DISCUSSION

5.0 Discussion

Bacterial food-borne pathogens are an important food safety issue worldwide. Rapid and accurate identification of bacterial pathogens isolated from food samples is important both for food quality assurance and for tracing of outbreaks of bacterial pathogens within the food supplies. Rapid microbial identification methods have become widely used in both food and water microbiology laboratories. These rapid identification methods offer some important advantages over conventional methods, including reduced labor, reduced human error, increased sample throughput and faster turn around times for test results.

Food Regulation in India and Hazard Analysis Critical Control Point (HACCP)

In India, quality control with regard to food products is being enforced through various regulatory mechanisms like the Prevention of Food Adulteration Act (PFA), Agriculture Grading and Marketing (AGMARK), Fruit Products Order (FPO), etc. The Bureau of Indian Standards (BIS) launched a HACCP certification programme for the food industry. While efforts are being made to implement HACCP in the organised sector of the food industry, there is a need to implement HACCP in the unorganised sector also as it accounts for 70-80% of food produced and processed in India (ICMR Bulletin, 2000). Department of Health Ministry (Government of India) passed an enforcement law in 2005, for testing of bacterial pathogens in food and water 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 the field conditions. As per the new regulatory guidelines 2005, all the food and beverages that are available in open market should be free of bacterial pathogens. So, there is an urgent need to develop a cost effective assay, which can detect the above pathogens in field conditions. The main emphasis of this work is to develop a molecular screening protocol for detecting pathogens and to provide testing services at an affordable cost in the context of national interest.

The rapid, cost-effective and automated diagnosis of food-borne pathogens through out the food chain continues to be a major concern for the industry and public health. Because of these requirements, the PCR became a powerful tool in microbiological diagnostics during the last decade (Sachse, 2003). An international expert group of

the European Committee for Standardization has been established to describe protocols for the diagnostic detection of food-borne pathogens by PCR. A standardized PCR-based method for the detection of food borne pathogens should optimally fulfill various criteria such as analytical and diagnostic accuracy, high detection probability, high robustness (including an internal amplification control (IAC), low carryover contamination, and acceptance by easily accessible and user-friendly protocols for its application and interpretation (Malorny *et al.* 2003). The second generation of PCR methodologies, real-time PCR, has the potential to meet all these criteria by combining amplification and detection in a one-step closed-tube reaction.

To optimize pharmaceutical process control, corrective actions must be performed in real time, not after 7 or more days of manufacturing. Rapid methods will identify microbial contamination with detection times ranging from 90 min to 30 h allowing the monitoring of critical control points, reducing losses, and optimizing resources. A recent technical report by the Parenteral Drug Association (PDA) provided some information and guidelines for the evaluation, validation, and implementation of rapid microbiological methods (PDA, 2000). Food-borne illnesses are known to share a major part of infections caused among human beings. Infectious microbes either viable or non-viable could become harmful if they produce toxins. Factors like inadequate refrigeration, preparation of food in advance, cooking, infected food handling, poor hygiene, holding temperature, reheating, contaminated raw ingredients, cross-contamination and poorly maintained equipments are some of the major causes of food contamination. The emergence and dissemination of microbial food-borne pathogens is affected by factors related to the nature of pathogens, type of host, process of food production, food storage, during transportation and consumption. Besides this, there has been an increased concern over the use of genetically modified organisms (GMOs) in food products (Deisingh *et al.* 2005; Royal Society, 2005).

Water borne pathogens are another major source of disease across the globe; more than 1.2 billion people do not have access to safe drinking water, while others are exposed to significant risks to health because of contamination during transport or

storage (Kerwick *et al.* 2005). More than 250 million cases of water borne diseases are reported each year, resulting in more than 10 million deaths and nearly 75% of these water borne diseases cases occur in tropical countries. Interventions in hygiene, sanitation and water supplies proved to control these diseases. Universal access to safe drinking water and sanitation has been promoted as an essential step in reducing these preventable diseases (WHO, 1994; WHO, 2001).

In 2002, there was an outbreak of bacillary dysentery among tea plantation workers in Northern district of West Bengal, India. The causative organism was found to be *Shigella dysenteriae* 1 for the outbreak (Sarkar *et al.* 2003). Another important pathogen is *Salmonella* which causes localized infection to the intestinal epithelium, known as "non- typhoid salmonellosis", as well as the systemic infection "typhoid salmonellosis" (Garcia-Del Portillo *et al.* 2000). *Salmonella* spp. were isolated from 6 stool samples and in one blood sample among soldiers of an army unit located at high altitude (3300m) in western Himalayas in 1998. Epidemiologically, frozen fowl was traced as a probable incriminating food agent responsible for the outbreak (Singh *et al.* 1998). Numerous outbreaks were associated with faulty or unclean medical equipment (Stephenson *et al.* 1985; Srinivasan *et al.* 2003) and from environmental reservoirs (Foca *et al.* 2000). In 2003 and 2004, Delhi experienced two outbreaks of cholera. In 2003, cholera outbreak occurred in August which was considered a peak season for diarrheal infections whereas the 2004 outbreak was seen in April. *Vibrio cholerae* O1 serotype Inaba outnumbered the dominant serotype Ogawa in Delhi and its adjoining areas. Emergence of *V. cholerae* O1 serotype Inaba has been reported in many parts of the country (Taneja *et al.* 2005; Dutta *et al.* 2006; Pal *et al.* 2006).

Bacillus cereus is a spore-forming food-borne pathogen often associated with food products such as meat, vegetables, soup, rice, and milk and other dairy products. Between 1 and 20% of the total number of outbreaks of food infection in the world is caused by *B. cereus* (Kramer and Gilbert, 1989). In general, *Listeria monocytogenes* has been found in raw milk, soft cheeses, fresh and frozen meat, poultry, seafood, fruits, and vegetable products. *L. monocytogenes* has been shown to survive in foods for long periods (Jay, 1996). *V. parahaemolyticus* infection can cause gastroenteritis in humans, and the illness is most frequently associated with the consumption of raw

or undercooked seafood and seafood recontaminated with the bacterium after cooking (Rippey, 1994). The groups of foods commonly implicated in the appearance of cholera cases are: fish and shell fish, fruit and vegetables and other foods such as rice, potatoes, lentils, beans, egg, chicken, etc (Kaysner *et al.* 1992; Kaysner *et al.* 1994).

Water intended for human consumption should be both safe and wholesome. It also should also be easily accessible, adequate in quantity, free from contamination and readily available. The major hazard in drinking water supplies is microbial contamination, which is due to agricultural land wash, domestic sewerage, industrial effluents, improper storage and handling (Saha *et al.* 2006). Primary contamination in drinking water is mainly due to source of water supply, water storage and leakage of pipes and secondary due to man made such as improper handling, storage, distribution and serving methods (Tambekar and Banginwar, 2005). The potable water can be easily contaminated by incorrect method of storage or by dipping dirty dipper or the finger or by dirty glass, jug etc. and causing basic hygiene related diseases like diarrhea every year (Tambekar *et al.* 2006). The microorganisms that appear in the pharmaceutical raw materials can be the origin of certain diseases or may cause spoilage of the medicaments (Venkateswara Rao *et al.* 2008).

Street food vending is prevalent in India, with a large number of people from all ages and income groups consuming a variety of foods. Previous reports on street vended raw salad vegetables, sprouts and fruits have shown the presence of *Staphylococcus aureus* and *Shigella* spp. (Viswanathan and Kaur 2001; Pingulkar *et al.* 2001) in India. *Staphylococcus* spp. is an important food-borne pathogen, and staphylococcal food poisoning ranks among the most prevalent causes of gastroenteritis worldwide (Bryan *et al.* 1992). *Shigella* species are an important cause of diarrheal disease in developing countries (Vargas *et al.* 1999).

Based on the above information about contamination sources and out breaks different categories of samples were selected in this study for the detection of bacterial pathogens. The food categories were fish and fishery products, meat and meat products, chicken and chicken products, milk and milk products, bakery products,

egg and egg products, vegetable and vegetable products, cereal and cereal products and starch products. The water sources were river and sewage water (raw water), municipal tap water and packaged drinking water and the pharmaceutical samples were various raw materials used in active process and finished products. The food samples were collected from various manufacturing industries, street vendors, local markets and testing laboratories in and around twin cities of Hyderabad, R.R District, Andhra Pradesh. Water samples were collected in and around areas of Hyderabad, Bangalore and Chennai. Pharma samples were collected from testing laboratories from and surrounding areas of Hyderabad.

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 pathogens can be detected 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). The common isolation strategy for *Escherichia coli* and *Salmonella* involves the use of one of several combinations of selective broths and agars, typically with indicators for enzymatic activities. These isolation methods require expert judgment and further testing to confirm the presence of these pathogens (Jennifer *et al.* 1998).

Culture based methods have limitations, including duration of incubation, antagonistic organism interference, lack of specificity and poor detection of slow-growing or nondividing microorganisms (Wingender and Flemming, 2004). In this study, Indian and International (IS/ISO) standard specifications were followed for culturing food and water samples, whereas in the case of pharma contaminants, United States pharmacopeia (USP) procedures were followed for the isolation and identification of bacterial pathogens. In all the above-mentioned methods a preenrichment/enrichment step was followed by morphological identification on

selective media and further confirmation by biochemical identification and serological tests. All the steps of these test procedures take 5-7 days to confirm the specific bacterial pathogen.

Rapid methods of pathogen testing have been gaining increasing interest in the food industry. These methods include antibody-based assays, genetic amplification methods and newer sensor development. 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). Genetic amplification methods such as the PCR and nucleic acid sequence based amplification (NASBA) have made it possible to significantly reduce assay times while maintaining a high level of sensitivity and specificity. These methods are also able to distinguish closely related species, which most antibody tests could not. Nevertheless, such amplification tools are currently used only as screening techniques prior to traditional culturing methods. This is due to the fact that Government recalls are not based on PCR assays alone. PCR assays, which use DNA as a target, are usually not quantitative with regard to viable organisms.

Several PCR based detection methods have been proposed for food and water-borne pathogens to replace the time consuming classical techniques (Candrian, 1995). Specific primers for the detection of *S. aureus* have been directed to the nuc gene encoding thermostable nuclease (Brakstad *et al.* 1996), enterotoxin genes (Mantynen *et al.* 1997), tst gene (shock syndrome), genes coding for exfoliative toxin A and B (eta and etb respectively) (Jennifer *et al.* 1998), the 16–23 rDNA spacer region (Saruta *et al.* 1997) and the 23S rDNA (Straub *et al.* 1999). In addition to the analysis of foods, PCR was also successfully applied for detection and identification of pathogenic organisms in clinical and environmental samples (Olsen, 2000).

Species-specific primers were designed in the study to the conserved regions of respective bacterial genomes. The target genes were 16S rRNA for *E. coli*, Inv A for *Salmonella* spp., mRNA nuclease for *S. aureus*, ipaH for *Shigella* spp., toxA for *V. cholerae*, PrfA for *L. monocytogenes*, bceT for *B. cereus*, PR72H DNA for *V.*

parahaemolyticus, membrane protein putataive gene for *S. faecalis* and oprL gene for *P. aeruginosa.* The PCR conditions were standardized for all the bacterial pathogens tested in the current study, in a uniplex PCR. The primers used were specific for the pathogens selected (Figure 19). The specificity of the primers was further confirmed by sequencing the amplified PCR product. The specificity and sensitivity of the primers and the similarity of annealing temperatures at which the pathogens amplified, has enabled us in designing the multiplex PCR.

Traditionally, identification of *Listeria* is achieved by initial *in vitro* culture followed by biochemical characterization of resultant isolates. Apart from being slow and expensive, this testing scheme can generate potentially variable results. The application of nucleic acid amplification technology has vastly improved the sensitivity and specificity of *Listeria* (Sallen *et al.* 1996; Graham *et al.* 1997; Bubert *et al.* 1999). In particular, with PCR primers derived from both conserved and species-specific variable portions of the shared iap gene, six *Listeria* species-specific products can be amplified (Bubert *et al.* 1999).

da Silva Filho (1999) described PCR amplification for algD GDP mannose for identification of *P.aeruginosa.* This PCR assay was tested on 182 isolates of *P. aeruginosa* and 20 isolates of other bacterial species, and demonstrated 100% specificity and sensitivity. The test was also able to detect *P. aeruginosa* directly in clinical samples such as sputum or throat swabs obtained from cystic fibrosis patients. Danbing *et al.* (2000) developed a PCR-based assay that targets the tuf gene, which could detect most enterococcal species. Lavenir *et al.* (2007) developed a PCR procedure, which targets the ecfX gene for the screening of *P. aeruginosa* strains. This test was specific to *P. aeruginosa* strains and did not amplify DNA from any of the other *Pseudomonas* species tested. The ecfX PCR screening was validated on environmental DNA extracts. These investigations suggest a preferential colonization of water rather than soil environments by *P. aeruginosa.*

In the present study, the specificity of the primers used in the multiplex PCR assay revealed the absence of non-specific amplification with other food, water and pharma contaminants such as *Burkholderia cepacia, Brevundimonas diminuta, Citrobacter*

freundii, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas fluorescence*, *P. putida*, *P. stutzeri*, *P. cepacia*, *Staphylococcus intermedius*, *S. epidermidis*, *S. haemolyticus*, *S. warneri*, *Vibrio alginolyticus*, *V. vulnificus*, *V. minicus*, *V. fluvialis*, *V.fischeri*, *Streptococcus agalactiae*, *S. pyogenes*, *S. lactis*, *S. cremoris* and presence of *Bacillus. cereus*, *Escherichia.coli*, *Salmonella* spp., *S. aureus*, *Shigella* spp., *P. aeruginosa*, *S. faecalis*, *Vibrio cholerae*, *V. parahaemolyticus and Listeria monocytogenes*. The sequencing results were submitted to the NCBI and the accession numbers are shown in Table 26. The specificity of the selected primers to the respective bacterial pathogens and the absence of nonspecific amplification in the present study suggest that the primers selected can be used for the screening of bacterial pathogens.

Detection of *Shigella* by PCR with *virA* primers could be a useful hazardous test because its sensitivity, would allow the detection of 1 fematogram of DNA or 1 to 10 cells in 10 ml of sample (Bej *et al.* 1990; 1991). In the present study, the sensitivity of detection is as low as 1.56 ng of DNA for the multiplex PCR for all the pathogens tested (Figures 26 -29).

Although many conventional culture media and enrichment regimes have been proposed for isolating *Salmonella* species from food or environmental samples (Alcaide *et al.* 1984), they are still difficult to culture, detect, or enumerate from the complex microbial communities of natural ecosystems. The ability of *Salmonella* spp. to enter a viable but nonculturable state after lengthy exposure to soil or groundwater under ambient conditions of temperature and low nutrient concentration may contribute to this difficulty (Roszak and Cohwell, 1987).

Bacteria that are naturally present in feed could have reduced viability due to, low water activity, high salt concentrations and unfavourable pH, freezing, or heating. As a result, for most microorganisms nonselective enrichment increases the probability of recovery and detection of damaged cells (Fleet, 1999). To increase the number of cells and dilute feed inhibitors, an enrichment step at 37°C for 18 h in Buffered Peptone Water (BPW) was followed, a commonly used nonselective medium for enrichment (Anonymous, 1999). A pre-enrichment step was included to increase the

sensitivity and to dilute any inhibitory substances present in the samples. The food and water samples were enriched in nonselective medium i.e. tryptic soy broth yeast extract medium for a period of 6-8 h. All bacteria could be isolated even at a lower inoculum (<10 cfu) in artificially contaminated food and water samples (except in raw water). In case of conventional methods, different enrichment and selective broths were used for identification as per the specifications. Enrichment prior to PCR has been reported to increase the number of target microorganisms and to minimize the risk of detecting DNA from dead cells (Sharma and Carlson, 2000).

Most PCR assays carried out currently include a minimum of 6 to 8 h pre-cultivation step (Guo *et al.* 2000; Oliveira *et al.* 2002; Wang and Yeh, 2002; Fratamico and Strobaugh, 2003; Oliveira *et al.* 2003; Ellingson *et al.* 2004; Myint *et al.* 2006; Patel *et al.* 2006) that can increase the sensitivity to one digit number of CFU, which is much better than the immunoassays that preenrich the samples for 24 to 30 h but still obtain minimum detection limits of 103 CFU (Soumet *et al.* 1999); Liu *et al.* 2001; Chen and Durst, 2006). Samantha and Arvind (2004) recorded that as few as four cfu of *Salmonella*/25 g of produce can be detected after 16 h of enrichment in buffered peptone broth. These assays were carried out entirely in sealed PCR tubes, enabling a rapid and high-throughput detection of *Salmonella* species in a large number of food and environmental samples.

The importance of resurgence and recovery procedures availed in food industry has been overlooked in the isolation techniques of pharmaceutical microbiological studies (Ray, 1986). Sample pre-enrichment is the most vital step during isolation of *Salmonella* spp. from pharma samples. To optimize PCR detection of *S. typhimurium*, particularly in minimal bacterial load samples, a different type of preenrichment broth was performed. BPW was previously used to enhance the recovery of *Salmonella* spp. in food samples using conventional and PCR methods (Gouws *et al.* 1998).

When pharmaceutical raw materials and products contaminated with mixed bacterial cultures of *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. typhimurium* were pre-enriched in lactose broth with and without Tween-20, 10 of 25 samples did not show the

presence of the *Salmonella* spp. The presence of non-*Salmonella* influencing the performance of the PCR-based assay was reported (Jimenez *et al.* 2001). But when the same samples were enriched in BPW, all the samples were positive for *Salmonella*. Jimenez *et al.* (2001) revealed that all ten samples that were negative for PCR in lactose broth were found to be positive with BPW enrichment medium. BPW enrichment medium has increased *S. typhimurium* growth resulting in rapid PCR detection.

In this study, for pharmaceutical sample analysis, trypticase soy broth containing 4% Tween-20 and 0.5% soy lecithin were used for enrichment of *E. coli*, *S. aureus*, and *P.aeruginosa* and BPW for *S. typhimurium*. The detection limit achieved for pharmaceutical samples were < 2 cfu/gm. *E. coli* O157:H7 is a major strain, which causes food-borne outbreaks all over the world. The detection and correct identification of this strain are important parts of food hygiene. Traditional methods for the identification of *E. coli* O157:H7, such as biochemical and serotype tests, used to take 5 to 7 days. In recent years, some molecular methods were developed to detect and identify this food-borne pathogen, such as PCR and enzyme-linked immunosorbent assay. PCR is a rapid and easy-to-use method and can provide a preliminary characterization (Fortin, 2001).

PCR has been widely accepted as a rapid and sensitive method for the detection and identification of bacteria. The mPCR (multiplex-PCR) method has the additional advantages of a lower economic cost and rapid processing. The detection of three of the bacterial strains, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes,* has been described in artificially inoculated raw beef using a multiplex-PCR (Alarcon *et al.* 2004). mPCR detection has also been tested on *E. coli* O157:H7 and *Salmonella* spp. (Fratamico and Strobaugh, 2003; Jin *et al.* 2004). Therefore, PCR-based procedures have been favored as rapid and highly specific methods, where the detection and identification can be completed with in 24 h, without the need for isolating pure cultures (Hill, 1996). The mPCR detection also costs less and requires less time for the detection of multiple species than with sequential rounds of uniplex PCR. Moreover, mPCR techniques for the simultaneous detection of two to four bacterial pathogens have been demonstrated (Kong *et al.*

1995; Brasher *et al.* 1998; Phuektes *et al.* 2001; Lim and Lee, 2002; Kong *et al.* 2002; Gilbert *et al.* 2003). For the simultaneous detection of more than one pathogen, the differences in growth requirements and rates must also be considered. Vidal *et al.* (2004) developed a multiplex PCR for detection of three categories of diarrheagenic *E. coli eae*, *bfp*, *stII*, and *lt* genes. With this method, enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, and enterotoxigenic *E. coli* were identified in fecal samples from patients with hemorrhagic colitis, watery diarrhea, or hemolytic-uremic syndrome and from food-borne outbreaks.

In this study, the multiplex PCR assay is developed for the simultaneous detection of *E. coli, Salmonella* spp., *Staphylococcus aureus* and *Shigella* spp. in the first set of food samples; *Vibrio cholerae, V. parahaemolyticus, Bacillus cereus* and *Listeria monocytogenes* in the second set of food samples; *Escherichia coli, S. aureus, V. cholerae, Streptococcus faecalis* and *Pseudomonas aeruginosa* in water samples and *E.coli, Salmonella* spp., *S.aureus* and *P.aeruginosa* in Pharma samples. Moreover, commercially available food, water and pharma samples have not been investigated with respect to the presence of these potential pathogens by molecular biology methods in Indian scenario, which have the potential to pose substantial health risks. Here, the development of mPCR technique is reported for simultaneous detections, in one tube, of four potential pathogens in above mentioned sample categories using a single step. This mPCR procedure may provide a more cost-effective and rapid detection approach than other currently available techniques.

The potential Turn Around Time (TAT) for the PCR method provides a marked advantage over conventional methods. The analysis time needed for the enumeration of *Staphylococcus aureus* by the PCR method takes only 24 h when compared with the conventional methods which take 96 h as described in the International Standards Organization (ISO) (Dogan *et al.* 2002). In the present study, multiple bacterial pathogens are detected in four multiplex reactions in less than 24 h. Cui *et al.* (2003) assessed with fresh ground beef samples inoculated with varying concentrations of *E. coli* O157:H7. When a 6 h enrichment step was incorporated, the detection limit was 1 CFU g^{-1} beef. The total time required from beginning to end of the procedure was 12 h. Jimenez *et al.* (2001) also showed simultaneous detection of *E.coli, S. aureus,*

P.aeruginosa and *A. niger* with detection levels <10 CFU/g or ml in pharma samples using RoboCycler 96-gradient PCR.

To assess the detection limits in our assays, the samples were spiked with known quantities of different pathogens. These artificial contaminated food samples were subjected to multiplex PCR. The sensitivity of the assay was as low as <10 cfu/25g for food-borne pathogens (Lane 1 of Figure 32, Table 29; Figure 33, Table 30); The samples that were negative by the conventional methods of detection, found positive by multiplex PCR, thus confirming the sensitivity of this PCR method. The detection limits of multiplex PCR in case of water samples were < 10 cfu/100ml (Lane 2 & 3 of Figure 32, Table 32) and for pharma samples the detection limits were < 2 cfu/g (Figure 36, Table 34).

Few countries have established legal limits on the number of *L. monocytogenes* that are permissible in foods, especially ready-to-eat products, whereas others have suggested guidelines or criteria that do not have legal standing. Any ready-to-eat food that contains *L.monocytogenes* can be considered adulterated and thus be subject to recall and/or seizure. The European Community directive on milk and milk-based products specifies zero tolerance for soft cheeses and the absence of the organism in 1g of other products. The International Commission on Microbiological Specification for Foods suggested that if this organism does not exceed 100 organisms/g of food at the point of consumption, the food is considered acceptable for individuals who are not at risk (Jay, 1996).

In India as per the new regulatory guidelines (Food safety and standards Bill, 2005) of food safety, all the foods and beverages that are available in open market should be free of major food and water-borne pathogens such as *E. coli, Salmonella* spp., *Shigella* spp., *S. aureus, V. cholerae, V. parahaemolyticus, B. cereus, L. monocytogenes, S. faecalis and P. aeruginosa.* As per USP guidelines the pharmaceutical raw materials and finished products should be free from indicator pathogens such as *E. coli, Salmonella* spp., *S. aureus* and *P. aeruginosa.*

To assess the applicability of the mPCR in the field conditions, possibly

contaminated samples were analysed and compared with the conventional culturing methods. In case of possibly contaminated samples, out of 205 food samples tested, 80% were positive for *E. coli,* 28% for *Salmonella* spp., 68% for *S. aureus* and 24% for *Shigella* spp. by species specific multiplex PCR, where as conventional methods are positive for 59% *E.coli,* 20% of *Salmonella* spp., 48% for *S. aureus* and 11%. *Shigella* spp.(Figure 30, Table 27). Out of the 150 food samples tested, percentage of positive samples for *B. cereus, L. monocytogenes, V. cholerae* and *V. parahaemolyticus* were 11, 3, 1 and 1 by conventional method and 15, 4, 1 and 2 by mPCR respectively (Figure 31, Table 28). Out of 221 water samples tested for possible contamination of water-borne pathogens, 39% were positive for *E.coli,* 16% for *S.aureus,* 23% for *P.aeruginosa,* 4% for *S.faecalis* and 9% for *V.cholerae* by conventional methods whereas by multiplex PCR 43% for *E.coli,* 18% for *S.aureus,* 31% for *P.aeruginosa,* 10% for *S.faecalis,* and 16% of samples were detected positive for *V. cholerae* (Figure 34, Table 31). Out of 70 samples tested, our results indicated that only 9, 1, 7 and 4 percent of pharma samples were positive by conventional methods for *E. coli, Salmonella* spp., *S. aureus* and *P. aeruginosa* respectively, where as by mPCR 11, 4, 11 and 8 percent of samples were positive (Figure 35, Table 33).

Multiplex PCR method is rapid and the level of sensitivity achieved in our experiments is applicable to the practical survey of microbial contamination in food, water and pharma samples. A major outcome of the study is the development of a multiplex PCR to detect multiple pathogens using compatible primers. The implications of the present study are promising and choice of primers in PCR can be extended to detect contaminant bacterial pathogens present in food, water, pharmaceuticals, raw materials, and cosmetic finished products. When compared with standard conventional microbiological methods, these technologies provide rapid and reliable microbiological monitoring of raw materials, finished products, and water systems allowing faster corrective actions and sample release.