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The Socio Biological Need of Rapid, Specific and Sensitive Tests For Detection of Coliforms in Food and Drinking Water

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Abstract

In developing countries, the contamination of drinking water sources is very common. The regular monitoring of water quality is not an easy task in developing countries. To achieve this objective, rapid and qualitative tests such as presence-absence (P-A) test, H_2S test and test based on substrate specificity fluorogenic and chromogenic media, PCR and immunological test ELISA were introduced. One of the latest techniques used in rapid detection of pathogenic agent in water and food is fluorogenic and chromogenic media. These media are very specific and their component act as substrate for specific enzyme. In this review we study the fluorogenic and chromogenic media in rapid identification of pathogenic microorganisms in water and food in recent decade. Rapid, sensitive and specific tests eliminate the need of subculture and further biochemical test for identification of pathogenic organisms and at a very short period of time pathogenic organisms can be identified. This review describes some recent developments in rapid, specific and sensitive tests in microbiological diagnostic.

Key word: Fluorogenic, Chromogenic, Culture media, Rapid, Specific, P-A, H_2S, Household water, Total coliform

INTRODUCTION

In developing countries, the contamination of drinking water sources is very common (Feachem *et al*., 1983, Suman *et al*., 2003). However, the regular monitoring of water quality is not an easy task in developing countries. To achieve this objective, rapid and qualitative tests such as presence-absence (P-A) test (Clark, 1967), H_2S test (Manja *et al*., 1982) and substrate specificity test (Feng and Hartman, 1982; Moberg, 1985) were introduced. A characteristic feature of the coliform group is their ability to ferment lactose, with the production of acid and gas (APHA, 1992), but these assays do not distinguish faecal coliforms from non-faecal coliforms. One important method used to distinguish faecal coliforms from total coliforms is elevated temperature test proposed by Eijkman (1904). The specificity of the test has been enhanced somewhat by an increase in the temperature of incubation, most of the coliforms grow at 35^{O}C, but only faecal coliforms grow at about 45^{O}C (Weiss and Humber, 1988). However, the elevated temperature effect does not distinguish *E. coli* from other thermotolerant coliforms such as *Klebsiella, Citrobacter* and *Enterobacter* species (Ramteke *et al*., 1992a; Ramteke, 1995). The latter three organisms can be isolated readily from soil, sediments and organic industrial wastes, but only *E. coli* is normally present in human faecal matter (Leclerc *et al*., 1981) and is generally considered a more reliable sanitary indicator (Caplenas and Kanarek, 1984).

Most coliforms are present in large numbers among the intestinal flora of humans and other warm-blooded animals, and are thus found in fecal wastes. As a consequence, coliforms, detected in higher concentrations than pathogenic bacteria and are used as an indicator organism of the potential presence of entero-pathogens in water environments. The use of the coliform group, and more specifically *E. coli*, as an indicator of microbiological water quality dates from their first isolation from feces at the end of the 19th century. Coliforms are also routinely detected in diversified natural environments, as some of them are of telluric origin, but drinking water is not a natural habitat for them. The presence of coliforms in drinking water must be considered as a threat or indicative of microbiological water quality deterioration. Positive total coliform

samples in a treated water indicate ineffectiveness of treatment, loss of disinfectant, breakthrough (McFeters *et al*., 1986), intrusion of contaminated water into the potable water supply (Clark *et al*., 1996) or regrowth problems (LeChevallier, 1990) in the distribution system.The use of the coliform group as an indicator of the possible presence of enteric pathogens in aquatic systems has been a subject of debate for many years. Many authors have reported waterborne disease outbreaks in water meeting the coliform regulations (Payment et al.,1991; Moore *et al.*, 1994; MacKenzie *et al*., 1994; Gofti *et al.*, 1999). However, the purpose of this review is not to discuss the indicator concept, but rather to identify the rapid methods currently in use or which can be proposed for the monitoring of coliforms in drinking water.

Enterobacteriaceae, *Esherichia coli* coliform are the most infectious bacteria of food and water. The detection and its quantification of this emerging pathogen is therefore an important task for microbiological food and clinical diagnostic laboratories. Traditional methods for bacterial detection like biochemical test have been used for long. These methods consume time and materials. Previous study on coliforms and specially *E. coli* made it possible to identify them as microbial contaminants marker in food and water. The presence of *E.coli* in drinking water and food indicates that these materials are contaminated with other enteric pathogens. Hence their isolation and enumeration have great importance in the determination of food hygiene (Muller et al., 2001). Standard ISO 6579 2003 (Microbiology of food and animal feeding stuffs – Horizontal method for detection of *Salmonella* spp.) includes four stages of the detection process and depending on the need to obtain confirmations, it lasts for 5 to 7 days: 1. Pre-enrichment in non-selective liquid medium; 2. Selective enrichment in liquid media; 3. Plating on selective media; 4. Serological and biochemical identification of suspected colonies.

Presence-Absence (P-A) test

The use of the P-A concept and P-A tests for fecal indicator bacteria, primarily coliforms, fecal coliforms and *E. coli*, has a history that goes back more than four decades (Clark, 1968). Considerable effort in the form of expert analysis and judgement went into the development and implementation of P-A tests for these microbes in drinking water. Much of this effort included consideration of the wealth of available historical data on the occurrence of these indicator bacteria in municipal drinking water, based on the frequency of positive results

(fecal indicator presence) in 100-ml volumes of drinking water and the acceptability (or risk) of drinking waters based on these observed frequencies. These analyses led to current guidelines and standards for the microbial quality of drinking water based on positive P-A test results.

The P-A test is an inexpensive test for rapid qualitative detection of bacterial indicators of faecal pollution, developed by Clark (1967) as a means to monitor drinking water systems. This test was considered by several workers as an alternative to the multiple tube (Most Probable Number) and membrane filtration (MF) method for monitoring water samples (Clark, 1967; 1969; Pipes and Christians, 1984).

The test is more appropriate for the rural environment by filling the test bottle directly from the pump or tap up to the pre calibrated 100 ml mark and transporting back to the laboratory for incubation at ambient temperature (30-37°C). Very little technical knowledge is required for performance of the test, and with a minimum of facilities and trained manpower highly contaminated water samples were screened (Dutka, 1990).

H_2S paper strip test

In 1975, Allen and Geldreich showed that the presence of coliforms in water was also associated with hydrogen sulphide (H_2S) producing organisms. In 1982, Manja *et al*. developed a simple paper-strip method to screen for bacteriological contamination of potable waters. This study, and several subsequent studies, have found that the H_2S test gave generally good agreement with the standard Most Probable Number (MPN) and membrane filtration methods commonly used for determining the presence and number of coliform and faecal coliform organisms (Hazbun and Parker 1983; Dutka 1990; Castillo *et al*. 1994; Martins *et al*. 1997; WHO 2002). As noted in a multi-country inter comparison study summarised by Dutka (1990), this test is "an ideal tool for testing rural and isolateddrinking water supplies".Bacteria can produce hydrogen sulphide through the anaerobic catabolism of cysteine,an amino acid containing the sulfahydryl group, or by the use of elemental sulphur or some oxidised sulphur compounds as the terminal electron acceptor in their metabolic processes. All members of the *Enterobacteriacae*group are capable of the former while the latter occurs in dissimilatory sulphate-reducing bacteria. The H_2S test uses a medium with thiosulphateas a sulphur source

and ferric ammonium citrate as an "indicator," only certain enteric bacteria will produce hydrogen sulphide resulting in the development of a black precipitate. Hydrogen sulphideis produced by the reduction of thiosulphate and then reacts with the ferric salt to form an insoluble black ferrous sulphide precipitate. Members of the Enterobacteriacae group such as *Salmonella, Citrobacter, Clostridia, Klebsiella* and *Proteus* are all able to produce hydrogen sulphide in such a medium.

The H_2S paper strip test was first developed as a field testing procedure by Manja and co-workers in 1982. They found the test inexpensive, the medium used can be stored for extended periods under ambient conditions, and it can be run by minimally skilled operators in remote areas. Another advantage is that the test can be completed under ambient temperatures of 25-30°C.

β-D-GLUCURONIDASE (GUD) AND SUBSTRATE SPECIFIC CULTURE MEDIUM

Approximately 97% of *E. coli* strains were found to possess the enzyme β-D-glucuronidase (GUD) while almost all other coliforms lack this enzyme (Kilian and Bulow, 1979). Several techniques for detection of *E. coli* are based on the enzymatic hydrolysis of fluorogenic and chromogenic substrates for GUD (Manafi *et al.*, 1991; Eaton *et al.*, 1995). Recently, several studies have showed that the 4-methyl-umbelliferyl-GUD (MUG) assay comparable to standard methods for the detection of *E. coli* from surface waters, wastewaters and sewage samples (Freier and Hartman, 1987; Mates and Shaffer, 1989; Clark *et al.*, 1991). Studies also indicated non-interference of heterotrophic plate counts in detection of *E. coli* in MUG amended media (Edberg *et al.*, 1988; 1990; Clark *et al.*, 1991). Several commercial bacteriological media have been introduced for the specific detection of *E. coli* in food, clinical and environmental samples (Ramteke and Suman, 2002).

In recent times, great emphasis has been placed on the detection, enumeration or quantification of microorganisms using synthetic enzyme substrates or fluorogenic dyes. This subject has been reviewed extensively by several workers (Watson, 1976; Boscomb, 1980; Manafi and Kneifel, 1991; Manafi, 1996). Fluorogenic dyes most commonly used are 8-anilino-11-naphthalene-sulfonic acid (ANS) and acridine orange (AO).

MUG is now widely used for the identification of *E.coli* in food and water (Hartman, 1989; Gautheir *et al*., 1991; Manafi *et al*., 1991). Since the fluoresces of 4-MU is known to be pH dependent (Goodwin and Kavanagh, 1950), the pH of the growth medium should be within the range of neutral to slightly alkaline (Maddocks and Greenam, 1975; Freier and Hartman, 1987). Identification system for *E. coli* based on D-glucuronidase activity, possible substrate and their reactions is given in Table I.

Many of these substrates (chromo- and fluorogenic) have been incorporated into media or detection kits for rapid and direct identification of *E. coli* and are commercially available (Table II).

McFeters *et al*. (1993) compared the performance of three of these media, Colilert™ (Environetics), Coliquick™ (Hach) and Colisure™ (Millipore), with traditional membrane filtration and lauryl sulphatetryptose (MPN, LST/MPN) techniques for detecting low levels of chlorine-injured coliforms and *E. coli* in water. These three methods gave results within 48 h that were not statistically different from the LST/MPN results.

RESULT:

Table I
Identification systems for *E. coli* based on D-glucuronidase activity and possible substrates and their reactions.

Substrate (Tests)	End product	Colour	Reference
p-Nitrophenyl-□-D glucuronide (PNPG) (Rosco tablets, Rosco Denmark)	*p*-Nitrophenol	Yellow	Kilian and bullow, 1979; Hansen and Yourassowsky, 1984
Phenolphthalein □-D-glucuronide (PHEG)	Phenolphthalin	Red	Bulhler*et al*., 1951; Rod *et al*., 1974
5-Bromo-4-chloro-3-indolyl-□-D-glucuronide (xGLUC) Indoxyl-□-D-glucuronide (IBDG) (Petrifilm*E. coli* count plate, 3M; system 5, 4, 3, Lad M; chromeagar*E. coli* m-caliblue, Hach),	Indoxyl	Blue	Frampton and Restaino, 1993

4-Methylumbelliferyl-□-D-glucuronide (Fluorocultmedia and Bacident*E. coli*, Merck; Rapidec coli and uriline IO, bio Mevieuse; MUG Plustest, Difco; RIM *E. coli*, Austin Biological; Rapid detect *E. coli*,organonTeknika; MUG tube test, Remel)	Indigo 4-MU	Blue flurosence	Hartman, 1989 Frampton and Restaino, 1993
5-Bromo-6-chloro-3-indolyl-β-d-glucuromide (magenta-glc)	Magenta	Red	Manafi*et al.*, 1991
6-chloro-3-indolyl-β-D-glucuronide (Salmon-glc)	Salmon	Pink	Manafi*et al.*, 1991

Table II
Commercially available media for the simultaneous detection of coliforms and *E. coli.*

Medium	Substrate/colour		Selective agent	Manufacturer
	Coliform	*E. coli*		
FluorocultLMX broth	X-Gal/blue-green	MUG/blue fluores.	Lauryl sulphate	Merck (Germany)
Colilert	ONPG/Yellow	MUG/blue fluores.	—	Environetics (USA)
Coliquick	ONPG/Yellow	MUG/blue fluores.	—	Hach (USA)
Colisure	red	MUG/blue fluores.	—	Millipore (USA)
Solid Media EMX agar	XGAL/blue	MUG/blue fluores.	Bile Salt	Buotest (Germany)
C-EC-MF agar	XGAL/blue	MUG/blue fluores.	Bile salt	Merck (Germany)
Chromocult	Slmon Gal/Red	XGLVC/blue-violet	—	Tergitol 7Biomeriens (France)
Coli-ID	XGAL/blue	—/Rose-Violet	—	Biomeriens (France)
Chromagar ECC	red	XGLUC/blue	—	Chromagar (France)
Identification strip				
Coli complete	XGAL/blue	MUG/blue fluores.	—	Biocontrol (USA)

Glucuronidase assay for the detection of *E. coli* was evaluated from environmental water samples, using LT broth with MUG, *E.coli* broth with MUG and auto analysis coliert (AC) procedure (Shadix and Rice, 1991). However, little is known about the performance of chromogenic based media such as Chromocult, Coli ID-, Coli complete and chromoagar as these media are relatively new and have not been evaluated in treated sewage (Miescier and Cabelli, 1982).

PCR Kits for Food Microbiology

There are two broad types of detection method:

End-point PCR detection takes place, when the amplification process is complete. Typically, agarose gel electrophoresis, followed by staining with fluorescent ethidium bromide is used to detect the amplified DNA fragments. This method is time consuming and not sensitive enough to measure the accumulated DNA copies accurately, so can only give a qualitative result.

Most food testing applications employ a method known as real-time PCR detection – combining the amplification and detection stages of the process so that amplification is monitored continuously during the exponential phase. Real-time detection is more accurate and the result can also be quantified.

Real-time PCR detection methods

The simplest method is to use intercalating fluorescent dyes, such as SYBR Green. These fluoresce only when bound to double-stranded DNA and the increase in fluorescence can be measured at each cycle. Unfortunately, these dyes bind to all double-stranded DNA present, including any non-specific PCR products. This makes it difficult to quantify the result accurately. The problem can be overcome to some extent, but only by adding an extra stage at the end of the PCR process.

A second, more accurate and reliable method is to use fluorescent reporter probes. This method utilises an additional primer, the probe, which also binds specifically to the target DNA sequence during annealing. Probes have a fluorescent reporter dye at one end and a quencher dye, which inhibits fluorescence, at the other. During the extension stage the probe is broken apart by the DNA-polymerase and begins to fluoresce more strongly. The fluorescence emitted can be measured at each cycle and increases in proportion to the number of target sequence copies produced. To quantify the assay, the cycle at which the fluorescence intensity rises above the background level is recorded for each test sample and for a set of standards run at

the same time. A standard curve can then be constructed. The amount of target DNA present in the sample can be calculated from the standard curve. Since the probe only binds specifically to the target DNA, non-specific PCR products are not detected.

Although PCR is very sensitive method but detection of the amplified fragment using gel electrophoresis is limited to the end point analysis only. Real-time PCR method allows built in product detection (both quantitative and qualitative) during the entire reaction period including exponential phase of the amplification reaction. RT- PCR is so named as one can continuously monitor the development of amplicons in a fluorimeter. SYBR-Green or other fluorescent labeled probes that emit lights during amplification are widely used in Real-time PCR (Dinesh and Ambarish, 2009). The emitted light signals corresponding to DNA amplification recorded at frequent intervals generating a curve showing product generation. The more targets DNA amplifies in the sample, the earlier amplicons can be detected and the peak curve is generated. The specificity, however, relies on the use of a specific probe (Tichopad et al., 2003). Baggi et al. (2005) have used Real Time-PCR for rapid detection of diarrheagenic *E. coli* using SYBR Green Dye and best sensitivity and specificity was observed. However, in order to validate the data or incorporate the data one should be very particular about the similarities of test

conditions, test parameters and sequence data of target genes if the experimental set up does not belong to same laboratory. For the detection of food samples 5' nuclease multiplex PCR can also be employed. The method uses the 5' nuclease activity of Taq Polymerase (Holland et al., 1991; Exner et al., 2002; Fach et al., 2003).

Enzyme-linked immunosorbent assay (ELISA)

ELISA is one of the most commonly used immunological methods for the detection of water and food borne pathogens. Sandwich ELISA is the most effective form of ELISA whereby it involves two antibodies (Zhao et al., 2014). The primary antibody is usually immobilized onto the walls of the microtiter plate wells. The target antigen like bacterial cells or bacterial toxins from the food and water sample binds to the immobilized primary antibody and the remaining unbound antigens are removed by washing. After that, an enzyme-conjugated secondary antibody is added and it will bind to the antigen and the remaining unbound antibodies are removed. The complex consisting antigen sandwiched between two antibodies is formed and it can be

detected by adding a colorless substrate which will be converted into a colored form in the presence of the enzyme (Zhang, 2013). There are different types of enzymes which can be used in ELISA, some of the most commonly used enzymes include horseradish peroxidase (HRP), alkaline phosphatase and beta-galactosidase (Yeni et al., 2014).

Many studies have been performed using the sandwich ELISA for rapid detection of foodborne pathogens. For example, Kumar et al. (2011) performed the detection of pathogenic *Vibrio parahaemolyticus* in seafood with sandwich ELISA, using monoclonal antibodies against the TDH-related hemolysin (TRH) of pathogenic *Vibrio parahaemolyticus*. The detection limit of this assay was 10^3 cells of pathogenic *Vibrio parahaemolyticus*. Commercial ELISA test kit such as BIOLINE *Salmonella* ELISA Test is also available for the detection of *Salmonella* in food products. The detection limit of this test kit was 1 CFU/25 g sample with minimum four of the 20 food matrixes tested (Bolton et al., 2000). ELISA is also commonly used for the detection of toxins present in foods such as *Clostridium perfringens* α, β, and ε toxin, staphylococcal enteroxins A, B, C, and E, botulinum toxins and *Escherichia coli* enterotoxins (Aschfalk and Mülller, 2002; Zhao et al., 2014).

The immunological method is easy and rapid to perform and suitable for large numbers of samples during routine drinking water surveys. In combination with growth in the pre enrichment medium, the test is particularly designed to detect coliform bacteria. Analysis of drinking water by the standard method requires a minimum of 2 days and a maximum of 5 days. In contrast to the standard method, signs of lactose degradation in samples and time-consuming confirmatory tests are not needed with the immunological method. With this test it is possible for rapid analysis of drinking water for the presence of Enterobactenaceae in only 24 h. (Hubner *et al*., 1992).

CONCLUSION

Water is the basic requirement for human survival. Safe and ready to drink water is very important for human health, whatever the drinking water is essential phenomenon for domestic and food production. The hygienic water with filled of minerals supply and sanitation, is the better management of water resources that can be boost up the countries economical growth and easily contribute to the poverty reduction. In Indian perspective the safe drinking water is challenge for govt. and NGOs. Because the water is concern to every

aspect of human day today activities directly or indirectly. It is fundamental right of health to drink safe water and wellbeing of society. Rapid detection and identification of microorganisms is extremely important in microbiology. In general, fluorogenic and chromogenic substrates have proved to be a powerful tool, utilizing specific enzymatic activities of certain microorganisms. By incorporation of synthetic fluorogenic or chromogenic substrates into primary selective media, enumeration and detection can be performed directly on the isolation plate. The introduction of many of these media and identification tests has led to improved accuracy and faster detection of target organisms, often reducing the need for isolation of pure cultures and confirmatory tests.

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