INTRODUCTION

1.0 Introduction

Pure water, fresh air, healthy food and safe medicines are required for quality human life. A critical aspect of water, air, food and drug safety is the absence of pathogenic microorganisms. Presence of pathogens in the supply chain of food, water and pharma samples represents a significant health risk. Contamination of water, food and pharma products with pathogens such as *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Vibrio cholerae* and *V. parahaemolyticus*, at any stage of production or processing or marketing could pose a potential health risk. These pathogens will affect the human health in many significant ways.

Bacillus cereus is a Gram-positive spore-forming rod that is ubiquitous in the environment. It has been implicated in many food-borne outbreaks involving cooked foods such as rice, meat loaf, turkey loaf and mashed potatoes (Drobniewski, 1993). In addition to these types of foods, *B. cereus* is a common contaminant in dairy products (Becker *et al.* 1994). Conventional methods for the identification of *B. cereus* consist of biochemical tests and microscopic analysis of cell morphology. Microscopic analysis is necessary since the closely related *Bacillus thuringiensis* has similar biochemical characteristics. Polymerase chain reaction (PCR) assay which can discriminate *B. cereus* from *B. thuringiensis* based upon two single-base differences within cereolysin AB gene (Kim *et al.* 2000).

The typical indicators such as faecal coliforms, *E. coli* and enterococci give a good estimate of the health risks only in the case of drinking water, for which there is zero tolerance to faecal contamination (James and Evison, 1979; Maier *et al.* 2000). So the detection of faecal contaminants should be obtained simultaneously to the identification of the sources of pollution. Identification of enterococci through conventional methods, i.e., by determining phenotypic characters, is complicated and often requires 48 to 72 h (Ruoff *et al.* 1990; Devriese *et al.* 1993). The phenotypic identification of some enterococcal species may be occasionally difficult or even impossible because these species lack typical characteristics. More rapid and accurate methods would be helpful for microbiology laboratories. Several DNA based methods

for the specific detection of *E. faecalis* or *E. faecium* have been reported (Robbi *et al.* 1996; Satake *et al.* 1997; Cheng *et al.* 1997).

The presence of *Escherichia coli* in foods is of regulatory significance for indicating faecal contamination and with some strains of this bacterium. The widely used Most Probable Number (MPN) method described by the International Standards Organization (ISO) enumerates coliforms on the basis of their ability to ferment lactose. With this method, after incubation of serially diluted samples at 35°C for 24–48 h in Lauryl Sulphate Tryptose (LST) broth, positive (presence of gas) tubes are further sub-cultured in *E. coli* (EC) broth and incubated at 44.5° or 45.5°C for another 24–48 h. The presence of gas in the tubes is taken as an indication of faecal coliforms. Discrimination of *E. coli* beyond this initial step requires additional testing. This takes up to 4 days for the determination of faecal coliforms and an additional 6 days by the Food and Drug Administration (FDA) method, or 2 days by the ISO method, for confirmation of organism as *E. coli* (Andrews *et al.* 1987; Anon, 1993).

PCR is a powerful molecular biology technique that was introduced to facilitate the detection of *E. coli* virulence factors (Nataro and Kaper, 1998); however, its direct application to fecal specimens is impeded by the presence of inhibitors in such crude materials (Lou *et al.* 1997). For this reason, most studies using this technique worked with isolated colonies and/or extraction and partial DNA purification (Pass *et al.* 2000).

Listeria monocytogenes has been established as a food-borne pathogen and has become a major concern to the food industry and health authorities. The association between the consumption of cooked and chilled ready-to-eat poultry products and several cases of listeriosis in England (Anonymous, 1989) and the United States (Barnes *et al.* 1989), together with the finding that *L. monocytogenes* was present in 12 to 27% of such products at the retail level (Gilbert *et al.* 1989), indicates that ready to- eat and inadequately reheated chilled foods may constitute a public health risk.

To monitor the incidence of *L. monocytogenes* in foods, reliable methods must be developed for the rapid detection of the organism. Conventional methods are tedious and are variable in their results. Once selective enrichment procedures have been

optimized, methods for the rapid identification of food isolates must be designed (McLauchlin, 1989). The suggested technique is PCR for the analysis of food samples which detects the hlyA and iap genes of *L. monocytogenes* (Anonymous, 1989). HlyA encodes the well-recognized virulence factor listeriolysin 0 (Audurier and Martin, 1989). The iap gene is a presumptive virulence gene which is thought to code for an invasion-associated protein (Anonymous, 1990).

Pseudomonas aeruginosa is considered one of the most problematic Gram-negative bacterium in all sorts of water systems. To confirm the presence of *P. aeruginosa* or *Salmonella* spp., as long as 2–11 days are generally required (De Vos *et al.* 1997). However, PCR-based approaches offer distinct advantages over culture and other standard methods for the detection of microbial pathogens. PCR is specific, sensitive, rapid, and accurate enough to detect small amounts of target nucleic acid in water and waste water samples (Toze, 1999).

Salmonella is an important food and water-borne pathogen associated with acute gastrointestinal illnesses around the world. The infective dose can be as low as 15–20 cells (Feng, 1992). The conventional method for detection of salmonellae by isolation and identification of the organisms usually requires 3 to 5 days, and hence in most instances it cannot achieve the primary objective of timely intervention (Tsui *et al.* 1996). Standardized diagnostic procedures to detect the presence of *Salmonella* in food samples (ISO 6579:2002) are mainly based on microbiological culturing methods, which in general require up to 5 days (Stewart *et al.* 1998). In order to reduce the time demand, alternative techniques like immunological assays (Bolton *et al.* 2000) and molecular methods (Heller *et al.* 2003) have been applied to detect *Salmonella* in various samples.

Shigella can contaminate several kinds of foods, including raw vegetables, milk, poultry, and some dairy products mainly through human transmission (Wachsmuth and Morris, 1989). Therefore, as with other pathogenic microorganisms, it is important that the presence of *Shigella* be detected in foods. Traditionally, the detection test of food-borne microorganisms (hazard test) is made by plating a food homogenate on highly selective media, although in the case of some bacteria a

preenrichment step is required. After several days of incubation, the presence or absence of the microorganism or the number of colonies is determined. This plating technique, based on the phenotype of the bacteria, is labor-intensive and can take several weeks to obtain results (Hill, 1996). On the other hand, rapid, highly sensitive, and specific techniques based on genetic characteristics have been developed. DNA probe hybridization and PCR are the best known of these techniques for the detection and identification of food-borne microorganisms (Olsen *et al.* 1996).

Staphylococcus aureus is one of the most common agents in bacterial food poisoning outbreaks. It is also a major causative pathogen of clinical or subclinical mastitis of dairy domestic ruminants. Poultry, meat and egg products as well as milk and milk products have been reported as common foods that may cause staphylococcal food poisoning (Le Loir *et al.* 2003). Routine detection of *S. aureus* in food is usually carried out by traditional methods based on morphological and biochemical characterization. These methods are time consuming and tedious. The advent of nucleic acid based assay systems like PCR has led to the emergence of improved, expedient and reliable methods of microbial identification and surveillance (Scheu *et al.* 2004).

Cholera is usually transmitted by ingestion of contaminated water and foods. The outbreaks of food-borne cholera have been noted quite often in the past 30 years; seafood, including molluscan shellfish, crustaceans and finfish are most often incriminated in food-borne cholera cases in many countries (Albert *et al.* 1997; Rabbani and Greenough, 1999). Conventional microbiological methods for identifying *Vibrio cholerae* in foods are reliable; however, they require several days to complete and thus may result in considerable loss of perishable foods (Food and Drug Administration, 1992). The PCR has the potential to detect microbial species by amplification of gene sequences unique to that organism. A rapid PCR method has been developed for determining the presence of enterotoxigenic *V. cholerae* in water (Chakraborty *et al.* 1999).

Vibrio parahaemolyticus is a halophilic bacterium that occurs naturally in estuarine environments worldwide. It is one of most important food-borne pathogens in Asia,

causing approximately half of the food poisoning outbreaks in Taiwan, Japan and Southeast Asian countries (Kaysner and DePaola, 2001). The most common method to detect *V. parahaemolyticus* is a culture procedure using enrichment media and subsequent isolation on selective plating media. Since the conventional detection method requires 2 to 3 days, a more rapid method is required. In recent years, workers have developed PCR-based assays that target one or more of the genes i.e tlh, tdh and trh for identification of *V. parahaemolyticus* in various types of samples (Kaufman *et al.* 2002).

Traditional culture methods for the isolation of the pathogens from water, food and pharma samples are relatively inefficient because the organisms are often found in low numbers and their growth may be impaired by sub-lethal injuries and competition from other bacteria present in these substances (Tollison and Johnson, 1985). Traditionally, the detection test for these pathogens is made by plating a food homogenate on highly selective media, although in the case of some bacteria a pre enrichment step is required. After several days of incubation, the presence or absence of the microorganism or the number of colonies is determined (Hayes, 1985). This plating technique, based on the phenotype of the bacteria, is labor-intensive and can take several weeks to obtain results (Hill *et al.* 1985). Culture based methods have limitations, including duration of incubation, antagonistic organism interference, lack of specificity and poor detection of slow-growing or non-dividing microorganisms (Wingender and Flemming, 2004).

PCR is a rapid *in vitro* procedure for enzymatic amplification of specific DNA sequences which increases the number of copies of the target sequence. This allows increased sensitivity of detection of a DNA sequence present in trace amounts in mixed populations (Kwok *et al.* 1988). The interlaboratory variation due to personnel, reagents, minor temperature or pH fluctuations and thermal cyclers will have a minimum affect on the performance of the PCR method (Amir *et al.* 2004). PCR is deemed to be more reliable than conventional identification since it is based on stable genotypic characteristics rather than relying on biochemical or physiological traits, which can be genetically unstable (Lawrence and Gilmour, 1994). Several investigators have developed PCR and DNA probe techniques for the detection of

pathogenic bacteria (Karunasagar *et al.* 1995; Lampel and Kornegay, 2000; Nguyen *et al.* 2004).

In the present work, a multiplex PCR technique is developed for simultaneous detection of potential bacterial pathogens in contaminated food, water and pharma samples. Moreover commercially available samples have not been investigated in India for bacterial pathogens by multiplex PCR. Department of Health Ministry (Government of India) had passed an enforcement law for testing these bacterial pathogens and invited all nationally recognized testing laboratories for developing pathogen detection tools which will be rapid, sensitive, specific, less time consuming and cost effective, particularly in field conditions. The main emphasis of this work is to develop molecular methods for detecting bacterial pathogens and to provide testing services at an affordable cost in the context of national interest. Taking cognizance of the above developments, the following objectives have been taken up.

Objectives

- To analyze the possibly and artificially contaminated samples by conventional methods
- To design species specific primers to the conserved regions of the bacterial pathogens.
- To develop a PCR assay and examining the selectivity and specificity of the primers.
- To develop a multiplex PCR assay to identify food, water and pharma contaminants.
- To evaluate the complete PCR-based method with artificially and possibly contaminated samples.
- To compare the performance of the developed PCR method with the conventional culture method.