SUMMARY

6.0 Summary

The detection of pathogenic bacteria is the key to identification and prevention of problems related to health and safety. Legislation is particularly tough in areas such as the food and pharma industry, where failure to detect an infection may have terrible consequences. In spite of the real need for obtaining analytical results in the shortest time possible, traditional and standard bacterial detection methods may take up 5 to 7 days to yield an answer. This is clearly insufficient, and many researchers have recently directed their efforts towards the development of rapid methods for detection. In addition to the health risk associated with contaminated food and pharma samples, there is often devastating economic impact to the manufacturer.

Traditional culture methods for the isolation of 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. 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. This plating technique, based on the phenotype of the bacteria, is labor-intensive and can take several weeks to obtain results. These isolation methods require expert judgment and further testing to confirm the presence of these pathogens.

Rapid methods of pathogen testing have been gaining increasing interest in the food and pharma industry. These methods include antibody-based assays, genetic amplification methods and newer sensor development. Traditional plating methods following enrichment can take days to yield results, while newer rapid methods require hours. Techniques such as immunomagnetic separation (IMS) and polymerase chain reaction (PCR) including real time PCR have paved the way for rapid and sensitive detection of bacterial pathogens, and advances in nanobiotechnology have allowed for miniaturization of devices. Despite the recent advances in pathogen detection, there still exists many challenges and opportunities to improve the current technology.

In the present study, both conventional culturing and PCR methods are employed to detect pathogenic bacteria in food, water and pharma samples. The main objectives of this research work are

- 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 possibly and artificially contaminated samples.
- To compare the performance of the developed PCR method with the conventional culture method.

Procurement of samples

In this study the food samples were collected from various manufacturing industries, street vendors, local markets and testing laboratories in Hyderabad and its surrounding areas; water samples from river water, sewage water, municipal water supply used for drinking purpose and packaged drinking water from different manufacturing units in Hyderabad, Chennai and Bangalore and their surrounding areas and pharma samples from various testing laboratories in Hyderabad and the surrounding areas.

Conventional analysis

In this study, we followed Indian and International (IS/ISO) standard specifications 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 the above-mentioned methods a preenrichment/enrichment step was followed by morphological identification on selective media and further confirmed by biochemical identification and serological tests. This whole procedure requires 5-7 days to confirm the specific bacterial pathogen.

Food-borne pathogens

A total of 205 possibly contaminated samples consisting of 40 fish and fishery products, 30 meat and meat products, 30 chicken and chicken products, 30 milk and milk products, 25 bakery products, 25 egg and egg products and 25 vegetable and vegetable products and 90 artificially contaminated food samples including 20 fish and fishery products, 15 meat and meat products, 15 chicken and chicken products, 10 milk and milk products, 10 bakery products, 10 egg and egg products, 10 vegetable and vegetable products were screened for *Escherichia coli, Salmonella* spp., *Staphylococcus aureus* and *Shigella* spp.

In second set, a total of 150 possibly contaminated samples consisted of 60 fish and fishery products, 25 meat and meat products, 10 chicken and chicken products, 25 milk and milk products, 5 bakery and bakery products, 10 vegetable and vegetable products, 10 cereal and cereal products and 5 starch products and 50 artificially contaminated food samples including 15 fish and fishery products, 15 chicken and chicken products, 10 meat and meat products, 7 milk samples and 3 vegetable and vegetable products were screened for *Vibrio cholerae, V. parahaemolyticus, Bacillus cereus* and *Listeria monocytogenes.*

The methodology employed for the conventional analysis of food-borne pathogens is as follows:

Escherichia coli – As per IS:5887(Part-I)-1976 (Reaffirmed 2005)
Salmonella spp. – As per IS: 5887 (Part-III)-1999(Reaffirmed 2005)/ISO 6579:1993
Staphylococcus aureus- As per IS:5887(Part-II)-1976 (Reaffirmed 2005)
Shigella spp.– As per IS:5887(Part-VII)-1999 (Reaffirmed 2005)

Bacillus cereus – As per IS:5887(Part-VI)-1999 (Reaffirmed 2005)/ ISO 7932:1993
Vibrio spp. (*V.cholerae* and *V.parahaemolyticus*)– As per IS:5887(Part-V)-1976 (Reaffirmed 2005)
Listeria monocytogenes – As per IS:14988 (Part-1)-2001/ ISO 11290-1-1996

Water-borne pathogens

A total of 141 possibly contaminated samples including 50 raw water, 41 tap water and 50 packaged drinking water samples and 110 artificially contaminated water samples including 50 raw water, 30 tap water and 30 packaged drinking water samples were screened for *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus faecalis* and *Vibrio cholerae*.

Standard isolation protocols employed for the water-borne pathogens are mentioned below.

Escherichia coli – As per IS:5887 (Part-I)-1976 (Reaffirmed 2005)
Staphylococcus aureus- As per IS:5887 (Part-II)-1976 (Reaffirmed 2005)
Vibrio cholerae – As per IS:5887 (Part-V)-1976 (Reaffirmed 2005)
Pseudomonas aeruginosa – As per IS:13428-2005
Streptococcus faecalis – As per IS:15186-2002/ISO 7899-2-2000

Pharma contaminants

A total of 70 possibly contaminated samples consisting of 18 lactose, 10 nicotinamide, 8 sodium starch glycollate, 6 xanthan gum, 6 gelatin, 6 maize starch, 4 micro crystalline cellulose, 3 ranitidine HCL, 3 mannitol, 3 talc purified and 3 ibuprofen suspensions and 50 artificially contaminated pharma samples consisting of 9 magnesium stearate, 8 aluminium hydroxide gel, 6 talc purified, 6 lactose, 6 gelatin, 5 maize starch, 4 attitude hand and body cream, 3 Nutrilite daily tablets and 3 simethicone emulsions were screened for *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The methodology adopted for the isolation of pharma contaminants is as follows:

Escherichia coli – As per USP 2006

Pseudomonas aeruginosa – As per USP 2006

Salmonella spp. – As per USP 2006

Staphylococcus aureus – As per USP 2006

PCR analysis

Species-specific PCR primers were developed to detect all the bacterial pathogens in the food, water and pharma samples. The primers were synthesized to the conserved regions of the respective bacterial pathogens. The target genes were 16S rRNA for *Escherichia coli*, Inv A for *Salmonella* spp., m-RNA nuclease for *Staphylococcus aureus*, ipaH for *Shigella* spp., toxA for *Vibrio cholerae*, PR72H DNA for *V. parahaemolyticus*, bceT for *Bacillus cereus*, PrfA for *Listeria monocytogenes*, membrane protein putative gene for *Streptococcus faecalis* and oprL gene for *Pseudomonas aeruginosa.*

The standard positive cultures were used for standardizing the PCR conditions.

The optimal concentration of the primers, $MgCl_2$ concentration and conditions for the PCR amplification were standardized in a uniplex PCR reaction. A multiplex PCR assay allows the simultaneous detection of multiple pathogens in a single reaction, using smaller amounts of reagents and less time to set up and analysis than simplex PCR, thus making it more applicable to routine diagnostic use. Based on Uniplex PCR results and annealing temperatures, four different multiplex PCRs were tested for the detection of food, water and pharma contaminants.

Multiplex PCR 1 (Food 1) – Designed for the detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Shigella* spp.

Multiplex PCR 2 (Food 2) – Designed for the detection of *Bacillus cereus*, *Listeria monocytogenes*, *Vibrio cholerae* and *V. parahaemolyticus*

Multiplex PCR 3 (water) – Designed for the detection of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus faecalis* and *Vibrio cholerae*

Multiplex PCR 4 (pharma) – Designed for the detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa*

The detection of multiple bacterial pathogens in a single reaction was achieved by annealing temperatures between 55°C-57°C, 2.5-5.0 picomoles of primer with1.5 mM $MgCl_2$. The sensitivity of the detection was as low as 1.56 ng DNA in a multiplex PCR reaction. 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 could be used for screening the bacterial pathogens.

After standardizing the multiplex PCR conditions, the replicate test samples used in conventional analysis were processed for screening bacterial pathogens in food, water and pharma samples by multiplex PCR.

Conventional culture method Vs Multiplex PCR

Culture based methods have limitations, including duration of incubation interference with antagonistic organism, lack of specificity and poor detection of slow-growing or non-dividing microorganisms. The multiplex PCR reaction could overcome the incubation time and would provide a cost-effective and rapid screening protocol.

Possibly contaminated samples were analyzed for food, water and pharma contaminants.

Food-borne pathogens

Out of 205 samples, *E. coli* was detected in 80%, *Salmonella* spp. in 28%, *S. aureus* in 68% and *Shigella* spp. in 24% of samples by species specific multiplex PCR, whereas in the case of conventional method 59%, 20%, 48%, 11% of samples tested were positive for *E. coli*, *Salmonella* spp., *S. aureus* and *Shigella* spp. respectively. *B. cereus*, *L. monocytogenes*, *V. cholerae* and *V. parahaemolyticus* were detected

positive in 11%, 3%, 1%, 1% of samples by conventional method and 15%, 4%, 1% and 2% of samples by multiplex PCR respectively in 150 tested food samples.

Water-borne pathogens

In the present study, 221 water samples were tested for possible contamination of pathogens such as *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, *Streptococcus faecalis* and *Vibrio cholerae*, out of which 33%, 16%, 23%, 4% and 9% of samples were positive by conventional methods and 43%, 18%, 31%, 10% and 16% of samples were positive by multiplex PCR respectively.

Pharma contaminants

In this study, 70 samples were tested by conventional culture and multiplex PCR methods for the presence of indicator pharma contaminants. The results showed that only 9%, 1%, 7% and 4% of samples were positive by conventional methods, whereas by multiplex PCR 11%, 4%, 11% and 8% of samples were positive for *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* respectively.

Artificially contaminated samples were used for determining the detection limits of food, water and pharma contaminants both by conventional method and multiplex PCR. The detection limits in this assay were as low as <10 cfu/25g for food-borne pathogens, <10 cfu/100ml for most pathogens in water samples and <2 cfu/gm for pharma samples in a multiplex PCR, where as in the case of conventional method the detection limits were >10 cfu/25g for food-borne pathogens, >10 cfu/100ml for water-borne pathogens and >2 cfu/gm for pharma samples.

The devised multiplex PCR method provides a promising option for the rapid identification of multiple bacterial pathogens in hours rather than days required for conventional cultural methods. Multiplex PCR can also improve the detection level due to its high sensitivity with low cost.