International Journal of Multidisciplinary and Current Research

Research Article

ISSN: 2321-3124
Available at: http://ijmcr.com

Detection of Water-borne Pathogenic Bacteria: Where Molecular Methods Rule

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Accepted 04 April 2014, Available online 15 April 2014, **Vol.2** (**March/April 2014 issue)**

Abstract

Environmental reservoirs are the potential sources of pathogenic E. coli. These are ideal habitat for survival, growth, and proliferation of E. coli as they are rich in nutrients. A wide range of different animals harbor pathogenic E. coli, which are consistently associated with human and animal diarrheal infection. Infected cattle typically excrete pathogenic E. coli. These discharges are released into the aquatic environment or storm water runoff. The surface water carries a number of pathogenic bacteria when used for the drinking purposes. Apart from these, the water is treated in municipal treatment plants and supplied through pipelines for drinking purposes. The processed clean water sometimes also gets contaminated due to leakage of nearby sewer lines. Highly prevalent diarrheagenic E. coli variants are present in the surface and potable waters in India. The consumption of unsafe water in developing countries causes a number of disease outbreaks. The detection of target pathogens needs improvements to overcome existing drawbacks and should be based on simple, sensitive and specific methodologies. Polymerase Chain Reaction is one of the latest techniques in modern biology which is effective in the detection of these pathogens. Real Time PCR technique has advantages over conventional methods in terms of rapidity, high specificity and sensitivity. The real time PCR uses primer pair and molecular probe (TaqMan, Molecular Beacon, Scorpion etc.) for the sensitive detection of pathogens.

Keywords: *E. coli, Water, Detection, PCR, Real Time PCR*

Introduction

Water pollution, an inevitable consequence of urbanization and industrialization has major public health and socio-economic impact. Increase in globalization of trade and human movement facilitates urban crowding, favoring host-to-host contact and the dissemination of emerging infectious diseases (Ford 2006; Van *et al.,* 2007). Two third of the total drinking water consumed worldwide is derived from various surface water sources that may easily be contaminated microbiologically by sewage discharges or fecal loading by domestic or wild animals defecation, malfunctioning of septic trenches, storm water drainage, municipal wastes and industrial effluents (Hamner *et al.,* 2006; Orsi *et al.,* 2007).

Globally surface, ground and potable water are receiving pollutants from feacal origin (Watkinson *et al.,* 2007, Meusburger *et al.,* 2007). These water harbor pathogen such as STEC, ETEC and Salmonella etc. (Fong *et al.,* 2007, Jyoti et al, 2010). Currently majority of Indian rivers and their tributaries, the major source of potable water supply are polluted due to drainage of sewerage, solid wastes and industrial effluents into the rivers and streams with out any treatment. Each of the 23-macro cities in India generates about 1500-million liters sewage water/day. Only 10% of this is treated while the rest drains into the rivers and other dumping grounds (DAE 2005).

Pollution Control Board of India reported that bacterial contamination continued to be critical for water bodies, due to discharge of untreated domestic and industrial wastewater from the urban centers of the country during 1995 to 2006. The receiving water bodies also do not have adequate water for dilution, leading to increase in bacterial pollution load. This is mainly responsible for water borne diseases (CPCB 2005). Millions of people living along the rivers are exposed to microbial contaminants, while taking bath and using polluted water for domestic purposes (Hamner *et al.,* 2007; Servais 2007). In recent years, an increase in incidences of illness attributable to recreational and drinking water exposure has been observed. A safe supply of drinking water depends upon use of either protected water sources, or properly selected and operated series of interventions, use of appropriate treatment technologies capable of detecting and reducing pathogens, and finally prevention of re-contamination in distribution system.

Sources of Pathogenic Microorganism and Transmission

Most pathogens in drinking water are generally of fecal origin and animal wastes (Leclerc *et al.,* 2002). Although a wide range of different animals harbor pathogenic *E. coli*, which are consistently associated with human and animal diarrhoeal infection. Infected cattle typically excrete between 10^2 and 10^5 CFU pathogenic *E. coli* per gram of faeces (Fukushima and Seki, 2007). These discharges may be released directly into the aquatic environment or indirectly through storm water run off. Moreover, during supply through pipeline, processed clean potable water also gets contaminated due to leakage of nearby sewer line. Therefore, there is need for monitoring of pathogenic microorganism in urban and rural environment of developing world.

Regulation and Risk Assessment

Health based targets provide the basis for the application of the National and International Standards for surface and potable water quality for community and household supplies. These guidelines intend to support the development and implementation of risk management strategies to ensure the safety of drinking water supplies (Table 1). Water quality guidelines and standards, recommended by various authorities and might be similar to ensure the minimum health risk, but differ due to economic and technical capabilities, perceptions of acceptable risks of infections in rural and urban environment (Dechesne and Soyeux, 2007). Worldwide regulatory authorities provide international and national microbial water quality standards and issue directive to control microbial pollution of aquatic resources (Hamner *et al.,* 2006).

Table 1 Bacterial contamination regulations and guidelines for drinking water.

Country	Total coliform	*E. coli*
India	0/100ml (95% should not contain any coliform organisms in 100ml)	0/100ml (100%)
USA	0/100ml (95%), a consecutive sample from the same site must be coliform free /, <5% positive	0/100ml (100%)
Canada	0/100ml (90%) none should contain more than 10 CFU/100ml,a consecutive sample from the same site must be coliform free	0/100ml (100%)
EEC	0/100ml, or MPN<1	0/100ml (100%)
IS 10500	0/100ml	0/100ml (100%)
WHO	0/100ml (95%)	0/100ml (100%)
OECD	0/100ml	0/100ml (100%)
UK	0/100ml (95%)	0/100ml (95%)
EC	0/100ml	0/100ml

Source: Ministere de la sante, 1996; OECD 1999; Parsons 2000; USEPA, 1990, 2003; WHO 1994.

Surface water qualities standards used by different water regulatory agencies are based on the concentration of indicator organisms such as the Canadian Council of the Ministers of the Environment (CCME) water quality standard is 100 and 200 FC $100mL^{-1}$ for recreational water uses, respectively (CCME 1999). The USEPA standard for freshwater recreational water is that the geometric mean of at least five samples during a 30-day period must not exceed 126 *E. coli* $100mL^{-1}$ with no one sampled exceeding 235 *E. coli* $100mL^{-1}$. Several states also have bacterial standards that depend on season, which correspond with seasonal water (USEPA 2003).

The Pathogenic Variants of 'Indicator' Organism, *E. coli*

E. coli are Gram-negative, nonspore-forming, oxidase-negative, rod-shaped, facultative anaerobic bacteria that ferment lactose by β-D-galactosidase to acid and gas within 24 - 48 h at 36 ± 2°C (Ashbolt *et al.,* 2001). *E. coli* produce acid and gas after lactose fermentation at 44.5 ± 0.2°C within 24 ± 2h. In addition *E. coli* did not grow on citrate agar produces indole from tryptophan and acidic end products when they are grown in methyl red-Voges-Proskauer broth. However, Manafi (2000) reported that *Klebsiella oxytoca,* which is indole positive, could yield false positive *E. coli* results with the standard confirmation procedure. This was resolved by the use of gas production at 44.5°C for *E. coli* confirmation tests. Generally, *E. coli* is not associated with adverse health effects. However, under certain conditions pathogenic *E. coli* may cause urinary tract infections, bacteraemia, meningitis and diarrhoeal disease in humans (Nataro and Kaper, 1998; Gyles 2007).

Diarrhoeagenic strains of *E. coli* can be classified into at least six different categories with corresponding distinct pathogenic schemes such as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC)**.** Current situation in which there are now at least 11 recognized pathotypes of *E. coli* in humans (Kaper *et al.,* 2004). Strains with in each pathotype are characterized by shared virulence traits and can typically be further distinguished by O (lipopolysaccharide) and H (flagellar) antigens (Kaper *et al.,* 2004).

Enterotoxigenic E. coli (ETEC)

Fecal contamination of water and food sources is the main factor for high incidence ETEC infections**.** ETEC strains cause diarrhoea disease in infants as well as humans through the action of enterotoxins, *LT* (heat-labile enterotoxin) and *ST* (heat-stable enterotoxin). These strains produce an *LT* or *ST* enterotoxin alone or both (Quadri *et al.,* 2005; Turner *et al.,* 2006). The heat labile enterotoxins are oligomeric protein and consist of two major serogroups, namely LT-I and LT-II (Nataro and Kaper, 1998). Heat labile enterotoxins (*LT-I*) is similar to the cholera toxin, it is consist of one A subunit and five identical B subunits (Nataro and Kaper, 1998; Negy and Fekete, 2005). The later binds to the ganglioside GM_1. The A subunit is responsible for the enzymatic activity of the

toxin by activating adenylate cyclase resulting in an intracellular increase in cyclic adenosine monophosphate (cAMP) concentrations. This leads to a decrease in sodium absorption by villous cells and subsequent active chloride secretion by crypt cells thereby resulting in osmotic diarrhoea toxin (Kaper *et al*., 2004).

Heat stable enterotoxin is small, monomeric toxin comprising two unrelated classes, namely *STa* and *STb* (Nataro and Kaper, 1998; Negy and Fekete, 2005). The receptor for *STa* is guanylate cyclase C and binding results in increased intracellular cyclic guanylate monophosphate (cGMP) levels leading to stimulation of chloride secretion or inhibition of sodium chloride absorption and subsequent intestinal fluid secretion leading to diarrhea. ETEC has great importance for developing countries due to its association with surface and potable water borne diarrheal disease outbreaks.

Enteropathogenic E. coli (EPEC)

EPEC is a major etiological agent of infant diarrhoea in developing countries (Varma *et al.,* 2000; Prasannan *et al.,* 2001). The EPEC serotypes are responsible for diarrhoeal diseases in humans and animals. Although they do not produce any classic toxins but harbor a number of virulence factors (Nataro and Kaper, 1998). The intimin protein is involved in colonization of the intestinal mucosa in humans and animals and production of characteristic attaching and effacing (AE) lesions (Kaper 1996; Trabulsi *et al.,* 2002). *E. coli* strains that produce AE lesions and carry the *eaeA* gene but not shiga toxin genes are designated as enteropathogenic *E. coli* (Nataro and Kaper, 1998; Kaper *et al.,* 2004).

In addition, strains expressing plasmid EPEC adherence factor (EAF) mediated by bundle-forming pili (*bfpA*) have been defined as typical EPEC and strains lacking the EAF-plasmid have been designated as a typical EPEC (Prasannan *et al.,* 2001; Kaper 1996; Trabulsi *et al.,* 2002; Chen and Frankel, 2005).

Enteroaggregative E. coli (EAEC)

Enteroaggregative *E. coli* strains are associated with persistent diarrhoea in human particularly in children worldwide (Nataro 2005). EAEC strains are a highly heterogeneous group consisting of about 90 identified serotypes, the most common being O15:H18, O44:H18, O77:H18, O111:H12, O125, and O126 (Okeke and Nataro, 2001). EAEC are defined by their aggregative adherence to intestinal epithelial cells in a characteristic "stacked-brick" pattern (Nataro and Kaper, 1998). During pathogenesis, EAEC adhere to the intestinal mucosa by plasmid encoded aggregative adherence fimbria (AAF/I–AAF/III), leading to inflammation causing mucosal toxicity and increased production of a mucus biofilm and cytokine release (Huang *et al.,* 2004; Torres *et al.,* 2005). Other virulence factors of EAEC are plasmid encoded enterotoxin (PET) (Eslava *et al.,* 1998) and enteroaggregative heat stable toxin 1 (EAST-1) (Kuhnert *et al.,* 2000).

Enteroinvasive E. coli (EIEC)

EIEC was first described in 1944, later identified as *E. coli* O124 associated with diarrhoea diseases bloody diarrhoea being typical of EIEC infection, and the occurrence being high in developing countries (Lan *et al.,* 2004; Sarantuya *et al.,* 2004)**.** EIEC strains are difficult to distinguish from *Shigella* spp. and other *E. coli* strains. Both plasmid and chromosomal genes confer pathogenicity in EIEC strains. The chromosomal locus *kcpA* activates transcription of the plasmid borne gene *virG*, which encodes an outer membrane protein required for directional movement through cytoplasm (Nataro and Kaper, 1998; Lan *et al.,* 2004). EIEC strains invade and multiply within enterocytes, causing the death of host cell (Escobar-Paramo *et al.,* 2003; Thiem *et al.,* 2004).

Enterohemorrhagic E. coli (EHEC)

Enterohaemorrhagic *E. coli* (EHEC), verotoxin producing *E. coli* (VTEC) or Shiga-toxin producing *E. coli* (STEC) are different names for the same group of organism. EHEC infections in humans can result in bloody diarrhoea. Approximately, 2 - 7% of cases of EHEC infection may develop in to hemolytic uremic syndrome (HUS) that can be fatal (Nataro and Kaper, 1998). *E. coli* O157:H7 is the most commonly occurring member of this group. Genes involved in EHEC pathogenesis are similar to those implicated for EPEC, except for the presence of *stx*-encoding phage on the EHEC chromosome (Nataro and Kaper, 1998). A cluster of genes involved in the generation of attaching and effacing lesions are chromosomally carried on a "pathogenicity island" named the locus of enterocyte effacement. This pathogenicity island also carries the *eaeA* gene, which is responsible for the generation of an outer membrane protein called intimin that is necessary for attachment to enterocytes. Some of the STEC strains harbor a 97 kb plasmid (pO157), encoding possible additional virulence genes such as *hlyA* (EHEC-*hlyA*) that acts as a pore-forming cytolysin on eukaryotic cells, the bifunctional catalase peroxidase (KatP), the *etpD* gene cluster that probably encodes a type II secretion pathway and the secreted serine protease (*EspP*) (Schmidt *et al.,* 1999). The identification of DNA sequences (*rfb* operon for O-antigen synthesis) unique to *E. coli* 0157 serotype have been shown to serve as molecular markers for detection by PCR (El Syed Zaki and El-Adrosy, 2007). EHEC strains have been documented to have a very low infectious dose of 1 - 10^2 CFU (Sharma *et al.,* 2006).

E. coli O157:H7 is the predominant serotype of STEC in the United States, Canada, the United Kingdom, and Japan, but in continental Europe, Australia, and Latin America, non-O157: H7 serotypes are much more common than *E. coli* O157:H7 (Kaper and O'Brien, 1998).

Sorbitol fermenting *E. coli* O157: $H7^-$ strains are also increasingly associated with diarrhoea, HC and HUS in several European countries, Argentina, Chile, South Africa, the United States and Australia (Nataro and Kaper, 1998; Johnson *et al.,* 2004).

Shiga toxins are most important pathogenic factors in EHEC contains two major toxins, called *stx1* and *stx2*, which share approximately 55% aminoacid homology, these are similar to the one produced by *Shigella dysenteriae* serotype 1, (Nakao and Takeda, 2000; Golurmelon *et al.,* 2006). While, *stx1* is highly conserved, *stx2* has 11 distinct variants (Nakao and Takeda, 2000), with *stx2c, stx2d, stx2e*, and *stx2f* being the most frequently reported but the presence of *stx2* and *stc2c* has more significant correlation with human disease (Nakao and Takeda, 2000; Bidet *et al.,* 2005). Shiga toxins are proteins formed by one catalytic subunit (A) of 32 kDa and five B subunits. The A subunit cleaves a single adenine residue from the 28S rRNA component of the eukaryotic ribosomal 60S subunit. The B subunits mediate binding to cells through interaction with globotriaosylceramide (Gb3) expressed on epithelial and endothelial cell membranes. The action of the toxin inhibits the activity of ribosomal RNA, causing protein synthesis to cease and producing cell death (O'Brien *et al.,* 1992). *Stx* genes are produced in the colon and travel by the bloodstream to the kidney, where they can damage renal endothelial cells and occlude the microvasculature through a combination of direct toxicity and induction of local cytokine and chemokine production, resulting in renal inflammation (Tarr *et al.,* 2005).

Distinguishing characteristics of *E. coli* O157:H7 growth studies in trypticase soy broth indicate that the organism grows well between 30 - 42°C, having generation times between 0.49 h and 0.64 h at 37°C and 42°C, respectively (Padhye and Doyle, 1991). The procedures used to detect fecal coliforms in food use incubation temperatures of 44 - 45°C that fail to detect *E. coli* O157:H7. *E. coli* O157:H7 do not ferment or produce acid from D-sorbitol within 24h and lack glucoronidase activity (Manafi and Kremsmaier, 2001). The most common media is sorbitol MacConkey (SMAC) agar is used for isolation of *E. coli* O157:H7 in water and food resources (Fujisawa *et al.,* 2002). The addition of Cefixime and tellurite to SMAC agar permits the selective growth of *E. coli* O157:H7 and *Shigella sonnei* strains but inhibits the growth of most of the other *E. coli* strains. However, CT-SMAC culture does not detect *E. coli* O157: H^- and non-O157 STEC serotypes, which generally ferment sorbitol.

Strategies for Detection of *E. coli* Pathotypes

The problem of detection and monitoring of microbial pathogens in drinking water is being viewed in a global perspective. The Strategies for detection and identification of microorganisms from any type of niche can be conceptualized into phenotypic (based on protein, carbohydrate, lipid or other bio-molecules produced by target organisms) or genotypic (based on nucleic acids). Various biochemical methods, such as coliform assay, new chromogenic or fluorogenic media based on β-galactosidase (total coliforms) or β-glucuronidase and immunoassay are widely used for detection of *E. coli* in water food and clinical samples. But these methods have many technical limitations (Manafi and Kremsmaier, 2001). Nucleic acid based detection methods offer novel, more sensitive and specific ways of detecting microorganisms associated with surface and drinking water.

The Polymerase chain reaction (PCR) is a rapid technique with high specificity and sensitivity for the identification of target organisms. Multiplex PCR is a technology that can amplify more than two gene sequences in single tubes (Shelton *et al.,* 2006). As STEC and ETEC have potential to contaminate the surface and potable water, it is useful to detect them simultaneously. The applications of singleplex and multiplex PCR for detection of STEC and ETEC have been reported (Lothigius *et al.,* 2007). However, quantitative detection of target genes is not feasible in singleplex and multiplex PCR assays because amplified products can be only visualized in agarose gels after the completions of PCR (Kubista *et al.,* 2006; Espy *et al.,* 2006).

The rep-PCR DNA fingerprinting is used to examine sources of feacal pathogenic bacteria impacting beaches and waterways (Johnson *et al.,* 2004). But this technique has limitations in throughput, allowing for analysis of only about 400 *E. coli* isolates per week. Several hurdles including recovery to non-viable organism are encountered in conventional and molecular methods used for detection of pathogens.

Studies are needed to confirm the superiority of new method for acceptance by appropriate regulatory agencies. An international expert meeting of Interlaken 1998 concluded that the application of molecular methods has to be considered in a framework of a quality management for drinking water. The utility of Polymerase Chain Reaction (PCR) is evidenced by guidelines issued by NCCLS (1999) encouraging use in clinical laboratories for bacterial identification.

Molecular tools can be developed to identify organisms that would not be detected in current culture techniques and to track new pathogenic entities, including variants of otherwise pathogenic microorganisms (Nataro and Kaper 1998; Orsi *et al.,* 2007). A more useful method would be one that permits evaluation of multiple targets within a single sample, preferably in a real time to monitor hazardous shifts in pathogen population.

Concentrations and Re-Concentrations of Pathogenic Bacteria in Water

The monitoring and identification of a pathogen from water samples remain difficult due to presence of large

numbers of harmless background micro-flora rather than target microorganism (Fukushima *et al.,* 2007). The biggest problems are the lack of a consistent method for simultaneous concentration and detection of a particular microorganism from water samples. Another common difficulty is the broad variation in recoveries of microorganism, particularly from water samples with high turbidity levels (. Very large volumes of water (10 to 100 liters of raw water and up to 1,000 liters of finished water) have been recommended for testing to ensure adequate protection (Rochelle *et al.,* 1999).

Information regarding the capabilities, time requirements and limitations of each method is intended to serve as a basis to aid research and development of rapid non-cultural enrichment techniques and overall rapid detection of pathogenic organisms. Several methods have been applied, individually or incombination with other methods, such as centrifugation (Bernhardt *et al.*, 1991), filtration (Fernandez-Astorga *et al.,* 1996), cationic and anionic-exchange resins (Turpin *et al.,* 1993), aqueous two-phase partitioning (Pedersen *et al.,* 1998), immobilized lectins (Payne *et al.,* 1992) and immunomagnetic separation (Safarik *et al.,* 1999) for bacterial concentration in water and food samples to reduce time of cultural enrichment.

It has been suggested that if bacteria could be easily separated, purified and concentrated from a biological sample, rapid detection technologies such as PCR and real-time PCR could have more applications. Berry and Siragusa (1997) have reported the use of hydroxyapatite to concentrate indigenous bacteria from meat slurries and environmental samples. Many techniques have limitations in detection of pathogenic entities in water resources. The lack of selective or differential cultivation methods, limitations in serological characterizations, resistance to chlorination and filtration exhibited by water-borne pathogens indicate the need for less labor intensive, rapid and specific techniques. Methods that individually or in combination can rapidly enrich bacteria from water samples in less time than cultural enrichment are needed.

The application of PCR for waterborne pathogen detection can deliver high sensitivity, specificity and speed in culture independent mode. However, PCR based pathogen detection techniques suffer from inherent low throughput design, thereby numerous reaction are required to monitor pathogenic microorganisms in water and aquatic macrophytes. Moreover mispriming and gel electrophoresis based detection methods decrease specificity and sensitivity of PCR based monitoring (Kubista *et al.,* 2006). Real-time PCR with molecular probe such as Molecular beacon, TaqMan, FRET, Scorpion can be exploited for detection of pathogens in surface water and potable water. Such methodologies can aid real-time monitoring for development of strong early warning systems, reliable field diagnostics, symptom treatments and more cost effective remediation. Therefore, in the present study, a profile of virulence determinants of antimicrobial resistant representative diarrhoeagenic *E. coli* (STEC and ETEC) was generated for surface water and potable water isolates to identify prevalent genes responsible for virulent behavior of these pathotypes, prevalent in Indian environment. Real-time florescent probes were designed and *in-silico* validated prior to laboratory use. Further, applicability of these probes can be tested for culture independent detection and quantitative enumeration of representative diarrhoeagenic *E. coli* (in very low concentration) in environmental samples (aquatic macrophytes, surface and potable water).

Emerging Molecular Tool: Real-Time PCR

Real-Time Polymerase Chain Reaction is a powerful advancement of the basic PCR, through use of fluorescent detection strategies allows the quantitation of nucleic acid in a reaction without post-PCR analysis and minimizing the possibility of cross-contamination between analyses, while providing rapid, continuous data collection and decreased time requirements for results (Hanna *et al.,* 2005; Kubista *et al.,* 2006; Espy *et al.,* 2006). Real time PCR has high specificity, speed and sensitivity in pathogen detection, with detection limits<100 copies of specific genes present in complex sample (Amar 2007). The advantage and limitations of Real-time PCR are given (Table 2).

Table 2: Advantages and Limitations of Real-Time PCR

Advantages	Limitations
▪ Wide dynamic range of quantification (7-8 logarithmic decades) ▪ High analytical sensitivity (<5 copies; 1fg to 10 pg bacterial DNA per PCR reaction) ▪ Better precision (<2% standard deviation) ▪ Closed system to reduce risk of contamination ▪ No post PCR processing ▪ Lower turnaround time ▪ Increased throughput ▪ Multiplexing capabilities	▪ PCR product increases exponentially cannot monitor amplicon size. ▪ Variation increase with cycle number ▪ Maximum of four simultaneous reactions ▪ Overlap of emission spectra ▪ Increased risk of false negative in pathogen detection (particularly for new emerging or highly variable pathogens) ▪ Non-specific binding (SYBR green analysis)
Source: Hanna *et al.,* (2005)	

Recently, a new multicolor combinational probe technology has been used to increase the number of targets at a time that can be distinguished in a single real-time PCR for rapid and reliable species specific detection (Huang *et al.,* 2007). In real-time PCR, fluorescent reporter is used to monitor PCR reaction as it occurs. During the exponential phase in real-time PCR experiments a fluorescence signal threshold is determined at which point all samples can be compared using one of the numerous real-time thermocyclers available now. This threshold is calculated as a function of

the amount of background fluorescence and is plotted at a point in which the signal generated from a sample is significantly greater than background fluorescence (Kubista *et al.,* 2006; Espy *et al.,* 2006).

Detection Formats

Mechanism of real time PCR technique depend on the basis of fluorescent reporter can be classified on the basis of fluorescent dyes such SYBR Green and real-time probe. The real-time probe can also divided into hydrolysis and hybridization probe. The most relevant hydrolysis probes are TaqMan, MGB TaqMan and hybridization probes are FRET, Molecular beacon and Scorpion probe etc.

Specific Dye

SYBR Green Dye

SYBR Green I is an asymmetrical cyanine dye used as a nucleic acid stain and binds to double-stranded DNA, resulting DNA-dye-complex absorbs blue light (λ_{max} = 498 nm) and emits green (λ_{max} = 522 nm) (Zipper *et al.,* 2004). Once bound, the dye emits a fluorescent signal depending on amplified product, which is more than 1000 times greater than that emitted by unbound dye. Advantages of SYBR Green include the relative ease with which it can be applied to existing PCR assays relatively lower cost, and ease of assay design. Melting curve analysis after the amplification to verify that the fragment obtained is the correct sequence is another advantage of SYBR green I. The primary disadvantage of the SYBR Green I dye chemistry is that it may generate false positive signals; *i.e.*, SYBR Green I dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences (primer dimmers, mispriming). SYBR Green I has been applied successfully in real-time PCR (McCrea *et al.* 2007) and more recently, in the demanding quantification of dsDNA in crude extracts of environmental samples that is usually hampered by a variety of quenching processes.

Real-Time Fluorescent Probes: Sequence-Specific Hydrolysis Probes

The hydrolysis probes are those probes, which are cleaved by the proof reading activity of DNA polymerase during the elongation phase of primers, yield a real time measurable fluorescence emission. The best-known hydrolysis probes are TaqMan and minor groove binder (MGB) probes both developed by Applied Biosystems.

TaqMan Probe

TaqMan probes are short oligonucleotides (20 - 30 base pairs) contain reporter fluorophore at the 5'- end and a quencher fluorophore at the 3'- end, which capitalizes on the 5’ -exonuclease activity of Taq Polymerase to cleave a labeled hybridization probe during the extension phase of PCR (Holland *et al.,* 1991). During DNA amplification in the PCR process, the TaqMan probe hybridizes to the template and is digested by the 5' - 3' endonuclease activity of the *Taq*DNA polymerase as the PCR primer is extended. The TaqMan probe is digested only if DNA replication occurs, separating the reporter and quencher molecules. PCR products are monitored during increasing florescent signal exponentially with successive PCR amplification cycles (Holland *et al.,* 1991). Advantages of TaqMan probes include specific hybridization between probe and target where is required to generate fluorescent signal, distinguishable reporter dyes, which allows amplification of more than two distinct sequences in one reaction tube and eliminated post-PCR processing, which reduces assay labor and material cost.

The primary disadvantage of the TaqMan chemistry is that the synthesis of different probes is required for different sequences. A quantitative TaqMan PCR has been developed to quantify *Enterococcus faecalis* as Indicator in recreational waters (Santo Domingo *et al.,* 2003) in ≤ 2 hr.

Hybridize Probes

Molecular beacon

Molecular beacon is a short oligonucleotide (25 - 40 nucleotides) that forms a hairpin structure with a loop and stem. The loop is designed to hybridize to a 15 - 20 nucleotides portion of the target DNA sequence while the either side of loop, there are another 5 or 6 nucleotides, which are complementary to each another. A fluorescent reporter molecule is placed at the 5’- end of the Molecular beacon and a non-fluorescent quencher is placed at 3’- end. During annealing, binding of Molecular beacon to its target leads to increased fluorescence, due to separation of reporter and quencher, which directly corresponds to accumulation of product (Tyagi and Kramer, 1996). Molecular beacons have an advantage over conventional nucleic acid probes because they have a higher degree of specificity with better signal-to-noise ratios (Park *et al.,* 2000). Another advantage would include better quantification, less dependence on optical geometry and less interference due to photo bleaching that could affect optical measurements.

Florescence Resonance Energy Transfer Probes

FRET probes are labeled with different fluorescent dyes and are designed to anneal in a head-to-tail orientation to the target DNA between the PCR primers (Wittwer *et al.* 1997). Each probe has a single label either a donor fluorophore at the 3’- end or an acceptor fluorophore at 5’- end. Fluorophores are so chosen basis of emission spectrum of one overlaps significantly with the excitation spectrum of the other. During FRET, the donor fluorophore excited by a light source, transfers its energy to an acceptor fluorophore when positioned in the direct

vicinity of the former. The FRET probes must be blocked at their 3'- end using phosphate moiety to prevent the extension during the annealing step. During annealing, the two probes hybridize adjacent to their target sequence and thus the excitation energy is transferred by FRET from the donor to acceptor probe, allowing the acceptor dye to dissipate fluorescence at a different wavelength (Bernard and Wittwer, 2002).

Scorpion Probes

Scorpion probes is used in a fluorescence based method for real - time PCR analysis, which is ideal for all diagnostic applications such as pathogen detection, SNP analysis, and gene expression analysis. It consists of a specific probe sequence held in hairpin loop structure by complementary stem sequences on the 5' and 3' sides of probe. A quencher attached to the 3' end of the loop quenches the fluorophore attached to the 5' - end. The 3'- end of loop is linked to the 5'- end of primer via non-amplifiable stopper moiety such as hexa ethylene glycol (HEG). In addition, the probe sequence is complementary to an internal region of the sequence extended by the adjacent primer (Whitcombe *et al.,* 1999). After extension of the primer during PCR amplification, the specific probe sequence is able to bind to target sequences. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched and an increase signal is observed (similar to Molecular beacons). The PCR stopper prevents read through, which could lead to opening of the hairpin loop in the absence of the specific target sequence. Such read would lead to the detection of non-specific PCR products e.g. primer dimmers or mispriming events.

A common drawback of this probe systems is that it generates unwanted signal due to probe destruction by decrease-of-quench (e.g., by unintentional hydrolysis of the probes by the *Taq* DNA polymerase) or by formation of secondary structures of the probes that lead to a decrease in quench. Further, the cost and expertise required for validation of real-time PCR probes for real-life samples is not a trivial exercise. Hence, *in-silico* development and comparison of real-time PCR probe chemistry can be first step in selection of appropriate chemistry for real-time detection of pathogens.

Acknowledgment

We wish to express our sincere acknowledgement to Dr. Ashok Kumar Chauhan, President, RBEF parent organization of Amity University Madhya Pradesh (AUMP), Mr. Aseem Chauhan, Additional President, RBEF and chairman of AUMP; Lt. Gen. V.K. Sharma, AVSM (Retd.), Vice Chancellor of AUMP, Gwalior, for providing their valuable support, necessary facilities and encouragement throughout the work.

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