Review of Literature
Food is essential for life of everyone. Therefore, the safety of food we consume always brings much attention. Consumption of food contaminated with pathogens may pose a risk since in some cases a very low infective dose is enough to cause illness. *Campylobacter jejuni, Listeria monocytogenes, Salmonella, Staphylococcus aureus* and *Verotoxigenic E. coli* have been recognized as most frequent cause of food poisoning. Several studies and reviews have highlighted the contribution of changing demographics in the United States with the increased risk of food borne illness. In the United States, incidence of food borne illness is documented through FoodNet (a reporting system used by public health agencies that captures food borne illness in over 13% of the population). The bacterial agents responsible for most cases of food borne illness are *Salmonella, Campylobacter, and Shigella* from ten pathogens tracked by FoodNet. The estimated number of cases and mortality rate are considered for bacterial, viral, and parasitic cases of food borne illness, *Salmonella* causes 31% of food related deaths, followed by *Listeria* (28%), *Campylobacter* (5%), and *Escherichia coli O157:H7* (3%) (IFT, 2004). In addition, food contaminated with antibiotic resistant bacteria could be a major threat to public health as the antibiotic resistance determinants can be transferred to other pathogenic bacteria, causing compromise in the treatment of severe infections (AFIC, 2003; Anon, 2005; Iroha et al., 2011).

The identification of pathogen associated with food borne illness can provide the information about the source of the outbreak. In this view, early detection is becoming increasingly important as countries move towards industrialization. Rapid investigation of food borne disease outbreaks is crucial to prevent them from taking on massive proportions. Therefore, a rapid method not only provides instant or real time results but also reduces the identification time (Mandal et al., 2011).

Conventional methods normally used are time consuming and sometimes show difficulty in identifying pathogen. Immunological methods like ELISA are the earliest and probably most prevalent format and antibody assays used for pathogens or toxin detection in food including 16-24h enrichment. However, the incubation time for agglutination, immunodiffusion and turbidometric assays are very short. The molecular biology principle methodologies emerging for the rapid diagnosis of food poisoning are polymerase chain reaction (PCR) based, that amplify and detect DNA/RNA of the pathogen. The technique may significantly reduce the detection
time for pathogens in food and faecal samples, compared with traditional culture methods (Mandal et al., 2011). This review study is focused on prevalence and the use of rapid assays for five food borne bacterial pathogens; *Campylobacter jejuni*, *L. monocytogenes*, *Salmonella*, *S. aureus* and Verotoxnic *E. coli*.

### 2.1 *Campylobacter jejuni*

*Campylobacters* were observed in stools of diarrhoeic infants in Germany as early as 1880. However, the first recognized identification was made in 1913 in sheep abortion whereas confirmatory assays were carried out in 1918 from aborted bovine foetuses. Due to spiral appearance the organisms were originally assigned to the genus *Vibrio*, and were named as *Vibrio fetus*. The genus *Campylobacter* was proposed in 1963, as it was realized that the organisms were not able to utilize sugars and had a varied G+C content to that of *Vibrio spp*. *C. jejuni* and *C. coli* were named by Veron and Chatelain in 1973 which cause Seterocolitis. Now, the genus *Campylobacter* has been classified under family *Campylobacteriaceae* (Trachoo, 2003; Ryan and Ray, 2004; Skirrow, 2006; Boyson, 2012).

Campylobacteriosis has been recognized as the cause of typical muscular pain, headache and fever (known as the “febrile prodrome”) followed by watery or bloody diarrhoea, abdominal pain and nausea. Symptoms may last 1 day to 1 week or longer (usually 5 days). Excretion of the organism in stools occurs on average for 2 to 3 weeks and is mostly self-limiting. Hospitalization has been reported in up to 13% of cases. The attack rate is around 45%. In long term effect Campylobacteriosis is cause of chronic sequelae in the form of Guillain-Barre syndrome (GBS). The frequency of GBS resulting from Campylobacteriosis has been estimated as 0.1% (Altekruse et al., 1999) and can occur one to three weeks after enteritis. In treatment of Campylobacteriosis, erythromycin or norfloxacin are usually recommended. Strains resistant to erythromycin and norfloxacin have been isolated from a small number of Campylobacteriosis cases in New Zealand, whereas infection may have acquired overseas (Lake et al., 2007; CDMP, 2012).

#### 2.1.1 Status of *C. jejuni* in all over World and incidences in foodstuffs

*Campylobacter* are the major cause of diarrhoea in Europe, US and other industrialized countries. In England and Wales, with over 50,000 reported cases in 1997, Campylobacteriosis was the most commonly reported cause of human
gastroenteritis cases and most of the Campylobacter isolates reported were C. jejuni and C. coli (Frost et al., 1999). In Ireland, the National Disease Surveillance Centre (NDSC) reported 2085 human cases of Campylobacteriosis in 1999, with an incidence rate of 57.5 cases per 100,000 populations (Whyte and Igoe, 2000). The national surveillance system of Norway reported that the incidence of Campylobacter infection had almost doubled from 1992 to 1997 and in past years, reports of Campylobacteriosis had increased sharply and reached up to 63.9 cases per 100,000 population in 2001 (Kapperud et al., 2003). According to a report on Campylobacter infections, approximately 1 million cases of food borne illness due to Campylobacter spp. occur annually in the USA (Adedayo and Kirkpatrick, 2009). Unicomb et al., (2009) reported summary of outbreaks from 2001-2006 in Australia. A total 33 outbreaks were noticed, affecting 457 persons.

Campylobacters have been isolated from food items such as poultry meat, raw milk, pork, beef, lamb, and seafood (Duffy et al., 2001; Gharst et al., 2006; Madden et al., 2007; Milnes et al., 2008; Little et al., 2008; Bostan et al., 2009; EFSA, 2010b; Sammarco et al., 2010; Lawes et al., 2012; Rahimi et al., 2012a). Few studies have explored the presence and survival of Campylobacter spp. in milk products. The preparation processes of Brie and Camembert cheeses or hard and semi-hard cheeses seem unfavourable to Campylobacters (Bachmann and Spahr 1995; Medeiros et al. 2008), and the survival of C. jejuni in yoghurt is poor (Birk and Knochel, 2009). Campylobacter jejuni is the leading cause of human bacterial food borne illness worldwide (Wesley et al., 2000). Campylobacter is also the second most common causative agent of food borne outbreaks in EU, even though most reported cases of Campylobacteriosis are sporadic in nature (Roasto et al., 2010).

2.1.2 Status of Campylobacter in India

In a study, the carriage rate of C. jejuni in diarrhoeic handlers and apparent healthy handlers was 16.6% and 18.8% respectively. Carriage rate for C. coli among healthy handlers was determined to be 2.2% (Rathore, 1989). Studies carried out in rural population near Calcutta, the isolation rate was found to 11.5% in diarrhoeal cases and 11.1% from non-diarrhoeal cases (Sen Gupta et al., 1991). In another study, Rathore (1989) has reported 8.5% isolation rate of C. jejuni from cattle faeces and 20% and 53.3% isolation rate from sheep and pig faeces from Kolkata, respectively.
Khan and Khanna (1992) and Khurana and Kumar (1996) reported 20% isolation rate from Bareilly (UP) and Hisar (Haryana) respectively. From Calcutta, 1.9% isolation rate of *Campylobacter* *spp.* was reported from chevon (Das et al, 1996). In another study, Singh (1998) reported 8.33% isolation rate of *C. jejuni* from chicken meat surface swabs and 6.94% from chicken meat in Ludhiana (Punjab). In another study, no *C. jejuni* was isolated from chicken meat products as well as goat and sheep meat. Garbyal (2000) has shown 37.41% positive chicken samples from Bareilly (UP). Kumar et al., (2001) reported that *C. jejuni* is also present in vegetables. Khan and Khanna (1992), and Garbyal (2000) have reported 2% isolation rate of *Campylobacter* *spp.* from Bareilly (UP) respectively. Kownhar et al., (2007) reported *Campylobacter jejuni* among hospitalized HIV infected versus non-HIV infected patients with diarrhoea in southern India. A prospective case-control study was conducted to determine the association between *Campylobacter jejuni* infection and childhood Guillain-Barre syndrome in the Indian population. The study found that 27.7% patients were infected with *C. jejuni* (Kalra, 2009). The isolation of *C. jejuni* was also reported in human stool samples with frequency of 8.5% from Meghalaya and Assam hospital (Rizal et al., 2010). In the last decade, investigators have reported varying incidence of *C. jejuni* in different foodstuffs including sea foods from India (Bandekar et al., 2005; Singh et al., 2009; Patyal et al., 2011; Singh et al., 2011).

### 2.1.3 Detection of *Campylobacter jejuni* from foods

#### 2.1.3.1 Conventional methods

A number of media formulations have been proposed for the isolation of the *Campylobacters* by various workers, and their number probably exceeds that for any other group of bacteria (Correy et al., 1995). The isolation of "related *Vibrio*" (as *Campylobacter* were then known) was based on the use of membrane filtration followed by subculture onto nutritionally rich blood agar, before the medium of Dekeyser et al., (1972). The method used by Dekeyser and co-workers in 1972 included a combination of centrifugation, filtration through a 0.65 µm membrane filter and the plating of the filtrate onto a selective agar.

Clark and Duffy (1978) used enrichment medium for transport and enrichment of samples of perpetual liquid for *C. fetus*. The medium constituted of 300µg/ml of 5-flourouracil, 100 IU/ml of polymyxin-B sulphate, 50 µg/ml of brilliant green, 3
µg/ml of nalidixic acid and 100 µg/ml of cyclohexamide as an antifungal agent. In a trial, in which 89 samples were collected from 5 infected bulls and transported and cultured in the medium, *C. fetus* subsp. *fetus* was demonstrated in 77 samples by culture method. Rothenberg et al., (1984) compared Doyle and Roman Enrichment Broth (DREB), Park and Stankiewiez Enrichment Broth and newly developed Enrichment Broth for the isolation of *C. jejuni* from raw chicken and found that Doyle and Roman Enrichment Broth showed greatest selectivity. DREB and Park Enrichment Broths were compared for recovery of *C. jejuni* from food. No significant differences were found between the results obtained with the two broths (Heisick et al., 1984). In another study to compare several enrichment and direct isolation media to evaluate their suitability for detection and enumeration of five strains of *C. jejuni* in refrigerated (5°C) chicken meat, it was found that Campy Brucella Agar (CBAP), Blood Free *Campylobacter* Medium (BFCM) are superior than Modified Butzler Agar (MBA) and Doyle and Roman Enrichment Broth (Beuchat, 1985). Kakkar and Dogra (1990) used candle jar for creating microaerophilic conditions for growth of *Campylobacter* from rectal swabs and successfully isolated *C. jejuni*, *C. coli* and *C. lardis*. Shobha et al., (1992) designed modified internal gas generating system for creating microaerophilic conditions for isolation of *Campylobacter* spp. by using sodium borohydride, sodium bicarbonate and citric acid. They further compared their results by using gas pack for providing microaerophilic conditions and reported that *Campylobacter* was efficiently isolated by using both methods.

A pilot study was conducted to compare Blaserwang, Butzler and Skirrow's media for isolation of *Campylobacter* from poultry and found that Skirrow's medium gave maximum recovery of the organisms (Kumar et al., 2001). A blood and antibiotic free differential, Kapadnis–Baseri (KB) medium was formulated and tested for isolation of *Campylobacter* spp. (Basersalehi et al., 2004). Barua and Rathore (2006a), prepared a modified selective enrichment broth and blood free selective agar medium and compared it with Preston and Park and Sanders media for the isolation of *C. jejuni* and found that isolation rates of *C. jejuni* were higher than that of Park and Sanders and Preston media, which was 57.4%, 50.0% and 39.8% respectively. Ghazwan et al., (2009) described three media selective for *Campylobacter*: Campy-Cefex (CD), Modified Charcoal Cefoperazone Deoxychocolate Agar (mCCDA) and Skirrow media. An enrichment broth media supplemented with trimethoprim,
rifampicin, polymyxin B, cefoperazone, amphotericin was used. Later enriched cultures were transferred onto blood agar base supplemented with 7% lysed horse blood and vancomycin, polymyxin B and trimethoprim (Khanzadi et al., 2010).

2.1.3.2 Immunoassays

The immunofluorescent assays including fluorescent antibody technique (FAT), nanoparticles-based immunoassay, multiplexed sandwich chemiluminescent enzyme immunoassay and fluorescent nanoparticles probe immunoassay and PCR-enzyme-linked immunosorbent assay (ELISA) have been described (Hong et al., 2003; Chemburu et al., 2005; Watson and Galan, 2008; Mortensen et al., 2011).

2.1.3.3 Polymerase chain reaction for detection of *Campylobacter* spp.

Since its discovery, polymerase chain reaction has impacted virtually all areas of microbiology and in particular it has been used in detection of microbial pathogens in a wide range of sample types.

The first report of a PCR assay for the detection of *Campylobacter* spp. in foods was made by Giesendorf et al., (1992) who described a PCR assay for the rapid and sensitive detection of *Campylobacter* spp. in chicken products. The assay was applied to the detection of *Campylobacter* spp. in naturally contaminated as well as artificially inoculated samples of chicken skin following enrichment of the sample in Preston broth for 18 h. Korolik et al., (2001) evaluated a multiplex PCR assay for the rapid detection of *Campylobacter* spp. and *C. jejuni*. Kulkarni et al. (2002) compared selective culture, membrane filtration and polymerase chain reaction for the detection of *Campylobacter* from stool samples. Out of 343 samples examined, 17 were found positive by selective culture, 12 by membrane filtration and 20 by PCR method. Out of total 23 samples found positive by one or more method, 18 were identified as *C. jejuni* by PCR, whereas selective culture could identify only 14. In a study, Inglis and Kalischuk (2003) used PCR for detecting *Campylobacter* directly for bovine faeces without enrichment.

The species identification of *Campylobacter* generally requires 4 to 7 days to confirm the *Campylobacter* spp. Culture also requires special laboratory care, including micro-aerobic conditions, a specific temperature, and enrichment media. In addition, sometimes differentiating between *C. jejuni* and *C. coli* with conventional biochemical methods is problematic, because of close similarity between these two species.
species. *Hippuricase* activity is the only marker known to enable distinction between them. Several investigators developed PCR assays for specific identification of *Campylobacter jejuni* from *Campylobacter coli* by targeting various genes including hippuricase gene (Collins et al., 1996; On and Jordan, 2003; Khanzadi et al., 2010; Wangroongsarb et al., 2011). Linton et al., (1997) reported a primer set HIP400F & HIP1134R for specific amplification of *hippuricase* gene (*hip’O’) for *C. jejuni* detection in faecal samples. Persson and Olsen (2005) described 3 gene (aspartokinase *asp*, hippuricase *hip’O’* and 16S rDNA) based multiplex PCR for simultaneous detection of *C. jejuni* and *C. coli*. Workman et al., (2005) reported use of PCR to detect *Campylobacter spp.* in pet dogs and chicken meat. Few years back, four genetic markers were selected from the completely sequenced genomes of *C. jejuni* strains 81-176 (Hofreuter et al., 2006), RM1221, and NCTC 11168 using comparative genomics (Chaudhuri et al., 2008), and primers were designed for the detection of these markers, which were *ggt*, the γ-glutamyl transpeptidase gene; *dmsA* (*Cju34*), a subunit of the putative tripartite anaerobic *dimethyl sulfoxide* (*DMSO*) oxidoreductase (*DMSO/trimethylamine N-oxide reductase*) gene; *Cj1585c*, coding for a putative oxidoreductase; and *CJJ81176-1371*, a putative *serine protease* gene. A comparative analysis was made between cultural and PCR based assay for the detection of *C. jejuni* in food and faecal samples (Singh et al., 2011). Recently, a rapid, PCR-based method for the detection of *Campylobacter* from bootsocks was evaluated and found to be extremely sensitive and convenient, with results being available within 24 hours of sample collection (Merga et al., 2012).

### 2.1.4 Antibiotic resistance and sensitivity

Worldwide, there is continuous increase in number of antibiotic resistant *Campylobacter* strains. The increasing number of human infections by antimicrobial resistant strains of *C. jejuni* has made the clinical management of Campylobacteriosis increasingly difficult (Snelling et al., 2005).

Most of the *Campylobacter* strains have been found to be resistant to fluoroquinolone group of antibiotics, which are most frequently used antibiotics in human beings. Primary resistance to quinolone therapy in humans was firstly observed in early 1990's in Asia and in European countries like Sweden, the Netherlands, Finland, Spain and the UK (Allos, 2001). The resistance coincided with initiation of the administration of the fluoroquinolone antibiotic enrofloxacin to food
animals like broiler chicken (Altekruse et al., 1998; Allos, 2001). It has been reported that a large number of fluoroquinolone resistant Campylobacter infections in humans are the result of fluoroquinolone use in poultry house. Strongest evidence in support of this statement comes from a recent study of human Campylobacteriosis in Australia, where despite regular clinical use of fluoroquinolones and normal rates of Campylobacter infections, there are no confirmed cases of domestically acquired FQ-resistant Campylobacteriosis, because in Australia, fluoroquinolone are prohibited in poultry production (Price et al., 2005; Unicomb et al., 2009).

Fallon et al., (2003) studied antimicrobial resistant pattern of 78 C. jejuni strains to eight antibiotics by disc diffusion assay. The higher rates of resistance were recorded to ampicillin (35%) followed by 20.5% to tetracycline, 20.5% nalidixic acid and 17.9% to ciprofloxacin. Multi drug resistance against two or more antibiotics was observed in 30.7% of C. jejuni strains. Ge et al., (2003) determined antimicrobial susceptibilities of 378 Campylobacter isolates and found that resistance to tetracycline was highest (82%) followed by resistance to doxycycline (77%), erythromycin (54%) nalidixic acid (41%) and ciprofloxacin (35%).

In another study, isolates of Campylobacter spp. from conventional broiler and turkey farm, where antibiotics were routinely used and isolates from organic broiler farm where antimicrobials were never used, were compared for antimicrobial resistance pattern. It was found that less than 2% of isolates from organically raised poultry were resistant to fluoroquinolones, while 46% and 67% of isolates from conventionally raised broiler and turkey, respectively were resistant to fluoroquinolones. In addition, among isolates obtained from conventionally raised poultry than that of organically raised poultry, a high frequency of resistance to erythromycin, clindamycin, kanamycin and ampicillin was reported (Luangtongkum et al., 2006).

Few years back, a large scale survey was conducted; C. jejuni isolates in seven European countries during the period from 2004 to 2007 were screened for antimicrobial resistance pattern. The average tetracycline resistance varied between 23% and 33% while nalidixic acid resistance from 23% and 35% (EFSA 2010a). Majority of C. jejuni and C. coli isolates are intrinsically resistant to a number of antibiotics, including bacitracin, novobiocin, rifampicin, streptomycin B, trimethoprim, vancomycin and usually cephalothin. Various combinations of these
antibiotics are used as selective agents in media for the selective isolation of *Campylobacter* (Snelling et al., 2005; Ghazwan et al., 2009; Khanzadi et al., 2010). In a recent study on prevalence of antibiotic resistance among *Campylobacters* from various meat sources, the isolates were resistant to ciprofloxacin and nalidixic acid (32.7% each) (Rahimi et al., 2012a).

### 2.2 Listeria monocytogenes

In 1911, Professor G. Hulphers described a bacterium that caused necrosis of the liver in rabbits, and because of its characteristic affinity to liver, it was named *Bacillus hepatis*. Later, Pirie in 1940 changed the name to *Listeria monocytogenes*. In 1948, *L. monocytogenes* was placed under the same name in the 6th edition of Bergey's Manual of Determinative Bacteriology. The first reported isolation of *L. monocytogenes* from sheep was done in 1929 and the first reported human isolation was done in 1929 by Nyfeldt (Odjadjare, 2010). Until 1961, *L. monocytogenes* was the only recognized species in the genus *Listeria*. Currently, there are six species under the genus *Listeria*; *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, and *L. ivanovii* subsp. *ivanovii*, and *L. ivanovii* subsp. *londonsiensis* (Adzitey and Huda, 2010).

*Listeria monocytogenes*, a gram positive, facultative anaerobic and non-acid fast bacterium, is a non-spore forming rod that expresses a typical tumbling motility at 20-25°C. It is widely distributed throughout the environment and is considered hardy bacteria because of their ability to grow over a wide range of temperatures (1-45°C), pH (4.1-9.6) and osmotic pressures, which allows them to survive longer under adverse conditions (Adzitey and Huda, 2010).

*L. monocytogenes* causes several illnesses in human beings and animals; the annual incidence of human Listeriosis ranges from 1.6 - 6 cases per million people (Rocourt et al., 2000). Predominant clinical manifestations of human Listeriosis are meningitis, encephalitis, cerebritis and septicemia; hospitalization rate in this group is as high as 91% in untreated cases and mortality rate upto 30% in patients receiving therapy. In addition, endocarditis, pericarditis and pyogenic abscesses (local inflammatory reactions in various organs) have been documented. Immunocompromised patients, neonates and pregnant women are more susceptible to Listeriosis. However, the disease can also develop in seemingly healthy individuals.
Listeria species have been isolated from various plant and animal food products associated with many Listeriosis outbreaks; therefore, contaminated food are considered a primary source of transmission of infection in sporadic cases as well as outbreaks (Adzitey and Huda, 2010). Major outbreaks of human Listeriosis are on increase globally. The first investigated outbreak occurred in 1981 in Maritime Provinces of Canada where 7 adults and 34 prenatal cases of Listeriosis resulted due to consumption of contaminated coleslaw with case fatality rate of 44% (18 deaths). Later on, many food borne outbreaks (involving pasteurized, whole milk and milk products) of Listeriosis have been reported (Amagliani et al., 2004; Curtis, 2007). A case control study (Olsen et al., 2005) established in a multistate outbreak of L. monocytogenes infection linked to delicatessen Turkey meat. Cases of infection caused by these isolates were associated with 4 deaths and 3 miscarriages. In 2007 an outbreak of L. monocytogenes infections occurred associated with pasteurized milk from a local dairy, in Massachusetts (MMWR, 2008). This outbreak illustrated the potential for contamination of fluid milk products after pasteurization and the difficulty in detecting outbreaks of L. monocytogenes infections. Similarly, due to consumption of ‘Quargel’ cheese a multinational outbreak of Listeriosis due to L. monocytogenes has been reported in Austria and Germany. The outbreak comprised 14 cases (including five fatalities) infected by a serotype 1/2a Listeria monocytogenes, with onset of illness from June 2009 to January 2010. A second strain of L. monocytogenes serotype 1/2a spread by this product could be linked to further 13 cases in Austria (two fatal), six in Germany (one fatal) and one case in the Czech Republic, with onset of disease from December 2009 to end of February 2010 (Fretz et al., 2010).

In another case study of Listeriosis outbreak, in dairy cattle, with a high case mortality and acute death after onset of symptoms was investigated using gross pathology and bacteriologic approaches, including molecular characterization of a clinical Listeria monocytogenes isolate. Recently, in a herd of 315 animals, 9 animals showed clinical symptoms consistent with Listeriosis, including 3 animals that died within 2-4 days after acute onset of clinical signs, 4 animals that were euthanized, and 2 that survived (Bundrant et al., 2011). In Colorado, United States, Multistate
outbreak of Listeriosis caused by *L. monocytogenes*, linked to whole Cantaloupes from Jensen farms were reported (CDC, 2011).

### 2.2.1 Incidence of *L. monocytogenes* in foodstuffs

The organism has been found to be present in wide variety of dairy products. Out of these, cheese has been most extensively examined because of its known association with Listeriosis (Dumen et al., 2008; Latorre et al., 2009; Jami et al., 2010). *L. monocytogenes* has the ability to survive the manufacturing and ripening processes of various types of cheese. In raw milk the overall world wide incidence of *L. monocytogenes* appears to be less than 5%. The incidence rates have been reported to vary from country to country; 4 - 4.2% in USA, 1.3% in Canada, 1.0-1.5% in Scotland and 10% in UK. *L. monocytogenes* has also been reported from sheep and goat milk from Yugoslavia and United Kingdom (Wong, 2010; Latorre et al., 2009; Torres-Vitella et al., 2012).

A wide variety of meat samples (particularly their surfaces) have been found to be contaminated with *L. monocytogenes* (Nayak et al., 2010). However, the pathogen has also been detected in the deeper muscle tissues of beef, pork and lamb roasts. The organism was probably present in the muscle tissues at the time of slaughter (Beloeil et al., 2003; Sanna et al., 2010). The incidence of *L. monocytogenes* in fresh meat has been reported to vary from 0% to 94%, in contrast to 1-30% in ready to eat meat products (Asma et al., 2009; Nayak et al., 2010; Zhu et al., 2012). *L. monocytogenes* is one of the important etiology of bovine, cattles and ewes mastitis (Winter et al., 2004) and the excretion of the organism in the milk for a long period has been reported. Therefore, the dairy products have been particularly vulnerable to contamination of Listeria (Schoder et al., 2003; Kargar and Ghasemi, 2009; Jami et al., 2010; Yadav et al., 2010; Konosonoka et al., 2012).

Chicken seems to be heavily contaminated with *L. monocytogenes* as surveys show contamination rates ranging from 20 to 60% (Mahmood et al., 2003; Paszkowska et al., 2005; Stonsaovapak and Boonyaratanaakornkit, 2010). Studies show that, UK has reported the prevalence of *L. monocytogenes* from fresh chicken to be 60.0%, 50% in Taiwan, and 56.3% in US (Wong, 2010; Cook et al., 2012).

The prevalence in tropical fresh fish, smoked fish and sea foods has been reported to vary from 0 to 30% (Fuchs and Surendran, 1989; Salihu et al., 2008;
Stonsaovapak and Boonyaratanakornkit, 2010; Pinto et al., 2010). A relatively high incidence of the organism (6-36%) in ready to eat cold smoked salmon and cooked fish products have been reported (Embarek, 1994; Salihu et al., 2008). L. monocytogenes have been reported to be always associated with fresh or frozen fish and fish based sea foods (Salihu et al., 2008; Zarei et al., 2012).

2.2.2 Status of L. monocytogenes in India

In India, the first isolation of the organism was credited to Vishwanathan and Ayyar (Parihar, 2008) from sheep. Later, on several reports poured in indicating the occurrence of disease in many species of domestic animals. The organism was successfully isolated from Listeriosis cases of sheep and goats (Barbuddhe et al., 2000), buffalo (Nayak et al., 2010), and ready to eat meat products (Asma et al., 2009). Kaur et al., (2007) have also reported the isolation of organism from human cases. The prevalence of L. monocytogenes in fish and fish products has been also notified by several investigators (Karunasagar and Karunasagar, 2000; Parihar et al., 2008; Swetha et al., 2012).

In the last decade, the emergence of L. monocytogenes as an important food borne pathogen has prompted investigators to explore the incidence of this organism from foods in India. The presence of organism in foods of animal origin viz. sheep, goat, poultry and buffalo meat has been reported with the incidence rate below 10% (Barbuddhe et al., 1999, 2002; Vijayakrishna et al., 2000; Nayak et al., 2010; Saikia and Joshi, 2010). Studies on prevalence of L. monocytogenes in milk by Bhilegaonkar et al., (1997) and Barbuddhe et al., (1997, 2002) have revealed an overall incidence of L. monocytogenes to be 3-6% in cow and buffalo milk (Kalorey et al., 2008). However, the occurrence of this organism was very high (25%) in the cow milk collected from private dairy farm. Yadav et al., (2010) reported 3.5% of L. monocytogenes from mastitic milk of cows and buffaloes. The organism frequently recovered from the various types of cheese and is considered as most common source. Various literatures are available on incidence of L. monocytogenes from cheese and their products (Sharma et al., 2012).

In addition to the reports from the foods of animal origin, the presence of L. monocytogenes has also been reported from fresh vegetables such as coriander and spinach. (Kapoor et al., 1995; Dhanashree et al., 2003).
2.2.3 Detection of *L. monocytogenes* from foods

2.2.3.1 Conventional methods

Several cultural media and methods currently exist for detection and enumeration of *L. monocytogenes*. However, suitability of any specific method or medium is influenced by the purpose of analysis and the type of sample being analysed. Thus, the media devised for isolation of the organism from relatively uncontaminated clinical samples have been found to be of only limited use in the food and feeds, which often contain heavy load and wide variety of microflora (Amagliani et al., 2007). Isolation and identification of *L. monocytogenes* as well as limiting its presence and proliferation in foods have been the focal point of international investigations (Adzitey and Huda, 2010).

Among the earliest methods used for the recovery of *L. monocytogenes* from foods and environmental samples, was the use of cold enrichment and incubation at 4°C (Donnelly and Nyachuba, 2007). However, greatest drawback of this method was the long duration (upto 3 months) required for the identification of the organism. Later, on various other media providing semi anaerobic conditions were devised to overcome this lacunae *viz.* Tryptose Phosphate Broth with Furacin and Trypaflavine Nalidixic Acid Cyclohexamide Broth (TNCB). The incorporation of certain specific selective agents such as nalidixic acid and acriflavin into the enrichment media have been shown to reduce the time required for isolation of the organism (Wong, 2010).

In USA, two enrichment procedures are commonly used for detection of *L. monocytogenes*. These are enrichment broth described by Lovett et al., (1987) and University of Vermont (UVM) Broth (Asma et al., 2009) used by FDA and USDA, respectively. The FDA procedure has been designed specifically for dairy products, whereas the USDA procedure has been recommended primarily for meat and poultry products (USDA, 2002; Hitchins, 1998). The USDA enrichment procedure has been found to be slightly superior to the FDA procedure for detection of *L. monocytogenes* from foods (Navas et al., 2007). Several other broths such as Fraser Broth (Fraser and Sperber, 1988), Polymyxin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol Egg Yolk (PALCAM) Broth (Nayak et al., 2010) have also been developed for selective growth of *L. monocytogenes* from various samples.
Resuscitation procedures have also been standardized to recover the sublethally injured cells of *L. monocytogenes* due to heating or freezing, like modified UVM (MUVM) and Universal Pre-enrichment Broth. These media negate the detrimental effect of acidic pH. Various chemicals that either scavenge oxygen or degrade hydrogen peroxide are also incorporated into the enrichment broth (Anonymous 1996b, 2004b). Addition of catalase, a hydrogen peroxide scavenger and oxyrase to media at concentrations of 400 µg/ml and 0.01 unit/ml, respectively, have been found to be superior over other scavenging chemicals for resuscitating heat stressed *L. monocytogenes* (Barbuddhe et al., 2000; Zhou et al., 2010).

In addition to the above mentioned media, several useful media have been used for *L. monocytogenes* including Al-Zoreky Sandine *Listeria* medium (ASLM) (Al-Zoreky and Sandine, 1998), Dominguez-Rodriguez Agar (DRA) (Barbuddhe et al., 2000), Polymyxin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) agar (Dumen et al., 2008), modified Vogel Johnson agar (MVJ), MVJ Modified further (MVJM) (Barbuddhe et al., 2008), and Agar *Listeria* according to Ottaviani and Agosti (ALOA) (Reissbrodt, 2004), Oxford agar and Modified Oxford agar (Amagliani et al., 2004; Amagliani et al., 2007; Dumen et al., 2008; Pinto et al., 2010).

The current *L. monocytogenes* isolation methods are based on less time-consuming procedures, involving selective enrichment and selective plating. Standard methods for detection and enumeration of *L. monocytogenes* issued by FDA, IDF and ISO are widely used by food and environmental laboratories worldwide (Allerberger 2003; Jeyaletchumi et al., 2010). Since the 1950’s potassium tellurite, lithium chloride, nalidixic acid, acriflavin, polymyxin B, moxalactam and ceftazidime have been the most recognized selective agents in isolation of *Listeria* species (Donnelly and Nyachuba 2007). In addition, blood or chromogens can be used as selective agents or indicator substrates to differentiate *Listeria spp.* (Leclercq, 2004; Greenwood et al. 2005; Jeyaletchumi et al., 2010; Madajczak et al., 2012).

### 2.2.3.2 Immunoassays

Amongst the most commonly used immunological methods for rapid detection of *L. monocytogenes* from foods are immunofluorescent assays and enzyme immunoassays. The immunofluorescent assays including fluorescent antibody
technique (FAT), nanoparticles-based immunoassay, multiplexed sandwich chemiluminescent enzyme immunoassay and fluorescent bioconjugated nanoparticles probe immunoassay have been described (Khan et al., 1977; Magliulo et al., 2007; Yang et al., 2007; Wang et al., 2010) while Enzyme Linked Immunosorbent Assay (ELISA) have also been used frequently in large samples. Serological assays for epidemiological surveys and more sensitive and rapid detection protocols in ELISA are also in use for detection of *L. monocytogenes* (Barbuddhe et al., 2000; Boerlin et al., 2003; Palumbo et al., 2003; Wong, 2010).

### 2.2.3.3 Polymerase chain reaction (PCR)

In the first reported PCR for identification of *L. monocytogenes* the *hly* sequence was used (Bessesen et al., 1990). The positive reaction was confirmed by the agarose gel electrophoresis and by probing with fragment internal to the defined 606 base pair PCR product. This PCR was used to detect *L. monocytogenes* in water, whole milk and human cerebrospinal fluid. Another *inlA* gene polymorphism based PCR-RFLP has been described for screening potentially noninvasive *L. monocytogenes* strains. PCR-amplified *inlA* fragments were digested using the restriction endonuclease *Alu*I, and PCR-RFLP fragments were resolved by electrophoresis in 3.5% agarose gel. The position of restriction fragments was normalized using an internal DNA size marker (Lyautey et al., 2007). Listeriolysin O (LLO) has been shown to be a major virulence factor in *L. monocytogenes* and is not found in other *Listeria* spp. (Dumen et al., 2008). Therefore, Numerous PCR based studies or methods based on *listeriolysin* or *haemolysin* (*hly*) gene have been described for detection of *Listeria monocytogenes* (Rawool et al., 2007; Kargar et al., 2009; Brankica et al., 2011).

Multiplex PCR assays, where parts of the two various genes are amplified simultaneously, have also been evolved to detect *L. monocytogenes*. Rawool et al., (2007) developed multiplex PCR where parts of four different genes (*iap* gene, *hly* gene, *actA* and *plcA*) were amplified simultaneously. A multiplex PCR was attempted with two sets of primers, each comprising four virulence-associated genes. One of the set consisted of genes *plcA* encodes a phosphatidylinositol phospholipase C, *actA* encodes a metalloprotease and a surface actin polymerization protein, *hlyA* encodes a haemolysin (listeriolysin) and *iap* (p60 gene) and the second set consisted of *prfA* (regulatory gene), *actA*, *hlyA* and *iap*. All the primer sets were found to be specific
to the target genes as they specifically amplified the PCR products of those genes (Kaur et al., 2007). However, the protocol was used in clinical samples and the adaptability of this protocol in food samples was not determined. Jami et al., (2010) described another multiplex-PCR assay, using prs primers specific for putative phophoribosyl pyrophosphate synthetase gene that amplify a 370 bp fragment of the putative phophoribosyl pyrophosphate synthetase gene of Listeria spp. and LM lip1 primers specific for the prf A gene that amplify a 274 bp fragment of the prf A gene for the detection of L. monocytogenes from food samples. Thus numerous mPCR protocols for Listeria and Listeria spp. detection have been developed well (Kumar et al., 2012; Gambarin et al., 2012).

2.2.4 Antibiotic resistance in L. monocytogenes

Srinivasan et al. (2005) found that most L. monocytogenes were resistant to ampicillin and rifampicin. These results for penicillin, tetracycline, and chloramphenicol were similar with those reported by various investigators (Vela et al., 2001; Li et al., 2007; Arslan and Ozdemir, 2008). The antibiotic susceptibility patterns of L. monocytogenes were determined using the broth micro dilution method of the National Committee for Clinical Laboratory Standards (NCCLS) against various antibiotics. The resistance breakpoint concentrations used were 512 µg/ml for sulfomethoxazole, 4 µg/ml for ciprofloxacin, and 16 µg/ml for tetracycline. As expected, all 91 isolates were resistant to nalidixic acid. One isolate (1.1%) from smoked ham was found to be resistant to ciprofloxacin. Fifteen isolates (16%) exhibited tetracycline resistance. Sulfomethoxazole resistance was detected for 55 isolates (60%). No resistance was observed for any L. monocytogenes isolate to ampicillin, gentamycin, penicillin G, or trimethoprim (Shen et al., 2006).

In another study, a commercially made antibiotic susceptibility disk was used to check the antibiotic susceptibility pattern of Listeria monocytogenes isolates. The ampicillin resistance was profound among ten isolates which were randomly selected. The study showed that peflacin, tarivid and chloramphenicol remained most active against this bacterial pathogen (Nwachukwu et al., 2009). A study conducted in Botswana, antimicrobial susceptibility testing was performed on all the 57 confirmed L. monocytogenes isolates. Of these isolates, 31 (54.39%) were found to be resistant to one or more antibiotic. Resistance against penicillin G, sulphamethaxozole/trimethoprim, chloramphenicol, and tetracycline were observed in
42.11, 29.82, 28.30, and 22.81%, respectively, while no resistance was observed for fusidic acid, erythromycin, methicillin, ampicillin and cephalothin. Of all the food products tested, frozen cabbage and salads recorded the highest diversity of resistance patterns. From the resistance patterns, only one pattern (penicillin G and tetracycline) was common among all the food products that tested positive for _L. monocytogenes_ (Morobe et al., 2009). The antimicrobial susceptibilities of the 64 isolate of _Listeria spp_. were also examined by the standard disk diffusion method. In the study, _Listeria spp_. were found resistant to penicillin (6.3%), chloramphenicol (3.1%) and tetracycline (1.6%), but sensitive to amoxicillin, vancomycin, ampicillin, rifampicin and sulphamethaxozole (Stonsaovapak et al., 2010).

### 2.3 Salmonella

The genus _Salmonella_ was originally created by medical bacteriologist to include antigenically related organisms that gave rise to a certain type of illness in human and animals. Later, it became clear that _Salmonellae_ had many common biochemical characteristics. The genus _Salmonella_ includes not only the familiar pathogens of mammals but also other serotypes formerly considered to be biochemically aberrant types. It is now considered to comprise two species; _S. enterica_ and _S. bongori_ (Malorny et al., 2009).

The principal clinical syndromes associated with _Salmonella_ infection are enteric (typhoid) fever and gastroenteritis. Enteric fever is a protracted systemic illness that results from infection with exclusively human pathogens, _S. typhi_ and _S. paratyphi_. Clinical manifestations include fever, abdominal pain, transient diarrhoea or constipation, and occasionally maculopapular rashes. The pathological hallmarks of enteric fever are mononuclear cell infiltration and hypertrophy of the reticuloendothelial system, including the intestinal Payer’s patches, mesenteric lymph nodes, spleen, and bone marrow. Without treatment, mortality is 10%–15%. In contrast, many non-typhoidal _Salmonella_ strains, such as _S. enteritidis_ and _S. Typhimurium_, infect a wide range of animal hosts, including poultry, cattle, and pigs, and usually cause self-limited enteritis in humans. In certain inbred mouse strains, however, _S. Typhimurium_ infection produces an illness resembling enteric fever that serves as an experimental model for systemic _Salmonella_ infections. All _Salmonella_ infections begin with the ingestion of organisms from contaminated food or water (Miller and Pegues, 2000; WHO, 2012b).
Salmonellosis is an important food borne infective disease worldwide occurring mostly as sporadic cases, in families or as outbreaks. In 2006, a total of 160,649 confirmed cases of human Salmonellosis were reported in the EU (Anonymous, 2007c). The incidence was 34.6 cases per 100,000 populations, ranging in the countries from none to 235.9 cases per 100,000 populations. In the USA, during 1998-2002 Salmonellosis was reported as most common cause of food borne outbreaks and illnesses (Funk, 2009). However, it is generally estimated that the real number of infections are significantly higher. FoodNet, a US initiative as part of the CDC’s Emerging Infections Program, estimated 1.4 million annual cases of human non-typhoid Salmonellosis (Voetsch et al. 2004). Poultry and poultry products have been the most commonly implicated food to cause infection in humans (Loongyai et al., 2010). Although meat and meat products, milk and milk products, and water have also been associated with large outbreaks of Salmonellosis (Bansal et al., 2006; Bhunia et al., 2009; Nicolay et al., 2010). Dominguez et al., (2002) isolated seven various Salmonella serotypes from chicken meats from various retail stores in Spain. The most abundant serotype isolated was Salmonella subspecies enterica serovar Enteritidis, which agreed with the fact that this serotype was associated with outbreaks in humans in Spain in 1999. From 1985 to 1999, eggs and egg-containing foods were the primary vehicles of S. enterica serovar Enteritidis infection, having been implicated in 80% of the known sources of S. enterica serovar Enteritidis outbreaks reported to CDC (Patrick et al., 2004; Schroeder et al., 2005). A multistate outbreak of Salmonella enterica subspecies enterica serotype Typhimurium in 2003 was linked to the consumption of raw milk from a dairy in Ohio where raw milk was sold by the glass or in milkshakes (Mazurek et al., 2004). In 2008, during March and April, an outbreak due to S. enterica serotype Muenster, was associated with consumption of goat’s cheese in France (Van-Cauteren et al., 2009). Among the 21 cases of infection, 16 reported consumption of goat's cheese in the days prior to symptoms. The investigation incriminated goat's cheese from producer as being the most likely source of the outbreak. S. Muenster was isolated from both cases and the incriminated goat's cheese. In a large study conducted in Morocco during 2002-2005, a total of 0.91% prevalence of Salmonella was observed from various foods and sea foods (Bouchrif et al., 2009). In 2012 CDC reported 8 food borne outbreaks in United States due to various species or serovars of Salmonella (CDC, 2012a).
2.3.1 Incidences in foodstuffs

It has been described that large outbreaks are possible even if a small percentage of the implicated food products are contaminated and extremely low levels of *Salmonella* are present in food (Angulo et al., 2008). There are several reports of isolation of *Salmonella* from various meat (poultry, chicken, beef, cattle) and meat products like sausages and cooked meat (Nicolay et al., 2010; CDC, 2012b). The isolation of *Salmonella* has also been reported from milk and milk products (Bansal et al., 2006). Milk borne Salmonellosis has been associated with raw, heat treated or inadequately pasteurized milk (Olsen et al., 2004). Milk products like cheese also have been implicated in numbers of outbreaks (Fontaine et al., 1980; D’Aoust, 1985; Maguire et al., 1992; Synnot et al., 1998). Ice-creams (Nassib et al., 2003), milk powder (Deeb et al., 2010) and cream cakes (Solhan et al., 2011) have also been reported as source of *Salmonella*.

2.3.2 Status of *Salmonella* in India

Numerous reports on the prevalence of *Salmonella* in food samples in India have been accounted from a variety of foods including beef (Agarwal et al., 1999), poultry and poultry products (Selvaraj et al., 2010; Kumar et al., 2011), meat (Kumar et al., 2010), milk and milk products (Bansal et al., 2006; Singh, 2010). The presence of *Salmonella* has also been reported from various fast foods (Kakkar and Udiipi, 2002) and fruits, fruit juices and vegetables (Bansal et al., 2006; Kumar et al., 2010).

Isolation studies conducted in various parts of India revealed presence of *Salmonella* in man and animals (Saikia et al., 2002). The prevalence of *Salmonella* from fish (20% - 90%) has been observed in South India (Kumar et al., 2010). Rajendran and co-workers have reported water containing *Salmonella* along with *E. coli* and *Vibrio* from the tsunami affected villages and relief shelters. *Salmonella* Paratyphi B isolated from analysis of two well water samples (Rajendran et al., 2006). Interestingly, five serovars, *Salmonella* Newport, *Salmonella* Paratyphi B, *Salmonella* Teko, *Salmonella* Virchow and *Salmonella* Saintpaul were reported from ready-to-eat betel leaves (Paan) and in water used for soaking betel leaves in North Indian cities (Singh, 2006). Saroj et al., (2008) firstly attempted to assess the genetic diversity of *Salmonella enterica* serovar Typhimurium isolates from sprouts and fish samples from India. Recently, Gunasegaran et al., (2011) conducted study to
determine the prevalence of *Salmonella* in the curries and to characterize the antibiotic sensitivity of the isolates. Investigators have also reported *Salmonella* from various food sources including poultry and seafoods (Bhowmick et al., 2012).

### 2.3.3 Detection of *Salmonella* from foods

#### 2.3.3.1 Conventional isolation methods

To determine the presence of potentially low levels of *Salmonellae* in foods, methods involving a series of sequential cultural steps have been developed (Gilbert et al., 2010). Rappaport-Vassiliadis Soy Peptone (RVS) Broth has been used as a selective enrichment medium for the isolation of *Salmonella* from food, environment specimens and from faeces where malachite green acts as selective agent. The medium is not useful while suspecting *Salmonella* Typhi. Another liquid medium Tetrathionate Broth has been used for selective enrichment of *Salmonella* but it is found inhibitory to *S. Typhi*, *S. Pullorum*, and *S. Gallinarum*. The enzymatic digest of casein and enzymatic digest of animal tissue act as nitrogen, carbon, vitamins, and amino acids source. Selectivity is increased by the combination of Sodium thiosulphate and tetrathionate, which suppresses commensal intestinal organisms. Tetrathionate is formed in the medium upon addition of the iodine and potassium iodide solution. Organisms containing the enzyme tetrathionate reductase proliferate in the medium. Bile salts, as selective agents, suppress coliform bacteria and inhibit Gram-positive organisms. Calcium carbonate neutralizes and absorbs toxic metabolites (Global Salm-Surv, 2003; Neogen, 2004). Selenite Cystine Broth is based upon the formula of Selenite Broth described by Leifson, with the addition of cystine. The FDA has proposed Selenite Cystine Broth as an enrichment medium for detecting *Salmonella* in food materials. The AOAC, USP, and APHA have recommended Selenite Cystine Broth as a selective enrichment medium for *Salmonella spp*. The medium inhibits the growth of other Gram-negative bacilli. Sodium Selenite is the selective agent against Gram-positive bacteria and most enteric Gram-negative bacilli whereas L-cystine acts a reducing agent (Neogen, 2008).

Several solid agar media also have been described for the rapid and selective isolation of *Salmonella* from foods and clinical sources. Brilliant Green Agar (BGA) contains brilliant green exhibits indicative ability to ferment lactose and sucrose and has been used for isolation of *Salmonella* from food and clinical sources by various
investigators. Phenol red is the pH indicator, which changes from yellow to red at pH 6.8 - 8.4. Therefore, lactose negative and sucrose negative bacteria like *Salmonella* grow as red-pink, white opaque colonies surrounded by brilliant red zones in the agar (Global Salm-Surv, 2003).

Xylose Lysine Deoxycholate (XLD) agar also has been described for selective isolation of *Salmonella*. Sodium deoxycholate is the selective agent and phenol red is the pH indicator. *Salmonella* suspect colonies grow as red colonies with a black centre. The use of novobiocin has been described as more useful in selective isolation of *Salmonella* (Global Salm-Surv, 2003; Ruban et al., 2010).

Several workers described MacConkey’s agar for isolation of *Salmonella*, based on principal that bile salts and crystal violet largely inhibit the growth of the gram-positive microbial flora. Lactose and the pH indicator neutral red are used to detect lactose degradation. Lactose-negative colonies were colourless while lactose-positive colonies grow red surrounded by a turbid zone due to decrease in pH by the precipitation of bile acids (Rostagno et al., 2005). Hektoen Enteric agar is a moderately selective and differential medium and has been used by various investigators for the isolation of *Salmonella* from both clinical and nonclinical specimens. Hektoen Enteric agar was developed in 1967 by King and Metzger of the Hektoen Institute in order to increase the recovery of *Shigella* and *Salmonella* organisms. The selective nature of Hektoen Enteric Agar is due to the incorporation of bile salts in the formulation. These substances inhibit gram-positive organisms but can also be toxic for some gram-negative strains (Becton-Dickinson and Company, 2006). *Salmonella-Shigella* agar containing neutral red as pH indicator is a modification of the Deoxycholate Citrate agar described by Leifson is recommended for testing clinical and food specimens for the presence of *Salmonella spp.* and some *Shigella spp.* (Neogen, 2004; Ruban et al., 2010).

An international standard method of ISO (EN-ISO 6579:2002), by using various enrichment broths and agar media has been described for detection of *Salmonella*. The method involves non selective pre-enrichment in Buffered Peptone Water (BPW), selective enrichment in Rappaport-Vassiliadis Soy (RVS) Broth and Muller-Kauffmann Tetrathionate Novobiocin (MKTTn) Broth, plating on the selective solid medium Xylose Lysine Deoxycholate (XLD) agar and a second selective solid medium such as Brilliant Green agar and a final serological and
biochemical confirmation. The same procedure, without the MKTTn step, is used in the NMKL71 method, which is the standard method for Salmonella detection in the Nordic countries for food but also for feed. The Modified Semisolid Rappaport Vassiliadias (MSRV) method, Draft Annex D of EN-ISO 6579:2002 is based on migration of motile Salmonella through the selective medium. It has been shown in several studies of naturally infected or artificially contaminated food or faecal samples that MSRV is, in most cases, more sensitive than the standard methods. However, there are also studies showing that MSRV is less sensitive when compared to other enrichment media (Koyuncu and Haggblom, 2009).

2.3.3.2 Immunoassays

Immunoassays like FAT (Thompson and Wells, 1971), RIA (Ibrahim et al., 1986) and Enzyme immunoassays (Swaminathan and Ayres, 1980) have been used for detection of Salmonella in foods. But these assays are time consuming, less sensitive, specific and very complex (Blackburn, 1993). The serotyping of Salmonella involves the characterization of surface antigens, O and H antigens, according to the Kauffman-White scheme. O antigens are characterized by a slide agglutination assay (Patel, 2007). An immunological method referred as immuno-magnetic separation (IMS) and has been used extensively (Malorny et al., 2009). Enzyme-linked immunosorbent assays (ELISA) using various (combinations of) antigenic components of Salmonella spp. have been described (Thomas, 2010).

2.3.3.3 Polymerase chain reaction (PCR)

The first PCR assay for specific detection of Salmonella DNA has been published in early 90’s. The target was the oriC gene. Later, another PCR assay based on the invA gene including a comprehensive set of 630 Salmonella and 142 non Salmonella strains was performed. These primers showed the highest selectivity in a comparison study and were selected for a comprehensive international validation study. The PCR assay includes an internal amplification control (IAC, An IAC is a non-target DNA sequence present in the same sample reaction tube which is co-amplified simultaneously with the target sequence) which became mandatory for diagnostic assays. Many other targets and primer sets have been published differing in their target genes, detection limits, and accuracies. In some cases, the strain collections used for validation did not include all seven known subspecies of
Salmonella enterica and Salmonella bongori and lacked epidemiologically important isolates (Malorny et al., 2009). PCR technology is one of the most promising of the rapid microbiological methods for the detection and identification of bacteria in a wide variety of samples. Several polymerase chain reaction (PCR) methods for detecting Salmonella have been employed utilizing specific gene sequences as targets (Eid, 2010; Mollenkopf et al., 2011).

The various gene sequences which have been targeted for designing of primers and replicons are oriC (McCarthy et al., 2009) gene coding for DNA binding protein (Bej et al., 1994), agfA gene encoding aggregative fimbrae (Loongyai et al., 2010), ompC gene encoding outer membrane protein (Kwang et al., 1996), 16S rRNA (Trkov and Avgustin, 2003), hilA encoding hyperinvasive locus (Akbarmehr, 2010), invA (Shi et al., 2012), and spv encoding serovar associated plasmids (Amini et al., 2010). However the invA gene of Salmonella contains sequences unique to this genus and has been proved as suitable PCR target, with potential diagnostic applications (Shanmugasamy et al., 2011).

2.3.4 Antibiotic resistance in Salmonella spp.

The indiscriminate use of antibiotics to treat Salmonella infection has lead to the emergence of the multiple drug resistance towards Salmonella and other enteric pathogens. The study of the antimicrobial resistance of Salmonella spp. has been proposed as priority to the food industry mainly because most infections with antimicrobial resistant Salmonella are acquired by eating contaminated foods, especially foods of animal origins (White et al., 2001). These investigators isolated 13 various serotypes of Salmonella spp. from retail ground meats in the Washington D.C. area. They found that 84% of their isolates were resistant to at least one antimicrobial including ceftriaxone, the drug of choice for treating Salmonellosis in children. In an Irish study 9 various serovars of Salmonella were isolated from local raw retail chickens and imported chicken portions. Among the isolates, the most resistant to antimicrobials was Salmonella enterica serovar Typhimurium definitive type DT104, which can cause severe illness. The isolate was resistant to ampicillin, amoxyclav, sulfonamide, chloramphenicol, tetracycline, trimethoprim and streptomycin (Wilson, 2004). In both studies, ciprofloxacin was found effective against all isolates which is a fluoroquinolone, and are the drugs of choice for treatment of invasive gastrointestinal infections in many parts of the world (Moller et al., 2003). Chloramphenicol,
enrofloxacin, nalidixic acid, norfloxacin, gentamycin, trimethoprim, streptomycin, nitrofurantoin and co-trimoxazole were found to give positive response against *Salmonella* infection whereas ampicillin, tetracycline and furazolidone were found to be ineffective antibiotics (Gashe and Mpuchane, 2000; Rahman et al., 2002; Saikia et al., 2002).

However, fluoroquinolone resistant strains of *Salmonella* spp have been isolated and are becoming a great concern for public health (Guerra et al., 2003). A study carried out in Portugal, revealed that out of the isolated 10 serotypes of *Salmonella* from retail chickens, 50% were resistant to nalidixic acid (a quinolone) and enrofloxacin (a fluoroquinolone) (Antunes et al., 2002). The first outbreak of fluoroquinolone resistant *Salmonella* infection in the US was reported in 2001, this outbreak was caused by *S. enterica* serotype Schwarzengrund (Olsen et al., 2001). The US Food and Drug Administration (FDA) withdrew on September 12, 2005 its approval for use of fluoroquinolones in treating poultry (Doyle and Erickson, 2006). This action was based on the fact that use of fluoroquinolones in poultry promotes the development of fluoroquinolone resistant *Campylobacter* that can be transferred to humans and become a hazard to human health (CDC, 2006).

In the US, The National Antimicrobial Resistance Monitoring System (NARMS) for enteric bacteria was established as collaboration between CDC, FDA, and USDA to monitor antimicrobial resistance among food borne enteric bacteria isolated from humans, foods and animals in 1996 (FDA, 2006). Its latest published report in 2003 states that among retail meats, 27 serotypes of *Salmonella* were found. Among these, serovars Heidelberg, Saint Paul and Typhimurium were the most common. It was also found that these serotypes isolated were more resistant to streptomycin and tetracycline than any other antimicrobial (FDA, 2006). Kumar et al., (2010) reported 100% resistance towards methicillin, ciprofloxacin, nitrofurantoin, novobiocin, vancomycin from various foods (fish and meat) and fruits (banana, grapes) sources. In the same study, 100% sensitivity towards norfloxacin was also reported. The results suggested that ciprofloxacin resistant strains were emerging. In 2012, Indian Network for Surveillance of Antimicrobial Resistance Group (INSAR) reported antibiogram of *Salmonella enterica* serovar Typhi and *S. enterica* serovar paratyphi. In this report, a total of 3275 isolates of Salmonellae causing enteric fever were included. There were 2511 *S. enterica* serovar Typhi
strains (430 in 2008, 694 in 2009 and 1387 in 2010) and 764 *S. enterica* serovar Paratyphi A strains (311, 217 and 236 in 2008, 2009 and 2010 respectively) during the study period. These strains were isolated predominantly from blood culture. Few isolates from pus, stool and urine also were also included in the study (INSAR, 2012).

### 2.4 *Staphylococcus aureus*

The first normal description of genus *Staphylococcus* was given by Rosenbach in 1884. Later clear differentiation was described on DNA based composition by Silvestri and Hill (Peacock, 2010). *Staphylococcus aureus* has been recognized as one of the most common causes of food borne infections in most of the countries of the world (Zahoor and Bhatia, 2007). Common symptoms of Staphylococcal intoxication include nausea, vomiting, retching, abdominal cramping, sweating, chills, prostration, weak pulse, shock, shallow respiration, and subnormal body temperature. The most common symptoms experienced with Staphylococcal food poisoning are nausea, vomiting, diarrhoea and abdominal cramping. These symptoms usually appear within 1-6 h after consuming infected food. However, the onset and severity of illness are usually dependent on the amount of contaminated food eaten, the amount of toxin ingested and the susceptibility of individual to it. Growth of enterotoxigenic strains to equal or more than $10^5$ CFU per gram food is generally considered necessary to produce enough to induce illness. A wide variety of foods have been observed with the transmission of *Staphylococcus aureus*, including meat, eggs, bakery and dairy products. *S. aureus* usually contaminate the food during the handling stage after cooking (Abubakar et al., 2007; Addis et al., 2011; Torres-Vitella et al., 2012).

Staphylococcal enterotoxins (*se*) cause severe gastroenteritis (inflammation of the intestinal tract lining). Several antigenically various protein enterotoxins exist. To date, *se* A, B, C1, C2, C3, D, E, G, H, I, and J have been identified (Balaban and Rasooly, 2000). The effective dose of enterotoxins to cause intoxication in humans is unclear (Mitchell and Wong, 2003). Enterotoxin A is the most toxic and the one most commonly involved in Staphylococcal food poisoning outbreaks. Data from outbreaks involving enterotoxin A indicate that less than 1μg of toxin can result in illness (Medina et al., 2005). The most common toxins implicated in Staphylococcal food poisoning are *se*A to *se*J, which cause 95% of all outbreaks (Abubakar et al., 2007). *S. aureus* is characterized by gram-positive cocci, 0.5-1.5 μm in diameter, arranged singly, in pairs or in irregular ‘grape like’ clusters. They are found to be non-motile.
and non-spore forming. The Staphylococcal cell wall has been reported resistant to lysozyme and sensitive to lysostaphin, which specifically cleaves the pentaglycin bridges of Staphylococcus spp. Staphylococcus aureus found to grow in a wide range of temperatures (7°C to 48.5°C with an optimum of 30°C to 37°C), pH (4.2 to 9.3, with an optimum of 7 to 7.5) and sodium chloride concentrations up to 15% NaCl (Peacock, 2010).

Staphylococcus aureus is one of the most common causes of food borne infections in most of the countries of the world (Zahoor and Bhatia, 2007). Centers for Disease Control and Prevention (CDC) in 2007 showed that the number of MRSA infections in clinical environment was doubled nationwide. The annual number of the infected cases increased from approximately 127,000 cases in 1999 to 278,000 in 2005, and the annual death number also increased from 11,000 to more than 17,000 in the same time period. When comparing with patients without normal S. aureus infections, a 2004 study indicated that in the United States the patients who suffered from MRSA infections had approximately three times the length of hospitalization and three times the total cost (Noskin et al., 2005; Klein et al., 2007). In 1995, there was an estimated 13,989 cases of infectious intestinal illness caused by S. aureus in England and Wales (Mitchell and Wong, 2003). S. aureus has long been recognized as a pathogen in neonates. Staphylococcus aureus remains one of the most feared micro-organisms. Methicillin-resistant S. aureus (MRSA) has long been a common pathogen in healthcare facilities (Von Eiff et al., 2007; Agrawal et al., 2008). Methicillin resistant S. aureus (MRSA), commonly associated with hospital infections, caused a food borne outbreak when a delicatessen employee prepared coleslaw. Assays concluded that the employee carried the outbreak strain of MRSA, which was presumably transferred from a nursing home that the employee frequently visited (Jones et al., 2002). S. aureus has been reported transiently in the oropharynx (Smith et al., 2001), faeces (Arvola et al., 2006), nostrils and skin of human (Djouhri Bouktab, 2011; Verhoeven et al., 2012).

2.4.1 Incidences in foodstuffs

S. aureus does not cause illness by the bacteria itself unlike other common food borne pathogens such as E. coli or Salmonella. Instead, the enterotoxin produced by S. aureus can lead to food poisoning under certain conditions when the food has been contaminated by S. aureus (Dinges et al., 2000; Pereira et al., 2009). Since S.
*S. aureus* is ubiquitous food contamination with the pathogen is common (Kitai et al., 2005; Normanno et al., 2005; Pu et al., 2009). Various food products including meat products, eggs, dairy products, vegetables and other processed food such as sandwich fillings or chocolate eclairs are frequently contaminated by *S. aureus* and incriminated for *S. aureus* food poisoning (Normanno et al., 2005). Since *S. aureus* can colonize on various sites of animals such as pig or cow asymptomatically. Therefore, these animals may serve as reservoir and/or a transmission vehicle of spreading *S. aureus* and MRSA. Food products derived from the animals may be contaminated with *S. aureus* or MRSA during slaughtering and processing (Vanderhaeghen et al., 2010). An outbreak of Staphylococcal food poisoning by enterotoxin H was reported in raw milk used in the preparation of mashed potato (Jorgensen et al., 2005). While in another outbreak, enterotoxic *Staphylococcus aureus* was reported in raw milk from yaks and cattles in Mongolia (Tsegmed et al., 2007). *S. aureus* and MRSA have been isolated from meat or dairy products in several countries including United States, Netherlands, Italy, Australia, Japan and China (Kitai et al., 2005; Normanno et al., 2005; Pereira et al., 2009; Pu et al., 2009; Yan et al., 2012).

### 2.4.2 Status of *S. aureus* in India

Singh and Kulshrestha (1993) conducted a study on prevalence of *Staphylococcus aureus* in fish and fish products sea food samples purchased from various markets of India, *viz.* Bombay, Delhi, Calcutta, Bijnore and Bareilly. Isolates of *S. aureus* could be isolated from sea foods of Bombay, Bareilly and Delhi fish markets from fresh water fish, dried fish, dried prawn and fish eggs samples. Raghavan (2003) isolated *S. aureus* in the study of shrimp’s feeds during the screening of incidence of human pathogenic bacteria of commercial and farm-made feeds used in the various shrimp culture systems of south India. In India, Agrawal et al., (2008) isolated *S. aureus* from open fractures, bedsores and wounds clinically suspected to be infected, swabs from orthopaedic hospital, Jabalpur (M P). The report showed 18.91% incidence of *S. aureus*. A number of pathogenic and potentially pathogenic bacteria including *Staphylococcus* have been isolated from milk and fishes in India (Sugumar et al., 2001; Baruah et al., 2008; Khesar et al., 2008). A broad study on important bacterial food contaminants with respect to their isolations, biotyping, serotyping, pathogenicity in various common laboratory animals was undertaken in a quest to develop a reliable technique for their early detection by
Sharma and Singh (2008). In this investigation, a total of 939 samples from various parts of the State of Himachal Pradesh, India were collected including milk and milk products, meat (mutton, poultry, and chevon), and fish. The isolation of *S. aureus* was positive in all the food samples studied. It was highest in raw milk (41.35 per cent). Street vended fruit juices analyzed from Mumbai city, India showed absence of *S. aureus* in all the lime juice samples studied, whereas 50% of sugarcane juice and 20% of carrot juice showed contamination by *S. aureus* (Mahale et al., 2008). Saikia and Joshi (2010) reported *S. aureus* (20%) in a study of pathogenic contaminants in various parts of chicken meat collect from local meat markets of North-East India. Recently, in a study *S. aureus* was recovered from various food sources viz. meat, chicken waste, raw milk, bakery products etc. (Niveditha et al., 2012).

2.4.3 Detection of *Staphylococcus aureus* from foods

2.4.3.1 Conventional isolation methods

Various media have been developed to isolate *Staphylococci* in presence of other organisms. In the past, isolated colonies were required subsequent culturing to assay coagulase production. Several investigators described a variety of media selective for gram-positive bacteria (Columbia CNA, Remel, Lenexa, KS, U.S.) or media selective for MRSA (CHROM agar MRSA). On the CHROM agar plate, MRSA appeared as distinctly round, mauve-coloured colonies. Previously, CHROM agar has been shown to be a highly sensitive and specific media for the detection of MRSA (Malhotra et al., 2010). *Staphylococcus* medium 110 (SM110), also known as stone gelatin agar, has been widely used by various workers. SM 110 contains peptone as a source of carbon, nitrogen, vitamins and minerals. Yeast extract supplies B-complex vitamins which stimulate bacterial growth. Sodium chloride, in high concentration, inhibits most bacteria other than *Staphylococci* whereas lactose and D-mannitol serve as carbohydrates. Pathogenic *Staphylococci* (coagulase-positive *Staphylococci*) typically resist the high salt concentration and form colonies with a yellow-orange pigment. These organisms typically ferment mannitol and produce acid, and liquefy gelatine, producing zones of clearing around the colonies (Difco, 2010).

Potato Dextrose Agar (PDA) and Mannitol Salt Agar (MSA) are based on high salt concentration and mannitol fermentation. Chapman in 1945 developed the
Mannitol Salt Agar for isolation of *Staphylococci* which was based on the fact that *S. aureus* can withstand the osmotic pressure created by 7.5% NaCl, on the other hand this NaCl concentration inhibits the growth of most of the other gram-positive and gram-negative bacteria. Additionally, MSA contains mannitol and uses phenol red as a pH indicator (pK = 7.8) in the medium. At pH levels below 6.9, the medium is a yellow colour. In the neutral pH ranges (6.9 to 8.4), the colour is red; while above pH 8.4, the colour of phenol red is pink. When mannitol is fermented by a bacterium, acid is produced, which lowers the pH and results in the formation of a yellow area surrounding an isolated colony on MSA. A non-fermenting bacterium that withstands the high salt concentration would display a red to pink area due to peptone breakdown (Becton and Dickinson, 2005, 2010; Bachoon and Dustman, 2008; Neogen, 2008).

Baird-Parker in 1962, reported Baired Parker Agar (BPA) which was found to be particularly appropriate for the enumeration of coagulase positive *Staphylococci*. The growth of *Staphylococci* is favored by sodium pyruvate and glycine. Accompanying microflora is inhibited by lithium chloride, potassium tellurite (added extemporaneously), as well as a high concentration of glycine. Enrichment with egg yolk aids in identification by showing the action of lecithinase. The characterization of *Staphylococcus aureus*, black colonies on BPA due to the reduction of tellurite to telluride, surrounded by clear halos, may be complemented by the coagulase assay and optionally by the deoxyribonuclease and phosphatase assays (Neogen, 2010).

Few years back, Chromogenic media for *S. aureus* isolation was found to have unique property with high sensitivity of more than 95%, making it possible for accurate and easy identification of the coagulase positive *S. aureus* with purple colonies which in contrasts with other species exhibiting blue or colorless colonies (Manafi et al., 2004; Tauchi and Nakamura, 2005; Tavakoli, 2006). Later, several chromogenic media have been reported for the selective and specific isolation and identification of MRSA (Graveland et al., 2009; Nonhoff et al., 2009).
2.4.3.2 Immunoassays

Staphylococcal enterotoxins have been classified as members of the pyrogenic toxin superantigen family because of their biological activities and structural relatedness (Balaban and Rasooly, 2000; Dinges et al., 2000).

Double immuno-diffusion assays have been described for detection of capsular serotypes and surface polysaccharide serotype (Ma et al., 2004; Von Eiff et al., 2007). EIA has also been reported for detecting Staphylococcal enterotoxin (se). However, many drawbacks impair the development and use of these techniques (Schlievert, 2007). The ELISA test will not detect the other Staphylococcal enterotoxins, which partly explains some discrepancies that have arisen in the analysis of food extracts from Staphylococcal food poisoning outbreaks. Another drawback is the low specificity of some marketed kits, where false positives may occur depending on food components. Other interferences are associated with endogenous enzymes, such as alkaline phosphatase or lactoperoxidase (Hennekinne et al., 2010). Among them, only extraction followed by dialysis concentration has been approved by the EU to extract Staphylococcal enterotoxin from food (European community, 2007).

The presence of SEs in food using commercial EIA kits designed to detect seA to seE (Bennett, 2005; Hennekinne, 2007). Yeh et al., (2010) described the development of an immunoassay using an antibody-silver nanoparticles (Ab-AgNP) conjugate as a catalyst for the silver enhancement reaction. An EZ-Step MRSA rapid kit, a novel screening test for methicillin-resistant Staphylococcus aureus (MRSA) has been described to direct detection of penicillin-binding protein 2a from MRSA positive clinical blood culture samples (Shin et al., 2010). Some other immunoassay developed also by using various antibodies (Sandhu et al., 2012; Kumar et al., 2012).

2.4.3.3 Polymerase chain reaction (PCR)

Several studies have been conducted for designing primers and replicons targeting cap8A-B (Park et al., 2006), nuc gene (Brakstad et al., 1992), seA to seJ genes among S. aureus (Johnson et al., 1991; McLauchlin et al., 2000; Akineden et al., 2001; Rosec and Gigaud, 2002; Becker et al., 2003). Onasanya et al., (2003) reported genetic fingerprinting and phylogenetic diversity among Staphylococcus aureus isolated from human, pig, cow, soybean in Nigeria.
The PCR has also been applied in investigating prevalence of enterotoxin genes (seA-seE) in pathogenic *S. aureus* isolated from crude milk (Chapaval et al., 2006). Similar enterotoxin genes were also reported in cow, sheep and goat raw milk and sheep cheese (Tkaaikova et al., 2003). An investigation carried out to genotypically characterize *Staphylococcus aureus* isolated from bovine mastitis cases. They have used oligonucleotide primers that amplify genes encoding *coagulase* (*coa*), *clumping factor* (*clfA*), *thermonuclease* (*nuc*), *enterotoxin A* (*entA*), and the gene segments encoding the immunoglobulin *G* binding region and the *X* region of *protein-A* gene (*spa*) (Kalorey et al., 2007).

A multiplex PCR technique was described for the detection of major food borne pathogens like *Salmonella* spp., *Shigella* spp., *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* from ready-to-eat (RTE) food in Korea (Yun et al., 2008). Several other studies have also been conducted on simultaneous detection of *S. aureus* with other important food borne pathogens. PCR assays that can screen for 18 Staphylococcal enterotoxin (se) genes have been developed and the distribution of these genes was examined in enterotoxigenic coagulase-positive *Staphylococci*, including reference strains and isolates that have been collected from foods and Staphylococcal food poisoning outbreaks (SFPOs) in France since the 1980s. A total of 28 strains displaying multiple enterotoxin genotypes were selected for further mRNA expression kinetics studies (Hennekinne et al., 2010). Several other workers have described the use of PCR in detection of *S. aureus* from various human, animal and food sources (Parreira et al., 2009; El-Jakee et al., 2010; Sowmya 2012).

### 2.4.4 Antibiotic resistance in *S. aureus*

Before the first antimicrobial was discovered, the mortality of *S. aureus* bacteremia was over 80%, and more than 70% of patients developed metastatic infections (Lowy, 2003). In early 1940s, the introduction of penicillin significantly increased the survival rate of *S. aureus* infections. However, only 2 years later, the first penicillin resistant *S. aureus* was isolated in a clinical environment, and since then resistance pattern has been spreading from hospital to community (Chambers, 2001). Penicillin is inactivated by penicillinase (beta (β)-lactamase) (Lowy, 2003). Methicillin, as the first β-lactamase resistant penicillin, was used to treat *S. aureus* infection in 1961. The first methicillin-resistant *S. aureus* (MRSA) was identified in the United Kingdom in the same year. It appeared in the United States in 1981 among
intravenous drug users (Healy et al., 2004). Although there is no exact definition for MRSA currently, MRSA is commonly referred to as multidrug resistant \textit{S. aureus} or oxacillin-resistant \textit{S. aureus} (ORSA). It is a special group of \textit{S. aureus} that have acquired the ability to resist a large group of antimicrobials called the beta-lactams, including penicillin, methicillin, dicloxacillin, oxacillin and cefoxitin. In the United States, a significant increase in the number of MRSA infections has been observed during the past decade. A 2007 report from Centers for Disease Control and Prevention (CDC) showed that the number of MRSA infections in clinical environment have doubled nationwide. The annual number of the infected cases increased from approximately 127,000 cases in 1999 to 278,000 in 2005, and the annual death number also increased from 11,000 to more than 17,000 at the same time period (Klein et al., 2007).

The various isolates of \textit{S. aureus} show various characteristics features in their susceptibility/resistance towards antibiotics. Singh and Kulshrestha (1993) reported 100% sensitivity to polymyxin-B, gentamycin, cephalaxin, whereas, only one isolate from fish and fish products was resistant to erythromycin. Agrawal et al., (2008) reported sensitivity of \textit{S. aureus} isolates against cefoperazone, ticarcillin, tobramycin, ciprofloxacin, cloxacillin, cefotaxime, ceftriaxone. The isolates showed resistance towards amoxicillin, ampicillin, amikacin, penicillin, methicillin, nalidixic acid, azithromycin, nitrofurantoin, clindamycin. Recently, in a study in Ethiopia, \textit{S. aureus} isolates demonstrated a sensitivity of 100% to chloramphenicol followed by gentamycin (91.7%), kanamycin (88.9%) and streptomycin (86.1%). In contrast, isolates were highly resistant to penicillin (94.4%), trimethoprim and sulfamethoxazole (58.3%) and amoxicillin (36.1%) (Abera et al., 2010). Recently, some investigators reported higher percentage of multidrug resistant \textit{S. aureus} (34.8%) from various milk sources (Alian et al., 2012).

2.5 \textit{Escherichia coli} and Verotoxic \textit{E. coli}

Theobald Eschrich isolated an organism Bacterium coli, commonly now known as \textit{Escherichia coli} while attempting to isolate the etiologic agent of cholera in 1985 from faeces of neonates. Later, it was recognized as a predominant flora in the intestine of human and all warm blooded animals, causing distinct diarrhoeal and extra-intestinal diseases. Seven various types of pathogenic \textit{E.coli} have been reported viz. Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Enteroinvasive (EIEC),
Enteroaggregative (EAEC), Enterohaemorrhagic/Verotoxic/Shiga toxin producing \textit{E.coli} (EHEC/VTEC/STEC), Diffusely adherent (DAEC) and Cytolethal distending toxin (CDT) producing \textit{E.coli}. Among these VTEC/STEC is one of the important groups of \textit{E.coli} frequently implicated in food borne episodes. The term Verotoxic or Vero cytotoxigenic or Vero cytotoxin producing \textit{E.coli} (VTEC) is derived from the observation that these strain produce cytopathic effects on Vero (African green monkey kidney) cells. The name Shiga toxin producing or shiga toxigenic \textit{E.coli} (formerly shiga like toxin producing \textit{E.coli}) comes from the fact that one of the cytotoxins produced is identical to the \textit{shiga toxins} (Stx) produced by \textit{S. dysentriae}. A proposal to cover all these cytotoxins has been made to use the name \textit{shiga toxin} but the terms VTEC and STEC are interchangeable (Lahti, 2003; AFRC, 2007).

Haemorrhagic colitis or "ischemic colitis" is a distinct clinical syndrome that is represented typically with abdominal cramps and watery diarrhoea followed by a haemorrhagic discharge resembling lower gastrointestinal bleeding. Haemorrhagic uremic syndrome was firstly described as a distinct clinical entity. HUS is defined by a triad of features; acute renal failure, thrombocytopenia, and micro-angiopathic haemolytic anemia. HUS has been reported in a variety of clinical and epidemiological settings, and several various agents, including drugs, chemicals, toxins, and microbes. Thrombotic thrombocytopenic purpura (TTP) first described in 1924, TTP closely resembles HUS in its clinical-pathological features, but differs in showing prominent neurological signs and fever (Kok et al., 2001; Islam et al., 2008).

VTEC have most probably been present in the environment for many years as unrecognized causative agents of human morbidity and mortality, as in the outbreak caused by \textit{E. coli} O111 strains in USA in the 1950s (Belnap and O'Donnell 1955). The involvement of non-O157:H7 VTEC strains in human food borne outbreaks have increased dramatically in the past decade. In Australia and Argentina, non-O157 VTEC infections appear to be more common than O157:H7 infections, and in Germany non-O157 VTEC serotypes have replaced O157:H7 as the VTEC most commonly isolated in HUS cases. The most common non-O157 VTEC serogroups associated with human diseases are O26, O111, O128, and O103. Infections by O111: H in Italy and Australia are well documented, as infections by O111:H2 in Germany, by O111:H8 in the USA, by O103:H2 in France, the USA and Germany, by O145:H5 in Japan and by O104:H21 in the USA. In December 2005, an outbreak of HUS
associated to O26 VTEC contaminated unpasteurized Camembert cheese was recognized in France. More than 50 serotypes other than STEC O157 are capable of producing Haemorrhagic colitis and HUS (Hussien et al., 2001). In Italy, in 2005 six children infected with O26 VTEC developed HUS in Salerno Province and one of them died (Bonardi et al., 2005). The O:H serotypes of human VTEC isolates have been reported by various workers worldwide (Scotland et al., 1987, 1998; Smith et al., 1987; Bettelheim et al., 2003; Akter et al., 2005; Bugarel et al., 2012).

2.5.1 Incidences of Verotoxic E. coli in foodstuffs

Among the various groups of E. coli, Verotoxin producing E. coli (VTEC) have emerged as important food borne pathogens in humans (Mckee et al., 2003) because it can be transmitted through the consumption of contaminated food or food products, water, faecal-oral route. Verotoxin producing E.coli (VTEC) was first recognized as human enteric pathogen in 1982, when a major form of VTEC serotypes O157:H7 caused two major outbreaks of haemorrhagic colitis in Oregon and Michigan, USA (Wani et al., 2004). The outcome of several research works have suggested that the dairycattle among animals are an important reservoir of VTEC and thus, pose the risk of transmission of these potentially dangerous organisms to human beings through contaminated milk or meat. Milk becomes contaminated on the farm by various operations or by cross contamination at the processing centre (McKee et al., 2003; Karmali et al., 2010). In view of the pathogenic nature of this organism, the presence of this organism in food products including milk and meat products and water are of considerable public health significance. Numerous meat products like ground meat, hamburgers, fresh pork sausages and kababs etc. are reported to harbour E. coli with varying prevalence (Rathore et al., 2002; Mishra, 2004; Bhong, 2006; Dhanashree and Mallya, 2008; Manna et al., 2010). In Trinidad, 3.6% of bulk milk samples were found to contain Verotoxic E. coli. Of these E. coli O157 was isolated from 3 (19.1%) samples (Al-Charrakh and Al Muhana, 2010). Recently a study carried out on prevalence of STEC in meat and traditional dairy products in Iran. E. coli non-O157, E. coli O157:NM and E. coli O157:H7 were isolated from 14 samples (7%), 3 samples (1.5%) and 1 sample (0.5%) of the 201 dairy products, respectively (Rahimi et al., 2012b).

Verotoxic E. coli have been associated with Haemorrhagic urinary syndrome (HUS) and urinary tract infections have been reported. The serotype O157 had been
reported from the largest outbreak in UK occurring in central Scotland where more than 500 cases of infection and 21 deaths were reported. However, more than 100 non O157 serotypes of *E. coli* have been recognized as VTEC. (Scheutz et al., 2000; Willshaw et al., 2001; Gerber et al., 2002; Liptakova et al., 2005; Bonyadian, et al., 2010). The serotype O26:H11, O76:H7, O146:H5, Orough: H12, O118:H12 and O128ab:H2 have been reported in diarrhoeal or HUS patients from England (Willshaw et al., 2001). The serotypes O6,O8, O15, O20, O25, O27, O36, O44, O63, O78, O127, O146 and O167 have been reported as VTEC from human faecal samples associated with diarrhoea (Akter et al., 2005; Allison and Hanson, 2010; Rivero et al., 2010).

### 2.5.2 Status of Verotoxic *E. coli* in India

Several studies have reported varying degree of presence of Verotoxic *E. coli* in various foods in India. Various food products viz. kababs, khoa, paneer, burfi, peda have also shown to harbour Verotoxic *E.coli* (Rathore et al., 2002; Purushottam et al., 2003). In a study, 89.19% of 37 isolates from man, animals and foods of animal origin were found to be Verotoxic VCA; they also demonstrated the presence of virulent genes including *vt*1, *vt*2, *eae* and *hly*A gene (Rathore, 2000). The organism has been isolated from fruits, vegetables (Singh et al., 1996b), variety of ready to eat foods including sausage, kababs, cheese, ice cream, burfi, peda, khoa and sweet etc. (Ahmed and Sallam, 1991; Singh et al., 1996a; Rathore et al., 2002), various raw meats viz. chicken, mutton and beef (Banerjee et al., 2001; Chattopadhya et al., 2003), and water (Shrivastava et al., 2004; Hamner et al., 2007; Ram et al., 2008). Several investigators have also reported VTEC from human diarrhoeic samples (Nair, 2002; Sehgal et al., 2008). In recent years, the serotypes O1, O2, O3, O4, O6, O10, O12, O15, O18, O20, O21, O23, O25, O48, O60, O73, O84, O86, O101, O111, O138, O139, O140, O148, O152, O153, O156, O158, O159, O160, O168, O169, and O172 have been reported from stool and meat samples (Dhanashree and Mallya, 2008; Allison and Hanson, 2010). Most of them found to be pathogenic to human and animals.
2.5.3 Detection of Verotoxic *E. coli*

### 2.5.3.1 Conventional methods

EHEC is a subgroup of Verotoxin producing *E. coli* and O157: H7 strain is the prototype of this subgroup. Detection of *E. coli* O157: H7 is based on sorbitol fermentation and glucoronidase activity. *E. coli* O157: H7 neither ferments sorbitol within 24h nor shows fluorescence under UV light when cultivated on a medium containing MUG (4-methyl umbeliferyl glucoronidase). Thus, culture methods for its detection depend on non-fermentation of sorbitol and a glucoronidase negative activity. Sorbitol MacConkey agar supplemented with MUG (SMAC-MUG) can be used for initial screening of the pathogen after enrichment of samples. However, this isolation protocol does not give clear idea about the presence of several non O157:H7 Verotoxic *E.coli* (Aldus et al., 2003). For the isolation and confirmation of VTEC, conventional isolation of *E. coli* on MacConkey agar/EMB agar and subsequent confirmation by Verotoxicity assay or immunoassays is most commonly used. Enrichment broth media containing selective antibiotics and chemicals such as cefixime, novobiocin, tellurite, cefsulodin etc. have been used for enrichment prior to plating on the solid medium (AFRC, 2007).

### 2.5.3.2 Tissue culture assay

Vero cytotoxicity assay (VCA) i.e. cytopathic effect on Vero cells is one of the most commonly used methods to identify Verotoxic *E. coli*. Since, the pioneering work in 1977 by Konowalchuk and co-workers, faecal and other supernatant preparations have been successfully applied to Vero cells and the characteristic cytopathic effects were observed (Karmali et al., 1983). The ultimate assay to detect the Vero cytotoxicity of a potential toxin known as Vero cytotoxin is whether it affects Vero cells, with the production of specific cytotoxic effects. Studies have confirmed the value of direct assaying for Vero cell cytotoxicity (Cermelli et al., 2002). Several workers have used toxin assay in microtitre tissue culture plates for detection of cytotoxic effect (Akter et al., 2005; Al-Charrakh and Al-Muhana, 2010).

### 2.5.3.3 Immunoassays

Over the years, a number of immunological methods (enzyme immunoassays, colony blot and passive agglutination assays) for detection of VTs have been developed. The eight various EIA/ELISA assays for VT detection were developed and
commercially distributed (Scheutz et al., 2001). During the past decade, a number of Enzyme-linked immunosorbent assay (ELISA) have been developed including commercial kits. Lipopolysaccharide (LPS) antigen of various serogroups of VTEC have been used for detection of specific antibodies in patients serum but it has limited application as antigen is serogroup specific (Bhong, 2006). Numerous isolation methods using antibodies specific to particular VTEC serogroups are available. Immunomagnetic separation (IMS) method also has been used for the recovery of target cells by using paramagnetic beads. There is no standardized protocol for other VTEC, however, IMS has been shown to be useful in recovery of specific serogroups from food and faecal samples (AFRC, 2007).

Ge et al., (2002) combined PCR with ELISA to develop a sensitive and specific method for VTEC detection in foods. Parreira and Gyles, (2002) to identify avian VTEC, developed a similar assay. Premier-EHEC (Meridian Bioscience, Inc., Cincinnati, OH, USA) ELISA detects VT in specimens by an immunological method and has been successfully used to screen eight specimens for VTEC (Kirchgatterer et al. 2002; Klein et al., 2002). The use of nucleic acid lateral flow immunoassays (NALFIA) as biosensors for the detection of verotoxin gene among Shiga toxin producing E. coli has also been reported. In NALFIA, nucleic acids can be captured on the lateral flow test strips either in an antibody-independent or antibody-dependent manner (Noguera et al., 2011). Recently, Clotilde et al., (2011) used the luminex technology that detects multiple analytes in a single 50 ml sample. The immunoassay was developed for simultaneously serotyping E. coli O157 and detecting stx1 and/or stx2 using commercially available monoclonal antibodies coupled to carboxylated magnetic microbeads.

2.5.3.4 Polymerase chain reaction (PCR)

Polymerase chain reaction can be utilized to detect the virulence associated genes of pathogenic E. coli. Crude lysates or DNA extracts from single colony, mixed broth cultures or direct extract of faeces or foods can be used to detect bacterial DNA. PCR technique has been utilized for the detection of verotoxin (vt1 and vt2) genes, eae genes, E-hly genes etc. from various types of foods, faeces and clinical samples (Gannon et al., 1992; Schmidt et al., 1995; Sandhu et al., 1996; Bonnet et al., 1998; Ziebell et al., 2002; Chattopadhyya et al., 2003; Yadav et al., 2007; Ramamurthy et al., 2008; Bonyadian et al., 2010).
PCR combined with ELISA (Ge et al., 2002) to develop a sensitive and specific detection method for VTEC in foods. The principle of the method is the incorporation during the PCR amplification process, of dioxygenic labelled dUTP and a biotin-labelled primer specific for the vt genes. The labelled PCR products, bound to streptavidin-coated wells of a microtitre tray through the biotin, are then detected by an ELISA technique. This was also used to identify avian VTEC (Parreira and Gyles 2002). PCR was found to be rapid, sensitive and highly specific method to detect vt gene within in a working day (Nazmul, 2008). The assay has been described to be promising, reliable and rapid method for detection of \textit{E. coli} from milk and milk products (Purushottam et al., 2003; Al-Charrakh and Al-Muhana, 2010).

2.5.4 Antibiotic resistance among Verotoxic \textit{E. coli} \\

The emergence of resistant bacterial strains against antimicrobial agents has now become one of the major public health concerns in both in developed and developing countries in recent years. The problem is aggravated in developing countries due to uncontrolled use of antibiotics (Amabile-Cuevas, 2010). \textit{E. coli} strains were found to be sensitive to ciprofloxacin, gentamycin, amikacin, chloramphenicol, nalidixic acid, enrofloxacin (Dubey et al., 2001; Kumari et al., 2002; Chattopadhyay et al., 2003; Al-Charrakh and Al-Muhana, 2010). Whereas, resistant to ampicillin, tetracycline (Bettelheim et al., 2003; Chattopadhyay et al., 2003; Al Charrakh and Al Muhana, 2010) and cephalaxin (Sharma et al., 1995). Raji et al., (2006) reported that out of 11 \textit{E. coli} isolates, 81.8% were resistant to amoxyclav, 54.5% to neomycin and 36.3% to sulphamethaxole. In another study by Dhanashree and Mallya (2008) 77.5%, 55.0%, 32.5% and 17.5% resistance towards the ampicillin, cephoxitin, cefuroxime and cephotaxime respectively was reported from meat samples.

2.6 Enumeration of microbial load in food samples \\

In most developing countries, food safety systems are dysfunctional and, despite increasing concern from consumers, India is not exception. A recent internet discussion pointed out that regulation isn’t working, adulteration is widespread, testing inadequate, corruption rampant, rules not effective or followed and there are major hygiene and safety problems in all areas of food production and retailing (Solution Exchange, 2008). Therefore, intermittent microbial analysis and constant
monitoring are necessary and continue to produce hygienic and wholesome meat, milk and milk products to ensure safe public health.

The various investigators are observing microbiological quality of beef, raw chicken, fish, milk and milk products, regularly. Mukhopadhyay et al., (2008) reported the total aerobic plate count (TAPC) and coliform count (TCC) from beef and chevon. The TAPC ranged from log₁₀ 5.4 to 8.5 cfu g⁻¹ whereas TCC ranged from log₁₀ 3.0 to 7.5 cfu g⁻¹ in various samples of beef. Kumar et al., (2011) assessed the microbial quality of ready to eat meat product sold in Prabhani city, India. The author reported total aerobic count of 2-4.0 x10⁶ cfu g⁻¹ whereas 1.8-5.3x10⁶ cfu g⁻¹ faecal coliform counts. The aerobic colony counts in meat pies usually prepared from beef ranging from 0.2 x10⁹ cfu g⁻¹ to 8.0 x10⁹ cfu g⁻¹ observed by Falooa et al., (2011). In this study, mean count of total coliform was obtained as 7.6 x10⁶ cfu g⁻¹. Several other investigators also investigated the various products derived from beef (Kandeepan and Biswas, 2007; Selvan, et al., 2007)

Yousuf et al., (2008) reported the total aerobic plate count (TAPC) and coliform count (TCC) from shrimp and prawn. The TAPC and TCC ranged from 2.04 x 10² – 4.5 x 10² cfu g⁻¹ and 5.4 x 10² - 8.5 x 10⁵ cfu g⁻¹ respectively. Jha et al., (2010) reported total plate count from 49 x10³ cfu g⁻¹ to 139 x 10³ cfu g⁻¹ in various fishes. In the same study, author also reported total coliform count of 39 x10³ cfu g⁻¹ to 140 x 10³ cfu g⁻¹. Prabakaran et al., (2011) assessed the microbiological quality of a fish processing plant. They reported total plate count of <10 cfu g⁻¹ to 65 x 10² cfu g⁻¹.

Ruban and Fairoze (2011) reported total viable count (TVC) from 3.8±0.01 to 5.3±0.07 in raw chicken meat processed in different conditions. The microbial profile of broiler meat was observed by Javadi and Safarmashaei (2011). They assessed the total bacterial count and coliform count of log₁₀ 5.06 cfu g⁻¹and log₁₀ 4.03 cfu g⁻¹ respectively.

In a study, on microbiological monitoring of raw milk and yoghurt, the aerobic bacterial counts ranged between 5.0 x 10⁴ to 2.2 x 10⁶ cfu ml⁻¹ from raw milk and yoghurt samples were reported (El-Diasty and El-Kaseh, 2008). Lingathurai and Vellathurai (2010) found mean total plate count (TPC) of 12.5x10⁶ cfu ml⁻¹ from fresh cow milk collected from different farms. Batool et al., (2012) reported average total viable count (TVC) of log₁₀ 4.5 cfu ml⁻¹ from raw milk whereas log₁₀ 1.3 cfu ml⁻¹
from pasteurized milk. Investigators also assessed coliform count which was found \( \log_{10} 3.5 \text{ cfu ml}^{-1} \) in raw milk samples.

The several health agencies have established the microbial criteria of important bacterial pathogens including \textit{Campylobacter}, \textit{E. coli}, \textit{L. monocytogenes}, \textit{Salmonella} and \textit{S. aureus} for various foods in developed (Gilbert et al., 2000) and developing (FSSAI, 2011) countries. However these pathogens are found involved in various food and food products from all over the world including India (Jorgensen et al., 2002; Luber and Bartelt, 2007; Rosenquist et al., 2006; Rindhe et al., 2008).