Presence and viability of enterotoxigenic *Escherichia coli* (ETEC) in aquatic environments

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Till Karin och Malin

Ty det finns ingen vän som en syster
i storm eller lugnt väder
att liva den som gråhet ser
att hämta den som vilse går
att lyfta den som faller ner
att stärka den som står
/Christina Rossetti

ABSTRACT

Presence and viability of enterotoxigenic *Escherichia coli* (ETEC) in aquatic environments

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Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, Sweden, 2009

Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of diarrhoea among children in developing countries and in travelers to these regions. The bacteria are spread via contaminated water and food, and surface and drinking water in developing countries has been found to harbor these organisms. The standard methods for detection of ETEC include culturing and subsequent detection of ETEC enterotoxins by ELISA or the corresponding genes by PCR or DNA-DNA hybridisation. Identification of ETEC based on culturing of specimens may be unsuitable when analysing water samples for ETEC since it has been shown that enteric bacteria that enter the stressful environment of water can convert to a dormant, so called "viable but non-culturable" (VBNC) state.

In this thesis we have developed a real-time PCR assay with primers against the enterotoxin genes of ETEC for detection and quantification of ETEC bacteria in different types of water samples. The assay was proven to be highly specific for ETEC and allow detection of as few as three bacteria. The sensitivity was found to be considerably higher compared to phenotypic methods when analysing water samples from an endemic area in Bangladesh, where 67 % of the samples were positive for ETEC with the real-time PCR assay compared to 15 % based on culturing followed by toxin detection with ELISA.

The survival of ETEC bacteria was evaluated in sea- and freshwater microcosm experiments. Clinically isolated ETEC strains were incubated in the different water types for 12 weeks and the morphology, culturability and expression of virulence factors and housekeeping genes were studied over time. We could show that the ETEC bacteria remained intact and expressed virulence and housekeeping genes, despite the fact that all strains in seawater and one of the strains in freshwater were non-culturable *in vitro* using standard culturing media. It was also shown that ETEC was ingested by and survived in mussels at different temperatures indicating that consumption of contaminated seafood might be a route of infection for ETEC.

To show that the VBNC ETEC from the water microcosms were potentially viable the different strains were used to infect infant mice. Results showed that all strains from the seawater microcosms and one of six strains from freshwater were able to regain their culturability in mice suggesting that VBNC forms of ETEC present in water may be infectious. These results may be of importance for public health since previously used diagnostics based on culturing methods cannot detect VBNC forms of the bacteria and hence the risk of getting infected with ETEC might have been underestimated.

Keywords: Enterotoxigenic *Escherichia coli*, ETEC, real-time PCR, aquatic environments, VBNC, resuscitation, infant mouse model

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ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I – IV):

I **Å. Lothigius**, A. Janzon, Y. Begum, Å. Sjöling, F. Qadri, A.-M. Svennerholm and I. Bölin
Enterotoxigenic *Escherichia coli* is detectable in water samples from an endemic area by real-time PCR.
J Appl Microbiol. 2008;104(4):1128-36

II **Å. Lothigius**, Å. Sjöling, A-M. Svennerholm, and I. Bölin
Survival and Gene Expression of Enterotoxigenic *Escherichia coli* during Long Term Incubation in Sea Water and Freshwater.
J Appl Microbiol. 2009; *In Press*

III B. Hernroth , **Å. Lothigius** and I. Bölin
Factors Influencing Survival of Enterotoxigenic *Escherichia coli*, *Salmonella enterica* (serovar Typhimurium) and *Vibrio parahaemolyticus* in Marine Environments.
Accepted for publication in FEMS Microbiology Ecology

IV **Å. Lothigius**, S. Attridge, A-M. Svennerholm and I. Bölin
Resuscitation of Viable but Non-Culturable Forms of Enterotoxigenic *Escherichia coli* in an Infant Mouse Model.
Submitted

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ABBREVIATIONS

cDNA	Complementary DNA
CFU	Colony forming units
CF	Colonization factor
CT	Cholera toxin
DAEC	Diffusely adherent *E. coli*
DNA	Deoxyribonucleotide acid
EAEC	Enteroaggregative *E. coli*
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
GM_1	Monosialotetrahexosylganglioside; receptor for CT and LT
HNW	High nutrient water
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
Ig	Immunoglobulin
LB	Luria-Bertani
LNW	Low nutrient water
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleotide acid
RT	Reverse transcriptase
SLC	Sven Lovén Centre for Marine Science
Spp	Species (plural)
ST	Heat-stable enterotoxin
UV	Ultraviolet light
vPCR	Viability PCR
VBNC	Viable but non-culturable
WHO	World Health Organization

INTRODUCTION

ENTERIC BACTERIA

Most of the enteric bacteria belong to the family of *Enterobacteriaceae* which is the largest of medically important gram-negative bacilli with more than 130 described species (74). These bacteria are found worldwide in soil, water and vegetation and are usually part of the intestinal normal flora of most animals and humans.

Many of the bacteria in this family can live in the gut without causing any health problems but some bacteria almost always cause infections with symptoms like vomiting, diarrhoea and fever. People usually get infected with enteric bacteria as a result of poor hygienic conditions, such as inadequate sanitation and contaminated food and drinking water, which is common in developing countries. Infections with enteric bacteria are one of the major causes of childhood morbidity and mortality in the developing world today (93, 109) and acute infectious diarrhoea is estimated to cause 2 million deaths each year (1, 105).

Important enteric pathogens

In the genus *Salmonella*, *Salmonella enterica* is the most common species associated with human disease. *S. enterica* is further divided into several serogroups (or serovars) based on their cell surface antigens. The most common human pathogens are the serovars *S. enteriditis* and *S. typhimurium* causing gastroenteritis, sometimes referred to as salmonellosis (74, 107). Most infections with *Salmonella* are acquired by consumption of contaminated food (108). The bacteria are invasive and replicate in the small intestinal epithelium (107, 127) causing cell death, inflammation and secretory responses.

Shigella species cause gastroenteritis (shigellosis) and in severe cases also bacterial dysentery with humans as the only reservoir (74). *Shigella* is also an invasive bacterium that invades the epithelial cells of the large intestine but it can also produce a toxin, Shiga toxin, which inhibits protein synthesis. The dysenteric form is characterised by bloody diarrhoea caused by necrosis and inflammation in the colon.

Campylobacter belongs to the family of *Spirillaceae* (motile, spirally, curved rods). The bacterium is one of the most common causes of food-borne, diarrhoeal illness in the world. Contaminated poultry is the major source of infection, but the mechanisms of pathogenesis is not well established (128). Some strains of *Campylobacter* have invasive potential but no obvious virulence factors other than the cytolethal distending toxin (CDT) has been revealed (89).

The genus *Vibrio* consist of many species of curved bacilli of which *V. cholera*, *V. parahemolyticus* and *V. vulnificus* are most commonly infecting humans (74). Infection with *V. cholera* cause gastroenteritis and the source of infection is most often contaminated food and water. *V. parahaemolyticus* and *V. vulnificus* cause gastroenteritis and wound infections and these species are naturally existent in coastal marine environments and can therefore be transmitted via consumption of contaminated seafood (91).

The most common and important species of the genus *Escherichia* is *Escherichia coli* (74). *E. coli* colonise the gastrointestinal tract of infants within a few hours after birth and is the predominant facultative anaerobe of the normal flora throughout our lives (35). However, the bacterium is also responsible for a variety of diseases like urinary tract infections (UTI), sepsis, meningitis and gastroenteritis.
There are six different groups of pathogenic *E. coli* that cause diarrhoeal disease (77). These pathogenic groups have evolved through the acquisition of virulence genes and pathogenicity islands by horizontal gene transfer in the normal flora. Some of the groups differ more than 30 % in their genetic material compared to non-pathogenic *E. coli* (86, 125).

- Diffusely adherent *Escherichia coli* (DAEC) attach to the epithelium of the small intestine and stimulate elongation of the microvilli which cause a watery diarrhoea (74).
- Enteroaggregative *Escherichia coli* (EAEC) is associated with persistent diarrhoea in children in developing countries EAEC produce a hemolysin and a toxin, similar to the ST toxin of ETEC, that stimulate intestinal secretion and it also attaches to the intestine, shortening the microvilli with decreased fluid absorption as a result. EAEC cause disease in humans only (107, 124).
- Enterohemorrhagic *Escherichia coli* (EHEC) are the most common pathogenic *E. coli* in developed countries (74). The bacteria are also called shiga-like toxin producing- or

verotoxin producing *E. coli* named after the cytotoxins produced by the bacteria (128). EHEC attach to the epithelial cells in the large intestine and induce cytoskeleton remodeling by injection of effector proteins by a type III secretion system. The very potent toxins produced can cause severe diseases like hemorrhagic colitis and hemolytic uremic syndrome due to local and systemic absorption of the toxins (107).

- Enteroinvasive *Escherichia coli* (EIEC) is closely related to *Shigella* and is able to invade and destroy the epithelium in the large intestine, resulting in watery diarrhoea and in severe cases also dysentery (74).
- Enteropathogenic *Escherichia coli* (EPEC) causes infant diarrhoea in developing countries (74). The bacteria produce its own receptor, Tir, which is injected by a type III secretion system into the cell and subsequently inserted in the epithelial cell membrane. Several other proteins are also injected into the host cell causing redistribution of actin and changes in cell morphology, similar to EHEC (18, 128). The result is destruction of microvilli and thereby malabsorption and diarrhoea.
- Enterotoxigenic *Escherichia coli* (ETEC) is the most common one among the diarrhoeal *E. coli*, particularly in children in developing countries. A more detailed description of ETEC will follow below.

ENTEROTOXIGENIC *ESCHERICHIA COLI*

History

ETEC was first recognised in Calcutta in 1956 (27) when *E. coli* isolates from persons with a cholera-like disease were injected into rabbit ileal loops and were found to cause a massive fluid accumulation. More than ten years later, *E. coli* isolates from patients with a cholera-like illness were found to produce a toxin that was responsible for the diarrhoea (93) which later was confirmed by oral challenge of human volunteers (32). The fact that ETEC is a separate group of diarrhoeal bacteria was thus discovered rather recently (113).

ETEC were more frequently found in children than in adults and today, this bacteria is estimated to cause 380.000 deaths, accounting for one fifth of all deaths in children under the age of five (65).

Metabolism

ETEC is a chemoheterotroph, facultative anaerobic bacteria that can derive energy by aerobic respiration if oxygen is present but it can also switch to fermentation or anaerobic respiration under anaerobic conditions. The nutritional requirements of the bacteria are simple. This versatility enables *E. coli* to adapt to both intestinal (anaerobic) and extraintestinal (aerobic or anaerobic) milieus. In the laboratory, ETEC can grow on a variety of different media and it has been shown that the bacteria can survive for long periods in the environment (70).

ST and LT toxins

The virulence mechanisms of ETEC include two toxins; heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). ETEC can produce one or both of these toxins. The toxin genes are situated on plasmids and the plasmid profiles in ETEC show great variability.

The heat-stable enterotoxin is a low-molecular-weight protein that consists of 18 or 19 amino acids. ST can be divided into two groups; STa (or STI) and STb (or STII). STa is produced by ETEC strains infecting both humans and animals whereas STb is primarily associated with disease in animals, although recent studies indicate that it might also be associated with human disease (77, 81). STa is further subdivided into STh (STaI) and STp (STaII) named after their initial discovery in humans and pigs, respectively. The toxins are encoded by the transposon-associated *estA* (STh) and *estB* (STp) genes and the amino acid sequences are very similar, only one amino acid differentiates the two proteins (107).

The receptor for the ST toxins is guanylate cyclase in the small intestine. Binding to the receptor results in increased levels of cyclic GMP which stimulate chloride secretion and inhibit NaCl absorption, resulting in net intestinal fluid secretion (Fig. 1) (77, 107).

The heat-labile enterotoxin exists in two forms; LTI is associated with both human and animal disease and LTII which has only been found in ETEC strains infecting animals (107). The LTI (hereafter referred to as LT) is a so called AB_5 toxin with five binding subunits (LTB) and one active subunit (LTA). LT toxin is very similar to cholera toxin (CT) with 80 % protein sequence identity. In fact, LT and CT bind to the same receptor (GM_1 ganglioside) and have the same enzymatic activity. Binding to the receptor results in increased levels of intracellular cyclic AMP (cAMP) which stimulate chloride channels in the apical membrane,

leading to secretion of electrolytes and water and inhibition of NaCl absorption in the intestine (Fig. 1) (77, 107). Also, studies have suggested that the enterotoxins can influence the enteric nervous system in the gut, resulting in increased motility and fluid secretion from the intestine (71).

Colonization factors

ETEC use colonization factors (CFs) to attach and bind to receptors on cells in the small intestine. There are more than 25 identified CFs in ETEC strains infecting humans and they are designated as coli surface antigen (CS) with a number in their order of identification, with the exception of CFA/I. The CFs are mainly fimbrial or fimbrillar structures and the genes of some of the CFs have been shown to be plasmid borne (38, 129). Some CFs are more common than others, CFAI, CS1 to CS7, CS17 and CS21 are the most common CFs present on diarrhoeagenic strains and are found on ETEC strains all over the world (92). However, CFs are not detected on all ETEC strains. The reason for this might be absence of CFs, loss of CF properties or lack of detection methods. Recent studies on ETEC colonisation suggest that LT toxin itself can act as a colonization factor binding to structures on the epithelial cell surfaces (58, 72).

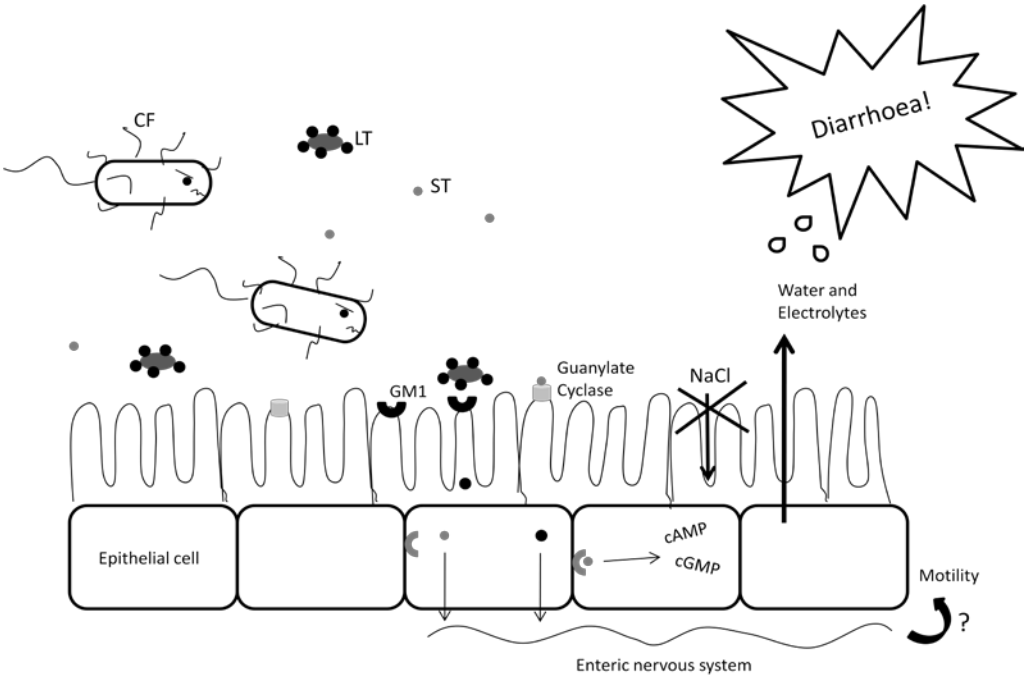


Figure 1. *Virulence factors and mode of action of ETEC in the gut.*

ETEC disease

ETEC disease is referred to as cholera-like and can vary from very mild to severe and there are also short-term, asymptomatic carriers of the bacteria (11, 93). ETEC diarrhoea usually has a sudden onset with an incubation period of 14 – 50 hours. The symptoms are watery diarrhoea, vomiting and in rare cases fever. The illness last for 3 to 4 days and is self limiting but in the more severe cases hospitalising might be needed. Treatment of ETEC diarrhoea includes intravenous rehydration therapy and/or oral rehydration solutions. Antibiotics are not commonly used in treatment of ETEC diarrhoea due to the time demanding diagnosis and lack of controlled studies to provide recommendations. Also, childhood diarrhoea is usually a result of several bacterial and viral agents (63, 93) and the effect of antimicrobials is difficult to study in children.

Diagnostics

ETEC bacteria are not fastidious and grow on a variety of different media. Differentiation of ETEC from other *E. coli* is achieved by identifying the specific virulence factors. There are many, both genotypic and phenotypic, as well as physiologic methods that can be used for detection of ST and LT toxins and CFs in various types of samples. Initially, the golden standards for identification of ETEC were the physiological models; rabbit ileal loop model for LT (27) and the infant mouse model for ST (28). These assays are time demanding and require skilled personnel. Tissue cultures has also been used for detection of LT (30) but later on simpler methods were introduced when the enzymed-linked immunosorbent assay (ELISA) was developed (132). ELISA is a phenotypic method that requires culturing of the bacteria before testing for presence of enterotoxins. The technique has been widely used for detection of LT using microtiter GM1 ganglioside methods and this technique has also been further evolved into the inhibition-ELISA for detection of ST (117). Both ELISA and inhibition ELISA, based on monoclonal antibodies against LT and ST, are today used in many laboratories for detection and identification of ETEC. However, sensitive and specific molecular techniques have been developed and are in many cases preferable due to reduced time of the tests. DNA-probes derived from plasmids encoding ST and LT are used for detection of the enterotoxin genes in DNA-DNA hybridisation assays (52).

Serotyping has also been used to identify and characterise ETEC strains (85). Determination of O serogroups associated with the lipopolysaccharides in the cell wall and H serogroups of the flagella are commonly used for other pathogenic *E. coli* but is somewhat difficult since more than 78 O groups and 34 H groups have been identified among ETEC (93). There are no serotype that dominate and the huge number of combinations of O and H groups makes serotyping less suitable for identification of ETEC. The most common diagnostic methods for ETEC are summarised in Table 1.

Polymerase chain reaction (PCR) is also used in ETEC diagnosis. PCR is a molecular method for amplification of a specific DNA sequence that has revolutionised molecular biology. It is an extremely efficient method; millions of copies of any specific sequence can be made, even from a complex mixture of DNA. Two DNA binding oligonucleotides, called primers, are mixed with a DNA polymerase enzyme, nucleotides that serve as building blocks and the DNA sequence that is to be amplified in a reaction tube. The mixture undergoes several rounds of replication and in each replication the number of DNA sequences is doubled. After *n* rounds of replication, 2^n copies of the target have, theoretically, been produced.
PCR is a sensitive and specific method that can be used for diagnosis on both clinical and environmental samples and is widely used for detection and identification of ETEC and a variety of primers against enterotoxin genes and colonization factor genes are described in the literature (112). PCR assays can also be performed with several primer pairs in each run (multiplex PCR) and in this way target several genes at once (101).

Table 1. *Summary of some of the most common diagnostic methods for ETEC.*

Method	**Sensitivity**	**Specificity for ETEC**	**Quantitation**	**Time**
Animal models	Low	Very low	No	1-2 days
Culture	Very low	Low	Possible	1 day
ELISA	Low	High	No	1-2 days
Conventional PCR	High	High	No	< 1 day
Multiplex PCR	High	High	No	< 1 day
Real-time PCR	Very high	High	Yes	< 1 day

Recently, real-time PCR with the possibility to quantitate the bacteria in the sample has been introduced in ETEC research (69, 99).

Real-time PCR (51) is based on the same principles as PCR but the amplification of the DNA target is measured in real-time. The nucleotides are fluorescently labeled and emit lights, which is measured by the instrument, when binding in to their target. The increase in fluorescence for each cycle is plotted against the number of cycles performed. After a certain number of cycles (threshold cycle or C_t) the change in light emission becomes significantly higher than the background fluorescence, which is dependent on the initial concentration of DNA target. In this way, it is possible to determine the concentration of target sequence in the sample tested. Real-time PCR is often referred to as quantitative PCR (qPCR) and is now frequently used in microbiology when quantifying bacterial genes or genomes in a variety of samples (66). It is a very sensitive method and can target species specific sequences or parts of genes depending on the design of the primers. It is however important to remember that the method detects all DNA and cannot discriminate between live or dead bacteria, or viable but non-culturable (VBNC) forms of bacteria.

The methods used for diagnosis of ETEC infection varies from one laboratory to another and is usually dependent upon the capability of that laboratory. In developing countries, culture on selective media, e.g. MacConkey agar plates followed by phenotypic methods are more common since they are usually relatively easy to set up. However, since the virulence genes are situated on plasmids, these can be lost, silenced by mutations or repressed by regulatory genes and hence, genotypic methods may therefore be a preferable alternative, or at least a complement to the phenotypic methods. For these reasons, many laboratories in developing countries are not able to identify ETEC in clinical and environmental samples and ETEC is not always included in their routine diagnostics (93).

Immunity and protection

The incidence and prevalence of ETEC disease decrease with increasing age suggesting that immunity against the bacteria is developed (12). Infection with ETEC triggers the production of secretory immunoglobulin A (SIgA) in the intestine which may give protective immunity by preventing bacterial adhesion and toxin activity. Systemic IgA and IgG antibodies against the CFs, LT toxin and O antigen is also seen but the main protection against ETEC diarrhoea is thought to be locally produced SIgA antibodies against the CFs (116). ST toxin is non-immunogenic and does not give natural protection against re-infection with ST producing ETEC.

There is a great need for a safe and efficient vaccine against ETEC to reduce childhood mortality. The strategies for vaccine development are based on specific colonization factors. A vaccine containing the most common CFs (CFAI and CS1 to CS6) would provide up to 80 % protection (93). One vaccine candidate that has been tested on human volunteers is the inactivated whole-cell vaccine which contains killed ETEC bacteria expressing the most important CFs together with the cholera toxin B subunit (2). This vaccine was proven safe and gave rise to significant IgA responses in the intestine when given to Swedish adult volunteers. However, in a study in Egypt the same vaccine did not give significant protection in 6 – 18 months old children (116). Other vaccine strategies are the live ETEC vaccines with strains expressing different CFs and with a detoxified LT toxin. Recently, it has been shown that ETEC strains overexpressing CFs induce strong serum IgA and IgG responses and they may be useful as candidate strains for an oral ETEC vaccine (119).

Transmission and epidemiology

ETEC is one of the main causes of childhood diarrhoea in developing countries and also the major cause of travelers' diarrhoea in these areas. The bacteria is spread via contaminated water and food and, in developing countries where the sanitation is insufficient, ETEC is often a major cause of diarrhoea. According to WHO, one third of the world's population lack water to meet their daily needs (1).

In developing countries such as Bangladesh, ETEC bacteria have been isolated from surface waters and transmission of the bacteria may occur while bathing or using the water for household purposes (8). Studies on ETEC strains isolated from surface waters in Bangladesh

showed that these strains were comparable to clinically isolated ETEC strains in both toxin- and CF-type and in pulsed-field electrophoretic analyses (93). These results support the theory that surface waters may be a source of survival and transmission of ETEC as has been observed for *V. cholerae*.

Studies from Bangladesh, Egypt and Brazil, where ETEC is endemic, show that ETEC diarrhoea and asymptomatic infections increase during the warm periods of the year. In Bangladesh, ETEC infections follow a typical seasonal pattern with one peak during the hot moths of April – June and one peak after the heavy monsoon rains in September – October (Fig. 2) (93).

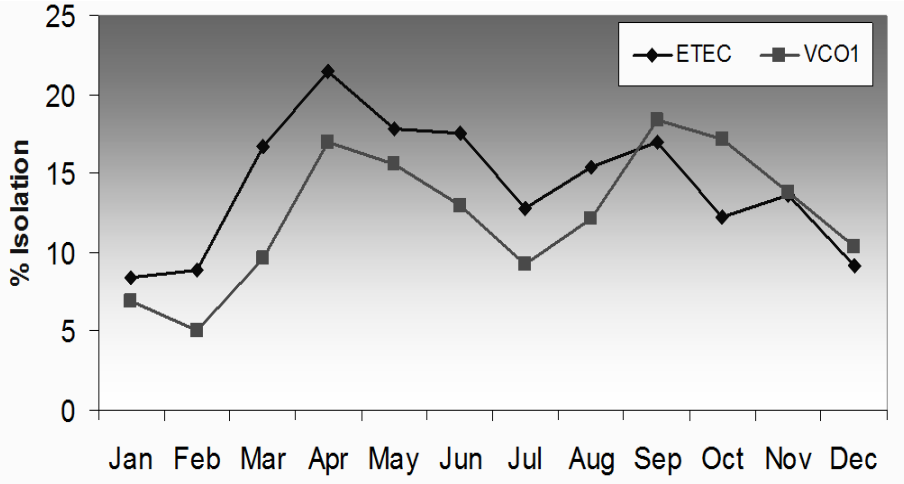


***Figure 2**. Isolation of ETEC* (♦) *and Vibrio cholera* O1 (■) *from diarrhoeal stools of children less than 5 years of age at ICDDR,B between 1996 – 2002 (93). Stools obtained from 2 % systematic sampling.*

Travelers visiting these areas are also at greater risk to get infected during the epidemic peaks. There have also been reports on outbreaks of ETEC diarrhoea onboard cruise ships (26). According to CDC (Center for Disease Control in USA, www.cdc.gov) there were 16 reported outbreaks of ETEC disease between 1996 and 2003 in the US. The incidence of ETEC diarrhoea in the US is approximately 79.500 cases per year compared to several hundreds of million cases in the whole world. In Sweden there is no compulsory registration of ETEC disease and it is therefore difficult to estimate the incidence of ETEC diarrhoea in the country. However, a prospective study from 1997 on adult patients with acute diarrhoea in Sweden showed that 8% of the patients were infected with ETEC (118).

The genotype of ETEC may differ in different parts of the world (17, 46) and it has been shown that specific clones of ETEC can circulate in defined areas for a period of time (78).

ETEC in water

The primary bacterial indicator used for assessment of microbial contamination of water consists of the coliform group. *E. coli* is one of the most common coliform bacterial types and detection of *E. coli* is a definite evidence of fecal pollution (34, 115).

E. coli has been extensively studied in the environment and it has been shown that the bacteria can survive for long periods of time in water (24, 37, 64, 76, 90). Although pathogenic forms of *E. coli* share many basic properties with *E. coli* in the normal flora, they differ enough in their genetic material to deserve to be studied separately.

The survival response of *E. coli* in different types of water as well as in environments associated with aquatic milieus, i.e. sand, has been studied (4, 76, 102, 103, 120) but still, more research in the subject is needed. When exposed to aquatic environments, the bacteria face a number of hostile factors, e.g. biotic (competition, predation) and abiotic (pH, light, salinity, oxidative stress, temperature, osmotic pressure and nutrients deficiency) and in order to survive the bacteria need to save energy by a number of different stress responses (102, 103). These mechanisms include downregulation of genes involved in cell division and nucleotides biosynthesis and upregulation of genes involved in energy metabolism, chemotaxis and motility (103). Several studies point out that the survival of enteric bacteria in water is enhanced by lower temperatures (25, 102, 104, 110). Contrary, visible and UV light greatly affect the survival of enteric bacteria in water in a negative fashion (76, 102, 120).

Different pathogenic forms of *E. coli*, including ETEC, have been isolated from drinking and surface water samples (96) and among the pathogenic *E. coli* types, EHEC is the most well-studied in aquatic environments (4, 126). This is probably due to the risk of animal-derived EHEC in runoff from agricultural land into watercourses.

Even though it is known that ETEC is transmitted via contaminated water and that the bacteria has been isolated from drinking and environmental water in endemic countries (8, 69, 95, 96), little is still known about the mechanisms of survival and if these bacteria are still infectious. Most studies on ETEC in aquatic environments have focused on detection and isolation of the bacteria (8, 67, 69, 94) and antibiotic resistance in isolated ETEC strains (95, 96).

The largest villain in ETEC disease is poor water quality and sanitation problems. Control and prevention of transmission of ETEC would help solve the problem, but in developing countries where the access of clean water is very limited, this is not easily achieved.

VIABLE BUT NON-CULTURABLE BACTERIA

The ability to culture bacteria on routine media in the laboratory has long been considered proof of viability. However, studies over the last 20 years have shown that this is not always true. When bacteria are subjected to a new environment they may adapt and employ a variety of genetic mechanism in order to survive (22). These bacteria are able to retain their metabolic function with energy saving strategies but cannot grow on standard media with available methods. The term used for this state of the bacteria is viable but non-culturable (VBNC) or active but non-culturable (ABNC) (61). The environmental factors that might induce the state of VBNC include changes in temperature, nutrients, salinity, osmotic pressure and pH (82).

This dormant stage is not new for microbial ecologists who recognised the limitations to isolate and grow bacteria in nature long ago (22, 44, 130) but, the VBNC term is still debated. Some studies suggest that VBNC forms of bacteria are either dead or of no significance (3, 13, 14) whereas others believe that pathogenic bacteria entering the VBNC state are not only viable but also infectious (6, 68, 90). It has been shown that enteric bacteria, like *E. coli, Campylobacter jejuni*, *Salmonella* and *Vibrio* species can enter this dormant stage under starvation conditions (76, 82, 120) but little is still known about the mechanisms for survival and the ability to cause disease by these bacteria. Some studies suggest that the reason for the inability to grow on standard media is that the high concentrations of nutrients in the media might be toxic for the bacterial cells and that free radicals are produced after exposure which might prevent colony development on the media (84). Much effort has been made to convert non-culturable bacteria back to “normal”, often by changing the nutrients in the media and gradually switch from low to high nutrient content but few of these studies show sufficient evidence of resuscitation of these organisms (61).

AIMS OF THE STUDY

The overall aim of this thesis was to study the presence of ETEC in various water environments and subsequently analyse morphological and molecular characteristics of these bacteria in relation to disease. This work also aims to answer whether water, or organisms living in water, such as plankton or mussels, may be reservoirs of ETEC and thus a source for human infection.

The specific aims were:

- To develop sensitive and specific methods for detection and quantification of ETEC in various types of water samples.
- To study survival, gene expression and toxin production of ETEC during long term incubation in sea- and freshwater.
- To evaluate if waterliving organisms, like mussels, can ingest the bacteria and act as a reservoir for ETEC.
- To evaluate if VBNC forms of ETEC are induced under stressful conditions, like incubation in water.
- To study if VBNC forms of ETEC can regain their culturability in an infant mouse model.

MATERIAL AND METHODS

Detailed descriptions of the methods used can be found in paper I-IV included in the thesis. Here, the most important methods and techniques used in the study are discussed.

COLLECTION, TREATMENT AND ANALYSIS OF WATERSAMPLES

The prevalence of ETEC in drinking and environmental water samples from Dhaka, Bangladesh was studied by two different methods; ELISA and real-time PCR.

Drinking water samples were collected from households in Mirpur, a slum area in Dhaka between October 2005 and March 2006. The water is chlorinated prior to distribution via pipelines to a central water pump in Mirpur where the inhabitants collect their water and store it in pots and jars in their homes for up to 72 hours (Fig 3). Drinking water samples were collected from these pots and jars from different households.

Figure 3. *People collecting water from a central water line in Mirpur, a slum area in Dhaka, Bangladesh. Photo: Anders Janzon*

Environmental water samples from ponds and lakes were collected during the same time period. The samples were collected at the surface level, approximately 20 cm below the water surface.

All water samples were collected in volumes of 150 ml in sterile flasks and transported on ice to the Centre of Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Environmental water samples were filtered through a Whatman filter to remove large particles and then through a 0.22 µm Millipore filter. Drinking water was directly filtered through the 0.22 µm filter. One half of the Millipore filters were used for culturing of *E.coli* by pre-incubation in MacConkey broth and thereafter 100 µl of the broth cultures were spread on MacConkey agar plates. From each plate, 52 lactose fermenting colonies with *E. coli* morphology were tested for ST and LT enterotoxins by GM1 ELISA and inhibition ELISA (7, 112). This part of the experiment was done at ICDDR,B.

The second halves of each filter were used for DNA extraction. Each filter was put in an microcentrifuge tube and sent to University of Gothenburg. For DNA extraction, the filters were put in DNA lysis buffer overnight whereafter DNA extraction was performed as described (69). The purified DNA was tested for ETEC enterotoxin genes in real-time PCR with primers against the genes of STh, STp and LT. Standard curves of the genes tested were included in each run in order to quantify the number of ETEC cells in each sample. (69). The treatment of the water samples in paper II is summarised in figure 4.

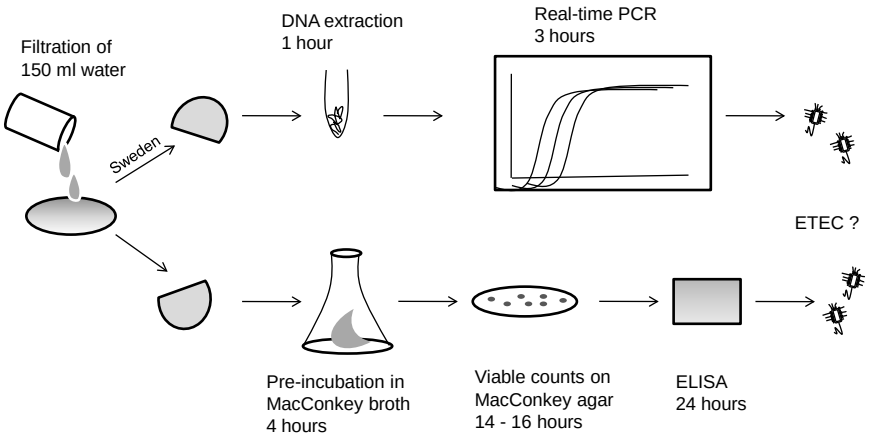


Figure 4*. Schematic picture of the processing of water samples from Mirpur, Bangladesh and the approximate time of analysis.*

In addition to the water samples from Bangladesh, samples were also collected from Moçambique, Guatemala and Djibouti. These samples were treated in the same way as above

but with the exception that the ELISA analyses were not performed, although culturing was done to determine if there were culturable *E. coli* bacteria in the water samples. These analyses are part of ongoing project with collaborating laboratories and are yet not presented in any publications or manuscripts.

WATER MICROCOSMS

In order to study ETEC in aquatic environments, water microcosms were set up using sea- and freshwater. Seawater was taken from the water system of Sven Lovén Centre for Marine Sciences (SLC), Fiskebäckskil (SLC; former Kristineberg Marine Research Station) where seawater from 30 m depth with a salinity of ca 33 PSU (practical salinity units) is continuously pumped from the Gullmarn Fjord outside SLC. The pH of the sea water was 7.81. In addition, microcosms experiments were carried out using seawater with different nutrient status; high nutrient water (HNW) and low nutrient water (LNW) collected from Maputo, Moçambique (paper III).

Freshwater for the microcosms was collected from Ören, an oligotroph-mesotroph lake 8 km east of Gränna, and the collection was done at the surface level. The pH of the freshwater was 6.00. Sea- and freshwater was sterile filtered through 0.22 µm filters into cell culturing bottles or glass flasks and ETEC bacteria were inoculated into the microcosms in order to measure culturability, viability, morphology, toxin production and gene transcription when exposed to aquatic environments.

The culturability of ETEC bacteria were measured during the experimental period by spreading samples from the microcosms on different agar plates and counting of colony forming units (CFU). CFUs were compared with the total number, as determined by qPCR, and the number of intact ETEC cells, as determined by vPCR (see "Extraction of DNA and RNA), in each sample in order to determine if ETEC switch into a VBNC state. In addition, mRNA was extracted and cDNA synthesised and analysed in real-time PCR with primers against toxin genes, CF genes and housekeeping genes (70) in order to evaluate if virulence genes and housekeeping genes were transcribed during the incubation. The morphology of ETEC bacteria in sea- and freshwater was studied by electron microscopy on samples from each microcosm (70).

Culturability of ETEC was also studied in seawater at different temperatures (8 ºC and 18 ºC) and with different nutrient status (HNW and LNW) by incubation of bacteria in the different water types. Survival was measured after one and two weeks by spreading of samples from the microcosms onto horse blood agar plates and determination of CFUs (paper III).

The influence on culturability of ETEC (E20738A, LT, CS17) by phyto- and zooplankton was evaluated by addition of the dinoflaggelate *Scrippsiella trochoidea* and/or the copepod *Paraeuchaeta norvegica* to seawater microcosms (250 ml). The microcosms were incubated for 22 hours at 8 ºC and 18 ºC after which the samples were filtered through a series of filters (200 µm, 20 µm and 0,22 µm) for collection of the different size fractions of the bacteria and plankton. Each filter was rinsed in 1 ml of PBS of which 100 µl was spread onto horse blood agar plates and the culturability of ETEC was measured as described. In addition, DNA was extracted from each fraction and analysed in real-time PCR with primers against the *eltB* gene as described.

EXTRACTION OF DNA AND RNA

DNA was extracted from all samples using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and kept at 4 ºC for short term storage and – 20 ºC for long term storage. When extracting DNA from mussels, a few different techniques were evaluated for optimal DNA recovery; Phenol-chloroform extraction (20), E.Z.N.A.® Mollusc DNA Kit (Omega Bio-Tek, NA, USA), QIAamp DNA Stool Mini Kit (Qiagen) and the DNeasy Blood and Tissue kit of which the latter gave the highest DNA yield from homogenised hepatopancreas of the mussels. However, when analysing the diluted and undiluted DNA in real-time PCR for quantification of ETEC bacteria, we could conclude that inhibitory substances were present in the samples. Also, when spiking homogenised hepatopancreas with known concentrations of ETEC bacteria, the DNA yield after DNA extraction varied significantly between duplicate samples. Therefore, DNA extraction and real-time PCR analyses were excluded from the experiments with mussels.

By treating samples with ethidium monoazide bromide (EMA) before extracting DNA from the samples, it was possible to eliminate DNA from bacteria with a non-intact cell membrane (79). EMA can penetrate dead cells and bind to intracellular DNA upon exposure to light, preventing its amplification via PCR. This is a method that is frequently used for viability

testing together with qPCR, sometimes referred to as viability PCR (or vPCR) (29, 39, 55, 88, 121, 122). In this study, EMA was used in order to be able to distinguish between the total number of ETEC cells and the number of intact ETEC cells when quantifying the bacteria in real-time PCR.

For RNA extraction, all samples were immediately stabilised in lysis buffer (TE-buffer with 400 µg ml^{-1} lysozyme and Buffer RLT (Qiagen) with 1 % β-mercaptoethanol), RNA-later or RNAprotect Bacteria Reagent (both from Qiagen) whereafter RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.
RNA samples were divided into two aliquots, one was used for cDNA synthesis, performed with the Quantitect Reverse Transcripton kit (Qiagen) and the other was used as a reverse transcriptase negative control (-RT control) in the subsequent real-time PCR assays.

REAL-TIME PCR

Real-time PCR (qPCR) primers targeting virulence genes and housekeeping genes (69, 70) were designed using the software Primer Express (Applied Biosystems, Foster City, CA, USA). To ensure specificity of the primers, all primer sequences were analysed in a BLAST search (NCBI genome database) against all available genomes. The primers were also tested in qPCR against a number of different enteric bacteria as well as against a number of well characterised ETEC bacteria (69, 70) to ensure that the primers detect the specific target genes in ETEC and that no unspecific products were produced.

Standard curves were developed with serial dilutions of known concentrations of ETEC genomes or from known concentrations of PCR products from the genes tested. By using the standard curves based on whole genomes, it was possible to quantify the number of bacteria in each sample. This was mainly done when analysing DNA from water samples, DNA from mouse intestines or bacterial cultures with unknown concentrations of ETEC bacteria.
The standard curves based on PCR products were used when analysing cDNA from samples in order to determine the gene expression levels of ETEC in subsequent samples.
By combining the two different standard curves in the qPCR assays, it was possible to determine the gene expression level per bacteria in each sample.

MUSSELS AND HAEMOCYTES

The marine mussel *Mytilus edulis* is widely distributed in European waters and often used as a model species for pollution studies (33). It was earlier believed that this species were not capable of ingesting free bacteria (75) but recent studies show that blue mussels filtrate and selectively ingest bacteria (50). The mussels filter water through the gills and capture small particles, like microorganisms, in the water. From here, the particles are either rejected or transported to the stomach. However, the innate immune system of the mussels is also able to kill microorganisms by phagocytosis and secretion of antimicrobial peptides (87).

In this study, we used *Mytilus edulis* as a model to test if ETEC bacteria can be taken up and concentrated by mussels. The experiments were carried out at SLC, Fiskebäckskil where facilities for these types of studies are available. Blue mussels were captured from 2 m depth outside KMF and cleaned from epiphytes on their surfaces. The mussels were divided into groups of 14 and placed in buckets with a grid base in 2.5 L of seawater (salinity: 33 PSU) at 8 ºC or 18 ºC. ETEC bacteria (E20738A, LT, CS17) were then added to the buckets to a final concentration of 4×10^8 L^{-1} alone or with the addition of 2.8×10^6 L^{-1} of the phytoplankton *Rhodomonas salina* (kindly provided by the dept. of Marine Botany, Göteborg University). The experiments were carried out in thermoconstant rooms in the darkness. Three mussels from each treatment group were taken out after 0, 6, 22 and 44 hours and the hepatopancreas of the mussels were dissected, weighed and homogenised (Fig 5). Recovery of ETEC bacteria was determined by spreading of serial dilutions of homogenised hepatopancreas onto Drigalski agar plates and counting of CFUs after 24 hours at 37 ºC. In addition, DNA was extracted from the homogenised material as described for analysis in real-time PCR.

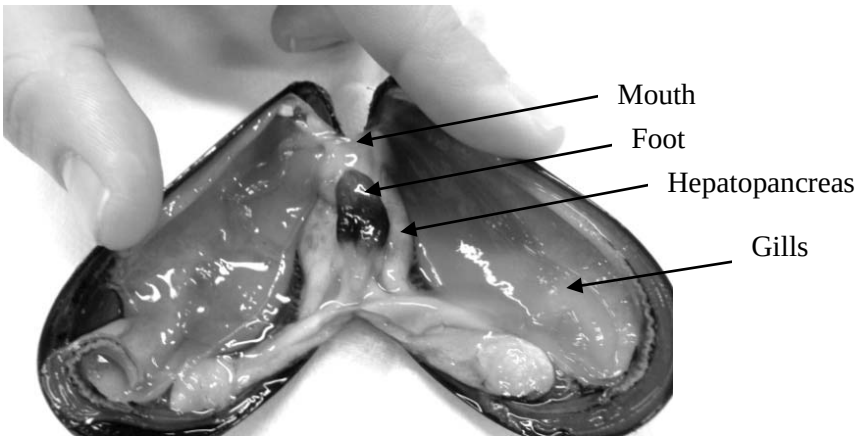


Figure 5. *Opened blue mussel, Mytilus edulis. The hepatopancreas is situated under the foot of the mussel. Photo: Åsa Lothigius*

INFANT MOUSE MODEL

The infant mouse model has earlier been used for detection of heat stable enterotoxin (ST) activity (28, 41) but only in a few studies on ETEC infection. This animal model has both advantages and drawbacks. Mouse models are cheap and a number of different mouse strains are available. On the other hand, the intestinal milieu of mice is different from humans and colonization factors of human ETEC strains are host specific and their function in animal models is not characterised. Also, human ETEC strains rarely cause disease in mice. There are few good models for ETEC infection and the most reliable method is studies on human volunteers.

In this thesis, the infant mouse model was used to study growth of clinical isolates of ETEC and resuscitation of VBNC forms of subsequent ETEC strains.

Based on studies at our laboratory with infant mice infected with *Vibrio cholerae* showing good colonisation of the bacteria and also studies on VBNC forms of other enteric bacteria (5, 6, 83), Swiss outbread CD1 mice were chosen as model animals. All animals in this study were treated and housed under specific-pathogen free conditions as approved by the Ethical Committee for Laboratory Animals in Gothenburg (ethical permit 110-2008). Infant mice at the age of approximately three days, with a weight of 2.4 – 2.8 g, were separated from their mothers and starved for 4 hours before orally infected with ETEC bacteria. The bacteria were administrated orally into the stomach with an eye needle and a syringe, carefully so that no fluid ended up in the airways of the mice. Twenty-four to 48 hours after infection the mice were sacrificed and the intestines, except for the caecum, were taken out and homogenised in PBS. Recovered ETEC bacteria were measured as CFUs on horse blood agar plates and streptomycin plates from the intestines. Also, DNA was extracted from the intestines and analysed in qPCR for quantification of total ETEC cells as previously described (69).

RESULTS AND COMMENTS

Development and application of a real-time PCR for detection and quantification of ETEC (paper I)

Detection and characterisation of ETEC in developing countries is usually based on culturing of the samples and analyses of ST and LT in GM1-ELISA and inhibition-GM1-ELISA (112). With the increasing knowledge of VBNC forms of enteric bacteria in the environment the purpose of this study was to develop a molecular method that was not dependent on the growth of bacteria. Real-time PCR (qPCR) had previously shown to be sensitive and specific for toxin producing *E. coli* in clinical samples and cultures (53, 99). The idea was to use the method for detection and quantification of ETEC in water samples. The qPCR assay developed was based on detection of the enterotoxin genes *estA* (STh), *estB* (STp) and *eltB* (LT). Analyses showed that the designed primers were specific for ETEC toxin genes when tested against a number of well characterised ETEC strains and a variety of enteric bacteria, including the other pathogenic *E. coli* (UPEC, EHEC and EPEC) (69).

Two different standard curves were developed. They were based on either whole genomes, for quantification of total number of cells, or PCR products of the enterotoxin genes, for quantification of genes or gene transcripts, of ETEC. By combining the two different standard curves it was possible to determine the toxin gene copy number per bacteria for 35 well characterised ETEC strains. Since the toxin genes are situated on plasmids, their number per cell may vary depending on type of strain and growth conditions. Also, the genes of ST and LT are flanked by insertion regions (106, 114) and may therefore be transferred to other sites in the genome or to other plasmids. However, the results showed that the number of gene copies per bacteria were stable during the culturing conditions used here (LB broth for 4 hours in 37 ºC at 150 rpm). Also, gene copy numbers did not vary between strains carrying one or more of the enterotoxins (STh, STp and LT). In summary, the number of toxin genes per ETEC bacteria were; STp: 1, STh: 2 and LT: 4. These results can be explained by two theories; either the toxin genes are situated on the same plasmid but with different gene copy numbers, or the genes are on separate plasmids with a constant plasmid copy number in each bacteria. There is however, a recent study showing that ETEC strain H10407, a very well studied strain isolated in 1971 (113), carries a plasmid containing both the gene for LT (*eltA*) and the gene for STp (*estIa*) as determined by sequencing of the Ent plasmid (80). The

organisation of toxin genes and the plasmid contents of clinically isolated ETEC strains need to be further studied to get a clearer picture of the correlation between toxin genotypes and plasmid content.

Researchers at ICDDR,B had previously been successful in isolating ETEC strains from environmental water samples in Bangladesh (8). When those were compared with isolates from patients at the hospital in Dhaka a correlation of toxin types and CFs between the isolates from patients and water was found (7). However, there was a risk to miss positive water samples containing low numbers of ETEC due to the culturing and ELISA-steps in the detection procedure.

One of the goals with the qPCR assay for ETEC was to increase the sensitivity when analysing water samples presumably containing ETEC bacteria. The sensitivity of the qPCR assay was estimated by analysing DNA extracted from microscopically counted and diluted bacteria. Likewise, the sensitivity of the whole assay (filtration of water, extraction of DNA from filters and qPCR) was determined by spiking water samples with known numbers of ETEC bacteria before filtering and DNA extraction.

The detection level of the qPCR was three bacteria per reaction, as compared to previous qPCR assays for ETEC detecting between 10 – 100 bacteria per reaction (10, 43, 99). The sensitivity of the whole assay showed that approximately 50 % of the bacteria added to the water before filtration and DNA extraction could be detected in qPCR, suggesting a loss of bacteria during the filtering and DNA extraction procedure. Attempts were made to increase the DNA yield by washing the filters with PBS followed by collection by centrifugation. However, as direct extraction of DNA from the filters was shown to give a 1000 times higher yield (57), this method was used when analysing the water samples from Bangladesh, as well as from other endemic countries.

Thirty-nine water samples from Bangladesh were analysed with the newly developed assay. The results were compared with culturing of the filters and testing of *E. coli* colonies in ELISA and inhibition ELISA for ETEC toxins (112). Twenty-six of these were positive for ETEC DNA in qPCR but only six samples were positive for ETEC enterotoxins in the ELISA assays (69). Also, significantly more samples were positive for ETEC DNA during the epidemic season compared to the endemic period (87 % vs. 52 %) which was in agreement with previous studies where the prevalence of ETEC disease was estimated by isolation of the bacteria from diarrhoeal stools in Dhaka, Bangladesh (93).

One sample was positive for LT toxin in ELISA but negative in qPCR. The reasons for this could be several; uneven distribution of bacteria on the filter, one half is used for DNA extraction and the other for culturing, contamination of the filter/ELISA procedure or inhibition in the qPCR. In other respects, the qPCR results were in agreement with the ELISA results but with far more positive samples, indicating that this is a more reliable method to use when testing water samples for ETEC bacteria. However, the culturing steps in the ELISA assay have the advantage that it is possible to store isolated ETEC strains for further genotypic and phenotypic analyses which is not possible to do on DNA extracted directly from the water samples.

The ELISA assays have long been the golden standards for detection of ETEC in various types of samples. However, the results from this study suggest that the qPCR is a more sensitive method for detection of ETEC in water. Also, the qPCR allows for quantification of the bacteria in the sample which is not possible with the ELISA method. It would be possible to use conventional PCR to analyse DNA extracted from water samples but the sensitivity of conventional PCR is lower than that of qPCR. The detection limit for ETEC in conventional PCR was estimated to 50 – 500 bacteria (paper I) and many of the water samples from Bangladesh, positive for ETEC DNA, would probably have been missed if this method had been used.

In some of the samples from Bangladesh, positive for ETEC in qPCR, the number of ETEC bacteria was as high as 1300 ml^{-1}. Theoretically, it would be sufficient to drink a few glasses of such contaminated water to become infected. The infectious dose of ETEC has been estimated to 10^6 - 10^{10} bacteria but it should be remembered that these are studies done on human adult volunteers. Children, who are the main victims of ETEC disease probably require a considerably lower dose to develop diarrhoea (93). However, when evaluating the qPCR assay, it is important to remember that the PCR detects all DNA in the sample, both from dead and live bacteria. The question raised from this is whether ETEC can survive in different types of water for longer periods and, if so, are they still infectious?

In addition to the studies on water samples from Dhaka, water samples were analysed with the same methodology in Moçambique (a SIDA financed Minor Field Study by Malin Lothigius and Johanna Bengtsson, Linköping University, unpublished results). Water was collected at three sites around the city of Maputo from waste stabilisation ponds of the Infulene River, from the Massingir dam and from the Umbeluzi River. Water from these sites is regularly

used for household and irrigation. In total, 49 samples were collected and 35 % of these were positive for ETEC DNA in qPCR analyses at our laboratory in Gothenburg.

The qPCR assay was also implemented at Laboratorio Diagnostico Molecular, Guatemala where the Ministry of Health is concentrating on research on the spread of infectious diseases. In Guatemala, drinking water was collected from taps in hostels and schools in the city of Antigua and even though the sampling of water was done in a more primitive way, by filling plastic bags and transporting them in picnic cooler boxes to the lab, the assay was reliable and results showed that 29 % of the samples were positive for ETEC DNA (Å. Lothigius and O. Torres, unpublished results). In Guatemala, the qPCR is now being used in larger field studies.

The assay has also been used in field trials in Djibouti in eastern Africa with several positive ETEC samples from wells and ponds as a result (J. Maslin, unpublished results).

Survival of ETEC in sea- and freshwater microcosms and formation of VBNC bacteria (paper II and III)

Analysing the water samples from Bangladesh with our qPCR assay resulted in 67 % of samples positive for ETEC DNA but as mentioned before, the method cannot discriminate between live, dead or possible VBNC forms of ETEC. From a public health point of view and to further strengthen the reliability of the qPCR assay it was important to evaluate if ETEC can survive in water and if so, are they capable of causing infection? The ideal would of course have been if we could study ETEC bacteria in the same environments as they are found, i.e. water in endemic countries, but this was not possible within the frame of our project and instead these experiments were conducted at Göteborg University and SLC with water collected in Sweden.

The use of microcosms to study microorganisms in aquatic environments is common and a convenient way of controlling biotic and abiotic factors that may influence the behaviour of the organisms studied (25). Here we used microcosms with either seawater or freshwater collected as previously described. The water was sterile filtered in order to minimise the influence of biotic factors in the samples. Six clinical ETEC isolates were then incubated in the water microcosms for 12 weeks (paper II). Samples were taken from the microcosms and analysed for culturability, total cells, intact cells, toxin production, virulence gene expression and morphology (Fig. 6) (70).

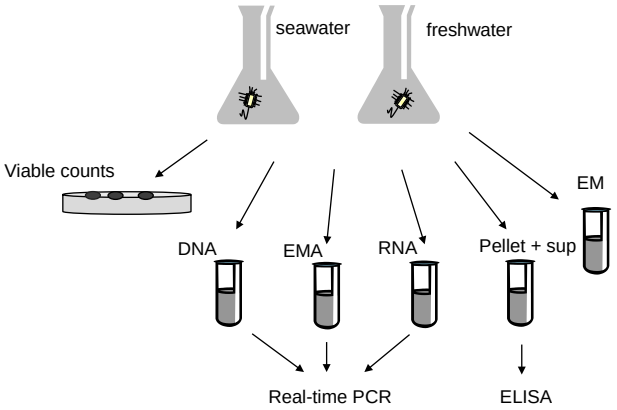


Figure 6. *Schematic picture of the processing of samples from sea- and freshwater microcosms (paper II). Sup = supernatant.*

The culturability of ETEC showed great variation between the different water types. In freshwater microcosms, the bacteria were culturable throughout the experiment but in seawater microcosms, four out of six strains could not be cultured on blood agar plates after 12 weeks. Attempts were made to culture these strains on M9 defined minimal media and also to pre-culture the samples in LB broth for four hours before spreading onto agar plates but none of these improved the recovery of culturable bacteria.

However, the total number of ETEC cells in the samples, as determined by qPCR, did not change much over time and when comparing these results with the number of intact cells as determined by vPCR, it was clear that a majority of the cells in sea- and freshwater still had an intact cell membrane (Fig. 7, paper II).

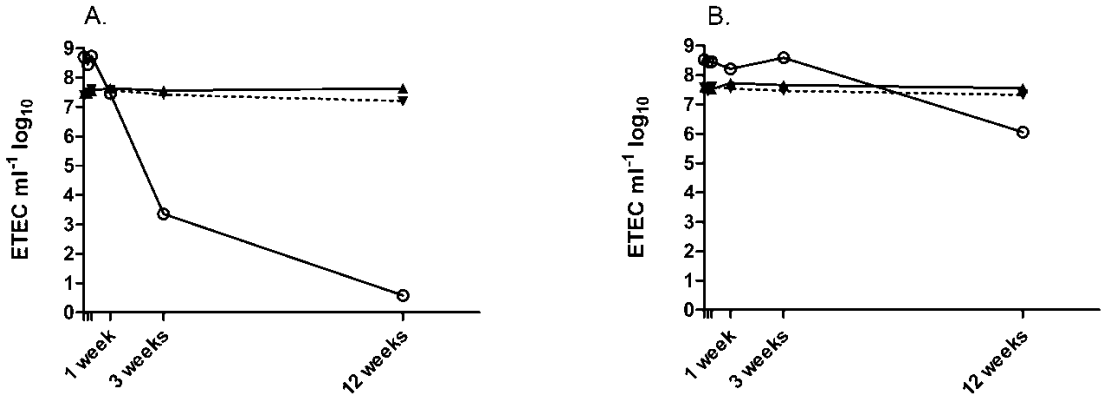


Figure 7. *Culturability (CFUs)* (○)*, total cells* (▲) *and intact cells* (▼) *in sea- (A.) and freshwater (B.) microcosms. The number of ETEC bacteria in each sample is presented as the mean of the 6 strains analysed (paper II).*

Also, when analysing the gene expression of virulence genes and housekeeping genes it was shown that virulence genes and the genes of 16S and gapA were transcribed in both water types, despite the fact that some of the strains were not culturable (paper II). It should be noted that gene expression of *gapA* was somewhat higher in seawater compared to freshwater, suggesting that ETEC strains in seawater had a higher metabolic activity than the corresponding strains in freshwater microcosms. The expression of enterotoxin genes were downregulated whereas the colonization factor genes were upregulated in water compared to when cultured in CFA broth. The reason for this might be that the bacteria are preparing for re-colonisation if favourable conditions should return but the regulation of the CF genes needs to be further investigated in order to fully explain this upregulation. However, transcription of the gene of CFAI was not detected at all. Since there were no indications of sources of error in the qPCR and all control samples were positive the only explanation for this negative result is that the gene of CFAI, unlike the other CFs, was not expressed in water samples.

In this case, gene expression was determined by absolute quantification related to the number of intact cells in the sample. Gene expression analyses may also be performed by relative quantification related to a housekeeping gene but, since gene expression levels, also for housekeeping genes, change under different culturing conditions in bacteria (45, 103), absolute quantification was considered a better alternative. A recent study, also using absolute quantification, on gene expression in VBNC *V. cholerae* showed that the expression of 16S rRNA was reduced under the VBNC state compared to exponentially growing cells (42). In our study, we could see an upregulation of 16S rRNA in VBNC compared to exponentially growing ETEC bacteria. Despite these differences, rRNA expression is considered as an indicator of cellular activity and could therefore be used as a viability marker (42).

Based on the facts that the number of intact ETEC cells remained stable in water, gene expression was detected and the culturability was lost or greatly impaired we concluded that ETEC cells were viable under these stressful conditions which support the VBNC theory that has been described for other enteric bacteria (22, 82, 90, 131).

However, it should be mentioned that even though the enterotoxin genes were transcribed, no production of toxins could be detected when analysing the pellets and supernatants from the water samples in ELISA and inhibition ELISA. The reason for this has not been determined but the ELISA and inhibition ELISA are not very sensitive and require high numbers of toxin producing bacteria (112). If toxins were produced in small amounts by the intact bacteria in the water samples, it is possible that these were not detected by the ELISA assays due to too low sensitivity in the tests.

Besides the differences in culturability of ETEC in seawater and freshwater, one other variance was the morphology of the bacteria. In seawater microcosms, the bacterial cells became elongated and thin in comparison to freshwater microcosms where the cells adopted a round shape indicating that seawater had a greater impact on the shape, probably due to the osmotic pressure. These morphological alterations of the cells became more pronounced at the end of the incubation period. Figure 8 shows electron micrographs of ETEC bacteria after the initial experiment where two ETEC strains were incubated in seawater and freshwater for six months (70).

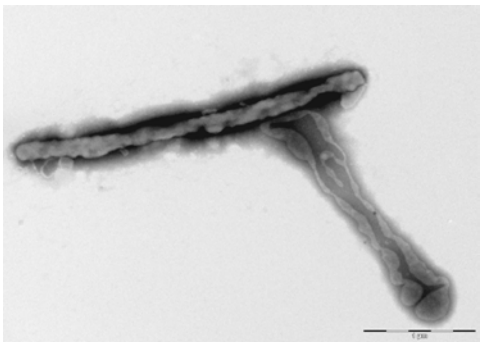
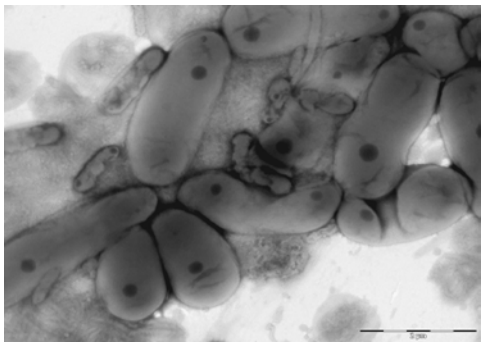
A.  B. 

Figure 8. *Electron micrographs of ETEC strain ST64111 in seawater (A) and freshwater (B) microcosms after six months of incubation. A: Bar = 1 µm, B: Bar = 2 µm.*

Major stress factors for enteric bacteria in aquatic environments are salinity, light and temperature (64, 76, 102). The influence of temperature on culturability of ETEC in seawater was tested by incubation in microcosms at 8 ºC and 18 ºC showing a significantly improved culturability at the lower temperature (paper III). This enhanced stability at lower temperatures has been seen for other enteric bacteria in aquatic environments (25, 37, 102, 104, 110) and may be explained by a decreased metabolic activity as a energy saving strategy. In this experiment, only one ETEC strain was used and in order to further evaluate the influence of temperature on the culturability of ETEC, more strains, with different virulence factor profiles should be included.

In addition to low temperature, the culturability of ETEC was also significantly enhanced by a high nutrient content in the water (paper III). We did not focus on any specific nutritional compounds in the water but rather the culturability in a more general sense comparing eutrophic and oligotrophic conditions. Other studies have shown that *E. coli* culturability in

seawater is not considerably affected by a reduction of nutrients (120) but in this study we could conclude that eutrophic conditions had a positive impact on the culturability on ETEC. This was supported by results from sewage water samples from waste stabilisation ponds in Moçambique that had more samples positive for ETEC than samples from the other collections sites (Lothigius, M and Bengtsson, J., unpublished).

The survival of bacteria in water is not only dependent on abiotic factors. It has for example been shown that *V. cholerae* can be associated to zooplankton and that outbreaks of cholera strongly correlate with the seasonal occurrence of algal blooms since zooplankton are grazing phytoplankton (98). Zooplankton-associated *V. cholerae* attach to the surface of the plankton to utilise their chitinacious exoskeleton as a source of nutrients (19, 47, 54). Such a relationship between *Vibrio* and zooplankton, both of which are natural inhabitants of the ocean might be a co-evolutionary phenomenon. Contrary, ETEC bacteria are considered as contaminants in water and unlikely to have coevolved with marine organisms. However, the association between *Vibrio* and plankton is thought to favour the survival of the bacteria in aquatic environments (54, 56, 98) and, since ETEC infection follows a similar seasonal pattern as cholera infection, we wanted to evaluate if ETEC bacteria can be associated or increase their culutrability by incubation together with plankton. For this purpose, ETEC was incubated together with phytoplankton and zooplankton at different temperatures (8 and 18ºC) and after separation of bacteria and plankton, the culturability of ETEC was determined. The ETEC strain used was culturable in both phytoplankton- and zooplankton fractions but there were no significant differences between the different incubations, although ETEC seemed to be favoured by the presence of plankton at 18 ºC where a higher total recovery of ETEC bacteria was found (B. Hernroth and Å. Lothigius, unpublished results). This is in contrast to the previous study where ETEC had a significantly enhanced culturability at the lower temperature. This indicates that plankton had a positive impact on ETEC culturability. Unfortunately, there was a great loss of bacteria when washing the filters containing the different plankton fractions which made it difficult to interpret results from the experiments. It would be of interest to further develop the filtration assay in order to determine the ratio between culturable and VBNC forms of ETEC associated with plankton and also to include more strains in the analysis. In that way it would be possible to assess if ETEC survival is favoured together with plankton.

Uptake in mussels and influence of bivalve haemocytes (Paper III)

Agricultural runoffs and sewage discharge are increasing the pathogen load in coastal waters and marine environments are often considered as a source of infection with enteric pathogens (73). In marine milieus, bivalves have been reported to accumulate pathogens from the surrounding water acting as a vector for transmission of enteric bacteria (91, 97, 100). Since ETEC were shown to be viable after incubation in seawater at different temperatures, alone or in association with plankton, it would not be far-fetched to argue that ETEC, like other enteric bacteria, could be transmitted to humans via consumption of contaminated bivalves. This study aimed to investigate if bivalves could act as a vector also for ETEC and if the bacteria were affected by the bivalve immune defense.

The risk of getting infected with ETEC by consumption of contaminated bivalves has not been addressed before and here we could show that ETEC indeed can be taken up by mussels (*M. edulis*). The mussels were fed with ETEC or ETEC plus phytoplankton (*R. salina*) at two different temperatures (8 ºC and 18 ºC). The uptake of ETEC bacteria was significantly higher at 18 ºC (paper III) but the presence of phytoplankton did not increase the ingestion of ETEC (unpublished results). Blue mussels has an optimum particle size for uptake between 7 – 100 µm (75) but, as has been shown in previous studies, the mussels were able to ingest high numbers of bacteria also when not co-fed with particles of the more convenient size of phytoplankton (50). We did however see a faster clearance of the bacteria when mussels were co-fed with plankton, probably because of an increased metabolic activity (Fig. 9, unpublished results).

The bacteria were culturable after being extracted from the mussel hepatopancreas throughout the experiment (44 hours) at both temperatures but unfortunately it was not possible to quantify the bacteria with qPCR due to inhibitory substances in the samples. It would be of value to refine the DNA extraction and qPCR assay in order to evaluate the viability of ETEC after ingestion by the mussel.

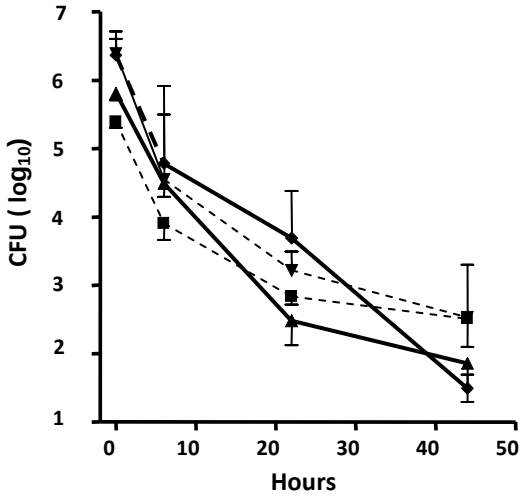


Figure 9. *Uptake and depuration of ETEC in blue mussels (Mytilus edulis) determined by viable counts.* (▼) *ETEC 18 ºC,* (♦) *ETEC 18 ºC + plankton,* (■) *ETEC 8 ºC,* (▲) *ETEC 8 ºC + plankton (mean ± S.D.; n = 3).*

The metabolic activity of mussels is affected by both the availability of food and by the surrounding temperature. Temperature also affects the immunological response in blue mussels (40, 48, 49) which might promote the elimination of microorganisms.

The immune defence in mussels consists of innate immune cells that are able to kill microorganisms by phagocytosis and production of antimicrobial peptides (87). In this study, *in vitro* experiments showed that bivalve haemocytes from *Mytilus edulis* and *Ostrea edulis* had a higher bactericidal capacity on ETEC compared to that on *Vibrio parahaemolyticus* (paper III). The fact that *V. parahaemolyticus* had a higher resistance against the haemocytes may not be surprising since this is a bacteria commonly found in marine environments and it was even shown that the bacteria were able to grow in the presence of haemocytes from *M. edulis* (paper III). However, the effect of bivalve haemocytes on ETEC survival has not been tested before and the results presented in paper III might therefore contribute to the overall picture of ETEC survival in marine environments.

The role of mussels in the transmission of ETEC disease, based on the results presented here, should be interpreted with care. Even though there was clear evidence for uptake and persistence of ETEC in the mussels, the *in vitro* studies on the other hand showed that bivalve hemocytes had a bactericidal capacity on ETEC. If mussels act as vectors for ETEC or if they function as a biological filter remains to be proven until further studies can evaluate the long term persistence as well as the virulence of ETEC bacteria after ingestion. It should, however, be mentioned that the survival of ETEC in mussels was similar to that of *Salmonella enterica* which is well known to be spread via contaminated seafood (36) and ETEC should be considered when assessing the risk of marine environments as a source of enteric infection.

VBNC ETEC are able to resuscitate in an animal model (paper IV)

The initial studies of this thesis showed that ETEC can survive in sea- and freshwater microcosms and in seawater with different nutrient statuses from an endemic area. There was also substantial evidence for formation of VBNC ETEC in the water microcosms. Still, it was unclear whether these VBNC forms were able to colonise and cause disease. To prove that the VBNC forms are infectious and evaluate the significance of these dormant forms of ETEC bacteria it was necessary to set up an *in vivo* model.

There are no golden standard models for colonisation studies of ETEC but based on results on colonisation with VBNC forms of other enteric bacteria (5, 6, 83), the infant mouse model was chosen for this purpose. The infant (or suckling) mouse model had previously been used in a few studies on ETEC infection (9, 31, 41) and here we aimed to use this model in order to evaluate if VBNC ETEC were able to grow in the intestines of these animals.

The six clinical ETEC isolates were again incubated in seawater and freshwater microcosms showing similar values for culturability and viability as in paper II. The strains were also tested for growth in infant mice after cultivation in CFA broth for four hours. ETEC were administrated orally and the intestines were removed and analysed for bacterial survival and growth (Fig. 10). All strains were recovered from the intestines of infant mice after 24 and 48 hours and when further analysing two LT-producing strains with different CF profiles (CS7 and CS17 respectively) it was shown that the growth was dose dependant with an optimal infection dose of 10^4 and 10^6 bacteria for the two strains. Based on these results we decided to use one ml of the water microcosms, corresponding to 4×10^4 – $1{,}4 \times 10^6$ bacteria as determined by qPCR (paper IV), for infection of the mice. This range in infectious dose has also been used in other studies on resuscitation of VBNC bacteria (59, 83). In paper II the endpoint for the experiments was 12 weeks and this timepoint was also chosen here. At this point, all strains in seawater microcosms had switched into a VBNC state but in freshwater microcosms, five out of six strains were still culturable at CFUs of $4{,}7 \times 10^2$ – 1×10^4 ml^{-1}. The recovery of ETEC bacteria did not increase significantly after 24 hours in the intestine and considering the general condition of the mice, 24 hours was chosen as incubation time.

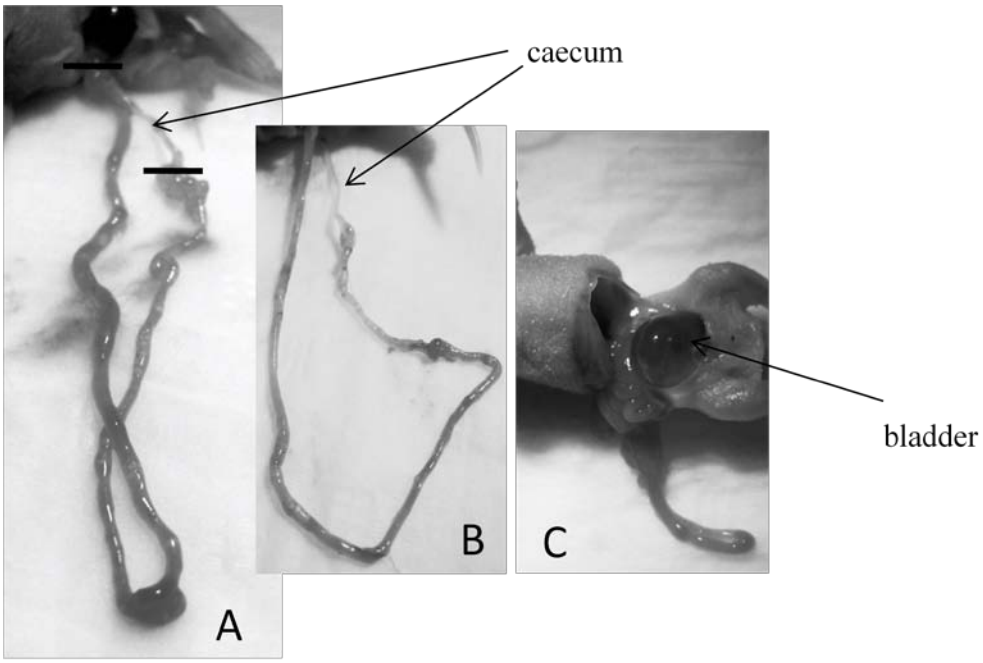


Figure 10. *Intestines of infant mice 24 hours after infection with ETEC. The intestines of infected mice were swollen (A) compared to uninfected animals (B) and the bladder was filled with urine (C). The lines represent the positions of where the intestine was cut. The piece between the lines (A) was used for culturing and extraction of DNA. Photo: Åsa Lothigius*

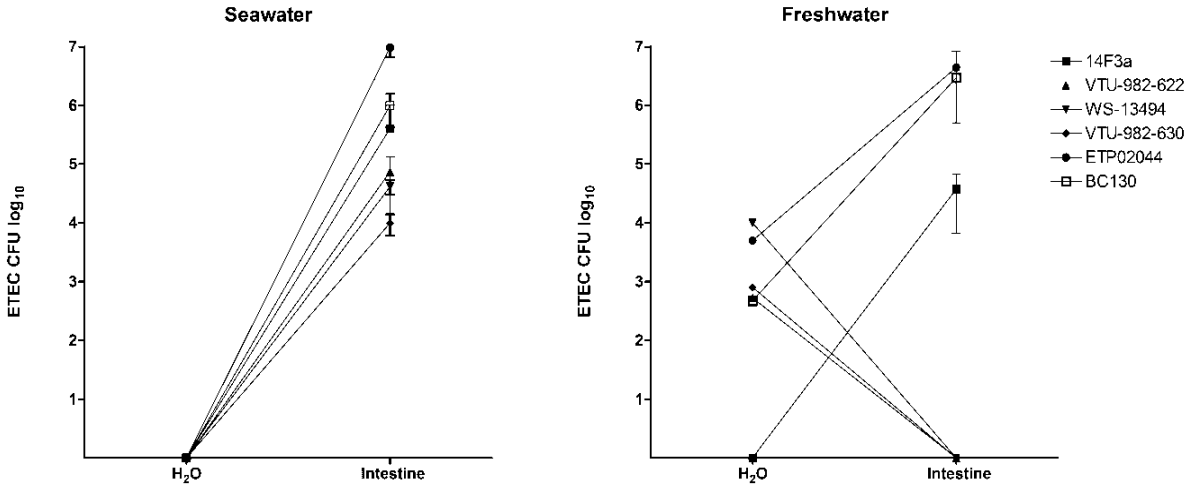


Figure 11. *Culturability of clinically isolated ETEC strains in sea- and freshwater microcosms (ETEC ml^{-1}) and of the corresponding strains 24 hours after infection in infant mice (ETEC/intestine) (paper IV).*

Three out of six strains in freshwater microcosms regained or increased their culturability whereas the other three strains were not recovered from the mouse intestines. In contrast, all six ETEC strains from seawater microcosms regained their culturability 24 hours after infection in mice (Fig. 11, paper IV).

The inoculation dose of ETEC from water microcosms might seem rather low and perhaps a higher dose of ETEC should have been tested in order for all the strains to regain their culturability from freshwater. However, no correlation between culturability in freshwater, or the amount of VBNC bacteria, and the ability to grow in the intestines were seen for the strains. Previous studies on resuscitation of VBNC bacteria indicated that culturable cells need to be present in order for dormant cells to regain their culturability *in vivo* (5) but in our study, the presence of culturable cells did not seem to enhance the ability to resuscitate in the infant mice.

Studies showing resuscitation in animal- and human models have been done with *Vibrio* spp. that are well known to enter a VBNC state in aquatic environments (6, 23, 61, 83) but to our knowledge, no similar analysis has been conducted on ETEC VBNC. Here, we could for the first time show that these non-culturable forms of ETEC were able to regain their culturability in an animal model. Recent findings suggest that resuscitation of dormant bacteria is required in order to prove that these bacteria are truly VBNC (60). Based on that statement, we can therefore conclude that ETEC bacteria indeed can enter a VBNC state under aquatic conditions.

However, although VBNC ETEC has the ability to resuscitate and grow in the intestines of infant mice, this does not prove that they are pathogenic for humans. The attempts that were made to study the gene expression and production of virulence factors of these VBNC strains *in vivo* failed. A recent study on ETEC toxin gene expression showed that there were no significant differences between gene expression *in vivo* and *in vitro* (111). If this is true, this might also be the reason for not detecting any virulence gene expression in our study but it should also be remembered that it is technically difficult to extract RNA from *in vivo,* e.g. diarrhoeal stool samples. We were unable to detect any phenotypic expression of CFs in dot blot assays on homogenised intestines with monoclonal antibodies against the CFs of interest. The reason for this might be that proteins from the mouse intestines blocked the CF antigens as well as difficulties in handling the rather small intestines. Also, the dot blot assay requires a rather high number (10^9 bacteria ml^{-1}) of expressing bacteria for a positive signal (112).

The toxin activity of ST can be measured in the infant mouse model by fluid accumulation in the intestines. In this study, we could see that the intestines were swollen (Fig. 10) but no measurements of fluid were done. In future studies of VBNC ETEC in infant mice, it would be valuable to study toxin activity as well as to further develop methods to study virulence expression of VBNC ETEC in order to determine the impact of VBNC ETEC for humans. Another possibility is to study VBNC ETEC in human volunteers as has been done with *V. cholerae* (23) and in that way measure the pathogenicity of VBNC ETEC.

GENERAL DISCUSSION

The lack of clean water and adequate sanitation is the world's largest cause of illness and it has been estimated that more than two million people, most of them children, die every year from water-borne infections (16, 65, 93). In 2000, the United Nations (UN) Millennium Declaration emphasised the need for all countries to stop the unsustainable exploitation of water resources and one of the goals is to reduce by half the proportion of people without access to safe drinking water by 2015 (www.un.org).

Many of the deaths caused by water-borne infections, like diarrhoea, may be prevented by appropriate vaccines and water sanitation programs (21). It has been estimated that nearly half of the deaths from diarrhoea every year could be prevented through an understanding and implementation of basic hygiene, and investments in sanitation infrastructure and education programs are important tools in the prevention of water-borne diseases (www.un.org).

Research about the mechanisms of transmission of infectious diseases is important and the possibility to determine if a water source is contaminated with diarrhoeal pathogens may prevent disease outbreak in that area.

One of the initial aims of this project was to develop sensitive methods for detection and quantification of ETEC in water, including possible non-culturable forms of ETEC. This goal has been fulfilled since a highly sensitive and specific real-time PCR (qPCR) assay allowing detection and quantification of ETEC in various water samples was established.

The choice of detection strategy for ETEC may reflect personal preferences but also available resources. Many laboratories in developing countries are using methods based on isolation of ETEC strains by culturing and subsequent detection of enterotoxins in ELISA, or the corresponding genes by PCR or probe-hybridisation assays for detection of ETEC (112). However, the possibility to quantify even very low numbers of ETEC bacteria using the qPCR assay has resulted in increased use also in developing countries and our qPCR (paper I) is now used in several different laboratories.

Our findings of ETEC DNA in water samples from endemic countries without the possibility to grow the bacteria from the same samples raised the question of the viability of ETEC after long term storage in water. The qPCR assay may be a valuable tool in ETEC diagnostics, but if the DNA detected originate from dead bacteria the assay might be less useful for identification of sources of ETEC infection. The possibility that ETEC may persist in water as VBNC forms was likely since this has been shown for a large number of food and waterborne

microbial pathogens (60, 61) but this possibility had not been tested for ETEC before. In this work we have shown evidence for VBNC formation of clinical ETEC isolates in both sea- and freshwater microcosms. The majority of the bacteria retained an intact cell membrane and both ST and LT enterotoxin genes were transcribed, although the strains lost the capacity to grow on standard culturing media. However, to designate bacteria as VBNC, a resuscitation protocol is required. Furthermore, if VBNC forms do not regain their culturability, they do probably not play a role as infectious agents.

Our results (paper II) indeed support that ETEC may form VBNCs during prolonged incubation both in sea- and freshwater. In particular, seawater seemed to have a greater influence on the formation of VBNC ETEC and we found a greater downregulation of toxin genes in seawater than in freshwater. When infecting infant mice with the ETEC strains incubated during three months in water, we anticipated that strains from the seawater microcosms would show less growth in intestines than corresponding strains incubated in freshwater. However, the strains from the seawater microcosms, in which all bacteria showed to be in a VBNC state, were able to resuscitate in the mouse intestines, whereas some of the strains from freshwater microcosms could not be recovered although they were culturable *in vitro*.

It is known that the RpoS (σ^S) transcription factor controls the expression of a large number of genes involved in cellular responses to a diverse number of stress factors (102, 123). The *rpoS* gene is the most dominant gene involved in seawater survival in *E. coli* and it may also be important in reprogramming dormant bacteria, enabling them to better colonise the host as has been suggested for uropathogenic *E. coli* (15).

The infant mouse model showed to be useful for studies of the putative culturability of VBNC ETEC in research purpose but may be less appropriate for demonstration of ETEC in water samples. For such studies an *in vitro* method would have the benefit to isolate specific strains for future studies on bacterial properties, such as antibiotic resistance. However, media normally used for culturing of ETEC cannot be used for isolation of VBNC ETEC. Since the VBNC ETEC were able to grow in mouse intestines it might be possible to develop an *in vitro* culturing method resembling *in vivo* conditions allowing resuscitation of VBNC ETEC.

It is more likely that ETEC is spread via freshwater, like drinking water and water from ponds and rivers that are used for household purposes in developing countries, than via seawater. Nevertheless, we have shown that ETEC can persist in a viable form to the same degree in seawater as in freshwater. We could even show that ETEC strains incubated in seawater was

more efficient in growing in infant mouse intestines than corresponding strains from freshwater microcosms. However, studies on the prevalence of ETEC in seawater in endemic areas are lacking and ETEC has not to our knowledge been isolated from natural seawater samples.

The transmission of ETEC bacteria and routes of infection might be different depending on the type of water source. My view of the different transmission pathways of ETEC are shown in figure 12. I believe that transmission via freshwater is more common than via seawater. Large amounts of surface water from freshwater sources are used for household purposes in developing countries and water scarcity as well as lack of resources to properly clean water force people to consume contaminated water. Freshwater is not only used for drinking but also for food preparation, crop irrigation, personal hygiene, cleaning, washing and waste disposal that all may expose people for contaminating pathogens.

Seawater is usually not used directly for household purposes but nevertheless, in countries where seafood constitutes a major part of the daily food intake, like in Moçambique, there is a risk to get infected by ingestion of contaminated shellfish. Our results that ETEC can be ingested and survive in mussels suggest that this might also include infections with ETEC (paper III).

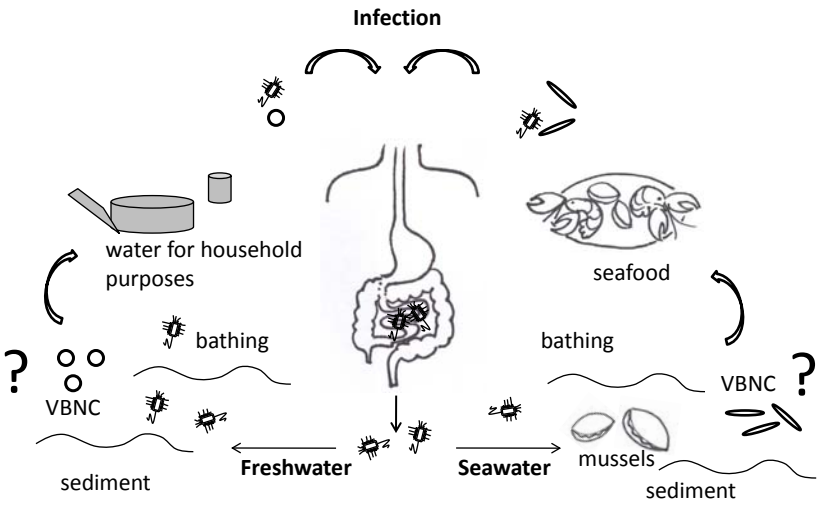


Figure 12. *Sketch of the possible transmission pathways of ETEC bacteria after dissemination in water. The elongated shapes represent VBNC ETEC in seawater and the round shapes VBNC ETEC in freshwater.*

The seasonality of ETEC disease in developing countries follows the same pattern as seen for *V. cholerae* (62, 93). The fact that water samples positive for ETEC were primarily found during the epidemic season in Bangladesh (paper I) may be explained by either of two

theories. One is that fecal pollution of the water might be more common during the warm and rainy seasons. Unfortunately, we did not obtain any data on total CFUs of *E. coli* from the analyses in endemic regions which would have given a picture of the fecal bacterial load in the waters at different time points. Another possibility is that ETEC survival and growth is enhanced during the warm and rainy periods and that the floods, e.g. in Bangladesh, are supplying water sources with nutrients that might promote ETEC viability. In this study, high nutrient water seemed to favour ETEC culturability (paper III) but this can probably not explain the seasonality of ETEC disease in developing countries.

CONCLUSIONS

In conclusion, we have developed a highly sensitive and specific assay for detection and quantification of ETEC in water and by using this method shown that waterborne transmission of the bacteria is likely, since ETEC DNA were found in different types of freshwater samples from endemic areas in Bangladesh (paper I), Guatemala and Moçambique. In the studies on marine organisms as a reservoir for ETEC we could show evidence for a possible marine transmission pathway (paper III) but this area needs further attention to evaluate the virulence of the bacteria in these milieus.

This thesis has also provided evidence for VBNC formation of ETEC in aquatic environments (paper II) and we could show that these dormant forms are able to regain their culturability in an animal model (paper IV). These results suggest that VBNC forms of ETEC present in water may be infectious, but further research is however needed in order to evaluate the importance of VBNC ETEC in human disease.

Although the specific aims of this thesis were fulfilled, the overall goal to evaluate the persistence and infectious potential of ETEC in aquatic environments is far from reached. The fact that ETEC can survive in a marine environment rather raises more questions than I had from the start of this project. It is therefore hoped that this thesis may encourage more studies on the role of ETEC in aquatic environments which may help understand the spread of ETEC disease and hopefully give tools for disease prevention.

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