CHAPTER II
LITERATURE REVIEW

2.1 Quarantine of export fresh vegetables

Thailand exports fresh vegetables to Japan, USA, United Kingdom, Germany, Australia and other countries (Katenil, 2000), approximately 3,300 million Baht/year and tendency seems to be increased in the future (Department of Foreign Trade, 2007). The important export vegetables are asparagus, onion-garlic, baby corn and okra, which were increased up to 39.26%, 14.56%, 65.90% and 15.99%, respectively in 2007 (Manager Weekly, 2007). Norway has prohibited the importing of fresh vegetable (such as peppermint, lemon grass, coriander, stink weed, sweet basil, ka-yaeng, acacia and puk-preaw) in July 2005 because of contamination of *E. coli* and *Salmonella* spp. (Jonngao, 2005). European Union countries had warned and rejected the importing of fresh vegetable in October – December 2006 and January-February 2007, because of *Salmonella* spp. contamination in vegetable, *i.e*., kaffir leaves, sweet basil, yard-long bean, coriander, peppermint, lemon grass and snake eggplant (Office of Commercial Affair, 2007). In 1998 to 2003, vegetables and fruits exported to USA detained at 439, 293, 321, 332, 85 and 63 times/year, respectively (Knowledge Network Institute of Thailand, No date). In 26 May - 8 July 2005, the products had been detained and emergency-warned again in many countries such as Finland, Iceland, Norway, Sweden and United Kingdom at 11, 10, 14, 1 and 8 times/country, respectively (Department of Agriculture, 2008). In 2009, a notification that detected contamination of *E. coli*, *Salmonella* and *Campylobacter* totaling 11 times from Norway, which contaminated in the morning glory, *Acacia pennata* (cha-om) leaves, cauliflower, baby corn, asparagus, neem leaves, okra, banana leaves (Neawna, 2010). Because of vegetable rejection, Thailand has to control the quality of export products to Norway, Iceland and European Union countries. Kinds of vegetable which have to be detected for contamination of *E. coli* and *Salmonella* spp. were coriander (*Coriandrum sativa* Linn.), stink weed (*Eryngium foeidum* Linn.), holy basil

(*Ocimum sanctum* Linn.), sweet basil (*Pcimum basilicum* Linn.), ka-yang (*Limophila aromatica* (Lamk) Merr.), peppermint (*Mental cordifolia* Opiz.), puk-peaw (*Polygonum odoratum* Lour.), green shallot (*Allium cepa* var. aggregatum), Chinese celery (*Apium graveolens* Linn.), chinese chive leave and flower (*Allium tuberosum* Roxb.), Acacia pennata (*Acacia pennata* (L.) Willd. subsp. insuavis Nielsen), lemon glass (*Cymbopogon citratus* (DC.) Stapf), convolvulus (*Ipomoea aquatica* Forsk.), water fern (*Marsilea crenata* Presl.), water mimosa (*Neptunia oleracea* Lour.), centella (*Centella asiatica* Urban), Piper sarmentosum (*Piper sarmentosum* Roxb.), amaranth (*Amaranthus lividus* Linn.), yard long bean (*Vigna sesquipedalis* Koern), asparagus (*Asparagus officinalis* L.), chili pepper (*Capsicum frutescens* Linn.) and Ceylon spinach (*Basella alba* Linn.). These vegetables have to pass the plant quarantine and they have to be certified before exporting by Department of Agriculture. The certified vegetables for export must contaminate with *E. coli* less than 100 CFU/g and not detected in 25 g for *Salmonella* spp.

2.2 Characteristic and pathogenesis of *E. coli*

2.2.1 Biological and biochemistry

E. coli is now classified as part of domain: Eubacteria, class: gamma-proteobacteria, order: Enterobacteriales, family: Enterobacteriaceae, genus: *Escherichia* and species: *coli*. It is a Gram-negative, facultative anaerobic and non-spore forming. Cells are typically rod-shaped and are about 2 micrometers (μm) long and 0.5 μm in diameter, with a cell volume of 0.6-0.7 μm^3 (Figure 1). Optimal growth of *E. coli* occurs at 37°C (98.6°F) but some laboratory strains can multiply at temperatures of up to 49°C (120.2°F) (Fotadar *et al*., 2005). Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen and amino acids, and the reduction of substrates such as oxygen, nitrate, dimethyl sulfoxide and trimethylamine N-oxide. Strains that possess flagella can swim and are motile. The flagella have a peritrichous arrangement (Darnton *et al*, 2007).

E. coli and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process led to the spread of the gene encoding shiga toxin from *Shigella* to *E. coli* O157:H7, carried by a bacteriophage.

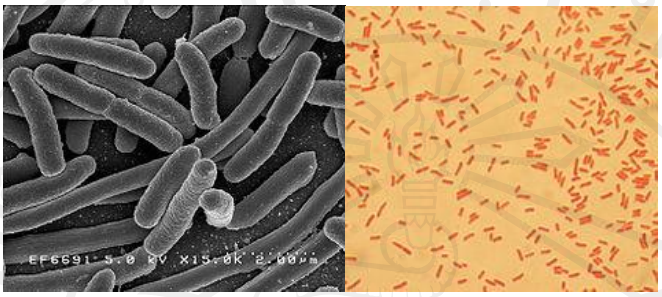

Figure 1 *E. coli* bacteria, the most prevalent Gram-negative flora in the intestine

Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O_2. Under anaerobic conditions, it will grow by means of fermentation, producing characteristic "mixed acids and gas" as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extra intestinal (aerobic or anaerobic) habitats.

E. coli can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a unicellular organism. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or it can stop swimming and grow fimbriae that will specifically attach it to a cell or surface receptor. In response to change in temperature and osmolarity, it can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances. With its complex mechanisms for regulation of metabolism, the bacterium can survey the chemical contents in its environment in advance of synthesizing any enzymes that metabolize these compounds. It does not

wastefully produce enzymes for degradation of carbon sources unless they are available, and it does not produce enzymes for synthesis of metabolites if they are available as nutrients in the environment.

E. coli is a consistent inhabitant of the human intestinal tract, and it is the predominant facultative organism in the human GI tract; however, it makes up a very small proportion of the total bacterial content. The anaerobic *Bacteriodies* species in the bowel outnumber *E. coli* by at least 20:1. However, the regular presence of *E. coli* in the human intestine and feces has led to tracking the bacterium in nature as an indicator of fecal pollution and water contamination. As such, it is taken to mean that wherever *E. coli* is found there may be fecal contamination by intestinal parasites of humans.

2.2.2 Pathogenesis of *E. coli*

Over 700 antigenic types (serotypes) are recognized based on O, H and K antigens. Serotyping is still important in distinguishing the small number of strain that actually cause disease. *E. coli* is responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal disease (gastroenteritis). These three diseases depend on a specific array of pathogenic (virulence) determinants (Todar, 2007).

2.2.2.1 Urinary tract infections

Uropathogenic *E. coli* cause 90% of the urinary tract infections (UTI) in anatomically-normal, unobstructed urinary tracts. The bacteria colonize from the feces or perineal region and ascend the urinary tract to the bladder. Bladder infections are 14-times more common in females than males by virtue of the shortened urethra. The typical patient with uncomplicated cystitis is a sexually-active female who was first colonized in the intestine with a uropathogenic *E. coli* strain. The organisms are propelled into the bladder from the periurethral region during sexual intercourse. With the aid of specific adhesions, they are able to colonize the bladder.

The adhesion that has been most closely associated with uropathogenic *E. coli* is the P fimbria (or pyelonephritis-associated pili [PAP]). The letter designation is derived from the ability of P fimbriae to bind specifically to the P blood group antigen which contains a D-galactose-D-galactose residue. The fimbriae bind not only to red

cells but to a specific galactose disaccharide that is found on the surfaces uroepithelial cells in approximately 99% of the population.

The frequency of the distribution of this host cell receptor plays a role in susceptibility and explains why certain individuals have repeated UTI caused by *E. coli*. Uncomplicated *E. coli* UTI virtually never occurs in individuals lacking the receptors

Uropathogenic strains of *E. coli* possess other determinants of virulence in addition to P fimbriae *E. coli* with P fimbriae also possess the gene for Type 1 fimbriae and there is evidence that P fimbriae are derived from Type 1 fimbriae. In any case, Type 1 fimbriae could provide a supplementary mechanism of adherence or play a role in aggregating the bacteria to a specific manosyl-glycoprotein that occurs in urine.

Uropathogenic strains of *E. coli* produce siderophores that probably play an essential role in iron acquisition for the bacteria during or after colonization. They also produce hemolysins which are cytotoxic due to formation of transmembranous pores in host cells. One strategy for obtaining iron and other nutrients for bacterial growth may involve the lysis of host cells to release these substances. The activity of hemLsins is not limited to red cells since the alpha-hemolysins of *E. coli* also lyse lymphocytes and the beta-hemolysins inhibit phagocytosis and chemotaxis of neutrophils.

Another factor thought to be involved in the pathogenicty of the uropathogenic strains of *E. coli* is their resistance to the complement-dependent bactericidal effect of serum. The presence of K antigens is associated with upper urinary tract infections, and antibody to the K antigen has been shown to afford some degree of protection in experimental infections. The K antigens of *E. coli* are “capsular” antigens that may be composed of proteinaceous organelles associated with colonization (e.g., CFA antigens), or made of polysaccharides. Regardless of their chemistry, these capsules may be able to promote bacterial virulence by decreasing the ability of antibodies and/or complement to bind to the bacterial surface, and the ability of phagocytes to recognize and engulf the bacteria cells. The best studied K antigen, K-1, is composed of a polymer of N-acetyl neuraminic acid (sialic acid), which besides being antiphagocytic, has the additional property of being an antigenic disguise.

2.2.2.2 Neonatal meningitis

Neonatal meningitis affects 1/2,000-4,000 infants. Eighty percent of *E. coli* strains involved synthesize K-1 capsular antigen (K-1 is only present 20-40% of the time in intestinal isolates). *E. coli* strains invade the blood stream of infants from the nasopharynx of GI tract and are carried to the meninges. The K-1 antigen is considered the major determinant of virulence among strains of *E. coli* that cause neonatal meningitis. K-1 is a homopolymer of sialic acid. It inhibits phagocytosis, complement, and responses from the host's immunological mechanisms. K-1 may not be the only determinant of virulence; however, as siderophore production and endotoxin are also likely to be involved. Epodemilogic studies have shown that pregnancy is associated with increased rates of colonization by K-1 strains and that these strains become involved in the subsequent cases of meningitis in the newborn. Probable, the infant GI tract is the portal of entry in the blood stream. Fortunately, although colonization is fairly common, invasion and the catastrophic sequelae are rare. Neonatal meningitis requires antibiotic therapy that usually includes ampicilin and a third-generation cephalosporin.

2.2.2.3 Intestinal disease caused by *E. coli*

As a pathogen, *E. coli* of course, is best known for its ability to cause intestinal diseases. Five classes (virotypes) of *E. coli* that cause diarrhea diseases are now recognized: Enterotoxigenic (ETEC), Enteropathogenic (EPEC), Enteroinvasive (EIEC), Enterohaemorrhagic (EHEC) and Enteroaggregative (EAEC). Each class falls within a serological subgroup and manifests distinct features in pathogenesis. Each of class has characteristics and virulence details difference (Table 1).

2.2.3 Epidemiology of gastrointestinal infection

Transmission of pathogenic *E. coli* often occurs via faecal-oral transmission (Gehlbach *et al*., 1973). Common routes of transmission include: unhygienic food preparation, farm contamination due to manure fertilization, irrigation of crops with contaminated grey water or raw sewage (Heaton and Jones, 2008), feral pigs on cropland, or direct consumption of sewage-contaminated water (Chalmers *et al*., 2000). Dairy and beef cattle are primary reservoirs of *E. coli* O157:H7 and they can carry it asymptomatically and shed it in their faeces (Bach *et al*., 2002a).

Food products associated with *E. coli* outbreaks include raw ground beef, raw seed sprouts or spinach, raw milk, unpasteurized juice, unpasteurized cheese and foods contaminated by infected food workers via faecal-oral route.

According to the U.S. Food and Drug Administration, the faecal-oral cycle of transmission can be disrupted by cooking food properly, preventing cross-contamination, instituting barriers such as gloves for food workers, instituting health care policies so food industry employees seek treatment when they are ill, pasteurization of juice or dairy products and proper hand washing requirements.
Shiga toxin-producing *E. coli* (STEC), specifically serotype O157:H7, have also been transmitted by flies (Alam and Zurek, 2004), as well as direct contact with farm animals (Rahn *et al*., 1998; Trevena *et al*., 1999), petting zoo animals (Heuvelink *et al*., 2002), and airborne particles found in animal-rearing environments (Varma *et al*., 2003).

Symptoms of *E. coli* start about 7 days after infection by the germ. The first sign is severe abdominal cramps that start suddenly. After a few hours, watery diarrhea starts. The diarrhea causes fluids lose and electrolytes (dehydration). This makes to feel sick and tired. The watery diarrhea lasts for about a day. Then the diarrhea changes to bright red bloody stools. The infection makes sores in your intestines, so the stools become bloody. Bloody diarrhea lasts for 2 to 5 days. It might have 10 or more bowel movements a day (Familydoctor, 2007).

Table 1 Enteric *E. coli* (EC) are classified on the basis of serological characteristics and virulence properties.

Name	Hosts	Description
Enterotoxigenic *E. coli* (ETEC)	causative agent of diarrhoea (without fever) in humans, pigs, sheep, goats, cattle, dogs, and horses	ETEC uses fimbrial adhesins (projections from the bacterial cell surface) to bind enterocyte cells in the small intestine. ETEC can produce two proteinaceous enterotoxins: the larger of the two proteins, LT enterotoxin, is similar to cholera toxin in structure and function. the smaller protein, ST enterotoxin causes cGMP accumulation in the target cells and a subsequent secretion of fluid and electrolytes into the intestinal lumen. ETEC strains are non-invasive, and they do not leave the intestinal lumen. ETEC is the leading bacterial cause of diarrhoea in children in the developing world, as well as the most common cause of traveler's diarrhea. Each year, ETEC causes more than 200 million cases of diarrhoea and 380,000 deaths, mostly in children in developing countries.
Enteropathogenic *E. coli* (EPEC)	causative agent of diarrhea in humans, rabbits, dogs, cats and horses	Like ETEC, EPEC also causes diarrhoea, but the molecular mechanisms of colonization and aetiology are different. EPEC lack fimbriae, ST and LT toxins, but they utilize an adhesin known as intimin to bind host intestinal cells. This virotype has an array of virulence factors that are similar to those found in *Shigella*, and may possess a shiga toxin. Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation. EPEC cells are moderately invasive (i.e. they enter host cells) and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to "attachment and effacement" is likely the prime cause of diarrhoea in those afflicted with EPEC.
Enteroinvasive *E. coli* (EIEC)	found only in humans	EIEC infection causes a syndrome that is identical to Shigellosis, with profuse diarrhoea and high fever.
Enterohemorrhagic *E. coli* (EHEC)	found in humans, cattle, and goats	The most famous member of this virotype is strain O157:H7, which causes bloody diarrhoea and no fever. EHEC can cause hemolytic-uremic syndrome and sudden kidney failure. It uses bacterial fimbriae for attachment.

Table 1 Enteric *E. coli* (EC) are classified on the basis of serological characteristics and virulence properties (continued).

Name	Hosts	Description
Enterohemorrhagic *E. coli* (EHEC)	found in humans, cattle, and goats	(*E. coli* common pilus, ECP) is moderately invasive and possesses a phage-encoded Shiga toxin that can elicit an intense inflammatory response.
Enteroaggregative *E. coli* (EAEC)	found only in humans	So named because they have fimbriae which aggregate tissue culture cells, EAEC bind to the intestinal mucosa to cause watery diarrhea without fever. EAEC are non-invasive. They produce a hemolysin and an ST enterotoxin similar to that of ETEC.

Source : Anon. (2011a).

2.3 Characteristic and pathogenesis of *Salmonella* spp.

2.3.1 Biological and biochemistry

Salmonella is closely related to the *Escherichia* genus, it is classified as kingdom Bacteria, class Gamma Proteobacteria, order Enterobacteriales, family Enterobacteriaceae, and genus *Salmonella* Lignieres 1900 species. It is rod-shaped, Gram-negative, non-spore forming, predominantly motile enterobacteria with diameters around 0.7 to 1.5 μm, lengths from 2 to 5 μm, and flagella which project in all directions (i.e., peritrichous) (Figure 2). They are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Most species produce hydrogen sulfide (Clark and Barret, 1987), which can readily be detected by growing them on media containing ferrous sulfate, such as TSI. Most isolates exist in two phases: a motile phase I and a nonmotile phase II. Cultures that are nonmotile upon primary culture may be switched to the motile phase using a Cragie tube (Ryan and Ray, 2004). Salmonellae live in the intestinal tracts of warm-and cold-blooded animals. Some species are ubiquitous. Other species are specifically adapted to a particular host. In humans, *Salmonella* are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection/intoxication.

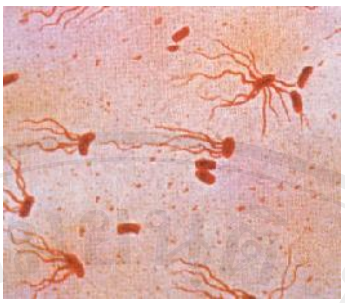

Figure 2 *Salmonella typhi*, the agent of typhoid. Gram stain.

2.3.2 *Salmonella* nomenclature

The genus *Salmonella* is a member of the family *Enterobacteriaceae.* It is composed of bacteria related to each other both phenotypically and genotypically. *Salmonella* DNA base composition is 50-52 mol% G+C, similar to that of *Escherichia, Shigella,* and *Citrobacter.* The bacteria of the genus *Salmonella* are also related to each other by DNA sequence. The genera with DNA most closely related to *Salmonella* are *Escherichia, Shigella,* and *Citrobacter.* Similar relationships were found by numerical taxonomy and 16S ssRNA analysis.

Salmonella nomenclature has been controversial since the original taxonomy of the genus was not based on DNA relatedness; rather names were given according to clinical considerations, e.g., *Salmonella typhi, Salmonella cholerae-suis, Salmonella abortus-ovis,* and so on. When serological analysis was adopted into the Kauffmann-White scheme in 1946, a *Salmonella* species was defined as "a group of related fermentation phage-type" with the result that each *Salmonella* serovar was considered as a species. Since the host-specificity suggested by some of these earlier names does not exist (e.g., *S. typhi-murium, S. cholerae-suis* are in fact ubiquitous), names derived from the geographical origin of the first isolated strain of the newly-discovered serovars were next chosen, e.g., *S. london, S. panama, S. stanleyville.*

Subsequently, it was found that all *Salmonella* serovars form a single DNA hybridization group, i.e., a single species composed of seven subspecies, and the nomenclature had to be adapted. To avoid confusion with the familiar names of serovars, the species name *Salmonella enterica* was proposed with the following names for the subspecies:

enterica I

salamae II

arizonae IIIa

diarizonae IIIb

houtenae IV

bongori V

indica VI

Each subspecies contains various serovars defined by a characteristic antigenic formula. Since this formal Latin nomenclature may not be clearly understood by physicians and epidemiologists, who are the most familiar with the names given to the most common serovars, the common serovars names are kept for subspecies I strains, which represent more than 99.5% of the *Salmonella* strains isolated from humans and other warm-blooded animals. The vernacular terminology seems preferred in medical practice, e.g., *Salmonella* ser. Typhimurium (not italicized) or shorter *Salmonella* (or *S.*) Typhimurium.

2.3.3 Antigenic structure

As with all *Enterobacteriaceae,* the genus *Salmonella* has three kinds of major antigens with diagnostic or identifying applications: somatic, surface and flagellar. Somatic (O) or cell wall Antigens Somatic antigens are heat-stable and alcohol-resistant. Cross-absorption studies individualize a large number of antigenic factors, 67 of which are used for serological identification. O factors labeled with the same number are closely related, although not always antigenically identical.

Surface (Envelope) Antigens: Surface antigens, commonly observed in other genera of enteric bacteria (e.g., *Escherichia coli* and *Klebsiella),* and may be found in some *Salmonella* serovars. Surface antigens in *Salmonella* may mask O antigens, and the bacteria will not be agglutinated with O antisera. One specific surface antigen is well known: the Vi antigen. The Vi antigen occurs in only three *Salmonella* serovars (out of about 2,200): Typhi, Paratyphi C, and Dublin. Strains of these three serovars may or may not have the Vi antigen.

Flagellar (H) Antigens: Flagellar antigens are heat-labile proteins. Mixing salmonella cells with flagella-specific antisera gives a characteristic pattern of agglutination (bacteria are loosely attached to each other by their flagella and can be dissociated by shaking). Also, antiflagellar antibodies can immobilize bacteria with corresponding H antigens.

A few *Salmonella enterica*serovars (e.g., Enteritidis, Typhi) produce flagella which always have the same antigenic specificity. Such an H antigen is then called monophasic. Most *Salmonella* serovars, however, can alternatively produce flagella with two different H antigenic specificities. The H antigen is then called diphasic. For example, Typhimurium cells can produce flagella with either antigen i or antigen 1,2. If a clone is derived from a bacterial cell with H antigen i, it will consist of bacteria with i flagellar antigen. However, at a frequency of 10^{-3}- 10^{-5}, bacterial cells with 1,2 flagellar antigen pattern will appear in this clone.

2.3.4 Pathogenesis of *Salmonella* infections in humans

Salmonella infections in humans vary with the serovar, the strain, the infectious dose, the nature of the contaminated food and the host status. Certain serovars are highly pathogenic for humans; the virulence of more rare serovars is unknown. Strains of the same serovar are also known to differ in their pathogenicity. An oral dose of at least 10^5*Salmonella* Typhi cells are needed to cause typhoid in 50% of human volunteers, whereas at least 10^9 *S.* Typhimurium cells (oral dose) are needed to cause symptoms of a toxic infection. Infants, immunosuppressed patients, and those affected with blood disease are more susceptible to *Salmonella* infection than healthy adults.

In the pathogenesis of typhoid, the bacteria enter the human digestive tract, penetrate the intestinal mucosa (causing no lesion) and are stopped in the mesenteric lymph nodes. There, bacterial multiplication occurs, and part of the bacterial population lyses. From the mesenteric lymph nodes, viable bacteria and LPS (endotoxin) may be released into the bloodstream resulting in septicemia. Release of endotoxin is responsible for cardiovascular “collapsus and tuphos” (a stuporous state-origin of the name typhoid) due to action on the ventriculus neurovegetative centers.

Salmonella excretion by human patients may continue long after clinical cure. Asymptomatic carriers are potentially dangerous when unnoticed. About 5% of patients clinically cured from typhoid remain carriers for months or even years. Antibiotics are usually ineffective on *Salmonella* carriage (even if salmonellae are susceptible to them) because the site of carriage may not allow penetration by the antibiotic.

Salmonellae may survive with sewage treatments if suitable germicides are not used in sewage processing. In a typical cycle of typhoid, sewage from a community is directed to a sewage plant. Effluent from the sewage plant passes into a coastal river where edible shellfish (mussels, oysters) live. Shellfish concentrate bacteria as they filter several liters of water per hour. Ingestion by humans of these seafoods (uncooked or superficially cooked) may cause typhoid or other salmonellosis. Salmonellae do not colonize or multiply in contaminated shellfish.

Typhoid is strictly a human disease. The incidence of human disease decreases when the level of development of a country increases (i.e., controlled water sewage systems, pasteurization of milk and dairy products). Where these hygienic conditions are missing, the probability of fecal contamination of water and food remains high and so is the incidence of typhoid.

The people of Thailand suffered from diarrhea, food poisoning and dysentery are approximately 64,678, 7,656 and 908, respectively, in January- March 2005. Almost of them were reported to be children and elderly persons. The quantity of patients found in each region over the county were as; Northeast > Central> North> South (Manager online, 2005; BEC News, 2007).

2.3.4.1 Foodborne *Salmonella* toxic infections

Salmonella toxic infections are caused by ubiquitous *Salmonella* serovars (e.g., Typhimurium). About 12-24 hours following ingestion of contaminated food (containing a sufficient number of *Salmonella*), symptoms appear (diarrhea, vomiting, fever) and last 2-5 days. Spontaneous cure usually occurs.

Salmonella may be associated with all kinds of food. Contamination of meat (cattle, pigs, goats, chicken, etc.) may originate from animal salmonellosis, but most often it results from contamination of muscles with the intestinal contents during evisceration of animals, washing and transportation of carcasses. Surface contamination of meat is usually of little consequence, as proper cooking will sterilize it (although handling of contaminated meat may result in contamination of hands, tables, kitchenware, towels, other foods, etc.). However, when contaminated meat is ground, multiplication of *Salmonella* may occur within the ground meat and if cooking is superficial, ingestion of this highly-contaminated food may produce a *Salmonella* infection. Infection may follow ingestion of any food that supports

multiplication of *Salmonella* such as eggs, cream, mayonnaise, creamed foods, etc., as a large number of ingested salmonellae are needed to give symptoms. Prevention of *Salmonella* toxic infection relies on avoiding contamination (improvement of hygiene), preventing multiplication of *Salmonella* in food (constant storage of food at 4°C) and use of pasteurized and sterilized milk and milk products. Vegetables and fruits may carry *Salmonella* when contaminated with fertilizers of fecal origin, or when washed with polluted water.

The incidence of foodborne *Salmonella* infection/toxication remains reletavely high in developed countries because of commercially-prepared food or ingredients for food. Any contamination of commercially-prepared food will result in a large-scale infection. In underdeveloped countries, foodborne *Salmonella* intoxications are less spectacular because of the smaller number of individuals simultaneously infected, but also because the bacteriological diagnosis of *Salmonella* toxic infection may not be available. However, the incidence of *Salmonella* carriage in underdeveloped countries is known to be high.

Salmonella epidemics may occur among infants in pediatric wards. The frequency and gravity of these epidemics are affected by hygienic conditions, malnutrition and the excessive use of antibiotics that select for multiresistant strains.

2.3.4.2 *Salmonella* Enteritidis infection

Egg-associated salmonellosis is an important public health problem in the United States and several European countries. *Salmonella* Enteritidis, can be inside perfectly normal-appearing eggs, and if the eggs are eaten raw or undercooked, the bacterium can cause illness. During the 1980s, illness related to contaminated eggs occurred most frequently in the northeastern United States, but now illness caused by *S.* Enteritidis is increasing in other parts of the country as well.

Unlike eggborne salmonellosis of past decades, the current epidemic is due to intact and disinfected grade A eggs. *Salmonella* Enteritidis silently infects the ovaries of healthy appearing hens and contaminates the eggs before the shells are formed. Most types of *Salmonella* live in the intestinal tracts of animals and birds and are transmitted to humans by contaminated foods of animal origin. Stringent procedures for cleaning and inspecting eggs were implemented in the 1970s and have made salmonellosis caused by external fecal contamination of egg shells extremely rare.

However, unlike eggborne salmonellosis of past decades, the current epidemic is due to intact and disinfected grade A eggs. The reason for this is that *Salmonella* Enteritidis silently infects the ovaries of hens and contaminates the eggs before the shells are formed.

Although most infected hens have been found in the northeastern United States, the infection also occurs in hens in other areas of the country. In the Northeast, approximately one in 10,000 eggs may be internally contaminated. In other parts of the United States, contaminated eggs appear less common. Only a small number of hens seem to be infected at any given time, and an infected hen can lay many normal eggs while only occasionally laying an egg contaminated with *Salmonella* Enteritidis.

A person infected with the *Salmonella* Enteritidis usually has fever, abdominal cramps, and diarrhea beginning 12 to 72 hours after consuming a contaminated food or beverage. The illness usually lasts 4 to 7 days, and most persons recover without antibiotic treatment. However, the diarrhea can be severe, and the person may be ill enough to require hospitalization. The elderly, infants, and those with impaired immune systems (including HIV) may have a more severe illness. In these patients, the infection may spread from the intestines to the bloodstream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics.

2.4 Source of *E. coli* and *Salmonella* spp.

E. coli and *Salmonella* spp. are found in soil, surface water, plants, organic fertilizer, warm-blood animals and humans. Johnson *et al.* (2003) reported that *E. coli* O157:H7 and *Salmonella* spp. were found in the surface water from the Old Man River watershed in southern Alberta, Canada. In this irrigated region in which intensive agricultural practices have flourished, prevalence of *E. coli* O157:H7 and *Salmonella* spp. in water samples was 0.9% (n=1483) and 6.2% (n=1429), respectively. While date examined at a regional level show a relationship between high livestock density and high pathogen levels in south Alberta, statistical analysis of point source data indicates the predicted manure output from bovine, swine and poultry feeding operations was not directly associated with ether *Salmonella* spp. or *E. coli* O157:H7 prevalence.

Microbiological analyses of fresh fruit and vegetable produced by organic and conventional farmers presented that the average coliform counts in both farming systems were 2.9 log most probable numbers per gram. The percentages of *E. coli*-positive samples in conventional and organic produce were 1.6% and 9.7% respectively. Organic lettuce had the largest prevalence of *E. coli* (22.4%) compared with other produce types. Organic samples from farms that used manure or compost aged less than 12 months had a prevalence of *E. coli* 19 times greater than that of farms that used older materials. *E. coli* O157:H7 was not detected in any produce samples, whereas *Salmonella* spp. was isolated from one organic lettuce and one organic green pepper (Diez *et al*., 2004).

2.5 Method for detection of *E. coli* and *Salmonella* spp.

Several methods have been used for detection and enumeration of *E. coli* and *Salmonella* spp. There are many certifications such as AFNOR, AOAC, ISO, FIL/IDE etc. These devised 2 main methods are conventional and rapid methods.

2.5.1 Conventional methods for detection of *E. coli* and *E. coli* O157

2.5.1.1 Multiple tube fermentation (MTF)

This is a simple method to perform and expresses results as a most probable number (MPN) of bacteria in the original samples based on statistical analysis of the number of tube/dilutions demonstrating growth and fermentation of specific substances in the media. It dose not give true enumeration. However, it is non-specific fermentation of lactose which resulted in inaccurate MPN values. There is also a risk of inhibition from contaminating organisms. This test is useful for complex and turbid matrices that cannot be filtered. For a simple presence/absence test single incubations are performed (Standing Committee of Analysis water method).

2.5.1.2 Membrane filter method

Membrane filtration of the sample is included in the method of choice for isolation and detection of *E. coli* from water in many approved methods. This method allows visualization of resultant colonies and subsequent enumeration. The method is limited by the turbidity of the sample to be tested because very turbid samples can block the filter. To overcome this range of serial dilutions (possibly containing a

reducing agent, e.g., cysteine hydrochloride to limit damage to bacterial cells from oxygen and free radicals) may be filtered. The UK Standing Committee of Analysts method for detection of O157 from water utilises Filter Aid. This is a solution of diatomaceous earth which when used with a sterile absorbent pad acts as a coarse filter. Also in development are methods which agitate the membrane filter when filtering turbid solutions but the concern is that the bacterial cells may be damaged in this process.

In the approved methods utilizing membrane filtration the sample is filtered through 0.45 μm membrane filter to trap the bacteria and the filter incubated on selective medium. Many different selective media have been used following membrane filtration. In the USA, the American Public Health Association (APHA) recommend mEndo agar for enumeration of coliforms in water, waste water and foods. The nutrients in this medium are casein, peptone, yeast extract and lactose. Lactose-fermenting colonies appear red with a metallic sheen due to the production of aldehydes. ISO 9308-1(1988) recommends the use of Lactose TTC in the membrane filtration method for detection and enumeration of *E. coli*. The medium contains Tergitol (sodium heptadecylsulphate) and positive lactose-fermenting colonies appear yellow/orange: yellow due to acid production detected by the indicator, Bromothymol blue, and orange due to weak reduction of 2, 3, 5-Triphenyltetrazolium chloride (TTC).

Problems have occurred if the organisms being recovered are stressed or sub-lethally injured, for example, during water treatment processes. Sartory (1995) refer Warnes and Keevil (2004) suggested that the addition of 0.01-0.1% (w/v) sodium pyruvate could reduce this effect. More recently, methods in the UK for the analysis of drinking water have used membrane lauryl sulphate broths and agars. However, there is currently no universal medium for the isolation of *E. coli* from different environmental samples.

2.5.1.3 Chromogenic and fluorogenic substrates

Chromogenic and fluorogenic substrates produce colour and fluorescence, respectively, upon specific enzyme cleavage and are widely used in selective culture media. The principal substrates and the 2 major enzymes involved in the detection of *E. coli* have been described previously (see above). The inclusion of these substrates,

together with the selective nature of the media (reducing the number of background microflora), have resulted in increased sensitivity and rapidity (Gaudet *et al.*, 1996). It may be easier to distinguish specific colonies earlier using these media. Observation of fluorescent colonies is relatively easy using a basic UV illumination chamber. Dogen *et al.* (2002) describe effective use of fluorogenic broths for the detection of *E. coli* in foods (MPN). As with any enzymic reaction, conditions of pH and temperature must be optimal for the enzyme and substrates to function and these conditions must be specified in the protocol. For example, fluorogenic substrates are usually quenched at low pH and require neutral conditions in the medium. In the detection of clostridia using fluorogenic substrates which are cleaved by acid phosphatase neutral pH also results in activation of alkaline phosphatase giving, false positive results.

2.5.1.3.1 Agar media and broths

There are many commercially available chromogenic and fluorogenic media available as agar plates or broths, e.g., Colisure, m-Coliblue, ColiComplete. Colisure® broth (IDEXX) simultaneously detects coliforms and *E. coli* in water utilising their ability to hydrolyse chlorophenyl red b-D-galactopyranoside (CPRG; sample turns yellow to red/magenta) or MUG (sample fluoresces). The manufacturer suggests that Colisure® can detect coliforms and *E. coli* at 10 CFU in100 mL within 24 hours when incubated at 35±0.5ºC. It can also be used with the Quanti-Tray MPN system. EPA included Colisure® in its proposal to update analytical methods for biological pollutants in ambient water, however, the manufacturer declined to conduct the study and the product has therefore not been approved in the final rule (Federal, 2003). This may be because the product is being superceded by Colilert®.

2.5.1.3.2 Colilert®/Quantitray® technology

The Colilert® assay system (IDEXX Laboratories) has been accepted by the US EPA under their 40 CFR part 136 final rule for ambient water (Federal, 2003). Colilert® can be used for presence/absence samples utilising chromogenic (ONPG) and fluorogenic (MUG) substrates to simultaneously detect total coliforms and *E. coli* after incubation at 35ºC for 24 h. However, its strength lies in being adaptable to a semi-automated MPN method: this involves incubation of sample and defined substrate media in proprietary multiwell plates rather than tubes. The

technology is based on the IDEXX Quanti-Tray and Quanti-Tray/2000 formats to provide easy, rapid and accurate counts of coliforms, *E. coli* and enterococci. The IDEXX Quanti-Tray and Quanti-Tray/2000 are semi-automated quantification methods based on the Standard Methods Most Probable Number (MPN) model. The Quanti-Tray® Sealer automatically distributes the sample/reagent mixture into separate wells. After incubation, the number of positive wells is converted to an MPN using a table provided. Quanti-Tray uses 51 wells and provides counts from one to 200 per 100 mL. The medium formulation is suggested to suppress up to 2 million heterotrophs per 100 mL; this could pose a challenge for analysing *E. coli* in sludge, soil and biowaste samples with high microbial background flora. Quanti-Tray/2000 uses 97 wells of two different sizes and counts from one to 2,419 per 100 mL, with a far better 95% confidence limit than a 15-tube serial dilution. IDEXX considers the technology to be superior to MPN and at least as good a performance as MF, with a greater counting range (Table 2). Total hands-on time is less than one minute per test. We consider that the if the Colilert® system is to be use for sludge, soil, and biowaste analysis then the Quanti- Tray/2000â format should be the preferred option because of its wider counting range.

Table 2 Comparison of Quantitray, MPN and MF techniques.

Method	Lower counting Range /100 mL	Upper counting Range 100/mL
Quanti-Tray	<1	200
Quanti-tray/2000	<1	2,419
5-Tube MPN	<1.1	16
10-Tube MPN	<1.1	23
Membrane filtration	<1	80

Source: Modified from Warmer and Keevil (2004)

Colilert® is claimed to be able to detect 1 *E. coli* /100 mL, provide a less subjective interpretation, compared to counting colonies on agar, and identify 50% fewer false positives and 95% fewer false negatives than the standard membrane filtration (MF) method. The multiple well format gives greater precision than

conventional 5-tube:3 dilution MPN methods with a MPN of <1 giving a range of lower and range at 95% confidence limits of 0 and 3.7 bacteria. Colilert® has 75% lower equipment cost than membrane filtration and is claimed to be 25-50% less expensive than traditional methods. The reagent packs have up to an 12-month shelf life and the comparatively rapid 24-hour test saves incubator space. IDEXX claim to be able to detect coliforms and/or *E. coli* in drinking water in under 24 hours. IDEXX also sell Colilert-18® which has an enhanced formulation to detect coliforms and *E. coli* in 18 hours, improving workflow in large laboratories by reading afternoon samples the next morning. Samples need to be pre-warmed for presence/absence samples, if they are not already at 33-38ºC, but not for Quanti-Tray® or Quanti-Tray®/2000 samples. The sample should be placed in a 35ºC water bath for 20 minutes or a 44.5ºC water bath for 7-10 minutes. This pre-warming time is part of (not in addition to) the 18-hour incubation period for Colilert- 18®. The company claim that the Colilert-18® / Quanti-Tray®/2000 technology has 95% less equipment costs than membrane filtration (MF). Colilert-18® is the only US EPA-approved 18-hour test and is included in the US Standard Methods for Examination of Water and Wastewater.

2.5.2 Conventional methods for detection of *Salmonella* species

A diverse range of methods have been developed over the years to detect *Salmonella,* primarily in foodstuffs and water supplies both as a routine monitoring of food and water quality and in the event of an outbreak detection of the contamination source. Because the infectious dose is very low in humans, the sensitivity of the methods used have to be high. This is further complicated by the fact that numbers of *Salmonella* in contaminated water, food and wastes are usually greatly outnumbered by other organisms of faecal origin e.g. *E. coli* and enterococci, and organisms naturally occurring in the environment including *Citrobacter* and *Proteus* spp. Any *Salmonella* that are present may be sub-lethally stressed and require incubation in a highly nutritious non-selective medium (enrichment) prior to further processing. A range of methods have been developed to detect *Salmonella* in food and water which rely on standard culture methods and biochemical confirmation tests. A number of these have been standardized and are used routinely (Table 2). They all rely on the principal 4 stages outlined below:

2.5.2.1 Sampling and release of the bacterium from the matrix

Usually a 10-30 g sample of food or waste is diluted approximately 1/10 in a buffer containing surfactant (e.g. non-ionic detergent) and mixed in a homogenizer, stomacher or more recently Pulsifier (Microgen Bioproducts Ltd.) which employs combining shock waves and intense stirring. All these processes are used to ensure that the bacteria are completely released from the matrix and adequately dispersed to prevent clumping before the pre-enrichment stage.

2.5.2.2 Pre-enrichment

This stage allows small numbers of potentially environmentally stressed bacteria to recover and grow before the use of selective media. Because non-selective, highly nutritious media are used at this stage, often Buffered Peptone Water (BPW), there is a risk of overgrowth of other contaminants which could actually inhibit the growth of any *Salmonella*. The timing and temperature are therefore important at this stage to ensure the *Salmonella* can still be recovered. Usually an 18-24 hour incubation at 37^{o}C is adequate. In spite of these difficulties it is not advisable to use selective media from the outset because if the *Salmonella* cells are sub-lethally stressed they would not recover on selective media and produce a false negative test result. This has practical difficulties for developing a rapid assay taking less than 24 hours (see later).

2.5.2.3 Selective enrichment

Usually samples from the pre-enrichment broths are inoculated into selective broths. There is considerable dispute as to which selective agents give the best recovery and the choice depends on the matrix (food type e.g. meat; water or sludge), conditions of sampling and the species of *Salmonella* under investigation. The selective medium specified for use by the food industry onhighly contaminated foods in the USA and for food and water samples in the UK is Rappaport- Vassiliadis (RV) medium. Broths are incubated at a higher temperature of 41.5ºC for 24 and also 48 hours.

Yanko *et al.* (1995) refer Warnes and Keevil (2004) however described the use of Tetrathionite Brilliant Green Broth recovering more *Salmonella* from activated sludge, compost and anaerobically digested biosolids. Brown and Keevil (2000) refer Warnes and Keevil (2004) described a method for detection of *Salmonella* in human

sludge, cattle and pig slurries which omits the pre-enrichment broth but after filtration of the sample onto 0.45 μm cellulose nitrate membranes the latter are incubated on filter pads soaked in Tetrathionate broth (Oxoid, European formulation) prior to growth on selective solid media. The inclusion of novobiocin is very important to suppress background competitors such as *Proteus* spp. which can reduce tetrathionate and impair the value of the medium to grow Salmonella. Novobiocin is included in the enrichment media specified in several of the draft MF and presence/absence CEN methods for detecting *Salmonella* in sludge (Table 3).

2.5.2.4 Agar media

In the majority of methods the RV broths are then used to inoculate solid selective media, for example desoxycholate citrate agar (DCA), xylose lysine desoxycholate citrate agar (XLD), Brilliant green Agar (BGA), bismuth suphite agar (BSA), Salmonella-Shigella agar (SS), or mannitol lysine crystal violet brilliant green (MLCB). Many of these contain selenium salts, brilliant green and malachite green to inhibit the growth of other *Enterobacteriaceae* and detect the production of hydrogen sulphide (although some serotypes are negative) by the *Salmonella*. More recently used formulations are XLT4 (Tate, 1990) which contains xylose, lysine, lactose, sucrose, phenol red and the surfactant, Tergitol, which has given improved recoveries of *Salmonella* from meat from poultry farms.

Rambach agar uses a new phenotypic characteristic which is the formation of acid from propylene glycol and hydrolysis of X-Gal to differentiate *Salmonella* species from other *Enterobacteriaceae.* Rainbow agar (Biolog) can isolate and differentiate the widest range of *Salmonella* species, including *S. typhi,* and can detect the weakest to the strongest hydrogen sulphide producers. A combination of 2 or 3 of the above media is used routinely by UK PHLS and SCA published methods for the detection of *Salmonella* in water and food (Table 2). The membrane filtration methods for sludge and wastes recommended by CAMR/ University of Southampton and CEN 308 method 2 , and CEN 308 method 1 (liquid enrichment) used Rambach agar. Other novel media are being developed all the time and those that incorporate a combined preenrichment/ selective enrichment or result in reducing the time necessary to obtain a result (i.e. become more rapid) are described later.

2.5.2.5 Biochemical and serological confirmation

To confirm that the resultant colonies on selective agars are *Salmonella* a range of biochemical and serological tests are usually performed, e.g., urease test, growth on iron sugar medium, phage typing and latex bead agglutination (LBA). The immunolabelling methods fall into several broad categories, summarized as immunofluorescence assay (IFA), enzyme immunoassay (EIA: also commonly referred to as enzyme-linked immunosorbent assay; ELISA) and latex bead agglutination (LBA). IFA can be used for the direct specific detection of original samples, providing the assay is sufficiently sensitive for the concentration of target organism present, and after sample concentration (filtration or immunocapture) or amplification (pre-enrichment) steps.

EIA (ELISA) and LBA are used more often for rapid post-broth and agar colony screening to confirm the identity of the target organism, augmented by conventional serotyping (see later). IFA offers the ability to specifically detect pathogens *in situ*, particularly where sub-lethally damaged or VNC bacteria are suspected. Once the antibody has been produced, the method is quick and inexpensive. The IFA has been combined with CTC-detected respiration to determine the identity and physiological status of *E. coli* O157 in water (Pyle *et al*., 1995 refer Warnes and Keevil, 2004). Fluorescently-labelled antibodies have been used to screen wastewater for *Salmonella* (Desmonts *et al*., 1990 refer Warnes and Keevil, 2004). The method is rapid but requires that there is no cross-reactivity with other species, that the target epitope is expressed and conserved in the test environment and there are sufficient numbers of cells for observation by microscopy or cell cytometry.

The traditional culture methods for *Salmonella* detection described above can take up to 6 days for an accurate confirmation. The worldwide incidence of salmonellosis is increasing and with increasing pressures on the food industry bound by law to produce safe foodstuffs there is a great need for new, rapid and sensitive methods for detection of *Salmonella*. The majority of methods for the detection of *Salmonella* have been devised to isolate the organism from food and water. These methods can be adapted to a different matrix such as soil and biosolids but modifications may have to be made.

2.5.3 Rapid methods for detection of *E. coli* and *E. coli* O157

2.5.3.1 Fluorogenic substrates (Warnes and Keevil, 2004)

Colifast (Colifast Systems,Oslo) is an automated system utilising fluorogenic substrates but does have an enrichment step for the recovery of stressed organisms and measures the fluorescence directly. This usually recommend for bathing waters. Suwansonthichai and Rengpipat (2003) described the enumeration of *E. coli* from frozen black tiger shrimp using conventional MPN and rapid methods Chromocult, Fluorocult and Petrifilm plates. Matner *et al.* (1990) compared Petrifilm *E.coli* Count and plate (PEC) and AOAC MPN method to determine the efficacy of the PEC method to detect *E. coli* and coliforms in 115 inoculated chess samples, 94 vegetables samples, in 100 naturally contaminated poultry samples. The PEC method was compared to two other coliform plate count methods. The 24 h PEC method is as good as pr better than the AOAC MPN method for the detection *E. coli*. In addition, quantitative results suggest that the PEC method may be more sensitive than the 9 tube MPN method for the detection of very low number of *E. coli*. Comparable coliform results were obtained. Vaill *et al*. (2003) enumerated *E. coli* with Petrifilm Plates compared to standard methods. *E. coli* counts in environmental water samples enumerated with Petrifilm were significantly correlated ($R>0.9$; slope = 0.9-1.0; $p<0.01$) with counts obtained with three commonly used methods, m-TEC (Becton Dickin- son, Sparks, MD), m-ColiBlue (Hach, Loveland, CO), and Colilert-18/IDEXX Quanti-Tray 2000 (IDEXX, Westbrook, ME). Blue colonies on Petrifilm plates were most reliably identified as *E. coli* when accompanied by gas formation, as determined by characterization of the colonies on MacConkey agar plates (PML Microbiologicals, Mississauga, ON, Canada) and by polymerase chair reaction (PCR) with *E. coli*–specific primers. The main disadvantage of Petrifilm plates for environmental water testing is the small volume (1 mL per sample) that can be tested; however, the plates appear to be suitable for screening and locating sites that exceed criteria for total body and partial body contact. Simplicity of use and storage, reliability, and relatively low cost, make Petrifilm plates suitable for volunteer-based and educational water quality monitoring applications, particularly when using as a preliminary screening method to identify problem sites.

Table 3 Isolation and detection of *Salmonella*.

Origin of method	Matrix method devised for:	Sample size	Summary of method	Comments/ validated etc.
EA/SCA UK 2002	Water	100 mL treated	Filter the pre enrich on BPW 37ºC 24 hr Select. Enrichment on Rapp. Vass. Med 41.5ºC 24 hr and 48 hr. Subcut at each time point to sel agars XLD, BGA, flagellar test, iron sugar etc	
PHIS SOP UK W7	Water	1000 mL	BPW 37ºC 24 hr Select. Enrichment on Rapp. Vass. Med 41.5ºC 24 hr and 48 hr. Subcut at each time point to sel agars XLD, BGA, MLCB flagellar test, iron sugar etc	
F13	Food	25 g	Stomach in Buffered Peptone Water leave 18 hr (pre enrich) Selective enrich in Rapp- Vass + selenite cys 22 hr Subcut to sel agar XLD, BGA, MLCB	
Ston/CAMR	Sludge	25 g	Stomacher Dilns filtered and placed on resus pad soaked in tetrathionite broth + iodine Filter onto rambach agar	
CEN TC 308 Method 1 DIN Liquid enrichment	Sludge	10 g DM	10g + 90mL tryp Homogenise 2 mins Primary enrichment in selcys 36ºC 20 hr (dilns) Secondary enrich Rapp Vass Subcut to Rambach XLD Confirm urea, indole MPN	Validated
Method 2 UK Pt 1 Membrane filtration 6 log drop	Sludge	25 g wet weight	Stomacher MTSB (Novobiocin)* Fuller dilns Resus Tetrathionite broth 36ºC 16 hr Rambach, spry and UV	
Method 3	Sludge, soil, slurry	20 g wet weight	20 g in 200mL sterile Na Cl Shake 20 hr 2ºC Dilns onto Rapp Vass 20 hr 42ºC then subcut ontoXLD,BPLA – Most Prob No. test	
ISO 6579 (2002)	Food		Muller Kauffman tetrathionate novobiocin broth, RVS broth then XLD agar	

Source: Warmer and Keevil (2004)

2.5.3.2 Cell cytometry

Cell cytometry relies on a stream of liquid flowing as discrete microdroplets through a laser beam. Optical signals are detected whenever a particle, either unlabelled or fluorescently labeled, passes through at rates exceeding 10,000 per second. The types of information available include size, shape, and labeled RNA, DNA and surface antigen content. The data are collected for comparison of

parameters such as size versus fluorescent intensity. Incorporation of a fluorescently activated cell sorter (FACS) allows gates to be set of say size versus fluorescence and each particle which gives a positive signal within the gate can be deflected to a collector to provide a specific separation and quantification procedure. The specificity of fluorescently labeled antibodies has been exploited to detect and purify microorganisms such as *E. coli*, *L. pneumophila*, spores of *Bacillus anthracis* and oocysts of *Cryptosporidium parvum* by flow cytometry.50, 51. The coupling of flow cytometry with the use of 16S or 18S rRNA fluorescent probes has been advocated to facilitate the quantification of specific microorganisms from environmental samples.

2.5.3.3 Laser scanning

A system that addresses the need for rapid detection and identification of microorganisms from environmental samples has been developed by Chemunex (Maisons Alfort, France). The ChemScan RDI is based on direct fluorescent labelling of viable organisms trapped on a 25 mm diameter membrane, coupled with an ultra-sensitive laser scanning and counting system. The high level of sensitivity of the solid phase cytometer means that a single cell on a membrane can be detected. The use of fluorescently-labeled antibodies, enzyme substrates or nucleic acid probes provides the specificity for ChemScan to identify and enumerate target microorganisms without the need for enrichment. FITC-labeled *C. parvum* oocysts can be counted within 3 minutes before visual observation of the presumptive positives by epifluorescence microscopy. Of concern is that non-culturable cells can be detected by this method. Viability can be assessed by incubating with fluorochrome esters which fluoresce when the substrate is actively taken up by viable cells and intracellular esterases release the fluorochrome. The technology is now ready for application to untreated and treated wastes, provided good fluorescent antibody and oligonucleotide reagents are available, and trapped non-target cells do not interfere. The latter may be unlikely when looking for low numbers of a pathogen against a high background in sewage sludge, unless IMS or selective enrichment is undertaken first. Reynolds *et al.* (1999) refer Warnes and Keevil (2004) observed that one advantage of isolating target organisms on a membrane was that interfering substances such as clay particles could be washed away before incubation with antibody reagents.

2.5.3.4 Immunological methods

These methods are based on the interaction between specific antibodies (polyclonal or monoclonal) and antigens and it is this very specificity which can limit the effectiveness of the method. The method usually takes the form of antibodies fixed to a solid phase such as a multiwell plate (ELISA), flow through grid, and dipstick or magnetic beads as in the case of immunomagnetic separation (IMS). The antibodies capture the specific bacterial cells or surface antigens and positive binding is detected by either growth in culture media or addition of further antibodies conjugated to enzymes. The addition of the enzyme substrate, usually chromogenic, results in a detectable color change in ELISA and dipstick. In complex matrices including sludges, immunological methods may be inhibited by large numbers of contaminating organisms and large amount of debris in the samples.

IMS is used primarily to isolate the bacterium of choice from a complex matrix especially where there are low numbers in the sample or after an enrichment step. However, IMS is the method of choice for the isolation of *E. coli* O157 from moderate turbidity water supplies in the UK Standing Committee of Analysts (method F) following selective enrichment in buffered peptone water or modified tryptone soy broth. The selectivity of the antibodies used dictates the recovery efficiency and this method recommends Dynal Dynabeads or their equivalent e.g. Aureon Biosystems. The method is effective because it results in the isolation of the O157 serotype from the high numbers of contaminating other serotypes of *E. coli.* Once the organisms have been isolated they are plated onto selective media. This method is expensive but effective for complex matrices and more competitors are now available which could eventually affect the price. Recent evidence does suggest that false negatives can be reported due to the loss of surface antigen in stressed serotype O157 especially in environmental samples where there are starvation conditions.

PATHIGEN (Igen) is a commercially available assay for *E. coli* O157 in food and environmental samples. Like Dynabeads, magnetic beads are coated with antibodies to *E. coli*. The bacteria in the sample bind to the beads and a second antibody which has a fluorescent tag binds also. Positive samples are detected by flow cytometry. The product was evaluated by Norpath laboratories UK who claim it was 100 times more sensitive than selected dipstick and ELISA methods.

Tu *et al.* (2001) captured *E. coli* O157 using antibody conjugated to alkaline phosphatase. This complex was then immunomagnetically captured and the degree of enzyme plus substrate catalysis measured. There are several commercially available ELISA including TECRA screen for serotype O157 and Assurance EIA (BioControl Laboratories) which is an enzyme immunoassay detecting O157 in food and environmental samples. Bio Control have also developed EHEC8 enrichment medium for isolation of serotype O157 from beef. VIDAS produce automated enzyme linked immunofluorescence systems. Itoh *et al.* (2002) described a filtration ELISA where bacterial cells were directly filtered into a 96 well tray. SafePath is an ELISA test for O157 serotype. Other immunological tests include latex agglutination tests for the confirmation of colonies isolated by conventional culture techniques (microgen Bioproducts,Oxoid,Unipath,Meridian diagnostics). IFA details Oxoid have a EIA to detect the heat stable enterotoxin (ST) which uses a synthetic peptide toxin analogue and monoclonal antibodies.

PATHATRIX (Matrix Microscience) is a novel method for detection of a range pathogenic bacterium in food samples. The PATHATRIX system is a patented technology that relies on the use of antibody coated paramagnetic particles to selectively bind and purify the target organism from a comprehensive range of complex food matrices. It is unique in that it is the only microbial detection system that can analyze the entire 225 mL + 25 g sample simultaneously by re-circulating the sample through a “capture phase” where the antibody coated magnetic beads are immobilized. By providing heat to the system the organisms can be cultured and captured simultaneously, thus increasing the method sensitivity. Once captured and concentrated the sample is now ready for use with a variety of detection methods: either direct plating onto the appropriate selective media and incubated or tested using one of the following; COLORTRIX; FLURATRIX (fluorescence microscopy); serology; PCR; ELISA; and/or DNA probe. There are two available formats to provide maximum flexibility and sample throughput to match customers specific requirements. The “3 Hour” format is intended for same day sample processing (no pre-incubation of sample required) whilst the “30 minute” format is intended for high throughout sample processing (following overnight incubation). Data from internal and external validation studies, e.g., AOAC trials have demonstrated that

PATHATRIX system is significantly more sensitive than many of the current standard methods, at low spike levels 1-10 CFU/25 g sample. The company claim to have launched the world's fastest commercially available method for the detection of *E. coli* O157 in food samples.

For 25 g samples, the test can be completed, from start to finish, in just over 5 hours. For 375 g samples, the test can be completed in 6¾ hours. The new test combines two of Matrix's proprietary technologies, PATHATRIX and COLORTRIX. The PATHATRIX system is designed for the rapid detection and positive identification of microbiological food contaminants, while COLORTRIX is a screening system, which provides presence/absence results within 15 minutes. The PATHATRIX/COLORTRIX method, which is capable of detecting a single CFU in a 25 g sample, is proving particularly popular with the beef market, where accurate, rapid testing can significantly enhance productivity and is critical for QA. To undertake the test, a 25g food sample is homogenised with 225 mL of growth media in a stomacher and incubated for 4½ hours. PATHATRIX capture reagent, which consists of *E .coli* specific antibody coated magnetic particles, is then added directly to the sample. The sample is loaded onto the PATHATRIX workstation, connecting the sample to the circulatory system in preparation for the Capture-Culture step. Once loaded, PATHATRIX is pre-programmed to run for 30 minutes and on completion of the run, the *E. coli* cells are bound onto the phase by the capture reagent. Residual debris and non-specific binding are removed during a single wash step. The captured pathogen complexes are then concentrated into a small volume. i.e., 200 l using a magnetic rack. A COLORTRIX antibody/enzyme is then added to the concentrate for 5 minutes before being diluted with 1 mL of wash buffer and magnetic removal of the of the bead/bacteria complexes. After a further two washes, half the concentrate is removed and added to a second reagent. The sample is then left for 5 minutes to develop color. A blue color indicates a presumptive positive, while a clear sample is recorded as a presumptive negative. Should a positive result be recorded, the sample remaining in the wash vessel is plated on the appropriate agar media, while a negative indicates that no further action is required.

A recently-announced product is the GridCount for the enumeration of bacteria in sewage sludges and other complex materials (MicroScience Technologies

Ltd., Edinburgh). This is a quantitative immunoassay that uses polymer grid bars coated, on one side only, with millions of micron-sized dots containing antibody. The grid is placed in a screw cap tube with sludge and shaken. Bacteria present are recognized by the specific antibody, in this case *E. coli* O157, and become attached. The open grid design prevents fouling with suspended solids and ensures an even coating. Thus the bacteria are captured in a two-dimensional array, which facilitates further interrogation. The simplest method is to culture the grid upside down on an agar plate. Within two hours, daughter cells from the fixed bacteria transfer on to the plate. The grid is then removed (and can be kept for further analysis, or disposed of). After overnight incubation the bacterial colonies, arrayed in grid formation, are counted. This can be done automatically using, for example, the Symbiosis Acolyte machine. The method can be used in conjunction with selective or chromogenic media, although this is not necessary since the antibody on the grid acts as a selective reagent. Alternatively, the grid can be stained and scanned directly using, for example, the Chemunex ChemScan RDI. The capacity of the grid used in culture mode is around 500 colonies. The capacity of the grid in scan mode is 500,000 colonies. The lower resolution in culture mode arises from the need to allow space for growth of the colonies. New versions of GridCount for total *E. coli* and *Salmonella* are planned shortly.

2.5.3.5 Lateral flow devices and dipsticks

There have been rapid advances with lateral flow devices, including Visual Immunoprecipitate assay (VIP; BioControl System Inc.) and gold labeled immunosorbent assay (GLISA) technologies such as the Merck Singlepath range for *E. coli* O157, *Salmonella* etc. The *Salmonella* test is carried out after a selective enrichment of the sample from one selective enrichment culture (taking 24-48 h). The end result is thus available two days sooner saving material and labor costs. A VTEC test for O157 is available, and yields a yes /no result in 20 minutes following overnight enrichment culture. It therefore provides the required detection in less than 24 hours. Another development of the technology is the Duopath range which is used to detect the VT1 and VT2 verocytotoxins produced by VTEC. The technologies claimed to be:

- **Fast:** Simple to handle, definite results from culture within 20 minutes.

- **Easy to use:** Simply apply the sample and read off the yes/no result.
- **Safe:** Definite test results with an additional positive control. Specially adapted enrichment media guarantee precise and reliable test results.
- **Economic:** Rapid results help save laboratory costs and investments in automation; a faster product release gives you a head start in the marketplace.

Bown and Keevil (2000) referred Warnes and Keevil (2004) evaluated two lateral flow devices for their suitability for rapid immunological detection of O157 in sludge: the Morningstar O157 7 hour test and the BioControl VIP. Initially, pure cultures of *E. coli* O157 were grown to test the response of the Morningstar kit. Following overnight growth 200 mL of the culture was applied to the well region of the device. A strong positive reaction was elicited in the observation window after 10 minutes incubation at room temperature. Spiked samples (equivalent to 10 cells of *E. coli* O157 per gram of sludge) were stomached and then enriched for 6 hours before the immunoprecipitation assays. At 4 hours enrichment no signal was detectable, however at 6 hours a strong signal was present. When this experiment was repeated using the VIP assay only a weak signal was detectable after 6 hours enrichment. If samples were enriched for 7 hours then the signal was stronger. The sensitivity varied for each kit detecting $>10^6$ and 10^3-10^4 of enriched cells in the VIP and Morningstar kits, respectively. A survey of 5 treatment works in one region of the UK was undertaken to assess the device performance. When tested by the VIP assay all samples were negative. Repeat of the experiment using Morningstar kits indicated a very strong positive in the Works A sample and a very weak positive in the Works B sample, all other samples were negative. These analyses were repeated with fresh samples over several days. This suggested the value of the Morningstar kit for same day screening of routine sludge samples for this important pathogen. Another flow device finding favour to detect *E. coli* O157 meat, milk and juices is the SAS *E. coli* O157 (SA Scientific, distributor M-Tech Diagnostics). Consequently, flow device technology shows promise and may be applicable for rapid detection of Salmonella. However, a full parallel study comparing culture methods with the flow devices should be considered as considered as part of a true evaluation of a range of flow devices and dipstick types. There are several dipstick rapid tests available for

use in water and food matrices. Quix Rapid *E. coli* O157 strip test (distributor M-Tech Diagnostics) has been advocated for the detection of serotype O157 in human faeces and will be available soon. The literature suggests that this should be a promising test, claiming to give results in 5 minutes and is applicable to field testing. Further claims are that the detection level is 3.4 x 10^4 CFU/mL with 99% specificity and 100% sensitivity for all strains of *E. coli* O157. This company also just released a Quix immuno-chromatographic assay for analysis of enrichment broths.

2.5.3.6 Nucleic Acid based methods

Rapid advances in molecular biology and molecular taxonomy are making it clear that many microorganisms exist in the environment, including potable water and wastewater, which cannot presently be cultured. Research has indicated that only approximately 1% of the bacteria in potable water can be cultured, yet they appear metabolically active. This non-culturability is presumed to be because:

- they are of previously unidentified genera/species whose physiology and growth requirements are not understood (implying that conventional laboratory growth media contain inappropriate nutrients), or
- they are environmentally stressed due to nutrient limitation, extremes of temperature, pH, redox, osmolarity etc., or to the presence of disinfectants such as chlorine, and may be termed viable but nonculturable (VNC) using routine laboratory media.

Knowledge of these non-culturable species is important because in the environment and the built environment they play a role in biofilm formation (causing biofouling, heat loss or corrosion of pipework, but making important contributions to wastewater treatment processes) and might also provide a shelter for bacteria causing infectious diseases. In particular, sub-lethally damaged or VNC pathogens such as VTEC, *Salmonella, Shigella* or *Campylobacter* spp. may be present in untreated and treated wastes and, although possibly remaining capable of causing infection, are undetectable by routine culture.

Where microorganisms can be cultured from low nutrient environments on specialized media, such as low nutrient R2A media, there is frequent disagreement over their identity when characterized using commercial API, BIOLOG and VITEK

biochemical databases (as discussed previously). Even then, they make take 7-10 days to grow before identification.

However, 16S and 23S rRNA sequences provide a unique signature for each prokaryotic species. Phylogenetic analysis of the rRNA sequences can be used to identify recovered bacteria in relation to well characterized strains, or the creation of new genera (e.g. within the α, β, or γ subclasses of the Proteobacteria or Eubacteria) The development of *in situ* hybridization with rRNA-targeted oligonucleotide probes has allowed rapid identification of bacteria within their natural habitat. Furthermore, where species are nonculturable, strain specific rRNA probes can be produced (using conserved primers as original templates to amplify the variable regions for sequence analysis; to determine their abundance *in situ*. Fluorescence *in situ* hybridization (FISH) relies on the presence of sufficient rRNA to bind to the labeled oligonucleotides and produce a bright fluorescence. Early studies correlated the ribosome content and growth rate of *S. typhimurium* and this data has been extrapolated to, or found to be reproducible with, data obtained from other species. The detection of cells is dependent therefore on the number of ribosomes and, hence, their physiological state. In microorganisms with a low rRNA concentration, including VNC, ribosome content can be increased by pre-incubating samples in a nutrient medium such as yeast extract or R2A medium, in the presence of a DNA gyrase inhibitor such as pipemidic acid to inhibit cell division. Fidelity and specificity of probe binding also can be problematic, requiring incubation with a specific concentration of formamide to maintain stringency. However, the recent advent of protein nucleic acids may solve this problem since these molecules are more flexible than conventional nucleic acids and bind better to curves and hairpin loops in the rRNA. Detection of hybridization may be by direct or scanning microscopy or flow cytometry. There may be limitations if the sample is taken from a nutrient starved environment which affects the ribosomal content. These methods are useful, even if it is detection of rRNA cannot be truly correlated to viability.

Several rRNA probes are now commercially available (GeneTrak). However, the search for a specific rRNA probe sequence for *E. coli* remains elusive. *E. coli* has been detected in freshwater biofilms with FISH using a 23S rRNA probe. The probe sequence (Gam 42a) is complementary to a selected region in the 23S rRNA of the

bacteria grouped in the gamma-subclass of *Proteobacteria*, and is therefore not specific for *E. coli* or its serotypes. This group also contains other enterobacteria, *Acinetobacter* and *Pseudomonas* spp. To improve specificity constructed a 24-mer oligonucleotide probe (termed "Colinsitu"), complementary to a piece of the *E. coli* 16S rRNA. They tested its sensitivity by and specificity by visualizing *E. coli* cells by in situ hybridization and epifluorescence microscopy. The fluorescent dye-labeled probe was able to stain cells of *E. coli*, *Shigella* spp. and *E. fergusonii*. *Shigella* spp. are known to belong to the *E. coli* genomospecies and *E. fergusonii* is the nomenspecies closest to *E. coli* by DNA-DNA hybridization. The probe did not stain any strain of 169 other genomospecies of the family Enterobacteriaceae or of a few other species frequently encountered in the environment. Revivification without cell division allowed the visualization of *E. coli* cells in contaminated water. They concluded that ISH using the Colinsitu probe is a potential tool for the confirmation of (atypical) *E. coli* in reference centers and the rapid (3-6 h) detection and enumeration of *E. coli* in urine specimens, contaminated water and food. More work is needed to include ISH in the routine laboratory.

The development of rapid methods, and methods which requires enrichment and culture, is limited by how short incubation times can be before limits of detection drop to unacceptable levels. The use of molecular methods which could be used instead of lengthy culture incubations appears to be a promising direction to take in the development of rapid methods. However, these methods are only going to be efficient if the specificity of the genetic probes are high enough, especially in samples with a high bacterial load and the method is not hampered by inhibitory substances in complex environmental samples. It is also very difficult to quantify molecular methods as the amount of genetic material generated by the method has to be calibrated back to bacterial cell numbers. Recently the use of real-time PCR which can be related to the true bacterial cycle and gives some degree of quantification. This method has been improved by the introduction of fluorescent gene specific probes. Results from molecular methods unlike culture methods do not assess whether the bacteria are viable in the samples tested. The method may be applied after an enrichment step but the result still cannot determine if the organisms are alive. O'Hanlon *et al*. (2004) developed real-time PCR detection procedure for *E.*

coli O111, O26 and O157 from minced meat. Strains of each of *E. coli* O26, *E. coli* O111 and *E. coli* O157 were inoculated at ca 10-20 CFU/g into minced retail meat and enriched for 6 h at 41.5°C as follows: *E. coli* O26 in tryptone soya broth (TSB) supplemented with cefixime (50 μg/l), vancomycin (40 mg/l) and potassium tellurite (2.5 mg/l); *E. coli* O111 in TSB supplemented with cefixime (50 μg/l), and vancomycin (40 mg/l); *E. coli* O157 in *E. coli* broth supplemented with novobiocin (20 mg/l). DNA was extracted from the enriched culture, and detected and quantified by real-time PCR using verotoxin (*vt*2 and *vt*2) and (O157 *per* gene, O26 *fli*C-*fli*A genes and O111 *wzy* gene) specific primers. The methods outlined were found to be sensitive and specific for the routine detection of *E. coli* O111, O26 and O157 in minced beef.

A real-time PCR with molecular beacons was capable of detecting *E. coli* O157:H7 when $>10^2$ CFU/mL was present in the samples, and as few as 1 CFU/mL in raw milk and apple juice was detected after 6 h of enrichment (Fortin *et al*., 2001). McKillip and Drake (2000) used a molecular beacon probe designed to hybridize with a region of the *stx*2 gene to detect *E. coli* O157:H7 in artificially contaminated skim milk. Spano *et al*. (2005) reported real-time PCR method for detected *E. coli* O157:H7 which rapid and sensitive may be useful to monitor the persistence of verocytotoxin-producing E. coli in general and to assess the effectiveness of wastewater treatment.

2.5.4 Rapid methods for detection of *Salmonella* spp.

2.5.4.1 Modification of existing culture methods

These usually include a pre-enrichment step in BPW or TSB and may also include a selective enrichment step. The modifications are devised to improve specificity at the end of procedure and may not result in reduced procedure time.

Although rapid end-point tests (e.g. lateral flow devices) enable *Salmonella* to be detected in only a few minutes, even the most sensitive of them requires at least 10^4 cells /mL of broth. Most naturally contaminated foodstuffs or environmental samples contain far fewer stressed cells/mL, making the initial enrichment phase essential. To enable stressed *Salmonella* to be isolated from food in under 48 hours, Oxoid has recently launched the S.P.R.I.N.T *Salmonella* kit which combines pre-enrichment and selective enrichment in a single incubation stage, so that the time

taken to complete this step is reduced by half. The kit uses plastic bags, containing slow release capsule of selective enrichment ingredients, and is therefore suitable for use with samples first homogenized in a Stomacher or Pulsifier. When used with an endpoint test such as Salmonella Chromogenic Medium, BAX Salmonella or Oxoid Salmonella Rapid Test, a result can be achieved within 48 hours.

In a similar way, Merck have introduced Salmosyst broth which is a 2 step pre-enrichment and selective enrichment procedure. Pignato *et al*.86 used Salmosyst broth as a combined pre-enrichment/selective enrichment broth and Rambach agar for isolation. They found that in artificially contaminated ground beef *S. enteritidis* was detected at a concentration of 10 CFU per 25 g.

2.5.4.2 Immunomagnetic separation (IMS)

Latterly, researchers have attempted to overcome the problems of interference from the background matrix, lack of sensitivity of detection and the long process of enrichment by using selective separation with antibodies liganded to magnetic particles. There are 2 principal companies involved in immunomagnetic separation (IMS) of pathogens, Dynal and IDG, and both supply kits which require pre-enrichment of samples in broth culture before capture on superparamagnetic polystyrene beads linked to antibodies. The beads are designed to replace the use of selective enrichment broths, and produce about the same degree of enrichment within 30 minutes as opposed to 24 hours. At the appropriate time, powerful magnets draw the beads to one side of the incubation tube allowing the supernatant containing unwanted material to be aspirated. The beads can then be washed before further analysis of the captured pathogens by PCR, ELISA, staining and microscopy, or culture. The technology involved in coupling monoclonal or polyclonal antibodies to magnetic beads for the IMS techniques is well established and has been used to detect salmonellae in food (Luk and Lindberg, 1991), and biotoxoids and bacterial spores (Gatto-Menking *et al*., 1995). More recently it has been advocated for the detection of low numbers of *C. parvum* and *Giardia lamblia (intestinalis)* in potable water, post-filtration, and forms the basis of UK and US EPA Methods 1622 and 1623. IMS has also found favour for the selective detection of *E. coli* O157 in food and water (Okrend *et al*., 1992) and faeces (Chapman *et al*., 1996); the detection limit was 1-2 CFU/g sample. Cubbon *et al*. (1996) found that IMS detection of O157 in faecal

samples was more sensitive than culture and compared well with PCR. The main problem when using the IMS technique is the number of sorbitol non-fermenters other than *E. coli* O157 that adhere non-specifically to the magnetic beads. Recovery of the pathogen from enrichment broth is enhanced by using antibody-coated magnetic beads and non-specific binding of other organisms is reduced by washing beads with phosphate buffered saline containing 0.002-0.05% Tween 80 (Anon, 1994 referred Hanko, 2000). A new approach to molecular labelling without a PCR step is molecular labelling, using for example DNA oligonucleotide probes linked to biotin (e.g. LightOn Salmonella, Aureon, Vienna). The assay is performed very similarly to a regular ELISA procedure. The detection and confirmation is done with luminescence labelled reagent rather than colorimetric. The nucleic acid based hybridization yields the specificity required for immediate confirmation. Once the probes hybridize to the overnight culture of target cells, they can be labelled with streptavidin-linked to an enzyme producing fluorescence or light for sensitive detection. The sensitivity by light detection is about 100-fold more sensitive than the colorimetric detection in ELISA procedures. The sample is read by the 96 well Mediators PhL, an ultrasensitive luminometer, in less than 2 minutes. No target amplification is necessary. The assay is suitable for the detection and confirmation of all *Salmonella* spp., either as picked colonies from agar culture, or directly from enriched samples treated with Salmonella A-*Beads*. The *Salmonella* A*Beads*™ are polydisperse 1.5 μm cluster type paramagnetic particles covalently coupled with antibodies raised against surface epitopes of *Salmonella.* They are designed for the fast and specific isolation of *Salmonella* from food and environmental samples. Any food, feed and environmental samples that contain a minimum of 100 *Salmonella* per mL of sample will yield a positive result. A-*Beads*™ coupled with an antibody to *Salmonella* will bind specifically to *Salmonella* in a mixed flora sample matrix. Using MagnetOnTM, the target bacteria is isolated from the sample matrix and is ready for subsequent detection. The recovery of *Salmonella* from the sample is significantly increased and yields almost pure *Salmonella* bacteria for optimal detection signal. Following enrichment, the *Salmonella* A-*Beads*™ will detect 1 viable organism in a 25g sample if present. The bacterial RNA is released from the bacteria upon lysis and is captured by an immobilized DNA-oligonucleotide. The

captured RNA is hybridized with a fluorescein-labelled detection probe. This probe is detected by an alkaline phosphatase conjugated anti-fluorescein antibody. The action of alkaline phosphatase on the fluorescein substrate causes decomposition of a chemiluminescent intermediate and the energy released is emitted as light, which can be measured in the 96 well microplate luminometer. The system is also applicable to detection of *E. coli* O157. Several methods have used an immunomagnetic separation (IMS) technique to concentrate the cells from a complex matrix, usually food, before proceeding with molecular or immunological steps. Wang and Slavik (1999) used an 18 hour pre-enrichment broth followed by IMS and flow cytometry on washings of chicken carcasses.

The PATHATRIX system described earlier for *E. coli* O157 detection is also suitable for *Salmonella.* MATRIX MicroScience Ltd. has launched a unique rapid detection and positive identification system, which simultaneously tests for *Listeria* spp and *Salmonella* spp contamination in food samples. Previously, tests for each pathogen have had to be conducted separately.Giving completed test results in just 40 hours, the new PATHATRIX Dual test, has received AOAC RI Validation after an extensive evaluation process at Campden & Chorleywood Food Research Association (CCFRA). MATRIX's PATHATRIX system has also received AOAC accreditation for the individual testing of *E. coli* O157, *Listeria* spp and *Salmonella* spp. As a result of the Dual test, laboratories will no longer have to conduct two separate tests, weigh both sets of samples and prepare two sets of selective media.

The single sample requires only addition of Buffered Peptone Water and the process achieves significant savings in terms of both equipment and consumables. Utilizing the proven PATHATRIX technology, the method requires less than two minutes hands-on time per test. Viable cultures are produced during the test allowing full and detailed analysis of any positive results. A standard 25 g food sample is homogenized with 225 mL of growth media in a stomacher and is incubated overnight. PATHATRIX capture reagent, which consists of antibody coated magnetic particles specific to the target pathogen, are then added directly to the sample. The sample is loaded onto the PATHATRIX workstation using a MATRIX proprietary consumable pack, connecting the sample to the circulatory system in preparation for the Capture-Culture step. Once loaded, the PATHATRIX workstation is pre-

programmed to run for 30 minutes at the desired incubation temperature. Upon completion of the run, the target microorganisms are bound onto the phase by the capture reagent. Residual debris and nonspecific binding are removed during a single wash step. The capture phase is disconnected from the system and the capture reagent/pathogen complexes are eluted by washing the phase into a vessel. The captured pathogen complexes are then concentrated into a small volume. i.e., 200 L using a magnetic rack. The sample can be plated directly onto selective media and incubated overnight for visualization the following morning. In the case of the new Dual test, the single sample is simply split over two plates, each containing the appropriate media for the target pathogen. The standard PATHATRIX test enables colonies to be viewed within 40 hours from point of sample without the interference from other non-target organisms that are seen in conventional tests.

2.5.4.3 Immunoscreening

A large proportion of immunolabelling is used as culture confirmation of species identity following enrichment and agar culture, using enzyme immunoassay (EIA) (also called enzyme linked immunosorbent assay; ELISA) or latex bead agglutination (LBA) augmented with serotyping and phage typing. However, these techniques can also be used without the agar culture step to speed up the recovery/detection time. A typical format for EIA or ELISA involves the coating of rabbit polyclonal or mouse monoclonal antibody to the wells of microtitre plates followed by introduction of the test sample. Protein or polysaccharide (lipopolysaccharide) antigens present in the sample are bound immunologically by the antibody. After washing to remove unbound material, enzyme-conjugated affinity-purified antibody specific to the target antigen is added. Following a second washing step to remove unbound enzyme-conjugated antibody, enzyme substrate is added and the incubation proceeds until stopped e.g. by addition of acid or alkali which also helps develop the product colour. Typical enzymes used include alkaline phosphatase (with p-nitrophenyl phosphate as substrate) and horseradish peroxidase (with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate and hydrogen peroxide). Most EIA kits have a sensitivity of approximately 10^6 CFU/mL organisms, and therefore usually require a concentration step (filtration, IMS and/or pre-enrichment). Pre-

enrichment in appropriate medium may be obligatory if there are concerns that epitope expression, e.g. flagellar antigen, is affected by the environment.

Many rapid manual immunoscreening assays are commercially available to detect *E. coli* O157 and *Salmonella* spp. These tests are performed on heat-killed culture broth after 24 hours for *E. coli* O157 and 40-48 hours for *Salmonella* spp. Microtitre well-based ELISAs (e.g. Microgen Salmonella ELISA) and dipstick ELISAs (e.g. Lumac Salmonella Path-Stick) are also commercially available for these pathogens and are recommended for wastewater treatment. Organon Teknika company have gone a step further by introducing an ELISA for *E. coli* O157 incorporating immuno-capture beads (EHEC-Tek). Recently, the manual ELISA tests have been adapted for use in automated instruments and greatly increase the capacity of a laboratory to perform up to 100,000 tests per annum on one instrument. ELISA has been used to detect the presence of Enterotoxigenic *E. coli* in water 76 and *S. enteritidis* in raw sewage, sludge and wastewater (Brigmon *et al*., 1992). 77 ELISA technology is maturing rapidly and can be included in 96-well plates for automated reading and software manipulation. However, a major disadvantage of the technique is the lack of sensitivity. A minimum of 10^5 cell/mL *S. enteritidis* are required to generate a clear signal against the background. LBA provides the least technically demanding method and, as the name suggests, relies on the agglutination of microscopic latex beads which are liganded with a specific polyclonal or monoclonal antibody to an epitope expressed by the microorganism. The preparations become cloudy or clump, which can be seen against a dark background. Sensitivity of detection varies from 10^2-10^6 cells/mL, depending on the avidity of the antigen-antibody reaction. For example, *E. coli* O157 can be detected with latex bead agglutination using antibodies raised against the lipopolysaccharide O157 antigen, reversed passive latex agglutination and passive haemagglutination. Isolates can also be serotyped with antisera and phage typed, as demonstrated successfully by Rahn *et al*.(1978) refer Warnes and Keevil (2004) a detailed study of *E. coli* O157:H7 persistence in human and animal faeces. However, Brehm-Stecher and Johnson (2004) (Food Research Institute) have developed a simplified DNA based FISH protocol that yields bright genus-specific hybridisation results in 10 minutes. They also demonstrated the compatability of FISH with the use of the respiratory substrate

5- cyano-2,3-ditolyl tetrazodium chloride (CTC), a red fluorescent viability indicator. They used flow cytometry and fluorescence microscopy to evaluate the fluorescent cells. This work gave simultaneous detection and viability determination of *Salmonella* in food. As with any molecular method the specificity of the probes is the key but if this method could be properly validated it could potentially be of great value in the screening of food, environmental and medical samples for salmonella.

2.5.4.4 Lateral flow devices

As discussed for *E. coli*, there have been rapid advances with lateral flow devices, including Visual Immunoprecipitate assay (VIP; BioControl System Inc.) and gold labelledimmunosorbent assay (GLISA) technologies such as the Merck Singlepath range for *E. coli* O157, *Salmonella* etc. The *Salmonella* test is carried out after a selective enrichment of the sample from one selective enrichment culture (taking 24-48 h) and yields a yes /no result in 20 minutes. The end result is thus available two days sooner saving material and labour costs. Techra Salmonella Visual Immunoassay (VIA) kit includes a single selective enrichment step and takes 22 hours to get results. This method has undergone large scale trials in food industry and is now an AOAC official method

2.5.4.5 Biochemical testing profile

Improvement is biochemical screenings do not really reduce the length of the total testing protocol but they do improve the sensitivity and specificity. The major tests available are the API (BioMerieux), Microbact (med Vet, supplied by Microgen Bioproducts) and the Automated VITEK. These systems are used predominantly to identify isolated colonies of bacteria and yeasts by comparing biochemical activities under defined growth and assay conditions i.e. detecting the biochemical phenotype. The results are compared to databases of standard strains but, because of market forces, have concentrated on identification of human pathogens from a clinical environment rather than a wide range of microorganisms from the environment at large.

The API systems (bioMerieux, Basingstoke) measure activities based on species characteristics known for many years, including catalase, oxidase, nitrate reduction, urease, indole production, and sugar fermentation activities to produce coloured reactions. The API 20E Enterobacteriaceae system comprises 20 tests

including ornithine decarboxylase, arginine dihydrolase, melibiose and inositol fermentation, and citrate utilisation. A 10 test system is also available. The API 20E and the similar Sensititre autoidentification system were both poor in discriminating between species of the genera *Klebsiella, Enterobacter* and *Serratia* (Barr *et al*., 1989). A later study showed that the API 20E identified 77% of *Enterobacteriaceae* strains correctly at the end of the initial incubation and subsequently identified >95% correctly when the additional tests were performed, as recommended by the manufacturer (O'Hara, 2005). The system was particularly good at identifying *E. coli* strains, as well as most *Salmonella enteritidis* and *Shigella* spp. The API 20E system has been shown to identify many yellow colonies of a typical *E. coli* isolated on m-FC medium from stream water; many of these strains were ONPG-positive and some produced gas in lactose medium at 44.5°C (Rychert and Stephenson, 1981). However, comparing fatty acid methyl ester (FAME) analysis with the use of API 20E gave good identification of approx. 30% of coliforms isolated on MacConkey agar from river water (Brown and Leff, 1996). Microgen Bioproducts now market the Microbact 12E and 24E Gram-negative identification system from Medvet (Mugg and Hill, 1981).

Automated systems are also produced; they include the Rapid ID 32E for *Enterobacteriaceae*, which gives results in 4 hours, and the VITEK AutoMicrobic system which uses a series of comparison cards for either detection, identification or susceptibility testing. The VITEK GNI card gave good identification (93%) of members of the *Enterobacteriaceae* within 4 to 18 hours (O'Hara *et al*., 1993) while the VITEK EPS card gave good discrimination of *Salmonella, Shigella* and *Yersinia* spp. (99.5%) after 4-8 hours incubation (Imperatrice and Nachamkin, 1993). This card is composed of 3 sections, with 10 wells in each section, allowing the user to test 3 different colonies on each card.

By contrast, the BIOLOG identification system (Don Whitley) employs a redox dye, tetrazolium violet, as an indicator of substrate utilization. The cell's metabolism of the test substrate results in the formation of NADH, which, in order to be deoxidized, passes electrons to the dye via an electron transport chain to produce a purple formazan. The system is thus able to detect the ability to metabolize a range of carbohydrates, amino acids, peptides, and carboxylic acids incubated in a 96 well

format for automated reading. Active growth in the wells is not required. The GN Microplate system has been used to identify over 600 Gram negative strains, including each of the 4 *Shigella* species (47-93% identification), *E. coli* (80% identification) and a range of *Salmonella* spp. (7-100% identification) (Holmes *et al*., 1994). For greater discrimination, BIOLOG introduced the ES Microplate system to characterise and/or identify different strains *of E. coli* and *Salmonella* spp. It is up to the user to build their own database, as described for *Legionella* spp. using the GN Microplate system (Mauchline and Keevil, 1991).

The metabolic phenotype of microorganisms is subject to the variability of growth and assay conditions. A trial organized by Yorkshire Environmental circulated 23 isolates to 40 participating UK and European laboratories to assess reproducibility between laboratories for biochemical identification of coliform bacteria. Only 3 isolates were consistently identified by all of the participants (Anon, 1998 refer Warnes and Keevil, 2004). Indeed, up to 9 different genera were reported for a single sample.

2.5.4.6 Impedance

Impedance technology is a rapid; automated qualitative technique which measures in a medium the conductance change induced by bacterial metabolism (Silley and Forsythe, 1996). The detection time is a function of both initial microorganism concentration and growth kinetics in a given medium. Specificity is incorporated into the technique by including either selective agents into the incubation broth and/or specific substrates. Thus, Easter and Gibson (1985) described an impedance technique in which changes in electrical conductance due to reduction by Salmonellae of trimethylamine-N-oxide were monitored. By contrast, Bullock and Frosham (1989) pre-enriched Salmonellas from contaminated confectionery in skimmed milk before 24 hour impediometry in lysine-iron-cystine-neutral red broth in a Bactometer 123 system (Bactomatic Ltd., Henley). The authors found that the inclusion of novobiocin (0.15 μg per well) eliminated false positive results due to *Citrobacter freundii* or *Enterobacter cloacae*. Pridmore and Silley (1998) refer Warnes and Keevil (2004) used the Rapid Automated Bacterial Impedance Technique (RABIT, Don Whitley, Shipley) to detect total coliforms, thermotolerant coliforms and enterococci in domestic sewage and 70% industrial sewage from 2 wastewater

treatment works. The coliforms were detected in Whitley MacConkey broth at 37°C and 44°C using the direct impedance technique. The majority of faecal coliform results were obtained within 7 hours (10^3 CFU/mL) compared to 24 hours using membrane filtration on MLSB, and without the need for serial dilution of samples and manual reading of plates.

The indirect impedance technique allows the use of components inappropriate in the direct method on account of high basal conductance. This method is based on the detection of carbon dioxide released by microorganisms into the culture medium, and which is absorbed in an alkaline solution in contact with the electrodes of the tubes. Blivet *et al*. (1998) proposed a new medium named KIMAN (Whitley Impedance Broth basal medium supplemented with 3 selective components: potassium iodide, malachite green and novobiocin,). This medium supported the growth of *Salmonella* serotypes, while inhibiting non-salmonella strains in pure culture, and was appropriate for the indirect impedance technique. As mentioned previously, the use of novobiocin is very important to suppress background competitors such as *Proteus* spp. and is included in the enrichment media specified in several of the draft MF and presence/absence CEN methods for detecting *Salmonella* in sludge.

2.5.4.7 Molecular probes

There is a confusing array of *Salmonella* primers for use in PCR techniques currently available. Lofstrom *et al.* (2004) used PCR to detect *Salmonella* in animal feeds. DeMedici *et al.* (2003) refer Warnes and Keevil (2004) compared using PCR and ELISA after a pre-enrichment step in meat samples and were able to detect 1-10 cells per 25 g. Gado *et al.* (2000) refer Warnes and Keevil (2004) used 2 primers (INVA 1/INVA) in a PCR, following pre-enrichment and selective broths, but experienced sensitivity problems.

The Bax system (DuPont) is the first commercially PCR-based approved method for food (AOAC Certified USA). The pre-enrichment has to produce at least 1000 cells per mL of culture. A lysate is made of this suspension followed by PCR with specific primers. The manufacturers claim this test is 10 to 100 times more sensitive than immunoassays. It takes 4 hour to obtain a result. The BAX Salmonella

test is now commercially available (e.g. UK distributor is Oxoid) for food and may be applicable to sludge, soil and biowastes.

The GENE-TRACK nucleic acid hybridization assay has been compared to conventional culture (Meckes and MacDonald, 2003) in the detection of *Salmonella* spp. in biosolids. The method has been used before successfully on foods. All samples were homogenized, pre-enriched in BPW followed by incubation in RV broth. Samples from these broths were tested by the molecular probe or sub-cultured onto selective agars or broths. There was some discrepancy between the two testing laboratories as each had slightly different protocols and the original samples were different (one tested poultry farm washings, the other biosolids before and after treatment. They concluded, however, that both methods gave equivalent results but the probe yielded results in 52 hours as compared to 120 hours for the culture methods. There was no indication of sensitivity and they did not use spiked samples.

Unfortunately *Salmonella* cannot be detected at the species or genus level using rRNA probes as there is no suitable oligonucleotide probes published to discriminate amongst the other coliforms of the δ subclass of Proteobacteria. Lin and Tsen (1995) confirmed this lack of specificity using 3 probes for the V3 to V6 region of the 16S rRNA gene of *S. enteritidis* and the cross reactivity with other coliforms. This lack of specificity was confirmed by Perry- O'Keefe *et al*., (2001). However, Nordentoft *et al*. (1997) selected an 18-mer oligonucleotide probe nn the basis of the 23S rRNA gene sequences representing all of the *S. enterica* subspecies and *S. bongori.* The specificity of the probe was tested by in situ hybridization to bacterial cell smears of pure cultures. Forty-nine of 55 tested *Salmonella* serovars belonging to subspecies I, II, IIIb, IV, and VI hybridized with the probe. The probe did not hybridize to serovars from subspecies IIIa (*S. arizonae*) or to *S. bongori*. No cross-reaction to 64 other strains of the family *Enterobacteriaceae* or 18 other bacterial strains outside this family was observed. The probe was tested with sections of formalin-fixed, paraffin-embedded tissue from experimentally infected mice or from animals with a history of clinical salmonellosis. In these tissue sections the probe hybridized specifically to *Salmonella* serovars, allowing for the detection of single bacterial cells. The development of a fluorescence-labelled specific oligonucleotide probe makes the FISH technique a promising tool for the rapid identification of *S.*

enterica in bacterial smears, as well as for the detection of *S. enterica* in histological tissue sections. Similarly, it could have value as apart of a rapid method for detecting *Salmonella* in sludge, soil and biowastes.

Duam *et al*. (2002) used real-time PCR detected *Salmonella* in suspect foods from outbreak of acute gastroenteritis among 109 attendees of a church picnic in Kerr County, Texas, was reported. A 5'-nuclease PCR assay was used to screen for *Salmonella* in nine food items from the buffet line. Barbeque chicken B tested positive for *Salmonella*, and no amplification was detected in the remaining food items. These PCR findings were consistent with culture results and were confirmed by direct nucleotide sequencing. *Salmonella enterica* serotype Panama was cultured from both food and patient stool samples.

Nam *et al*. (2005) developed and evaluated a SYBR Green 1 real-time PCR method for the specific detection of *Salmonella* spp. in dairy farm environmental samples. Previously reported 119-bp *invA* gene was selected for specificity, and 124 *Salmonella* spp. including type strains and 116 non-*Salmonella* strains were evaluated. All *Salmonella* strains tested were *invA*-positive and all non-*salmonella* strains yielded no amplification products. The melting temperature (T_m=79 °C) was consistently specific for the amplicon. Correlation coefficients of standard curves constructed using the threshold cycle (C_T) versus copy numbers of *Salmonella* Enteritidis showed good linearity in broth (R^2=0.994; slope=3.256) and sterilized milk (R^2=0.988; slope=3.247), and the minimum levels of detection were $>10^2$ and $>10^3$ colony forming units (CFU)/mL, respectively. To validate the real-time PCR assay, an experiment was conducted with both spiked and naturally contaminated samples. Lagoon water, feed/silage, bedding soil, and bulk tank milk samples obtained from dairy farms were spiked 10^0 to 10^5 CFU/mL of *Salmonella* Enteritidis. Sensitivities for detecting *Salmonella* in these sources were with 10^3 to 10^4 CFU/mL of inoculums in broth without enrichment. Detection limits were reduced to <10 CFU/mL of inoculums in broth after 18 h enrichment. Ninety-three environmental samples including fecal slurry, feed/silage, lagoon water, drinking water, bulk tank milk, farm soil, and bedding soil were analyzed for the presence of *Salmonella* by real-time PCR, results were compared with those obtained by conventional culture methods. All

samples analyzed were negative for *Salmonella* by both real-time PCR and standard culture method. No false positive or false negative results were detected.

Perelle *et al*. (2004) reported the performance of PCR assay amplifying 285-bp of the *inv*A gene of *Salmonella* spp. through an international ring-trial involving four participating laboratories. They have compared PCR-enzyme-linked immunosorbent assay (PCR-ELISA) and LightCycler real-time PCR assay (LC-PCR) with the standard ISO 6579 bacteriological reference method. The two PCR tests incorporated an internal amplification control (IAC) co-amplified with the *inv*A gene of *Salmonella* to monitor potential PCR inhibitors and ensure successful amplification. The selectivity study involved 84 *Salmonella* and 44 non-*Salmonella* strains and the samples tested were represented by 60 artificially-contaminated samples of fish, minced beef and raw milk, and 92 naturally-contaminated milk and meat samples. When using either PCR-ELISA or LC-PCR assays, only *Salmonella* strains were detected. PCR-ELISA and LC-PCR assays gave with pure *Salmonella* cultures the same detection limit level of 10^3 CFU/mL, which corresponds respectively to 50 and 10 cells per PCR tube. Data on artificially contaminated samples indicated that both PCR methods were able to detect after enrichment less than five *Salmonella* cells in 25 g of food, giving 100% concordance with the ISO 6579 reference method. The results on naturally contaminated samples demonstrated that despite certain inhibition problems, LC-PCR and PCR-ELISA assays were highly specific and sensitive, and provide a powerful tool for detection of *Salmonella* in food samples.

2.6 Sources of pathogenic organisms in agricultural products

Fecal wastes from domestic animals, wildlife and humans are applied to the soil surface and to varying extents are incorporated into the soil. These fecal wastes can also enter water systems by direct contamination of the water or through seepage or surface runoff. Humans contaminate water sources through poorly processed sewage effluents, malfunctioning septic tanks and seepage from sanitary landfills. Domestic and wild animals contaminate water by defecation in unprotected surface water, through runoff and as a result of seepage of water through soil that contains an excessive amount of animal feces. A wide variety of pathogenic and non-pathogenic

viruses, bacteria and parasites are normally contaminate in the feces of wild and domestic animals and humans as well as in soil and water. Only a small number of animal pathogens in feces, water and soil have the potential to infect humans and domestic animals (Table 4). These pathogens are of great concern to the public, who usually expose to them through the consumption of fecal contaminated food or water.

The population of wildlife is also a potential source of water and soil contamination. Some types of wildlife congregate in herds and flocks and can contribute to extensive pathogen contamination to the water and soil in certain location and times of the year.

Bacterial, parasitic and viral pathogens in human fecal waste have the greatest potential to cause infection in other humans. Raw sewage is processed to varying degrees before it is distributed on soil or discharged into water systems. Failure to appropriate process of human sewage probably poses the greatest threat to human health however, it is often impossible to identify sources of food and water contamination. Animal feces may contain pathogens infectious to both humans and animals. As a result food animals are incriminated in many waterborne and foodborne outbreaks. It is critical for human health, animal health and agriculture sustainability reasons that water and food supplies must be protected from contamination by animal feces.

Table 4 Prevalence of Enteric pathogens in humans, cattle, pigs and poultry.

Pathogens	Human	Cattle	Pigs	Poultry
Salmonella spp	1%	0-13%	0-38%	10-100%
E. coli 0157:H7	1%	16%	0.4%	1.3%
Campylobacter jejuni	1%	1%	2%	100%
Yersinia enterocolitica	0.002%	<1%	18%	0%
Giardia lamblia	1-5%	10-100%	1-20%	0%
Cryptosporidium spp	1%	1-100%	0-10%	0%

Source: Olson *et al*., (1997)

2.6.1 Enteropathogenic *E. coli*.

Enteropathogenic *Escherichia coli* are present in the feces of humans and animals. Both animals and humans are responsible for pollution of lakes and streams. Although *E. coli* may be found in feces, water and soil, only a small proportion (<1%) are potentially harmful strains. Most strains of *E. coli* inhabit the intestines of healthy animals and humans and are harmLess and in many cases beneficial. The harmful strains are called enterotoxogenic *E. coli* (ETEC) and the most common of these is *E. coli* O157:H7. *E. coli* O157:H7 and some other strains, produce potent toxins that can cause severe illness in humans. The combination of letters and numbers in the bacterium name refers to specific surface proteins that distinguish it from the harmLess types of *E. coli*. The organism can be found in the feces of some healthy cattle (Laegreid *et al*., 1999; Hancock *et al*., 1997; Zhao *et al*., 1995; Tauxe, 1997). Other domestic animals (sheep, pigs,) and wildlife (e.g. deer) can also harbour *E. coli* O157:H7 (Tauxe, 1997). The prevalence of *E. coli* O157:H7 has been reported to be between 0.4% and 7.5% of healthy pigs and up to 1.5% of pork meat samples. Verotoxin producing *E .coli* O157:H7 are only rarely isolated (Tauxe, 1997). This is contrast to cattle where 15% of cattle shed *E. coli* O157:H7 and up to 99% produce toxin. The risk to humans occur when it contaminates food (meat, milk) or drinking water supplies (Cole *et al*., 1999; Cieslak *et al*., 1993). Other sources of contamination include juice and uncooked greens (lettuce, sprouts) that have been contaminated with animal feces containing *E. coli* O157:H7 (Tauxe, 1997). The bacteria can be transmitted from one infected human to another through poor hygiene.18 Humans rarely carry the bacterium however young children can shed the bacterium for one to two weeks after an infection. Ingestion of just a few organisms can lead to disease.

E. coli O157:H7 infection may cause severe bloody diarrhea and abdominal cramps. Symptoms can also be mild to none at all. Usually the illness resolves in 5 to 10 days. In young children and the elderly, the infection can cause a serious complication called hemolytic uremic syndrome where the red blood cells are destroyed and the kidneys fail to function. This occurs in about 2% to 7% of the cases. These patients may die from this complication or have life-long kidney failure, seizures, blindness or high blood pressure. As pigs rarely excrete verotoxin producing

E. coli O157:H7, pig manure is considered a very low risk for human infections. Arthur *et al*. (2010) evaluated contamination of *E. coli* O157:H7 in beef feces at high levels (>10^4 CFU/g). Islam *et al*., (2004c) studies were done to determine the fate of *E. coli* O157:H7 in manure compost-amended soil on carrots and green onions grown in an environmentally controlled growth chamber. *E. coli* O157:H7 cell numbers decreased within 64 days by 3 log CFU/g in soil and soil beneath the roots of green onions and by more than 2 log CFU/g on onions. *E. coli* O157:H7 survived better during the production of carrots, with a 2.3 log CFU/g reduction in soil and a 1.7 log CFU/g reduction on carrots within 84 days.

2.6.2 *Salmonella* spp.

Salmonella is a bacterium that is widespread in the intestines of mammals, birds and reptiles. Salmonellosis is caused by many species of *Salmonella* and is characterized by three major symptoms-septicaemia, acute enteritis and chronic enteritis. The disease is observed in all animals and occurs worldwide. The incidence of salmonellosis in man has increased in recent years and animals have been incriminated. Feces of infected animals can contaminate animal feed, water, milk, fresh and processed meats and plant and animal products (Ekperigin and Najaraja, 1998). In swine, the prevalence of *Salmonella* spp in the feces has been reported in Quebec to be between 8 and 25% (Letellier, 1999).

The disease is endemic on certain farms with sporadic outbreaks. Subclinical infections are observed in older animals. Stress such as transportation, crowding and mixing can precipitate clinical signs and shedding of the bacterium. People infected with *Salmonella* develop diarrhea, fever and abdominal cramps 12 to 72 hours after infection (Tauxe, 1997). The illness lasts for less than a week and most individuals recover without treatment. The diarrhea in some people is so severe that it may require hospitalization and the infection can occasionally spread to the blood stream and other body sites. These patients may die of the infection. The elderly, infants and people with an impaired immune system are most susceptible to a severe illness. About 40,000 cases of salmonellosis are reported in Canada and the United States each year with about 1,000 people dying of acute salmonellosis each year (Tauxe, 1997). Salmonellae have become increasingly resistant to a variety of antimicrobial

agents and there are concerns that some of this resistance is associated with antimicrobial use in agriculture and human medicine (Tauxe, 1997).

A new strain of *Salmonella typhimurium* (called type DT104) has recently been recognized as a significant cause of diarrhea in animals and humans in Canada, the US and Europe21. This strain is of particular concern as it is resistant to several antibiotics that have traditionally been used to treat salmonellosis. The prevalence of *Salmonella typhimurium* DT 104 has been increasing in the past few years (Elperigin *et al*., 1998; Gynn *et al*., 1998).

Salmonella is usually transmitted to humans by eating foods contaminated with animal feces (Tauxe, 1997; Elperigin *et al*., 1998; Letellier *et al*., 1999; Gynn *et al*., 1998). Meat is usually contaminated at the slaughter house. Food may also be contaminated by food handlers who do not thoroughly wash their hands before touching food. The *Salmonella* bacterium is destroyed by cooking. Contaminated manure used to fertilize unprocessed foods (lettuce, sprouts, mushrooms) may be a source of human infection. Water may be contaminated by animals defecating in water or through runoff following heavy rainfall. Human infections may occur when unprocessed water is consumed or when the water is used to wash uncooked foods (Xiao, 1994; Olson *et al*., (1997).

Islam *et al*., (2004b) experimented three different types of compost, PM-5 (poultry manure compost), 338 (dairy cattle manure compost), and NVIRO-4 (alkaline-pH-stabilized dairy cattle manure compost), and irrigation water were inoculated with an a virulent strain of *Salmonella enterica* serovar Typhimurium at 10^7 CFU/g and 10^5 CFU/mL, respectively, to determine the persistence of salmonellae in soils containing these composts, in irrigation water, and also on carrots and radishes grown in these contaminated soils. A split-plot block design plan was used for each crop, with five treatments (one without compost, three with each of the three composts, and one without compost but with contaminated water applied) and five replicates for a total of 25 plots for each crop, with each plot measuring 1.8-4.6 m. Salmonellae persisted for an extended period of time, with the bacteria surviving in soil samples for 203 to 231 days, and were detected after seeds were sown for 84 and 203 days on radishes and carrots, respectively. *Salmonella* survival was greatest in soil amended with poultry compost and least in soil containing alkaline-pH-stabilized

dairy cattle manure compost. Survival profiles of *Salmonella* on vegetables and soil samples contaminated by irrigation water were similar to those observed when contamination occurred through compost. Hence, both contaminated manure compost and irrigation water can play an important role in contaminating soil and root vegetables with salmonellae for several months.

2.7 Composting of animal manures

Manure that has undergone appropriate treatment to inactivate human pathogens can be a safe soil amendment for use in agriculture. However, incomplete treatment of manure can lead to survival of human pathogens that could contaminate produce in the field and, ultimately, lead to foodborne-illness for those who eat the produce. Composting involves decomposition of organic matter by microorganisms that create a humus-like material for use as a soil amendment. Advantages to composting include. Composting involves decomposition of organic matter by microorganisms that create a humus-like material for use as a soil amendment. Advantages to composting include: improving soil structure and thus encouraging root development and making the soil easier to cultivate; providing plant nutrients to soil that enables the increased uptake of nutrients by plants; aiding water absorption and retention by the soil; binding of synthetic agricultural chemicals and thus minimizing contamination of groundwater supplies; and substantial reduction, if not elimination, of pathogenic microorganisms. Disadvantages of composting organic material include: loss of nutrients, such as nitrogen, during the process; the significant time, equipment, labor, land required for composting; and offensive odors generated during composting. Composting may occur under both aerobic (involving large amounts of oxygen) and anaerobic (involving the absence of oxygen) conditions but the organisms involved in the former system generate substantial amounts of heat while the organisms in the latter system do not.

2.7.1 Composting process

Composting proceeds in predictable stage. During different stages, temperature and nutrients availability vary and affect the kinds and numbers of microorganisms that develop. Initially, the pile is at approximately the ambient temperature. The composting material warms through the mesophilic temperature

range (15-40°C) as the microorganisms become more active. Therefor, microbial activity raises the temperature of the pile to thermophilic temperatures (41-77°C). This is considered the most productive stage of composting.

Mesophilies and thermophilies are microbes adapted to mesophilic and thermophilic conditions, respectively. Composting proceeds at a much faster rate under thermophilic conditions. Eventually, readily-available substrates within the feedstock are exhausted, temperatures gradually return to the mesophilic range, and curing begins. The following section expands on the microbiology of each stage **(**Erickson *et al*., 2010).

2.7.1.1 Initial Stage.

The process of transporting and manipulating the feedstock for composting exposes the organic matter to additional sources of microorganisms, all of which may contribute toward initiating the composting process. Initially, mesophiles predominate and proceed to decompose the readily-degradable sugars, proteins, starches, and fats typically found in undigested feedstocks. The availability of easily-usable organic substances enables the proliferation of the fastest-growing microorganisms, the bacteria. Mesophilic bacteria, therefore, dominate initial decomposition. These bacteria release heat from the breakdown of large amounts of easily-degraded organic matter. This heat begins to raise the temperature within the pile due to the high insulating capacity of a properly-sized compost pile. Within just hours, the temperature of the compost pile can rise above the 41°C thermophilic thresholds.

2.7.1.2 Active Stage

As the compost reaches higher temperatures, thermophiles begin to dominate the bacterial community. The active stage is typically the stage where most of the organic matter is converted into carbon dioxide and humus, and the microorganism population grows. The thermophilic population continues generating more heat by decomposing the remaining organic matter. Due to limitations with isolation techniques, laboratory studies have only been able to isolate a few genera of bacteria from the thermophilic stage (*Bacillus*, *Clostridium* and *Thermus*), but many microorganisms remain to be discovered and described. In a properly ventilated composting pile, the temperature will be maintained between approximately 55°C and 68°C. Fortunately, pathogens such as human viruses and infectious bacteria are

typically unable to persist in such a hostile environment. The higher temperatures will ensure rapid organic matter processing while simultaneously providing optimal conditions for the destruction of human and plant pathogens as well as weed seeds.

Because the composting pile is cooler on its outer surface, periodic mixing of the outer layer into the pile is essential for maximum pathogen and seed kill. Mixing or turning the pile also helps ventilate it by increasing the size and number of air pores. This is important because in an unventilated compost pile, the temperatures can exceed 71.1°C, effectively stopping all microbial activity. The air pores also serve as passages for oxygen to enter the pile. Microbes require oxygen to efficiently break down organic matter.

2.7.1.3 Overheating

If a pile does overheat, surpassing approximately 76.7°C, most microbes will be destroyed and microbial activity will virtually cease. Surviving microorganisms are typically those able to survive as *spores*. The spores will germinate when the composting pile returns to a more favorable temperature. These spores are thick-walled structures that are formed by the microorganism under stress such as heat, cold, drought, and low nutrient conditions. After overheating, the composting pile will cool to a mesophilic state, requiring the activity of mesophilic microorganisms to return the pile to thermophilic conditions. If the composting pile is low in readily-utilizable organic substrates, the pile may not be able to support the microbial activity needed to return to thermophilic conditions. In such a case, it may be necessary to supplement the composting pile with additional feedstock to ensure maximal degradation and pathogen removal. An overheated composting pile may return to thermophilic temperatures through the germination and activity of spore-forming microorganisms, and through the infiltration of microorganisms from the outer surface of the composting pile where the temperature was less extreme.

2.7.1.4 Curing Stage

A properly-functioning composting pile will eventually deplete itself of a majority of the easily-degradable organic substrates leaving some cellulose, but mainly lignins and humic materials. Bacteria are generally considered less adept at metabolizing these remaining compounds. Consequently, the bacterial population will decline in numbers as compared to fungi and actinomycetes. Because less heat is

generated at this point, the temperature of the composting pile will slowly fall to mesophilic temperatures. With the return of mesophilic conditions, the final curing stage of composting begins.

During the curing stage, the fungi and actinomycete populations predominate, while the bacterial population may decline somewhat. Fungi and actinomycetes proliferate on the remaining less-degradable organic matter such as chitin, cellulose and lignin. These compounds are more persistent because they are insoluble in water and, due to their size and chemical complexity, cannot pass into the bacterial cell. Thus, degradation of these compounds requires the use of extracellular enzymes.

Once the complex organic compounds are broken down into smaller and more soluble forms, they can enter the cell and become food and energy for the microorganism. Microbes able to produce extracellular enzymes suitable for breaking down recalcitrant materials will have a selective advantage at this point in the composting process. A novel feature of many of the extracellular enzymes common in fungi is that they are capable of breaking down a wide range of compounds that would otherwise require several specific enzymes, a feature not commonly found in a single microorganism. Fungi, though they grow and reproduce more slowly than bacteria when food is readily available, are well suited for exploiting an environment rich in complex recalcitrant organic compounds like those found in the compost during the curing stage.

The curing process can vary in duration; a longer curing period provides more assurance that the compost is free of pathogens and phytotoxins. If the compost is incompletely cured (i.e., not stable), it maintains a higher microbial activity, leading to increased oxygen consumption. When unstable compost is applied in the field, it can thereby decrease the supply of oxygen available to plant roots. In addition, immature compost can contain higher levels of soluble organic matter (i.e., organic acids), which can lead to toxicity problems for certain horticultural applications, such as seed germination. As the curing stage continues, there is a gradual increase in the humus fraction. Humus is a complex class of chemicals that result from the incomplete degradation of organic matter. Humus is among the most resistant compounds to degradation in nature. It is also one of the major mechanisms for the retention of nutrients (e.g., nitrogen, phosphorus) and micronutrients (e.g., copper,

zinc, iron, manganese, calcium) in the soil. Because humic compounds retain micronutrients and water so well, they are often the site for the highest biological activity, including microorganisms, protozoans, invertebrates (e.g., worms, springtails) and plants.

2.7.2 Compost microorganism

2.7.2.1 Sources

The microorganisms needed for composting are found throughout the natural environment. They are present in compost feedstock as well as in the water, air, soil and machinery the feedstock and compost are exposed to during processing. These sources ensure a high diversity of microorganisms which helps maintain an active microbial population during the dynamic chemical and physical processes of composting, such as shifts in pH, temperature, water, organic matter and nutrient availability. Only on rare occasions will the addition of microorganisms be warranted.

2.7.2.2 Microbe Types and Requirements

The microbiological components of compost consist of bacteria and fungi. Because of their unique nature, *actinomycetes* are discussed here as a third microbiological component, though in actuality actinomycetes are a particular kind of bacteria. The majority of microorganisms responsible for the formation of compost are aerobes in that they require or work best in the presence of oxygen. Many difficulties associated with composting may be traced to insufficient oxygen levels to support the decomposition of compost feedstock. Compost microbes also require a moist environment because they live in the water films surrounding composting organic matter particles. A 50 to 60 percent moisture content is optimal.

2.7.2.3 Fungi

Fungi form their individual cells into long filaments called hyphae. Fungal hyphae are larger than actinomycetes and may be more easily seen with the naked eye. They penetrate throughout the composting material, decomposing both chemically and mechanically the more recalcitrant organic matter fraction such as lignins and cellulose. Fungal hyphae physically stabilize the compost into small aggregates, providing the compost with improved aeration and drainage. Fungi number between 0.01 and 1 million propagules per gram of soil. About 70,000

different species of fungi have been described worldwide, but an estimated 1 million additional species remain undiscovered and undescribed. Ecologically, fungi play a vital role in breakdown of dead plant materials.

2.7.2.4 Bacteria

The most numerous biological component of compost is the bacteria. Although they often can exceed 1 billion microorganisms per gram of soil, bacteria (with the exception of actinomycetes) do not contribute as much to the overall microbiological mass as fungi because of their relatively small size. Although bacteria (with the exception of actinomycetes) exist as individuals and do not form filaments, they also contribute to the stabilization of aggregates through the excretion of organic compounds that bind adjacent organic matter and soil particles together. Bacteria are typically associated with the consumption of easily-degraded organic matter. They are the dominant population throughout the entire composting process whereas the actinomycetes and fungi typically proliferate in the later stages.

2.7.2.5 Actinomycetes

While actinomycetes are visually similar to fungi in that they have networks of individual cells that form filaments or strands, they are actually a type of bacteria. These filaments allow for a colony of actinomycetes to spread throughout a compost pile where they are typically associated with the degradation of the more recalcitrant compounds. Actinomycetes number between 0.1 and 10 million propagules per gram of soil. Their filaments contribute to the formation of the stable organic aggregates typical of finished compost. Actinomycetes are tolerant of lower moisture conditions than other bacteria and are responsible for the release of geosmin, a chemical associated with the typically musty, earthy smell of compost.

2.7.3 Types of composting systems

2.7.3.1 Active Aerated Static Pile (Susangka1 and Chaerul, 2009)

In this method, a blower is used to force or draw air through the pile. No turning of the material is required once the pile is formed. As the pile is not turned, particular attention must be given to the blending of the material with structural amendments to maintain porosity throughout the composting period. It is important to achieve a homogeneous mixture and not compact the material with machinery

while constructing the pile, so that air distribution is even and no anaerobic areas develop causing sections of uncomposted material.

2.7.3.2 Passive Aerated Static Pile

In this method, the need for turning is eliminated as perforated open-ended pipes embedded across the base of the windrow allow air diffuse through the material. As the material is not turn over, particular attention must be given to the size, structure, moisture and porosity of the material. Commonly, straw and wood chips amendments are well achieve to use for good structure of the pile. Because there is no turning over, the raw materials must be thoroughly mixed before windrow formation and care must be given not to compact the material while building the windrow. Generally, the windrow performance is in a size of 1.5 m high and 3 m wide and the aeration pipes are lined at the base of the compost pile.

2.7.3.3 Windrow

The material is arranged in long narrow piles 3-6 m wide and 1-3.6 m high. The width of the windrow is largely determined by the size of the machine used to turn the windrow. The size of the windrow for the maintenance of aerobic conditions is determined by the porosity of the material. Large windrows will quickly become anaerobic in the core, requiring constant turning, while windrows which are too small will not attain the required temperatures for efficient composting and the destruction of weed seeds and pathogens. Turning frequency depends on the rate of the composting reaction. Temperature, oxygen concentration and odors are good indicators for turning.

2.7.3.4 Vermicomposting

Vermi-composting, also known as vermiculture, is a simple technology using the natural digestion process of redworms and earthworms to break down organic material. From the moment it hatches, a worm can consume daily its body weight in organic matter such as vegetables, fruit, leaves, grass, meat, fish, sludge, cardboard, and paper. The waste is continuously turned and mixed as the worms burrow through the medium. Worm castings contain high concentrations of nitrates, potassium, calcium, phosphorous, and magnesium and can be applied instead of chemical fertilizers in some agricultural practices. Castings also contain many worm eggs which continue to enrich the soil when it is applied.

2.7.4 Factors affecting composting

2.7.4.1 Aeration

Aerobic composting requires large amounts of O, particularly at the initial stage. Aeration is the source of O, and, thus, indispensable for aerobic composting. Where the supply of O is not sufficient, the growth of aerobic micro-organisms is limited, resulting in slower decomposition. Moreover, aeration removes excessive heat, water vapour and other gases trapped in the pile. Heat removal is particularly important in warm climates as the risk of overheating and fire is higher. Therefore, good aeration is indispensable for efficient composting. It may be achieved by controlling the physical quality of the materials (particle size and moisture content), pile size and ventilation and by ensuring adequate frequency of turning.

2.7.4.2 Moisture

Moisture is necessary to support the metabolic activity of the micro-organisms. Composting materials should maintain moisture content of 40-65 percent. Where the pile is too dry, composting occurs more slowly, while moisture content in excess of 65 percent develops anaerobic conditions. In practice, it is advisable to start the pile with moisture content of 50-60 percent, finishing at about 30 percent.

2.7.4.3 Nutrients

Micro-organisms require carbon (C), nitrogen (N), phosphorus (P) and potassium (K) as the primary nutrients. Of particular importance is the C:N ratio of raw materials. The optimal C:N ratio of raw materials is between 25:1 and 30:1 although ratios between 20:1 and 40:1 are also acceptable. Where the ratio is higher than 40:1, the growth of micro-organisms is limited, resulting in a longer composting time. A C:N ratio of less than 20:1 leads to underutilization of N and the excess may be lost to the atmosphere as ammonia or nitrous oxide, and odor can be a problem. The C:N ratio of the final product should be between about 10:1 and 15:1.

2.7.4.4 Temperature

The process of composting involves two temperature ranges: mesophilic and thermophilic. While the ideal temperature for the initial composting stage is 20-40°C, at subsequent stages with the thermophilic organisms taking over, a temperature range of 41-70°C may be ideal. High temperatures characterize the aerobic composting process and serve as signs of vigorous microbial activities. Pathogens are normally

destroyed at 55°C and above while the critical point for elimination of weed seeds is 62°C. Turnings and aeration can be used to regulate temperature.

2.7.4.5 Polyphenols

Polyphenols include hydrolysable and condensed tannins (Schorth, 2003). Insoluble condensed tannins bind the cell walls and proteins and make them physically or chemically less accessible to decomposers. Soluble condensed and hydrolysable tannins react with proteins and reduce their microbial degradation and thus N release. Polyphenols and lignin are attracting more attention as inhibiting factors. Palm *et al*. (2001) suggest that the contents of these two substances be used to classify organic materials for more efficient on-farm natural resource utilization, including composting.

2.7.4.6 pH value

Although the natural buffering effect of the composting process lends itself to accepting material with a wide range of pH, the pH level should not exceed eight. At higher pH levels, more ammonia gas is generated and may be lost to the atmosphere.

2.7.5 Maintaining a compost pile

Turning the compost pile on a regular basis (weekly or so) will help ensure that enough oxygen gets to all parts of the pile to encourage a quick decomposition rate. A pitchfork is a good tool to use to turn the compost pile. When the pile is turned regularly, you will notice that the contents change their appearance each time. By the third turning, the original contents should not be recognizable. Keep in mind that when the original contents are shredded, decomposition will occur more quickly, also.

Water is needed too. Sources from Cornell Cooperative Extension indicate that a moisture content of 40-60 percent is a good amount of water that does not interfere with the aeration process. How does a gardener know when the magic number has been reached? Usually the "squeeze" test is a good indicator. The material should feel damp to the touch, with just a drop or two of liquid expelled when the material is tightly squeezed in the hand.

A compost pile can be just that-yard, garden and food waste that is put into a pile in your yard. A good size for a compost pile is 3 feet high by 3 feet wide by 3 feet deep. This is large enough to hold the heat but small enough to allow air to get

into the center of the pile. Many people will use fencing of some kind to contain the pile. There is also what is known as "compost pockets." These are places in your garden where the fruit and vegetable wastes from the kitchen that were collected in a plastic container or bucket are buried. The holes should be about 10-15 inches deep and not larger than 2 feet across. Bury the scraps into the hole, covering them with at least 8 inches of soil **(**Mochamer, 2010**).**

2.7.6 Intrinsic and extrinsic properties of the composting process to control foodborne pathogens

The primary factor responsible for inactivation of foodborne pathogens during aerobic composting of animal manures is heat; thus, developing and holding of temperatures above 55°C (131°F; 3 days for static piles or bioreactors and 15 days for turned windrows) has been considered the minimum threshold for this purpose. In addition to temperature, other chemical, biological and physical factors during composting also influence pathogen inactivation. Examples include volatile acids, ammonia, microbial competition, drying and UV light. Aerobic composting of manures is a complex process but typically starts by mixing one or more carbon amendments with a nitrogen-rich material to produce a nutrient-rich environment favorable for the growth of microorganisms. The compost material is then placed into piles, windrows or containers that provide a sufficient mass for self-insulation. During composting, the process follows a predictable succession of stages. During the initial stages when temperatures are 35 to 45°C, mesophilic bacteria (bacteria that grow best at temperatures from 30 to 45°C) predominate and their metabolic activity may be accompanied by a decrease in pH due to the accumulation of volatile organic acids, such as acetic and lactic acids. When organic acid levels decline and the pH begins to increase due to the production of ammonia, the temperature increases (50 to 70°C) and marks the thermophilic stage during which thermophilic microorganisms (microorganisms that thrive at an optimal growth temperature of 55 to 75°C) dominate. If oxygen levels become low or the temperature approaches 70°C at any time during this active composting period, the temperature will decrease because microbial activity declines. Turning the pile or applying forced aeration, however, will revitalize the system and temperatures will increase again. Another consequence of heat generation is that moisture is removed from the compost heap and surfaces

become drier. Eventually, the microbial activity slows down and the temperature will decrease and stabilize. A curing or maturation period then follows the active composting stage. During this period, compost material continues to be broken down but at a much slower rate by the dominant microbial community of fungi and actinomycetes (Tiquia *et al*., 2002).

One of the major characteristics of composting systems that affects pathogen inactivation is temperature and moisture stratification (Shepherd *et al*., 2007), and this characteristic would be accentuated during winter composting. A gradation of temperature zones exists from the interior (high temperatures, moist conditions) to the exterior (ambient temperature, dry conditions) and thus a gradation in the population of surviving pathogens also occurs. Interestingly, significant correlations between moisture content and the temperature distribution within compost piles have been reported (Gotaas, 1956): when the moisture content is high, the high temperature zone extends closer to the surface than when the moisture content is low. The status of these conditions is significant because moist heat, in general, is more destructive to pathogens than dry heat. To circumvent disparities in stratification, turning of compost heaps is often recommended in order to expose the material to the thermal temperatures. In one mathematical model, it was predicated that at least three turns are required for windrow composting to ensure that less than 0.2% of the raw material remained in the 'cold' part of the heap (Haug, 1993). An overlooked weakness of this solution, however, is that recontamination of interior portions from contaminated sites (i.e. surface compost material or turning equipment) would occur during turning of the material.

In the absence of turning, the contribution of chemical and physical factors, other than heat, are more dominant in pathogen inactivation at sites near the surface of compost piles. For example, in slightly acidic compost systems (pH ~ 5.5 to 6.0), inactivation of both *Salmonella* spp. and *Listeria monocytogenes* occurred with very little increase in temperature and was attributed to an increase in volatile acids (Erickson, 2009a,b). Ammonia generated during composting is another chemical that has bactericidal properties (Michel *et al*., 1998). On the surface of compost piles, pathogens are exposed to solar radiation and very dry conditions, either of which can result in their inactivation (Erickson *et al*., 2010).

An implicit assumption made with time-temperature guidelines for pathogen inactivation is that inactivation is not dependent upon the rate at which that temperature is achieved. Extended exposure to non-lethal temperatures above 40°C, however, has been shown to generate heat-shock proteins that aid in the survival of the organism at higher temperatures (Cebrian *et al*., 2008). Such conditions may have occurred in those cases where pathogens were detected in finished composts that were determined to have received the appropriate time-temperature conditions.

Differences in the amounts of heat generated among compost systems are in large part dependent on the feedstocks incorporated into the compost preparations (Mote *et al*., 1980). In general, raw materials are blended to an initial moisture content of 40 to 60% and a carbon:nitrogen (C:N) ratio of 20:1 to 40:1 to serve as nutrients for the types of microbes that produce the most desired form of compost. Carbon amendments vary in their availability to microorganisms. For example, carbon from cellulose within straw is much more available to microorganisms than is the carbon from lignin within woody materials. Hence, compost heaps made with straw will heat more rapidly than those made with wood chips. Even when the same carbon amendment is used, differences in heat generation can occur when the carbon becomes more available through increases in the amendment's surface area. Similarly, the carbon in older feedstocks would likely be more readily available due to microbial decomposition that already has occurred to some extent. Manure stockpiling, prior to composting, can also affect the rate of heat generation during composting as the nutritional composition of this material for compost microbes would have changed from its initial fresh state. Since composting may include a very diverse group of feedstocks with a wide range of nutritional constituents affecting microbial metabolism, it would be difficult to avoid situations in which pathogens can be exposed to temperatures conducive to their production of heat-shock proteins.

A critical component to the breakdown of organic materials and subsequent generation of heat in compost systems is the non-pathogenic indigenous microflora that metabolize available nutrients. Manure and not the carbon amendment, is the primary source of the microbial community (Green *et al*., 2004). In general, high microbial diversity is considered fundamental for an efficient and satisfactory composting process; however, differences in microbial composition exist among

manures. For example, in poultry-manure compost, the bacterial community is comprised of a more divergent group of species that utilize a more diverse group of substrates than the microbes associated with cattle- manure compost (Wang *et al*., 2007). In spite of these differences, ample levels and diversity of microflora are present in manure such that commercial inoculants, (non-pathogenic microbial cultures added to the green compost) and accelerant chemicals (ammonium sulfate) rarely affect the thermophilic phase of the composting process (Regan, 1998).

In addition to metabolic heat generated at interior locations of compost piles, indigenous microflora may also affect the fate of pathogens in compost mixtures through other mechanisms. These include production of antimicrobials like lactic acid or bacteriocins. Moreover, the role of antimicrobials may be more important when heat does not play a dominant role, such as at surface locations or compost that is curing, i.e., in the later stages of composting when relatively little heat is generated. Pathogen populations when present in compost typically represent only a small fraction of the total microbial population. As a result of this imbalance, pathogens are at a competitive disadvantage compared to the total microbial population, especially when available growth nutrients are limited at the later stages of composting. Indigenous microbes may also affect pathogen survival through the production of antimicrobial agents; in one study, the growth of *Salmonella* was suppressed in non-sterilized composted biosolids compared to sterilized samples (Sidhu *et al*., 2001)