CHAPTER 7

Bacterial Agents of Foodborne Illness

7.1 *AEROMONAS HYDROPHILA*

7.1.1 Introduction

Currently, *Aeromonas* (principally *A. hydrophila*, but also *A. caviae* and *A. sobria*) has the status of a foodborne pathogen of emerging importance. Like *Listeria monocytogenes*, *Plesiomonas*, and *Yersinia enterocolitica*, it has attracted attention primarily because of its ability to grow at chill temperatures, prompting the concern that any threat it might pose will increase with the increasing use of chilled foods (Table 7.1). Present uncertainty over its significance however, is reflected in much of the information available which does not, as yet, present a coherent picture. It was first isolated from drinking water by Zimmerman in 1890 and the following year from frog's blood by Sanarelli. They called their isolates *Bacillus punctata* and *Bacillus hydrophilus* respectively and it was not until the 1930s that the genus *Aeromonas* was first described. Although the taxonomy is still not settled, more recent studies have led to the recognition of two major groups within the genus: the Salmonicida group, which contains the non-motile *Aeromonas salmonicida* and several

Table 7.1 *Reported lag times and growth rates of psychrotrophic pathogens at chill temperatures*

	Lag time (days)			*Generation time (h^{-1})*	
Temperature (°C)	**0–1**	**2–3**	**5**	**0–1**	**4–5**
Aeromonas hydrophila	>22	6–10	3–4	>49	9–14
Listeria monocytogenes	3–33	2 –8	1–3	62 –131	13–25
Yersinia enterocolitica	3	2.4	–	25	20

sub-species, and the Hydrophila–Punctata group containing a number of motile species, including *A. hydrophila A. sobria*, and *A. caviae*.

A. salmonicida is not a human pathogen but causes diseases of freshwater fish which can be an important economic problem in fish farming. Members of the Hydrophila group can cause extra-intestinal infections, commonly in the immunosuppressed or as a result of swimming accidents where the skin is punctured. The first report of gastroenteritis due to *Aeromonas* came from Jamaica in 1958, but evidence of its ability to cause gastroenteritis in otherwise healthy individuals is patchy.

Epidemiological investigations in several countries have reported higher rates of isolation of aeromonads from patients with diarrhoea than from control groups, although this does not necessarily indicate a causal relationship. In one study, the incidence of *A. hydrophila* in American travellers to Thailand with diarrhoea was significantly higher than in unaffected individuals. Interestingly, isolation rates in the Thai population were similar for both groups of patients suggesting that *Aeromonas* may be a cause of 'travellers diarrhoea' in these regions.

Good supporting evidence from sources other than epidemiological studies has proved difficult to obtain. In a feeding trial involving 50 volunteers with doses as high as 5×10^{10}, only two cases of diarrhoea resulted, although a laboratory accident has been reported where approximately 10^9 cells were ingested by a worker mouth pipetting who later suffered acute diarrhoea.

7.1.2 The Organism and its Characteristics

Aeromonads are Gram-negative, catalase-positive, oxidase-positive rods which ferment glucose. They are generally motile by a single polar flagellum.

A. hydrophila is neither salt ($<5\%$) nor acid (min. pH ≈ 6.0) tolerant and grows optimally at around 28 °C. Its most significant feature with regard to any threat it may pose in foods is its ability to grow down to chill temperatures, reportedly as low as -0.1 °C in some strains. Its principal reservoir is the aquatic environment such as freshwater lakes and streams and wastewater systems. The numbers present will depend on factors such as the nutrient level and temperature but can be as high as 10^8 cfu ml^{-1} in a relatively nutrient rich environment such as sewage. Although it is not resistant to chlorine, it is found in potable water, where it can multiply on the low level of nutrients available in piped water systems. It has also been isolated from a wide range of fresh foods and is a transient component of the gut flora of humans and other animals.

7.1.3 Pathogenesis and Clinical Features

Gastroenteritis associated with *Aeromonas* occurs most commonly in children under five years old. It is normally mild and self-limiting mostly characterized by profuse watery diarrhoea, although dysenteric stools may sometimes be a feature. Vomiting is not usually reported.

Aeromonas spp., particularly *A. hydrophila* and *A. sobria*, produce a range of potential virulence factors including a number of distinct cytotoxic and cytotonic enterotoxins. Most clinical strains of *A. hydrophila* and *A. sobria* produce aerolysin, a heat-labile, β-haemolytic, cytotoxic enterotoxin with a molecular mass of 52 kDa. Three cytotonic enterotoxins have also been described which act like cholera toxin, stimulating accumulation of high levels of cAMP within epithelial cells. Only one of these shows any marked structural similarity to cholera toxin as measured by cross reactivity with cholera toxin antibodies.

7.1.4 Isolation and Identification

In some instances enrichment media such as alkaline peptone water are used, but where high numbers are present direct plating is usually sufficient. Species of the Hydrophila group grow on a wide range of enteric media but may often be misidentified as 'coliforms' since many strains can ferment lactose. Most cannot ferment xylose and this is a useful distinguishing feature used in several media. As well as bile salts, ampicillin is used as a selective agent in media such as starch ampicillin agar, blood ampicillin agar and some commercial formulations. Colonies which give the characteristic appearance of *Aeromonas* on the medium concerned and are oxidase-positive are then confirmed with biochemical tests.

7.1.5 Association with Foods

Apart from their possible role in gastroenteritis, food and water are also probably the source of the severe extra-intestinal *Aeromonas* infections associated with immunocompromised individuals.

Aeromonads of the Hydrophila group have been isolated from a wide range of fresh foods including fish, meat, poultry, raw milk, and salad vegetables as well as water. The ability of some strains to grow at very low temperatures can lead to the development of high numbers under chill conditions and they can be an important part of the spoilage flora of chilled meats.

They are unlikely to survive even mild cooking procedures but may be introduced as post-process contaminants from uncooked produce or contaminated water.

7.2 *BACILLUS CEREUS* AND OTHER *BACILLUS* SPECIES

7.2.1 Introduction

An early report associating food poisoning with *Bacillus* spp. was made in 1906 when Lubenau described an outbreak in a sanatorium where 300 inmates and staff developed symptoms of profuse diarrhoea, stomach cramps and vomiting. A spore forming bacillus was isolated from meatballs from the incriminated meal. Although Lubenau named the organism *Bacillus peptonificans*, the properties he described resemble those of *B. cereus*. Subsequently, aerobic spore formers were implicated in a number of outbreaks in Europe and between 1936 and 1943 they were suspected of causing 117 of 367 cases investigated by the Stockholm Board of Health.

Bacillus cereus was not conclusively established as a cause of food poisoning until 1950, after the taxonomy of the genus had been clarified. Hauge described four outbreaks in Norway involving 600 people. The food vehicle was a vanilla sauce which had been prepared a day in advance and stored at room temperature before serving. Samples of the sauce later tested contained from 2.5×10^7 to 1.1×10^8 *B. cereus* ml^{-1}. This classic report and many of the early ones from Europe described an illness in which diarrhoea was the predominant symptom. It is now known that *B. cereus* is responsible for two distinct types of foodborne illness: a relatively late-onset, 'diarrhoeal syndrome' and a rapid-onset, 'emetic syndrome', first described in 1971 in the UK.

Since 1975 a number of other *Bacillus* species have been associated with foodborne illness. In these episodes, tests have failed to find known pathogens but food remnants and/or clinical specimens have yielded high numbers of *Bacillus* spp. Far less common than outbreaks featuring *B. cereus*, they usually involve very closely related species such as *B. licheniformis* and *B. pumilis* or *B. subtilis*. *B. thuringiensis* has also been reported as causing an outbreak in Canada.

Overall, the reported number of cases of foodborne illness due to *Bacillus* spp. in the UK is much lower than those for *Salmonella*. Since 1992 there have been up to 10 outbreaks each year involving a total of 67 cases. Such statistics though, are likely to underestimate the true level far more than those for *Salmonella* since the data come only from outbreaks and there is no estimate of sporadic cases. In some Northern European countries however the organism appears to have far greater importance. It accounted for 33% of total bacterial food poisoning cases in Norway between 1988 and 1993, 47% in Iceland (1985–1992), 22% in Finland (1992) and 8.5% in the Netherlands (1991). In Denmark, England and Wales, Japan, the USA and Canada the figure ranges between 0.7 and 5.0%.

7.2.2 The Organism and its Characteristics

Members of the genus *Bacillus* are Gram-positive, aerobic, spore-forming rods, though they do, on occasion, display a Gram-negative or variable reaction. Their taxonomy is quite complex and has been subject to considerable revision in recent years. The genus still contains about 80 species, those causing food poisoning being *Bacillus cereus*, a number of species very closely related to *B. cereus* and *Bacillus subtilis*.

Bacillus cereus is facultatively anaerobic with large vegetative cells, typically 1.0 μm by 3.0–5.0 μm in chains. It grows over a temperature range from 8 to 55 °C, optimally around 28–35 °C, and does not have any marked tolerance for low pH (min. 5.0–6.0, depending on the acidulant) or water activity (min. ~0.95).

Spores are central, ellipsoidal in shape and do not cause swelling in the sporangium. As a spore former, *B. cereus* is widely distributed in the environment and can be isolated from soil, water and vegetation. This ubiquity means that it is also a common component of the transient gut flora in humans. The spores show a variable heat resistance; recorded D values at 95 °C in phosphate buffer range between around 1 min up to 36 min. Resistance appears to vary with serovar.

In the UK, a serotyping scheme based on the flagellar (H) antigen has been devised, based on a set of 29 agglutinating antisera raised against outbreak and non-outbreak strains isolated from foods. In about 90% of outbreaks it is possible to serotype the causative organism, although only about half of environmental isolates are typable. There does not appear to be a strong association between the two different types of *B. cereus* food poisoning and particular serotypes. Some have been associated with both types of syndrome, although in a study of 200 outbreaks of the emetic syndrome from around the world, serotype 1, which possesses markedly greater heat resistance than other serotypes, was isolated from implicated foods, faeces or vomitus in 63.5% of cases.

7.2.3 Pathogenesis and Clinical Features

Symptoms of the diarrhoeal syndrome resemble those of *Clostridium perfringens* food poisoning. The onset of illness is about 8–16 h after consumption of the food, lasts for between 12 and 24 h, and is characterized by abdominal pain, profuse watery diarrhoea and rectal tenesmus. Nausea and vomiting are less frequent.

The emetic syndrome resembles the illness caused by *Staphylococcus aureus*. It has a shorter incubation period than the diarrhoeal syndrome, typically 0.5–5 h, and nausea and vomiting, lasting between 6 and 24 h, are the dominant feature.

Table 7.2 *Chatacteristics of the two types of disease caused by* Bacillus cereus

	Diarrhoeal syndrome	*Emetic syndrome*
Infective dose	10^5–10^7 (total)	10^5–10^8 (cells g^{-1})
Toxin produced	In the small intestine of the host	Preformed in foods
Type of toxin	Protein(s) 3 components MW37, 38, 46 kDa	Cyclic peptide MW 1.2 kDa
Heat stability	Inactivated 56 °C, 5 min	Stable 126 °C, 90 min
pH stability	Unstable < 4 and > 11	stable 2–11
Incubation period	8–16 h (occasionally > 24 h)	0.5–5 h
Duration of illness	12–24 h (occasionally several days)	6–24 h
Symptoms	Abdominal pain, watery diarrhoea and occasionally nausea	Nausea, vomiting and malaise sometimes followed by diarrhoea, due to additional enterotoxin production?
Foods most frequently implicated	Meat products, soups, vegetables, puddings/sauces and milk/milk products	Fried and cooked rice, pasta, pastry and noodles

Adapted from Granum and Lund, *FEMS Microbiol. Lett*., 1997, **157**, 223 – 228

Both syndromes are caused by distinct enterotoxins (Table 7.2). A number of toxins have been associated with the diarrhoeal syndrome but illness appears to be associated primarily with production in the gut of two three-component enterotoxins: a haemolytic enterotoxin HBL consisting of three proteins B, L_1 and L_2 and a non-haemolytic enterotoxin NHE. Some strains produce both HBL and NHE though others contain the genes for only one. The toxins, which are sensitive to heat and proteolytic enzymes such as trypsin and pepsin, are produced in the late exponential/early stationary phase of growth. Like *C. perfringens* toxin, they exert their effect by binding to epithelial cells and disrupting the epithelial membrane, though the precise mechanisms of action are thought to be different. Though the toxins can be produced in food, their sensitivity to low pH and proteolysis, and the relatively long incubation period associated with illness indicate that toxin production in the gut is primarily responsible for the observed symptoms.

The emetic toxin, cereulide, is a 1.2 kDa cyclic peptide that is acid and heat resistant. Closely related to the potassium ionophore valinomycin, cereulide is a dodecadepsipeptide consisting of three repeats of a unit containing 2 aminoacids and 2 oxyacids (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (Figure 7.1). The toxin is produced in the food in the late exponential to stationary phase of growth and is thought to act by binding to and stimulating the vagus nerve.

Pathogenic features of the illness caused by the other *Bacillus* spp. are not known. The short incubation periods recorded in outbreaks (from

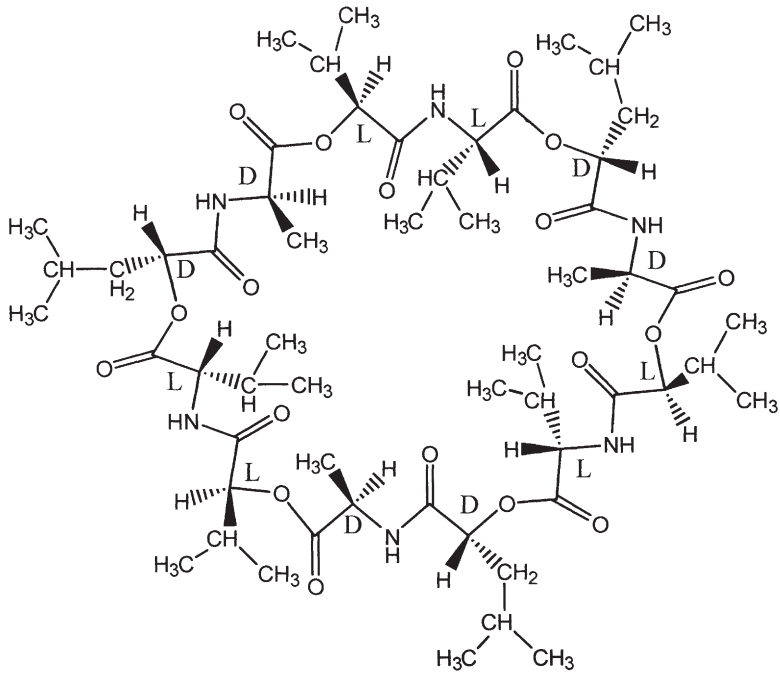

Figure 7.1 *Cereulide, the emetic toxin of* B. cereus

$<$1 h up to 11 h) suggest an intoxication, though no toxin has been isolated and described as yet.

7.2.4 Isolation and Identification

In an outbreak of *Bacillus cereus* food poisoning, implicated foods will contain large numbers ($>10^5\,g^{-1}$) of organisms so enrichment techniques are not needed. The same is true of faecal or vomitus specimens and a non-selective medium such as blood agar (sometimes with the addition of polymyxin as a selective agent to suppress Gram-negatives) is commonly used. *B. cereus* can be identified after 24 h incubation at 37 °C by its characteristic colonial morphology of large (3–7 mm diameter), flat or slightly raised, grey-green colonies with a characterisitc granular or ground-glass texture and a surrounding zone of α or β haemolysis. To confirm the identity of a blood agar isolate or to isolate smaller numbers of *B. cereus* from foods, a more selective diagnostic agar is necessary. Several of these have been proposed which have a number of common features. Polymyxin/pyruvate/eggyolk/mannitol/bromothymol blue agar (PEMBA) is one widely used example. It includes polymyxin as a selective agent and where yeasts and moulds are likely to be a problem actidione may also be included. On PEMBA, *B. cereus* produces typical

crenated colonies which retain the turquoise-blue of the pH indicator (bromothymol blue) due to their inability to ferment mannitol, they are surrounded by a zone of egg-yolk precipitation caused by lecithinase activity. Pyruvate in the medium improves the egg-yolk precipitation reaction and a low level of peptone enhances sporulation. Colonies of *B. cereus* can be confirmed by a microscopic procedure combining a spore stain with an intracellular lipid stain. Spores appear green in a cell with red vegetative cytoplasm and containing black lipid globules. Biochem-ical confirmation can be based on an isolate's ability to produce acid from glucose but not from mannitol, xylose and arabinose.

Commercial kits are available which claim to detect the diarrhoeal enterotoxin though they have limited use. One detects the L_2 unit of HBL, though some outbreak strains do not produce this toxin. The other detects a protein present in NHE but not in the HBL complex.

7.2.5 Association with Foods

The ability to produce spores resistant to factors such as drying and heat means that the food-poisoning bacilli are widely distributed in foods. In most circumstances however they are only a small part of the total flora and are not present in numbers sufficient to cause illness.

Heat processing will select for spore formers and a number of surveys have reported a higher incidence of *B. cereus* in pasteurized and other heat-processed milks (typically 35–48% of samples positive) compared with raw milk ($\sim$9% positive). In most of these cases the numbers detected were low ($<10^3\,ml^{-1}$), but when pasteurized milk or cream are stored at inadequate chill temperatures *B. cereus* can grow and cause the type of spoilage known as 'sweet curdling' or 'bitty cream'. Despite this, milk and dairy products are rarely associated with illness caused by *B. cereus*, although dried milk has been implicated in outbreaks when used as an ingredient in vanilla slices and macaroni cheese. A possible explanation is that though liquid milk is an excellent growth medium for the organism, toxin production is not favoured. One study in Sweden has linked this with the low aeration in static packs of milk.

The ability of spores to resist desiccation allows their survival on dried products such as cereals and flours. In the Norwegian outbreaks described above (Section 7.2.1), the cornflour used to thicken the vanilla sauce was implicated. Moderate heating during preparation would not inactivate the spores and subsequent extended storage of the high-a_w sauce at ambient temperature was conducive to spore germination and outgrowth.

The emetic syndrome is particularly associated with starchy products such as rice and pasta dishes. In the UK, its association with cooked rice has been sufficiently marked for it to earn the soubriquet 'Chinese

Restaurant Syndrome'. The typical scenario is where rice is prepared in bulk, in advance. Spores, commonly those of the more heat-resistant serotype 1, survive precooking to germinate, grow and produce the emetic toxin in the product during storage. This would be prevented by chilling to below 8 °C, but the rate of cooling in the centre of a bulk of cooked rice, even if transferred to chill storage, can be slow enough for growth and toxin production to occur. Reheating the rice prior to serving will not inactivate the emetic toxin and render the product safe.

A wider range of foods have been implicated with the diarrhoeal syndrome including meat products, soups, vegetables, puddings and sauces. Dried herbs and spices used in food preparation can be an important source of *B. cereus* and this has often been cited as a reason for a relatively high incidence of *B. cereus* food poisoning in Hungary, where between 1960 and 1968 it was the third most common cause of food poisoning accounting for 15.2% of persons affected. More recent figures suggest that its relative importance has declined somewhat but whether this is due to changes in culinary practices, improvements in hygiene, decreased contamination of spices or a statistical artefact is not known.

Meat pies and pasties are common vehicles for the other food-poisoning bacilli along with a range of processed meats and meat and rice dishes. Baked goods such as bread and crumpets have been involved in a number of *B. subtilis* outbreaks. Although *B. subtilis* is responsible for the defect known as ropey bread where spores surviving baking degrade the loaf 's internal structure and produce a sticky slime, this does not always prevent people from eating it. In 1988, a bakery in the Isle of Man omitted propionate from their bread in order to claim for it the virtue of being free from artificial preservatives and thereby more healthy. As a result, nine people developed nausea, vomiting, diarrhoea, headache and chills 10 min after consuming ropey bread containing more than 10^8 organisms gm^{-1}.

7.3 *BRUCELLA*

7.3.1 Introduction

The genus *Brucella* is named after Sir David Bruce who in 1887 recognized it as the causative organism of undulant fever (brucellosis, Malta fever, Mediterranean fever). Each of the four species that are human pathogens is associated with a particular animal host, *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (pigs), and *B. canis* (dogs) (Table 7.3). Brucellosis is principally contracted from close association with infected animals and is an occupational disease of farmers, herdsmen, veterinarians and slaughterhouse workers. It can also be contracted by consumption of milk or milk products from an infected animal, although the risk is lower. The

Table 7.3 *Differential characteristics of* Brucella *species*

	B. abortus	*B. canis*	*B. melitensis*	*B. suis*
5% CO_2 required	+	−	−	−
H_2S produced	+	−	−	+
Urease	weak	strong	weak	strong
Growth on dye medium:				
Basic fuchsin ($1{:}10^5$)	+	−	+	−
Thionin ($1{:}10^5$)	−	+	+	+

illness has been effectively eliminated from the United States, Scandinavia, the UK and other countries by campaigns to eradicate the organism in the national dairy herds through a programme of testing, immunization of young calves and compulsory slaughter of infected cattle.

7.3.2 The Organism and its Characteristics

Brucella are Gram-negative, catalase-positive, oxidase-positive, short oval rods (0.3 μm × 0.4 μm) which are non-motile and usually occur singly, in pairs, or, rarely, in short chains. It grows optimally around 37 °C and is killed by heating at 63 °C for 7–10 min. When shed in the milk of an infected animal it can survive for many days provided the acidity remains low (< 0.5% as lactate).

7.3.3 Pathogenesis and Clinical Features

Brucellosis is a protracted and debilitating illness characterized by an incubation period of from one to six weeks followed by a chronic, relapsing fever with accompanying lassitude, sweats, headache, constipation, anorexia, pains in the limbs and back, and weight loss. After the temperature has returned to normal for a few days, another bout of fever may ensue and such episodes recur a number of times over several months. Treatment is commonly with a mixture of tetracycline and streptomycin.

It is a facultative parasite and can live intracellularly or in extracellular body fluids. During the febrile stage, caused by circulating endotoxin, the organism may be isolated from the bloodstream but in the majority of laboratory-confirmed cases diagnosis is based on serological tests rather than cultural techniques.

7.3.4 Isolation and Identification

Brucella are quite fastidious organisms and do not grow in conventional laboratory media. Liver infusions or calf serum are normally added. The

organism grows slowly and cultures are normally incubated for three weeks before they are considered negative. In view of this, testing foods for the organism is not practically feasible or useful. Cattle are tested for the presence of antibodies to the organism in the 'Ring Test'. Stained antigen is mixed with the test milk, if antibodies to *Brucella* are present (indicative of infection) then they will cause the antigen to clump and rise with the milkfat on standing to form an intense blue-violet ring at the top of the milk.

7.3.5 Association with Foods

Although brucellosis has sometimes been associated with the consumption of inadequately cooked meat from an infected animal, raw milk or cream are the principal food vehicles. *Brucella* is readily killed by normal milk pasteurization conditions so there is no risk from pasteurized milk or products made from it. Cheeses made from unpasteurized milk can sometimes pose a problem since the organism can survive the cheese-making processes and subsequent storage in the product.

7.4 *CAMPYLOBACTER*

7.4.1 Introduction

Campylobacter has been known as a veterinary problem since the early years of the 20th Century when the original isolate, known then as *Vibrio fetus*, was associated with infectious abortion in sheep and cattle. In 1931 the species *Vibrio jejuni* was described as the cause of winter dysentery in calves and in 1946 a similar organism was isolated from blood cultures of patients in a milk-borne outbreak of acute diarrhoea. Later King, working with human blood isolates, distinguished two groups on the basis of their optimum growth temperature. One group corresponded to *Vibrio fetus* and the second, 'thermophilic', group, which grew best at 42 °C, came from patients with preceding diarrhoea.

Both groups differ from the cholera and halophilic vibrios biochemically, serologically and in their mol% G + C ratio, and were reclassified into the new genus *Campylobacter* in 1963.

In the 1970s, with the development of suitable selective media, it was established that *Campylobacter jejuni*, and to a lesser extent *Campylobacter coli*, are a major cause of diarrhoeal illness, rivalling and even surpassing *Salmonella* in importance in many countries. *Campylobacter laridis*, *C. concisus* and *C. hyointestinalis* have also been isolated occasionally from patients with diarrhoea and *C. pylori*, now reclassified as *Helicobacter pylori*, has been associated with gastritis and stomach and duodenal ulcers.

The *Campylobacter*-like genus *Arcobacter* is frequently associated with abortion and enteritis in cattle and pigs. Two species, *Arcobacter butzleri* and *A. cryaerophilus*, have also been implicated in human infections causing diarrhoea, bacteraemia and other extra-enteric infections.

7.4.2 The Organism and its Characteristics

Campylobacters are non-sporeforming, oxidase-positive, Gram-negative rods. Cells are pleomorphic and may be 0.5–8 μm in length and 0.2–0.5 μm in width. Log-phase cells have a characteristic slender, curved or spiral shape and one or more polar or amphitrichous flagella which confer a rapid, darting motility and may be an important feature in pathogenesis (Figure 7.2). As cultures age, spiral or curved bacilli are replaced by round forms.

Campylobacters cannot ferment or oxidize sugars and are oxygen-sensitive microaerophiles, growing best in an atmosphere containing 5–10% carbon dioxide and 3–5% oxygen.

All *Campylobacter* species grow at 37 °C; *C. jejuni* and *C. coli* have optima at 42–45 °C but cannot survive cooking or pasteurization temperatures (D_{55} 2.5–6.6 min). They do not grow below 30 °C and survive poorly at room temperature. Although their viability declines during chill or frozen storage, they may nevertheless persist under these conditions for prolonged periods; survival has been recorded in milk and water at 4 °C after several weeks storage and in frozen poultry after several

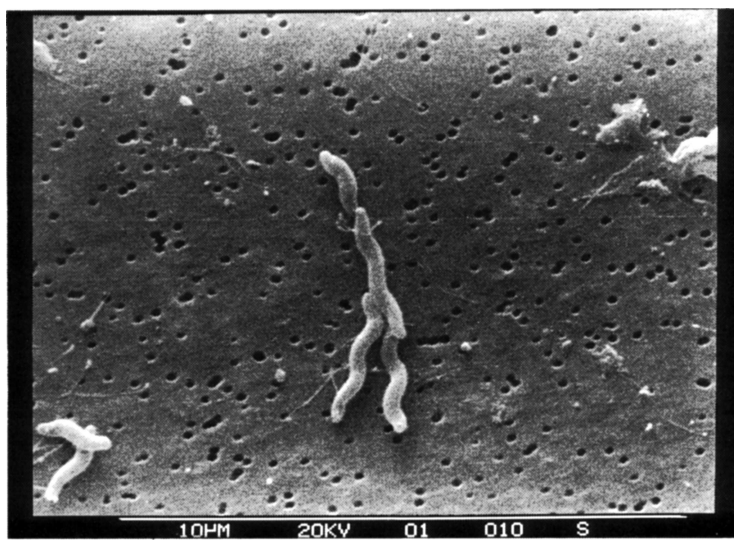

Figure 7.2 Campylobacter jejuni (Photo S. Boucher)

months. They are also particularly sensitive to other adverse conditions such as drying and reduced pH.

The principal environmental reservoir of pathogenic campylobacters is the alimentary tract of wild and domesticated animals and birds and it is a commonly found commensal of rodents, dogs, cats, dairy cattle, sheep, pigs, poultry and wild birds. The high optimum growth temperature of *C. jejuni* and *C. coli* could be an adaptation to the higher body temperature of birds and reflect their importance as a primary reservoir of the organism. Asymptomatic human carriage also occurs.

Though they would not appear to survive particularly well outside an animal host, campylobacters can be commonly isolated from surface water. Survival is enhanced by low temperatures and studies conducted in Norway have shown that strains of *C. jejuni*, *C. coli* and *C. laridis* remained viable in unchlorinated tap water at 4 °C for 15 days (10 days at 12 °C) and 10–15 days in polluted river water at the same temperature (6–12 days at 12 °C).

Under adverse environmental conditions campylobacters have been reported to adopt a 'viable non-culturable' state where the organism cannot be isolated by cultural methods but nevertheless remains infective. Evidence for this is conflicting, one study has shown that viable non-culturable *C. jejuni* can revert to a culturable state by passage through an animal host but others have failed to observe this effect.

Studies in the United States and Europe have isolated *Arcobacter* species from pork and poultry meat, though the rates of isolation vary widely (0.5 to 97%). Physiologically, *Arcobacter* species differ significantly from *Campylobacter* and *Helicobacter* in being both aerotolerant and capable of growth at 15 °C, attributes which could give them a considerable advantage when it comes to foodborne transmission. However the evidence for an association between *Arcobacter* and diarrhoeal disease in humans remains circumstantial at present.

7.4.3 Pathogenesis and Clinical Features

Enteropathogenic campylobacters cause an acute enterocolitis which, in the absence of microbiological evidence, is not easily distinguished from illness caused by other pathogens. The incubation period is from 1 to 11 days, most commonly 3–5 days, with malaise, fever, severe abdominal pain and diarrhoea as the main symptoms. The diarrhoea produces stools containing 10^6–10^9 cells g^{-1}, which are often foul-smelling and can vary from being profuse and watery to bloody and dysenteric. Gastrointestinal symptoms are sometimes preceded by a prodromal stage of fever, headache and malaise which lasts about a day. The diarrhoea is self-limiting and persists for up to a week, although mild relapses often occur. Excretion of the organism continues for up to 2–3 weeks. Vomiting is a less common feature.

Complications are rare although reactive arthritis can develop and *Campylobacter* has been shown to cause the serious neurological disease, Guillain–Barre syndrome.

As with other pathogens the infective dose will depend upon a number of factors including the virulence of the strain, the vehicle with which it is ingested and the susceptibility of the individual. Young adults (15–24 years old) and young children (1–4 years) appear particularly susceptible. In an outbreak at a boys' school in England caused by contamination of a water-holding tank with bird droppings, the infective dose was estimated as 500 organisms and in a separate study, a similar dose in milk caused illness in a volunteer. Motility, chemotaxis and the corkscrew morphology of the cells are all important factors in the virulence of *Campylobacter*, enabling it to penetrate the viscous mucus which covers the epithelial surface of the gut. Studies with *C. jejuni* have demonstrated a chemotactic response toward the sugar L-fucose, a number of amino acids, and intestinal mucus from mice and pigs. Although *Campylobacter* does not normally possess fimbriae it probably possesses other adhesins that enable it to adhere to epithelial cells once the mucosal barrier has been penetrated. The production of peritrichous 'pili-like' appendages when cells are grown in the presence of bile has been observed. A nonpiliated mutant did not show any reduced adherence or invasion in a cell line although it did produce significantly reduced disease symptoms in an animal model.

There is considerable uncertainty as to the precise mechanisms by which *Campylobacter* causes illness. It has been shown to be invasive in cell cultures and a number of toxins with cholera-like or cytotoxic activity have been described. Their role in pathogenesis however has been brought into question following the complete sequencing of the *Campylobacter* genome which has failed to identify sequences associated with known toxins. One exception is the so-called cytolethal distending toxin which has also been reported in a small number of *E. coli* and *Shigella* strains. There is evidence that some of the pathogenic features of the illness are the result of the body's inflammatory response to invasion by the organism, a factor also implicated in illness caused by *Salmonella*.

7.4.4 Isolation and Identification

Although most of the isolation procedures and media used were designed for *C. jejuni*, they are also suitable for *C. coli* and *C. laridis*.

Pathogenic campylobacters have a reputation for being difficult to grow but in fact their nutritional requirements are not particularly complex and they can be grown on a number of peptone-based media including nutrient broth. Where problems can sometimes arise is in their sensitivitiy to oxygen and its reactive derivatives. Although pathogenic

campylobacters possess catalase and superoxide dismutase, the accumulation of peroxides and superoxide in media during storage or incubation can inhibit growth. For this reason an incubation atmosphere of 5–6% oxygen with about 10% carbon dioxide and media containing oxygen scavenging compounds such as blood, pyruvate, ferrous salts, charcoal and metabisulfite are commonly used.

To isolate the relatively low numbers of cells that may be present in foods, several selective enrichment media are used which include cocktails of antibiotics such as polymyxin B, trimethoprim and others as selective agents. In many cases cells isolated from food or other environmental sources have been sub-lethally injured as a result of stresses such as freezing, drying or heating and, as a result, are more sensitive to antibiotics and toxic oxygen derivatives. This can mean that they will not grow on the usual selective media unless allowed a period for recovery and repair in which case a resuscitation stage of 4 h at 37 °C in a non-selective environment is recommended.

After selective enrichment for 24 and 48 h under microaerobic conditions at 42–43 °C, samples are streaked on to selective plating media. These normally contain a nutrient-rich basal medium supplemented with oxygen scavengers such as blood and/or FBP (a mixture of ferrous sulfate, sodium metabisulfite, and sodium pyruvate), and a cocktail of antibiotics similar to those used for selective enrichment. It is important to store pre-prepared media under nitrogen, at 4 °C and away from light to reduce the build-up of toxic oxides.

Colonies are non-haemolytic and have a rather unimpressive flat, watery appearance with an irregular edge and a grey or light-brown coloration. Suspect colonies are examined microscopically for motility and morphology and subjected to a range of tests after purification. *C. jejuni*, *C. coli*, and *C. laridis* are catalase and oxidase positive, reduce nitrate to nitrite, grow at 42 °C but not at 25 °C microaerobically and cannot grow aerobically at 37 °C. *C. laridis* is resistant to nalidixic acid while *C. jejuni* and *C. coli* are not. *C. jejuni* and *C. coli* can be distinguished by the ability of the former to hydrolyse hippurate. Various typing schemes have been proposed for epidemiological investigations. Biotyping based on biochemical tests and the more discriminating serotyping schemes of Lior and Penner have been used most frequently but not routinely. Molecular methods such as ribotyping and flagellin gene typing have been used but pulsed-field gel electophoresis has become the subtyping method of choice due to its sensitivity and discriminatory power.

7.4.5 Association with Foods

The incidence of *Campylobacter* infection is characterized by large numbers of sporadic cases rather than single source outbreaks. Infection

can be acquired by a number of routes. Direct transmission person-to-person or from contact with infected animals, particularly young pets such as kittens or puppies, has been reported, as have occasional water-borne outbreaks. However, food is thought to be the principal vehicle.

As a common inhabitant of the gastrointestinal tract of warm-blooded animals, *Campylobacter* inevitably finds its way on to meat when carcasses are contaminated with intestinal contents during slaughter and evisceration. Numbers are reduced significantly as a result of chilling in the abattoir; the incidence of *Campylobacter*-positive beef carcasses in Australia was found to decrease from 12.3% to 2.9% on chilling and a similar survey of pig carcasses in the UK found a decrease from 59% down to 2%. This is primarily a result of the sensitivity of *Campylobacter* to the dehydration that takes place on chilling. Subsequent butchering of red-meat carcasses will spread the surviving organisms to freshly cut, moist surfaces where viability will decline more slowly.

Poultry carcasses which cool more rapidly due to their size suffer less surface drying when air-chilled and this, probably coupled with the surface texture of poultry skin, enhances survival. Surveys in Australia, the UK and the USA have found 45%, 72% and 80% respectively of chilled poultry carcasses at the abattoir to contain *Campylobacter*.

The incidence of campylobacters on retail meats in several countries has been found to vary from 0–8.1% for red meats and from 23.1–84% for chicken. Adequate cooking will assure safety of meats but serious under-cooking or cross-contamination from raw to cooked product in the kitchen are thought to be major routes of infection.

Despite its frequent occurrence in poultry, eggs do not appear to be an important source of *Campylobacter*. Studies of eggs from flocks colonized with *C. jejuni* have found the organism on around 1% of egg shells or the inner shell and membranes. Prolonged survival on the dry egg surface is unlikely and egg albumin has been shown to be strongly bactericidal.

Milk can contain *Campylobacter* as a result of faecal contamination on the farm or possibly *Campylobacter* mastitis. The bacterium cannot survive correct pasteurization procedures and the majority of outbreaks, many quite large, have involved unpasteurized milk. More than 2500 children aged 2–7 years in England were infected by consumption of free school milk which is thought to have by-passed pasteurization. In Switzerland, a raw-milk drink was associated with an outbreak at a fun-run which affected more than 500 participants. It is not on record whether any personal best times were achieved that day.

Post-pasteurization contamination may always re-introduce the organism to milk. For example, pecking of doorstep delivered pasteurized milk by birds of the crow family has been strongly implicated in a number of cases of *Campylobacter* enteritis in the UK. Dairy products other than

fresh milk do not pose a threat due to the low resistance of *Campylobacter* to conditions of reduced pH or a_w.

Other foods recognized as potential sources of *Campylobacter* infection include shellfish and mushrooms. *C. jejuni* and *C. coli* were detected in 14% of oyster flesh tested, although 2 days depuration was sufficient to cleanse oysters artificially contaminated with 800 cfu of campylobacters g^{-1}. An outbreak in the USA was ascribed to raw clams.

7.5 *CLOSTRIDIUM BOTULINUM*

7.5.1 Introduction

Because of its severity and distinctive symptoms, botulism is the form of bacterial food poisoning for which we have the earliest reliable reports.

In 1793 in Wildbad, Wurttemburg, 13 people fell ill and 6 later died after eating Blunzen, a type of sausage made by packing blood and other ingredients into a pig's stomach. The sausage had been boiled and then smoked, after which it was considered stable at room temperature for several weeks and suitable for consumption without reheating.

Several further incidents of *Wurstvergiftung*, or sausage poisoning, were recorded in the years that followed, usually associated with sausages that contained animal components other than muscle tissue. This prompted a local district medical officer, Justinius Kerner, to undertake a study of the disease which became known as botulism (Latin: *botulus* = sausage). Kerner noted several important features including the facts that heating was an essential precondition for the development of toxicity in sausages and that small sausages or those containing air pockets were less likely to become toxic.

It was not until 1896 that the micro-organism responsible was isolated and described by van Ermengem, Professor of Bacteriology at the University of Ghent and former pupil of Robert Koch. This was a result of his investigation into an outbreak of botulism where 34 members of a music club in Belgium ate raw, unsmoked ham. Several noted that the ham had a slightly 'off' flavour akin to rancid butter but was otherwise unremarkable. About a day later, 23 of the group fell ill and 3 died within a week.

Van Ermengem established that botulism resulted from the consumption of food containing a heat-labile toxin produced by an obligately anaerobic, spore-forming bacillus which he called *Bacillus botulinus*. He further demonstrated that toxin would not be produced in the presence of sufficient salt, that it was resistant to mild chemical agents and was not uniformly active against all animal species.

Although much of the early evidence suggested that botulism was confined to meat products, it was later found to occur wherever foods

and their processing offer conditions suitable for survival and growth of the causative organism. It was identified with icthyism, a paralytic illness associated with the consumption of raw, salted fish, known in Russia since 1880, and in 1904 an outbreak of botulism in Darmstadt, Germany was caused by canned white beans.

7.5.2 The Organism and its Characteristics

Van Ermengem's original designation was superseded in 1923 when the organism responsible for botulism was reclassified as *Clostridium botulinum*. The cells are Gram-positive, motile with peritrichous flagella, obligately anaerobic, straight or slightly curved rods 2–10 μm long, and form central or subterminal oval spores.

Strains of *C. botulinum* display sufficient variety of physiological and biochemical characteristics to be inconsistent with their inclusion in a single species. In this instance however, taxonomic rectitude has been sacrificed to avoid any possibility of confusion over nomenclature with potentially fatal consequences. The most important common feature of the species is the production of pharmacologically similar neurotoxins responsible for botulism. Eight serologically distinct toxins are recognized (A, B, C_1, C_2, D, E, F, and G, though C_2 is not a neurotoxin), a single strain of *C. botulinum* will usually only produce one type, although there are exceptions. In 1985, certain strains of *C. barati* and *C. butyricum* responsible for cases of infant botulism (Section 7.5.3) were found to produce similar neurotoxins, although they have not been implicated in any foodborne cases of botulism.

Physiological diversity within the species *C. botulinum* is recognized by its division into four groups (Table 7.4) and molecular studies based on DNA homology and ribosomal RNA sequences have confirmed this grouping. Group I strains are culturally indistinguishable from the non-toxigenic species *Clostridium sporogenes* which can sometimes serve as a useful and safe model in laboratory studies. They are strongly proteolytic and will often betray their presence in food by partial disintegration of the product and a slight rancid or cheesy odour. Unfortunately despite these warning signs the potency of the toxin is such that the amount ingested on sampling the food has often proved sufficient to cause illness. Group I strains are not psychrotrophic and are therefore of little concern in adequately refrigerated products. They do, however, produce the most heat-resistant spores and can pose a problem when foods that depend upon a heating step for their stability and safety are underprocessed.

In contrast, Group II strains represent a greater potential hazard in chilled foods. They are non-proteolytic with native protein, can grow and produce toxin down to about 3 °C and produce spores with a low resistance to heat. They also tend to be more susceptible to inhibition

Table 7.4 *The physiological subdivision of* Clostridium botulinum

Group	*Toxin types*	*Proteolytic*	*Lipolytic*	*Saccharolytic*	*Psychrotrophic (*min.growth temp.*)*	*Inhibition by salt (a_w)*	*Heat resistance*	*Pathogenicity*
I	A, B or F	+	+	+	− (10–12 °C)	10% (0.94)	+ (D_{121} 0.1–0.25 min)	Humans
II	B, E or F	−	+	+	+ (3–5 °C)	5% (0.975)	− (D_{80} 0.6–3.3 min)	Humans
III	C_1, C_2 or D	−	+	+	− 15 °C	3%	±	Usually animals and birds
IV	G	+	−	−	12 °C	>3%	No data	Humans

by salt (Table 7.4). The rate of growth and toxin production at the lower temperature limit is slow and will be reduced still further by any other factors adverse to growth. Experimental studies have indicated that storage periods of 1–3 months are necessary for toxin production at 3.3 °C, although this period can be markedly reduced at higher temperatures still within the chill range. Vacuum-packed herrings inoculated with 100 spores per pack became toxic after 15 days storage at 5 °C.

Most cases of botulism in humans are due to toxin types A, B or E and the incidence of other types in human illness is extremely rare. Group III strains producing toxin types C and D are usually associated with illness in animals and birds. Type G toxin has been incriminated in human illness largely as a result of its isolation at autopsy from people who had died suddenly and unexpectedly. Since botulism was not necessarily the cause of death in these cases, and there have been no reports of the presence of type G in foods, its role in foodborne illness is questionable. Group IV strains which produce type G toxin and some non-toxigenic clostridia have been re-designated as a new species, *Clostridium argentinense*.

Although it is found occasionally, growing in the alimentary tract of birds and mammals, *C. botulinum* is essentially a soil saprophyte. It occurs widely, although the geographical distribution is not uniform. Surveys conducted in the United States found type A to be the most common in the Western States, rare in the Mississippi Valley but less so along the Eastern Seaboard where type B was predominant. This distribution was reflected in outbreaks of botulism in the United States in the period 1950–1979; when 85% of those west of the Mississippi were due to type A toxin and 63% of those to the east were due to type B. In European soils type B tends to be more common than type A.

Aquatic muds provide a moist, anaerobic, nutrient-rich environment in which clostridia can flourish, so isolation of *C. botulinum* from these sources is more frequent than from soils. The psychrotrophic type E has been particularly associated with this environment in regions such as western North America, Japan and the Baltic sea coasts. As a consequence, type E is often responsible for outbreaks of botulism where fish is the vehicle.

The minimum pH at which *C. botulinum* will grow depends very much on factors such as temperature, water activity and the acid used to adjust the pH. The consensus has long been that a pH around 4.7 represents an absolute minimum and this fact has had important practical implications for the canning industry (see Chapter 4). Non-proteolytic strains have a lower acid tolerance and are generally inhibited at pH 5.0–5.2. Reports have appeared of growth and toxin production at pH values as low as 4.0 in protective, high-protein containing media but this does not reflect the situation in acid canned foods which are generally low in protein. In cases where botulism has occurred in foods where acidity is an important protective hurdle, such as canned fruits, it has been as a result of other

organisms, yeasts or moulds, growing in the product and increasing the pH.

The maximum pH for growth is 8.5–8.9 and the toxin is unstable at alkaline pH values. This is generally an unimportant feature of the organism's physiology since nearly all foods are slightly acidic. It may be significant however in some North American fermented fish products occasionally associated with botulism where the usual increase in pH on fermentation would be a protective factor.

7.5.3 Pathogenesis and Clinical Features

Three types of botulism are recognised: foodborne botulism, infant or infectious botulism and wound botulism. Only in the first type is food invariably involved.

Foodborne botulism is an example of bacterial food poisoning in its strictest sense: it results from the ingestion of an exotoxin produced by *Clostridium botulinum* growing in the food. The botulinum toxins are neurotoxins; unlike enterotoxins, which act locally in the gut, they affect primarily the cholinergic nerves of the peripheral nervous system.

Experiments in animals have shown that toxin ingested with food and surviving inactivation is absorbed in the upper part of the small intestine and reaches the bloodstream *via* the lymphatics. It binds to the nerve ending at the nerve–muscle junction, blocking release of the acetylcholine responsible for transmission of stimuli, thus producing a flaccid paralysis.

Initial symptoms of botulism occur anything from 8 h to 8 days, most commonly 12–48 h, after consumption of the toxin-containing food. Symptoms include vomiting, constipation, urine retention, double vision, difficulty in swallowing (dysphagia), dry mouth and difficulty in speaking (dysphonia). The patient remains conscious until, in fatal cases, shortly before the end when the progressive weakness results in respiratory or heart failure. This usually occurs 1–7 days after the onset of symptoms. Surviving patients may take as long as 8 months to recover fully.

The clinician can do little to mitigate the effect of toxin already adsorbed at the neuromuscular junction, although neuromuscular blockade antagonists such as 4-aminopyridine have produced transient improvements. Survival is therefore critically dependent on early diagnosis and treatment, principally by alkaline stomach washing to remove any remaining toxic food, intravenous administration of specific or polyvalent anti-toxins to neutralize circulating toxin, and mechanical respiratory support where necessary.

The mortality rate is usually high (20–50%), but will depend on a variety of factors such as the type of toxin (type A usually produces a higher mortality than B or E), the amount ingested, the type of food and the speed of treatment.

The botulinum toxins are the most toxic substances known, with a lethal dose for an adult human in the order of 10^{-8} g. They are high molecular mass (150 kDa) proteins and can be inactivated by heating at 80° C for 10 min. In culture, they are produced during logarithmic growth as complexes and released into the surrounding medium on cell lysis. In the smallest of these complexes, the M complex, neurotoxin is accompanied by a similar-sized protein with no apparent biological activity, while in the larger L complex, an additional haemagglutinin component is also present. It appears that the neurotoxin is synthesized as a single chain protoxin which is activated by proteolytic cleavage to produce a molecule consisting of light (M_r 50 kDa) and heavy (M_r 100 kDa) chains linked by a disulfide bridge (Figure 7.3). Where the organism does not itself produce appropriate proteolytic enzymes, protoxin can be activated by the gut enzyme trypsin. More extensive proteolysis will lead to toxin inactivation so that, although the structure of the natural complex affords some protection, the lethal oral dose of toxin A in mice is 10^4–10^5 times that observed when administered intraperitoneally. The heavy chain is responsible for specific binding to neuronal cells and cell penetration by the light chain. The light chain is a zinc endopeptidase which is activated by reduction of the interchain disulfide bond.

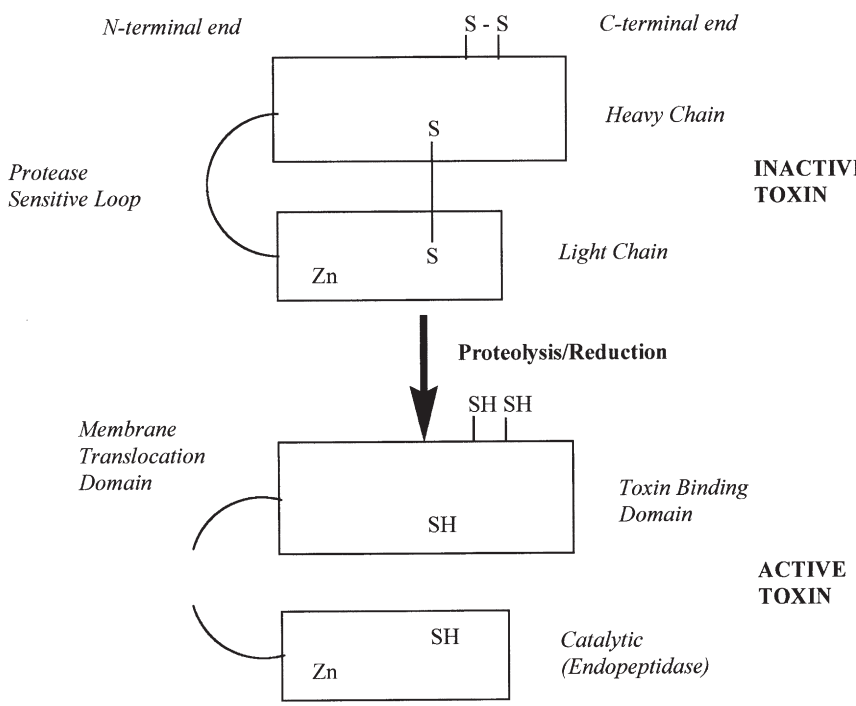

Figure 7.3 *Production and activation of botulinum toxin*

The endopeptidase then cleaves components of the docking and fusing complex of the synaptic vesicle, the vesicle that contains the neurotransmitter acetylcholine. The particular protein attacked and the specific peptide linkage hydrolysed varies with the type of toxin. Toxin types B, D, F, and G each hydrolyse a different peptide bond on vesicle associated membrane protein, also known as synaptobrevin. Types A and E attack different linkages on the synaptosome-associated protein, SNAP-25, and type C_1 degrades both SNAP-25 and syntaxin.

It has been shown, at least for types C and D, that the genetic information coding for toxin production is associated with a temperate bacteriophage. This persists in the bacterial cell as a prophage; its DNA incorporated and replicating with the bacterial chromosome without causing lysis. This lysogenic state occurs widely among bacteria in nature, usually without changing the micro-organism's characteristics, but sometimes, as here, it is associated with the production of toxins. Another example is the production of diphtheria toxin by *Corynebacterium diphtheriae*.

Infant botulism differs from the classical syndrome in that it results from colonization of the infant's gut with *C. botulinum* and production of toxin *in situ*. It was first described (in 1976) and is most frequently reported in the United States, although cases have occurred in Australia, Canada, Europe and South America. Up to 2005 there had been 6 confirmed cases of infant botulism in the UK, mostly involving type B toxin producers.

It occurs mostly in infants aged 2 weeks to 6 months, particularly around the time that non-milk feeds are introduced. At this stage the infant's gut microflora is not fully developed and is less able to outcompete and exclude *C. botulinum*. Since it only requires the ingestion of viable spores, environmental sources other than food may be involved and those foods that do act as vehicles need not be capable of supporting growth of the organism. Honey has been associated with several cases of infant botulism in the USA and some surveys have found viable spores of *C. botulinum* in 10% of the samples examined. Consequently it is thought inadvisable to feed honey to children less than a year old.

The illness is characterized by neuromuscular symptoms related to those of classical botulism and diagnosis can be confirmed by the isolation of the organism and its toxin from the faeces. Although implicated in a small proportion (4%) of cases of sudden infant death syndrome in the United States, the mortality rate is low in treated cases.

Wound botulism is caused by a subcutaneous infection with *C. botulinum*. This can result from trauma, but in recent years has been more commonly associated with intravenous drug use. Accidental overdoses of botulinum toxin during its cosmetic use to remove facial wrinkles have also caused occasional cases.

7.5.4 Isolation and Identification

In view of the metabolic diversity within the species selective media are of limited use in the isolation of *C. botulinum* and identification is based on the ability of typical colonies to produce toxin in culture.

C. botulinum will often constitute only a small proportion of the total microflora so enrichment or pre-incubation is necessary to improve the chances of isolation. Sometimes enrichment cultures are heated prior to incubation to eliminate non-sporeforming anaerobes. However, depending on the heating regime used, 80 °C for 10 min is commonly cited, this may also eliminate the less heat resistant strains of *C. botulinum* and is therefore often omitted.

After enrichment in a medium such as cooked meat broth at 30 °C for 7 days, the culture is streaked on to fresh horse-blood or egg yolk agar and incubated anaerobically for 3 days. Characteristic smooth colonies, 2–3 mm in diameter with an irregular edge and showing lipolytic activity on egg-yolk agar (type G excepted) are transferred into a broth medium to check for toxin production.

A technique has been described that simplifies this procedure by incorporating antitoxin into the agar medium so that toxin-producing colonies are surrounded by a zone of toxin–antitoxin precipitate.

Despite the development of a range of *in vitro* immunoassay procedures for toxin, the mouse neutralization test (Figure 7.4), remains the most sensitive (a typical lethal dose of toxin for a mouse is a few picograms). However, the distressing nature of the test guarantees its eventual replacement as soon as immunoassay amplification systems have been sufficiently improved.

A suspect toxin extract is divided into three portions: one, to serve as control, is heated at 100 °C for 10 min to destroy any toxin present; a second is treated with trypsin to activate any protoxin that may be there; and a third is untreated. Each of the portions is injected intraperitoneally into 2 mice and the mice observed over 4 days for the development of typical symptoms of laboured breathing and the characteristic 'wasp-waist' appearance. The presence of toxin is confirmed by protection of mice with polyvalent antitoxin and the toxin type can be identified using monovalent antisera.

7.5.5 Association with Foods

Four common features are discernible in outbreaks of botulism.

(1) The food has been contaminated at source or during processing with spores or vegetative cells of *C. botulinum*.

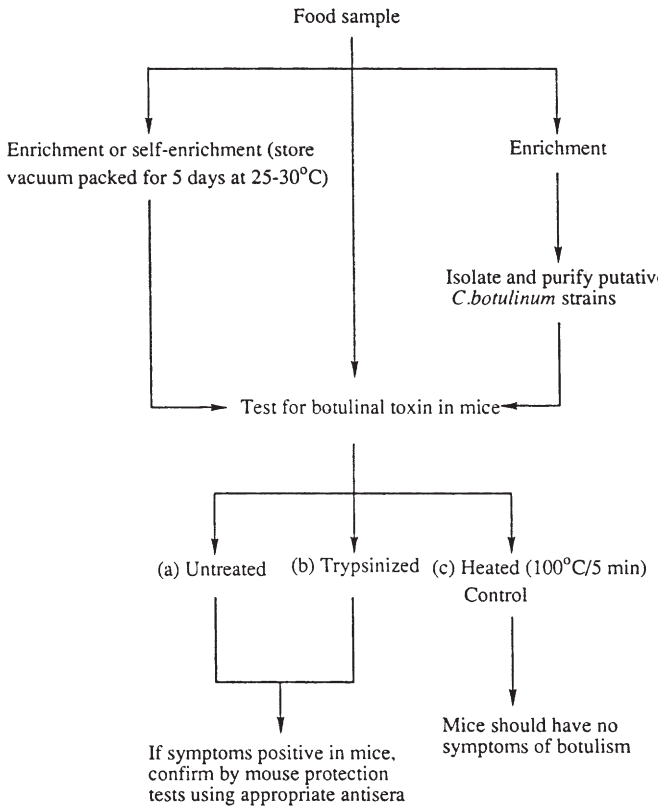

Figure 7.4 *Mouse neutralization assay for botulinum toxin*

(2) The food receives some treatment that restricts the competitive microflora and, in normal circumstances, should also control *C. botulinum*.
(3) Conditions in the food (temperature, pH, E_h, a_w) are suitable for the growth of *C. botulinum*.
(4) The food is consumed cold or after a mild heat treatment insufficient to inactivate toxin.

Since low-acid canned foods can fulfil all the above criteria, it has been necessary for the canning industry to introduce stringent process control measures to ensure safety (see Chapter 4). When canned foods are produced as a small-scale, domestic activity however, greater variability and less rigorous control are clearly potential sources of problems. In the United States, where home-canning is more widely practised than elsewhere, inadequately processed products, particularly vegetables, are the most common cause of botulism. Between 1899 and 1981 there were 522 outbreaks associated with home-canned products, including 432

involving vegetables. This compares with 55 outbreaks over the same period caused by commercially canned products; the majority occurring before 1925.

A variety of foods have been associated with botulism in the UK and they are frequently home-produced rather than commercial products [Table 7.5(a)]. Fortunately the incidence is extremely low, though slightly higher rates have been reported in some other European countries [Table 7.5(b)].

Table 7.5a *Foodborne botulism in the United Kingdom*

Year	*Number of deaths/cases*	*Food vehicle*	*(Home produced)*	C. botulinum *toxin type*
1922	8/8	Duck paté	(No)	A
1932	1/2	Rabbit and pigeon broth	(Yes)	?
1934	0/1	Jugged hare	(Yes)	?
1935	4?/5?	Vegetarian nut brawn	(Yes)	A
1935	1/1	Minced meat pie	(Yes)	B
1949	1/5	Macaroni cheese	(Yes)	?
1955	0/2	Pickled fish	(?)	A
1978	2/4	Canned salmon	(No)	E
1987	0/1	Rice and vegetables, shelf-stable airline meal	(No)	A
1989	1/27	Hazelnut puré added to yoghurt	(No)	B
1998	1/2	Bottled mushrooms	(Yes)	B
2003	1/1	Polish sausage	(Yes)	B
2004	0/2	Travel/Hummus	(?)	–
2005	0/1	Polish preserved pork	(Yes)	–

Adapted from *Eurosurveillence*, vol.4, Jan 1999

Table 7.5b *Botulism in Europe (number of cases/year)*

	1988	*1989*	*1990*	*1991*	*1992*	*1993*	*1994*	*1995*	*1996*	*1997*	*1998*[a]
Belgium	0	2	1	0	1	1	0	0	1	3	1
Denmark	0	0	0	0	0	0	0	2	0	0	1
England and Wales	0	27	0	0	0	1	1	0	0	0	2
Finland	0	0	0	0	0	0	0	0	0	0	0
France	4	6	11	3	5	10	13	7	5	8	NA
Germany	39	15	15	23	4	17	13	11	12	9	19
Greece	0	0	0	0	0	0	0	0	0	0	0
Italy	53	54	45	12	26	39	26	41	58	32	26
Scotland	0	0	0	0	0	0	0	0	0	0	0
Spain	8	8	10	5	12	9	7	6	7	9	11
Sweden	0	0	1	2	0	0	2	1	1	0	0
The Netherlands	0	0	0	0	0	0	0	0	0	0	0

[a] January – October.NA = not available
Data for Austria, Ireland and Portugal not available
Adpated from *Eurosurveillance*, vol. 4, Jan 1999

Fish can be contaminated with *C. botulinum*, particularly type E, from the aquatic environment and uncooked fish products have been responsible for several outbreaks of type E botulism.

Smoked fish consumed without reheating has generally been hot-smoked so control of *C. botulinum* depends on microbial inactivation by heat plus the inhibitory effects of salt, smoke constituents and surface drying. With the advent of refrigeration, the severity of the salting and smoking stages has been reduced in line with the perceived consumer preference for a less strongly flavoured product. In the early 1960s, two outbreaks of type E botulism in North America associated with vacuum-packed, hot-smoked fish caused considerable alarm and led to Canada banning the importation of all types of packaged fish. A similar outbreak in Germany in 1970 was caused by smoked trout from a fish farm. At first it was feared that vacuum packing, an emerging technology at that time, was responsible by providing an anaerobic environment in which *C. botulinum* could flourish.

It transpired that the problem was compounded of several factors. The salting and smoking treatments had been insufficient to eliminate *C. botulinum* or inhibit its growth during storage. A minimum salt concentration (in the water phase) of 3% and an internal temperature not less than 63 °C during smoking are recommended. The product had also been subjected to severe temperature/time abuse allowing *C. botulinum* to grow and produce toxin. The product should have been stored at temperatures below 4 °C. Finally, vacuum packing had improved the product shelf-life by inhibiting the normal spoilage microflora of bacteria and moulds which would have indicated that the product was inedible.

Fish products that are consumed raw after a fermentation process have also caused occasional problems, for example I-sushi (see Chapter 9). In 1986 in the Canadian Northwest Territories an outbreak of type E botulism was recorded after consumption of a meal comprising raw fish, seal meat and fermented seal flipper. The latter had been prepared by packing the product in a plastic bucket, covering with seal fat and leaving it outside the house to ferment. The process differed from normal in that the product was stored for 7 days instead of the usual three and the weather had been unseasonably warm. It was claimed that the seal flipper had an unusual taste and subsequent investigation established the presence of *C. botulinum* type E in the product. In Europe, the Norwegian fermented trout rak-orret has also been responsible for outbreaks of botulism.

The long association of botulism with meat products in Europe has already been noted and inadequate curing of meats still gives rise to occasional problems in some European countries. Outbreaks of botulism in the UK are relatively infrequent. The largest outbreak this century occurred in 1989 when 27 people fell ill and one died. In this outbreak the

vehicle was hazelnut yoghurt. The pH of yoghurt is too low for toxin production *in situ*, but the toxin (type B) had been produced in the hazelnut puree which was inadequately heat processed.

Soil contamination is a major source of *C. botulinum* in foods and one to which vegetables, particularly root crops, are inevitably prone. Three outbreaks of type A botulism in the United States have been attributed to potato salad where cooked or partly cooked potatoes had been stored for several days at ambient temperatures and under anaerobic conditions before further processing. In 1987 an airline passenger in Europe contracted type A botulism from a prepacked vegetable salad. Important features in these outbreaks were temperature abuse and anaerobiosis created by vacuum packing or wrapping in aluminium foil. In 2006 a number of cases were reported in the USA and Canada caused by pasteurised carrot juice, presumably as a result of inadequate refrigeration of the product.

7.6 *CLOSTRIDIUM PERFRINGENS*

7.6.1 Introduction

Clostridium perfringens, formerly *welchii*, has been known as a cause of the serious wound infection, gas gangrene, since 1892 when it was first described by the American bacteriologist Welch. Although accounts appeared shortly after, in 1895 and 1899, linking it with outbreaks of gastroenteritis in St. Bartholomew's Hospital, London, it was not until the mid-1940s that outbreaks associated with school meals in England (1943) and pre-cooked chicken dishes in the USA (1945) firmly established *C. perfringens* as a cause of food poisoning.

The species is classified into five types, designated A–E, based on the production of four major exotoxins, α, β, ε, and ι, and eight minor ones (Table 7.6). *C. perfringens* type A which is responsible for food poisoning and gas gangrene produces only the α major toxin which has lecithinase (phospholipase C) activity. Its ability to hydrolyse lecithin and some other phospholipids plays an important role in the pathogenesis of gas gangrene;

Table 7.6 *Classification of* Clostridium perfringens *based on major exotoxin production*

Type	*Toxin*			
	α	β	ε	ι
A	+	−	−	−
B	+	+	+	−
C	+	+	−	−
D	+	−	+	−
E	+	−	−	+

by attacking cell membranes it produces local tissue disruption in the wound and its absorption into the circulation causes a serious toxaemia. It does not however have any role in the food poisoning syndrome.

C. perfringens type C which produces α and β toxins causes enteritis necroticans, a more severe, but far more rare, enteric disease in which the β toxin damages the intestinal mucosa causing necrosis. Illness is preventable by active immunization against the β toxin. Outbreaks were reported in Germany in 1946 and 1949, but it is nowadays particularly associated with Papua New Guinea where it is known as pigbel. Symptoms of abdominal pain and bloody diarrhoea develop several days after a high-protein meal, often pork consumed on festive occasions. Low levels of intestinal proteases are a predisposing factor in victims. This could arise from a poor diet low in protein, as with the European outbreaks after the Second World War, and may be compounded in Papua New Guinea by protease inhibitors consumed with other foods in the diet such as sweet potatoes.

C. perfringens type A ranks below *Salmonella* as a cause of outbreaks of bacterial food poisoning in the UK. Total cases numbered less than one-tenth the number of salmonella cases between 1980 and 2005. Reported outbreaks of *C. perfringens* food poisoning have declined in recent years from an average total of 28 per year in the period 1992-1998 to an average of 9 p.a. in the period 1999-2005. The total numbers of people made ill in these outbreaks are shown in Figure 7.5. These do not include sporadic cases which the Infectious Intestinal Disease Study in England recognised as being ''quite common''.

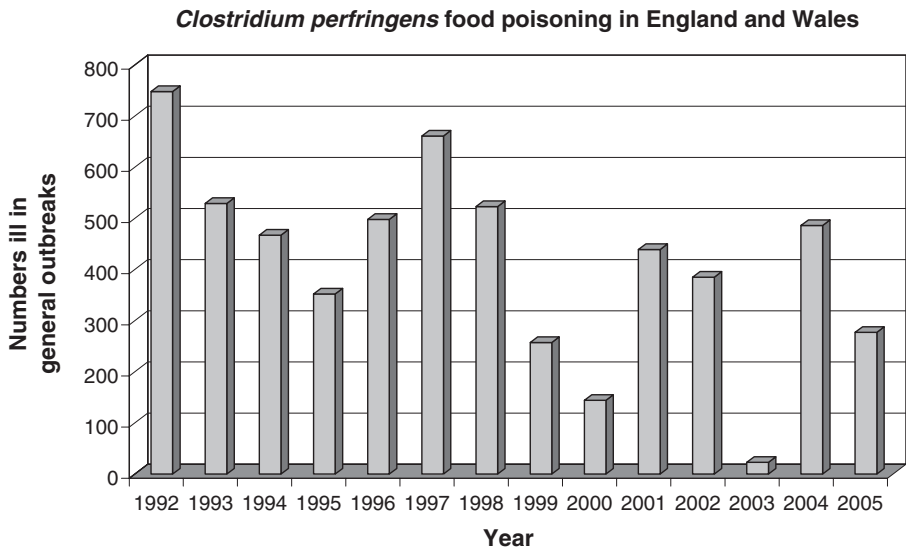

Figure 7.5 Clostridium perfringens *food poisoning in England and Wales*

7.6.2 The Organism and its Characteristics

Clostridium perfringens is a Gram-positive, rod-shaped anaerobe which forms oval subterminal spores. It differs from most other clostridia in that the relatively large rods (1×3–$9\,\mu m$) are encapsulated and non-motile. Though a catalase-negative anaerobe, *C. perfringens* will survive and occasionally grow in the presence of oxygen.

Growth occurs over the temperature range 12 to 50 °C although it is very slow below about 20 °C. At its temperature optimum, 43–47 °C, growth is extremely rapid with a generation time of only 7.1 min at 41 °C. Vegetative cells show no marked tolerance to acid (minimum pH 5, optimum 6.0–7.5), have a minimum a_w for growth of 0.95–0.97, depending on the humectant, and will not grow in the presence of 6% salt.

The heat resistance of vegetative cells is comparable to that of non-sporeforming bacteria with D values at 60 °C in beef of a few minutes D values of spores at 100 °C show a wide inter-strain variation with recorded values from 0.31 min to more than 38 min. This may, in part, be due to differences in the culture methods used since some workers included lysozyme in their media to improve the recovery of heat-damaged spores.

Distribution of type A *C. perfringens* is widespread in the environment. In soil, where it can be found at levels of 10^3–$10^4\,g^{-1}$, it persists much longer than types B,C,D, and E which are obligate animal parasites and of more limited distribution. It can be isolated from water, sediments, dust, raw and processed foods and is a common inhabitant of the human gastrointestinal tract. Spore counts of 10^3–$10^4\,g^{-1}$ are common in faeces from healthy individuals and surveys in Japan and the UK have found levels of up to 10^8–10^9 cfu g^{-1} in healthy elderly people in long-stay care.

7.6.3 Pathogenesis and Clinical Features

C. perfringens food poisoning is generally a self-limiting, non-febrile illness characterized by nausea, abdominal pain, diarrhoea and, less commonly vomiting. Onset is usually 8 to 24 h after consumption of food containing large numbers of the vegetative organism; the median count of *C. perfringens* in foods implicated in UK outbreaks is $7 \times 10^5\,g^{-1}$ and the required ingested dose has been variously estimated at 10^6–10^8 cfu. In otherwise healthy individuals, medical treatment is not usually required and recovery is complete within 1–2 days, although occasional fatalities occur in the very old or debilitated.

Ingested vegetative cells that survive the stomach's acidity pass to the small intestine where they grow, sporulate and release an enterotoxin. The enterotoxin is synthesized by the sporulating cells, although low levels of production have been observed in vegetative cultures. The toxin is closely associated with the spore coat, but is not thought to be an

important structural component, and is released into the intestinal lumen on lysis of the sporangium.

Toxin production can also occur *in vitro*. Low levels have been detected in foods, including a sample involved in an outbreak, and a few reported cases with incubation periods less than 2 h suggest that, on occasion, ingestion of pre-formed toxin may cause illness. This is generally held to be rare however.

The enterotoxin is a 35 kDa protein with an isoelectric point of 4.3. It is inactivated by heating in saline at 60 °C for 10 min and is sensitive to some proteolytic enzymes. It acts like cholera toxin by reversing the flow of Na^+,Cl^-, and water across the gut epithelium from absorption to secretion, though it does so by a different mechanism. Rather than increase the level of intracellular cyclic nucleotides, it acts at the cell membrane. It first binds to specific protein receptors on the epithelial cell and is then inserted into the cell membrane producing morphological changes within a few minutes. It changes cell permeability, inhibits synthesis of cell macromolecules, and produces pores in the membrane of the cell which eventually dies as a result of membrane damage. Illness tends to be relatively mild and short-lived since the toxin affects primarily the cells of the villus tip, which are the oldest intestinal cells and therefore replaced much sooner than younger cells.

Diagnosis of *C. perfringens* food poisoning is normally based on a number of factors:

(i) case history and symptoms;
(ii) large numbers ($>10^6\,g^{-1}$) of *C. perfringens* spores in the patient's faeces;
(iii) large numbers of vegetative cells of the same serotype in the incriminated food ($>10^6\,g^{-1}$);
(iv) presence of enterotoxin in faeces.

Faecal count data should be treated with some caution since the level of excretion can be very high in aged, healthy, institutionalized patients.

7.6.4 Isolation and Identification

In the investigation of outbreaks, enrichment culture is rarely necessary since *C. perfringens* will invariably be present in high numbers in implicated foods or clinical samples. Similarly, in routine quality assurance of foods, there is generally little value in being able to detect very low numbers in view of its ubiquity in the environment. In the examination of foods, the total count (vegetative cells plus spores) is determined but with faecal specimens a spore count, obtained after heating a suspension at 80 °C for 10 min, is also performed.

The most commonly employed selective plating media used to enumerate *C. perfringens* employ antibiotic(s) as the selective agent and sulfite reduction to produce black colonies as the differential reaction. The most popular combinations are tryptose/sulfite/cycloserine (TSC) medium and oleandomycin/polymyxin/sulfadiazine/perfringens (OPSP), incubated anaerobically for 24 h at 37 °C. A better diagnostic reaction is obtained if pour plates are used since colonies on the agar surface of spread plates can appear white.

Suspect colonies can be confirmed by the absence of motility, their ability to reduce nitrate to nitrite, lactose fermentation, and gelatin liquefaction. A traditional confirmatory test, the Nagler reaction, which looks for the production of α-toxin and its neutralization by antitoxin on lactose egg-yolk agar is less favoured now because of its lower specificity and the occurrence of lecithinase-negative strains.

Serotyping based on capsular antigens is employed for epidemiological purposes. Most isolates from outbreaks can be serotyped and this can be usefully supplemented by typing with bacteriocins, particularly when serotyping is not possible.

A number of methods are available for the detection of enterotoxin. Traditional biological tests such as the ligated ileal loop and mouse challenge have been superseded by more sensitive, rapid, convenient and humane serological techniques. A commercially available kit employs reverse passive latex agglutination. The name derives from the fact that in a standard agglutination assay, soluble antibody reacts with a particulate antigen such as bacterial cells. In a reversed passive latex agglutination assay, a soluble antigen reacts with antibody attached to latex particles. These play no part in the reaction and are therefore passive, but they do provide a visual signal when they cross-link as a result of the antigen–antibody reaction.

7.6.5 Association with Foods

For an outbreak of *C. perfringens* food poisoning, the typical scenario includes the following events:

(i) a meat dish containing spores of *C. perfringens* is cooked;
(ii) the spores survive the cooking to find themselves in a genial environment from which much of the competitive flora has been removed;
(iii) after cooking, the product is subjected to temperature/time abuse, such as slow cooling or prolonged storage at room temperature. This allows the spores to germinate and multiply rapidly to produce a large vegetative population;

(iv) the product is either served cold or reheated insufficiently to kill the vegetative cells. Some of the ingested cells survive through into the small intestine where they sporulate and produce enterotoxin.

From the above outline it is clear that *C. perfringens* food poisoning is more likely to occur where food is being prepared some time in advance of consumption and that adequate refrigeration is the key to its control.

Most cases (>70% in the United States and >87% in England and Wales) are associated with meat products such as stews, meat gravies, roast joints and pies. This is partly due to the frequent association of the organism with meats, but major contributory factors are the low redox potential, mode of preparation and consumption which can give *C. perfringens* the opportunity to multiply to dangerous levels.

Cured meats are rarely involved in *C. perfringens* food poisoning. This is a fine example of the hurdle concept in action (see Section 3.4); individual preservative factors such as salt content, nitrite level and heat processing are insufficient on their own to assure safety but effectively control growth of *C. perfringens* in combination.

Most outbreaks occur in connection with institutional catering such as schools, old people's homes and hospitals. The association between *C. perfringens* and hospital food in particular goes back a long way, to the outbreaks at St. Bartholomew's Hospital in the 1890s, but here at least there are signs of progress. In the UK, the 1991 Richmond Report on the safety of food noted that outbreaks in hospitals fell from a peak of more than 20 per year in the 1970s to about half this number in the 1980s: a decline attributed to improvements in facilities and staff training.

7.7 *ENTEROBACTER SAKAZAKII*

7.7.1 Introduction

Enterobacter sakazakii is an emerging opportunistic pathogen associated particularly with sporadic life threatening infections in low birth-weight infants, though it can cause disease in all age groups. The first recorded cases occurred in 1958 in Europe and the United States. Up until 2005 there had been about 75 cases of *Ent. sakazakii* infection reported worldwide. In many cases, particularly those reported up to the mid-1980s, the source of the infection was unknown but in more recent years contaminated infant milk formula has been recognised as the principal source of the organism in clinical infections.

7.7.2 The Organism and its Characteristics

Enterobacter sakazakii is a Gram-negative, motile member of the Enterobacteriaceae. It was originally classified as a strain of *Enterobacter*

cloacae distinguishable from other members of the species by production of a yellow water diffusible pigment on tryptone soy agar. In 1980 it was renamed *Enterobacter sakazakii* in honour of the eminent Japanese bacterial taxonomist Riichi Sakazaki.

It is a typical mesophile and can grow between 6° and 47 °C. As with many species, its heat resistance varies between strains. Some workers have described the heat sensitivity as similar to other Enterobacteriaceae while others have reported slightly higher resistance. A typical published D value measured in reconstituted dried milk at 60 °C was 2.5 minutes with a z value of 5.8 °C. *Ent. sakazakii* appears to be relatively more resistant to low a_w stress than other Enterobacteriaceae and this may be a significant factor in its transmission.

7.7.3. Pathogenesis and Clinical Features

Ent. sakazakii is the recognised cause of severe infections in infants characterised by meningitis, cerebritis, bacteraemia and necrotising enterocolitis. Infection is associated with a high mortality rate of 50% or more and severe long term, irreversible sequelae occur in most survivors. These include quadriplegia and impaired sight or hearing. The most common predisposing factors for infection are low birth-weight or premature birth.

7.7.4. Isolation and Identification

The severity of the illness and an apparently low infectious dose have led to very stringent criteria applying to the presence/absence of *Ent. sakazakii* in powdered infant formulae. Thus cultural detection follows a similar scheme to *Salmonella* detection involving pre-enrichment, and selective enrichment stages prior to the use of selective agars. Agar media can be selective and diagnostic for Enterobacteriaceae in general followed by biochemical testing to confirm the identity of isolates, although selective media have also been developed to detect a key biochemical characteristic of the organism such as its ability to produce α-glucosidase.

A variety of molecular methods have also been developed for the identification and typing based on PCR and ribotyping, pulsed-field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD)

7.7.5. Association with Foods

Ent. sakazakii appears widespread in the environment and has been isolated from water, soil and vegetation as well as the contents of household vacuum cleaners. Powdered infant formula foods have been

established as a common cause of infection although occasional cases in adults and infants not exposed to powdered infant formula indicate that this is not invariably the vehicle.

Pasteurisation is an effective control measure; conventional pasteurisation conditions of 72 °C for 15 seconds would produce more than a 10 log reduction in the number of survivors, assuming a D_{60} of 2.5 minutes and $z = 5.8$ °C. It seems that the organism is most likely to enter the product as a result of post pasteurisation contamination.

Surveys of powdered infant formulae have shown contamination rates ranging between 0 and 14% but generally, when it occurs, levels of contamination are low ranging from 0.36 cfu/100g to 66 cfu/100g. It may be that the infectious dose is very low in the very vulnerable patients affected, but poor hygienic practices during reconstitution and prolonged storage of the reconstituted product allowing bacterial multiplication have been identified as significant risk factors.

7.8 *ESCHERICHIA COLI*

7.8.1 Introduction

Since 1885, when it was first isolated from childrens' faeces and described by the German bacteriologist Theodor Escherich, scientific attention has been lavished on *Escherichia coli* to such an extent that it is today probably the best understood free-living organism.

E. coli is an almost universal inhabitant of the gut of humans and other warm-blooded animals where it is the predominant facultative anaerobe though only a minor component of the total microflora. Generally a harmless commensal, it can be an opportunistic pathogen causing a number of infections such as Gram-negative sepsis, urinary tract infections, pneumonia in immunosuppressed patients, and meningitis in neonates. Its common occurrence in faeces, ready culturability, generally non-pathogenic character, and survival characteristics in water led to the adoption of *E. coli* as an indicator of faecal contamination and the possible presence of enteric pathogens such as *S.* Typhi in water. This usage has been transferred to foods where greater circumspection is required in interpreting the significance of positive results.

Strains of *E. coli* were first recognized as a cause of gastroenteritis by workers in England investigating summer diarrhoea in infants in the early 1940s. Until 1982, strains producing diarrhoea were classified into three types based on their virulence properties: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enterotoxigenic *E. coli* (ETEC). They are not very common causes of foodborne illness in developed countries, but an important cause of childhood diarrhoea in less developed countries.

ETEC is also frequently associated with so-called traveller's diarrhoea. However since 1982, enterohaemorrhagic *E. coli* (EHEC) particularly associated with serotype O157:H7 has been recognized as the cause of a number of outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome, particularly in North America, where foods such as undercooked ground meat, raw milk and fresh produce have been implicated. An exponential rise in isolations of O157:H7 was reported in Canada between 1982 and 1986 and a study in the UK between 1985 and 1988 suggested that the increased reporting of isolations there (118 in England and Wales and 86 in Scotland) represented a real increase. The number of cases in the UK continued to increase until 1997 and has fluctuated between 600 and 1000 isolations per year since then. Other European countries have also reported increased isolation rates.

Two further types of *E. coli* are recognized as causes of diarrhoea, primarily in children. Termed enteroaggregative *E. coli* (EaggEC) and diffusely adherent *E. coli* (DAEC), they have characteristic patterns of adherence to Hep-2 cells in culture. The emergence of these numerous pathotypes of *E. coli* is thought to reflect the plasticity of the organism's genome. The acquisition, loss or rearrangement of genetic elements introduces new pathogenicity and virulence characterisitics and the different pathotypes represent strains sharing common virulence determinants.

7.8.2 The Organism and its Characteristics

Escherichia is the type genus of the Enterobacteriaceae family and *E. coli* is the type species of the genus. It is a catalase-positive, oxidase-negative, fermentative, short, Gram-negative, non-sporing rod. Genetically, *E. coli* is very closely related to the genus *Shigella*, although characteristically it ferments the sugar lactose and is otherwise far more active biochemically than *Shigella* spp. Late lactose fermenting, non-motile, biochemically inert strains of *E. coli* can however be difficult to distinguish from *Shigella*.

E. coli can be differentiated from other members of the Enterobacteriaceae on the basis of a number of sugar-fermentation and other biochemical tests. Classically an important group of tests used for this purpose are known by the acronym IMViC. These tested for the ability to produce:

(i) indole from tryptophan (I);
(ii) sufficient acid to reduce the medium pH below 4.4, the break point of the indicator methyl red (M);
(iii) acetoin (acetylmethyl carbinol) (V); and
(iv) the ability to utilise citrate (C).

Table 7.7 *The IMViC tests*

	Indole	*Methyl Red*	*Voges Proskauer*	*Citrate*
Escherichia coli	+	+	−	−
Shigella	V	+	−	−
Salmonella Typhimurium	−	+	−	+
Citrobacter freundii	−	+	−	+
Klebsiella pneumoniae	−	−	+	+
Enterobacter aerogenes	−	−	+	+

Since production of mixed acids and acetoin are alternative pathways for the metabolism of pyruvate, most species of the Enterobacteriaceae are either VP positive or methyl red positive. In the IMViC tests, most strains of *E. coli* are indole and methyl red positive and VP and citrate negative (Table 7.7). The tests are still used for identification purposes but nowadays usually as part of the larger range of tests available in modern miniaturized test systems.

E. coli is a typical mesophile growing from 7–10 °C up to 50 °C with an optimum around 37 °C, although there have been reports of some ETEC strains growing at temperatures as low as 4 °C. It shows no marked heat resistance, with a D value at 60 °C of the order of 0.1 min, and can survive refrigerated or frozen storage for extended periods. A near-neutral pH is optimal for growth but growth is possible down to pH 4.4 under otherwise optimal conditions. The minimum a_w for growth is 0.95.

A serotyping scheme for *E. coli* based on lipopolysaccharide somatic O, flagellar H, and polysaccharide, capsular K antigens was proposed by Kauffman in the 1940s. As currently applied in the O:H system, principal serogroups are defined by O antigens and then subdivided into serotype on the basis of H antigens. Strains of each category of pathogenic *E. coli* tend to fall within certain O:H serotypes, so the scheme plays an important role in detecting pathogens as well as in epidemiological investigations.

7.8.3 Pathogenesis and Clinical Features

There are four major categories of diarrhoeagenic *E. coli* based on distinct, virulence properties.

7.8.3.1 Enterotoxigenic E. coli *(ETEC).* Illness caused by ETEC usually occurs between 12 and 36 h after ingestion of the organism. Symptoms can range from a mild afebrile diarrhoea to a severe cholera-like syndrome of watery stools without blood or mucus, stomach pains and vomiting. The illness is usually self-limiting, persisting for 2–3 days, although in developing countries it is a common cause of infantile diarrhoea where it can cause serious dehydration.

The ingested organism resists expulsion from the small intestine with the rapidly flowing chyme by adhering to the epithelium through attachment or colonization factors in the form of fimbriae on the bacterial cell surface. These can have different morphology and be either rigid (6–7 nm diameter) or flexible (2–3 nm diameter) structures composed of 14–22 kDa protein subunits. They are mannose resistant, *i.e.* they mediate haemagglutination in the presence of mannose, and particular colonization fimbriae are restricted to certain O:H serotypes. They are encoded on plasmids which frequently also encode for the diarrhoeagenic toxins.

Two toxin types are produced: the heat-stable toxins (ST), which can withstand heating at 100 °C for 15 min and are acid resistant, and the heat-labile toxins (LT) which are inactivated at 60 °C after 30 min and at low pH. LTI bears a strong similarity to cholera toxin; it consists of five B subunits (M_r 11.5 kDa) which are responsible for binding of the toxin to the epithelial cells and an A subunit (M_r 25 kDa) which is translocated into the epithelial cell where it activates adenylate cyclase. The subsequent increase in cAMP levels then inhibits Na^+, Cl^- and water absorption by the villus cells and stimulates their loss from intestinal crypt cells thus leading to profuse watery diarrhoea. LTII toxin produced by certain ETEC strains has similar biological activity to LTI but does not cross react with antiserum to LTI or cholera toxin.

Two types of ST have been recognized; the most common, ST_A, is a low molecular weight, poorly antigenic polypeptide of less than 20 amino acids produced from a 72 amino acid precursor. Its resistance to heat, low pH and proteolytic digestion probably derive from its compact three-dimensional structure which contains at least 3 disulfide linkages. It acts by stimulating the production of cGMP by guanylate cyclase in epithelial cells. The mechanism of action of ST_B, which can be distinguished from ST_A by its inability to produce fluid secretion in the intestines of suckling mice, is not known but does not appear to operate through the stimulation of cyclic nucleotide production.

7.8.3.2 Enteroinvasive E. coli *(EIEC)*. Infection by EIEC results in the classical symptoms of an invasive bacillary dysentery normally associated with *Shigella*. Like *Shigella*, EIEC invades and multiplies within the epithelial cells of the colon causing ulceration and inflammation, though EIEC strains do not produce Shiga toxin. Clinical features are fever, severe abdominal pains, malaise and often a watery diarrhoea which precedes the passage of stools containing blood, mucus, and faecal leukocytes. Invasiveness is determined by a number of outer membrane proteins which are encoded for on a large plasmid ($\approx$140 MDa). The infective dose of EIEC appears to be substantially higher than for *Shigella* and this is thought to be a reflection of the organism's greater sensitivity to gastric acidity.

7.8.3.3 Enteropathogenic E. coli *(EPEC)*. When the properties of ETEC and EIEC were established it was noted that these strains were rarely of the same serotypes first associated with *E. coli* diarrhoea in the 1950s. Subsequent investigation of some of these earlier strains in most cases failed to demonstrate the property of enteroinvasiveness or the ability to produce ST or LT and yet they retained the ability to cause diarrhoea in volunteers.

Symptoms of EPEC infection, malaise, vomiting and diarrhoea with stools containing mucus but rarely blood, appear 12–36 h after ingestion of the organism. In infants, the illness is more severe than many other diarrhoeal infections and can persist for longer than two weeks in some cases. Pathogenesis is related to the ability of EPEC strains to adhere closely to the enterocyte membrane and produce the so-called attaching and effacing lesions. This is a complex and fascinating process mediated by the genes encoded on a 35 kb pathogenicity island called the locus of enterocyte effacement (LEA). Binding to the enterocytes occurs in three stages: non-intimate association mediated by pili, attachment or signal transduction, and then intimate contact. During this process the bacteria facilitate their own binding by producing a series of changes in the underlying enterocytes. A bacterial type III secretion system translocates another LEA encoded protein, Tir, into the enterocyte where it is incorporated into the cell's membrane. There it acts as a receptor for an outer membrane bacterial protein, intimin, which mediates close contact. The attachment stage is accompanied by increased levels of intracellular Ca^{2+}, release of inositol phosphates and activation of tyrosine kinase, an enzyme which phosphorylates tyrosine residues on intracelluar proteins. Following this the enterocytes accumulate filamentous actin as they form pedestal-like surface structures on which the bacteria rest. This results in deformation and loss of some microvilli; events which are thought to cause diarrhoea by disrupting the balance between absorption and secretion in the small intestine.

7.8.3.4 Enterohaemorrhagic E. coli *(EHEC)*. EHEC, sometimes also known as Verotoxin-producing *E. coli* (VTEC), was first described in Canada where in some areas it rivals *Campylobacter* and *Salmonella* as the most frequent cause of diarrhoea. *E. coli* O157:H7 is the most common EHEC serotype reported, although others do occur. Non-motile (H negative) O111 and O157 are more common in Australia for example. EHEC has attracted attention not only because foodborne transmission is more common than with other diarrhoeagenic *E. coli*, but because the illness it causes can range from a non-bloody diarrhoea, through haemorrhagic colitis, to the life threatening conditions haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP).

Haemorrhagic colitis is typically a self-limiting, acute, bloody diarrhoea lasting 4–10 days. Symptoms start with stomach cramps and watery diarrhoea 1–2 (sometimes 3–8) days after eating the contaminated food and, in most cases, progress over the next 1–2 days to a bloody diarrhoea with severe abdominal pain. It can be distinguished from inflammatory colitis by the usual lack of fever and absence of leukocytes in the stools. It affects mainly adults, with a peak incidence in the summer months, and can be life-threatening in the elderly.

Haemolytic uraemic syndrome is characterized by three features, acute renal failure, haemolytic anaemia (reduction in the number of red blood cells) and thrombocytopaenia (a drop in the number of blood platelets), sometimes preceded by a bloody diarrhoea. It is most common in children among whom it is the leading cause of acute renal failure in western Europe and North America. Approximately 10% of children under 10 with symptomatic *E. coli* O157 infection go on to develop HUS; half will require kidney dialysis and the mortality rate is generally 3–5%. In 70 cases seen in London between 1980 and 1986 the fatality rate was 6%, with 13% of cases showing some long-term kidney-damage. In one outbreak in a North American nursing home, the fatality rate among the 55 affected residents was 31%.

Thrombotic thrombocytopaenic purpura is a less common complication which is largely confined to adults. It is related to HUS but causes less kidney damage and includes fever and neurological symptoms resulting from blood clots in the brain.

Attachment is an important factor in virulence and O157:H7 strains possess the LEA pathogenicity island and adhere by a mechanism similar to EPEC, characterised by intimate attachment of the bacteria to the epithelial cells and effacement of the underlying microvilli.

EHEC strains produce the cytotoxin Verotoxin (so-called because of its ability to kill Vero (African Green Monkey Kidney) cells). Studies have revealed the presence of at least two toxins VTI and VTII which because of their similarity to Shiga toxin (see Section 6.6) have also been called Shiga-like toxins, SLTI and SLTII. It has been proposed that the nomenclature for these toxins be rationalised as Shiga family toxins so that the prototype toxin Shiga toxin is designated Stx, and SLTI and II become Stx 1 and Stx 2 respectively. Stx 1 bears the closest resemblance to Shiga toxin; it cross reacts with antisera to Shiga toxin, is also composed of A (M_r 32 kDa) and B (M_r 7.7 kDa) subunits and the B units are structurally identical. The B units bind specifically to the glycolipid receptor, globotriaosylceramide (Gb_3), on the eukaryotic cell surface and the susceptibility of the kidney in O157 infections may be due to higher levels of these receptors in kidney glomeruli. Following binding, the toxin is internalized by endocytosis and the A subunit activated. It then hydrolyses the *N*-glycosidic bond of a specific adenosine residue

in 28S rRNA thus stopping protein synthesis in the cell. Stx 2 also comprises an A and B subunit but these are larger than in Stx 1 (M_r 35 kDa and 10.7 kDa respectively) and do not cross-react immunologically, though they do share a 60% amino acid sequence homology, with Shiga toxin. Both toxins have been shown to be phage encoded in a number of strains.

7.8.4 Isolation and Identification

Selective techniques for *E. coli* mostly exploit the organism's tolerance of bile and other surfactive compounds, a consequence of its natural habitat, the gut. Aniline dyes and the ability of many strains to grow at temperatures around 44 °C are also used as selective agents.

The first selective and differential medium was that originally devised by MacConkey in 1905. It has been variously modified since but its essential characteristics have remained unchanged. Bile salts (and sometimes the aniline dye, crystal violet) act as inhibitors of Gram-positive and some fastidious Gram-negative bacteria. Lactose is included as a fermentable carbohydrate with a pH indicator, usually neutral red. Strong acid producers like *Escherichia, Klebsiella*, and *Enterobacter* produce red colonies, non-lactose fermenters such as *Salmonella, Proteus*, and *Edwardsiella*, with rare exceptions produce colourless colonies. MacConkey agar is not however strongly selective and will support the growth of a number of non-Enterobacteriaceae including Gram-positives such as enterococci and staphylococci.

Eosin/methylene blue agar is a popular selective and differential medium in North America. The aniline dyes eosin and methylene blue are the selective agents but also serve as an indicator for lactose fermentation by forming a precipitate at low pH. Strong lactose fermenters produce green-black colonies with a metallic sheen.

A biochemical feature of *E. coli* increasingly being used in diagnostic media is β-glucuronidase activity, which is possessed by around 95% of *E. coli* strains but by only a limited number of other bacteria. A fluorogenic or chromogenic glucuronide is incorporated into a conventional medium and enzyme activity detected by the production of colour or fluorescence. Most widely used is the fluorogen 4-methylumbelliferyl-β-D-glucuronide (MUG) which is hydrolysed to produce fluorescent 4-methylumbelliferone.

Suspect colonies from selective and differential media can be confirmed by further biochemical testing.

Detection of *E. coli* O157:H7 is based on phenotypic differences from most other serotypes: its inability to ferment sorbitol on MacConkey sorbitol agar and absence of β-glucuronidase activity in most strains. Presumptive *E. coli* O157:H7 from these tests must then be confirmed serologically for which a latex agglutination kit is commercially available.

Identification of diarrhoeagenic *E. coli* can be based on detection of their associated virulence factors. For example, procedures are available to detect the ST and LT of ETEC serologically, and the *LTI* and *Stx* genes in ETEC and EHEC using gene probes and the polymerase chain reaction (PCR).

7.8.5 Association with Foods

Faecal contamination of water supplies and contaminated food handlers have been most frequently implicated in outbreaks caused by EPEC, EIEC and ETEC. A number of foods have been involved, including a coffee substitute in Romania in 1961, vegetables, potato salad, and sushi. In the United States, mould-ripened soft cheeses have been responsible for outbreaks in 1971, associated with EIEC in which more than 387 people were affected, and in 1983, caused by ETEC (ST). *E. coli* would not be expected to survive well in a fermented dairy product with a pH below 5 but, where contamination is associated with mould-ripening, the local increase in pH as a result of lactate utilization and amine production by the mould would allow the organism to grow.

Outbreaks caused by EHEC serotype O157:H7 have mostly involved undercooked ground meat products and occasionally raw milk. Cattle seem to be an important reservoir of infection and O157:H7 has been isolated from 0.9–8.2% of healthy cattle in the UK. Other surveys have isolated the serotype from 3.7% (6/164) samples of retailed fresh beef and a significant percentage (1–2%) of other fresh meat products such as pork, poultry and lamb.

There have been a number of very large outbreaks around the world and their public impact has often been dramatic. Six hundred people became ill and four children died in a major US outbreak in 1993 caused by undercooked beef hamburgers. This caused a major public outcry over meat hygiene and resulted in, amongst other things, the introduction of new meat-labelling regulations.

In August 1997, a cluster of cases in Colorado prompted the largest food recall in US history when more than 12 000 tons of ground beef were recalled.

A large outbreak in Scotland in 1996 had a similar impact in the UK. Nearly 500 were affected and 20 elderly patients died. The cause was thought to be cross-contamination of cooked meats from raw meat in a butcher's shop and the resultant enquiry produced a tightening of regulation.

The failures that led to these outbreaks were generally simple breakdowns of basic food hygiene. With both raw milk and ground beef products, the primary cause has been a failure to heat process/cook the products adequately. While it is true that intact cuts of meat such as

steaks can often be consumed safely when the interior is undercooked, this is because microbial contamination is usually a surface phenomenon. Comminution of the meat, however, will mix surface contaminants into the middle of products and they will therefore need cooking throughout to ensure microbial safety. The USDA has produced regulations specifying that the centre of beef hamburgers should reach on cooking: 71.1 °C (160 °F) instantaneously for consumers and 68.3 °C (155 °F) for 16 seconds in food service operations.

Outbreaks of EHEC have been reported with other foods. Lettuce has been associated on several occasions and unpasteurised apple juice was the vehicle in a large outbreak in the US. In the summer of 1996, an epidemic in Japan involved over 9000 cases and 12 deaths in children. The largest outbreak during the epidemic, in Sakai City, involved 5700 people and was associated with contaminated radish sprouts, and the same vehicle was implicated in a further outbreak the following year. Alfalfa sprouts were also implicated in an outbreak in the US.

Outbreaks caused by acidic foods such as apple juice and fermented sausages, and laboratory studies with mayonnaise, remind us of the potential for bacteria to survive for prolonged periods at pH values that do not permit growth, particularly when the product is refrigerated. EHEC does appear to have a more marked ability to survive at low pH values than some other bacteria and this may also account for the relatively low infectious dose, 2–2000 cells, recorded in outbreaks.

7.9 *LISTERIA MONOCYTOGENES*

7.9.1 Introduction

L. monocytogenes is the only important human pathogen among the six species currently recognized within the genus *Listeria*, although *L. seeligeri*, *L. welshimeri*, and *L. ivanovii* have occasionally been associated with human illness. It was first described by Murray in 1926 as *Bacterium monocytogenes*, the cause of an infection of laboratory rabbits where it was associated with peripheral blood monocytes as an intracellular pathogen, and it has since been established as both an animal and human pathogen. As an important veterinary problem, it causes two main forms of disease: a meningoencephalitis most common in adult ruminants such as sheep and cattle, and a visceral form more common in monogastrics and young ruminants which attacks organs other than the brain causing stillbirth, abortion and septicaemia. Listeriosis in sheep increased in Britain from 86 recorded incidents in 1979 to 423 in 1988. This was partly due to the increased size of the national flock over that period but has also been attributed to changes in silage-making

techniques towards greater use of big-bale ensilage. In big-bale ensilage, the silage is made in large plastic bags rather than in a single large clamp. Rupture of the bag or inadequate sealing at its neck can allow mould growth to occur on the lactic acid present increasing the silage pH to a value at which *L. monocytogenes* can flourish.

Human listeriosis is described in more detail in Section 7.9.3 below. The recent widespread concern it has caused is largely attributable to the realization that food is a major source of the infection (a possibility first suggested as long ago as 1927), the psychrotrophic character of the organism, and the high mortality rate of the illness. Reported incidence of human listeriosis increased in several countries during the 1980s, but remains generally low when compared to other foodborne infections such as salmonellosis. For example, in England and Wales reported cases of listeriosis peaked in the late 1980s at around 300 per year while reports of *Salmonella* and *Campylobacter* infections numbered nearly 27 500 and 29 000 respectively. Reported cases of listeriosis dropped in 1990 and 1991 to 118 and 131 respectively; a decrease attributed to the effect of Department of Health advice to the immuno compromised and pregnant to avoid soft cheeses and to reheat certain chilled foods adequately and to withdrawal of contaminated paté from a single manufacturer (Figure 7.6). Reported incidence of human listeriois in England and Wales remained at around 100 cases p.a. until 2001 when numbers increased to more than 200 cases p.a. in 2003 and 2004. Unlike the earlier peak in 1988, this was marked by an increase in non-pregnancy associated cases. In the United States, the Center for Disease Control (CDC) has estimated an annual incidence of around 1700 cases resulting in 450 adult deaths and 100 foetal and postnatal deaths.

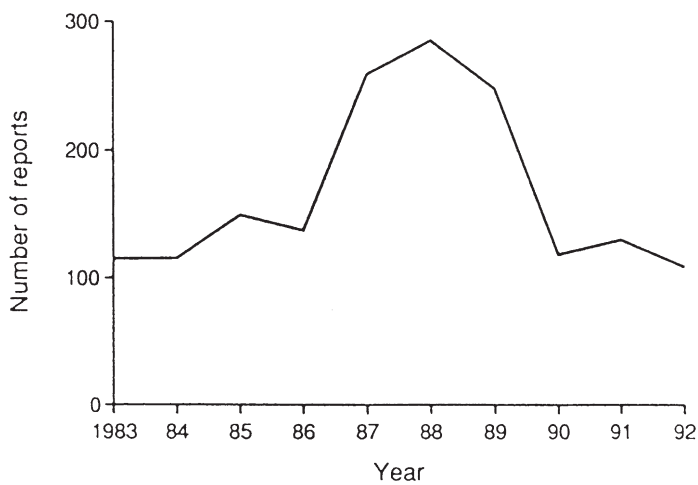

Figure 7.6 *Human listeriosis in England and Wales*

7.9.2 The Organism and its Characteristics

L. monocytogenes is a Gram-positive, facultatively anaerobic, catalase-positive, oxidase-negative, non-sporeformer. The coccoid to rod shaped cells (0.4–0.5 μm × 0.5–2.0 μm) cultured at 20–25 °C possess peritrichous flagella and exhibit a characterisitic tumbling motility. Colonies on tryptose agar viewed under oblique illumination have a characteristic blue–green sheen.

L. monocytogenes elaborates a 58 kDa β-haemolysin, listerolysin O, which acts synergistically with the haemolysin produced by *Staphylococcus aureus* to give enhanced haemolysis on blood agar. This reaction forms the basis of a useful diagnostic test to distinguish *L. monocytogenes* from *L. innocua*, and is known as the CAMP test after Christie, Atkins, and Munch-Peterson who first described the phenomenon with group B streptococci.

L. monocytogenes will grow over a wide range of temperature from 0–42 °C with an optimum between 30 and 35 °C. Below about 5 °C growth is extremely slow with lag times of 1 to 33 days and generation times from 13 to more than 130 h being recorded. The thermal survival characteristics of *L. monocytogenes* have received considerable attention following an outbreak in the United States associated with pasteurized milk and the suggestion that the organism could survive commercial pasteurization conditions. Despite some conflicting data in the literature, it appears that the heat resistance of *L. monocytogenes* is similar to that of other non-sporeforming Gram-positives with a typical D_{60} of a few minutes and a D_{70} of a few seconds. It was proposed that *L. monocytogenes* cells in contaminated milk are protected from heat by their intracellular location within milk leucocytes but subsequent studies have failed to demonstrate any significant effect.

Models of the thermal inactivation of *L. monocytogenes* in milk have indicated that conventional HTST pasteurization achieves a reduction of 5.2 log cycles in the number of survivors; an acceptable safety margin assuming low numbers of the organism present on the incoming milk.

Growth of all strains is inhibited at pH values below 5.5 but the minimum growth pH is dependent on both strain and acidulant and has been variously reported as between 5.6 and 4.4. *L. monocytogenes* is also quite salt tolerant being able to grow in 10% sodium chloride and survive for a year in 16% NaCl at pH 6.0.

The organism is ubiquitous in the environment. It has been isolated from fresh and salt water, soil, sewage sludge, decaying vegetation, and silage. Its prolonged survival in the environment has been demonstrated in one study where the level of *L. monocytogenes* in sewage sludge sprayed on to agricultural land remained unchanged for more than 8 weeks. Asymptomatic human and animal carriage is also common with

reports of isolation of the organism from the faeces of, among others, cattle, pigs, sheep, chickens, turkeys, ducks, crustaceans, flies and ticks. In a study of faecal carriage in human population groups, it was isolated from 4.8% of healthy slaughterhouse workers, 1.2% of hospitalized adults, 1% of patients with diarrhoea, and 26% of household contacts of listeriosis patients.

7.9.3 Pathogenesis and Clinical Features

Its ubiquity in the environment suggests that human exposure to *L. monocytogenes* must be frequent. Incidence of infection is however low since invasive infection will result only if a susceptible individual is exposed to a sufficiently high dose of a virulent strain.

Estimates of the minimum infective dose are always fraught with difficulty and this is certainly the case with *L. monocytogenes*. It is thought to be relatively high since foods implicated in outbreaks have been found to contain numbers in excess of 10^3 cfu g^{-1}.

Incubation periods for the disease have varied from 1 day to as long as 90 days with a typical incubation period of a few weeks; a situation which makes the identification of food vehicles difficult if not often impossible.

Symptoms of the disease, which is most likely to develop in pregnant women, the very young or elderly and the immunocompromised, can vary from a mild, flu-like illness to meningitis and meningoencephalitis.

In pregnant women, it most commonly features as an influenza-like illness with fever, headache and occasional gastrointestinal symptoms, but there may be an associated transplacental foetal infection which can result in abortion, stillbirth, or premature labour.

Listeriosis in the newborn can be an early-onset syndrome, which occurs at birth or shortly afterwards, or a late-onset disease appearing several days to weeks after birth. Early-onset illness results from *in utero* infection, possibly through the aspiration of infected amniotic fluid, and is characterized by pneumonia, septicaemia and widely disseminated granulomas (abscesses). Meningitis is rare.

In the late-onset syndrome, meningitis is more common, 93% (39 of 42) of late-onset cases in Britain between 1967 and 1985 had evidence of infection of the central nervous system. Infection may occur from the mother during passage through the birth canal, but some may also be acquired after delivery. A study in the UK found a lower mortality rate for late-onset disease (26%) than for early-onset listeriosis (38%).

Listeriosis in non-pregnant adults is usually characterized by septicaemia, meningitis and meningoencephalitis, but can also include endocarditis. It is particularly associated with those with an underlying condition which leads to suppression of their T cell mediated immunity, so that malignancies or immunosuppression (after renal transplantation,

for example) are often predisposing factors. Although not a common infection in AIDS patients, its incidence is around 300 times that in the general population. Other conditions such as alcoholism, diabetes and cirrhosis can act as predisposing factors, but illness does occur in otherwise healthy individuals who account for about 18% of adult cases in England and Wales.

Adult listeriosis has a high mortality rate, figures calculated using data for 1989 gave values around the world of between 13 and 34%. Early treatment with antibiotics, normally ampicillin, with or without an aminoglycoside, or chloramphenicol, is essential but in the most severe forms, the prognosis remains poor.

L. monocytogenes is a facultative intracellular pathogen which like *Mycobacterium, Brucella,* and others can survive and multiply in cells of the monocyte–macrophage system. The organism penetrates the gut either by crossing the Peyer's patches or by invading enterocytes. This process of endocytosis is promoted by a number of virulence factors including internalin, an 800 amino acid bacterial surface protein encoded by a chromosomal gene *inlA*, other products of the so-called *inl* family of genes, and p60, a 60 kDa extracellular protein. Internalization results in the bacteria being enclosed in a phagosome. In order to multiply intracellularly the organism must survive in the phagosome and escape rapidly before it fuses with a lysosome, a process which would kill the bacteria. Pathogenic *L. monocytogenes* produce listeriolysin O (LLO) a 58 kDa haemolysin which breaks down the lipid bilayer of the phagosomal membrane allowing the bacteria to escape from the phagosome. Most *L. monocytogenes* infecting epithelial-type cells do this and are released into the cytoplasm. Only 10% of those invading monocytes are successful, the remaining 90% being destroyed.

During intracellullar replication, actin polymerizes around the bacterial surface, a process which propels the bacterial cell around the host cell and into adjacent cells, thereby spreading the organism while avoiding the host's immune system. The bacterial cells reach the mesenteric lymph nodes and are disseminated around the body *via* the blood. The liver plays an important role in eliminating the organism and controlling the infection. Infection of hepatocytes causes an intense inflammatory reaction. Polymorphonuclear cells (neutrophils) destroy infected hepatocytes forcing them to release the bacteria which are in their turn destroyed. Where infection is not controlled in the liver, it can be further disseminated by the blood to the central nervous system or placenta causing more severe illness.

7.9.4 Isolation and Identification

Low-temperature enrichment at 4 °C is the traditional technique for isolating *L. monocytogenes* from environmental samples, but the

increased interest in routine isolation of the organism from foods has led to its replacement by more rapid, selective enrichment procedures based on antibiotic cocktails as selective agents and incubation at near-optimal growth temperatures.

Selective agars have likewise relied on a combination of selective agents such as lithium chloride, phenylethanol and glycine anhydride and antibiotics. Identification of presumptive *Listeria* colonies was based on microscopic examination of plates illuminated from below at an incident angle of 45° (Henry illumination), when they appear blue–grey to blue–green. Some media avoid the use of this technique by incorporating aesculin and ferric ammonium citrate so that *Listeria* colonies appear dark brown or black as a result of their ability to hydrolyse aesculin.

Confirmation of *L. monocytogenes* requires further biochemical testing including sugar-fermentation tests to distinguish it from other *Listeria* species and, in particular, the CAMP test to differentiate *L. monocytogenes* from *L. innocua*. Specific miniaturized test kits have been produced to simplify this procedure including one which replaces the CAMP test, which is not always easy for the inexperienced to interpret, with one for acrylamidase activity (*L. monocytogenes*, negative; *L. innocua*, positive). Enzyme-linked immunosorbent assay (ELISA) and gene probe kits are also available.

L. monocytogenes may be serotyped according to a scheme based on somatic and flagellar antigens. This is of limited epidemiological value since the majority of human cases of listeriosis are caused by just three of the thirteen serotypes identified (1/2a, 1/2b, and 4b). Phage typing and molecular typing techniques can however be used to assist in epidemiological investigations.

7.9.5 Association with Foods

Its widespread distribution in the environment and its ability to grow on most non-acid foods offer *L. monocytogenes* plenty of opportunity to enter the food chain and multiply.

The transmission of listeriosis by food was first convincingly demonstrated in an outbreak that occurred in the Maritime Provinces of Canada in 1981. The outbreak involved 41 cases in all. Of the 34 perinatal cases, there were 9 stillbirths, 23 neonatal cases with a mortality rate of 27%, and 2 live births of healthy infants. The mortality rate in adult cases was 28.6%. Coleslaw was implicated as the result of a case control study and *L. monocytogenes* serotype 4b (the outbreak strain) was isolated from a sample of coleslaw in a patient's refrigerator. It was not possible to isolate the organism at the manufacturer's plant but it transpired that a farmer who supplied cabbages to the manufacturer also

kept sheep, two of whom had died of listeriosis. The cabbage had been grown in fields fertilized by fresh and composted manure from the sheep and the harvested cabbages had been stored in a large shed through the winter – factors thought to account for the introduction of the organism and its multiplication to dangerous levels.

Raw vegetables, in the form of a garnish containing celery, tomatoes and lettuce, were also implicated on epidemiological grounds in an outbreak that occurred in eight Boston hospitals in 1979.

Surveys in the UK, the United States, Australia and elsewhere have reported a high frequency of isolation of *L. moncytogenes* from meats and meat products, where serotype 1 generally predominates. A number of sporadic cases of listeriosis have been associated with products such as pork sausage, turkey frankfurters, cook-chill chicken, and chicken nuggets.

L. monocytogenes is relatively resistant to curing ingredients and has been found in a range of delicatessen meats such as salami, ham, corned beef, brawn and paté. In an Australian survey 13.2% of samples were found to be positive, largely as a result of cross-contamination in the shop. In Britain in 1989/90, high levels on vacuum-packed ham and on paté, from which serotype 4b was isolated, prompted the recall of both products from the market. Pork tongues in aspic were identified as the original source of a large outbreak in France caused by serotype 4b. Between March and December 1992, 279 cases were reported with 63 deaths and 22 abortions.

Dairy products such as raw and pasteurized milk and soft cheeses have been associated with a number of major outbreaks of listeriosis. The overall incidence of *L. monocytogenes* in raw milk derived from surveys in Australasia, Europe and the United States averages at around 2.2%, although one Spanish study reported an incidence in excess of 45%. Pasteurized milk was responsible for an outbreak in Massachusetts in 1983 involving 42 adult and 7 perinatal cases with an overall mortality rate of 29%. The milk had come from farms where bovine listeriosis is known to have occurred at the time of the outbreak. It was the absence of evidence of improper pasteurization at the dairy that gave rise to the concern that *L. monocytogenes* might display marked heat resistance in some instances (see Section 7.9.2 above).

Soft cheeses are also frequently contaminated with *L. monocytogenes*. In 1985 there was an outbreak in California in which a Mexican-style soft cheese which had been contaminated with raw milk was the vehicle. One hundred and forty-two cases were recorded comprising 93 perinatal and 49 adult cases with an overall mortality rate of 34%. This outbreak served to focus attention on soft cheeses and there have since been other incidents identified in which they have been implicated, including a major outbreak covering the period 1983–87 with 122 cases and 31 deaths associated with the Swiss cheese Vacherin Mont d'Or.

This association with soft cheeses appears to be due to the cheese ripening process. *L. monocytogenes* survives poorly in unripened soft cheeses such as cottage cheese but well in products such a Camembert and Brie. During the ripening process, microbial utilization of lactate and release of amines increase the surface pH allowing *Listeria* to multiply to dangerous levels. There have also been two European outbreaks of listeriosis in 1998 and 2003 and a major product recall in the United States in 2004 associated with butter, hitherto considered a relatively low risk food.

7.10 MYCOBACTERIUM SPECIES

7.10.1 Introduction

The genus *Mycobacterium* consists largely of harmless environmental organisms but is best known as the cause of two of the most feared and ancient of human diseases, tuberculosis (TB) and leprosy. TB, described by John Bunyan as 'Captain of these men of death', can sometimes be foodborne and is therefore of more concern to us here.

There is archaeological evidence to suggest that TB was endemic in much of the world from ancient times but with the rise in urbanisation between the 18th and 20th centuries it became epidemic in many areas, killing millions. Death rates in Europe and the United States peaked in the 19th century when it has been estimated that 30% of all deaths under the age of 50 in Europe were due to TB. By the late 20th century, a combination of improved social conditions, childhood immunization, screening and effective chemotherapy had reduced the incidence of TB in the developed world to the point where public health officials talked confidently of eliminating the disease altogether. This optimism proved unfounded as we have seen increasing numbers of cases since the late 1980s in groups such as AIDS patients and the socially disadvantaged, as well as the emergence of drug resistant strains. In the world's poorer countries, tuberculosis has always remained an important cause of morbidity and mortality. In 1990 the WHO and International Union against Tuberculosis and Lung Disease estimated that one-third of the world's population was infected with the tubercle bacillus and there were 7–8 million new cases each year.

Human illness is primarily associated with *Mycobacterium tuberculosis* which is thought to account for 98% of cases of pulmonary TB and 70% of non-pulmonary forms. It is spread person to person by aerial transmission of droplets produced by an infected person coughing, sneezing or spitting. *Mycobacterium bovis* is very closely related to *Myco. tuberculosis* but causes tuberculosis in cattle and other animals as well as in humans. It too is spread by respiratory aerosols between animals, and from animals

to humans, but can also be transmitted to humans by milk and, to a lesser extent, by meat from tuberculous animals.

Mycobacterium paratuberculosis causes paratuberculosis, otherwise known as Johne's disease, in cattle and it has been suggested that it may be implicated in the etiology of Crohn's disease in humans. This remains to be established, but if so, consumption of infected milk may be a possible route of transmission.

7.10.2 The Organism and its Characteristics

Mycobacterium species are generally non-fastidious, Gram-positive, non-sporeforming, pleomorphic aerobes 1–4 μm in length. *Myco. bovis* is mesophilic and is not heat-resistant, being readily killed by normal milk pasteurization conditions.

A special feature of mycobacteria is the chemical composition of their cell walls. These have a high lipid content made up of esterified mycolic acids, complex branched-chain, hydroxy lipids with the general formula $R^1CHOH.CHR^2.COOH$ (where R^1 and R^2 are very long aliphatic chains), and, as a result, the wall is very hydrophobic and waxy. This confers a number of important properties on the organisms. For example, uptake of nutrients from aqueous solution is impeded making them very slow growing so that it often takes more than a week for growth to be apparent on solid media. They are also very resistant to drying and therefore can persist and remain infectious in the environment for long periods. The cell wall is more resistant to degradation by lysosomal enzymes in phagocytes enabling the pathogenic mycobacteria to survive and grow in macrophages. Its hydrophobic nature also makes the cells rather difficult to stain. However, once stained they are very resistant to decolourization and have the characteristic diagnostic property of 'acid fastness'. This was first noted by Ehrlich in 1882 and is detected using the Ziehl–Neelsen staining procedure in which cells are stained with hot carbol fuchsin, and mycobacteria, if present, will resist subsequent decolourization with acid alcohol.

7.10.3 Pathogenesis and Clinical Features

Most forms of tuberculosis are chronic taking months or even years before recovery or death. The commonest clinical signs include fever, chills and weight loss, but other symptoms present depending on the organs involved. The tissue damage produced is not a direct result of microbial activity as the infecting organism does not produce toxins, but is a consequence of the body's immune response to the organism.

In foodborne tuberculosis, *M. bovis* enters the body through the intestinal tract and the primary infection usually occurs at the mesenteric

lymph nodes. The bacteria are engulfed by macrophages and are then isolated in nodules called tubercles or granulomas which are mainly composed of a dense accumulation of activated macrophages and lymphocytes. For many people this is as far as the infection proceeds, the development of the tubercle is checked by surrounding it with a fibrous wall and it then calcifies to a yellow gritty mass. In others, however, illness ensues when the tubercle liquefies causing local tissue necrosis and releasing the bacteria to spread infection around the body.

7.10.4 Isolation and Identification

Tuberculous lesions can be identified in meat animals by post-mortem inspection of carcasses but disease can also be identified in the live animal (and humans) using the tuberculin test. In this, the animal exhibits delayed hypersensitivity to injection of tuberculin, a protein preparation from *Myco. bovis*. In clinical specimens mycobacteria can be identified directly on the basis of their acid-fast reaction in the Ziehl–Neelson stain when the organisms appear red and the surrounding tissue blue. The organisms can be cultured on simple media but are very slow growing.

7.10.5 Association with Foods

In 1900 at the London Congress on Tuberculosis, Robert Koch caused consternation when he concluded that the risk of transmission of bovine tuberculosis to humans was so slight that he did not deem it advisable to take any measures against it. The impact of this from the world's leading bacteriologist and discoverer of the tubercle bacillus can only be imagined – especially on John McFadyean who was due to talk on the same subject two days later! Nonetheless, when his turn came he felt compelled to 'offer some criticism on the pronouncement of one, the latchet of whose shoes I am not worthy to unloose'. He pointed out that in post-mortem examinations of hundreds of children in London and Edinburgh, primary infection appeared to have occurred through the intestines in approximately 28% of cases, and that 2% of all cows in Britain had tuberculosis of the udder and were excreting the bacillus in their milk.

Pooling of milk increased the incidence of *Myco. bovis* so that in the 1920s and 1930s the organism could be isolated from 5–12% of milk samples and the high rates of TB in children due to *Myco. bovis* were attributed to the consumption of unpasteurized milk. In North America, compulsory pasteurization regulations were introduced in a number of the large cities from about 1910 and had a marked effect reducing the incidence of bovine TB in children. In the UK there was considerable resistance to the introduction of milk pasteurization, but the available evidence suggests that its later and more gradual introduction had a

similar effect. By 1944, all London's milk was pasteurized and the death rate from abdominal tuberculosis in children was 4% of what it had been in 1921 when there was no pasteurization. In contrast, the death rate in 1944 in rural areas, where pasteurization was less extensively practised, was 10 times the London rate.

Though there has been considerable recent concern about the world-wide resurgence of TB, the contribution of foodborne transmission is probably insignificant in the developed world where it is effectively controlled by the testing and elimination of infected cattle, rigorous meat inspection and milk pasteurization. This may not be true in many developing countries such as those of Africa where the extent of human tuberculosis caused by *Myc. bovis* is not known and there is wide-spread consumption of unpasteurized milk and, in some areas, raw meat products.

7.11 *PLESIOMONAS SHIGELLOIDES*

7.11.1 Introduction

Plesiomonas shigelloides is the only species of the genus whose name is derived from the Greek word for neighbour; an allusion to its similarity to *Aeromonas*. Its position as a causative agent of foodborne illness also bears some similarity to *Aeromonas*. It is not normally recovered from human faeces, except in Thailand where a carriage rate of 5.5% has been reported. The association with diarrhoea is largely based on its isolation from patients suffering from diarrhoea in the absence of any other known pathogens and the strongest of this evidence has come with isolation from several patients in the same outbreak. However volunteer feeding trials have failed to demonstrate a causal link.

7.11.2 The Organism and its Characteristics

A member of the family Enterobacteriaceae (previously classified in the Vibrionceae), *P. shigelloides* is a short, catalase-positive, oxidase-positive, Gram-negative rod. It is motile by polar, generally lophotrichous flagella in contrast to *Aeromonas* and *Vibrio* which are monotrichous. It grows over a temperature range from 8–10 °C to 40–45 °C with an optimum at around 37 °C. It is not markedly heat resistant and is readily eliminated by pasteurization treatments. Growth is possible down to pH 4.5 and the maximum salt concentration it will tolerate is between 3 and 5% depending on other conditions.

The organism is ubiquitous in surface waters and soil, more commonly in samples from warmer climates. Carriage in cold-blooded animals such as frogs, snakes, turtles, and fish is common and it has

been isolated from cattle, sheep, pigs, poultry, cats and dogs. It is not normally part of the human gut flora.

7.11.3 Pathogenesis and Clinical Features

Cases of *P. shigelloides* infection are more common in warmer climates and in travellers returning from warmer climates. The usual symptoms are a mild watery diarrhoea free from blood or mucus. Symptoms appear within 48 h and persist for several days. More severe colitis or a cholera-like syndrome have been noted with individuals who are immunosuppressed or have gastrointestinal tumours.

Little is known of the pathogenesis of *P. shigelloides* infections. Motility appears to be an important factor and evidence has been presented for an enterotoxin causing fluid secretion in rabbits' ligated ileal loops.

7.11.4 Isolation and Identification

The relatively recent growth of interest in *P. shigelloides* is reflected in the use of 'second-hand' media in its isolation. Alkaline peptone water and tetrathionate broth have both been used for enrichment culture of *P. shigelloides* at 35–40 °C and salmonella–shigella and MacConkey agars have been used as selective plating media. Selective plating media have been developed such as inositol/brilliant green/bile salts, *Plesiomonas* agar. Isolates can be readily confirmed on the basis of biochemical tests.

7.11.5 Association with Foods

Fish and shellfish are a natural reservoir of the organism and, with the exception of one incident where chicken was implicated, they are the foods invariably associated with *Plesiomonas* infections. Examples have included crab, shrimp, cuttle fish and oysters.

7.12 *SALMONELLA*

7.12.1 Introduction

Most salmonellas are regarded as human pathogens, though they differ in the characteristics and the severity of the illness they cause. Typhoid fever is the most severe and consequently was the earliest salmonella infection to be reliably described. This is credited to Bretonneau, the French physician who is also regarded as the founder of the doctrine of the aetiological specificity of disease. During his life, he published only one paper on typhoid, or 'dothinenterie' as he called it, in 1829, and his treatise on the subject was only published in 1922 by one of his descendants.

In 1856, the English physician William Budd concluded that each case of typhoid is epidemiologically linked to an earlier case and that a specific toxin is disseminated with the patients faeces. To support his proposition he demonstrated that treating the excreta of victims with chlorinated lime (bleaching powder) reduced the incidence of typhoid. The typhoid bacillus was first observed by the German bacteriologists Eberth and Koch in 1880 and four years later Gaffky succeeded in its cultivation. The paratyphoid bacilli, responsible for the clinically similar condition, paratyphoid fever, were first isolated by Achard and Bensaud (1896) and by Gwyn (1898), and confirmed as culturally and serologically distinct from the typhoid bacillus by Schottmüller in 1901. Other salmonellas were isolated during the same period; Salmon and Smith (1885) isolated *Bacillus cholerae-suis* from pigs with hog cholera, a disease now known to be viral in origin, and similar bacteria were isolated from cases of foodborne infection and animal disease. The genus *Salmonella* was finally created in 1900 by Lignières and named in honour of D.E. Salmon, the American veterinary pathologist who first described *Salmonella cholerae-suis*.

Salmonellas are now established as one of the most important causes of foodborne illness worldwide. In Europe in 1989 the annual incidence of salmonellosis was around 50 per 100 000 inhabitants in most countries, though actual figures varied from below 10 in the case of Luxembourg to more than 120 in Hungary and Finland. Data collected by the European surveillance system Enter-net reported an increase in the annual total for salmonellosis in 12 European countries from 41 870 in 1995 to 55 278 in 1997. Since the contribution of England and Wales to these figures was 29 314 in 1995 and 32 596 in 1997 it suggests that the effectiveness of data collection and/or food hygiene are far from uniform across these countries. In the United States in 1998–2001 the incidence was 15.1 per 100 000.

On the basis of DNA/DNA hybridization, the genus *Salmonella* was recognized to contain a single species, *S. enterica* (formerly known as *S. cholerae-suis*), which comprises seven subspecies. One of these subspecies, which is relatively unimportant as a cause of human infection and accounts for less than 1% of *Salmonella* serovars, has been proposed for elevation to species status as *S. bongori*.

The Kauffman–White serotyping scheme has proved the most useful technique for differentiating within the genus. This describes organisms on the basis of their somatic (O) and flagellar (H) antigens, and by capsullar (Vi) antigens (possessed by *S. typhi*, *S. dublin* and occasional strains of *S. paratyphi* C). In 1941 the scheme contained 100 serovars and the number has since risen to the current level of more than 2400.

The taxonomic nomenclature of the genus is rather different from that of other genera. Many of the different serovars were named as if they

were distinct species. The earliest to be described were given species epithets derived from the disease they caused, either in humans (*S. typhi*, *S. paratyphi* A and B), or in animals (*S. typhimurium, S. cholerae-suis*, or *S. abortusovis*). Limitations in this approach led to the use of serovar names based on the geographical location of the first isolation, for example *S. dublin, S. montevideo*, *S. minneapolis*, and even *S. guildford*. This has some advantage over the use of long serological formulae but since 1966 has only been applied to serovars of subspecies I (*S. enterica* subsp. *enterica*) which accounts for more than 59% of the 2400 serovars known and the vast majority (>99%) of human isolates.

To introduce some taxonomic rectitude the non-italicized serovar name is used after the species name so that *S. typhimurium* becomes *S. enterica* subsp. *enterica* ser. Typhimurium or, more concisely, *Salmonella* Typhimurium. By retaining the old serovar name much of the potential for confusion inherent in other schemes is reduced. In the case of other subspecies which comprise mainly isolates from the environment and cold-blooded animals, the serovar formula is used after the name of the subspecies, e.g. *Salmonella fremantle* would be *S. enterica* subsp. *salamae* ser.42;g,t:-.

7.12.2 The Organism and its Characteristics

Salmonellas are members of the Enterobacteriaceae. They are Gram-negative, non-sporeforming rods (typically 0.5 μm by 1–3 μm) which are facultatively anaerobic, catalase-positive, oxidase-negative, and are generally motile with peritrichous flagella.

Growth has been recorded from temperatures just above 5 °C up to 47 °C with an optimum at 37 °C. Salmonellas are heat sensitive and are readily destroyed by pasteurization temperatures. *S.* Senftenberg 775 W is the most heat resistant serotype at high a_w and has a D_{72} in milk of 0.09 min (*S.* Typhimurium $D_{72} = 0.003$ min). Heat resistance has been shown to be enhanced by sub-lethal heat shocking at 48 °C for 30 min and can also be markedly increased in low a_w media, for example *S.* Typhimurium has a D_{70} of 11.3–17.5 h in chocolate sauce. In frozen foods, numbers of viable salmonella decline slowly, the rate decreasing as the storage temperature decreases.

The minimum a_w for growth is around 0.93 but cells survive well in dried foods, the survival rate increasing as the a_w is reduced. The minimum pH for growth varies with the acidulant from 5.4 with acetic acid to 4.05 with hydrochloric and citric acids. Optimal growth occurs around pH 7.

It was noted in Section 7.12.1 above that the most important technique for sub-dividing the genus is the serotyping scheme of Kauffman and White. This does not provide a complete account of the antigenic

structure of each salmonella, but does provide a workable scheme using antigens of diagnostic value. In the case of the more common serotypes such as *S*. Typhimurium and *S*. Enteritidis a more discriminating scheme of classification is required for epidemiological purposes and this is provided by phage typing.

This was first applied to *S*. Typhi where most strains could be classified into one of 11 phage types using a set of phages that acted only on bacteria possessing the Vi antigen. A high degree of correlation has been observed between phage type and epidemic source. Similar successful phage typing-schemes have been developed for, among others, *S*. Typhimurium, which employs 36 phages to distinguish at least 232 definitive types currently recognized, *S*. Enteritidis and *S*. Virchow.

Biotyping according to biochemical characteristics has sometimes proved useful in epidemiological investigations where it can supplement phage typing or subdivide a large group of otherwise untypable strains. This has proved most useful for *S*. Typhimurium where Duguid's scheme based on 15 biochemical tests has identified 184 full biotypes.

Plasmid profiling based on the isolation and separation of plasmids by electrophoresis on agarose gels has also met with some success as an epidemiological tool. One notable example of its use was in the early 1980s when it was used to identify a strain of *S*. Muenchen responsible for an outbreak in the United States where the food vehicle was marijuana. The plasmid profile was sufficiently distinctive and stable to allow the outbreak strain to be distinguished from strains of other serotypes and non-outbreak strains of *S*. Muenchen. A number of other molecular typing techniques described in Chapter 10 have been used with *Salmonella* including pulsed field gel electrophoresis (PFGE).

Salmonellas are primarily inhabitants of the gastrointestinal tract. They are carried by a wide range of food animals, wild animals, rodents, pets, birds, reptiles, and insects, usually without the display of any apparent illness. They can be disseminated *via* faeces to soil, water, foods and feeds and thence to other animals (including humans).

Most salmonellas infect a range of animal species but some serotypes are host adapted such as *S*. Enteritidis PT4, *S*. Pullorum and *S*. Gallinarum in poultry and *S*. Cholerae-suis in pigs. In these cases direct animal-to-animal transmission can be more important and vertical transmission may occur – parents infecting offspring. For example, *S*. Enteritidis PT4 can pass from breeding flocks to newly hatched broiler and egg-laying chicks *via* transovarian infection of the egg or its shell.

7.12.3 Pathogenesis and Clinical Features

Salmonellas are responsible for a number of different clinical syndromes grouped here as enteritis and systemic disease.

7.12.3.1 Enteritis. Gastrointestinal infections are predominantly associated with those serotypes which occur widely in animals and humans. They can range in severity from asymptomatic carriage to severe diarrhoea and are the most common type of salmonellosis.

At any one time human illness is usually associated with a limited number of serotypes; in the UK only about 200 serotypes may be reported in any one year. Currently *S.* Enteritidis, and *S.* Typhimurium, are the most common, accounting for about three-quarters of laboratory reports. Other relatively more common serotypes are *S.* Virchow, *S.* Infantis and *S.* Newport.

The incubation period for salmonella enteritis is typically between 6 and 48 h. The principal symptoms of mild fever, nausea and vomiting, abdominal pain and diarrhoea last for a few days but, in some cases, can persist for a week or more. The illness is usually self-limiting but can be more severe in particularly susceptible groups such as the very young, the very old and those already ill. One example of this is the outbreak which occurred in the Stanley Royd Hospital in the UK in 1984 where about 350 patients and 50 staff were affected and 19 of the patients died.

Ingested organisms, which survive passage through the stomach acid, adhere to the epithelial cells of the ileum *via* mannose-resistant fimbriae. They are then engulfed by the cells in a process known as receptor mediated endocytosis. The ability of salmonellas to enter non-phago-cytic cells is a property essential to their pathogenicity. Our understanding of the molecular basis of this process has increased considerably with the discovery that it is largely encoded on a 35–40 kb region of the chromosome, described as a pathogenicity island. This region of the DNA encodes a complex secretion system for the proteins required in the signalling events which subvert the host cell and ultimately lead to bacterial uptake. Known as a type III or, in some cases, a contact dependent secretion system, such systems are also present in a number of other enteropathogens such as *Shigella*, *Yersinia*, enteropathogenic and enterohaemorraghic *E. coli*. Phylogenetic analysis and their base composition suggest that these regions of DNA may have been acquired from another micro-organism as a block; an event which clearly marks an important evolutionary step towards pathogenicity. Endocytosed salmonellas pass through the epithelial cells within a membrane-bound vacuole, where they multiply and are then released into the lamina propria *via* the basal cell membrane. This prompts an influx of inflammatory cells leading to the release of prostaglandins which activate adenylate cyclase producing fluid secretion into the intestinal lumen. The picture is a little more complex than this since there are at least four other pathogenicity islands also contributing to the overall pathogenicity of the organism.

As a general rule, the infectious dose of salmonella is high, of the order of 10^6 cells, but this will vary with a number of factors such as the

virulence of the serotype, the susceptibility of the individual and the food vehicle involved. A number of outbreaks have occurred where epidemiological evidence points to an infective dose as low as 10–100 cells. This appears to be particularly associated with more susceptible individuals such as children and the elderly, and with fatty foods such as cheese, salami and chocolate. In an outbreak in Canada where the vehicle was cheddar cheese it was found to contain 1.5–9.1 cells per 100 g. It seems likely that the high fat content in some foods affords the bacteria some protection from stomach acidity. A low infective dose (<200) was also indicated in a waterborne outbreak in the early 1970s. In this case fat was clearly not a factor, but the more rapid transit of water through the stomach may have served a similar purpose.

After symptoms have subsided, carriage of the organism and its passage in high numbers in the stools may occur for a few weeks, or occasionally months.

7.12.3.2 Systemic Disease. Host-adapted serotypes are more invasive and tend to cause systemic disease in their hosts; a feature which is linked to their resistance to phagocytic killing. In humans, this applies to the typhoid and paratyphoid bacilli, *S.* Typhi, and *S.* Paratyphi A, B, and C, which cause the septicaemic diseases, enteric fever.

Typhoid fever has an incubation period of anything from 3 to 56 days, though it is usually between 10 and 20 days. Invasive salmonellas penetrate the intestinal epithelium and are then carried by the lymphatics to the mesenteric lymph nodes. After multiplication in the macrophages, they are released to drain into the blood stream and are then disseminated around the body. They are removed from the blood by macrophages but continue to multiply within them. This eventually kills the macrophages which then release large numbers of bacteria into the blood stream causing a septicaemia. In this, the first phase of the illness, the organism may be cultured from the blood. There is a slow onset of symptoms including fever, headache, abdominal tenderness and constipation and the appearance on the body of rose red spots which fade on pressure.

During the second stage of the illness, the organism reaches the gall bladder where it multiplies in the bile. The flow of infected bile reinfects the small intestine causing inflammation and ulceration. The fever persists but with the onset of a diarrhoea in which large numbers of the bacteria are excreted with the characteristic 'pea soup' stools and, to a lesser extent, with the urine. In more serious cases, haemorrhage of the ulcers may occur and perforation of the intestine leading to peritonitis. In milder cases, the ulcers heal and fever falls with recovery after 4–5 weeks.

Unlike the more localized enteric infections, typhoid is usefully treated with antibiotics such as chloramphenicol, ampicillin and amoxycillin.

After remission of symptoms, a carrier state can persist for several months and occasionally years as parts of the gall bladder are colonized and bacteria are discharged intermittently with the bile into faeces. This occurs more commonly in women and the elderly and there have been a number of typhoid carriers who have achieved some notoriety as a result of their condition and its consequences. These include the 'Strasbourg Master Baker's Wife', the 'Folkestone Milker', and, probably best known of all, 'Typhoid Mary'. Mary Mallon worked as a cook in a number of households and institutions in the New York area at the beginning of the 20th century. She first attracted the attention of the authorities when she disappeared after an outbreak of typhoid fever in a family for whom she had been working. When she was eventually tracked down by following a trail of outbreaks in places she worked, she was forcibly detained by the New York City Health Department for three years. Despite an undertaking not to work as a cook or handle food on her release, she disappeared again, assumed a false name, and started work as a cook. In 1915 she was working at a New York hospital when a typhoid outbreak occurred in which 25 people were affected and two died. She failed to return from leave, but was later found and held at a hospital on North Brother Island until her death, from a stroke, in 1938, aged 70.

Nowadays chronic carriers can be treated with antibiotics, but in particularly recalcitrant cases cholecystectomy (surgical removal of the gall bladder) is necessary.

A number of non-human adapted serotypes such as *S*. Blegdam, *S*. Bredeny, *S*. Cholerae-suis, *S*. Dublin, *S*. Enteritidis, *S*. Panama, *S*. Typhimurium, and *S*. Virchow can also be invasive in susceptible individuals. They can cause less severe forms of enteric fever and septicaemia, and focal infections at a wide variety of sites around the body such as the heart, appendix, gall bladder, peritoneum, lungs, urinary tract, brain, meninges and spleen. Localization is more likely to occur at sites where there is pre-existing disease or damage and some sites of infection are associated with particular population groups such as meningitis in infants, pneumonia in the elderly, and osteomyelitis in patients with sickle-cell anaemia.

7.12.4 Isolation and Identification

Methods for the isolation and identification of salmonellas in foods have arguably received more attention than those for any other foodborne pathogen. Using traditional cultural techniques, a five-stage procedure has emerged as the widely accepted norm. This is outlined in Figure 7.7.

Pre-enrichment in a non-selective medium increases the recovery rate of salmonellas by allowing the repair of cells which have been sublethally

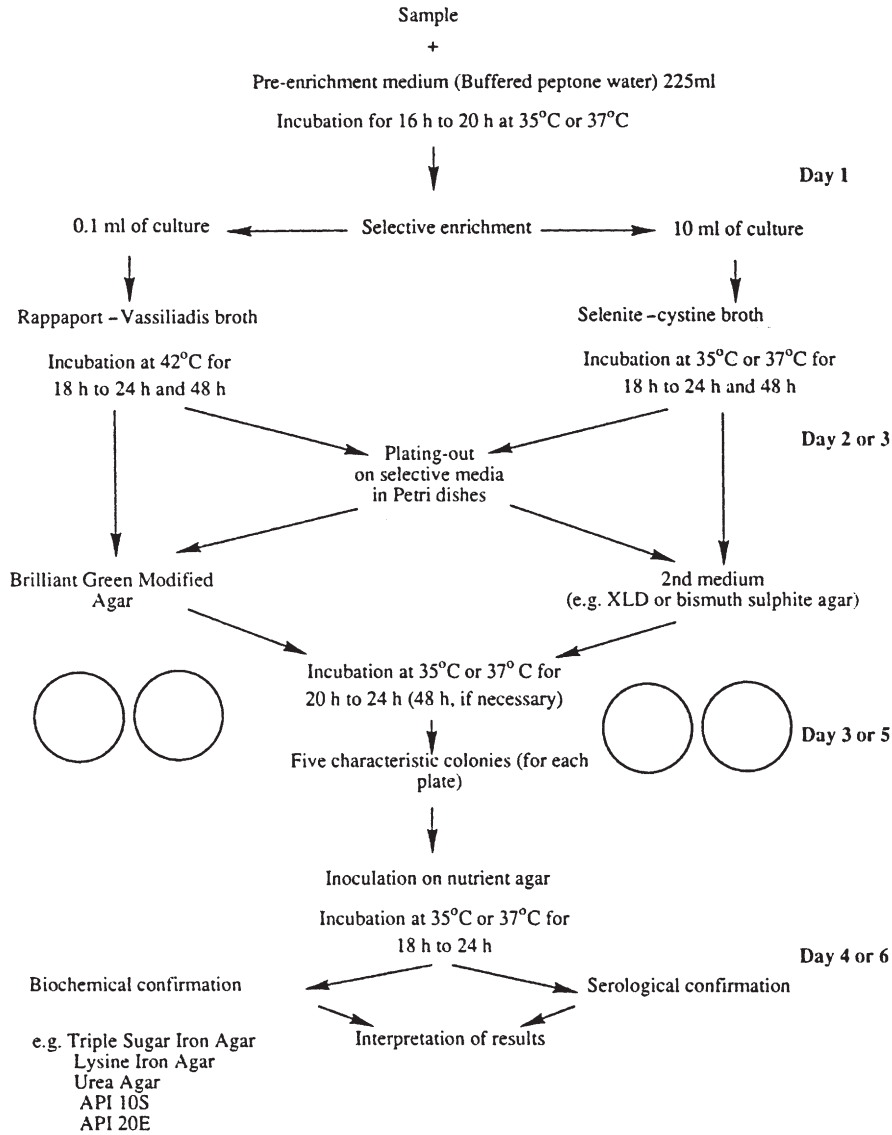

Figure 7.7 *Traditional cultural protocol for isolation of* Salmonella *from food*

damaged. Such damage can result from any exposure to adverse conditions that might occur during food processing, such as chilling, freezing, or drying, and increases the cell's sensitivity to selective agents used in media in subsequent stages of the isolation procedure. Failure to include a resuscitation step could therefore result in the non-detection of cells that might recover and cause infection if the food is mishandled.

The selective enrichment stage is intended to increase the proportion of salmonella cells in the total microflora by allowing them to proliferate

while restricting growth of other micro-organisms present. To this end a number of different media have been proposed employing selective agents such as bile, brilliant green, malachite green, tetrathionate and selenite. The most widely used are selenite–cystine broth, which contains cystine to stimulate growth of salmonellas; Muller–Kauffman tetrathionate broth, containing tetrathionate, brilliant green, and bile; and Rappaport–Vassiliadis (RV) broth, which contains malachite green, magnesium chloride and a slightly reduced pH as selective factors. Since they differ in their selectivity, two broths are usually used in parallel; commonly a combination of the less selective selenite–cystine broth and one of the others.

From the selective enrichment broths, cultures are streaked on to selective and differential solid media. Once again it is usual to use two different media in parallel. The selective agents used are bile salts or deoxycholate and/or brilliant green and the diagnostic reaction is usually provided by the inability of most salmonellas to ferment lactose and/or the production of hydrogen sulfide. In choosing the media to use, it is advisable to select two based on different diagnostic reactions to ensure that atypical strains, for instance lactose-positive ones, will not be missed.

Presumptive salmonellas from selective plating media must be confirmed by biochemical testing and serologically by agglutination with polyvalent O antisera.

The whole protocol is rather complex and lengthy, requiring at least four days for a negative result. In view of this, a number of procedures have been described which attempt to simplify the procedure and reduce the elapsed time involved. Two of these employ the motility of salmonellas which means that they would fail to detect non-motile salmonellas (incidence $<0.1\%$).

In one, a conventional pre-enrichment culture is inoculated into an elective medium, salmonellas swim into a compartment containing a selective medium and from there into one containing a diagnostic medium. A diagnostic medium giving the appropriate colour change is then tested for ability to agglutinate latex particles coated with salmonella antibodies. A positive result indicates a presumptive salmonella, which must then be confirmed by conventional serological and biochemical testing using a sub-culture from the diagnostic medium. With this technique, presumptive identification of a salmonella is obtained within 42 h compared with 3–4 days by the traditional cultural method.

In another system, salmonella detection is by formation of an immunoprecipitate as *Salmonella* antibodies diffusing down through a medium meet salmonellas swimming up from a chamber containing a selective medium.

Impedance–conductance techniques (see Chapter 10) have been successfully applied to the detection of salmonellas. The original medium of

Easter and Gibson comprises a modified selenite–cystine broth containing dulcitol and trimethylamine oxide (TMAO). Salmonellas are able to ferment dulcitol and reduce TMAO to the base trimethylamine. This increases the conductivity of the medium and provides the basis for detection. The detection time is reduced if the samples are pre-enriched in a medium containing dulcitol and TMAO to induce the relevant enzymes. In a comparison using 2586 samples of milk powder, this method was found to be as effective as a traditional cultural method but with considerable savings of time and labour. With a 24 h pre-enrichment step, *Salmonella*-negative samples can be detected within 48 h.

A number of modifications to the original medium and protocol have been described. These include the incorporation of a *Salmonella*-specific bacteriophage in a parallel sample to demonstrate that observed changes in electrical properties are in response to salmonella; the replacement of dulcitol with mannitol or deoxyribose in order to detect dulcitol-negative salmonellas; and the use of detection media based on lysine decarboxylase activity.

ELISA and gene probe kits for the detection of salmonellas are also available, but like all the techniques described, they require a certain threshold concentration of salmonellas. One approach to avoid or curtail the enrichment steps that this usually entails is immunomagnetic separation. *Salmonella* antibodies are attached to magnetic particles which are added to a liquid culture containing salmonellas which are then captured by the antibodies. The beads with adhering *Salmonella* cells can then be readily separated from the culture with a magnet, achieving a substantial enrichment in minutes. Their presence can then be confirmed using conventional media or one of the more rapid techniques.

7.12.5 Association with Foods

Salmonellosis is described as a zoonotic infection since the major source of human illness is infected animals. Transmission is by the faecal–oral route whereby intestinal contents from an infected animal are ingested with food or water. A period of temperature abuse which allows the salmonellae to grow in the food and an inadequate or absent final heat treatment are common factors contributing to outbreaks.

Meat, milk, poultry, and eggs are primary vehicles; they may be undercooked, allowing the salmonellas to survive, or they may cross-contaminate other foods that are consumed without further cooking. Cross-contamination can occur through direct contact or indirectly *via* contaminated kitchen equipment and utensils.

Human carriers are generally less important than animals in the transmission of salmonellosis. Human transmission can occur if the faecally contaminated hands of an infected food handler touch a food

which is then consumed without adequate cooking, often after an intervening period in which microbial growth occurs. This was the cause of a major outbreak affecting an international airline in 1984. The outbreak involved 631 passengers and 135 crew and was due to contamination of an aspic glaze by a member of catering staff who returned to work after illness but was still excreting *Salmonella* Enteritidis PT4.

Direct person-to-person spread by the faecal–oral route is also possible but is usually restricted to institutional outbreaks such as occur in hospitals, old people's homes, and nurseries.

Food animals may acquire salmonella infection on the farm from wild birds and rodents, but the principal sources are other animals, which may be symptomless excreters, and contaminated feeding stuffs (Figure 7.8). Measures that can be taken to minimize transmission between animals on the farm include good animal husbandry, protection of feeds and water from contamination, hygienic disposal of wastes, and maintenance of a generally clean environment. Transfer of *Salmonella* between animals is particularly associated with situations where animals may be stressed and crowded such as during transport, at markets, and when in lairage at the slaughterhouse. It is best minimized by avoidance of overcrowded conditions, ensuring a clean environment, and otherwise limiting the stress to animals on such occasions.

An important factor in maintaining the cycle of *Salmonella* infection in food animals has been the practice of using animal by-products as animal feeds such as meat and bone meal. The heat process that these materials undergo in their conversion to feeds should destroy any salmonellas present. Nevertheless they are subject to post-process contamination either in the plant or on the farm by contact with unprocessed material or with bird and rodent faeces. The importance of animal feeds

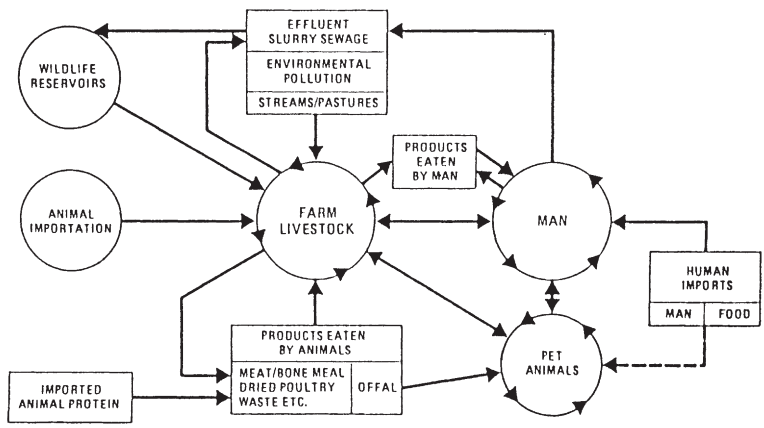

Figure 7.8 *The* Salmonella *cycle of infection*
(Reproduced with permission from WHO, 1983)

as a source of *Salmonella* should decline following the 1996 ban on feeding mammalian-derived protein to all farm animals.

In the UK, the major source of *Salmonella* infection is poultry and poultry products. Here the problem is not confined to horizontal transfer of the organism between animals but also includes vertical transmission of host-adapted serotypes from the breeding flocks to their progeny. Particularly noteworthy in this respect is *S*. Enteritidis PT4 which has been responsible for the rise in salmonellosis since 1985 (Figure 7.9). Isolations of *S*. Enteritidis increased 14-fold between 1981 and 1988, while those of *S*. Typhimurium less than doubled, and *S*. Enteritidis is now the commonest serotype recorded. Poultry was the food most commonly implicated in outbreaks of salmonellosis in 1986 and 1987 but in 1988 and 1989, eggs were the most frequent vehicle. This remained the case in 1995 and 1996. Most outbreaks were associated with raw eggs in products such as home-made mayonnaise and ice cream or, in one instance, a 'body-building' drink.

Contamination of eggs with salmonellas is a long-recognized problem but in most cases this was due to contamination of the eggshell exterior with faecal material in the hen's cloaca or after laying in the nest or battery. The shell could then contaminate the contents when the egg was broken. This is a particular problem when breaking large quantities of

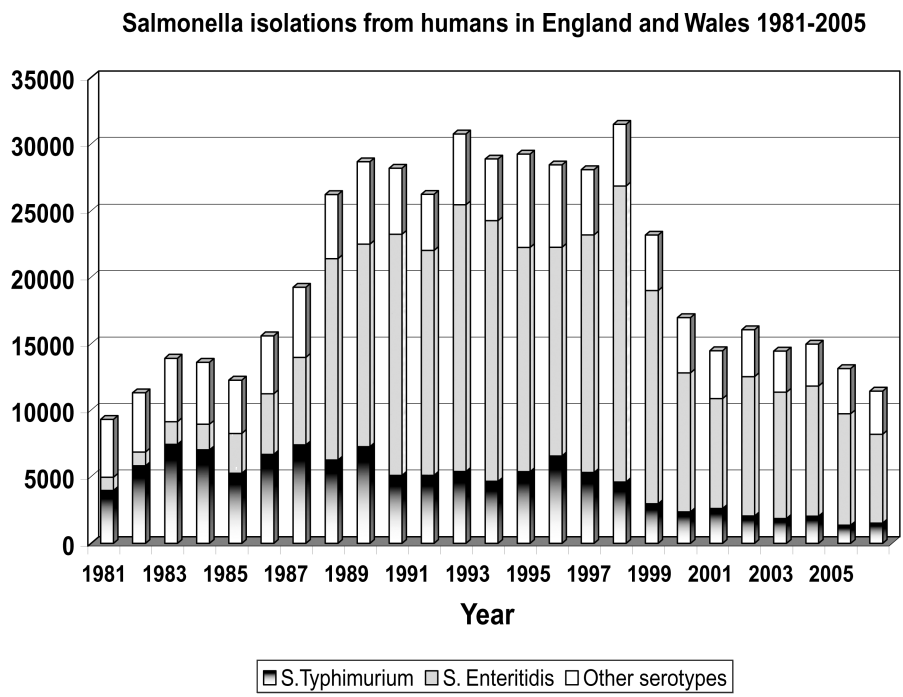

Figure 7.9 Salmonella *in humans. England and Wales 1981–2005.*

eggs where it is difficult to avoid some contamination with shell fragments. During the Second World War, there were a number of outbreaks attributed to dried whole egg powder and a survey conducted in 1961/2 found 16% of frozen whole egg samples to contain salmonellas. This led to the introduction in 1963 of regulations requiring that liquid whole egg be pasteurized at 64.4 °C for 2.5 min. The prescribed heat treatment also inactivates the yolk enzyme α-amylase and provides the basis of a simple test to ensure that the regulations have been complied with.

In the more recent cases however, contamination of the yolk of intact hen's eggs has also been indicated. In the UK and in other European countries, particularly Spain, this problem has been associated with *S.* Enteritidis PT4 but other phage types (PT8 and PT13a) have been reported to cause similar problems in the United States. It is thought that these organisms infect the bird's ovaries and oviduct and thereby contaminate the egg contents. The temperatures reached in the yolk during mild cooking procedures such as 'soft boiling' or light frying are probably insufficient to kill the organism and the fat content of the yolk may protect the organism from gastric acidity.

The precise extent of this problem is difficult to determine, but one survey found *Salmonella* in the contents of one in a thousand eggs from flocks associated with human illness. When one considers that 30 million eggs are eaten daily in the UK, then eggs are clearly an important source of human infection.

The massive increase in salmonella infections which started in 1985 prompted a number of new biosecurity measures to exclude salmonella and reduce the number of infections on egg and poultry farms. Compulsory bacteriological monitoring of all commercial egg-laying and breeding flocks was introduced and if *S.* Enteritidis was isolated the birds were required to be slaughtered. This practice has now been stopped although breeder flocks infected with either *S.* Enteritidis or *S.* Typhimurium are still subject to compulsory slaughter. General hygiene practices on farm were introduced or improved but probably the single most significant intervention was the introduction of vaccination of broiler breeder flocks (1994) and commercial laying flocks (1996) against *S.* Enteritidis. As a result of these measures, surveillance studies have shown the contamination rates in retail frozen poultry in the UK declined from 79% in 1984 to 41% in 1994 to 11% in 2001. The equivalent figures for chilled poultry were 54%, 33% and 4% respectively. The prevalence of *S.* Enteritidis and/or *S.* Typhimurium in laying hen flocks in the UK in 2004-2005 was found to be 8% compared to an EU average of 20.3%. Figures for individual member states ranged from zero in Norway, Sweden and Luxembourg to more than 50% in Spain, Poland and the Czech Republic.

The positive effect of these interventions can be seen in the substantial decline in human *Salmonella* infections since 1997. In particular

infections caused by *S*. Enteritidis PT4 decreased from 10,056 in 1998 to 2693 in 2003. Hidden by the overall figures however there was an almost doubling of infections caused by *S*. Enteritidis non PT4 from 3548 in 2000 to 7065 in 2003. This was attributed to imported raw shell eggs used largely in catering.

Salmonella Typhimurium definitive phage type (DT) 104 emerged initially in cattle in the UK but has since been reported in poultry, sheep, pigs and horses. It is now the second most prevalent salmonella in humans in England and Wales with reported isolations increasing more than 16 fold to 4006 in 1996. Ninety six percent of isolates in 1996 were multiresistant, displaying resistance to four or more antimicrobials. Twenty one percent were **R**-type ACSSuSpT which have chromosomal resistance genes to Ampicillin, Chloramphenicol, Streptomycin, **Su**lphonamide, **Sp**ectinomycin and **T**etracyclines. Since 1994 there has been an increasing number of isolates with additional resistances to trimethoprim, nalidixic acid and to quinolone antibiotics such as ciprofloxacin. Quinolones are used in the treatment of salmonellosis and typhoid fever in humans and the emergence of resistance may be linked to their veterinary use since 1993 to combat salmonellosis in cattle, pigs and poultry. Increased isolation rates of multiresistant DT104 have also been reported from other European countries, such as Germany where DT104 accounted for more than 10% of 10 000 human isolates, and the United States. The organism also appears to be unusually virulent since hospitalization and fatality rates respectively twice and ten times those of other foodborne salmonella infections have been reported.

Though the primary source appears to be foods of animal origin such as poultry and unpasteurized milk, cross-contamination has led to a bewildering variety of foods being implicated in DT104 outbreaks. These include chicken drumsticks, tuna and salmon sandwiches, ham, cod roe, scotch eggs, unpasteurized milk, roast beef, apple crumble and coleslaw.

Raw milk will inevitably contain *Salmonella* and any slight nutritional advantage it may have over pasteurized milk is far outweighed by the very real risk of salmonellosis (and campylobacteriosis). Outbreaks in a number of countries have been associated with pasteurized milk that has been inadequately processed or subject to post-process contamination. *Salmonella* is unable to grow in dried milk but is able to survive and resume growth when the milk is reconstituted. *S*. Ealing was responsible for an outbreak in the UK in 1985 where the vehicle was a dried baby-milk. The organism had contaminated the insulation surrounding a spray drier and penetrated the drying chamber itself through a small defect in the chamber wall. Rigorous cleaning and disinfection were unable to eliminate the contamination from the spray drier which was eventually decommissioned.

Fish and fish products are only occasionally associated with salmonellosis, although fish meal for animal feed often contains *Salmonella* as a

result of contamination from rodents and birds. Filter-feeding shellfish harvested from polluted waters and frozen precooked prawns have been identified as higher risk products.

Since birds, rodents, insects, infected food handlers or infected foods can all contaminate foods directly or indirectly, potential food vehicles for salmonella are numerous. Contaminated cocoa beans which had been processed into chocolate were responsible for outbreaks of *S*. Eastbourne in the United States and Canada, of *S*. Napoli and *S*. Montevideo in England, and *S*. Typhimurium in Scandinavia. Although the production of chocolate involves a heating stage, this was insufficient to kill all the salmonellas present, possibly as a result of a protective effect from the cocoa butter.

Desiccated coconut is used in a range of confectionary products and was identified as a hazard following cases of typhoid and salmonellosis in Australia. In 1959/60, a survey of desiccated coconut imports into the UK from Sri Lanka revealed that 9% of samples contained *Salmonella*. In response, the introduction and enforcement of regulations in Sri Lanka to improve production hygiene have now reduced the contamination rate dramatically.

Other plant products such as salad vegetables have been associated with occasional outbreaks of typhoid and salmonellosis. Use of polluted irrigation water or human and animal manure as fertilizer can be important contributory factors in such cases.

7.13 *SHIGELLA*

7.13.1 Introduction

The genus *Shigella* was discovered as the cause of bacillary dysentery by the Japanese microbiologist Kiyoshi Shiga in 1898. It consists of four species *Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii* and *Sh. sonnei*, all of which are regarded as human pathogens though they differ in the severity of the illness they cause. *Sh. dysenteriae* has been responsible for epidemics of severe bacillary dysentery in tropical countries but is now rarely encountered in Europe and North America where *Sh. sonnei* is more common. *Sh. sonnei* causes the mildest illness, while that caused by *Sh. boydii* and *Sh. flexneri* is of intermediate severity.

Although *Shigella* is relatively inactive biochemically when compared with *Escherichia* species, studies of DNA relatedness have demonstrated that they do in fact belong to the same genus. The separate genera are retained however, because, unlike *Escherichia*, most strains of *Shigella* are pathogenic and a redesignation might cause confusion with potentially serious consequences.