31 Enterobacteriaceae III: Salmonella

INTRODUCTION

The genus *Salmonella* consists of bacilli that parasitise the intestines of a large number of vertebrate species and infect human beings, leading to enteric fever, gastroenteritis, septicemia with or without focal suppuration, and the carrier state.

The most important member of the genus is *Salmonella* Typhi, the causative agent of typhoid fever. The typhoid bacillus was first observed by Eberth (1880) in the mesenteric nodes and spleen of fatal cases of typhoid fever and was isolated by Gaffky (1884). It came to be known as the Eberth–Gaffky bacillus or *Eberthella typhi*. Salmon and Smith (1885) described a bacillus which was believed to cause hog cholera (mistakenly, as it is a viral disease). This bacillus, later called *S.cholerae-suis,*, was the first of a series of similar organisms to be isolated from animals and human beings, the genus *Salmonella*. It was subsequently realised that the typhoid bacillus also belonged to this group, in spite of minor biochemical differences, and it was redesignated *S.*Typhi, the genus *Eberthella* having been abolished.

Salmonellae currently comprise above 2000 serotypes or species, all of them potentially pathogenic. For practical and clinical purposes, Salmonellae may be divided into two groups:

- **Typhoidal**: The enteric fever group, consisting of the typhoid and paratyphoid bacilli that are exclusively or primarily human parasites; and
- **Non-typhoidal**: The food poisoning group, which essentially comprises animal parasites but which can also infect human beings, producing gastroenteritis, septicemia or localised infections.

Morphology

Salmonellae are Gram-negative rods, about 1–3 × 0.5 μm in size. They are motile with peritrichate flag-

Salmonella

Clinical Case A 10-year-old boy was admitted to the Pediatrics ward with a history of remittent fever which increased gradually in a step-ladder pattern over the previous 10 days. He had taken antipyretics and ciprofloxacin, prescribed by a local private practitioner. At presentation to the hospital, he complained of lack of appetite, pain in the abdomen and lethargy. On examination, he was found to have fever with anemia and hepatosplenomegaly. A blood sample was obtained for culture and serology. His blood culture was positive for *Salmonella* typhi and the Widal test was negative. However, the IgM antibody test for *S.*Typhi was positive. His isolate was resistant to ciprofloxacin and so he was treated with ceftriaxone. He responded after 10 days of therapy.

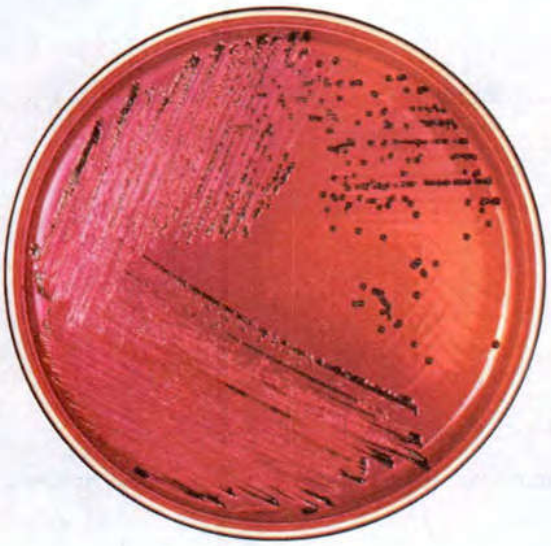

Fig. 31.1 Salmonella on XLD media

ella, except for *S.*Gallinarum and *S.*Pullorum, which are always non-motile. Non-motile mutants of other types may sometimes be found. They do not form capsules or spores but may possess fimbriae.

Cultural characteristics

Salmonellae are aerobic and facultatively anaerobic, growing readily on simple media over a pH range of 6–8 and temperature 15–41°C (optimum 37°C). Colonies are large, 2–3 mm in diameter, circular, low convex and smooth. They are more translucent than coliform colonies. On **MacConkey agar**, they grow as non-lactose fermenting colonies. On **deoxycholate citrate** media and **XLD** (xylose lysine deoxycholate), colonies show black heads due to H_2S production (Fig. 31.1). On the **Wilson** and **Blair** bismuth sulphite medium, jet black colonies with a metallic sheen are formed due to the production of H_2S. *S.*Paratyphi A and other species that do not form H_2S produce green colonies.

Selenite F and **tetrathionate** broth are commonly employed as enrichment media.

Biochemical reactions

Salmonellae ferment glucose, mannitol and maltose, forming acid and gas. An important exception is *S.*Typhi, which is anaerogenic. Lactose, sucrose and salicin are not fermented. Indole is not produced. They are MR positive, VP negative and citrate positive. *S.*Typhi and a few other salmonellae do not grow in Simmons' citrate medium as they need tryptophan as the growth factor. Urea is not hydrolysed. H_2S is produced, except by *S.*Paratyphi A, *S.*Choleraesuis and some other species.

Table 31.1 *Biochemical characteristics of typhoid and paratyphoid bacilli*

	Glucose	Xylose	d-Tartrate	Mucate
*S.*Typhi	A	d	A	d
*S.*Paratyphi A	AG	–	–	–
*S.*Paratyphi B	AG	AG	–	AG
*S.*Paratyphi C	AG	AG	AG	–

The enteric fever group may be classified biochemically (Table 31.1).

Resistance

The bacilli are killed at 55°C in one hour or at 60°C in 15 minutes. Boiling or chlorination of water and pasteurisation of milk destroys the bacilli. In polluted water and soil, they survive for weeks and in ice for months. Cultures may be viable for years if prevented from drying. They are killed within five minutes by mercuric chloride (1:500) or 5% phenol.

Classification and nomenclature

Kauffmann–White scheme: Salmonella has traditionally been named and identified using the Kauffmann–White scheme. Inclusion in the genus is based on common biochemical properties. Classification within the genus takes place by antigenic characterisation. This scheme depends on the identification, by agglutination, of the structural formulae of the O and H antigens of the strains (Table 31.2).

This is also used for the identification of isolates. According to this, salmonellae are initially classified into serological groups, based on the presence of distinctive O antigen factors, which are designated 1, 2, 3, etc. Strains possessing factor 2 belong to group A, factor 4 to group B, factor 9 to group D and so on. Within each group, differentiation of serotypes is by identification of phase 1 and 2 flagellar antigens. Earlier serogroups were designated by capital letters, A to Z, and as more were added, they were assigned numbers—currently 51–67. Within each group, differentiation of serotypes is by identification of phase 1 and 2 flagellar antigens. Sometimes, serotypes may have to be further differentiated. Thus, *S.*Gallinarum and *S.*Pullorum cannot be distinguished serologically but can be identified by biochemical reactions.

Table 31.2 *Kaufmann–White scheme: Illustrative examples**

Serogroups	Serotype	Antigen O	Antigen H	
			Phase I	Phase II
A	*S.paratyphi A*	1, **2**, 12	a	–
B	*S.paratyphi B*	1, **4**, 5, 12	b	1, 2
	S.typhimurium	1, **4**, 5, 12	i	1, 2
	S.chester	**4**, 5, 12	e, h	e, n, x
C1	*S.paratyphi C*	6, **7**, (Vi)	c	1, 5
	S.cholerae-suis	6, **7**	c	1, 5
C2	*S.muenchen*	6, **8**	d	1, 2
D	*S.typhi*	**9**, 12, (Vi)	d	–
	S.enteritidis	1, **9**, 12	g, m	–
	S.gallinarum	1, **9**, 12	–	–
E1	*S.anatum*	3, B	e, h	1, 6

*As the Kauffman–White scheme was created before modifications in nomenclature, this table has retained the earlier style. However, in the text, current nomenclature is used.

(*S*.Gallinarum is anaerogenic and ferments dulcitol, unlike *S*.Pullorum.) Important pathogens such as *S*.Typhi, *S.paratyphi A* and *B*, and *S.typhimurium* can be further typed for epidemiological purposes by phage susceptibility, biochemical properties, antibiogram and molecular typing.

The classification and nomenclature of salmonellae has undergone modification over the years. Modern taxonomical techniques have shown that all the members of the genus Salmonellae are very closely related in a genetic, phylogenetic and evolutionary sense. Variations in properties such as antigenic structure, biochemical reactions and host preferences exhibited by different strains can therefore be considered as intraspecies divergences.

DNA hybridisation studies have shown that there are two species in the genus *Salmonella:*

1. **Species enterica** – which is further divided into six subspecies
   - I – *S.enterica* subsp. *enterica*
   - II – *S.enterica* subsp. *salamae*
   - IIIa – *S.enterica* subsp. *arizonae*
   - IIIb – *S.enterica* subsp. *diarizonae*
   - IV – *S.enterica* subsp. *houtenae*
   - VI – *S.enterica* subsp. *indica*

   Most human infections are caused by subspecies *enterica* and rarely by *arizonae*.
2. **Species bongori** (earlier subspecies V)

All these species are further divided into more than 2500 serovars or serotypes. The salmonella serotype is unique in that each serotype is considered as a species. The genus name is given followed by the word 'serotype' and then the serotype name, for example, *Salmonella* typhi is written as **Salmonella enterica subspecies enterica serovar Typhi** or in short, *Salmonella* Typhi or *S*.Typhi (the serovar is not written in italics and also starts with a capital letter). The nomenclature system is based on recommendations from the WHO Collaborating Centre.

Antigenic structure

Salmonellae possess the following antigens based on which they are classified and identified:

- Flagellar antigen H
- Somatic antigen O
- Surface antigen Vi, found in some species

Several strains carry fimbriae. Fimbrial antigens are not important in identification but may cause confusion due to their non-specific nature and widespread prevalence among enterobacteria.

H antigen: This antigen present on the flagella is a heat labile protein. It is destroyed by boiling or by treatment with alcohol but not by formaldehyde. When mixed with antisera, H suspensions agglutinate rapidly, producing large, loose, fluffy clumps. The H antigen is strongly immunogenic and induces antibody formation rapidly and in high titres following infection or immunisation. The flagellar antigen is of a dual nature, occurring in one of two phases.

O antigen: The somatic O antigen is a phospholipid–protein–polysaccharide complex which forms an integral part of the cell wall. It is identical to endotoxin. It can be extracted from the bacterial cell by treatment with trichloracetic acid, as first shown by Boivin (and therefore called the **Boivin antigen**). Treatment with

phenol splits off the protein moiety, removing the antigenicity but retaining the toxicity of the complex.

The O antigen is unaffected by boiling, alcohol or weak acids. When mixed with antisera, O antigen suspensions form compact, chalky, granular clumps. O agglutination takes place more slowly and at a higher optimum temperature (50–55°C) than H agglutination (37°C). The antibody to the O antigen is cross-reactive while that to the H antigen is a more reliable indicator.

The O antigen is not a single factor but a mosaic of two or more antigenic factors. Salmonellae are classified into a number of groups based on the presence of characteristic O antigens on the bacterial surface.

Vi antigen: Many strains of *S.*Typhi fail to agglutinate with the O antiserum when freshly isolated. This is due to the presence of a surface polysaccharide antigen enveloping the O antigen. Felix and Pitt, who first described this antigen, believed that it was related to virulence and gave it the name Vi antigen. It is analogous to the K antigens of coliforms. It is heat labile. Bacilli that are not agglutinable with the O antiserum become agglutinable after boiling or heating at 60°C for one hour. It is also destroyed by N HCl and 0.5 N NaOH. It is unaffected by alcohol or 0.2% formaldehyde.

Originally observed in *S.*Typhi, the Vi antigen with similar antigenic specificity is present in *S.*Paratyphi C and *S.*Dublin, as well as in certain strains of *Citrobacter* (the Bethesda–Ballerup group). The Vi antigen tends to be lost on serial subculture. The Vi polysaccharide acts as a virulence factor by inhibiting phagocytosis, resisting complement activation and bacterial lysis by the alternative pathway and peroxidase mediated killing. In human volunteer experiments, strains possessing the Vi antigen were found to cause clinical disease more consistently than those lacking it.

The Vi antigen is poorly immunogenic and only low titres of antibody are produced following infection. No Vi antibody is induced by the phenolised vaccine, though low titres are produced by the alcoholised vaccine. The protective efficacy of the Vi antigen is demonstrated by the success of the purified Vi vaccine for typhoid now in routine use. Detection of the Vi antibody is not helpful for diagnosis and hence the Vi antigen is not employed in the Widal test. The antibody disappears early in convalescence. Its persistence indicates the development of the carrier state. The Vi antigen affords a method of epidemiological typing of *S.*Typhi strains based on specific Vi bacteriophages.

Antigenic variations

The antigens of salmonellae undergo phenotypic and genotypic variation:

H–O variation: This variation is associated with the loss of flagella. When salmonellae are grown on agar containing phenol (1:800), flagella are inhibited. This change is phenotypic and temporary. Flagella reappear when the strain is subcultured on media without phenol. Rarely, salmonellae may lose their flagella by mutation. A stable non-motile mutant of *S.*Typhi is the 901-O strain which is widely employed for the preparation of O-agglutinable bacterial suspensions. Generally, the loss of flagella is not total and only a diminution in the number of flagella and the quantity of the H antigen occurs. Flagellated cells are found in small numbers in such cultures. To obtain a population of motile cells rich in H antigen from such cultures, selection may be carried out by using Craigie's tube (Fig. 31.2). This consists of a wide tube containing soft agar (0.2%) at the centre of which is embedded a short, narrow tube open at both ends in such a way that it projects above the agar. The strain is inoculated carefully into the inner tube. After incubation, subcultures withdrawn from the top of the agar outside the central tube will yield a population of motile cells. Instead of Craigie's tube, a U-tube of soft agar may be employed, inoculation being made into one limb and subculture taken from the other.

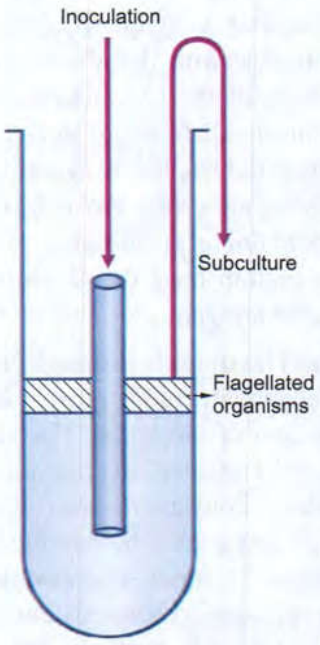

Fig. 31.2 Craigie's tube

Phase variation: The flagellar antigens of most salmonellae occur in one of two phases, that is, the flagella may exhibit one or the other of two alternative sets of antigens, defined by two separate sets of genes in the bacterial genome. Phase 1 antigens are either specific for a species or shared by a few species only. Hence it is called the **'specific'** phase. Phase 2 antigens are widely shared and hence this is called the **'non-specific'** or **'group'** phase. Phase 1 antigens are designated a, b, c, d, etc., and after z, as z1, z2, etc. Phase 2 antigens are far fewer and are termed 1, 2, etc. In some species, antigens belonging to Phase 1 may occur as Phase 2 antigens (for example, e, n, x, z15). Strains that possess both phases are called **diphasic**. Some, like *S.*Typhi, occur only in Phase 1 and are called **monophasic**.

A culture will contain cells with the flagellar antigens of both phases, but generally one or the other phase will predominate so that the culture is agglutinated only by one of the phase antisera. For serotyping of *Salmonella* isolates, it may be necessary to identify the flagellar antigens of both phases. A culture in Phase 1 can be converted to Phase 2 by passing it through a Craigie's tube containing specific Phase 1 antiserum, and the reverse conversion achieved by using Phase 2 antiserum.

V–W variation: Fresh isolates of *S.*Typhi generally carry a surface layer of Vi antigen that completely masks the O antigen. Such bacilli are agglutinable with the Vi antiserum but not with the O antiserum. This is called the **V form**. After a number of subcultures, the Vi antigen is completely lost. Such cultures are not agglutinable with the Vi antiserum but readily agglutinable with the O antiserum. This is called the **W form**. Intermediate stages during the loss of the Vi antigen, when the bacillus is agglutinable with both Vi and O antisera, are called **VW forms**.

Other Vi-containing bacilli such as *S.*Paratyphi C and *S.*Dublin seldom have the O antigen completely masked by the Vi antigen.

S–R variation: The smooth-to-rough variation is associated with a change in colony morphology and loss of the O antigen and of virulence. The colony becomes large, rough and irregular. Suspensions in saline are autoagglutinable. Conversion into R forms occurs by mutation. R forms may be common in laboratory strains maintained by serial subcultivation. S–R variation may be prevented to some extent by maintaining cultures on Dorset's egg media in the cold, or ideally by lyophilisation.

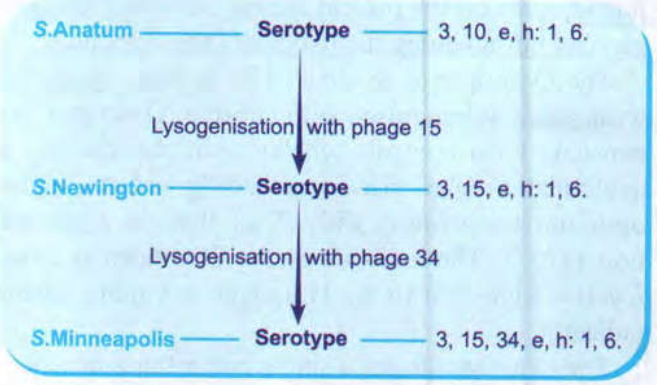

Fig. 31.3 Phage conversion of *Salmonella* serotypes

Mucoid colonies, associated with the development of a new mucoid or 'M' antigen, have been described with *S.*Paratyphi B and some other species.

Variations in the O antigen: Changes in the structural formulae of the O antigen may be induced by lysogenisation with some converting phages, resulting in the alteration of serotypes. Thus, *S.*Anatum is converted into *S.*Newington by one phage and the latter into *S.*Minneapolis by another phage (Fig. 31.3).

Pathogenicity

Salmonellae are strict parasites of animals or humans. *S.*Typhi, *S.*Paratyphi A and usually, but not invariably, *S.*Paratyphi B are confined to human beings. Other salmonellae are parasitic in various animals—domestic animals, rodents, reptiles—and birds. Some species are host adapted, *S.*Abortus-equi found only in horses, *S.*Abortus-ovis in sheep and *S.*Gallinarum in poultry. Others such as *S.*Typhimurium have a wide host range, affecting animals, birds and humans. Infection in animals may vary from an asymptomatic condition to fatal and sometimes epizootic disease. *S.*Typhimurium and *S.*Enteritidis cause fatal septicemia in rats and mice.

Salmonellae cause the following clinical syndromes in humans:

- Enteric fever
- Gastroenteritis or food poisoning
- Septicemia, with or without local suppurative lesions

ENTERIC FEVER

The term enteric fever includes typhoid fever caused by *S.*Typhi and paratyphoid fever caused by *S.*Paratyphi A, B and C.

Typhoid fever was once prevalent all over the world and was not well demarcated from other prolonged fevers. A detailed study of the disease was presented by Bretonneau (1826), who identified the intestinal lesions. The name typhoid was given by Louis (1829) to distinguish it from typhus fever. Budd (1856) pointed out that the disease was transmitted through the excreta of patients. Eberth (1880) described the typhoid bacillus and Gaffky (1884) isolated it in pure culture. Its causative role was confirmed by Metchnikoff and Besredka (1900) by infecting apes experimentally. *S.*Paratyphi A was isolated by Gwyn (1898), *S.*Paratyphi B (*S.schottmulleri*) by Achard and Bensaude (1896) and *S.*Paratyphi C (*S.hirschfeldii*) by Uhlenhuth and Hubener (1908) from cases resembling typhoid fever.

The infection is acquired by ingestion. In human volunteer experiments, the ID50 was found to be about 10^3 to 10^6 bacilli. On reaching the gut, the bacilli attach themselves to the microvilli of the ileal mucosa and penetrate to the lamina propria and submucosa. They are phagocytosed there by polymorphs and macrophages. The ability to resist intracellular killing and to multiply within these cells is a measure of their virulence. The genes responsible for this reside on a 'pathogenicity island'. They enter the mesenteric lymph nodes, where they multiply and, via the thoracic duct, enter the bloodstream. Transient bacteremia follows, during which the bacilli are seeded in the liver, gall bladder, spleen, bone marrow, lymph nodes, lungs and kidneys, where further multiplication takes place. Towards the end of the incubation period, there occurs massive bacteremia from these sites of multiplication, heralding the onset of clinical disease.

As bile is a good culture medium for the bacillus, it multiplies abundantly in the gall bladder and is discharged continuously into the intestine where it involves Peyer's patches and the lymphoid follicles of the ileum. These become inflamed, undergo necrosis and slough off, leaving behind the characteristic typhoid ulcers. Ulceration of the bowel leads to the two major complications of the disease—intestinal perforation and hemorrhage. During the 3–4 weeks that normally constitute the course of the disease, the intestinal lesions undergo healing.

Clinical course

The incubation period is usually 7–14 days but may range 3–56 days and appears to be related to the dose of infection. The clinical course may vary from mild undifferentiated pyrexia (ambulant typhoid) to a rapidly fatal disease (*Case*).

- **Onset** is usually gradual, with headache, malaise, anorexia, a coated tongue and abdominal discomfort with either constipation or diarrhea.
- The typical features are **step-ladder pyrexia**, with relative bradycardia and toxemia.
- A soft, **palpable spleen** is a constant finding. Hepatomegaly is also common.
- '**Rose spots**' that fade on pressure appear on the skin during the second or third week but are seldom noticeable in dark-skinned patients.

Complications

The most important complications are intestinal perforation, hemorrhage and circulatory collapse. Some degree of bronchitis or bronchopneumonia is always found. Some develop psychoses, deafness or meningitis. Cholecystitis, arthritis, abscesses, periosteitis, nephritis, hemolytic anemia, venous thromboses and peripheral neuritis are other complications. Osteomyelitis is a rare sequel.

Convalescence is slow. In about 5–10 per cent of cases, relapse occurs during convalescence. The relapse rate is higher in patients treated early with chloramphenicol (15–20 per cent).

*S.*Paratyphi A and B cause paratyphoid fever which resembles typhoid fever but is generally milder. *S.*Paratyphi C may also cause paratyphoid fever but more often it leads to frank septicemia with suppurative complications. Other salmonellae have on occasion been reported to cause enteric fever. These have included *S.*Dublin, *S.*Barielly, *S.*Sendai, *S.*Enteritidis, *S.*Typhimurium, *S.*Eastbourne, *S.*Saintpaul, *S.*Oranienburg and *S.*Panama. Infection with *Alkaligenes faecalis* may sometimes cause a similar clinical picture.

Epidemiology

Typhoid fever has been virtually eliminated in the developed countries during the past several decades, mainly as a result of improvements in water supply and sanitation, but it continues to be endemic in the resource limited nations of the world. The control of paratyphoid fever has not been so successful. The distribution of paratyphoid bacilli shows marked geographical differences. *S.*Paratyphi A is prevalent in India and other Asian countries, Eastern Europe and South America,

S. Paratyphi B in Western Europe, Britain and North America and *S.* Paratyphi C in Eastern Europe and Guyana.

Enteric fever is endemic in all parts of India. An incidence of 500–980 per 100,000 population has been reported in different studies varying with age and geographical area. Worldwide, 22 million cases are estimated to occur annually, with 600,000 deaths (highest concentration in Asia)! The proportion of typhoid to paratyphoid A is about 10:1. Paratyphoid B is rare and C very rare. The disease occurs at all ages but is probably most common in the age group of 5–20 years. The age incidence is related to the endemicity of the disease and the level of sanitation.

Carriers: The source of infection is a patient or, far more frequently, a carrier. Patients who continue to shed typhoid bacilli in feces for three weeks to three months after clinical cure are called **convalescent carriers**. Those who shed the bacilli for more than three months but less than a year are called **temporary carriers** and those who shed the bacilli for over a year are called **chronic carriers**. About 2–4 per cent of patients become chronic carriers. Development of the carrier state is more common in women and in the older age groups (over 40 years). Some persons may become carriers following inapparent infection (symptomless excretor). The shedding of bacilli is usually intermittent. The bacilli persist in the gall bladder or kidneys and are eliminated in the feces (fecal carrier) or urine (urinary carrier), respectively. Urinary carriage is less frequent and is generally associated with some urinary lesion such as calculi or schistosomiasis.

Food handlers or cooks who become carriers are particularly dangerous. The best known of such typhoid carriers was Mary Mallon (**'Typhoid Mary'**), a New York cook who, over a period of 15 years, caused at least seven outbreaks affecting over 200 persons.

Carriers occur with paratyphoid bacilli also. While *S.* Paratyphi A occurs only in human beings, *S.* Paratyphi B can infect animals such as dogs or cows, which may act as sources of human disease.

Typhoid fever occurs in two epidemiological types. The first is **endemic** or **residual typhoid** that occurs throughout the year though seasonal variations may sometimes be apparent. The second is **epidemic** typhoid, which may occur in endemic or non-endemic areas. Typhoid epidemics are water-, milk- or food-borne.

Laboratory diagnosis

Bacteriological diagnosis of enteric fever consists of isolation of the bacilli from the patient and the demonstration of antibodies in his/her serum.

1. Specimen: Blood is collected for culture, as is urine and stool. Serum is obtained for the Widal test. The choice of specimen depends on the duration of illness (Fig. 31.4).

2. Blood culture: A positive blood culture is diagnostic. Bacteremia occurs early in the disease and blood cultures are positive in approximately 90 per cent of cases in the first week of fever. Blood culture is positive in approximately 75 per cent of cases in the second week, 60 per cent in the third week and 25 per cent thereafter till the subsidence of pyrexia. Blood cultures rapidly become negative on treatment with antibiotics.

Method: About 5–10 ml of blood is collected by venipuncture and inoculated into a culture bottle containing 50–100 ml of 0.5 per cent bile broth along with a standard blood culture media (Fig. 31.5). Blood contains substances that inhibit the growth of the bacilli and hence it is essential that the broth be taken in sufficient quantity to provide at 1:10 dilution of blood. The addition of liqoid (sodium polyanethol sulphonate) counteracts the bactericidal action of blood.

After overnight incubation at 37°C, the bile broth is subcultured on MacConkey agar. Pale non-lactose fermenting colonies that may appear on this medium are further characterised by biochemical tests.

Subculture repetition: If the first culture is negative, subculture should be repeated and culture declared

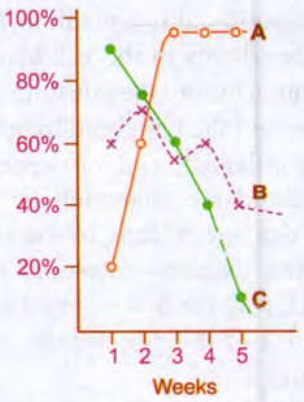

Fig. 31.4 Laboratory diagnosis of typhoid fever. The approximate percentages of tests found positive during different stages of the disease (weeks 1–5). A. Widal agglutination. B. Feces culture. C. Blood culture.

negative only after incubation for 10 days. To eliminate the risk of contamination during repeated subcultures, and also for economy and safety, **Castaneda's method** of culture may be adopted. In this, a double medium is used. The bottle of bile broth has an agar slant on one side. After inoculation of blood, the bottle is incubated in the upright position. For subculture, the bottle is merely tilted so that the broth runs over the surface of the agar. It is reincubated in the upright position. If salmonellae are present, colonies will appear on the slant.

Serotyping by slide agglutination: A loopful of the growth from an agar slope is emulsified in two drops of saline on a slide. One emulsion acts as a control to show that the strain is not autoagglutinable. If *S.*Typhi is suspected (that is, when no gas is formed from glucose), a loopful of typhoid O antiserum (factor 9/group D) is added to one drop of bacterial emulsion on the slide, and agglutination looked for after rocking the slide gently. Prompt agglutination indicates that the isolate belongs to Salmonella group D. Its identity as *S.*Typhi is established by agglutination with the flagellar antiserum (anti-d serum). Quite often, fresh isolates of *S.*Typhi are in the V form and do not agglutinate with the O antiserum. Such strains may be tested for agglutination against the anti-Vi serum. Alternatively, the growth is scraped off in a small amount of saline, boiled for 20 minutes and tested for agglutination with the O antiserum. Where the isolate is a non-typhoid Salmonella (producing gas from sugars), it is tested for agglutination with O and H antisera for groups A, B and C. For identification of unusual serotypes, the help of the **National Salmonella Reference Centre** should be sought. In India it is located at the Central Research Institute, Kasauli. The reference centre for salmonellae of animal origin is at the Indian Veterinary Research Institute, Izatnagar.

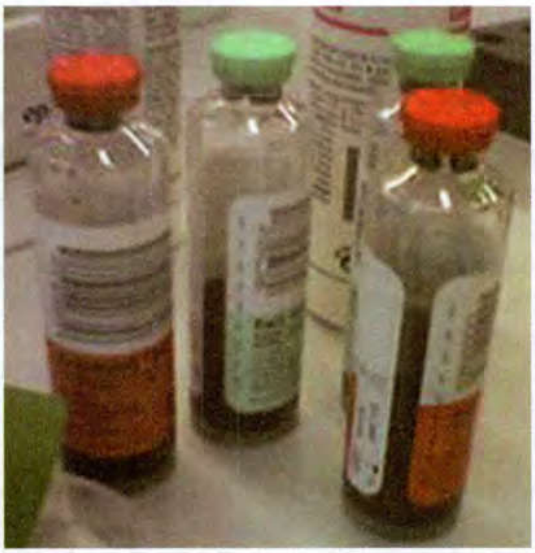

Fig. 31.5 Blood culture bottles

3. Clot culture: An alternative to blood culture, in clot culture, 5 ml of blood is withdrawn from the patient into a sterile test tube and allowed to clot. The serum is pipetted off and used for the Widal test. The clot is broken up with a sterile glass rod and added to a bottle of bile broth. The incorporation of streptokinase (100 units per ml) in the broth facilitates lysis of the clot. Clot cultures yield a higher rate of isolation than blood cultures as the bactericidal action of the serum is obviated. Another advantage is that a sample of serum also becomes available. Even though agglutinins may be absent in the early stages of the disease, the Widal test provides a baseline titre against which the results of tests performed later may be evaluated.

4. Feces culture: Salmonellae are shed in feces throughout the course of the disease and even in convalescence, with varying frequency. Hence, fecal cultures are almost as valuable as blood cultures in diagnosis. A positive fecal culture, however, may occur in carriers as well as in patients. The use of enrichment and selective media and repeated sampling increase the rate of isolation. Fecal culture is particularly valuable in patients on antibiotics as the drug does not eliminate the bacilli from the gut as rapidly as it does from blood.

Fecal samples are plated directly on **MacConkey, DCA** or **XLD** and **Wilson–Blair media**. The last is highly selective and should be plated heavily. On MacConkey and DCA media, salmonellae appear as pale colonies and on XLD, they appear pink with a black centre (Fig. 31.1). On the Wilson–Blair medium, *S.*Typhi forms large black colonies, with a metallic sheen. *S.*Paratyphi A produces green colonies due to the absence of H_2S production.

For enrichment, specimens are inoculated into one tube each of selenite and tetrathionate broth, and incubated for 12–18 hours before subculture onto plates.

5. Urine culture: Salmonellae are shed in urine irregularly and infrequently. Hence, urine culture is less useful than the culture of blood or feces. Cultures are generally positive only in the second and third weeks and then only in about 25 per cent of cases. Repeated sampling improves the rate of isolation. Clean voided urine samples are centrifuged and the deposit inocu-

lated into enrichment and selective media, as for fecal culture.

6. Other materials for culture: Isolation may be obtained from several other sources but they are not usually employed. **Bone marrow** culture is valuable as it is positive in most cases even when blood cultures are negative. Culture of **bile** obtained by duodenal aspiration is usually positive and may be employed for the detection of carriers. Other materials which may yield isolation at times are rose spots, pus from suppurative lesions, CSF and sputum. At autopsy, cultures may be obtained from the gall bladder, liver, spleen and mesenteric lymph nodes.

7. Serology:

Widal reaction: This is a test for the measurement of H and O agglutinins for typhoid and paratyphoid bacilli in the patient's sera.

Two types of tubes are generally used for the test:

- A narrow tube with a conical bottom (**Dreyer's agglutination tube**) for H agglutination
- A short round-bottomed tube (**Felix tube**) for O agglutination

Procedure: Equal volumes (0.4 ml) of serial dilutions of the serum (from 1/10 to 1/640) and the H and O antigens are mixed in Dreyer's and Felix agglutination tubes, respectively, and incubated in a water bath at 37°C overnight. Some workers recommend incubation at 50–55°C for two hours, followed by overnight incubation at room temperature. Control tubes containing the antigen and normal saline are set to check for autoagglutination. The agglutination titres of the serum are read. H agglutination leads to the formation of loose, cotton-woolly clumps, while O agglutination is seen as a disc-like pattern at the bottom of the tube. In both, the supernatant fluid is rendered clear.

The antigens used in the test are the H and O antigens of *S.*Typhi and the H antigens of *S.*Paratyphi A and B. The paratyphoid O antigens are not employed as they cross-react with the typhoid O antigen due to their sharing of factor 12. The H agglutinable suspension is prepared by adding 0.1% formalin to a 24-hour broth culture or saline suspension of an agar culture. For preparing the O suspension, the bacillus is cultured on phenol agar (1:800) and the growth scraped off in a small volume of saline. It is mixed with 20 times its volume of absolute alcohol, heated at 40–50°C for 30 minutes, centrifuged and the deposit re-suspended in saline to the appropriate density. Chloroform may be added as a preservative. It is important to use standard smooth strains for antigen preparation. The strains usually used are *S.*Typhi 901, 'O' and 'H'. Each batch of antigen should be compared with a standard. Readymade Widal kits of stained antigens available commercially are now widely used.

Interpretation of results: The results of the Widal test should be interpreted taking into account the following:

- The agglutination titre will depend on the **stage** of the disease. Agglutinins usually appear by the end of the first week, so blood taken earlier may give a negative result. The titre increases steadily till the third or fourth week, after which it declines gradually.
- Demonstration of a **rise in titre** of antibodies by testing two or more serum samples is more meaningful than a single test. If the first sample is taken late in the disease, a rise may not be demonstrable. Instead, a fall in titre may be seen in some cases.
- The results of a **single test** should be interpreted with caution. It is necessary to obtain information on the distribution of antibody levels in 'normal population' in different areas to determine the cut-off titre.
- Agglutinins may be present on account of prior disease, inapparent infection or **immunisation**. Therefore, the mere presence of agglutinin in the Widal test should not be taken as proof of typhoid fever.
- **H agglutinins** persist longer than **O agglutinins**. Serum from an individual immunised with the TAB vaccine will generally have antibodies to *S.*Typhi and *S.*Paratyphi A and B, while in case of infection antibodies will be seen only against the infecting species.
- Persons who have had prior infection or immunisation may develop an **anamnestic response** during an unrelated fever. This may be differentiated by repetition of the test after a week. The anamnestic response shows only a transient rise, while in enteric fever the rise is sustained.
- Bacterial suspensions used as antigens should be **free from fimbria**. False positive results may occur otherwise.
- Cases treated early with **antibiotics** may show poor agglutinin response.

IgM detection kits: They can be useful in the diagnosis of infection in the initial weeks in a primary infection when a single serum sample is available. They detect IgM antibodies to lipolpolysaccharide or outer membrane protein antigens.

8. PCR-based tests: These are also sensitive but not widely available.

9. Demonstration of circulating antigen: Typhoid bacillus antigens are consistently present in the blood in the early phase of the disease, and also in the urine of patients. The antigen can be demonstrated by sensitised **staphylococcal coagglutination** test. *S.aureus* (**Cowan I strain**) which contains protein A is stabilised with formaldehyde and coated with *S.*Typhi antibody. When a 1% suspension of such sensitised staphylococcal cells is mixed on a slide with serum from patients in the first week of typhoid fever, the typhoid antigen present in the serum combines with the antibody attached to staphylococcal cells, producing visible agglutination within two minutes. The test is rapid, sensitive and specific but is not positive after the first week of the disease.

10. Other laboratory tests: A white cell count is useful. Leucopenia with relative lymphocytosis is seen. Eosinophils are said to be absent but in the tropics, with a high incidence of helminthic infestation, eosinophils are usually present.

Diagnosis of carriers

The detection of carriers is important for epidemiological and public health purposes. Laboratory tests are also useful in screening food handlers and cooks to detect the carrier state.

- Identification of fecal carriers is by **isolation** of the bacillus from feces or from bile. The frequency and intensity of bacillary shedding vary widely and it is essential, therefore, to test repeated samples. Cholagogue purgatives increase the chance of isolation. For the detection of urinary carriers, repeated urine cultures should be carried out.
- The **Widal reaction** is of no value in the detection of carriers in endemic countries. Demonstration of antibodies to Vi antigens has been claimed to indicate the carrier state. While this is useful as a screening test, confirmation should be made by culture.
- The tracing of carriers in cities may be accomplished by the '**sewer-swab**' technique. Gauze pads left in sewers and drains are cultured, and by tracing positive swabs, one may be led to the house harbouring a carrier.
- Another technique of isolating salmonellae from sewage is by **filtration through Millipore membranes** and culturing the membranes on highly selective media such as Wilson and Blair media.

Typing methods

Bacteriophage typing: Intraspecies classification of *S.*Typhi for epidemiological purposes was made possible by bacteriophage typing, first developed by Craigie and Yen (1937). They found that a bacteriophage acting on the Vi antigen of the typhoid bacillus (Vi phage II) was highly adaptable. As phage typing of *S.*Typhi depends on the presence of Vi antigens, a proportion of strains (Vi negative) will be **untypeable**. The phage type is stable. Apart from helping in tracing the source of epidemics, phage typing also provides information on the trends and patterns in the epidemiology of typhoid at the local, national and international levels. The **National Salmonella Phage Typing Centre** for India is located at the Lady Hardinge Medical College, New Delhi. Among *S.*Typhi phage types, A and E1 are more common in India, while among the *S.*Paratyphi A phage, types 1 and 2 are the most common. However the lack of discriminatory power limits the utility of phage typing as an epidemiological tool.

Antibiogram: This is also a stable typing method but lacks discriminatory power.

Molecular methods: Currently, more discriminating genotyping methods like pulse field gel electrophoresis, multilocus enzyme electrophoresis, IS-200 profiling and random amplified polymorphic DNA analysis have been employed for epidemiological typing.

Prophylaxis

Typhoid fever can be effectively controlled by:

General measures, such as improvements in sanitation and provision of protected water supply. Many developed countries have been able to eliminate the risk by these measures, but occasional outbreaks do appear due to unforeseen lapses.

Vaccines:

- **TAB vaccine:** Specific prophylaxis with the heat killed typhoid bacillus vaccine was developed and successfully field tested by Almroth Wright during the Boer war in South Africa. The TAB vaccine which came into general use later contained *S.*Typhi, 1000 million and *S.*Paratyphi A and B, 750 million each per ml killed by heating at 50–60°C and preserved in 0.5 % phenol.

  Dose schedule: The vaccine is given in two doses of 0.5 ml subcutaneously at an interval of 4–6 weeks. Local and general reactions lasting one or two days

are quite frequent. Such reactions may be avoided if the vaccine is administered in a dose of 0.1 ml intradermally. In non-endemic areas, vaccination is recommended for troops, medical and paramedical personnel. In endemic areas, vaccination is recommended for all children in whom a single dose might give adequate protection, which may be maintained for several years by the booster effect of repeated natural subclinical infections. The killed vaccines do not provide cell-mediated immunity.

- **Live oral vaccine:** A live oral vaccine has been introduced after successful field trials. The live oral vaccine (**typhoral**) is a stable mutant of *S.*Typhi strain Ty2 1a, lacking the enzyme UDP-galactose-4-epimerase (Gal E mutant). On ingestion, it initiates infection but 'self-destructs' after four or five cell divisions, and therefore cannot induce any illness.

  Dose: The vaccine is an enteric-coated capsule containing 10^9 viable lyophilised mutant bacilli. The course consists of one capsule orally, taken an hour before food, with a glass of water or milk, on Days 1, 3 and 5. No antibiotic should be taken during this period.
- **Vi vaccine:** The injectable vaccine (typhim-Vi) contains purified Vi polysaccharide antigen (25 µg per dose) from *S.*Typhi strain Ty2. It is given as a single subcutaneous or intramuscular injection, which causes only minimal local reaction.

  Both the oral and Vi vaccines are recommended only for those over five years of age, the same dose being used for children and adults. In both cases, protection is stated to commence 2–3 weeks after administration and lasts for at least three years, after which a booster may be given. Both vaccines are effective and only their relatively high cost stands in the way of their wider use.

  Typhoid bacilli are primarily intracellular parasites, and cell-mediated immunity rather than humoral antibodies may be more relevant in protection against the disease. Cell-mediated immunity develops during the course of the disease. Cellular immunity to the typhoid bacillus is common in populations in endemic areas. Absence of CMI has been claimed to indicate susceptibility. The killed vaccines currently used do not stimulate CMI.

Treatment

Specific antibacterial therapy for enteric fever became available only in 1948 with the introduction of chloramphenicol, which continued as the sheet anchor against the disease till the 1970s, when resistance became common. Ampicillin, amoxycillin and cotrimoxazole were the other drugs found useful in the treatment of typhoid fever but current strains show resistance to these also. At present, ciprofloxacin is the drug of choice or, in case of resistance, ceftriaxone is given.

Carriers: While antibacterial therapy has been effective in the treatment of cases, it has been disappointing in the treatment of carriers. A combination of antibacterial therapy along with the vaccine has been tried for the eradication of the carrier state. This combination has also been used to prevent relapses. Elimination of the carrier state may require heroic measures such as cholecystectomy, pyelolithotomy or nephrectomy.

Drug resistance

Though occasional resistant strains had been identified in the laboratory, resistance to chloramphenicol did not pose any problem in typhoid fever till 1972, when resistant strains emerged in Mexico and in Kerala (India).

In India, chloramphenicol-resistant typhoid fever appeared in epidemic form first in Calicut (Kerala) in early 1972. It became endemic and was confined to Kerala till 1978. Subsequently such strains carrying drug resistant plasmids appeared in many other parts of India. Though resistant to chloramphenicol, such strains were initially sensitive to ampicillin, amoxycillin, cotrimoxazole and furazolidone, which were successfully used for treatment. By late 1980s, typhoid bacillus strains resistant to many or all of these drugs began to spread to most parts of India. The drugs useful in the treatment of such multiresistant typhoid cases were the later fluoroquinolones (such as ciprofloxacin, pefloxacin, oflaxacin) and the third-generation cephalosporins (such as ceftazidime, ceftrioxone, cefotaxime). Furazolidone is still active against most isolates. Now several isolates of typhoid bacilli are once again sensitive to chloramphenicol.

SALMONELLA GASTROENTERITIS

Salmonella gastroenteritis (more appropriately enterocolitis) or food poisoning is generally a zoonotic disease, the source of infection being animal products. It may be caused by any salmonella called non-typhoidal salmonellae. In most parts of the world, *S.*Typhimurium is the most common species. Some other common spe-

cies are *S.* Enteritidis, *S.* Haldar, *S.* Heidelberg, *S.* Agona, *S.* Virchow, *S.* Seftenberg, *S.* Indiana, *S.* Newport and *S.* Anatum.

Sources of infection

Human infection results from the ingestion of contaminated food. The most frequent sources of salmonella food poisoning are poultry, meat, milk and milk products. Of great concern are eggs and egg products. Salmonellae can enter through the shell if eggs are left on contaminated chicken feed or feces, and grow inside. Human carriers do occur but their role is minimal when considered in relation to the magnitude of infection from animals. Even salads and other uncooked vegetables may cause infection if contaminated by manure or by handling. Food contamination may also result from the droppings of rats, lizards or other small animals. Gastroenteritis may occur without food poisoning, as in cross-infection in hospitals.

Pathogenesis

Clinically, the disease develops after a short incubation period of 24 hours or less, with diarrhea, vomiting, abdominal pain and fever.

- It may vary in severity from the passage of one or two loose stools to an acute cholera-like disease.
- It usually subsides in 2–4 days, but in some cases, a more prolonged enteritis develops, with passage of mucus and pus in feces, resembling dysentery.
- In a few, typhoidal or septicemic type of fever may develop.

Laboratory diagnosis

This is made by **isolating** the salmonella from the feces. In outbreaks of food poisoning, the causative article of food can often be identified by taking a proper history. Isolation of salmonellae from the article of food confirms the diagnosis.

Control of salmonella food poisoning requires the prevention of food contamination. Food may become contaminated at various levels, from natural infection in the animal or bird, to contamination of the prepared food. Proper cooking of food destroys salmonellae.

While enteric fever is a major problem only in developing countries, salmonella food poisoning is largely a problem for developed nations. This is due to the differences in food habits and living conditions between them and also because food production, packaging, storage and marketing have become industries in the developed countries while they still remain agricultural in the developing world.

Treatment of uncomplicated, non-invasive salmonellosis is symptomatic. Antibiotics should not be used. Not only do they not hasten recovery but they may also increase the period of fecal shedding of the bacilli. However, for serious invasive cases, antibiotic treatment is needed.

SALMONELLA SEPTICEMIA

Certain salmonellae, *S.* Choleraesuis in particular, may cause septicemic disease with focal suppurative lesions, such as osteomyelitis, deep abscesses, endocarditis, pneumonia and meningitis. Antecedent gastroenteritis may or may not be present. The case fatality may be as high as 25 per cent.

Salmonellae may be isolated from blood or from pus from the suppurative lesion. Feces culture may also sometimes be positive. Septicemic salmonellosis should be treated with chloramphenicol or other appropriate antibiotics as determined by sensitivity tests.

MULTIRESISTANT SALMONELLAE

R factors conferring multiple drug resistance have become widely disseminated among salmonellae. The clinical significance of this phenomenon was first observed during studies of human and veterinary infections with drug-resistant *S.* Typhimurium phage type 29 in England in the 1960s. Human infections were initially gastroenteritis due to spread from infected animals, through food. Subsequently, some salmonellae appear to have changed their ecology in some ways. From being responsible for zoonotic infections only, as in the past, some multiresistant salmonellae have now become important agents of hospital cross-infections. Such nosocomial salmonellosis manifests, particularly in neonates, as septicemia, meningitis and suppurative lesions. Diarrhea may not always be present.

In India, several hospital outbreaks of neonatal septicemia caused by multiresistant salmonellae have occurred in recent years. Mortality in neonates is very high unless early treatment is started with antibiotics to which the infecting strain is sensitive.

RECAP

- The genus *Salmonella* belongs to the family Enterobacteriaceae. It is a Gram-negative, facultatively anaerobic, motile bacillus that is catalase positive and oxidase negative.
- Many serologically distinct species exist. These species have now been unified into one common species, *Salmonella enterica*, with the previously separate species now being referred to as serovars.
- The serovars causing typhoid fever include *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Paratyphi A and *Salmonella enterica* serovar Paratyphi B.
- The *Salmonella* species responsible for enteric fever are spread only from human to human. Water contaminated with feces is a common source, especially during natural disasters when the quality of drinking water is compromised.
- For diagnosis, samples of feces or blood are cultured and identified by biochemical characteristics and by slide agglutination tests using reference antibody to the O, H, and Vi (capsular polysaccharide) antigens.
- Some patients become carriers, harbouring the bacteria in their tissues without symptoms and passing organisms in their feces for years.
- Enteric fever can be prevented by proper personal hygiene, consumption of safe drinking water and vaccines. Antibiotics are needed to treat sick individuals and to eliminate the carrier state. There is currently a live oral vaccine available for immunisation.
- The Salmonella species responsible for food poisoning are transmitted by ingestion of food contaminated with feces from infected animals or humans; eggs and poultry are the most common animal sources.
- There are many serovars implicated in food poisoning, the prominent ones being *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis.
- The disease is prevented by good hygiene (hand washing) and by elimination of animal reservoirs.

ESSAYS

1. Classify enterobacteria. Give an account of the morphology, cultural characteristics and pathogenicity of *S.*Typhi.
2. Enumerate the organisms causing PUO and explain the pathogenesis and laboratory diagnosis of enteric fever.

SHORT ANSWERS

1. Slide agglutination for the identification of salmonella
2. Detection of typhoid carriers
3. Media used for the culture and identification of salmonella
4. Widal test

SHORT NOTES

1. Vi antigen in *S.*Typhi
2. Non-typhoidal *Salmonella*