CHAPTER 5: DISCUSSION

5.1 Isolation and identification of *E. coli*

In the isolation of *E. coli*, the selective medium, Chromocult Coliform Agar (CCA) used was not reliable. Overgrowth of competitive microorganisms was observed which could be due to the lack of specificity of the culture medium. However, CCA was reported to be efficient for *E. coli* detection by Byamukama *et al*. (2000).

In this study, EMB medium has been used to further confirm the *E. coli* isolates due to inefficiency of the CCA. EMB agar eliminate the growth of *Enterobacter*, *Klebsiella* and it provides favorable conditions for growth of *E. coli* and improves its proliferation in particular compared to other lactose-positive bacteria. *E. coli* produces large amounts of acid from lactose and gave very dark, metallic sheen colonies on EMB agar, which helped to discriminate *E. coli* from the accompanying *Klebsiella* and *Enterobacter*. The growth of other Gram-positive microorganisms was inhibited by the dyes (eosin and methylene blue) contained in EMB agar. As in Chromocult coliform, since it allows the growth of other coliforms as well the tendency for false positive results was high.

High positive correlation between total coliform and *E. coli* counts were obtained, which suggest that total coliform is a reliable water quality indicator in this study. Identification of total and fecal coliforms is practical and relatively inexpensive, and therefore they serve as useful risk indicators that fecal waste and associated pathogens

may be present (Hoyer *et al*., 2006). Hoyer *et al*. (2006) also noted that total coliform may not be the best indicator of fecal contamination because total coliform testing can sometimes result in high counts from naturally occurring, nonfecal coliform bacteria found in association with soil, submersed aquatic plants and water. Likewise, fecal coliform testing can be confounded by false positives that come from nonfecal bacteria (such as *Klebsiella* spp.) that are also found in soil and water.

In the biochemical tests, two isolates out of 29 isolated *E. coli* showed positive results for citrate utilization. Ishiguro and Sato (1979) also reported isolates of citrate positive variants of *E. coli* from man and domestic animals which otherwise were typical *E. coli* in their biochemical characteristics. Besides that, Lee and Choi (1983) reported citrate utilizing variants of *E. coli* from fecal samples. Dubey *et al.* (2001) observed that *E. coli* strains isolated from diarrheic goats failed to utilize citrate in Simmon's medium but were positive for using citrate as a sole carbon source when tested in Christensen's citrate agar.

Besides that, false positive results were obtained for detection of *E. coli* in biochemical tests due to the presents of high number of *Klebsiella* in some samples. *Klebsiella* which is also a member of coliform group shares similar morphological and biochemical characteristics with *E. coli* and other coliforms. Thus in this study, the monoplex PCR method had to be conducted to reconfirm the identification of the *E. coli* isolates. According to API 20E results, most of the *Klebsiella* detected were *Klebsiella pneumoniae*, which is one of the common gram-negative bacteria found in environment and in mammalian mucosal surfaces. Pneumonias that are caused by

Klebsiella pneumoniae are difficult to control and mortality rates have even been reported as up to 50% after antibiotic treatment (Straus, 1986).

5.2 Survival of *E. coli* and total coliform at different holding time

The comparison of colony count of total coliform and *E. coli* at different time showed an obvious drop of microbial population in samples analyzed after 1 week of collection. Thus, it is proven that analysis of biological parameters must be initiated within their specific holding times. According to the 16th Edition of Standard Methods (1985) bacterial testing should begin as soon as possible to avoid unpredicted changes in the microbial population. Guardabassi *et al*. (2002) indicated that bacteria density will drop as the environmental sample is held prior to its analysis. Thus, the magnitude of this effect can be minimized by a proper storage of the samples and fast analysis procedures. The time and conditions of sample storage between sampling and analysis period is very important. The goal of sample preservation is to maintain sample integrity between collection and analysis and should limit biological, chemical and physical changes to the sample. Containers and packing should be chosen to protect the samples from cross contamination, breakage or leakage. Storage time should be considered as a whole, both during transport and in the laboratory, so that the analysis is carried out before the sample is beyond its shelf life.

In general, refrigeration will reduce biological activity and moisture will induce microbial activity unless the temperature is very low. Refrigeration will also results in losses of volatile materials and generally slow down some chemical reactions

depending on the system being considered. These factors may also influence the survival of microbiological activity at these temperatures. Sinton *et al*. (2000) stated that *E. coli* in water is very sensitive to sunlight inactivation, but the effects can be reduced by using containers with suitable light filtration properties.

Another reason for the drop in colony count of isolates could be due to the frequent process of freezing and thawing. Parker and Martell (2002) reviewed survival of microorganisms under freezing conditions. Bacteria can be injured or die as a result of rapid chilling without freezing, freezing, storage at low or sub-zero temperatures, and subsequent warming. For bacteria that have been frozen and thawed, both the rates of cooling and warming affect survival.

In this study, *E. coli* was not found in most of the coastal water samples. The survival of cells of *E. coli* in sea water is low, indicating that predators and competitors organisms found in sea water contribute to the rapid death of the bacterium (Carlucci *et al*., 1960). Many factors affect the survival times of *E. coli* in water as discussed above, with much shorter survival times in seawater than in freshwater (Fujioka *et al*., 1981).

5.3 PCR confirmation of *E. coli* isolates

In the monoplex PCR assay, unspecific bands and intense primer dimer were observed even though the conditions used were well optimized by a previous student. The failure to reproduce identical results despite using similar reagent concentrations and amplification conditions maybe due to the different brands of PCR reagents used. Thus, modification of primer concentration was done as high concentration of primer leads to unspecific bands.

The use of higher concentrations of primers in PCR assays does not improve the amplification of the desired PCR product but raising their concentration only results in the creation of primer dimers or will lead to non-specific primer binding and the creation of spurious, undesirable PCR products. Raising the primer concentration does not therefore cause an increase in the effective concentration of the primers. Low primer concentration generally ensures cleaner product and lower background. However, to amplify short PCR target sequences, careful calculation of the optimum primer concentration is required. In order to generate the required number of PCR product molecules, a greater number of primers may be needed. Therefore, concentration of primers higher than 1µM may be necessary, and desirable, for short target sequences.

5.4 Prevalence of virulence genes

The presence of ETEC strain in one of the sample in this study is of public health interest. ETEC belongs to a heterogeneous family of lactose fermenting *E. coli*, belonging to a wide variety of O antigenic types with colonization factors which allow the organism to readily colonize the small intestine and cause diarrhea (Qadri *et al*., 2005). The diseases caused by ETEC vary from minor discomfort to a severe cholera-like syndrome. ETEC are acquired by ingestion of contaminated food and water, and adults in endemic areas evidently develop immunity.

Qadri *et al*. (2005) has reported that ETEC is a major cause of diarrheal disease where drinking water and sanitation are inadequate and transmission may occur while bathing or using water for food preparation. Also stated that these forms of transmission are common in area where it is endemic both in local population and in international travelers to these areas. In the present study ETEC was detected in sample collected near jetty, the residence of foreign workers with poor sanitary management which agreed to Qadri's findings. Recently, Alhaj and coworkers (2007) developed a multiplex PCR for the simultaneous detection of diarrheagenic *E. coli* belonging to STEC or EPEC.

Overall, out of 50 samples, only 29 samples were positive for presence of *E.* coli and only 1 isolate was pathogenic. Thus, it is important to understand the dynamics of *E. coli* in aquatic systems. Fecal coliforms are a broad category of bacteria that are present in the intestinal tracts of humans and other warm-blooded animals. Of these

fecal coliforms, *E. coli* is probably the most widely known. Human feces may consist of as much as 5-50% of these bacteria (Laws, 1993). In waters contaminated with human feces, the proportion of enteropathogenic *E. coli* is probably less than 1% and the percentage of persons excreting enteropathogenic *E. coli* is no more than 1-10% (Geldreich, 1972). Therefore, the high counts of fecal coliforms and/or *E. coli* that may be observed within surface waters are not necessarily pathogenic strains, but show the potential for the presence of pathogenic strains. With the fact that fecal coliform bacteria including *E. coli* are relatively short lived and rarely reproduce in most aquatic systems, the fecal coliform bacteria levels within surface waters fall relatively quickly. Thus, pathogenic bacteria may be found in the environment, but with much less frequency than the contaminants and opportunistic pathogens.

5.5 Antimicrobial Susceptibility test

On the basis of antibiotic susceptibility, only the ETEC strain was multiple resistant to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole. Multi drug resistance is increasing in ETEC due to widespread use of chemotherapeutic agents (Quadri *et al*., 2005). Sayah *et al*. (2005) reported that *E. coli* isolates from domestic species were resistant to larger number of antimicrobial agents compared with isolates from human excretions and surface water. Livestock operations and human seepage is the main source for the contamination of surface water with resistant bacteria (Sayah *et al*., 2005). Another study found that livestock contributed more than humans to fecal coliform contamination of surface water and that reducing livestock access to surface water reduced the fecal coliform levels by an average of 94% (Hagedorn *et al*., 1999).

However, in this study antimicrobial resistance pattern was not a useful marker as most of the strains were sensitive to the tested antibiotics. This could be due to the geographical location of the samples where no livestock farms were found nearby the sampling sites.

5.6 Genomic diversity among isolates based on REP-PCR

As stated by others (Cornuet *et al*., 1999 and Whittam *et al*., 1996) the *E .coli* isolates in this study were highly diverse. Anderson *et al*. (2006) noted that the unstable and diverse nature of *E. coli* populations observed is due to the survival rates of *E. coli* strains in environmental waters which can differ significantly according to strain, where some exhibit prolonged persistence in the culturable state in environmental waters. With regard to microbial source tracking methods, the dependence of *E. coli* population diversity on the host species, the great sampling effort required to obtain a representative sample in some host species. The temporal variability observed in all host types, and the lack of host specificity suggest that library-based microbial source tracking methods utilizing *E. coli* experience major logistical limitations (Anderson *et al*., 2006).

In this study, the results of REP-PCR analysis have proven that REP-PCR fingerprinting is a promising molecular typing tool for *E. coli*. Analysis of the dendrogram shows that the REP-PCR system successfully distinguished among the isolates classifications. REP-PCR DNA fingerprint analysis was also useful for differentiating between *E. coli* isolates obtained from different sources as most of the

clinical isolates and food isolates were separated in their own clusters. Since genotypic analyses are less subject to environmental effects than phenotypic analyses, REP-PCR may be a method of choice for differentiating and grouping *E. coli* isolates from human food and environment. According to Hahm *et al*. (2003), REP-PCR based fingerprintings methods are relatively easy and convenient compared to AFLP or PFGE. As it is true for all PCR based methods, optimal conditions must be used to ensure reproducibility of data (Tyler *et al*., 1997).