water type, dummies, family size and haemoglobin concentration) for first study

Predictors	Odds ratio	p-value	95% confidence interval for OR
Water type			
Tap water (baseline)	1		1 to 1
Bottle water	1.755	0.078	0.938 to 3.284
Well water	0.924	0.873	0.348 to 2.449
Storage water	7.055	0.001	2.199 to 22.643
Dummies			
No dummy use (baseline)	1		1 to 1
Dummy use	2.893	0.000	1.689 to 4.855
Family size			
1 child (baseline)	1		1 to 1
2 – 4	2.786	0.046	1.019 to 7.620
5 – 9	3.566	0.018	1.242 to 10.23
10 – 11	9.633	0.003	2.208 to 42.010
Haemoglobin concentration			
≤ 9 (Baseline)	1		1 to 1
9.1 – 10.9	1.411	0.475	0.549 to 630
11.0 – 11.9	0.889	0.803	0.354 to 2.233
≥ 12	11.921	0.000	3.419 to 41.553

We also tested dummy use and fever shows no association ($\chi^2 = 0.0$, p-value=1.00).

Finly we tested the number of motion per also shows no evidence of association with fever ($\chi^2 = 1.86$, p-value=0.394).

We are looking at association between vomiting and factors such as stool duration, number of motion, presence of pathogens, type of feeding and age using the chi square test as previously. No evidence of association between vomiting and stool duration ($\chi^2 = 3.28$, p-value=0.350).

For the number of motions no evidence no was found for this sample ($\chi^2 = 0.564$, p-value=0.754).

For the presence of pathogens no association was found with vomiting in the sample ($\chi^2 = 2.34$, p-value=0.311).

4.10.3.2 Univariate analysis from second study

As previously for study 1, we will carry out univariate analyis by means of the chi square test. The test is showing a strong association between fever and stool duration, where longer periods favour the presence of fever ($\chi^2 = 23.3$, p-value<0.001). See appendix 4.

For the effect of number of motion per day the test is showing no significant effect ($\chi^2 = 2.27$, p-value=0.322). The presence of pathogen does not seem to significantly affect fever ($\chi^2 = 2.94$, p-value=0.230). Feeding type is significantly associated with fever as shown by the chi square test ($\chi^2 = 9.56$, p-value=0.023). There is no evidence to show that vomiting is associated with fever ($\chi^2 = 1.84$, p-value=0.175).

Older children seems associated with fever ($\chi^2 = 8.38$, p-value=0.015).

The use of dummies has no effect on the presence of fever as shown by the chi square test ($\chi^2 = 1.88$, p-value=0.170).

There is a significant association between stool duration and vomiting for this sample; ($\chi^2 = 20.45$, p-value=0.001). The number of motion per day is marginally significant ($\chi^2 = 5.51$, p-value=0.064).

The presence of pathogen is significantly associated with vomiting in this sample especially rotavirus infection; ($\chi^2 = 18.19$, p-value=0.001). There is no evidence that feeding type is associated with vomiting; ($\chi^2 = 2.16$, p-value=0.539).

4.10.3.3 Multivariate analysis from first study

In this section we will carry out a multiple logistic regression to show the adjusted effect on fever of all the factors we used in the univariate analysis. The effect will be displayed as odds ratios and their 95% confidence intervals. See appendix 4.

After adjustment it appears that the effect of stool duration on fever shows some significance especially for longer period above 3 days, but even a period of 2 to 3 days has p-value relatively small (p-value =0.112). This effect has been observed in the univariate analysis where the p-value was relatively close to significance (p=0.064). Age is showing an increasing risk but not significantly. Vomiting and feeding type are also showing increasing risk of fever but not significantly. Motion on the other hand is showing a decreasing risk although not significant, that is the more motion the less likely a presence of fever. All these results need to be checked in the analysis using data from study 2. The sample size could play a role in reinforcing the power of the significance test. See table 25 and 26.

4.10.3.4 Multivariate analysis from second study

In this section we will carry out a multiple logistic regression to show the adjusted effect on fever of all the factors we used in the univariate analysis as before in study 1. The effect will be displayed as odds ratios and their 95% confidence intervals. Age, stool duration, vomiting, number of motion and feeding type will be used as the

Table 25 Logistic regression summary of first study (fever)

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
	Age	1.104	0.068	0.993	1.228
Stool duration	1 day (baseline)	1		1	1
	2 – 3	7.195	0.116	0.614	84.343
	4 – 6	12.693	0.041	1.115	144.503
	>=7 days	14.757	0.035	1.206	180.496
Vomiting	Vomit No (baseline)	1		1	1
	vomit Yes	1.502	0.325	0.669	3.373
Motion/ day	4 - 6 (baseline)	1.00		1.00	1.00
	7 – 9	0.623	0.475	0.170	2.284
	>=10	0.850	0.814	0.219	3.297
Type of feeding	Breast (baseline)	1		1	1
	Bottle	6.579	0.282	0.213	203.463
	Mixed	5.269	0.230	0.349	79.582
	Solid	3.232	0.306	0.341	30.604

Table 26 Logistic regression summary second study (fever).

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
	Age	1.137	0.000	1.076	1.201
Stool duration	1 day (baseline)	1		1	1
	2 – 3	2.485	0.105	0.827	7.466
	4 – 6	7.039	0.000	2.373	20.881
	>=7 days	8.877	0.000	2.790	28.251
Vomiting	Vomit No (baseline)	1		1	1
	vomit Yes	1.657	0.038	1.028	2.672
Motion/ day	4 - 6 (baseline)	1		1	1
	7 – 9	1.083	0.849	0.477	2.458
	>=10	0.873	0.746	0.385	1.980
Type of feeding	Breast (baseline)	1		1	1
	Bottle	11.129	0.013	1.677	73.864
	Mixed	10.095	0.002	2.399	42.486
	Solid	6.409	0.002	1.939	21.181

confounder factors to predict the presence of fever. See appendix 4.

The logistic regression is showing that when the factors are used together in a regression model some effects become more significant. All the factors are showing significant effect except the number of motion per day. The confidence interval are now less wide than those of the study 1, and this can be explained by the larger number of cases included. Stool duration has an increasing effect, the longer the duration the more likely a presence of fever. Older children seem more exposed to fever than younger ones. See tables 27 and 28.

Table 27. The logistic regression summary of first study (vomiting).

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
	Age	0.942	0.160	0.868	1.024
Stool duration	1 day (baseline)	1		1	1
	2 – 3	1.905	0.501	0.292	12.446
	4 – 6	1.786	0.541	0.278	11.463
	>=7 days	3.499	0.208	0.497	24.606
Fever	No (baseline)	1		1	1
	Yes	1.438	0.368	0.652	3.173
Motion/ day	4 - 6 (baseline)	1		1	1
	7 – 9	0.527	0.308	0.153	1.808
	>=10	0.549	0.362	0.151	1.992
Type of feeding	Breast (baseline)	1		1	1
	Bottle	0.048	0.075	0.002	1.357
	Mixed	0.088	0.072	0.006	1.247
	Solid	0.158	0.115	0.016	1.567

Table 28. the logistic regression summary of second study (vomiting)

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
	Age	.838	.000	.764	.921
Stool duration	1 day (baseline)	1		1	1
	2 – 3	5.204	.038	1.091	24.819
	4 – 6	.910	.906	.191	4.341
	>=7 days	1.744	.512	.330	9.207
Fever	No (baseline)	.854	.703	.379	1.921
Motion	Yes 4 - 6 (baseline)	1		1	1
	7 – 9	.290	.068	.077	1.096
	>=10	.261	.036	.075	.915
Pathogens	Bacteria (baseline)	1		1	1
	Rotavirus	2.679	.390	.284	25.313
	Parasite	10.913	.055	.948	125.610
Feeding type	Breast (baseline)	1		1	1
	Bottle	.002	.000	.000	.042
	Mixed	.004	.000	.000	.054
	Solid	.009	.000	.001	.086

4.11 Summary

In the second study, rotavirus peaked during the winter (cold season) while bacteria and parasites peaked during the summer and autumn seasons. Breast feeding of Libyan children was found to be very low in patients (4.2%) and 15.3% of controls. In the second study 11.4% of patients were breast fed compared to 24% of controls. Dummies used is the risk factor for occurs of diarrhoea in Libyan children (OR: 2.5 95% CI: 1.42-4.59, $p<0.0007$) in the first study and (OD: 3.28, 95% CI: 2.24-4.90, $p<0.001$ in the second. Other risk factor is bottle feeding (OR: 6.256, 95%, CI: 3.509-11.152, $p<0.001$) in the first study and (OR: 4.146, 95%, CI: 3.066-5.605, $p<0.001$) in the second. No differences between patients and controls for water supply were established in the first study but in the second study there was a variation in use of storage water ($P<0.05$). Animal contact was not significant different between patients and controls in either study ($p>0.05$). The age distribution of patients with variation enteric pathogens differed in both studies. In the first study, the prevalence of bacterial infections was higher than in the second for infection of patients aged < 6 moths 18/37 (48.6%). The prevalence of parasite infections was higher for infection of patients aged > 12 m 4/7 (57.1%) in the first study and 30/62 (48.4%) in the second.

Rotavirus infection in the second study was greater than in the first study for infectin of patients aged 6 to 12 m 52/98 (53.1%).

The presence of pathogens is significantly associated with vomiting especially rotavirus ($\chi^2 = 18.19$, p-value=0.001).

There is a significant association between stool duration and vomiting ($\chi^2 = 20.45$, p-value=0.001).

Feeding type is significantly asssociated with fever as shown by the McNemer, chi sequare test ($\chi^2 = 9.56$, p-value=0.015) in the second study.

Chapter 5

General Discussion and Conclusions

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 The background to these studies

Two studies were performed to detect aetiology of acute diarrhoea in hospitalized children aged under five years in Tripoli, Libya. In the first study, two hundred and thirty-six stool samples were obtained. Of these, 118 were from patients admitted for acute diarrhoeal disease, and 118 age and sex-matched concurrent controls were admitted for other reasons. In the second study, eight hundred and ten stool specimens were collected 405 from patients and the same number from controls. All were admitted to the Aljala Children's Hospital, Tripoli, Libya. The first study was conducted between August 1st 1997 and 31st October 1997 and the second study was conducted between January 1st 2003 and 31st December 2003. One or more enteric pathogens were isolated from 55.9% of patients and 13.6% of controls in the first study and 68.1% of patients and 12.6% of controls in the second study.

5.2 The aims of these studies include:

1. Establishing a relationship between clinical symptoms and the aetiology of acute diarrhoea. .
2. Finding the factors contributing to the cause of acute diarrhoea besides the age group (s) that are prone to infection.
3. Finding the relationship between the cause of acute diarrhoea and time of year it occurs (seasonal variation).
4. Using new methods to diagnose the aetiology of acute diarrhoea not used in Libya e.g. selective media, API and PCR.
5. Increasing the detection rate of the aetiology of acute diarrhoea.

5.3 The results obtained in these studies show that:

Aetiological agents were detected in 55.9% of diarrhoeal patients in the first study and 68.1% in the second. Bacteria were the most common agents detected in 43.3% patients in the first study, whereas rotavirus was the most common agent identified in 30.4% patients in the second study. Parasites were detected in 8.4% of patients in the first study and 19.3% in the second. These findings suggest that rotavirus GE remains a common disease in hospitalized Libyan children and causes a substantial burden of disease.

In the second study, API 20E and API Campy were adopted to identify bacteria isolated by cultures and the number of confirmed cases decreased from 142 to 112. Rotavirus peaked during the winter season (cold months) while bacteria and parasites peaked during the summer and the beginning of autumn (hot months).

PCR technique was adopted for the first time in identification of Campylobacter in Libya, where all *Campylobacter* spp. were identified (*Campylobacter jejuni* and *Campylobacter coli*) as the same as detected by API Campy technique. The total number was 24 cases.

Libyan children under six months of age were the most susceptible to acute diarrhoea requiring admission to hospital. This was noted in 46.6% children among the first study and 43.2% among the second. The highest prevalence of acute diarrhoea was in children aged one year.

Exclusive breast feeding of Libyan children was of a very low percentage, merely 4.2% of patients and 15.3% of controls in the first study and 11.4% of patients and 17.7% of controls in the second. Children using dummies were about 2.5 times more at risk of diarrhoea than the non users in the first study (OR: 2.5, 95% CI: 1.42-4.591, $p<0.001$) and (OR: 3.28, 95% CI: 2.24-4.90, $p<0.001$) in the second, this fell to children being 3 times much at risk of contracting diarrhea. Other risk factor is bottle

feeding, in the first study, the odds of having the diarrhea for bottle feeding is 5 times higher than those who are breast fed.

Watery stool was the most common stool consistency of diarrhoeal patients in both studies consisting of 58.5% in the first study and 56.6% of the second.

Fever was the most clinical feature associated with acute diarrhoea for both studies. In the first study, the percentage of patients who had fever was 72.9% and in the second study this amounted to 71.6%.

Moderate dehydration was the most common degree of dehydration. The percentage of patients who had this at admission was 51.7% of patients in the first study and 55.5% in the second. Isotonic dehydration was the most common type of dehydration in both studies. The percentage of patients totaled 76.3% of the first study and 75.8% of the second.

In Libya, oral rehydration therapy is still not extensively used. From the history and medical records of patients, it was remarked that ORT was used for rehydration of patients in a very low percentage of cases. This equaled 8.5% in the first study and 13.8% of the second study.

5.4 Aetiological agents

In the first study, microbiological agents were obtained from the stool specimens of 118 patients. Of those 55 (55.9%) were identified as either single or mixed infections. The detection of microbiological agents from the control group was 16/ 118 (13.6%) as a single infection.

In the second study, aetiological agents were detected in 68% of patients as either single or mixed infections and in 12.6% of controls as a single infection. The aetiological agents were detected in patients which were significantly different of the controls (P <0.05) in both studies. The highest rate of detection in

the second study may be due to the dissimilar protocols and duration of both studies, the protocol in the second study included more investigations. The first study was carried out at the end of summer and the beginning of autumn while the second was conducted throughout the year. The sample size of the second study was also much larger than that of the first study. Other studies have displayed similar results in developing countries. In northern Jordan, the aetiological agents of acute diarrhoea were identified in 66.4% of children < 5 years of age. A single enteric pathogen was detected in 50.9% of patients and multiple pathogens were detected in 15.5%. (Youssef *et al.* 2000). In Uruguay, the detection of diarrhoeal pathogens were discovered in 63.8% of patients and 34.4% of controls (Torres *et al.* 2001). In Brazil, intestinal pathogens were detected in stool specimens among 72.8% of patients and 21.5% of controls from children aged < 5 years with and without diarrhoea (Souza *et al.* 2002). In Tanzania, the aetiology of acute diarrhoea was 67.6% in stool patients under 5 years old and 35.7% of controls (Vargas *et al.* 2004). In Vietnam, the aetiological identification of pathogens in faecal samples of children below five years of age with diarrhoea was 67.3% of patients (Vu Nguyen *et al.* 2006). In developed countries, the results from the studies vary. For example: in Denmark, potential pathogens were found in 54% of patients and 22% of controls (Olesen *et al.* 2005). In Switzerland, isolation of enteric pathogens from specimens of children with and without diarrhoea were found in 50.3% of patients and 18% of controls (Pabst *et al.* 2003).

5.4.1 Bacteria

Bacteria were the most common micro-organisms detected in the first study and rotavirus in the second. The high prevalence of bacterial isolation in the first study compared to the second, may be due to the former being performed

over a short period and excluding the winter season where rotavirus had peak incidence. Another explanation could be that API was not adopted for confirmation tests in the first study, as the number of bacteria was isolated by cultures and identified by Gram stain and morphological characteristics whilst some bacteria were excluded as they were not identified by API test in the second study. *Escherichia coli* was the most common agent isolated in 22.9% of patients and 4.2% of controls in the first study and 16.5% of patients and 2.7% of controls in the second study. The over-detection of *E. coli* in the first study may include non- pathogenic *E. coli* living in normal flora because it is not confirmed by serotyping or API 20E tests or other confirmation tests due to a lack of adequate diagnostic laboratory tools in Tripoli Central Laboratory at the time of the first study being performed.

The results are similar to other studies conducted in developing countries. In Northern Jordan, *E. coli* was the most common bacterial infection leading to acute diarrhoea in children (28.7%; Youssef *et al.* 2000). In Brazil *E. coli* was also the most common bacterial isolated from stool specimens of children with acute diarrhoea (18.2%; Orlandi *et al*. 2006). In developed countries, the prevalence of bacteria was lower than in developing nations. In Denmark, among all sampling tested, a bacterial pathogen was found in 17% of cases and 4% of controls. *E. coli* was 6.7% (Olesen *et al*. 2005). In Libya, the prevalence of bacterial infection was similar to prevalence in developing nations and higher than that of developed countries.

Campylobacter was detected from the stools of Libyan children with acute diarrhoea. This was 9/118 (7.6%) patients, 1/118 (0.8%) of controls of the first study and 24/405 (6.0%) and 4/405 (1.0%) of the second. There was great disparity between the two studies despite using API Campy in the second. The incidence of *Campylobacter* in Libyan children in these studies is similar to a previous study carried out between September 1992 and August 1993 in which *Campylobacter*

prevalence was similar to the results obtained here for patients but different in controls (6% of patients, 3% of controls; Ghenghesh *et al.* 1997). *Campylobacter* isolation rates in developing countries range from 5 to 20% (Oberhelman and Taylor 2000). In Uruguay, the prevalence was 7.4% of patients (Torres *et al.* 2001). In Egypt, *Campylobacter's* isolation rate was 9.0% from stool specimens taken from < 5-years-olds with acute diarrhoea (Rao *et al.* 2001). In Jordan, *Campylobacter* was detected in 8.4% of children with diarrhoea and 0% of controls (Nimri *et al.* 2004). In England, the prevalence of *Campylobacter* spp. was 12.2% (Tompkins *et al.* 1999). In Switzerland, the prevalence was 3.3% of children with diarrhoea and 0% of children without it (Pabst *et al.* 2003). In Denmark, prevalence was 3.8% of patients and 0.5% of controls (Olesen *et al.* 2005). The studies conducted in developing countries demonstrated frequent human-animal contact. The presence of animals and poor hygiene conditions within a house are risk factors (Rao *et al.* 2001). In Libya, animal contact was very little and raw chicken was unlikely to play a role in infection by *Campylobacter* because traditional Libyan food requires meat to be well-cooked. The possible factors for infection may be via contaminated water or by consumption of un-pasteurized milk.

Proteus was discovered in stool specimens of patients in 7.6% and 1.7% of controls of the first study and 2.2% of patients and 0.7 of controls of the second study. The prevalence of the first study was very high when compared with the second. The explanation for this is that detection of *Proteus* in the first study depended upon morphological features of colonies on solid media where the colonies were non-lactose ferments, had a fish-like smell and were very motile, and by Gram stain showed gram-negative rods and were not confirmed by other tests while the second study API 20E confirmed the detection.

Klebsiella was detected in stool specimens in 4.2% of patients and 0.8% of controls in the first study and 3% of patients and 0.7% of controls in the second. Again *Klebsiella* was identified by morphological features on solid media where the colonies were large, raised, moist and viscid. On the MacConkey medium, colonies were pink in colour, produced gas from fermented lactose and were non-motile. By Gram stain, gram-negative bacilli. *Klebsiella* was not confirmed by other confirmation tests in the first study and had higher prevalence than the second study. The API 20E was confirmed and two types were detected. *Klebsiella pneumoniae* and *Klebsiella oxytoca.* These latter both commensals of the human gastro- intestinal tract, have been reported to be an occasional cause of diarrhoea in humans (Arora *et al.* 1983). *Klebsiella pneumoniae* may induce diarrhoea through the production of an STa enterotoxin similar but not identical to *E. coli* STa (Guarino *et al.* 1989). However, the role of these toxins in the pathogenesis of *klebsiella*-associated diarrhoea has not been clarified (Guerin *et al.* 1998). Study used the heamoagglutination method where types of fimbriae were identified. Above 90% in investigated *klebsiella* strains revealed the presence of fimbriae (Maczynska *et al.* 2003). Over 70% strains demonstrated a high level of adherence to cell lines. These results suggest that among *Klebsiella* strains in the gastrointestinal tract are multi- resistant strains with a high ability to adhere. These may represent a potential source of nosocomial infection (Maczynska *et al.* 2003). In Italy a total of 85 strains Gram-negative bacilli were screened for virulence factors, *Proteus* and *Klebsiella* were identified in 48/85 ((56.5%). These organisms were isolated in pure or dominant culture and in the absence of other intestinal pathogens from children with acute diarrhoea. On the other hand the production of cytotoxic factor was identified for 14 strains examined; in particular the frequency of cytotoxic strains among *Proteus* (Caprioli *et al.* 1985). *Proteus* and *Klebsiella* are free-living saprophytes and are not usually thought of as causing diarrhoea. They can be isolated

from soil, water, sewage and may be found in the intestine in many healthy individuals. The possible pathogenic role of these bacteria in causing diarrhoea should be investigated. When comparing between results of bacterial isolated by ordinary cultures and API 20E in the second study, there was 76.5% of bacterial isolated by cultures confirmed by API 20E. This difference may be due to the identification of bacterial isolation by morphology of colonies and Gram stain being insufficient to confirm that bacterial isolated is not precisely the type of bacteria, but there is a need to confirm this by conducting other tests e.g. serotyping or phenotyping. These were not available due to a lack of proper diagnostic tools in Tripoli central laboratory. The comparison between *Campylobacter* spp. were isolated by selective medium and identified by API Campy in the second study, 88.9% of *Campylobacter* spp. were isolated by selective medium. The difference between the other bacteria isolated by ordinary medium and *Campylobacter* spp. isolated by selective medium (CCDA) was very distinct because the selective medium is specific and growth of other bacteria not usually occur. The variation between API 20E (detected at 76.5%) of other bacteria isolated by ordinary medium and API Campy (detected at 88.9%) of *Campylobacter* spp isolated by selective medium may be due to difficulty in obtaining pure subculture on ordinary medium especially for cases of multiple infection where many bacteria were unconfirmed by API technique in the first study.

The reason for the *Salmonella* spp. not being discovered in our studies was because of a dearth of diagnostic tools in Tripoli central laboratory e.g. selenite broth to enrich growth before culture on Salmonella-Shigella medium and also lack of serotyping or phenotyping. In a study carried out in Libya in (Ali *et al.* 2005) *Salmonella* spp. was detected. The protocol for that study differs from our studies, especially the age of the children involved. In that study children were aged from a few days to 12 years. The children participating were older than in our studies where the children attended

primary school and might contract food poisoning from eating contaminated food there. The children who participated in our studies were of preschool age. In a recent study carried out in Hanoi, Vietnam, no *Salmonella* spp. was isolated, even when conventional methods were used in combination with ELISA, immunoseparation and PCR (Vu Nguyen *et al* 2006).

Despite the identification of several risk factors associated with *Campylobacter* infection by numerous case-control studies, the sources of the majority of human cases of Campylobacteriosis remain unknown (Cowden 1992). Rapid, sensitive and specific methods for detecting enteropathogenic *Campylobacters* are essential to detect and confirm *Campylobacter* spp. It is appropriate to utilise other methods to detect cultures. PCR provides a molecular approach for detecting micro-organisms by specific amplification of nucleic acids. Assays using this technology have been developed for a range of pathogenic and environmental micro-organisms, including *Campylobacter* (Giesendorf *et al*. 1992, Wang *et al*. 1992 and Hazeleger *et al*. 1994).

The majority of *Campylobacter* PCR assays utilize primers designed from sequence data for the 16S RNA or flagellin genes of *Campylobacter jejuni* (Giesendrof *et al.* 1992 and Oyofo *et al*. 1992). Despite the wide distribution of homologous genes throughout prokaryotes, such assays appear specific for *Campylobacter jejuni* or its closest relatives. This specificity appears to be due to the large phylogenetic distance between campylobacter and other bacterial taxa, with functionally identical genes having quite dissimilar DNA sequence identities (Oyofo *et al*. 1992). Primers used in this assay have been designed from previously uncharacterized *Campylobacter jejuni* two-component regulator gene (Jackson 1995).

Several Campylobacter PCR methods cannot differentiate *Campylobacter jejuni* from *Campylobacter coli* (Giesendrof *et al*. 1992 and Oyofo *et al.* 1992), and specification of amplified strains by restriction enzyme analysis is a novel feature of the assay

adopted for this study. The characteristic digestion patterns appear highly stable and reproducible for the two most common pathogens, *Campylobacter jejuni* and *Campylobacter coli* (Jackson *et al*. 1996).

Restriction analysis was not employed in this study. Digestion of this amplimer with restriction enzymes produced simple restriction fragment length polymorphism (RFLP) within the amplification products which are characteristic of the species of *Campylobacter jejuni* and *Campylobacter coli.* Simple restriction fragment length polymorphism of the digested PCR products can be used to differentiate the amplified species, without the requirement for species-specific DNA probing (Jackson *et al.* 1996). When commencing work, the amount of hyperLadder V marker) 25 µl was taken and the marker spaced bands were joined together, especially the lower bands due to the large amount used (25 µl). After adjustment of the amount of HyperLadder V to 5 µl, the banding on the gel was move district and identification of the appropriately sized restriction fragment was possible at between 250 and 300 bp (theoretically 256 bp). The large bends in figure 6 was due to overloading with DNA. HyperLadder V produces 12 regularly spaced bands, ranging from 25 to 500 bp.

The use of selective media for isolation of *Campylobacter* followed by phenotypic tests is the standard method of *Campylobacter* detection in diagnostic laboratories. The results of some studies using PCR for detection of *Campylobacter* spp. in clinical specimens illustrated that PCR detected twice as many positives as culture and, further more, the results from this study indicated that PCR was a sensitive and specific method for detection of *Campylobacter* in human faecal specimens (Vanniasinkam *et al.* 1999). Other studies have demonstrated that filtration techniques may be more sensitive than culture techniques (Kaijser and Mégraud. 1992).

The PCR technique is still unfamiliar to Libyan laboratories where they have only recently started to use a diagnostic procedure in Tripoli central laboratory for

confirmation of HIV diagnosis. Thus this is the first time where *Campylobacter* have been isolated from stool specimens collected from Libyan children by culture and identity confirmed by PCR.

This study demonstrated that *Campylobacter* isolated by selective culture when identified using API Campy, the number reduced from 27 to 24, and all of *Campylobacter* (24) which were identified by API Campy. and confirmed by PCR.

The incidence of *Campylobacter* in diarrhoeal disease may not be accurate because culture is considered to be insensitive for detection of sub-lethally damaged, un-culturable but viable cells, and low numbers of organisms which may be present in some stool specimens (Jones *et al.* 1991).

For determination of the true incidence of *Campylobacter* in diarrhoeal disease in Libya, more studies are required using culture on selective media, phenotypic tests, filtration technique, API Campy and PCR.

5.4.2 Rotavirus

Rotavirus is the second major cause of acute diarrhoea in children after bacteria in developing nations, whereas in developed countries rotavirus is the major cause and bacteria are the second most frequent cause. The incidence of rotavirus in Libyan children was higher in the second study at approximately 30%, whilst in the first study this was 15.3%. The difference of prevalence of rotavirus between two studies was obvious, because the first study was conducted for 3 months at the end of summer and beginning of autumn when rotavirus infection was very low while the second study was conducted during the course of a full year and rotavirus infection was very high during the winter season. Another recent study in Zliten, a small urban city in Libya showed that the prevalence of rotavirus represented 26.6% of patients with diarrhoea among children aged from a few days up to 12 years (Ali *et al.* 2005). The difference was probably because the age of the children involved in that

study being older than those of our studies (from 1 month to 12 years). Similar results were obtained from studies which were conducted in developing countries. In Jordan, rotavirus was the most common enteropathogen (32.5% of patients; Youssef *et al.* 2000). In England, the prevalence of rotavirus infection was 39% (Crowley *et al.* 1997). In Argentina rotavirus infection of hospitalised children was 35.3% of patients (Giordano *et al.* 2001). On the Colombian Northern Coast, rotavirus was discovered in 36.6% of diarrhoeal children (Urbina *et al.* 2003). In Tunisia, rotavirus was found in 20% of children (Fodha *et al.* 2006). Among Egyptian children, clinic–based surveillance of two physicians' clinics rotavirus was detected among 36% in the first clinic and 46% in the second (Wierzba *et al.* 2006). By contrast, in Denmark, the prevalence of rotavirus was 14% (Olesen *et al.* 2005). In the United States, rotavirus was the most common pathogen recorded for 18% and 19% of diarrhoea-associated hospitalisation in 1997 and 2000 (Malek *et al.* 2006). In Switzerland, the prevalence of rotavirus was 18.7% of diarrhoeal children (Pabst *et al.* 2003). The incidence of rotavirus disease was observed to be similar in both developed and developing countries, suggesting that adequate control may not be achieved by improvements in water supply, hygiene and sanitation (Parashar *et al.* 2003a). Rotavirus in the second study was the most common aetiological agent detected in hospitalized Libyan children (30.4%). The strategy for DDC should be to introduce a rotavirus vaccine into the immunisation programme to prevent severe or fatal rotavirus infection, by lowering the rate of infection, decreasing morbidity and mortality caused by diarrhoea and also decreasing of hospitalisation of children due to diarrhoea.

5.4.3 Parasites

The prevalence of parasite infection is higher in the second study (19.5%) and (8.4%) in the first study. The occurrence of *Entamoeba histolytica* was very much higher in the second. *Ent. histolytica* was the most common parasite cause of acute

diarrhoea in Libyan children and represented 64.6% of parasite infections. In Egypt, among patients with intestinal protozoa, 16.2% had *Ent. histolytica/dispar* by stool examination of stained smears (El-kadi *et al.* 2006). Other studies have revealed similar results in developing countries. In the Colombian Northern Coast, *Ent. histolytica* infection was detected in 10% (Urbina *et al.* 2003). In Jordan, *Ent. histolytica* was detected in 10.5% (Youssef *et al.* 2000). In Bangladesh, the isolation rate of *Ent. histolytica* was 10% (Haque *et al.* 2003a).

The classic stool ova and parasite examination by light microscopy is insensitive and cannot differentiate *Ent. histolytica* from the non-pathogenic *Ent. dispar*.

In the present study, *Ent. histolytica* was detected by light microscopy and other tests eg. ELIZA or PCR were not used. In Egypt, a recent survey using antigen detection tests displayed a higher isolation rate of *Ent. histolytica* (Abd-Alla *et al.* 2002). The detection rates of *Ent. histolytica* and other parasites by advanced techniques is required to determine accurate prevalence, especially differentiation of pathogenic from non-pathogenic species and treatment should only be prescribed for patients who have pathogenic parasite infections.

Giardia lamblia showed a similar increase in the second study being 3.7% higher than the first study (1.7%). *G. lamblia* was detected in another recent study in Libya among 1.2% of children (Ali *et al.* 2005). Similar results were obtained in other countries. In Uruguay, the prevalence rate was 3.6% (Torres *et al.* 2001). In contrast, other studies illustrated higher prevalence rates. In Tanzania this was 14.5% (Vargas *et al.* 2004), and 11% in India (Haque *et al.* 2003b), and then in Colombia 12.4% (Manrique-Abril *et al.* 2006). *G. lamblia* is the second parasitic infection after *Ent. histolytica* among Libyan children.

Cryptosporidium was identified in 2.5% of patients and 0.8% of controls in the first study and 3.2% of patients and 0.7% of controls in the second. The incidence of

Cryptosporidium in these studies was for lower than that of a previous study in Libya which reported a high incidence at 29.5% in patients and 23.1% of controls (Ghenghesh *et al*. 1997). The variation between our studies and the latter may be due to the different techniques used to detect *Cryptosporidium*. The study yielding such a high incidence rate adopted the modified Ziel-Neelson acid-fast stain. With this method, faecal debris and some yeasts occasionally occupy the primary stain providing false positive results (Baxby and Blundell 1983). The method adopted in my study was more selective (Nicols and Thom 1984). A recent study in Libya gave the prevalence of *Cryptosporidium* as 13% (Ali *et al*. 2005). The technique utilised for detection of *Cryptosporidium* was the same as that used in a previous study. Other studies performed in developing countries revealed the incidence of *Cryptosporidium* as similar to our study. In Jordan this was 1.5% (Youssef *et al*. 2000), and in Bangladesh 8.4% (Haque *et al.* 2003b). In Egypt, the prevalence vas higher than our studies and detected was 15% (El-Mohamady *et al.* 2006). In contrast, for studies in other developing countries, the rates were much higher. In Uganda prevalence was 25.0% (Tumwine *et al*. 2003). In developed countries, the incidence proves to be very low 1.7% in Denmark (Olesen *et al*. 2005) and 6% in UK (Crowley *et al*. 1997).

The detection of parasite infections using advanced techniques e.g. ELIZA, PCR will allow accurate determination of parasite infection rates.

5.5 Seasonal distribution

The first study was conducted over a relatively short period (3 months) and no seasonal variation could be determined. The second study was performed during a whole year and seasonal variation could, in this instance be determined. The highest frequency of isolation of enterobacteriae was in the summer season and parasites were detected more frequently in the summer and autumn, whereas the incidence of

infection with rotavirus infection was higher in the winter. Other studies in many countries have demonstrated the same seasonal variation. In a study in Bangladesh, the incidence of diarrhoea showed a greater rate in the months of March-August than was the case in September-February (Haque *et al.* 2003a). In Nepal, the prevalence of diarrhoea was common in the summer season at 61% of diarrhoeal cases (Ono *et al.* 2001). In Tanzania, the prevalence of diarrhoea was higher in the dry season of July-September than any other season (Vargas *et al*. 2004). In Egypt, rotavirus-associated diarrhoea peaked in late summer to early winter, while bacterial agents were prevalent during summer (Wierzba *et al*. 2006).

Bacteria were more frequently isolated in the summer because Libya is located in a temperate region, where the temperature is much higher in the summer. Many factors may contribute to bacteria being the leading cause of diarrhoea in the summer months, probably because hot weather encourages the growth of most pathogenic organisms (bacteria and parasites) on food and drink, which easily contaminated, especially if not placed in the refrigerator or left uncovered. During the summer period there is an increase in outdoor cooking, and picnics, where food on many occasions remains slightly raw which can lead to many health problems. In addition summer is also the breeding season for flies that act as mechanical vectors of enteropathogens to contaminate food and water. Various other studies supply similar results to our studies, regarding seasons of bacteria. In Denmark, bacterial infections tended to occur during summer and autumn (Olesen *et al*. 2005). In this study, *Campylobacter* isolations were higher in July-August and occurred later than in Egypt, where the incidence was higher in the warmer months between May and August (Rao *et al*. 2001).

The seasonal variation in frequency of rotavirus infections differed markedly from that of bacterial and parasite infections. Rotavirus was most frequently detected in the winter season (62/123; 50.4%). Reasons for this may stem from the high degree of

contact children have with other children or adults, especially in enclosed and overcrowded areas. Rotavirus is disseminated and transmitted through the community in the winter faster than is the case in the summer season. The growth of rotavirus in the winter is faster than the summer because the low temperature and dry air are suitable for optimum growth of rotavirus. The seasonal variation of rotavirus in some countries was similar. For instance, in Bangladesh (Albert *et al.* 1999), Nepal, (Ono *et al.* 2001), Denmark, (Fischer 2001), Argentina, (Giordano *et al.* 2001), Venezuela, (Salinas *et al.* 2004) and the United States (Malek *et al.* 2006). Other countries contrasted with this e.g. in Brazil children were hospitalised with severe rotavirus-associated gastroenteritis with 72% of the cases occurring in June and July (Carneirio *et al.* 2005). Again, in Brazil, rotavirus- associated diarrhoea also occurred in June and July (Nadya *et al.* 2005). In Egypt, the incidence rates of rotavirus diarrhoea was greater during late summer and early winter (Wierzba *et al.* 2006). Most countries had rotavirus admissions all year round, and rotavirus generally reached a seasonal winter peak in both temperate and tropical climates (Kane *et al.* 2004).

The seasonal variation of parasite infection was similar to that for bacteria where the highest numbers were detected in the hot months. In studies in various nations, the incidence was similar to our study. In Uruguay, *Cryptosporidium* has a seasonal presentation at the end of summer and autumn (Torres *et al.* 2001). In Tanzania, a high proportion of *G. lamblia* was isolated during the rainy season, while *Ent. histolytica* isolation was higher in the dry season (Vargas *et al.* 2004).

In Bangladesh, *Ent. histolytica*-associated diarrhoeal incidence did not demonstrate a seasonal relationship (Haque *et al* .2003a).

5.6 Descriptive statistics

Libyan children under one year of age were the most susceptible to acute diarrhoea

requiring admission to hospital. A difference between the two studies (78.8% in the first study and 70.1% in the second) was noted which may have been due to increased breast feeding in the second study. Similar results have been reported in many countries. In most developing nations the incidence of diarrhoeal diseases are greatest among children younger than 1 year of age, and thereafter rates decline (Kosek *et al.* 2003). In Jordan, the prevalence of acute diarrhoea was higher in children under one year at 69.1% (Youssef *et al.* 2000). There is a difference between the present study and one performed in Denmark where only 27% of children with diarrhoea were aged under one year and incidence was highest in the age group < 2 years old (68% of children; Olesen *et al.* 2005). In analysis of a 2-year study of 1249 admissions with GE in Tripoli Children's hospital, infants comprised 92% of patients below 1 year and 59% were aged 6 months or younger (Ben Halim. 1991). A further study also conducted in Libya showed that the prevalence of diarrhoea was 85% of children aged below one year (Ghenghesh *et al.* 1997). These results were in contrast to many studies, especially those done in developed countries, where diarrhoeal incidence was at its highest among the age group of two-year olds. In developed countries, children receive good care from child health care programmes, and enjoy good environmental and socioeconomic conditions. In the age group of < 6 months and under one year old Libyan children, the incidence of acute diarrhoea was still very high when compared with other countries and this may be due to failure of breast feeding and control. diarrhoeal disease programmes.

The incidence of acute diarrhoea in both studies was slightly higher in males than in females. The ratio of male: female was (1.3:1) in the first study and (1.2:1) in the second. Similar results were noted in a previous study in Libya where the male : female ratio was 1.2:1 (Ghenghesh *et al.* 1997). Gender did not bear a significant role in causing diarrhoea in Libyan children. In many countries the sex distribution is not

much different. In Jordan, the sex distribution of children demonstrated a male: female ratio of 1.3:1 (Youssef *et al.* 2000). In Bangladesh this was 1:0.7 (Albert *et al.* 1999). In Nepal, the male to female ratio was 1.7:1 (Ono *et al.* 2001). In Australia, the male to female ratio was 1.2:1 (Tenkate and Stafford 2001). The slight variance may be due to the natural distribution of males and females in those countries or that parents tend to show greater care for males than they do for females, as is the case in the majority of developing countries.

Breast feeding of Libyan children had a very low percentage. Only 4.2% of patients and 15.3% of controls were exclusively breast fed. The total number of mothers who breast fed their children was 23/236 mothers (9.7%) in the first study and 11.4% of patients and 24% of controls i.e. 143/810 mothers (17.7%) in the second. In the second study the total number of mothers who breast fed their children was greater. The difference between the two studies regarding breast feeding was approximately 8%. Another study in Libya demonstrated that 97% of patients admitted to the children's hospital for GE were wholly artificially fed (Ben Halim. 1991). Early artificial feeding may be responsible for the high incidence of acute diarrhoea in Libyan infancy especially among the age group 7 to 12 months which represented 43-46% of patients. Children who were breast fed were substantially less likely to suffer diarrhoea compared with their non-breast fed counterparts (p <0.05). The above suggest that breast feeding will protect younger children from contracting diarrhoea. Many studies have established a relationship between breast feeding and protection from diarrhoea. In Brazil, the chance of presenting with diarrhoea was 64% higher in children younger than six months who were not breast fed. When compared to children who were exclusively breast fed, the chance of presenting with diarrhoea increased to 82% in those who were not breast fed. Breast feeding and exclusive breast feeding was found to be a protective factor against diarrhoea in the first six months of life (Vieira *et al.*

2003). In England, in a case-control study of diarrhoeal disease, after adjustment for confounders, breast feeding was associated with significantly less diarrhoeal disease. Associations were striking even in infants aged > or = 6 months (Quigley *et al.* 2006). A study in Egypt illustrated that exclusive breast feeding was protective by reducing asymptomatic infections, symptomatic infections, and clinical severity of diarrhoeal episodes (Mahmud *et al*. 2001).

In Libya, the CDD programme, where necessary, should be activated by health education especially breast feeding for protection of infants particularly younger infants from birth to 6 months old, where the majority of this age group were found to suffer from acute diarrhoea in both studies. In Denmark, formula milk or ready made food containing milk powder was associated with diarrhoea, whereas being breastfed was associated with decreased risk of diarrhoea (Ethelberg *et al*. 2006). In Turkey, more children in the hospitalised group had high frequency of stool of diarrhoea, frequency of vomiting, malnutrition, dehydration and fever, and were not breastfed on admission compared to the control group (Yalcin *et al*. 2005).

By excluding the percentage of patients and controls who were exclusively breast fed and did not drink water, there was little difference between patients and controls with the type of water supply in either study. Thus the type of water supply was found not to play a significant role in causing diarrhoea among Libyan children. When comparing between the first and second studies, we noted the water supply of tap and bottle water were used more for children attending hospital in the second study than the first, with the difference being approximately 23%. One explanation is that water prior to 2000 was stored as most residents in Tripoli city had personal wells or collected and stored water in their homes because bottled water was very expensive. Subsequent to 2000, most houses in Tripoli were connected to the general network of water sources, when water of the Great Man- Made River reached Tripoli city and also

bottled water became less expensive. In the first study, usage of well and storage water was not significantly different between patients and controls because water of the Great Man-Made River did not supply Tripoli city at the time when the first study was carried out while in the second study, there was a statistically significant difference ($p<0.05$). Accordingly, families must be encouraged to cease use of wells and storage water supplies especially for administering to infants.

The patient and control groups of the second study who used a dummy reflected a decrease in the number of children (36.3% and 16% respectively), compared with the first study (57.6% and 18.6% respectively). The explanation for this may be due to the increased number of children in the second study who were breast fed. The patients group who used a dummy were substantially more likely to contract diarrhoea compared with the controls ($P < 0.05$) of the first study and ($P < 0.05$) of the second. Use of dummies may increase the risk of diarrhoea because they are easy to contaminate which may lead to the transmission of pathogens, especially in families with poor hygiene. Moreover there is a relationship between breast feeding and the use of dummies because when the child is breast fed, dummies are not normally used whereas when the child is bottle fed the child invariably also uses a dummy.

Education in Libya is now generally satisfactory since the government has attempted to eradicate illiteracy within the last thirty years by opening schools everywhere, particularly in rural and desert communities by encouragement of adult return to learning within adult education centres and by legislation so that parents are responsible by law if their children fail to attend school until they have at least completed intermediate education level. In the first study, the illiteracy rate remained high (21.2% of mothers of patients and 17.8% of mothers of controls). In the second study, the illiteracy rate was slightly lower (15.8% of mothers of patients and 12.6% of mothers of controls). One explanation is that illiterate mothers of children involved in

these studies may have been older than other educated mothers because years ago parents tended to manifest sexual discrimination between male and female education, the girls being far less likely to be educated. However, a mother's education had no effect upon the occurrence of diarrhoea in Libyan children in either study here.

5.7 Clinical observation

The history and nature of stools taken from children suffering from acute diarrhoea is very important, because it will provide indications of whether the diarrhoea is mild, moderate or severe and assessment of characteristics of stool is a key feature in determining potential pathogens causing an acute diarrhoea episode. Liquid non-bloody stools are associated predominantly with a watery stool due to small intestinal dysfunction process, whereas bloody diarrhoea and pus (dysenteric) or bloody stools are associated as a rule with an inflammatory or invasive process of the colon. Most viral agents and bacteria that release enterotoxins do not generally cause bloody diarrhoea (Podewils *et al*. 2004). Mucous can be present in both situations and should be distinguished from pus. The ability to obtain an aetiological diagnosis in a child with diarrhoea is largely dependent on the quality of the stool sample, the experience and skill of the microbiologist, and resources available for stool evaluation. In these studies watery stools were more frequent (58.5% in the first study and 56.6% in the second). This would indicate that the most likely causes of diarrhoea in both studies were rotavirus and *E. coli*. Studies from other countries illustrated that watery stool was more common in children with acute diarrhoea. In Jordan, watery stool represented 91.7% of children hospitalised with acute diarrhoea with 10.9% of bloody stools (Yuossef *et al*. 2000).

Libyan mothers were prone to seek medical assistance if the child had fever or vomiting. Over 70% of patients had fever reported at admission in both studies. Most

studies from other nations e.g. Denmark further noted that fever was the most frequently observed clinical feature associated with acute diarrhoea in 56% of patients (Olesen *et al.* 2005). Fever is a common occurrence and is usually associated with invasive pathogens and rotavirus infections. Fever was the most frequent clinica feature associated with acute diarrhoea in Libyan children.

Vomiting is one of the primary reasons for admission of a child to hospital because he/she is unable to obtain fluids and food orally and these will need to be replaced intravenously. Vomiting is the consequence of disturbed motor activity of the stomach. Vomiting is observed more frequently in viral diarrhoea and illness caused by ingestion of preformed bacterial toxins (Podewils *et al.* 2004). In Jordan, vomiting was presented in 77.1% of patients and was the most frequently observed clinical symptom in children hospitalised by diarrhoea (Youssef *et al.* 2000).

Despite these clues, detecting the causative agent of diarrhoea in an individual patient based on clinical manifestations alone is normally problematic. In the first study, just over half the patients had vomiting at admission. Vomiting was the second most frequently observed clinical feature associated with acute diarrhoea in both studies. The number of patients who had fever associated with vomiting was 32% in the first study and 35% in the second. Vomiting is a significant clinical factor for hospital admission and IVT administered to patient. If a child with mild or moderate dehydration is treated with TVI and assessment after that period for to take design for admission or not, that may result in unnecessary hospital admission.

All children presenting acute diarrhoea should be accurately assessed for dehydration. Dehydration and its complications are invariably the cause of death in acute diarrhoea cases especially among infants. The fundamental aim of treatment of dehydration is to administer fluids to correct this situation. Estimation of the type and degree of dehydration at admission is crucial in order to commence proper treatment according

to current WHO guidelines and additional practice guidelines for treatment of acute gastrointestinal illness in children (American Academy of Pediatrics. 1997). Because the most frequent type of dehydration is isotonic, measurement of serum electrolytes is required in only those children who have either moderate dehydration with a typical clinical history or findings, or in children with severe dehydration. The type of dehydration according to the serum sodium value at admission in both studies were comparable to studies conducted previously and studies performed in other countries. In the first study, isotonic dehydration was 76.3%, hypotonic dehydration was 13.5%, and hypertonic dehydration was 10.2% and 79.2%, 11.5 and 9.3% respectively in the second study. In Tunisia, hypertonic dehydration was 11.51% of children with acute diarrhoea (Chouchane *et al*. 2005). The percentage of cases of hypertonic dehydration declined by 2.5% in the second study, but there was still a marked difference between the two studies. The explanation for this is that in 1997 Tripoli city water supplies were extremely salty, whereas in 2000 the Great Man-Made River water supply was established endowing the city with low salt water (Anon. 1994). The degree of dehydration in the first study was 32.2% for mild, 51.7% for moderate and 16.1% for severe and 33.1%, 55.5% and 11.4% respectively in the second study. The variation between both studies was obvious in the degree of severe dehydration which decreased in the second study, perhaps as a result of mothers who brought their child to the doctor early before the dehydration became much worse. No patient who was deteriorated or shocked participated in either study, because such cases will be admitted directly to the ICU. In developing countries, where severe dehydration is more prevalent because there are many factors which contribute to severe dehydration: the mother stops breast feeding, there is no use of ORS especially for infants, there is cessation of administering any fluids or solid or semi-solid food, delay to bring the child to the medical centre or hospital, profuse watery stool and high frequency. The

level of serum electrolytes (sodium, potassium and chloride) during acute diarrhoea especially in severe dehydration cases, will be disturbed but, when the dehydration is corrected, the electrolytes will return to normal levels. It is critical to determine the serum sodium, potassium and chloride level as soon as possible after admission of the patient, because hypocalaemia (potassium below normal level) or hypercalaemia (potassium above normal levels) and hyponatraemia (sodium below normal level) or hypernatraemia (sodium above normal level) will require different intravenous drips to correct dehydration to prevent any associated complications.

In both studies, the serum electrolytes of patients in 75.8 to 78.8 % were within normal ranges and residual percentage was corrected properly. No patient suffered complications due to electrolytes disturbance because most patients were rehydrated by IVT (86.2%) and others by ORT. Normally mothers had brought their children to hospital early before the child developed severe dehydration or electrolyte disturbance.

The protocol of treatment of acute diarrhoea in recent years has altered according to WHO recommendations, where mild and moderate dehydration are treated by using ORT, a part from patients with vomiting where IVT is used. This is also adopted when severe dehydration is present. The effective use of ORT has saved millions of lives around the world. In the first study, the percentage of patients treated for dehydration by ORS was only 8.5% and 91.5% of patients received IVT. In the second study, the percentage of patients treated for dehydration by ORT was 13.8% and 86.2% of patients received IVT. There was a slight increase in the use of ORT in the second study but, generally, in Libya ORS is still not extensively used. In both studies, approximately 11% of patients were treated by ORT and the percentage of patients where moderate dehydration was diagnosed was approximately 32.5%. This is in contrast with WHO recommendations. Many factors may be responsible for excessive

use of IVT. Firstly, mothers provide an incorrect history especially of vomiting and frequency of diarrhoea. This may be because they consider the treatment is superior if their child is admitted and IVT is administered. Secondly, many authorities suggest that children who are assessed as being moderately dehydrated should be given IVT when they could be rehydrated orally according to WHO recommendations. Thirdly, there was an over- estimate of dehydration by junior doctors at admission which may lead to unnecessary hospital admission and over-treatment with IVF. This result is similar to a study done in Thailand (Gusbi 1991). In many other countries a high percentage of ORT was utilised. In Jordan, ORS was administered much more regularly than in Libya and rehydration of patients during hospitalisation was 58.1% (Youssef *et al.* 2000). In Saudi Arabia, mothers used ORS in greater than 40% of cases (Bani *et al.* 2002).

5.8 Age distribution and clinical features in patients with different enteric pathogens

In both studies, prevalence of acute diarrhoea in Libyan children was greatest among of patients were aged < 6 months (48.6% and 45.6%), and previous studies from other countries yielded similar results. For example, Libyan children < 6 m were more susceptible to bacterial infections because the breast-feeding percentage was very low particularly for infants aged < 4 m, whereas bottle feeding was normally adopted. These may not have been prepared under hygienic conditions e.g: hand washing, cleaning of bottles, use of sterilising tablets, or heating of water when mixing it with milk powder. In this age most of the infants receiving bottle feed further used dummies (also easily contaminated).

Rotavirus incidence was higher in both studies compared with other pathogens in children aged from 6 to 12 months (45.4% and 53.1%). In Venezuela, the prevalence

is similar to our study for detection of rotavirus in children aged one year at 46.3% (Salinas *et al.* 2004).

The incidence of rotavirus infection is lower in infants < 6 months because the factors affecting spread of rotavirus infection by environmental factors is not similar to bacterial spread, rotavirus spread mainly occurs in communities by close contact.

Parasite prevalence was greater in both studies compared with other pathogens in children aged > 12 months (57.1% and 48.4%). The prevalence of parasites in these studies increased with the age of the children because they had more contact with the community and began to eat and drink individually. The results of both studies were similar to studies performed in other countries.

Fever was the most common clinical feature of acute diarrhoea associated with rotavirus infection of patients (81.8%) of the first study and (77.7%) of the second. Bacterial infection was the second pathogens where fever was associated with acute diarrhoeal infections in the two studies. Parasite infections were lower than rotavirus and bacteria associated with acute diarrhoeal infection.

Vomiting was the second clinical feature of rotavirus infection associated with acute diarrhoea in both studies. In the first study, vomiting was reported in 72.7% of cases and 75.5% of the second. Other studies proved to be quite dissimilar to our studies. In Denmark, for instance vomiting was the most clinical feature associated with acute diarrhoea of children and was very high at 93% (Olesen *et al.* 2005).

Dehydration is the most significant clinical feature needing careful examination of the child to detect the type and degree of dehydration prior to starting treatment. In our studies mild dehydration was noted in patients with parasite infections (100%) except *Ent. histolytica* at 50% among patients with acute diarrhoea. Parasite infections, loose mucus of stool consistency was more common than watery stool and vomiting additionally was lower than rotavirus and bacterial infections. Moderate dehydration

was more common in *Campylobacter* infection in the first study and *Proteus* in the second. Severe dehydration was more common in rotavirus infection in both studies. Abdominal cramps was the most common in *Cryptosporidium* 2/3 (66.7) of patients and in 3/6 (50%) of patients with *Campylobacter* infection in the first study. In the second study, abdominal cramps were again reported as a more common clinical feature in patients with *Cryptosporidium* infection in 8/13 (61.5%) and *Campylobacter* in 9/16 (56.3%) patients. Other studies reported *Cryptosporidium* as the leading clinical feature associated with acute diarrhoea in children.

Watery stools were commonly noted in rotavirus infection associated with acute diarrhoea in both studies at 72.7% and 75.5% respectively. Other studies provided similar results. In Vietnam, watery stools was high in 81.1% of patients with rotavirus infection associated with acute diarrhoea (Vu Nguyen *et al*. 2004). In Jordan, watery stools of patients with rotavirus infection was high in 96.2% of patients with acute diarrhoea (Youssef *et al*. 2000). Mucus present in the stools of patients with acute diarrhoea was common in patients with *Cryptosporidium* and *Campylobacter* infections in almost 70% of cases in both studies.

Stool mixed with blood was common in *Campylobacter* infection in our studies in 33.3% and 31.3% of patients respectively.

The mean frequency of motion per day was greatest in patients with rotavirus infection in both studies (7.3 and 7.9 respectively). Rotavirus was the most common pathogens causing watery diarrhoea in our studies. The episodes of stool per day will be more frequent. Many other studies were similar to ours. In Vietnam, patients with acute diarrhoea caused by rotavirus infection, watery stool was in 81.1% of patients and the mean motion per day was 7.78. (Vu Nguyen *et al*. 2004).

The mean duration of diarrhoea/days was longest in patients with *Cryptosporidium* infection which caused acute diarrhoea in both studies (7.2 and 7.4 respectively).

Cryptosporidium was the most likely cause of prolonged duration of acute diarrhoea and was responsible for alteration to chronic diarrhoea e.g. in Bangladesh the mean duration of *Cryptosporidium* infection was 9 days. This study involved 48 patients with acute diarrhoea, 6 *Cryptosporidium*-infected cases who presented with diarrhoea and continued to suffer from it during the follow-up period after 14 days when it became persistent diarrhoea (Khan *et al*. 2004). My study protocols did not involve follow-up of cases of diarrhoea and patients with persistent diarrhoea were not examined further.

5.9 CONCLUSIONS

From the results of both studies and from discussions we can conclude:

Aetiological agents were detected in 66/118 (55.9%) of diarrhoeal patients in the first study and in 276/405 (68.1%) of the second. Bacteria were the most common agents detected in 43.3% of patients in the first study, whereas rotavirus was the most common agent identified in 30.4% of patients in the second study. Parasites were detected in 8.4% of patients and 3.4% of controls in the first study and this increased to 19.3% of patients and 4.7% of controls in the second. These findings indicate that rotavirus GE remains a common disease in hospitalized Libyan children and causes a substantial burden of disease.

API 20E and API Campy were utilised to identify bacterial isolates in the second study and concluded that here and the number of confirmed bacterial causes decreased from 142 (35.1%) to 112 (27.7%).

Rotavirus peaked during the winter season (cold months) while bacteria and parasites peaked during the summer and at the beginning of the autumn (hot months).

PCR technique was utilised for the first time in identification of Campylobacter in Libya, where *Campylobacter* spp. were identified (*Campylobacter jejuni* and

Campylobacter coli) as the same as detected by API Campy technique and the number of cases involved 24.

Libyan children below six months of age were the most susceptible to acute diarrhoea requiring admission to hospital. This was observed in 55 (46.6%) children of the first study and 175 (43.2%) of the second. The highest prevalence of acute diarrhoea was in children aged one year where the number was 94 (78.8%) of the first study and 284 (70.1%) of the second.

Exclusive breast feeding among Libyan children was of a very low percentage, only 4.2% of patients and 15.3% of controls in the first study and 11.4% of patients and 24.0% of controls in the second. Both studies illustrated that most children who received breast feeding were older than 6 months. Bottle feeding represented a high percentage of patients fed in the two studies. In the first study, there were 62.7% and in the second there were 53.8%.

The use of dummies among Libyan infants was quite considerable. In the first study this was 57.6% of patients and in the second study 36.3%. However breast feeding slightly increased and bottle feeding and dummy usage decreased in the second study. Nevertheless Libyan infants remained susceptible to acute diarrhoea. The risk of contracting diarrhoea in Libyan children using dummies was about 3 times greater than among non-users in the first study and about 2 times greater in the second study. This means that when the number of children who used dummies in the second study decreased the risk also decreased.

Watery stool was the most common stool consistency of diarrhoeal patients in both studies. This involved 58.5% of the first study and 56.6% of the second.

Fever was the most clinical feature associated with acute diarrhoea in either study. In the first study, the number of patients who had fever was 86 (72.9%) and 290 (71.6%) of the second study.

Moderate dehydration was the most common degree of dehydration. The number of patients who had this level at admission was 61 (51.7%) in the first study and 225 (55.5%) in the second. Isotonic dehydration was the most common type of dehydration across the two studies. The number of patients was 90 (76.3%) for the first study and 307 (75.8%) for the second.

In Libya, oral rehydration therapy is still not extensively used. From the history and medical records of patients, ORT was adopted for their rehydration and was at a very low percentage. In the first study this was 8.5% and 13.8% in the second.

Breast feeding, use of dummies and bottle feeding are the most common factors associated with the occurrence of diarrhoea in Libyan children (especially infants).

5.10 Recommendation and future studies

From the results of both studies and discussion, recommendations will be classified to three levels:

First level: health authority:

1. Support and activate Child Health Care programmes, especially Control Diarrhoeal Disease (CDD) programme.

2. Health education via mass media e.g. Radio, TV and directly to high school students, particularly girls' colleges.

3. Short courses for health personnel: health workers, maternity and child health care and health visitors for training in CDD programme, where children first seek medical assistance in health centers.

4. Promotion of National Surveillance Programme not solely for diarrhoeal diseases but additionally for other children's diseases.

5. Support doctors and microbiologist to perform studies and researches for diarrhoeal diseases by modern diagnostic laboratories and budget to allow

continuation of further studies and research.

6. Enhancing the availability and accessibility of ORS with greater health education regarding its use.

7. Observation and of sources of water supply by periodical analysis both chemical and bacteriological to prevent contamination of water that can lead to diarrhoea and other diseases.

8. Introduce rotavirus vaccine through expanding vaccination programme after performing epidemiological studies on rotaviruses by serological and genetic surveys to detect rotavirus strains within Libya.

Second level: health personnel

1. Promotion by health education of mothers by messages to be undertaken to correct faulty maternal practices during diarrhoeal episodes in their children, especially for feeding of children and to encourage and promote breast feeding.

2. To use ORT for treatment of dehydration according to WHO recommendations and IVT to be restricted to cases of severe dehydration or vomiting alone.

3. Hospital admission restricted solely to severe cases or for a child who has serious vomiting to prevent unnecessary hospitalisation and to decrease cross-infections.

4. Restriction of overuse of antibiotics and their use only in selected cases (*Shigella)* or other bacterial or parasite causing bloody diarrhoea following stool culture or stool analysis to avoid antibiotic resistance.

Third level: mothers

1. Advice to mothers to breast feed younger babies, especially infants for at least 6 months. There are numerous studies demonstrating that exclusively breast feeding will protect children from diarrhoea when compared to children who were received only

bottle fed. Mothers need to be advised to continue breast feeding and to delay weaning until two years which is better for the child's health.

2. Early referral for medical attention if the child has frequent diarrhoea (more than 8/day) or suffers bloody diarrhoea or severe vomiting.

3. Women who have children must have ORS sockets in their homes and should know how to mix and administer them to prevent dehydration.

4. Infants should not be in contact with any person who has diarrhoea because the most acute diarrhoea is caused by enterovirouses and transmission from person to person by contact.

5. If there is contraindication (medical reason) for breast feeding, the bottle feeding must be prepared under general health precautions with the use of a safe water supply, very clean or sterilised bottle, hand washing by soap prior to preparing the feed to minimise infection.

Further diarrhea studies are required

In Libya, study and research on diarrhoea is still scant and faces many problems.

1. No cooperation occurs between clinician and microbiologist to work to gather data for performing studies and researches not just for diarrhoeal disease but for all diseases.

2. There is no budget for study and research and much of the available budget is spent upon treatment for diseases.

3. Many laboratories in Libya do not function well because there is a lack of adequate diagnostic laboratory tools (aquipments and materials) and professional technicians are required for repair and maintenance of equipment and quality controls.

4. Training of microbiologists and technicians in the proper use of modern diagnostic techniques and use of highly advanced equipment is required.

5. A Communicable Disease Surveillance Centre needs to be established.

Future studies

1. Wide studies in the community and a clinical base for detection of incidence and prevalence rates for the pathogens causing this need to be implemented. Acute diarrhoea in Libyan children especially the pathogens are not detected by the sensitive and modern diagnostic techniques obtain accurate results.

2. Performing studies for rotavirus to detect serotype and genotype before intrducing rotavirus vaccine.

3. New studies to detect morbidity and mortality related to acute diarrhoea in children because the current figures pertain to 1988.

4. Performing studies to detect the accuracy of incidence of hypertonic dehydration.

5. To detect complications of acute diarrhoea by the follow-up of children who have acute diarrhoea, especially those with serious complications e.g.; Guillan-Barré Syndrome, Haemolytic Uraemic Syndrome, and Disseminated Intravascular Coagulopathy.

References & Appendixs

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Appendix 1

Case sheet

Case number……

Date of admission……..

Name of patient……..

Address……

Age…….. Sex…….

Mother education ……Family size ……

Type of feeding: Breast……. Bottle…… Mixed……..Solid

Use of teat ……. Type of water supply……Animal contact……

No. of loose motion/day. ……Duration…….

Stool appearance: Loose……..Watery……..Mucous……Bloody……

Fever……Grade…….pattern……duration……..

Vomiting ……Frequency……Duration…….

Abdominal pain or crumb……

Dehydration……Type: Isotonic……Hypotonic……Hypertonic …….

Degree dehydration……. Mild……. Moderate……Severe……..

Associated infection……Chest.infection……Meningitis……..Urinary tract infection……. Other infection……

Investigation: Urea……Na……K…….. Hb……

Treatment: Oral rehydration therapy……IV drip Therapy……. ...Both……

Body weight: On admission……On discharge…….

Date of discharge……..Duration of stay……

Comments……

Appendix 2

Laboratory results

Case number ……. Date……

Name ……..

Age…….

Sex……..

Department (Unit)…….

Microscopic examination of stool:

WBC…….RBC…….Others…….

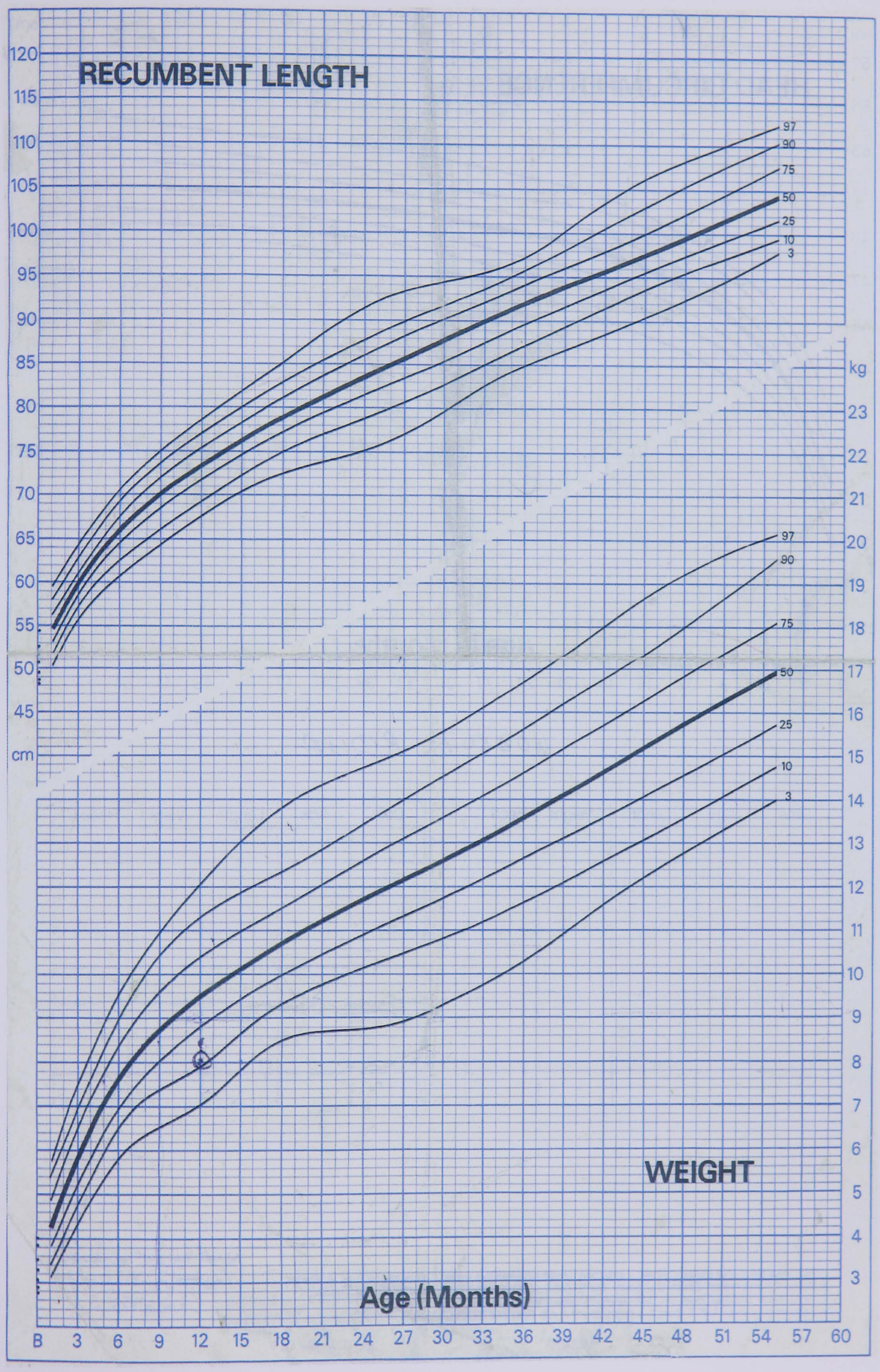
Ova…… Cyst……parasite…….

Type of bacterial isolation…….

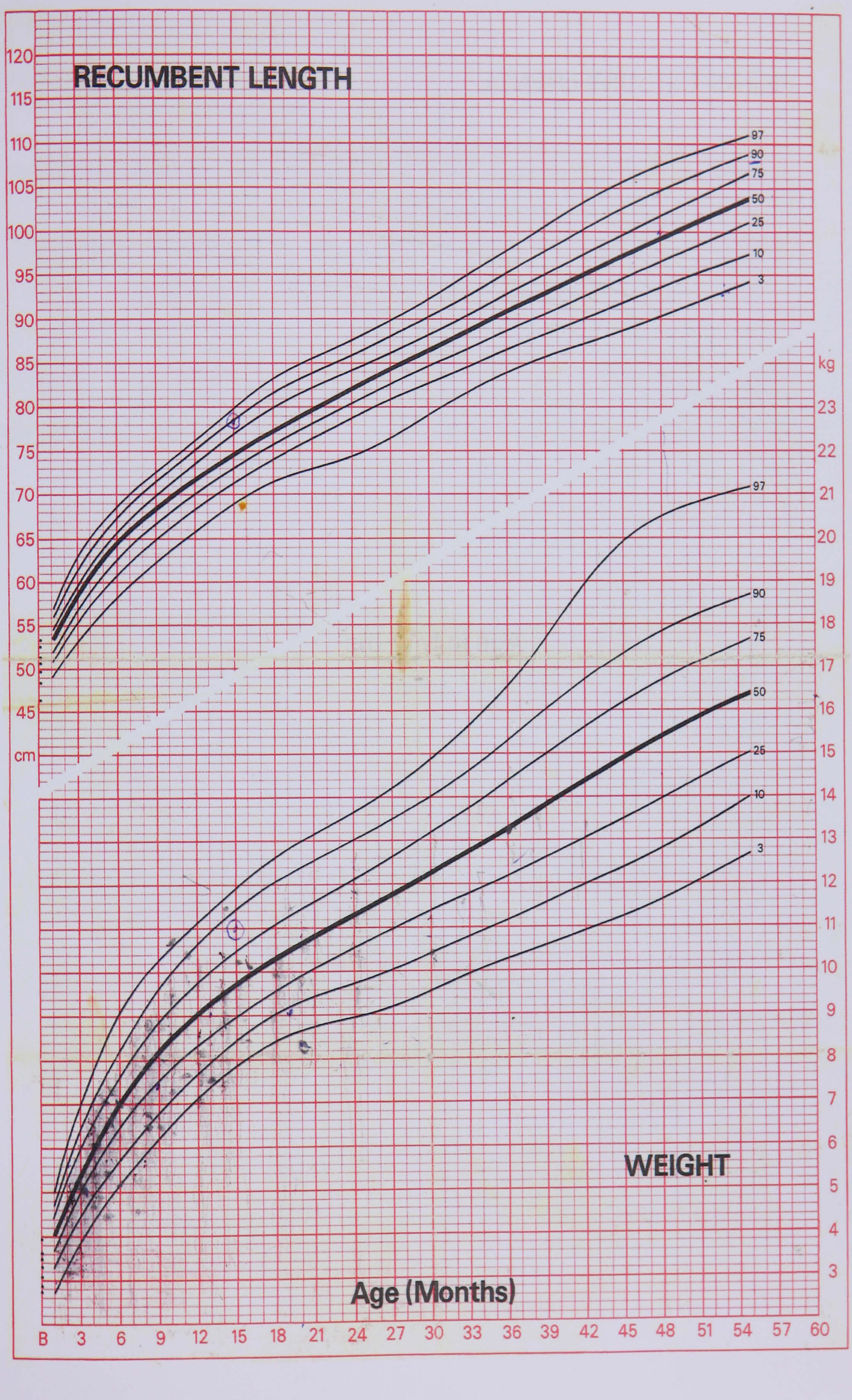
Rotavirus Assay……..Result of test……

Appendix 3

Standard charter percentile of Libyan children (male)



Standard charter percentile of Libyan children (female)



Appendix 4

Univariate analysis of first study

Fever and Stool Duration

			Stool Duration				
			1 day	2 - 3	4 - 6	>=7 days	Total
Fever	No	Count	17	22	8	6	53
		%	53.1%	39.3%	34.8%	85.7%	44.9%
	Yes	Count	15	34	15	1	65
		%	46.9%	60.7%	65.2%	14.3%	55.1%
Total		Count	32	56	23	7	118
		%	100%	100%	100%	100.0%	100%

Fever and Pathogen

			Pathogen			
			Bacteria	Rotavirus	Parasite	Total
Fever	No	Count	17	6	3	26
		%	45.9%	54.5%	60.0%	49.1%
	Yes	Count	20	5	2	27
		%	54.1%	45.5%	40.0%	50.9%
Total		Count	37	11	5	53
		%	100.0%	100.0%	100.0%	100%

Fever and Feeding type

			Feeding type				
			Breast	Bottle	Mixed	solid	Total
Fever	No	Count	2	35	12	4	53
		%	40.0%	47.9%	42.9%	33.3%	44.9%
	Yes	Count	3	38	16	8	65
		%	60.0%	52.1%	57.1%	66.7%	55.1%
Total		Count	5	73	28	12	118
		%	100.0%	100%	100%	100%	100%

Fever and Vomiting

			Vomiting		
			No	Yes	Total
Fever	No	Count	29	24	53
		%	50.9%	39.3%	44.9%
	Yes	Count	28	37	65
		%	49.1%	60.7%	55.1%
Total		Count	57	61	118
		%	100%	100%	100%

Fever and age

			Age			
			Patients aged < 6 m	Patients aged 6 - 2 m	Patients aged > 12 m	Total
Fever	No	Count	21	20	12	53
		%	44.7%	46.5%	42.9%	44.9%
	Yes	Count	26	23	16	65
		%	55.3%	53.5%	57.1%	55.1%
Total		Count	47	43	28	118
		%	100.0%	100.0%	100.0%	100%

Fever and Dummies

			Dummies		
			No	Yes	Total
Fever	No	Count	22	31	53
		%	44.0%	45.6%	44.9%
	Yes	Count	28	37	65
		%	56.0%	54.4%	55.1%
Total		Count	50	68	118
		%	100%	100%	100%

Fever and Number of motion motion/day

			Number of motion/day			
			4 - 6	7 - 9	>=10	Total
Fever	No	Count	31	17	5	53
		%	50.8%	41.5%	33.3%	45.3%
	Yes	Count	30	24	10	64
		%	49.2%	58.5%	66.7%	54.7%
Total		Count	61	41	15	117
		%	100.0%	100.0%	100.0%	100%

Multivariate analysis of first study

Logistic regression summary

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
	Age	1.104	0.068	0.993	1.228
Stool duration	1 day (baseline)	1		1	1
	2 - 3	7.195	0.116	0.614	84.343
	4 - 6	12.693	0.041	1.115	144.503
	>=7 days	14.757	0.035	1.206	180.496
Vomiting	Vomit No (baseline)	1		1	1
	vomit Yes	1.502	0.325	0.669	3.373
Motion/ day	4 - 6 (baseline)	1.00		1.00	1.00
	7 - 9	0.623	0.475	0.170	2.284
	>=10	0.850	0.814	0.219	3.297
Type of feeding	Breast (baseline)	1		1	1
	Bottle	6.579	0.282	0.213	203.463
	mixed	5.269	0.230	0.349	79.582
	solid	3.232	0.306	0.341	30.604

Univariate analysis of Second study

Fever and Stool Duration

			Stool Duration				
			1 day	2 - 3	4 - 6	>=7 days	Total
Fever	No	Count	63	62	20	15	160
		%	55.3%	32.6%	27.0%	55.6%	39.5%
	Yes	Count	51	128	54	12	245
		%	44.7%	67.4%	73.0%	44.4%	60.5%
Total		Count	114	190	74	27	405
		%	100%	100%	100%	100.0%	100%

Fever and Number of motion/day

			Number of motion motion/day			
			4 - 6	7 - 9	>=10	Total
Fever	No	Count	81	67	12	160
		%	40.1%	42.1%	29.3%	39.8%
	Yes	Count	121	92	29	242
		%	59.9%	57.9%	70.7%	60.2%
Total		Count	202	159	41	402
		%	100%	100%	100%	100%

Fever and Pathogen

			Pathogen			
			Bacteria	Rotavirus	Parasite	Total
Fever	No	Count	51	19	7	77
		%	39.8%	48.7%	63.6%	43.3%
	Yes	Count	77	20	4	101
		%	60.2%	51.3%	36.4%	56.7%
Total		Count	128	39	11	178
		%	100.0%	100.0%	100.0%	100%

Fever and feeding type

			Feeding type				
			Breast	Bottle	Mixed	Solid	Total
Fever	No	Count	6	100	32	22	160
		%	42.9%	45.5%	36.4%	26.5%	39.5%
	Yes	Count	8	120	56	61	245
		%	57.1%	54.5%	63.6%	73.5%	60.5%
Total		Count	14	220	88	83	405
		%	100.0%	100%	100%	100%	100%

Fever and Vomiting

			Vomiting		
			No	Yes	Total
Fever	No	Count	85	75	160
		%	43.1%	36.1%	39.5%
	Yes	Count	112	133	245
		%	56.9%	63.9%	60.5%
Total		Count	197	208	405
		%	100%	100%	100%

Fever and age

			Age			
			Patients aged < 6 m	Patients aged 6 - 2 m	Patients aged > 12 m	Total
Fever	No	Count	68	56	36	160
		%	43.0%	45.5%	29.0%	39.5%
	Yes	Count	90	67	88	245
		%	57.0%	54.5%	71.0%	60.5%
Total		Count	158	123	124	405
		%	100.0%	100.0%	100.0%	100%

Fever * Dummies Crosstabulation

			Dummies		
			No	Yes	Total
Fever	No	Count	69	91	160
		%	35.8%	42.9%	39.5%
	Yes	Count	124	121	245
		%	64.2%	57.1%	60.5%
Total		Count	193	212	405
		%	100%	100%	100%

Multivariate analysis of second study

Logistic regression summary

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
Age		1.137	0.000	1.076	1.201
Stool duration	1 day (baseline)	1		1	1
	2 – 3	2.485	0.105	0.827	7.466
	4 – 6	7.039	0.000	2.373	20.881
	>=7 days	8.877	0.000	2.790	28.251
Vomiting	Vomit No (baseline)	1		1	1
	vomit Yes	1.657	0.038	1.028	2.672
Motion/ day	4 - 6 (baseline)	1		1	1
	7 – 9	1.083	0.849	0.477	2.458
	>=10	0.873	0.746	0.385	1.980
Type of feeding	Breast (baseline)	1		1	1
	Bottle	11.129	0.013	1.677	73.864
	Mixed	10.095	0.002	2.399	42.486
	Solid	6.409	0.002	1.939	21.181

Univariate analysis of first study

Vomiting and Stool Duration

			Stool Duration				
			1 day	2 - 3	4 - 6	>=7 days	Total
Vomiting	No	Count	16	28	8	5	57
		%	50.0%	50.0%	34.8%	71.4%	48.3%
	Yes	Count	16	28	15	2	61
		%	50.0%	50.0%	65.2%	28.6%	51.7%
Total		Count	32	56	23	7	118
		%	100%	100%	100%	100.0%	100%

Vomiting and Number of motion motion/day

			Number of motion/day			
			4 - 6	7 - 9	>=10	Total
Vomiting	No	Count	31	20	6	57
		%	50.8%	48.8%	40.0%	48.7%
	Yes	Count	30	21	9	60
		%	49.2%	51.2%	60.0%	51.3%
Total		Count	61	41	15	117
		%	100%	100%	100%	100%

Vomiting and Pathogen

			Pathogen			
			Bacteria	Rotavirus	Parasite	Total
Vomiting	No	Count	19	3	3	25
		%	51.4%	27.3%	60.0%	47.2%
	Yes	Count	18	8	2	28
		%	48.6%	72.7%	40.0%	52.8%
Total		Count	37	11	5	53
		%	100.0%	100.0%	100.0%	100%

Multivariate analysis of first study

Logistic regression summary

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
Age		0.942	0.160	0.868	1.024
Stool duration	1 day (baseline)	1		1	1
	2 – 3	1.905	0.501	0.292	12.446
	4 – 6	1.786	0.541	0.278	11.463
	>=7 days	3.499	0.208	0.497	24.606
Fever	No (baseline)	1		1	1
	Yes	1.438	0.368	0.652	3.173
Motion/ day	4 - 6 (baseline)	1		1	1
	7 - 9	0.527	0.308	0.153	1.808
	>=10	0.549	0.362	0.151	1.992
Type of feeding	Breast (baseline)	1		1	1
	Bottle	0.048	0.075	0.002	1.357
	mixed	0.088	0.072	0.006	1.247
	solid	0.158	0.115	0.016	1.567

Univariate analysis of second study

Vomiting and Number of motion/day

			Number of motion motion/day			
			4 - 6	7 - 9	>=10	Total
Vomiting	No	Count	102	82	13	197
		%	50.5%	51.6%	31.7%	49.0%
	Yes	Count	100	77	28	205
		%	49.5%	48.4%	68.3%	51.0%
Total		Count	202	159	41	402
		%	100%	100%	100%	100%

Vomiting and Pathogen

			Pathogen			
			Bacteria	Rotavirus	Parasite	Total
Vomiting	No	Count	65	9	10	84
		%	50.8%	23.1%	90.9%	47.2%
	Yes	Count	63	30	1	94
		%	49.2%	76.9%	9.1%	52.8%
Total		Count	128	39	11	178
		%	100.0%	100.0%	100.0%	100%

Vomiting and Feeding type

			Feeding type				
			Breast	Bottle	Mixed	Solid	Total
Vomiting	No	Count	9	110	40	38	197
		%	64.3%	50.0%	45.5%	45.8%	48.6%
	Yes	Count	5	110	48	45	208
		%	35.7%	50.0%	54.5%	54.2%	51.4%
Total		Count	14	220	88	83	405
		%	100.0%	100%	100%	100%	100%

Vomiting and age

			age			
			Patients aged < 6 m	Patients aged 6 - 2 m	Patients aged > 12 m	Total
Vomiting	No	Count	70	69	58	197
		%	44.3%	56.1%	46.8%	48.6%
	Yes	Count	88	54	66	208
		%	55.7%	43.9%	53.2%	51.4%
Total		Count	158	123	124	405
		%	100.0%	100.0%	100.0%	100%

Multivariate análisis of second study

Logistic regression summary

		Odds ratio	p-value	95.0% C.I.for OR	
				Lower	Upper
	Age	.838	.000	.764	.921
Stool duration	1 day (baseline)	1		1	1
	2 – 3	5.204	.038	1.091	24.819
	4 – 6	.910	.906	.191	4.341
	>=7 days	1.744	.512	.330	9.207
Fever	No (baseline)	.854	.703	.379	1.921
	Yes				
Motion	4 - 6 (baseline)	1		1	1
	7 – 9	.290	.068	.077	1.096
	>=10	.261	.036	.075	.915
Pathogens	Bacteria (baseline)	1		1	1
	Rotavirus	2.679	.390	.284	25.313
	Parasite	10.913	.055	.948	125.610
Feeding type	Breast (baseline)	1		1	1
	Bottle	.002	.000	.000	.042
	Mixed	.004	.000	.000	.054
	Solid	.009	.000	.001	.086