COMPARISON OF BACTERIOLOGICAL METHODS FOR DETECTING TOTAL COLIFORMS AND *ESCHERICHIA COLI* IN WATER

A THESIS PRESENTED BY

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DECLARATION

This thesis contains an account of my own work performed at the Department of Botany, Faculty of Science, University of Peradeniya, under the supervision of Dr. (Mrs.) C.L. Abayasekara, Dr. G.B.B. Herath and Dr. D.R.I.B. Werellagama. It describes the results of my own independent research except where due references have been made in the text. No part of this thesis has been submitted previously in candidature for a degree of this or any other University.

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Bacteriological analysis of water, using indicator bacteria is a routine practice around the world to assure the microbiological quality of water. The current study investigated the performance of four alternative methods compared with the reference Sri Lanka Standard-Multiple Tube Fermentation (SLS-MTF) method, for the detection and enumeration of total coliforms and *Escherichia coli* in different water sources (tap water, bottled water, well water, surface water and effluent water-to cover a wide range of contamination levels), collected from different geographical areas. The four alternative methods were American Public Health Association (APHA)-MTF method, Colilert-MTF method, Sri Lanka Standard -Membrane Filtration (SLS-MF) method and m-ColiBlue24-MF method. Colilert and m-ColiBlue24, which are enzymatic methods, detect total coliforms and *E. coli* simultaneously, by the activity of enzymes β-D galactosidase (in total coliforms) and β-D glucuronidase (in *E. coli*), while the other methods are based on lactose fermentation.

Variance analysis results revealed that Colilert, m-ColiBlue24 and SLS-MF methods detected significantly higher ($p \leq 0.05$) total coliform counts, compared to the SLS-MTF method. In *E. coli* detection, Colilert ($p \leq 0.05$) and m-ColiBlue24 ($p \leq 0.1$) methods detected significantly higher counts compared to SLS-MTF method. Simple Linear Model showed, the three alternative methods detected several folds higher total coliforms counts than SLS-MTF method (3.8, 1.75 and 1.55 times higher counts were detected by Colilert, m-ColiBlue24 and SLS-MF methods respectively). For *E. coli*, 2.93, 1.83 and 1.35 times higher counts (than SLS-MTF) were detected by m-ColiBlue24, Colilert and SLS-MF methods respectively. ISO performance criteria (sensitivity, specificity, efficiency, false positive and false negative ratios), showed the two enzymatic and SLS-MF methods were superior to the conventional SLS-MTF method. Method performances by paired count evaluations showed inconclusive results due to inadequate valid data resulted by contaminations and missing data during subculturing.

Confirmational rates obtained for both bacterial types were higher in three alternative methods, than SLS-MTF method. Values being: for coliforms, Colilert (78.2 %), SLS-MF (75.2 %), m-ColiBlue24 (72.1 %) and SLS-MTF (71 %) and for *E. coli,* Colilert (66.6 %), m-ColiBlue24 (50 %), SLS-MF (50 %) and SLS-MTF (37.5 %).

Cost comparison showed that MTF methods (SLS, APHA and Colilert) were more expensive compared to MF, for drinking water analysis. The most economical method for drinking water analysis was SLS-MF method (6.7 times cheaper than SLS-MTF); followed by m-ColiBlue24 (2.1 times cheaper). For surface water analysis SLS-MF method was the cheapest (4.3 times cheaper), followed by the Colilert method (2.1 times cheaper) than the SLS-MTF method.

Conventional MTF methods (SLS and APHA) showed several drawbacks such as, need for longer incubational periods, confirmational tests, more labour and subjective nature of results interpretation. In comparison, Colilert was very efficient in all aspects. Among MF methods, m-ColiBlue24 was more efficient than the conventional SLS-MF method with the advantages of simultaneous detection of both types of bacteria, absence of heavy background growth or atypical colony formation, easy preparations and easy result interpretations, less time and labour requirement, etc.

Bacteriological identifications revealed 60 % identifications of non-coliforms by the SLS-MTF method. In contrast, Colilert, m-ColiBlue24 and SLS-MF methods identified more than 60 % of coliform bacteria. Identifications showed that, even the drinking water sources tested were contaminated with fecal (pathogenic) organisms, suggesting threats on drinking water quality in Sri Lanka.

In conclusion, results of the current study revealed that the conventional SLS-MTF method is less efficient, compared to the Colilert, m-ColiBlue24 and SLS-MF methods. SLS-MF method was the most economical method for analyzing both drinking and surface water samples. m-ColiBlue24 and Colilert methods, with their superior performance could be recommended as alternative methods for analyzing drinking water and surface water samples respectively, when cost is not the limiting factor.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. INTRODUCTION

Water can be considered the foodstuff consumed in the greatest quantity around the world. Therefore, it comes as no surprise that the health risks associated with consumption of contaminated water are of great interest (Eckner, 1998). Many of the water borne infectious diseases are caused due to pathogenic bacteria excreted by people suffering from or carrying the disease (Tebutt, 1983). Among the pathogens disseminated in water sources, enteric pathogens are the most frequently encountered (Rompre *et al.*, 2002). Some of them lead to severe and sometimes life-threatening infections such as typhoid, cholera, hepatitis caused by hepatitis A virus and diseases caused by *Shigella* species and *Escherichia coli* O157:H7. Others are typically associated with less severe outcomes, such as diarrhoeal diseases (WHO, 2008). As a consequence, sources of fecal pollution in waters devoted to human activities must be strictly controlled (Rompre *et al.*, 2002).

In addition, one of the most important assignments, from a public health perspective, in water safety assurance is to assess that effective processing steps have been followed to assure management of waterborne disease outbreaks with a microbial etiology. Therefore, assessing microbial contamination in a water supply, whether distributed by pipeline or bottled, constitutes a major commitment of public health professionals. The success of such effort is demonstrated by the virtual disappearance of waterborne disease outbreaks such as cholera and typhoid fever that were once a menace to the public. Worldwide regulations have been promulgated that ensure the implementation of natural and artificial barriers to control waterborne pathogens and monitoring systems for assessing the efficacy of the adopted intervention strategy rapidly and reliably (Leclerc *et al.*, 2001).

As Tebutt (1983) suggested, detection of the specific pathogenic bacteria excreted by infected people, is not easy since it is necessary to utilize its characteristic behavior by supplying special selective media and/or incubation conditions appropriately. Further, as

Gerba *et al.* (2000) explain, this is often a tedious, difficult and time-consuming task. Therefore, rather than test specifically for individual pathogens, regulatory agencies and treatment facilities test for 'indicator organisms', whose presence suggests the possibility of contamination with human feces (Covert *et al.*, 1989). These indicator organisms are normally present in high numbers in the feces of humans and other warm blooded animals. Their occurrence at specified levels in drinking water points to inadequate decontamination of water or its recontamination (Mossel, 1982). It is due to loss of disinfectant, break-through, intrusion of contaminated water into the potable water supply or regrowth problems in the distribution systems (Rompre *et al.*, 2002). The most widely used of these indicators are 'coliform bacteria' in general (Covert *et al*, 1989), 'fecal coliforms', (Lewis and Mak 1989; APHA, *et al.,* 1998; Doyle and Erickson, 2006) and more specifically the homiotherm enteric bacterium, *Escherichia coli* (Fricker and Fricker, 1996; Niemi *et al.*, 2001; Rompre *et al.*, 2002).

The concept of indicator bacteria for monitoring bacteriological quality of water has a century old history (Eckner, 1998; Niemi *et al.*, 2001; NRC, 2004), and later it was authenticated by the worlds leading regulatory agencies such as World Health Organization (WHO), United States Environmental Protection Agency (US EPA), European Union Drinking Water Directive (EU DWD), etc., for drinking water monitoring. Further, the use of coliforms was later expanded and adopted for ambient, recreational, and shellfish waters and continues to focus on identification of fecal contamination in water (NRC, 2004). Regulatory agencies of individual countries have the authority for regularizing the standards for water quality assessment for each country. In Sri Lanka, bacteriological quality of water is detected according to the recommendations published by the Sri Lanka Standards Bureau, under the guidance of the WHO (SLS 614: Part 2: 1983). The use of coliforms was later expanded and adopted for ambient, recreational, and shellfish waters and continues to focus on identification of fecal contamination in water (NRC, 2004). Wastewater treatment facilities around the world also monitor their effluents to ensure that their disinfection procedure is effective and the treated wastewater is pathogen free before releasing into the receiving waters.

1.1. Coliform bacteria as indicators of water quality

1.1.1. Application of indicator bacteria concept for water quality monitoring

The first epidemiological outbreak causing cholera in London, England, was found to be due to consumption of sewage contaminated well water (John Snow, 1854). Leclerc *et al.* (2001), reports on similar outbreaks due to contaminated drinking water throughout the world: Sweden (in 1966); USA (in 1968 and 1975); Japan (in 1977), leading to investigate on causative pathogenic agents. However, some characteristic features of pathogens, such as excretion in low numbers, low survival rates in the environment, discrete dispersion in water, clumped nature, etc., have made them difficult to culture in artificial media (WHO, 2008). As a result, an alternative approach was required to detect pathogenic contamination in water. This was the main reason of introducing indicator bacteria concept for microbiological water quality assessment. As Bonde (1966) has suggested, an ideal indicator must possess some specific criteria such as, simultaneous presence in higher concentrations; higher resistance to disinfectants and to the aqueous environment; growth performance in simple media; characteristic and simple reactions for unambiguous identification; random distribution in the sample; independent growth with other organisms; specificity to desired target organism; precision and adequate sensitivity. Coliforms have been identified as more reliable indicator organisms in this regard, since they show many of the above criteria (NRC, 2004).

According to Leclerc *et al.* (2001), the first routine examination of water has been reported as early as 1885, and for the first time, the bacterial indicator concept has been introduced in 1891 in London, England. The term coliform or *coli-aerogenes* bacteria to designate the indicators of fecally contaminated water have been first used by British bacteriologists (Lewis, 1917). Since then, terms commonly and indiscriminately used were coliforms, colon group, *Escherichia-Aerobacter* group or *coli-aerogenes* group. Later, thermotolerant or fecal coliforms, in addition to *E. coli*, have been differentiated from total coliforms, as more specific indicators of fecal pollution (Leclerc *et al*, 2001). The use of the coliform group and more specifically *E. coli* as indicators of microbiological water quality dates from their first isolation from feces at the end of the

19th century (Rompre *et al.*, 2002). For more than 100 years, U.S. public health personnel have relied extensively on an indicator organism approach to assess the microbiological quality of drinking water (NRC, 2004). In 1914, the U.S. Public Health Service has adopted the enumeration of coliforms as a more convenient standard of sanitary significance (Feng *et al.*, 2002).

Although coliform is not a taxonomic classification, the term is used to describe a group of bacteria showing common biochemical characteristics. Therefore, most definitions of coliforms are essentially based on biochemical characterization. According to Standard methods for the examination of water and wastewater (Part 9221 and 9222; APHA *et al*, 2005), coliform group members are described as;

1. all aerobic and facultative anaerobic, Gram negative, non- spore forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35 °C (multiple-tube fermentation technique; Section 3.1). or
2. all aerobic and many facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that develop a red colony with a metallic sheen within 24 hours at 35 °C on an Endo-type medium containing lactose (membrane filter technique; Section 3.2).

The definitions of members of the coliform group have recently been extended to include other characteristics, such as β-galactosidase-positive reactions (Part 9223; APHA *et al.*, 1998) (enzyme substrate test, Section 4.2). According to Rompre *et al.* (2002), the definition of coliform bacteria differs slightly on the country or on the organization in charge of the microbiological monitoring regulations. As he has stated, in Canada, the definition is the same as in the US, and differs in some European counties. Eg: the French Standardization Association (NFT90-413 and NFT90-414; AFNOR (1990), in Rompre *et al.*, 2002). AFNOR (Association De France Normalization) describes total coliforms as; rod-shaped, non-spore forming, Gram negative, oxidase-negtive, aerobic or facutatively anaerobic bacteria that are able to grow in the presence of bile salts or other replacement surface active agents having an analogous growth inhibitory effect and that ferment lactose with gas and acid (or

aldehyde) formation with 48 hours at 37 ± 1 °C. Further, thermotolent coliforms have the same fermentation properties as total coliforms but at a temperature of 44 ± 0.5 °C and *E. coli* as a thermotolerant coliform which produces indole from tryptophane at a temperature of 44 ± 0.5 °C, giving a positive methyl red test and unable to produce acetyl-methyl carbinol and not using citrate as the sole carbon source.

These coliform bacteria are included in the family Enterobacteriaceae, which consists of thirty genera and one hundred and fifteen species and sub species (Holt *et al.*, 2000).

1.1.2. Family Enterobacteriaceae

According to the 9th edition of Bergey's Manual of Determinative Bacteriology (2000), these bacteria are Gram-negative straight rods, which are motile by peritrichous flagella. Enterobacteriaceae are facultatively anaerobic and chemoorganotrophic, having both a respiratory and a fermentative type of metabolism. Most species grow well at 37 °C; while some species grow better at 25-30 °C and are often more active metabolically at these temperatures. D-glucose and other carbohydrates are catabolized with the production of acid and gas. Enterobacteriaceae are oxidase negative and catalase positive and reduce nitrates. They are distributed worldwide and can be found in soil, water, fruits, vegetables, grains, flowering plants and trees, and animals ranging from worms and insects to humans. There is substantial heterogeneity in ecology, host range and pathogenic potential for humans and animals, insects and plants. A number of species cause diarrheal diseases including typhoid fever and bacillary dysentery. Many species not normally associated with diarrheal disease are often referred to as opportunistic pathogens. Most of these, as well as the species causing diarrheal disease, can cause a variety of extra intestinal infections including bacteremia, meningitis and urinary tract, respiratory, and wound infections. Enterobacteriaceae are responsible for about 50 % of nosocomial infections, most frequently caused by *E. coli, Klebsiella* sp, *Enterobacter* sp, *Proteus* sp, *Providencia* sp, and *Serratia marcescens*. However, differentiation of genera in Enterobacteriaceae could not be easily depicted using a single table, since large number of biochemical tests are involved in the identification of more than 115 named species and subspecies (Holt *et al.*, 2000). If an organism is not identified with certainty,

additional tests are available to differentiate among subspecies and species within a given genus, or genera. Appropriate identification can be performed with miniaturized, multi-biochemical reactions such as api 20 E, since they allow a reliable differentiation between the various members of the coliform group (Willcox *et al.*, 1980).

1.1.3. Coliforms of intestinal or fecal origin

The gastrointestinal tract of humans and animals is colonized by complex microbial populations, referred to as microbiota (Leclerc *et al.*, 2001). The composition of the normal microbiota of the gastrointestinal tract of human and animals is a climax community (Anon, 1976). In man, the numbers of intestinal bacterial cells when counted microscopically is constantly in excess of 1011 per ml, containing up to 500 different strains. By using strictly anaerobic techniques and appropriate media, about 60–90% of the intestinal bacterial cells have been isolated in pure culture (Conway, 1997). By using more advanced detection and enumeration techniques such as molecular phylogenetic methods, it has been found that the human microbiota show a characteristic association compared to other animals (Leclerc *et al.*, 2001). Studies conducted by Finegold in 1983, on the composition of human colon flora, have illustrated that the bacteria isolated from lumen contents were predominantly of obligate anaerobic bacteria (belonging to the taxa *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Lactobacillus*, and *Clostridium*) and facultative bacteria, including *Enterococcus* and *E. coli*. This study has also reported that, coliforms are the most common of the Gram-negative facultative anaerobes found in human stools. The commensal *E. coli* strains that inhabit the large intestine of all humans and warm blooded animals comprise about 1 % of the total biomass and are in constant flux in the enteric lumen as well as attached to wall enterocytes. Nevertheless, they consistently exceed the less numerous *Citrobacter*, *Klebsiella*, and *Enterobacter* species. The stability of microbiota composition is evident at the level of bacterial species: *E. coli* are permanent members of the intestinal community, whereas the presence of other genera, *Citrobacter*, *Klebsiella*, and *Enterobacter*, bears a transient character (Leclerc *et al.*, 2001).

1.1.3.1. *Escherichia coli* as fecal indicators

The unique, fecal *E. coli*, which is the classical inhabitant of the intestinal tract of warm-blooded animals is restricted to this habitat and may not or only very poorly grows in receiving waters, although it is quite adapted to grow in foods with pH 7 (Mossel, 1995). *E. coli* was first isolated by Theodor Escherich in 1885 and named as "Bacterium coli commune". Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immuno compromised hosts. There are also pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans, eg. *E. coli* O157:H7. In 1892, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination (Feng *et al.*, 2002). *E. coli* is excreted in feces and can survive in fecal particles, dust and water for weeks or months (Quinn *et al.*, 1999). Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it was easier to isolate than known gastrointestinal pathogens. Hence, the presence of *E. coli* in food or water became accepted as indicative of recent fecal contamination and the possible presence of pathogens (Rice *et al.*, 1991; Feng *et al.*, 2002). Many authors have showed that prolific growth at an elevated temperature is characteristic of *E. coli*. However, as Leclerc *et al.* (2001) have stated, the optimum growth temperature is 37–38 °C and the maximum is 47 °C, whereas the most discriminating is 41–42 °C. According to Dufour (1977), aerogenesis is not a characteristic unique to *E. coli* at an elevated temperature (44–46 °C). Anaerogenic (do not produce gas in the fermentation of carbohydrates) strains are as common in *E. coli* as in other coliforms. The appropriate identification can be easily performed with the miniaturized, multi-biochemical reactions such as api 20E (Willcox *et al.*, 1995), when they allow a reliable differentiation between the various members of the coliform group (Leclerc *et al.*, 2001).

Properties of *Escherichia coli*

E. coli is often subdivided serologically or by the presence of virulence factors to identify and characterize epidemiologically pathogenic strains. Complete serotyping includes somatic (O), capsular (K), and flagellar (H) antigens. Typical *Escherichia*

species could also be easily differentiated from other genera by chemical testing. However, it is difficult to differentiate metabolically inactive *E. coli* strains from shigellae, which are metabolically inactive, nonmotile biogroups. Especially for a biogroup of *E. coli* strains that are atypically lactose negative, nonmotile and anaerogenic. Another biogroup of *E. coli* is negative in reactions for lysine decarboxylase, arginine dihydrolase, and ornithine decarboxylase, which makes them similar to *Enterobacter (Pantoea) agglomerans* and other species that are negative in these tests (Leclerc *et al.*, 2001). Differential reactions for separating *E. coli* and other *Escherichia* species from these genera are depicted in the 9th edition of Bergey's Manual of Determinative Bacteriology (1994). *E. coli* strains sometimes exhibit atypical reactions in a variety of tests including H_2S, citrate, urease, KCN, adonitol, inocitol, and indole, making it essential to consider the overall biochemical profile rather than specific "key" reactions before eliminating *E. coli* from consideration.

Pathogenicity of *Escherichia coli*

Generally, *E. coli* strains that colonize the human bowel are harmless commensals. However, within the species there are fully pathogenic strains that cause distinct syndromes of diarrheal disease because they possess virulence factors such as enteroadhesins or enterotoxins (Leclerc *et al.*, 2001). However, in other parts of the body *E. coli* can cause serious diseases, such as urinary tract infections, bacteraemia and meningitis. Several classes of enteropathogenic *E. coli* have been identified on the basis of different virulence factors, including enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (WHO, 2008).

Human health effects

EHEC serotypes, such as *E. coli* O157:H7 and *E. coli* O111, cause diarrhoea that ranges from mild and non-bloody to highly bloody, which is indistinguishable from haemorrhagic colitis. Between 2% and 7% of cases can develop the potentially fatal haemolytic uraemic syndrome (HUS), which is characterized by acute renal failure and

haemolytic anaemia. Children under 5 years of age are at most risk of developing HUS. The infectivity of EHEC strains is substantially higher than that of the other strains. ETEC produces heat-labile or heat-stable *E. coli* enterotoxin, or both toxins simultaneously, and is an important cause of diarrhoea in developing countries, especially in young children. Symptoms of ETEC infection include mild watery diarrhoea, abdominal cramps, nausea and headache. Infection with EPEC has been associated with severe, chronic, non-bloody diarrhoea, vomiting and fever in infants. EPEC infections are rare in developed countries, but occur commonly in developing countries, with infants presenting with malnutrition, weight loss and growth retardation. EIEC causes watery and occasionally bloody diarrhoea where strains invade colon cells by a pathogenic mechanism similar to that of *Shigella* (WHO, 2008). In most well studied waterborne outbreaks, described in the United States and in Sweden, the causal organisms belonged to the enteropathogenic *E. coli* class. In developed countries contamination of water have occasionally also led to outbreaks of enterotoxigenic *E. coli* infections (Leclerc *et al*, 2001).

1.2. Water quality guidelines and regulations

Water quality guidelines and regulations have been established, to ensure that all human beings within a country have access to safe drinking water. These guidelines (benchmarks that should be followed) and regulations (enforceable by law), put forward by the international and national regularity agencies are subjected to continuous periodical revisions to improve the sanitary quality of water.

The quality of potable water is generally controlled through a combination of protection of water sources, control of treatment processes and management of the distribution and handling of the water. Guidelines are appropriate for national, regional and local circumstances, which require adaptation to environmental, social, economic and cultural circumstances and priority setting (WHO, 2004). The use of indicator bacteria for monitoring fecal contamination of water is subject to strict governmental regulations (Rompre *et al.*, 2002).

1.2.1. World Health Organization (WHO) guidelines

The WHO guidelines outline a preventive management framework for safe drinking water' that comprises five key components: health based targets (based on an evaluation of health concerns); system assessment (from source through treatment to the point of consumption); operational monitoring in the drinking water supply; management plans for system assessment, monitoring, operational, system improvement and a system of independent surveillance to verify components. Microbial quality is one of the most important aspects of WHO water quality guidelines. WHO has introduced 'Water safety plans' (WSP), for monitoring the efficiency of control measures using appropriately selected determinants. WHO has recommended a final verification of water, using methods, procedures or tests undertaken by the supplier, surveillance agencies or combination of the two. It includes testing of source water, water immediately after treatment and in distribution systems or stored household water. For verification of the microbial quality of drinking water, WHO recommends testing for *E. coli* as an indicator of fecal pollution, since it provides conclusive evidence of recent fecal pollution (Table 1.1). However, total coliforms were also accepted water quality indicators in the 1980s and 1990s (WHO, 1983 and 1996) (Appendix 1: Tables 1, 2). Such water quality verification complements operational monitoring and assessments of contamination risks. Testing for thermotolerant coliform bacteria is also recommended as an acceptable alternative in many circumstances. In the majority of cases, monitoring for indicator bacteria provides a high degree of safety because of their large numbers in polluted waters (Appendix 1: Table 3). However, under certain circumstances, pathogens more resistant to conventional environmental conditions or treatment technologies may be present in treated drinking water in the absence of *E. coli*. Therefore, WHO has also recommended the use of resistant microorganism such as enterococci, *Clostridium perfringens* (spores) and bacteriophages as fecal indicators of water (WHO, 2004; 2008).

Table 1.1 The current WHO guideline values for verification of microbial quality [a]

Organisms	Guideline value
All water directly intended for drinking	
E. coli or thermotolerant coliform bacteria [b,c]	Must not be detectable in any 100-ml sample
Treated water entering the distribution system	
E. coli or thermotolerant coliform bacteria [b]	Must not be detectable in any 100-ml sample
Treated water in the distribution system	
E. coli or thermotolerant coliform bacteria[b]	Must not be detectable in any 100-ml sample

(WHO, 2008)

[a] Immediate investigative action must be taken if *E. coli* are detected.
[b] Although *E. coli* is the more precise indicator of fecal pollution, the count of thermotolerant coliform bacteria is an acceptable alternative. If necessary, proper confirmatory tests must be carried out. Total coliform bacteria are not acceptable indicators of the sanitary quality of water supplies, particularly in tropical areas, where many bacteria of no sanitary significance occur in almost all untreated supplies.
[c] It is recognized that in the great majority of rural water supplies, especially in developing countries, fecal contamination is widespread. Especially under these conditions, medium-term targets for the progressive improvement of water supplies should be set.

1.2.1. US Environmental Protection Agency (EPA) guidelines

According to US EPA regulations, a system that operates at least 60 days per year, and serves 25 people or more or has 15 or more service connections, is regulated as a public water system in the US, under the safe drinking water act (originally passed by the US Congress in 1974). If a system is not a public water system as defined by US EPA's regulations, it is not regulated under the safe drinking water act, although it may be regulated by state or local authorities (EPA, 1990).

Under the safe drinking water act, EPA requires public water systems to monitor for coliform bacteria. If any is positive for total coliform, the same sample must be analyzed for either fecal coliform or *E. coli*, which are indicators of contamination with animal waste or human sewage (EPA, 1990).

1.2.1.1. Total Coliform Rule (TCR)

The Total Coliform Rule which was published in 1989 has been revised as: *Corrections and Technical Amendments, 6/19/90 and Partial Stay of Certain Provisions (Variance Criteria) 56 FR 1556-1557, Vol 56, No 10.* This revision has been done with the purpose of improving public health protection by reducing fecal pathogens to minimal levels through control of total coliform bacteria, including fecal coliforms and *Escherichia coli* and has become effective in 1990. The rule consists of both health goals (MCLGs) and legal limits (MCLs) for the presence of total coliforms in drinking water (Table 1.2).

Table 1.2 US EPA guidelines for bacteriological quality of drinking water

Contaminant	**MCLG[a]**	**MCL[b]**	**Potential health effects from ingestion of water**	**Sources of contaminant in drinking water**
Total coliforms (including fecal coliform and. *E.coli*)	zero	5.0%[c]	Not a health threat in itself; it is used to indicate whether other potentially harmful bacteria may be present[d]	Coliforms are naturally present in the environment; as well as in feces; fecal coliforms and *E. coli* only come from human and animal fecal waste.

US EPA, 1990

[a] Maximum Contaminant Level Goal (MCLG) - The level of a contaminant in drinking water below which there is no known or expected risk to health. MCLGs allow for a margin of safety and are non-enforceable public health goals.

[b] Maximum Contaminant Level (MCL) - The highest level of a contaminant that is allowed in drinking water.

[c] more than 5.0% samples total coliform-positive in a month. (For water systems that collect fewer than 40 routine samples per month, no more than one sample can be total coliform-positive per month.). Every sample that has total coliform must be analyzed for either fecal coliforms or *E. coli* if two consecutive TC-positive samples, and one is also positive for *E.coli* fecal coliforms, system has an acute MCL violation.

[d] Fecal coliform and *E. coli* are bacteria whose presence indicates that the water may be contaminated with human or animal wastes. Disease-causing microbes (pathogens) in these wastes can cause diarrhea, cramps, nausea, headaches, or other symptoms. These pathogens may pose a special health risk for infants, young children, and people with severely compromised immune systems.

1989 Total Coliform Rule (TCR)

The TCR (54 FR 27544-27568, June 29, 1989, Vol. 54, No. 124) requires all public water systems to monitor for the presence of total coliforms in the distribution system. For drinking water, total coliforms are used to determine the adequacy of water treatment and the integrity of the distribution system. The absence of total coliforms in the distribution system minimizes the likelihood that fecal pathogens are present. Thus, total coliforms are used to determine the vulnerability of a system to fecal contamination. The TCR requires systems to monitor for total coliforms at a frequency proportional to the number of people served (Appendix 1: Table 4). If any sample tests positive for total coliforms, the system must perform the two additional tests;

- Further test that culture for the presence of either fecal coliforms or *E. coli*;
- Take one set of 3-4 repeat samples at sites located within 5 or fewer sampling sites adjacent to the location of the routine positive sample within 24 hours; and take at least 5 routine samples during the next month of operation.

1.2.3. Sri Lanka Standard (SLS) bureau guidelines

This Sri Lanka Standard has been authorized for adoption and publication by the council of the bureau of Ceylon standards on 20.12.1983, after the draft finalized by the Drafting Committee on potable water approved by the Agricultural and Food Products Divisional committee with the assistance from WHO and the Department of Health and Society Security of the Ministry of Housing and local Government of the United Kingdom.

1.2.3.1. Specification for potable water

This specification covers the quality of water used for drinking purposes. As specified in the Part 2 (SLS 614: Part 2: 1983), of this standard, bacteriological examination of water is necessary in determining its fitness for use for human consumption, and for food processing industries.

Part 2: Bacteriological requirements

The bacteriological requirements for potable water are based on the examination of several samples taken from the supply source under different conditions. The samples obtained as prescribed in section 4 of the standard, when examined by the methods given in Appendix A (SLS 614: Part 2: 1983) of the standard, shall comply with the following requirements;

a) Pipe borne public water supplies

- Throughout any year, 95 percent of the samples shall not contain any coliform organisms in 100 ml.
- None of the samples examined shall contain more than 3 coliform organisms per 100 ml (Amendment No. 1 Approved on 07.06.1988).
- None of the samples examined shall contain *E. coli* in 100 ml.

b) Individual or small community supplies

- None of the samples examined shall contain more than 10 coliform organisms per 100 ml on repeated examination (Amendment No. 1 Approved on 07.06.1988).
- No sample shall contain *E. coli* in 100 ml

1.3. Different methods used to detect coliform bacteria

Most of the indicator applications rely on biological measurements of bacteria. The classical laboratory techniques presently used for these measurements are primarily culture based, involving quantification of a metabolic or growth response after a suitable incubation period in an appropriate substrate. Culture based methods have been used for more than 100 years in water and related areas of environmental microbiology and have been considered adequate to provide quantification of indicator bacteria (NRC, 2004). As Eckner (1998) reports, these methods have been developed in the early 1900s to assess water quality with regard to public health by enumerating coliforms. Coliforms which normally occur in the intestines of all warm blooded animals are excreted in great numbers in feces. This group of bacteria includes *Escherichia, Citrobacter, Enterobacter* and *Klebsiella* species, which are relatively easy to detect. Specifically this group includes all aerobic and facultatively anaerobic, gram-negative, non-spore-forming, rod shaped bacteria that produce gas upon lactose fermentation in prescribed culture media at specified incubation temperatures (Gerba *et al.*, 2000).

The US EPA (1990) and APHA *et al.* (1995; 1998; 2000) have approved several methods for coliform detection: Multiple Tube Fermentation (MTF) also termed as Most Probable Number (MPN) and Membrane Filtration (MF) are the most commonly used two standard methods, based on lactose fermentation. WHO (2008) has also approved these two methods according to ISO standards, 9308-1:1990 Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive *Escherichia coli* – Part 1:Membrane filtration method and 9308-2:1990 Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive *Escherichia coli* – Part 2: Multiple tube (most probable number) method. In addition to these two conventional methods, other methods based on defined substrate technology by using chromogenic and fluorogenic media based on the detection of enzymatic activities present in coliforms and *E. coli* bacteria have also been approved by the US EPA (1990) and APHA *et al.* (1995; 1998; 2000).

1.3.1. Conventional Techniques

1.3.1.1. Multiple Tube Fermentation (MTF) technique

In the early 20th century, Eijckman used this method for the first time, to detect *E. coli* by observing gas production in glucose broths at elevated temperatures (Buckalew *et al.*, 2006). Later, it has been approved as a standard bacteriological analytical method throughout the world (US EPA, 1990; APHA *et al.*, 1995, 1998 and 2000). In Sri Lanka, Bureau of Ceylon Standards (1983) has also published Multiple Tube Fermentation (MTF) technique as a standard method to detect bacteriological quality of water in Sri Lanka. The MTF is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases (Rompre *et al.*, 2002). Results of the examination of replicate tubes and dilutions are reported in Most Probable Number (MPN) of organisms present. This number based on certain probability formulas, is an estimate of the mean density of coliforms in the sample. The precision of each method depends on the number of tubes used. Bacterial density can be obtained by a formula or from a MPN table (formed based on the assumption of a Poison distribution) using the number of positive tubes in the sample dilutions. The 3-tube MTF test is used for testing most foods. The 5-tube MTF is used for water, shellfish and shellfish harvest water testing and there is also a 10-tube MTF method that is used to test bottled water or samples that are not expected to be highly contaminated (APHA *et al.*, 1998; 2000).

The method consists of inoculating a series of tubes (filled with lauryl tryptose broth) with appropriate decimal dilutions of the water sample. Production of gas, acid formation or abundant growth in the test tubes after 48 hours of incubation at 35 °C constitutes a positive presumptive reaction (APHA *et al.*, 1995; 1998; 2000). All tubes with a positive presumptive reaction are subsequently subjected to a confirmation test. The formation of gas in brilliant green lactose bile broth fermentation tube at any time within 48 hours at 35 °C constitutes a positive confirmation test for total coliforms. For presumptive total coliform test, both lactose and lauryl tryptose broths could be used as presumptive media. However, lactose broth media are reported to have interference with high number of non-coliform bacteria (Evans *et al*, 1981).

Simultaneous incubation of peptone water tubes at 44 °C for 24 hours for the formation of gas, followed by indole reaction with Kovac's reagent constitutes a positive test for *E. coli* (APHA *et al.,* 1998; 2000; Sri Lanka Standards 1983). The fecal coliform test using EC medium can determine total coliforms that are of fecal origin: the production of gas after 24 hours of inoculation at 44.5 °C in an EC broth medium is considered as a positive result. Use of EC -4-methylumbelliferyl β-D glucuronide (MUG) broth, incubated at 44.5 °C for 24 hours will confirm *E. coli* by exhibiting fluorescence under a long wavelength UV light (APHA *et al.*, 1998; 2000).

The results of the MTF technique expressed in terms of the most probable number (MPN) is a statistical estimate of the mean number of coliforms in the sample (Rompre *et al.*, 2002). It is assumed that each viable organism will show growth and differential reaction appropriate to the organism and the medium used (Sri Lanka Standards, 1983). The MPN of the organisms in the original sample could be estimated from the number of tubes giving positive reaction. Statistical tables of probability are used for this purpose (Sri Lanka Standards, 1983).

1.3.1.2. Membrane Filtration (MF) technique

The Membrane Filtration (MF) technique is described as a highly reproducible method, which could be used to test relatively large sample volumes than MTF procedure (APHA *et al.*, 1995; 1998; 2000). In this method, a water sample is filtered through a sterile filter having a pore size of 0.45μm, on which, the bacterial cells will be retained. By incubating the filter on a selective medium typical countable colonies can be observed (Rompre *et al.*, 2002). Hence, MF method yields numerical results more rapidly (APHA *et al.*, 1995; 1998, 2000). Many media and incubation conditions for the MF method have been tested for optimal recovery of coliforms from water samples (Rice *et al.*, 1996; Schets *et al.*, 2002; Niemela *et al.*, 2003). Among these the most widely used media are the M-Endo-type medium in North America (APHA *et al.*, 1995) and the Tergitol-TTC medium in Europe (Niemela *et al.*, 2003). In Sri Lanka, LES Endo medium is used as recommended by the Bureau of Ceylon Standard (Sri Lanka Standards, 1983). Coliform bacteria form red colonies with metallic sheen on an Endo-type medium containing lactose (incubation at 35 °C /24 hours for total coliforms) (APHA *et al*, 1995; 1998; 2000); or yellow-orange

colonies on Tergitol-TTC media (incubation at 37 °C for 24 hours to detect total coliforms and at 44.5 °C for 48 hours to detect fecal coliforms respectively) (according to ISO 9308-1, Schets *et al.*, 2002). Incubation of filters at 44.5 °C for 24 hours on enriched lactose medium (M-FC) was proposed by APHA *et al* (1995; 1998; 2000) for the detection of fecal coliforms. Because of the addition of rosolic acid salt reagent and the elevated incubation temperature allows only few non coliform colonies to develop on the M-FC medium (APHA *et al.*, 1995; 1998; 2000), while enhancing the growth of fecal coliforms.

Verification of total coliforms is recommended by lactose fermentation or by using cytochrome oxydase test and ONPG (ortho-nitrophenyl-β-D galactopyranoside) to avoid false-positive and false-negative results. (APHA *et al.,* 1995; 1998). Fecal coliforms and *E. coli* specifically are verified by inoculating in to EC broth (incubation at 44.5 °C for 24 hours) and EC-MUG broth (incubation at 35 °C for 24 hours) respectively.

1.3.1.3. Drawbacks of conventional methods

The conventional laboratory techniques used for bacterial detection and identification are primarily culture based methods, involving quantification of a metabolic or growth response after a suitable incubation period in an appropriate substrate (NRC, 2004). Further, these conventional culture based methods may be limited by their incubation period, since most require 24 hours or longer, during which time the public is potentially exposed to a health risk (NRC, 2004). Since the biochemical tests used in these conventional methods are not fully specific, many additional tests are required to obtain specific confirmation results (Rompre *et al.*, 2002). Further, the identification up to species level is not possible. Hence, a complete analysis requires an additional 24 to 72 hours for the final result (Edberg *et al.*, 1988). In addition, the production of gas in lactose fermentation is not always relevant, since many strains (including *E. coli*), may or may not ferment lactose, due to various environmental factors (Fricker *et al.*, 1996; Edberg *et al.*, 1997 and 2000; Leclerc *et al.*, 2001). Further, lactose-positive and lactose-negative biotypes have been isolated irrespective of their origin (Leclerc *et al.*, 2001). Furthermore, a significant proportion of the coliforms are reported to be anaerogenic (fail to produce gas when fermenting lactose) (Edberg *et al.*, 1997 and 2000; Fricker *et al.*, 1996) and according to Fricker *et al* (1994) approximately 10 % of coliforms do not ferment lactose.

1.3.2. Other methods

Recent and forecasted advances in microbiology, molecular biology, and analytical chemistry has made it timely to investigate on new and emerging advance technologies to facilitate rapid and accurate detection of indicator bacteria. As a result, alternative techniques based on particular enzymatic reactions (Fricker *et al.*, 1997; Eckner, 1998), molecular techniques (such as immunological, polymerase chain reaction and in-situ hybridization), direct cell counting methods, etc., were developed for rapid and sensitive detection and enumeration of coliform bacteria compared to conventional techniques (Rompre *et al.*, 2002).

1.3.2.1. Enzymatic methods

The use of microbial enzyme profiles to detect indicator bacteria was found to be an attractive alternative to the conventional MTF and MF methods with various limitations. Enzymatic reactions are rapid and sensitive, and can be group, genus or species specific, depending on the enzyme targeted (Rompre *et al.*, 2002). The most commonly used enzymes are β-D galactosidase, β-D galactosidase permease and β-D glucuronidase (Fricker *et al.*, 1997; Eckner., 1998; Rompre *et al.*, 2002; Pitkanen *et al.*, 2007) present in the cells of coliform bacteria. In this method, specific chromogenic and fluorogenic substrates used in this method (Edberg *et al.*, 1988; Edberg *et al.*, 1989) are reported to produce colour and fruorescence, respectively upon cleavage by specific enzymes (Rompre *et al.*, 2002). The enzymatic techniques for bacteriological analysis are also designated as autoanalysis since a colour change (chromogenic) is produced by the target microbe(s) with no necessity for cofirmatory tests or technologist labour. Performing the test is simple without involving sophisticated equipment or complex procedures. The specific colour changes denote the target microbe(s) (Edberg *et al.*, 1988).

Detection and enumeration of *E. coli* and total coliforms are reported elsewhere in the world, using the enzymes β-D glucuronidase and β-D galactosidase respectively (Fricker and Fricker, 1996). The enzyme β-D glucuronidase (GUS) discovered in the bacterium *E. coli* catalyses the hydrolysis of β-D glucopyranosiduronic derivatives into their corresponding aglycons and D-glucuronic acid. Further, β-D glucuronidase catalyses the

breakdown of lactose into galactose and glucose and has been used mostly for enumerating the coliform group (Rompre *et al.*, 2002). Different chromogenic and fluorogenic substrates have been used for bacterial diagnostics (Tripeta and Edberg, 1984; Piscicotta *et al.*, 2002). They have observed that the use of these substrates has led to improved accuracy and faster detection by using a single medium instead of using several media at different steps of the procedure. For the detection of β-D glucuronidase in *E. coli* several chromogenic substrates have been used. ρ-nitrophenyl-β (PNBG) (Tripeta and Edberg, 1984), indoxyl-. β-D glucuronide (IBG) (Haines *et al.*, 1993), the commercial chromogenic medium CHROMagarECC, have been used to detect both *E. coli* and coliforms simultaneously (Alonso *et al.*, 2004). Further, Geissier *et al.* in 2000 have studied the use of two commercial preparations of chromogenic Chromocult Coliform agar and fluorogenic Fluorocult LMX broth for the simultaneous detection of total coliforms and *E. coli*. Most frequently, the fluorogenic substrate 4-methylumbelliferyl β-D glucuronide (MUG) and the chromogenic substrate o-nitrophenyl β-D galactopyraniside (ONPG) have been used (Trepeta and Edberg, 1984; Edberg *et al.*, 1988; Covert *et al.*, 1989; Edberg *et al.*, 1989; Lewis and Mak, 1989; Palmer *et al.*, 1993; Fricker *et al.*, 1997; Eckner, 1998; Piscicotta *et al.*, 2002; Niemela *et al.*, 2003; Pittkanen *et al.*, 2007, etc.,) to detect *E. coli* and total coliforms respectively.

Presence/absence techniques and enumeration by multi-tube techniques using enzymatic methods

The incorporation of 4-methylumbelliferyl β-D glucuronide (MUG) into lauryl tryptose broth in MTF technique for the rapid detection and immediate confirmation of *E. coli* in water is reported by APHA *et al.* (1998) and Feng and Hartmen (1982). Visualization of blue-white fluorescence in methylumbelliferone formed due to the hydrolysis of MUG in *E. coli* positive tubes has been the tool for identification. Edberg and Edberg (1988) proposed using a combined substrate technology with substrate ONPG for the constitutive enzyme β-D galactosidase present in all coliforms and the substrate MUG for the specific detection of *E. coli*. The defined substrate method was basically constituted as a presence/absence test. Colour change from colourless to yellow after incubation at 35 °C is taken as a positive test for total coliforms, due to the hydrolysis of ONPG. Any yellow

colour tubes fluorescing under long wave (365nm), indicates the presence of *E. coli*. No additional confirmatory tests are needed. Also, the sensitivity has proved equal sensitivity with classical methods with greater specificity (Rompre *et al.*, 2002). Further studies reported by several scientists (Edberg *et al.*, 1988; Edberg *et al.*, 1989; Covert *et al.*, 1989; Lewis and Mak, 1989; Geissler *et al.*, 1989; Niemela, 2003) have proven the sensitivity and efficiency of using defined substrate technology (DST) for bacteriological studies of water.

Later, several commercial media have been developed based on the defined substrate technology; Colilert [IDEXX laboratories, Westbrook, Maine, USA]; Colisure [Millipore Corporation, Bedford, MA, USA]; Coliquick [Hach, Loveland, CO, USA] (Rompre *et al.*, 2002). Most of these are available for a presence/absence response and for enumeration by MTF technique. The most widely used among them is the Colilert test, which utilizes the ONPG and MUG (Rompre *et al.*, 2002). The detection tests using DST, using chromogenic and fluorogenic substrates are more rapid, sensitive and need no confirmatory steps (Pitkanen *et al.*, 2006). Since, DST needs less labour, they are reported to be cost effective than conventional methods (Fricker and Fricker, 1996).

Membrane filtration technique conjugated to enzymatic detection of coliforms

Reports are available for the incorporation of MUG into agar media to detect the presence of β-D glucuronidase activity of *E. coli* (Entis and Boleszczuk, 1990; Brenner *et al.*, 1993). Another study reported by Trepeta and Edberg (1983) suggests the direct incorporation of MUG in to modified MacConkey agar to directly detect the presence of β-D glucuronidase in *E. coli*. The methods were shown to be sensitive, rapid, selective and specific (Trepeta and Edberg, 1983; Brenner *et al.*, 1996).

Commercial preparations of modified MF agar media with specific chromogenic and/or fluorogenic substrates for the detection of β-D glucuronidase and of β-D galactosidase now available around the world include, Chromocult Coliform agar (Merck, Darmstagt, Germeny) (Schets *et al.*, 2002; Pittkanen *et al.*, 2007), Fluorocult *E. coli* Direct agar (Merck, Germeny) (Rompre *et al.*, 2002). Further, modified liquid culture media consisting of chromogenic substrates to detect those two specific enzymes are

commercially available; m-ColiBlue24 broth (Hach, USA) (Schauer *et al.*, 2007) is one such commercial preparation. Rapid, accurate sensitive estimates and simultaneous detection of total coliforms and *E. coli* are some of the advantages of these plate count methods compared to the conventional MF methods.

Direct determination of enzymatic activity by fluorometry

Rapid assays with β-D glucuronidase and β-D galactosidase for the detection of *E. coli* and total coliforms without any cultivation steps have been reported (Trepeta and Edberg 1984; George *et al.*, 2000). Direct enzymatic detection of fecal coliforms within 30 minutes also has been successful using fluorogenic substrate MUG (George *et al*, 2000). In addition, an automated analyzer (Colifast CA-100 [Colifast systems, Oslo, Norway)]has been developed on the basis of enzymatic properties of coliforms, which takes only two hours for detection (Berg *et al.,* 1998). However, this automated Colifast test is usually recommended for bathing water analysis. Therefore, fluorometric method is thus a direct determination of the enzymatic activity with high coliform abundance (surface water) and involves a growth phase for samples with low coliform abundance [drinking water] (Rompre *et al.*, 2002).

The above mentioned enzymatic methods involve incorporation of chromogenic or fluorogenic substrates to agar or to liquid media. Most of these methods are rapid, accurate and more sensitive compared to conventional methods. Furthermore, the advent of increasingly sophisticated and powerful molecular biology techniques provide new opportunities and alternative approaches to improve upon present indicators both culture and non-culture methods (NRC, 2004).

1.3.2.2. Molecular methods

Molecular methods have been developed for the detection of coliforms with high degree of sensitivity and specificity without the need for a complex cultivation and additional confirmation steps (Rompre *et al.*, 2002). They do not require incubation to culture bacteria because they can directly quantify existing cellular or sub-cellular structural properties of cells. Therefore, these methods have the potential to be more rapid than

culture methods, providing results in as little as minutes to a few hours rather than the typical overnight incubation time for culture methods (NRC, 2004). Further, the presence of bacterial cells in viable but non culturable (VBNC) states which can not be detected using routine culture based-methods could be detected using molecular methods (Baudart *et al.*, 2002). Some of these nucleic acid-based methods employ amplification schemes in which a small amount of indicator genetic material is replicated up to a billion fold for easy detection. They also have the potential to be less expensive, making direct measurement of pathogens more economically feasible. Furthermore, molecular methods can be coupled with or linked to microbial culture methods in ways that can increase sensitivity, decrease detection time, and provide conclusive and rapid confirmation of identity and infectivity (NRC, 2004). Several molecular based techniques are available for the specific detection of coliforms over the world. However, the use of molecular methods in developing countries is still minimum, due to poor laboratory facilities, lack of skilled technical staff and higher chemical costs.

1.3.2.3. Immunological methods

Immunological methods are based on the specific recognition between antibodies and the antigens and the high affinity that is characteristic of this recognition reaction. This characteristic feature is being used to detect antigens at family, genus, species or serotype levels (Rompre *et al.*, 2002). The properties of the antigen-antibody complex is used; to perform immunocapture of cells or antigens by enzyme-linked immunosorbant assay (IMS or ELISA), or to detect targeted cells by immunofluorescence assay (IFA) or immuno-enzyme assay (IEA). An ELISA technique has been developed using a monoclonal antibody against the enterobacterial common antigen (ECA) for the detection of coliforms (Rompre *et al.*, 2002). Immunofluorescence assay (IFA), which allows the identification and enumeration of a single specific cell, has been performed for the detection of fecal coliforms by applying a direct immunofluorescence microscopy technique (Zaccone *et al.*, 1995).

1.3.2.4. Nucleic acid-based methods

Most of the nucleic acid methods use molecular hybridization properties, which involve the complementary sequence recognition between a nucleic probe and a nucleic target. The Polymerase chain reaction (PCR) and the in situ hybridization (ISH) methods are more frequently used nuclic acid based methods for the detection of coliforms in water samples (NRC, 2004).

Polymerase Chain Reaction (PCR) method

Amplification of a targeted DNA fragment is achieved in PCR by cyclic replication (*in vitro* or *in situ*) with olygonucleotide primers catalyzed by a DNA poymerase (*Taq* polymerase). This technique has been described for the detection of total coliforms and *E. coli* (Rompre *et al.*, 2002).

***In situ* Hybridization (ISH) techniques**

In this method, oligonucleotide probes are used to detect the complementary nucleic acid sequences. Since these probes are specific to selected nucleic acid sequences of a given microorganism, species or group, detection of specific microorganisms is possible. Several commercial preparations of oligonucleotide probes are available for bactreriological analysis (Rompre *et al.*, 2002). Earlier, in situ hybridization relied on radioactive probes to detect the probe target hybrid. Later work on r RNA in situ hybridization used fluorescent-labeled nucleotide probes to detect hybridization (FISH), which is a highly specific detection method at a cellular level (Pernthaler and Amman, 2004). However, FISH can have some limitations in detecting enteric bacteria under environmental pressures such as starvation and oxidative stress. Therefore, combined Direct Viable Count method (DVC) and FISH has been successful in detecting viable but nonculturable (VBNC) enterobacerial cells in fresh water and drinking water systems (Baudart *et al.*, 2002). Although FISH is currently considered as a highly specific and easier cellular detection method, the identification of the target sequence is difficult. Also its use in detection of non-phylogenetically identified organisms such as coliforms is not successful.

1.4. Detection and enumeration of coliforms and *Escherichia coli* for bacteriological quality of water in Sri Lanka

Bacteriological analysis of water is one of the most sensitive analytical parameter, which is used in assuring the quality of drinking water in Sri Lanka. In Sri Lanka, bacteriological analysis is conducted to assure and confirm the Sri Lanka Standards (SLS) of water quality.

1.4.1. Sri Lanka standards for water quality assessment

As described in the section 1.2.3, the Sri Lanka Standards Bureau has published specifications on bacteriological quality for pipe borne drinking water. This section covers the specifications for public water supplies and community water supplies (SLS 614: Part 2: 1983), in accordance with the WHO water quality guidelines (WHO, 1983). Later, in 1988 this standard has been amended for the public and small community potable water supplies as depicted in the Table 1.3.

1.4.2 Amendment (No.1 approved on 07.06.1988) of SLS 614: Part 2: 1983

Table 1.3. SLS standards for pipe borne water, Amendment No. 1

Category	Clause	Original value	Amended value
Public water supplies	3.1.2	maximum 10 coliforms/100 ml	maximum 3 coliforms/100 ml
Small community supplies	3.2.3	maximum 20 coliforms/100 ml (on repeated examination)	maximum 10 coliforms/100 ml

After this revision, no other amendments have been introduced to the potable water standards in Sri Lanka, although the WHO guidelines have been modified in 2004 (WHO 1990; 2004).

In addition to these standards, in 2003 the SLS has also established standards for bottle water quality (Table 1.4).

Table 1.4. SLS standards for bottled water

Standard	n	c	m	M	Method
Packed bottled drinking water					
SLS 894: 2003					
Total coliforms/ 100 ml	10	1	0	10	SLS 614 Part 2
E. coli/ 100 ml	10	0	0	-	SLS 614 Part 2
Aerobic Plate count (APC)/ml at 37 °C for 72 hrs	5	4	100	1×10^4	SLS 516 Part 1
Natural mineral water:					
SLS 1038:2003					
Total coliforms/ 250 ml	4	1	0	2	SLS 614 Part 2
E. coli/ 250 ml	4	0	0	-	SLS 614 Part 2
Aerobic Plate count (APC)/ml at 20-22 °C for 72 hrs (250 ml)	4	0	100	-	SLS 516 Part 1
Aerobic Plate count (APC)/ml at 37 °C ±1 for 24 hrs (250 ml)	4	0	20	-	SLS 516 Part 1
Fecal streptococci (200 ml)	4	1	0	2	SLS 516 Part 1
Pseudomonas aeruginosa (250 ml)	4	1	0	2	SLS 516 Part 1
Anaerobic chlostridia (50 ml)	4	1	0	2	SLS 516 Part 1

n - number of samples to be tested
c - maximum allowable samples yielding values between m and M
m - limit below which a count is acceptable for any sample
M - limit above which a count is unacceptable for any sample

1.4.3. Currently practiced techniques in Sri Lanka

Sri Lanka Standards Bureau (SLS 614: Part 2: 1983) recommends two standard methods to detect the presence of coliform bacteria. Viz; the Multiple Tube Fermentation (MTF) and the Membrane Filtration (MF) method based on lactose fermentation ability of the coliform group. SLS 614: Part 2: 1983 standard covers the details of sampling techniques,

media preparation, methodology and culture requirements etc. All government and non-government institutes are strictly adhered to the SLS standards and follow the recommended MTF or MF procedures for detecting bacteriological quality of water. According to SLS 614, the results obtained by these two methods are not comparable, since MF does not indicate gas formation from lactose fermentation. However, saving of time, labour and glassware in the MF are considered as advantages compared to the MTF method (SLS 614: Part 2: 1983).

CHAPTER 2

COMPARISON OF DIFFERENT BACTERIOLOGICAL METHODS

PART-A

PRELIMINARY STUDY ON COMPARISON OF FIVE BACTERIOLOGICAL METHODS FOR DETECTING AND ENUMERATION OF TOTAL COLIFORMS AND *E. COLI*

2.1. INTRODUCTION

As reported elsewhere, the MF and MTF techniques based on fermentation of lactose, producing sheen colonies, gas, or acid and gas, are the most commonly used conventional methods for detecting and enumeration of coliform bacteria for water quality assessment around the world. However, both methods require multi step confirmational and completed tests after the initial observation of a positive presumptive result (Edberg *et al.*, 1988; Covert *et al.*, 1989; Edberg *et al.*, 1989; Lewis and Mak, 1989; Eckner, 1998). These tests will require an additional 24-72 hours, making a water analysis extending up to 2-4 days to obtain a complete final result (Edberg *et al.*, 1988; Edberg *et al.*, 1989). Therefore, these conventional tests are considered as time-consuming methods (Lewis and Mak, 1989; Geissler, et al., 2000; Baudart *et al.*, 2002). Further, these conventional methods are laborious which require media preparation, sterilization, etc., and also involved with handling of large amounts of glassware and equipment (especially in MTF method).

Traditionally, the detection of *E. coli* has been based on lactose fermentation with gas production at an elevated temperature (44 °C) and by indole reaction Niemi *et al.* (2001). Although prolific growth at an elevated temperature is characteristic of *E. coli*, anaerogenic strains are also common (Leclerc *et al.*, 2001). According to Niemi et al (2001), the value of gas production as the criterion for confirmation of *E. coli* is not very appropriate due to the presence of anaerogenic *E. coli*. Non-gas-producing strains of *E.*

coli have been reported to approach 10% of the *E. coli* population (Edberg *et al.*, 1997). Further, the identification of anaerogenic lactose-fermenting coliforms, reported by the same author, has revealed that more than 90% belonged to one of the four coliform genera, *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter*. The production of indole at 44 °C is not exclusive to *E. coli* since some stains of *Klebsiella,* notably *K. oxycota* are known to produce a positive reaction in this test (Fricker *et al.*, 1996). In order to resolve this, Niemela *et al.* (2003) have proposed the use of gas production at 44.5 °C as one of the *E. coli* confirmation tests. However, some strains of *E. coli* have been reported to be indole negative (Fricker *et al.*, 1996). As they further stated, not all *E. coli* have the ability to grow at elevated temperatures and in particular, EC O157:H7, which causes haemorrhagic colitis in humans cannot grow or grow poorly at this elevated temperatures. Therefore, the conventional techniques are more confusing in detecting *E. coli*. The public health implications of these findings continue to be debated, but most specialists argue that anaerogenic coliforms are of no more, nor less, sanitary significance than aerogenic ones (Edberg *et al.*, 1997; 2000).

In addition, the subjective nature of results interpretation in analysis (Edberg *et al.*, 1988) is another limitation found among conventional methods. As Evans, *et al.* (1981) has stated, when using conventional methods, the estimates of coliform density from a single sample may show variability. In addition, the duration of incubation, interference from antagonistic organisms, lack of specificity to the coliform group and a weak level of detection of slow growing or stressed coliforms are some of the other limitations found in these two methods (Rompre *et al.*, 2002).

Further, according to Edberg *et al.* (1988), several inherent properties of the two methods have limited the ability of public health officials to make decisions regarding the health risk. Most restricting were the time required to obtain a definitive health based answer, the inability to differentiate fecal from total coliforms without either the performance of tests specifically for fecal coliforms or identification of bacteria and the subjective nature of interpretation of the analytical methods.

In response to the above-mentioned limitations, new techniques were developed based on technology originally designed to identify microbes by the analysis of their constitutive

enzymes using defined substrates (DS) (Edberg *et al.*, 1988). The incorporation of defined substrates such as para nitrophenyl β-D glucuronide (PNBG) (Trepeta and Edberg, 1984), 4-methylumbelliferyl β-D glucuronide (MUG) (Shadix and Rice, 1991; Feng and Hartman, 1982; Moberg, 1985) in to conventional MTF and MF media also gained popularity from 1990s up to date. Other detection methods also exist in various levels of development and application. However, as Rompre *et al.*, (2002) has suggested that, criteria like detection limit and sensitivity, time required for results and lab outlays (skilled labour and cost) should be considered when introducing these new techniques in to routine analysis of water samples.

The first comparison study conducted by Edberg *et al.* in 1988 involved comparison of the standard MTF method with a DS method (Autoanalysis Colilert /AC) for simultaneous enumeration of total coliforms and *E. coli*. They have found that the AC was sensitive as the standard MTF; more accurate; not interfered by heterotrophic bacteria; easy to inoculate and interpret and able to analyze both total coliforms and *E. coli* simultaneously, without further confirmational steps. In 1989 they were able to get the approval from the US EPA for the use of Autoanalysis Colilert (AC) as an alternate system for detecting coliform bacteria. Swedac the Swedish laboratory accreditation body also approved the use of Colilert for analysis of all water sources in Sweden (Eckner, 1998). Further, it (colilert) has also been included in the Standard Methods for the Analysis of Water and Wastewater (APHA *et al.*, 1995; 1998; 2000).

More comparison studies involving defined substrate technology/enzymatic activities of coliform bacteria have been conducted extensively throughout the past few decades (Table 2.1). Most of these comparisons were based on simple analysis on variance statistics (Lightfoot and Maier, 1998).

Table 2.1 Literature review on method comparison for detecting and enumeration of total coliform bacteria and *E. coli* in different water sources

Methods/media compared	Types of water sources used for comparison	Research outcome	Reference
LTB, BGLB/ MTF, with Autoanalysis Colilert (AC) /MTF	Deep and shallow wells, springs, rivers, surface reservoirs	No statistical difference between AC and MTF. Colilert was sensitive as standard MTF, specifically enumerated 1 total coliform per 100 ml within 24 h	Edberg, *et al.*, 1988
Standard method (SM) Presence-Absence (P-A) method with Autoanalysis Colilert (AC) P-A method	Deep and shallow wells, springs, rivers, surface reservoirs	No statistical difference between AC and SM. Overall agreement rate between AC P-A and SM P-A was 94 %. Subculturing confirmed total coliforms for yellow tubes and *E. coli* for fluorescing tubes. No effect from heterotrophic plate count bacteria in Colilert method	Edberg, *et al.*, 1989
Colilert /MTF, Colilert (AC) P-A, M-Endo LES/MF, LTB, BGLB/MTF, LTB P-A method	Natural springs, surface waters, well waters, cisterns, treated coagulated settles water, public drinking water supply distribution system	All the methods were comparable in detecting total coliforms; Heterotrophic bacteria did not interfere in Colilert method; No statistical difference in	Covert, *et al.*, 1989

		precision of all methods	
M-endo LES/MF with Colilert (AC) P_A method	Samples from building fixtures and fire hydrants after flushing procedures.	Presumptive total coliform results of two tests agreed 97 % and confirmation test 98.5 %. No statistical difference in detecting total coliforms; *E. coli* could not be isolated from fluorescing tubes	Lewis and Mak, 1989
LMX broth (P-A) method with Colilert (AC) P-A method	Ground and surface water supplies, partially treated water, marginally chlorinate water, final distribution water	No statistical difference in detecting total coliforms and *E. coli* by both methods; Total coliform detection LMX> AC; *E. coli* detection AC> LMX. Confirmation rates for total coliforms AC 100 %, LMX 84.3 %; For *E. coli* AC 100 %, LMX 90.4 %	Fricker and Fricker, 1996.
MLSB/MF, Colilert-24/MTF, Colilert-18/MTF	Drinking water, disinfected sewage effluent	Total coliform detection: Colilert> MLSB; *E. coli* detection: Colilert = MLSB; no significant difference between Colilert-24 and Colilert-18. Colilert is easier, less time consuming	Fricker, *et al.*, 1997.
M-Endo-LES	Drinking water, bathing	Colilert was more	Eckner,

agar/MF and lactose broth/MPN with Colilert method	water	sensitive than MF and MTF and equally sensitive for *E. coli* to MF and MTF for drinking water; coliform detection in bathing water was anomalous due to confirmation difficulties; *E. coli* detection in bathing water was similar by all 3 methods	1998
LS broth/MTF, LMX broth/MTF, CC agar/MF, CC-CFS/MF	Marine water	Coliform detection: LMX, CC, LS broth recovered 2.63, 1.95, 1.90 times as CC-CFS. *E. coli* detection: no statistical difference among methods. background growth was les on CC-CFS	Geissier, *et al.*, 2000
Differential coliform agar/MF, LES endo agar/MF, Lactose Tergitol TTC agar/MF	Shallow well waters	Number of typical colonies: Differential coliform>LES endo>Lactose Tergitol TTC; confirmation rates-LES endo (92 %), Lactose Tergitol TTC (75 %), Differential coliform (74 %)	Niemi, *et al.*, 2001
LTTC/MF with Colilert-18/MTF,	Surface water, half treated drinking water,	LTTC had heavy background growth;	Schets *et al.*, 2002

Lauryl Sulfate Agar (LSA)/MF, Chromocult Coliform agar/MF, *E. coli* Direct Plating (DP)/MF method	fully treated drinking water spiked with surface water	Colilert-18 higher counts with wide group of coliforms; For *E. coli*, DP was the best; Colilert produced low counts and false negative results	
ISO 9308.1 (Tergitol TTC)/MF with Colilert/Quanti-Tray/MTF	Treated drinking water, groundwater, surface water	Colilert detected significantly higher total coliform and *E. coli* counts; Recovery of *E. coli* was significantly higher and no confirmation needed with Colilert.	Niemela, *et al.*, 2003
MF and MTF (APHA, 9221 B,C; 9222B,D; 9230B,C)	Beach water samples	Total coliform counts were high with Colilert and Colilert-18 than MF and MTF methods; fecal coliforms 12 % lower in Colilert (due to specificity); Colilert had 90% agreement with MTF/MF when data were categorical analyzed.	Noble *et al.*, 2004
M-Endo, M-FC /MF , LTB/BGLB/MPN, with Colilert method (Quati-Tray 2000,	Open marine beach water, estuary water, water from flowing creeks.	Total coliform detection: Colilert> MF/MTF; 40 % false positives in verification; Fecal coliforms: Colilert 10 % less than MF/MTF	Griffith, *et al.*, 2006

Colilert-18)			
ISO 9308-1 (LTTC)/MF with LES Endo/MF, Colilert-18/MTF, CC agar/MF, HECM/MF, CECM/MF	Groundwater, bathing water, spiked tap water and well water	Coliform counts in LES Endo, Colilert, CC, HECM and CECM were higher than LTTC; *E. coli* counts were higher in LTTC; background growth highest on LTTC; Colilert, CC and CECM suggested as potential alternative media	Pitkänen, *et al.*, 2006
M-FC/MF, Coliert/MF	Environmental waters	*E. coli* counts by Colilert were positively correlated with M-FC confirmed counts; Colilert is simple, quick, easy to quantify, tha M-FC. Therefore, Colilert i precise and a versatile method	Buckalew, *et al.*, 2006
Colilert/MTF, Colilert-18/MTF, Colisure/MTF, mColiblue-24/MF, Readycult Coliform medium 100/MF, Coliscan, E*Colite, Chromocult/MF, MI agar/MF,	Ground water from three geographically and chemically different sources; spiked with *Serratia, E. coli, Klebsiella, Citrobacter, Enterobacter*	Test methods varied in their ability to recover specific coliform organisms; At low pH and alkalinity and with high heterotrophic bacterial growth affected the growth of coliforms in Colisure and m-Coliblue 24, Colilert and MI agar could suppress the growth of	Schauer, *et al.*, 2007

Colitag		*Aeromonas*, while other media could not; chemical characteristics of media and heterotrophic growth affected the performance of media.	

MTF- Multiple Tube Fermentation
MF- Membrane Filtration
LTB- Lauryl Tryptose Broth
BGLB- Brilliant Green Lactose Broth
MLSB- Membrane Lactose Sulfate Broth
LS- Lactose Sulfate
CC- Chromocult Coliform agar
ISO-International Standards Organization
APHA-American Public Health Association
LTTC- Lactose Tergotol-7 agar
HECM- Harlequin ™ E. coli/Coliform Medium
CECM- Chromogenic E. coli/Coliform Medium

2.1.1. Currently available bacteriological analytical methods in Sri Lanka and their limitations

Sri Lanka Standards Bureau (SLS 614: Part 2: 1983) recommends two standard methods to detect the presence of coliform bacteria namely, the Multiple Tube Fermentation (MTF)/Most Probable Number (MPN) method and the Membrane Filtration (MF) method, based on lactose fermentation ability of the coliform group. SLS 614: Part 2: 1983 standard covers the details of sampling techniques, media preparation, methodology, culture requirements, etc. All government and non-government institutes are strictly adhered to the SLS standards and follow the recommended MTF or MF procedures for detecting bacteriological quality of water. Hence, the Sri Lanka National Water Supply and Drainage Board, all universities, other governmental and non-governmental institutes involved in water quality testing (eg: MRI, ITI, SGS Laboratories) currently use the two standard methods as stipulated by the Sri Lanka Standards Bureau (Table 2.2).

However, these two methods are conventional methods, consisting of lot of inherent limitations as described above (Chapter 2, Section 2.1). Therefore, analysis of large number of samples at the same time is a tedious task, especially in cases like epidemiological outbreaks and other emergency conditions due to natural disasters (eg: Tsunami outbreaks, flood conditions, etc.,) or other physical damages occurring in the

distribution systems. As a result, management of epidemiological outbreaks and other sanitary problems from contaminated water is complicated in Sri Lanka, due to delaying of test results.

However, these two techniques have not been properly investigated for their accuracy in Sri Lanka. Further, comparison studies between the two methods have not been done following standard procedures (personal communications with government officials, 2006, 2007). In addition, different laboratories use different brands of media and different procedures for media preparation and even different practices in the same detection procedure (personal communications with laboratory staff of different institutions). As an example, different regional laboratories of the Sri Lanka National Water Supply and Drainage Board use either MTF or MF in their routine analysis using media from different manufacturers. Therefore, uniformity among laboratories in Sri Lanka is another important factor to be considered for quality assurance among water testing laboratories.

Table 2.2 Bacteriological analytical methods used in different institutes of Sri Lanka

Institute	Method used	
Sri Lanka Standard Institute	MPN	MF *
Industrial Training Institute (ITI)	MPN	MF *
National water Supply and Drainage Board (NWS & DB)		
Rathmalana (Central Laboratory)	MPN	
Ambathale	MPN	
BOI (Colombo)		MF
Sarasaviuyana (Peradeniya)		MF
BOI (Awissawella)		MF
Kegalle		MF
Bandarawela		MF
Anuradhapura		MF
Medical Research Institute (MRI)	MPN	MF
National Institute of Health Services, Kaluthara	MPN	
National Building and Research Organization (NBRO)	MPN	
City Analysts' Laboratory, Municipal Council, Kandy	MPN	
City Analysts' Laboratory, Municipal Council, Colombo	MPN	

* only on request by customers

Another factor noticed during lab visits was the inadequate practical knowledge of the involved laboratory staff. For example, determining of proper dilutions while performing MF method was not clear to most of the laboratory staff. Majority of them are not properly trained and they are not updated on currently emerging technologies in water microbiology. Further, the conventional way of thinking and their attitudes towards the modern technology is not satisfactory, compared to other countries of the world. It is very unfortunate that, sharing of knowledge or collaborative work among water laboratories in Sri Lanka is very poor. Another common weakness identified was, the lack of confirmatory testing when testing with MF method. During the enumeration of bacteria, typical/atypical colonies and background growth are not considered as important. Therefore, there is a tendency of obtaining misleading data due to inaccurate interpretations. Lacking of expertized technical staff and training programmes on this specialized area are major constraints in developing quality assurance of bacteriological testing in Sri Lanka. However, most of the technical staff involved in water quality testing in different institutes is keen on adopting more efficient, rapid and easy methods (personal communications with laboratory management and technical staff members in selected institutes, 2007). Most of them complained about the time lag, intense preparations required and other difficulties encountered with the conventional methods.

Further, sensitivity testing of the two conventional methods for analyzing different source water types under various conditions has not been properly investigated in Sri Lanka. Up to now, introducing of new techniques in bacteriological testing in Sri Lanka has not been reported at National level. Therefore, this study investigated the performance of currently available conventional bacteriological methods compared with other alternative methods for detecting and enumeration of total coliform bacteria and *Escherichia coli* for an efficient bacteriological quality assurance in Sri Lanka.

2.2. OBJECTIVES OF THE STUDY

The main objective of this study was to investigate the most appropriate and sensitive bacteriological analytical method/s for detecting and enumeration of total coliform bacteria and *Escherichia coli* for bacteriological quality assurance of water and to recommend the best alternative method/s to the Sri Lanka Standards Institute.

In order to achieve this main objective, the research was conducted with the following specific objectives;

1. To compare four different bacteriological analytical methods with the conventional SLS reference method (MPN, SLS 614; Part 2: 1983) for the detection and enumeration of total coliforms and *Escherichia coli.*
2. To recommend the most sensitive and accurate microbiological methods for different types of analysis;
    a) Routine drinking water samples
    b) For different types of other water sources (bottled water, well water, surface water, sewage effluent)

Other scopes of the study;

1. To investigate the sensitivity of each method for detecting total coliforms and *Escherichia coli* by comparing their confirmation rates.
3. To identify the limitations (time required, other practical aspects, skilled labour, etc.,) involved with each method.
4. To do a cost benefit analysis for each method in order to find out the most economically feasible, efficient method.
5. Identification of certain bacterial species in different sources (by using biochemical tests and api 20 E identification strips).
6. To find out the relationship between api 20E identification strips and conventional biochemical tests in identification of bacteria.

2.3. MATERIALS AND METHODS

A preliminary study was conducted to examine the performance of several alternative methods compared to the Sri Lanka Standard MTF method (by reviewing previous studies by other researches as described in the Table 2.1 on comparison of microbiological methods), using simple statistical analysis of variance.

2.3.1. Samples used for analysis

Samples were collected from different sources in Kandy, the hill capital of Sri Lanka (between 7° 18' N and 80^0 39' E; elevation: 510 m from mean sea level; average monthly temperature: 23 °C- 27.5 °C; annual rainfall: 1500 mm), with the objective of obtaining a wide range of bacterial populations. The selected sources were; tap water, bottled drinking water, well water, river water and wastewater effluent.

Tap water was collected from the University water supply scheme from a tap at the Department of Botany, University of Peradeniya and confirmed for residual chlorination by using DPD tablets (Hach Company, USA). 1 l plastic drinking bottled water samples of one particular brand commonly available in Kandy district, previously found to be contaminated with fecal bacteria (unpublished data, Department of Botany, Faculty of Science, University of Peradeniya, 2007) were collected as bottled water samples. Non-disinfected well water samples were collected from an open well (in Kandy) with no cover but protected with a 90 cm high outer ring wall. River water samples were collected from River Mahaweli at the Peradeniya water intake of the National Water Supply and Drainage Board, near Kandy. Effluent samples were collected from Hanthana Wastewater Treatment Plant, Kandy, prior to final disinfection.

Water samples were collected regularly on a monthly basis from February 2007 to March 2008, following the guidelines described by the Standard Methods for the Examination of Water and Wastewater (APHA et al., 1995) and Sri Lanka Standard 614: Part 2: 1983 (Bureau of Ceylon Standards, 1983). 1 l sterilized glass bottles were used for collecting tap water and well water samples. River water and effluent water were colleted in to

sterilized 250 ml glass bottles and bottled drinking water in 1 l plastic bottles was used for analysis.

2.3.2. Bacteriological analysis using different bacteriological methods

Collected samples were transported to the laboratory under cold conditions (4 °C) and the analysis was done within 24 hours after collection.

Four bacteriological analytical methods were compared with the Multiple Tube Fermentation (MTF)-SLS reference method (SLS 614: Part 2: 1983) for the detection and enumeration of total coliforms and *E. coli*. The alternative methods included, two MTF methods (American Standards Method, APHA *et al.,* 1995; Colilert method, IDEXX, 2000) and two MF methods, the SLS-MF (SLS 614: Part 2: 1983) and m-coliBlue24 method (Hach, USA). Each sample was analysed in two replicates with appropriate dilutions as explained in Table 2.3. Different sample volumes of water were mixed with different volumes/weights of media appropriately (Table 2.3). To obtain the correct dilution for different water types, several trial experiments were conducted and obtained readable counts. Therefore, the dilutions given in the table 2.3 were found to be correct for each water type.

2.3.2.1. Multiple Tube Fermentation (MTF) techniques

SLS reference method (SLS 614: Part 2: 1983)

Sri Lanka Standards MTF method (SLS 614: Part 2: 1983) was used as the reference method. It consists of two basic tests; a presumptive test followed by a confirmed test for detecting total coliform and *E. coli* bacteria in water.

Presumptive test

Different sample volumes with appropriate dilutions were inoculated in to tubes of double strength (DS)/single strength (SS) MacConkey (Himedia, India) medium (Table 2.3) with inverted Durham tubes (five-tube MPN). All the tests were conducted in replicates. Inoculated fermentation tubes were then incubated at 36.0 °C ± 0.5 °C (Genlab INC 190 C

UK) and were examined for the production of acid (by changing its colour from red to orange yellow) and gas in inverted Durham tubes, after 24 to 48 ± 2 hours or earlier. All tubes showing acid and gas (or gas only) at the top of the Durham tube were recorded as presumptive positives and continued for the confirmation step. All the tubes with no acid and gas (or gas) formation were recorded as presumptive negative tests.

Table 2.3 Different sample and media volumes used in MTF methods

	MTF							
Source	**SLS**			**APHA**			**Colilert**	
	Vol./ml sample	Mac Conkey	Tubes	Vol./ml sample	LTB	Tubes	Vol./ml sample	Tubes
Tap	50	50 (DS)	1 x 2	10	10 (DS)	10 x 2	20	5 x 2
Bottle	50	50 (DS)	1 x 2	-	-	-	10	3 x 2
	10	10 (DS)	5 x 2	10	10 (DS)	10 x 2	1	3 x 2
	1	5 (SS)	5 x 2	-	-	-	0.1	3 x 2
Well	10	10 (DS)	5 x 2	10	10 (DS)	5 x 2	10	3 x 2
	1	5 (SS)	5 x 2	1	10 (SS)	5 x 2	1	3 x 2
	0.1	5 (SS)	5 x 2	0.1	10 (SS)	5 x 2	0.1	3 x 2
	0.01	5 (SS)	5 x 2	0.1	10 (SS)	5 x 2	0.1	3 x 2
River	0.1	5 (SS)	5 x 2	0.1	10 (SS)	5 x 2	0.1	3 x 2
	0.01	5 (SS)	5 x 2	0.01	10 (SS)	5 x 2	0.01	3 x 2
	0.001	5 (SS)	5 x 2	0.001	10 (SS)	5 x 2	0.001	3 x 2
	0.0001	5 (SS)	5 x 2	0.0001	10 (SS)	5 x 2	0.0001	3 x 2
Effluen	0.01	5 (SS)	5 x 2	0.01	10 (SS)	5 x 2	0.01	3 x 2
t	0.001	5 (SS)	5 x 2	0.001	10 (SS)	5 x 2	0.001	3 x 2
	0.0001	5 (SS)	5 x 2	0.0001	10 (SS)	5 x 2	0.0001	3 x 2
	0.0000			0.0000			0.0000	
	1	5 (SS)	5 x 2	1	10 (SS)	5 x 2	1	3 x 2

DS- double strength medium SS- single strength medium

Confirmation test

Two or three loopfulls from each of the presumptive positive tubes were subcultured into two sets of Brilliant green lactose bile broth (BGLB, Himedia, India)) tubes and peptone water tubes (preheated to 44.5 °C). One set of the inoculated BGLB and peptone water (Himedia, India) tubes was incubated for 6-24 hours at 44.5 ± 0.1 °C, and the other set of BGLB tubes was incubated at 36 ± 1 °C for 24 to 48 ± 2 hours. Tubes were examined for the production of gas for the confirmation of total coliforms in BGLB tubes. Confirmation of *E. coli* was done using the Indole reaction, by adding 0.2-0.3 ml of Kovac's reagent (Himedia, India) to the peptone water tubes. Immediate appearance of a red colour ring on the upper layer was considered as a positive indole reaction. Number of positive tubes for each set of the appropriate volumes of the sample was recorded and the relevant MPN value was calculated per 100 ml of the sample by using MPN tables for total coliforms and *E. coli* separately.

American Standards method (APHA *et al.*, 1998)

Presumptive test

Different sample volumes with appropriate dilutions were inoculated in to double strength (DS)/single strength (SS) Lauryl tryptose broth (LTB, Himedia, India) tubes (Table 2.3) with inverted Durham tubes (five-tube MPN). All the tests were conducted replicated. Inoculated fermentation tubes were then incubated at 36.0 °C ± 0.5 °C (Genlab INC 190C, UK) and were examined for the growth (turbidity change) and gas in inverted Durham tubes, after 24 to 48 ± 2 hours or earlier. All tubes showing acid and gas sufficient to fill the concavity at the top of the Durham tube were recorded as presumptive positives were followed with the confirmation step. All the tubes with no acid and gas (or gas) formation were recorded as presumptive negative tests.

Confirmation of coliforms and *E. coli*

Two to three loop fulls from each of the presumptive positive tubes were sub-cultured into Brilliant green lactose bile broth (BGLB) and were incubated for 24-48 hours at 36 ± 0.5 °C. Formation of gas in any amount in the inverted vial was considered a positive confirmed result for total coliforms. Growth from Lauryl tryptose tubes were inoculated to EC-MUG broth (Hach, USA) and the tubes were incubated for 24± 2 hours at 44.5± 0.1 °C. Tubes showing fluorescence after keeping under long wave (366nm) ultra violet (UV) illumination were confirmed as *E.coli* positive tubes.

Number of positive tubes for each set of the appropriate quantities of inoculum for total coliforms and *E.coli* were recorded and the relevant MPN value was calculated per 100 ml of the sample by using MPN tables for total coliforms and *E. coli* separately.

Colilert method (IDEXX, USA)

Three tube MPN technique was followed as described in the *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 1995) by using Colilert medium (IDEXX, USA) using the same dilutions used in the other two MTF methods. Colilert powder was mixed with appropriate volumes of water samples (Table 2.3). Appropriate weights of Colilert powder (IDEXX) were mixed with appropriate volumes of each dilution of the test samples. Sterilized distilled water was added to each tube to obtain the final volume and mixed well (colourless after mixing) and were incubated at 36 ± 0.5 °C for 24 hours or less.

A yellow colour after incubation was considered as a positive total coliform test and florescence under UV illumination (366 nm) was considered as *E. coli* positive.

Confirmation of total coliforms and *E. coli*

Although further confirmation is not required according to the manufacturer, a percentage of total coliform and *E. coli* positive tubes were further confirmed using conventional biochemical tests. 20 % of the total coliform positive tubes (which changed from colourless to yellow) were confirmed for total coliforms by inoculating 2 to 3 loop fulls of the culture in to BGLB (Himedia, India) tubes incubated at 36 ± 1 °C for 22 ± 2 h. All tubes showing gas formation were considered as total coliform bacteria. 20 % of the

yellow and fluorescing tubes (total coliform and *E. coli* positive) were confirmed for *E. coli* by inoculating into BGLB and peptone water tubes and incubated at 44.0 - 44.5 °C for 22 ± 2 h. Similarly, 10 % of the yellow but non-fluorescing tubes (total coliform positive but *E. coli* negative) were tested for false negative *E. coli* confirmation. Confirmation of *E. coli* was done by observing gas in BGLB and by the indole reaction as described above. Number of yellow colour and fluorescing tubes were converted in to MPN counts using MPN tables and recorded as cfu per 100 ml of water for total coliforms, and for *E. coli* respectively.

2.3.2.2. Membrane Filtration (MF) techniques

SLS -MF method (SLS 614: Part 2: 1983)

Most of the major laboratories of the Sri Lanka National Water Supply and Drainage Board follow the MF technique as stipulated by the Sri Lanka Standards Bureau (SLS 614: Part 2: 1983). Therefore, this method was considered as one of the alternative methods compared to the SLS-MTF method and referred to as SLS-MF method in this study. Media preparation and inoculation were conducted according to the SLS 614. Appropriate volumes and dilutions of water samples (Table 2.4) were filtered through the membrane filtration apparatus (Pyrex, Germany) using sterilized membrane filters (Sartorius, Germany) with 0.45 µm pore sizes. (Appropriate volumes of sample were selected by conducting trial experiments for different source water types). Membrane filters were aseptically placed on pre sterilized absorbent pads (Sartorius, Germany), saturated with 3 ml of M-endo medium (Himedia, India) and 3 ml of M-FC medium (Himedia, India) and were incubated at 36 ± 1 °C and at 44.5 °C (for 24 hours), for the detection of total coliforms and *E. coli* respectively.

Table 2.4 Different sample and media volumes used in MF methods

	MF methods		
	SLS-MF		m-Coliblue24
	M-endo	M-FC	
Source	sample/ml	sample/ml	sample/ml
Tap	100	100	100
Well	5	10	10
	10	20	0.1
			0.01
Bottle	100	100	100
River	0.01	0.01	0.01
	0.001	0.001	0.001
	0.0001	0.0001	0.0001
Effluent	0.0001	0.0001	0.0001
	0.00001	0.00001	0.00001

Enumeration of total coliforms and *E. coli*

Typical red colour colonies with metallic sheen on M-endo medium were counted as total coliforms. Both pink/red colour colonies without metallic sheen were also considered as total coliforms. Presence of atypical colonies which were cream or white in colour was recorded. If any background growth was observed it was also recorded. Blue colour colonies formed on M-FC medium were counted as *E. coli* colonies. Atypical colonies of different colours were also recorded. Both total coliform and *E. coli* counts were converted in to colony forming units (cfu)/cells per 100 ml of water.

m-ColiBlue24 method (Hach, 2000)

Appropriate volumes and dilutions of water samples were filtered through the membrane filter as described in 2.3.2.2 and the membranes were placed on pre sterilized absorbent pads saturated with 2.5 ml of m-ColiBlue24 (pre prepared) medium (Hach. 2000). Since the absorbent pad was not fully soaked with the medium it was further soaked by using sterilized distilled water (about 1 ml) and were incubated at 36 ± 1°C (for 24 hours).

Enumeration of total coliforms and *E. coli*

Typical red colour colonies and blue colour colonies appeared on the membranes were counted and recorded as total coliforms (red + blue colonies) and *E. coli* (blue), in cfu per 100 ml of water.

2.3.2.3. Confirmation of total coliforms and *E. coli* in MF methods

20 % of selected typical colonies formed on M-endo and m-ColiBlue24 media were confirmed for total coliforms. Test was performed by using the conventional total coliform confirmational test, by inoculating small portions of colonies in to BGLB tubes and incubating at 37 °C for 24-48 hours. Formation of gas in the Durham tube was considered as positive total coliform confirmed tests. In addition to typical colonies, atypical colonies and background growth on M-endo also were checked for confirmtion using the same procedure.

Similarly 20 % of typical colonies formed on M-FC and m-ColiBlue24 media were subjected to confirmation. In confirmation, small portions of blue colour colonies formed on M-FC and m-ColiBlue24 media were inoculated in to peptone water and incubated at 37 °C for 24h. 0.2-0.3 ml of Kovac's reagent were added to the peptone culture and mixed gently. Immediate appearance of a red colour in the upper layer was recorded as a positive indole reaction for the confirmation of *E. coli*. Atypical colonies and background growth formed on M-FC medium were also confirmed for *E. coli*.

Confirmation rates

Confirmation Rate of each method was calculated as follows;

MPN Confirmation rate = $\frac{\text{Number of positive confirmation tubes}}{\text{Number of positive presumptive tubes}} \times 100$ .....(a)

MF Confirmation rate = $\frac{\text{Number of positive confirmation tests}}{\text{Number of colonies tested for confirmation}} \times 100$ .....(b)

2.3.3. Seasonal variation of bacterial counts

Variation of bacteriological counts was recorded in relation to the rainfall and temperature variation during the experimental time period.

2.3.4. Cost comparison among different methods

Chemical cost, preparation time, equipment and glassware requirement and labour hours was calculated for each method and a comparison was made (The cost data are for the years 2007-2009).

2.4. RESULTS

2.4.1. Bacteriological counts obtained with different methods of analysis

Total coliform and *E. coli* counts present in each sample, collected from different sources were obtained by averaging the counts of two replicates when analyzed by different methods at each sampling time (different months). Table 2.5 shows the mean total coliform counts obtained throughout the experimental period by different methods, when all the water sources were grouped together. Mean (arithmetic) values were obtained by dividing the sums of counts in each method by, the number of samples with valid results.

Table 2.5 Summary of total coliform counts in all water types by different methods (Preliminary study)

Method	Total number of analysis (# = 600)	*Mean of total coliform counts (cfu/100 ml)	Standard Deviation	Minimum (cfu/ 100 ml)	Maximum (cfu/ 100 ml)
SLS	40 x 2 [b=8]	1.1 x10^5	± 3.4 x 10^4	2.5	1.9 x 10^5
APHA	40 x 2 [a=2, b=6]	1.5 x 10^5	± 5.0 x 10^4	2.2	2.5 x 10^5
Colilert	46 x 2 [a=2]	3.1 x 10^5	± 7.9 x 10^4	0.55	3.4 x 10^6
M-endo	38 x 2 [a=5, b=5]	3.9 x 10^5	± 1.1 x 10^5	0.5	6.6 x 10^6
M-coli	43 x 2 [a=5]	2.7 x 10^5	± 5.0 x 10^4	6.0	1.9 x 10^6

Total number of samples (N) = 600; (sources = 5; replicates = 2); (methods = 5); trials = 12
Tap water samples (12 x 2) excluded (duo to lack of contamination)
Number of samples included in analysis n = 48 (60 – 12)
[a] = missing values due to *too numerous to count (TNTC)* values
[b] = missing values due to experimental errors
* = arithmetic mean

Although 600 numbers of analyses were done for total coliform bacteria, the total events could not be included in data analysis due to several reasons. As depicted in the table 2.5, only 414 analytical data were used in the analysis. This was due to excluding tap water samples (12 x 2), as contamination by coliform bacteria was absent in the samples tested throughout the experimental time. In a few cases, the counts were not readable due to too

numerous to count values ([a]). In such cases, all the tubes were positive in MPN methods, or the plates were overgrown by coliform bacteria in MF methods. In a few other cases, the plates were covered by background growth as a mat. Further, in other plates, atypical colonies were overgrown on typical colonies, making it difficult to count typical colonies. Such observation could also be due to selection of lower dilutions for the analysis. If further dilutions were done, countable numbers of tubes and colonies could have been obtained. In a few other samples, the counts were not readable possibly due to some experimental errors ([b]) such as media performance problems (differences observed with the recommended pH values, recommended amounts of medium, etc.,) and the quality of distilled water used in the laboratory, experienced at the beginning of the experiments. The above experimental errors were overcome in the subsequent studies.

Data obtained for total coliform bacteriological counts in water samples were different when analyzed by five different methods (Table 2.5). Zero counts obtained by tap water (throughout the study period) and few bottle water samples were excluded in the analysis. Both minimum (for bottle water) and maximum countable total coliform values were obtained by M-endo medium, in comparison to other methods. When considering the mean total coliform counts in all water sources, SLS method gave the minimum numbers (1.1×10^5), while the maximum was obtained by M-endo method (3.9×10^5) (Table 2.5).

The differences of alternative methods compared to the reference SLS-MTF method was statistically analyzed using Least Squares Mean separation test as described in the section 2.4.1.2.

Table 2.6 shows the mean *E. coli* counts obtained throughout the experimental time by different methods, when all the water sources were grouped together.

Minimum *E. coli* counts were obtained by APHA method and the maximum was recorded by M-FC, in comparison to the other methods. When considering the mean *E. coli* counts in all water sources, SLS method gave the minimum counts (4.4×10^4), and the maximum of 1.5×10^5 were obtained by the two enzymatic methods Colilert and m-ColiBlue24 (Table 2.6).

Table 2.6 Summary of *E. coli* counts in all water types by different methods (Preliminary study)

Methods	Total number of analysis (# = 600)	*Mean *E. coli* counts (cfu/100 ml)	Standard Deviation	Minimum (cfu/ 100 ml)	Maximum (cfu/ 100 ml)
SLS	$40 \times 2^{(b=8)}$	4.4×10^4	$\pm 2.4 \times 10^4$	4.0	1.3×10^5
APHA	$40 \times 2^{(a=4, b=4)}$	9.6×10^4	$\pm 3.4 \times 10^4$	1.0	1.4×10^5
Colilert	$46 \times 2^{(a=4)}$	1.5×10^5	$\pm 3.1 \times 10^4$	4.1	1.1×10^6
M-FC	$38 \times 2^{(a=5, b=5)}$	8.7×10^4	$\pm 2.8 \times 10^4$	86.0	1.2×10^6
M-coli	$43 \times 2^{(a=5)}$	1.5×10^5	$\pm 5.0 \times 10^4$	12.5	1.0×10^6

Total number of samples (N)= 600; (sources=5); (replicates = 2); (methods = 5); trials = 12
Tap water samples (12 x 2) excluded (duo to absence of contamination)
Number of samples included in analysis n = 48 (60 – 12)
[a] = missing values due to *too numerous to count (TNTC)* values
[b] = missing values dué to experimental errors
* = arithmetic mean

2.4.1.1. Bacteriological counts in different sources by different methods

To study the method performance in relation to different water source, the counts of both bacterial types detected by different methods were considered separately for each water source and analyzed separately.

Total coliform counts in different sources by different methods

As described before, there was no total coliform contamination in tap water samples, throughout the experimental time period. Total values of the total coliform counts detected in different source water types, throughout experimental time period, when analyzed by five different methods, are presented in Figure 2.1. Means, Standard deviations, minimum and the maximum counts in different water sources by different methods are depicted in Table 2.7

Bottled water samples: Total coliform counts varied from $0 - 3.3 \times 10^2$ cfu/100 ml in replicate counting. Total counts were differently enumerated by five methods. The minimum countable value was detected by APHA (1.1 x 10 cfu/100 ml), and the maximum, by m-ColiBlue24 (4.9×10^2 cfu/100 ml). m-ColiBlue24 method enumerated

more than 25 times higher counts than that of the SLS and APHA methods (Figure 2.1-a). Similarly, the highest mean counts were detected by m-ColiBlue24 method which is 40 times higher than that of the SLS and APHA methods (Table 2.7).

Well water samples: Total coliform counts ranged from 1.1 cfu/100 ml (APHA) to 9 x 10^3 cfu/100 ml (m-ColiBlue24) during the experimental time period. Mean and total counts were highest in m-ColiBlue24 method compared to other methods. Mean counts were nearly 10 times higher (Table 2.7), while total counts were nearly 7 times higher SLS and APHA methods (Figure 2.1-b).

River water samples: The counts ranged from 4.0 x 10^2 (APHA) to 1.0 x 10^6 (M-endo), during the analysis. The highest mean counts were given by m-ColiBlue24, which was 7 times higher than the lowest counts given by APHA and SLS methods. Total counts given by m-ColiBlue24 method was 6 times higher than the SLS counts (Figure 2.1-c).

Effluent water samples: The highest total coliform counts were detected in effluent samples and it ranged from 2.0 x 10^3 (SLS) to 6.6 x 10^6 (M-endo), during the analysis. The highest and the lowest mean counts were detected by SLS and M-endo (Table 2.7), which was nearly 3 times higher than the SLS. The highest total counts were given by M-endo, which was nearly 3 times higher than the lowest SLS counts (Figure 2.1-c).

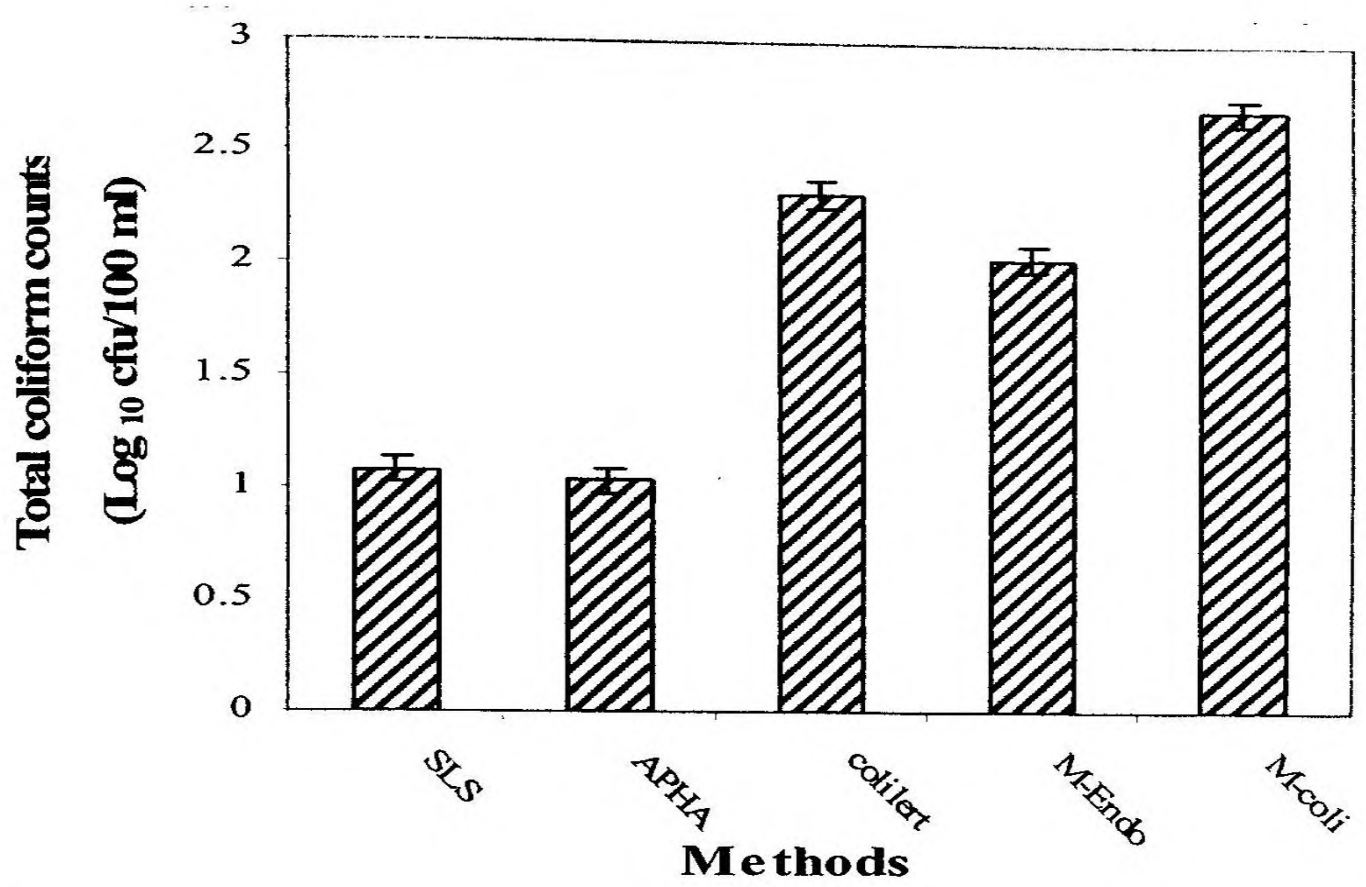
The differences were statistically analyzed and presented in the section 2.4.1.2.

Table 2.7. Summary of total coliform counts in different water types by different methods (Preliminary study)

Source	Methods	Sample #	Mean total coliform counts (cfu/100 ml)	Standard Deviation	Minimum (cfu/ 100 ml)	Maximum (cfu/ 100 ml)
Bottle	**SLS**	12 x 2	1.0	± 1.6	0	9.0
	APHA	12 x 2	0.9	± 1.5	0	5.5
	Colilert	12 x 2 [a=1]	1.9 x 10	± 5.4 x 10	0	1.9×10^2
	M-endo	12 x 2 [a=3]	1.2 x 10	± 2.5 x 10	0	8.2 x 10
	M-coli	12 x 2	4.1 x 10	± 9.1 x 10	0	4.9×10^2
Well	**SLS**	12 x 2	5.3×10^2	$\pm 6.8 \times 10^2$	5.0	1.8×10^3
	APHA	12 x 2	2.7×10^2	$\pm 4.3 \times 10^2$	1.1	1.7×10^3
	Colilert	12 x 2	1.7×10^3	$\pm 2.1 \times 10^3$	8.0	1.6×10^3
	M-endo	12 x 2 [a=3]	3.1×10^3	$\pm 3.6 \times 10^3$	1.3×10^2	7.0×10^3
	M-coli	12 x 2 [a=2]	4.3×10^3	$\pm 6.8 \times 10^2$	1.2×10^2	9.0×10^3
River	**SLS**	12 x 2	2.3×10^4	$\pm 4.4 \times 10^4$	7.0×10^2	1.6×10^5
	APHA	12 x 2	9.3×10^3	$\pm 9.9 \times 10^3$	4.0×10^2	3.0×10^4
	Colilert	12 x 2 [a=1]	9.7×10^4	$\pm 1.4 \times 10^5$	4.0×10^3	4.6×10^5
	M-endo	12 x 2 [a=3]	1.4×10^5	$\pm 3.0 \times 10^5$	8.0×10^3	1.0×10^6
	M-coli	12 x 2 [a=1]	1.8×10^5	$\pm 3.1 \times 10^5$	1.5×10^4	1.0×10^5
Effluent	**SLS**	12 x 2 [a=2]	4.5×10^5	$\pm 5.9 \times 10^5$	2.0×10^3	2.5×10^6
	APHA	12 x 2 [a=1]	7.1×10^5	$\pm 9.1 \times 10^5$	4.0×10^3	3.2×10^6
	Colilert	12 x 2 [a=2]	1.3×10^6	$\pm 1.2 \times 10^6$	9.0×10^3	4.6×10^6
	M-endo	12 x 2 [a=3]	1.4×10^6	$\pm 2.1 \times 10^6$	3.0×10^4	6.6×10^6
	M-coli	12 x 2 [a=1]	8.9×10^5	$\pm 6.2 \times 10^5$	2.0×10^4	2.0×10^6

[a] = missing values due to *too numerous to count (TNTC)* values [b] = missing values due to experimental errors

(a)


Total coliform counts
(Log 10 cfu/100 ml)
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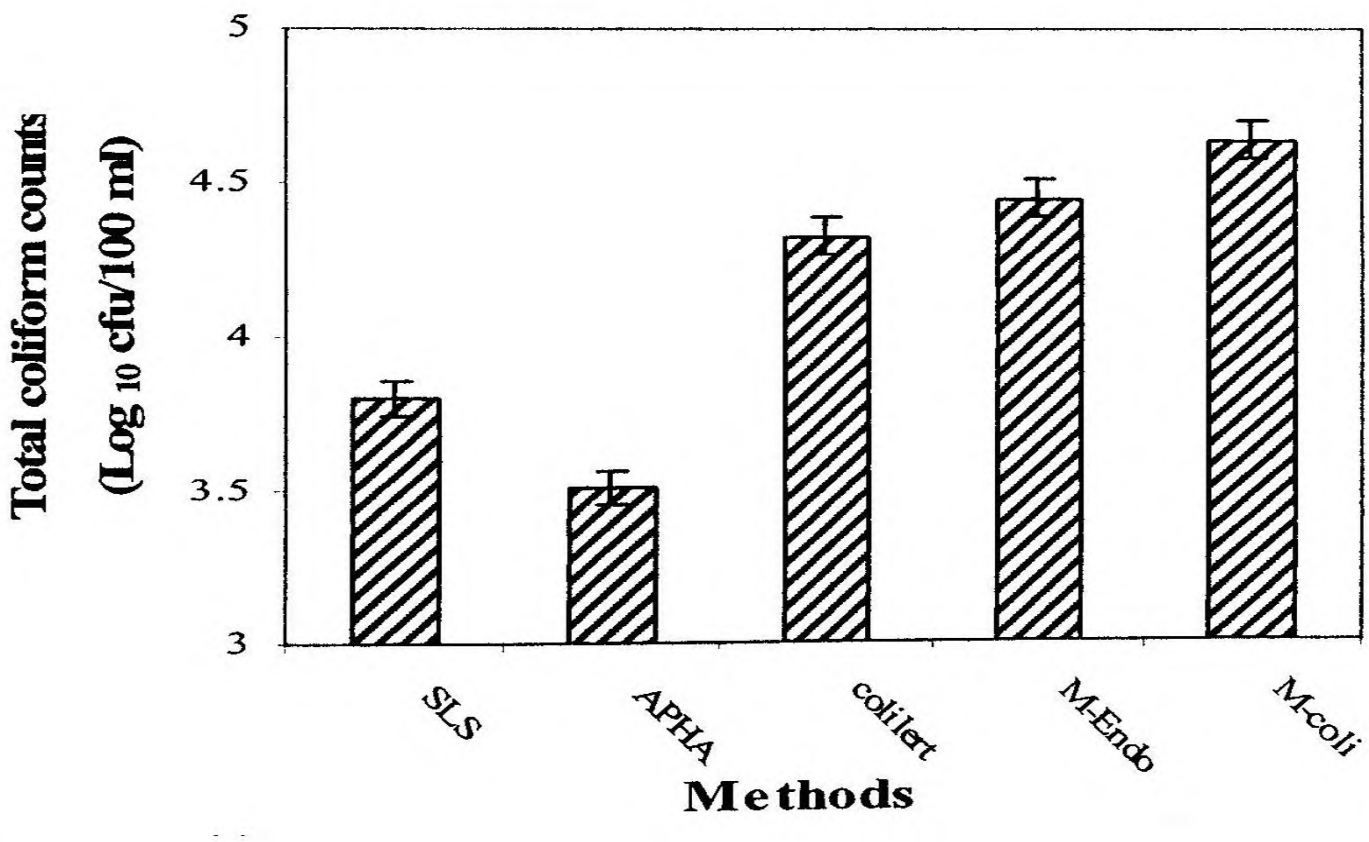
2
1.5
1
0.5
0
SLS
APHA
colilert
M-Endo
M-coli
Methods

(b)


Total coliform counts
(Log 10 cfu/100 ml)
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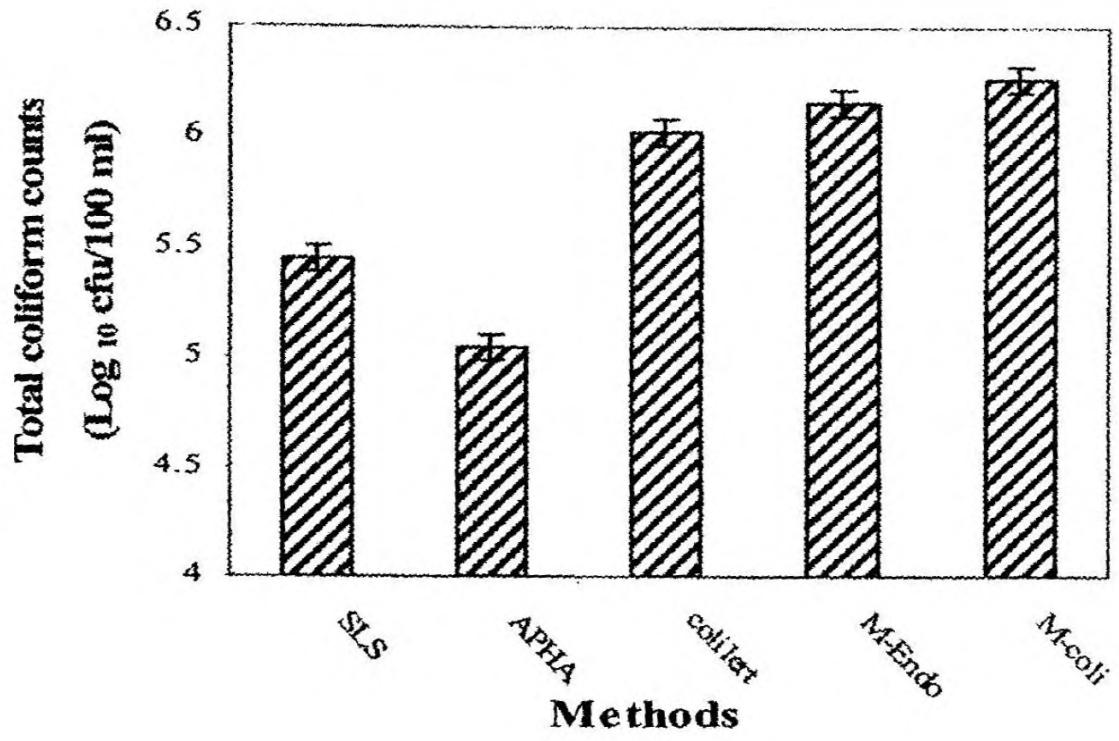
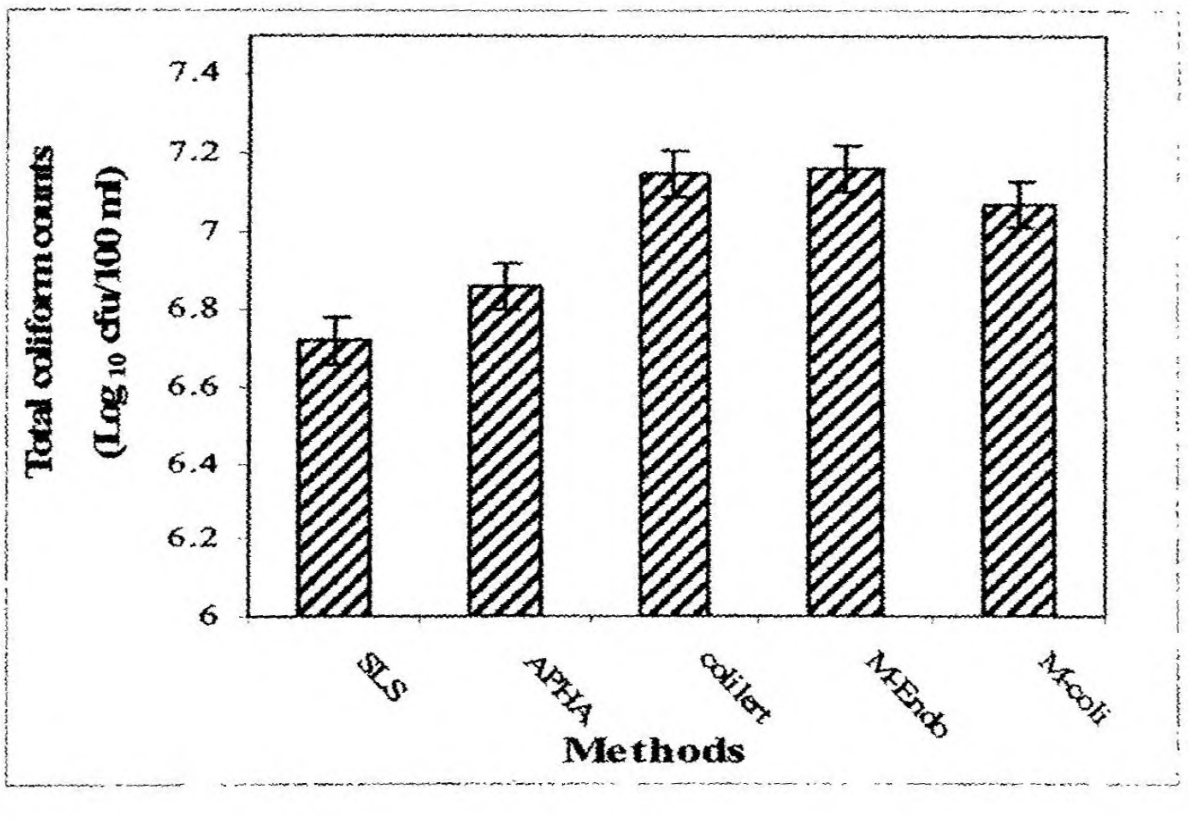
4.5
4
3.5
3
SLS
APHA
colilert
M-Endo
M-coli
Methods

(c)



(d)



(a) Bottle water
(b) Well water
(c) River water
(d) Effluent water

Figure 2.1 Total values of total coliform counts (log $_{10}$ values) in different water sources obtained by five different water testing methods (Preliminary study)

E. coli counts in different sources by different methods

There was no *E. coli* contamination in tap water samples, throughout the experimental time. Total *E. coli* counts detected in different source waters throughout the experimental time were plotted in Figure 2.2 a-d. Summary of *E. coli* counts are given in Table 2.8.

Bottled water samples: *E. coli* were present in only 3 occasions and detected by only two methods (Colilert and M-FC) throughout the experimented time frame and the counts ranged from 0 – 1.5 x 10^2 cfu/100 ml in replicate counting. Mean counts and the total counts were higher in M-FC than in Colilert (Table 2.8 and Figure 2.2 a). Since m-ColiBlue24 plates showed too numerous counts, obtaining valid data was not possible for analysis. In contrast, both APHA and the SLS methods were unable to detect any *E. coli* bacterium, present in bottle water samples throughout the preliminary study.

Well water samples: *E. coli* counts ranged from 1.0 cfu/100 ml (Colilert) to 6 x 10^2 cells/cfu/100 ml (M-colibliue-24) during the experimental time period. Highest and the lowest mean counts were recorded m-ColiBlue24 and SLS respectively (Table 2.8). The lowest total counts were recorded by APHA and SLS, while the highest was given by m-ColiBlue24 (Figure 2.2-b).

River water samples: The counts ranged from 4.0 x 10^2 (APHA) to 1.0 x 10^5 (M-FC), during the analysis. The highest and the lowest mean counts were given by SLS and M-FC respectively (Table 2.8). Total counts were also highest in M-FC method, while SLS showed the lowest count (Figure 2.2-c).

Effluent water samples: The highest *E. coli* counts were also recorded from effluent samples and it ranged from 2.0 x 10^3 (SLS) to 4.0 x 10^6 (m-ColiBlue24), during the analysis. The highest and the lowest mean counts were detected by m-ColiBlue24 and SLS (Table 2.8) respectively. The highest and the lowest total counts were also detected by m-ColiBlue24 and SLS methods respectively (Figure 2.2-d).

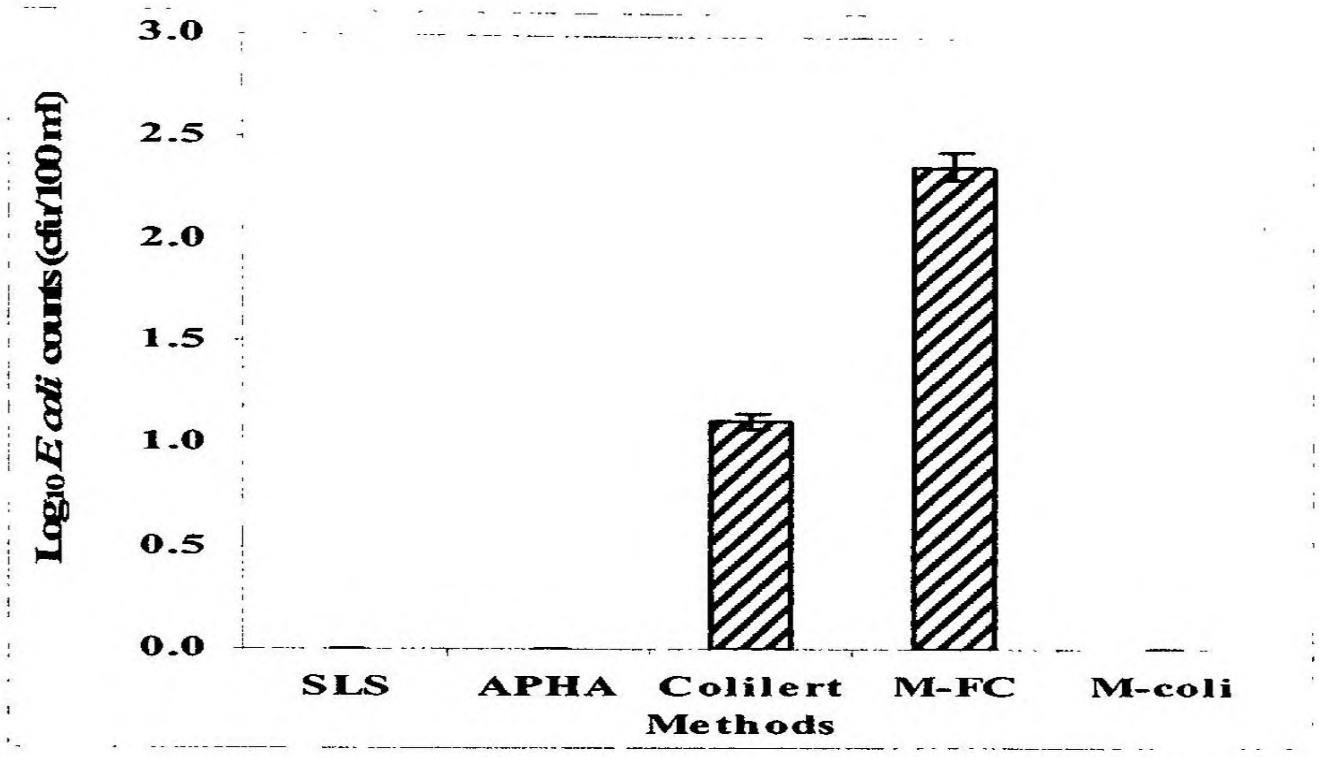
Table 2.8 Summary of *E coli* counts in different water types by different methods (Preliminary study)

Source	Methods	Sample #	Mean *E. coli* counts (cfu/100 ml)	Standard Deviation	Minimum (cfu/ 100 ml)	Maximum (cfu/ 100 ml)
Bottle	**SLS**	12 x 2	0		0	0
	APHA	12 x 2	0		0	0
	Colilert	12 x 2	1.1	± 2.9	1.1	6.9
	M-FC	12 x 2	1.9 x 10	± 4.5 x 10	8.6 x 10	1.5×10^2
	M-coli	12 x 2 [a=2]	-	-	0	-
Well	**SLS**	12 x 2 [b=3]	6.5 x 10	± 5.8 x 10	1.8	1.3 x 10
	APHA	12 x 2 [a=1]	6.7 x 10	± 5.0 x 10	3.5	1.8×10^2
	Colilert	12 x 2 [a=1]	1.2×10^{-2}	$\pm 1.5 \times 10^2$	1.0	1.7×10^2
	M-FC	12 x 2 [a=1, b= 2]	3.2 x 10	$\pm 2.4 \times 10^2$	2.6	4.1×10^2
	M-coli	12 x 2 [a=1]	2.3×10^2	$\pm 2.8 \times 10^2$	5.0 x 10	6.0×10^2
River	**SLS**	12 x 2 [b=3]	3.7×10^3	$\pm 3.1 \times 10^3$	1.0×10^2	1.1×10^4
	APHA	12 x 2 [a=1, b=2]	4.5×10^3	$\pm 4.7 \times 10^3$	4.0×10^2	1.4×10^4
	Colilert	12 x 2 [a=1]	6.6×10^3	$\pm 8.3 \times 10^3$	9.0×10^2	2.8×10^4
	M-FC	12 x 2 [a=2, b=2]	1.4×10^4	$\pm 1.1 \times 10^4$	3.0×10^3	1.0×10^5
	M-coli	12 x 2 [a=1]	6.1×10^3	$\pm 5.2 \times 10^2$	1.0×10^2	2.0×10^4
Effluent	**SLS**	12 x 2 [a=2, b=2]	3.4×10^5	$\pm 4.2 \times 10^5$	2.0×10^3	1.3×10^6
	APHA	12 x 2 [a=2, b=2]	4.8×10^5	$\pm 5.2 \times 10^5$	4.0×10^3	1.4×10^6
	Colilert	12 x 2 [a=2]	4.5×10^5	$\pm 4.2 \times 10^5$	2.3×10^3	1.1×10^6
	M-FC	12 x 2 [a=2, b=1]	4.9×10^5	$\pm 6.1 \times 10^5$	3.0×10^3	1.4×10^6
	M-coli	12 x 2 [a=1]	5.9×10^5	$\pm 7.6 \times 10^5$	3.0×10^3	4.0×10^6

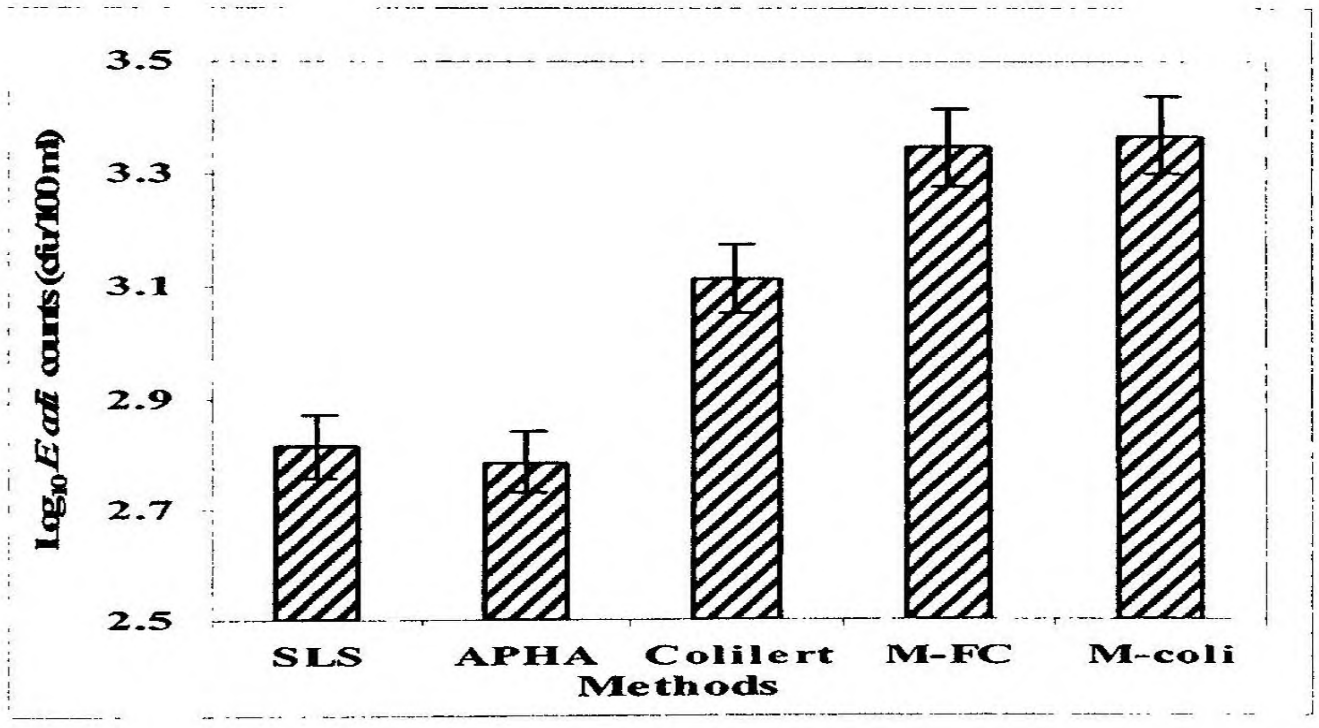
[a] = missing values due to *too numerous to count (TNTC)* values

[b] = missing values due to experimental errors

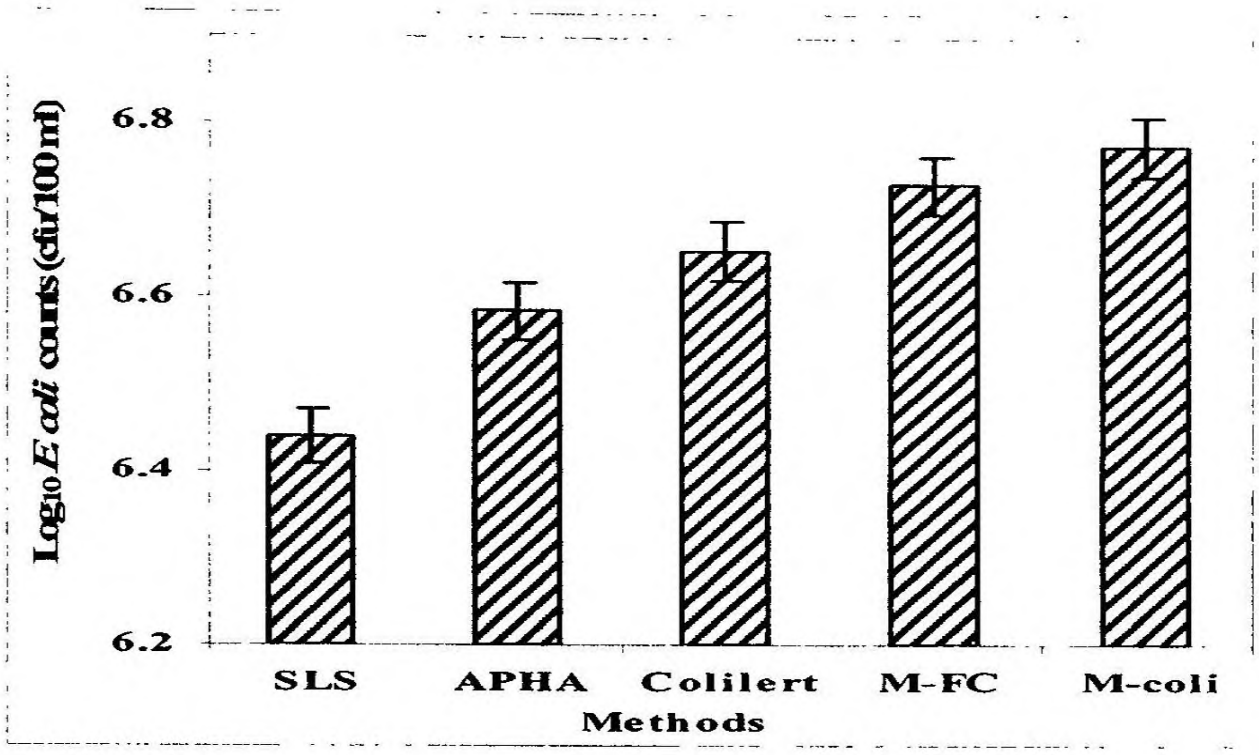
(a)


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Log10 E coli counts (cfu/100ml)
SLS
APHA
Colilert
M-FC
M-coli
Methods

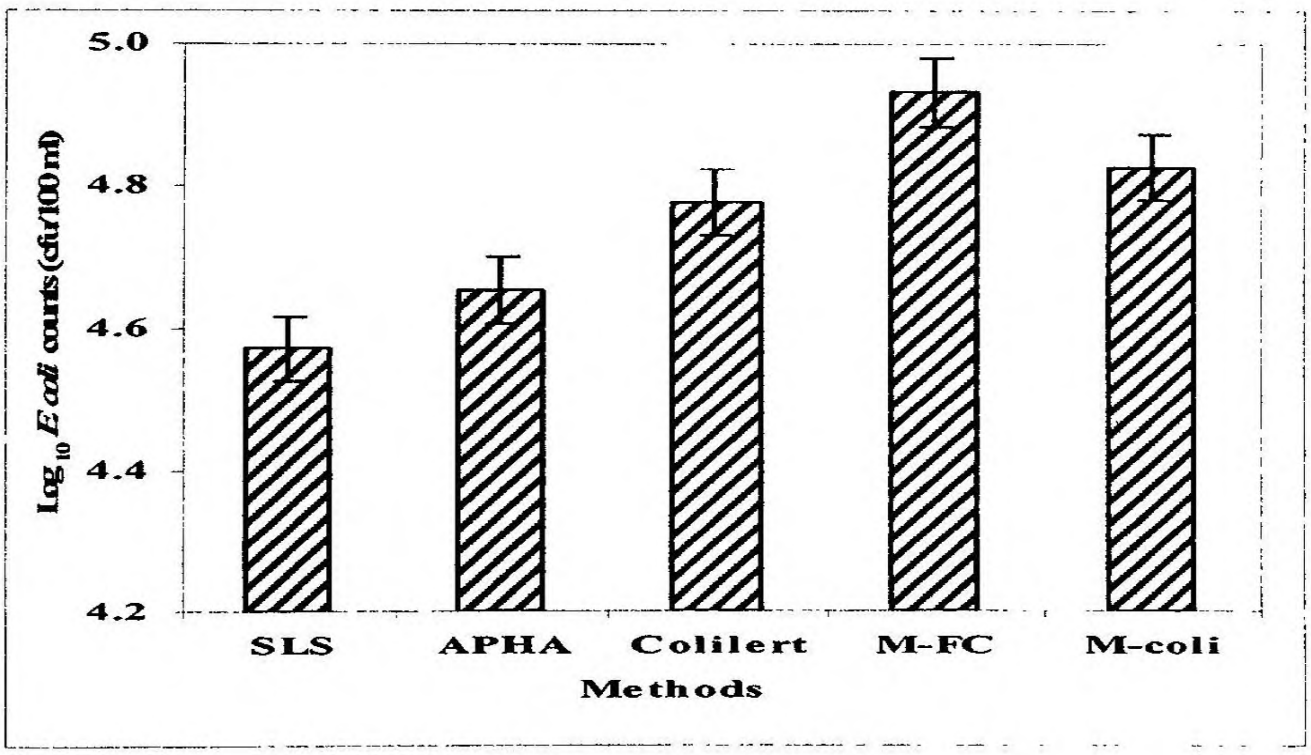
(b)


3.5
3.3
3.1
2.9
2.7
2.5
Log10 E coli counts (cfu/100ml)
SLS
APHA
Colilert
M-FC
M-coli
Methods

(c)



(d)



(a) Bottle water
(b) Well water
(c) River water
(d) Effluents

Figure 2.2 Total *E. coli* counts (log $_{10}$ values) in different water sources obtained by five different water testing methods (Preliminary study)

The differences were statistically analyzed and presented in the section 2.4.1.2.

2.4.1.2. Statistical analysis

SAS System for Windows V8 software (SAS Institute Inc., SAS Online Doc®, Version 8, Cary, NC) was used for statistical analysis of data.

Analysis of variance among source water types in detecting total coliforms and *E. coli*:

To test whether the source water types used in the study were different or not (with respect to contamination level), the data were analyzed using General Linear Model (GLM) test. The Null Hypothesis used was, 'There is no effect from source water types, on bacteriological counts'. If the probability values (p) obtained were, $p \leq 0.05$, the differences were considered statistically significant. For analysis, both total coliform and *E. coli* counts obtained for different water sources, during the whole experimental time period were considered separately. Tap water source has been excluded by the model, due to zero contaminations. The probability values obtained for total coliform bacteria and *E. coli,* were ≤ 0.05.

Analysis of variance among different methods in detecting total coliforms and *E. coli*:

General Linear Model (GLM) test was used to test whether the five methods used in the study were significantly different or not. The Null Hypothesis used was, 'There is no effect from methods used, on bacteriological counts'. If the probability values (p) obtained were, $p \leq 0.05$, the differences were considered statistically significant. For analysis, both total coliform and *E. coli* counts obtained by five different methods, during the whole experimental time period were considered separately. The probability values obtained for total coliform bacteria and *E. coli,* were below 0.05.

Method comparison for the detection and enumeration of bacteria

To obtain the most reliable method, among the five different methods to detect and enumerate bacteria, the probability differences of the different methods were analysed by

using Least Squares Mean Separation Test. The analysis was performed as a pre-planned test, by comparing four alternative methods with the SLS-MTF reference method. For the analysis, all water types were grouped together and blocked. The mean values of five methods were compared by using Complete Randomized Block Design (CRBD), to observe whether the alternative methods used were significantly different with the SLS-MTF reference method in enumerating both bacterial types separately.

Comparison of alternative four methods with the reference SLS-MTF method for the detection of bacteria

Reference SLS-MTF method was compared with the four alternative methods by using Least Squares Mean Separation Test for the detection and enumeration of the total coliform bacteria and *E. coli* present in all water types. The differences were considered statistically significant, in cases where the p-value was ≤ 0.05. Both CFU and MPN results were transformed in to logarithmic values for statistical analysis. Results are summarized in Table 2.9.

According to the p-values in the table 2.9, three methods (Colilert, NWS&DB and m-ColiBlue24) were significantly different with the SLS-MTF reference method in detecting both total coliforms and *E. coli*, when all water sources were grouped together. However, APHA method did not show significant differences in detecting total coliforms or *E. coli,* when all water sources were grouped together.

Table 2.9 Results of the least squares mean separation test to compare alternative methods with SLS-MTF reference method (Preliminary study)

Comparison	p-value for comparison with SLS	
	Total coliforms	*E. coli*
SLS /APHA	0.8226	0.1436
SLS /Colilert	0.0025*	0.0002*
SLS /M-endo	0.0014*	<.0001*
SLS /M-coliBlue24	<.0001*	<.0001*

* $p \leq 0.05$

Comparisons of bacteriological counts in different water sources

The differences of alternative four methods in comparison to the reference SLS-MTF method in different sources, was performed separately by using the same mean separation test (Table 2 10). In analysis, each water source was blocked and analyzed separately by using a complete randomized block design. The differences were considered significant when $p \leq 0.05$.

Total coliform counts in different water sources

As depicted in the Table 2.10, Colilert, SLS-MF (M-endo medium) and m-CoiBlue24 methods were significantly different with the reference SLS-MTF method in detecting total coliform bacteria in bottle water, well water and river water sources. APHA method was not significantly different with the reference method in detecting total coliforms in any of the water sources tested. In effluent samples, only m-CoiBlue24 method showed a significant difference with the reference method. All other alternative methods showed no significant difference with SLS-MTF method in detecting very high concentrations of total coliform counts in effluent samples.

***E. coli* counts in different water sources**

In bottle water samples, Colilert and SLS-MF (M-FC medium) methods were significantly different with the SLS-MTF reference method for enumerating *E. coli* (Table 2.10). However, m-CoiBlue24 method was not included in the statistical analysis, due to too numerous to count (TNTC) colony numbers were observed on plates. In well water samples, all the alternative methods, except APHA method detected significantly higher counts of *E. coli* than the SLS-MTF reference method. In river water samples M-FC and m-CoiBlue24 methods were significantly different at 95% confidence levels ($p<0.05$), while the Colilert method was significantly different with SLS-MTF, at 90% of confidence level ($p<0.1$). APA was not significantly different wit SLS-MTF. In effluent samples, only two enzymatic methods Colilert and m-CoiBlue24 was able to detect *E. coli* counts at significantly higher levels than the SLS-MTF method, while the APHA and the

SLS-MF methods were not significantly different with the reference method in detecting *E. coli*.

Table 2.10 Results of mean separation test for comparison of four methods with SLS-MTF reference method for enumeration of bacteria in different sources (Preliminary study)

Water source	Comparison	p-value for comparison with SLS	
		Total coliforms	*E. coli*
Bottle			
	SLS/APHA	0.1714	-
	SLS/Colilert	0.0100*	0.0009*
	SLS/M-endo[1]/M-FC[2]	0.0003*	<.0001*
	SLS/M-coliBlue24	0.0016*	-
Well			
	SLS/APHA	0.3049	0.2134
	SLS/Colilert	0.0064*	0.0104*
	SLS/M-endo[1]/M-FC[2]	0.0001*	<.0001*
	SLS/M-coliBlue24	<.0001*	<.0001*
River			
	SLS/APHA	0.4512	0.7593
	SLS/Colilert	0.0016*	0.0928**
	SLS/M-endo[1]/MF-C[2]	0.0055*	<.0001*
	SLS/M-coliBlue24	<.0001*	0.0003*
Effluent			
	SLS/APHA	0.7253	0.7238
	SLS/Colilert	0.1220	0.0259*
	SLS/M-endo[1]/M-FC[2]	0.3591	0.1229
	SLS/M-coliBlue24	0.0427*	0.0008*

* p .05 ** p ≤0.1 [1] M-endo medium – for total coliforms [2] M-FC medium – for *E. coli*

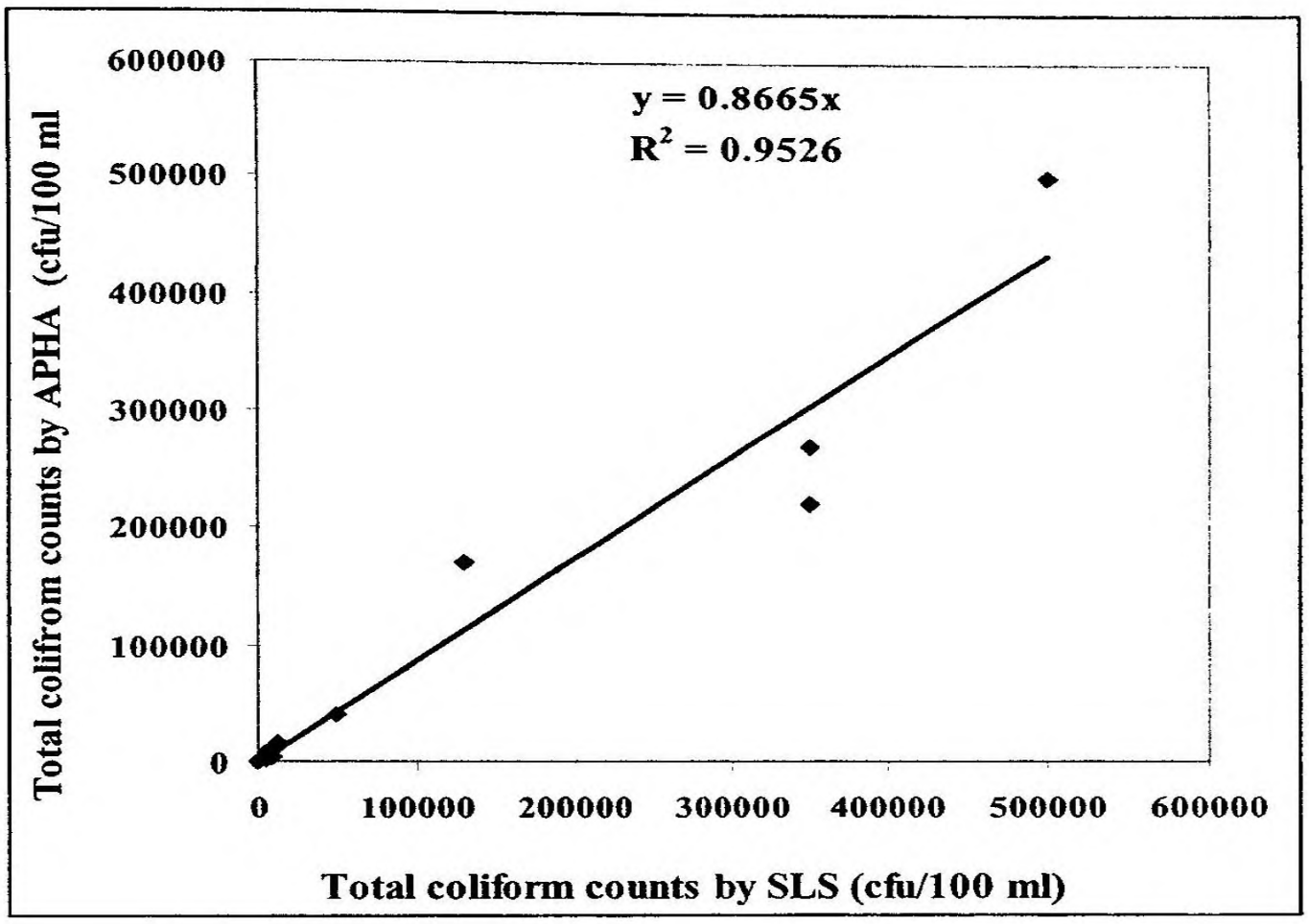
Simple linear model for analyzing the relationships between SLS and the other alternative methods

The relationships between different alternative methods with the SLS-MTF reference method, was analyzed by using the Simple Linear Model test. The relationship was obtained by, $y = mx + c$ equation and the relationship was considered efficient when the R^2 value was closer to 1.0.

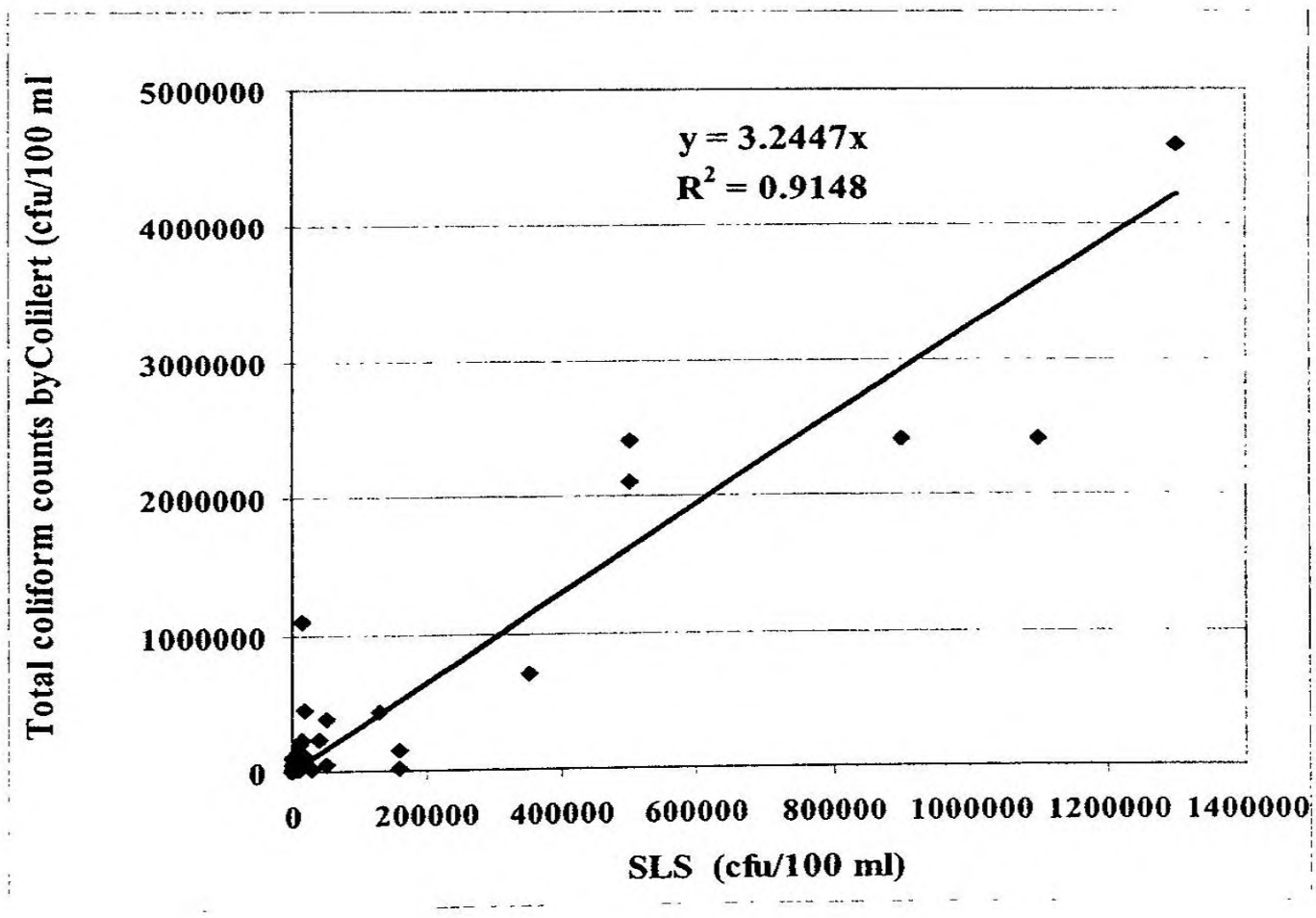
Simple linear relationships between SLS and the other alternative methods in detecting total coliform bacteria

The linear relationships between SLS-MTF reference method with the other alternative methods are shown in Figure 2.3. As depicted in the Figure 2.3-a, although there is a linear relationship between SLS-MTF and APHA (R2 = 0.9526), the counts obtained by APHA are lower than the SLS counts (y = 0.866x). In contrast, all the other alternative methods showed significantly positive linear relationships with the reference SLS-MTF method (Figure 2.3 –b, c, d and e respectively for Colilert, M-endo and m-ColiBlue24 methods). Among them, the most efficient positively significant relationship was observed between SLS-MTF and the m-ColiBlue24 method, having a R^2 value of 0.9463, which means that the variation of SLS counts could explain the variation of Colilert counts by more than 94 % accuracy and m-ColiBlue24 could detect 1.8333 times higher counts (y = 1.8333x), than the SLS-MTF counts. M-endo medium also could detect 1.3693 times higher counts than SLS- MTF. However the highest total coliform counts could be obtained by the Colilert method, which was 3.2447 times higher than the SLS-MTF counts.

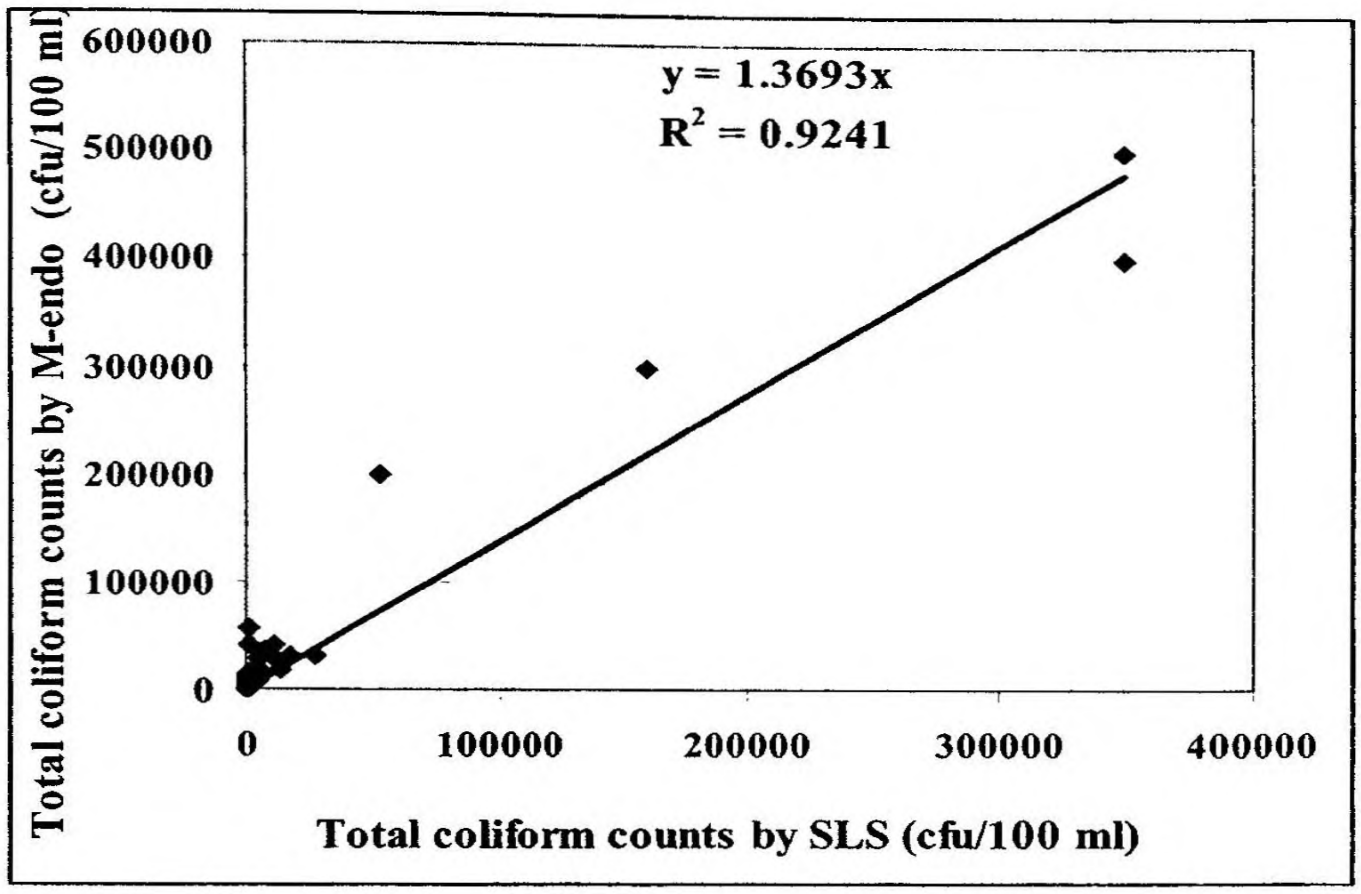
(a)


y = 0.8665x
R² = 0.9526
Total colifrom counts by APHA (cfu/100 ml
Total coliform counts by SLS (cfu/100 ml)
600000
500000
400000
300000
200000
100000
0
0
100000
200000
300000
400000
500000
600000

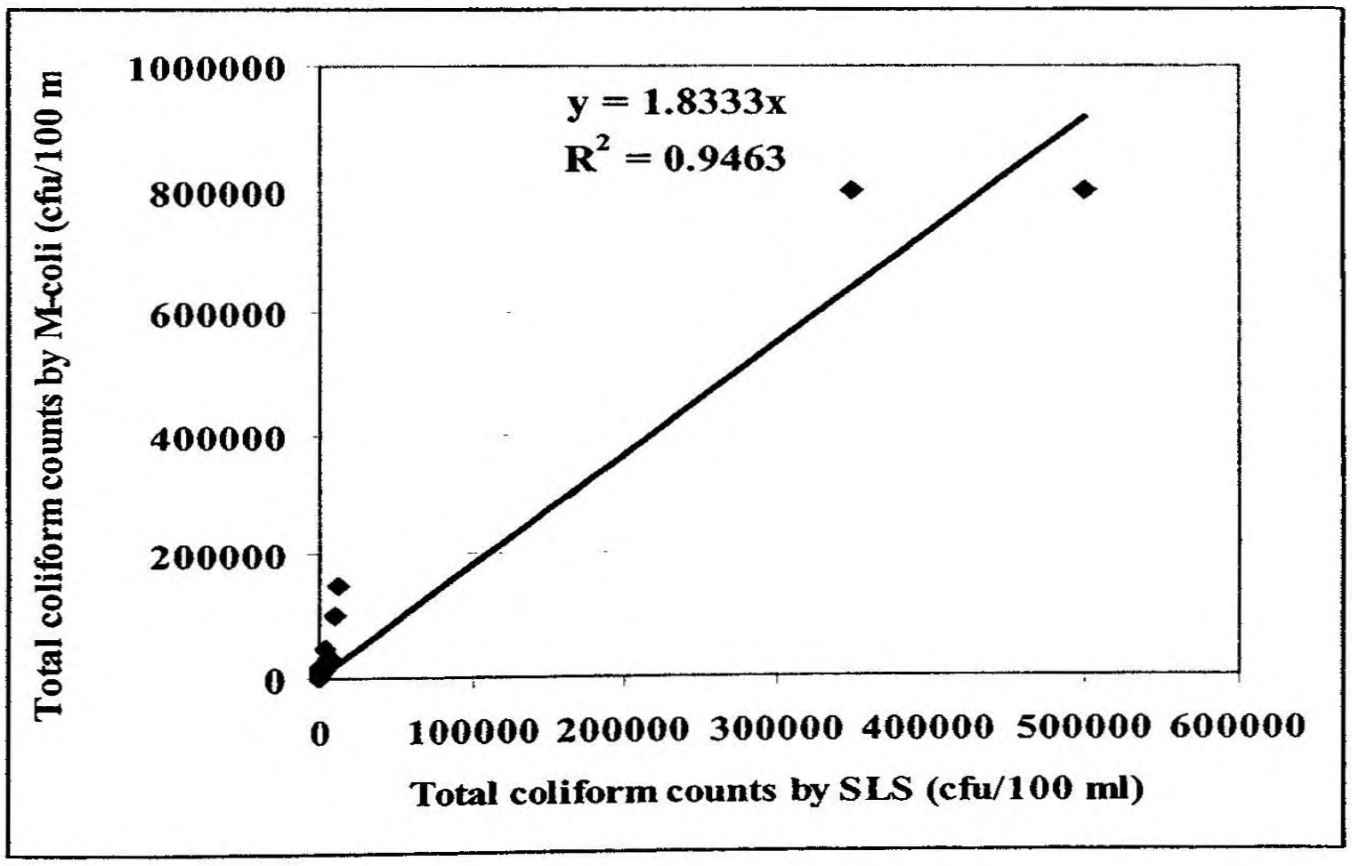
(b)


y = 3.2447x
R² = 0.9148
Total coliform counts byColilert (cfu/100 ml
SLS (cfu/100 ml)
5000000
4000000
3000000
2000000
1000000
0
0
200000
400000
600000
800000
1000000
1200000
1400000

(c)



(d)



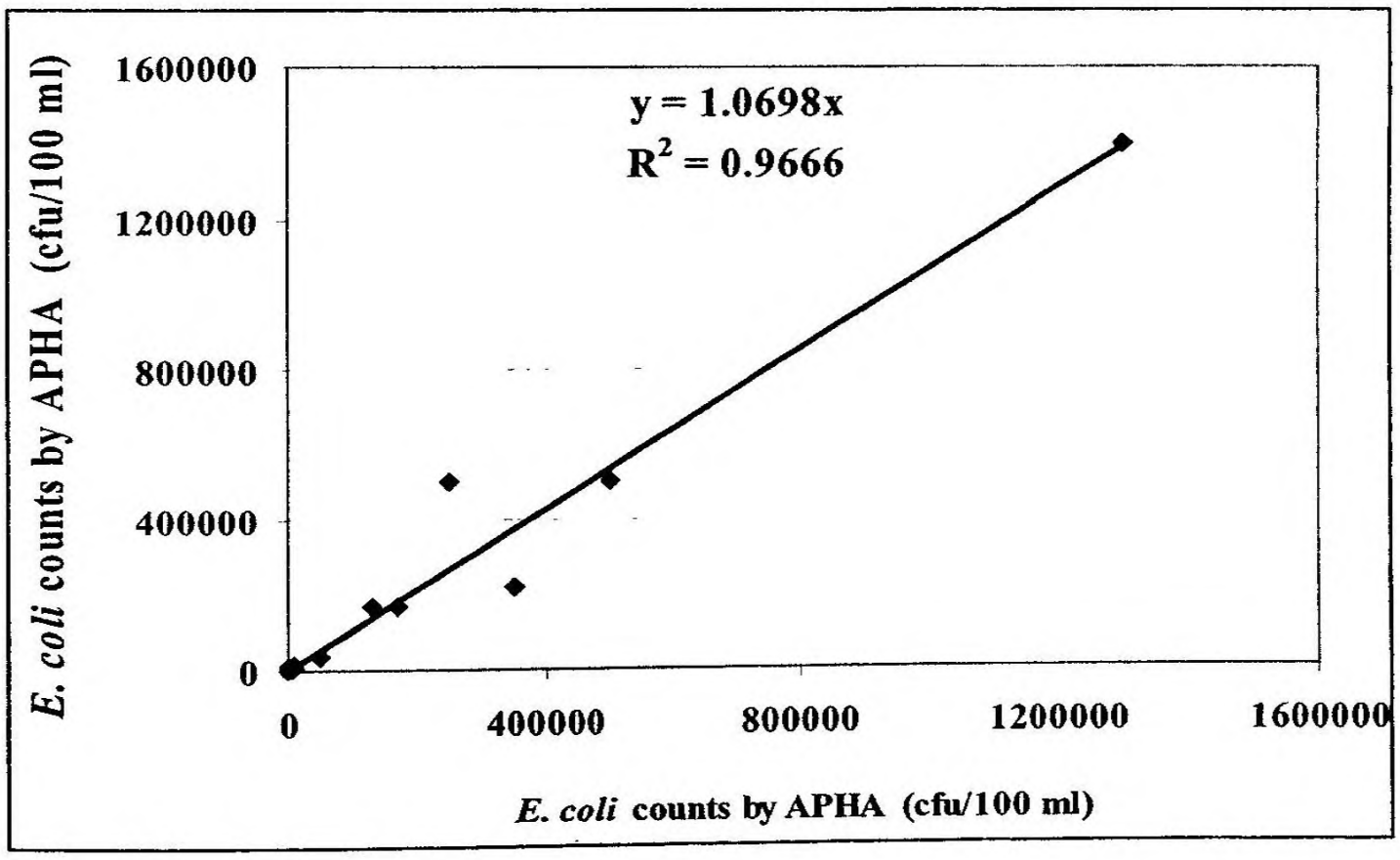
(a) –SLS/APHA
(b) –SLS/Colilert
(c) –SLS/M-endo
(d) –SLS/M-coli

Figure 2.3 Simple Linear Model for comparing SLS and alternative methods for detecting total coliforms (Preliminary study)

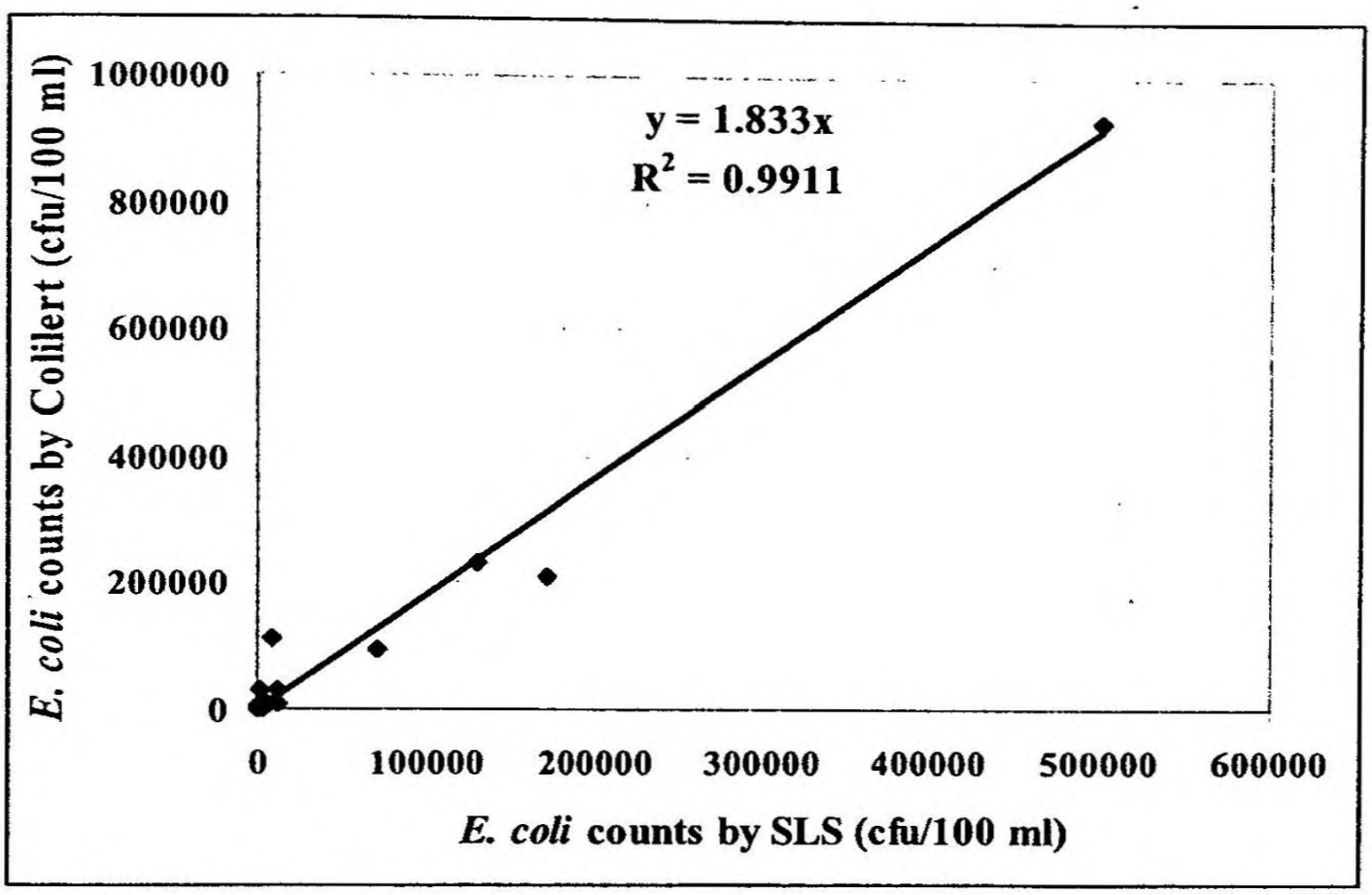
Simple linear relationships between SLS and the other alternative methods in detecting *E. coli*

The linear relationship between SLS-MTF reference method with the other alternative methods are shown in Figure 2.4. In contrast to total coliform detection, all the alternative methods including APHA, showed positive linear relationships with SLS-MTF method in detecting *E. coli* bacteria Figure 2.7 - a, b, c and d for APHA, Colilert, M-FC and m-ColiBlue24 respectively). Among them the most efficient model was shown by Colilert and SLS-MTF having a R^2 of 0.9911, which means that 99% of the SLS variation could explain the variation of Colilert counts in the relationship y = 1.833x. Further, the highest counts were detected by the m-ColiBlue24 method, .which was 2.27 fold higher than that of the SLS-MTF counts.

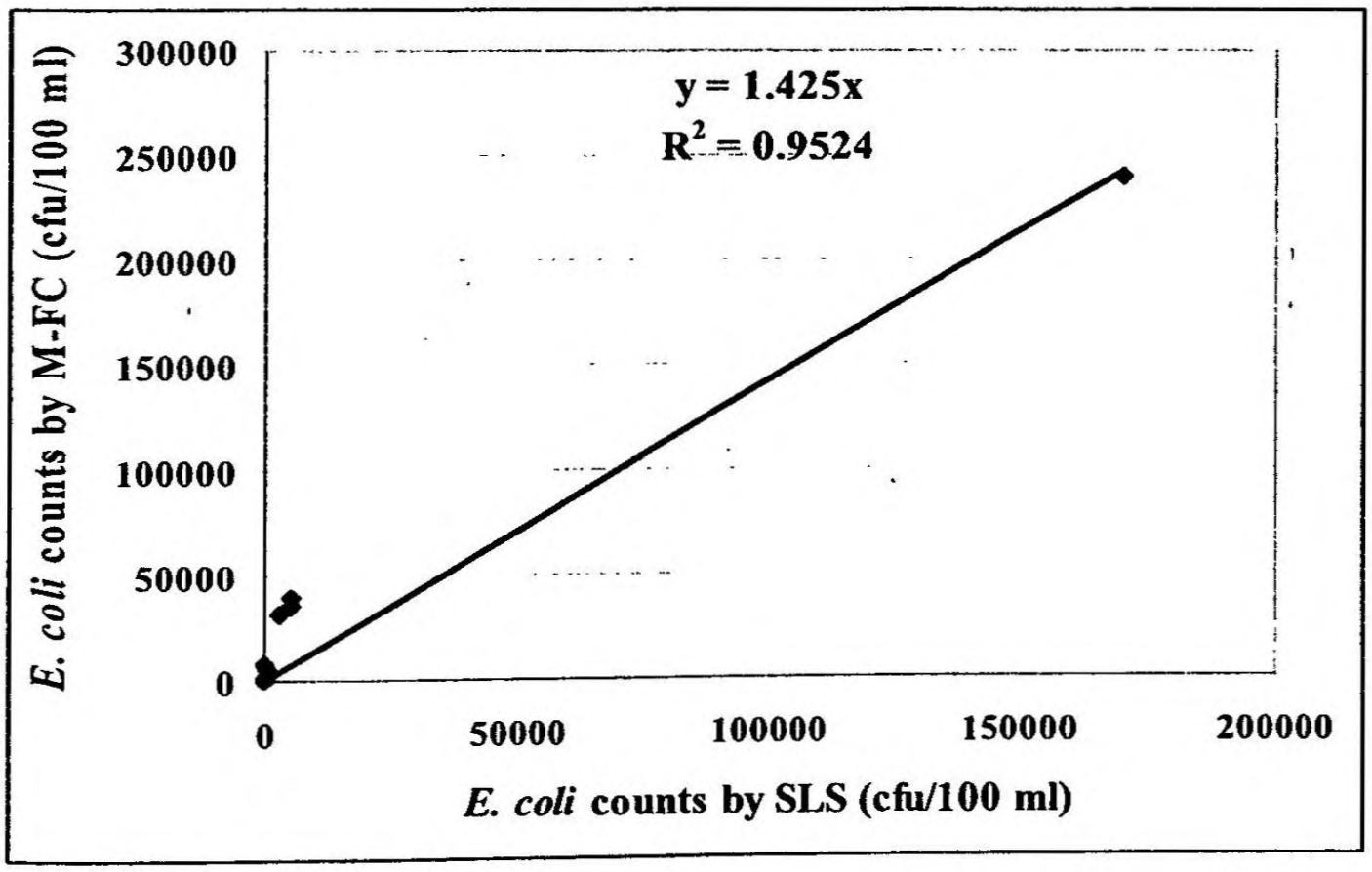
(a)



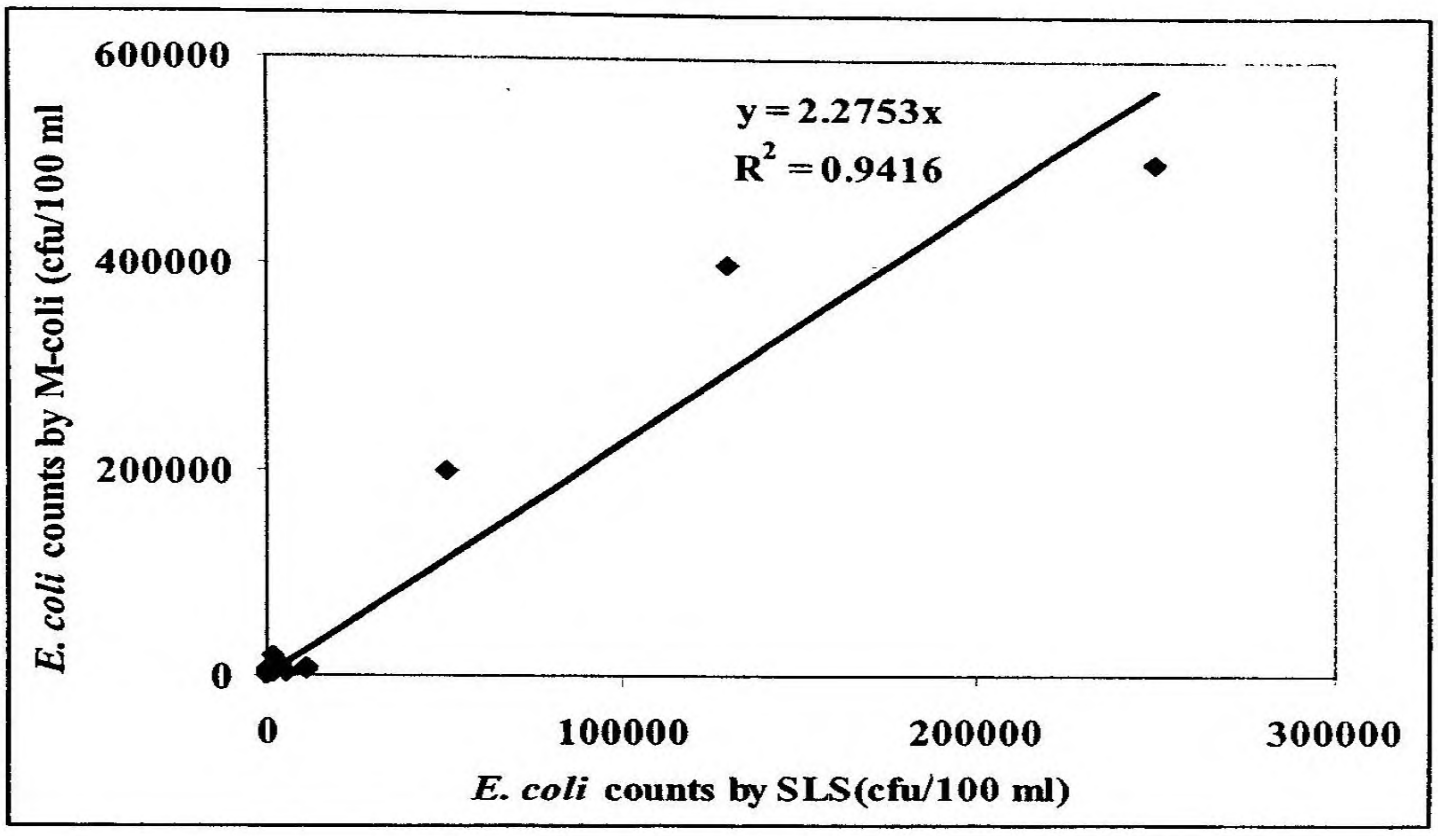
(b)


y = 1.833x
R² = 0.9911
E. coli counts by Colilert (cfu/100 ml)
1000000
800000
600000
400000
200000
0
0
100000
200000
300000
400000
500000
600000
E. coli counts by SLS (cfu/100 ml)

(c)


y = 1.425x
R² = 0.9524
E. coli counts by M-FC (cfu/100 ml)
300000
250000
200000
150000
100000
50000
0
0
50000
100000
150000
200000
E. coli counts by SLS (cfu/100 ml)

(d)



(a) –SLS/APHA
(b) –SLS/Colilert
(c) –SLS/M-endo
(d) –SLS/M-coli

Figure 2.4 Simple Linear Model for comparing SLS and alternative methods for detecting *E. coli* (Preliminary study)

2.4.2. Confirmation rates of bacteria in different methods

2.4.2.1. Confirmation rates of total coliforms

Confirmation rates for total coliform bacteria were obtained as described in the section 2.3.2.3 under Materials and Methods. In the three MPN methods (SLS, APHA and Colilert), confirmation rates were obtained by using numbers of presumptively positive total coliform tubes (Plate2.1) and the confirmed number of total coliform tubes (Plate 2.2) as described in the equation (a) in section 2.3.2.3. In SLS and APHA methods, all the presumptively positive tubes and all the confirmed positive tubes were included in the calculation. However, in Colilert method, only 20 % of the positive yellow tubes were used for additional confirmation test using BGLB medium (conventional confirmation test) as described in 2.3.2.1. Confirmation test results of MPN methods are shown in Table 2.11 (a)

In MF methods, a percentage (20 %) of typical (Plate 2.3-a.) and atypical colonies (Plate 2.3-b.) were used for confirmation testing. Atypical colony formation and background growth were observed only on M-endo medium. There was no such observation on m-ColiBlue24 plates (Plate 2.4). Results obtained are shown in Table 2.11 (b). The water sources were considered together in confirmation testing.

Among MPN methods, the highest confirmation rate was given by Colilert method (92.8 %.). Among MF methods, m-ColiBlue24 method showed the highest total coliform percentage from the total bacteriological count. On the other hand, M-endo medium formed atypical colonies which were also confirmed as total coliform bacteria. Further, the disturbing background growth appeared on M-endo also contained 55 % of total coliform bacteria depicting the low sensitivity of M-endo medium in detecting total coliform bacteria compared to the m-ColiBlue24 method.

Table 2.11 Confirmation test results for detecting total coliforms (Preliminary study)

(a) MPN Methods

Method	Total number of presumptive tubes	Number of confirmed tubes	Confirmation rate %
SLS	480	370	77.0
APHA	303	245	80.8

Method	Number of yellow tubes used for confirmation	Number of confirmed tubes	Confirmation rate %
Colilert	84	78	92.8

(b) MF Methods

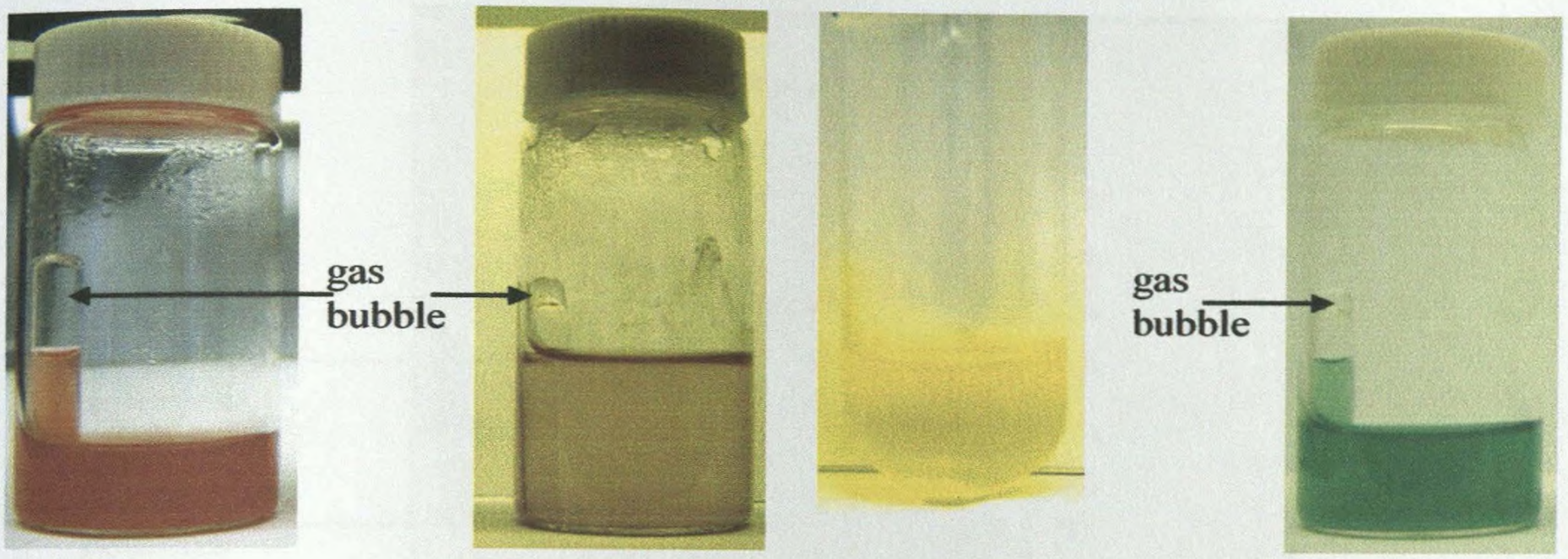
Method	Number of colonies used for confirmation	# Confirmed tests	Confirmation rate %
SLS-MF	Typical (red metallic sheen/red/pink)	108	88	81.4
(M-Endo)	Atypical (cream)	20	15	75.0
	Background growth	20	11	55.0
m-ColiBlue24	Typical red	144	128	88.8

2.4.2.2. Confirmation rates of *E. coli*

Among MPN methods, *E. coli* positive confirmed tubes of SLS and APHA methods were not reconfirmed by the conventional Indole test, where as 20 % of the fluorescent *E. coli* positive tubes and 1 % of non fluorescent tubes (Plate 2.5) of Colilert method were reconfirmed for *E. coli* (using Indole test) (Plate 2.7). Among MF methods [on M-FC (Plate 2.6) and M-coliblue-24 media], 20 % of typical and atypical colonies were confirmed for *E. coli.* Atypical colony formation was observed only on M-FC medium (Plate 2.8), while there were no such observations on m-ColiBlue24 medium. Results of *E. coli* confirmation tests are shown in Table 2.12.

E. coli were present in 80.8% of the yellow fluorescing tubes in Colilert method. However, in addition to these typical yellow fluorescing tubes, yellow, but non-fluorescing tubes also contained *E. coli* (25 %).

Among MF methods, m-ColiBlue24 showed the highest *E. coli* rate being 93.5 %. However, in M-FC plates atypical colonies and background growth were also confirmed as *E. coli*, in addition to the typical blue colour colonies having a 67.2 % of *E. coli*. This indicates that the M-FC medium is less sensitive compared to m-ColiBlue24 medium in detecting *E. coli* in water samples.

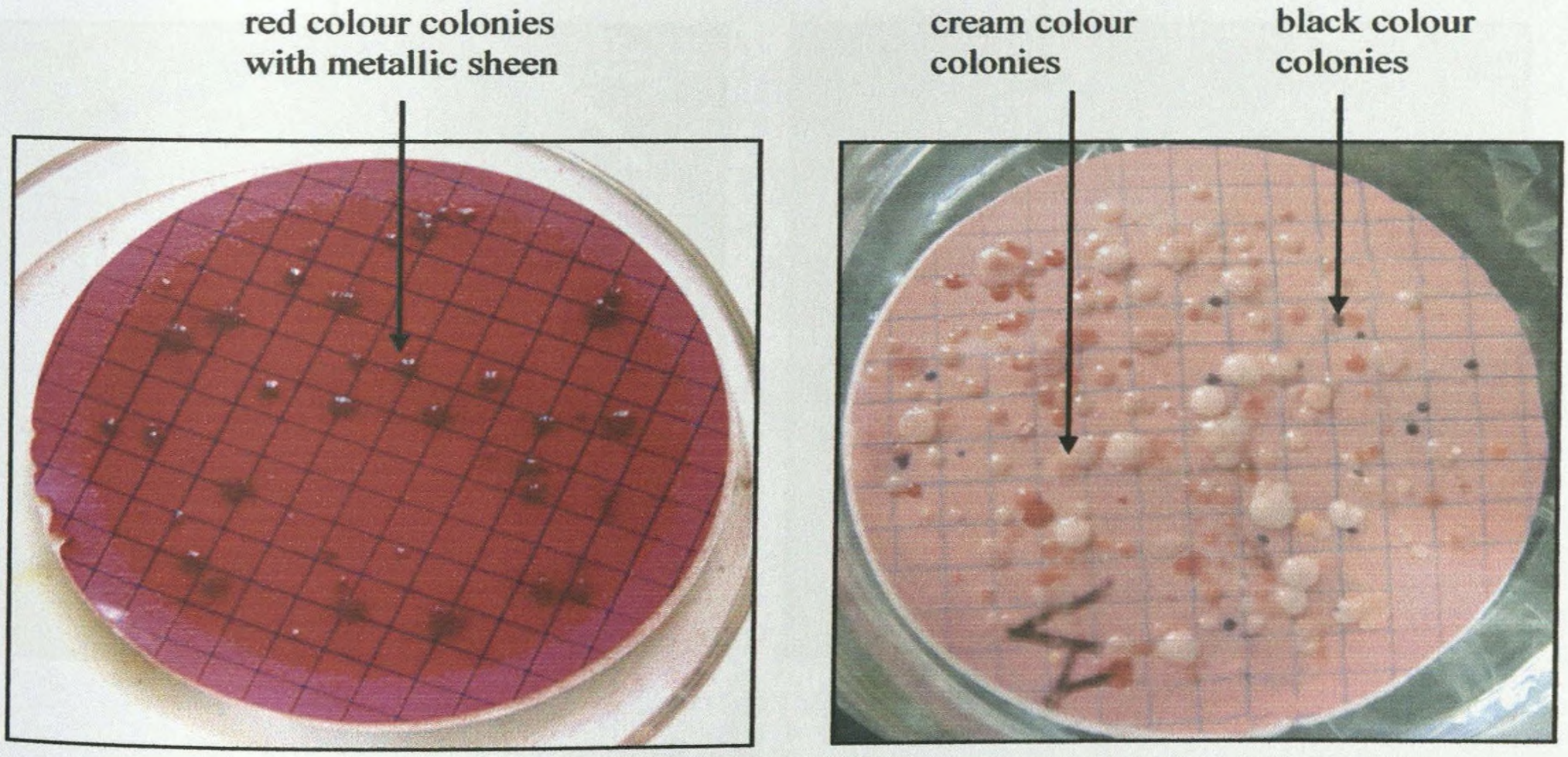


(a) (b) (c) Positive tube with BGLB broth

(a) Positive tube with Mac Conkey broth
(b) Positive tube with Lauryl tryptose broth
(c) Positive tube (yellow colour) with Colilert medium

Plate 2.1 Presumptively positive total coliform tubes

Plate 2.2 Confirmed total coliform tube



(a) (b)

(a) Typical colonies (red with metallic sheen)
(b) Atypical colonies (cream colour)

Plate 2.3 Total coliform results on M-endo plates

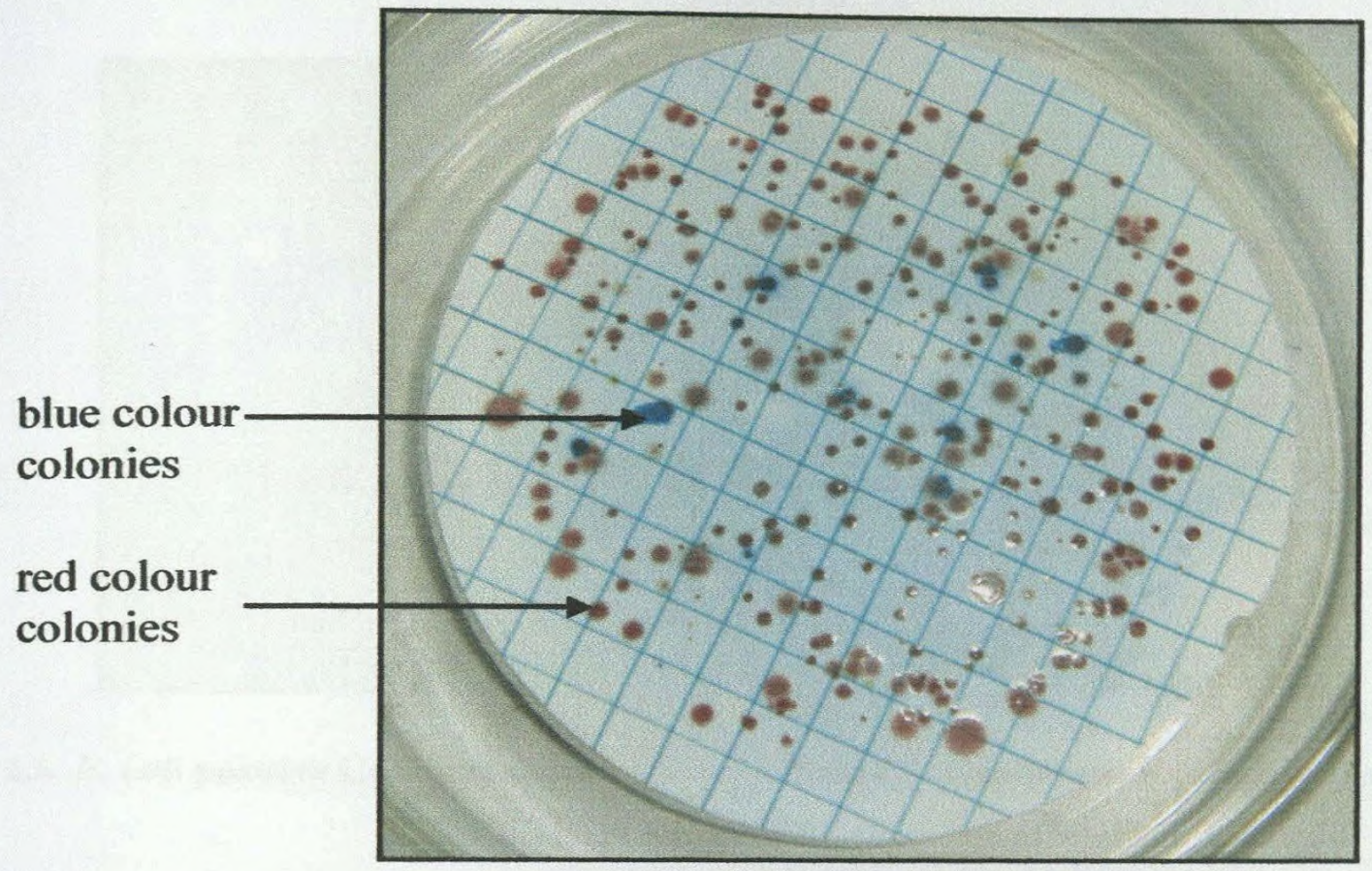
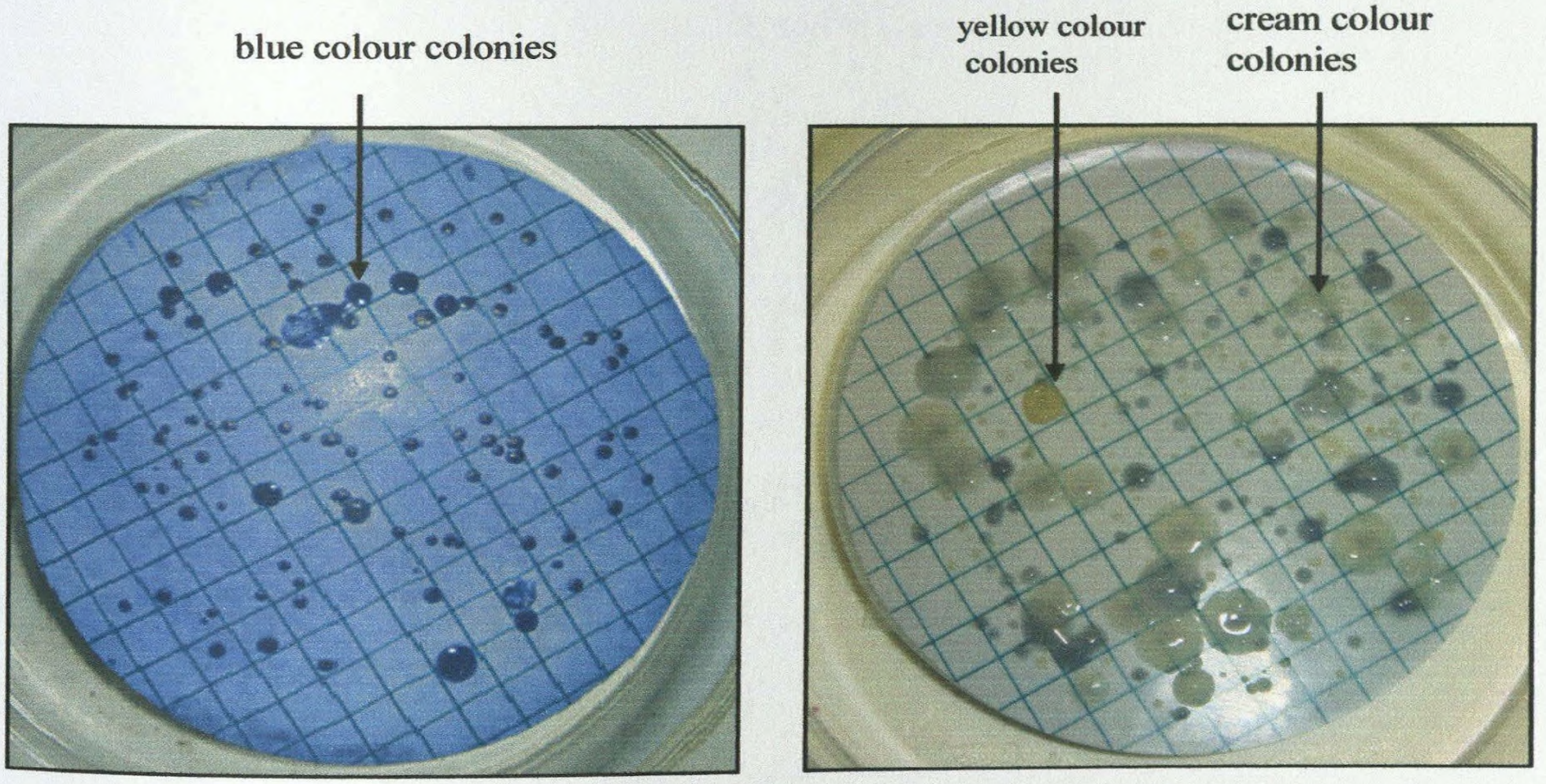


Plate 2.4 m-ColiBlu24 plate with typical colonies



(a) Typical blue colour colonies

(b) Atypical colonies (yellow and cream colour)

Plate 2.5 Fecal coliform results on M-FC plates

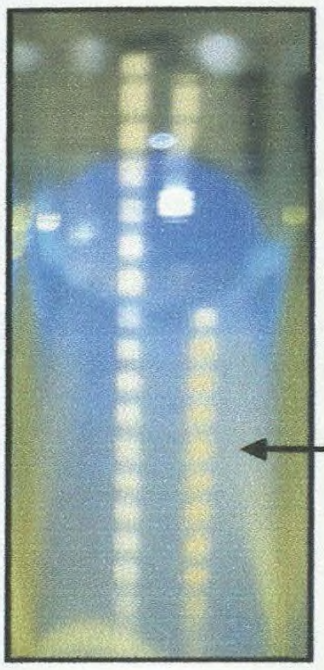


Plate 2.6 *E. coli* positive Colilert tube

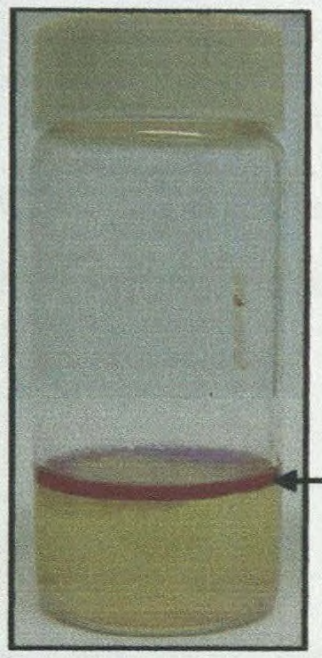


Plate 2.7 Confirmed *E. coli* test result (Indole reaction)

Table 2.12 Confirmation test results for detecting *E. coli* (Preliminary study)

(a) Colilert Method

Number of tubes used for confirmation		Number of confirmed tubes	Confirmation rate %
Yellow with fluorescence	68	55	80.8
Yellow without fluorescence	8	2	25.0

b) MF Methods

Method	Number of colonies used for confirmation		Number of confirmed tests	Confirmation rate %
SLS-MF	Typical (blue)	58	39	67.2
(M-FC)	Atypical (cream)	28	16	57.1
	Background growth	27	10	37.0
m-ColiBlue24	Typical blue	78	73	93.5

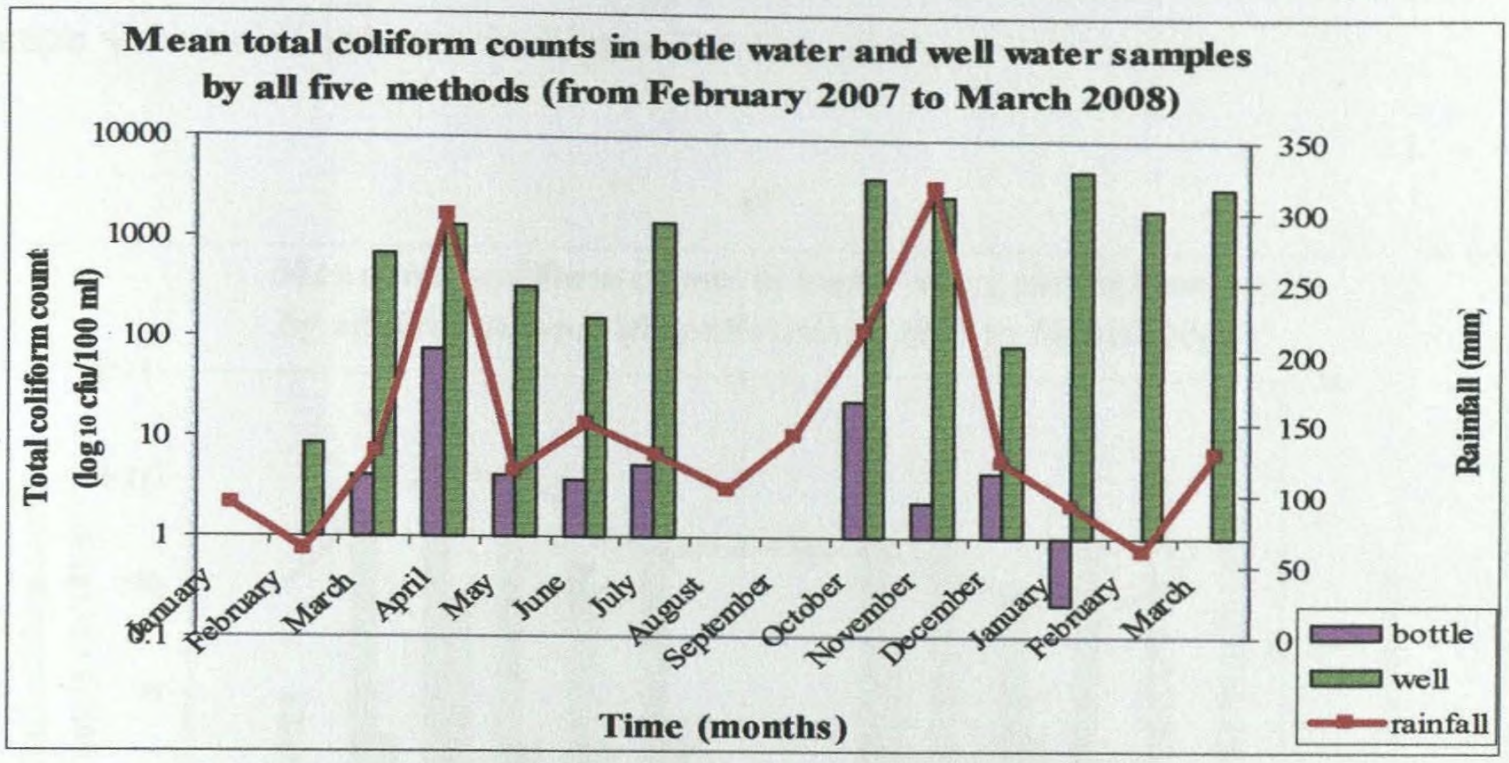
2.4.3. Seasonal variation of bacterial counts

2.4.3.1. Seasonal variation of total coliform counts

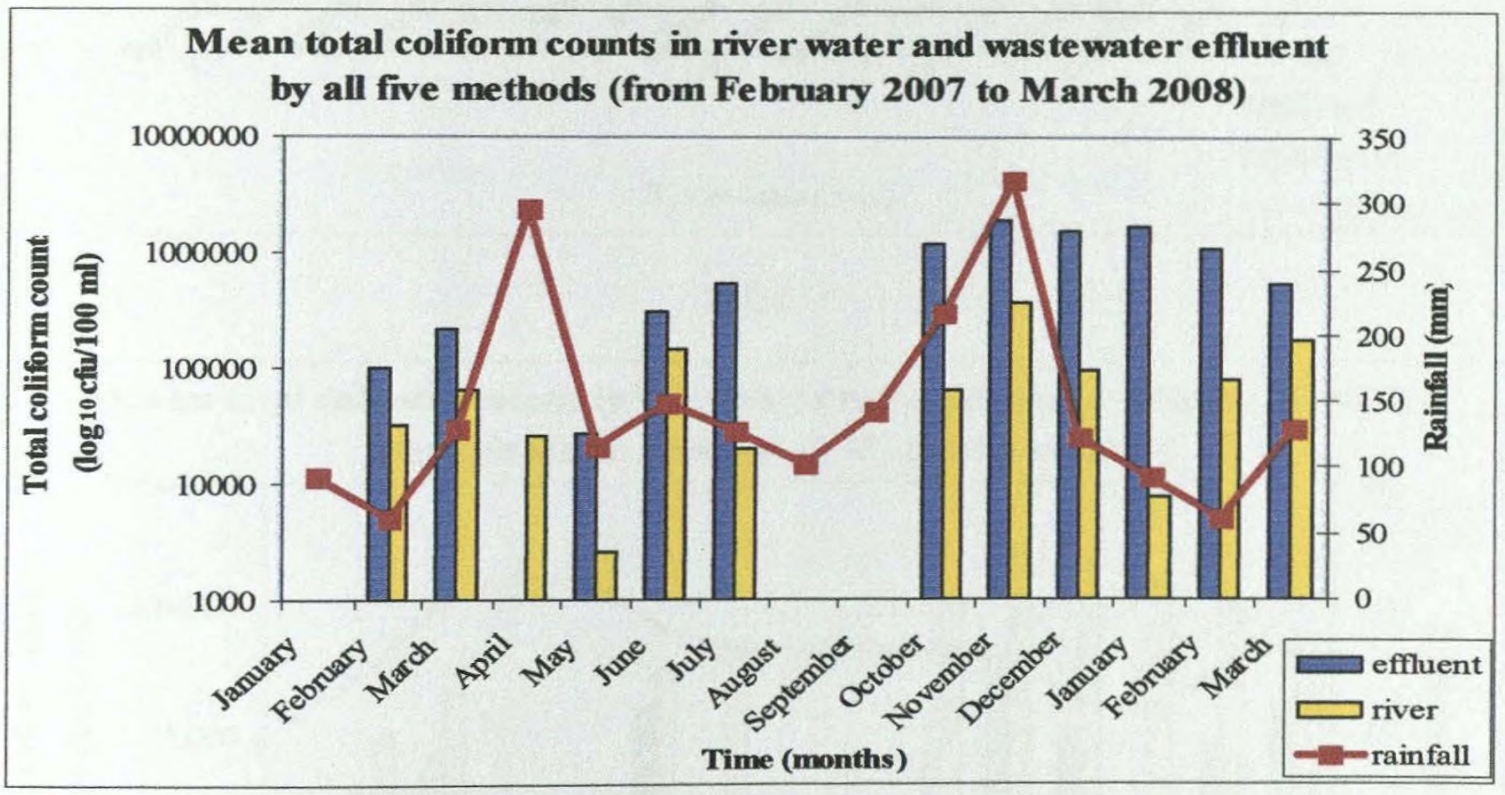
Effect of rainfall on seasonal variation of total coliform counts

As shown in Figure 2.5, mean monthly rainfall in Kandy, varied between 50 – 350 mm, (data obtained during a nine years time period from year 2000 to 2008). Two dry seasons (rainfall, < 150mm) from March to May and from October to November; and two wet seasons (rainfall 150-350 mm) from June to September and December to February were observed during the experimented time period. Total coliform counts in each water type varied with time during the 12 months period (Figure 2.5. a and b).

(a)



(b)



(a) Bottle water and well water **(b) River water and effluent water**

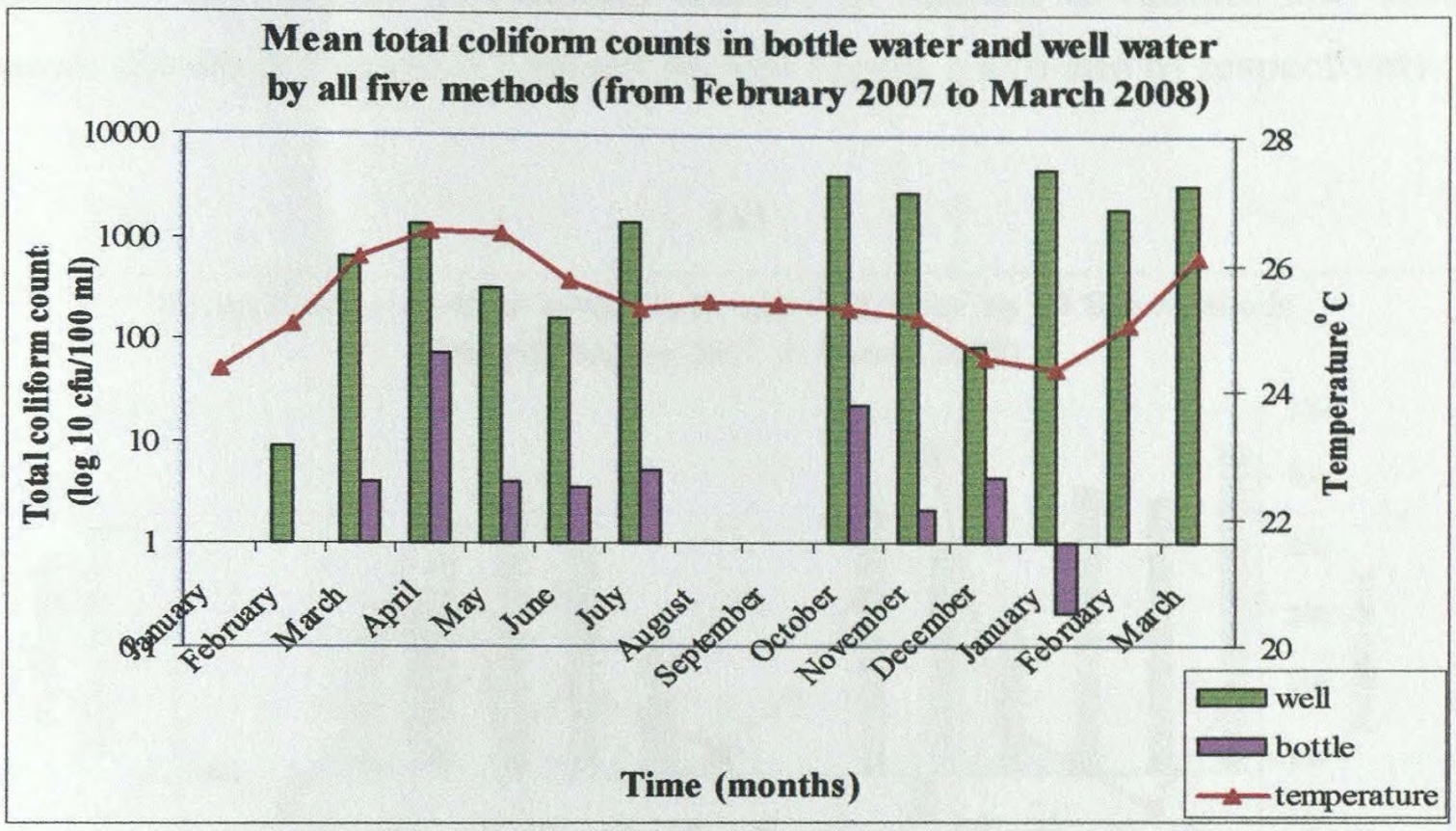
Figure 2.5 Effect of rainfall on seasonal variation of total coliform bacteria

Effect of temperature on seasonal variation of total coliform counts

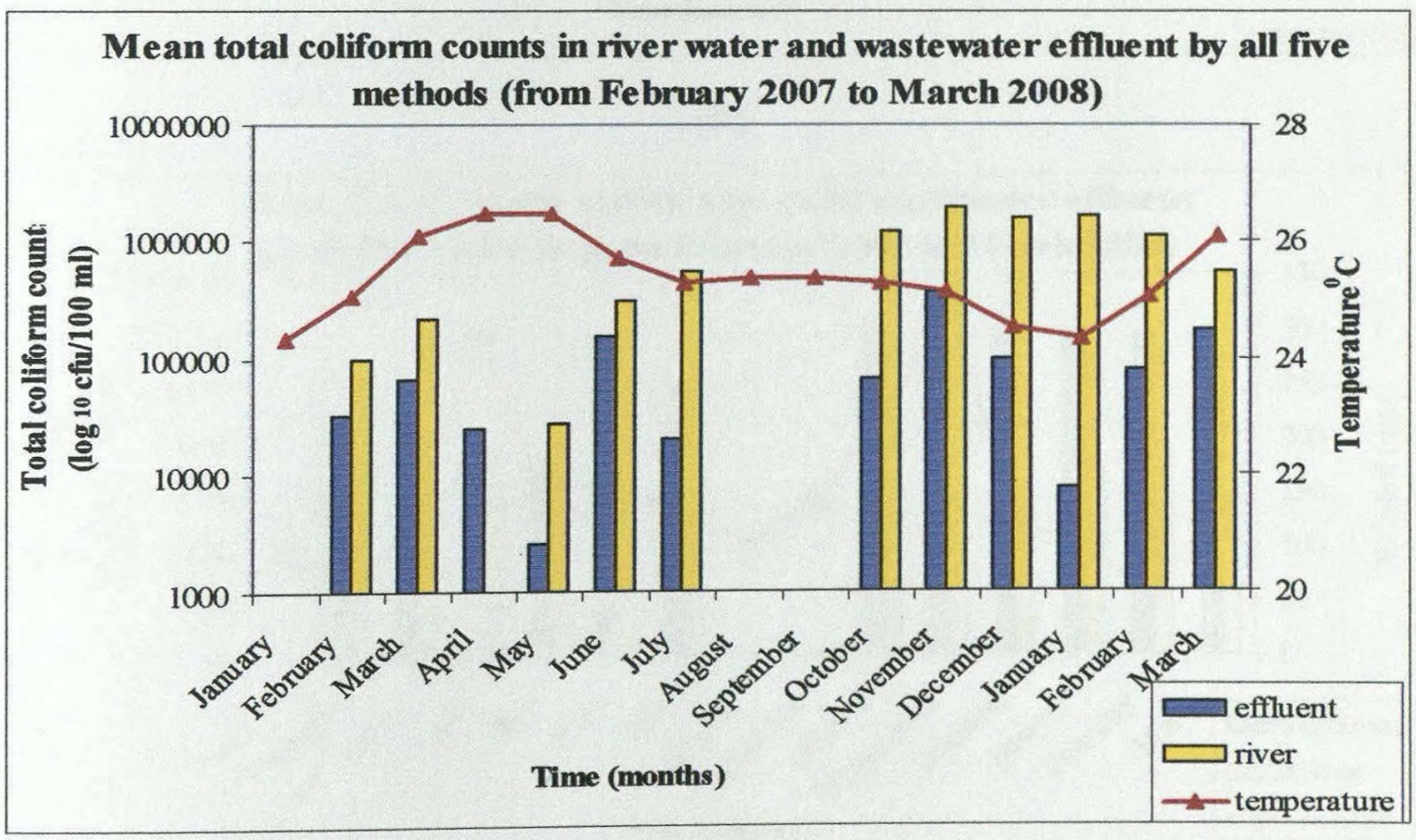
As shown in figure 2.6, variation of mean monthly temperature was only 2.16 °C, within a year (data obtained during a nine years time period from 2000 to 2008) in Kandy. From March to June, temperature values were higher (around 26 °C compared to other months

(between 24-25 °C). Variation of total coliform counts in different sources in relation to temperature variation is shown in Figure 2.6 (a and b).

(a)



(b)



(a) Bottle water and well water **(b) River water and effluent water**

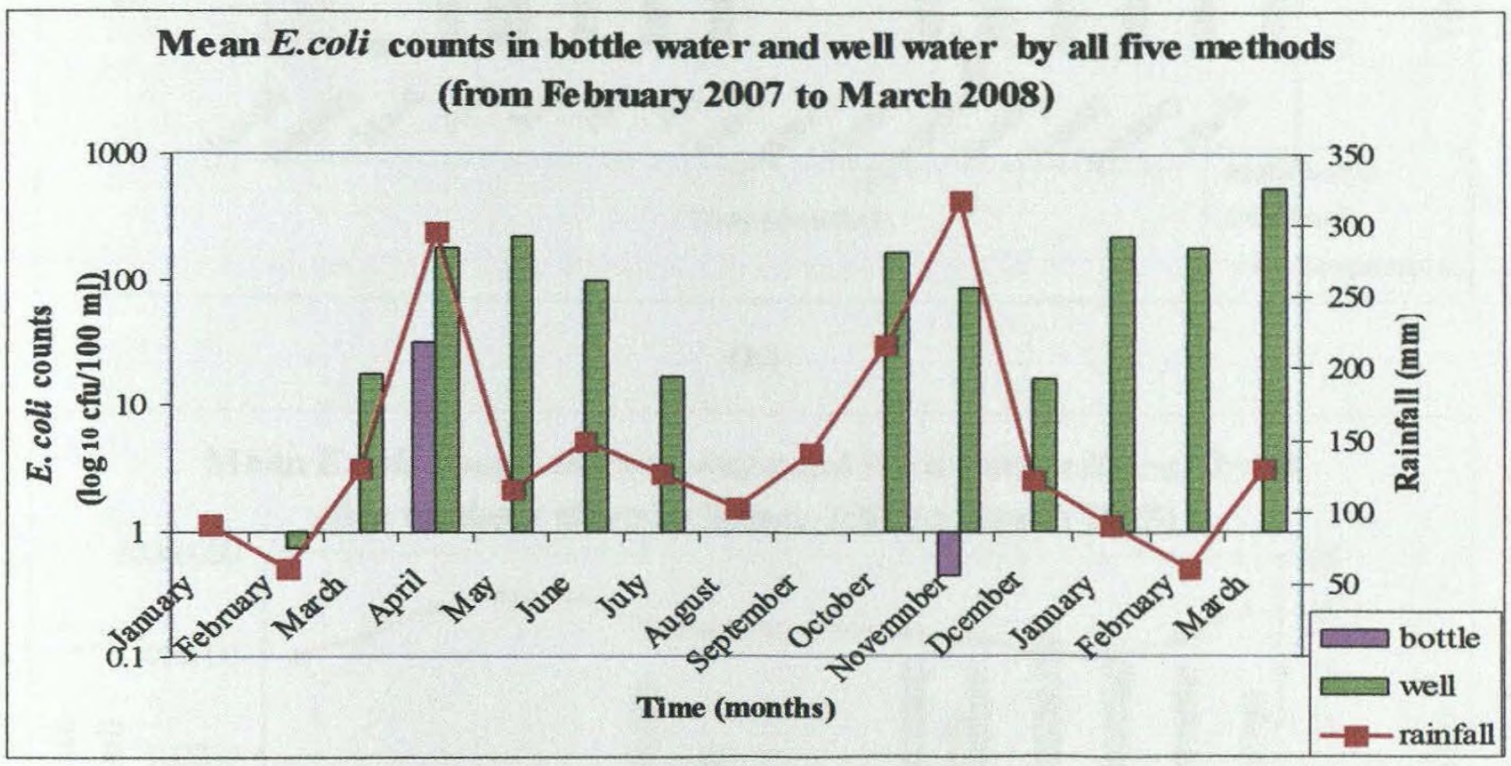
Figure 2.6 Effect of temperature on seasonal variation of total coliform bacteria

2.4.3.2. Seasonal variation of *E. coli* counts

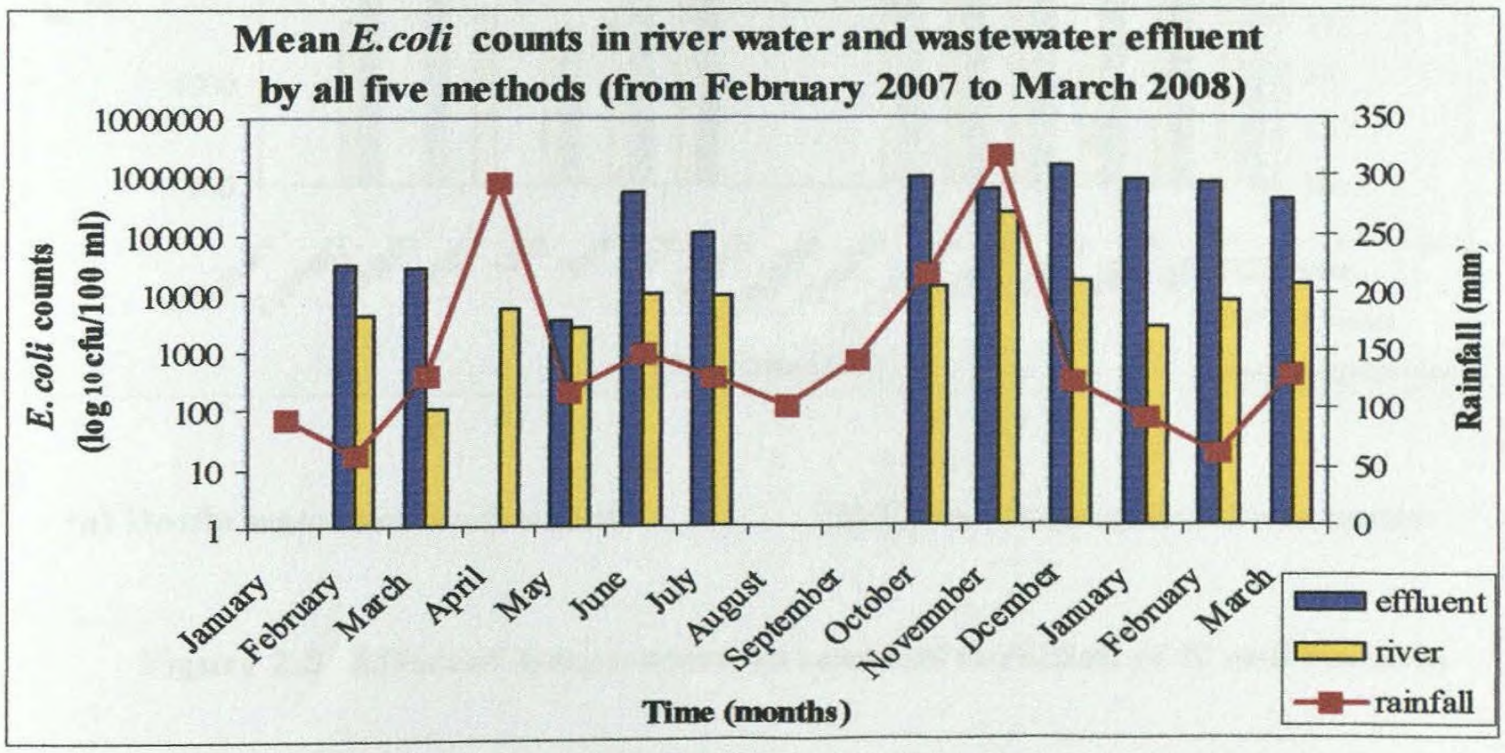
Effect of rainfall on seasonal variation of *E. coli* counts

Variation of *E. coli* counts in different sources in relation to rainfall and temperature variation are shown in Figures 2.7 (a and b), and Figure 2.8 (a and b) respectively.

(a)



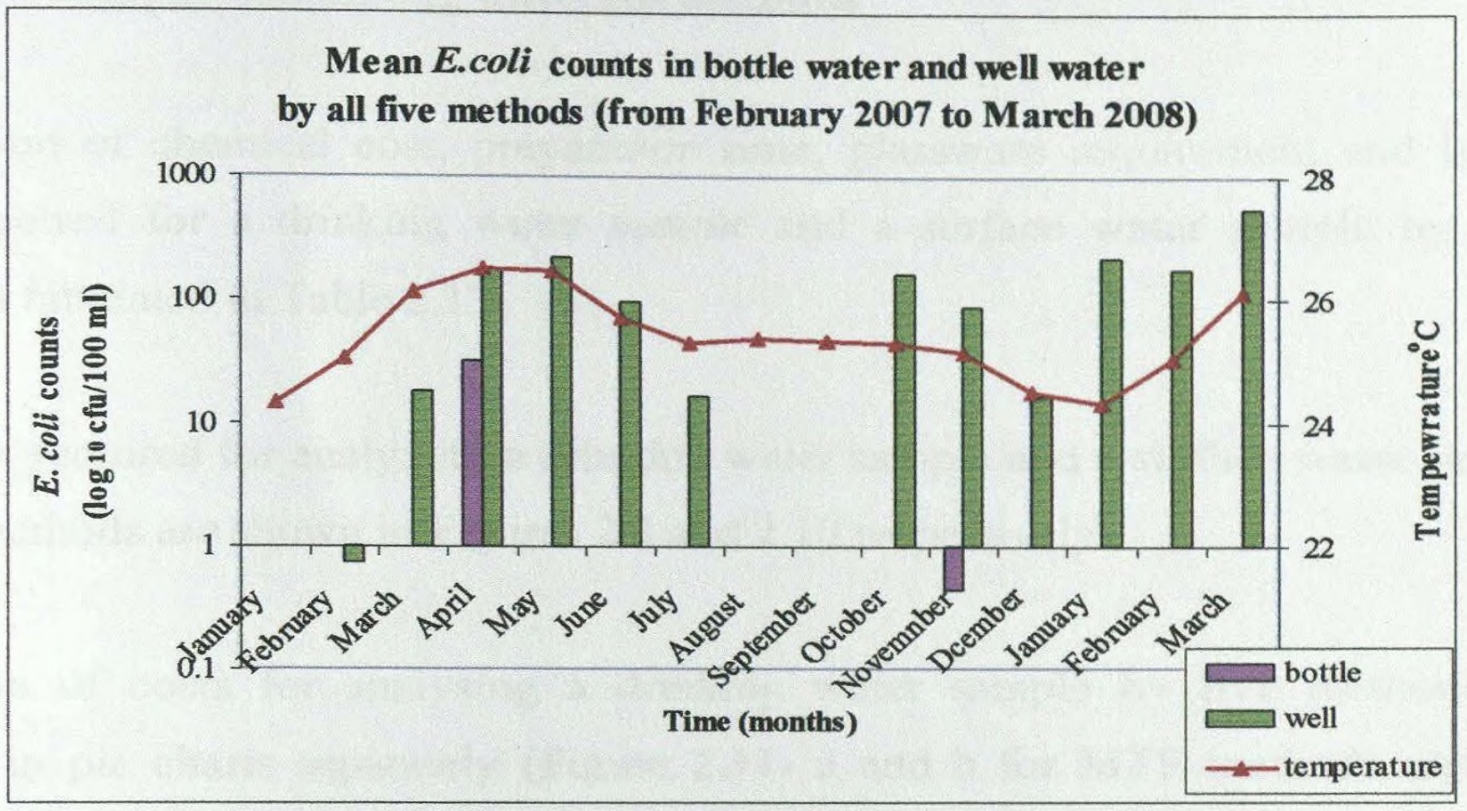
(b)



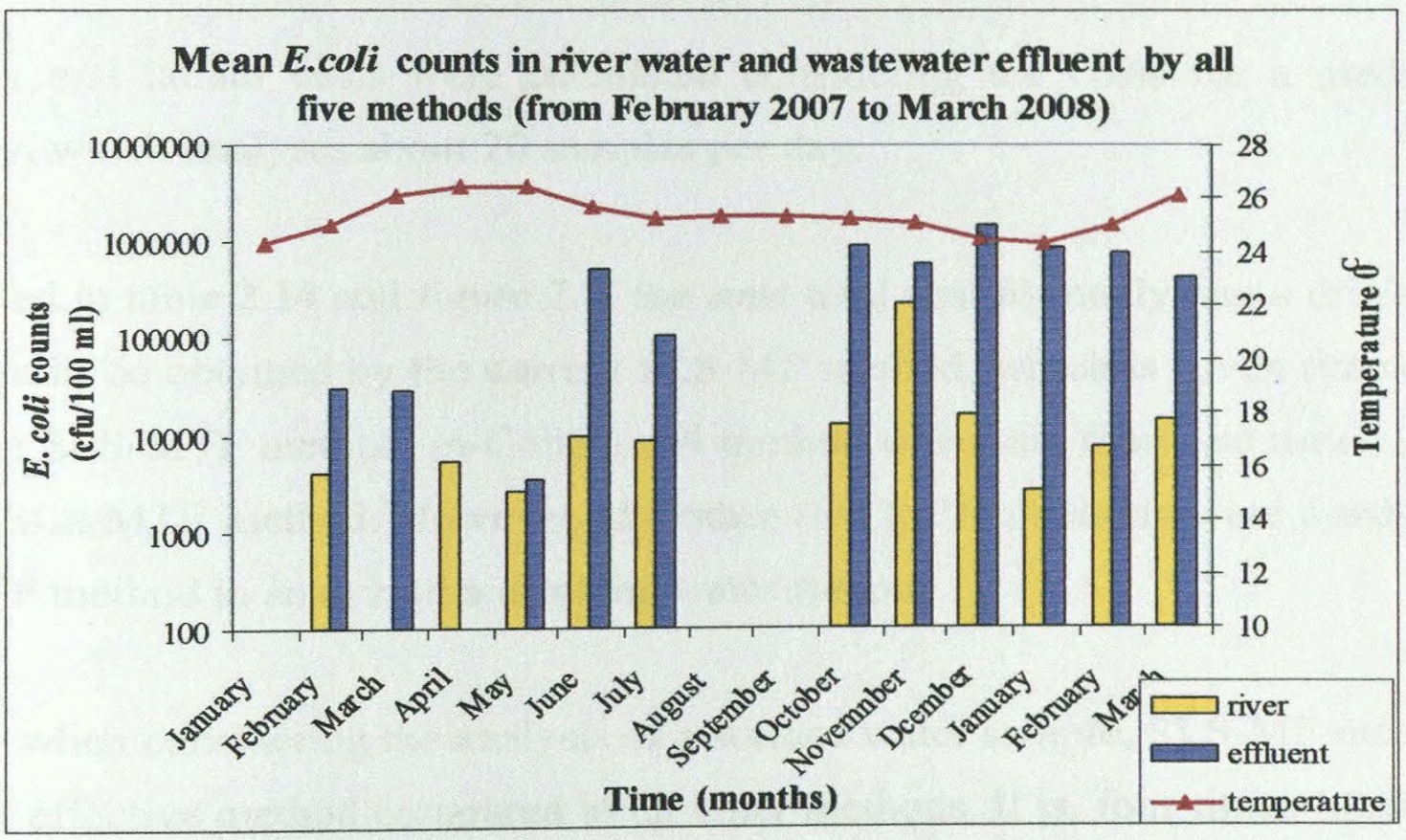
(a) Bottle water and well water **(b) River water and effluent water**

Figure 2.7 Effect of rainfall on seasonal variation of *E. coli* bacteria

(a)



(b)



(a) Bottle water and well water **(b) River water and effluent water**

Figure 2.8 Effect of temperature on seasonal variation of *E. coli* bacteria

2.4.4. Cost comparison among different methods

Comparison of chemical cost, preparation time, glassware requirement and labour hours required for a drinking water sample and a surface water sample by each method is tabulated in Table 2.13.

Total cost required for analyzing a drinking water sample and a surface water sample by five methods are shown in Figures 2.9 and 2.10 respectively.

Proportion of costs for analysing a drinking water sample by five methods are depicted in pie charts separately (Figure 2.11- a and b for MTF methods and MF methods respectively).

Electricity and labour costs were calculated considering the costs for a medium scale laboratory, which analyses about 20 samples per day.

As depicted in table 2.14 and figure 2.9, the least total cost for analyzing a drinking water sample could be obtained by the current SLS-MF method, which is seven times less than that of the SLS-MTF method. m-ColiBlue24 method was more than two times less costly than the SLS-MTF method. However, the other two MPN methods were costly than the SLS- MTF method in analyzing a drinking water sample.

Similarly when considering the analysis of a surface water sample, SLS-MF method is the most cost effective method compared to all other methods. It is, four times less than SLS-MTF method. Colilert method is two times less costly than SLS-MTF method for analyzing a surface water sample, while other two methods are not cost effective than SLS-MTF. The highest cost is shown by the APHA method (Figure 2.10).

When comparing the total time required for a drinking water sample analysis, the longest duration is shown by the two MTF methods, SLS and APHA. The shortest time duration is required by the Colilert method. similar results were given for analyzing a surface water sample (Table 2.13).

Amount of glassware requirement is much higher in both standard MTF methods (SLS and APHA), compared to all other methods. The minimum amount is of glassware required by the chromogenic m-ColiBlue24 method for both drinking water and surface water analysis (Table 2.13).

Table 2.13 Comparison and cost analysis per sample by the use of five different test methods (2007-2009)

	SLS-MTF		APHA		COLILERT		SLS-MF		M-COLI	
	D	S	D	S	D	S	D	S	D	S
Cost (Rs/=)										
Chemicals	1479	2408	2532	3460	2500	1125	30	90	600	1800
Membrane filters							180	270	90	270
Time/hrs.										
Preparation of glassware	2.5	2.5	2.5	2.5	1.5	1.5	1.5	1.5	1.5	1.5
Preparation of media	2.5	2.5	2.5	2.5	0.08	0.25	2.5	2.5	-	-
Inoculation	0.16	0.25	0.16	0.25	0.08	0.24	0.16	0.48	0.08	0.24
Results recording					24	24	48	48	36	36
a. Presumptive test	48	48	48	48						
b. Confirmation test	48	48	48	48						
Electricity needed time/hrs.										
Sterilizing glassware	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Sterilizing media	1.5	1.5	1.5	1.5			1.5			
Glassware required										
Tubes	22	30	10	30	10	30				
Petri dishes							4	12	2	6

D- Drinking water S- Surface water

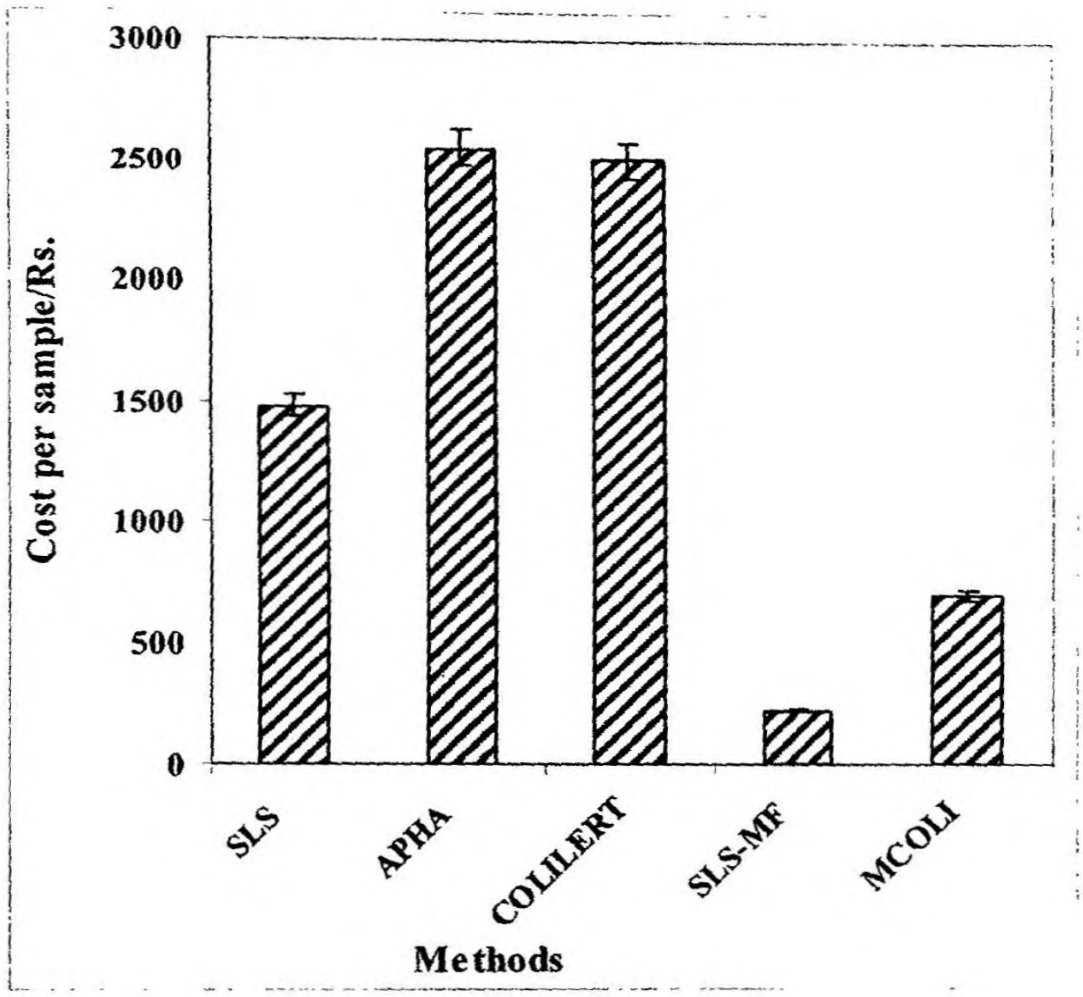


Figure 2.9 Total cost for analyzing a Drinking Water sample by five different methods

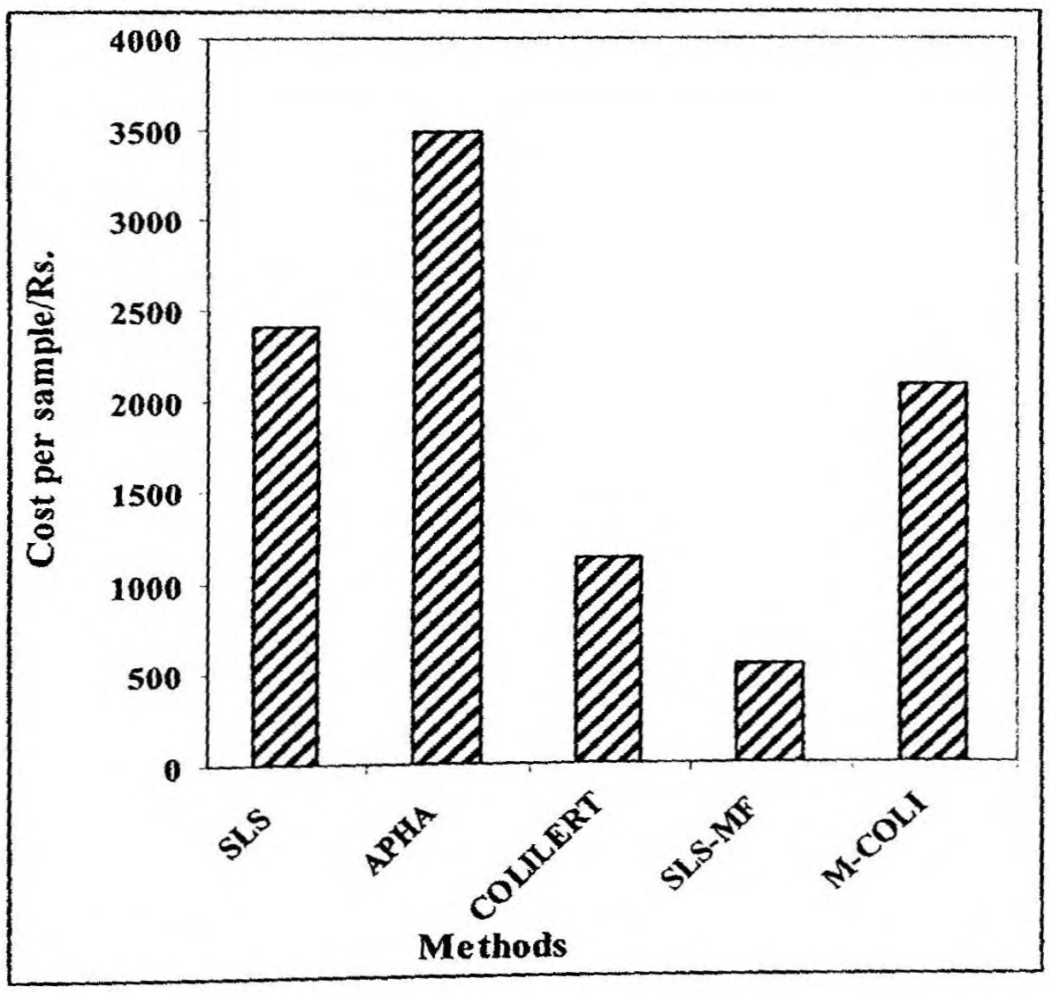
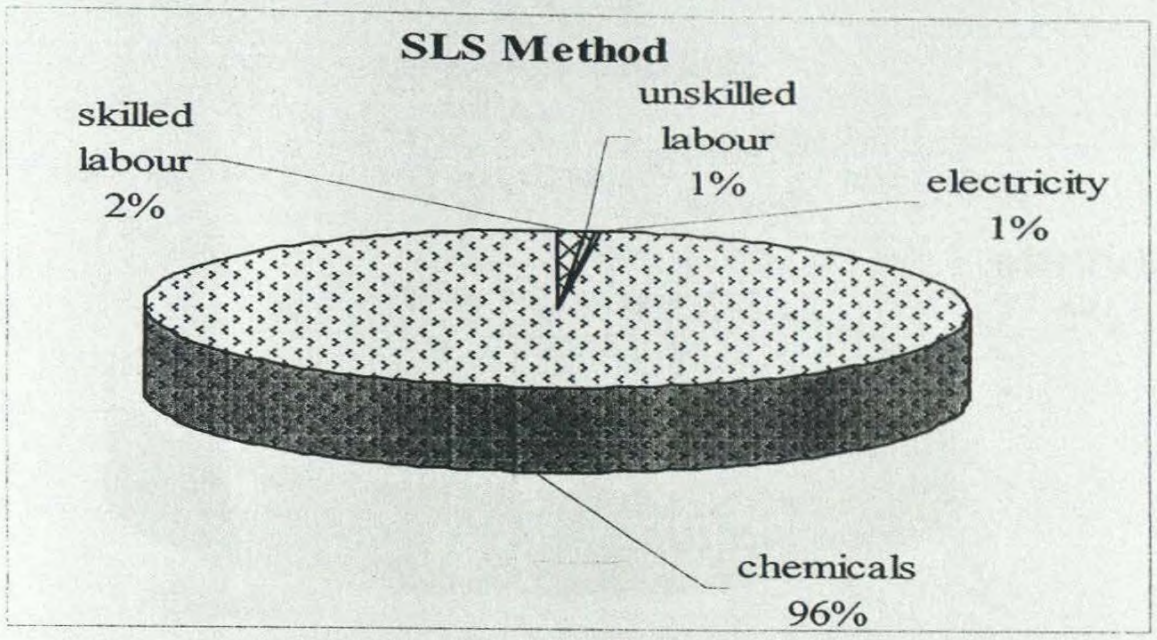


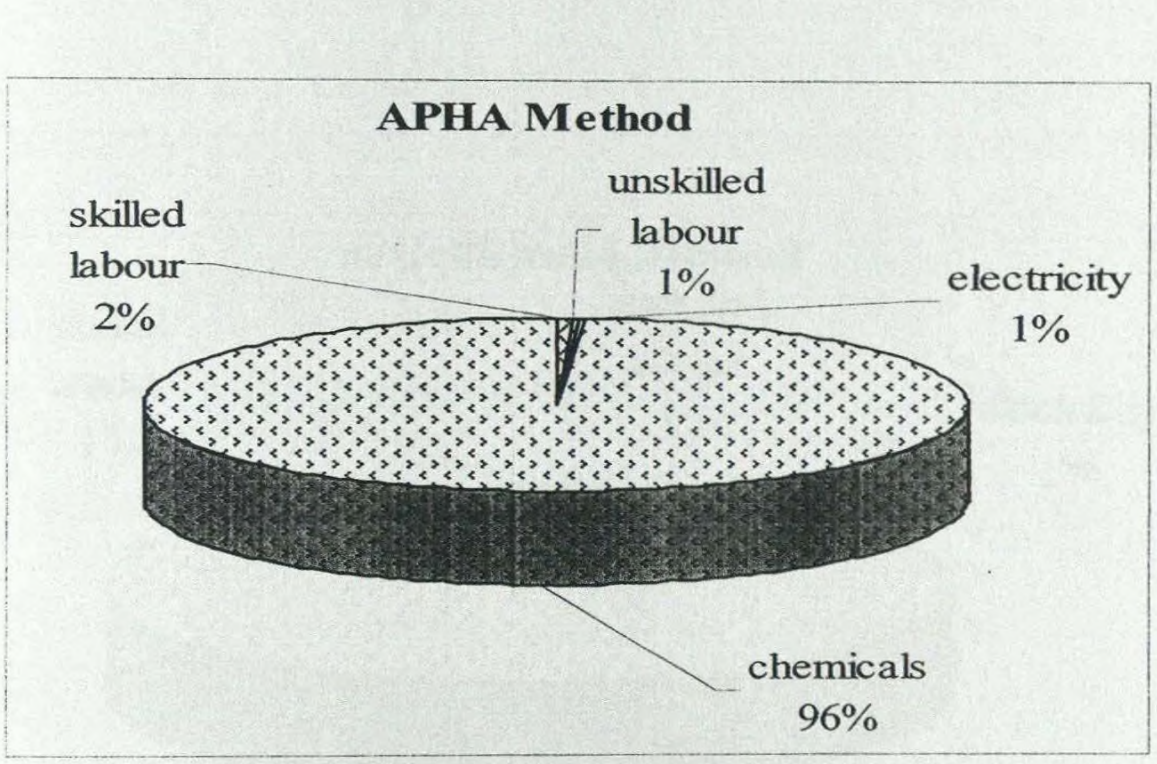
Figure 2.10 Total cost for analyzing a Surface Water sample by different methods

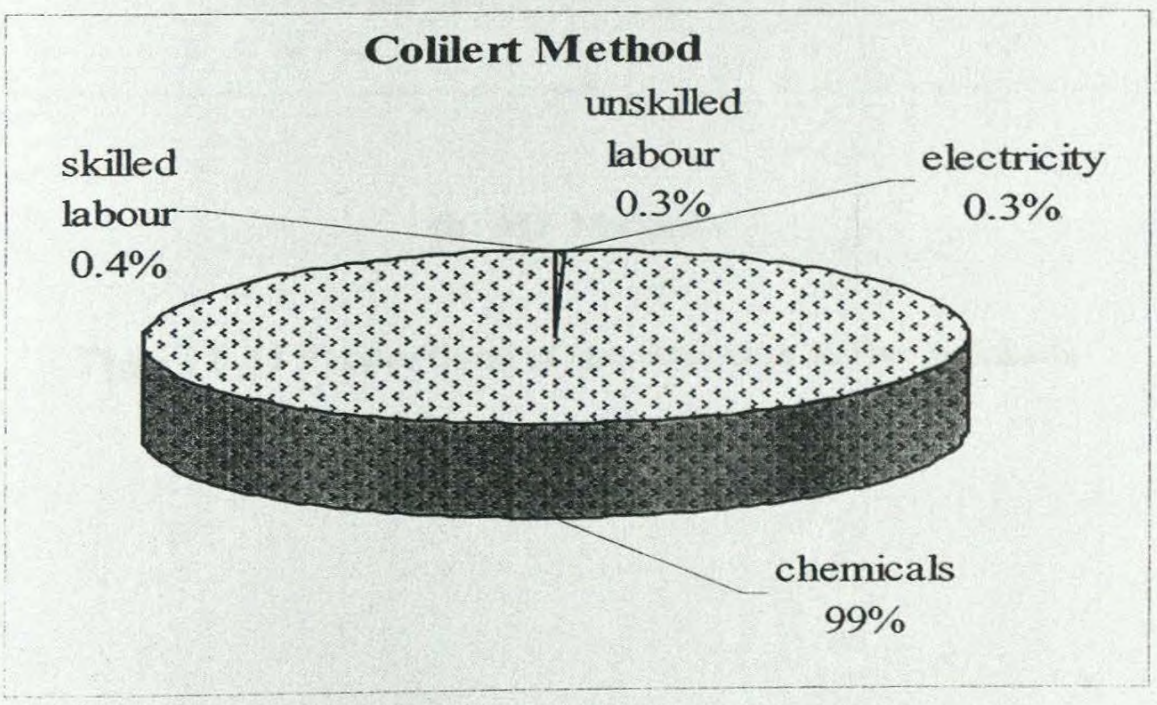
When considering the proportions of each cost items for analyzing a drinking water sample, the highest proportion was required for chemicals in all five methods. However, the rate of this proportion varied among methods; the minimum and the maximum were recorded by SLS-MF and Colilert methods respectively. Colilert method was less labor-intensive, compared to all other methods.

Table 2.14 Proportion of total cost per drinking water sample

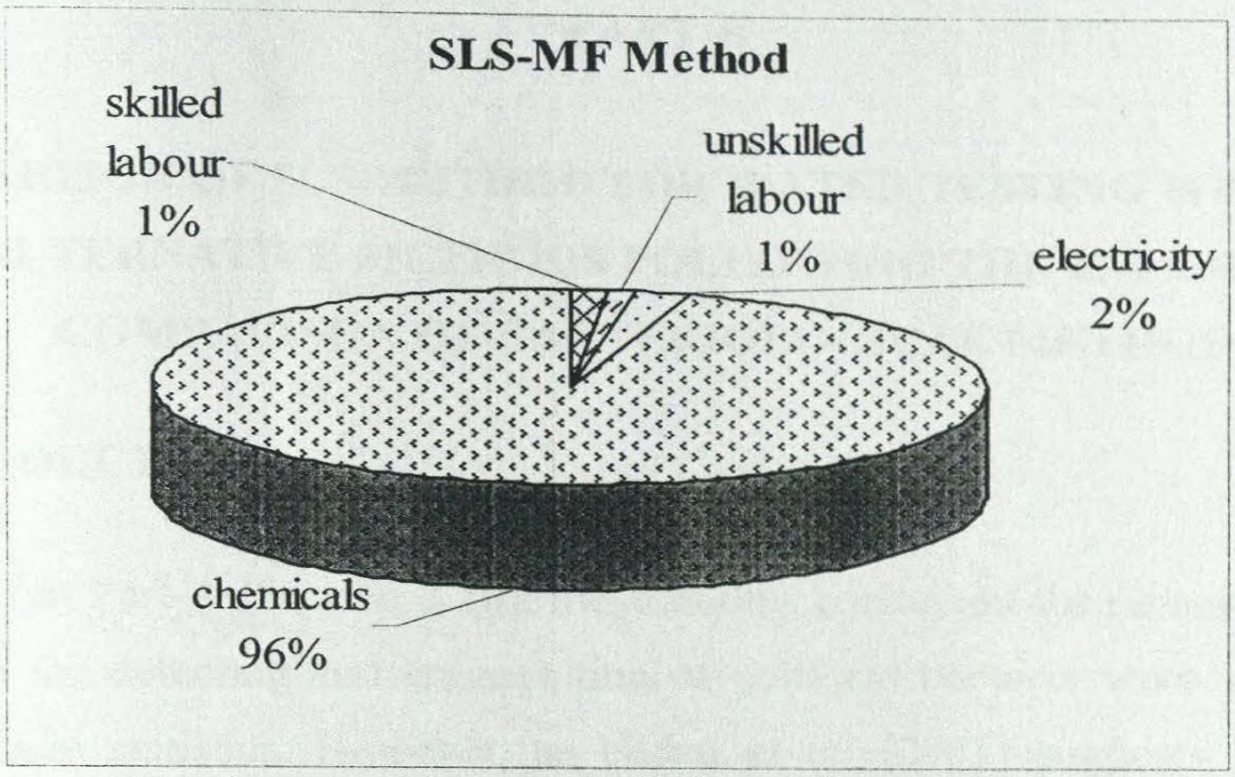
	Method				
	SLS-MTF	**APHA**	**Colilert**	**SLS-MF**	**M-coli**
Electricity/Rs	15.00	15.00	3.00	3.00	3.00
Skilled labour/Rs.	5.00	5.00	2.50	2.50	2.50
Unskilled labour/Rs.	5.00	5.00	2.50	5.00	2.50
Chemicals + (membrane filters in MF methods) /Rs	1454.00	2532.00	2500.00	210.00	690.00
Total cost per sample/Rs	**1479.00**	**2557.00**	**2508.00**	**220.50**	**698.00**

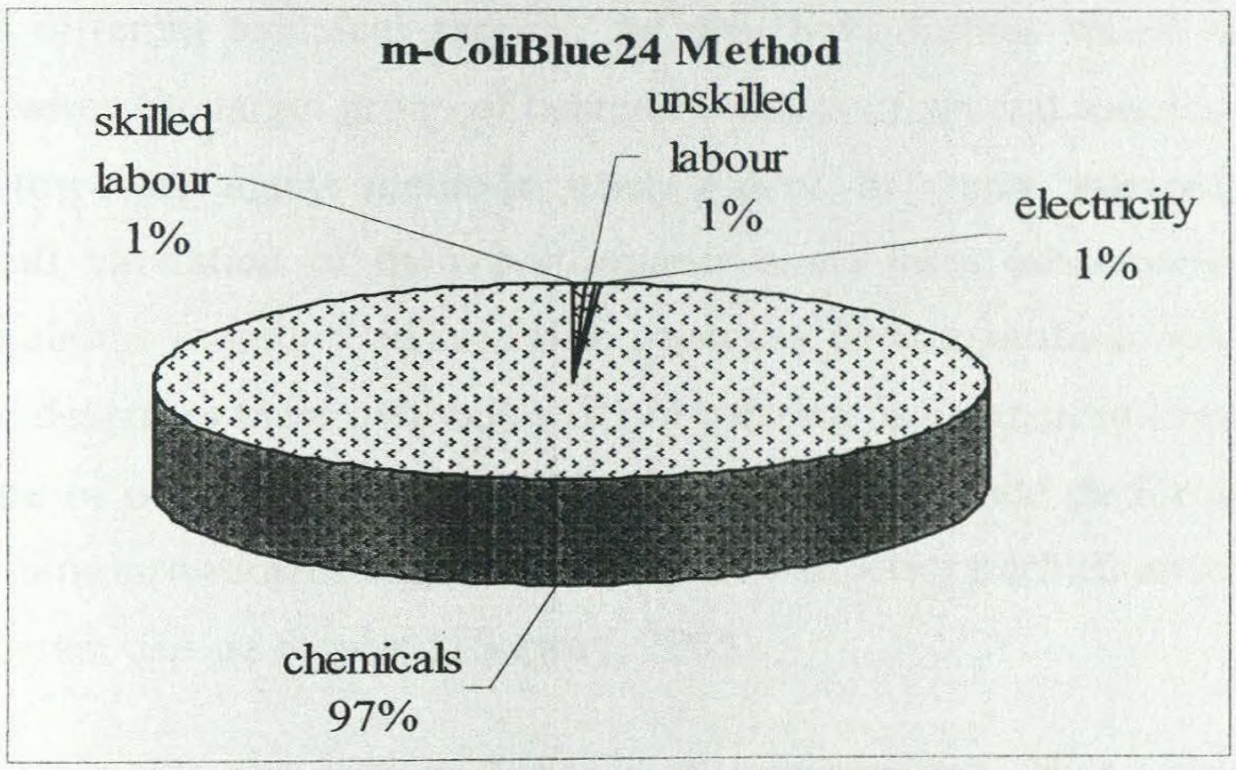






(a) MPN Methods





(b) MF Methods

Figure 2. 11 Proportions of the total cost in five methods

PART-B

COMPARISON OF SLS METHOD FOR WATER TESTING WITH THREE OTHER ALTERNATIVE METHODS FOLLOWING THE ISO CRITERIA FOR COMPARISON OF BACTERIOLOGICAL METHODS

2.5. INTRODUCTION

As described in Part-A (Section 2.10), most studies conducted for comparison of method performance for detecting and enumerating of coliform bacteria were mainly based on simple variance statistics. However, as Niemi *et al.* (2001) suggests it is difficult to introduce new methods into routine use when their validation doesn't cover all types of samples and different technical aspects. As they have further stated the task becomes complicated when the target group of bacteria consists of several species as in the case of coliforms. However, many methods used widely in water microbiology have not undergone full validation of their performance since most laboratories have accepted performance claims in published scientific papers or from manufacturers (Sartory, 2005). As he further describes these methods will not perform as claimed in every laboratory and for every type of matrix. Therefore, verification of the claimed performance, and where appropriate, demonstration of equivalent or inferior/superior performance compared to the method in current use are required (Sartory, 2005).

However, the guidance on assessment of performance characteristics for water microbiological methods on comparing the performance of two methods is very limited. USEPA (1995) has published a procedure for comparison of two presence/absence (P/A) methods (USEPA, 1995). These approaches have been successfully employed for P/A methods where the results are simply positive or negative and only a limited set of data is required for statistical analysis to demonstrate superior or inferior performance of one method against another. However, comparing two quantitative methods is more complicated since distribution of microbes is not homogenous. Further, the stage of growth, state of stress response and metabolic status at the time of analysis will impact on the response of target bacteria in quantitative methods requiring selective growth. All these factors result in a significant natural variability in recovery of micro-organisms from

water which must be taken into account when devising analytical and statistical protocols for comparing quantitative methods, particularly when the numbers that are encountered in routine samples tend to be very low, as in the case of drinking water monitoring (Sartory, 2005).

However, in 1980s or early 90s there were no published procedures for demonstrating equivalency between various methods. Later, in the year 2000, the International Standard Organization (ISO) published, a technical report on Water quality-Guideline on Validation of Microbiological Methods (*ISO/TR 13843*). The standard describes the information required for the derivation of the numerical and descriptive specifications of a method. These developments have provided a sound framework for the validation and verification of performance of new methods prior to adoption by a water microbiology laboratory (Sartory, 2005).

The ISO/TR 13843 (ISO 2000E) defines several terms;

a) **Primary Validation:** an exploratory process with the aim of establishing the operational limits and performance characteristics of a new, modified or otherwise inadequately characterized method.
b) **Secondary Validation ("Verification")**: will find out the suitability of a new method introduced in to a particular laboratory.

   For verification of methods, a number of natural samples, analyzed as split samples or replicate dilution series with duplicate counting were required to verify expected counting performance. There was no recommendation on the number of natural samples that should be analyzed for verification of performance, but about 30, covering the range of water types or matrices typically analyzed by the laboratory, is reasonable.
c) **Confirmation**: confirmation of target bacteria are essential and *ISO/TR 13843* has recommended that 100 presumptive positives and up to 50 non-target presumptive isolates should be verified (using appropriate biochemical or serological protocols).

Performance characteristics related to specificity and selectivity

Performance characteristics relate to the relative proportions of colonies or tubes assumed positive or negative on the basis of the first impression (presumptive) compared with the 'truth' after verification. After n verification tests have been made results are divided in to four categories:

a) number of presumptive positives found positive (true positives)
b) number of presumptive negatives found positive (false negatives)
c) number of presumptive positives found negative (false positives)
d) number of presumptive negatives found negative (true negatives)

Frequency table

		Presumptive count		
		+	-	
Confirmed count	+	a	b	a + b
	-	c	d	c + d
		a + c	b + d	n

Performance characteristics

1) **sensitivity** = a/(a + b), the fraction of the total positives correctly assigned in the presumptive count
2) **specificity** = d/(c + d), the fraction of the total negatives correctly assigned in the presumptive count
3) **false positive rate** = c/(a + c), the fraction of the observed positives wrongly assigned

   false negative rate = b/(b + d), the fraction of the observed negatives wrongly assigned [total no. of tests = a + b + c + d = n]
4) **efficiency** E = (a + d)/n fraction of colonies or tubes correctly assigned

Therefore, adoption of a new method in a laboratory could only be recommended after the successful performance of a new method satisfying the verification exercise. If, the new method were to replace the one already being used by a laboratory it is recommended to assess the new method against the current method, and to generate verification of performance data at the same time. Using this type of data it would be beneficial to explain to the customers to change the existing method due to greater recovery or specificity/selectivity or rapid testing of the new method (*ISO/TR 13843*, ISO 2000E).

Later, in year 2004, ISO has established the criteria for comparison of microbiological methods by the ISO standard 17994: 2004, which specifies the basic requirements for an equivalency experiment

2.5.1. ISO criteria for establishing equivalence between microbiological methods (ISO 17994: 2004)

2.5.1.1. Samples

The most important basic requirement of equivalency trials is a wide range of samples, collected from different geographical areas. Participation of several laboratories is recommended. Results of the comparison are only valid within the range of sample types studied. Samples should be pre-selected and they must contain enough bacteria since the likelihood of scoring a zero count is small. Sample should represent types that are included in the scope of both methods. Natural samples are ideal. Appropriate sample also be prepared by dilution, spiking, or mixing of different kinds of water to achieve the desired population in a suitable density.

It is difficult to determine the exact number of samples required for a valid comparison. It will depend on the actual difference observed, on the experimental standard deviation and on the difference considered significantly. Introduced 'maximum acceptable deviation' and the expanded uncertainity will decide the required number of samples. If the data are found inadequate for deciding that the methods are 'not different, more samples will be needed for comparison. However,

the minimum sample number specified for a comparison study is 30 (ISO 17994: 2004).

2.5.1.2. Methods

For comparison studies, two MF methods, two MTF methods or two P/A methods could be used. Also comparisons between MF method and a MTF method or counting method (MF or MTF) with a P/A method is described. Selecting appropriate numbers of samples could be done by applying appropriate formulas. The minimum specified number is 30.

2.5.1.3. Confirmation testing

There should be well-separated 10-30 colonies for confirmation. Otherwise dilutions should be carried out. For MTF and P/A confirmation of all presumptive results is recommended. Confirmation of bacteria should be done using standard confirmation tests.

2.6. MATERIALS AND METHODS

2.6.1. Samples for analysis

Water samples were collected from seven selected districts (situated in five provinces), in order to cover wide geographical areas of the country. Sources were selected following the ISO criteria 17994 (2004) for establishing equivalence between microbiological methods. Selected sources were; bottled drinking water, well water, surface water (rivers/streams/lakes) and effluents from different wastewater treatment plants,

Selected districts were; Kandy, Nuwaraeliya (in Central Province); Ratnapura (Sabaragamuwa Province); Kurunegala (North Western province); Anuradhapura, Dambulla (North Central Province) and Colombo (Western province). Geographical information of the five selected provinces are given in the table 2.15.

Table 2.15 Geographical information of the five provinces

Province	Location	Soil type	Rainfall (yearly /inches	Mean monthly temperature /°C
Central	Kandy	red yellow podsolic soils, immature brown loams and reddish brown latosolic soils	50-60	23
	Nuwaraeliya	red yellow podsolic soils with dark B horizon and red yellow podsolic soils with prominent A1 horizon	50-60	15
	Dambulla	reddish brown earths and immature brown loams	< 30	28
North Central	Anuradhapura	reddish brown earths and immature brown loams	< 30	28
North Western	Kurunegala	red yellow podsolic soils with strongly mottled sub soils, low humic glay soils, red yellow podsolic soils with soft and hard laterite, regosols on old red and yellow sands	35-40	27
Sabaragamuwa	Ratnapura	red yellow podsolic soils and red	>125	25
Western	Colombo	red yellow podsolic soils with soft and hard laterite soils and bog to half bog soil	40-50	27
	Awissawella	yellow podsolic soils with semi prominent A1 horizon	100-125	

Department of Meteorology, Sri Lanka, 2009

Sampling

Sampling was carried out in all selected districts (Plate 2.8) from various sources as in table 2.16. Sampling was done from randomly distributed sites in a particular province by using a grid with equal squares, kept on district maps. Samples were collected following the standard methods described in the section 2.3 of Chapter 2. Water samples were collected from 3 surface water bodies and 3 drinking water wells from each district. When collecting bottle water samples, district from which the bottle was purchased was not considered since the source of origin was more important for bacteriological contamination of water. Effluent samples were collected mainly from wastewater treatment facilities of leading hotels and other domestic wastewater treatment facilities. Sampling was conducted from July 2008 to June 2009.

Table 2.16 Water samples collected from different geographical areas of the country

Province	District	Bottle water	Well water	Surface water	Effluents
Central	Kandy	15	3	river/stream_ 2 lakes-1	5
	Nuwaraeliya	_	3	lakes-2	_
North Central	Anuradhapura	_	3	lakes_3	
	Dambulla	_	1 (tube well)	_	2
North Western	Kurunegala	_	3	lakes-2	_
Sabaragamuwa	Ratnapura	5	3	river/stream_ 4	1
Western	Colombo	5	1	river/stream_ 3	_
	Total	**25**	**17**	**17**	**8**

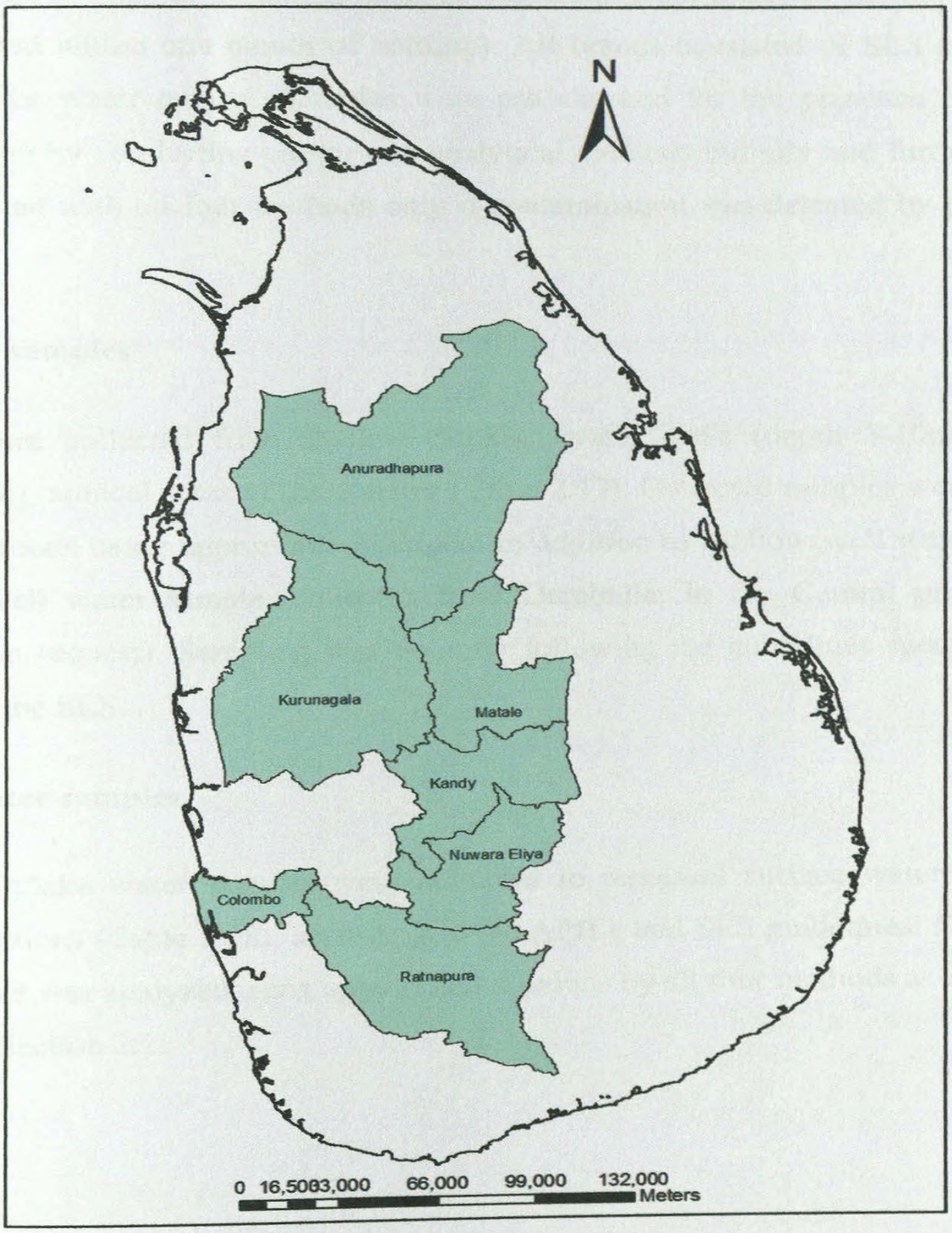


Plate 2.8 Distribution of sampling districts

Bottled water samples

Different brands of drinking bottle water were collected from shops irrespective of their manufactured or selling district. Twenty five brands were collected which were filled from different sources such as natural springs or dug wells from different districts. (samples were collected within one month of bottling). All brands consisted of SLS certification (SLS 894) for water quality. Samples were pre checked for the presence of coliform contamination by conducting one or two analytical methods initially and further analysis was conducted with all four methods only if contamination was detected by preliminary testing.

Well water samples

Samples were collected from shallow drinking water wells (depth 3-10m) covering different geographical areas of the country (Table 2.17). Collected samples were analyzed by four methods using appropriate dilutions. In addition to shallow well water samples, one tube well water sample, collected from Dambulla, in the Central province was analyzed (on request). Sampling was done by following the guidelines specified in the APHA and the SLS.

Surface water samples

River/stream/lake water samples were collected to represent surface water sources, at various locations (Table 2.18), according to the APHA and SLS guidelines. Each surface water sample was analyzed using appropriate dilutions by all four methods as described in Chapter 2, Section 2.1.

Table 2.17 Well Water samples collected from different geographical areas of the country

Distritct	Location	Sample name	Description closed/ with a ring wall (height/m)	Soil type
Kandy	Gelioya	KD1	0.9	Reddish brown latosolic,
	Kossinna	KD2	0.8	immature brown loams, low
	Gannoruwa	KD3	Closed	humic glay and red yeliow podsolic
Nuwaraeliya	Meepilimana	NE1	0.9	Red yellow podsolic soils with
	Ambewela	NE2	1	prominenet A1 horizon and red
	Pattipola	NE3	1	yellow podsolic with dark brown B horizon
Dambulla	Kandalama	TW1	tube well (closed)	Reddish brown earth and low humic glay soils
Anuradhapura	Jaffna Junc.	AP1	1	
	Mihinthale	AP2	0.9	
	Matale Junc.	AP3	1	
Kurunegala	Kalugamuwa	KG1	1	Red yellow podsolic soils with
	Gepallawa	KG2	1	strongly mottled sub-soil, red
	Malkaduwawa	KG3	0.9	yellow podsolic, low humic gly, reddish brown latosolic and regosol solis
Ratnapura	Atale	RP1	1	Red yellow podsolic, red yellow
	Seethawaka	RP2	1	podsolic soils with semi
	Dehiowita	RP3	0.8	prominent A1 horizon and low humic glay soils
Colombo	Dehiwala	CB1	1	Red yellow podsolic with soft
	Pitakotte	CB2	1	and hard laterite, low humic gley
	Malabe	CB3	0.9	and regosol solis

Table 2. 18 Surface water samples collected from different geographical areas of the country

District	Type	Sample name	Source	Sampling location
Kandy	river	KDR1	Mahaweli	Gannoruwa bridge
	stream	KDS1	Mahaoya	Near 'Gajasinghe' bridge
	lake	KDL1	Kandy Lake	Opposite to 'Awanhala' restaurant
Nuwaraeliya	lake	NEL1	Gregory Lake	Near the main road
	lake	NEL2	Black Pool	Near the main road
	stream	NES1	Not known (Malabe)	Malambe bridge
Anuradhapura	lake	APL1	Basawakkulama Lake	Near the army camp
	lake	APL2	Nuwara Wewa	Near the 'Sorowwa'
	stream	APS1	Malwathu oya	Near the bridge (on the way to Jethawanaramaya)
Kurunegala	lake	KGL1	Kurunegala Lake	Closer to the water intake
	lake	KGL2	Jayawadane Wewa	Closer to the bathing area
	steam	KGS1	Not known (Gepallawa)	Bridge at Gepallawa
Awissawella	stream	RPS1	Ritigala Oya	Near the Ritigala bridge
	stream	RPS2	Gurugoda Oya	Near the Gurugoda bridge
Ratnepura	stream	RPS3	Seethawaka Ganga	Near the Seethawaka bridge
Colombo	river	CBR1	Kelani river	Kelani bridge
	stream	CBS2	Diyawannawa oya	Opposite to the Parliament building
	stream	CBS3	Not known (Nugegoda)	Nugegoda town

Effluent water samples

Effluent samples were collected from number of wastewater treatment plants, designed for treating domestic wastewater (Table 2.19). Five samples were collected from Kandy; three samples of them were from wastewater treatment plants established in leading hotels in Kandy, one sample from a domestic wastewater treatment plant, designed for treating wastewater from a housing scheme in Kandy and the other from a treatment facility, treating wastewater released from a university dormitory. Of the other three samples, one was collected from an industrial zone, where industrial wastewater directed to the same domestic wastewater treatment plant after on-site pre-treatment. Other two samples were collected from hotel wastewater treatment plants located in Dambulla area. Appropriate dilutions were analyzed using four methods as described in Chapter 2, Section 2.2.

Table 2. 19 Effluent water samples

District	Type	Origin	Sample name
Kandy	Anaerobic filter	hotel	KDE1
	Package treatment plant (aerobic and anaerobic)	hotel	KDE2
	Package treatment plant (aerobic and anaerobic)	hotel	KDE3
	Trickling filter	Hanthana housing scheme	KDE4
	Constructed wetland	University dormitory	KDE5
Anuradhapura	Activated sludge	hotel	APE1
	Activated sludge	hotel	APE2
Ratnapura	Oxidation ditch	BOI Industrial zone	RPE1

2.6.2. Bacteriological examination of water

Collected samples were transported to the laboratory under cold conditions (4 °C) and the analysis was done after the temperatures of water reached room temperature. Each sample was analysed in two replicates with appropriate dilutions (Section 2.3 of Chapter 2).

Three bacteriological analytical methods were compared with the SLS-MTF reference method (SLS 614: Part 2: 1983) for the detection and enumeration of total coliforms and *Escherichia coli*. Considering the results obtained in the preliminary study of this research, APHA method was omitted in comparison, since it was not significantly different with SLS-MTF method for the detection of total coliform bacteria. Further, APHA is a conventional method consisting of inherited disadvantages characteristic to MPN methods. Other alternative methods included, Colilert-MTF and two MF methods; SLS-MF method and the m-ColiBlue24 method.

Bacteriological analysis was conducted as described in Section 2.3 of Chapter 2.

2.6.3. Confirmation of bacteria

Pure isolated colonies were used for confirmation of bacteria. Pure colonies were obtained by subculturing on Tryptic Soy Agar (TSA) (Oxoid) plates. Loopfulls taken from selected presumptive positive tubes of MTF methods were streaked on TSA and incubated for 24 hours at 37±1 °C. Selected (isolated) presumptive positive colonies on membrane filters were subcultured by streaking as described above and isolated colonies were obtained on TSA. At least three tubes from each MPN method and at least five colonies from a MF plate were subcultured (per sample) several times on TSA, until separated colonies with similar morphological features appeared. Those were considered as pure cultures and they were tested with three basic biochemical tests, done for confirming the bacteria belonging to family Enterobacteriaceae. They were, Gram's, Catalase and Oxidase tests.

Gram's test was done by placing a drop of 3 % potassium hydroxide on a glass slide, on to which an isolated colony from a TSA plate was gently mixed. A positive test was indicated by the formation of a continuous string like appearance when raised up with the wire loop. Catalase test was performed by mixing up a colony from a culture plate with a

wire loop on to a drop of 3 % Hydrogen peroxide. Emergence of oxygen bubbles was considered a positive reaction. Oxidase test was performed by applying a small portion of a pure colony on an Oxidase strip with a sterilized toothpick. Purple colouration within 10 seconds was considered a positive reaction.

2.6.3.1 Confirmation of total coliform bacteria

All Gram negative, Catalase positive and Oxidase negative colonies were confirmed as coliform bacteria included in the family Enterobacteriaceae.

2.6.3.2 Confirmation of *E. coli* bacteria

Pure cultures showing positive reactions for all three tests isolated from presumptive *E. coli* positive tubes and membranes were tested with indole reaction by inoculating in to tryptone water tubes. After incubation at 37±1 °C, 2 to 3 drops of Kovac's reagent was added and positive confirmed tests were obtained by formation of a red colour ring over tryptone water.

All positively confirmed cultures and some negatively confirmed cultures were stored by freezing for further studies. For storing, cultures were inoculated in to Eppendorf tubes, containing double strength Brain Heart Infusion broth (Oxoid) or double strength Tryptic Soy Broth (Merk) and incubated for 24 hours at 37 °C. The cultures were stored at –20 °C, after overlaying with 40 % glycerol on the broth.

2.7. RESULTS

Bacteriological data obtained for different source water types, collected from different geographical areas were analysed by following the same procedures as described in the section 2.3 (Part-A), of this chapter. In addition, the results were also analysed using the ISO criteria for comparison of bacteriological methods (Appendix 2: Sections 2.1, 2.2). Sections 2.7.1 and 2.7.2 describes the data interpretation and analysis following the same principles as described in the section 2.4.

2.7.1. Bacteriological counts by different methods

Total coliform and *E. coli* counts present in each sample, from different sources collected from different geographical areas were obtained by averaging the counts enumerated of two replicates for each sample. Summary of total coliform and *E. coli* counts are presented in tables 2.20 and 2.21 respectively.

For total coliform analysis, 314 valid data, out of 344 analyses were remained (Table 2.20) due to too numerous to count (TNTC) data, however, there were no missing data due to experimental errors. In this experiment also, the total coliform bacteriological counts in water samples were different when analyzed by four different methods like in the case of the preliminary test. Minimum (2 cfu/100 ml) and the maximum (1.1×10^6 cfu/100 ml) total coliform counts were obtained by SLS and Colilert methods respectively. The lowest mean count was detected by SLS, as in the case of the preliminary study. However, the highest mean count was recorded by Colilert, compared to M-endo in the preliminary study.

Table 2.20 Summary of total coliform counts in all water types by different methods

Method	Total number of analysis (# = 344)	Mean of total coliform counts (cfu/100 ml)	Standard Deviation	Minimum** (cfu/100 ml)	Maximum (cfu/100 ml)
SLS	41 x 2 ($^{a=2}$)	7.3×10^4	$\pm 1.6 \times 10^4$	2.0	7.8×10^4
Colilert	40 x 2 ($^{(a=3}$)	9.8×10^5	$\pm 2.6 \times 10^5$	9.5	1.1×10^6
M-endo	38 x 2 ($^{(a=5}$)	2.9×10^5	$\pm 3.9 \times 10^5$	3.5 x 10	1.2×10^5
M-coli	38 x 2 ($^{(a=5}$)	5.7×10^5	$\pm 1.3 \times 10^5$	3.7 x 10	7.2×10^5

Total number of samples (N= 43); (sources = 4; replicates = 2); (methods = 4)
[a] = missing values due to *too numerous to count (TNTC)* values
** = Zero counts were excluded

In respect to *E. coli* enumeration there were 314 valid data (Table 2.21) for analysis due to same reasons explained above. The minimum *E. coli* count (1.0 cfu/100 ml) was enumerated by SLS, and the maximum (7.2×10^5) was enumerated by m-ColiBlue24

medium. When considering the means of *E. coli* counts, the lowest count was recorded by SLS (3.4×10^4) and the highest *E. coli* counts (3.3×10^5) were obtained by Colilert method reproducing the same results as in the preliminary test. Further, m-ColiBlue24 method produced similar counts with Colilert in the preliminary study, while it was comparatively less in this study.

Table 2.21 Summary of *E. coli* counts in all water types by different methods

Method	Total number of analysis (# = 344)	Sum of total coliform counts (cfu/100 ml)	Standard Deviation	Minimum** (cfu/ 100 ml)	Maximum
SLS	41 x 2 ([a=2])	9.3×10^3	$\pm 2.6 \times 10^3$	1.0	1.5×10^4
Colilert	40 x 2 [(a=3)]	8.8×10^4	$\pm 2.7 \times 10^4$	1.1	1.5×10^5
M-endo	38 x 2 [(a=5)]	2.5×10^4	$\pm 7.4 \times 10^3$	2.0	1.2×10^5
M-coli	38 x 2 [(a=5)]	3.9×10^4	$\pm 1.4 \times 10^4$	8.0	7.2×10^5

Total number of samples (N= 43); (sources = 4; replicates = 2); (methods = 4)
[a] = missing values due to *too numerous to count (TNTC)* values
** = Zero counts excluded

2.7.2. Bacteriological counts in different sources by different methods

Total coliform counts in different water sources by different methods

Total values of the total coliform counts obtained for different water sources, when analyzed by four different methods are depicted in figure 2.12- a, b, c, d. Mean counts, standard deviations, minimum and maximum counts detected by different methods for each sources water type are presented in the table 2.22.

Bottled water samples: Among the bottle water samples (25 brands) tested, only one brand purchased from Kandy, was contaminated by total coliforms. Two other brands, which showed contamination in preliminary tests, were negative when analyzed by all four methods. All the other remaining brands (22 numbers), analyzed within the experimental time period, were not contaminated by total coliform bacteria.

Total coliform counts varied from 2.0 – 2.3 x 10^3 cfu/100 ml in replicate counting by different methods. The highest mean counts were detected by m-ColiBlue24 method (2.3x 10^3 cfu/100 ml), which is thousand times higher than that of the SLS method (Table 2.22). However, the differences among the Colilert, M-endo and m-ColiBlue24 methods were negligible, compared to counts of those three methods with that of SLS-MTF counts.

Well water samples: Total coliform counts ranged from 2.0 cfu/100 ml (SLS) to 3.1 x 10^4 cfu/100 ml (m-ColiBlue24) during the experimental period. The highest mean counts were detected by m-ColiBlue24 method compared to other methods. It was nearly 6 times higher than SLS method (Table 2.22), which was a similar result with the preliminary study. However, both M-endo and m-ColiBlue24 methods gave similar counts when adding all values together (total count) (Figure 2.12-b).

Surface water samples: The counts ranged from 1.4 x 10^2 (SLS) to 3.8 x 10^6 (M-endo), during the analysis. The highest and the lowest mean and total counts were given by Colilert and SLS methods respectively (Table 2.22 and Figure 2.12-c). The difference was more than 8 times higher.

.

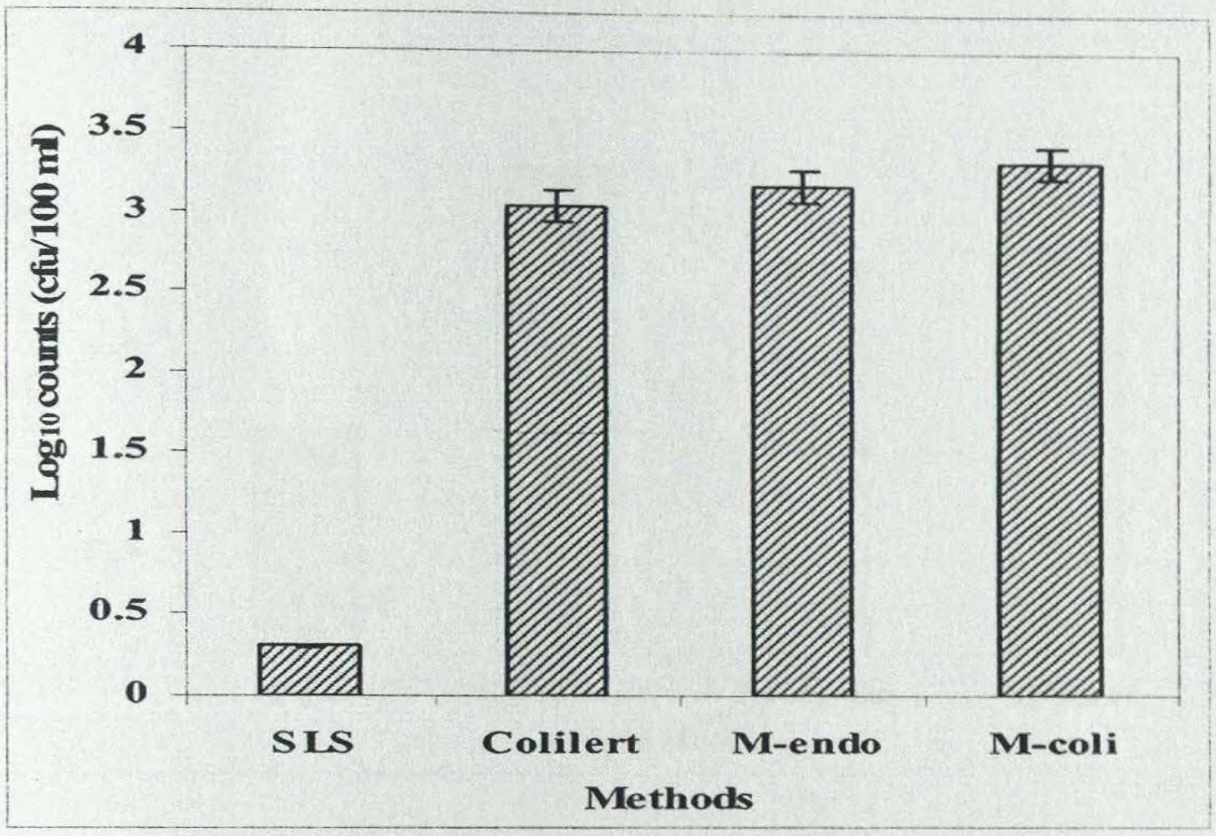
Effluent water samples: The highest total coliform counts were detected in effluent samples. Counts ranged from 7.0 x 10 (SLS) to 7.2 x 10^6 (m-ColiBlue24), during the analysis. The highest and the lowest mean counts were detected by SLS and m-ColiBlue24 (Table 2.22) Both Colilert and m-ColiBlue24 methods showed the highest total counts (Figure 2.1-c). Total value of m-ColiBlue24 was 8 times higher than the SLS-MTF value.

Table 2.22 Summary of total coliform counts in different water types by different methods

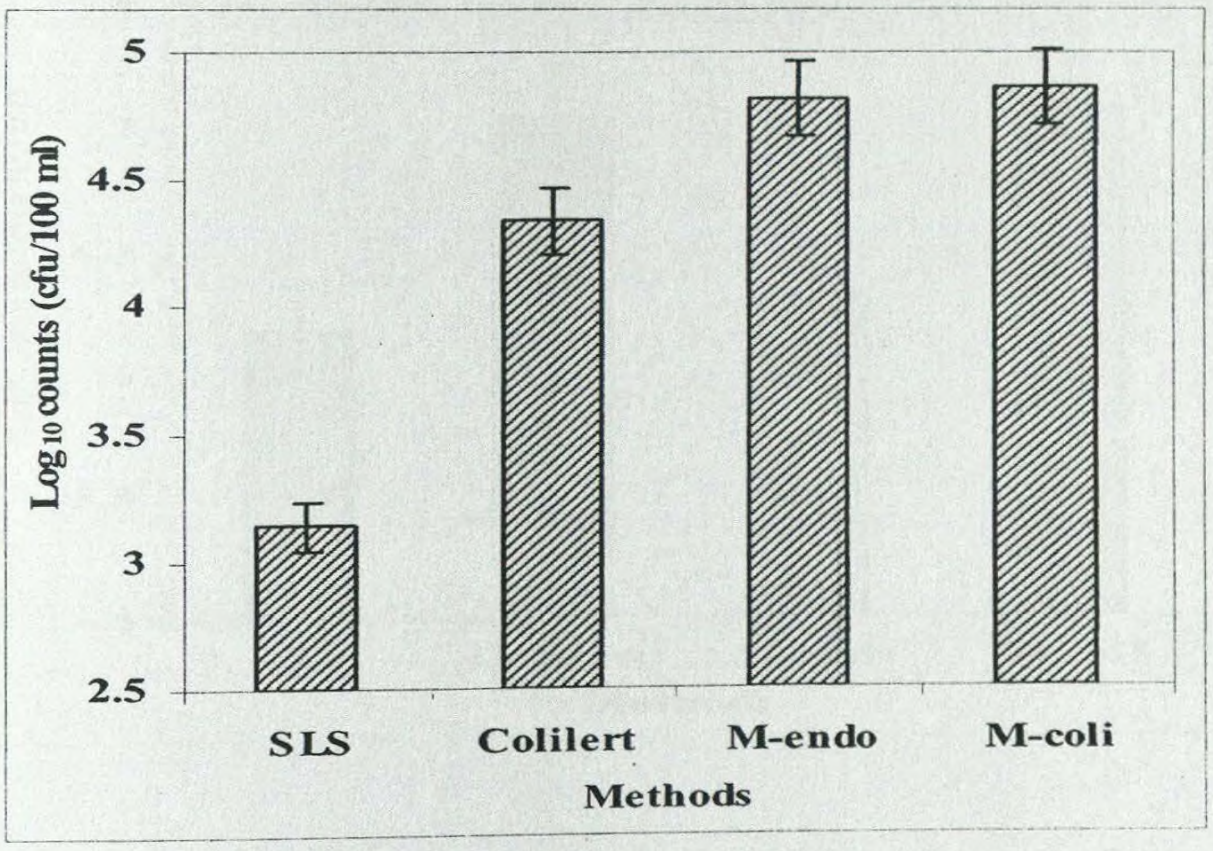
Source	Methods	Sample #	Mean total coliform counts (cfu/100 ml)	Standard Deviation	Min.** (cfu/ 100 ml)	Max. (cfu/ 100 ml)
Bottle	SLS	1 x 2	2.0	± 0	2.0	2.0
	Colilert	1 x 2	1.1×10^3	± 0	1.1×10^3	1.1×10^3
	M-endo	1 x 2	1.5×10^3	$\pm 2.8 \times 10$	1.9×10^3	2.0×10^3
	M-coli	1 x 2	2.0×10^3	$\pm 3.6 \times 10^2$	1.7×10^3	2.3×10^3
Well	SLS	12 x 2	1.1×10^2	$\pm 9.3 \times 10$	2.0	2.4×10^2
	Colilert	12 x 2	1.8×10^3	$\pm 2.9 \times 10^3$	1.8×10	1.1×10^4
	M-endo	12 x 2 [a=1]	5.5×10^3	$\pm 6.1 \times 10^3$	3.5×10^2	2.0×10^4
	M-coli	12 x 2 [a=1]	6.0×10^3	$\pm 8.5 \times 10^3$	3.7×10^2	3.1×10^4
Surface	SLS	17 x 2	7.5×10^4	$\pm 1.3 \times 10^5$	1.4×10^2	4.6×10^5
	Colilert	17 x 2 [a=1]	1.5×10^6	$\pm 3.5 \times 10^6$	1.6×10^2	1.1×10^7
	M-endo	17 x 2 [a=1]	5.2×10^5	$\pm 4.6 \times 10^5$	5.5×10^2	1.2×10^6
	M-coli	17 x 2 [a=2]	6.5×10^5	$\pm 6.5 \times 10^5$	1.2×10^3	3.8×10^6
Effluent	SLS	8 x 2 [a=2]	1.8×10^5	$\pm 2.6 \times 10^5$	7.0×10	7.8×10^5
	Colilert	8 x 2 [a=1]	1.3×10^6	$\pm 2.2 \times 10^6$	1.1×10^4	6.0×10^6
	M-endo	8 x 2 [a=3]	2.5×10^5	$\pm 2.1 \times 10^5$	9.9×10^2	5.9×10^5
	M-coli	8 x 2 [a=1]	1.5×10^6	$\pm 2.3 \times 10^6$	1.3×10^3	7.2×10^6

[a] = missing values due to *too numerous to count (TNTC)* values
** = Zero counts excluded

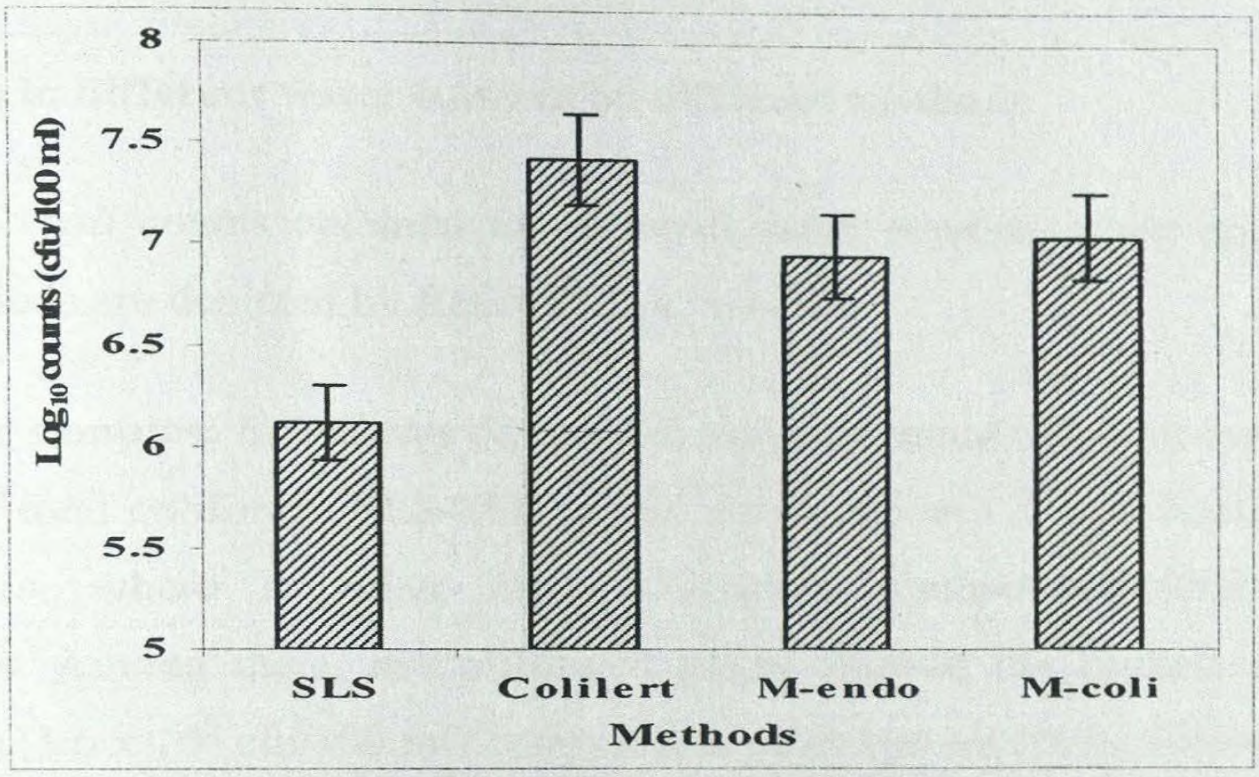
(a)


Log10 counts (cfu/100 ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
SLS
Colilert
M-endo
M-coli
Methods

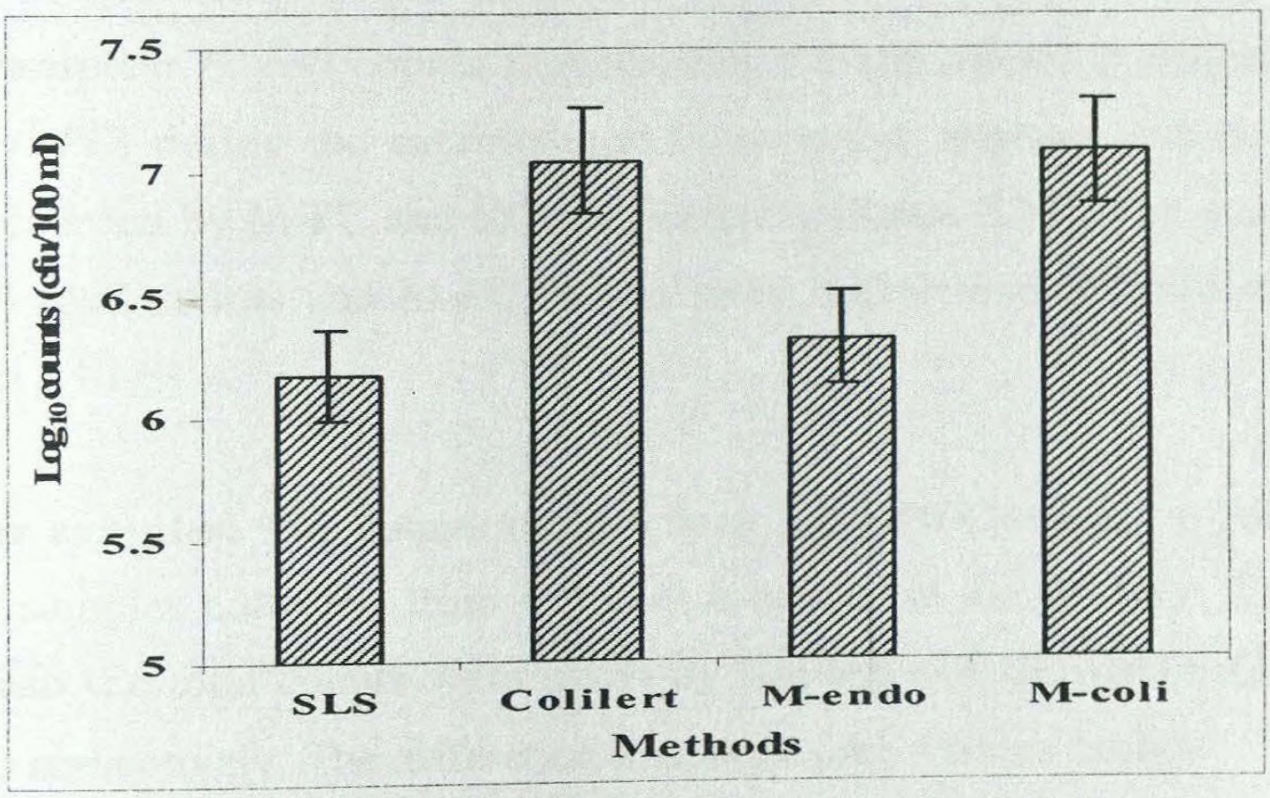
(b)


Log10 counts (cfu/100 ml)
5
4.5
4
3.5
3
2.5
SLS
Colilert
M-endo
M-coli
Methods

(c)



(d)



(a) Bottled water
(b) Well water
(c) River water
(d) Effluents

Figure 2.12 Total values of total coliform counts (log $_{10}$ values) in different water sources by different methods

The differences were statistically analyzed and presented in the section 2.4.1.2. by using Least Squares Mean Separation Test.

E. coli counts in different water sources by different methods

Sum of the *E. coli* counts obtained for different water sources, when analyzed by four different methods are depicted by figure 2.13-a, b, c, d.

Bottled water samples: *E. coli* was detected in only one brand of bottle water samples, as in the case of total coliforms. SLS-MTF could not detect any *E. coli* bacterium in bottle water samples, where as other three alternative methods detected at different concentrations. Among them, m-ColiBlue24 plates showed the highest typical *E. coli* colony counts (1.6×10^3 cfu/100 ml) compared to other two methods. However, mean and the total counts among the three methods were not much different (Table 2.23, Figure 2.13-a).

Well water samples: *E. coli* counts ranged from 1.1 cfu/100 ml (Colilert) to 1.3×10^2 cfu/100 ml (M-FC) during the experimental time period. Highest and the lowest mean counts were recorded by M-FC and SLS respectively (Table 2.23). The same pattern was observed with total counts and M-FC counts were higher than 8 times than SLS-MTF counts (Figure 2.13-b).

Surface water samples: The counts ranged from 3.0 (M-FC) to 6.1×10^5 (Colilert), in surface water samples collected from different locations in the country. The highest and the lowest mean the total counts were given by Colilert and SLS-MTF (Table 2.23 and Figure 2.13-c) respectively. The difference was more than 4 times higher.

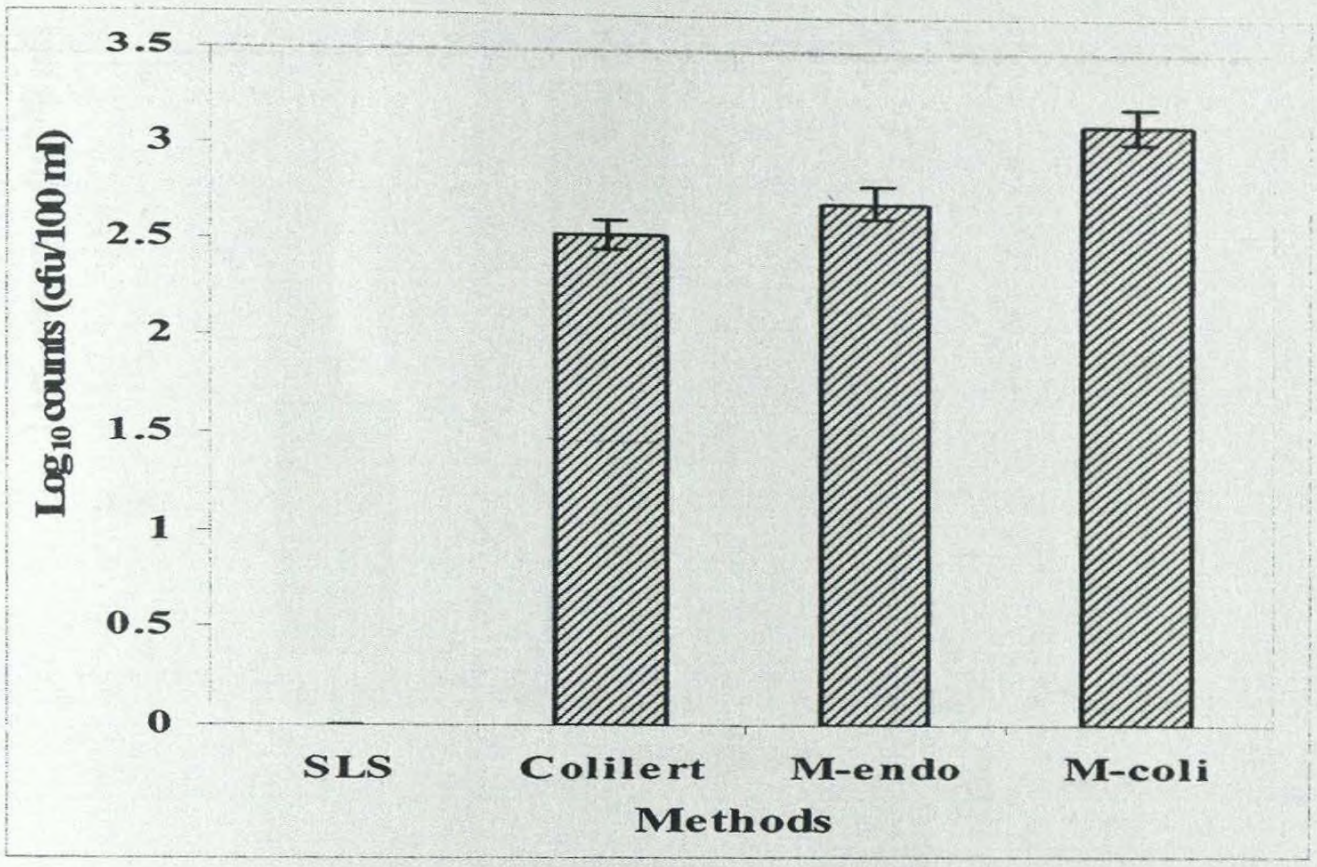
.

Effluent water samples: The highest *E. coli* counts were recorded from effluent samples as in the preliminary study, and the it ranged from 3.2×10 (SLS) to 1.6×10^6 (Colilert), in different effluent samples. The highest and the lowest mean counts were detected by Colilert and SLS-MTF (Table 2.23). Colilert was 8 times higher than the SLS method. Similarly the highest and the lowest total counts were given by Colilert and SLS methods. (Table 2.23 and Figure 2.13-d).

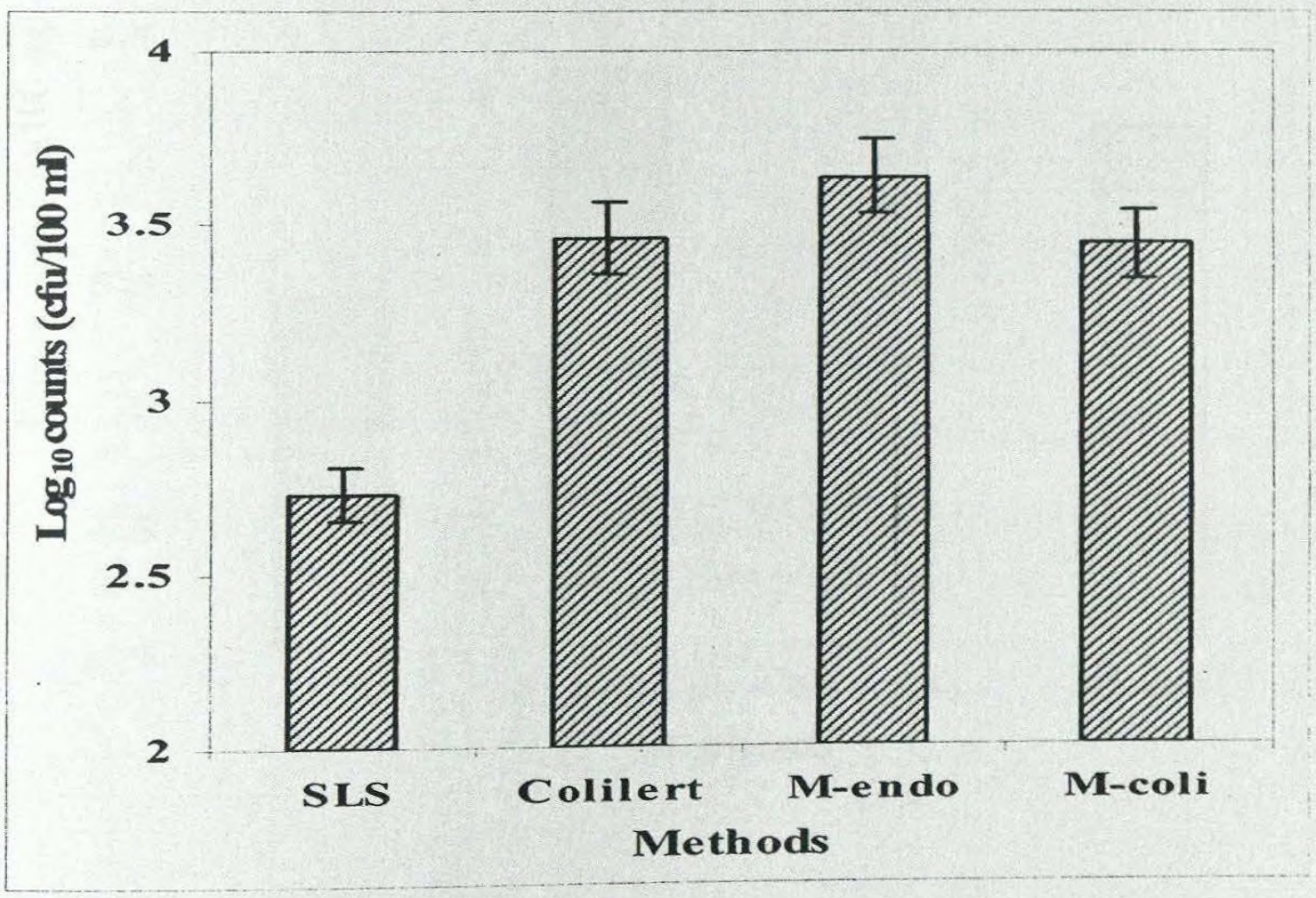
.Table 2.23 Summary of *E. coli* counts in different water types by different methods

Source	Methods	Sample #	Mean *E. coli* counts (cfu/100 ml)	Standard Deviation	Min. (cfu/ 100 ml)	Max. (cfu/ 100 ml)
Bottle	SLS	1 x 2	0.0	-	0	0
	Colilert	1 x 2	3.4×10^2	$\pm 1.7 \times 10^2$	2.2×10^2	4.6×10^2
	M-FC	1 x 2	5.2×10^2	$\pm 6.0 \times 10^2$	9.0×10	9.4×10^2
	M-coli	1 x 2	1.3×10^3	$\pm 4.3 \times 10^2$	1.0×10^3	1.6×10^3
Well	SLS	12 x 2	4.4×10	$\pm 4.0 \times 10$	2.0	1.3×10^2
	Colilert	12 x 2	2.4×10^2	$\pm 3.9 \times 10^2$	1.1	1.1×10^3
	M-endo	12 x 2 (a=)	3.6×10^2	$\pm 4.9 \times 10^2$	2	1.3×10^3
	M-coli	12 x 2 (a=)	2.3×10^2	$\pm 2.4 \times 10^2$	2.5	6.8×10^2
Surface	SLS	17 x 2	4.4×10^3	$\pm 7.5 \times 10^3$	4.0	2.7×10^4
	Colilert	17 x 2 (a=1)	5.9×10^4	$\pm 1.5 \times 10^5$	2.3×10	6.1×10^5
	M-endo	17 x 2 (a=1)	1.8×10^4	$\pm 4.7 \times 10^4$	3.0	1.5×10^5
	M-coli	17 x 2 (a=2)	6.8×10^3	$\pm 1.1 \times 10^4$	5.9×10	3.9×10^4
Effluent	SLS	8 x 2 (a=2)	3.5×10^4	$\pm 4.9 \times 10^4$	3.2×10	1.5×10^5
	Colilert	8 x 2 (a=1)	2.9×10^5	$\pm 5.3 \times 10^5$	2.2×10^2	1.6×10^6
	M-endo	8 x 2 (a=3)	8.7×10^4	$\pm 1.4 \times 10^5$	3.0×10^2	4.2×10^5
	M-coli	8 x 2 (a=1)	1.7×10^5	$\pm 3.0 \times 10^5$	4.5×10^2	8.7×10^5

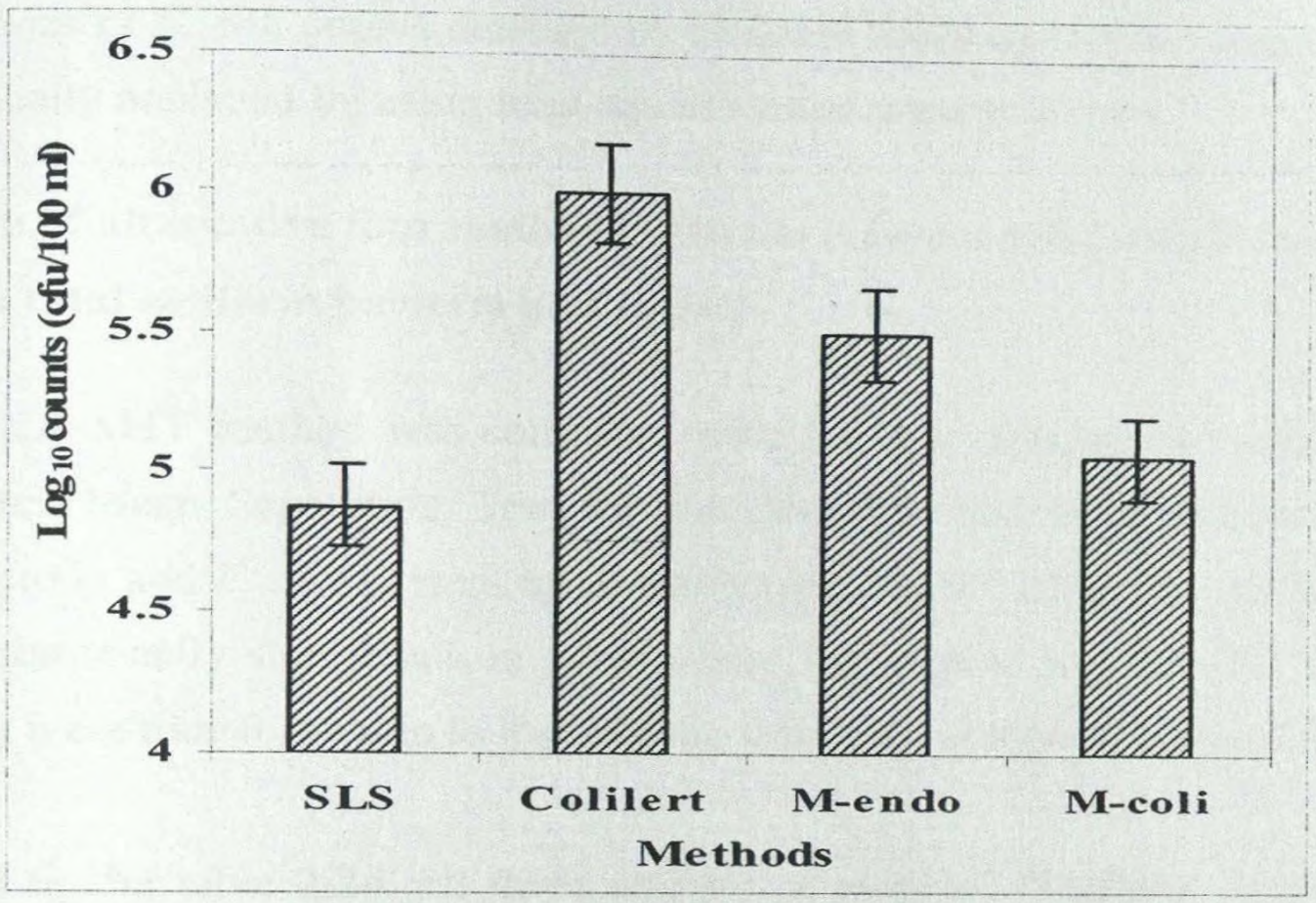
(a)


3.5
3
2.5
2
1.5
1
0.5
0
Log10 counts (cfu/100 ml)
SLS
Colilert
M-endo
M-coli
Methods

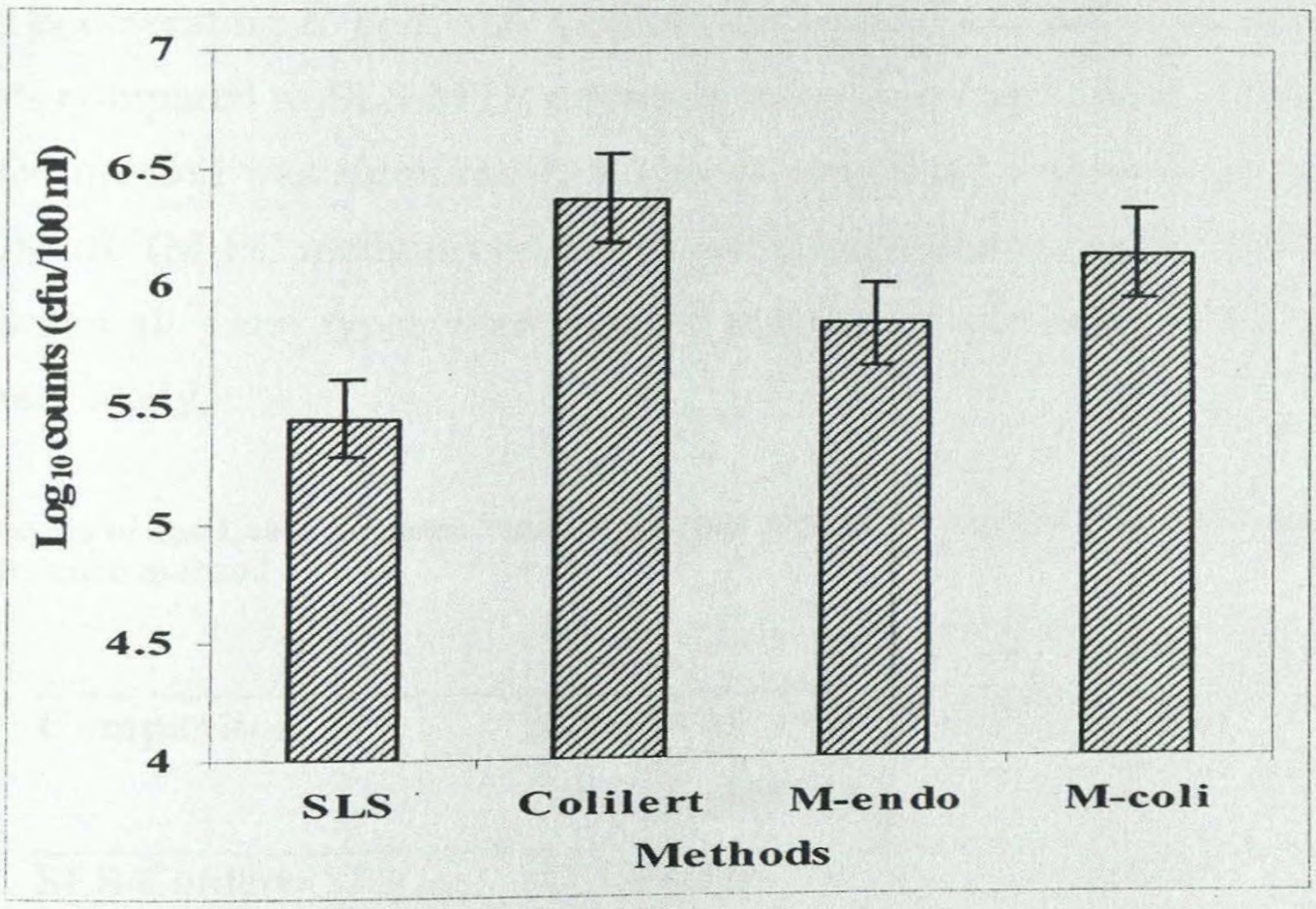
(b)


4
3.5
3
2.5
2
Log10 counts (cfu/100 ml)
SLS
Colilert
M-endo
M-coli
Methods

(c)



(d)



(a) Bottled water
(b) Well water
(c) River water
(d) Effluents

Figure 2.13 Total *E. coli* counts in different water sources by different methods

The differences of *E. coli* counts detected by different methods in four source water types were statistically analyzed by using least squares mean separation test.

Comparison of alternative four methods with the reference SLS-MTF method for the detection of total coliform bacteria and *E. coli*

Reference SLS-MTF method was compared with the four alternative methods by using Least Squares Mean Separation Test for the detection and enumeration of the total coliform bacteria and *E. coli* present in all water types (Table 2.24). The differences were considered statistically significant, in cases where the p-value was ≤ 0.05. Both MF and MPN results were transformed in to logarithmic values for statistical analysis.

As depicted in the table 2.24, all three alternative methods (Colilert, SLS-MF and m-ColiBlue24) were significantly different with the SLS-MTF reference method in detecting total coliforms bacteria, when all water sources were grouped together ($p \leq 0.05$). However, in enumerating *E. coli*, only Colilert and method was able to detect significantly higher counts compared to SLS-MTF reference method at a 95 % confidence level, while m-ColiBlue24 method was significantly higher at a confidence level of 90 % ($p \leq 0.1$). In contrast, SLS-MF (M-FC medium) was not significantly different with SLS-MTF method ($p = 0.15$), when all water types were grouped together, which was not the case found in the preliminary study.

Table 2.24 Results of the Least Squares Mean Separation test to compare alternative methods with SLS-MTF reference method

Comparison	p-value for comparison with SLS	
	Total coliforms	*E. coli*
SLS/Colilert	0.0021*	0.0280*
SLS/M-endo/MFC	<.0001*	0.1581
SLS/M-coliBlue24	<.0001*	0.0823**

* $p \leq 0.05$. ** $p \leq 0.1$

Comparisons of bacteriological counts in different water sources

Comparison of four alternative methods with the reference SLS-MTF method for the detection of both total coliform bacteria and *E. coli* in different water sources was performed similarly as explained in the section 2.3 of this chapter. Results are shown in the Table 2.25.

Total coliform counts in different water sources

According to Table 2.25, all the alternative methods (Colilert, SLS-MF (M-endo medium) and m-ColiBlue24methods) were significantly different with the reference SLS-MTF method in detecting total coliform bacteria in bottle water, well water and in surface water sources. In contrast, non of the alternative methods were significantly different with SLS-MTF method in detecting total coliforms in effluent samples at 95 % confidence level. However, the two-enzymatic methods (Colilert and m-ColiBlue24) were able to detect significantly higher total coliform counts at 90 % confidence level in effluent samples, where M-FC could not detect any higher counts at least at 10 % confidence level ($p = 0.3659$).

***E. coli* counts in different water sources**

When considering the detection and enumeration of *E. coli*, all three alternative methods were able to detect significantly higher *E. coli* concentrations at a 95% confidence level in bottle water source, compared to the conventional SLS-MTF method However, in non of the other water sources alternative methods could not detect significantly higher counts of *E. coli* at 95% confidence level, where as, M-endo and m-ColiBlue24 methods in well water samples, and Colilert method in surface water samples were able to detect significantly higher counts at a confidence level of only 90% respectively. In the preliminary study, counts on M-FC medium were statistically different with SLS-MTF in surface water samples, at 90 % confidence level. In effluent samples, *E. coli* enumeration was not statistically different by any of the alternative methods compared to the SLS-MTF method. However, in the preliminary study, two enzymatic methods were able to detect

high concentrations of *E. coli* at significant levels compared to the conventional SLS-MTF method

Table 2.25 Results of the Least Squares Mean Separation Test for Comparison of three methods with SLS-MTF reference method

Water source	Comparison	p-value for comparison with SLS	
		Total coliforms	*E. coli*
Bottle			
	SLS/Colilert	<.0001*	<.0001*
	SLS/M-endo[1]/M-FC[2]	<.0001*	<.0001*
Well			
	SLS/Colilert	0.0017*	0.1806
	SLS/M-endo[1]/M-FC[2]	<.0001*	0.0980**
	SLS/M-coliBlue24	<.0001*	0.0695**
Surface			
	SLS/Colilert	0.0189*	0.0807 **
	SLS/M-endo[1]/M-FC[2]	0.0012*	0.5316
	SLS/M-coliBlue24	0.0007*	0.4065
Effluent			
	SLS/Colilert	0.0678**	0.1308
	SLS/M-endo[1]/M-FC[2]	0.3659	0.5934
	SLS/M-coliBlue24	0.0843**	0.2337

* $p \leq 0.05$ ** $p \leq 0.1$ [1] M-endo medium – for total coliforms [2] M-FC medium – for *E. coli*

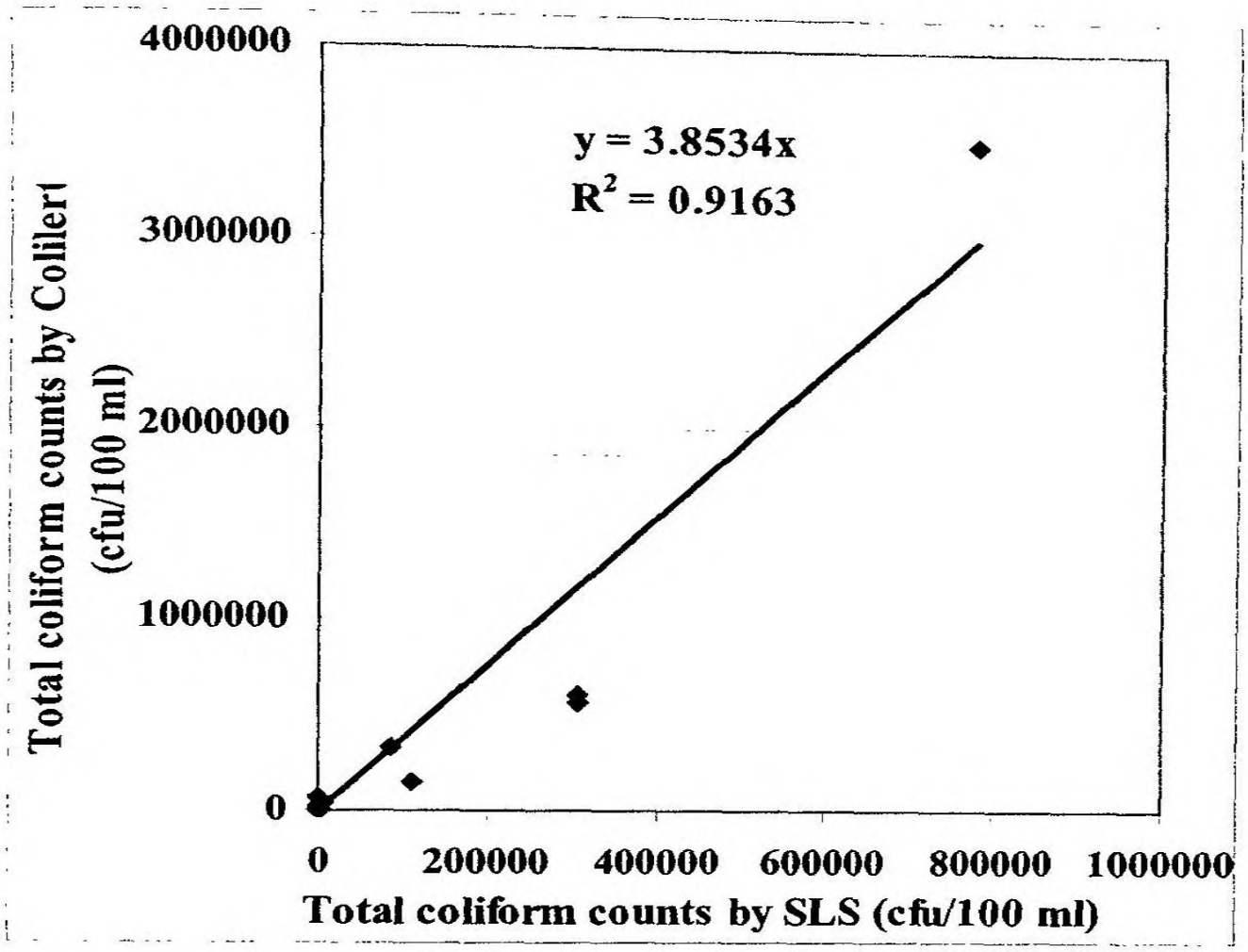
Simple linear model for analyzing the relationships between different methods

The relationship between different alternative methods with the SLS-MTF reference method, was analyzed by using the Simple Linear Model test as described in section 2.4.1.2 of this chapter. Results of the linear relationships between SLS-MTF reference method with the other alternative methods for the detection and enumeration of total coliform bacteria and *E. coli* are shown in Figures 2.14.and 2.15 respectively. Figures a, b, and c depict the linear relationships for Colilert, M-endo (for total coliforms)/M-FC (for *E. coli*), and M-coli with SLS-MTF reference method respectively.

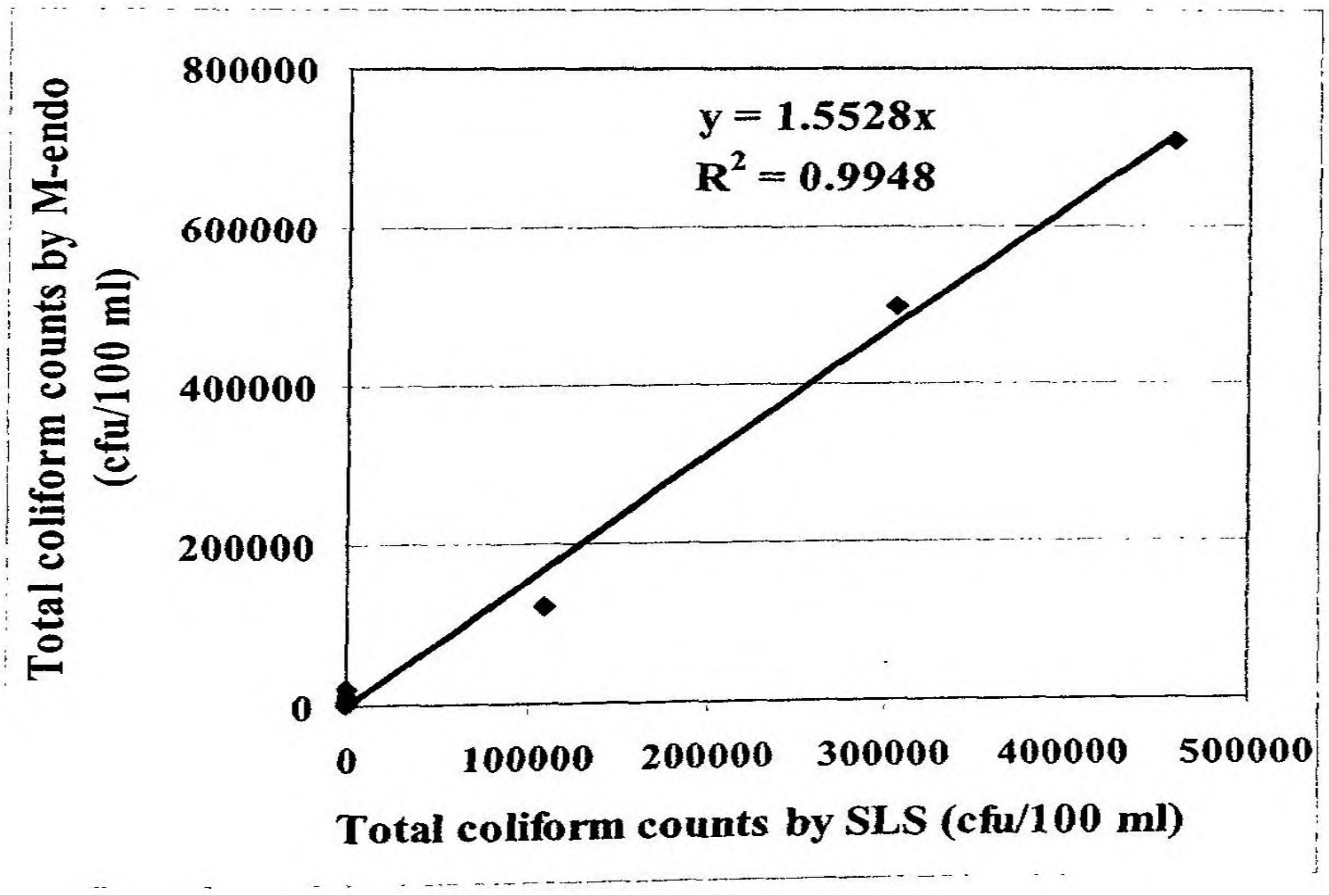
Simple linear relationships for detecting total coliform bacteria

As depicted in the Figure 2.14-a, b and c, all three alternative methods showed positive significant linear relationships when computed with SLS-MTF reference method. Among them, the linear relationship between SLS-MTF with M-endo was the most significant relationship ($R^2 = 0.9948$), compared to other two relationships. However, according to the simple linear model the highest counts compared to SLS-MTF counts could be obtained by the Colilert method, when applied to the equation $y = 3.8534x$. Therefore, the Colilert counts are more than 3.8534 times higher than that of the SLS-MTF method. M-ColiBlue24 could detect 1.7534 times higher counts and M-endo could detect 1.5528 times higher total coliform counts than the SLS-MTF method respectively.

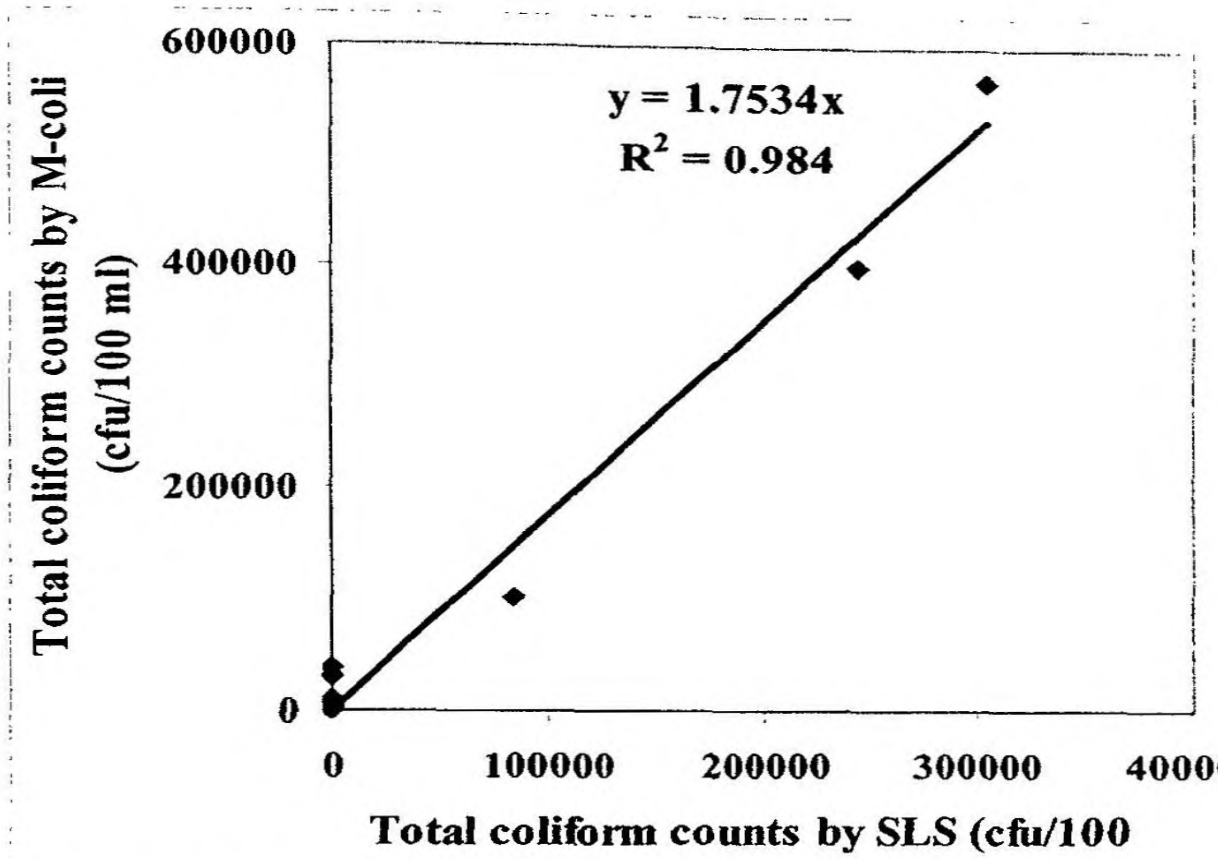
(a)


y = 3.8534x
R² = 0.9163
Total coliform counts by Colilert (cfu/100 ml)
4000000
3000000
2000000
1000000
0
0
200000
400000
600000
800000
1000000
Total coliform counts by SLS (cfu/100 ml)

(b)


y = 1.5528x
R² = 0.9948
Total coliform counts by M-endo (cfu/100 ml)
800000
600000
400000
200000
0
0
100000
200000
300000
400000
500000
Total coliform counts by SLS (cfu/100 ml)

(c)



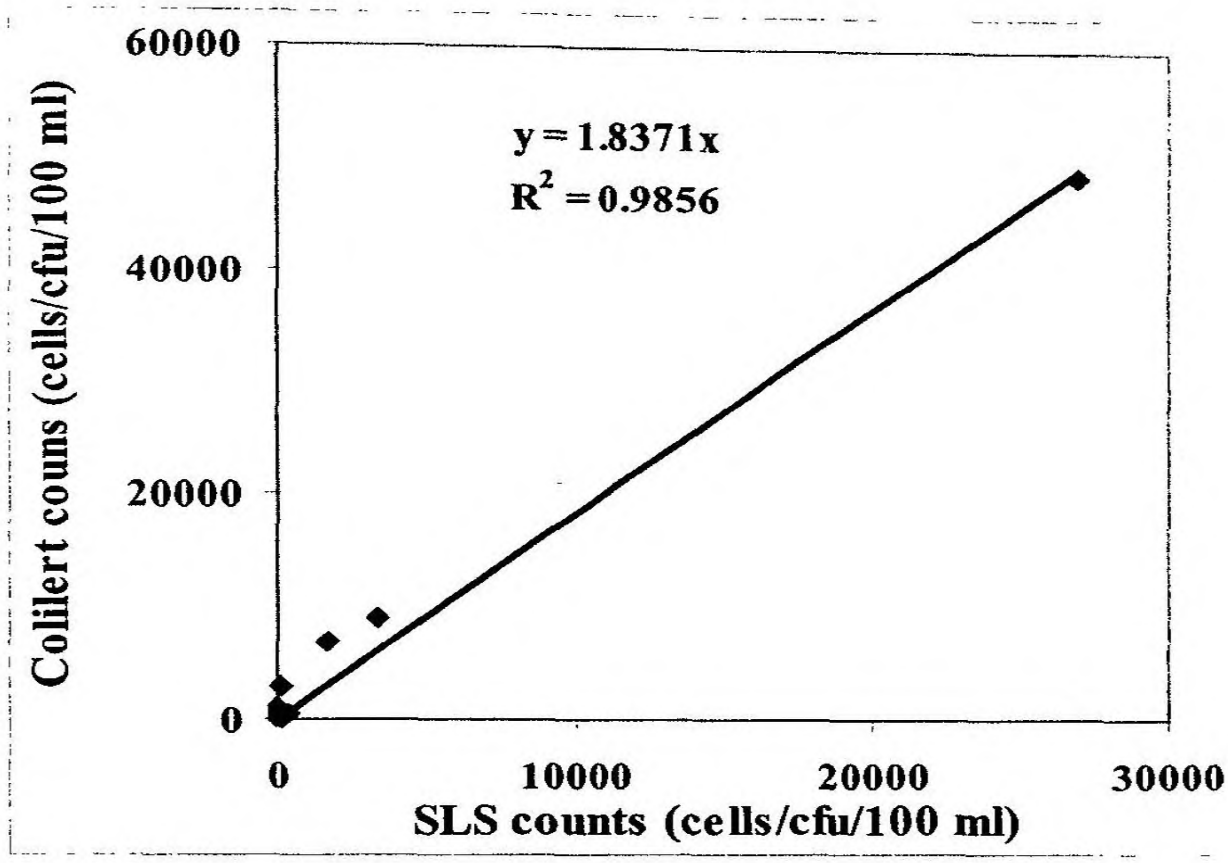
(a)- SLS-MTF/Colilert
(b)- SLS-MTF/M-endo
(c)- SLS-MTF/M-coliblue24

Figure 2.14 Simple Linear Model for comparing SLS method and alternative methods for detecting total coliforms

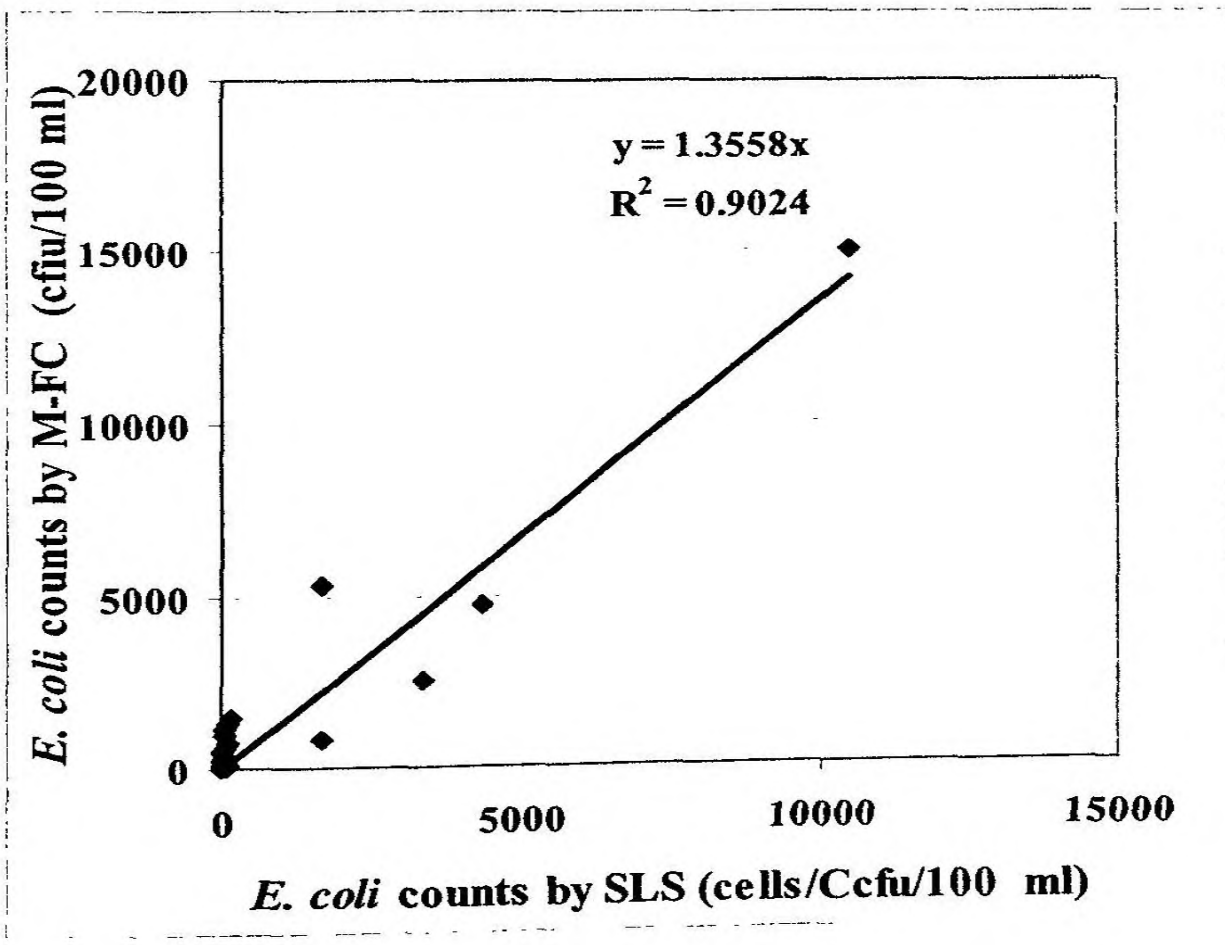
Simple linear relationships for detecting *E. coli*

As shown in Figure 2.15, all the alternative methods resulted in positive linear relationships with SLS-MTF method in detecting *E. coli* bacteria (Figure 2.15 - a, b, and c for Colilert, M-FC and m-ColiBlue24 respectively). Among them the most efficient relationships were shown by Colilert and m-ColiBlue24 having R^2 values 0.9856 and 0.9835 respectively. Compared to other two methods the highest counts were given by m-ColiBlue24 method, when all the water sources were grouped together ($y = 2.9355x$). Colilert method detected 1.8371 times higher counts and M-FC detected 1.3558 times higher E. coli counts that the conventional SLS-MTF method.

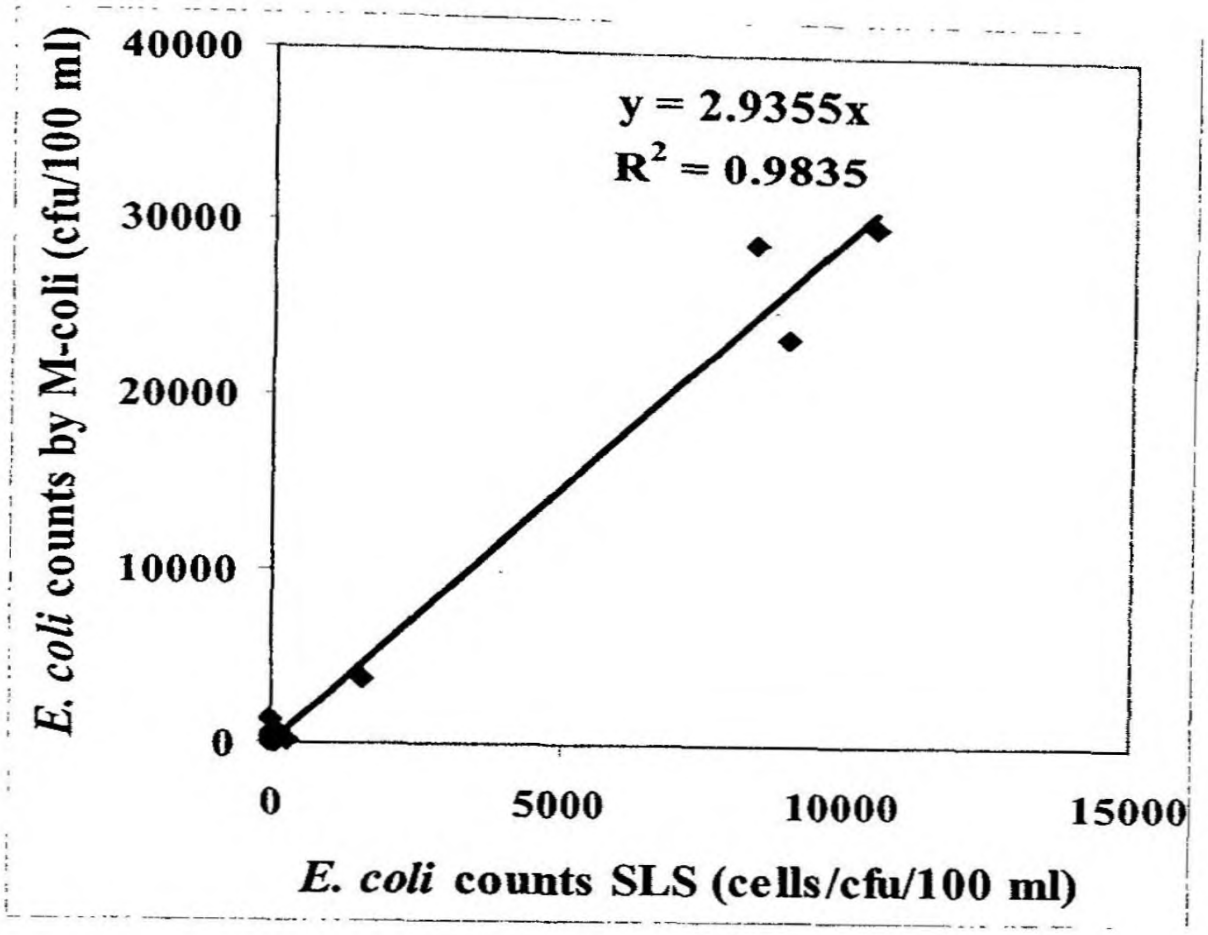
(a)


60000
40000
20000
0
Colilert couns (cells/cfu/100 ml)
y = 1.8371x
R2 = 0.9856
0
10000
20000
30000
SLS counts (cells/cfu/100 ml)

(b)


20000
15000
10000
5000
0
E. coli counts by M-FC (cfiu/100 ml)
y = 1.3558x
R2 = 0.9024
0
5000
10000
15000
E. coli counts by SLS (cells/Ccfu/100 ml)

(c)



(a)- SLS-MTF/Colilert
(b)- SLS-MTF/M-endo
(c)- SLS-MTF/M-coliblue24

Figure 2.15 Simple Linear Model for comparing SLS method and alternative methods for detecting *E. coli*

2.8.3. Confirmation rates of bacteria by different methods

Confirmation rates for total coliform bacteria were obtained as described in the section 2.6.3 under Materials and Methods in this chapter. Calculations were done following the equations 'a' (for MPN methods) and 'b' (for MF methods) in section 2.3.2.3 in Part-A of this chapter. Confirmation test results for total coliforms (negative Oxidase test) and *E. coli* (negative Oxidase test and positive Indole test) by different methods are shown in Tables 2.26 and 2.27 respectively.

Confirmation rates of total coliforms

When considering total coliform bacteria, the highest confirmation rate (78.2 %) was given by Colilert method. In contrast, the lowest confirmation rate was given by SLS-MTF method (71.1%). A similar observation was made in the preliminary study, with

highest values in Colilert and the lowest values in SLS-MTF methods respectively. However, the confirmation rates were lower in this experiments compared to the values obtained in the preliminary study. In this experiment, the confirmation test was performed only for isolated pure colonies grown on Tryptic Soy Agar plates, whereas, in the preliminary study, it was done directly from the positive presumptive test tubes or for colonies that appeared on plates. Further, the confirmation test procedures followed in the two experiments were also different as described in the sections 2.3.3 and 2.5.3 of this chapter. For confirmation of total coliform colonies, Brilliant Green Lactose Bile Broth (conventional method) was used in the preliminary study, while the Oxidase test using Oxidase reagent (ISO method) was used in the subsequent experiment.

In this experiment, typical and atypical colonies formed on M-endo were isolated by streaking on TSA plates and the isolated colonies were tested for confirmation. Confirmation tests showed, both typical and atypical presumptive colonies that appear on M-endo media consists of both total coliform bacteria and non-coliform bacteria. Among the atypical cream colour colonies isolated, 60% were confirmed as total coliforms, while the typical red metallic sheen/pink colour colonies consisted of 74 % of total coliform bacteria, belonging to the family Enterobacteriaceae. In contrast, there was no atypical colony formation on m-ColiBlue24 plates. Total coliform confirmation rate in m-ColiBlue24 method was 72.1 % (Table 2.26).

Table 2.26. Confirmation test results for detecting total coliforms

Method	Type of observation	Confirmation rate %
SLS	Gas formation/(and acid formation)	71.0
Colilert	Yellow colouration	78.2
	Colourless	0.0
M-endo	Typical red metallic sheen/pink colonies	75.1
	Atypical cream	60.0
M-coli	Typical red	72.1

Confirmation rates of *E. coli*

Compared with the preliminary test results, *E. coli* confirmation rates were comparatively lower in this experiment. However, during the preliminary study, *E. coli* positive SLS-MTF tubes were not further confirmed, where as in this experiment, confirmed *E. coli* tubes (Indole positive) were further reconfirmed following the ISO confirmation test. In the ISO confirmation test, isolated cultures should be Oxidase negative with a Indole positive reaction. The table 2.27 shows the confirmation test results, after performing both reactions. According to the results, the highest confirmation rates were given by Colilert method (66.6 %). Further, in Colilert method, yellow but non- fluorescing tubes did not contain any *E. coli*. That means only typical positive tubes (yellow and fluorescing) tubes contained *E. coli* bacteria. However, in the preliminary study, yellow but non-fluorescing tubes also contained 25 % of *E. coli*. m-ColiBlue24 and SLS-MF (M-FC medium) methods showed 50 % of confirmed *E. coli,* out of the typical blue colour colonies. However, the atypical colonies (cream colour and yellow colour colonies) formed on M-FC plates were not confirmed as *E. coli* (confirmation rate 0 %).The lowest *E. coli* confirmation rate was recorded by the SLS-MTF method (37.5 %), similar with the total coliform confirmation test results.

Table 2.27. Confirmation test results for detecting *E. coli*

Method	Type of observation	Confirmation rate %
SLS	Positive Indole reaction	37.5
Colilert	Fluorescence	66.6
	Non fluorescing yellow	0.0
M-FC	Typical blue colonies	50.0
	Atypical cream	0.0
	Atypical yellow	0.0
M-coli	Typical blue	50.0

2.8.4. Performance of methods according to ISO criteria

Performance of methods was assessed by following the ISO criteria for Guidance on validation of microbiological Methods (ISO/TR 13843). As described in the section 2.5 in this chapter, the performance characteristics of four methods were analyzed using the observations made in different methods for two types of bacteria separately. For analyzing performance characteristics, the following parameters were used (section 2.5. of this chapter).

a) number of presumptive positives found positive (true positives)
b) number of presumptive negative found positive (false negatives)
c) number of presumptive positives found negative (false positives)
d) number of presumptive negatives found negative (true negatives)
n) total number of tests

Observations done with four methods when analyzing total coliform bacteria are presented in the table 2.28.

Table 2.28 Observations done for total coliform bacteria by different methods

Parameter	SLS	Colilert	M-endo	M-coli
a	54	61	56	62
b	-*	0^	6	0^
c	22	11	18	24
d	-*	10	4	0^
n	**76**	**82**	**84**	**86**

* presumptive negatives were not confirmed ^ presumptive negatives were absent

The minimum number of confirmation tests done for confirming total coliforms in four method was 100 (Table 2.29). However, some of the cultures streaked on TSA (Tryptic

Soy Agar) did not show sufficient growth to obtain pure cultures, even with several attempts of sub culturing. In addition, a considerable proportion of the plates had to be discarded due to contaminations (17 %, 18 %, 20.5 % and 15 % in SLS-MTF, Colilert, M-endo and m-ColiBlue24 methods respectively). Contamination was difficult to overcome during the isolation procedure even with several precautions (such as fumigating the lab, taking several precautions in cleaning and sterilizing plates and needles, taking extra precautions in sub culturing process and media preparation, etc.,).

Table 2.29 Summary of total coliform data

Description	Method			
	SLS	Colilert	M-endo	M-coli
number of samples analyzed	42	42	42	42
sum of presumptive tubes or colonies	614	554	3181	3004
number of tubes or colonies included in the confirmation	102	100	102	106
number of confirmed cultures (oxidase negative/O-)	54	61	56	62
number of contaminated cultures,	18	18	21	16
number of discarded cultures due to poor isolation/no growth	8	10	7	6

Performance of methods in detecting total coliforms

Obtaining all performance criteria was not possible for each method, according to the ISO criteria, since every required parameter could not be analyzed in each method during the study, due to practical difficulties. In SLS-MTF method, negative presumptive tubes were not used in confirmation testing. Therefore, there were no valid results for b and d parameters. Further, there was no atypical colony formation in m-ColiBlue24 method, making no valid results for b and d (Table 2.28). There were no atypical results with the use of the Colilert method because all positive tubes showed a colour change. Therefore,

obtaining performance criteria such as 'sensitivity, specificity and false negative rates were not successful for all methods concerned (Table 2.30).

However, with the available data, certain performance criteria were obtained as depicted in the table 2.30. According to the results, m-ColiBlue24 medium has the highest sensitivity (1.0), and M-endo showed 0.9 of sensitivity. Specificity values were also obtained only for Colilert and SLS-MF (M-endo) methods. The specificity of Colilert method (0.47) was higher than that of the M-endo medium (specificity = 0.18). False positive rate was highest in SLS-MTF method, which indicates the low performance compared to other methods. False positive rate was lowest in Colilert method when detecting total coliform bacteria, which is a good performance character of the method. Further, the false negative rate was zero in both enzymatic methods (Colilert and the m-coliblue-24) as the two methods did not show any presumptively negative result. Furthermore, the highest efficiency among the methods was shown by Colilert method, compared to the other methods. M-endo showed a 0.64 efficiency, which is considerably a low value. m-ColiBlue24 method showed a 0.73 efficiency, which is in between. However, the efficiency of SLS-MTF could not be predicted accurately due to lack values for the 'd' parameter (Table 2.28).

Table 2.30 Performance of different methods for total coliform bacteria

Performance character	Method			
	SLS-MTF	Colilert	M-endo	M-coli
Sensitivity = a/(a + b)	- *	1^	0.90	1^
Specificity = d/(c + d)	- *	0.47	0.18	- ^
False positive rate = c/(a + c)	0.29	0.15	0.24	0.28
False negative rate = b/(b + d)	-	0	0.6	0
Efficiency E = (a + d)/n	0.71*	0.81	0.64	0.73^

* presumptive negatives were not confirmed ^ presumptive negatives (atypical colonies) were absent

Performance of methods in detecting *E. coli*

Observations done with four methods when analyzing *E. coli* are presented in the table 2.31.

Table 2.31 Observations done for *E. coli* by different methods

Parameter	SLS-MTF	Colilert	M-FC	M-coli
a	3	8	8	8
b	0*	0	0	0^
c	19	28	18	21
d	0*	10	2	0^
n	**22**	**46**	**28**	**29**

* presumptive negatives were not confirmed ^ presumptive negatives (atypical colonies) were absent

The minimum number of confirmation tests done for confirming *E. coli* by the four methods was 46 (Table 2.32). However, as in the case of total coliforms, some of the cultures streaked on TSA (Tryptic Soy Agar), did not resulted in sufficient growth to obtain pure cultures, even with several attempts of sub culturing. Further, contaminations (20.8 %, 28.0 %, 17.4 % and 26.5 % in SLS-MTF, Colilert, M-endo and m-ColiBlue24 methods respectively) by other contaminants were also observed in *E. coli* isolation tests too.

Table 2.32 Summary of *E. coli* data

Description	Method			
	SLS-MTF	Colilert	M-FC	M-coli
number of samples	42	42	42	42
sum of presumptive tubes or colonies	309	313	2602	1764
number of tubes or colonies included in the confirmation	48	51	46	49
number of Oxidase negative cultures	8	12	16	16
number of Indole positive cultures	3	8	8	8
number of contaminated cultures,	10	16	8	13
number of discarded cultures due to poor isolation/no growth	6	7	4	7

As in the case of total coliforms, it was difficult to obtain all performance criteria for each method even with *E. coli*. In SLS-MTF method, negative presumptive tubes were not used in confirmation testing. Therefore, there were no valid results for 'b' and 'd' parameters. There were no atypical colony formation in m-CoiBlue24 method, creating zero values for 'b' and 'd' (Table 2.31). Therefore, obtaining performance criteria 'sensitivity, specificity and 'false negative rate' were not successful when all methods were concerned, when testing for *E. coli* (Table 2.33).

From the available data, certain performance criteria were obtained as in the case of total coliforms. As depicted in the table 2.33, all alternative methods obtained a value of 1.0 for sensitivity. In SLS-MTF method, if presumptive negative cultures were tested a valid result could have been obtained. Specificity in *E. coli* detection was 0.26 in Colilert, where as it was only 0.1 in M-FC medium.

The highest false positive rate was obtained by SLS-MTF method, which indicates the low performance compared to other methods. Further, the minimum false positive rate was given by Colilert method as in the case of detecting total coliform bacteria. False positive rates were 0.72 and 0.68 in m-CoiBlue24 and M-FC respectively. Furthermore,

the false negative rate was zero in all alternative methods which is again a good performance character of a method. The highest efficiency among the methods was shown with Colilert method (0.39), compared to the other methods. It was 0.36 and 0.28 in M-FC and m-ColiBlue24 methods respectively, while the minimum rate was obtained with the reference SLS-MTF method.

Table 2.33 Performance of different methods for *E. coli*

Performance character	Method			
	SLS-MTF	Colilert	M-FC	M-coli
Sensitivity = a/(a + b)	*	1.0	1.0	1.0^
Specificity = d/(c + d)	*	0.26	0.1	^
False positive rate = c/(a + c)	0.86	0.60	0.68	0.72
False negative rate = b/(b + d)	-	0	0	0
Efficiency E = (a + d)/n	0.13*	0.39	0.36	0.28^

* presumptive negatives were not confirmed ^ presumptive negatives (atypical colonies) were absent

2.8.4.1. Detection of different groups of bacteria by different methods

Confirmation tests showed that, total coliforms and *E. coli* were detected in different amounts by different methods (Tables 2.26 and 2.27). Further, other groups of bacteria such as Oxidase positive bacteria and Gram positive bacteria were also detected by all tested methods, in addition to the coliform group of bacteria. Confirmation rates gave the proportions of different bacterial groups detected by different methods.

Confirmation rates of different groups of bacteria

As depicted in the table 2.34, the highest total coliform detection rate (78.2 %) was shown by Colilert method, while the SLS-MTF method has shown the lowest (71%) confirmation rate. Further, the SLS-MTF method detected 4 % of Gram positive bacteria,

where as none of the alternative methods detected Gram positive bacteria during total coliform detections. When considering Oxidase positive bacterial rates, the highest proportion was recorded by the m-ColiBlue24 method, while SLS-MTF being the second highest. The lowest amount of Oxidase positives was detected by the Colilert method (18.1 %) Altogether, the highest non-coliform ratio was recorded with the SLS-MTF method, compared to the alternative methods. The lowest proportion of non-coliform bacteria was detected by the Colilert method.

Table 2.34 Percentages of confirmed coliform and non-coliform bacteria detected by different methods

Methods	Oxidase (-)/ total coliform confirmation rate %	Oxidase (+) rate %	Gram (+) rate %	Total non-coliform rate %
SLS-MTF	71.0	25.0	4.0	29.0
Colilert	78.2	18.1	0	18.1
M-endo	75.7	24.3	0	24.3
M-coli	72.1	27.9	0	27.9

As depicted in the table 2.35, M-FC medium was able to detect the highest Oxidase negative bacterial percentage (61.5), compared to the other three methods in detecting *E. coli*. The lowest (36.3 %) was recorded by the conventional SLS-MTF method. In contrast to the results obtained for total coliforms, the highest Oxidase positive rate (53.5 %) was shown by Colilert method, while the lowest was recorded by the M-FC (38.4 %). However, the highest Gram-positive rate (13.6 %) was also given by SLS-MTF, similar with total coliform detection results. Although Colilert method also reported a very little amount of Gram positives (3.5 %), M-FC and m-ColiBlue24 did not detect any Gram-positive bacteria in the process of *E. coli* detection.

Table 2.35 Percentages of non-*E. coli* bacteria detected by different methods in detecting *E. coli*

Methods	Oxidase (-) rate %	Oxidase (+) rate %	Gram (+) rate %	Total non coliform rate %
SLS-MTF	36.3	50.0	. 13.6	63.6
Colilert	42.8	53.5	3.5	57.0
M-FC	61.5	38.4	0	38.4
M-coli	55.1	44.8	0	44.8

2.8.4.2. Comparison of methods according to ISO criteria

Each alternative method was compared with the reference SLS-MTF method for the detection of total coliform bacteria and *E. coli* separately, following the ISO criteria for establishing equivalence between microbiological methods (ISO 17994: 2004).

In each comparison, confirmed counts from the same sample obtained on the alternative method were paired to confirmed counts obtained on the reference medium (SLS-MTF). Both MF and MPN results were rounded up to the nearest whole number before calculations. After natural logarithm transformation of the paired counts data, the relative difference between compared methods in each sample were calculated using the equation 2.1.1.(Appendix 2, Section 2.1), (with ai and bi being the paired counts) as well as the mean relative difference, using the equation 2.1.4.(Appendix 2, Section 2.1) (the sum of relative differences divided by the number of samples) and expanded uncertainty, using the equation 2.1.7. (Appendix 2, Section 2.1) and the standard uncertainty of the mean (s) were calculated, using the equation 2.1.6. (Appendix 2, Section 2.1) (ISO 17994, 2004, as explained in Appendix 2, section 2.1.).

Results obtained with method comparison using paired counts are shown in tables 2.36 and 2.37 for total coliform counts and *E. coli* counts respectively.

Evaluation of Method comparison

For evaluation of the performance of alternative methods, in relation to the reference SLS-MTF method for the enumeration of two bacterial types, was done by using the equations given in the section 2.2 (Appendix 2).

Expanded Uncertainty (equation 2.1.7, Appendix 2, Section 2.1) and the Confidence intervals (of the expanded uncertainty around the mean) (equations 2.1.8 and 2.1.9; Appendix 2, Section 2.1), were obtained as explained in the ISO standard (Section 2.2, Appendix 2).

A value of 10 % as the maximum acceptable deviation from zero (D) was used in this study and the evaluations were done two-sided as explained in ISO standard (ISO 17994-2004).

Evaluation was done by using the calculated confidence interval values (x_I and x_{II}), following the relationships given in the ISO standard ('Method Evaluation' in section 2.2, Appendix 2).

Results of evaluation with method comparison according to ISO criteria are given in tables 2.38 and 2.39, for total coliforms and *E. coli* respectively.

According to the comparison results for total coliform detection (Table 2.36), the highest mean relative difference (6.33) was obtained in the comparison of SLS-MTF with the SLS-MF (M-endo). Highest standard uncertainty (85.2), highest standard uncertainty of the mean (14.4) and the expanded uncertainty (28.8) were found in the comparison of SLS-MTF with the Colilert method, compared to other three comparisons.

Table 2.36 Results of the Method comparison with SLS methods for enumeration of total coliform bacteria

Parameter	Method comparison		
	SLS-MTF/Colilert	SLS-MTF/M-endo	SLS-MTF/M-coli
$\bar{x}$	2.97	6.33	0.70
s	85.2	67.77	69.67
s$\bar{x}$	14.4	12.97	6.08
U	28.8	24.74	22.90

$\bar{x}$= Mean Relative differences; s = Standard uncertainty;

S $\bar{x}$= Standard uncertainty of the mean; U= Expanded uncertainty;

Looking at the method comparison results for *E. coli* detection (Table 2.37), the highest mean relative difference (27.72) was found in the comparison of SLS-MTF with the SLS-MF (M-FC), similarly to the total coliform detection results. In contrast to results obtained for total coliforms, for *E. coli* detections, the highest standard uncertainty (15.2), highest standard uncertainty of the mean (40.01) and the expanded uncertainty (30.25) too were found in the comparison of SLS-MTF with the SLS-MF (M-FC) method, compared to other three comparisons.

Table 2.37 Results of the Method comparison with SLS method for enumeration of *E. coli*

Parameter	Method comparison		
	SLS-MTF/Colilert	SLS-MTF/M-FC	SLS-MTF/M-coli
$\bar{x}$	17.32	27.72	19.8
s	13.39	15.12	13.09
s$\bar{x}$	34.65	40.01	34.65
U	26.19	30.25	26.19

$\bar{x}$ = Mean Relative differences; s = Standard uncertainty;

s$\bar{x}$= Standard uncertainty of the mean; U= Expanded uncertainty;

Evaluation results obtained for the method comparison in detecting total coliform bacteria are depicted in the Table 2.38. In all three comparisons, the lower limits (X_L) are having minus values (< 0) and the upper limits (X_H) exceed the acceptable 10 % (+ D). When this situation is related to the method evaluation, it agrees with the equation 2.2.4 (Appendix 2). As it explains, the evaluation for this type of relationship is "Inconclusive".

It also suggests that even though there is a variation of all alternative methods with the reference SLS-MTF method, the variation exceeds the acceptable range (D value > 10 %).

In the current study even with more than 30 comparison results (which satisfy the ISO requirement), (ISO, 17994, 2004), the evaluation results obtained were 'inconclusive' (meaning, data are insufficient for a decision) in all three comparisons, because the range of the deviation (D) exceeded 10 %. Therefore, to overcome this limitation, ISO criteria, recommends increasing the number of samples examined leading to narrow down the variation range below 10 %. Then it will be able to obtain a final conclusion on the comparison study (relating to the evaluation relationships explained in the section 2.2. (Appendix 2), using the compared bacteriological methods to detect total coliform bacteria in water.

Table 2.38 Results of the evaluation test for comparison of alternative methods with SLS reference method for numeration of total coliform bacteria

Parameter	**Method comparison**		
	SLS-MTF/Colilert	**SLS-MTF/M-endo**	**SLS-MTF/M-coli**
x_L	-25.8	-25.83	-22.20
x_H	31.8	31.78	23.61
Evaluation	Inconclusive	Inconclusive	Inconclusive

x_L = Lower limit; x_H = Upper limit

Similarly with total coliform evaluation results, comparison results were analyzed for *E. coli* detection by three alternative methods in comparison to the reference SLS-MTF method (Table 2.39). According to the table 2.39, the evaluations obtained for *E. coli* detection were also 'inconclusive' as in the case of total coliform bacteria. However, in analyzing *E. coli* detections, the number of compared paired counts was not exceeding 30 comparisons due to several unavoidable circumstances (such as obtaining plates with insufficient growth, without pure colonies or with contaminations), leading to comparatively low number in confirmation testing (compared to total coliforms). Therefore, the range of the lower limits and the upper limits were exceeding the 10 % of maximum acceptable deviation from zero (D), suggesting that even there is a variation of all the alternative methods with the reference SLS-MTF method, in detecting *E. coli*, the range is too wide. Therefore, similarly as with total coliforms, the range of variation should be further narrowed down to obtain valid evaluation results. In order to obtain valid evaluation data, the number of samples should be further increased (as recommended by the ISO, 17994, 2004), which will facilitate the increasing of valid paired counts for comparisons. Therefore, the final conclusion of this comparison study (using ISO criteria for detecting *E. coli* bacteria in method comparison) was that, more precise results could be obtained in method comparison, if the number of samples is increased for testing with different methods for the detection and enumeration of *E. coli* for water quality assessment in Sri Lanka.

Table 2.39 Results of the evaluation test for comparison of alternative methods SLS reference method for enumeration of *E .coli*

Parameter	**Method comparison**		
	SLS-MTF/Colilert	**SLS-MTF/M-FC**	**SLS-MTF/M-coli**
x_L	-8.86	-2.52	-6.39
x_H	43.52	57.97	46.00
Evaluation	Inconclusive	Inconclusive	Inconclusive

x_L = Lower limit; x_H = Upper limit

2.8. DISCUSSION

Method comparison

Both the preliminary and the secondary phases of this study, showed that the different methods used to analyze total coliform bacteria and *E. coli* in different water sources have enumerated both bacterial types in different amounts, regardless of the sampling locations. This observation shows that the methods selected in this study though not at same level but have demonstrated their performances favorably, in enumerating both bacterial types, from a geographically diverse set of samples, in Sri Lanka.

When considering bacteriological counts in both phases of the study, the lowest mean counts in detecting both total coliforms and *E. coli* were shown by the conventional SLS-MTF reference method. The method comparison done with the least squares mean separation test has also proved that all the alternative methods used (Colilert, SLS-MF, m-ColiBlue24, in both phases), except the APHA-MTF method (included only in the preliminary study), were significantly different with the SLS-MTF reference method in detecting total coliform bacteria when all the water sources were grouped together. There are several possible reasons for this observation. One possibility might be, due to the presence of anaerogenic (without producing gas) lactose fermenting coliforms in most of the samples analyzed, as suggested by Eckner (1998). Therefore, the SLS and APHA-MTF methods, which are based on conventional lactose fermentation, might not be able to detect all coliforms, in water samples, especially the ones that cannot produce gas in fermenting lactose (Leclerc, *et al.*, 2002). This observation was further confirmed by the weak relationship obtained by the simple linear model between SLS-MTF and APHA-MTF methods.

In contrast, the simple linear relationship results for enzymatic methods, Colilert and the m-ColiBlue24 methods produced much better results in total coliform detections with several folds higher counts, in relation to the SLS-MTF method. (3.85 and 1.75 fold

higher counts by Colilert and m-ColiBlue24 methods respectively) Similar observations were also recorded by Noble, *et al.*, (2004), in which they have found 1.8 times higher counts with Colilert compared to the conventional MTF (APHA) method (using lauryl tryptose broth). Further, Schets, *et al.*, (2004) has also reported comparatively higher total coliform counts by Colilert method than the lactose containing MF medium. As Eckner, (1994) reported, the Colilert method has enumerated significantly higher ($p = 0.1$) total coliform counts compared to Swedish MTF method, when analyzing different water sources including environmental water samples. Similar results were also obtained by Covert, *et al.*, (1989), where they have observed significantly higher total coliform counts by the Colilert method, compared to the conventional 10-tube MTF (APHA) method, for analyzing different types of water sources including drinking water.

The enzymatic method Colilert, can detect β-D galactosidase enzyme present abundantly in total coliform bacteria (Pikanen, *et al.*, 2006, Edberg, *et al.*, 1988)) in water. According to Schets, *et al* (2004), coliform bacteria have both *lacY* (for β-galactosidepermease enzyme) and *lacZ* (for β –galactosidase enzyme) genes. Certain coliforms have only *lacY* gene, which is responsible for lactose fermentation, while, the other coliforms do not have *lacY,* but do have *lacZ.* Those bacteria do not ferment lactose, but can use Colilert substrate ONPG (o-nitrophenyl β-D galactopyranoside). In their study, larger proportion of bacteria have contained only *lacZ* gene, resulting in higher total coliform counts with Colilert.

Another reason for higher recoveries of total coliforms and *E. coli* with the enzymatic methods in present study, could be due to the presence of stressed cells, which are unable to grow in culture media; but which maintain their viability (viable but non-culturable bacteria), and still capable of metabolic activity (Niakaeen, *et al.*, 2009). Several studies have shown that, the bacteria could be metabolically active, even they were not detected by the cultivation techniques commonly used (George, *et al.*, 2000; Caruso, *et al.*, 2002). Comparison of an enzymatic assay (Colisure test) with standard methods techniques for detecting bacteria subject to chlorine stress by McFeters *et al.*, (1997) has demonstrated more recovery of chlorine-injured total coliform and *E. coli* by enzymatic assay, resulting

in a more realistic estimate of the actual population of indicator bacteria in tested water supplies.

However, the current study, the M-endo medium, which is not an enzymatic method, was also able to detect significantly higher counts of total coliforms compared to SLS-MTF method, in both the preliminary and detailed study. Simple linear model showed that, M-endo and SLS-MTF methods had positive relationships demonstrating higher counts by M-endo, in relation to SLS-MTF method during both preliminary and detailed studies. Among the membrane filter media, M-endo has been reported to show superior sensitivity and selectivity, by producing equivalent results to MTF (APHA), even with chlorine-stressed coliforms (Tobin *et at.*, 1980). Although there is no recorded information available on comparisons of M-endo with Mac Conkey medium (SLS-MTF), the results of this study could be attributed to the efficiency of detecting the acid reaction by M-endo medium, due to its higher specificity and sensitivity than the Mac Conkey medium in detecting the lactose fermentation reaction.

In the current study, m-ColiBlue24 method also showed a similar trend with M-endo medium, in detecting total coliform bacteria in both phases of the study. Both M-endo medium and m-ColiBlue24 showed significantly higher ($p<0.05$), total coliform counts compared to the SLS-MTF method during both phases, and also the simple linear relationship with SLS-MTF were also positive with these two methods. Similar observations have been demonstrated by Grant, (1997), in comparison of M-endo with m-ColiBlue24, for detecting total coliform bacteria from a geographically diverse set of samples. In his study, also M-endo and m-ColiBlue24 have performed similarly in detecting total coliform bacteria with no significant differences between the two methods. However, comparisons done with standard MTF with m-ColiBlue24, for detecting total coliform bacteria were not available. m-ColiBlue24, is an enzymatic method which is supplemented with a chromogen to detect *E. coli* simultaneously with total coliforms on the same membrane filter (Grant, 1997, Schauer, *et al.*, 2007).

Furthermore, in SLS-MTF method, mostly, a positive test was based on detecting gas formation, since the acid reaction was not prominent in most of the tubes. Generally in

APHA method only gas formation with turbidity in luaryl tryptose broth is mentioned as a positive presumptive test, while acid formation is not indicated (APHA, *et al.*, 1998). Therefore, absence of gas formation in tubes is usually considered as negative presumptive tests causing false negative results in conventional MTF methods, including SLS and APHA.

In contrast to this observation occurring in lactose-based media, enzymatic methods such as Colilert show distinct colour reactions (colourless to yellow) in total coliform positive tubes due to the detection of the enzyme β-D galactosidase present in most total coliform bacteria (Edberg, *et al.*, 1998, Eckner, 1998). According to Niemela, *et al.* (2003), Colilert can show higher total coliform counts due to the detection of β–galactosidase in cells as some total coliforms could not ferment lactose by primary isolation due to the presence of inhibitory agents in the conventional culture media. Further, as Edberg, *et al.*, (1988) describes, in conventional media, several secondary reactions must occur before a change in the indicator is visible. The target microbe must transport the substrate (e.g., lactose) through the cell membrane, transform the substrate to glucose, metabolize glucose through the glycolytic cycle to pyruvate, and then convert pyruvate to the desired end product, either acid or gas. Because conventional testing requires the microbe to go through many steps to yield a positive visible end point, a number of anomalous results may occur, such as false-negative gas producers (i.e., anaerogenic *E. coli*) or false-positive tests (i.e., acid from lactose by some clones of *Aeromonas hydrophilia*). However, ONPG-positive noncoliforms do not yield a positive Colilert test, because the formula does not support their metabolism. Therefore, the detection of the β-galactosidase system of heterotrophs will occur only at extremely high microbial concentrations (>20 to 100,000/ml). This phenomenon is seen with approximately 10% of members of the genus *Aeromonas* (Edberg, *et al.*, 1988). As Edberg, *et al* (1989) suggests, unlike o-nitrophenyl-4-D-galactopyranoside tests used for species identification, which depend on bacterial inoculations of 10^7/ml and measure passive β-galactosidase, the Colilert test uses o-nitrophenyl-β-D-galactopyranoside as a defined substrate (Edberg and Edberg, 1988). Therefore, unless there are very large numbers of *Aeromonoas* spp. (>10^4 to 10^5/ml) present in the initial drinking-water sample, false-positive Colilert tests will not be formed. This number of *Aeromonas* spp. is normally unlikely to be encountered in

drinking-water samples but if found, should be considered a public health threat because of the association of *Aeromonas* spp. with waterborne disease (Moyer, *et al.*, 1987). Another reason for higher counts produced by Colilert might be due to the ability of Colilert to detect injured coliforms, which are viable but unable to grow within 24 h, (Bucklow, 2006; Edberg and Edberg, 1989). Therefore, MTF methods based on enzymatic methods can detect higher total coliform concentrations compared to the conventional lactose based methods (Griffith, *et al.*, 2006, Noble, *et al.*, 2004).

E. coli enumeration test results also showed that, the SLS-MTF method and the APHA-MTF methods detected the lowest mean counts compared to other methods, when all water sources were grouped together in both phases of the study. Least squares mean separation test results showed that all alternative methods, except the APHA-MTF method were significantly different with SLS-MTF method, in defecting *E. coli* during the preliminary study. However, APHA-MTF detected comparatively higher *E. coli* counts than the SLS-MTF method when all water sources were considered. The simple linear relationship also showed 1.06 times higher counts in APHA-MTF, than the SLS-MTF method. During the second phase of the study, only the two enzymatic methods showed significant difference in detecting *E. coli*. Among the two, only Colilert was significantly different at 95 % confidence level, where as m-ColiBlue24 was significantly different only at 90 % confidence level. M-FC medium has not measured significantly different counts compared to SLS-MTF method, when all the sources were grouped together. Simple linear model showed that, the m-ColiBlue24 method detected 2.93 times higher *E. coli* counts, while M-FC showed 1.35 times higher *E. coli* counts than SLS-MTF method.

The conventional SLS method uses the ability of *E. coli* to hydrolyze the amino acid, tryptophan, in to indole, pyruvic acid and ammonia by tryptophanase enzyme present in E. coli cells. Peptone water, used in the confirmation tests contains tryptophan, which is utilized by *E. coli* and produce a red colour, in reaction with the Kovac's reagent (para-dimethylaminobenzaldehyde in alcohol) after incubating at 44.5 ^{0}C (or 35-37 ^{0}C according to literature) for 24-48 hours. However, the low detection levels of *E. coli* in SLS method, might be due to the missed *E. coli*, by the false negative results (gas negative), shown in the presumptive tests. Another reason might be the low performance

of the peptone water broth. In contrast to the SLS-MTF method, APHA-MTF method was able to detect considerably higher concentration of *E. coli* (5.2 x 10^3 cell/100 ml) compared to SLS-MTF method, even though the difference was not significant (at $p>0.5$). In APHA method, *E. coli* is detected by the EC-MUG broth, which can detect the *E. coli* by cleaving the substrate 4-methylumbelliferyl β-D glucuronide (MUG) in to a fluorogenic product, by the activity of β–glucuronidase present in *E. coli* cells. Therefore, the detection of *E. coli* using EC-MUG should possibly be higher compared to the SLS-MTF. However, due to the false negative results shown in the presumptive coliform test with lauryl tryptose broth, observations might have missed the total *E. coli* cells used in the *E. coli* confirmation test, producing lower counts in this study.

Colilert method detected significantly higher (1.83 times higher counts during both phases of the study) counts of *E. coli* in this study. A few previous researches have compared the conventional MTF methods with the enzymatic methods for the enumeration of *E. coli*. In most of the reported studies, *E. coli* concentrations detected by Colilert method showed no significant differences with the conventional MTF methods, in contrast to the results obtained in the current study (eg: APHA-MTF method: Edberg, *et al.*, 1990 for surface water; Noble, *et al.*, 2004 for beach water samples; Griffith, *et al.*, 2006 for ambient water samples and the Swedish-MTF method: Eckner, 1994 for different water sources including environmental waters). However, the difference observed in the current study, may be due to the high rate of detection of *E. coli* by the enzymatic activity. Another possible reason could be due to the detection of false positives from the interference by non-coliform organisms, such as *Aeromonas*, *Vibrio, Pseudomonas*, and *Flavobacteria* spp., (Griffith, *et al.*, 2006), which are known to metabolize MUG (Pisciotta, *et al.*, 2002, Landre, *et al.*, 1998, Davies, *et al.*, 1995, Hidalgo, *et al.*, 1977), and found abundantly in tropical environmental waters, with high temperatures (Fricker, *et al.*, 1996). However, as Edberg, *et al.*, (1989) suggested, Colilert has not produced false-positive *E. coli* tests during their survey for drinking water samples. The results reported have shown that the Colilert method was sensitive and specific for the simultaneous detection of total coliforms and *E. coli* in drinking water samples in their study.

When considering the detection of *E. coli* by the M-FC medium, conventional SLS-MTF method, M-FC was able to detect significantly higher counts of *E. coli*, only during the first phase, while it failed during the second phase. It might be due to the effect of media performance on water samples collected from different geographical locations with different physiological features of water, which could not be analyzed during this study. However, this same observation has been also reported by Griffith, *et al.,* (2006), when comparing the method performance of M-FC, with the standard APHA-MTF method, to detect *E. coli,* present in ambient coastal water samples. Similarly according to their findings, *E. coli* counts, by the two methods were not significantly different with each other. Another comparison study by Noble, *et al* (2004) also proved the same as Griffith, *et al.*, when using storm water affected coastal waters.

The enzymatic method, m-ColiBlue24 was able to enumerate significantly higher counts of *E. coli,* during both phases of the study. The simple linear relationships also proved, 2.27 times and 2.93 times higher counts (during the two phases of the study) of *E. coli* by m-ColiBlue24, than the conventional SLS-MTF method. This could be due to the detection of the enzyme β–glucuronidase present in *E. coli* cells, by the chromogen, 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid present in m-ColiBlue24 medium (Grant, 1997). This observation also proves the explanation by Pitkanen, *et al* (2007) that the enzymatic detection of *E. coli* is mostly higher than the lactose fermentation reaction detected by the conventional MTF methods. Although reports on similar work using Mac Conkey broth and the m-ColiBlue24 was not available in literature, the results of the current study indicated that the m-ColiBlue24 is an alternative approach that could provide better and more rapid information for the assessment of microbial quality of water. It could simultaneously detect total coliforms and *E. coli* from a water sample within 24 h and hence, will provide utilities a quick measure of whether a sample has been subjected to fecal contamination.

Further, this finding is in agreement with other studies, which have compared the classical *standard methods* procedures with enzymatic commercial kits. MI agar is one commercial preparation which has also shown higher performances compared to the conventional MTF methods (Edberg *et al.*, 1990; Clark *et al.*, 1991; Brenner *et al.*, 1993; Brenner *et al.*,

1996). The results of a study by Nikaeen, *et al* (2004), have indicated that the enzymatic LMX broth assay was comparable to the MTF method for the detection of total coliforms and *E. coli* in drinking water. The overall results for total coliforms and *E. coli* tests have showed that LMX has recovered 1.4 and 1.18 times as many total coliforms and *E. coli*, respectively, as the MTF method. However, statistical analysis demonstrated no significant difference between the two methods. A similar finding has been noted in the quantitative determination of total coliforms and *E. coli* in marine waters by Geissler, *et al* (2000). The higher recovery level of total coliforms and *E. coli* by the LMX has been explained by the presence of stressed cells which were unable to growth in culture media; but which maintain their viability (viable but non-culturable bacteria) and still capable of metabolic activity. A number of chemical and physical factors involved in drinking water treatment, including disinfection, can cause sublethal injury to coliform bacteria (Rompre *et al.*, 2002; Bitton, 2005). Several studies have shown that bacteria could be metabolically active even when they were not detected by the cultivation techniques commonly used (Pommepuy *et al.*, 1996; George *et al.*, 2000; Caruso *et al.*, 2002). Comparison of an enzymatic assay (Colisure test) with standard methods techniques for detecting bacteria subject to chlorine stress by McFeters *et al.*, (1997) showed more recovery of chlorine-injured total coliforms and *E. coli* by enzymatic assay, resulting in a more realistic estimate of the actual population of indicator bacteria in water supplies.

Confirmation of bacteria

When considering the confirmation rates of both total coliforms and *E. coli*, a distinct decrease was observed during the second phase of the study. As explained in the Materials and Methods section of this chapter, the confirmation testing of the second phase was done, following the ISO methods, compared to the conventional procedure followed in the first phase. In the ISO method, isolation of bacteria by streaking on agar plates (TSA) is an essential step, which was not done in the first phase. This would have been a major reason to select bacteria in isolation, where as in the preliminary study, the chance of obtaining a total coliform bacterium or an *E. coli* cell is quite easy, by inoculating loopfuls

from MTF tubes or directly inculcating from colonies on MF pates in to confirmation test tubes. Therefore, the confirmation results obtained by the detailed study were considered as more accurate than in the preliminary study. Hence, the results obtained by the detailed study will be discussed below.

Total coliform confirmation rates were different among the methods, and the highest (78.2 %) was shown by the positive yellow tubes of the Colilert method, when all water sources were grouped together. The results of this study was comparatively lower with most of the previous studies in confirming of total coliform bacteria detected by Colilert method in different types of water sources: 93.7% in subtropical fresh water samples (Chao, *et al.*, 2004; 100% in different water sources including, ground and surface water supplies, partially treated water, marginally chlorinated water and final distribution water (Fricker and Fricker, 1996); 98.5% for water samples from building fixtures and fire hydrants after flushing procedures (Lewis and Mak, 1989); 90% in different water sources including, ground water, well water, bathing water and spiked tap water samples (Pikanen, *et al.*, 2007). In the current study, false positive rate of Colilert was 15 %. Further, the colourless tubes did not have any total coliform bacteria making the false negative rate zero. Hence, the sensitivity of the Colilert method was 100 % in detecting total coliform bacteria. However, in the current study, 18.1 % of yellow tubes contained Oxidase positive bacteria. This phenomena might be due to the presence of non-coliform Oxidase positive bacterial species such as *Aeromonas, Pseudomonas* and *Flavobacteria spp.* which are commonly found in tropical waters and can give a positive Colilert test (Piscicotta, *et al.*, 2002, Davies, *et al.*, 1995, Edberg, *et al.*, 1988). According to Landre, *et al* (1998), aeromonads, which are known to constitute a fraction of the aquatic heterotrophic population, are not efficiently suppressed by Colilert reagent within 4 weeks of shelf-life expiry. Further, it is shown that very low levels of aeromonads are capable of giving a false positive reaction at longer incubation periods. Therefore, they have concluded that the presence of *Aeromonas* could mediate a false-positive Colilert reaction when examining drinking water samples. Covert, *et al* (1989) found that *Serratia* spp., *Haffnia alvei*, *Vibrio fluvialis*, and *Aeromonas* spp. constituted 25 % of isolates after 24 hours and 40.8 % of isolates after 28 hours of incubation with Colilert. These four groups of noncoliforms constitute the main sources of false positive results in Colilert. However, in

the current study the Colilert method did not detect any Gram positives bacteria, confirming previous results obtained by Pikanen, *et al* (2007). Further, compared to all other methods, Colilert detected the lowest rate (18.1%) of non-coliform bacteria in the current study, when all water sources were grouped together.

M-endo medium detected the second highest confirmed total coliforms (75.1 %), compared to m-ColiBlue24 method and the conventional-SLS-MTF methods. Being a specific and sensitive medium, M-endo might have specifically detect more total coliforms than the other lactose based media. However, the atypical cream colour colonies formed on M-endo plates were also confirmed as total coliforms (60%). Therefore, even though M-endo was sensitive (0.9) in detecting total coliforms, its specificity was 0.18 in this study. Further, it showed a false positive rate of 24 %, which is still lower rate, compared to the previous record 26.8 %, by Grant (1997), but higher than the 11.7 % obtained by Cenci, *et al* (1993) and the 14.9 % obtained by Evans, *et al* (1981). According to Covert, *et al* (1989), *Serratia* spp., *Haffnia alvei*, *Vibrio fluvialis*, and *Aeromonas* spp. are the main reasons of the false positive results obtained in total coliform media including M-endo medium. In present study, the efficiency of the M-endo medium in detecting total coliforms was 0.64, which is comparatively low with the Colilert method.

The total coliform confirmation rate detected in m-ColiBlue24 method was 72.1 % in this study. However, there were no atypical colony formation observed in this study, compared to the former finding done by Grant (1997), in which he has observed 1.6 % of untargeted colorless colonies. However, no other report was found on studies done with m-ColiBlue24 confirmation rates. Due to the absence of atypical colonies on m-ColiBlue24 plates, the sensitivity was 100 % in detecting total coliforms in present study. However, it showed 28 % false positive total coliforms, which is very close to 29 % in the former report by Grant (1997). These false positive results might be due to the detection of non-coliform species as described above by Covert, *et al* (1989). However, in the current study, there were no Gram positives detected by m-ColiBlue24 method, which is a similar observation done with Colilert and M-endo media in this study. It shows that the m-ColiBlue24 is specific only to gram negative bacteria, which is a positive feature observed by this method. However, the efficiency of m-ColiBlue24 in detecting total coliforms in

this study was 0.73, which was comparatively lower with the Colilert method, but higher the other lactose based methods.

When considering *E. coli* confirmation rates, the highest rate (66.6 %) was given by the Colilert method. m-ColiBlue24 and M-FC medium (of the SLS-MF method), enumerated 50 % of *E. coli*, out of the detected blue colour typical colonies, while the minimum (37.5 %), was confirmed by the conventional SLS-MTF method. The comparatively lower *E. coli* rates in enzymatic methods observed in the current study, might be due to the presence of contaminants, such as humic acids and suspended solids, which have the potential to interfere with this method in natural ambient water samples (Griffith, *et al.*, 2004). Further, the natural samples also contain native bacteria, such as *Aeromonas, Vibrio, Pseudomonas*, and *Flavobacteria spp.*, which have been shown to produce positive reactions in substrates containing 4- methylumbelliferyl- β -glucuronide (MUG) and can lead to false positives in the Colilert test (Pisciotta, *et al.* 2002, Landre, *et al.* 1998, Davies, *et al.* 1995, Hidalgo *et al.* 1977). Further, as Davies, *et al.* (1994) suggested, some alga and plant material at high concentrations may significantly interfere with the detection and enumeration of coliform bacteria by methods, which are based on the production of, β-D-galactosidase and β-D-glucuronidase enzymes. For algae, this will depend on the numbers of cells present and may be significant when there are algal blooms. The significance of the production of β-galactosidase and, β-D-glucuronidase enzymes by aquatic plants and macroalgae is less easily interpreted, as it is difficult to estimate concentrations at which plants may realistically release the enzymes into the environment.

Comparatively low *E. coli* rates given by M-FC method in this study might also be due to the detection of non target bacteria by M-FC medium. The non-coliform percentage detected by M-FC was 50 %, which consisted with the Oxidase positive bacteria. As described above for Colilert method, the Oxidase positive non-coliform bacteria such as *Aeromonas, Pseudomonas, Vibrio* and *Flavobacteria* might be detected by M-FC medium, in addition to the fecal *E. coli* bacteria. Further non-typical colony formation was also observed in M-FC medium, though they were not confirmed as *E. coli*. That means, most of the fecal bacteria other than *E. coli,* such as *Klebsiella, Citrobacter* and

Enterobacter spp. (Leclerc, *et al.*, 2002), might have been detected by the M-FC medium. However, Gram-positive bacteria have not been isolated by M-FC method, in this study, which is a positive characteristic feature of the method. Hence, the sensitivity of M-FC was 1.0 in detecting *E. coli*, which is also similar with Colilert. However, the false positive rate 68 % was comparatively higher than the Colilert method. Efficiency of the method was also low compared to the Colilert method.

The overall *E. coli* confirmation rate detected by m-ColiBlue24 was 50 %, which is similar with M-FC, and closer to Colilert. Although *E. coli* confirmation rates by m-ColiBlue24 method were not found (in numbers) from previous studies, m-ColiBlue24 method was reported superior in detecting *E. coli* from a geographically diverse set of samples (Grant, 1997), compared to other lactose based MF media. m-ColiBlue-24 has performed better than those media, as measured by sensitivity, specificity, false positive and false negative rates. Further, as he reports detection of *E. coli* colonies have been easier with m-ColiBlue24, because it was not necessary to transfer filters either from one medium to another or between incubators or to use a UV lamp, in their study. Therefore, he suggests that m-ColiBlue24 is a useful medium for coliform analysis because of its ease of use and nominal cost.

The lowest confirmation rate of *E. coli* was given by the classical SLS-MTF method. Further, it showed the highest false positive rate (86 %) compared to all other methods. Although details of confirmation rates in Mac Conkey broth were not available, the low specificity of the medium to detect *E. coli* might be one reason for those observations. Therefore, this medium might have detected more non-coliform bacteria than the coliform bacteria. It is also indicated by the higher rates of oxidase positive bacteria (50 %). Further, surprisingly highest proportion (13.6 %) of Gram-positive bacteria was also detected by this medium in present study. Unfortunately, these results could not be compared with other similar work, due to lack of information. However, this method was found to detect more of non-coliform bacteria when using for *E. coli* enumerations, in the current study. The overall non-coliform bacterial percentage was 63.6%, which was the highest among the four methods compared.

Seasonal variation of bacteria

In bottled water samples and well water samples, total coliform counts were higher during wet periods than that of the dry periods. A similar pattern was observed in river water samples, during the second wet period where as it was not clearly observed during the first wet period. Further, in river water samples, the counts were low during both dry seasons compared to wet seasons. This pattern was not observed in effluent samples. The counts remained high during the dry seasons as well.

In bottled water samples, total coliform counts were comparatively higher during the months with high temperatures. However, in well water samples, the counts were related to increase of temperature only during the first few months of the year, whereas the counts increased with time although the temperatures were relatively low. In river water samples, total coliform counts were lower in high temperature months compared to low temperature months. However, there were no considerable positive relationships between counts and the temperature. Similarly in effluent samples, the counts were higher even when the temperature values were low. These results show that the total coliform counts are influenced more by the rainfall, than the temperature compared to other studies conducted in temperate countries (Niemi, *et al.,* 1997), where the temperature variations are much greater (from -10 °C to 25 °C) than in the tropical countries. Further, the temperature variation in Kandy, where the sampling was conducted was limited to a small range showing no effect on the bacteriological quality of water, including river and well water sources.

E. coli counts in each water type varied with time during the 12 months period. As shown in Figure 2.10, in bottled water samples, *E. coli* contamination was observed in the rainy seasons of the year, while no contamination was detected during dry periods, by all five methods (bottled water samples were collected within one month of manufacture). A similar pattern was observed in well water samples and river water samples, where *E. coli* counts were relatively higher during wet seasons compared to the dry seasons. In contrast, *E. coli* counts did not show a relationship to the rainfall pattern in wastewater effluent

samples throughout the experimented time period. As depicted in Figure 2.11, *E. coli* contamination of bottled water samples were not much affected by temperature throughout the year since *E. coli* were detected in high temperature and low temperature months. However, in well water samples *E. coli* counts were increasing with the increasing of temperature during the first few months of the year. Subsequently the counts decreased with time and the next increase was observed in relation to the increasing of temperature at the beginning of the year 2008. In river water samples, *E. coli* counts were lower in high temperature months than in low temperature months. However, there was an increase with increasing of the temperature at the beginning of the year 2008. *E. coli* counts in wastewater effluent samples were not much affected by temperature, since even during low temperature months the counts were high. Further, the counts were comparatively low during high temperature months. As described above for total coliform observations, *E. coli* are also not affected much by temperature in a tropical country like Sri Lanka, in contrast to temperate countries (Niemi, *et al.,* 2002), However, the rainfall could have contaminated source waters due to surface run off with heavy rain falls. Similarly, in well water samples, rainfall could enhance the ground flow of water with bacterial contaminations, causing high counts during the rainy seasons. It emphasizes that, there is a need to protect the drinking wells from surface contamination.

Cost-benefit analysis of the methods tested

Tables 2.14, 2.15 and figures 2.9 and 2.10 show the results obtained for the cost-benefit analysis of the five methods.

The study suggests, that the most economical method to analyze bacteriological quality of a drinking water sample is, the SLS-MF method in the Sri Lanka's context, compared to all other methods. Anyhow, with the less labour, electricity and less time requirement, the enzymatic method m-ColiBlue24 method was placed second. Considering the easy handing, easy interpretations and especially the rapidness of the test, m-ColiBlue24 method is the most efficient method, which could be recommended for special drinking water analysis such as bottled water. Further, this method has proved greater detection limits of total coliform and *E. coli*, compared to the SLS-MF method, in this study.

However, for routine analysis, SLS-MF method might be beneficial, compared with the high cost, longer time and complexity of the conventional MTF methods. Moreover, when considering easy handling, reduced skilled labour, easy interpretation, higher accuracy and higher detection levels, Colilert method is the most efficient method among the MTF methods. Further, the Colilert method with added advantages compared to all other methods, could measure even very low concentrations of bacteria present in drinking waters with less contaminations like bottled water. However, it's comparatively higher cost must be taken in to consideration in replacing methods for drinking water analysis.

When considering analysis of surface water samples also, SLS-MF method is the cheapest method. However, being more accurate and with more advantages as described above, the Colilert method becomes the next economical method in Sri Lanka's conditions for analyzing surface water samples. Therefore, the Colilert method could be used efficiently for bacteriological quality analysis in surface water samples with high contaminations. In contrast, highly contaminated water will increase the chemical cost and the workload in SLS-MF method. It also can cause loss of data, due to inappropriate dilutions used generally in MF methods. However, since Colilert pre-prepared medium can be used as a MTF method, these errors will not occur with the Colilert method.

Method comparison by ISO criteria

The observations made following the ISO criteria revealed that all the comparisons resulted in 'inconclusive' evaluations. Even though more than hundred confirmation tests were coducted as prescribed, problems such as contamination of plates, loss of cultures due to poor growth or poor isolation caused lowering of paired count data, which was the major constraint in the comparison study causing inconclusive evaluations. Similar problems were encountered with *E. coli* confirmation tests. These results suggested a requirement for increasing the number of samples by all four methods, as described by ISO criteria. Since there were no previous records on similar work done by using SLS-MTF method, results obtained in this study could not be compared with any other studies. However, one such study done previously, by Pitkanen, *et al* (2007) by following the ISO

criteria, has reported inconclusive evaluations even with comparatively higher sample numbers.

Since this types of research was conducted in a developing country like Sri Lanka (probably for the first time), the above mentioned practical problems could not be easily overcome with the available limited facilities. However, if these problems could be successfully addressed in the future, validity and efficiency of microbiological method comparisons would be enhanced. Therefore, collaboration with different institutes involved in water quality industry would be very important for improving future work, since this involves complicated work, which may not be possible to be undertaken by a single laboratory. However, since it is evident that the conventional methods currently used in Sri Lanka are more costly and inefficient, it is very essential to conduct further research with newly emerging, efficient and low cost microbiological methods in the world.

CHAPTER 3

ISOLATION AND IDENTIFICATION OF BACTERIA FROM DIFFERENT WATER SOURCES

3.1. INTRODUCTION

As discussed, the conventional bacteriological methods available for testing fecal contamination enumerate coliform bacteria by detecting gas and/or acid by the lactose fermentation reaction. In these methods of testing, Bile, sodium desoxycholate, Brilliant green, detergents and other ingredients are used to limit the growth of other bacteria, especially the detection of Gram-positive bacteria (Niemi *et al.*, 2001). However, the recently developed methods rely on β-D galactosidadse and β-D glucuronidase activity for detections instead of lactose fermentation (Manafi *et al.*, 1991). These new media are also supplemented with chemicals inhibiting the growth of non-coliform bacteria (Fricker and Fricker, 1996). According to Schauer, *et al.*, (2007), since 2002, the United States Environmental Protection Agency (USEPA) has approved ten new enzyme-based methods for the detection of total coliforms and *E. coli* in drinking water. However, these enzyme-based methods also show differences in detecting coliforms and also, in said to be suppressing non-coliform bacteria such as *Aeromanas* spp., a common cause for producing 'false positive' results (Schauer, *et al.*, 2007).

The current study included two enzyme-based methods, together with the conventional lactose-based methods selected for the comparison. However, the selected four bacteriological methods (SLS-MTF, Colilert, SLS-MF and m-ColiBue24 method), though intended to detect bacteria of the coliform group, also detected certain non-coliform bacteria, as revealed by the confirmation tests. As suggested by Niemi *et al* (2001), the reliability and accuracy of a method in detecting coliform bacteria is a concern when introducing new methods. Further, introducing new methods into routine use is difficult, when their validation becomes much complicated in detecting coliform bacteria, consisting of numerous numbers of species. Furthermore, result interpretation become difficult when methods yield different spectra of coliform species (Niemi, *et al.*, 2001).

Therefore, several studies have been conducted in the past to elucidate the strengths and weaknesses of both enzyme-based methods and lactose based methods, in detecting coliform bacteria by isolation and identification of bacteria from different water sources. Table 3.1 shows the details of certain reported studies.

Table 3.1 Literature review on bacteriological identification studies

Comparison methods/media	**sources of water used for comparison**	**Results**	**Reference**
Colilert /MTF, Colilert (AC) P-A, M-Endo LES/MF, LTB, BGLB/MTF, LTB P-A method	Natural springs, surface waters, well waters, cisterns, treated coagulated settled water, public drinking water supply	api 20E tests have identified 48 isolates from which 44 were coliforms and 4 non-coliforms from the positive Colilert tests. (Identifications have not been done with the other media).	Covert, *et al.*, 1989
Standard method (SM) Presence-Absence (P-A) method with Auto-analysis Colilert (AC) P-A method	Deep and shallow wells, sprigs, rivers and surface reservoirs	api 20E system has identified 100 of them 54 coliform bacteria from the isolates detected by Colilert (AC), and SM-P/A method, respectively	Edberg, *et al.*, 1989
m-ColiBlue24 and M-endo	Surface water, one effluent source, one potable water source	api 20E tests have identified 25.2 % and 11.4 % coliform bacteria and 26.8 % and 29 % non-coliform bacteria from m-ColiBlue24 and M-endo media respectively.	Grant, 1997
LMX broth, Chromocult	Marine water	api 20E identification has revealed 83 % total coliforms and 17%	Geissier, *et al.*, 2000

Coliform (CC) agar, Standard – MTF method		*Aeromonas* species in LMX broth and, 40 % total coliforms and 53 % *Aeromonas* species in CC agar.	
Differential coliform agar/MF, LES endo agar/MF, Lactose Tergitol TTC agar/MF	Shallow well waters	Coliform identification rates were 81.6 %, 68.1% and 77.6 % in Tergitol, Differential coliform, and LES endo media respectively, when identified by API20E tests.	Niemi, *et al.*, 2001
Colilert-18	River water samples	Fatty acid methyl ester (FAME) profile has identified 64.5 %, and the api 20E strips have identified 76.5% as *E. coli* from the positive Colilert-18 wells.	Chao, *et al.*, 2004
10 enzyme-based methods: Colilert, Coliert-18, Colisure, m-ColiBlue24, Readycult Coliforms 100, Coliscan, E*Colite, Chromocult, MI agar, and Colitag	Geographically and chemically different ground water sources in the US	In Presence/Absence testing, Colilert-18, Colisure, m-ColiBlue24, Readycult and E*Colite methods failed to detect *E. coli* at varying failure rates. *Aeromonas* suppression rates were differed among the 10 methods, depending on the strain.	Schauer, *et al.*, 2007
German Standard MTF, ISO 9308-1, Colilert-18	Drinking water reservoirs in Germany	api 20E identification resulted in 95%, 29% and 15% coliform detections by Colilert-18, ISO 9308-1 and conventional German Standard method respectively.	Kampfer, *et al.*, 2008

In addition, Niemi, *et al.,* (2001) have demonstrated that, certain Oxidase negative colonies confirmed to be coliforms, have been identified as Oxidase positives by bacteriological identification tests using api 20E test kits. This observation highlights the fact that reliability of the confirmation tests should also be handled with care. Therefore, bacteriological identification will be a much more reliable source of interpretation as regard to performance, than that of the general confirmation tests, in assessing bacteriological quality of water.

3.2. OBJECTIVES

Considering the factors mentioned above, the current study attempted;

1. To identify bacterial isolates for each method to obtain a clear picture regarding their performance, reliability and the specificity in detecting bacteria in the coliform group.
2. To study the differences among the spectra of coliform bacteria detected by each method.
3. To investigate the interference from other non-coliform bacteria, giving rise to false positive results in each method to identify the bacterial spectra present in different water sources.

3.3. MATERIALS AND METHODS

3.3.1. Isolation of bacteria

Bacterial isolation was performed using Tryptic Soy agar (TSA, Oxoid, UK) as explained in the section 2.5.3, of Chapter 2. The three basic standard confirmation tests (Gram's test, oxidase test and catalase tests) conducted for coliform bacteria, were performed with each isolate from different sources by the four methods.

Stock cultures of all isolates were made as follows, for further identification. In preparing stock cultures, the isolated pure colonies were inoculated in to Eppendorf tubes, containing Brain Heart Infusion broth (Oxoid, UK) or double strength Tryptic Soy broth (Oxoid, UK), and incubated for 24 hours at 37 °C. Four replicate stock cultures were prepared from each isolate, and stored at –20 °C, after overlaying with 40 % glycerol.

3.3.2 Identification of bacteria

One or two Eppendorf tubes from each stock culture were thawed, and the tubes were centrifuged for a few seconds to obtain a concentrated cell mass. Subsequently the TSA (Oxoid, UK) plates were streaked from the concentrated cell mass to obtain pure colonies required for identification tests. All plates were incubated at 37 °C for 24 hours to obtain pure cultures. The three basic confirmation tests, the Gram's test, Oxidase test and the Catalase test were performed for further clarification. Subsequently the other standard biochemical tests used for identification of bacteria were performed as described below (3.1.3). Identification of bacteria was also performed by using commercially available api 20E (bioMérieux) rapid identification strips. Results of both these tests were recorded. However, due to contamination issues at the laboratory, and also due to missing isolates in the identification procedure, each and every sample analyzed by all methods could not be identified simultaneously. Therefore, identifications were performed only with the successfully isolated colonies of certain samples and not always with all four methods.

3.3.2.1. Biochemical tests

The common biochemical tests, generally performed for the isolated pure colonies on TSA plates, for identification of bacteria belonging to the family Enterobacteriaceae were conducted for bacterial identification. Those tests include; TSI, Urease, MR-VP, Citrate utilization, Hydrogen Sulphide production, Indole production, ONPG and the Motility tests. However, when the identification was found to be non-coliforms (which gave oxidase positive test), certain additional tests such as Gelatin liquefaction test, Pigment test, Growth at different temperatures, etc., were performed. In addition to these tests, an additional Gram staining was also performed for certain cultures, which showed anomalous results. All biochemical tests were performed by following the standard microbiological procedures.

1. Triple Sugar Ion Agar (TSI) test

This test was used as the initial test, to differentiate coliform bacteria with non-coliform bacteria, depending on the reaction given. The media were poured as slants and was

inoculated by stabbing to the butt followed by streaking of the slant surface. The bacteria therefore were exposed both to anaerobic conditions (butt) and aerobic conditions (slant). Phenol red was used as an indicator. Caps of the tubes were not tightly closed in order to facilitate gas exchange. Depending on the reactions shown with TSI slants (Oxoid, UK), further tests were conducted.

Table 3.2 Reactions shown on TSI medium

Reaction	TSI reaction					Conclusions
	Growth	Slant	Butt	Gas	H_2S	
1	+	acid	acid	+	-	Perform further tests for enteric bacteria
2	+	alkaline	(acid)	+	+	”
3	+	alkaline	acid	variable	-	”
4	+	acid	(acid)	+	+	”
5	+	alkaline	alkaline	-	-	Perform further tests for non-enteric bacteria
6	+	acid	acid	-	-	”
7	-	-	-	-	-	”

( ) obscured by H_2S in butt

2. Urease test

Urease agar (Oxoid, UK) slants were inoculated and observed for the hydrolysis of urea by the enzyme urease (colour change to pink), present in particular species in the family Enterobacteriaceae.

3. Methyl Red and Voges-proskauer tests

The MR-VP broth (Oxoid, UK) containing dextrose as the carbohydrate source, was inoculated and observed for the methyl red and Voges-proskauer reactions separately.

4. **Methyl Red test:** Methyl red indicator was added to a one half of the MR-VP broth after incubation at 37 ^{0}C for 24 hours. Fermentation of dextrose was observed by the colour change to red, due to the lowering of pH, below 5.

5. **Voges-Proskauer test:** The other half of the MR-VP broth was incubated for a further 48 hours at 37 °C, and the Butanediol fermentation was observed by adding VP 1 and VP 11 reagents. The development of a pink or a red colour after agitation was considered a positive reaction for the production of acetoin.

6. Citrate utilization

Simmons Citrate agar (Oxoid, UK) containing citrate as the sole carbon source and ammonium salts as the sole nitrogen source, was used for inoculation at 37 °C for 24 hours. A positive reaction was noted by a colour change from green to deep blue (indicator- bromo thymol blue), due to the increase of pH by metabolizing citrate and releasing ammonia in to the medium.

7. Indole test (Tryptophan hydrolysis)

Here the Tryptone water (Oxoid, UK) tubes were inoculated and incubated at 37 °C. After 24 hours of incubation, few drops of Kovac's reagent (para-dimethylaminobenzaldehyde in alcohol) were added to observe the production of a red colour ring in the reagent layer, resulting from the production of Indole, due to hydrolyzing tryptophan (present in tryptone water), by the activity of tryptophanase enzyme present in certain bacterial species of family Enterobacteriaceae.

8. H_2S production

H_2S production was observed in the inoculated TSI agar (Oxoid, UK) slants, after incubation at 37 °C for 24 hours. A positive reaction was indicated with a colour change from yellow to black, detected by ferrous sulfate included in the TSI medium.

9. ONPG test

ONPG test was performed by inoculating in to tubes with Colilert medium (IDEXX, Maine, USA), containing ONPG (o-nitrophenyl β-D galactopyranoside), as a substrate. The bacteria consisting of the enzyme β-D galactosidase, turned Colilert tubes from colourless to yellow.

10. Motility test

Bacteria motility was observed using the 'hanging drop' method. A small portion of a 24 hours grown culture was taken with a sterilized wire loop and dispersed in a drop of distilled water, on a cover-slip. Vaseline was applied on the four corners of the cover-slip. Then, a cavity slide (in inverted position) was carefully kept on the cover-slip to stick at the corners of the cover slip. The cavity slide was then turned to the write position (upward) and the motility of bacteria was observed under a light microscope (10 x 40).

11. Gelatin liquefaction

Oxidase positive cultures grown on TSA plates (producing a bluish colour or not), were suspected to be *Pseudomonas*. This was confirmed with the gelatin liquefaction test. *Pseudomonas* species have the ability to produce proteolitic enzymes under this test and inoculating them in nutrient gelatin tubes, incubated at 37 °C for 24-48 hours can confirm this as under refrigeration, the positive cultures did not solidify.

12. Pigment test

The bacterial cultures, suspected to be in the genus *Pseudomonas*, were tested for the pigments too. This facilitates the identification of their species. 2-3 ml of chloroform was added to a well-grown culture (3-5 days) and was shaken vigorously. The pigments present in each culture were detected in the chloroform layer and also observed for fluorescence, under a UV light.

13. Growth at different temperatures

Suspected *Pseudomonas* cultures were inoculated on TSA plates and their growth was observed at different temperatures, by incubating at 5 °C, 25 °– 30 ° C and 42 °C respectively for species differentiation.

3.3.2.2. Identification using api 20 E identification strips

Pure colonies (well separated) on TSA plates were selected and emulsified in 5 ml sterilized distilled water, in a sterilized tube and mixed well using a vortex machine (Fisher FB 65000, UK) to obtain a homogenous suspension. Using a micropipette, this bacterial suspension was inoculated into twenty mini test tubes of the api 20 E strip, following the manufacturer's instructions; both the tube and the cupule were filled for the tests marked as CIT, VP and GEL. Only the tube was filled in the remaining tubes; anaerobiosis was created for the tests ADH, LDC, ODC, H_2S and URE by overlaying with sterilized mineral oil. To create a humid atmosphere, 5 ml of sterilized distilled water was distributed in honey-combed wells on the tray. Incubation box was closed with the lid and incubated at 37 °C for 18-24 hours.

After incubation, the strip was read referring the 'reading table' provided by the Biomerieux, Inc, USA. Reactions of the spontaneous reactions were recorded, and the TDA, VP and IND tests were performed by addition of TDA, VP 1 + VP 2 and IND reagents respectively, and the results were recorded. On the results sheet, the tests were separated into groups of 3 sets and values were summarized as instructed. By adding together, the values corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests of the api 20 E strip.

After this, the identification was performed using the database (V 4.0). The numerical profile was looked up in the Analytical Profile Index. The 7-digit numerical profile was entered manually via the keyboard in to the Identification software and submitted.

3.4. RESULTS

Results obtained for bacteriological identification, when performed with conventional biochemical tests and the api 20E rapid identification system are shown in Table 3.3. Biochemical tests were performed for 59 isolates from which 54 identifications were successful. api 20E tests were performed for 71 isolates of which, 50 successful identifications were obtained. Most of the unsuccessful identifications obtained in api 20 E tests were with Oxidase positive isolates.

By using the two identification systems, different spectra of coliform bacteria detected by each method were further studied. In addition, the interferences from other non-coliform bacteria, giving rise to false positive results in each method were investigated. Finally, the bacterial spectra present in different water sources tested during this study were identified.

3.4.1. Different bacterial spectra identified by different methods

As depicted in the table 3.3, all four methods identified 24 bacterial species including 13 coliform species and 11 non-coliform species. Out of the coliform bacterial identifications, 8 were fecal coliform species identified by api 20E and/or biochemical tests. Proportions of different bacterial types were calculated as percentage values, as given in the table 3.4. The different bacterial types (such as coliforms and non-coliforms) detected by each method were counted, divided by the total number of species detected and multiplied by 100, to obtain a percentage value.

When comparing the different bacterial spectra identified by each method, SLS method identified one total coliform species, three fecal coliform species and five non-coliform species in all water sources. As a proportion from the total number of detections, SLS detected only 40 % coliforms, with 60 % non-coliforms (Table 3.4). Colilert method has detected two fecal coliform species, one total coliform species and only two non-coliform species. As proportions, the coliforms were 60 % and the non- coliforms were 40 %. In M-endo medium, the bacterial spectrum consisted of three fecal coliform species, one total coliform species and four non-coliform species, with proportions of 73.3 % coliforms and 26.7 % non-coliforms. Bacterial spectrum detected by M-FC, consisted of four fecal

coliform species, one coliforms species and five non-coliform species. As proportions, coliforms and non-coliform were, 76 % and 24 % respectively. In m-ColiBlue24 method, bacterial spectrum consisted of one total coliform species, four fecal coliforms, and 12-non-coliform species. In proportion, the coliforms were 64.7 % and the non-coliforms were 35.3 %.

Table 3.3 Numbers of bacterial isolates detected by different methods

Identified bacterial isolate	SLS		Colilert		M-endo		M-FC		M-coliBlue24		Total	
	Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API
Klebsiella pneumoniae	1	0	1	0	3	0	4	0	3	0	12	0
Klebsiella pneumoniae ssp. *pneumoniae*	0	1	0	1	0	3	0	5	0	3	0	13
Klebsiella oxicota	0	0	0	0	0	0	1	2	0	0	1	3
E. coli	2	0	4	0	0	0	0	0	0	0	6	0
E. coli 1	0	4	0	2	0	1	3	3	0	0	0	10
Enterobacter sp.	0	0	0	0	0	0	0	0	1	0	1	0
Enterobacter sakazaki	0	0	0	0	0	0	0	1	0	1	0	2
Enterobcter cloacae	0	0	0	0	0	0	0	0	1	1	1	1
Citrobacter braakii	0	1	0	0	0	0	0	0	0	0	0	1
Citrobacter freundii	1	0	0	0	1	1	0	0	0	0	2	1
Kluyvera spp.	0	0	0	0	0	2	0	0	0	0	0	2
Pantoea sp.3	0	1	0	0	0	0	0	0	0	0	0	1
Rautella ornithinolytica	0	0	0	1	0	0	0	0	0	1	0	2
Serratia liquifaciens	0	1	0	0	0	0	2	0	0	0	2	1
Pseudomonas spp.	2	0	0	0	1	0	0	0	0	0	3	0
Pseudomonas aeruginosa	2	5	2	1	1	0	1	0	1	0	7	6
Pseudomonas fluorescence	2	0	0	0	1	0	0	0	0	0	3	0
Pseudomonas oryzihabitans	0	0	0	1	0	0	0	0	0	0	0	1
Aeromonas spp.	3	1	0	0	0	0	0	0	3	0	6	1
Aeromonas hydrophila	2	0	0	0	0	0	1	0	0	0	3	0
Aeromonas hydrophila ssp. *hydrophila*	1	0	0	0	0	0	0	0	0	0	1	0
Aeromonas salmonicida ssp.	0	0	0	0	0	0	1	0	0	0	1	0

salmonicida												
Salmonella spp.	0	0	0	1	0	0	0	0	0	0	0	1
Salmonella arizonae	0	0	0	0	0	0	0	0	1	0	1	0
Salmonella choleraesuis ssp. *arizonae*	0	0	0	1	0	1	1	0	0	1	1	3
Acinetobacter spp.	0	0	0	0	0	0	1	0	0	0	1	0
Acinetobacter baumanii	0	0	0	0	0	0	0	1	0	0	0	1
Unidentified	2	8	0	4	2	3	0	3	0	3	4	21
Total identified											54	50

Total coliform species	**5**
Fecal coliform species	**8**
Non-coliform species	**11**
Total number of species	**24**

Table 3.4 Percentages of different bacterial types isolated by different methods

Method	% Bacterial isolates		
	Total coliforms	Fecal coliforms	Non coliforms
SLS	6.7	33.3	60
Colilert	6.7	53.3	40
M-endo	13.3	60	26.7
M-FC	12	64	24
m-BoliBlue24	11.8	52.9	35.3

Among the coliform bacteria identified, the dominant species was *Klebsiella pneumoniae*, which was a fecal coliform species, detected by all four methods. M-endo, M-FC and m-ColiBlue24 methods detected the highest percentages of *Klebsiella pneumoniae*, compared to other two methods (Table 3.5). The next abundant species detected was *E. coli*, which is again a fecal coliform bacterium. *E. coli* was detected mostly by the Colilert method (percentage detection 33% - Table 3.5), compared to other methods. Among the non-coliform flora, the dominant species was *Pseudomonas aeruginosa*, mostly was detected by the SLS method (Table 3.5). The next abundant species was *Aeromonas* spp., again mostly detected by the SLS, Colilert and the m-ColiBlue24 methods.

During the present study, the widest spectra of coliform bacteria were detected by M-FC and m-ColiBlue24 methods compared to other methods. The widest spectra of non-coliform bacteria were detected by SLS, m-Coliblue24 and M-FC methods.

Specificity of methods in detecting coliform bacteria

According to above results and the results depicted in table 3.4, the specificity of methods in detecting coliform bacteria is in the order of; M-FC, M-endo, m-ColiBlue24, Colilert and finally the SLS, based on the proportions of coliform bacteria detected in the water sources tested in this study.

3.4.2 Bacterial spectra isolated from different sources

During the study, different bacterial spectra belonging to both coliform group and other non-coliform groups were isolated from different water sources (identified by api 20E and/or biochemical tests). Identified different spectra and their numbers (as detected by different methods) are depicted in tables 3.6 and 3.7 respectively.

Bottled water source:

In bottled water samples only two species belonging to the same genus were identified. Both were fecal coliform species belonging to the genus *Enterobacter*. Isolations done with m-ColiBlue24 and SLS methods were able to detect these organisms.

Enterobacter cloacae was identified both with the api 20E strips and the conventional biochemical tests (Plate 3.2), while *Enterobacter sakazaki* was detected only with the api 20E strips. The parallel biochemical tests identified only to the genus level.

Well water source:

Well water samples had the broadest spectrum of bacteria among all water sources tested in this study. It included five fecal coliform species, three total coliform species and eight non-coliform species. Among the fecal bacterial flora, *Klebsiella pneumoniae* (Plate3.3) was the most commonly isolated species by all methods. Biochemical tests identified the species, while api 20E test kits identified species to its subspecies level. Next abundant species detected was *E. coli* (Plate 3.4), confirming the fecal contamination of well water, collected from indicated provinces in Sri Lanka. *E. coli* species was identified as *E. coli* 1, by api 20E tests. In addition to these dominant species, fecal coliforms such as *Klebsiella oxicota, Citrobacter braakii, Citrobacter freundii, Enterobacter sakazaki* and total coliform species *Rautella ornithinolytica, Pantoea* spp. 3 and *Kluyvera* spp. too were identified. Among non-coliform bacteria, *Pseudomonas aeruginosa* was the dominant species found in well water samples identified by both biochemical and api 20E tests. Other non-coliform species include *Pseudomonas* spp., *Pseudomonas oryzihabitans, Aeromonas* spp., *Aeromonas*

hydrophila, Aeromonas hydrophila ssp. *hydrophila, Salmonella* spp., *Salmonella arizonae, Salmonella choleraesuis* ssp. *arizonae* and *Acinetobacter* spp.

Surface water source:

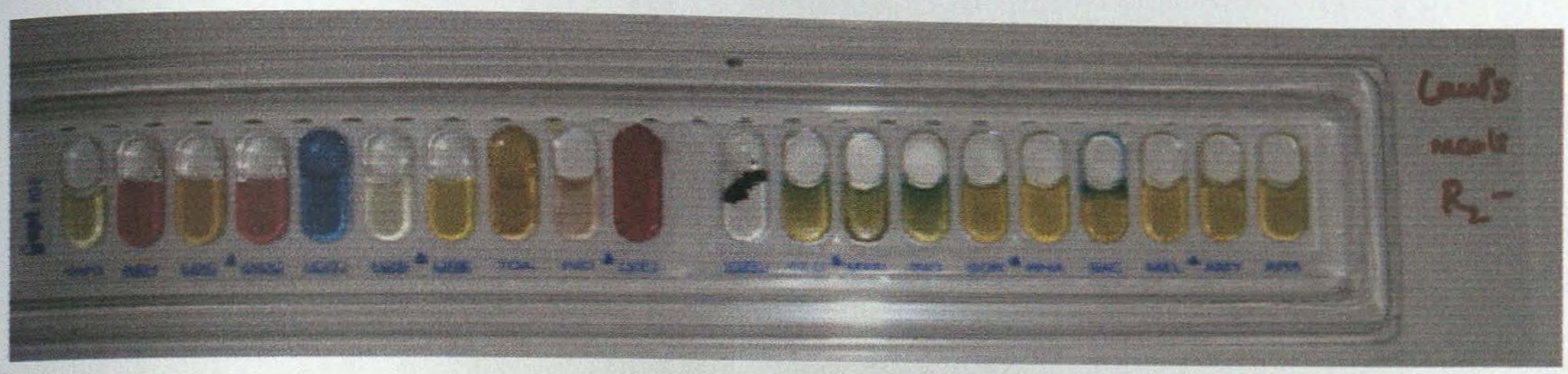
Bacterial spectrum identified in surface water samples was not diverse as in well water samples. Only three fecal coliform species were identified. Among the three identified coliform species, *E. coli* was the dominant species. Other than *E. coli*, *Klebsiella pneumoniae* and *Citrobacter freundii* too were present as fecal contaminants. Further, *Serratia liquifaciens* and *Rautella ornithinolytica* are the two total coliform species identified. However, the detection of non-coliform species was comparatively higher, with a diverse spectrum of seven species identified of which, *Pseudomonas aeruginosa* (Plate 3.5) being the dominant species. In addition, *Pseudomonas fluorescence*, *Aeromonas* spp., *Aeromonas salmonicida* ssp. *salmonicida, Acinetobacter baumanii* and *Salmonella choleraesuis* ssp. *arizonae* were present. Further, a Gram + bacterium was isolated from two surface water bodies, which was not identified at the present study.

Effluent water source:

Three coliform and three non-coliform species were identified from effluent water samples. *Klebsiella pneumoniae* and *E. coli* were the dominant fecal coliform species, while *Kluyvera* spp. was identified as the total coliform species. The three non-coliform species belonging to the genus *Pseudomonas* were identified as *Pseudomonas aeruginosa, Pseudomonas oryzihabitans* and another *Pseudomonas* spp.

Table 3.5 Percentages of dominant bacterial species isolated by different methods

	Percentage detection %									
Bacterial species	SLS		Colilert		M-endo		M-FC		m-ColiBlue24	
	Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API
Klebsiella pneumoniae	5	-	8	-	27	-	16	-	20	-
Klebsiella pneumoniae subsp. *Pneumoniae*	-	5	-	8	-	20	-	22	-	23
E. coli	10	-	33	-	-	-	12		-	-
*E. coli*1	-	18	-	17	-	7	-	13	-	-
Pseudomonas aeruginosa	10	23	17	8	9	0	4	0	7	0
Aeromonas sp.	15	5	0	0	0	0	0	0	20	0



api 20 E strip

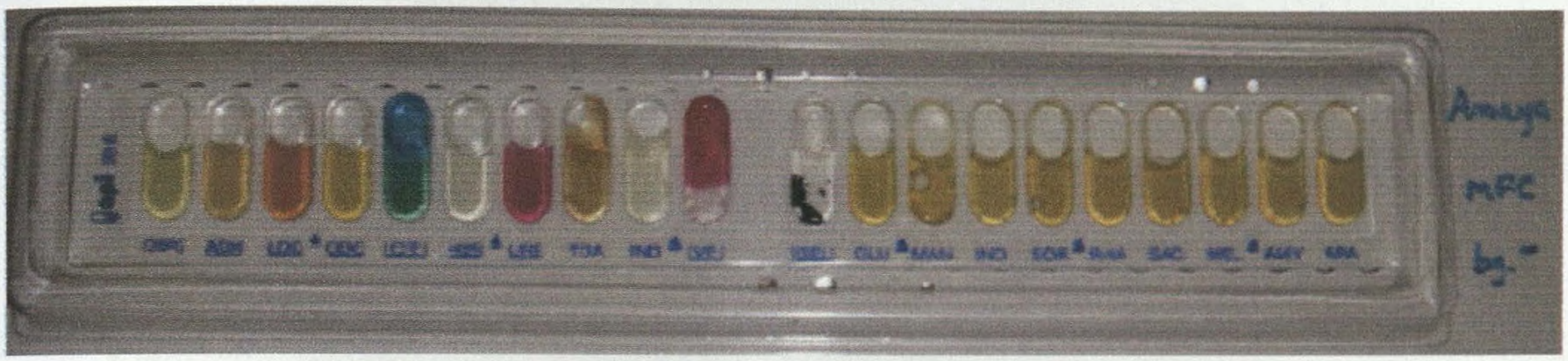
Biochemical test results

Plate 3.1 Identification results for *Enterobacter cloacae*

api 20 E strip

Biochemical test results

Plate 3.2 Identification results for *Citrobacter freundii*



api 20 E strip

Biochemical test results

Plate 3.3 Identification results for *Klebsiella pneumoniae* ssp. *pneumoniae*

Table 3.6 Different bacterial spectra isolated from different sources

Identified bacterial strains	Bacterial type	Source			
		Bottle	Well	Surface	Effluent
Klebsiella pneumoniae	FC	_	√	√	√
Klebsiella pneumoniae ssp. *pneumoniae*	FC	_	√	√	√
Klebsiella oxicota	FC	_	√	_	_
E. coli	FC	_	√	√	√
E. coli 1	FC	_	√	√	√
Enterobacter spp.	FC	√	_	_	_
Enterobacter sakazaki	FC	√	√	_	_
Enterobacter cloacae	FC	√	_	_	_
Citrobacter braakii	FC	_	√	_	_
Citrobacter freundii	FC	_	√	√	_
Kluyvera spp.	TC	_	√	_	√
Pantoea sp.3	TC	_	√	_	_
Rautella ornithinolytica	TC	_	√	√	_
Serratia liquifaciens	TC	_	_	√	_
Pseudomonas sp.	NC	_	√	√	√
Pseudomonas aeruginosa	NC	_	√	√	√
Pseudomonas fluorescence	NC	_	_	√	_
Pseudomonas oryzihabitans	NC	_	√	_	√
Aeromonas sp.	NC	_	√	√	_
Aeromonas hydrophila	NC	_	√	_	_
Aeromonas hydrophila ssp. *hydrophila*	NC	_	√	_	_
Aeromonas salmonicida ssp. *.salmonicida*	NC	_	_	√	_
Salmonella spp.	NC	_	√	_	_
Salmonella arizonae	NC	_	√	_	_
Salmonella choleraesuis ssp. *arizonae*	NC	_	√	√	_
Acinetobacter spp.	NC	_	√	√	_
Acinetobacter baumanii	NC	_	_	√	_

TC-total coliform FC- fecal coliforms NC- non coliforms

Table 3.7 Numbers of different bacterial species isolated from different sources

Source	Bacterial isolate	SLS		Colilert		M-endo		M-FC		M-coliBlue24		Total	
		Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API	Bioch.	API
Bottle	*Enterobacter cloacae*	1	_	_	_	_	_		_	1	1	2	1
	Enterobacter sakazaki		_	_	_	_	_		_		1	0	1
	Enterobacter spp.	1	-	-	-	--				1		2	0
Well	*Klebsiella pneumoniae* ssp. *pneumoniae*	-	1		1		2		2		1	0	7
	Klebsiella pneumoniae	1				2		1		1		5	0
	E. coli 1	1	2	3	2		0		0		0	4	4
	Klebsiella oxicota	-	0	-	0		0	1	2		0	1	2
	Pantoea sp.3	-	1	-	0		0		0		0	0	1
	Citrobacter braakii	-	1	-	0		0		0		0	0	1
	Enterobacter sakazaki	-	0	-	0		0		1		0	0	1
	Rautella ornithinolytica	-	0	-	1		0		0		0	0	1
	Citrobacter freundii	-	0	-	0	1	1		0		0	1	1
	Kluyvera spp.	-	0	-	0		1		0		0	0	1
O+	*Pseudomonas aeruginosa*	1	3	1	1		0		0	1	0	3	4
	Pseudomonas sp.	2		-								2	0
	Pseudomonas oryzihabitans	-	0	-	1		0		0		0	0	1
	Aeromonas sp.	1	1	-	0		0		0	1	0	2	1
	Aeromonas hydrophila	2		-				1				3	0
	Aeromonas hydrophila ssp. *hydrophila*	1		-								1	0

	Salmonella arizonae									1		1	0
	Salmonella choleraesuis ssp arizonae	-	0	-	1		0		0		0	0	1
	Acinetobacter spp.							1				1	0
	Unidentified	2	4	0	1	0	0	0	2		1	3	8
Surface	*E. coli* 1	1	1	-	0		1	2	3		0	3	5
	Serratia liquifaciens		1	-	0		0		0		0	0	1
	Citrobacter freundii	1	0	-	0		0		0		0	1	0
	Klebsiella pneumoniae			1						1		2	0
	Klebsiella pneumoniae ssp *pneumoniae*		0		1		0		0		1	0	2
	Rautella ornithinolytica										1	0	1
O+	*Pseudomonas aeruginosa*	1	1	1	1	1	0	1	0		0	4	2
	Pseudomonas fluorescence	2		-		1						3	
	Salmonella choleraesuis ssp arizonae		0		1		1		0		1	0	3
	Acinetobacter spp.							1				1	0
	Acinetobacter baumanii		0		0		0		1		0	0	1
	Aeromonas spp.	2								2		4	0
	Aeromonas salmonicida ssp. *Salmonicida*							2				2	0
	Unidentified	0	4	0	0	2	2		0	0	1	2	7
Effluent	*E. coli* 1		1	1	1		0	1	0	1	0	3	2
	Klebsiella pneumoniae					1		3		1		5	
	Klebsiella pneumoniae ssp. *pneumoniae*		0		0		1		3		1	0	5
	Kluyvera spp.		0		0		1		0		0	0	1
O+	*Pseudomonas* spp.					1						1	0
	Pseudomonas aeruginosa		1		0		0		0		0	0	1
	Pseudomonas oryzihabitans		0		1		0		0		0	0	1

3.4.3 Comparison of biochemical tests and api 20E tests in identification of isolates

Both biochemical tests and api 20E tests were not performed similarly in identification of bacteria, at certain instances of the study. During parallel identifications done by both identification systems, only 59.5 % gave similar identification results, at least to genus level from the 47 tests performed (Table 3.8). This was mainly due to the oxidase positive isolates tested in the present study. Most of the misidentifications or inconclusive results obtained by api 20E strips were for the Oxidase positive cultures, whereas conventional biochemical tests were able to identify most of those isolates at least to the genus level. However, the conventional biochemical tests failed in identifying some Oxidase negative coliform bacteria, which were identified by the api 20E strips (Table 3.8).

Table 3.8 Performance of biochemical tests and api 20E tests in parallel identifications

Total number of identifications done by both biochemical tests and api 20E tests	47
Percentage isolates identified to the species level by both tests	53.2 %
Percentage isolates identified to the genus level by both tests	6.3 %
Percentage isolates (Oxidase +) identified only by biochemical tests	27.6 %
Percentage isolates (Oxidase -) identified only by api tests	8.5 %

3.5. DISCUSSION

The yield and the spectra of coliform bacteria varied among the methods adopted in the current study. Each method compared had a characteristic species distribution of target bacteria and a typical level of non target bacteria. These observations confirm the previous results observed by Edberg, *et al.* (1989); Lewis, *et al.* (1989); Geisser, *et al.* (2000); Piscicotta, *et al.* (2002) and Kampfer, *et al.* (2008). A study conducted by Niemi, *et al.* (2001) on determining the numbers and distribution of coliform bacteria in well water samples, has revealed that each method compared had a characteristic species distribution of target bacteria and a typical level of interference of non-target bacteria. Further, they have reported that a high yield and a sufficient selectivity are difficult to achieve simultaneously.

In the current study, the highest percentage of coliform flora was shown by the M-FC medium of the SLS-MF method, suggesting the higher specificity of M-FC medium for the coliform group. Therefore, it could be suggested that the inhibitory substances used in the M-FC medium for suppressing the growth of non-coliform bacteria is effective. However to confirm this observation, further studies will be needed. There were no such previous records available in literature. The next specific medium in detecting coliform bacteria was M-endo of the SLS-MF method, which has detected 73.3 % of coliform bacteria, which is in contrast with the 11.4 % observed by Grant (1997). This might be due to the relatively higher abundance of coliform bacteria in tropical waters compared to the temperate bacterial flora. Another reason might be the higher detection rates of tropical coliform bacteria, than the temperate bacteria by M-endo medium. However, further studies will be required to confirm these ideas. When m-ColiBlue24 was used, the percentage detection was 64.8 % . This result is also comparatively higher compared to the 25.2 % reported by Grant (1997). The reasons for this observation might also be similar with the explanation for M-endo medium.

Colilert method detected 60 % of coliform bacteria during the current study. This was a comparatively low value compared with the 76.5 % reported by Chao, *et al.* (2004) and 95 %, reported by Kampfer, *et al.* (2008). This might be due to the detection of more Oxidase positive non-coliform species found abundantly in tropical waters as suggested by Fricker, *et al.* (1998). The lowest percentage (40 %) of coliform bacteria was detected by the SLS-MTF method. This could be attributed due to the fact that Mac Conkey medium is comparatively less specific for coliforms, than the other media tested in this study. Another factor might be, the high rate of false negatives due to the anaerogenic coliform bacteria present in the samples tested. The detection of Oxidase positive non-coliforms present abundantly in tropical waters, might be the other possible reason for this observation. Therefore, it could be concluded that the least specific medium for the detection of coliform bacteria in the tropical waters tested in this study is Mac Conkey broth of the SLS-MTF method. All the other alternative methods performed comparatively better than the SLS-MTF method in detecting coliform bacteria in the current study.

In bottle water samples, only two species were identified by the SLS and the m-ColiBlue24 methods. However, this result does not imply that the other methods cannot detect them.

This observation was due to the fact that, successful isolations were possible only with those two methods. Inadequacy of different brands with contaminations was a reason for limited isolations done from bottle water samples. However, there are reports on various bacterial isolations done from bottle water samples around the world.

However, a surprisingly higher diversity of bacterial flora was observed in well water samples, collected from different provinces of Sri Lanka in this study. The fecal bacterial flora consisted of five species, compared to 6 fecal coliform species identified by Niemi, *et al.*, (2001), in well water samples collected from different locations in Finland. However, the counts of fecal bacteria were very low in their study, compared to the higher counts in the current study. According to their explanation, the reported low counts are due to the dry weather and lack of surface run-off during their study. However, high counts of fecal bacteria were observed throughout the current study, even though most of the wells consisted of ring walls, preventing from surface run-off. Therefore, the fecal contaminations of well water in Sri Lanka might mainly be due to the contaminated subterranean water flow, through the toilet pits built relatively closer to the wells. Further, the heavy rainfall and the sloping terrain patterns could have a direct effect on the high rate of contaminations of fecal flora to the shallow wells in Sri Lanka. However, this observation should be further studied in detail to make a final conclusion on well water contamination in Sri Lanka. Further, the water abstraction rate from the well also has an effect on the contamination. When the abstraction is higher, the pollution by total coliforms could also be higher (personal communications with Dr. D.R.I.B. Werellagama, 2010).

Since some of these fecal flora are pathogenic (eg: some strains of *E. coli*, *Klebsiella pneumoniae* and *Enterobacter sakazaki*), consumption of well water as a source of drinking water, should be more of a concern. Therefore, in most of the developed countries, well water sources supplying drinking water for small communities are disinfected by chlorination (Niemi, *et al.*, 2001). However, being a developing country, boiling before consumption would be the most safe way of using well water for drinking purposes in Sri Lanka.

In addition to the fecal flora, total coliform species commonly isolated from tropical water were also present in the well water samples tested. However, the total coliforms identified in temperate countries were *Ervinia* spp., *Hafnia alvei, rahnella aquitilis, Serracia fanticola, Serracia liquifaciens, Serracia enterolitica, Yersinia enterolitica* and *Yersinia* spp. (Niemi, *et al.*, 2001), compared to *Rautella ornithinolytica, Citrobacter freundii* and *Kluyvera* spp. identified in collected well water samples in the current study.

Niemi, *et al.,* (2001) have isolated 13.4 %, Oxidase positive non-coliform bacterial population, compared to 34%, identified in the present study. However, Niemi, *et al.*, have not identified the isolated non-coliform species, while nine species were identified in the present study, while most of them were environmental species. However, among them certain pathogenic species such as *Pseudomonas* spp., *Aeromonas* spp., *Salmonella* spp., *Salmonella arizonae, Salmonella choleraesuis* ssp. *arizonae* and *Acinetobacter* spp. were also present.

When considering two identification systems used in this study, similarities as well as certain differences were observed in bacteriological identifications. Conventional biochemical tests were successful in 93.2 % of identifications, at least to the genus level, including Oxidase positive non-coliform species. The api 20E rapid identification system was able to identify 80.6 % bacterial species from the total identifications performed. The failed percentage was mostly due to the Oxidase positive bacteria. Although api 20E system is manufactured for the identification of members of the family Enterbacteriaceae, certain Oxidase positive bacteria could also be identified using the same system. According to the manufacturer's instructions, about 32 Oxidase positive species could be identified accurately by these test kits. Therefore, Oxidase positive isolates were also identified using the api 20E system in the present study (However, for identification of non-coliforms, use of api 20NE strips (if available) is more appropriate). In the present study oxidase positive bacterial species such as *Pseudomonas aeruginosa* was identified with accuracy of 99.9 %. Further, the Oxidase negative isolates accurately resembled the profiles described for different Enteroacteriaceae species by the manufacturer of the api 20E galleries and the 'absolute identification' was therefore excellent. However, in few cases identification to distinct species was hampered by the slight differences between the species when using the tests included in the galleries, resulting in 'low discrimination'.

CHAPTER 4

GENERAL DISCUSSSION

Bacteriological quality assurance is one of the prime issues with the current rate of water pollution. Investigations on sensitive and reliable bacteriological analytical methods are intensively being conducted around the world to overcome the weaknesses associated with the conventional bacteriological methods. Hence, newly emerging methods have been approved by the regulatory agencies such as United States Environmental Protection Agency (US EPA), and included in the standard methods published by American Public Health Association (APHA), etc. Further, most developed countries have already adopted the currently emerging methods (such as enzymatic methods), by comparing them with the available conventional bacteriological analytical methods (Griffith, *et al.*, 2006. Pitkanen, *et al.*, 2007, Noble, *et al.* 2003, Eckner, 1998, Palmer, *et al.* 1993, Edberg, *et al.* 1990, Covert, *et al.* 1989). These investigations have resulted in valuable information on the weaknesses and the strengths of different methods used to detect total coliforms and *E. coli*, especially in water samples collected from temperate countries. However, such information are very limited in the tropics, especially in Asia.

Therefore, the current study investigated the performance of different conventional and enzymatic methods, using tropical drinking and environmental water sources, in Sri Lanka. Results of the current study revealed that the different methods performed differently in detecting total coliform bacteria and *E. coli* in tropical water samples tested. Further, the bacteriological identifications showed that the bacterial spectra detected during the study, also differed with different methods. The results obtained in the preliminary study, when analyzed by variance statistics, revealed that the five methods tested were statistically different with each other and also the conventional SLS-MTF method was significantly different at $p<0.05$, with all alternative methods except with the APHA-MTF, which is also a conventional method. Based on these results, a detailed study was conducted by excluding the APHA-MTF method, during the second phase of the study. This step allowed a comparison study, by following the ISO criteria for comparison of bacteriological methods

in Sri Lanka and probably this being the initial such study in Asia. Bacteriological comparisons, following the ISO criteria was first reported by Niemela, *et al* in 2003 using fabricated samples created primarily from laboratory strains of bacteria seeded into clean matrices and then, by Pitkanen, *et al.* (2007), using natural non-disinfected water samples. Both studies were carried out in Europe.

The current study compared conventional methods as well as currently available enzymatic methods. The two enzymatic methods, Colilert and m-ColiBlue24 were selected based on their performance as reported in other similar studies, affordability and the availability in the market at the time of the commencement of work. The Sri Lanka Standard (SLS)-MTF method, using Mac Conkey and Brilliant Green Lactose Bile Broth, based on lactose fermentation was selected as the 'reference method' with the other 'alternative methods' during both phases of the study. Comparisons done at the preliminary phase of the study showed the strengths and the weaknesses of each method in detecting total coliforms and *E. coli*, in addition to the quantitative data on bacteriological counts. Based on these observations, certain positive and negative features of the methods were revealed.

When considering MTF methods, the interpretation of positive presumptive tubes were subjective in conventional SLS and APHA methods, compared to the enzymatic Colilert method. This was because, the detection of growth (turbidity), gas production and acid reaction were not distinctly clear in certain trials for SLS and APHA methods. On the other hand, Colilert gave a distinct yellow colour in total coliform positive tubes. The detection of fluorescence in Colilert tubes was comparatively easier than in EC-MUG tubes in APHA method, due to the turbidity of EC-MUG tubes. This observation confirms the previous results obtained by Shadix and Rice (1991), when they observed difficulty in detecting the fluorescence of the MUG reaction in lactose based MUG media due to turbidity caused by the heavy bacterial growth. Further, the Colilert method required less time and labour, less equipment and confirmation steps than SLS and APHA methods. Simultaneous detection of total coliforms and *E. coli* using Colilert was rapid, easy and efficient, which is very important when analyzing potable water samples. However, in this study, Colilert method was tested by the usual 3-tube MPN method and not by using the device QUANTRITRAY which converts the observations in to final MPN values. If this device could have been used, the Colilert system would have been much easier and faster.

Findings of the current study agree with the early findings made by Edberg, *et al.* (1988), who compared the conventional MTF method with Autoanalysis Colilert (AC) in a field trial. The autoanalytical nature of Colilert method is said to be more practical and efficient than the current coliform methods and less subjective than the Standard methods procedures. Availability of medium in powder form; shelf life for one year at room temperature; easy interpretation of results; user friendliness in the field; longer survival of bacteria (up to seven days) in Colilert tubes, are other advantages of Colilert method. Thus findings of the current study is similar to the observations of Edberg, *et al.* (1989), who have proved that the Colilert medium was sensitive, specific and easier than the conventional methods to detect total coliforms and *E. coli* in drinking water and other environmental waters. Shadix and Rice (1991) showed that the enzyme β–glucuronidase posses the accuracy and specificity required for the detection of *E. coli* in environmental waters. Their findings have also been corroborated with the results of other investigators (Robinson, 1984; Moberg, 1985), who have found 90 % or more *E. coli* from a variety of sources when using β–glucuronidase containing media. The strains of *E. coli* isolated from environmental samples in the study have showed a 95 % positivity rate for β–glucuronidase by the AC method. Further, they have also concluded that the AC system is a more rapid test procedure than the lactose fermentation method which produces results within 24 hours of initial inoculation. In addition, AC tubes have also detected anaerogenic strains of *E. coli,* which might be missed due to failure to produce gas in the presumptive phase of the conventional test. Fricker and Fricker (1996) have suggested that Colilert could even be used in small laboratories with limited facilities and resources. Results obtained by Pitkanen, *et al.* (2007) state that Colilert did not require confirmation with most types of water samples except bathing water, in their study.

Among MF methods, atypical colony formation and background flora on membranes incubated on different culture media have been reported in previous studies (Schauer, *et al.*, 2007, Pitkänen, *et al.*, 2007, Schets, *et al.*, 2002, Niemi, *et al.*, 2001, Grant, 1997). The background growth on M-endo and M-FC media poses disadvantages, since it appeared as a continuous mat on the membranes. In contrast, uniform red colour total coliform and blue colour *E. coli* colonies on m-ColiBlue24 medium made counting easier compared to colonies formed on M-endo and M-FC media with different morphologies throughout the current study. However, a comparison study done by Grant (1997), has reported atypical,

light pink coloured colonies on m-ColiBlue24 plates, in contrast to the observations in the current study. However, it should still be noted that many atypical colonies and background colonies were confirmed as total coliforms and *E. coli,* on M-endo and M-FC media respectively, in the current study. Further, as Pitkanen, *et al.* (2007) reported, Harlequin ™ *E. coli*/coliform (HECM) medium and LES Endo media have formed very few background flora compared to Chromocult Coliform agar (CC) and LTTC (Lactose based Tergitol-7 Agar) media. Further, colonies formed on Chromogenic *Escherichia coli*/Coliform (CECM) plates have exhibited bright colours, making it easy to differentiate between *E. coli*, coliforms and background flora. Counting on CC plates has been easier compared to LTTC plates due to colour reactions on CC medium.

One of the advantages observed in MF methods is that, large sample sizes as much as one litre could be analyzed by the MF method, while only much smaller aliquots can be analyzed by the MTF method. On the other hand, clogging of filters is a drawback in MF methods. In addition, the need of several dilutions in MF methods, makes additional effort and cost. Moreover, m-ColiBlue24 medium needed more dilutions compared to M-endo and M-FC media, due to its high sensitivity.

Variance analysis done separately with different water types gave an idea about the type of method suitable to detect indicator bacteria in different water sources. In bottle water samples, all alternative methods, (except the APHA-MTF) detected significantly higher total coliform counts compared to the SLS-MTF method, during both phases of the study. Further, the SLS-MTF method was not able to detect any total coliform bacteria in the bottled water samples during the first phase of the study. Further in detecting *E. coli* in bottle water samples, SLS-MTF (in both preliminary and secondary phases) and the APHA-MTF (preliminary study) failed to detect any *E. coli* in bottle water samples tested. Similarly in total coliform detection all the alternative methods, except APHA-MTF, were able to detect significantly higher *E. coli* counts in bottle water samples tested. Therefore, it can be concluded that, using SLS-MTF, to detect total coliform bacteria and *E. coli* in bottled water samples is not reliable. Unfortunately, all the bottle water samples tested in Sri Lanka, obtain their certification through the Sri Lanka Standards Institute, which uses the standard MTF method to analyze bacteriological quality of the bottle water samples. Therefore, it is probable that, relying on such conventional methods on confirming

bacteriological quality of bottle water posses a high risk factor in Sri Lanka, at present. Unpublished data by a research group currently working at the University of Peradeniya, using M-endo, M-FC_and m-ColiBue24 methods revealed contamination of bottled water, further confirming the results of the current study (Personal communications with W.H.M.A.T. Herath, 2010).

Considering well water samples, total coliform counts were significantly higher ($p \leq 0.05$) with all alternative methods (except APHA-MTF), compared to the SLS-MTF in both phases of the study. However, the *E. coli* counts of all alternative methods (except APHA-MTF), were significantly different at $p \leq 0.05$, during the preliminary phase of the study. In the secondary phase, SLS-MF and m-ColiBue24 methods showed significantly higher counts at $p \leq 0.1$. Although the counts obtained by Colilert method were higher than SLS-MTF, the difference was not significant when the samples contained water from different geographical areas of the country. The reason for this observation must be due to the presence of different bacterial flora in different samples used in the study. Another reason might be due to the presence of *E. coli* strains which do not produce fluorescence (Fricker, *et al.*, 1982) in Colilert tubes. However, as described before, if TNTC values were included in the analysis, alternative methods would have shown significantly higher *E. coli* counts than the SLS method. As results revealed, even without incorporating TNTC values, in analyzing bacteriological quality of well water samples, all alternative methods (except the APHA-MTF), performed better than the SLS-MTF method. Therefore, for obtaining accurate bacteriological counts in well water, which is also being used widely as a drinking water source in Sri Lanka, it is advantageous to use more sensitive and economical methods compared to the conventional, less sensitive, complex and more costly SLS-MTF method. However, depending on the severity of contamination, SLS-MF method or Colilert method could be proposed as better options for analyzing well water samples in Sri Lanka.

When considering the total coliform detection in surface water samples, all alternative methods except APHA-MTF showed significantly higher counts ($p \leq 0.05$), than the SLS-MTF method. In *E. coli* detection, Colilert method showed significantly higher counts ($p \leq 0.1$) compared to the SLS-MTF, during both phases of the study. However, SLS-MF method and m-ColiBlue24 method detected significantly higher *E. coli* counts only during the preliminary study, while the differences were not significant in the second phase. This

might be due to the differences in method performances in different water samples collected from different geographical areas. Further, when detecting very high concentrations of bacteria, differences of method performances could not be observed due to a unknown reason. However, these findings should still need clarifications for making conclusions. According to results obtained in the current study, in detecting bacteriological quality of surface water samples, Colilert method might be a better alternative method with its superior qualities such as sensitivity, simplicity and rapidity compared to the SLS-MF method.

The same observation was made with the effluent samples, where the counts exceeded $1x10^5$ cfu per 100 ml of water. Therefore, when analyzing total coliform bacteria, only the two enzymatic methods showed significantly different higher counts ($p \leq 0.1$), to the SLS-MTF method. Other methods were not significantly different, although the counts were higher. Similar results were obtained for *E. coli* detections at the preliminary study, while there were no significant differences among methods during the second phase. Therefore, for analyzing bacteriological quality of effluents, any optional method could be used depending on the simplicity and the cost effectiveness of the method. However, Colilert method could be proposed as an alternative method for effluent analysis too.

Based on the bacteriological counts obtained in both phases of the study, certain interesting findings were obtained. As revealed by the variance statistics during both phases, all the alternative methods used were able to detect higher counts of total coliform bacteria and the mean counts were significantly different ($p \leq 0.05$) with the conventional SLS-MTF method. Most probably this would be the first comparison attempt, which revealed the deficiencies of the currently used SLS-MTF method with other current methods using lactose fermentation and also with the newly emerging enzymatic methods in Sri Lanka. Similar observations were obtained with *E. coli* detections (when all water samples were grouped together) during the preliminary study, where the differences were significant at $p \leq 0.05$ with all the alternative methods. However, during the second phase, where water samples were collected from different geographical areas of the country, similar observation was achieved only with Colilert method at $p \leq 0.05$. With m-ColiBlue24, the difference was significant at $p \leq 0.1$ but, with M-FC the difference was not significant even

at p ≤ 0.1. However, the *E. coli* counts were comparatively higher compared with the conventional SLS-MTF method.

However, this observation would certainly be different and will confirm the efficiency of the alternative methods, if the *Too Numerous To Count* (TNTC) values were also included in the analysis. TNTC values were mainly obtained with the alternative methods other than the SLS method (Chapter 2, sections 2.3 and 2.6), confirming the higher detection levels of the alternative methods compared with to SLS. Therefore, if the TNTC values were included in the analysis, it will increase the mean and the total bacteriological counts of the alternative methods, probably affecting the significance values of the statistical analysis. Hence, the methods which showed higher p values ($p > 0.05$), will possibly show low probability values ($p \leq 0.05$). If so, all alternative methods at both phases of the study will show significantly higher counts than the reference SLS method.

Further analysis done by using simple linear relationships of all methods with the SLS-MTF method showed that all the alternative methods, except APHA-MTF, showed positive linear relationships with the SLS methods in detecting total coliform bacteria in both phases of the study. In detecting total coliform bacteria Colilert method detected more than three times higher counts than SLS-MTF method in both phases of the study. This amount was much higher magnitude compared with the SLS-MF and m-ColiBlue24 methods. Similarly in detecting *E. coli*, Colilert measured more than two times (on average, in two phases of the study) higher counts, than the SLS-MTF counts. Both SLS-MF and m-ColiBlue24 methods were also able to measure much higher counts than SLS-MTF, although the counts were less than two times higher. Therefore, in detecting total coliform bacteria and *E. coli*, Colilert method was superior to all other alternative methods. In detecting *E. coli*, both SLS-MF and m-ColiBlue24 methods performed quite similarly, according to the simple linear relationship analysis.

With the basic information gathered by the preliminary study on the method performance, the detailed study revealed more details on method performance criteria such as sensitivity, specificity, efficiency and other aspects of the tested methods. According to the results obtained by following the ISO criteria in the current study, the highest sensitivity in detecting total coliform bacteria was recorded by Colilert and m-ColiBlue24 methods.

Similar observations were obtained by Turki and Ziney (2009), in a comparison study with MTF and MF versus Colilert method, in analyzing drinking water samples. They have found that Colilert method as the most sensitive method among the three. However, compared to Colilert, MF method has shown higher specificity, in the same study (Turki and Ziney 2009), while the highest specificity for total coliforms was shown by the Colilert method during the current study.

Moreover, the highest false positive rate when detecting total coliforms was reported by the SLS-MTF method, compared to all other methods proving its less specificity to the coliform group. The highest false negative rate was shown by the M-endo medium of the SLS-MF method. This was because, most of the light pink or cream colour colonies also proved to contain total coliform bacteria in confirmation testing. Due to an unidentified reason these colonies did not appear as typical red colonies with a metallic sheen. Most of the total coliform colonies did not show the metallic sheen, even though, they were red in colour. According to APHA (APHA, *et al.,* 1998) and SLS 614 (Sri Lanka Standards Bureau, 1982), typical total coliform colonies are defined as red colour colonies with metallic sheen (on the whole colony/on the centre/on the periphery). However, the SLS 614 also recommends counting both typical (pink to dark red with metallic sheen) and atypical colonies (no explanation) to be counted as presumptive total coliform colonies.

The two enzymatic methods Colilert and m-ColiBlue24 methods did not produce any false negatives, since all positive total coliforms gave the distinct yellow colour or the red colour colonies in Colilert and m-ColiBlue24 methods respectively. However, the false negative rate could not be calculated in SLS-MTF method as the tubes without gas formation were not confirmed for the presence of total coliform bacteria, in this study. Therefore, this step could be recommended to be followed in a similar future research, since this information is important in assessing the method performance. Further, the current study revealed the most efficient method in detecting total coliform bacteria, as the Colilert method and secondly, the m-ColiBlue24 method. Therefore, the current study was able to find that the two enzymatic methods, Colilert and m-ColiBlue24 showed the best performance in detecting total coliform bacteria, compared to the lactose based SLS-MTF and the SLS-MF methods that are presently been used in Sri Lanka.

ISO performance criteria, analyzed to test the detection of *E. coli* bacteria proved that, Colilert, m-ColiBlue24 and SLS-MF methods showed the highest sensitivity for *E. coli*; the highest specificity was recorded with the Colilert method. According to the findings reported by Turki and Ziney (2009), Colilert was the most sensitive method, while the MF method showed the highest specificity for *E. coli* in their study. In the current study, the highest false positive rate in detecting *E. coli,* was also reported by the SLS-MTF method, as in the case of total coliforms. Colilert method reported the lowest false positive rates, confirming its higher performance in detecting *E. coli*, as reported by Shadix and Rice (1991). Furthermore, false negative rates were zero in Colilert, m-ColiBlue24 and the SLS-MF methods, for detecting *E. coli*. This was because, there were no *E. coli* in non-fluorescing yellow colour tubes in Colilert; no other colonies except blue colour colonies on m-ColiBlue24 plates and no *E. coli* in atypical cream colour or yellow colour colonies on M-FC plates (SLS-MF method). However, as previous studies report, Colilert has shown 30 % (Pitkanen, *et al.,* 2007) and 20 % (Schets, *et al.*, 2002) false negative rates, when detecting *E. coli* bacteria. According to the results of the current study, SLS-MTF method was the most weakly performing method, in detecting *E. coli* bacteria in water.

Further, the confirmation testing also proved the specificity of the enzymatic methods and SLS-MF method compared to the SLS-MTF methods. The lowest confirmation rates for both types of bacteria were recorded by the reference SLS-MTF method compared to Colilert, m-ColiBlue24 and SLS-MF method. Further, the highest non-coliform rate was reported with the SLS-MTF method, confirming its less specificity in detecting coliform bacteria. The lowest non-coliform percentage was recorded with the M-endo medium of the SLS-MF method. It agrees with the previous finding by Grant (1997), who has described the specificity of the M-endo medium in detecting total coliform bacteria. Moreover, the highest percentage of Gram positive bacteria was also reported by the SLS-MTF method, suggesting its low performance in detecting *E. coli* bacteria.

Therefore, when considering the qualitative and quantitative analytical information, all alternative methods, except APHA-MTF performed better than the SLS-MTF method in detecting and enumeration of both total coliform bacteria and *E. coli* in the water samples tested in the current study.

The bacteriological identification tests done in the current study, revealed that the bacterial flora detected may be dependant on the method, based on the characteristic features of method performance in detecting specific bacterial spectra. Further, the bacterial isolates identified by biochemical tests or api 20E tests depended on the sample of origin. However, in the current study, the selected isolates did not come from the same sample analyzed by all four methods, due to the limited number of pure bacterial cultures that remained as a result of the laboratory contaminations and missing isolates when sub culturing, If the same sample, analyzed by all methods was used for identifications, more fruitful information on method performance could have been obtained. However, identification results revealed that the proportion of non-coliforms were higher than that of the coliforms detected with SLS-MTF method. Therefore, even the bacteriological identification studies confirmed the less specificity and poor performance of SLS-MTF method in detecting coliform bacteria in water.

Cost-benefit analysis of the methods showed that, MF methods were more cost efficient than the MTF methods. The lowest total cost per sample for both drinking water and surface water samples was reported by the SLS-MF method, which is also a MF method. m-ColiBlue24 method was the next cost effective method in analyzing drinking water samples. Further, this method proved greater detection levels of total coliforms and *E. coli*, compared to the SLS-MF method, in this study. Moreover, when considering its easy handing, easy interpretations and especially the rapidness of the test, m-ColiBlue24 method could be proposed as one of the most efficient alternative methods, to analyze drinking water samples in Sri Lanka.

SLS-MF method was the cheapest method for analyzing surface water samples. The enzymatic Colilert method, even being a MTF method, was placed second, which is also a less labour-intensive, rapid, user-friendly and easy handling method Therefore, Colilert method would be one of the best alternative methods, to be used efficiently for bacteriological quality analysis in surface water and in other highly contaminated water samples in Sri Lanka.

As per the information gathered during this study, Sri Lanka National Water Supply and Drainage Board (NWS & DB) charges a reasonable price (Rs. 350/=) for analyzing

bacteriological quality of a water sample, which is very close to the cost obtained in this study. However, certain private institutions charge very high amounts, even for MF methods, which are generally less costly than MTF methods. For instance, a particular institute charges Rs. 2800/= per sample for MTF method, which is reasonable. However, the same institute charges Rs. 2000/= per sample even for MF method, which is very unreasonable.

In conclusion, the bacteriological methods examined in the current study performed differently, independent on the water source or the sampling location. Different strengths and weaknesses of the methods in detecting total coliform bacteria and *E. coli* were identified. Qualitative and quantitative analyses of the current study proved that the existing conventional SLS-MTF method has many limitations in detecting and enumeration of total coliform bacteria and *E. coli* in water. In contrast, other alternative methods showed superior performance in detecting both bacterial types accurately. These alternative methods were more sensitive, specific, efficient and also cost efficient in certain instances. Further, based on the contamination level and water sources, appropriate methods could be selected depending on cost effectiveness and the other performance criteria such as sensitivity, specificity, efficiency and other common features like simplicity, speediness of the test, user-friendliness, etc. In comparison, the currently existing MF method, which is been practiced by the NWS & DB also proved many advantages compared to the SLS-MTF method in the current study. Therefore, for routine bacteriological quality analysis, SLS-MF method, which is currently in use, might be a better alternative than the conventional SLS-MTF method.

CHAPTER 5

FUTURE ASPECTS AND RECOMMMENDATIONS

5.1 INTRODUCTION

Bacteriological quality assurance of water sources, using indicator bacteria is one of the top priorities in the world including Sri Lanka. The main water related regulatory bodies in Sri Lanka, are currently adhered to conventional methods for water quality testing. However, many weaknesses and limitations involved with these conventional methods were identified during this study.

Further, certain information gathered on water quality analysis during this study revealed that, several problems are currently involved at present in Sri Lanka. One factor noticed during laboratory visits was the inadequate practical knowledge of the laboratory staff. For example, determining of proper dilutions while performing MF method was not clear to most of the laboratory staff. Majority of them are not properly trained and they are not updated on currently emerging technologies in water microbiology. Further, the conventional way of thinking and their attitudes towards modern technology is not satisfactory, compared to other countries of the world. It is very unfortunate that, sharing of knowledge and conducting collaborative work among water laboratories is very poor or absent in Sri Lanka. Another common weakness identified was, the lack of confirmatory testing, when performing MF method. During the enumeration of bacteria, typical/atypical colonies and background growth are not considered as important in laboratories visited. Therefore, there is a tendency for obtaining misleading data due to inaccurate interpretations. Lack of expert technical staff and training programmes on this specialized area are major constraints in developing quality assurance of bacteriological testing in Sri Lanka. However, most of the technical staff involved in water quality testing in different institutes are keen on adopting more efficient, rapid and easy methods (personal communications with laboratory staff members in selected institutes, 2007). Most of them complained about the time taken, intense preparations and other difficulties encountered with the conventional methods.

5.2. Future aspects

Research and development

Results of this research revealed that the currently practiced methods for bacteriological water quality assessment in Sri Lanka suffer from several drawbacks compared to the newly emerging technologies. Further, the conventional methods are relatively complex by nature, which are labour intense, require more glassware, more preparations, confirmational steps, longer time periods (at least 3-4 days in MTF method) leading to inefficient and slow processes, compared with the modern technologies which require less labour, limited amount of glassware, less preparations due to readily available media, ability to obtain results within one day for both total coliforms and *E. coli* simultaneously leading to rapid efficient possesses.

Moreover, as revealed by the current study (and also revealed by many previous work, as discussed in other chapters of this thesis), the ISO performance criteria such as sensitivity, specificity, efficiency and detection levels are also comparatively higher with the newly emerging methods, than the currently available methods in Sri Lanka. Therefore, it is timely that the methods currently in use in Sri Lanka be replaced in order to obtain more accurate results within a relatively less time period (probably, even at a low cost). Further, these efficient methods are also simple and can even be handled by unskilled personnel. Furthermore, the latest technologies could even easily be used in the field, in cases where laboratory facilities are not available, or even during epidemiological out breaks or in certain natural disasters such as Tsunami situations or flood conditions, where proper disinfection facilities are not available and water quality problems are very frequent. If early warning of water quality deterioration could be announced by using the above motioned bacteriological testing methods in the field, officials could take immediate action to prevent further contamination of source waters.

Need for National field evaluation studies

Considering the above mentioned facts, it could be suggested to conduct research, based on national field evaluations. In order to achieve this, collaboration of related institutes such as Sri Lanka Standards Institute, National Water Supply and Drainage Board, Ministry of Health and other bodies such as universities, research institutes (eg. MRI, IFS, etc) and water quality testing laboratories (eg. ITI, NBRO, CEA, etc.) would be necessary.

a) Intercalibration studies

Conducting intercalibration studies for comparability of the currently available methods practiced by all institutes, for routine bacterial monitoring of water could be suggested. Assessment of comparability of results among different laboratories that conduct routine bacterial monitoring using two bacterial indicator (total coliforms and *E. coli*) measurement methods would be possible (since the methods are not uniform around the country). For instance, as mentioned in previous chapters, the procedures, brands of the media used, obtaining and interpretation of results are different from laboratory to laboratory. Therefore, it is recommended to have standard uniform procedures, same brands of media (could be selected after conducting research on media performance and cost effectiveness), uniform way of results interpretation, etc. This type of work will evaluate the reliability of methods through verification of target organisms, and identified common causes of error in determining bacterial concentrations for water quality monitoring purposes.

b) Comparison studies

Further comparison studies could be conducted with other different enzymatic methods (Colisure, MI agar, LMX agar, etc.) or any other newly introduced cost effective methods using different types of water sources collected from different geographical areas of the country. It will also enable to investigate further new methods with superior qualities. Further, the cost benefit analysis will investigate their cost effectiveness compared to the conventional methods.

Technology development of laboratories

As discussed earlier, most of the laboratory staff involved in water quality monitoring is not updated with the emerging technologies around the world. Although the laboratories are not as equipped like in developed countries, technology development with the existing facilities will be possible. To facilitate this proper guidance for all laboratory staff should be given. Workshops, skill development laboratory training programs and seminars could be organized, in order to disseminate the knowledge on standard technologies and currently emerging techniques with the help of local as well as foreign resource persons. Participation in foreign training programs could be encouraged and facilitated by the government. All these activities will enhance the dissemination of updated knowledge, while sharing experiences from different working environments. The final aim of these activities will be to introduce uniform laboratory techniques, which could be more reliable than the existing different laboratory procedures.

Improvements of national water quality monitoring schemes

As discussed in previous chapters, the most versatile standard indicator organisms used to detect contamination of water are, coliform bacteria. Although several novel technologies are being developed and are being currently in use around the world, Sri Lanka has not adopted any new technology, after introducing the bacteriological water quality standards by the Sri Lanka Standards Bureau in 1982. After 28 years of introduction, the standards and the methods that exist are same, without any major revisions or updates, compared to other countries. For instance, WHO and the US EPA have revised their water quality standards and guidelines, for drinking water and other source waters. Further, they have already introduced new criteria for different water sources such as beach water, swimming pools water intakes (providing raw water for drinking water facilities), and other water bodies used for recreational purposes. Therefore, introducing new water quality guidelines and standards should be recommended by the SLS and other accepted bodies for these sources as well.

Furthermore, revisions (in the least once in 10 years) and introducing of new criteria for water quality assurance in Sri Lanka should be considered with more attention and also need to be addressed immediately.

This must include guidelines, standards and also technologies for improving water quality assurance in Sri Lanka. Based on the information gathered and findings obtained during the current study, a few recommendations and suggestions could be forwarded for consideration.

5.3 Recommendations for water quality testing in Sri Lanka

As mentioned earlier, Sri Lanka Standards bureau, being the standardization institute of Sri Lanka, is adhered to the water quality standards stipulated as early as 1980 s by the Word Health Organization. However, WHO and other regularity agencies like US EPA, are revising and updating their water quality standards and guidelines appropriately with new technological developments, the current rate of environmental pollution and increased rates of population expansion causing contamination of water resources. Furthermore, these agencies are also recommending the use of currently emerging modern technologies for water quality monitoring in the world. Therefore, based on the results of the current study, it is timely that Sri Lanka identified the most appropriate methods for bacteriological testing of water, particularly for different types of water sources.

Recommendations for different water sources

Based on results obtained in the current study, following appropriate methods were identified depending on their efficiency and cost benefits to assess different types of water sources for bacteriological testing in Sri Lanka.

a) Drinking water analysis

According to the cost benefit analysis in the current study, the most economical method identified for drinking water assessment was, SLS-MF method (currently in use by the

NWS & DB, Sri Lanka), which is 7 times cheaper than the currently used SLS-MTF method. The drinking water sources tested in the current study were bottled water and well water (since tap water was free of contamination). For both drinking water sources, detection levels of SLS-MF method were not much different compared with the two enzymatic methods.

However, the ISO criteria for method performances were superior with the Colilert and m-ColiBlue24, compared to the SLS-MF method. Further, when considering the cost efficiency, m-ColiBlue24 method obtained the second place (3 times cheaper than SLS-MTF). Therefore, considering the performance criteria, cost efficiency and added advantages such as requirement for less labour, less time, less electricity and user friendliness, m-ColiBlue24 method could be recommended as the most appropriate method for analyzing drinking water samples in Sri Lanka, based on the outcome of the current study.

Although Colilert method showed superior method performances and other added advantages compared to the conventional methods, it was costly compared to the m-ColiBlue24 method for analyzing drinking water samples. However, if the cost factor is not considered, Colilert method could also be recommended as a more sensitive, specific and efficient method than the conventional MTF or MF methods currently in use, in Sri Lanka.

b) Surface water analysis

According to the cost comparison, the most economical method for surface water analysis was found to be SLS-MF method, with the cost being 5 times lower compared to the SLS-MTF method. The next economical method identified was the enzymatic, Colilert method with more superior qualities compared to the conventional MTF or MF methods. Further, according to the detection levels, Colilert was able detect comparable or even higher (results obtained in the detailed study) total coliform and *E. coli* counts than the SLS-MTF, SLS-MF or enzymatic m-ColiBlue24 methods. Therefore, considering its added advantages such as user-friendliness, requirement for less labour, less time and less electricity, easy results interpretation and also its superior method performance (according to ISO criteria),

Colilert method could be recommended for the surface water analysis in Sri Lanka, based on results of the current study.

c) **Wastewater effluent analysis**

The contamination levels of effluent water samples (before final disinfection) were not very high compared with the surface water source in this study. Based on the cost benefit analysis, the most economical method for analyzing effluent water was also, SLS-MF method. Therefore, SLS-MF method could be recommended for effluent water sample analysis However, with the difficulties of obtaining correct dilutions in MF methods, use of MTF with Colilert would be more advantageous, minimizing the additional costs with SLS-MF method. Therefore, for analyzing highly contaminated water sources such as wastewater effluents, Colilert would be a good alternative method, compared to the conventional methods.

Table 5.1 summarizes the recommendations, based on the results of the current study.

Table 5.1 Recommendations for analyzing bacteriological quality of different water sources

Water source	**Most economical method**	**Most efficient method ***	**Cost comparison**
Drinking water	SLS-MF	m-ColiBlue24	3 times as SLS-MF
Surface water	SLS-MF	Colilert	2 times as SLS-MF
Wastewater effluent	SLS-MF	Colilert	2 times as SLS-MF

* Based on user friendliness, requirement for less labour and time, no media preparation, efficiency, better result interpretation, superior method performance (ISO criteria) and higher bacterial detection levels

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APPENDIX 1

Water quality guidelines

Table 1 WHO guidelines for bacteriological quality of drinking water (WHO, 1983)

Organisms	Unit	Guideline value
A) Piped water supplies		
1. Treated water entering the distribution system		
Fecal coliform bacteria	number/100 ml	0
Coliform bacteria	number/100 ml	0
2. Untreated water entering the distribution system		
Fecal coliform bacteria	number/100 ml	0
Coliform bacteria	number/100 ml	0[1]
Coliform bacteria	number/100 ml	3[2]
3. Water in the distribution system		
Fecal coliform bacteria	number/100 ml	0
Coliform bacteria	number/100 ml	0[3]
Coliform bacteria	number/100 ml	3[2]
4. Un piped water supplies		
Fecal coliform bacteria	number/100 ml	0
Coliform bacteria	number/100 ml	10[4]

(WHO, 1983)

[1] in 98% of samples taken throughout any 12-month period

[2] in an occasional sample, but not in consecutive samples

[3] in 95% of samples taken throughout any 12-month period

[4] should not occur frequently

Table 2 WHO guidelines for bacteriological quality of drinking water (WHO, 1996[1])

Organisms	Guideline value
All water directly intended for drinking	
E. coli or thermotolerant coliform bacteria [b,c]	Must not be detectable in any 100-ml sample
Total coliform bacteria	Must not be detectable in any 100-ml sample
Treated water entering the distribution system	
E. coli or thermotolerant coliform bacteria [b]	Must not be detectable in any 100-ml sample
Total coliform bacteria	Must not be detectable in any 100-ml sample
Treated water in the distribution system	
E. coli or thermotolerant coliform bacteria[b]	Must not be detectable in any 100-ml sample
Total coliform bacteria	Must not be detectable in any 100-ml sample In the case of large supplies, where sufficient samples are examined, must not be present in 95% of samples taken throughout any 12-month period

(WHO, 1996)

1. Immediate investigative action must be taken if either *E. coli* or total coliform bacteria are detected. The minimum action in the case of total coliform bacteria is repeat sampling; If these bacteria are detected in the repeat sample, the cause must be determined by immediate further investigation.
2. Although *E. coli* is the more precise indicator of fecal pollution, the count of thermotolerant coliform bacteria is an acceptable alternative. If necessary, proper confirmatory tests must be carried out. Total coliform bacteria are not acceptable indicators of the sanitary quality of rural water supplies, particularly in tropical areas where many bacteria of no sanitary significance occur in almost all untreated supplies.

It is recognized that, in majority of rural water supplies in developing countries, fecal contamination is widespread. Under these conditions, the national surveillance agency should set medium-term targets for the progressive improvement of water supplies (WHO, 1996).

Table 3 Examples of high detectable concentration (cfu per litre) of enteric pathogens and fecal indicators in different types of source waters (WHO, 2008).

Pathogen or indicator group	Lakes and reservoirs	Impacted rivers and streams	Wilderness rivers and streams	Groundwater
Campylobacter	20–500	90–2500	0–1100	0–10
Salmonella	—	3–58000	1–4	—
E. coli	10000–10000000	30000–1000000	6000–30000	0–1000
Viruses	1–10	30–60	0–3	0–2
Cryptosporidium	4–290	2–480	2–240	0–1
Giardia	2–30	1–470	1–2	0–1

Table 4 US EPA guidelines for routine water monitoring frequencies in public water systems (TCR, 1989)

Total coliform	*E. coli*	Monitoring requirements	
		population	Samples/month
0/100 ml (95%) a consecutive sample from the same site must be coliform-free	0/100ml (100%)	25-1000	1 sample/month
0/100 ml (95%) a consecutive sample from the same site must be coliform-free	0/100ml (100%)	96,001-130,000	100 samples/month

US EPA (1990)

US EPA recommends to disinfect all surface water sources serving drinking water for public since they are more vulnerable to contamination rather than ground water.

APPENDIX 2

ISO criteria for establishing equivalence between microbiological methods (ISO 17994: 2004)

2.1. Calculations

i) Basic relative differences

$x_i = \ln(a_i) - \ln(b_i) \times 100\ \%$.....2.1.1 ($a_i$, b_i) = paired non zero confirmed counts

$x_i = \ln(a_i + 1) \times 100\ \%$.....2.1.2 ($a_i$, 0) = confirmed count with zero values

$x_i = -\ln(b_i + 1) \times 100\ \%$.....2.1.3 (0 , b_i) = confirmed count with zero values

ii) Mean relative differences

$\bar{x} = \sum x_i / n$.....2.1.4 n = number of samples x_i = relative difference in sample i

iii) Standard uncertainty (Standard deviation)

$$s = \sqrt{\Sigma(x_i - \bar{x})^2 / n-1}.....2.1.5$$

iv) Standard uncertainty of the mean (Standard error)

$$s\bar{x} = s / \sqrt{n}.....2.1.6$$

v) Expanded uncertainty

$U = ks\ \bar{x} = 2s / \sqrt{n}$.....2.1.7 coverage factor $k = 2$

v) Confidence intervals (of the expanded uncertainty around the mean)

Lower limit $x_L = \bar{x} - U$.....2.1.8

Upper limit $x_H = \bar{x} + U$.....2.1.9

Method evaluation

Two-sided evaluation

i) Methods "not different"

$-D \leq x_L \leq 0$ and $0 \leq x_H \leq +D$.....2.2.1 D = maximum acceptable deviation

ii) **Methods "different"**

$x_L > 0$ or $x_H < 0$.....2.2.2

iii) Inconclusive

Data are insufficient for a decision when

$x_L < -D$ and $x_H > 0$ or 2.2.3

$x_L < 0$ and $x_H > +D$.....2.2.4 more samples should be examined.

iv) Indifferent

The methods are significantly different, but the difference is too small to be of practical significance.

$x_L > -D$ and $x_H < 0$ or.....2.2.5

$x_L > 0$ and $x_H < +D$.....2.2.6

One-sided evaluation

Only the lower value of the maximum acceptable deviation (-D) is considered in the evaluation.

i) Methods "not different"

$-D \leq x_L \leq 0$ and $x_H > 0$.....2.2.7

ii) **Trial method: higher recovery**

$x_L > 0$ 2.2.8

iii) Trial method: higher recovery

$x_H < 0$ 2.2.9

iv) Inconclusive

Data are insufficient for a decision when

$x_L < -D$ and $x_H > 0$.....2.2.10 more samples should be examined.

v) Indifferent

The methods are significantly different, but the difference is too small to be of practical significance.

$x_L > -D$ and $x_H < 0$.....2.2.11

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