CHAPTER 2 THEORY AND LITERATURE REVIEWS

This chapter provides the theory and literature review related to this study including Thai food product, morphology and characteristic of *Escherichia coli*, yeast and mold traditional detection method and modern rapid method.

2.1 Thailand's Exported Food Products

Thailand is one of the world's largest producers of food products such as rice, canned tuna, frozen seafood, chicken and canned pineapple. In 2011, the value of Thai food exports increased 20% from the previous year owing to strengthened economic performance amongst major food importers such as the US, Japan, and ASEAN countries.

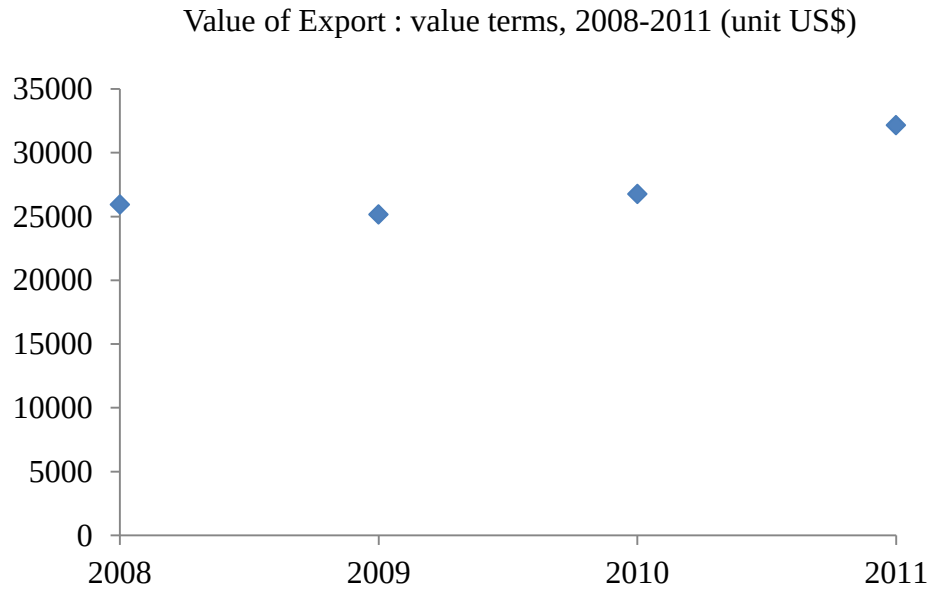

Figure 2.1 Thailand's Exported Food Products, 2008 –2011
(Source: *National Food Institute*)

Thailand's ambition to establish itself as the Kitchen of the World has dramatically increased awareness about, and focus on, safe and durable processed food. Therefore high

levels of safety and quality assurances are being implemented. Even the 9000 odd cottage and small enterprises maintain the highest standards of quality and hygiene which is the key to their competitiveness and access to some of the world's most demanding markets which have stringent and exacting access standard (Murray, 2007).

2.2 Food-Borne and Waterborne Diseases

Many microorganism contaminating food and water can cause acute gastroenteritis or inflammation of the stomach and intestinal lining. When food is the source of the pathogen, the condition is often called food poising. Gastroenteritis can arise in two ways. The microorganisms may actually produce a food-borne infection. That is, they may first colonize the gastrointestinal tract and grow within in, then either invade host tissues or secrete exotoxins. Alternatively the pathogen may secrete an exotoxin that contaminates the food and is then ingested by the host. This is sometimes referred to as a food intoxication because the toxin is ingested and the presence of living microorganisms is not required. Because these toxins disrupt the functioning of the intestinal mucosa they are called enterotoxins. Common symptoms of enterotoxin poisoning are nausea, vomiting and diarrhea.

Worldwide, diarrheal diseases are second only to respiratory diseases as a cause of adult death; they are the leading cause of childhood death, and in some parts of the world they are responsible for more years of potential life lost than all other causes combined. For example, each year around 5 million children (more than 13,600 a day) die from diarrheal diseases in Asia, Africa and South America. In the United States estimates exceed 10,000 deaths per year from diarrhea and an average of 500 childhood deaths are reported.

2.3 Morphology and characteristic of Yeast & Mold

Yeasts and moulds in food product can periodically cause problems, both economic and sensory. The food especially contain high levels of sugars and other nutrients, and they possess an ideal water activity for microbial growth; their low pH makes them particularly

susceptible to fungal spoilage, because a big part of the bacterial competition is eliminated since most bacteria prefer near neutral pH.

2.3.1 Yeasts

Yeasts are unicellular fungi that reproduce by budding, that is, by forming and pinching off daughter cells. (Fig 2.2) Yeast cells are much larger (about five to eight times) than bacterial cells. The best-known (and most useful) species is "bakers' yeast," *Saccharomyces cerevisiae*, used in bread making and in fermentations for wine and beer production.

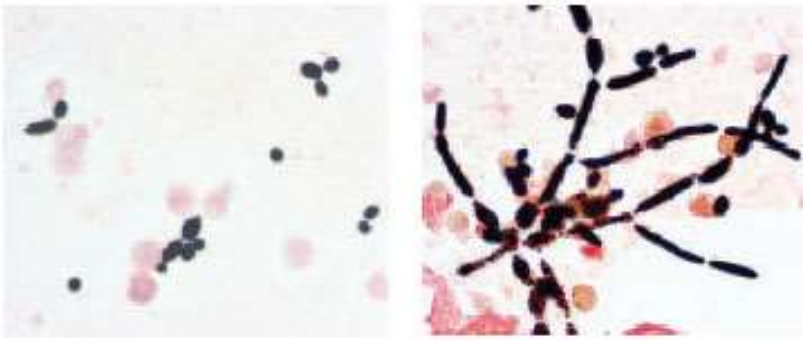

Figure 2.2 Gram stain of *Candida albicans* cells isolated from the blood culture of a patient. At left the yeast cells are budding, and at right, they have formed long, filamentous, irregularly staining hyphae

2.3.2 Mold

Molds are multicellular, higher forms of fungi. They are composed of filaments called *hyphae*, abundantly interwoven in a mat called the *mycelium*. Specialized structures for reproduction arise from the hyphae and produce *conidia* (also called *spores*), each of which can germinate to form new growth of the fungus. The visible growth of a mold often has a fuzzy appearance because the mycelium extends upward from its vegetative base of growth, thrusting specialized hyphae that bear conidia into the air. This portion is called the *aerial* mycelium. You have often seen this on moldy bread or other food, and you have probably also noted that different molds vary in color (black, green, yellow) because of their conidial pigment.

Figure 2.3 Colonies of three *Aspergillus species.* Some molds may be recognized by the color of their spores (conidia). Clockwise from left: *A. flavus* (yellow), *A. fumigatus* (smoky gray-green), *A. niger* (black)

Most of the thousands of species of yeasts and molds that are found in nature are saprophytic and incapable of causing disease. Indeed, many are extremely useful in the processing of certain foods (such as cheeses) and as a source of antimicrobial agents. *Penicillium notatum*, for example, is the mold that produces penicillin.

2.4 Impact of Yeast & Mold infection

The large and diverse group of microscopic foodborne yeasts and molds (fungi) includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile environmental requirements. Although the majority of yeasts and molds are obligate aerobes (require free oxygen for growth), their acid/alkaline requirement for growth is quite broad, ranging from pH 2 to above pH 9. Their temperature range (10-35°C) is also broad, with a few species capable of growth below or above this range. Moisture requirements of foodborne molds are relatively low; most species can grow at a water activity (a_w) of 0.85 or less, although yeasts generally require a higher water activity.

Both yeasts and molds cause various degrees of deterioration and decomposition of foods. They can invade and grow on virtually any type of food at any time; they invade crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage. They also grow on processed foods and food mixtures. Their detectability in or on foods depends on

food type, organisms involved, and degree of invasion; the contaminated food may be slightly blemished, severely blemished, or completely decomposed, with the actual growth manifested by rot spots of various sizes and colors, unsightly scabs, slime, white cottony mycelium, or highly colored sporulating mold. Abnormal flavors and odors may also be produced. Occasionally, a food appears mold-free but is found upon mycological examination to be contaminated. Contamination of foods by yeasts and molds can result in substantial economic losses to producer, processor, and consumer.

Several foodborne molds, and possibly yeasts, may also be hazardous to human or animal health because of their ability to produce toxic metabolites known as mycotoxins. Most mycotoxins are stable compounds that are not destroyed during food processing or home cooking. Even though the generating organisms may not survive food preparation, the preformed toxin may still be present. Certain foodborne molds and yeasts may also elicit allergic reactions or may cause infections. Although most foodborne fungi are not infectious, some species can cause infection, especially in immunocompromised populations, such as the aged and debilitated, HIV-infected individuals, and persons receiving chemotherapy or antibiotic treatment.

2.5 Thai FDA Revising Yeast and Mold Level in Foods

Thai Food and Drug Administration (FDA) have regulated yeast and mold in food products. Due to the no-tolerance level imposed by the Thai FDA on these contaminants, disputes between the Thai FDA and Thai food manufacturers and importers have arisen with the latter arguing that yeast and mold free food is not realistic as there are also natural or unavoidable yeast and mold in some foods that present no health hazards for humans. In addition, the weather conditions in Thailand, humid and warm, are perfect for yeast and mold to multiply. Therefore, the Thai FDA is proposing to amend its regulations to address these concerns by revising the level of yeast and mold in some food categories taking into consideration the fact that some products, even those manufactured under “Good Manufacturing Practice” guidelines, will contain certain levels of yeast and mold that don’t present a food safety risk. The revised notifications notified to the WTO are G/SPS/N/THA/191, 192, 193, 31/Rev.1, 33/Rev.1, and 34/Rev.1 on September 28-29 and

target six food categories beverages in sealed containers, coffee, tea, chocolate, weight control foods, and electrolyte drinks. It is to note that the amended regulations are trade enhancing and will facilitate trade of U.S. agricultural products. The revision of level of yeast and mold found in foods would ease the import process for those six product groups, which are categorized under specifically-controlled and standardized food categories. To register those products with the Thai FDA, the importers require submitting the lab analysis result of yeast and mold. The exports of those six product groups from the US to Thailand valued over $27 million in 2009.

The proposed revised level of yeast and mold required the amendment of six MOPH notifications currently defined non-tolerance level of yeast and mold in foods as follows:

- Notification of the Ministry of Public Health No. 83 B.E. 2527 (1984) Re: Chocolate dated 15th November B.E. 2527 (1984).
- Notification of the Ministry of Public Health No.121 B.E. 2532 (1989) Re: Weight Control Food dated 23rd May B.E. 2532 (1989).
- Notification of the Ministry of Public Health No. 195 B.E. 2543 (2000) Re: Electrolyte Drinks dated 19th September B.E. 2543 (2000).
- Notification of the Ministry of Public Health No. 196 B.E. 2543 (2000) Re: Tea dated 19th September 2543 (2000).
- Notification of the Ministry of Public Health No. 197 B.E. 2543 (2000) Re: Coffee dated 19th September 2543 (2000).
- Notification of the Ministry of Public Health No. 214 B.E. 2543 (2000) Re: Beverages in Sealed Container dated 19th September 2543 (2000).

The method of analysis for yeast and mold must comply with the Bacteriological Analytical Manual (BAM) Online, U.S. Food and Drug Administration (updated version) or equivalent method. All notifications will be come into effect as from the day following date of their publication in the Government Gazette. The new proposed level of yeast and mold allowed to be found in foods are show in Table 2.1

Table 2.1 The new proposed level of yeast and mold allowed to be found in foods

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
1. Chocolate and its products	Free of mold for 1g of product	Less than 100 CFU/g
2. Coffee (Product definition can be found in the MOPH Notification No.197)		
• UHT or sterilized ready to drink coffee	Free of yeast and mold	Less than 1 CFU/ml
• Other than UHT or sterilized ready to drink coffee	Free of yeast and mold	Less than 100 CFU/ml
• Ready to drink coffee in dry form dissolved as per instruction on the package	Free of yeast and mold	Less than 100 CFU/g
3. Weight Control Food		
• UHT or sterilized weight control in liquid form	Free of yeast and mold	Less than 1 CFU/ml
• UHT or sterilized weight control in concentration, dry, and semi-solid form	Free of yeast and mold	Less than 10 CFU/g
• Other than UHT or sterilized weight control in liquid, concentration, dry, and semi-solid form	Free of yeast and mold	Less than 100 CFU/g
4. Electrolyte Drinks		
• UHT or sterilized electrolyte drinks	Free of mold	Less than 1 CFU/ml
• Other than UHT or sterilized electrolyte drinks	Free of mold	Less than 100 CFU/ml
• Electrolyte drink in dry form dissolved as per instruction on the package	Free of mold	Less than 100 CFU/g

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
5. Beverages in Sealed Container		
• Water with dissolved Carbon dioxide or oxygen	Free of yeast and mold	Less than 1 CFU/ml
• UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 1 CFU/ml
• UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 1 CFU/ml
• Other than UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/ml
• Other than UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/ml
Beverages in concentrated form and needs to be diluted before consumption:		
• UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 10 CFU

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
• UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 10 CFU/g
Beverages in concentrated form and needs to be diluted before consumption:		
• Other than UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g
• Other than UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g
Beverages in dry form:		
• Beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g
• Beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
6. Tea		
• UHT or sterilized ready to drink tea	Free of yeast and mold	Less than 1 CFU/ml
• Other than UHT or sterilized ready to drink tea	Free of yeast and mold	Less than 100 CFU/ml
• Ready to drink tea in dry form dissolved as per instruction on the package	Free of yeast and mold	Less than 100 CFU/g

(Source: *MOPH Notifications on the Revised of yeast and mold Level Allowed to be Found in foods*)

2.6 Enumeration of Yeasts and Mold in Food

The dilution plating and the direct plating methods may be used to detect fungi in foods. The direct plating method is more efficient than the dilution plating method for detecting individual mold species, including most of the toxin producers, but it is less effective in detecting yeasts. It is also used to determine whether the presence of mold is due to external contamination or internal invasion.

2.6.1 Conventional Plate Count Method

The basis of methods used for the testing of yeast and mold in foods is very well established, and relies on the incorporation of a food sample into a nutrient medium in which microorganisms can replicate thus resulting in a visual indication of growth. Such methods are simple, adaptable, convenient and generally inexpensive. However, they have two drawbacks: firstly, the tests rely on the growth of organisms in media, which can take many days and result in a long test elapse time; and secondly, the methods are manually oriented and are thus labor intensive.

2.6.1.1 Spread-plate method.

Aseptically pipette 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculums with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

2.6.1.2 Pour-plate method.

Use sterile cotton-plugged pipette to place 1.0 ml portions of sample dilution into prelabeled 15 x 100 mm Petri plates (plastic or glass), and immediately add 20-25 ml tempered Potato Dextrose Agar. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. After adding sample dilution, add agar within 1-2 min; otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate.

2.7 Pathogenic *Escherichia coli*

Theodor Escherichia first described *E. coli* in 1885, as Bacterium coli commune, which he isolated from the feces of newborns. It was later renamed *Escherichia coli*, and for many years the bacterium was simply considered to be a commensally organism of the large intestine. It was not until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants (Schulze et al., 2006).

The GI tract of most warm-blooded animals is colonized by *E. coli* within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant. The human bowel is usually colonized within 40 hours of birth. *E. coli* can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period (weeks to months), and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal flora. The basis for these shifts and the ecology of *Escherichia coli* in the intestine of humans are poorly understood despite the vast amount of information on almost every other aspect of the organism's existence. The entire DNA base sequence of the *E. coli* genome has been known since 1997.

E. coli is the head of the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultatively anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. The *Enterobacteriaceae* are among the most important bacteria medically. A number of genera within the family are human intestinal pathogens (e.g. *Salmonella*, *Shigella* and *Yersinia*). Several others are normal colonists of the human gastrointestinal tract (e.g.*Escherichia*, *Enterobacter* and *Klebsiella*), but these bacteria, as well, may occasionally be associated with diseases of humans.

2.7.1 Physiology

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.

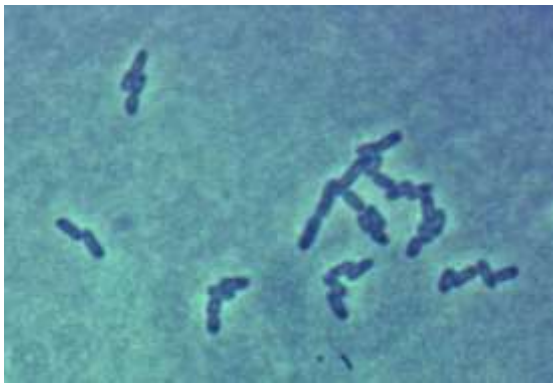

Figure 2.4 Unstained cells of *E. coli* viewed by phase microscopy. about 1000X magnification. CDC

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.

2.7.2 *Escherichia coli* in the Gastrointestinal Tract

The commensal *E. coli* strains that inhabit the large intestine of all humans and warm-blooded animals comprise no more than 1% of the total bacterial biomass. The *E. coli* flora is apparently in constant flux. One study on the distribution of different *E. coli* strains colonizing the large intestine of women during a one year period (in a hospital setting) showed that 52.1% yielded one serotype, 34.9% yielded two, 4.4% yielded three, and 0.6% yielded four. The most likely source of new serotypes of *E. coli* is acquisition by the oral route. The intestinal strains tend to displace one another about three or four times a year.

2.7.3 Pathogenesis of *E. coli*

Over 700 antigenic types (serotypes) of E. coli are recognized based on O, H, and K antigens. At one time serotyping was important in distinguishing the small number of strains that actually cause disease. Thus, the serotype O157:H7 (O refers to somatic antigen; H refers to flagellar antigen) is uniquely responsible for causing HUS (hemolytic uremic syndrome). Nowadays, particularly for diarrheagenic strains (those that cause diarrhea) pathogenic E. coli are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic E. coli usually requires that the isolates first be identified as E. coli before testing for virulence markers.

Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis). The diseases caused (or not caused) by a particular strain of E. coli depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses. These are summarized in Table 2.2 and applied to the discussion of pathogenic strains *E. coli* below.

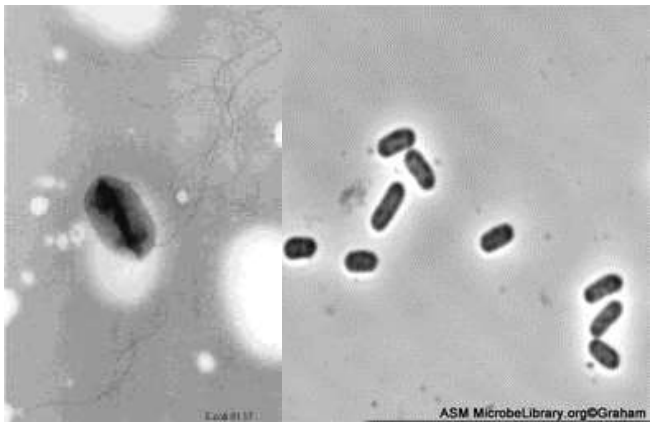

Figure 2.5 Unstained cells of *E. coli* O157:H7

2.7.4 Traveler's Diarrhea and *Escherichia coli* Infections

E. coli may cause diarrheal disease by several mechanisms, and six categories or strains of diarrheagenic *E. coli* are now recognized: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EHEC), enteropathogenic *E. coli* (EAggEC), and diffusely adhering *E. coli* (DAEC).

The enterotoxigenic *E. coli* (ETEC) strains produce one or both of two distinct enterotoxins, which are responsible for the diarrhea and distinguished by their heat stability: heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). The genes or ST and LT production and for colonization factors are usually plasmid-borne and acquired by horizontal gene transfer. ST binds to a glycoprotein receptor that is coupled to guanylate cyclase on the surface of intestinal epithelial cells. Activation of guanylate cyclase stinulates the production of cyclic guanosine monophosphate (cGMP), which leads to the secretion of electrolytes and water into the lumen of the small intestine, manifested as the watery diarrhea characteristic of an ETEC infection. LT binds to specific gangliosides on the epithelial cells and activates membrane-bound adenylate cyclase, which leads to increased production of cyclic adenoside monophosphate (cAMP) through the same mechanism employed by cholera toxin. Again, the result is hypersecretion of electrolytes and water into the intestinal lumen.

The eteroinvasive *E. coli* (EIEC) strains cause diarrhea by penetrating and multiplying within the intestinal epithelial cells. The ability to invade the pithelial cells is associated with the presence of a large plasmid; EIEC may also produce a cytotoxin and an enterotoxin.

The enteropathogenic *E. coli* (EPEC) stains attach to the brush border of intestinal epithelial cells and cause a specific type of cell damage called effacing lesions. Effacing lesions or attaching-effacing (AE) lesions represent destruction of brush border microvilli adjacent to adhering bacteria. This cell destruction leads to the subsequent diarrhea. As a result of this pathology, the ters AE *E. coli* is used to describe true EPEC strains. It is now known that AE *E. coli* is an important cause of diarrhea in children residing in developing countries.

The enterohemorrhagic *E. coli* (EHEC) strains carr the genetic determinats for attaching-effacing lesions and Shiga-like toxin production. The attaching-effacing lesion causes hemorrhagic colitis with severe abdominal pain and cramps followed by bloody diarrhea. The Shiga-like toxins I and II (also called verotoxins 1 and 2) have also been implicated in two extraintestinal diseases; hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. It is belived these toxins kill vascular endothelial cells. A major form of EHEC is the *E. coli* O157:H7 that has caused many outbreaks of hemorrhagic colitis in the Unted States since it was first recognized in 1982. Currently there are a minimum of 20,000 *E. coli* O157:H7 cases and 250 deaths in the United States each year.

The enteroaggregative *E. coli* (EAggEC) strains adhere to epithelial cells in localized regions, forming clumps of bacteria with a “stacked brick” appearance. Convectional extracellular toxins have not been detected in EAggEC, but unique lesions are seen in epithelial cells, suggesting the involvement of toxins.

The diffusely adhering *E. coli* (DAEC) strains adhere over the entire surface of epithelial cells and usually cause disease in immunologically naïve or malnourished children. It has been suggested that DAEC may have an as yet undefined virulence factor.

Diagnosis of traveler's diarrhea caused by *E. coli* is based on past travel history and symptoms. Laboratory diagnosis is by isolation of the specific type of *E. coli* from feces and identification using DNA probes, the determination of virulence factors, and the polymerase chain reaction. Treatment is with fluid and electrolytes plus doxycycline and trimethroprim-sulfamethoazole. Recovery is usually without complications. Prevention and control involve avoiding contaminated food and water.

Table 2.2 Bacteria That Cause Acute Bacterial Diarrheas and Food Poisonings

Organism	Incubation Period (Hours)	Vomiting	Diarrhea	Epidemiology	Pathogenesis	Clinical Features
Staphylococcus aureus	1-8 (rarely, up to 18)	+++	+	Staphylococci grow in meats, dairy and bakery products and produce enterotoxins.	Enterotoxins act on receptors in gut that transmit impulse to medullary centers; may also act as superantigens.	Abrupt onset, intense vomiting for up to 24 hours, recovery in 24-48 hours. Occurs in persons eating the same food. No treatment usually necessary expect to restore fluids and electrolytes.
Bacillus cereus	2-16	+++	++	Reheated fried rice causes vomiting or diarrhea.	Enterotoxins formed in food or in gut from rowt of *B. cereus*.	With incubation period of 2-8 hours, mainly vomiting. With incubation period of 8-16 hours, mainly diarrhea.
Clostridium perfringens	8-16	±	+++	Clostridia grow in rewarmed meat dishes. Huge numbers ingested.	Enterotoxin produced duing sporulation in gut, causes hypersecretion.	Abrupt onset of profuse diarrhea; vomiting occasionally. Recovery usual without treatment in 1-4 da Many clostridia in cultures of foo and feces of patients.

Organism	Incubation Period (Hours)	Vomiting	Diarrhea	Epidemiology	Pathogenesis	Clinical Features
Clostridium botulinum	18-24	±	Rare	Clostridia grow in anaerobic foods and produce toxins.	Toxin absorbed from gut and blocks acetylcholine release at neuromuscular junction	Diplopia, dyshagia, dysphonia, difficulty breathing. Treatment requires clear airway, ventilation, and intravenous polyvalent antitoxin. Exotoxin present in food and serum. Mortality rate high.
Escherichia coli (enterotoxigenic strain)	24-72	±	++	Organisms grow in gut and are a major cause of traveler's diarrhea.	Heat-labile (LT) and heat-stable (ST) enterotoxins cause hypersecretion in small intestine.	Usually abrupt onset of diarrhea; vomiting rate. A serious infection in newborns. In adults, "traveler's diarrhea" is usually self-limited n 1-3 days.
Vibrio parahaemolyticus	6-96	+	++	Organisms grow in seafood and in gut and produce toxin, or invade.	Toxin causes hypersecretion; vibrios invade epithelium; stools may be bloody.	Abrupt onset3of diarrhea in groups consuming the same food, especially crabs and other seafood. Recovery is usually complete in 1-3 days. Food and stool cultures are positive.

Organism	Incubation Period (Hours)	Vomiting	Diarrhea	Epidemiology	Pathogenesis	Clinical Features
Salmonella spp. (gastroenteritis)	8-48	±	++	Organisms grow in gut.	Superficial infection of gut, little invasion. Infective dose $>10^5$ organisms.	Gradual or abrupt onset of diarrhea and low-grade feer. Nausea, headache, and muscle aches common. No antimicrobials usless systemic dissemination is suspected. Stool cultures are positive. Prolonged carriage is frequent.
Salmonella typhi (typhoid fever)	10-14 days	±	±	Bacteria invade the gut epithelium and reach the lymph nodws, liver, spleen, and gallbladder.	Symptoms probably due to endotoxins and tissue inflammation. Infective dose $\geq 10^7$ organisms.	Initially fever, headache, malaise, anorexia, and muscle pains. Fever may reach 40C by the end of the first week of illness and lasts for 2 or more weeks. Diarrhea often occurs, ad abdominal pain, cough, and sore throat may be prominent. Antibiotic therapy shortens duration of the illness.

Adapted from: www.realrawmilkfacts.com

2.8 Microbial Detection Methods

Available bacterial detection methods can be divided into two groups which are traditional methods based on culturing and biochemical test and the techniques developed to shorten the detection time such as real time PCR, immunoassay or molecular technique.

2.8.1 Conventional microbiological techniques

The convectional microbiological techniques are based on the established method of incorporating food samples into nutrient media and incubating for a period of time to allow the microorganisms to grow. The detection or counting method is then a simple visual assessment of growth. These methods are thus technically simple and relatively in expensive, requiring no complex instrumentation. The methods are however very adaptable, allowing the enumeration of different groups of microorganisms.

Before testing, the food sample must be converted into a liquid from in order to allow mixing with the growth medium. This is usually done by accurately weighing the sample into a sterile container and adding a known volume of sterile diluent (the sample to diluents ratio is usually 1:10); this mixture is then homogenized using a homogenizer that breaks the sample apart, releasing any organisms into diluents. The correct choice of diluent is important. If the organisms in the sample are stressed by incorrect pH or low osmotic strength, then they could be injured or killed, thus affecting the final result obtained from the microbiological test. The diluent must be well buffered at a pH suitable for the food being tested and be osmotically balanced. When testing some food (e.g. dried products) which may contain highly stressed microorganisms, then a suitable recovery period may be required before the test commences, in order to ensure cells are not killed during the initial phase of test procedure (Davis and Jones, 1997)

The enumeration of organisms in samples is generally done by using plate count, or most probable number (MPN) methods. The former are the most widely used, whilst the latter tend to be used only for certain organisms (e.g. *Escherichia coli*) or groups (e.g. coliforms).

2.8.1.1 Plate count method

The plate count method is based on the deposition of the sample, in or on agar layer in a Petri dish. Individual organisms or small groups of organisms will occupy a discrete site in the agar, and on incubation will grow to form discrete colonies that are counted visually. Various types of agar media can be used in this form to enumerate different types of microorganisms. The use of a non-selective nutrient medium that is incubated at 30°C aerobically will result in a total viable count or mesophilic aerobic count. By changing the conditions of incubation to anaerobic, a total anaerobe count will be obtained. Altering the incubation temperature will result in changes in the type of organism capable of growth, thus showing some of the flexibility in the convectional agar approach. If there is a requirement to enumerate a specific type of organism from the sample, then in most cases the composition of the medium will need to be adjusted to allow only that particular organism to grow. There are three approaches used in media design that allow a specific medium to be produced: the elective, selective and differential procedures.

Elective procedures refer to the inclusion in the medium of reagents, or the use of growth conditions, that encourage the development of the target organisms, but do not inhibit the growth of other microorganisms. Such reagents may be sugars, amino acids or other growth factors. Selective procedures refer to the inclusion of reagent or the use of growth conditions that inhibit the development of non-target microorganisms. It should be noted that, in many cases, selective agents will also have a negative effect on the growth of the target microorganism, but this will be less great than the effect on non-target cells. Example of selective procedures would be the inclusion of antibiotics in a medium or the use of anaerobic growth conditions. Finally, differential procedures allow organisms to be distinguished from each other by the reactions that their colonies cause in the medium. An example would be the inclusion of a pH indicator in a medium to differentiate acid –

producing organisms. In most cases, media will utilise a multiple approach system, containing elective, selective and differential components in order to ensure that the user can identify and count the target organism.

The types of agar currently available are far too numerous to list. For details of these, the manuals of media manufacturing companies (e.g. Oxoid, LabM, Difco and Merck) should be consulted.

2.8.1.2 MPN method

The second enumerative procedure, which is the Most Probable Number (MPN) Method or a Durham Test. In principle, this Durham Test relies on the lactose fermentable characteristic (APHA, 1992). The Most Probable Number (MPN) Method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are inoculated into broth media. Analysts score the number of gas positive (fermentation of lactose) tubes, from which the other 2 phases of the assay are performed and then uses the combinations of positive results to consult a statistical tables, to estimate the number of organisms present. Typically only the first 2 phases are performed in coliforms and fecal coliform analysis, while all 3 phases are done for *E. coli*. The 3-tube MPN test is used for testing most foods. The 5-tube MPN is used for water, shellfish and shellfish harvest water testing and there is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly contaminated (APHA, 1998).

As indicated earlier, this method is used only for particular types of test and tends to be more labour and materials intensive than plate count methods. In addition, the confidence limits are large even if many replicates are studied at each dilution level. Thus the method tends to be less accurate than plate counting methods but has the advantage of greater sensitivity.

2.8.1.2.1 MPN - Presumptive test for coliforms, fecal coliforms and *E. coli*

Weigh 50 g food into sterile high-speed blender jar. Frozen samples can be softened by storing it for ≤18 h at 2-5°C, but do not thaw. Add 450 mL of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades. Prepare decimal dilutions with sterile Butterfield's phosphate diluent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm or vortex mix for 7 s. Do not use pipette to deliver <10% of their total volume. Transfer 1 mL portions to 3 LST tubes for each dilution for at least 3 consecutive dilutions. Hold pipette at angle so that its lower edge rests against the tube. Let pipette drain 2-3 s. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

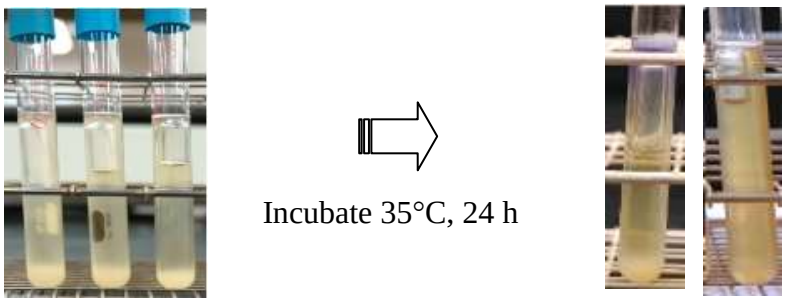

Figure 2.6 Presumptive tests for coliforms, fecal coliforms and *E. coli*

Incubate LST tubes at 35°C. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 h. Perform confirmed test on all presumptive positive (gas) tubes.

2.8.1.2.2 MPN - Confirmed test for coliforms

From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at 35°C, because most coliforms grow at 35°C (Weiss et al., 1983) and examine for gas production at 48 ± 2 h. Calculate most probable number (MPN) of coliforms based on proportion of **confirmed** gassing LST tubes for 3 consecutive dilutions.

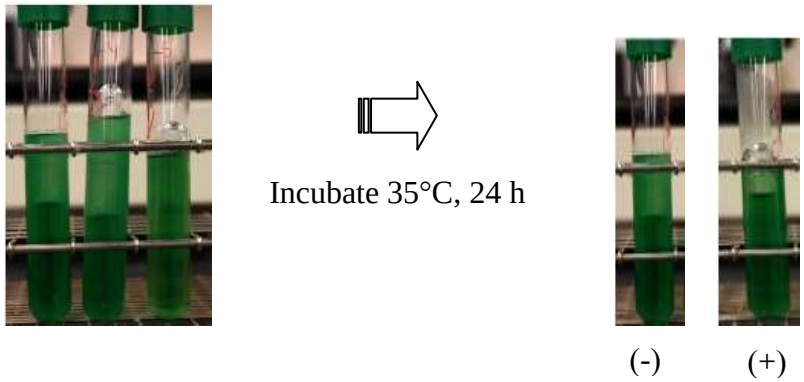

Figure 2.7 Confirmed test for coliforms

2.8.1.2.3 MPN - Confirmed test for fecal coliforms and *E. coli*

From each gassing LST tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). EC medium was chosen because : the medium contains bile salts, which inhibit most spore – forming or gram – positive bacteria capable of fermenting lactose. (Warren et al., 1978) Incubate EC tubes 24 ± 2 h at 45.5 °C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis.

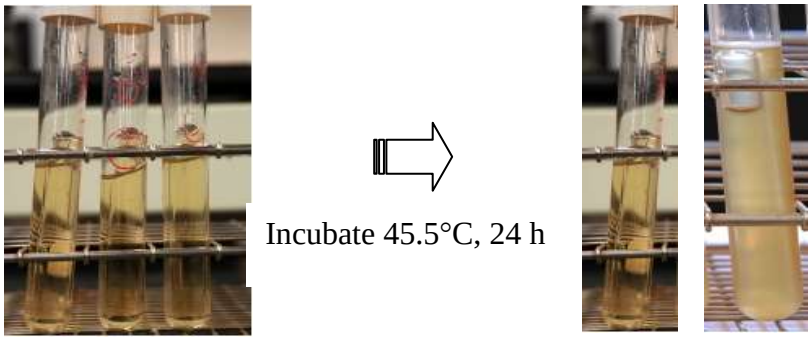

Figure 2.8 Confirmed test for fecal coliforms and *E. coli*

2.8.1.2.4 MPN - Completed test for *E. coli*.

To perform the completed test for *E. coli*, gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 18-24 h at 35°C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to **5** suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 h at 35°C and use for further testing.

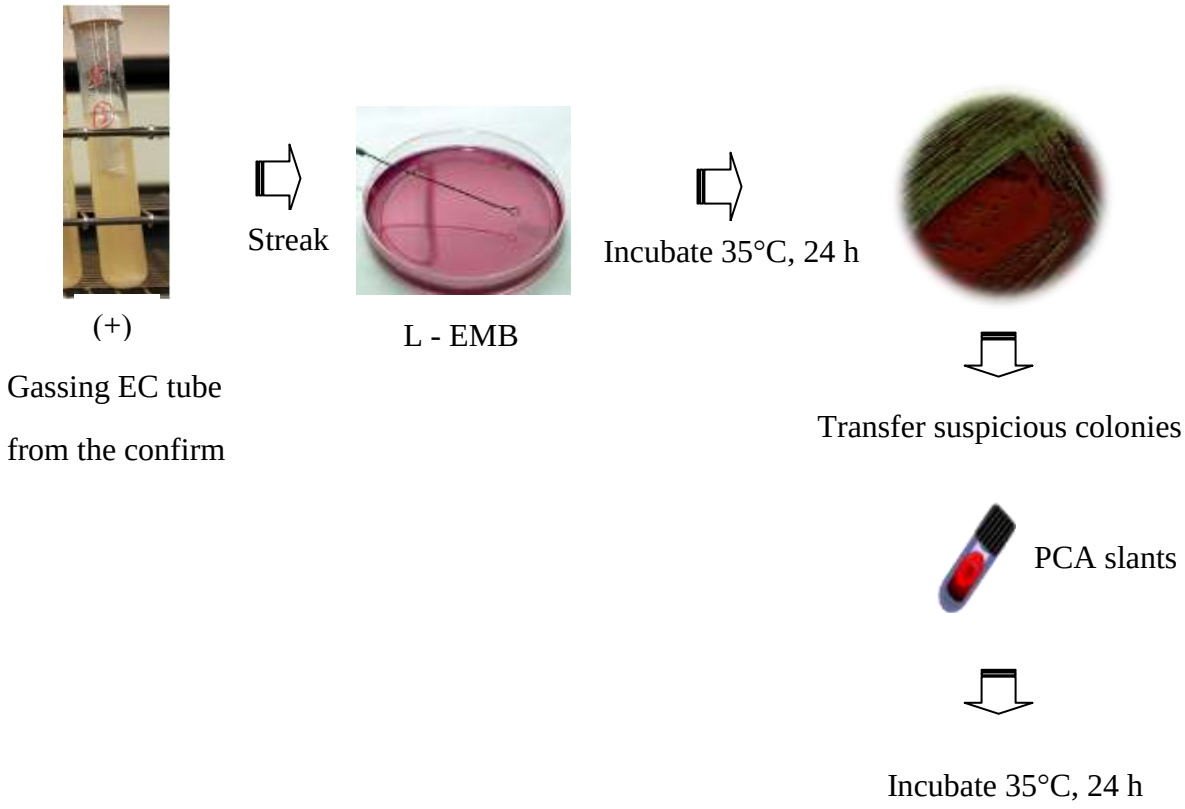

Figure 2.9 Completed test for *E. coli*

2.8.2 The Modern Rapid Method of Yeast & Mold *and Escherichia coli* Detection

2.8.2.1 The Enzyme – based Method

New techniques have been developed recently for detection and differentiation of bacteria. They are based on the utilization of chromogenic and/ fluorogenic substrates for detection of activities of specific enzymes. Theses sensitive methods have led to improved accuracy and faster detection and may be performed by using the microbiological growth media that contain enzyme substrates linked to a chromogen (colour reaction), fluorogen (fluorescent reaction) or a combination of both. The target population is characterized by enzyme systems that metabolize the substrate (sugar or amino acid) to release the chromogen/fluorogen. This results in a colour change in the medium and/or fluorescence

under long wave UV light. The incorporation of such fluorogenic or chromogenic enzyme substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain micro-organisms. (Schonenbrucher et al., 2008 : Perry and Freydiere, 2007 : Greenwood et al., 2005 and Manafi, 1996, 2000)

The Chromogenic enzyme substrates are compounds which act as the substrate for specific enzymes and change colour due to the action of the enzyme. In general, based on their chemical reaction, four groups of chromogenic compounds can be distinguished and recently described by Manafi (1998). Indolyl derivatives are water soluble and heat stable and the mostly used derivatives such as 5-bromo-4-chloro-3-indolyl (X), 5-bromo-6-chloro-3-indolyl (magenta) or 6-chloro-3- indolyl (salmon) showing no diffusion on the agar plate.

The Fluorogenic enzyme substrates generally consist of a specific substrate for the specific enzyme such as sugar or amino acid and a fluorogen such as 4-methylumbelliferone, being able to convert UV light to visible light. Methylumbelliferyl-substrates are water soluble, highly sensitive and very specific. Because of their pH-dependance, strong diffusion in solid media and the need of UV - light, the use of these substrates is limited.

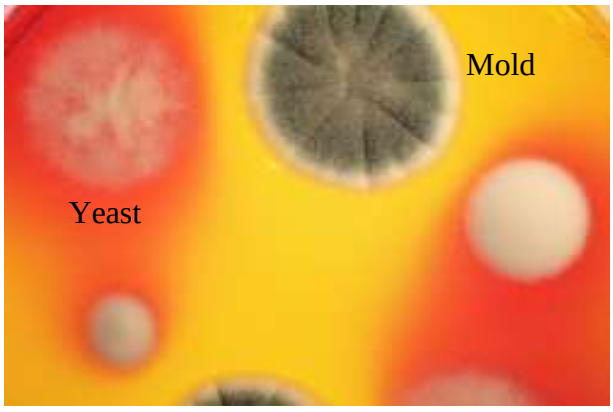

Figure 2.10 Chromogenic culture media products available for the isolation and identification of yeasts and mold in food products

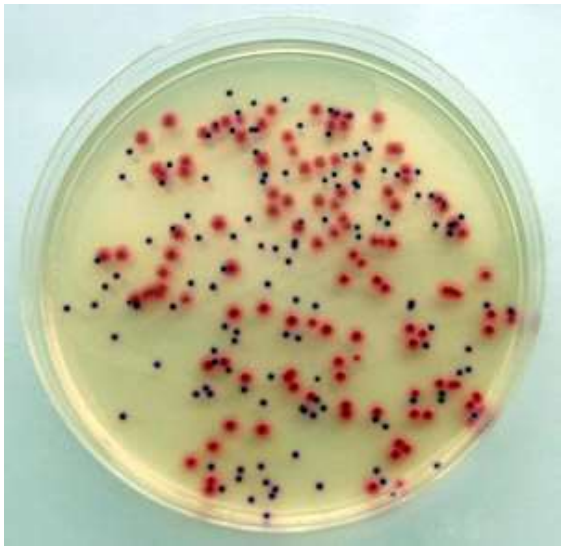

Figure 2.11 *E. coli*/coliform Selective Agar is a chromogenic medium for the detection and enumeration of *E. coli* and other coliforms (important hygiene indicators) from food and water samples. *E. coli* colonies turn a distinctive cherry red color whereas coliforms colonies turn blue

2.8.2.2 Rapid and automated methods

The general interest in alternative microbiological methods has been stimulated in part by the increased output of food production sites. This has resulted in the following greater numbers of samples being stored prior to positive release- a reduction in analysis time would reduce storage and warehousing costs. A greater sample throughput being required in laboratories- the only way that this can be achieved is by increased laboratory size and staff levels, or by using more rapid and automated method. A requirement for a longer shelf-life in the chilled foods sector- a reduction in analysis time could expedite product release thus increasing the shelf-life of the product. The increased application of HACCP procedures- rapid methods can be used in HACCP verification procedures.

Table 2.3 Pros and cons of conventional and rapid methods in bacterial detection

Conventional methods	**Rapid methods**
- Lower cost compared with molecular tools	- Fast detection
- Time consuming	- Time consuming
- Labor intensive	- Expensive cost/analysis

There are a number of different techniques referred to as rapid methods and most have little in common either with each other or with the convectional procedures that they replace. The methods can generally be divided into quantitative and qualitative tests, the former giving a measurement of the number of organisms in a sample, the latter indicating only presence or absence. Laboratories considering the use of rapid methods for routine testing must carefully consider their own requirements before purchasing such a system. Every new method will be unique, giving a slightly different result, in a different timescale with varying levels of automation and sample throughput. In addition, some methods may work poorly with certain types of food or may not be able to detect the specific organism or group that is required. All of these points must be considered before a method is adopted by a laboratory. It is also of importance to ensure that staff using new methods are aware of the principles of operation of the techniques and thus have the ability to troubleshoot if the method clearly shows erroneous results.