Coliform in Shrimp Farms: Total Content and Detection of Enterovirulent *Escherichia coli* Strains by Polymerase Chain Reaction

A thesis

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Examination Roll No: MS-110720
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BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
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MARCH, 2014

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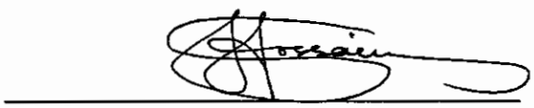

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DEDICATED TO MY

BELOVED PARENTS

TITLE

Coliform in Shrimp Farms: Total Content and Detection of Enterovirulent *Escherichia coli* Strains by Polymerase Chain Reaction

DECLARATION

This thesis report is the original research work of the authors, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the written concurrence of the concerned supervisor. Legal action would be taken in any case of infringement regarding the declaration.

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Abstract

The presence of coliform indicates that *Citrobacter, Enterobacter, Escheriechia, Hafnia* and *Klebsiella* as well as other pathogenic bacteria such as *Salmonella* sp., *Shigella* sp. and *Vibrio* sp. might be present. In Bangladesh, traditional shrimp culture farms are highly susceptible to contamination with pathogenic bacterial species because of lacking proper sanitation near to these farms. However, the aim of the present study was to quantify coliform as well as enterovirulent groups (EHEC, enterohaemorrhagic, EPEC, enteropathogenic and ETEC, enterotoxigenic) of *E. coli* in different shrimp farms in Khulna district, Bangladesh. Water samples were collected in winter (November to February, 2011-2012) and summer (May to August, 2011-2012) seasons. All the farms were contaminated with coliform but *E. coli* was present only in 55% farms. The number of coliform was varied from season to season and also farm to farm. In winter season, average number of coliform and *E. coli* in hygienic farms were 4.94×10^2 (cfu/mL) and 0 (cfu/mL) whereas that in unhygienic farms were 8.17×10^2 (cfu/mL) and 4.15×10^2 (cfu/mL), respectively. However, in summer the number were larger than winter. In the summer, the number of coliform and *E. coli* in hygienic farms were 82.65×10^2 (cfu/mL) and 8.51×10^2 (cfu/mL) whereas that in unhygienic farms were 111.32×10^2 (cfu/mL) and 45.27×10^2 (cfu/mL), respectively. Using cultural, biochemical and PCR technique, 76 *E. coli* were identified. Among them, EPEC and ETEC groups were 22% and 29%, respectively whereas EHEC group was absent. Based on the results, it might be concluded that 55% shrimp farms of Khulna district were contaminated by feces and unsafe for shrimp production.

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Chapter One

INTRODUCTION

1: INTRODUCTION

Bangladesh is rich in natural shrimp resources and nearly 60 species of shrimp (saltwater) and prawn (freshwater) occur in Bangladesh and they are contributing in fishing for hundreds of years. In the early seventies, Bangladesh entered the world's export market for shrimp and since then this crustacean has suddenly become a very high-priced commodity. In recent decades, due to an increased international demand, shrimp has become one of the most important export products of Bangladesh. The shrimp sector has undergone dramatic changes in terms of area, production, and marketing (DOF, 2009). At present, Shrimp industry is one of the most important contributors for economic sustenance and is the second largest export commodity of the country. In the 2010-11 fiscal years, the total production of shrimp is 7.82% of total fish production and about 239460 mt. in which 52.05% is from culture basis. Bangladesh has earned 3568.2 cores Tk. by exporting 54891 mt. frozen shrimp in the 2010-11 fiscal years (DOF, 2012).

The leading shrimp farming areas of Bangladesh are the Bagerhat, Khulna and Satkhira Districts in the south-western region, Cox's Bazar District in south-eastern region, and to some extent Pirojpur District in the south-central region. Experts and fisheries resource planners predict that all leading shrimp areas are unlikely to experience similar expansions (MPO, 1987). The main cultured species is the tiger shrimp locally known as *bagda* chingri, which scientific name is *Penaeus monodon* that contribute about 77% of export earning of shrimp sector. It is a marine shrimp and is cultivated in brackish water. There is ample demand in the international market for shrimp and Bangladesh is blessed with an environment congenial for shrimp production. However, the industry is fraught with many obstacles at present (USAID, 2005).

Shrimp farming concentrated largely in tropical developing countries for export to the west, has experienced spectacular growth over recent decades. Currently, aquaculture industry in Bangladesh and other parts of the world especially southwest region has been facing serious problems due to microbial diseases. In aquatic environments, diseases in fishes and shrimps are caused by opportunistic pathogens (Lundin et al., 1996). In culture conditions, various diseases are found which are acting like as natural disaster. Bacterial and viral diseases have most serious losses occurred. Like fish, shrimp lack of

antigen/antibody system and so cannot be vaccinated in the way fish can be. Prevention is the only cure.

Hence, disease is inevitable in these uncontrollable culture models. In addition, the use of antibiotics to control bacteria population and maintain healthy environment for prawn culture becomes popular. A wide range of antibiotics is now being used to treat bacterial diseases and to control bacterial population in the hatcheries and prawn farms (Lightner, 1996). The potential consequences of used antibiotics for treatment may arise various antibiotic resistant bacteria. The phenomenon resistance was transfer to pathogenic bacteria, and led to reduce efficacy of antibiotic treatment for disease caused by the resistant pathogens (Frappaolo et al., 1986). Moreover, all the fish have normal bactericidal activity in their blood serum. Current state of knowledge regarding fish health is still greatly needed. Few attempts have been taken in order to assess the bacterial population in aquatic environment and their involvement in causing disease. Representatives of upwards of 25 bacterial genera have been implicated at various times as pathogens of fresh water and/ or marine shrimp and fish species (King, 1997).

Freshwaters contaminated by fecal material from man and animal may contain a large variety of human pathogenic microorganisms. Health protection programs require estimating the level of contamination of aquatic systems by these microorganisms. Since the systematic search of all potential strains of entero-pathogens is not feasible, the enumeration of various indicator bacteria is usually used to evaluate the fecal contamination of waters and, the sanitary risk associated with various utilization of the water (bathing, production of drinking water, etc.). For years, total coliforms and fecal coliforms were the most widely used indicators but more recently, the abundance of *Escherichia coli* has been shown to be more related to the sanitary risk than that of coliforms (Fewtrell and Bartram, 2001).

Coliforms are bacteria that are always present in the digestive tracts of animals, including humans, and are found in their wastes. They are also found in plant and soil material. Total coliforms include bacteria that are found in the soil, in water that has been influenced by surface water, and in human or animal waste. Fecal coliforms are the group of the total coliforms that are considered to be present specifically in the gut and feces of warm-

blooded animals. Faecal coliforms are the indicator of the presence of *E. coli* as well as other bacterial pathogens such as *Salmonella* sp., *Shigella* sp. *and Vibrio* sp. These organisms can be transmitted via digestive tract by contaminated water and may lead to diseases such as gastroenteritis, salmonellosis, dysentery, cholera and typhoid fever. Higher concentration of faecal coliforms in water will indicate a higher risk of contracting waterborne disease. For years, total coliforms and fecal coliform were the most widely used indicators but, more recently, the abundance of *E. coli* has been shown to be more related to the sanitary risk than that of coliforms (Fewtrell and Bartram, 2001). *E. coli* is the major species in the fecal coliform group. Of the five general groups of bacteria that comprise the total coliforms, only *E. coli* is generally not found growing and reproducing in the environment. Consequently, *E. coli* is considered to be the species of coliform bacteria that is the best indicator of fecal pollution and the possible presence of pathogens.

E. coli which are widely distributed in the aquatic environment have been universally accepted as an indicator of faecal pollution because of its presence in high numbers in the gut of mammalians. *E. coli* are a gram negative bacterium that is commonly found in the lower intestine of warm-blooded animals. Most of *E. coli* strains are harmless, but only a small percentage is pathogenic to humans. The widespread occurrence of waterborne infections of *E. coli* origin in humans has become one of the major health problems worldwide. To date, several types of enterovirulent *E. coli* have been recognized as the aetiologic agents of various gastrointestinal infections in humans. The most commonly encountered are those belonging to the enterohemorrhagic *E. coli* (EHEC), which produces Shiga-like toxin and attaching-effacing lesions; enterotoxigenic *E. coli* (ETEC) produce heat-stable or heat-labile enterotoxins; enteroinvasive *E. coli* (EIEC), that causes bacillary dysentery via invasion of intestinal epithelial cells or by cytotoxin and enterotoxin; and enteropathogenic *E. coli* (EPEC), whose mechanism of virulence is thought to be unrelated to the excretion of typical *E. coli* enterotoxins (Kong et al., 1999).

Types and levels of bacterial populations associated with farmed brackish water shrimp (*P. monodon*) are the important indicators for the assessment of quality and safety of shrimp. In addition, most diseases in *P. monodon* are caused by opportunistic pathogens which are prevalent in the rearing environment (Jayasree et al., 1999). However, identification of bacteria is essential for the diagnosis of diseases in shrimp. Traditionally,

detection and enumeration of bacterial pathogens have been largely based on the use of selective culture and standard biochemical methods. Such methods suffer from a number of drawbacks. First, pathogenic bacteria which normally occur in low numbers tend to incur large errors in sampling and enumeration (Fleisher, 1990). Second, culture-based methods are time-consuming, tedious, invariably monospecific (i.e. detecting only one type of pathogen), and low throughput. Third, many pathogenic organisms in the environment, although viable, are either difficult to culture or non-culturable (Roszak, 1987) but can still cause illnesses (Rahman et al., 1996) There are also many bacterial species that are not cultivable by standard methods.

Given the importance of *E. coli* in water and product quality monitoring, a number of culture-based and immunological methods have been developed for its rapid detection. A fluorogenic method based on the enzymic cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUG) has been reported (Edberg *et al.*, 1990). However, the method which is widely used has several disadvantages especially in relation to its lack of specificity. First, it does not distinguish pathogenic from nonpathogenic strains of *E. coli*; second, some strains of *Salmonella, Shigella* and *Yersinia* which are capable of splitting MUG will also be detected (Bettelheim, 1992); and third, phenotypically MUG-negative *E. coli* (Chang et al., 1989) for example, EHEC strains (Doyle and Schoeni, 1984) is not detected by the method. For the specific detection of pathogenic ETEC and EHEC strains which produce different types of enterotoxins, several ELISA-based methods have been developed. They include ELISAs for the detection of heat-labile LT (Ristaino et al., 1983), heat-stable STI (Carroll et al., 1990) and heat-stable STII (Urban et al., 1990) enterotoxins of ETEC; and verotoxins VT1 and VT2 (Downes et al., 1989) of EHEC strains. However, some of the tests are known to possess variable sensitivities, the reliability of which depends upon the relative levels of gene expression of the target gene products under selective culture conditions, which has the added disadvantage of rendering unculturable cells non-detectable (Roszak and Colwell, 1987).

In recent years, the use of the polymerase chain reaction (PCR) and gene probe technology has provided rapid and highly sensitive methods for the specific detection of pathogenic ETEC (Pickett et al., 1989; Victor et al., 1991) and EHEC strains (Woodward et al., 1992). More recently, various multiplex PCR protocols to simultaneously detect segments of

different toxin genes of ETEC and/or EHEC strains have also been developed. For example, multiplex detection of LT1/LT2 (Kong et al., 1995), LT1/ST1 (Stacy-Phipps et al., 1995) and LT1/ST2 (Tsen et al., 1998) genes of ETEC strains; LTI/VTI/VTII genes of ETEC and EHEC strains (Lang et al., 1994); and VT1/VT2/HlyA/EAE-A/Rfb0111/Rfb0157 genes of EHEC strains (Paton and Paton, 1998) have been reported.

In western and developed countries, shrimp is considered as a potential health risk food for consumer due to its intended uncooked/minimally processed direct consumption. Contamination of uncooked raw shrimps by *Salmonella* was reported in many previous studies (Ahmed and Anwar, 2007). Pathogens from shrimp may be transmitted to humans when they are eaten undercooked or minimally processed or when other foods, which have been cross-contaminated by pathogens from shrimp, are eaten (Khan et al., 2007). *Vibrio parahaemolyticus* is a common food borne pathogen in Japan where infections have been linked to the consumption of aquaculture fish such as shrimp (Khan et al., 2007).

Fish importers often complain that pathogenic bacteria are found in the sea foods imported from Bangladesh. Among them certain strains of *E. coli* are most important which are responsible for various types of life threading diseases. Strain O157:H7 is notorious for causing serious and even life-threatening complications such as hemolytic-uremic syndrome. This particular strain is linked to the 2006 United States *E. coli* outbreak due to fresh spinach. The O104:H4 strain is equally virulent. Antibiotic and supportive treatment protocols for it are not as well-developed. It has the ability to be very enterohemorrhagic like O157:H7, causing bloody diarrhea, but also is more enteroaggregative, meaning it adheres well and clumps to intestinal membranes. It is the strain behind the deadly June 2011 *E. coli* outbreak in Europe. Severity of the illness varies considerably; it can be fatal, particularly to young children, the elderly or the immunocompromised, but is more often mild. Earlier, poor hygienic methods of preparing meat in Scotland killed seven people in 1996 due to *E. coli* poisoning, and left hundreds more infected. *E. coli* can harbour both heat-stable and heat-labile enterotoxins. The latter, termed LT, contain one A subunit and five B subunits arranged into one holotoxin, and are highly similar in structure and function to cholera toxins. The B subunits assist in adherence and entry of the toxin into host intestinal cells, while the A subunit is cleaved and prevents cells from absorbing water, causing diarrhea. (Tauschek et al., 2002).

Coliform bacteria are considered as indicator organism. The presence of coliform and fecal coliform indicate that *Citrobacter, Enterobacter, Escheriechia, Hafnia* and *Klebsiella* as well as other pathogenic bacteria such as *Salmonella* sp., *Shigella* sp. and *Vibrio* sp. might be present. Traditional shrimp culture farms are highly susceptible to be contaminated with such pathogenic bacterial species because of lack of proper sanitation near to these farms. Shrimp contaminated with one of these harmful strains will not be accepted in international market that would lead adverse effect on shrimp industries in Bangladesh. Besides it, these pathogenic strains may also be responsible for different types of shrimp diseases. So, it is very important to focus a special research on total load of coliform, as well as quantification and charecterization of *E. coli* for the presence of enterovirulent strains found in aquatic food chain and aquatic environment in the coastal region of Bangladesh. With the advantage of PCR, it has become possible to identify these genes in bacterial isolates, offering the possibility of rapid diagnosis of the mechanism operating in specific *E. coli* infection.

Objectives of the Study:

The specific objectives of the study were:

- Enumeration of total coliforms in shrimp farms water ;
- Isolation of *E. coli* ;
- Cultural and Biochemical characterization of *E. coli* ;
- Identification of *E. coli* by PCR ; and
- Detection and quantification of enterovirulent *E. coli*

Chapter Two

REVIEW OF LITERATURE

2: LITERATURE REVIEW

Contamination is a very important aspect as this is the mode that most unwanted microorganisms may be transmitted onto seafood and other food products. Unwanted microorganisms may access food processing environments through raw material, personnel or mobile equipment such as forklifts, through leakage and openings in buildings or through pests and some pathogens may even become established in the processing plant and form niches where they can survive for long periods of time (Reij et al., 2003). Many of these microorganisms occur naturally in aquatic and general environments, and may be transmitted onto seafood before capture, during and after processing. Also, contamination via air can occur through dust particles or via aerosols which are formed especially when contaminated surfaces, floors or drains are sprayed with high pressure-jets, resulting in formation of droplets that can be suspended in the air (Den Aantrekker et al., 2003). Water is also a vehicle for transmission of many agents of diseases (Kirby et al., 2003).

Food-borne or waterborne microbial pathogens are leading causes of illness and death in less developed countries, killing an estimated 1.9 million people annually at the global level. Even in developed countries, it is estimated that up to one third of the population are affected by microbiological food-borne diseases each year. The factors involved in significant increase are generally agreed to include changes in animal production systems and in the food production chain. Both types of changes can cause corresponding changes in patterns of exposure to the pathogens and the susceptibility pattern of the human population (Schlundt *et al.*, 2004).

Many pathogenic bacteria are naturally present in aquatic environments (*Clostridium botulinum* type E, pathogenic *Vibrio* sp., *Aeromonas sp)* and the general environment (*C. botulinum* type A and B, *Listeria monocytogenes)* (Huss et al., 2000). Other microorganisms are of the animal/human reservoir (*Salmonella, Shigella, E. coli*, enteric virus) (Huss et al., 2000). Thus, there is always a possibility that these microorganisms may be passed on to the raw material during production and processing.

In general, when a healthy fish is caught, the flesh is sterile as its immune system prevents bacteria to proliferate easily whereas after death the fish's immune system collapses allowing easy access of microorganisms into the flesh (Huss, 1995). Some microorganisms have been found on the entire outer surface (skin and gills) and in the intestines of live and newly healthy caught fish (Huss, 1995). Liston (1980) estimated the total number of microorganisms to vary enormously from a normal range of 10^{5}-10^{7} cfu (colony forming units)/cm^{2} on the skin surface, whereas counts between 10^{3}-10^{9} cfu/g on the gills and intestines were found by Shewan (1962).

Pathogenic contamination of water has long been a concern to the public. Concern is increasing worldwide due to the outbreak of waterborne diseases (Craun, 1986). According to World Health Organization (WHO), diarrheal disease alone accounts for an estimated 4.1% of the total disability-adjusted life year (DALY) global burden of disease and is responsible for the death of 1.8 million people per year (WHO, 2004), while 88% of that burden is caused by consumption of unsafe drinking water. The infection of diarrheal is spread through contaminated food or drinking water. Diarrheal disease accounts for nearly 1.5 million of the 9 million children under the age of 5 who die needlessly each year across the world (UNIEF/WHO, 2009).

In recent years, scientists have identified a large number of pathogens responsible for waterborne disease outbreaks and researches have focused on identifying their sources, development of resistance to water disinfection, and removal from drinking water supplies. The common bacterial infections of water diseases include *E. coli* infection, cholera, and 4 typhoid fever. Bathing, contact recreation, washing, and drinking can result infection if the water is contaminated. Contact recreation and drinking uses are considered to be the two major routes of infection. *E. coli* outbreaks, most of which have been caused by a specific strain of *E. coli* bacteria such as *E. coli* O157:H7, have received much media coverage (WSDH, 2010). A number of *E. coli* O157:H7 outbreaks have been reported from recreational use of polluted waters, particularly in swimming pools that were not adequately chlorinated. Moreover, from 1982 to 2002, 15% outbreaks of *E. coli* O157:H7 were due to drinking water exposure, which resulted in 1290 illnesses (Reynolds, 2008). The acute diseases followed the infection include hemolytic uremic syndrome with possible long-term squealed (Pond, 2005) and *E. coli* O157 gastroenteritis.

Food security is a complex issue, where fish and fishery products are generally regarded as high risk commodity in respect of pathogen contents, natural toxins and other possible contaminants and adulterants. In addition fish, shellfish and fish products have occupied second position (next to readymade garment and knitwear) in the list of exportable commodities of Bangladesh, whereas frozen shrimp occupied 72.4% i.e. about US$ 428 million (FSYB, 2007). The Food and Agricultural Organization of the United Nations and the World Health Organization (FAO/WHO, 2000) state that illness due to contaminated food is perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity.

Shrimp and other fishes or seafood can be jeopardous for health and life by carrying pathogenic or potentially pathogenic bacteria like Enterobacteriaceae, Staphylococcus etc (Ayulo et al., 1994). Enterobacteriaceae is a large family comprising heterogeneous group of gram-negative rods as coliforms, Salmonella, Shigella, Yersinia and so on (Pelczar et al., 1998; Stanier et al., 1995, Tortora et al., 1995), and can be contaminated in shrimp or seafood from animal or human reservoir. Therefore, fresh shrimps should have a specification of 10 CFU/g (WHO specification) and <102 CFU/g (Thai specification) for coliforms, and <3 CFU/g for E. coli (Suwansonthichai and Rengpipat, 2003).

Coliform bacteria are part of the Enterobacteriaceae and live in the lower intestines of warm-blooded animals. They are gram-negative, oxidase-negative, non-spore forming rods, that ferment lactose with gas production at 35-37°C after 48h (WHO, 2008; Cabral, 2010). Coliforms can be found in aquatic environment, in soil and on vegetation. Although coliform bacteria are not usually pathogenic themselves, their presence indicates fecal contamination, perhaps accompanied by disease-causing pathogens. Since the 1920s, total 6 and fecal coliforms are the standard microbial indicators of water quality (Reynolds, 2003). Indicator microorganisms are microorganisms or a group of microorganisms indicative for the possible presence of pathogens whose presence in given numbers points to inadequate safety in processing (Mossel et al., 1995). In general, they are most often used to assess food sanitation (Jay, 1992).

Coliform bacteria were referred to belong to the genera *Escherichia*, *Citrobacter*, *Klebsiella* and *Enterobacter*, but this group is also including other genera, such as *Serratia* and *Hafnia*. Thus, the total coliform group includes both fecal and environmental species (WHO, 2008). Total coliform counts are not an accurate measure of fecal pollution since it can detect bacteria that have no connection with fecal pollution (Cabral, 2010). Moreover, many pathogens of public health concern do not behave like fecal indicators and have no absolute indicator of their presence, only a probability of their co-occurrence (Payment, 2011). For example, fecal coliforms are not a reliable indicator during anaerobic digestion because viral pathogens tend to have a greater survivability than fecal coliforms (NHDES, 2003).

Fecal coliform bacteria are a subgroup of coliform bacteria. They also appear in the intestinal tract of warm-blooded animals. Fecal coliforms, which are outside of a warmblooded host, have a shorter life expectancy compared to total coliform bacteria which are associated with the digestive tract of humans or animals. The bacteria in fecal category are usually nonpathogenic, but also include pathogens such as *E. coli* O157:H7. The existence of fecal coliforms indicates fecal contamination and of the potential presence of enteric pathogens, especially bacterial pathogens. For recreational waters, fecal coliform bacteria were the primary bacteria indicator until relatively recently, when U.S. EPA began recommending *E. coli* and enterococci as better indicators of health risk from water contact (USEPA, 2010). Some states, such as New Hampshire, are still using fecal coliforms as the indicator bacteria (NHDES, 2003).

Gram staining is an empirical method of differentiating bacterial species into two large groups, gram-positive and gram- negative, based on the chemical and physical properties of their cell walls. *Escherichia coli*, commonly abbreviated *E. coli*, is a large and diverse group of gram-negative rod-shaped bacteria that are commonly found in the lower intestine of warm-blooded organisms. *E. coli* can grow in the media with glucose as the only organic component while wild type *E. coli* have no growth factor requirement and can transform glucose into all macromolecular components for constructing the cell through metabolism (Todar, 2011). Moreover, *E. coli* are facultative anaerobe and then can grow in the presence or absence of O_2. Under anaerobic conditions they will grow by means of fermentation or anaerobic respiration in which NO_3, NO_2 or fumarate as final

electron acceptors for respiratory process (Todar, 2011). Thus, *E. coli* can adapt both the intestinal and extraintestinal environments with such characteristics.

At this time, there are over 700 genetically different types of *E. coli* that have been identified. For many years after the first description the in 19th century, *E. coli* were simply considered to be a commensal organism in the intestine. However, a strain of *E. coli* that was first shown to be the pathogen that caused an outbreak of diarrhea among infants in 1935. Diseases caused by various strains of *E. coli* include not only diarrhea but also urinary tract infections (UTIs) and neonatal meningitis. *E. coli* are responsible for 90 percent of UTIs, neonatal meningitis in 1/300 infants and an unknown number of diarrheal illnesses (Todar, 2009).

Among the pathogenic strains, *E. coli* O157: H7 from the intestinal tract of warm-blooded animals has attracted more attention. This strain is primarily spread to people by consuming unpasteurized milk or undercooked beef and can lead to severe diarrhea, hemolytic uremic with possible sequelae, *E. coli* O157 gastroenteritis. Uropathogenic strains of *E. coli* (UPEC) are the most common cause of nonhospital- acquired urinary tract infection. This unique group can enter into the urinary tract and ascend to colonize the bladder, causing cystitis. Or these bacteria may ascend the ureter to infect the kidneys causing pyelonephritis (Vigil et al., 2011).

Food plants and many other institutions require sanitary conditions in order to prevent microbial contamination. The continuous evaluation of these environments is particularly important in order to assure the safety and quality of products, and the number of microbial cells contaminating food surfaces must be determined for this assessment (Yamaguchi et al., 2003). Many methods have been developed to detect microorganisms, and although some methods of analysis are better than others, every method has certain inherent limitations associated with its use (Jay, 1992).

In the contact plate method, direct surface contact plates, petri dishes or contact slides with selective or general purpose agar media are applied to the surface to be examined, followed by incubation and counting of colony forming units (Huss, 2003). This technique can only be applied to flat surfaces, which is a limiting factor (Huss, 2003). The RODAC plate has been shown to be the method of choice when the surfaces to be examined are

smooth, firm, and nonporous while it is not suitable for heavily contaminated surfaces (Jay, 1992).

Ristaino et al. (1983) stated that the specific detection of pathogenic ETEC and EHEC strains which produce different types of enterotoxins, several ELISA-based methods has been developed. They include ELISAs for the detection of heat-liable LT. Same procedure was followed by Carroll et al. (1990) to detect heat-stable STI, Urban et al.,(1990) to detect heat-stable STII enterotoxins of ETEC and Downes et al. (1989) to detect verotoxins VT1 and VT2 of EHEC strains.

Bettelheim (1992) reported that the method which is widely used has several dis-advantages especially in relation to its lack of specificity. First, it does not distinguish pathogenic from nonpathogenic strains of *E. coli*. Second, some strains of *Salmonella, Shigella* and *Yersinia* which are capable of splitting MUG will also be detected. Lang et al. (1994) and Paton and Paton (1998) evaluated a various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC and/or EHEC strains have also been developed. For example, multiplex detection of genes ETEC strains; LTI/VTI/VTII genes of ETEC and EHEC strains and VT1/ VT2/HlyA/EAE-A/Rfb0111/Rfb0157 genes of EHEC respectively.

Kong et al. (1995) conducted a various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC and/or EHEC strains have also been developed. For example, multiplex detection of LT1/LT2. Same procedure was followed by Stacy-Phipps et al. (1995) to detect LT1/ST1 and Tsen et al. (1998) to detect LT1/ST2.

Kong et al. (1999) evaluated a very sensitive multiplex PCR system for the rapid detection and typing of ETEC, EHEC and possibly EPEC strains of *E. coli* in the aquatic environment. The target genes chosen for this investigation included: the PHO-A housekeeping gene (present in all *E. coli*); the LT1, LT2 and ST1 genes of ETEC; the VT1 and VT2 verotoxin, and EAE virulence genes of EHEC and EPEC, respectively. The results showed that the six sets of PCR primers were highly specific for their target genes and produced specific amplimers of the expected size from several control strains of *E. coli* - ATCC 35401 (LT1+/ST1+); SA53 (LT2+/VT2+); and O157 (VT1+/VT2+/EAE+).

Multiplex PCR analysis of seawater samples indicated the presence in all four samples of *E. coli* bacteria that were positive for LT1, ST1, VT1 and EAE virulence genes.

Prere and Fayet (2005) evaluated the genotype EHEC test a new rapid system based on DNA multiplex amplification and further hybridization for the detection of shigatoxin *stx1*, *stx2* genes and intimin *eae* gene by enteroinvasive *E. coli* (EIEC). *E. coli* strains of various serogroups isolated from children with acute gastroenteritis, hemorrhagic colitis or hemolytic–uremic syndrome were tested. The strains collection included 11 STEC/EHEC and nine EPEC. The same strains were tested with genotype EHEC. For all the strains, the hybridization banding pattern obtained by genotype EHEC correlated with their expected genotypic characteristics. No specific equipment is required, except a thermocycler. Absence of electrophoresis system, of ethidium bromide staining and imaging system is a clear-cut advantage of Genotype EHEC.

Dow et al. (2006) stated that *Escherichia coli* strains isolated in a Libyan hospital (20 from children with diarrhoea and 30 from healthy children) were investigated for their pathotypes and virulence traits. Altogether nine *eae*-positive (enteropathogenic *E. coli*, EPEC) and nine *agg*R-positive (enteroaggregative *E. coli*, EAEC) strains were identified. while six EAEC strains were identified from diarrhoeal and three from healthy children. Typical (*eae*+, EAF+, *bfp*+) EPEC strains (n=6) belonged to classical EPEC serogroups O55, O114, O127 and showed localized adherence on Hela cells. EAEC strains revealed genetic heterogeneity but uniformly adhered to HeLa cultures in an enteroaggregative adherence pattern. Antibiotic resistance frequently, characterized the strains. Sixty-eight percentages of the strains were resistant against at least one antibiotic and 30% harbored a class 1 integron independently of their clinical background.

Sorsa et al. (2007) stated that extra intestinal pathogenic *Escherichia coli* (ExPEC) are a major cause of urinary tract infections, sepsis, and neonatal meningitis; they recently detected short DNA fragments, which were significantly associated with the extraintestinal virulent phenotype. In this study, they identified four novel genomic DNA regions of the highly virulent uropathogenic *E. coli* strain JS299 carrying these previously identified DNA fragments. The prevalence of 15 previously uncharacterized genes was determined in a collection of clinical ExPECs and commensal *E. coli* strains by means of DNA microarray analyses. They identified DNA sequences shared by both ExPEC and

enteropathogenic *E. coli* (EPEC). The results support the idea of a considerable genetic variability among ExPEC strains and suggest that the novel genomic determinants contribute to the ExPEC virulence.

Bolanle et al. (2010) stated that the bacteriological status of the Victoria-Island beach and the Lagos harbour waters were studied. Assessment was made for microbial density, *Escherichia coli* and *Vibrio* to determine their suitability for shrimp culture. The Victoria-Island beach water was found to have a total viable count of between 2.0–2.5 $x10^2$ cfu/ml, and the Lagos harbour water 7.5–8.4 x 10^3 cfu/ml. *Escherichia coli* and *Vibrio* were absent in the Victoria-Island beach water, but present in the Lagos harbour water.

Chapter Three

MATERIALS AND METHODS

3: MATERIALS AND METHODS

3.1 Sampling area

In this study, water samples were collected in winter (November to February, 2011-2012) and summer (May to August, 2011-2012) seasons from 31 shrimp farms located at different sites of Khulna district. Using visual observation farms were categorized as farms in unhygienic condition (U) and farms in hygienic condition (H). Criteria for unhygienic and hygienic shrimp farm are described in Table 3.1. These samples were used to counting total coliform, isolation and detection of enterovirulent *E. coli* in the Nutraceuticals and Bioresource Technology Laboratory of Biotechnology and Genetic Engineering Discipline and also in the Biochemistry and Molecular Genetics Laboratory of Fisheries and Marine Resource Technology Discipline of Khulna University.

Table 3.1 Criteria for unhygienic and hygienic shrimp farm

Unhygienic shrimp farm	Hygienic shrimp farm
Hanging toilet was very close to farm or at the canal or rivers which is the main water source of the farm.	No hanging toilet, only concrete and closed toilet, if necessary.
Connection of sewerage line with the canal, which is the water source of the farm.	Water source is ground water or rain water.
Cow-shed and grazing land very close to farm.	No cow-shed or grazing land nearby farm.
Excessive and continuous use of cow dung as fertilizer or for plankton growth.	No direct and excessive use of cow dung or no use at all.
Dirty premises.	Clean and healthy environment.

3.2 Sampling procedure

For the purpose of sampling, sampling kit consisting of several sterile plastic vials, dropper, plastic bag, alcohol and marking pen were taken. Sufficient amount of water from each shrimp farm were collected; kept in a vial and tagged. At each time of collection hands were sterilized with alcohol and then the vials were partially filled with material, the vials were properly tied and labeled. Special care was taken to avoid contamination as far as practicable.

3.3 Preservation of sample

After collection, the samples were brought to the laboratory carefully and preserved in the refrigerator for immediate use; however for long term usage samples were stored at 4^{o} C.

3.4 Chemicals

Table 3.2: A list of chemicals used in the experiment, their purpose and source

Name of chemicals	Source	Purpose
1. Luria-Bertani (LB) broth	Difco Laboratories, MI, USA	Bacteria culture
2. Nutrient Agar	Oxoid	Slant
3. MacConkey Agar Eosin	Oxoid	Characterization
4. Methylene Blue agar	Oxoid	Characterization
5. DNAzol reagent	Invitrogen Life technologies, USA	DNA extraction
6. 100% Ethanol	Merck, Germany	DNA wash
7. Solid Sodium hydroxide	Merck, Germany	DNA solubilization
8. 5x Green Reaction buffer	Promega Corporation, USA	DNA amplification in a thermal cycler
9. dNTP mixture	Bioneer, Sheul, Korea	
10. Go taq DNA Polymerase	Promega Corporation, USA	
11. 6 pair primer	Bioneer, Sheul, Korea	
12. di-ionized water	di-ionized water	
13. Agarose powder	Bioneer, Sheul, Korea	Gel preparation
14. 50x TAE buffer	Bioneer, Sheul, Korea	Gel electrophoresis
15. Ethidium Bromide	Bioneer, Sheul, Korea	Gel preparation
16. 100 bp DNA ladder	Bioneer, Sheul, Korea	PCR Product evaluation

3.5 Experimental design:

Water samples which were collected at first from 15 shrimp farms in winter season were designed as WH_1, WH_2, WH_3, WH_4, WH_5 and UF_6 from hygienic farms category and WU_1, WU_2, WU_3, WU_4, WU_5, WU_6, WU_7, WU_8 and WU_9 from unhygienic farm category. In second phase, water samples which were collected from 16 shrimp farms in summer season were designed as SH_1, SH_2, SH_3, SH_4, SH_5, SH_6, SH_7 and SH_8 from hygienic farms category and SU_1, SU_2, SU_3, SU_4, SU_5, SU_6, SU_7 and SU_8 from unhygienic farm category. In the second step the chemical parameters of the collected samples were observed. The third step was involved the quantification of coliform using MacConkey agar media and isolation and identification of *E. coli* using different cultural and biochemical tests. The fourth step, the extraction of DNA was involved from the identified *E. coli*. In fifth step, quantitative and qualitative analysis of extracted DNA was done by using spectrophotometer and agarose gel electrophoresis. The sixth step was involved the amplification of target gene by PCR technology; this step involved in two phases, at first phase, all samples were checked for the presence of alkaline phosphate (Pho-A) and only *E. coli*. Positive samples were further checked for the presence of enterovirulent strains in next phase. In the final step, gel electrophoresis was performed to detect enterovirulent *E. coli* strains by observing clear bands through comparing with a 100 bp DNA ladder.

3.6 pH and salinity

The p^H and salinity of the collected samples were recorded in the laboratory as soon as possible after collection. P^H the collected water samples was measured using P^H meter and salinity was measured using salinity test meter.

3.7 Counting of coliform and isolation of *E. coli*

MacConkey agar is a selective media for coliform. So this media was used to enumerate total coliform. A known quantity of collected aqueous samples was spreaded on MacConkey agar media and the plates were incubated at 37 $^{\circ}$ C for 24 hours. Lactose positive colonies showed red color on MacConkey agar plate and the number of coliform was equal to the number of lactose positive colonies. Then red colonies on MacConkey

agar plate were finally selected and isolated for detection and characterization of *E. coli.* The selected red colonies were subcultured onto nutrient agar until the pure cultures with homogenous colonies were obtained.

3.8 Maintenance and Preservation of the selected strains

3.8.1 Short term preservation

Nutrient agar (NA) slants were used for short term preservation of selected red colonies. The colonies were inoculated in NA slant using a sterile loop and incubated at 37 ° C for 18-27 hours. After growth the slants were stored at 4 ° C for short term storage and for usage.

3.8.2 Long term preservation

For long term preservation, the selected colonies were inoculated into sterilized nutrient broth media and culture at 37 ° C for 12-16 hours. Then 850 µl cultures were transferred into one drum vial and 150 µl sterilized glycerol was added into the vial. Then mixer was mixed by vortex and stored at - 80 ° C for long term preservation.

3.9 Tests for detection of *E. coli*

3.9.1 Eosine Methylene Blue (EMB) agar medium

This is selective for gram negative bacteria (one of which is *E. coli*). When the selected red colonies were streaked on EMB plate, *E. coli* was identified with a characteristics green metallic sheen along the streak after a 24-48 hour incubation period. This is due to the lactose fermenting properties of *E. coli* which elicits a green sheen. This green metallic sheen indicates positive result.

3.9.2 Lactose fermentation test

Lactose is a carbohydrate source. Fermentation of lactose is demonstrated by the production of gas. To identify lactose fermenter, each test tube was filled with 9 ml of lactose broth solution. Then Durham's tubes were inserted upside down in all test tubes and they were gently shaken to remove air. All the test tubes were clogged with cotton and sterilized by auto-claving. Then 1 ml of 10 % lactose solution sterilized by petroleum ether was added in each test tube. After that selected strains were inoculated and incubated at 37

° C. Gas was produced by lactose fermenter strains within 48 hours which indicate positive result.

3.9.3 Methyl Red (MR) test

This test detects acid production to a sufficient degree (below P^H 4.5). Tubes were inoculated with 5 ml MR-VP medium and incubated at 37 ° C for 48 hours. After incubation 10 drops of methyl red reagent was added and recorded immediately. A red colour indicates that P^H has been reduced to 4.5 or less. A yellow colour indicates a negative reaction.

3.9.4 Voges-Proskauer reaction (VP test)

This test detects the formation of acetyl methyl carbonol (acetone) from glucose. Tubes were inoculated with 5 ml MR –VP medium and incubated at 37° C for 48 hours. After incubation 24 drops (1.2 ml) of Barrit's reagent A and 8 drops (0.4 ml) of Barrit's reagent B were added and carefully shaked for 30 second to 1 minute. The tubes were allowed to stand for 30 minutes. A bright pink or eosine red colour indicates positive reaction. In negative reaction rose colour remains.

3.9.5 Utilization of citrate

Perpose of this test was to differentiate organisms on the basis of their ability to ferment citrate as sole carbon source. This ability depends on citrate permiase. Simmon's citrate agar slants were inoculated by means of a stab and streak inoculation then incubated at 37 ° C for 24 hours. All agar slants were examined. For coloration bromothymol blue indicator was used. Blue coloration indicates positive result and green indicates negative result.

3.10 Characterization of *E. coli* by PCR

3.10.1 Experimental design

In the present study, the isolated *E. coli* was characterized by some strain specific genes which were amplified by Polymerase Chain Reaction (PCR). The target genes were chosen for this investigation included: Alkaline phosphate (Pho-A), housekeeping gene (present in all *E. coli*); the LT1, LT2 and ST1 genes of ETEC; the VT verotoxin of EHEC and EAE virulence genes of EPEC. In this study, six pairs (forward and reverse) of specific oligonucleotide primers that were designed from aforementioned six. These primers named PhoA-F, PhoA-R, ST1-F, ST1-R, LT1-F, LT1-R, LT2-F, LT2-R, VT-F, VT-R, EAE-F and EAE-R were used for each sample.

3.10.2 Preparation of medium and bacteria culture

For Luria-Bertani (LB) broth preparation, 2g LB was added into 100 ml of distilled water and mixed well. Then 5 mL LB broth was transferred into each test tube and autoclaved at 121°C for 20 minutes. Test tubes containing 5mL autoclaved LB broth were then inoculated with isolated *E. coli* and placed in a shaking incubator at 37° C with 200 rpm (rotation per minute) for overnight to culture. The overnight culture provided huge amount of bacteria were used for the extraction of DNA.

3.10.3 DNA extraction

The DNA isolation was done by using DNAzol reagent (Genomic DNA Isolation Reagent) followed by the protocols of Ausubel et al., (1990) and further optimized by Islam (2006). DNAzol reagent is a complete and ready-to-use reagent for the genomic DNA isolation from small or large volume of solid and liquid samples that normally combines both reliability and efficiency with simplicity of the isolation protocol. The procedure was completed within 30 minutes with 70-100% of DNA recovery (Appendix-B).

3.10.4. Determination of DNA concentration and Purity:

The concentration and purity of DNA samples were determined from the ratio of absorbance at A_{260} and A_{280} (absorbance at 260 nm and 280 nm) using a spectrophotometer against NaOH blank cuvette. DNA sample containing cuvette was washed properly before loading next sample. Thus, a list of data of the samples for two absorbencies was found and saved. The protocol used in this experiment was designed for a double-beam spectrophotometer. The DNA concentration and purity was determined by the following formulas:

1. Double-stranded DNA concentration (C), μg/ml = Absorbance at $A_{260} \times 50 \times 500$

2. Purity = Absorbance at A_{260} /Absorbance at A_{280}

3.10.5 Gel electrophoresis

Agarose gel electrophoresis was performed according to Sambrook et al., (1989) to be confirmed that DNA was extracted or not. Gel electrophoresis was performed using a horizontal electrophoresis apparatus (Bioneer, A-7020, Korea) at a constant volt of 100 with a 1.5% agarose gel incorporating ethidium bromide and 1xTAE buffer. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA size and band were determined by comparison with a DNA size marker (100-2000 bp DNA Markers) (Appendix-C).

3.11 Description of target genes and primers

3.11.1 Target genes

The target genes were chosen for this investigation included: Alkaline phosphate (Pho-A), housekeeping gene (present in all *E. coli*); the LT_1, LT_2 and ST_1 genes of ETEC; the VT1 and VT2 verotoxin, and EAE virulence genes of EHEC and EPEC, respectively.

3.11.2 Oligonucleotide primers

The target genes chosen of this experiment were: Alkaline phosphate (Pho-A), housekeeping gene (present in all *E. coli*); the LT1, LT2 and ST1 genes of ETEC; the VT (verotoxin) of EHEC and EAE virulence genes of EPEC. Six pairs of specific primers were chosen from gene sequence (Table 3.3) to amplify the target DNA fragments (genes). To compare the size of double stranded DNA from 100 to 2,000 base pairs, 100 bp designed DNA markers were used. The DNA marker consists of 13 double stranded DNA fragments ranging in sizes from 100 to 1,000 (Bioneer, Korea).

Table 3.3 Primers and expected size of PCR-amplified gene targets of pathogenic *E. coli*

Enterovirulent groups	**Target Gene**	**Primers**	**Product size (bp)**	**Accession No.**
House keeping gene	1. Alkaline phosphatase (PhoA)	Pho-F Pho-R	1371	M13345
Enterotoxigenic (ETEC)	2. Heat-stable toxin 1 (ST1)	ST1-F ST1-R	638	M25607
	3. Heat-labile toxin 1 (LT1)	LT1-F LT1-R	725	J01646
	4. Heat-labile toxin 2 (LT2)	LT2-F LT2-R	997	M17894
Enterohemorrhagic (EHEC)	5. Verotoxin (VT)	VT-F VT-R	1240	M36727
Enteropathogenic (EPEC)	6. Attachment and Effacement (EAE)	EAE-F EAE-R	1155	M58154

3.12 Target gene amplification by Polymerase Chain Reaction (PCR)

At first, the concentrations of all the extracted DNA samples were adjusted. The reactions were performed in a 0.2 ml PCR tube (Eppendrof, Humburg, Germany) in which 25 µl reaction mixture containing 2 µl DNA sample, 2 µl oligonucleotide primers, 5 µl 5X green reaction buffer, 2 µl 10 mM dNTPs mixture, 1 µl Taq DNA polymerase and 13 µl de-ionized distilled water. The 0.2 ml PCR tube containing reaction mixtures were then placed in a DNA thermal cycler (C1000^{TM}, BIO-RAD, USA) for polymerase chain reaction (Table 3.4).

Table 3.4: The component of reaction mixture and their compositions in 25 µl reaction mixture together with final concentration for the PCR

Component	**Final volume**	**Final concentration**
1. Go Taq DNA Polymerase* (5 units/µl)	1 µl	1.25 unit
2. 5X Green Reaction Buffer	5 µl	1x
3. 10 mM dNTP mixture (each dNTP 2.5 mM)	2 µl	1.0 mM
4. Primers (Pho, ST1, LT1, LT2, VT and EAE)	2 µl	1.0 µM
5. Template DNA	2 µl	0.25 µg/25 µl
6. Di- ionized water	13 µl	

*To prepare 1 unit *Taq* DNA polymerase, it was 4 times diluted with di-ionized water.

The PCR conditions for target DNA amplification were: initial extended step of denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute and elongation at 72°C for 1 minute. While running PCR reactions, a positive control was also kept. The positive control constitutes highly *E. coli* contamination which ensured all the chemicals in the PCR samples worked efficiently.

the 257 isolated red colonies, total 76 red colonies gave positive result on EMB Agar Test, Lactose Fermentation and MR Test and negative result on VP Test and Citrate Agar Test. So these colonies were counted as *E. coli*. The biochemical test results were given in Table 4.4 and Fig 4.2-Fig 4.6.

4.5 Isolation of DNA from identified *E. coli*

Diagnostic screening for the presence of *E. coli* by PCR starts with extraction of the DNA materials to be amplified. Representative samples are of primary importance in the analysis of pathogens. In the present experiment, DNAzol solution was used to extract total genomic DNA from biochemically identified as *E. coli*. Extracted and PCR amplified DNA samples of bacterial cells showed the clear band patterns during gel electrophoresis. The average A_{260}/A_{280} ratio of the extracted DNA materials was 0.67 ± 0.18 which is within 1. Thus, spectrophotometric analysis revealed the satisfying purity of extracted DNA samples from all the experimental shrimp farms. The final yield of DNA ranged between 0.36 - 0.87 µg/mL.

4.6 Conformation of *E. coli* by PCR technique

Pho-A gene is a house keeping gene which is present in all *E. coli*. So PCR technique was used to diagnosis of this gene for the conformation *E. coli*. The electrophoretic patterns of the extracted DNA samples are shown in Fig: 4.7- Fig 4.8. These figures were presented as a representative of the total samples. As shown in those figures, most of the samples produced a DNA band with varying degree of intensity and smearing pattern. The smearing indicated the presence of numerous smaller size DNAs resulting from the mechanical shearing of the centrifugation process and pipetting involved. During Pho-A testing, 1371bp band pattern was found which clearly indicates the presence of *E. coli*.

4.7 Enumeration of total *E. coli*

The isolates which showed positive result in Eosine Methylene Blue Agar Test, Lactose Fermentation Test, Methyl Red Test and negative result in Voges–Proskauer Test and Citrate Agar Test were selected for PCR for confirmation of *E. coli*. Then the number of *E. coli* was calculated as colony forming unit per milliliter (cfu/mL) sample and the results were shown in Table 4.2 & Table 4.3. Among 31 shrimp farms 17 farms that mean around 55% farms were contaminated with *E. coli*. The number of *E. coli* was decreased in winter

Category of farm	Farm no	Total coliform (cfu/mL)	Total *E. coli* (cfu/mL)	Average of total coliform (cfu/mL)	Average of total *E. coli* (cfu/mL)
Hygienic	SH_1	71.60×10^2	0	82.65×10^2	8.51×10^2
	SH_2	193.00×10^2	0		
	SH_3	73.00×10^2	0		
	SH_4	15.50×10^2	6.64×10^2		
	SH_5	184.50×10^2	61.50×10^2		
	SH_6	17.60×10^2	0		
	SH_7	22.00×10^2	0		
	SH_8	84.00×10^2	0		
Unhygienic	SU_1	79.60×10^2	9.86×10^2	111.32×10^2	45.27×10^2
	SU_2	70.00×10^2	50.00×10^2		
	SU_3	170.00×10^2	0		
	SU_4	75.00×10^2	42.85×10^2		
	SU_5	90.00×10^2	20.00×10^2		
	SU_6	165.00×10^2	91.66×10^2		
	SU_7	79.00×10^2	39.50×10^2		
	SU_8	162.00×10^2	108.33×10^2		

Table 4.4: Biochemical test results of the selected red colonies

Farm no	Number of selected red colonies	Identified *E. coli*	Total number of *E. coli*
WH_1	6	0	0
WH_2	8	0	0
WH_3	10	0	0
WH_4	10	0	0
WH_5	10	0	0
WH_6	8	0	0
WU_1	10	1/1/9	1
WU_2	10	0	0
WU_3	12	3/1/2	4
		3/1/5	
		3/1/7	
		3/1/12	
WU_4	13	4/2/4	6
		4/2/5	
		4/2/6	
		4/2/8	
		4/2/9	
		4/3/3	
WU_5	8	5/2/1	3
		5/2/6	
		5/2/8	
WU_6	8	10/3	3
		10/4	
		10/6	
WU_7	10	29/2/1	7
		29/2/2	
		29/2/3	
		29/2/4	
		29/2/5	
		29/3/6	
		29/3/7	

Continued

WU_8	10	30/2/1	8
		30/2/2	

		30/2/3	
		30/2/4	
		30/2/5	
		30/2/8	
		30/2/9	
		30/2/10	
WU_9	12	31/2/1	7
		31/2/3	
		31/2/4	
		31/2/6	
		31/2/7	
		31/2/8	
		31/2/9	
SH_1	5	0	0
SH_2	5	0	0
SH_3	5	0	0
SH_4	7	20/3/1	3
		20/3/6	
		20/3/7	
SH_5	7	21/3/1	3
		21/3/2	
		21/3/3	
SH_6	5	0	0
SH_7	7	0	0
SH_8	7	0	0
SU_1	10	16/2/1	3
		16/2/8	
		16/2/9	

Continued

SU_2	7	17/3/1	5

		17/3/2	
		17/3/3	
		17/3/4	
		17/3/5	
SU_3	5	0	0
SU_4	7	19/3/1	4
		19/3/2	
		19/3/3	
		19/3/4	
SU_5	7	22/3/6	2
		22/3/7	
SU_6	9	26/2/1	5
		26/2/3	
		26/2/4	
		26/2/6	
		26/2/7	
SU_7	10	27/2/1	5
		27/2/2	
		27/2/3	
		27/2/4	
		27/2/5	
SU_8	9	28/2/1	6
		28/2/2	
		28/2/3	
		28/2/4	
		28/2/6	
		28/2/9	

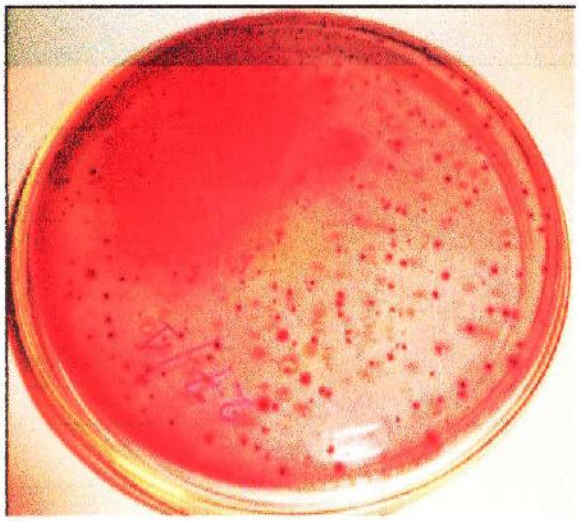

Fig.4.1. Red colonies on MacConkey agar Medium

Fig.4.2. Occurrence of green metallic sheen on EMB plate

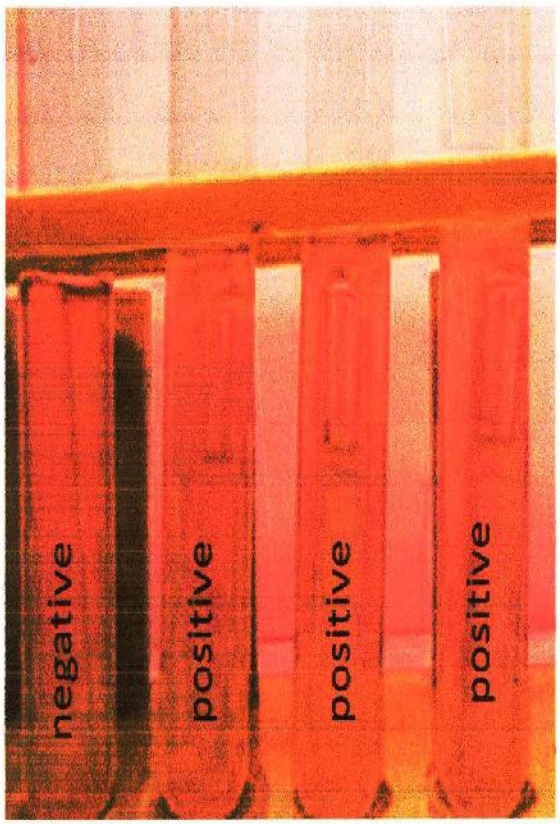

Fig.4.3. Lactose fermentation test

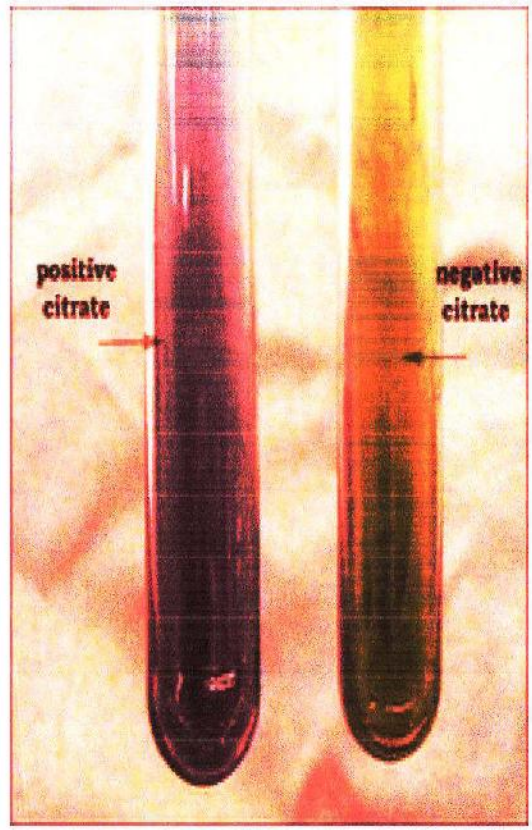

Fig.4.4. Citrate agar test

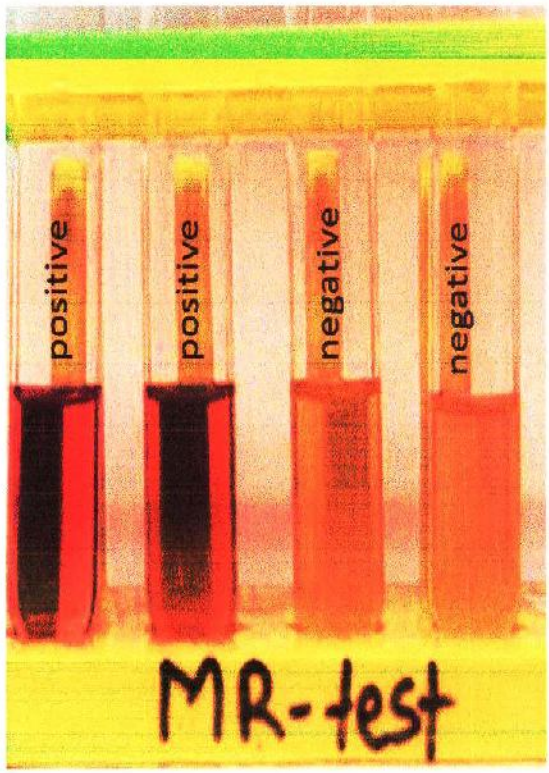

Fig.4.5. Methyl red (MR) test

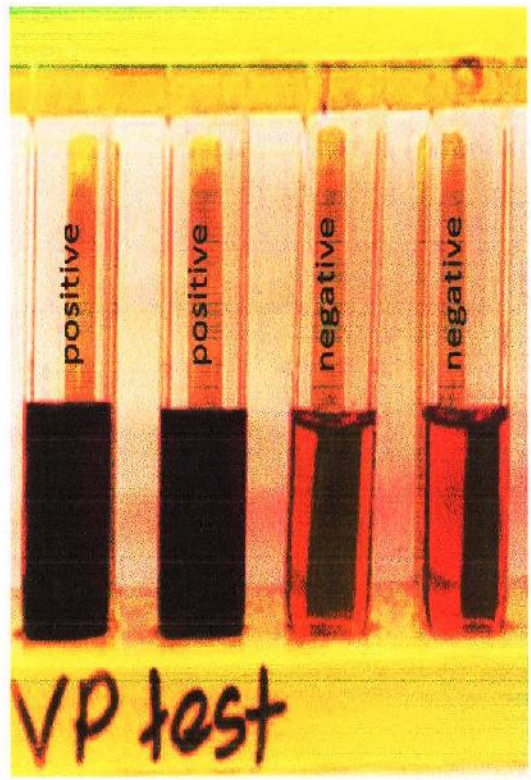

Fig.4.6. Voges–Proskauer (VP) test

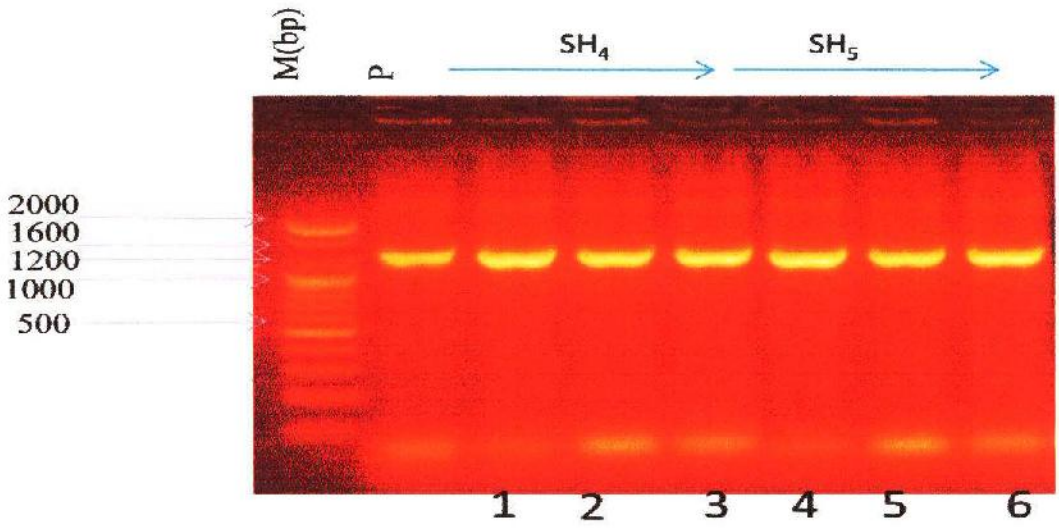

Fig 4.7: UV visualization of total genomic DNA extracted from six isolates of SH_4 and SH_5 shrimp farms following electrophoresis in a 1.5% agarose gel. Lane 1, 2, 3 indicates isolates 20/3/1, 20/3/6, and 20/3/7 of SH_4 farm and lane 4, 5, 6 are for isolates 21/3/1, 21/3/2, and 21/3/3 of SH_5 farm. Lane M and P represented 100 bp DNA ladder or marker, positive control respectively.

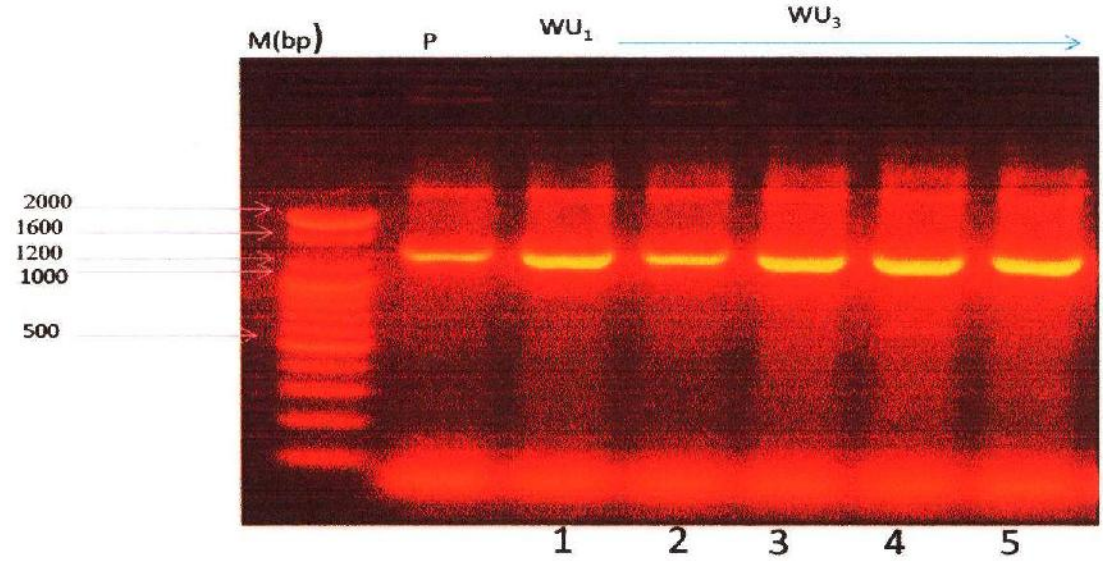

Fig 4.8: UV visualization of total genomic DNA extracted from five isolates of WU_1 and WU_3 shrimp farms following electrophoresis in a 1.5% agarose gel. Lane 1indicates isolate 1/1/9 of WU_1 farm and lane 2, 3, 4, 5 are for isolates 3/1/2, 3/1/9, 3/1/7, 3/1/12 of WU_3 farm. Lane M and P represented 100 bp DNA ladder or marker, positive control respectively.

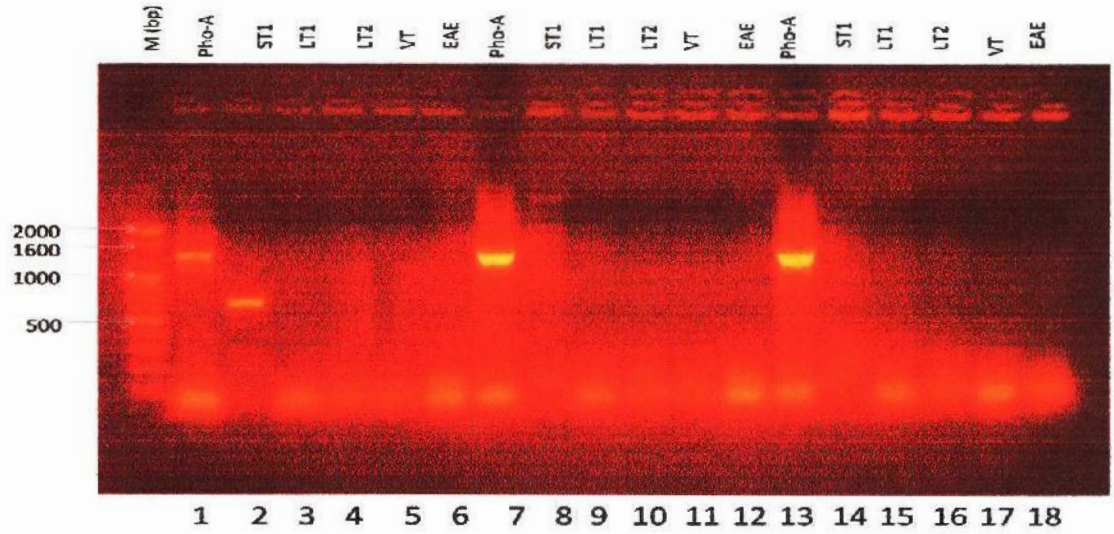

Fig 4.9: UV visualization of total genomic DNA extracted from three isolates of WU_4 shrimp farms following electrophoresis in a 1.5% agarose gel. Lanes 1 to 6, 7 to 12 and 13 to 18 are representation of 4/2/6, 4/2/5, and 4/2/4 isolates of WU_4 farm respectively where lane 1, 7, 13 for PhoA, 2, 8, 14 for ST1, 3, 9, 15 for LT1, 4, 10, 16 for LT2, 5, 11, 17 for VT and 6, 12, 18 for EAE; Lane M represents 100 bp DNA ladder or marker.

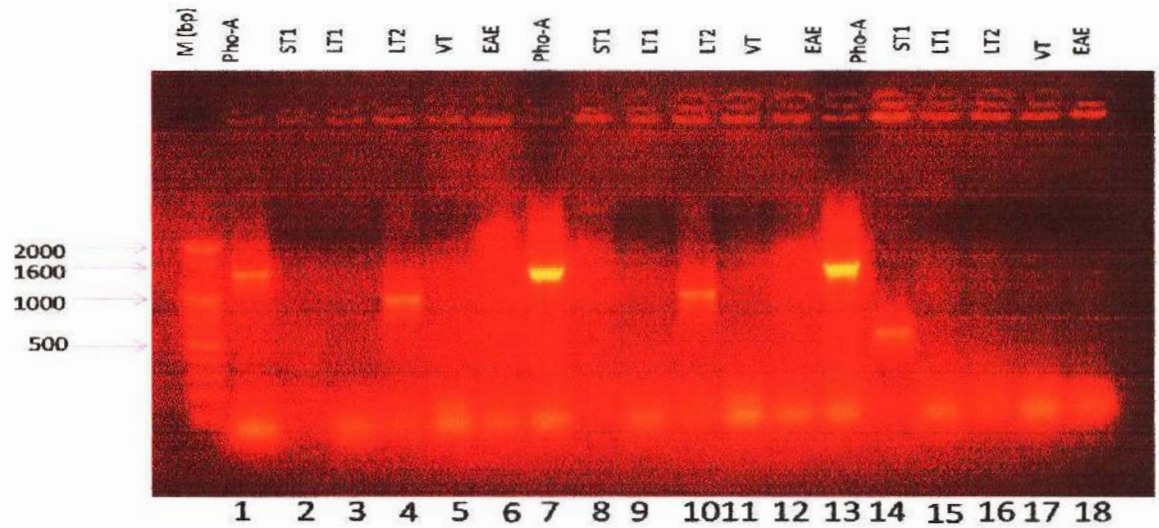

Fig 4.10: UV visualization of total genomic DNA extracted from three isolates of WU_5 shrimp farms following electrophoresis in a 1.5% agarose gel. Lanes 1 to 6, 7 to 12 and 13 to 18 are representation of 5/2/1, 5/2/6, and 5/2/8 isolates of WU_5 farm respectively where lane 1, 7, 13 for PhoA, 2, 8, 14 for ST1, 3, 9, 15 for LT1, 4, 10, 16 for LT2, 5, 11, 17 for VT and 6, 12, 18 for EAE; Lane M represents 100 bp DNA ladder or marker.

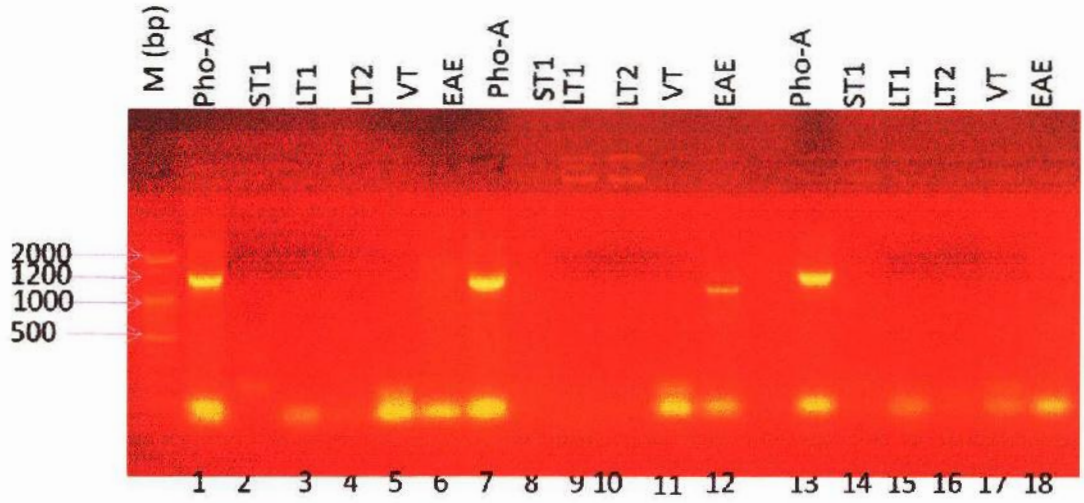

Fig 4.11: UV visualization of total genomic DNA extracted from three isolates of SU_2 shrimp farms following electrophoresis in a 1.5% agarose gel. Lanes 1 to 6, 7 to 12 and 13 to 18 are representation of 17/3/1, 17/3/2 and 17/3/3 isolates of SU_2 farm respectively where lane 1, 7, 13 for PhoA, 2, 8, 14 for ST1, 3, 9, 15 for LT1, 4, 10, 16 for LT2, 5, 11, 17 for VT and 6, 12, 18 for EAE; Lane M represents 100 bp DNA ladder or marker.

Table 4.5: PCR results summery of sampling farms in the winter and summer seasons

Group ⟶		House keeping gene	ETEC			EHEC	EPEC
Target genes ⟶		PhoA	ST1	LT1	LT2	VT	EAE
Farm category	Farm no						
Hygienic	WH_1						
	WH_2						
	WH_3						
	WH_4						
	WH_5						
	WH_6						
Unhygienic	WU_1	√					X
	WU_2						
	WU_3	√	X				
	WU_4	√	X				X
	WU_5	√	X		X		
	WU_6	√					X
	WU_7	√			X		X
	WU_8	√	X				X
	WU_9	√					X
Hygienic	SH_1						
	SH_2						
	SH_3						
	SH_4	√					
	SH_5	√					
	SH_6						
	SH_7						
	SH_8						
Unhygienic	SU_1	√	X				
	SU_2	√	X				X
	SU_3						
	SU_4	√			X		X
	SU_5	√			X		
	SU_6	√	X				X
	SU_7	√	X				X
	SU_8	√	X				X

√ Presence of *E. coli*

X Presence of enterovirulent *E. coli* strains

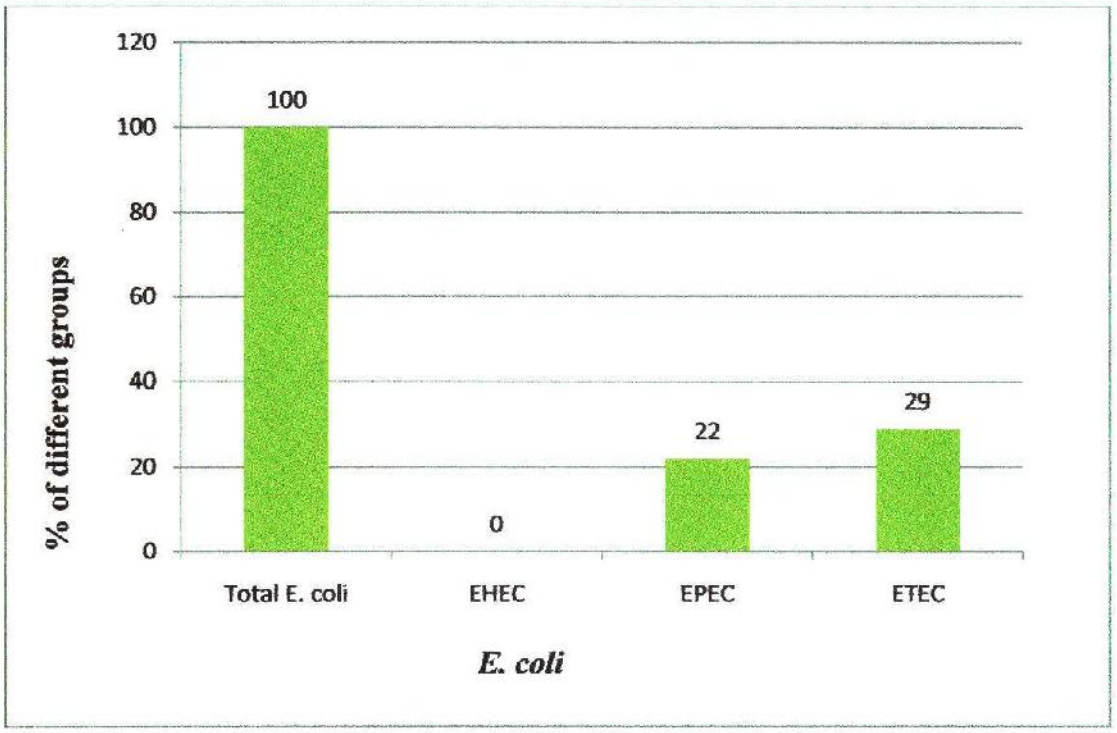

Fig 4.12: Percentage (%) of EHEC, EPEC and ETEC of *E. coli* in the shrimp farms.

Chapter Five

DISCUSSION

5: DISCUSSION

In any aquatic system, environmental parameters such as temperature, salinity, pH and dissolved oxygen play a foremost part in the distribution of bacteria (Palaniappan, 1982). But, pond being a confined environment the optimum environmental parameters (temperature 27-31°C; salinity 14 -25 ppt, pH 7.8-8.6 and DO 5.1 -5.9 ppm) could be maintained throughout the culture period by proper pond management involving water exchange and lime application. The bacterial loads might have changed due to the organic matter deposited at the pond bottom irrespective of environmental factors and water exchange, which was supported by Sharmila et al. (1996). They suggested that environmental parameters did not influence the distribution of bacterial load in the pond ecosystem because there was no dramatic change in the environmental parameters. Due to the influence of turbidity, heavy organic matter plays a major role in the distribution of bacterial population in pond water (Sharmila et al., 1996). High organic stuff is possible in shrimp culture ecosystem due to organic manure, fertilizers, high stocking density, feed waste, fecal matter, algal bloom and human interference (Moriarty, 1997; Lloberra et al., 1991).

According to Rozen and Belkin (2001), the survival of enteric bacteria in seawater is influenced by temperature (high impact), pH, salinity (low impact), nutrient availability (high impact), and light radiation (high impact). *E. coli* prefers acidic environments (pH 5). Seawater with a pH range of 7.5 to 8.5 impacts *E. coli* mortality. Fecal coliform (FC) exposed to lower pH values and temperatures have a higher survival rate.

Anderson et al. (1979) found *E. coli* cells to become nonviable within the first 2 days of exposure at 15 to 30 parts per thousand salinity, but at salinity levels of 10 parts per thousand or less; the cells experienced a slower death rate. Some scientists have demonstrated that viable but nonculturable cells are either dead or of no significance, while others have shown that they may still be infective (Rozen and Belkin, 2001). Therefore, the non-viable *E. coli* cells at 15 to 30 parts per thousand salinity reported by Anderson et al. (1979) may in fact pose a threat depending on how viability is understood and interpreted.

average salinity was zero. In other case, in summer season, average P^H was 7.0 for hygienic farms and 6.9 for unhygienic farms and average salinity was also zero. The number of coliform and *E. coli* varied from season to season and also farm to farm. In winter season, the average number of coliform and *E. coli* in hygienic farms were 4.94 × 10^2 (cfu/mL) and 0 (cfu/mL) whereas that in unhygienic farms were 8.17 × 10^2 (cfu/mL) and 4.15 × 10^2 (cfu/mL) respectively. In case of summer season, the average number of these bacteria was comparatively high. In this season, the average number of coliform and *E. coli* in hygienic farms were 82.65 × 10^2 (cfu/mL) and 8.51 × 10^2 (cfu/mL) whereas that in unhygienic farms were 111.32× 10^2 (cfu/mL) and 45.27 × 10^2 (cfu/mL) respectively.

In shrimp culture ecosystem , most of the bacteria play a negative role as they compete with shrimps for food and oxygen, causing stress and disease (Moriarty, 1997). Generally gram-negative bacteria were found to be the dominant forms in the shrimp culture ponds (Sung et al., 2003).

A number of microbial tests of fish and fish products are used by the industry for contractual and internal purposes and by the authorities to check that the microbiological status is satisfactory. Microbial indicators are often employed to assess food safety and sanitation. In the historical use of safety indicators, however, the pathogens of concern were assumed to be of intestinal origin, resulting from either direct or indirect faecal contamination. Thus, sanitary indicators (such as coliform) were used historically to detect faecal contamination of water and this practice was extended to foods (Jay, 1992).

The Total Coliform, *Salmonella* and *Vibrio* bacteria play a vital role in shrimp culture ecosystem (Ruangpan and Kitao, 1991) as they damage water quality causing diseases and mortality to the shrimp as primary and secondary pathogens. *Vibrio* sp. is the autochthonous flora of the coastal pond ecosystem (Ruangpan and Kitao, 1991). A sudden increase of bacterial load could develop bacterial infection directly and making shrimps susceptible to infection indirectly as found by Ping and Meimei (1994).

Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some strains can cause serious food poisoning in humans (Vogt and Dippold, 2005). The

harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, (Bentley and Meganathan, 1982) and by preventing the establishment of pathogenic bacteria within the intestine. *E. coli* and related bacteria constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination (Feng et al., 2002).

The present study was carried out to investigate the availability of coliform, *Eschericia coli*, and also enterovirulent *E. coli* strains in the shrimp farms. All shrimp farm's samples represented a moderate quantity of total coliform but not *E. coli.* Among the 31 farm 17 farms were contaminated with *E. coli.* Around 76 *E. coli* were isolated which were only found in samples WU_1, WU_3, WU_4, WU_5, WU_6, WU_7, WU_8, WU_9, SH_4, SH_5, SU_1, SU_2, SU_4, SU_5, SU_6, SU_7, SU_8 that indicates around 55 % shrimp farms are contaminated with *E. coli.*

For PCR detection of general strains of *E. coli*, a pair of primers targeted at the *E. coli* alkaline phosphatase (PHO-A) housekeeping gene (Chang et al., 1989), which has been previously demonstrated to be specific for *E. coli (*Kong et al., 1995) were used. The remaining 5 pairs of primers were targeted at the LT1 (Leong et al., 1995), LT2 (Pickett et al., 1989) and ST1 enterotoxin genes of ETEC; the VT1 (Paton et al., 1998) and VT2 verotoxin genes of EHEC; and the EAE virulence gene of EHEC and EPEC strains (Lightner, 1998). The VT primers which were targeted at two different stretches of DNA sequences within the VT1 and VT2 genes that share 100% sequence similarity were expected to amplify both or either of the genes from the DNAs of the corresponding EHEC strains. Similarly, the EAE primers which were targeted at two DNA domains that are highly conserved in the EAE genes of EHEC and EPEC strains were expected to amplify the targeted gene fragment from DNAs of either of those strains.

In the present study, at first 76 *E. coli* were isolated from different shrimp farms water using cultural and biochemical tests then detection experiment was performed in two steps. At first, all the isolated *E. coli* were checked through pho-A primer in a thermal cycler. A specific band for alkaline phosphatase gene (1371 bp position) was screened out to confirm the presence of *E. coli* in the sample during the evaluation of PCR products. As

alkaline phosphatase (PhoA) is the house keeping gene for *E. coli* it was available in both of studied apparently infected and uninfected farm category. In second step, only the *E. coli* positive samples were further tested by six pair primers to identify the presence of any enterovirulent groups in different samples.

Electrophoresis analysis of the PCR products using six pairs of primers revealed the presence of 1371 bp (Pho-A), 638 bp (ST1), 997 bp (LT2) and 1155 bp (EAE) DNA bands in 76 isolates of 17 different apparently infected shrimp farms (WU_1, WU_3, WU_4, WU_5, WU_6, WU_7, WU_8, WU_9, SH_4, SH_5, SU_1, SU_2, SU_4, SU_5, SU_6, SU_7 and SU_8). These band patterns clearly indicate the presence of different enterovirulent strains of *E. coli* in the shrimp farms. Among the 76 isolated *E. coli*, EPEC and ETEC groups were found which were 22% and 29%, respectively whereas EHEC group was absent.

EPEC groups were common in WU_1, WU_4, WU_6, WU_7, WU_8, WU_9, SU_1, SU_2, SU_4, SU_6, SU_7 and SU_8 shrimp farms. The presence of this enteropathogenic *E. coli* strains might be due to the poor sanitation system or contaminated water source from nearby farms or narrow canals which were observed during sampling. The pathogen is a causative agent of diarrhea in humans, rabbits, dogs, cats and horses (Toder, 2007). Like ETEC, EPEC also causes diarrhea, but the molecular mechanisms of colonization and etiology are different. EPEC lack fimbriae, ST and LT toxins, but they utilize an adhesin known as intimin to bind host intestinal cells. This virotype has an array of virulence factors that are similar to those found in *Shigella*, and may possess a shiga toxin. Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation. EPEC cells are moderately invasive (i.e. they enter host cells) and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to "attachment and effacement" are likely the prime cause of diarrhea in those afflicted with EPEC (Toder, 2007).

ETEC groups were found in WU_3, WU_4, WU_5, WU_7, WU_8, SU_1, SU_2, SU_5, SU_6, SU_7 and SU_8 shrimp farms. Poor management system, unhygienic environment was observed during sampling that might be the major reason for the presence of these virulent strains. In addition, a cow-shed and grazing ground was very close to the farm and the cowdung was used as fertilizer; therefore, there might be a chance of enterovirulent *E. coli* contamination. Karunasagar et al. (1999) reported cattle, sheep, pigs and chicken are thought to be the reservoir of ETEC and the zoonotic transmission of pathogen attains

from animal to man is thought to be occur. ETEC strains produce a cholera-like toxin called heat labile toxin (LT) and a second diarrhoeal toxin heat stable toxin (ST) (Karunasagar et al., 1999).

In the present experiment, there was no evidence of EHEC group in the sampling farms. It might be occurs due to the environmental variation of *E. coli* strain in the sampling area. Tave (1993) reported every organism requires a particular environment for growth and development. So, in this sense, the sampling area might be safer from the enterohemorrhagic *E. coli* strains; since *E. coli* frequently responses with the changing environment.

In the study of farms in hygienic condition category, only 1371 bp DNA band pattern (pho-A) was observed on electrophoresis gel in the isolates of SH_4 and SH_5 farms after first round (Pho-A) PCR test and no enterovirulent groups was evident after second round (all primers) PCR test. The results of PCR clearly indicate the potentiality of the PCR assay to identify and differentiate virulent *E. coli* strains in shrimp farm.

However considering higher count and prevalence of total coliforms and *E. coli* in almost all of the samples under investigation, it can be inferred that most of the shrimp farms are exposed to faecal contamination, indicating the contamination by other pathogens also and further suggested that these shrimps may contain bacterial toxin even after processing and may cause risk for health. The PCR test of pathogenic bacteria confirmed some pathogenic strain in the samples that show the unhygienic condition of the farms.

There is a substantial amount of information currently available on the advantages and disadvantages of total coliforms and other indicators of water quality (Gleeson and Gray, 1997; Ashbolt et al., 2001). It may be very difficult to get rid of these microorganisms from farm water as well as fish products and processing equipments when they originate from foodhandlers (humans) who handle the fish from capture to packaging of finished products. For a processor to maintain the number below or within exactly specified limits a GMP/GHP needs to be applied properly to get good results as well as the following suggestions may be made to prevent bacterial contamination of shrimps- (i) hygienic conditions for shrimp cultivation should be maintained, (ii) routine microbial analysis of feed is necessary before use, (iii) the feed manufacturer should maintain the microbial

quality, (iv) aseptic shrimp seed multiplication farm should be established, (v) water quality should be routinely tested and (vi) awareness should be developed among the personnel associated with this industry. This study will contribute to detect pathogenic bacteria in short time, so that one can understand the fact of bacteriological quality of fresh shrimps and shrimp farms, and to adopt strategies to improve the shrimp quality.

Chapter Six

CONCLUSION

6: CONCLUSION

In Bangladesh, shrimp cultivation and processing is one of the major sectors that earn a lot of foreign exchange every year and the sector is developing gradually. Of all the exportable agro-based primary commodities, shrimp is by far the most important. It alone contributes more than 70% to the total export earnings from all the agro-based products. There is ample demand in the international market for shrimp and Bangladesh is blessed with an environment congenial for shrimp production. However, the industry is fraught with many obstacles at present. Even export market of shrimp could be threatened if contaminated with pathogenic microbes such as *E. coli, Salmonella* sp., *Vibrio* sp. etc. In my present study, 31 shrimp farms were investigated and I observed that all shrimp farms were contaminated with coliform and around 55 % farms were contaminated with *E. coli*. 76 *E. coli* were also identified from shrimp farms using cultural, biochemical and PCR technique in which EPEC and ETEC groups were 22% and 29%, respectively whereas EHEC group was absent. In my study, it was also found that *E. coli* were present most of the hygienic and unhygienic farms but enterovirulent *E. coli* was present only the farm in unhygienic condition. This study revealed that 55% shrimp farms of Khulna district were contaminated by feces and most of the farms were vulnerable to contamination by harmful microorganisms and thereby unsafe for shrimp production. So the overall management system of shrimp culture must be developed immediately in order to avoid contamination of various pathogenic microbes in aquaculture farms as well as to increase the production performance and acceptability of our shrimp industry in the international market.

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APPENDICES

APPENDIX

Appendix-A: Composition of some of the media used in this course of work

Composition of MacConkey Agar Medium

Ingredient	Amount
Enzymatic Digest of Gelatin	17 g
Enzymatic Digest of Casein	1.5 g
Enzymatic Digest of Animal Tissue	1.5 g
Lactose	10 g
Bile Salts Mixture	1.5 g
Sodium Chloride	5 g
Neutral Red	0.03 g
Crystal Violet	0.001 g
Agar	13.5 g

Composition of Nutrient Broth Medium

Ingredient	Amount
Beef extract	3 g
Peptone	5 g
Sodium chloride	5 g
Distilled water	1000 ml

Composition of Nutrient Agar Medium

Ingredient	Amount
Beef extract	3 g
Peptone	5 g
Sodium chloride	5 g
Agar	18 g
Distilled water	1000 ml

Composition of Simmons Citrate Agar Medium

Source-Oxoid Ltd. Basingstoke, Hampshire, England

Ingredient	Amount
Ammonium di-hydrogen phosphate	1 g
Magnesium sulfate	0.2 g
Dipotassium phosphate	1 g
Sudium citrate	2 g
Sodium chloride	5 g
Bromothymol blue	0.08 g
Agar	18 g
Distilled water	1000 ml

Composition of MR-VP Broth Medium

Ingredient	Amount
Peptone	7 g
Dextrose	5 g
Potassium phosphate	5 g
Distilled water	1000 ml

Composition of Fermentation Broth Medium

Ingredient	Amount
Peptone	5 g
Beef extract	3 g
Carbohydrate	10 g
Bromothymol Blue	1.25 g
Distilled water	1000 ml

Composition of Eosin Methylene Blue Agar Media

Ingredient	Amount
Peptic digest of animal tissue	10 g
Dipotassium phosphate	2 g
Lactose	10 g
Eosin - Y	0.40 g
Methylene Blue	0.065 g
Agar	15 g
Distilled water	1000 ml

Biochemical test reagents

Barrit's reagent: For detection of acetylmethyl-carbinol

Solution A:

Ingredient	Amount
Alpha-napthol	5 g
Ethanol absolute	100 ml

Dissolve the alpha-napthol in the ethanol with constant stirring

Solution B:

Ingredient	Amount
Potassium hydroxide	40 g
Deionized water	100 ml

Dissolve the potassium hydroxide in 75 ml of distilled water. The solution will become warms. Allow cooling to room temperature and then add the remaining water.

Methyl Red Solution: For detection of acid

Ingredient	Amount
Methyl red	0.1 g
Ethyl alcohol (95 %)	300 ml
Distilled water	200 ml

Dissolve the methyl red in the 95% ethyl alcohol. Dilute to 500 ml with distilled water.

Appendix-B: DNA Extraction procedure

According to the Invitrogen manual for DNAzol Reagent, bacterial DNA was extracted from the cultured bacteria. DNAzoL Reagent is a complete and ready-to-use reagent for the isolation of genomic DNA isolation solid and liquid samples. The DNAzoL Reagent procedure is based on the use of a novel guanidine-detergent lysing solution which permits selective precipitation of DNA from a cell lysate. DNAzoL Reagent is an advanced DNA isolation reagent that combines both reliability and efficiency with simplicity of the isolation protocol. The DNAzoL Reagent protocol is fast and permits isolation of genomic DNA from a large number of samples of small or large volumes.

During the isolation, a biological sample is lysed (or homogenized) in DNAzoL Reagent and the genomic DNA is precipitated from the lysate with ethanol. Following an ethanol wash, DNA is solubilized in 8 mM NaOH. The procedure can be completed in about 30 min with DNA recovery of 70-100%. The isolated DNA can be used without additional purification for applications polymerase chain reaction (PCR). (Invitrogen, Life Technologies, USA; Ausubel et al., 1990; further optimized by Islam, 2006)

Stepes of DNA Extraction:

Summary of the following protocol:

1.	Lysis/Homogenization	1ml DNA_{ZOL} Reagent + 25-50 mg tissue, $1\text{-}3 \times 10^7$ cells, 0.1 ml liquid sample
2.	Centrifugation	10,000 RCF, 10 min
3	DNA Precipitation	Lysate + 0.5 ml 100% ethanol
4	DNA Wash	1 ml 75% ethanol
5	DNA Solubilization	100µl 8mM NaOH

Procedure

At the very beginning of extraction process, 2 ml (1 ml in each tube) cultured sample was centrifuged at 10000 rcf for 5 minutes with a centrifuge machine (Vision Micro High Speed Refrigerated Centrifuge, Model no: VS-15000 CFN II). At the end of centrifugation, clear supernatant layer was removed by a pipette (1000 µl) and deposited plate layer was kept for further procedure.

1. Lysis of cells: 500 µl DNAzol reagents were added to each tube of a sample pair. The plate was dissolved completely by gentle pipetting and the dissolved sample in pair tube was mixed up to keep into a single tube.

2. Centrifugation: The dissolved cells were centrifuged for 10 min at 10,000 rcf at 4°C or room temperature. Following centrifugation, the resulting viscous supernatant was carefully transferred to a fresh tube. This step removes insoluble tissue fragments, RNA, and excess polysaccharides from the lysate/homogenate. This process was performed in order to minimize RNA carry-over into the DNA.

3. DNA Precipitation: DNA from the lysate was precipitated by the addition of 0.5 ml of 100% ethanol per 1 ml of DNAzoL Reagent used for the isolation. Samples were mixed by inversion and store them at room temperature for 1-3 minutes. Then it was centrifuged for 10 minuts at 10000 rcf in cool temperature. DNA should quickly become visible as a cloudy precipitate. Carefully the supernatant was decanted leaving the DNA pellet near the top of the tube. The tubes were placed upright for 1 min and was aspirated the remaining lysate from the bottom of the tubes.

4. DNA Wash: The DNA precipitate was washed twice with 0.8-1.0 ml of 75% ethanol. At each wash, the DNA in ethanol suspended by inverting the tubes 3-6 times. The tubes were stored vertically for 0.5-1 min to allow the DNA to settle to the bottom of the tubes and remove ethanol by pipetting. At each wash, the DNA in ethanol was vortexed and was suspended by centrifugation for 10 minutes at 10000 rcf.

5. DNA Solubilization: After removing the ethanol, the DNA was air dried by storing in an open tube for 5-15 seconds. (If the DNA is exposed to air for more than a few seconds, it will be much more difficult to dissolve.). If the DNA was not properly dried, it was kept on a heatblock at 55°C for 5 minuts. The DNA was dissolved in 8 mM NaOH by slowly passing the pellet through a pipette tip. Use of the 8 mM NaOH was assured full solubilization of the DNA precipitate. 100 µl of 8 mM NaOH was added to the DNA precipitate to dissolve. Then it was centrifuged for 3 minutes at 10000 rcf.

Appendix C: Agarose gel electrophoresis determination of DNA integrity

Integrity of extracted DNA sample was studied by agarose gel electrophoresis as described by Sambrook et al. (1989). Briefly, electrophoresis was performed using a horizontal electrophoresis apparatus (Bioneer, Korea) at a constant volt of 100 with a 1.5% agarose gel containing 3 µl ethidium bromide in 150 ml 1x TAE buffer. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA size was determined by comparison with a DNA size marker (100 bp DNA ladder, Bioneer, Korea). DNA bands were visualized by UV illumination and photographed using the Gel Doc 1000 documentation system (Bio-Rad).

Materials

1. Electrophoresis buffer; TAE buffer (40 mM Tris- Acetate & 1mM EDTA, pH 8.0);
2. Agarose powder;
3. Ethidium bromide (NOTE: Ethidium bromide is a known mutagen and thus handled as a hazardous chemical — hand gloves were used while handling);
4. Gel loading buffer (BlueJuiceTM Gel Loading Buffer: 65% (w/v) sucrose, 10 mM Tris-HCI (pH 7.5), 10 mM EDTA, and 0.3% (w/v) bromophenol blue, Bioneer, Sheul, Korea);
5. DNA size marker;
6. An electrophoresis chamber, power supply, gel casting tray and sample combs (around which molten agarose is poured to form sample wells in the gel);
7. Electric balance (Mettler Toledo, B303-S, Germany);
8. Micro wave oven (Walton, Japan);
9. Digital camera (Canon G9, Japan); and
10. Conical flask, beaker, cylinder, parafilm, micro-pipette, micro tips and tissue paper.

Procedure

1. TAE buffer preparation: To prepare 1000 ml 1x TAE buffer solution from 50x TAE buffer 20ml TAE buffer and 980 ml distill water was mixed up to make 1000 ml 1x solution.
2. Agarose gel preparation
   a. Gel casting tray and samples comb were installed.

b. An amount of 2.25 g Agarose powder was weighted out using the electric balance and taken in a conical flask containing 150ml 1x TAE buffer, shake for a few times and then heated in a microwave oven for 2 minutes. To melt the powder properly, heated in the oven and shaking at a regular time (30s) interval.
c. The mixture was cooled to about 60°C by falling cool tap water on the wall of the conical flask with continuous jerking and by keeping at room temperature
d. 3 µl Ethidium bromide was added to the solution and mixed well;
e. The solution was poured into the gel casting tray containing sample comb and were allowed to solidified at room temperature; (Note: with decreasing temperature gel will be solidify rapidly thus step 1.4 to1.6 was done quickly without consuming high time.
f. After gel has solidified, the comb was removed, using care not to rip the bottom of the wells.
g. The gel could be stored at -4°C for further use.

3. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and was covered by adding approximately 450 ml TAE buffer;
4. 4 µl of the extracted DNA sample was mixed with 2 µl of loading buffer on a parafilm and were poured into a sample wells. By maintaining sample's serial number, this was done for every sample separately. To avoid cross-contamination, separate micro-tips were used for every sample.
5. Approximately 2 µl of colored DNA size marker was poured into first sample wells on gel ;
6. The electrophoresis chamber was covered with the lid;
7. The power supply was switched on, only volt was fixed at a constant value of 50, the machine was run until DNA migrated to the two-third of the gel towards the positive electrode (DNA was usually blue colored based on loading buffer);
8. After 25 to 30 minutes The power supply was switched off and the lid of the electrophoresis chamber was removed;
9. The gel, still in its plastic tray, was removed from the electrophoresis chamber and placed on an ultraviolet (UV) transilluminator.
10. The UV transilluminator was switched on, DNA banding pattern was visible;
11. The resulting banding pattern was captured by using the digital camera and software.

Appendix D: Biochemical characteristics of selected red colonies isolated from shrimp farms.

Farm no	strain no	Lactose fermentation test	Eosine Methylene Blue (EMB) test	Methyl Red (MR) test	Voges-proskauer reaction (VP) test	Citrate test
WH1	6/2/1	-	+	+	-	-
	6/2/2	-	-	-	-	-
	6/2/3	-	+	+	-	-
	6/2/4	-	+	+	-	-
	6/2/5	+	+	+	+	+
	6/2/6	-	+	+	-	-
WH2	7/1/1	-	-	+	+	+
	7/1/2	-	-	+	+	+
	7/1/3	+	-	+	-	-
	7/1/4	-	-	+	+	-
	7/1/5	-	-	+	+	+
	7/1/6	-	-	+	+	-
	7/1/7	-	-	+	-	-
	7/1/8	-	-	+	+	+
WH3	8/1	-	-	-	+	-
	8/2	-	-	+	-	-
	8/3	-	-	+	-	-
	8/4	-	-	-	+	+
	8/5	-	-	-	I	I
	8/6	-	-	+	-	-
	8/7	-	-	-	+	-
	8/8	-	-	-	+	+
	8/9	+	-	-	+	-
	8/10	-	-	+	-	-

Continued

WH4	9/1	-	-	+	-	-
	9/2	+	_	+	-	+
	9/3	_	_	+	_	+
	9/4	-	-	+	-	+
	9/5	-	-	+	-	-
	9/6	-	-	+	-	+
	9/7	-	-	+	-	-
	9/8	-	-	+	-	-
	9/9	-	-	+	-	-
	9/10	-	-	+	-	+
WH5	11/1	-	-	+	+	+
	11/2	-	-	-	+	-
	11/3	-	-	+	+	+
	11/4	-	-	-	+	+
	11/5	-	-	-	+	-
	11/6	-	-	+	-	+
	11/7	-	-	+	+	-
	11/8	-	-	-	+	-
	11/9	-	-	-	+	-
	11/10	-	-	-	+	-
WH6	12/1	-	-	-	+	+
	12/2	-	-	+	+	+
	12/3	-	-	-	+	+
	12/4	-	-	-	+	-
	12/5	-	-	-	+	+
	12/6	+	-	+	-	-
	12/7	-	-	+	-	+
	12/8	+	_	_	+	_

Continued

WU1	1/1/1	+	_	_	+	+
	1/1/2	_	_	_	+	+
	1/1/3	_	_	_	_	_
	1/1/4	_	_	_	+	+
	1/1/5	+	+	+	_	+
	1/1/6	-	+	+	-	-
	1/1/7	-	-	-	+	+
	1/1/8	-	-	-	-	-
	1/1/9	+	+	+	-	-
	1/1/10	_	_	_	+	+
WU2	2/2/1	-	-	-	+	+
	2/2/2	-	-	-	+	+
	2/2/3	-	-	-	+	+
	2/2/4	+	+	+	_	+
	2/2/5	-	-	-	+	+
	2/2/6	-	-	-	+	+
	2/2/8	-	-	-	+	+
	2/2/9	-	-	-	+	+
	2/2/10	-	-	-	+	+
WU3	3/1/1	-	-	-	+	-
	3/1/2	+	+	+	-	-
	3/1/3	_	+	+	_	_
	3/1/4	_	+	+	_	_
	3/1/5	+	+	+	_	_
	3/1/6	+	_	_	+	+
	3/1/7	+	+	+	-	-
	3/1/8	-	+	+	-	-
	3/1/9	+	-	+	-	-
	3/1/10	-	-	-	+	-
	3/1/11	+	-	+	-	+
	3/1/12	+	+	+	-	-

Continued

WU4	4/2/1	-	-	+	-	-
	4/2/2	+	-	-	+	+
	4/2/3	+	-	-	-	-
	4/2/4	+	+	+	-	-
	4/2/5	+	+	+	-	-
	4/2/6	+	+	+	-	-
	4/2/7	+	+	+	+	+
	4/2/8	+	+	+	-	-
	4/2/9	+	+	+	-	-
	4/2/10	+	-	-	-	-
	4/3/1	+	-	+	-	-
	4/3/2	-	-	+	-	-
	4/3/3	+	+	+	-	-
WU5	**5/2/1**	+	+	+	-	-
	5/2/3	-	-	-	+	-
	5/2/4	-	-	-	+	-
	5/2/5	+	+	+	-	+
	5/2/6	+	+	+	-	-
	5/2/7	-	-	-	+	-
	5/2/8	+	+	+	-	-
WU6	10/1	-	-	+	-	+
	10/2	-	+	+	-	-
	10/3	+	+	+	-	-
	10/4	+	+	+	-	+
	10/5	+	+	+	-	-
	10/6	+	+	+	-	-
	10/7	+	+	+	-	+
	10/8	+	+	+	-	+

Continued

WU7	**29/2/1**	+	+	+	-	-
	29/2/2	+	+	+	-	-
	29/2/3	+	+	+	-	-
	29/2/4	+	+	+	-	-
	29/2/5	+	+	+	-	-
	29/2/6	+	+	+	-	-
	29/2/7	+	+	+	-	+
	29/2/8	-	-	-	+	+
	29/2/9	-	-	-	+	+
	29/2/10	+	-	-	-	-
WU8	**30/2/1**	+	+	+	-	-
	30/2/2	+	+	+	-	-
	30/2/3	+	+	+	-	-
	30/2/4	+	+	+	-	-
	30/2/5	+	+	+	-	-
	30/2/6	-	-	-	+	+
	30/2/7	-	-	-	+	+
	30/2/8	+	+	+	-	-
	30/2/9	+	+	+	-	-
	30/2/10	+	+	+	-	-
WU9	**31/2/1**	+	+	+	-	-
	31/2/2	-	-	-	+	+
	31/2/3	+	+	+	-	-
	31/2/4	+	+	+	-	-
	31/2/5	+	-	-	+	+
	31/2/6	+	+	+	-	-
	31/2/7	+	+	+	-	-
	31/2/8	+	+	+	-	-
	31/2/9	+	+	+	-	-
	31/2/10	+	+	+	-	-
	31/2/11	-	-	-	+	+
	31/2/12	+	-	-	+	+

Continued

SH1	13/2/1	-	-	+	-	-
	13/2/2	-	-	-	+	+
	13/2/3	-	-	-	+	+
	13/2/4	-	-	+	+	–
	13/2/5	–	–	–	+	+
SH2	14/4/1	–	–	–	+	+
	14/4/2	–	–	–	+	+
	14/4/3	–	–	+	+	+
	14/4/4	–	–	–	+	+
	14/4/5	–	–	–	+	+
SH3	15/2/1	–	–	–	+	+
	15/2/2	–	–	–	+	+
	15/2/3	+	–	+	–	–
	15/2/4	–	–	–	+	+
	15/2/5	–	–	–	+	+
SH4	**20/3/1**	+	+	+	–	–
	20/3/2	–	–	–	+	+
	20/3/3	–	–	–	+	+
	20/3/4	–	–	–	–	–
	20/3/5	–	–	–	+	+
	20/3/6	+	+	+	–	–
	20/3/7	+	+	+	–	–
SH5	**21/3/1**	+	+	+	–	–
	21/3/2	+	+	+	–	–
	21/3/3	+	+	+	–	–
	21/3/4	–	–	–	–	–
	21/3/5	–	–	+	–	–
	21/3/6	–	–	–	+	+
	21/3/7	–	–	–	+	+

Continued

SH6	23/ 2/1	+	–	—	+	+
	23/ 2/2	–	–	+	+	+
	23/ 2/3	–	–	–	+	+
	23/ 2/4	+	—	–	+	+
	23/ 2/5	–	–	–	+	+
SH7	24/2/1	+	–	+	+	–
	24/2/2	–	–	–	+	+
	24/2/3	–	+	–	+	+
	24/2/4	–	–	–	+	+
	24/2/5	–	–	–	+	+
	24/2/6	–	–	–	–	–
	24/2/7	+	–	+	–	–
SH8	25/3/1	+	–	–	+	+
	25/3/2	–	–	–	+	+
	25/3/3	–	–	–	–	–
	25/3/4	–	–	–	+	+
	25/3/5	–	–	–	+	+
	25/3/6	–	–	–	+	+
	25/3/7	+	–	+	+	–
SU1	16/2/1	–	+	+	–	–
	16/2/2	–	+	+	–	–
	16/2/3	+	–	–	–	–
	16/2/4	–	–	–	–	–
	16/2/5	–	–	+	–	–
	16/2/6	+	+	+	–	–
	16/2/8	+	+	+	–	–
	16/2/9	+	+	+	–	–
	16/2/10	–	–	+	–	–

Continued

SU2	**17/3/1**	+	+	+	–	–
	17/3/2	+	+	+	–	–
	17/3/3	+	+	+	–	–
	17/3/4	+	+	+	–	–
	17/3/5	+	+	+	–	–
	17/3/6	–	–	–	+	+
	17/3/7	+	–	–	+	+
SU3	18/2/1	–	–	+	+	+
	18/2/2	–	–	–	+	+
	18/2/3	-	–	–	-	-
	18/2/4	+	–	+	+	-
	18/2/5	–	–	–	+	+
SU4	**19/3/1**	+	+	+	–	–
	19/3/2	+	+	+	–	–
	19/3/3	+	+	+	–	–
	19/3/4	+	+	+	–	–
	19/3/5	–	–	–	+	+
	19/3/6	–	–	+	+	+
	19/3/7	+	–	–	+	+
SU5	22/3/1	+	–	+	–	–
	22/3/2	–	–	–	+	+
	22/3/3	–	–	–	+	+
	22/3/4	–	–	–	+	+
	22/3/5	–	–	+	+	+
	22/3/6	+	+	+	–	–
	22/3/7	+	+	+	–	–
	22/3/8	–	–	–	+	+
	22/3/9	–	–	–	+	+

Continued

SU6	**26/2/1**	+	+	+	-	-
	26/2/2	–	–	–	+	+
	26/2/3	+	+	+	-	-
	26/2/4	+	+	+	-	-
	26/2/5	–	–	–	+	+
	26/2/6	+	+	+	-	-
	26/2/7	+	+	+	-	-
	26/2/8	–	–	–	+	+
	26/2/9	+	–	–	+	+
SU7	**27/2/1**	+	+	+	-	-
	27/2/2	–	–	–	+	+
	27/2/3	–	–	–	+	+
	27/2/4	+	+	+	-	-
	27/2/5	+	+	+	-	-
	27/2/6	+	+	+	-	-
	27/2/7	+	+	+	-	-
	27/2/8	–	–	–	+	+
	27/2/9	+	–	–	+	+
	27/2/10	–	–	–	+	+
SU8	**28/2/1**	+	+	+	–	–
	28/2/2	+	+	+	–	–
	28/2/3	+	+	+	–	–
	28/2/4	+	+	+	–	–
	28/2/5	–	–	–	–	–
	28/2/6	+	+	+	–	–
	28/2/7	–	–	–	+	+
	28/2/8	–	–	–	+	+
	28/2/9	+	+	+	–	–

'+' sign indicates positive result, '-' sign indicates negative result.

Appendix E: PCR results of isolated *E. coli*

Group →		House Keeping gene	ETEC			EHEC	EPEC
target genes →		PhoA	ST1	LT1	LT2	VT	EAE
Farm no	Isolate						
WH_1	0						
WH_2	0						
WH_3	0						
WH_4	0						
WH_5	0						
WH_6	0						
WU_1	1/1/9	+	–	–	–	–	+
WU_2	0						
WU_3	3/1/2	+	+	–	–	–	–
	3/1/5	+	–	–	–	–	–
	3/1/7	+	–	–	–	–	–
	3/1/12	+	+	–	–	–	–
WU_4	4/2/4	+	–	–	–	–	–
	4/2/5	+	–	–	–	–	–
	4/2/6	+	+	–	–	–	–
	4/2/8	+	+	–	–	–	–
	4/2/9	+	–	–	–	–	+
	4/3/3	+	–	–	–	–	–
WU_5	5/2/1	+	–	–	+	–	–
	5/2/6	+	–	–	+	–	–
	5/2/8	+	+	–	–	–	–
WU_6	10/3	+	–	–	–	–	+
	10/4	+	–	–	–	–	–
	10/6	+	–	–	–	–	+

Continued

Group ⟶		House keeping gene	ETEC			EHEC	EPEC
Target genes ⟶		PhoA	ST1	LT1	LT2	VT	EAE
Farm no	Isolate						
WU_7	29/2/1	+	–	–	–	–	–
	29/2/2	+	–	–	–	–	+
	29/2/3	+	–	–	+	–	–
	29/2/4	+	–	–	–	–	+
	29/2/5	+	–	–	–	–	+
	29/3/6	+	–	–	–	–	+
	29/3/7	+	–	–	–	–	–
WU_8	30/2/1	+	+	–	–	–	–
	30/2/2	+	–	–	–	–	–
	30/2/3	+	+	–	–	–	–
	30/2/4	+	–	–	–	–	+
	30/2/5	+	–	–	–	–	–
	30/2/8	+	–	–	–	–	–
	30/2/9	+	+	–	–	–	–
	30/2/10	+	–	–	–	–	–
WU_9	31/2/1	+	–	–	–	–	–
	31/2/3	+	–	–	–	–	–
	31/2/4	+	–	–	–	–	–
	31/2/6	+	–	–	–	–	+
	31/2/7	+	–	–	–	–	+
	31/2/8	+	–	–	–	–	–
	31/2/9	+	–	–	–	–	–
	31/2/10	+	–	–	–	–	–

Continued

Group ⟶		House keeping gene	ETEC			EHEC	EPEC
Target genes ⟶		PhoA	ST1	LT1	LT2	VT	EAE
Farm no	Isolate						
SH_1	0						
SH_2	0						
SH_3	0						
SH_4	20/3/1	+	–	–	–	–	–
	20/3/6	+	–	–	–	–	–
	20/3/7	+	–	–	–	–	–
SH_5	21/3/1	+	–	–	–	–	–
	21/3/2	+	–	–	–	–	–
	21/3/3	+	–	–	–	–	–
SH_6	0						
SH_7	0						
SH_8	0						
SU_1	16/2/1	+	+	–	–	–	–
	16/2/8	+	–	–	–	–	+
	16/2/9	+	–	–	–	–	–
SU_2	17/3/1	+	–	–	–	–	–
	17/3/2	+	–	–	–	–	+
	17/3/3	+	–	–	–	–	–
	17/3/4	+	–	–	–	–	–
	17/3/5	+	+	–	–	–	–
SU_3	0						

Continued

Group →		House Keeping gene	ETEC			EHEC	EPEC
Target gene →		PhoA	ST1	LT1	LT2	VT	EAE
Farm	Isolate						
SU_4	19/3/1	+	–	–	–	–	+
	19/3/2	+	–	–	–	–	–
	19/3/3	+	–	–	–	–	–
	19/3/4	+	–	–	–	–	–
SU_5	22/3/6	+	–	–	+	–	–
	22/3/7	+	–	–	–	–	–
SU_6	26/2/1	+	+	–	–	–	–
	26/2/3	+	+	–	–	–	–
	26/2/4	+	+	–	–	–	–
	26/2/6	+	+	–	–	–	–
	26/2/7	+	–	–	–	–	+
SU_7	27/2/1	+	+	–	–	–	–
	27/2/2	+	–	–	–	–	–
	27/2/3	+	–	–	–	–	–
	27/2/4	+	–	–	–	–	–
	27/2/5	+	–	–	–	–	+
SU_8	28/2/1	+	–	–	–	–	+
	28/2/2	+	–	–	–	–	–
	28/2/3	+	+	–	–	–	–
	28/2/4	+	+	–	–	–	–
	28/2/6	+	–	–	–	–	–
	28/2/9	+	+	–	–	–	–