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Detection of *Shigella* and enteroinvasive *Escherichia coli*, the causative agents of bacillary dysentery from environmental and clinical samples

Ph.D. DISSERTATION

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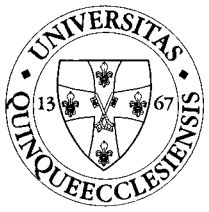

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To my brother,
my parents and
my grandparents

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This PhD dissertation is based on the following original communication (I-III.) The papers are referred to in the text by their Roman numerals. In addition, some previously unpublished data are also presented.

A. Published articles

I. Szakál D., I. Gadó, T. Pál (2001): A colony blot immunoassay to detect enteroinvasive *Escherichia coli* and *Shigella* in water samples. Journal of Applied Microbiology 90 (2): 229-236

II. Szakál D., Gy. Schneider, T. Pál (2003): A colony blot immune assay to identify enteroinvasive *Escherichia coli* and *Shigella* in stool samples. Diagnostic Microbiology and Infectious Disease 45(3): 165-71

III. Szakál D., T. Pál (2003): Comparison of media for the selective culture of enteroinvasive *Escherichia coli*. European Journal of Clinical Microbiology and Infectious Diseases 22:235-241.

B. Other communications (presentations, published abstracts)

1. Pál, T., R. Dhar, D. Szakál: An IpaC - specific colony blot immune assay to detect enteroinvasive *Escherichia coli* in contaminated faecal and water samples. *95th Annual meeting of the American Society for Microbiology Washington DC. 1995, Abstract:* Q-391: 265

2. Pál, T., R. Dhar, D. Szakál: Antigen-specific assays in the diagnosis of bacillary dysentery. *Posterday 1996, Kuwait University, Faculty of Medicine, Kuwait*

3. Szakál, D., T. Pál: Colony blot immunoassay to detect *Shigella* and enteroinvasive *Escherichia coli* strain in water samples. *11th Annual meeting of the Hungarian Society for Microbiology Szekszárd, Hungary, 1997. Abstract published in Acta Microbiol Immunol Hung*. 1998. **44**: 406

4. Szakál, D., T. Pál: Comparison of a colony blot immunoassay to PCR to detect *Shigella* in water samples. *International Medical Conference for Students and Young Doctors, Lublin, Poland, 1998.*

5. Szakál, D., Gy. Schneider, T. Pál: The aetiological diagnosis of bacillary dysentery with a colony immunoblot technique. *12th Annual meeting of the Hungarian Society for Microbiology, Miskolc, Hungary 1998. Abstract published in Acta Microbiol Immunol Hung.* 1999. **46**:126

6. Szakál, D., T. Pál: The effect of selective and selective-enrichment media on the growth of enteroinvasive *Escherichia coli*. *13th Annual meeting of the Hungarian Society for Microbiology Budapest, Hungary, 1999. Abstract published in Acta Microbiol Immunol Hung.* 2000. **47**:206

7. Schneider, Gy., D. Szakál, T. Pál: Detection of enterohaemorrhagic *Escherichia coli* serogroup O157 with colony blot technique. *13th Annual meeting of the Hungarian Society for Microbiology, Budapest, Hungary, 1999. Abstract published in Acta Microbiol Immunol Hung.* 2000. **47**:213

8. Schneider, Gy., D. Szakál, T. Pál, L. Emődy: Detection of enterohaemorrhagic *Escherichia coli* serogroup O157 from water samples with the help of a colony blot method. *International School for Molecular Biology, Microbiology and Science for Peace: Application of Molecular Biology in Microbiology, Medicine and Agriculture Smolenice (Bratislava), Slovakia, 1999. Abstract published in UNESCO-HUJ Institute of Virology, Abstracts, Science for Peace, ISMBM, 1999 p. 8.*

9. Szakál, D., K. Szőke, T. Pál The colony immunoblot method adapted for detecting *Shigella* and EIEC from milk. *14th Annual meeting of the Hungarian Society for Microbiology, Keszthely, Hungary, 2000. Abstract published in Acta Microbiol Immunol Hung.* 2001. **48**(2): 273.

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AP	Alkaline Phosphatase
CFU	Colony Forming Unit
DC	Desoxycholate Citrate Agar
EIEC	Enteroinvasive *Escherichia coli*
EDTA	Ethylendiamine Tetracetic Acid
ELISA	Enzyme Linked Immune Assay
EMB	Eosin Methilene Blue Agar
FCS	Fetal Calf Serum
GNB	Gram Negative Broth
HIV	Human Immunodeficiency Virus
HUS	Haemolytic Uraemic Syndrome
IL	Interleukin
Ipa	Invasion Plasmid coded Antigen
kb	kilobase
LDC	Lysine decarboxilase
LPS	Lipopolisaccharide
LT	Heat Labile enterotoxin
mdal	megadalton
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
SF	Selenite Faecal Broth
SS	Salmonella Shigella Agar
ST	Heat Stabile enterotoxin
TMP-SMX	Trimethroprim-Sulfamethoixazole
TNF	Tumour Necrosis Factor
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VMA	Virulence Marker Antigen
XLD	Xylose Lysine Desoxycholate Agar

1 INTRODUCTION

Bacillary dysentery is a global burden, as it is an important cause of morbidity and mortality amongst children in developing world, where endemic. It is also present in the industrialised countries in the form of outbreaks. The disease is caused by *Shigella* and enteroinvasive *E. coli* (EIEC) strains by the invading and multiplying in the epithelium of the human colon, causing ulcerations, resulting in diarrhoea with blood and mucus. These strains share a similar pathomechanism and genetic background, based on a virulence plasmid coding the virulence factors regulated by chromosomal genes. Occasionally, the disease can be fatal; its most severe form is caused by *S. dysenteriae* serotype 1. The bacteria have a very low infective dose, spread easily by personal direct contact through the faeco-oral route. In addition, contaminated water and food are also important transmitting routes of the disease. The emerging multiresistance to available antimicrobials, the lack of reliable vaccination, the increasing occurrence of the disease worldwide and the high incidence of the disease in populations at high risk all give strong reason to review and develop preventative measures. Besides vaccine development, one way of preventing the spread of the disease is the recognition of these pathogens as a necessary part of the maintenance and development of hygienic standards and sanitation. Therefore the detection and identification of the *Shigella* and EIEC strains from the environment, water and food, as well as from patients with dysentery and carriers, is of primary importance.

For the detection of the *Shigella* species, routinely used laboratory methods are available. These are based on culturing, biochemical and serological identification of the microbe. However, standard methods cannot differentiate EIEC from normal *E. coli* due to the lack of specific bio-, or serological markers, therefore EIEC remains undetected in most cases. Recently sophisticated molecular methods, DNA hybridisation and PCR assays have been successfully applied in its detection from etiological, water and food samples. These methods are highly specific and sensitive. They are however, due to their high cost and the requirement for highly trained staff, still unavailable in most routine laboratories. Assays based on the immunological detection have also been developed, presenting a simpler and cheaper way for smaller

laboratories with fewer staff with less equipment available, especially useful in the developing world where the disease is endemic.

In the 1980s a surface protein as described in our institute, the Virulence Marker Antigen, was shown to correlate with the virulence of these pathogens. This was later proved to be identical with the IpaC antigen coded on the virulence plasmid of virulent *Shigella* and EIEC strains. A polyclonal, later a monoclonal antibody based ELISA specific for this surface protein antigen was developed to detect *Shigella* and EIEC strains. The ELISA demonstrated high specificity and sensitivity when tested on clinical samples in several studies.

In the present study we have aimed to further develop the sensitivity of this ELISA by modifying it to a colony blot method. With the traditional ELISA only a limited number of colonies can be screened from the cultured sample. Therefore we aimed to improve the sensitivity of the immunodetection by developing a method that has the potential of directly screening significantly more colonies in the sample Additionally, we tried to apply this method to various environmental factors, like water and food, as well as clinical samples.

Samples, however, are routinely cultured to give a laboratory diagnosis. For the culture laboratories use different sets of various differentiating, mildly or highly selective and enrichment media for the isolation of enteric pathogens. However, there is no generally agreed protocol between different laboratories. The current study also aimed to investigate the behaviour of *Shigella* and EIEC strains on various selective and selective enrichment media and to determine the best choice of culturing media for culturing EIEC.

2 REVIEW OF THE LITERATURE

2.1. The significance of bacillary dysentery in the past and today

Bacillary dysentery, the invasive infection of the colon, is a disease recognised worldwide as a major burden in public health care. Caused by bacteria that invade the mucosal membrane of the intestines, the disease brings with it bloody diarrhoea that can ultimately kill the host. As it causes disease exclusively in humans, it could not have existed before and evolved together with civilization. Today dysentery is widespread in poor countries where dense populations depend on contaminated water supplies.

Already Hippocrates characterised in details a disease with bloody diarrhoea and referred to it as dysentery. Dysentery, as a disease that rears its head when people are crowded together in places with poor sanitation and food supply, nevertheless saw through the great wars and battles of history as one of the camp killers. Often called an army's "fifth column", it contributed to several historically significant epidemics recorded in connection with wars. One of the first epidemics described by Herodotus was probably an outbreak of dysentery, the Plague of Xerxes, which hit the Persian army. Charles Creighton, one of Britain's most learned medical historians wrote: "The Crusaders of the 11th - 13th centuries were not defeated so much by the scimitars of the Saracens as by the hostile bacteria of dysentery and other epidemics." "The summer of the first Crusade in 1099 was extraordinarily hot, the ill-prepared and rag-tag army of men and camp followers hampered by lack of fresh water and contaminated containers trudged along to their destiny, relieving themselves along the wayside or in the fields." During the Thirty Years War repeated epidemics struck in Germany, including typhus, plague and dysentery. In the Elizabethan period, nonexistent or poor plumbing was merely one of many sanitation factors that gave rise to the Black Death of the Middle Ages, as well as to dysentery, also directly related to human waste. Napoleon's attempt to invade Russia and the U.S. Civil War were both severely affected by shigellosis (Kiple 1993). The two World Wars and even the Gulf war were not exempt from it despite developments in medicine and warfare.

The two aetiological forms of dysentery, bacillary and amoebic, were differentiated at the end of the 20th century. The bacterium was first isolated in 1898 in Japan by Shiga from the stool of patients suffering from severe bloody diarrhoea (Shiga 1898). In 1913 Koger noted, that in refugee and war camps and in prisons bacteria were the cause of the disease, whereas in the tropical climate amoebae caused it. In 1950, at a Congress of the International Association of Microbiologists, it was suggested that the genus name of these bacteria so far referred to as dysentery bacterium or Bacillus dysentery should be *Shigella*.

According to the World Health Report estimates for 2000, despite recent improvements in medical-biology and epidemiology, from a total of 55.6 million deaths, over 17 million were ascribable to infectious and parasitic diseases. Diarrhoeal diseases, resulting in 2.1 million deaths per annum, are between the first three leading killers among infectious diseases preceded by lower respiratory infections (3.8 million) and HIV/AIDS (2.9 million), and followed by tuberculosis (1.6 million) and malaria (1 million) (WHO 2001). Data on morbidity is even more threatening, as diarrhoeal diseases of different aetiology were the leaders with 4 billion episodes in 1995, and the fifth among the leading causes of DALYs (Disability-Adjusted Life Year) with 62 million people in the year 2000 (WHO 1996, WHO 2001). Today in the tropical climate, especially in the overcrowded areas of the developing regions, bacillary dysentery has a great impact as being one of the most important forms of these infections. The annual number of estimated episodes throughout the world is 164.7 million, of which 163.2 million were in developing countries and 1.5 million in industrialised countries. Shigellosis is responsible for 5 million hospitalisations and 650,000 deaths per year. A total of 69 % of all episodes and 61 % of all deaths involving children under 5 years of age may be attributed to it (Kotloff 1999). In Hungary there have been a few thousand cases of bacillary dysentery in the past few years (Anon 1997). The severeness of the disease, high case fatality, person-to person spread, the increasing number of infected persons worldwide, and the emergence of multiple drug resistant strains call for a common effort for the recognition and prevention of the disease.

2.2. The causative agents of bacillary dysentery, biochemical and serological characteristics

Dysentery is a clinical entity, characterized by frequent passage of bloodstained mucopurulent loose stools. Aetiologically, it is divisible into two main categories: one is of amoebic and the other is of bacillary origin. Amoebic dysentery is caused by *Entamoeba histolytica* and presents only sporadic cases. Bacillary dysentery occurrs endemically or in the form of outbreaks. The etiologic agents are members of the genus *Shigella* and enteroinvasive *Escherichia coli* (EIEC) strains, which taxonomically belong to the genuses *Escherichia* and *Shigella* within the family of *Enterobacteriaceae*.

The causative agents of bacillary dysentery are Gram-negative, facultative anaerobic, coliform bacilli. *Shigellae* are 0.5-0.7 μm, non-capsulated, non-sporeforming, non-motile (no H antigen), rod-shaped bacteria. They are nutritionally simple to culture, however they are sensitive to acidic conditions and therefore easily destroyed due to the acidosis and the metabolic products of other bacteria in stools. They are metil red positives, do not produce urease or H_2S, the indol positivity varies and are either lactose negative or late lactose fermenters. They are sensitive to heat and disinfectants, and pasteurizing destroys them. They stay viable in stools for 2-3 days, in soil for 6-10 days, dried on objects like sheets even for weeks, in water for several months and in ice for 6-8 weeks. They are more resistant to the effect of the gastric acid/juice than other bacteria, which can explain its low infectious dose.

The genus *Shigella* consists of four major serological groups A through D according to their O antigens corresponding to the four species, respectively (Hoeprich 1977). In current taxonomy, these four serological subgroups are recognised as four species within the genus *Shigella*: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. The four distinct species can be differentiated on basis of serogrouping and biochemical analysis, mainly indol positivity, glucose, lactose and especially mannitol fermenting.

Group A is represented by *Shigella dysenteriae*, causing the most serious form of bacillary dysentery. Group A is present in 1% of the isolates in Hungary. Of the 12

serotypes, the most frequent is *Shigella* dysentery serotype 2, whereas the most pathogenic strain *S. dysenteriae* serotype 1 practically never occurs here (Echeverria et al 1991, Djuretic et al 1996). *Shigella dysenteriae* 1 secretes an exotoxin (Shiga toxin) that causes haemolytic uraemic syndrome and neurological disorders. Group B consists of *Shigella flexneri*, the most common cause of shigellosis in underdeveloped countries. Within the genus a further 13 *S. flexneri* serotypes are differentiated. Group C includes *Shigella boydii*, 18 *S. boydii* serotypes are known. The members of Group C are relatively rare, approximately 0.5 % of the total isolates. Most of them belong to serotype 1 (Prats et al 1985). Group D includes the single serotype *Shigella sonnei*, the most common cause of shigellosis in developed countries (Scerpella et al 1994). In Hungary, *S. sonnei* strains are the most frequent at present, preceding the *S. flexneri* isolates which predominated a few decades ago. In developing countries both *Shigella flexneri* and *Shigella sonnei* are isolated in a great number of cases and S. *dysenteriae* serotype 1 is usually endemic (Mathan et al 1984, Merson et al 1974, Haider et al 1990).

E. coli strains of the genus Escherichia are persistent inhabitants of the normal flora of the colon of humans and warm-blooded animal species. They are small rods, often with peritrich cilia and sometimes a polysaccharide capsule. Biochemical characteristics: lactose fermentive, sensitive to bile salts, not using citrate and malonate. They have endotoxin, as other Gram-negative bacteria. The serotyping is based on their somatic (O), capsular (K) and flagellar (H) antigens. Currently the number of O antigens identified has reached 173, K antigens 103 and H antigens 56.

While most strains are harmless, some can cause severe diseases. The pathogen strains are divided into two major groups, the extraintestinal and enteral groups. The extraintestinal infections – mainly urinary tract infections (UTI), septicaemia and neonatal meningitis – are usually caused by strains normally present in the stool, e.g. the uropathogenic *E. coli* (UPEC). Some of the strains of *E. coli*, if colonised in the intestines, are able to cause gastroenteritis. There are seven classes of enterovirulent diarrheagenic *E. coli* strains divided according to their pathomechanisms (Nataro and Levine 1994, Clarke 2001).

EHEC are enterohaemorrhaegic strains, producing verocytotoxins and Shiga-like toxins, causing haemorrhagic colitis and haemolytic uraemic syndrome (HUS). Of the strains *E. coli* O157:H7 serotype is most frequent.

ETEC are enterotoxinogenic strains, a major cause of travellers' diarrhoea. By their adherence and the production of either or both a heat stable and a heat labile enterotoxin (ST and LT) they cause cholera-like diarrhoea.

EPEC are enteropathogenic strains mainly causing infantile gastroenteritis with dehydration (colidispepsy) in children under 1, by adhering to the mucosa of the small intestine and producing the characteristic "attaching and effacing" lesions in the brush border of the microvillous membrane.

EAggEC are enteroaggregative strains, showing various types of adherence patterns (localised, diffuse or aggregative) are associated with acute and persistent diarrhoea, probably as a result of their toxin production.

EIEC are enteroinvasive strains, causing dysentery by similar pathomechanisms as *Shigella*. They are also positive in the Serény keratoconjunctivitis test, and harbour the same virulence factor, a 120-140 mD invasive plasmid. EIEC strains belong to certain serogroups, of which the most frequently occurring ones are types O28, O29, O112, O124, O136, O143, O144, O147, O152, O164 and O167 (Echeverria et al 1991). Recently new serotypes (for example O121, O171, O172) were also described as invasive. Several serotypes of EIEC show O antigenic relatedness or even identity with different *Shigella* serotypes (Ewing et al 1986, Wang et al 2001).

DHEC are diarrhoea associated haemolytic *E. coli*, also known as diffuse-adherent *E. coli* or cell-detaching DHEC because of their diffuse adherence pattern to cultured epithelial cells. Their virulence is mediated by hemolysin and cytotoxic necrotising factor 1. Their aetiological role in diarrhoea has been controversial. (Clarke 2001)

CDT is a cytolethal-distending toxin-producing *E. coli.* They cause the distension and eventual disintegration of cells of certain lines. The association of CDT- *coli* with diarrhoea is also not clear (Clarke 2001).

2.3. Clinical features of bacillary dysentery

The clinical syndromes of shigellosis range from asymptomatic infection to severe bacillary dysentery. After eating contaminated food, the onset of symptoms takes place in 1-7 day*s.* Dysentery can be suspected if diarrhoea lasts for more than two days, with abdominal pain, fever and toxicaemia, sometimes vomiting, and if the stool contains blood, mucus or pus. The disease lasts 5-6 days. It is often described as having two stages: starts with a mild form of watery diarrhoea, and within 1-2 days the clinical symptoms can develop to typical dysentery.

In the early stage the first symptoms are fever and abdominal cramps, watery diarrhoea in big volume, then the fever decreases, and stools are passed less frequently. After the ingestion of the microbe, a non-invasive colonisation and cell multiplication takes place in parallel with the production of enterotoxins by the pathogenic bacteria in the small intestine. Watery diarrhoea is attributed to the enterotoxic activity of Shiga toxin. Infants and young children often have high fever (hyperpyrexia) and neurological symptoms that can be attributed to the neurotoxic activity of the toxin (Krugman et al, 1977, Singh and Rodriguez 2002)

In the second stage after 1-2 days of incubation, the classical characteristics of bacillary dysentery develop. Abdominal pain, cramps and tenesmus, ineffectual and painful straining, accompanied with frequent passage of small volume bloody stools and mucoid discharge are typical. At this stage the microbes adhere to the tissue of the large intestine and invade it. The cytotoxic activity of Shiga toxin increases the severity of the disease. All patients with shigellosis have abdominal pain and fever; half of them discharge pus and forty percent pass blood in the stool. Sometimes diarrhoea dominates the entire duration of the disease (DuPont et al 1988, Munoz et al 1995). In case of S dysentery type 1, other complications besides prolonged diarrhoea may include rectal prolapse, seizures, leukemoid reaction, haemolytic uraemic syndrome (HUS) a

syndrome of haemolytic anaemia, Reiter's syndrome, sepsis and toxic megacolon. Without prompt effective treatment the case-fatality rate is 1% to 10% (Ashkenazi and Cleary 1992, WHO 1995)

Rectoscopic examinations reveal abnormally loose hyperaemic mucosal and increased mucus production. According to laboratory examinations bacteria are present in huge number in the acute phase of the disease. Bacillary dysentery results in histopathologic changes of the colon by creating superficial ulcerations of the large intestine due to the multiplication in the submucosa or in the lamina propria. These inflammatory, necrotic ulcers are situated perpendicularly for the longitudinal centreline of the bowel, in a circular pattern, covered with pus and fibrin. Lesions are most frequent in the rectum and ampullae (sigmoid portions) and their number decreases in the proximal direction. The colon can show gross colitis, mucosal erythema and oedema, with erythematous areas and luminal exudate on the mucous membrane. Ulcerations do not go beyond the lamina propria, however severe lesions and deep ulcers rarely with a pseudomembrane or pseudopolyposis occur. Microscopically the lamina propria shows typical inflammation with intensive cellular infiltration into the epithelium predominantly consisting of neutrophyls (Mandel et al 1995, Kertai 1989).

The stool contains microscopic or macroscopic blood or mucus. In most cases the presence of a great number of polymorphonucleic leukocytes can be observed in the stained stool. Especially in children with malnutrition, the disease can result in severe dehydration, loss of protein and in entheropathia. The occurrence of bacteraemia is rare (De Mol 1981, Chagla 1985, Trevett 1993). Convalescence from shigellosis is spontaneous in healthy persons, however it can be lethal in persons with a weak immune system (Levine et al 1973).

Dysentery caused by EIEC usually occurs within 12 to 72 hours following the ingestion of contaminated food. The illness is characterized by abdominal cramps, diarrhoea, vomiting, fever, chills, and a generalized malaise (Taylor et al 1988). Dysentery caused by this organism is generally self-limiting with no known complications. A common sequel associated with EIEC infection, especially in paediatric cases, is HUS,

thrombocytopenia, and acute renal failure, with pathological finding of thrombotic microangiopathy in kidney and renal cortical necrosis (McCarthy et al 2001).

2.4. Immunity

In endemic areas bacillary dysentery is primarily a children's disease, however this age specificity cannot be observed in non-endemic areas. Supplemented this with the results of sero-epidemiologic studies – as by the age of 5- 10 years people living in endemic areas have a high antibody titre against *Shigella* specific proteins – it might be possible that reoccurring infections produce a certain level of immunity. This, however, is weak and relative and, though the data is self-contradictory, serotype specific.

The protective immune mechanisms are unknown, optimal protection may depend on a combination of mucosal and systemic immunity (Sansonetti et al 1996, Echeverria et al 1988). Studies show that antibodies against proteins coded by the invasion plasmid can be found in human milk and colostrum, giving a protective effect to breastfed children (Hayani et al 1991, Cam et al 1992, Pál and Brasch 1987). *Shigella* infection induces strong anti LPS antibody responses in the serum and in secretions, although LPS-specific immunity is incomplete and of limited duration (DuPont et al 1972). Antibody responses to Ipa in adults and well-nourished children were higher than in malnourished ones (Oberhelman et al 1991). Presumably cell mediated immunity has an important role as shigellosis is caused by facultative intracellular parasites (Morgan et al 1984).

2.5. Virulence and pathogenesis of *Shigella* and EIEC strains

Shigella species and enteroinvasive strains of *E. coli* cause disease by invading and multiplying in the colonic epithelium and destroying the brush border creating superficial ulceration of the large intestine. The key step of pathogenesis is the invasion of these cells and avoiding the host's defence mechanisms. Today the steps of invasion can be examined with sophisticated techniques on cellular, molecular and genetic bases. These steps include: 1, the penetration of the epithelial cells of the colon by *Shigellae*; 2, the multiplication within theses cells; 3, intracellular and intercellular spread of the bacteria and finally 4, the destruction of the host cells.

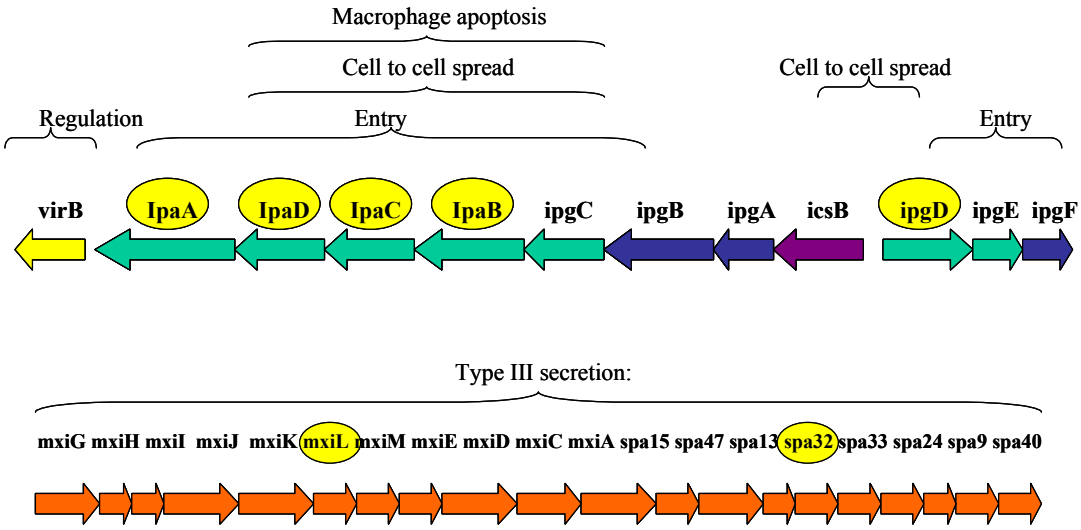
The invasiveness of *Shigellae* involves the attachment (adherence) and internalisation that is controlled by a large 220 kb extra-chromosomal DNA element, the invasion plasmid (Ip) (Sansonetti et al 1982, 1983a, Silva et al 1982, Hale et al 1983, 1985). This is based upon the following observations: 1, the loss of the plasmid leads to the loss of virulence; 2, inserting the plasmid into cells that originally did not contain it leads to the expression of the invasive phenotype (Sansonetti et al 1981).

The molecular weight of the invasion plasmid harboured by all virulent *Shigella* and EIEC strains is 140 Mdal with the exception of *S. sonnei*. The 120 Mdal plasmid of *S. sonnei* harbours the genes necessary for the synthesis of the lypopolysacharide (LPS) antigen, which can be found on the other *Shigella* and EIEC strains on the rfb locus of the chromosome. Thus, in case of *S. sonnei*, losing the plasmid results in SR transformation besides losing the invasive abilities (Sansonetti et al 1980). Although the endonuclease cleavage patterns of the invasion plasmids of different serotypes of *Shigella* species and EIEC do not show relatedness, considerable homology was observed in blotting hybridisation experiments using virulent plasmids as P-labelled probes (Sansonetti et al 1983b).

Characteristically coded by the virulence plasmid, at least three proteins mediate the entry of *Shigella* into the epithelial cells. The sequences required for invasive functions are localised within a 37-kilobase region (Maurelli et al 1985, Sansonetti 2001a). See Figure 1. One locus of this region is responsible for coding several polypeptides playing either directly or indirectly a role in invasion; the so-called invasion plasmid antigens (Ipa) designated A, B, C and D. IpaC is identical with the protein responsible for the antigen relationship between virulent enteroinvasive strains described in our institute, and called virulence marker antigen (VMA). The antigens IpaB, -C are directly responsible for invasion, and IpaD, putatively has adhesive function (De Geyter et al 1997, Barzu et al 1997, Menard et al 1993, 1994, Mounier et al 1997). This multi-gene virulence plasmid also harbours the genes of the so-called III-type secretion mxi/spa system functioning in the excretion of the polypeptide products of *ipa* genes (Andrews et al 1991, Venkatesan et al 1992, Sansonetti et al 2001a) and regulatory genes like virF

and virB (Adler et al 1989, Porter and Dorman 1997). The sequence analysis of the whole virulence plasmid was carried out by Venkatesan et al (2001).

Figure 1. *The invasion plasmid antigens in the virulence plasmid of Shigella and EIEC*



The sequences of the key genes are scattered on the plasmid of Shigella flexneri. A pathogenicity island of 30 kb contains genes responsible for the entry, cell to cell spread and macrophage apoptosis. It contains the invasion genes coding the plasmid antigens Ipa A, B, C and D and the type-III secretion system. The invasion plasmid antigens, ipaA, C, B and D are the key for the cell entry. They are located in a 30 kb pathogenicity island, together with the type three secretion system. After Sansonetti P. FEMS Microbiology Reviews. 2001 (25): 3-14.

The genetic relatedness of *Shigella* and EIEC strains was confirmed by a genetic study based on MLEE (multi-local enzyme electrophoresis), RAPD analysis (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) (Buysse et al 1995, Pupo et al 1997, Bando et al 1998). It was shown that EIEC strains, as well as other pathogenic *E. coli* strains are present in several clusters, scattered from each other on the dendogram showing genetic relationships. The implication is that these strains acquired the virulence factors independently, and the take up of the invasion plasmid was not clonal but lateral and happened several times during evolution. Reeves' group also analysed the genetic variation among the three main branches of *Shigella*, dating them on a molecular clock derived from the mutation rate of *E. coli*. Compared to normal *E. coli* strains, *Shigella* may replicate—and evolve—much faster because it spends less time outside its host. Putatively, *Shigella* arose as harmless *E. coli* strains acquired genetic material that enabled them to invade intestinal cells (Pupo 2000). Acquisition of invasiveness in the creation of pathogenic bacteria

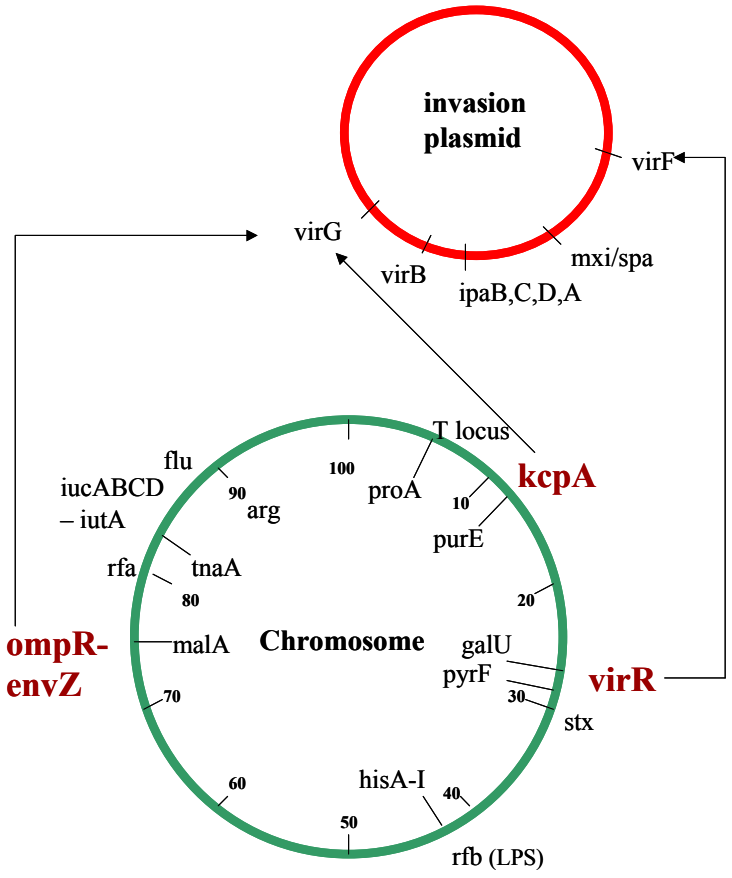
could be preceded by the formation of back holes, the deletion of chromosomal genes detrimental to pathogenic characteristics. For example the deletion of the region cadA coding the lysine decarboxylase, or a region called kcpA earlier in the vicinity of purE coding for a surface protease (Maurelli et al 1998).

The invasive ability of the *Shigella* and EIEC strains, the expression of the virulence genes is regulated by environmental conditions. The two most important environmental signals informing the microbe of the environment in the host system is temperature, and osmolarity sensed via the *ompR-envZ* system regularting the vir genes (Bernardini et al 1990, 1993). Under 37^{o}C invasivity decreases, at 30^{o}C it is fully deregulated. According to experiments, *S. flexneri* 2a, a *S. sonnei* and S. *dysenteriae* fully expressed their virulence at 37 ºC; they were invasive in Henle cells and positive in Serény test. At 35 ºC they showed a similar phenotype, whereas at 33 ºC only partial, and after incubation at 30 ºC Henle cell invasion or keratoconjunctivitis could not be observed. However, the invasive ability was restored if the bacteria grown previously at 30 ºC were further incubated for two hours at 37 ºC (Maurelli et al 1984a). VirR, a chromosome encoded central modulator regulates the virulence genes organised in regulons on the pINV according to the temperature. It represses their expression at 30 ºC or at low osmolarity (Maurelli and Sansonetti 1988, Hromockyj et al 1992). The primary event following the upshift of the temperature to 37 ºC is the synthesis of the pINV-encoded protein VirF, which triggers a regulatory cascade by directly activating *VirB* and *VirG* (Falconi et al 1998). On the other hand, *virB,* as an intermediary regulatory gene between *virF* and the ipa operon, directly activates the transcription of *ipaABCD* and the *mxi/spa* genes, which are not dependent on temperature (Tobe et al 1991). The strains' ability to bind to Congo Red is also temperature dependent - and corresponds to the presence of the invasive plasmid, haemolytic activity and thus invasiveness (Maurelli et al 1984b, Sasakawa et al 1986, Sakai et al 1986, Sharma et al 2001). Such dependence on temperature is characteristic of several other pathogen bacteria, like *Salmonella typhimurium, Yersinia pestis, Yersinia enterocolitica* and *Yersinia pseudotubercolosis* (Maurelli et al 1984 a, 1992).

For the full virulence of *Shigella* the presence of the virulence plasmid is not enough, in turn it is regulated by at least eight loci on the chromosome (see Figure 2.). These

virulence determinant products are either directly effecting the survival in the intestine, or cytotoxins that cause the severity of the disease, or regulate the expression of plasmid genes. Sansonetti and co-workers examined the pathogenicity of *E. coli* K-12 strain after transferring the virulence plasmid as well as chromosomal fragments of *Shigella flexneri*. *E. coli*, containing only the 140 Mdal plasmid of *Shigella,* showed invasiveness, but remained negative in the Serény test. Those hybrids containing both the plasmid and the chromosomal regions however, were positive in the Serény test as well. There was enough of the plasmid in vitro for the invasion of the HeLa cells, but in the animal assay, in vivo, also the chromosomal regions of *his*, *kcp*, *arg* and *mtl* loci were necessary. For the invasion of the epithelium the presence of all these regions are required (Hale 1991, Sansonetti et al 1983a).

Figure 2. *The virulence plasmid of Shigella is regulated by the chromosomal genes*



For the full virulence (e.g. Serény test) of Shigella the regulatory genes on the chromosome are required. KcpA, virR, ompR-envZ overregulate the invasion genes of the pathogenicity island of the virulence plasmid of virulent Shigella strains, which are responsible for the expression of invasion antigens required for in vitro epithelial cell invasion. The regulation is based on environmental signals like temperature and osmolarity. After Pál, PhD dissertation 1992, and Ashkenazi and Cleary, Shigella infections, in Textbook of pediatric infectious diseases, 1992, Vol 1, p. 639.

The molecular and cellular basis of pathogenesis has been studied by using in vitro grown mammalian cell lines, which are susceptible to infection. Undifferentiated, mainly HeLa and Henle-407 cell models played a basic role in the identification of virulence genes and in the characterisation of the invasion steps and the intracellular behaviour of *Shigellae* (Hsia et al 1993, Parsot 1994). Additionally, in vivo virulence tests, animal models of shigellosis can be used, such as the guinea pig keratoconjunctivitis test (Serény test), rabbit ileal loop assay, or the per os infection of rhesus monkeys (Serény 1955, Wood et al 1986, Wassef et al 1989, Oaks et al 1996).

In order to invade the intestinal mucosa, invasive bacterial pathogens must find a site of entry. Penetration of the host cell by the bacteria is accomplished by active endocytosis. Virulent bacteria trigger a mechanism similar to phagocytes in the epithelial cells, using the energy of both the host cell and the bacteria. The host cell develops pseudopodia around the bacterium. Upon contact with epithelial cells grown in culture, *S. flexneri* induces massive host cell cytoskeletal rearrangements of petal like structures termed ruffles. The bacteria are subsequently engulfed by invagination of the plasma membrane and at the same time activate the host cell signalling pathways. After being internalised in the cellular endosome, *Shigella* escapes into the host cytoplasm in 10-15 minutes by lysing the phagosomal vacuole and starts to multiply intensively in this much more optimal environment. *Shigella flexneri* can degrade the membrane of the phagosome in 30 seconds. Lysis of the phagosome is a requisite of the intracellular multiplication and is connected to the contact-haemolytic activity of the bacteria. The non-invasive cells are non haemolytic. The generation time of *S. flexneri* M90T is 40 minutes; in 4-5 hours 5 cells multiply to 500, which is a unique feature of *Shigella*. More than 20 years ago Ogawa already showed that 4 hours after the infection there are bacteria scattered everywhere in the cytoplasm of HeLa cells. (Baudry et al 1987, Parsot et al 1996, Tran Van Nhieu et al 1999, Vasselon et al 1991, Pál et al 1989a,b, Clerc et al 1986a, Sansonetti et al 1986).

By applying these findings on polarized epithelial cells it was thought that microbes trigger the steps described above from the lumen, entering through the apical surface of the cells in vivo. However, when infecting differentiated cells in vitro, it turned out that *Shigellae* were unable to infect the apical side of the differentiated cells, but they easily

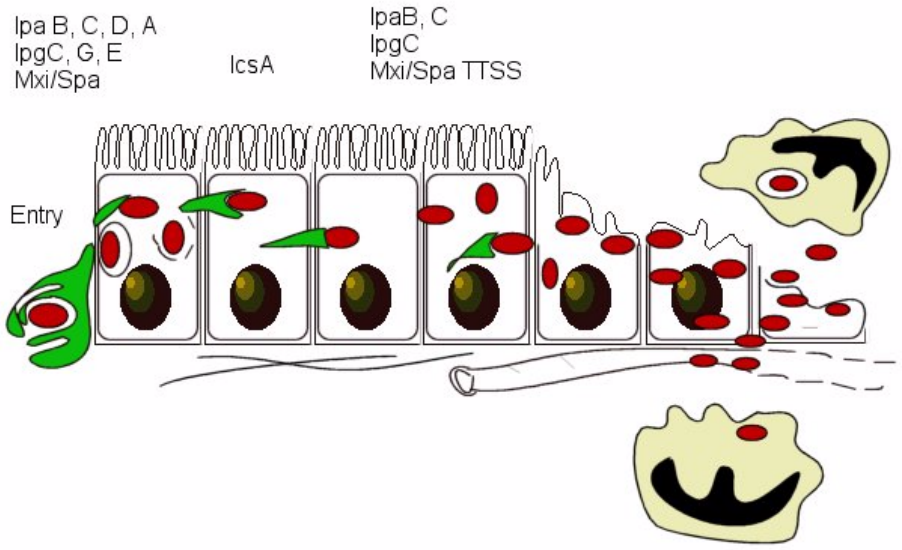
enter the freed lateral or basal sides (Sansonetti et al 1998). The endocytosis of the invasive bacteria is facilitated by the receptors of the host cell. In vivo where epithelial cells are situated in islands *S. flexneri* recognises the receptors on the basolateral (and not on the apical) pole of the host cells. Important components of these receptors are the integrins.

Another crucial factor of invasion is the ability of the organism to multiply and spread within the mucosal tissue. The intracellular spread of the bacteria is based on the connection of the host cytoskeleton and the *Shigella*. This was proved by cytochalasin treatment that inhibits the polymerisation of actin. The expression of a cell surface antigen IcsA coded on the plasmid (VirG) allows the spread within the cytoplasm and dissemination into adjacent cells. *Shigellae* move within the host cell by polar directional assembly of actin elicited on the bacterial surface by the protein IcsA. The product of the gene virG (icsA) located on the polar surface of the bacterial cell is necessary for polymerising the F-actin present in the cytoplasm. The polymerised F-actin envelops the bacterium and connects it with the host cell's actin structure. The assembled actin filaments propel the bacteria forward through the cytoplasm and into adjacent epithelial cells. The polymerised actin resembles a string drawn behind it on the immune-fluorescent EM photos. There has been more data gathered on the genetic basis of intra and intercellular spread. Although the entry of *Shigella flexneri* into epithelial cells is controlled by Rho family GTPases, they have no effect on intracellular motility. Two chromosomal loci were identified to be involved in the intercellular spreading, *vpsC* and *ispA* (Clerc et al 1987, Bourdet-Sicard et al 1999, Mournier et al 1999, Hong et al 1998). Thereby *Shigellae* infect, and by intracellular multiplication, kill the neighbouring cells, effectively avoiding antibody-mediated humoral immunity (similarly to *Listeria monocytogenes*). The infection is usually restricted to the mucosa; lymph node involvement and bacteraemia are uncommon.

On the basis of the findings described above Sansonetti and co-workers set up a new theory of the formation of bacillary dysentery. According to it, *Shigella* preferentially get through the epithelium of the colon by attaching to and invading the membranous epithelial cells (M cells), using them as ports of entry, from where they are able to infect the lateral epithelial cells (Isberg et al 2000). M cells can mainly be found in the

follicle-associated epithelium (FAE), which covers the isolated lymphoid nodules and aggregated lymphoid tissue (Peyer patches) in the ileum small intestine, the sites where mucosal immunity is generated. M cells typically are able to translocate macromolecules, particles and microorganisms from the intestine to the underlying lymphoid tissues (macrophages) – they thus sample and transport luminal foreign antigens. They can be identified by their poor brush borders and basolateral lymphocyte containing cytoplasmic pockets (Jepson et al 1998). The M cells deliver *Shigella* into lymphoid follicles where they encounter macrophages and other cells. *Shigella* - in contrast with Salmonella that multiplies in the phagocytic vacuole - can lyse the phagocytic vacuole (phagosome) and replicate in the cytoplasm. (Perdomo et al 1994, Sansonetti et al 1996, 1999, 2001b) See Figure 3.

Figure 3. *The inter- and intracellular spread of Shigella in the epithelium of the colon*



Shigella cells get through the M cells of the intestinal barrier, and enter the epithelial cells of the colon from their basolateral side by triggering phagocytosis. After lysis of the phagocytic vacuole Shigellae escape and spread intra - and intercellularly. The inflammatory response results in apoptosis of the epithelial cells. The toxins released by the pathogen are responsible for the destruction of the capillaries, resulting in blood in the faeces. Infected macrophages go under programmed cell death.

Infected macrophages lose cell viability and undergo morphological changes identical to apoptosis or programmed cell death, which is induced by the plasmid encoded IpaB protein. In parallel, macrophages are stimulated to excrete IL-1ß, which triggers the

acute inflammatory response. The release of IL-1ß and local TNFα, recruits inflammatory polymorphonuclear cells that migrate though the epithelium into the lumen, allowing massive entry of more bacteria into the mucosa by taking them up and channelling them to the basolateral side of the epithelial cells. As a result of this early inflammatory phase a focal disintegration in the epithelial line creates more and more lateral cell surface free to bacterial invasion. The cytotoxic factors released from the invaded macrophages and polymorphonuclear cells in this latter phase of the inflammation leads to the spreading of the apoptosis. The massive tissue destruction causes the mucosal abscesses and ulcers.

Experiments with HeLa cells showed that during *Shigella flexneri* infection bacteria can multiply rapidly in high number without inducing lysis of the host cell (Mantis et al 1996). Animal models of shigellosis suggest that the destruction of the colonic epithelial cell lining is due to this host immune response rather than the multiplication of bacteria within the cytoplasm of host cells. The process is usually self-terminating, because during the acute inflammatory response, the release of IL-18 induces IFN-γ, which is essential for the killing of *Shigella* along with the phagocytes inhibiting more cell infections. (Jones et al 1995, Sansonetti et al 1998, 2001b, Zychlinsky et al 1994, 1996)

Earlier, an exotoxin called Shiga toxin was thought to be responsible for destroying the host cells in the last step of invasion, however this hypothesis could not be proved. Shiga toxin is a very effective cytotoxin, produced in the biggest amount by *S. dysenteriae* 1 serotype. It is neurotoxic, lethal to animals, enterotoxic for ligated rabbit intestinal segments; and cytotoxic for vero, HeLa, and some selected endothelial cells (human renal vascular endothelial cells). It is encoded by chromosomal genes, with a two-domain (A-5B) structure similar to the Shiga-like toxin of enterohaemorrhagic *E. coli* (EHEC) (Kaplan et al 1998, O'Brien et al 1980, 1987, 1992).

According to current understanding, this toxin is responsible for the bloody diarrhoea occurring at the onset or during the disease. The diarrhoea is caused by its enterotoxic effect: the toxin adheres to small intestine receptors and blocks absorption of electrolytes, glucose and amino acids from the intestinal lumen. This contrasts with the

effects of cholera toxin and heat labile toxin, which block absorption of Na^+, but cause hypersecretion of water and ions of Cl^-, K^+ and HCO_3^- out of the intestine and into the lumen. Shiga toxin is responsible for the severity of bacillary dysentery: bloody diarrhoea is attributed to the cytotoxic effect by inhibiting protein synthesis of the capillary endothels. The B subunit of Shiga toxin binds the host cell glycolipid in the large intestine; A1 domain is internalised via receptor-mediated endocytosis and causes irreversible inactivation of the 60S ribosomal subunit. This results in cell death, microvasculature damage to the intestine, and haemorrhage. Shiga toxin is also thought to cause HUS. The considered signs of neurotoxic effect are fever and abdominal cramping (Sandvig 2001).

In spite of these effects described above, there is no evidence for its playing a role in the destruction of the epithelial cells invaded by *Shigella*. *Shigella flexneri* is able to destroy the host cells without producing Shiga toxin. It was shown in an invasion assay that when J774 macrophages were infected there was close correlation between the expressed phenotype of the cells and the destruction of the host cells. The concentration of intracellular ATP and lactate of *S. flexneri* decreased whereas the concentration of pyruvate increased. This finding can be attributed to that fermentation and cell respiration has stopped. By transmission EM it was shown that the bacteria that escaped from the phagosome are situated in the vicinity of the mitochondria of the host cell, sometimes attaching to them. The inner parts of the mitochondria are destroyed indicating that they were primarily targets of *Shigellae*.

Contrary to the infection of non-differentiated epithelial cells, detecting only the invasive ability of *Shigella*, other virulence tests demonstrating a more complex feature of pathogenicity are in use. Thus, the guinea-pig keratoconjunctivitis assay – or Serény test-, in which the conjunctiva of the animals is infected with the bacteria, has been used since the 1950s. The other alternative is – where available - the oral infection of primates with higher c.f.u. doses than the infectious human dose (Serény 1955, Wood et al 1986, Oaks et al 1996, Wassef et al 1989, Perdomo et al 1994).

2.6. Epidemiology of bacillary dysentery

2.6.1. Epidemiology of Shigella and EIEC

Shigellosis is a major cause of diarrhoeal disease throughout the developing world. Outbreaks are associated with low standards of sanitation and hygiene. In industrial countries it is not endemic any longer, however in developing countries this disease is still the major cause of childhood mortality (Mandel et al 1995). According to estimates 200 million diarrhoeic diseases are registered annually worldwide. The major cause of these is bacillary dysentery, mainly occurring in the paediatric age group (1-10 years old): toddlers aged 1 to 4 are the most likely to get shigellosis. Many cases are related to the spread of illness in child-care settings, and many more are the result of the spread of the illness in families with small children. The peak incidence of shigellosis is in children and young adults; it is rare in older adults, who usually get the disease from children (Cohen et al 1997). It is also rare in infants, because due to protective maternal immunity and breast-feeding the passively acquired IgG anti LPS confers protection to newborns up to 6 months of age. In the United States 15-20% of paediatric diarrhoea is estimated to be bacillary dysentery. In developing countries it is the leading cause of infant diarrhoea and mortality.

Bacillary dysentery has typical seasonality in the summer and autumn: 40-45% of the annual cases occur between August and October. Hypocrite recorded that when a dry winter is followed by a rainy spring, the number of dysentery cases increases rapidly. In tropical climates flies are an important transmitting factor. Bacillary dysentery shows 20-30 years periodicity. In Europe in the first quarter of this century *S. dysenteriae* predominated, between 1926 and 1938 *S. flexneri* did. Today *S. sonnei* is the major cause of the disease.

Shigella occurs naturally in humans and the higher primates such as monkeys and chimpanzees, so bacillary dysentery is spread from human to human via the faecal-oral route. Bacillary dysentery is a typical dirty hand disease, spreads easily and fast, often transmitted by direct contact - primarily by contaminated hands - as a result of its extremely low infectious dose (ID) (DuPont et al 1989, 1990). It is the most contagious

diarrhoeal disease, as few as 10 *Shigella dysenteriae* bacilli can cause clinical disease, whereas 100-200 bacilli are needed for *Shigella sonnei* or *Shigella flexneri* infection. It has a short, 1-3 day incubation period.

Shigellosis affects certain populations more than others. High-risk populations usually live in close communities where outbreaks can occur more frequently. In developing countries, amongst displaced populations the rate of morbidity and mortality is higher. As a result of war, famine, ethnic persecution, or any other sudden mass displacement of people, large, overcrowded populations are created in refugee camps with insufficient nutrition and sanitation. In spite of the development of modern warfare, communities in military camps, where it is impossible to maintain proper sanitation are still at high risk.

In industrialised countries, for example in the U.S., day-care facilities account for a large proportion of cases. Groups at increased risk include small children and toddlers in schools and day-care centres, elderly people or mentally retarded people in custodial institutions (Boyce et al 1982, Echeverria et al 1992, Kotloff et al 1999, Kourany and Vaquez 1969, Lee et al 1991, Punyaratabandh et al 1991, Sharp et al 1995). In a social institute interns were screened to test the transmission from person to person by contaminated hand/fingers. Both stool and swab samples from the fingers were taken from the patients. *Shigella* was isolated amongst 10% of these patients also from the fingers (DuPont 1970). In Great Britain annually 20,000 - 500,000 cases are registered, however the real number might be greater. Bacillary dysentery is mainly spread amongst toddlers and small schoolchildren, and its occurrence overlaps with the school year. It has been assumed that the cause of contamination is the toilet seats of the kindergartens and schools. This contamination originates from the stool of children with dysentery and is passed over to the hands of healthy children.

Because of its easy spread, its appearance should be reckoned with everywhere where personal hygiene is difficult to maintain. Travellers from industrialised countries to the developing world also face complications from diarrhoea, of which 1% is attributable to *Shigella*, often to multiresistant strains. *Shigella* strains are the second most frequent agents of the travellers' diarrhoea after enterotoxin producing *E. coli* (ETEC). (Adkins

et al 1990, Echeverria et al 1978, 1981). According to estimates EIEC strains cause a significant, up to 10% percent, amount of dysentery cases (Abuxapgui et al 1999, Echeverria et al 1992, Tamura et al 1996). EIEC strains occur mostly in the developing world, Southeast Asia, South America, and play a significant role in certain geographical areas, like Mexico (Taylor et al 1986, Wanger 1988, Echeverria et al 1989b 1992, Gordillo 1992, Pál et al 1997) EIEC strains are also regularly isolated in Europe and the US (Beutin et al 1997, Kétyi 1989, Wanger et al 1988). The real incidence and epidemiology of EIEC infections, however, is yet unknown, since due to the difficulties in the identification of this pathogen, EIEC often remains unrecognised.

Another feature that has also increased the significance of bacillary dysentery in the past few years is the increase of severe shigellosis and EIEC infections among patients with AIDS (Grant et al 1997, Batchelor et al 1996, Hickey et al 1993, Huebner et al 1993). This is threatening, if considering that by year 2020 – in case of the lack of an AIDS vaccine and free access to treatment – nearly half a billion people will be living with HIV/AIDS (WHO 1996.)

2.6.2. Waterborne diseases, pathogen microbes in water

According to the estimates of the World Health Organisation, 3.4 million people, mostly children die annually from water-related diseases (WHO 1996). At the beginning of 2000 2.4 billion people, two-fifths of the world's population, including the poorest in the world, lacked access to basic sanitation. 1.1 billion people, one-sixth of the world's population, lacked access to even improved water sources. The majority of these people live in Asia and Africa. These figures are all the more shocking when considering estimated global population growth. Two-thirds of mankind will be water-stressed by 2025. According to estimates by the UN Environmental Programme, to achieve adequate, universal water supply coverage by the year 2025, an additional 3 billion people will need to be provided with a water supply and more than 4 billion with sanitation (WHO 2000). A report prepared by the CIA states that there is a real prospect of water wars or water management by 2020: like now for oil, countries will fight with each other for water (Anon 2000).

The presence of disease-causing microorganisms in tap water typically results from poor water quality at source, lapses in disinfection and filtration treatment processes, or compromised distribution systems. Bacteria and viruses contaminate both surface and groundwater, whereas parasitic protozoa appear predominantly in surface water (Tardiff 1993). Most waterborne pathogens are enteric. They include parasitic agents such as *Cryptosporidium parvum*, *Giardia lamblia*, *Cyclospora* and *Entamoeba histolytica*; bacteria such as *Salmonella*, *Shigella*, *Campylobacter*, *Vibrio cholera*, enterovirulent *Escherichia coli*, *Aeromonas*, *Yersinia* and *Clostridium perfringens*; and viruses such as the enteroviruses, rotaviruses, parvoviruses, adenoviruses, caliciviruses and astroviruses (Tardiff 1993). Generally bacteria and protozoa induce gastrointestinal disorders, some even life-threatening diseases like typhoid and cholera. Viruses, besides gastroenteritis, can cause other severe diseases like aseptic meningitis, encephalitis, poliomyelitis, hepatitis, myocarditis and diabetes (Payment 1993). The real occurrence of waterborne diseases is difficult to estimate. Statistics are unreliable due to unreported cases or unknown etiology (Payment and Hunter 2001).

Waterborne diseases, although not endemically but rather in the form of outbreaks, are present in industrialised countries despite the well-developed communal water system. The Centrer for Disease Control (CDC) in Atlanta registered 21 food and water borne outbreaks between 1964 and 1968. Between 1971 and 1992 in the USA the total number of waterborne outbreaks was 684, with 164,158 cases of illness, 1,170 hospitalisations and 12 deaths. The second most frequently identified etiologic agent in waterborne outbreaks in the above mentioned period was *Shigella*, preceded by *Giardia lamblia*, and followed by Norovirus (formerly "Norwalk-like virus") (Craun 1994).

2.6.3. *Shigella* and EIEC as waterborne pathogens

Shigella and EIEC strains regularly cause food and waterborne epidemics. (Rasstegaeva and Mineev 1980, Reeve et al 1990, Todd 1997, Zaika et al 1996, Echeverria et al 1994, Fredlund et al 1987, Islam et al 1993). Under optimal conditions *Shigellae* can persist in water for a long time (Hoeprich et al 1977). Contamination of water can occur if the toilets are near wells, as well as when sewage pipes are directly run into lakes that feed these wells. Secondary transmission occurs frequently during outbreaks, especially if a

family member gets the disease. In such families 10% of the 1-4 year olds, and 20% of the older children got the disease. Long-term carriage may play an important role in water and food borne outbreaks.

Waterborne epidemics also originate from the contamination of surface waters used for recreational purposes (Simchen et al 1991, Egoz et al 1991, Lindell et al 1973, Kramer et al 1996, Moore et al 1993, Blostein 1991, Ewald et al 1991, Makintubee et al 1987, Faruque et al 2002). The spread of enteral pathogen microbes – as well as *Shigella* – in bathing water or in swimming pools is not uncommon. Unfortunately in the last 15 years in Hungary the occurrence of such outbreaks was significant and according to the number of patients this type of spread must be regarded as significantly affecting the epidemiological situation.

Both endemic and epidemic bacillary dysentery is present in developing countries. Most developing countries are at risk of epidemic dysentery caused by *S dysentery type 1*, which is the only cause of large-scale, regional outbreaks of dysentery. Since the late 1960s, pandemic waves of *S. dysenteriae type 1* dysentery have appeared in Central America, South and Southeast Asia and sub- Saharan Africa. An epidemic in Central America from 1969 to 1973 was responsible for more than 500,000 cases and 20,000 deaths. The epidemic in central and southern Africa began in 1979 and has affected at least nine countries (Kotloff et al 1999, WHO 1995). Bacillary dysentery, besides other diseases, is also a problem in industrialised countries like the US. According to Craun, between 1971 and 1992 in the USA *Shigella* caused 57 outbreaks and 9967 cases of illness as the second most frequent pathogen (Craun 1994).

The significance of waterborne outbreaks caused by EIEC was also proved by epidemiological studies in Hungary by Kétyi (1989). The epidemiology of EIEC was described on the basis of the O124 serogroup that was dominant in Hungary. EIEC strains causing a milder form of dysentery affected many thousands of people in the 1950-60's. They originated from waterborne outbreaks, probably due to the higher environmental resistance of EIEC. One of the differences from *Shigella* is in the age of patients. While *Shigella* infects babies and toddlers, EIEC causes a lower number of diseases amongst them. The target population of EIEC is all age groups. The number of

infections increases from the age of 3 onwards, the peak being between the ages of 7 and 14. No difference in gender was observed. Since EIEC is 10,000-fold less infectious and more resistant to environmental factors, than *Shigella*, food borne and waterborne diseases rather than person-to-person spread are more common. In Hungary between 1956 and 1966, fourteen major waterborne outbreaks were observed. The sources of these outbreaks were most often wells, or conduits, contaminated by sewage or by surface, direct or rainwater. EIEC also spreads directly but less frequently than *Shigella*, though it is related to hygienic conditions. Surveys show that in the North East part of the country, where sanitation is worst, infections occur more often. The long-term carriage of EIEC is more frequent when carriers were screened for amongst convalescents, even carriage for one year was observed.

2.6.4. Food poisonings, pathogen microbes in food

Food is a chemically complex matrix, and predicting whether, or how fast, microorganisms will grow in any given food is difficult. Several factors encourage, prevent, or limit the growth of microorganisms in foods; the most important are pH and temperature. The clinician or epidemiologist usually thinks of food poisoning as a primary cause in case of the sudden onset of such disease in a well-defined population after consuming the same food. The symptoms caused by different microbes involved in food poisoning are usually similar: nausea, vomiting and diarrhoea. Etiologic diagnosis can only be established after identifying the microbe in question, routinely by culturing.

Although food bacteriology and safety is a crucial point in the agenda of national and international organisations, there is no general agreement on the standardizing of laboratory detection methods for the investigation of food-borne outbreaks worldwide. In the world a wide range of bodies is involved in monitoring and researching microbial activities associated with food. These bodies, like the Food and Drug Administration (FDA), the World Health Organisation (WHO) in the US, the Food Standards Agency (FSA), the Institute of Food Research (IFR) in the UK establish food safety programmes, and carry out extensive research to reduce the incidence of food-borne disease. In Hungary food safety is monitored by the Institute of State Public Health and the Medical Officer Service (Állami Népegészségügyi és Tisztiorvosi Szolgálat,

ÁNTSZ). Currently, the International Commission on Microbiological Specifications for Foods (ICMSF) have been trying to provide guidance to government and industry on appraising and controlling the microbiological safety of foods.

Food is usually safe from pathogen microorganisms if produced from good quality stock. During the manufacturing processes special care must be taken to avoid contamination. Therefore it is desirable that food be processed according to Good Manufacturing Practice (GMP) (ICMSF, 1986).

The most common microbes isolated from food poisonings are *Staphylococcus aureus*, *Salmonella* and *Clostridium perfringens*. *Yersinia enterocolitica* and *Shigella sonnei* can also cause food borne disease. Food poisoning caused by *Clostridium botulinum* is rare. According to the proposed up-dated categorisation of food-borne pathogens of ICMSF, microorganisms fall into four categories: moderate, serious but not life threatening, severe hazard for general population and severe hazard for restricted population (Forsythe 2000). See Table 1. The most dangerous changes in the microflora of food are when it does not show any changes in appearance. Due to the metabolism of pathogen microbes, by-products may cause changes in the food making it inconsumable or even toxic.

When analysing food and drink it is examined by several different bacteriological tests. First the total number of viable germs is determined, including the number of bacteria, yeast and mould spores. Next, the number of coliform bacteria is determined. Finally the known specific microbes and the causative agent of the food poisoning are identified. There is no such assay that would be applicable for testing all types of food.
A great number and variety of microorganisms can be present in food, but their number can be reduced using different processes. High and low temperature, low pH and humidity, high salt and sugar concentration can be lethal for the microorganisms. These conditions, however, are favourable for the growth of yeast and mould, leading to the increase of mycotoxins in food.

Milk can be contaminated by bacteria during milking or processing. Regulation of milk processing has changed many times during the past 30 years, mainly in order to screen

for and eliminate *Mycobacterium bovis,* the causative agent of tuberculosis in cattle. With the introduction of milk pasteurisation this form of human tuberculosis has become very rare. Milk can be processed by the low temperature long time (LTLT) or high temperature short time (HTST) pasteurisation, by ultra-pasteurisation or sterilisation (Lewis et al 1999).

Table 1. *ICMSF microbiological hazards: proposed up-dated categorisation*[2]

Category[1]	Foodborne pathogens
Food poisoning organisms causing moderate, not life-threatening, no sequelae, normally short duration, self-limiting	*B. cereus* (including emetic toxin), *Cl. perfringens* type A, Norwalk-like viruses, *E. coli* (EPEC, ETEC), *St. aureus*, *V. cholerae* non-O1 and non-O139, *V. parahaemolyticus*
Serious hazard, incapacitating but not life-threatening, sequelae rare, moderate duration	*C. jejuni, C. coli*, S. *Enteritidis*, S. *Typhimurium*, *Shigellae*, hepatitis A, *L. monocytogenes*, *Cryptosporidium parvum*, pathogenic *Y. enterocolitica, Cyclospora cayetanensis*
Severe hazard for general population, life-threatening, chronic sequelae, long duration	Brucellosis, botulism, EHEC (HUS), S. *Typhi*, *S. Paratyphi*, tuberculosis, *Sh. dysenteriae*, aflatoxins, *V. cholerae* O1 and O139.
Severe hazard for restricted populations, life-threatening, chronic sequelae, long duration	*C. jejuni* O:19 (GBS), *C. perfringens* type C, hepatitis A, *Crytosporidium parvum*, *V. vulnificus*, *L. monocytogenes*, EPEC (infant mortality), infant botulism, *Ent. sakazakki*

[1]*Foodborne pathogens are divided into four groups category according to the severeness of the diseases they cause and the size of the population they affect*
[2]*This categorisation was proposed by the International Commission on Microbiological Specifications for Foods (ICMSF)*

The normal microflora of milk depend on the temperature: at between 15 and 30 ^{o}C *Streptococcus lactis* predominates and many other *Streptococcus* species and *Corynebacterium* are present, whereas at between 30 and 40 ^{o}C lactobacilli and coli dominate the microflora. At 45 ^{o}C the number of these thermophyl bacteria quickly increases. Gram-negative bacteria can be found in only a quarter of the samples, contaminating also via the same route (Collins 1989). Spoiled milk may contain *Pseudomonas*, *Achromobacter*, *Alcakigenes* and *Flavobacterium* species, which can all degrade fat and protein resulting in a special taste. *E. coli* produces gases during degrading lactose, causing milk to be gassy. *Streptococcus cremoris* and *Alkaligenes viscosus* produce capsid, resulting in slime formation. In stale milk *Oospora lactis* and yeast are present. *Pseudomonas aeruginosa* is responsible for blue milk, and *Serratia marcescens* is responsible for red milk. *Brucella abortus* is secreted in milk and can be detected by using test animals or by slide agglutination (Collins et al 1989).

2.6.5. *Shigella* and EIEC as food borne pathogens

The role food plays in transmitting *Shigella* is well established (Black et al 1978). Shigellosis accounts for fewer than 10% of the reported outbreaks of food borne illness in the U.S. Experimentally *Shigella*s survive in various foods. Under optimal temperature conditions, and without severe acidity, *Shigella*s may be recovered from milk, eggs, cheese and shrimp after 30 days (Hoeprich et al 1977). Contaminated milk and dairy products like cheese are most frequently the source in case of both *Shigella* and EIEC infections (Keogh et al 1971). Other associated foods can be salads (potato, tuna, shrimp, macaroni, and chicken), raw vegetables, and poultry and other foods that require a lot of mixing and handling and no further heat treatment. The most common means of spread is when a human carrier with poor sanitary habits handles liquid or moist food that is not then thoroughly cooked afterwards. The number of shigellosis attributable to food is unknown, but given the low infectious dose, it is probably substantial.

It is currently unknown what foods may harbour EIEC, but any food contaminated with human faeces from an ill individual, either directly or via contaminated water, could cause the disease in others. Outbreaks have been associated with hamburger meat and unpasteurised milk. The disease caused by EIEC is uncommon, but it may be confused with shigellosis and its prevalence may be underestimated. The relative frequency of EIEC food borne infections is small, or poorly documented since the detection of this organism in foods is extremely difficult because undetectable levels may cause illness. Several outbreaks in the U.S. have been attributed to EIEC of this organism. One outbreak occurred in 1973 and was due to the consumption of imported cheese. More recently, an outbreak on a cruise ship was attributed to potato salad, and an outbreak occurred in a home for the mentally retarded where subsequent person-to-person transmission occurred (Anon 1992)

2.7. Therapy, prevention and control of bacillary dysentery

2.7.1. Treatment with antibiotics, antibiotic resistance

As in the case of diseases with acute gastroenteritis, dehydration is a major problem. Dehydration is caused by loss of water in stools, increased evaporation through the skin due to fever and decreased fluid intake due to anorexia. Oral rehydration therapy with a solution containing salts should be enough; only severe cases need intravenous therapy. Special care must be taken with proper nutrition during shigellosis. Continued feeding is rather encouraged, because the disease does not affect of the small intestine much where most of the absorption of nutrients takes place. It is important to feed or breast-feed patients to prevent hypoglycaemia and weight loss. Antidiarrhoeal agents used for the symptomatic relief of abdominal pain, and for reducing the frequency of stool passage are likely to make the illness worse and should be avoided, because they may cause severe adverse events (WHO 1995).

Table 2. *Antimicrobials for the treatment of infections with Shigella dysentery*

Agent	Resistance		Cost	Availability
	S dysentery type 1	Other *Shigella*		
Ampicillin	Common	Variable	Medium	Wide
TMP-SMX	Common	Variable	Low	Wide
Nalidix acid	Increasing	Infrequent	Medium	Moderate
Pivmecillinam	Infrequent	Rare	High	Limited
Ciproflaxin	Rare	Rare	High	Limited
Norfloxacin	Rare	Rare	Medium	Limited
Enoxacin	Rare	Rare	High	Limited

1Cost: Low=<US$ 1.00; Medium= US$ 1-4.00; High=< US$ 5-30.00
Source: WHO 1995, Guidelines for the control of epidemics due to Shigella dysentery type 1.

Shigellosis can usually be treated with antibiotics, and persons with mild infections recover quickly without antibiotic treatment. Antimicrobial therapy shortens the duration of diarrhoea, fever, and period of communicability. The treatment of carriers, who are the major source of infectious organisms, is essential for the prevention of further spread of the bacteria. The antibiotics commonly used for such treatment are: ampicillin, trimethoprim/sulfamethoxazole (TMP-SMX, also known as Bactrim or Septra), nalidixic acid, or ciprofloxacin. The first choice of antimicrobial agent is

ciprofloxacin and azithromycin (Khan et al, 1997), alternatively TMP/SMX and ampicillin (resistance is common in Middle East, Latin America) (Gilbert et al, 2001).

Unfortunately some *Shigella* strains have become resistant to antibiotics. Therefore using antibiotics can actually make the bacteria more resistant, antibiotics are sometimes used selectively to treat only the more severe cases when many persons are affected by shigellosis. *Shigella* acquired resistance to sulfa drugs in the 1940s, to tetracycline and chloramphenicol in the 1950s, to ampicillin in the 1970s and to trimethroprim-sulfamethoixazole in the 1980s (WHO, 1997). Antibiotic resistance emerging among the S. *dysenteriae* 1 strains, which cause the most severe clinical features, mainly in Africa, South-Eastern Asia and South America is a major problem (Jamal et al 1998, Materu et al 1997, Taylor et al 1989, Hoge et al 1998). Table 2 (WHO 1995), presents a summary of the antibiotics in use, the developed resistance of *Shigella* serotypes and their cost and availability.

2.7.2. Vaccine development

The severity of the disease, its fast spread and high mortality and morbidity rates described above mean that there is an urgent need for a safe and efficacious vaccine. However, in spite of the extensive research over the past 40 years, no reliable vaccine for bacillary dysentery has been introduced. The development of a reliable vaccine has been hampered by three factors: (1) the ineffectiveness of parenterally injected inactivated whole-cell vaccines which led researchers to believe that serum antibodies do not confer immunity, (2) the lack of a suitable animal model and (3) the fact that there is only indirect evidence of immune mechanisms in humans (Passwell et al 2001, DuPont et al 1972 a, b, 1988, Lindberg and Pál 1993).

The ideal vaccine against shigellosis would be multivalent, orally administered in a single dose, well tolerated, inducing high level and long term protection and would also be easy to manufacture. Several different approaches have been used by different research groups developing candidate vaccines worldwide. These need to be evaluated for their efficacy and have now reached phase I, II or III clinical trials. At the meeting organised at the WHO in Geneva in 1996, priority was given to vaccine development

against *S. dysenteriae* type 1, which is associated with the most severe cases of bacillary dysentery (WHO 1997). Vaccination against EIEC has not been given importance, as EIEC causes less severe disease, and because the immune response is not clearly established, but supposedly different (Clarke et al 2001).

Due to the failure of the classic approach with whole cell vaccines or live attenuated parenteral vaccines, orally administered vaccines based on live attenuated strains have been developed. The recent vaccine candidate strains contain genetically engineered deletions in key enzymes in the metabolic pathway, or in virulence genes or their combination. Two live oral candidate vaccines developed in the Centre for Vaccines Development in Baltimore, USA. *S. flexneri 2a* and *S. sonnei* vaccines have been evaluated in phase I clinical study (Kotloff et al 2000, 2002). Another candidate using *S. dysenteriae 1* WRSd1 showed protection against challenge in animal models (Venkatesan et al 2002). One live oral *S. flexneri 2a* SC602 candidate attenuated in its intra and intercellular spread, developed at the Institute Pasteur in Paris, France showed promising results in a phase I study (Phalipon and Sansonetti 1995, Coster et al 1999). Another candidate vaccine based on a *S. flexneri Y* auxotrophic mutant was developed in Stockholm, Sweden, (Karnell et al 1991, Li et al 1992, Lindberg et al 1990).

A different approach, when the O antigen gene clusters of LPS of *S. dysenteriae 1* is expressed in a live attenuated vector has been used in Germany (Tzschaschel et al 1996), as well as in Switzerland, where the O antigen of *S. sonnei* and *S. dysenteriae 1* was expressed in cholera vaccine vector (Viret et al 1996).

Most recently, subunit vaccines have been constructed, in the form of conjugate vaccines, proteosomes or nucleoprotein vaccines. The NIH in Israel has developed a parenteral conjugate vaccine based on *S. sonnei*. This has been evaluated in a phase II study (Cohen et al 1997, Passwell et al 2001). A trivalent conjugate vaccine composed of the O-specific polysaccharide from *S. flexneri 2a*, *S. sonnei* and *S. dysentery type-1* covalently bound to carrier proteins was also developed by the same group (Taylor et al 1993). *S. sonnei* and *S. flexneri* proteosomes as nasal or oral vaccines (Fries et al 2001, Mallett et al 1995), and a parenterally administered nucleoprotein subcellular

(ribosomal) vaccine have been developed by WRAIR in the USA (Levenson et al 1995).

2.7.3. Personal hygiene, water and food safety

Until the introduction of a safe and efficacious vaccine the only measures of prevention are maintaining proper environmental facilities, and keeping personal standards of hygiene high. In developing countries where chlorination of the water supply is not general, special preventative measures must be taken. This starts with educating people about practicalities such as the proper disposal of human excreta and used diapers, the disinfection of clothing and disposal of bodies, and taking special care to keep *Shigella* and EIEC out of healthcare facilities. The simple act of washing one's hands with soap and water can reduce diarrhoeal disease transmission by one-third. Breast-feeding as way of preventing disease should also be encouraged (WHO 1995, 2000).

The only way to prevent the spread of shigellosis is the sanitary handling of food and beverages. Special measures are required, like avoiding leaving perishable foods unrefrigerated, cooking food thoroughly rather than eating raw food, protecting food from flies, avoiding preparing food when ill with diarrhoea or vomiting. Travellers to the developing world better keep the golden rules: "boil it, cook it, peel it, or forget it" (WHO 1995).

Safe drinking water is a global need, and proper sewage disposal and water chlorination are the most crucial and beneficial measures for the prevention of the spread of these pathogens. The chlorination of drinking water was introduced in the early years of the last century in Great Britain. It was subsequently adopted by the USA and other developed countries, resulting in the elimination of many waterborne diseases like cholera, typhoid, dysentery and hepatitis A. However, in developing countries water remains untreated or inadequately treated, the availability of safe drinking water is practically non existent, therefore these diseases are endemic and kill young and old. In these countries people need to be educated about how to store and treat water at home by boiling or chlorination. As stated at the 1992 First International Conference on the Safety of Water Disinfection, according to cost-benefit analysis the cost effectiveness of municipal water systems and water treatment for pathogens go way beyond the possible

decrease of mortality and morbidity, since it affects the productivity of industry and the whole economy (Christman 1998, Clark et al 1993).

The bacteriological screening of municipal and recreational water supplies for waterborne pathogens is critical in order to prevent outbreaks. In the USA, under the Environmental Protection Agency's (EPA) Total Coliform Rule, the presence or absence of e.g. *E. coli* as indicator bacteria, is used to confirm if drinking water is pathogen free and safe. The screening and isolation of microbes - *Shigella* and especially EIEC - from water presents difficulty in routine microbiological work. Water monitoring comprises of field investigation, chemical, bacteriological and biological testing. In Hungary the requirements for drinking water quality are regulated by the Hungarian Standards Institution, accessible in the document MSZ-450-3.

Table 3. *Allowed number of coliforms in water*

Bacteriological threshold limit	A	B	C
Parameters	Allowed quantities		
Number of coliforms in 100 ml	0	2	0
C.f.u. at 37 ^{o}C in 1 ml	20	100	500
C.f.u. at 20 ^{o}C in 1 ml	100	500	500

The Hungarian Standards Institution sets limitations on the allowed number of coliform bacteria in water in three categories.

Table 4*: Allowed number of enteric or pathogenic microorgamisms in water*

Parameters	Allowed quantities
Pseudomonas aeruginosa in 100	0
Faecal *Streptococcus* in 100 ml	0
E. coli or faecal coliform in 100 ml	0
Sulphite reducing anaerobe spore forming bacteria (*Clostridium*) in 50 ml	0
Enteric or other pathogen microorganisms in 5000 ml[1]	0
Phage of enteric bacteria in 100 ml	0

1Enteric or other pathogen microorganisms can be for example Campylobacter, Salmonella, Shigella, Staphylococcus aureus, pathogen fungi, protozoon, worm egg, and human pathogen virus.

The number of coliforms, their faecal origins, the number of *Enterococcus faecalis*, the presence of anaerobic and enteropathogen bacteria can be checked. Filtration of large volumes of water (2-5 litres) through a sterile membrane filter is used in these tests

(Anon. 1991, 1994). The limitations on the number of coliforms in 1 cm^3 water are summarized in Table 3 and 4.

2.8. Laboratory diagnosis in *Shigella* and EIEC infections

2.8.1. Traditional methods

The obvious prerequisite for successful treatment and prevention is the detection of the causative agents in clinical and environmental samples. The two forms of dysentery, amoebic and bacillary, can be distinguished by macroscopic and microscopic examination of the stool. The traditional diagnosis of bacillary dysentery is based upon microscopic examination and isolation of the organism by culturing it from the stools of infected individuals. Also the demonstration of invasiveness of isolates in tissue culture or in a suitable animal model is necessary (Lányi 1980, Nász 1988).

The procedure for isolation and detection of *Shigella* is well established, relatively simple and cheap. The suspicious colonies isolated on selective media are tested by established biochemical reactions, and slide agglutination. Their differentiation from the other members of the family *Enterobacteriaceae* is usually not a problem (Echeverria et al 1991b, Lányi 1980, Nász 1988).

Stool specimens and rectal swabs are cultured soon after collection or placed in appropriate transport medium. Routinely, samples are directly plated on mildly selective plates not inhibiting the normal flora of the colon and on other media with higher selectivity. Commonly used primary isolation media include MacConkey, Hektoen Enteric Agar, Eosine Methylene Blue, and Desoxycholate Citrate Agar, Xylose Lysine Desoxycholate Agar, Salmonella-*Shigella* Agar. These media contain bile salts to inhibit the growth of other Gram-negative bacteria and pH indicators to differentiate lactose fermenters (coliforms) from non-lactose fermenters such as *Shigellae*.

MacConkey agar (MAC) is a differential plating medium used in the isolation and differentiation of lactose-nonfermenting, gram-negative enteric bacteria from lactose-fermenting organisms. Hektoen Enteric Agar (HEA) is a differential selective agar that is useful for isolation of *Salmonella* and *Shigella.* It has an H_2S-indicator system for selecting H_2S-producing *Salmonella*, which produce blue-green colonies with a black centre. Eosine Methylene Blue is a differentiating medium, recommended for the

detection and isolation of the gram-negative enteric bacteria. Colonies of lactose fermenting organisms are differentiated from lactose negatives by eosin and methylene blue indicators. Desoxycholate citrate agar (DC) is a differential selective plating medium for the isolation of enteric pathogens, particularly *Shigella* and *Salmonella*. Colonies of lactose-nonfermenting strains are colourless. Xylose lysine desoxycholate agar (XLD) is a selective differential medium developed for isolating and differentiating gram-negative enteric bacilli, especially *Shigella* and *Providencia*. It is suitable for isolation of *Shigella* and *Salmonella* from stool specimens. Differentiation of these two species from non-pathogenic bacteria is accomplished by xylose and lactose fermentation, lysine decarboxylation, and hydrogen sulphide production. Salmonella-*Shigella* Agar (Bacto SS Agar) is used for isolating *Salmonella* and some *Shigella*. In SS Agar, bile salts and brilliant green inhibit gram-positive bacteria, most coliform bacteria, and the swarming phenomenon of *Proteus* spp. *Salmonella* and *Shigella spp.* are lactose non-fermenters and form colourless colonies. SS Agar is a highly selective medium; some *Shigella* strains may even not grow, therefore it is not recommended as the sole medium for primary isolation of *Shigella* (Anon 1998).

In parallel, samples can be enriched e.g. in selective–enrichment broth and further cultured on solid media. A liquid enrichment medium (Hajna Gram-negative broth) may also be inoculated with the stool specimen and subcultured onto the selective/differential agarose media after a short growth period. Bacto Selenite Broth or Selenite F (Faecal) Broth is used for enriching *Salmonella* spp. that may be present in small numbers and competing with intestinal flora during isolation procedures and for isolating *Salmonella* in foods. Sodium selenite inhibits the growth of gram-positive bacteria and many gram-negative bacteria (Anon 1998).

However, culturing *Shigella* on selective or selective enrichment media is rather difficult in comparison with that of *Salmonella* (Aroyo et al 1995, Moringo et al 1989), because *Shigellae* are more sensitive in their growth requirements and therefore more fastidious to culture (Hunt et al 1990, June et al 1993, King et al 1968, Morris et al 1970, Pitarangsi et al 1987, Satler and Gragas 1977, Serény 1967, Taylor 1965, Taylor and Schelhart 1967, 1968).

E. coli strains can also be easily identified, however, the identification of the diarrhoegenic *E. coli* strains and thus EIEC strains is extremely tedious to carry out via the traditional approach, or sometimes even impossible (Lányi 1980, Silva et al 1980, Echeverria et al 1991, Nataro et al 1998). The reason for this is that serological or biochemical markers are neither specific nor sensitive enough to identify the pathogen strains. We have to identify a pathogen, which is basically identical to the dominant members of the colon flora at the species level, with genus/species oriented diagnostic methods. On the other hand, although there are some O antigens that occur more frequently amongst the isolated pathogenic strains, the number of other different serogroups is continually increasing. The pathogen groups of *E. coli* appear in serologically well-defined groups. Dysentery-like symptoms are caused by the following serogroups: O28ac, O29, O112, O115, O124, O136, O143, O144, O152, O164, O167. In Hungary the most often isolated ones are O112, O124 and O143. The availability of the sera necessary for the serotyping is limited and they are very expensive. Thus most laboratories only - if at all - test for locally occurring serogroups, precluding new or other serogroups that arise more frequently at present. For example on the basis of this consideration, serogroup O124 is the most-looked for in Hungary.

The main difficulty is presented by the lack of such differentiating features of pathogen *E. coli* strains that could simplify testing. As shown in Table 5, there is no such biochemical reaction that could unequivocally differentiate the diarrhoeagenic strain from the non-pathogenic *E. coli* (Lányi 1980, Silva et al 1980). Some phenotypes often found amongst the isolates were described as lactose negative, lysine decarboxilase negative and non-motile. Unfortunately, these features are not general; EIEC strains are variable, expressing either *Shigella*-like or *E. coli*-like phenotype. The most often occurring and stable marker is lysine negativity, presumably due to the selective advantage provided by the lack of LDC activity.

The problem is that the routinely used enterobacterial media do not provide the possibility of screening for lysine negativity. Lack of motility is also often observed, but this feature makes it impossible to use it as a target marker in routine screening. The lack of lactose fermentation is another possibility because it is an often-occurring marker that is easy to test, however still not as common as lysine negativity, thus

unsuitable for pre-screening. As seen, routinely used bacteriological media cannot provide the possibility of screening these features either because these are not as universal, or because of their nature. These markers are rather useful for pre-screening only – otherwise many isolates could be lost.

Table 5. *Biochemical markers characteristic for invasive Shigella EIEC pathogens and normal apathogen E. coli*

Marker	*Shigella* spp.	*E. coli* spp.	EIEC
Urease	-	-	-
H_2S production	-	-	-
Voges-Poskauer	-	-	-
Indol production	+/-	+	+/-
Metil-red	+	+	+
Xylose	+/-	+/-	+/-
Motility	-	+	-/d
Christensen citrate	-	+/d	-/d
Na acetate	-	+/d	-/+
Na mucate	-	+	-/+
Lysine Decarboxilase (LDC)	-	+	-/d
Gas production from glycose	-	+	+/-
Fermentation:			
Glycose	+	+	+
Lactose	-/late	+	-/late
Sacharose	-/d	+/-	-/+
Salicin	-	+/-	-/+

There are no real biochemical markers that can fully differentiate between E. coli and EIEC. Even though lactose negativity, lysine decarboxilase negativity and, lack of motility is observed in a majority of EIEC isolates, these features are not general, therefore cannot be used for the identification of the pathogen strains.
d: different

Information about the possibilities of selective culturing of EIEC strains is very restricted. A few studies have been conducted to investigate the efficacy of different selective and enrichment media to recover *Shigella*, including media like HA, MAC, XLD, SS and DC and enrichment broths in many variations. Besides the investigation of Silva, comparative studies to examine the behaviour of EIEC have not been carried out extensively (Silva et al 1980). Like *Shigella*, EIEC infections also induce humoral immune response partly against protein, partly against LPS antigens. However, testing antibodies has no diagnostic value and has significance only in sero-epidemiological investigations (Cam et al 1993). On the basis of the above it is clear that with the

methods available to most laboratories at present, the likelihood of detecting EIEC strains is very limited. This is also the reason why our knowledge of the epidemiology and real occurrence of EIEC is so little.

Recent diagnostic methods aim to detect the pathogens on the basis of those features that differentiate them from the normal flora present in most clinical samples. According to the level of expression, these approaches fall into three categories: virulence test, molecular methods, and immunological detection.

Table 6. *Bioassays for invasive shigellosis*

Model	Effects
Oral feeding of sub-human primates	Clinical pictures similar to humans: diarrhoea, intestinal lesions with *Shigellae* in epithelial cells
Oral feeding of starved, opiated guinea pigs	Ulcerative lesions in intestinal tract and death
Injection of *Shigellae* into ligated illeal loops of rabbit	Fluid accumulation
Instillation of *Shigellae* to the eyes of rabbits, guinea pigs or mice	Invasion of corneal epithelial cells with production of keratoconjuncitvitis
Tissue culture cells (e.g. HeLa cells)	*Shigellae* within epithelial cells with destruction of cell monolayer

In vivo animal models and in vitro cell culture models can be used to demonstrate the invasiveness of isolated Shigella. These models also play a role in describing the virulence characteristics and the pathomechanism of the virulent Shigella and EIEC strains.

The so-called virulence tests belong to the first group of methods examining the full or partial expression of virulence. For *Shigella* and EIEC Serény developed a guinea pig keratoconjunctivitis assay, in which virulent *Shigella* strains inoculated into the conjunctival sac of the guinea pig elicit keratoconjunctivitis within three days (Serény 1955). Other assays are based on testing the invasion of the bacteria using epithelial cell cultures. Although these methods are highly specific their application in several routine diagnostic laboratories may present a problem due to their complexity, special requirements, such as an animal-house or the availability of cell culturing (Table 6.).

2.8.2. Molecular methods

The molecular approach aims to detect the genes coding certain virulence markers directly in the sample or after isolating the bacteria. These are the DNA hybridisation and the polymerase chain reaction (PCR) procedures. In PCR a virulence specific region is amplified to an amount that allows the detection by gel electrophoresis or even the quantification. For the amplification oligonucleotide primers of known sequences that hybridise with the required region are used. In the southern blot technique the virulence specific region of the target DNA in the sample hybridises with the known homological sequences.

As the genetic background of the virulence properties of *Shigella* strains have been discovered, gene probes for the invasiveness genes of both EIEC and *Shigella* spp. have also been developed. These have high significance because at the same time EIEC strains bearing virulence specific genetic markers identical to *Shigellae* also became detectable.

The first methods were based upon DNA hybridisation. The most widely spread test so far utilises a 17 kb EcoRI fragment of the invasive plasmid of the strains as a probe, at the beginning using isotope, nowadays non-isotope labelling. Besides, several, other DNA probes were applied successfully, for example using probes for the ipaC and ipaH regions. This method is usually used by hybridising spot cultures of the faeces with the specific probes. Molecular test therefore are often carried out on 3-10 isolated colonies, randomly selected from the samples, in the form of a macrocolony-blot. (Boileau et al 1984, Buysee et al 1995, Echeverria et al 1985, 1989, 1991b, Nataro et al 1998, Oberhelman et al 1993, Sethabutr et al 1985, 1993, Taylor et al 1986, 1988, Venkatesan 1988, 1989, Vieira 1991).

One of the first PCRs to detect *Shigella* and EIEC was based upon the amplification of the uid gene coding for the βglucuronidase enzymes and subsequent DNA blot hybridisation, with successful detection from water samples. The sensitivity of this method was determined by testing serial dilutions of genomic DNA from *E. coli*, by

amplifying with the *uidA* and *uidR* genes followed by DNA hybridisation with radiolabelled probes. As little as 10 fg genomic DNA could be detected which corresponds to one or two bacterial cells. The specificity of the primers was determined by testing DNA from other bacterial strains, showing no interference with the PCR amplification, suggesting that these regions are unique and conserved in *Shigella* and *E. coli* species (Bej et al 1991). Another early PCR was designed to detect these pathogens from food by the amplification of a 760 bp region in a HindIII fragment of the 220 kbp invasive plasmid (Lampel et al 1990). Extracting the nucleic acids directly from stool and environmental specimens, PCR can detect the pathogens even without culturing (Frankel et al 1990). This is clearly advantageous in case of water and food samples, where the number of pathogens can be relatively low, and during culturing in broth they could be overgrown by the microflora. The other advantage of the method is that due to the similarity in virulence genes it can detect both *Shigella* and EIEC strains (Venkatesan et al 1989, Echeverria et al 1991b, Faruque et al 2002, Villalobo and Torres 1998).

Later on other different PCR systems were developed and spread. Most of these use primers specific for the *ipaH* gene, which is a multicopy, both chromosomal and plasmid gene with a thus far unclear function (Fernandez-Prada et al 2000). Several other, *Shigella*/EIEC specific genes are also suitable for developing diagnostic methods based on PCR, even in multiplex form (Bej et al 1990ab, 1991, Frankel et al 1990, Jackson et al 1991, de Lamballerie et al 1992, Islam et al 1992,) Systems, detecting both *Shigella*/EIEC, or verotoxin and enterotoxin producing *E. coli* strains (VTEC, ETEC), plus ETEC producing ST and LT, or *Shigella*/EIEC, ETEC and Campylobacter have been successfully used (Frankel et al 1989, Oyofo et al 1996). For example Houng and co-workers developed a *Shigella* serotype specific multiplex system (Houng et al 1997).

Since the isolation of the pathogen is not carried out, the molecular approach does not distinguish between *Shigella* and EIEC, leaving the epidemiological questions unanswered. In addition, the success of the molecular approach depends on the ratio of the pathogen in the sample, or on the number of isolates that are tested. Although in dysentery cases the number of pathogens is usually high, in some cases, for example with less severe clinical symptoms or symptomless carriage of the strain, the number of

pathogens shed can be very low. If the ratio of the pathogen colonies on the culture plate is high enough, there is a greater chance of picking up the right clone for these tests. Different selective media are used to increase this ratio, however, very little is known about the behaviour of EIEC strains on these plates. According to Silva, Salmonella-*Shigella* (SS) was the most inhibitory medium for EIEC, amongst MacConkey (MAC), Xylose Lysine Desoxycholate (XLD) and Hektoen agars. In our experiments, we aimed to examine the efficacy of different selective medium in supporting the growth of EIEC, compared to *Shigella*, and normal *E. coli* strains as controls.

2.8.3. Immunological methods

The third group contains those approaches that aim to detect the virulence factor of the microbes itself. Pathogen bacteria could be identified or detected by the virulence specific antigens produced by them (Murray et al 1995). Probes specific for the capsid antigen, and assays detecting toxins according to their antigenicity belong to this approach. Methodically, gel precipitation, counter-immunoelectrophoresis, the different immunfluorescent methods, co-agglutination and ELISA are the methods most often in use. (Shiba 1998, Notermans et al 1991, Alexander et al 1985, Belmaaza et al 1986, Bibb et al 1984, Lu et al 1997, See et al 1989, Donohue-Rolfe et al 1986). The advantage of these methods is that they do not need the special and expensive equipment necessary for the molecular methods, and they are also technically simpler.

At the beginning of the 80's the antigenic relationship between each virulent *Shigella* and EIEC isolated was described in our institute, due to an antigen called at that time Virulence Marker Antigen (VMA) (Pál et al 1983). Pál and collaborators developed an ELISA using a polyclonal antibody specific for the so-called Virulence Marker Antigen (VMA) of *Shigellae* and EIEC. Rabbits were immunised with EIEC O143 enteroinvasive strain, and the resulting serum was absorbed with the non-virulent derivative of the same strain, thus not containing O and K antigens. The serum therefore was specific only for virulent *Shigella* and EIEC strains. 83 strains positive by the Serény virulence test were verified to be positive, whereas Serény negative strains such as *Salmonella*, *Yersinia enterolitica*, *Proteus*, *Klebsiella*, EPEC and ETEC were

negative tested by this ELISA. The Virulence Marker Antigen was later proved to be encoded on the invasion plasmid, and in the Western blot it was shown to be identical with the IpaC invasion protein. The VMA ELISA was proved to be effective and a simple way of detecting the invasive strains. It was used in a field-study in Thailand examining childhood dysentery. Altogether from 200 children 5276 *E. coli* strains were isolated, and 2363 analysed. 64 isolates were found to be positive by the VMA ELISA, corresponding to the results of the Ip specific DNA probe and Serény test (Pál et al 1985a,b, 1986, 1989c).

Since standardisation of an ELISA using a polyclonal antibody is rather difficult due to the many variants, besides the difficulty of producing it, a new version of the VMA ELISA, the MAIC-1 ELISA using monoclonal antibodies was developed, with the same sensitivity. It is highly specific, recognising all the invasive *Shigella* and EIEC strains but none of the non-invasive ones of other species and genera; it can therefore be a useful diagnostic method in the identification of bacillary dysentery (Floderus et al 1995, Pál et al 1997, 1998). This technique is simple and inexpensive enough to substitute for cumbersome virulence tests. However, this ELISA was only suitable for testing previously isolated colonies, not for primocultures, faecal or environmental samples, therefore its sensitivity was limited by the number of isolates tested.

Detection systems of isolated colonies of different other microorgamisms by immune sorbent assay have been used in a wide range. Dot blots have been applied for the identification of strains like SLT producing *E. coli* (Strockbine et al 1985) *Bordetella pertussis* (Gustafsson et al 1989), *Neisseria gonorrhoeae* (Schneider 1988), *Brucella abortus* (Roop et al 1987). The colony blot method can be used not only on isolated colonies but also directly on the sample spread on the culture plate, screening a greater number of bacteria. Recently, in this latter form, the colony blot method was developed for the identification of colonies of pathogens like *Clostridium botulinum* (Goodnough et al 1993), TSST-1 producing *Staphylococcus aureus* (See et al 1989), *Pediococcus acidilactici* (Bhunia et al 1992), *Rhodococcus equi* (Takai 1993), Shiga-toxin producing *E. coli* (Hull et al 1993) and SLT producing *E. coli* (Law et al 1994), *Salmonella* (Hoszowski et al 1996), *Listeria* (Wieckowska-Szakiel et al 2002) and *Legionella* (Steinmetz et al 1992).

The basic idea of our method originated from the experiments of Hull and co-workers. They had developed an immunoblot assay specific for the Shiga toxin to detect the Shiga toxin producing *Shigella* (Hull et al 1993). In our experiments, modifying their method using the MAIC-1 monoclonal antibody we explored the possibility of using this antibody in a model for the detection of *Shigella* and EIEC from clinical and environmental samples.

3 OBJECTIVES OF THE STUDY

The specific objectives of the present study were the following:

Short-term aims

1. To develop the colony immunoblot method for detecting *Shigella* and enteroinvasive *E. coli* strains
2. To apply the method for stool, water and food samples
3. To investigate the efficiency of the different selective and enrichment media in the diagnosis of enteroinvasive *Escherichia coli*

Long-term aims

4. To develop straightforward, affordable diagnostic methods to improve our knowledge on the significance and epidemiology of enteroinvasive *E. coli*.

4 MATERIALS AND METHODS

4.1. Bacterial strains (papers I-III)

Bacterial strains used in the present experiments are described in further details in the Materials and Methods section of individual articles.

As positive controls wild, fully virulent *S. flexneri 2a* strains 217-14-18 and YSH6000, Lac^+ EIEC isolates of serogroups O28 and O164, as negative controls *S. flexneri* 2a non-virulent derivate strains YSH6200, F-492, non-virulent *E. coli* O8, J53 *E. coli* K-12 were used.

In addition, 59 virulent *Shigella* strains representing all four species (33 *Shigella flexneri*, 18 *Shigella sonnei*, 3 *Shigella boydii* and 5 *Shigella dysenteriae* isolates), 80 virulent EIEC strains of serogroups O28, O29, O112, O121, O124, O136, O143, O152, O164, O167, O171, a further 100 strains of *Salmonella, Klebsiella, Enterobacter*, enterotoxigenic-, enterohaemorrhagic-, enteropathogenic *E. coli*, enterococcus, *Staphylococcus*, *Proteus*, *Pseudomonas*, were used for the development and application of the colony immunoblot method for environmental and clinical samples, and 53 non-enteric pathogen *E. coli* strains isolated from healthy adults, and 22 *Salmonella enterica* strains representing serovars Typhymurium, Enteritidis and Choleraesuis. Bacterial strains were stored in glycerine TSB at -80 ^oC.

The strains were partly from our strain collection and were partly donated by C. Sasakawa (Japan), S. Formal (USA), P. Echeverria (Thailand) and L. Trabulsi (Brazil).

4.2. Media (papers I-III)

For optimising the colony blot assay and for the artificial contamination of faecal and milk samples bacterial strains were grown in Tryptic Soy Broth (TSB, Scharlau Chemie) overnight at 37 ^oC on a rotary shaker. For the colony immunoblot method TSA plates, for the PCR Tryptic Soy Broth (TSB) was used. In order to prevent the overgrowth of colonies by swarming *Proteus* strains present in faecal specimens, all blots were prepared from Tryptic Soy Agar plates (TSA) made from TSB with 3 % agar-agar added (paper I). Stool and milk samples were cultured on Eosin Methylene Blue (EMB), Desoxycholat-citrate (DC) (both OXOID), and TSA plates. *Shigella*–like

colonies isolated from stool and food samples were verified on Nógrády-polytrop media.

To test the specificity of the immune assay blots were prepared from various plates inoculated with cultures of non-enteroinvasive strains, or with invasive strains mixed with *E. coli* J53 at different ratios.

For testing the growth of EIEC on different media, as differentiating plates EMB and McConkey (MAC), as selective plates DC, Salmonella-*Shigella* agar (SS) and Xylose-lysine decarboxylase (XLD) (all OXOID), as liquid selective-enrichment media Selenite F Broth and Gram Negative Broth (Scharlau) were used. The presence of the invasion plasmid of *Shigella* and EIEC strains was ensured by culturing on TSA plates containing Congo Red (Sigma) (Maurelli et al., 1984b).

4.3. MAIC-1 antibody (papers I-III)

IpaC specific MAIC-1 (Floderus et al., 1995) antibodies were produced by the hybrydome cells cultured in RPMI-1640 media without foetal calf serum (FSC) and the supernatant was used. Cells were grown up to confluent stage in 10% FSC cell culturing medium, then further on in media without FSC for 5-7 days. After centrifugation 0.1 % NaN_3 was added to the supernatant and stored at 4 ^{o}C. For each aliquot the optimal working dilution was determined by pre-testing both for the ELISA and the colony immunoblot method.

4.4. Virulence tests verifying enteroinvasive strains (papers I-III)

4.4.1. IpaC ELISA

The IpaC specific monoclonal antibody, MAIC-1 (Floderus et al., 1995), was used to test clones selected and inoculated into wells of flat bottom ELISA plates (Linbro, Flow Laboratories or Costar) containing 200 µl of TSB. When investigating artificially contaminated faecal or milk samples, 16 randomly selected colonies were tested from each specimen. At least one column of the ELISA plate was left non-inoculated as

negative control. ELISA plates were incubated overnight at 37 ^{o}C, during that pathogen microbes secreted the IpaC antigen and sensitised the wells. Next day wells were blocked by bovine serum albumin (BSA) and incubated for one hour in IpaC specific MAIC-1 antibodies and after a washing step anti-mouse horseradish peroxidase conjugate was added for one hour incubation at room temperature (DAKO). The reaction was stopped by adding 20% Sulphuric Acid, and results were read by an ELISA reader at 492 nm as described (Floderus et al., 1995; Pál et al. 1997).

4.4.2. Plasmid electrophoresis

The presence of the invasive plasmid was detected by the plasmid extraction method of Kado and Liu, followed by agarose-gel-electrophoresis in 1% agarose. Gels were stained with Ethydium-Bromide and visualised under a UV detector, photographs were taken by a Polaroid camera or image analysing computer system (BioCapture) (Kado and Liu 1981).

4.4.3. Cell invasion test

HeLa or HEp-2 cells were grown up to semi-confluent stage in 30 mm diameter cell culturing plates (Nunc) (Dulbecco's Modified Eagle's medium, GIBCO + 10 % FCS). Each plate was contaminated with 10^9 logarithmic phase bacteria cultured in TSB. Host-microbe contact was promoted by centrifugation of contaminated plates at low speed (700 rpm) for 10 minutes. After incubation for 60 minutes on 37 ^{o}C, 5 % CO_2 cells were washed four times in PBS, then fixed in methanol and stained with Giemsa. The presence of intracellular microbes in the cell was detected by immersion light microscopy by their characteristic morphologic feature (Pál et al 1989a).

When the aim was to select intracellular microbes for further culturing, after the incubation step cells were washed and further incubated in culturing liquid containing 40 μg/ml gentamycin to kill extracellular bacteria. Cells were dissolved by 0.1 % Triton and spread on TSA plates for culturing bacteria.

4.4.4. Guinea pig keratoconjunctivitis test (Serény test)

In order to select virulent clones of EIEC or *Shigella* for the artificial contamination of samples, or to assess the virulence of isolated strains from clinical specimens, strains were inoculated into the conjunctival sac of adult, out-bread guinea pigs, as described, then isolated back from the purulent keratoconjuctivitis (Serény, 1955).

4.5. Preparation of the bacterial cultures for the artificial contamination of samples (paper I-III)

Shigella and EIEC strains were previously tested for virulence by cell invasion test, plasmid detection or MAIC-1 ELISA. In order to ensure their invasive character, in all experiments, EIEC and *Shigella* colonies pigmented on Congo Red agar (Maurelli et al., 1984c) were used, only. Microbes used in spiking experiments were grown in TSB medium overnight at 37 ^{o}C a in rotary shaker thermostat, the resulting culture was centrifuged, washed and suspended in phosphate buffered saline, pH 7.2 (PBS). A standard curve was used to determine the number of colony forming units of the culture by measuring the optical density at 600 nm. Dilution series were prepared in PBS to spike water, faecal and milk samples.

4.6. The colony immunoblot method (papers I-III)

After incubation membranes were carefully taken off the plates carrying the antigens absorbed during the growth of colonies. Filters carrying the blots of colonies were incubated in 10 ml chloroform for 20 min to kill bacteria. This was followed by the extensive washing of the membranes in phosphate buffered saline (PBS) on an orbital shaker with 100 rpm (4 times 5 min in 20 ml aliquots) to remove the solvent and the bacterial debris.

After three rinses in PBS the membranes were incubated in 2 % skimmed milk for two hours at room temperature to block the free binding sites. A cell-free tissue culture supernatant diluted to 1:100 in 0.5 % skimmed milk containing the monoclonal antibody MAIC-1 (Floderus et al., 1995) was added to the filters for 1 hour, at room

temperature. This antibody specifically reacts with the invasion plasmid coded protein IpaC secreted by virulent *Shigella* and EIEC strains (Floderus et al., 1995). After three washing steps in PBS, anti-mouse Ig-alkaline phosphatase conjugate (DAKO) diluted 1:1000 was added, also for 1 hour at room temperature. The reaction was developed with Fast Red TR Salt - Naphtol AS MX Phosphate substrate (SIGMA-Aldrich GmbH) as previously described (Pál et al., 1989).

In case of faecal samples to eliminate the endogenous alkaline phosphatase (AP) activity regularly present in faecal samples (Horrigan and Danovitch, 1974) and interfering with the detection of the immune reaction (Hull et al., 1993) filters were soaked in 0.5 % Tween 20 for 10 minutes followed by 10 min at 100 ^{o}C in a hot oven. After blocking free binding sites the immune reaction was carried out as described.

4.7. Testing water samples (paper I)

Different amounts of sterile distilled water, physiological salt solution or tap water de-chlorinated with 80 mg 1^{-1} sodium tiosulphate were spiked with dilutions of virulent or non-virulent bacteria. The number of colony forming units for the spiking was extrapolated from the standard curve described above. Samples were filtered through a 0.45 μm nitrocellulose membrane of 47 mm diameter (Milliopore). Membranes were placed directly onto TSA plates for the colony immunoblot assay or into TSB for the PCR, and incubated overnight at 37 ^{o}C. When samples were spiked with high numbers of pathogen and non-pathogen microbes, the membranes were first placed onto selective DC plates and incubated overnight. The resulting culture was either further cultured for PCR or passaged onto 2-2 DC and EMB plates, or preparing an optimal suspension, re-filtered for the colony immunoblot.

4.8. Optimising the colony immunoblot method for faecal samples (paper II)

For the optimisation of the method for faecal samples three different blotting protocols were compared. In the replica-plating technique TSA plates were flood-inoculated with 500 μl aliquots of serially diluted samples and incubated overnight at 37 ^{o}C. Next day, a 82 mm nitrocellulose membrane (0.45μm, Sartorius) laid over a fresh TSA plate was replica-inoculated with a sterile velvet-disk from a master plate containing

approximately 500 colonies, and incubated at 37 ^{o}C, overnight (Hull et al., 1993). Alternatively, plates with similar colony counts were overlaid with the filters and the colonies were blotted at 37 ^{o}C for two hours. Finally, plates inoculated with a pre-determined dilution of the samples were covered with the filters and incubated overnight at 37 ^{o}C allowing the colonies to develop under the membranes. Orientation of plates and filters were marked for subsequent identification of colonies positive with the immune assay. Beyond flood-inoculation, when testing faecal samples, a set of TSA plates were also loop-inoculated with undiluted samples. After covering the plates with the filters they were incubated overnight at 37 ^{o}C. This procedure regularly resulted in approximately 200-300 isolated colonies.

4.9. Elimination of the enzyme activity of faecal samples (paper II)

A pre-treatment protocol of the membrane filters was developed to cover the possibility of samples having their own enzyme activity that would disturb the detection of the immune reaction (giving a false positive background). Faecal specimens, as well as IpaC positive and negative strains, were streak-inoculated onto TSA plates and covered by nitrocellulose membranes. After overnight incubation, the membranes were removed, treated with chloroform, washed and cut into strips perpendicular to the direction of inoculation. The strips were immersed into 0.1%, 0.5%, and 1% of Tween 20, Triton X 100, Formalin, β-Mercaptoethanol, into 100 mM EDTA, or into 1 mM Levamisole, respectively for 10 and 60 minutes, at room temperature. All chemicals were purchased from SIGMA-Aldrich GmbH. Other strips, placed on sheets of paper towels, were treated at 60 °C, 80 °C and 100 °C for 10 and 60 minutes in a hot oven. Chemical and heat treatment was also combined by exposing filters to the above solutions for 10 minutes at room temperature followed by incubation at 100 ^{o}C for 10 or 60 minutes. After this procedures membranes were rinsed three times in PBS. This was either followed by the immediate incubation in the enzyme substrate solution to detect any residual enzyme activity or, after incubation in skimmed milk, by treating the filters with the monoclonal antibody for the immune reaction.

4.10. Artificial contamination of faecal samples (paper II)

For the artificial contamination of faecal samples, both in the case of *Shigella* and EIEC, previously verified enteropathogen negative faecal samples were used.
For artificial contamination with *Shigella*, negative stool samples were obtained from the Baranya County Laboratory where they had been tested routinely enteric bacterial pathogens. These samples were cultured on EMB plates in order to select samples containing only lactose positive colonies. These were only used by us further on. Samples were diluted in 1:1 PBS and homogenised. 1-1 ml volumes were contaminated by virulent lactose negative *Shigella* strains. Stool samples were then spread on EMB and DC plates for conventional bacteriological tests and for TSA for the colony blot method. The TSA plates were covered by 80 mm diameter 0.45 μm pore size nitrocellulose membranes (Millipore) and incubated overnight at 37 ^{o}C degrees. From the DC and EMB plates *Shigella*-like lactose negative colonies were isolated after 18 hours of incubation and identified by the standard biochemical and serological methods.

For artificial contamination with EIEC, twenty faecal specimens, 10 of them with intrinsic AP activity, were obtained. Each sample was diluted to contain approximately 10^9 coliform CFU / ml, and subsequently contaminated with a serial dilution of a virulent EIEC strain of serogroup O164. The samples were submitted to three different tests: the *ipa*H-specific PCR, the IpaC-specific ELISA investigating 16 randomly selected colonies, and the colony immunoblot assay. For the latter method TSA plates were either flood-inoculated with samples further diluted to yield approximately 500 colonies per plate, or were loop-inoculated with the contaminated samples without further dilution.

4.11. Bacteriological examination of clinical faecal specimens (paper II)

165 faecal samples, including 44 repetitive specimens, from a total of 121 patients, received by the Microbiology Laboratory, Mubarak Al-Kabeer Hospital, Kuwait with the diagnosis of “diarrhoea”, “enteritis” or “dysentery” were processed according to the guidelines for hospital laboratories in Kuwait for common bacterial enteric pathogens, i.e. *Salmonella, Shigella, Campylobacter* and enteropathogenic *E. coli* (Johny et al.,

1994). Briefly, MacConkey (Oxoid) and salmonella-*Shigella* agar plates (Difco Laboratories) were inoculated before and after Selenite-F broth enrichment (Mast Laboratories). Colonies suspected for *Shigella* or *Salmonella* were inoculated into Kligler's iron agar slopes (Mast Laboratories) and into urea broth. Cultures showing reactions suggestive of *Shigella* or *Salmonella* were serogrouped with antisera (Murex Diagnostics). The species was confirmed in the Vitek automatic system using the Vitek GNI card (bioMerieux). The presence of enteropathogenic *E. coli* was investigated by the agglutination of ten colonies from the MacConkey plates with the diagnostic pooled sera 2, 3, and 4 for pathogenic *E. coli* (Murex Diagnostics). *Campylobacter* strains were isolated from Karmali's Campylobacter agar (Oxoid) incubated at 42 ^{o}C. The O antigen of EIEC strains identified by the immunoblot technique was determined by slide agglutination using immune sera prepared by injecting rabbits intravenously with boiled cells of EIEC strains of known serogroups.

4.12. Testing milk samples

Milk samples of 50-50 ml were artificially contaminated with *Shigella* or lactose positive EIEC, so that the samples contained 1-1000 bacterial cells per 1 ml. Different procedures were tested to process milk samples. Samples were either filtered through a nitrocellulose membrane filter or centrifuged at 4500 g for 10 minutes and a loopful of the pellet was spread onto TSA plates for the colony immunoblot assay, or EMB and DC for routine test. TSA plates were covered with 0.45 μm nitrocellulose membrane, and incubated overnight at 37 ^{o}C. The membranes were carefully removed the next day from the plates and the immune reaction was performed. After 18 hours of incubation 8-8 colonies were picked up randomly from the control EMB and DC plates and passaged to Congo Red TSA as microcolonies for further tests.

4.13. IpaH PCR (papers I-III)

A PCR system specific to the *ipa*H gene of *Shigella* or EIEC was used as a control method to indicate the presence of *Shigella* and EIEC strains. (Sethabutr et al., 1993; Venkatesan et al., 1989) To detect DNA sequences in water samples, membranes were transferred into Petri dishes containing 10 ml TSB and incubated overnight. The next

day one ml of the resulting culture was washed and centrifuged, and the pellet was extracted with 125 µl of DNA Extraction Reagent, (Perkin Elmer) by boiling for 30 minutes.

Faecal samples were inoculated onto TSA plates. The next day, approximately one quarter of the culture was suspended into 1 ml of distilled water and extracted as described (De Lamballerie et al. 1992, paper I). After centrifugation with 13,000 g for two minutes, the supernatants were used as samples in the PCR reaction with a pair of primers specific to the *ipa*H gene present on both the invasion plasmid and the chromosome of the pathogens (Sethabutr et al., 1993) using a PCR mix (GIBCO BRL). Electrophoresis of the amplified products was carried out in 1.2 % agarose gel, stained with Ethidium Bromide and visualised under UV light. Results were documented by taking Polaroid photographs or using an image analyser computer system (Biocapture).

4.14. DNA hybridisation (paper I)

The membranes covering the colonies grown overnight were hybridised at 65 ^{o}C with a 11.5 kbase EcoRI fragment as P^{32} probe of the *Shigella* invasion plasmid (Boileau et al 1984), as described (Milch et al. 1997).

4.15. Growth of isolated strains on solid media (paper III)

Strains were inoculated into Tryptic Soy Broth (TSB, Scharlau Chemie, Barcelona, Spain) and incubated overnight at 37 ^{o}C with shaking (180 rpm). Next day cells were washed once in phosphate buffered saline, pH 7.2, and suspended in the same buffer to yield approximately 500-800 colonies on TSA plates after spreading 100 µl aliquots onto them. Identical aliquots were also inoculated onto MAC, EMB, DC, SS and XLD plates, respectively, all purchased from OXOID (Basingstoke, Hampshire, England). The plating efficiency (PE) was expressed as the percentage of colonies on selective or differentiating plates compared to that on non-selective TSA plates. To compare the growth of pathogenic strains relative to that of the members of the normal flora an enrichment factor (EF) was calculated: The colony count of each strain on a selective plate, expressed as the percentage of its colony count seen on TSA, was divided by the

average of the same figures obtained from the 53 normal *Escherichia coli* isolates. All experiments were done in triplicate.

4.16. Growth of isolated strains in liquid media

The effect of liquid selective enrichment media was examined by studying the change of the c.f.u. value in the media. From the bacterial dilutions 10-10 µl was inoculated into 2-2 ml Selenite Broth or Gram Negative Broth and vortexed. Volumes of 30-30 µl were taken immediately from the inoculated broth to determine the c.f.u. of the culture at 0 hour. Dilution series were made in PBS from the inoculated broth before incubation. The inoculated broths were incubated for 6 hours at 37 ^{o}C in a non-shaking thermostat. Similarly, after incubation, dilution series (10^{-1} -10^{-8}) were made from the cultures as described above. From each dilution 10-10 µl was carefully distilled onto TSA plates and incubated at 37 ^{o}C overnight. The number of colony forming units was determined visually next day using a colony counter (Quebec).

4.17. Artificial contamination and selective culture of faecal samples (paper III)

Faecal samples from three healthy individuals were used throughout these experiments. The specimens did not yield any Lac^{-} or *Shigella*-like colonies on MAC, EMB, DC, SS and XLD plates. Samples were homogenized in PBS to contain ca. 0.9-1 x 10^{10} coliform cells/g faeces. Nine EIEC strains with different growth capacities on selective media (see Results) were mixed individually to all three faecal samples, in duplicates. In order to facilitate the recognition of EIEC Lac^{-} strains only were used in these experiments. The actual pathogen to non-pathogen ratio in the contaminated samples was set to be approximately 1:10 and 1:100, respectively, as checked by testing, with the IpaC-specific ELISA, 500 colonies randomly sampled from the non-selective TSA plates. From all MAC, EMB, DC, SS and XLD plates, respectively 10 Lac^{-} colonies (or all if less seen), and 10 Lac^{+} ones were also tested by ELISA. In all experiments, from all plates, all Lac^{-} colonies did, while none of the Lac^{+} ones tested gave a positive reaction to the IpaC-specific immune assay (data not shown). Therefore the first group was considered to represent EIEC, while the lactose fermenter ones were considered to be the members of the original, normal flora of the samples. For each strain an enrichment

factor was calculated by dividing the ratio of EIEC colonies on a particular differentiating or selective plate (i.e. that of the Lac^- colonies) by the ratio of the pathogen in the contaminated samples (i.e. the proportion of IpaC positive colonies on the respective, non-selective TSA plates).

4.18. Assessment of selective media in the recovery of EIEC from clinical faecal samples (paper III)

Two hundred and fifty faecal samples received by the Microbiological Laboratory of Mubarak Al-Kabeer Hospital, Kuwait with the clinical diagnosis of "diarrhoea" or "dysentery" were processed according to the standard laboratory protocols to search for common enteric pathogens (i.e. *Salmonella*, *Shigella*, *Campylobacter*) [22]. All samples were also subjected to the *ipa*H-specific PCR. Based on the results of PCR, and on that of the microbiological investigations specimens were grouped as PCR negative/*Shigella* negative; PCR positive/*Shigella* positive; and PCR positive/*Shigella* negative samples, respectively. This latter group was considered as "tentatively EIEC positive". No PCR negative/*Shigella* positive specimen was detected.

Simultaneously, the specimens were also suspended in PBS and spread onto the surface of two TSA, MAC, EMB, DC, XLD and SS plates respectively, and incubated for 18 hours at 37 ^oC. To prevent swarming of *Proteus* strains the agar concentration of TSA plates was increased to 3 % in these experiments. This, as was shown before [21], did not alter the colony-forming capacity, or the reaction with the IpaC specific antibodies of EIEC, *Shigella* or normal *E. coli* strains. Samples were also inoculated into Selenite (SF) and Gram Negative Broth (GNB) (both from Scharlau Chemie, Barcelona, Spain). After six, 12 and 18 hours of incubation at 37 ^oC these liquid media were sub-cultured onto a similar set of plates as used for primo-cultures. After overnight incubation the plates were saved at 4 ^oC for not more than 72 hours for subsequent IpaC ELISA until the PCR results became available. According to preliminary experiments with colonies of a set of 25 EIEC and 25 *Shigella* strains keeping the cultures at 4 ^oC for this period of time did not interfere with the isolates' reactivity in the subsequent immune assay (data not shown).

To asses the ratio of EIEC or *Shigella* colonies on the different media, from plates saved at 4 ^{o}C and grouped according to the PCR and culture results, 60 – 60 randomly selected colonies were subjected to the IpaC-specific ELISA. By picking all colonies from pre-marked areas of the plates random colony testing was strictly followed even from media indicating phenotypes (e.g. Lac^{-} or lysine decarboxylase negative colonies) suggestive of *Shigella* or EIEC. This was necessary to ensure the comparison between the proportions of pathogens on differentiating-selective media to that on non-differentiating, non-selective TSA plates. The figures obtained on this latter medium were considered to indicate the ratio of the pathogens in the samples. By comparing the ratios of IpaC positive colonies (i.e. EIEC or *Shigella*) identified from the differentiating or from selective plates to that found on non-selective TSA plates an EF for each media was calculated.

ELISA was carried out from all primo-culture plates and from sub-culture plates following 18 hours enrichment in liquid media, irrespective of the culture and PCR results. For PCR positive/*Shigella* negative (i.e. tentatively EIEC positive) samples colonies from plates inoculated after six and 12 hours of pre-incubation in SF or GNB were also subjected to ELISA. ELISA positive colonies were identified as *Escherichia coli* or *Shigella* by standard biochemical assays and by serotyping (Johny et al 1994).

4.19. Statistical analysis (papers I-III)

The sensitivity of the different techniques in detecting *Shigella* or EIEC in artificially contaminated water or faecal samples was compared using the McNemar's non-parametric test for two related dichotomous variables (Altman 1994).

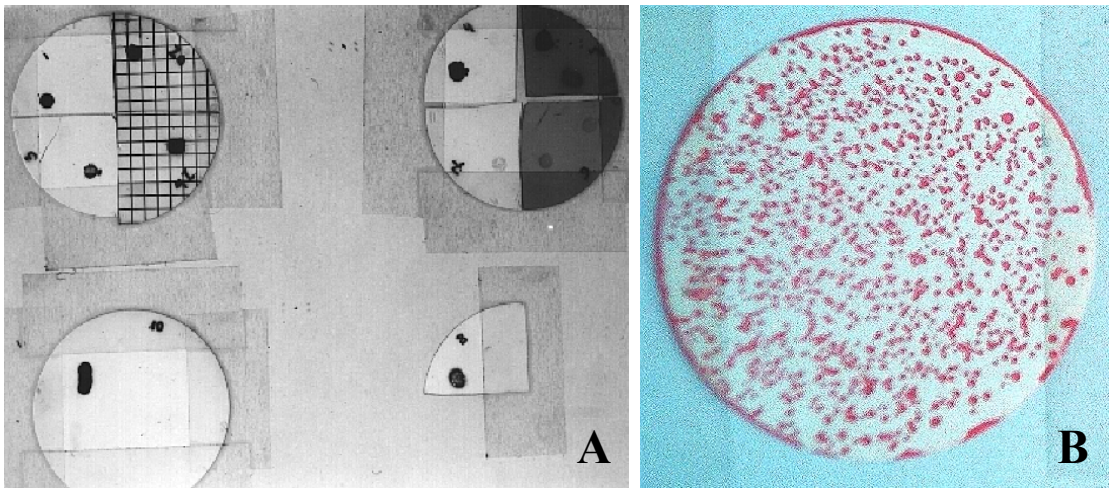
The statistical comparison of the different media was carried out using the Wilcoxon signed-ranks test and the Spearman's rank correlation test. The Mann-Whitney test was used to analyse the growth of different groups of strains cultured under the same conditions

5 RESULTS

5.1. Developing the *Shigella* /EIEC specific colony immunoblot method (paper I)

The first step was to examine whether *Shigella* and EIEC strains secret the IpaC antigen in an amount that would be detectable by the colony immunoblot technique. Virulent strains as microcolonies were inoculated onto various plates then covered with various membranes. Parallelly, membranes were placed on TSA plates and the strains were inoculated on to their upper side. After overnight incubation the membranes were processed by the immune reaction using the IpaC specific MAIC-1 antibodies. The result showed that on TSA plates enough IpaC was excreted and absorbed into the nitrocellulose membrane filters to give an intense colour reaction in the form of clearly visible dots with the substrate (see Figure 4.) When the colonies were growing covered by the membrane, the resulting red spots were more compact with a well-defined edge. However, when strains were inoculated on top of the membrane, the spots were rather dim – probably due to the diffusion of the IpaC antigen – therefore subsequently the inoculation under the membrane was performed.

Figure 4. *IpaC specific immunoblot assays of Shigella after the immune reaction*



As a result of intensive colour reaction with the substrate, red dots formed where the IpaC antigen of the colonies absorbed into the nitrocellulose membrane. (A) Macrocolonies of Shigella grown under various membranes. (B) Isolated colonies of Shigella grown on TSA..

20 *Shigella* and 80 EIEC strains were tested in the form of microcolonies by the colony blot method, all strains giving clearly visible and recognisable dots. *Shigella* strains were observed to give slightly stronger colour. There was no difference between the reactivity of Lac^- or Lac^+ EIEC strains. There were no false positive reactions when membranes not inoculated with invasive strains were processed, or when inoculated with non virulent or non pathogen bacteria, including 100 strains of *Salmonella*, *Klebsiella*, *Enterobacter*, enterotoxigenic-, enterohaemorrhagic-, enteropathogenic and normal, faecal *E. coli*, enterococcus, *Staphylococcus*, *Proteus* and *Pseudomonas* isolates. No difference was observed when contaminating sterile saline, distilled water or de-chlorinated tap water samples.

Besides TSA for the purpose of the colony immunoblot, other plates, more or less selective or selective-differentiating for enteric pathogens were tested. Although all the tested plates EO, Endo, MacConkey, DC and SS allowed the growth of *Shigella* or EIEC macrocolonies, the expression of IpaC antigen was more inhibited compared to TSA. In addition, the indicator dyes used in the selective plates interfered with the colour of the immune reaction. Therefore TSA was used for the colony blot method in subsequent experiments.

In stool as well as in environmental samples, the presence of highly motile *Proteus* strains can be predictable, and as they can easily overgrow other colonies due to their swarming, they might potentially inhibit the detection of pathogens. To avoid this kind of interference, instead of using chemical swarming inhibitors, we tried to increase the concentration of agar-agar of the TSA plates. As tested by using different TSA plates containing increasing concentration of agar-agar, it was shown that at 3% agar-agar concentration the swarming of *Proteus* strains was inhibited while the expression of IpaC stayed undisturbed. As a result of these findings, this TSA plate was used in the subsequent experiments.

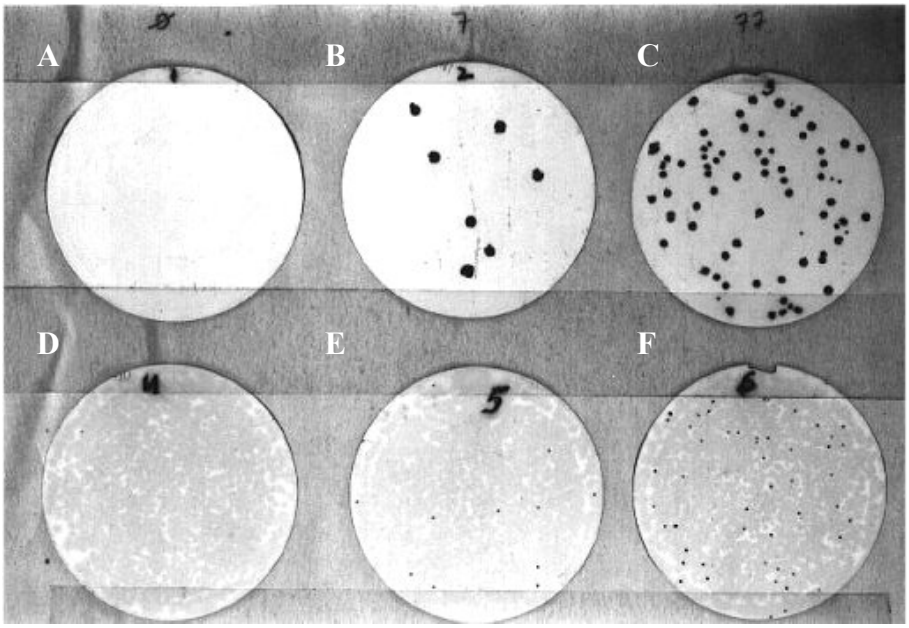
5.2. Applying the colony immunoblot method for different clinical and environmental samples

5.2.1. Applying the colony immunoblot method for water samples (paper I)

5.2.1.1. Optimising the colony immunoblot method for water samples

100 ml samples artificially contaminated with 1-30 *Shigella* or EIEC cells were filtered through nitrocellulose membranes. Membranes were subsequently incubated on TSA plates and after carrying out the immune reaction well defined red spots could be observed on the membrane showing the growth of the pathogen colony underneath. When filtering sterile liquid samples or ones not containing enteroinvasive pathogens, no colourisation of the membrane was observed. (Figure 5.)

Figure 5. *Water sample artificially contaminated with Shigella tested by the colony immunoblot method*



(A) 100 ml sterile water sample, (B) water sample containing 5-8 CFU Shigella, (C) water sample containing 50-80 CFU Shigella, (D) water sample containing only normal E coli, (E) water sample containing normal E coli plus 5-8 CFU Shigella, (F) water sample containing normal E coli plus 50-80 CFU Shigella.

When *Shigella* or EIEC was the only bacterium present in the water sample, even 1-5 cells could be detected by the colony immunoblot method, irrespective of the volume of

the liquid filtered. For example testing water samples of 10 litres contaminated with 1-5 *Shigella* cells, nine samples out of ten were positive by the colony blot method, while all were confirmed by PCR.

5.2.1.2. Detection of invasive bacteria in the presence of non-pathogenic strains

When the total cell count of contaminating bacteria was increased with non-pathogenic bacteria (i.e. *E. coli* or *Proteus*), the size of the colonies, and consequently the size of dots of *Shigella* or EIEC after the immune reaction, decreased proportionally. A total of 2000 cells of normal *E. coli* per sample in the *Shigella* or EIEC colonies were still visible as intense red pinpoint dots. Samples containing 2-5 *Shigella* cells and a gradually increasing number of non-pathogenic *E. coli* cells were tested by the colony immunoblot, PCR and DNA hybridisation methods (Table 7.). In the presence of 2-3 x 10^3 *E. coli* all three methods were able to detect the pathogenic bacteria in the samples, whereas PCR, as the most sensitive method, could detect them even in the presence of 2-3 x 10^4 non-pathogenic *E. coli* flora.

Table 7. *Identification of Shigella by IpaC specific colony immunoblot, by DNA probe and by PCR in the presence of non-pathogenic E. coli*

Number of cells in the sample		Assay results		
Shigella	*E. coli*	Colony blot	DNS probe	PCR
2-5	0	+	+	+
2-5	2-3	+	+	+
2-5	2-3x10^1	+	+	+
2-5	2-3x10^2	+	+	+
2-5	2-3x10^3	+	+	+
2-5	2-3x10^4	-	ND†	+
0	2-3x10^3	-	-	-

** 100 ml aliquots of 0.9 % NaCl were contaminated with the germs indicated*
†ND: Not done

5.2.1.3. Comparison of the sensitivities of the colony blot, the culture based method and PCR

It was also investigated whether the colony immunoblot method was applicable if the sample contained more than 2-3 x 10^3 non-pathogenic cells. Forty samples were contaminated with 2-4 *Shigella* or Lac^+ EIEC cells and 10^4 or 10^5 non-pathogenic cells. Samples were either tested directly, or after incubating them on selective DC plates, by the colony immunoblot method, by agglutinating selected colonies and the cell mass in an O-antigen-specific serum, and by PCR. It was shown that the colony immunoblot method could identify samples with the same sensitivity as the traditional method using a selective culture, but which gave the results, however, a day earlier. See results in Table 8.

Table 8. *Identification of EIEC by culture and by immunoblot with and without selective pre-culture*

				Number of positive samples identified					
				Without selective culture*			With selective culture†		
Bacterium	C.f.u. ml^{-1} in sample	Bacterium	C.f.u. ml^{-1} in sample	By culture‡	By blot§	*P*¦	By culture	By blot	*P*
Lac^+ EIEC	2-4	Lac^+ *E. coli*	10^4	1/40	21/40	<0.001	2/40	38/40	<0.001
	2-4		10^5	1/40	21/40	0.375	1/40	12/40	0.001

**Filter-collected cells were grown on TSA plates*
†Filter-collected cells were grown on DC plates
‡Culture of filter-collected cells were streaked onto 2 EO and 2 DC plates, respectively for side agglutination
§Culture of filter-collected cells were resuspended in two aliquots of 50 ml saline each to contain 2x10^3 cells and filtered onto two membranes followed by the immunoblot reaction
¦McNemar's test

None of the methods gave false positive results. PCR was the most sensitive technique used, recognising all the samples containing the pathogen in the presence of 2x10^3 non-pathogenic cells and recognising all samples after pre-culturing on selective DC plates if 2x10^4 cells were present. Without selective culturing, 38 of the 40 samples were positive in the later model (data not shown).

The colony blot method proved to be significantly more sensitive than the traditional method, especially when combined with selective culturing. In the presence of 2x10^4 non-pathogenic cells, 12 out of 40 samples were identified by the colony blot method whereas only 1 was positive by serotyping (p=0.001). In the presence of 2x10^4 cells the

difference between the two methods was significant even without selective pre-culture (21 vs. 1 of 40 contaminated samples).

5.2.2. Applying the colony immunoblot method for faecal samples (paper II)

5.2.2.1. Optimisation of the colony blot assay for testing faecal samples

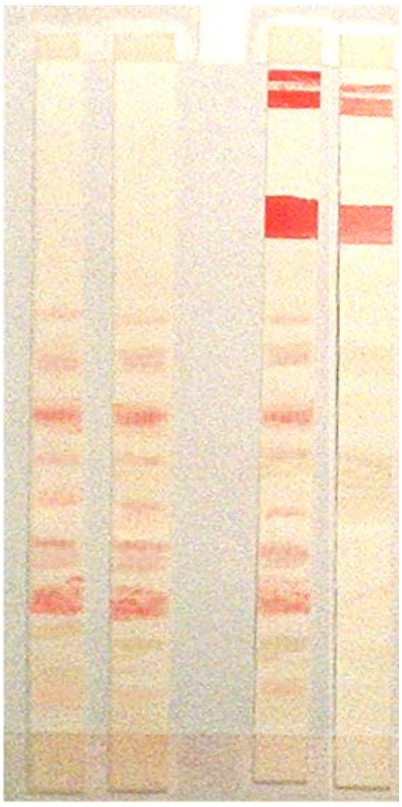
To establish an effective and simple protocol to transfer antigens secreted by EIEC and *Shigella* strains to nitrocellulose filters different blotting procedures were compared. All three protocols were tested, i.e. growing colonies on the surface of replica-inoculated nitrocellulose membranes, incubating filters placed over colonies for 2 hours at 37 ^{o}C, and growing colonies beneath the filters, respectively, resulted in colony prints clearly visible after the immune assay.

The intensity of the colour reaction on blots prepared by the 2 hour-long antigen-transfer procedure varied considerably when the same strains were tested repeatedly. On the other hand, no similar inter-assay variation was observed with the other two blotting techniques (data not shown). Therefore, after the initial experiments, the use of the 2 hour-long blotting protocol was discontinued. The colonies, and consequently their blots, were smaller when bacteria were grown covered by the filters compared to prints obtained when growing colonies on the membranes. However, this smaller size did not prevent the clear identification of positive colonies, as confirmed by subsequent IpaC-specific ELISA of 10 positive, and 10 negative colonies taken from plates containing mixtures of *E. coli* K12 and 10 EIEC or 10 *Shigella* strains, respectively (data not shown). Moreover, this procedure did not require the extra day needed to prepare the master plates for the replica-inoculation of the filters. Blots of the 80 EIEC and 59 *Shigella* strains were clearly marked by the immune reaction following this antigen transfer technique. The intensity of the colour reaction was slightly lower with most of the EIEC strains than that observed with the *Shigella* isolates. Nevertheless, it was clearly distinguishable from the negative reactions seen with all the 100 non-enteroinvasive strains tested. Therefore, during the subsequent experiments blots of cultures were prepared by allowing the colonies to develop while covered by the nitrocellulose filters.

5.2.2.2. Elimination of endogenous AP of faecal samples

Before applying the colony immunoblot method for testing faecal samples, a special treating procedure had to be developed to eliminate the interfering effect of the endogenous alkaline phosphatase activity found in normal stools - as well as that of the horse radish peroxidase - while keeping the reaction of the targeted antigen on the same level. Various treatments with heat and detergents were tested, including Tween, Triton, EDTA, Formalin, Mercaptoethanol and 10 mM Levamizol. The best combination was proved to be soaking the membranes in Tween 20 followed by dry heat at 100 ^{o}C each for 10 minutes (Figure 6.)

Figure 6. *Elimination of AP activity of faecal samples by pre-treatment*



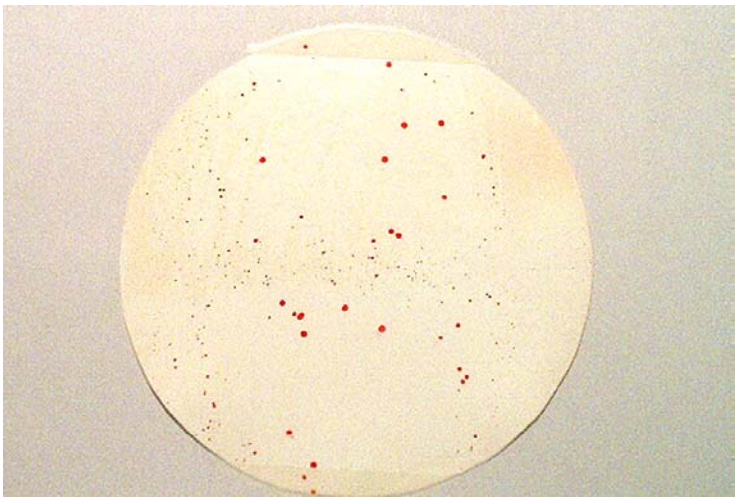
Row 1.: Blot of culture of virulent S. flexneri strain
Row 2.: Blot of culture of avirulent S. flexneri strain
Row 3.: Blot of culture of virulent EIEC strain
Rows 4-13: Blot of culture of normal stool samples
Column A: Incubated only with the substrate of alkaline phosphate
Column B: Incubated only with MAIC-1 antibody and the substrate of alkaline phosphate
Column C: Incubated with MAIC-1 antibody, conjugate and the substrate of alkaline phosphate
Column D: Incubated with MAIC-1 antibody, conjugate and the substrate of alkaline phosphate after treating in 0,5% Tween 20, and following 100 ^{o}C heat, each for 10 minutes

The effect of pre-treatmenting the membranes with a combination of Twin 20 and 100 ^{o}C dry heat each 10 minutes on the endogenous AP activity of stool and on the IpaC antigen of Shigella and EIEC strains.

The pre-treatment procedure was tested on samples artificially contaminated with varying number of *Shigella* cells. Samples were inoculated on TSA plates and covered with a nitrocellulose membrane. The following day, after incubation and pre-treatment,

the immune reaction was performed on the membranes. The picture of the immune reaction of a membrane is shown on Figure 7. The red small spots represent the *Shigella* colonies grown under the membrane during the overnight incubation.

Figure 7. *Colony immunoblot of a faecal sample containing 10^7 Shigella cells.*



The membrane was pre-treated with Tween20 and heat before the immuno developing in order to eliminate the AP activity of the faecal sample. The red spots are equivalent with the blots of the pathogen colonies of Shigella cells.

5.2.2.3 Sensitivity of the colony blot using artificially contaminated faecal samples

Twenty faecal specimens tested previously negative for enteric pathogens were diluted to contain approximately 10^9 coliform CFU / ml. A dilution series of a virulent EIEC strain of serogroup O164 was used to contaminate these negative samples with increasing germ counts. The samples were tested with the *ipa*H-specific PCR, with the IpaC-specific ELISA investigating 16 randomly selected colonies, and with the colony immunoblot assay. For the latter assay either flood-inoculation of further dilutions was used in order to yield approximately 500 colonies per plates, or TSA plates were loop-inoculated with the contaminated samples without further dilution.

No false positive result was obtained by any of these methods in any of the experiment when the negative samples were not contaminated with invasive pathogens. When contaminating samples artificially, PCR proved to be the most sensitive technique

identifying all pathogen-containing specimens even at 1:5000 ratio of contamination (Table 9). The least sensitive method was ELISA after testing a limited number of randomly selected colonies from the plates. Although all specimens containing EIEC as approximately 10 % of their total coliform flora were identified by this method, the performance of this diagnostic approach considerably decreased at lower rates of pathogen content.

However, the colony blot assay was able to detect IpaC-secreting colonies in all samples containing at least 1 % EIEC. Even at 1:250 and 1:500 rates of contamination, respectively, this latter technique significantly outperformed the method based on random colony selection ($p < 0.01$). As for the colony blot assay, flood-inoculation of the plates was more effective than the loop-inoculation technique, especially at the lower ratio of the pathogen (Table 9).

Table 9. *Sensitivity of different methods to identify EIEC in artificially contaminated faecal samples*

Approximate ratio of the pathogen*	Number of positive samples identified			
	PCR	ELISA†	Colony immunoblot	
			Flood-inoculation‡	Loop-inoculation§
1:10	20	20	20	20
1:100	20	8	20	20
1:250	20	1	18	16
1:500	20	0	12	7
1:1000	20	1	8	3
1:5000	20	0	1	1
0	0	0	0	0

**As compared to the CFU of coliforms in the specimen*
†Testing 16 randomly selected colonies
‡ Plates were inoculated with titrated dilutions of sample to provide approximately 500 colonies
§Plates were loop-inoculated with undiluted samples yielding approximately 200-300 colonies

It must be noted though that there was no significant difference statistically between these two methods at any ratio of pathogen content ($p > 0.05$) (Table 9). Therefore, since the flood inoculation had several drawbacks, the loop inoculation method was used in our subsequent experiments. Among these drawbacks were the extra time and material requirements for the preliminary determination of the CFU content of the samples (Hull et al., 1993).

5.2.2.4. Detection of EIEC and Shigella in clinical samples

One hundred and sixty-five faecal samples were collected at the Mubarak Al-Kabeer Hospital. These samples were investigated for enteric bacterial pathogens by standard laboratory methods, by the *ipa*H-specific PCR to detect the presence of *Shigella-,* or EIEC-related DNA sequences, and also tested by the IpaC specific colony blot assay.
Out of the 165 samples, 26 yielded non-typhoid *Salmonella* and 5 *Campylobacter jejuni* strains were isolated. Five samples associated with infantile diarrhoea were positive for *E. coli* strains expressing O antigens (2 strains of O111 and 3 of O86 serogroups). In 12 samples, all submitted from different patients *Shigella* strains (five *S. flexneri* and seven *S. sonnei*) were detected. None of the samples yielded more than one pathogen. The *ipa*H-specific PCR verified the presence of sequences specific to enteroinvasive strains in 17 samples including all the 12 yielding *Shigella* by culture.

The colony blot assay detected colonies expressing the IpaC in 16 samples, all of these also gave positive results by PCR. The 16 samples found to contain IpaC positive colonies included those from which *Shigella* had been simultaneously cultured. No PCR negative specimens tested positive with the immune assay. By locating colonies on the master plates with the aid of their respective blots, in 13 out of these 16 specimens, colonies expressing IpaC could be directly identified and isolated. In the remaining three samples the colony blot assay showed clear colour reaction at spots where the density of colonies was too high for the clear identification of the positive ones. However, by re-suspending cells in PBS from the respective area of the culture, and plating this suspension followed by a repeated colony blot assay we could isolate the pathogenic strains in these cases, too.

From the 12 samples shown to contain *Shigella* by culture, strains belonging to the same genus and species were recovered by isolating colonies marked by the immune assay. Beyond the 12 samples containing *Shigella*, the colony blot assay also identified four specimens which were also positive by PCR, but from which no enteric pathogens had been cultured. The isolation and identification of IpaC positive colonies revealed that these samples contained EIEC. These specimens derived from two patients, from samples submitted one and two days apart, respectively. From one of the patients an

EIEC strain expressing the O28 cell wall antigen, while from the other that of the O164 serogroup was isolated. All EIEC isolated were invasive, as shown in the guinea pig keratoconjunctivitis test. Both patients excreting EIEC had diarrhoea without microscopic blood or pus cells detected in the samples. One sample, repeatedly positive by PCR, did not yield any colonies reactive with the IpaC-specific antibody, even after testing blots of six loop-inoculated, and six flood-inoculated plates (*i.e.* testing over 4000 colonies). According to these results all together 2.4 % of the samples, received from 1.6 % of the patients, were positive for EIEC, as shown by the immunoblot technique.

5.2.3. Applying the colony immunoblot method for milk samples

5.2.3.1. Optimising the method for testing milk samples

Fifty ml aliquots of milk samples artificially contaminated with *Shigella* or EIEC cells were tested by the colony immunoblot method and compared to routine culturing. Filtering the milk samples through nitrocellulose membranes with a 0.45 μm diameter pore size was not successful due to the consistency of the samples. Instead filtering, centrifuging the samples at 4500 g for 10 minutes was considered to be the right method. A pellet of the samples was spread on TSA plates and covered with nitrocellulose membrane, which was proceeded by an immune reaction, giving a result in 24 - 48 hours. Samples not containing any pathogen cells or contaminated with *E. coli* J53, used as negative control, were always negative by the colony blot assay, verifying that there was no false positive reaction. The same result was shown when contaminating milk samples with *Shigella* cells.

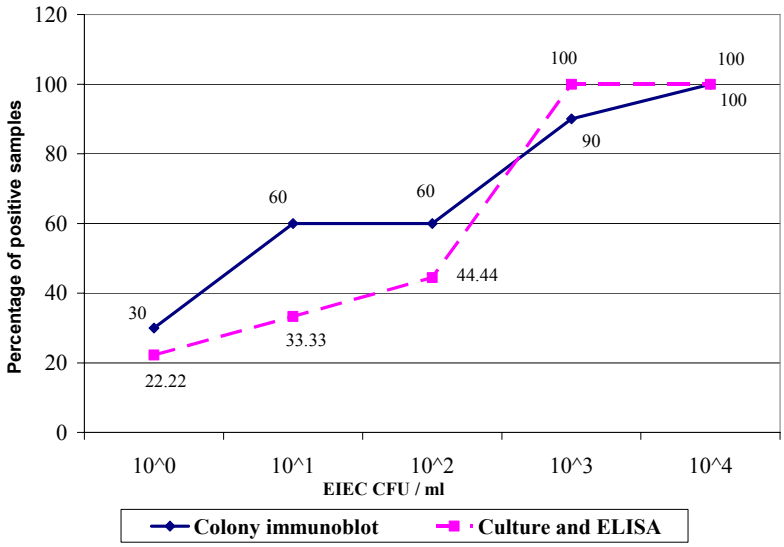
5.2.3.2. The sensitivity of the colony immunoblot method

After optimising the method for milk samples, the sensitivity of the method was compared to the traditional microbiological processing combined with the IpaC ELISA. Nine samples were contaminated with gradually increasing numbers of EIEC cells, 10^0, 10^1, 10^2, 10^3, 10^4 per ml, respectively. Of each sample, 16 randomly selected colonies

were tested by ELISA. The sensitivites of the various methods were shown to be equivalent. The pathogens were easily detected from samples with 10^2-10^4 /ml both by the traditional and the colony immunoblot method. Samples containing fewer pathogens (10^0, 10^1) were more difficult to identify by the traditional method, as 22.22% and 33.33% were identified by ELISA, as opposed to the 30% and 60% by the colony blot method, showing that the latter is more sensitive to detect a low number of pathogens (Figure 8.).

There was a significant difference between the times required by the traditional and the colony blot method. Testing milk samples by the traditional methods – culturing the pellet on routinely used differentiating and selective plates, and performing the IpaC specific ELISA on 16 randomly selected colonies - required a minimum of 72 hours. Performing the colony immunoblot method, from processing the samples to having the results required no more than 24 hours.

Figure 8. *Detecting EIEC in milk samples*



The sensitivity of the colony immunoblot and ELISA after culture was compared using milk samples artificially contaminated with a serial dilution of lac positive EIEC strain

5.3. Selective and enrichment media for culturing EIEC (paper III)

5.3.1. Growth of EIEC and other isolated strains as pure culture

The growth of pure cultures of EIEC, *Shigella* and *Salmonella* isolates on MAC, EMB, DC, SS and XLD plates was compared to that of *Escherichia coli* strains isolated from healthy individuals (Tables 10, 11). On MAC and on EMB plates EIEC exhibited growth characteristics similar to that of their non-pathogenic counterparts indicated by enrichment factor (EF) values around 1 (Tables 10, 11). Although on DC, SS and XLD plates EIEC strains suffered a 3 - 8 fold colony count reduction compared to their growth on non-selective TSA plates, the same figure for normal *Escherichia coli* was significantly higher, often over 100-fold. Consequently, as a group, EIEC grew significantly better on all of these media than did normal *Escherichia coli* strains ($P < 0.01$) exceeding the growth of this latter group by EF values 8.4, 10.9, and 4.2 on DC, SS and XLD plates, respectively (Tables 10, 11).

Table 10. *Growth of pathogens as pure cultures on differentiating and selective media: showing plating efficiency*

Strains	*n*	MAC Median (Range)	EMB Median (Range)	DC Median (Range)	SS Median (Range)	XLD Median (Range)
EIEC						
Total	78	77.5 (35.8-101.0)	81.9 (32.5-111.0)	16.1 (0.0-180.0)	24.1 (0.0-102.0)	9.4 (0.0-189.0)
Lac $^-$	54	75.0 (45.3-100.0)	78.9 (42.6-111.0)	16.3 (0.0-180.0)	25.8 (0.0-102.0)	10.1 (0.0-189.0)
Lac $^+$	24	87.2 (35.8-101.0)	85.8 (32.5-110.3)	15.5 (0.0-83.3)	23.4 (0.0-75.9)	8.5 (0.0-102.0)
Shigella	59	75.0 (41.0-98.3)	67.8 (23.6-98.3)	17.7 (0.0-121.2)	28.5 (0.0-95.8)	16.8 (0.0-146.9)
Salmonella	22	92.0 (60.0-120.0)	91.8 (64.3-112.2)	31.7 (0.0-244.0)	72.5 (7.4-184.0)	30.2 (0.0-186.9)
Normal *E. coli*	53	83.6 (54.3-102.3)	81.3 (31.2-111.0)	0.0 (0.0-24.0)	1.39 (0.0-8.0)	0.0 (0.0-12.8)

PE: Plating Efficiency, expressed as the percentage of colonies on the selective or differentiating plates compared to that on non-selective TSA plates

None of the selective media were significantly superior to the others in supporting the growth of EIEC as pure cultures. It was noteworthy that there was no significant difference between the growth of EIEC and *Shigella* strains, or between that of the Lac^- and Lac^+ EIEC isolates on any of the selective plates tested. On the other hand, *Salmonella* strains significantly exceeded the growth of all dysentery-causing bacteria on SS and XLD plates ($P < 0.05$).

Table 11. *Growth of pathogens as pure cultures on differentiating and selective media: showing enrichment factor*

Strains	*n*	MAC EF** Median (Range)	EMB EF Median (Range)	DC EF Median (Range)	SS EF Median (Range)	XLD EF Median (Range)
EIEC Total	78	0.96 (0.4-1.2)	1.0 (0.4-1.4)	8.4 (0.0-93.7)	10.9 (0.0-45.9)	4.2 (0.0-85.5)
Lac^-	54	0.93 (0.5-1.2)	1.0 (0.5-1.4)	8.5 (0.0-93.7)	11.6 (0.0-45.9)	4.5 (0.0-85.5)
Lac^+	24	1.0 (0.4-1.2)	1.1 (0.4-1.4)	8.1 (0.0-43.4)	10.5 (0.0-34.2)	3.8 (0.0-46.1)
Shigella	59	0.9 (0.5-1.2)	0.8 (0.3-1.2)	9.2 (0.0-63.1)	12.8 (0.0-43.1)	7.6 (0.0-66.4)
Salmonella	22	1.1 (0.7-1.4)	1.1 (0.8-1.4)	16.5 (0.0-127.0)	32.4 (3.3-82.8)	13.6 (0.0-84.5)
Normal *E. coli*	53	NA	NA	NA	NA	NA

*** EF: Enrichment Factor, calculated by comparing the rate of colony formation of pathogenic strains to the average of 53 non-pathogenic E. coli strains on the respective media*

The extensive variation in the growth of individual EIEC isolates on the media used (Tables 10, 11) suggested that there were isolates strongly inhibited by chemicals contained by these plates. In the current study, a "sensitive" EIEC strain was arbitrarily defined as the one with an EF < 2.0, i.e. growing less than twice as well than the average of normal *Escherichia coli* strains on any of the selective plates. On DC 29.5 %, on SS 15.4 % and on XLD 16.7 % of the EIEC strains fell into this category (data not shown). It was noteworthy that of the 27 EIEC strains with EF < 2.0 on any one of these plates, 16 also exhibited this phenotype on at least one other selective medium. The growth of EIEC strains on the different selective plates correlated well, as indicated

by rank correlation coefficient values 0.667, 0.614 and 0.496, when comparing the EF values of the individual isolates on DC and SS, on DC and XLD and on SS and XLD plates, respectively ($P < 0.01$).

Table 12. *The growth of EIEC in selective-enrichment media after 6 hours incubation*

Strains	*n*	Ratio of c.f.u. of EIEC before and after 6 hours incubation (± standard deviation)	
		SB	GNB
EIEC	80	-18,835 ± 58,19	33,96 ± 35,14
Shigella	42	163,72 ± 375,46	24,64 ±16,35
Salmonella	22	36,98 ± 47,33	64,38 ± 34,52
Faecal *E. coli*	52	-56,32 ± 103,71	28,85 ± 54,87

SB and GNB selective enrichment media were inoculated with EIEC strains and the germ count was calculated before after 6 hours incubation. The ratio and the standard deviation are shown.

As liquid selective enrichment media, Selenite Faecal Broth (SF) and Gram negative broth (GNB) were used to study whether these media selectively enhance the growth of EIEC strains against the normal flora members. The ratio of the germ counts of each strain before and after 6 hours of incubation in the liquid media was calculated as shown in Table 12. According to these results, it was concluded that media either inhibited or facilitated the growth of the isolates varying strain by strain, not proving to be significantly advantageous for the EIEC strains. Nevertheless, the effect of selective enrichment media was also studied when testing clinical faecal specimens, detailed below.

5.3.2. Recovery of EIEC from artificially contaminated faecal samples

Altogether nine Lac^- EIEC strains were chosen to infect faecal samples. When grown as pure cultures on DC, SS and XLD three of them were inhibited approximately to the same extent as were normal *Escherichia coli* isolates (i.e. EF < 2 on each of these media). Three strains were characterised by factors between 5 and 20, while for the last three ones DC, SS and XLD plates presented very moderate inhibition, only (EF above 30).

Considering the results with all the nine EIEC strains, as a group, MAC and EMB did not change considerably the proportion of EIEC colonies compared to the pathogen's representation in the contaminated samples (Table 13). On the other hand DC, SS and XLD provided a significantly better recovery of EIEC at both ratios of contamination (i.e. 1:10 and 1:100) ($P < 0.05$). DC and XLD yielded nearly identical results, and both of them clearly outperformed SS, particularly at the low (1:100) contamination rate ($P < 0.01$). In these cases, upon culture, DC and XLD increased the ratio of EIEC by more than ten times (i.e. over 10 % of the colonies on the plates were EIEC), while the median of the same figures for SS was 4.6, only (Table 13).

Table 13. *Enrichment of EIEC cultured from contaminated faecal samples*

Approximate ratio of contamination	Strains	Enrichment factor*, Median (Range)				
		MAC	EMB	DC	SS	XLD
1:10	All	1.2	1.0	3.2	2.0	3.2
	(n = 9)	(0.3 – 2.2)	(0.3 –2.5)	(0.1 – 8.0)	(0.1 – 5.6)	(0.2 – 6.7)
	"Sensitive	1.4	1.4	0.5	0.8	1.3
	" (n = 3)	(0.4 – 2.2)	(0.5 – 2.5)	(0.1 – 0.9)	(0.1 – 1.5)	(0.2 – 1.6)
1:100	All	1.3	1.3	12.3	4.6	10.3
	(n = 9)	(0.3 – 3.6)	(0.3 – 3.6)	(0.2 – 40.0)	(0.2 – 16.5)	(0.9 – 33.2)
	"Sensitive	1.4	1.8	1.6	0.8	3.1
	" (n = 3)	(0.6 – 1.9)	(0.3 – 2.5)	(0.2 – 3.2)	(0.2 – 1.9)	(0.9 – 3.5)

** The ratio of EIEC colonies on a selective plate compared to that of the pathogen in the sample (i.e. the percent of its colonies on non-selective TSA plate*

Analysing separately the results of the three "sensitive" isolates revealed, however, that these strains grew relatively poor on the selective plates also when recovered from faecal samples. At 1:10 ratio of contamination DC and SS plates actually suppressed the growth of most of these strains as compared to that of the normal flora (median EF values 0.5 and 0.8, respectively, Table 13), while, similarly to MAC and EMB plates, XLD did not decrease the proportion of two out of three strains. When EIEC cells represented about 1 % of the coliform flora of the sample (1:100 ratio of contamination), XLD moderately increased the ratio of the pathogens upon culture (median of EF 3.1), exceeding the performance of all other plates used (Table 13).

5.3.3. Culturing EIEC strains from clinical samples

Of the 250 faecal samples from patients with the diagnosis of "diarrhoea" or "dysentery" the *ipa*H specific PCR identified 22 specimens to contain DNA sequences specific to *Shigella* or EIEC. From 15 of them *Shigella* was isolated (eight *Shigella sonnei* and seven *Shigella flexneri* strains, respectively, each from different patients), while seven samples did not show enteric pathogens when investigated by the standard bacteriological methods. The specimens in this latter, PCR positive, but *Shigella* negative, group were considered as "tentatively EIEC positive".

All 22 PCR positive, as well as 50 PCR negative samples were further investigated by the IpaC-specific ELISA testing 60 randomly selected colonies from all plates with or without pre-culture in SF or GNB. No ELISA positive colonies were found in any of the PCR negative samples, while all *Shigella* containing samples were also identified by ELISA. However, the ELISA gave negative results with one of the *Shigella* negative but PCR positive specimens. From the remaining six PCR positive, *Shigella* negative samples, four O28 and two O164 EIEC strains were isolated. Two isolates of the former serogroup derived from a single patient from specimens received two days apart. No samples containing both *Shigella* and EIEC were found.

The proportions of EIEC colonies on the different plates are shown on Table 14. For EIEC, on the non-selective TSA plates reflecting the pathogen's ratio in the original sample, the values varied between 3.3 % and 41.6 % with a median of 13.3 %, not significantly different ($P > 0.05$) from the respective figures in samples containing *Shigella* (data not shown). As primo-culture media, DC and XLD plates were more effective to increase the ratio of EIEC than was SS agar (5.0 and 6.0 *vs.* 3.4 fold enrichment, Table 14) although the difference between the selective media did not reach the level of statistical significance. However, when compared to moderately selective MAC and EMB plates (with EF values 1.2 and 1.1) DC and XLD plates were significantly more effective ($P < 0.05$), while in the case of SS the difference was not significant. Consequently, using XLD plates as primary culture media, the representation of EIEC colonies reached at least 50 % for all samples tested, while inoculating specimens onto SS there were samples for which the same value did not

exceed 20 %. The respective figures for *Shigella* strains (data not shown) did not differ significantly from that of EIEC on any of the plates tested.

To examine whether liquid selective and enrichment media can support selectively the growth of EIEC strains as opposed to the normal flora of the faeces, Selenite F (SF) and Gram negative Broth (GNB) were tested. These liquid enrichment media variously either supported or inhibited the growth of EIEC, and never showed to give a selective advantage against the normal *E. coli* strains. Pre-culturing the specimens in GNB or in SF, did not alter significantly the recovery of EIEC, although SF was slightly inhibitory for some of the isolates (Table 14). The time of pre-culture in liquid media, varying between six to 18 hours, did not influence the results, either (data not shown).

Table 14. *Recovery of six EIEC strains from clinical specimens*

Media	% of colonies		Enrichment factor*	
	Median	Range	Median	Range
TSA**	13.3	3.3 – 41.6	1	NA
SF + TSA	23.3	3.3 – 46.6	1.2	1.0 – 1.8
GNB + TSA	20.8	11.6 – 50.0	1.4	1.2 – 4.0
MAC	*15.0*	10.0 – 33.3	1.2	0.8 – 3.5
SF + MAC	16.6	5.0 – 28.3	1.0	0.3 – 3.0
GNB + MAC	17.5	13.3 – 43.3	1.5	1.0 – 4.5
EMB	*14.1*	11.6 – 33.3	1.1	0.8 – 4.0
SF + EMB	18.3	5.0 – 41.6	1.4	0.3 – 6.0
GNB + EMB	18.3	13.3 – 43.3	1.4	1.0 – 4.5
DC	66.6	46.6 – 83.3	5.0	2.0 – 14.0
SF + DC	61.6	33.3 – 86.6	4.8	2.0 – 10.0
GNB + DC	78.3	46.6 – 90.0	6.1	2.1 – 14.0
SS	64.1	20.0 – 95.0	3.4	1.6 – 5.5
SF + SS	60.0	13.3 – 96.6	3.9	1.6 – 6.1
GNB + SS	63.3	31.6 – 96.9	3.9	1.7 – 9.5
XLD	85.5	53.3 – 98.3	6.0	2.3 – 18.0
SF + XLD	86.6	50.0 – 96.6	6.4	2.2 – 15.0
GNB + XLD	85.8	66.6 – 100.0	6.4	2.3 – 20.0

** The ratio of EIEC colonies on a selective plate compared to that of the pathogen in the sample*

*** The percent of EIEC colonies on TSA plates were considered to represent the pathogen's ratio in the samples*

6 DISCUSSION

As in the case of every infectious disease, in case of bacillary dysentery, the ideal detection method should be fast, simple and cheap, with high specificity and sensitivity. This is a prerequisite in order to diagnose the disease on time so that the patients get adequate treatment on time, to prevent the disease by screening the environment, to make early assumptions of the real epidemiological situation in order to facilitate proper preventative measures.

6.1. Diagnostic methods presently available for the detection of *Shigella* and EIEC strains (papers I-III)

Of the two groups of the causative agents of bacillary dysentery, the diagnostic protocol for *Shigella* is well established. However, it is still not sensitive enough in case of environmental and food samples. Molecular methods, like DNA hybridisation, or especially the introduction of the Polymerase Chain Reaction (PCR) achieve high enough sensitivity to overcome this problem, however, unfortunately these methods are too expensive to be available for every routine laboratory, especially in the developing world where this disease is endemic.

During the late 50's and early 60's it was found that *E. coli* strains isolated from patients with dysentery were also able to cause experimental keratoconjunctivitis in guinea pigs. Those strains were classified under many different names and lately designated as enteroinvasive *E. coli* (EIEC) and this name was universally accepted (Bando 1998). These strains are rather tiresome to isolate by the methods of the conventional microbiological diagnostics, mostly because these strains do not have biochemical and serological markers that would easily differentiate them from the other members of the normal flora of the colon. At present the detection and identification of diarrheagenic *E. coli,* and especially EIEC is problematic due to the lack of diagnostic methods combining easy and cheap technology with high sensitivity and specificity (Echeverria et al., 1991; Nataro and Kaper, 1998). The characteristics frequently associated with the phenotype of invasive *E. coli* are the following: the absence of lactose fermentation, the the lack of lysine and ornithin decarboxylase and mucinase, or

the non-motility. However, the combination of these features rather than any one of them alone is suggestive of EIEC (Abuxapgui et al., 1999; Silva et al., 1980). Therefore, as a large number of colonies should be tested in multiple biochemical reactions, biotyping has a limited value in screening for this pathogen. Serotyping in routine laboratories also seems to be inconvenient today as the number of *E. coli* serogroups with reported invasive characteristics has increased recently (Pál et al., 1998).The identification of these strains remains extremely tedious, because the virulence of the strain still needs to be confirmed, as neither biochemical markers nor any of the O antigens are specifically linked to the invasive character of the isolates.

The molecular methods can identify EIEC and other diarrhoeagenic strains with high specificity and sensitivity. These methods have the advantage of testing samples directly without culturing after extracting the DNA, or the direct testing of colonies, but have the disadvantage of certain limitations mentioned above. As a result of these diagnostic limitations, the causative agent of the disease remains of unknown etiology in many cases (Boileau et al., 1997; Echeverria et al., 1991; Frankel et al., 1990; Nataro and Kaper, 1998).

The ELISA test developed earlier by our group is specific for the virulence marker antigen of *Shigella* and EIEC strains. According to our previous experience, the VMA ELISA is an adequate method to overcome a few obstacles in the diagnosis of these pathogens. However, it is still not capable of detecting *Shigella* and EIEC strains from primo cultures as well as from filtered material in case of environmental specimens. In case of another enteric group of pathogens, the Shiga–like toxin producing enterohaemorrhagic *E. coli* (EHEC) the application of the colony immunoblot method was successful for faecal samples (Hull AE 1993, 177). Other pathogens have been successfully detected by the colony blot method, like the identification of colonies of *Clostridium botulinum* (Goodnough et al. 1993), TSST-1-producing *Staphylococcus aureus* (See et al., 1990), *Pediococcus acidilactici* (Bhunia and Johnson, 1992), *Rhodococcus equi* (Takai et al., 1993).

6.2. Developing the colony immunoblot method and its application to environmental and clinical samples (papers I, II)

The major objective of the present dissertation was to develop a *Shigella* and EIEC specific colony immunoblot assay, mainly on the basis of Hull and co-workers' results. In this study we have developed this assay and shown that it can also be applied for the specific and sensitive identification of EIEC and *Shigella* strains in water, faecal and milk samples. A monoclonal antibody specific to IpaC, a plasmid-coded protein of these pathogens was used in this assay (Floderus et al., 1995). This antibody was shown to be highly specific for invasive EIEC or *Shigella* strains, i.e. neither other pathogens nor nonpathogenic agents gave false positive reaction in the colony blot assay neither did negative controls without any agent.

The development of the colony immunoblot included the determination of the right parameters of the method as well as the membrane filter and the media and their application. None of the various membranes tried in the pre-tests gave as good results as the nitrocellulose membrane filter. Of the various media tested *Shigella* and EIEC grew on TSA the best, probably not only due to the indicator content of the other, mildly or highly selective plates. Supposedly, the additives that ensure their selective effect might interfere with the expression of the IpaC molecules, which is well known to be under strict environmental regulation. The problem presented by swarming *Proteus* strains that can be present in faeces and may easily overgrow the target pathogens, was successfully overcome by increasing the agar-agar concentration of the TSA plates, without effecting the expression of the IpaC antigens.

6.3. Application of the colony immunoblot method for water samples (paper I)

Water samples were filtered as per conventional methods when testing the microbiological status of water to determine pollution by determining the number of faecal indicator agents. The colony immunoblot method was applied successfully by the culture and subsequent immune reaction of the membranes. As shown when testing a series of water samples artificially contaminated with increasing number of pathogens and non-pathogenic background flora, the colony immunoblot method was shown to be

able to detect *Shigella* with the same sensitivity as the traditional microbiological methods. Besides, EIEC strains could also be detected with the same sensitivity, regardless of their serogroups. Therefore this is the first method - besides the mostly unavailable molecular techniques - that can foster direct and simple detection of EIEC colonies. Additionally the colony immunoblot method gave results a day earlier, which is an important factor when considering the treatment, prevention and etiological determination of the disease.

6.4. Application of the colony immunoblot method for faecal samples (paper II)

Similar results were gained when testing faecal samples, however the method had to be slightly modified. A pre-treatment method had to be developed to eliminate the activity of Alkaline Phosphatase Enzyme endogenously present in at least 20 % of the faecal specimens (own, unpublished data). Alkaline Phosphatase (AP) can be found in nature in a wide range, in species so far from each other as *E. coli* and *Homo sapiens.* The AP found in faeces, can be of three diferent origins (Lewis and Hodes 1968, MacAlister TJ 1972, Horrigan and Danovitch 1974):

- mostly from the epithelial cells of the colon
- from bacterial flora
- in small amounts from bile

Considering the expense and the required simplicity of the pre-treatment, the most effective way was to treat the membranes with the combination of Tween20 and heat, not effecting the expression of the IpaC antigens. This pre-treatment method was also effective in eliminating the activity of the Peroxidase enzyme, therefore applicable in any immunoblot method that applies an Alkaline Phosphatase or Peroxidase conjugate to detect pathogens in faecal specimens.

We tried to find the simplest protocol; therefore various methods of plate-inoculation and colony blotting were compared: flood inoculation, loop inoculation and replica plating. Loop-inoculation yielded fewer colonies for blotting of the plates than loop-inoculation with properly diluted samples (approximately 200-300 *vs.* 500). However,

loop-inoculation required only one plate per specimen, while when carrying out the flood-inoculation several plates had to be inoculated in order to find the proper sample dilution. Due to the difference in the number of resulting colonies on the plates, the sensitivity of loop inoculation was slightly less than that of the flood inoculation as shown with artificially contaminated samples (Table 9). Replica-plating, and subsequent growing of colonies on filters resulted in larger colony-print sizes than when allowing colonies to grow on plates covered by the membranes. As the latter protocol did not require a day to prepare the master plates for replica-inoculation while still yielding clearly visible colony blots, this protocol was chosen.

Hull and co-workers reported the detection of Shiga toxin producing *E. coli* in blots of mixed cultures at as low as 1:1000 to 1:5000 pathogen to non-pathogen ratios (Hull et al., 1993). With the protocol used in the present study, however, the sensitivity of the immunoblot technique was lower than that of the method used by Hull. This could probably be attributed to the fact that they used the more laborious replica-plating method that allowed more colonies to be screened.

However, it was proved that the compromises made concerning sensitivity to keep the protocol cheap, fast and straightforward did not adversely affect the performance of the immunoblot assay under field conditions. Among the 165 clinical specimens tested, 16 samples containing IpaC positive colonies were found by the colony immunoblot assay, just one specimen fewer than the 17 identified by the *Shigella* and EIEC specific PCR. As it can be expected, the ratio of the pathogen among the colonies present on the plate investigated determines the efficiency of the colony blot assay, as other assays, too. Apparently, in at least 16 out of the 17 PCR positive clinical samples the ratio was sufficiently high to find IpaC expressing colonies with the immunoblot method.

The immunoblot is a significantly more sensitive method than those based on testing randomly selected colonies with molecular, virulence, or immune assays. This can be attributed to the fact that the colony immunoblot assay can screen a few hundred colonies, whereas when using the above-mentioned approach, only a few, rather than a few hundred colonies are considered to represent the sample, which considerably reduces the sensitivity. This was clearly shown in this work even though as many as 16

randomly selected colonies, instead of the 3-5 recommended (Nataro and Kaper 1998), were tested by ELISA (Table 9).

As is well known from experience, severe bacillary dysentery cases are associated with a high amount of invasive bacteria in the faeces. However, there can be mild cases with significantly fewer pathogens, or even cases without clinical manifestation but shedding a low amount of the pathogens.

Our results do not exclude the latter: it can be possible that samples contained pathogens below the detection level of the immunoblot assay. Theoretically, this could be the case with the only PCR positive, but immunoblot, and culture negative sample encountered in this study. Alternatively, non-viable, disintegrating cells of the pathogen could also contaminate the sample with DNA fragments detected by molecular methods, but remaining undetectable with the colony immunoblot. In addition, as we have shown before, negative immune assays can also be the attributed to mutations resulting in the loss of IpaC expression, while molecular methods still could demonstrate the physical presence of virulence specific sequences (Pál et al., 1985). This could also be the case with the PCR system and the immune assay used in this study. The gene coding for the IpaC protein utilised by the colony blot assay is located extra-chromosomally only, while alleles of the *ipa*H gene, targeted by the PCR, are present both on the chromosome and on the invasion plasmid, (Parsot, 1994, Venkatesan et al., 1989). Although, based on these results and previous experience, it is supposed that an extremely low pathogen content may not be frequent in bacillary dysentery; its actual frequency needs to be determined in large-scale studies. If the clinical or epidemiological data suggest that the disease is caused by *Shigella* or EIEC, the sensitivity of the immunoblot assay could be further increased by blotting multiple, flood-inoculated plates from selected samples.

As the study of the clinical material was carried out in Kuwait, our results show the frequency of EIEC in that region, where EIEC was isolated from 1.6 % of diarrhoea cases. It is important to note, that this finding is consistent with other reports on the incidence of this pathogen from different parts of the world (Abuxapgui et al. 1999; Echeverria et al. 1989; Echeverria et al. 1992, Kétyi, 1989; Pál et al., 1997; Tamura et al. 1996).

6.5. Application of the colony immunoblot method for milk samples

As the means of transmitting the causative agents of bacillary dysentery can vary, the microbes can be found everywhere from contaminated food and water to air, toilets and household appliances etc. One of the many foods in which these pathogens can be found is milk (and other dairy products). In this study the colony blot method was applied to test artificially contaminated milk samples. The consistency of the milk samples (lipid and protein content) required a slight modification of the method, and as a result, the pellet of samples after centrifugation was inoculated onto TSA plates in a similar way to processing faecal samples. The detection of milk samples contaminated artificially with *Shigella* or EIEC by the colony blot method was successful compared to the traditional routine testing. The method's sensitivity was the same or even better according to our experiments, with the advantage that it gave reliable results much faster. Mention should be made of the superior sensitivity of the colony immunoblot method in the lower contamination level of the samples. This can be attributed to the fact that the colony blot method can screen more colonies (a few hundred) grown under the membrane than the number of isolated colonies, usually 8-16, screened by ELISA. To prevent foodborne outbreaks caused by *Shigella* and EIEC, further testing is required on other contaminated food, such as cheese, cottage cheese, yoghurt and ice-cream, as these are all well documented sources of such incidents.

6.6. Summary of the application of the colony blot method for environmental and clinical samples (papers I, II)

In summary, the IpaC-specific colony blot immune assay proved to be a suitable method of screening for EIEC and *Shigella* in liquid, faecal and milk specimens. As it is simple and cheap, does not require the setting up of well equipment laboratory, or trained staff, as in the case of molecular detection methods like DNA probes or PCR, it is easily applicable. Its time requirement does not exceed that of manual ELISA tests extensively used in several laboratories in countries of the developing word, where this disease is endemic. It is equally sensitive to ELISA, although it does not achieve the sensitivity of PCR performed on extracts of mixed stool cultures. On the other hand the colony immunoblot method is clearly superior to testing a limited number of randomly

selected colonies, i.e. the most frequently used diagnostic approach in laboratories equipped to carry out DNA hybridisation or virulence assays. The colony immunoblot method is highly efficient in screening a few hundred colonies simultaneously from the same sample, using one plate. The screening of this high number of colonies is practically unattainable with bio-, or serotyping.

Although the molecular methods have the advantage of directly testing the RNA or DNA extracts of the samples, they need simultaneous culturing in order to isolate the identified colonies for further investigations, like antibiotic sensitivity testing or epidemiological studies. Using the colony blot method, the pathogen colonies can be identified directly from the TSA plate used for culturing the sample under the membrane filter, and further investigations can be carried out without waiting for extra isolation. This advantage of the colony immunoblot method is of particular importance since EIEC is a pathogen present, but likely to be often unrecognised, in areas where it has only been occasionally reported before (Pál et al., 1997). For the further improvement of the methodology the possibility of combining antibodies specific to different virulence factors (e.g. IpaC and Shiga toxins) in a single colony blot assay to screen for various groups of diarrhoeagenic *E. coli* is currently under investigation.

6.7. Selective and enrichment media in the diagnosis of bacillary dysentery (paper III)

The conventional identification of *Shigella* from clinical samples is well established, however, the identification of Enteroinvasive *Escherichia coli* strains requires a more elaborate and sophisticated approach. EIEC can be identified by subjecting colonies isolated from the culture plate to molecular, immune or virulence assays specific to their invasive character (Echeverria 1991, Nataro JP 1998). The sensitivity of this diagnostic approach is determined by the ratio of EIEC colonies on the culture plate and also on the number of colonies selected randomly. Increasing the chosen number of colonies increases the costs and time requirements of the assay; therefore it is not practical in a routine laboratory. Some studies present the testing of maximum 20 colonies per sample, whereas Nataro and Kaper reported the minimum number when three colonies

were claimed to be sufficient to detect various groups of diarrhoeagenic *Escherichia coli* (Echeverria 1992, 1989, 1991, Taylor 1986, Nataro 1998).

The success in culturing the pathogen depends on the proportion of a pathogen's colonies present on the culture plates, which in turn depends on its ratio in the sample and also on the culture conditions used. No studies have been conducted previously to study the ratio of EIEC to normal flora in faecal samples, probably due to the fact that EIEC infections normally cause less severe clinical disease than *Shigella*, therefore it is not of primarily interest. In the present study it was shown that as a median value, the ratio of EIEC is 13.3 %, although in some cases as low as 3.3 % (Table 14). This finding is not significantly different from that seen for the 15 *Shigella*-containing samples. The ratio of the pathogen could even be lower as suggested by the lack of *Shigella* or EIEC among the over 2000 colonies tested by the IpaC-specific ELISA from one of the PCR positive samples. Our failure to detect any invasive pathogen from this particular specimen can, however, be explained by the high frequency of loss of the invasion plasmid, or disintegration of cells in the sample (Sethabutr O 1993). The ratios of EIEC colonies found in this study on TSA, MAC or on EO plates are shown in Table 14. According to these findings it can be concluded that random selection of three to five colonies, only, for virulence-specific assays from any of these plates could easily lead to false negative results.

When considering the effects of selective media on the growth of EIEC, it is worth bearing in mind that common enteric pathogens, like *Salmonella* or *Shigella* are known to be more resistant to chemicals used in selective media than the members of the normal flora (Dunn et al 1971, Hunt et al 1990, Taylor 1965, Taylor and Schelhart 1968, 1971, Hynes 1942, Isenberg et al 1969). This has not been investigated thoroughly in other studies before. As shown in our results this is also true for EIEC. Invasive *Escherichia coli* strains, in general, grew significantly better on highly selective plates than did their non-pathogenic counterparts of the same species (Tables 10, 11). Furthermore, as shown here for the first time, all dysentery-causing bacteria, irrespective of species (i.e. invasive *Escherichia coli* or *Shigella*), or of the biotype (*i.e.* Lac^- and Lac^+ strains of EIEC), as groups, were similar in their growth characteristics (Tables 10, 11).

Our results showed that DC, SS and XLD were significantly more effective than MAC or EMB plates to increase the ratio of EIEC in culture. This was supported by experiments both with isolated strains and with the attempt to recover EIEC from faecal samples (Tables 10, 11, 13). The comparison of selective plates, however, revealed, that DC and XLD were clearly superior to SS (Tables 13, 14). Of the six EIEC-containing (Table 14), and 16 *Shigella*-containing samples, none yielded less than 50 % pathogen colonies on XLD plates, while on SS there were specimens resulting in 20 % of EIEC colonies, only. Samples with lower pathogen contents than those seen in this study could also be encountered. Nevertheless, our data showed that selecting colonies from XLD for subsequent virulence-specific assays is more likely to ensure the identification of positive samples than random selection of colonies from SS plates.

Individual EIEC strains showed a broad variation in the colony forming capacities on selective plates. This suggested that, similarly to *Shigellae*, there were isolates more inhibited by these media than the majority of strains. Sensitive strains were strongly suppressed, indeed, by selective plates when recovered from faecal samples, too (Table 13). In these experiments also XLD provided the best results. Similarly to MAC and EMB, XLD plates were more effective to recover sensitive EIEC strains from faecal samples than DC or SS (Table 13). This is not surprising in the light of the similarities between the growth characteristics of EIEC and *Shigella* strains revealed by this study (Table 10, 11). XLD plates have often been noted to support the growth of "sensitive" *Shigella* strains apparently inhibited by other selective media (Hunt 1990, Taylor 1965, 1968, Isenberg 1969). However, the fact that on the different selective plates 15.4 % (SS) to almost 30 % (DC) of the EIEC strains grew rather weakly (EF < 2) suggests the need for a combination of plates with high and low selective power when processing clinical samples. This is also in accordance with the procedures generally recommended for the isolation of *Shigella* (Echeverria 1991b). The capacity of EIEC (and *Shigella*) strains to grow on the selective plates used do not relate to the plasmid-coded invasive character of the isolate, *per se*. In the present study, all EIEC and *Shigella* strains used were invasive.

Pre-incubating the samples in liquid enrichment media influenced the recovery of invasive bacteria slightly, only. As a tendency, pre-incubating samples in GNB moderately increased the recovery of EIEC, irrespective of the selective plates subsequently used, while SF was more inhibitory (Table 14).

The results of the three different experimental approaches, i.e. culturing pure strains, artificially contaminated faecal samples, and clinical specimens did not completely overlap. The three selective media did not differ significantly when culturing isolated cultures while DC and XLD outperformed SS when in recovering EIEC from faecal samples. The factors by which the growth of EIEC strains exceeded that of their non-pathogenic counterparts differed in the three groups of experiments (Tables 10-14). These differences can be explained with the different milieus the pathogens were exposed to under the various experimental circumstances.

When examining selective media for the purpose of isolating enteroinvasive *Escherichia coli* (EIEC) from clinical samples, we showed that EIEC strains behave exactly the same way on these media as *Shigella* strains regarding their culturing. This finding, however, although not surprising (due to the similarity of the pathogenicity of the two groups), is still a new finding. On the basis of these findings, that XLD was the best in recovering invasive *Escherichia coli* from faecal samples, the fact that it was more supportive for "sensitive" strains than were DC and SS plates, and finally, that it is frequently used with success by several laboratories to isolate *Shigella*, it is recommended that XLD should be used as a plating medium for the isolation of EIEC from clinical specimens. As samples may contain "sensitive" isolates it is also recommended that a less inhibitory medium, like MAC or EMB (Echeverria 1992, Silva 1980, Sethabutr 1993) should also be used in parallel. Random colony selection from these plates for molecular, immunological, or virulence assays should result in a better "hit-ratio" than if more inhibitory media, like SS, or if differentiating plates, like MAC, were used alone.

This draws many practical issues when considering the selective culture of samples associated with diarrhoea or bacillary dysentery. On the basis of these findings, it is recommended that beside the mildly selective media, even plates with high selectivity

(XLD) should be used, which, however can inhibit EIEC strains more than other medium but still have selective advantage for EIEC to the other normal members of the colon flora.

Our findings did correlate with the results of Silva's, i.e. that SS supports the growth of EIEC, however, in his studies this growth was not compared to the growth of the normal *E. coli*. Our findings showed that SS does not have a selective advantage to normal *E. coli*; therefore it is not applicable for the purpose of selective culture. According to our experience, the liquid selective and enrichment broths did not have this advantage either, therefore their primarily application is not recommended for the isolation of EIEC.

On the basis of the findings discussed above, the following protocol can be suggested in order to detect and isolate EIEC strains. The IpaC specific colony blot method should be carried out after culturing the sample on TSA plates, and/or the examination of isolates randomly selected from plates with low and always definitely high selectivity with the available EIEC specific methods like DNA hybridisation, ELISA, PCR, virulence tests.

7 CONCLUSIONS

1. A MAIC-1 monoclonal antibody based colony immunoblot method specific for the IpaC antigens of the causative agents of bacillary dysentery was developed.
2. The validation of the colony immunoblot assay was carried out to determine its specificity and sensitivity. The high specificity of this assay using the IpaC antigen specific monoclonal antibody MAIC-1 was proved. The sensitivity of this colony immunoblot assay was shown to be equal or slightly higher than that of the conventional methods, and lower than the molecular methods like PCR.
3. The method was successfully applied to testing environmental liquid samples. When filtering artificially contaminated water samples, even 1 bacterium cell was detectable from 10 litres of water after carrying out the colony immunoblot assay.
4. The method was also successfully applied to testing faecal samples. When examining primocultures of artificially contaminated or clinical faecal samples, it was clear that the sensitivity of the colony immunoblot assay is higher than that of the conventional microbiological methods.
5. A pre-treatment protocol using the combination of Tween-20 and heat treatment was established to eliminate the endogenous alkaline phospatase activity of the faecal samples that interfere with the immunodetection.
6. When testing food the method was successfully applied to examining artificially contaminated milk samples.
7. The selective media best applicable for the selective culture of EIEC was determined.
8. On the basis of the above results a diagnostic protocol was suggested that is applicable to detecting EIEC and *Shigella* strains even in laboratories with restricted resources. According to this protocol, besides the colony blot assay, highly selective media like XLD and also mildly selective, like MAC or EMB should be used in order to successfully culture invasive EIEC and *Shigella* strains.

8 SUMMARY

The importance of the detection and identification of the causative agents of bacillary dysentery, *Shigella* and EIEC strains, from the environment, water and food, as well as from patients with dysentery and carriers, is inevitable. The ideal detection method besides being sensitive and specific, should also be simple, cheap, in order to be available for routine use in less developed areas of the world where the disease is endemic.

The laboratory methods which are routinely used for the detection of *Shigella* are well defined, however, so far no simple method has been introduced for the routine detection of EIEC strains. As standard methods cannot differentiate EIEC from the normal *E. coli* due to the lack of specific bio-, or serological markers, only molecular methods like DNA hybridisation and PCR can detect these pathogens from clinical or environmental samples. Although these methods are highly specific and sensitive, yet lack the simplicity and low cost, therefore unavailable for most routine laboratories.

In the present study we further increased the sensitivity of a monoclonal antibody based, IpaC specific ELISA by modifying it to a colony blot method. We developed the colony immunoblot assay by determining the right parameters and media. Increasing the concentration of agar-agar of the TSA plates to 3%, without changing the expression of IpaC, successfully inhibited the swarming of *Proteus* strains. We applied the colony blot method to various samples including water, faeces and milk. For testing faecal samples, we developed a pre-treatment protocol to eliminate alkaline phosphatase activity in the samples. This protocol used a combination of heat and Tween 20 treatment of the nitrocellulose membranes. We compared the sensitivity of the assay to the traditional methods (culturing, bio and serotyping, ELISA) and the molecular methods (DNA hybridisation, the Polymerase Chain Reaction). The sensitivity of this method was shown to be equal to that of the traditional method based on culturing and bio or serotyping. The advantage of the colony blot assay to ELISA is that samples can be directly tested, and this method facilitates the screening of at least 200 times more colonies (maximum 2000) at a time. Additionally this method is simple, cost effective,

and relatively fast, as it gives results 24 hours earlier when compared to the time requirements of the conventional methods.

In this study we present first a comparative analysis of the growth of EIEC strains on various commercially available differentiating, selective and selective-enrichment media by culturing pure isolates, artificially contaminated faecal samples and clinical samples. EIEC strains were compared to other enteropathogenic strains as well as to the normal *E. coli* of the colon flora. MAC, EMB, DC, SS and XLD agars were compared to support the growth, and to facilitate the recovery of enteroinvasive *Escherichia coli* strains from artificially contaminated, as well as from clinical, faecal samples.

As pure cultures, *Escherichia coli* strains exhibited the same growth characteristics as did *Shigella* isolates. On the various selective plates they grew more weakly than *Salmonella* strains, but 4 – 10 times better than normal *Escherichia coli* isolates. XLD and DC plates were more effective in recovering enteroinvasive *Escherichia coli* from faecal samples than was SS. XLD, at the same time, was less inhibitory for "sensitive" strains than the other selective media. According to our results, the best choice of media for culturing EIEC was XLD, as a highly selective plate that inhibits the normal non-pathogenic flora of the colon effectively. These data show that XLD, in combination with MAC or EMB, provides the best chance of encountering enteroinvasive *Escherichia coli* when randomly selecting colonies from faecal cultures for subsequent molecular or immunological identifications assays.

Based on our results, we made a suggestion for a diagnostic approach for the detection of EIEC and *Shigella* to be used in routine laboratories. This protocol includes the use of the colony blot assay and/or the culture of samples on highly selective plates, like XLD in parallel with the mildly selectives.

9 ÖSSZEFOGLALÁS

A bakteriális vérhas, melyet a Shigellák és az un. enteroinvazív *Escherichia coli* (EIEC) törzsek okoznak, világszerte jelentős közegészségügyi probléma, ma is, elsősorban a fejlődő országokban, a hasmenéses megbetegedések egyik legfontosabb formája. A bacilláris dysenteria több mint két napig tartó fájdalmas hasmenéses enterális megbetegedés, lázzal és toxémiával jár, a széklettel pedig vér és/vagy nyálka ürül.

Az *Enterobacteriaceae* családba tartozó *Shigella* fajok és a pathogén enteroinvazív *E. coli* (EIEC) megegyező lefolyású bakteriális vérhast okoznak, megegyező pathomechanizmussal. A betegséget a bélfal nyálkahártya hámsejtjeinek inváziója révén okozzák. Az invázió egyes lépései 1, a baktérium behatolása a nem fagocitáló hámsejtekbe, 2, a baktérium megsokszorozóda a sejtben, 3, sejten belüli és sejtről sejtre való terjedése, 4, gazdasejt elpusztítása. Az invazivitáshoz szükséges polipeptideket vagy másnéven inváziós plazmid antigéneket (IpaA, B, C, D) egy 140 kb nagyságú plazmid egy régiója kódolja.

A terjedés általában közvetlen kontaktussal, a feco orális úton történik, azonban egyre növekvő számban fordulnak elő víz és élelmiszer okozta járványkitörések. A shigellózis, bár a fejlődő országokban ölt járványméreteket, a fejlett ipari országokban is felbukkan, ugyan már nem népbetegségként. A fejlődő országok elsősorban trópusi klímájuk és a nem megfelelő higiéniás viszonyok miatt alkalmasak a shigellózis járványszerű terjedésére. A *Shigella flexneri* fertőzések endémiásak, a S.*dysenteriae* pedig pusztító járványokat okoz. A shigellózis a fejlődő országokban a gyermekhalandóság egyik legfontosabb oka. A megelőzés és kontrol az egészségügyi intézkedéseken és a fejlett eset kezeléseken alapul, mivel hatékony vakcina kidolgozására még nem került sor. A prevenciót sürgeti az antibakerialis szerekkel szemben multirezisztens törzsek megjelenése, és az HIV fertőzések megnövekedése.

Mint minden fertőző betegség kapcsán, a bakteriális vérhas esetén is a korrekt, gyors, olcsó, érzékeny és specifikus mikrobiológiai diagnosztika elengedhetetlen feltétele a beteg kezelésének, a megelőzésnek, a járványtani helyzet felmérésének és a szükséges preventív intézkedések meghozatalának. A kórkép két kórokozó csoportja közül a

shigellák diagnosztikája kidolgozott, viszonylag egyszerű a hagyományos biokémiai és szerológiai módszerekkel, bár - különösen környezeti és élelmiszer minta esetén - nem kelloen érzékeny. A bélbaktériumok elkülönítésére kidolgozott Nógrády-féle polytropon egyszerre több biokémiai tulajdonság vizsgálható. Emellett polivalens és monoklonális ellenanyagokat használnak.

A kórkép további kórokozói, az enteroinvaziv *Escherichia coli* (EIEC) törzsek a hagyományos diagnosztika számára csak igen korlátozott mértékben hozzáférhetőek, tekintve, hogy a nem rendelkeznek a normál flóra tagjaitól őket egyértelműen elkülönítő biokémiai és szerológiai bélyegekkel. A rutin gyakorlatban az EIEC és más enteropathogén *E. coli* diagnosztizálására sorbitos McConkey táptalajról véletlenszerűen kiválasztott sorbit negatív telepeket, vagy kevert tenyészetbe belehúzva tömegből vett mintát agglutinálnak polivalens savókkal. Ezt követően agglutináció esetén térnek át a monoklonális savókkal történő agglutinációra.

A Shigellák diagnosztizálására a rutindiagnosztikai laborban elterjedt szelektív és differenciáló szilárd táptalajok az EMB és DC. Ezen kívül ismertek az XLD, SS, MAC. A folyékony táptalajban való dúsítás nem, vagy kevésbé terjedt el a gyakorlatban. Nagyon korlátozottak ismereteink az EIEC törzsek szelektiv tenyésztésének lehetőségeiröl. Silva szerint az EIEC növekedését az SS agar támogatta a legjobban.

A *Shigella* törzsek virulenciája genetikai hátterének feltárásával párhuzamosan már felmerült a lehetősége a törzsek molekuláris módszerekkel való felismerésének, illetve kimutatásának is. Az első módszerek a DNS hibridizáción alapultak, számos DNS próbát nagy sikerrel próbáltak ki. Az eljárást elsősorban makrokolónia telep-blot formájában használják a mintákból random válogatott klónokon. Ezt követően hamarosan elterjedtek a különböző PCR rendszerek is. Itt a legkiterjedtebben az ismeretlen funkciójú, de a plazmidon és kromoszómán is több kópiában megtalálható *ipa*H génre specifikus primereket használják. A mikróbák virulencia faktorainak megismerésével nyílt meg annak lehetősége, hogy a pathogén baktériumokat az általuk termelt, virulencia specifikus antigénjeik révén ismerjék fel. Ezen immunológiai eljárások előnye, hogy általában nem igénylik a molekuláris módszerekhez szükséges és költséges berendezéseket, és technikailag is egyszerûbbek.

Intézetünkben került kidolgozásra egy olyan ELISA eljárás, mely érzékenyen és specifikusan ismerte fel az EIEC (és *Shigella*) törzseket. Később ezt az eljárást módosítottuk a poliklonális ellenanyagot egy IpaC-specifikus monoklonális antitesttel (MAIC-1) helyettesítve. Az ELISA teszt azonban csak előzetesen izolált telepek tesztelésére volt alkalmas, primokultúrák vizsgálatára, és környezeti minták esetén a szuréssel feldolgozott anyagok tanulmányozására nem. A Shiga-like toxint termelő enterohaemorrhagiás *E. coli* (EHEC) esetén a telep immunoblot módszer ígéretes alkalmazásáról számoltak be székletminták esetén (177). Ebbol kiindulva választottuk disszertációnk egyik céljáva a *Shigella* / EIEC specifikus telep immunoblot módszer kifejlesztését, illetve az EIEC szelektív tenyészthetoségének vizsgálatát.

A telep immunblot módszer kidolgozása során meghatároztuk az eljárás paramétereit, és a szükséges táptalajokat. A kipróbált szelektív szilárd táptalajok közül a TSA lemezeken kapott eredményeket bizonyultak a legjobbnak. A lemezekben az agar-agar koncentrációját 3 % -ra emelve sikeresen oldottuk meg az esetleg jelenlévő *Proteus* törzsek rajzásának gátlását az IpaC kifejezodésének megváltoztatása nélkül. A széklet primokultura vizsgálatához kidolgoztunk egy olyan előkezelési eljárást, mely elroncsolja jelenlévő alkalikus foszfatáz aktivitást. Erre a célra legalkalmasabbnak a hő-, és Tween 20 kezelés kombinációját találtuk, mely nem befolyásolta az IpaC antigén kimutathatóságát. Ezzel az eljárással az egyes mintákba fellehető peroxidáz aktivitás is megszüntethető volt. Az immunblot módszer a székletminták esetén a shigellákat a hagyományos módszerekkel azonos érzékenységben mutatta ki. Emellett, ugyanilyen érzékenységgel mutathatók ki EIEC törzsek is szerocsoportjuktól függetlenül. Így - a sok helyütt nehezen hozzáférhető molekuláris módszerektől eltekintve - ez az első olyan eljárás, mely lehetővé teszi az EIEC telepek közvetlen, egyszerű azonsítását. Hasonló tapasztalattal jártak vizsgálataink a víz és tejmintákat illetően is. A vízminták esetében figyelemreméltó további eredmény volt az eljárás legalább egy nappal való rövidítése is.

A szelektív táptalajok vizsgálatakor megállapítottuk, hogy egyrészt az EIEC törzsek teljesen azonos módon viselkednek a *Shigella* törzsekhez hasonlítva tenyészthetőségük tekintetében. Ez a megállapítás - noha nem meglepő a két pathogén csoport

hasonlóságának ismeretében - mégis új. Gyakorlati következménye, hogy az EIEC szelektív tenyésztésre a kevéssé szelektív differenciáló lemezek (MAC, DC) mellett a nagy szelektivitású lemez (XLD) ajánlható, mely bár jobban gátolja az EIEC -et, mint a többi szelektív médium, a normál bélflóra tagjaival szemben az izolálást megkönnyítő előnyt biztosít az EIEC -nek. Bár mi is meg tudtuk erősíteni Silva korábbi megfigyelést, hogy az SS támogatja az EIEC növekedését, de az gyakorlatilag szelektív előnyt a normál *E. coli* -val szemben nem jelent, így a célra alkalmatlan. Tapasztalataink szerint szintén nem jelent előnyt a folyékony dúsítók használata sem.

Ezek alapján az EIEC felismerésére a következő protokoll javasolható: A primokulturából TSA lemezen IpaC specifikus telep immunoblot végzése, és/vagy kis szelektivitású differenciáló, és emellett feltétlenül nagy szelektivitású DC lemezről random válogatott laktóz pozitív és negatív telepek vizsgálata a rendelkezésre álló, EIEC -specifikus módszerekkel (hibridizáció, ELISA, virulencia tesztek).

Ebben a munkában végeredményben a következő célokat valósítottuk meg: a bakteriális vérhas kórokozóira specifikus, monoklonális antitest alapú telep immunoblot módszert dolgoztunk ki. A módszert sikerrel adaptáltuk széklet és környezeti folyadék mintákra. Kidolgoztunk egy, a széklet minták flórájának belső, az immundetektálással interferáló enzimaktivitásának eliminálásra szolgáló eljárást. Meghatároztuk az EIEC törzsek szelektív tenyésztésre alkalmas táptalajokat. Fenti eredmények alapján olyan diagnosztikus protokollra tettünk javaslatot, mely alkalmas az EIEC (és *Shigella*) törzsek felismerésére, és mely korlátozott lehetőségekkel rendelkező laboratóriumokban is megvalósítható.

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