4.8 Presumptive Epidemiological Cycle

With the available epidemiological information, it is now possible to work out a probable sequence of events in the epidemiology of HS. Figure 4.3 outlines this presumptive epidemiological cycle.

In an endemic area, after one outbreak of disease, a large number of surviving animals become latent carriers. They intermittently shed the organisms, the frequency diminishing with time. Since the herd immunity is also high, there are no fresh clinical cases. The first clinical case occurs when a shedder comes into contact with a susceptible animal, which will invariably be one born after the previous outbreak, or one introduced into the herd from elsewhere.

Movement of animals has frequently been associated with epidemics of HS, for example the 1993-94 outbreak in the Philippines (Molina et al. 1994). Movement of animals associated with the rice cultivation cycle is a common occurrence in Asia. In Sri Lanka an instance is on record where, under a river valley development program, a new human settlement was established in the endemic dry zone. Due to the limited number of cattle and buffaloes in this area and the improved husbandry practices, nearly 100% vaccination coverage was achieved. During the cultivation season, due to a shortage of draught power in the area, however, additional animals had to be brought in from elsewhere. An outbreak of HS occurred, but only the animals brought in from outside were affected.

Movement of animals can precipitate disease in two ways. Firstly, the animals being moved may be carriers and able to infect susceptible stock. Secondly, the animals being moved may be susceptibles, which may be infected from native immune carriers. In either case, explosive outbreaks could result. Thus, the chances of such contact will increase with time, as the susceptible population builds up.

Once the first clinical case occurs, more bacteria are shed and disseminated. Their survival in the environment and transmission to other animals depend on such factors as the climatic conditions, closeness of contact and hygiene. The magnitude of the outbreak that follows depends on the proportion of immune to nonimmune animals in the herd. Thus, in situations where occasional sporadic outbreaks occur, the build up of susceptible nonimmune animals can result in a major outbreak. Where regular, seasonal outbreaks occur, more animals are likely to be immune (through frequent exposure) and the outbreaks will be of a minor nature. The old belief that carrier animals break down into clinical cases, thereby initiating an outbreak, may not hold ground, since the available evidence shows that carrier animals are also immune.

Considering the large number of organisms that are required to give clinical disease under experimental conditions, it is not clear how a carrier could transmit such large numbers to a susceptible animal. Some of the questions that still exist in the epidemiology of HS are:

- What are the factors that cause activation of latent carriers? and
- What causes a susceptible animal to develop clinical disease with a presumably low dose of bacteria in natural transmission from a carrier?
Diagnosis

Overview

Clinical diagnosis
A clinical diagnosis of haemorrhagic septicaemia (HS) is based on a combination of clinical signs, gross pathological lesions and a consideration of relevant epidemiological parameters and other similar diseases prevalent in the locality.

Routine laboratory diagnosis
Routine laboratory diagnosis is by culture and serology. Material for laboratory diagnosis usually consists of blood or a long bone for bone marrow culture. Pure cultures are obtained from contaminated material by mouse inoculation and culture of the mouse blood. A positive diagnosis is available within 24–48 hours.

Other diagnostic tests
Additional serological, biochemical and molecular techniques are available as research and investigational tools.

If reporting is delayed and no material is available for culture and isolation at the time of investigation, antibody levels in surviving animals can be assayed as an indicator of HS infection.

5.1 Provisional Diagnosis

When a suspected outbreak of haemorrhagic septicaemia (HS) is reported, a provisional diagnosis can be made based on clinical signs and, if carcases are available, on the gross pathological lesions seen on postmortem examination. An investigation of relevant epidemiological parameters is also helpful. It is also important to consider other diseases prevalent in the locality that could account for the clinical signs observed, lesions and the number and pattern of deaths observed.

A provisional diagnosis is important since preventive measures to control the spread of the disease are required immediately, without waiting for laboratory confirmation. At the earliest opportunity, however, appropriate material should be collected, suitably packed and dispatched to the nearest laboratory where facilities are available for diagnostic tests to be performed. A combination of clinical signs, gross pathological lesions and epidemiological features, coupled with the isolation of the organism and identification of serotype, will help in arriving at a definitive diagnosis.

5.2 Clinical Diagnosis

As outlined in Chapter 3, the disease presents a variety of clinical signs, none of which, when taken individually, is specific for HS. Thus, the clinical picture should be considered as a whole, together with the pathological lesions and the epidemiological findings. As HS occurs mostly in animals reared under poor husbandry conditions, no clinical signs whatever may be observed in the first cases of an outbreak. Thus, the first reports may be of animals found dead suddenly, with no observed illness. Thereafter, upon closer observation, clinical signs will become evident. More advanced cases will show submandibular oedema spreading to surrounding areas, nasal discharge,
salivation and laboured breathing. Those in the early stages may be off their food and, if monitored twice daily, show a marked increase in rectal temperature.

The most obvious postmortem lesions are subcutaneous oedema and petechial haemorrhages, particularly on the base of the ventricle. The next most obvious lesions are those in the lungs, initially congestion, progressively moving towards consolidation and thickening of the interlobular septa giving rise to lobulation (see Section 3.4).

The clinical signs and pathological lesions must be combined with available epidemiological data. The classical epidemics of HS found in earlier records do not occur any longer, particularly in endemic countries, unless the disease is freshly introduced into virgin areas. Thus, the observed morbidity and mortality pattern has to be interpreted against the background of information, such as the species affected, vulnerable age group, endemicity of the location, season and vaccination history. The presence of any triggering circumstances, such as sudden or seasonal climate changes or movement of animals, also provides useful clues.

5.3 Differential Diagnosis

HS has to be differentiated from other diseases that present a similar syndrome and that are prevalent in the location where the disease has been reported. If the condition is an acute one characterised by sudden death, other diseases likely to cause sudden deaths, such as anthrax, rinderpest and black quarter, should be taken into account. Equally important are the noninfectious causes of sudden death such as lightning, snakebites and acute poisoning. The more protracted syndrome dominated by respiratory signs has to be differentiated from other forms of pasteurellosis caused by serotypes other than groups B and E, or by Pasteurella haemolytica. It must be borne in mind that the chronic or subacute pneumonic form of HS always displays a terminal septicaemia, which may not be a consistent feature with other serotypes.

5.4 Samples

5.4.1 Collection of samples

The ideal material for isolation of the specific pathogen is the tissues where it is most likely to be present in largest numbers and which are free, or contain minimal numbers, of other extraneous organisms such as contaminants and postmortem invaders. Tissues of a fresh carcase, preferably blood, fulfil this requirement. If facilities are available at the location of the carcase, a postmortem examination should be immediately carried out and blood drawn directly from the heart. The gross pathological lesions can be observed at this time. Alternatively, blood may be obtained by puncture of the jugular vein.

In regions where the disease occurs frequently, reporting systems may be suboptimal. Under tropical conditions, decomposition of the carcase may take place rapidly within a few hours and the blood may contain an abundance of postmortem invaders. It is, however, possible to isolate pasteurellae from blood collected from carcasses even 24 hours after death by biological screening using mice. In such situations, it is advantageous to collect and dispatch a long bone, which can be used for culture and biological examination of bone marrow. This can be done even from animals exhumed a few days after burial.

Material collected from clinically affected animals before death may not give consistent results. Nasal secretions may yield virulent pasteurellae upon culture. Blood will give positive cultures only in the terminal stage immediately before death. Much of the bacterial multiplication takes place in the carcase after death.

It is always better to dispatch to a laboratory material from more than one animal in an outbreak. Care must be taken to avoid collecting material from animals treated with antibiotics.

5.4.2 Dispatch of diagnostic samples

The fragile nature of pasteurellae and their poor survival outside the animal body has already been discussed in Chapter 3. Dhanda (1959a) reported that common contaminants such as Bacillus subtilis and Pseudomonas aeruginosa, and also B. anthracs, adversely affected the
viability of *P. multocida*. It has also been reported that when kept on cotton wool swabs and contaminated experimentally with small quantities of *B. subtillis* and staphylococci, pure cultures of the HS serotypes of *P. multocida*, or blood of infected animals, were rapidly overgrown by the contaminants at room temperature. When stored at refrigeration temperature, or when the swab was kept immersed in a transport medium, overgrowth by contaminants was arrested (De Alwis 1972, 1973). It was further reported that blood swabs collected from a carcass within six hours of death due to HS could be stored for four days at room temperature and up to one week in the refrigerator with consistent viable counts (within 0.5 log units). In swabs collected 24–30 hours after death, rapid overgrowth by contaminants occurred and no pasteurellae could be isolated on the third day. These observations have to be taken into account in determining methods for dispatch and storage of material during transport to a laboratory.

Blood should be packed in ice or placed in a suitable transport medium. If this is not possible, the swab should be dispatched at room temperature to the laboratory as quickly as possible. Long bones should be cleaned of all muscle tissue, and sent to the laboratory with minimum delay.

**Transport media**

Several transport media are now commercially available. The one used by De Alwis (1973) was a simple non-nutrient inert medium that contained disodium phosphate, thioglycollic acid and agar (0.4%) to give a semisolid consistency, and methylene blue as an indicator. Warner (1996) developed a transport enrichment medium with antibiotic and antifungal additives that promoted the survival of the pasteurellae and suppressed contaminants. Details of transport media are given in Appendix 1.

5.5 Laboratory Diagnosis

Laboratories in countries where HS is endemic receive a variety of specimen types and quality for diagnosis. Appropriate methods may therefore be needed to deal with these specimens. The serotypes of *P. multocida* that cause HS are not difficult to isolate, even in a laboratory with modest facilities. A wide range of laboratory diagnostic tests has been developed over the years. These include:

- culture and biological tests for isolation of the causative organism;
- biochemical and serological tests for identification of the organism and serotype;
- nonserological tests for presumptive identification of serotype; and
- molecular methods for strain differentiation within serotypes.

The conventional method of laboratory diagnosis is therefore based on isolation of the organism from animal tissues and identification by biochemical and serological methods. The nonserological tests and molecular techniques are useful investigatory tools but are not substitutes for the conventional tests. In instances where reporting is delayed and no material is available for culture and isolation at the time of investigation, high levels of antibody or, more specifically, rising antibody titres in surviving animals are useful indications in arriving at a presumptive diagnosis (see Section 5.11).

A routine laboratory diagnostic procedure that can be adopted in any laboratory in the endemic countries of Asia and Africa having modest facilities is illustrated in Figure 5.1.

If the material is uncontaminated, colonies can be isolated on a direct culture plate within 24 hours. These could be subjected to a rapid slide agglutination test. Based on colonial appearance, direct smear examination from culture plate and a positive rapid slide agglutination test, the laboratory could issue a positive report.

If the material is contaminated, as is often the case, a pure culture can only be obtained by mouse inoculation and culture of mouse blood. In addition to the above tests, pathogenicity to mice, and a mouse blood smear with an abundance of bipolar staining gram-negative coccobacilli, provide useful clues. Culture of the mouse blood and a rapid slide agglutination test on the culture confirms the diagnosis. Thus, within 24–48 hours, it is possible to issue a positive report from a laboratory. As a routine, this procedure has proved to be quite satisfactory. If further serotyping is considered
5.6 Routine Microbiological Procedures

5.6.1 Microscopic examination of stained blood smear

When blood smears from the dead animal (usually cattle or buffalo) are stained with methylene blue or Leishman stain, and Gram stain, they show the presence of bipolar staining, gram-negative short bacilli. This test alone is not sufficient to confirm diagnosis, however, as other gram-negative bacilli may present a similar appearance.

5.6.2 Isolation of pasteurellae

Culture

Pasteurellae grow on ordinary media such as nutrient agar, or enriched media such as tryptose agar or casein–sucrose–yeast agar (CSY agar), with or without 5% sterile blood. Blood of a young calf — the natural host — free of antibodies is preferred. Enrichment of the media and the addition of blood promotes growth.

A small volume of sterile physiological saline is mixed with the blood swab. For bone samples, the surface of the bone is cleaned with alcohol sterilised by flaming and cut open with a sterile saw. A small quantity of bone marrow is scooped out. One drop of this material (blood or bone marrow) is placed on the edge of a culture plate and spread with a flame-sterilised platinum loop, cooled by placing on the medium. Streak cultures are made at right angles to each other, sterlising and cooling the loop at each stage. This procedure helps to isolate individual colonies on the final streaks in cases when the inoculum is heavily laden with organisms, or on the initial spread area when it is only weakly laden with organisms.

Freshly isolated colonies on tryptose agar or CSY agar enriched with blood are approximately 2 mm in diameter after 24 hours at 37°C. Blood agar cultures yield colonies of approximately 1 mm and colonies on plain unenriched media devoid of blood may be smaller. Colonies on enriched media are smooth, nonmucoid, greyish, glistening and translucent.

Inoculation onto triple sugar iron (TSI) agar helps to differentiate pasteurellae from the common gram-negative enteric bacteria. Pasteurella gives a slow acid reaction with no gas, and no detectable production of hydrogen sulfide in this medium. Cultures are oxidase and catalase positive, produce indole and reduce nitrates, but fail to produce urease, utilise citrate, grow on McConkey agar medium or liquify gelatin. These biochemical tests are of diagnostic value.

Isolation by direct culture alone is only successful if there is little or no contamination and when the interval from collection to culture is short, or the material has been stored in transport medium or refrigerated. Otherwise, the plate may be overgrown with contaminants that will mask the pasteurellae.
Biological screening (mouse inoculation)

Since most swabs reaching diagnostic laboratories have a high proportion of extraneous bacteria, the best method to isolate the organism is by subcutaneous inoculation of a mouse with about 0.1–0.2 mL of a saline suspension of the blood or bone marrow sample prepared as described above for culture. If *P. multocida* types that cause HS are present, the mouse will die within 24 hours. Smears of the heart blood of the infected mouse will show an abundance of bipolar staining coccobacilli when stained with Gram, Leishman or methylene blue stains, the bipolar nature being more evident with the latter two stains (see Figure 5.2a,b). The blood of the mouse can also be cultured, and will yield pure cultures.

Type B pasteurellae that cause HS are highly virulent for mice, with a 50% lethal dose (LD50) of 1–10 viable organisms. Thus, this method is extremely useful in situations where the material has only a few viable organisms, even amidst numerous contaminants. Hence, the value of this biological test cannot be underestimated. Mouse inoculation also greatly facilitates the isolation of pasteurellae, even from nasopharyngeal swabs, tonsils, lymph nodes etc. in healthy carrier animals.

5.7 Conventional Serological Tests

Serological identification of pasteurella is based on the detection of the capsular and somatic antigens (see Chapter 2). Over the years, a variety of tests have been developed but the results of the different techniques are not strictly comparable. These methods are described in Appendix 2.

The following conventional tests are currently in use in diagnostic laboratories:
- rapid slide agglutination test (capsular);
- indirect haemagglutination test (capsular); and
- agar gel precipitation test (somatic).

5.7.1 Rapid slide agglutination test for capsular typing

This is a convenient test used routinely in diagnostic laboratories. It is based on the technique of Namioka and Murata (1961a) for simplified capsular typing using fresh cultures. A drop of physiological saline is placed on a clean slide. A single colony is picked from a fresh culture plate and is mixed with the saline to form a uniform suspension. A loopful of antisera type B:2 pasteurella hyperimmune sera prepared as described in Appendix 2 is thoroughly mixed with this suspension, gently warming the slide over a flame. A rapid, flaky agglutination appearing within a few seconds, with complete clearing of the background, indicates a positive test.

The above test should not be confused with the slide agglutination test for somatic typing developed by Namioka and Murata (1961b, c), which uses a suspension of HCl-treated cells as antigen.

5.7.2 Indirect haemagglutination test for capsular typing

In the indirect haemagglutination (IHA) test, the surface antigen of the organism is liberated by mild heat treatment. The cell residue is removed and the supernatant, which contains the antigen, is used to coat erythrocytes. Agglutination of the coated erythrocytes in serial 10-fold dilutions of hyperimmune rabbit antiserum is considered positive. A positive control may be set up using a known reference culture to prepare
antigen and coat the cells. An untreated erythrocyte suspension is used as the negative control. The serum used is the same as for the rapid slide agglutination test.

The original test described by Carter (1955) used fresh human 'O' erythrocytes. Subsequently, Carter and Rappay (1962) used formalinised human 'O' erythrocytes whilst Sawada et al. (1982) used glutaraldehyde-fixed sheep erythrocytes. Wijewardana et al. (1986a) modified this test using fresh sheep erythrocytes and suspensions of bacterial cells standardised on the basis of turbidity. An antigen titration was also carried out to standardise the amount of antigen used for coating the erythrocytes.

5.7.3 Agar gel precipitation test for somatic typing

The agar gel precipitation test (AGPT) for somatic typing is carried out basically according to the method of Heddleston et al. (1972) using Noble agar medium at a concentration of 0.9% in 8.5% sodium chloride. For antigen preparation, a concentrated harvest of pasteurella grown on a culture plate is made in 8.5% saline with 0.3% formalin. Antiserum for somatic typing is prepared in chicken. The test antigen is placed in a central well in the agar, with antisera against various somatic types in the peripheral wells. A positive result is indicated by diffusion bands of antibody-antigen precipitate, which develop overnight.

5.8 Other Serological Tests

In addition to the above conventional tests, which are necessary to confirm a diagnosis, several other serological tests have been developed.

5.8.1 Agar gel precipitation test for capsular typing

A simple immunodiffusion test has been described for capsular typing (Anon. 1981; Wijewardana et al. 1982). The gel medium consists of 1% Noble agar in 0.2 M phosphate buffer containing merthiolate at a final concentration of 1 in 10 000. As for the indirect haemagglutination test, rabbit hyperimmune sera prepared against whole cells and 56°C/30 minute supernatant are used as sources of antibody and antigen, respectively. Reference antiserum is usually placed in the centre well and test antigens in peripheral wells. Homologous antigen prepared from a reference strain placed in alternate peripheral wells serves as a positive control, and physiological saline or control serum from an unimmunised rabbit placed in one peripheral well serves as a negative control. Bands develop overnight.

5.8.2 Counter-immunoelectrophoresis

This test was originally developed by Carter and Chengappa (1981). The principle underlying the test, and the reagents used, are the same as for the AGPT. Parallel rows of wells are made on the medium on a slide. The wells on the side of the cathode are loaded with antigen while the wells on the side of the anode are loaded with antiserum. Electrophoresis is for 30 minutes, during which time precipitation bands appear. Results can be obtained much more rapidly by this method than by AGPT.

5.8.3 Coagglutination test

This test was developed by Rimier (1978). It is used to differentiate between type B and E strains that cause HS by a coagglutination test using antibody-coated staphylococci. The test has no distinct advantage over other methods and has therefore not gained popularity as a routine test in diagnostic laboratories.

5.8.4 Agglutination test for somatic typing

This test was developed in the early 1960s (Namioka and Murata 1961b,c) and at that time proved to be a useful supplement to Carter's capsular typing in the differentiation of strains associated with various disease conditions. The antigen preparation was made by treating cultures harvested from yeast–protease–cysteine agar plates (see Appendix 1) with normal HCl saline (0.85% saline in normal HCl) overnight. After several washes, the cell residue was suspended in phosphate buffered saline (PBS). Whole cell rabbit hyperimmune serum was used for slide agglutination tests.

When applied to P. multocida in general, this typing method is very complex as it involves a complex system of absorption of sera, in order to avoid cross-reactions
(see Chapter 2). However, since there were only two somatic types detected in capsular type B (6 and 11), and only one (6) within capsular type E, the identification of the HS strains is relatively easy, and unabsorbed sera can be used.

To facilitate interpretation, antisera prepared against three reference strains (Asian [6:B], African [6:E] and Australian [11:B] also known as strain 989) are used in agglutination tests with the three homologous antigens and the test antigen as shown in Table 5.1.

5.8.5 Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) for identification of *P. multocida* that cause HS was developed by Dawkins et al. (1990) in Australia (HS-antigen ELISA; see Appendix 3). These researchers examined 124 strains, consisting of 58 reference strains from collections in various laboratories and 66 field isolates, and reported a specificity of 99% and a sensitivity of 86%. This technique has proved useful for screening a large number of cultures from a collection, rather than occasional diagnosis of HS from specimens reaching a laboratory from time to time. In its present form, it has a serious limitation in that it fails to differentiate between the Asian (B:2) and African (E:2) types. This is not surprising as the antigen used in this test was the boiled antigen similar to that used in Heddleston’s somatic typing, where the somatic antigen 2 was common to both Asian and African types. From an epidemiological standpoint and for the purpose of initiating control measures in an outbreak, it is important to know the serotype.

5.9 Nonserological tests

Several non-immunological tests have been developed for the rapid identification of strains. These include the acriflavine flocculation test described by Carter and Subronto (1973) for identification of type D strains, the hyaluronidase decapsulation test for type A strains (Carter and Rundell 1975) and the hyaluronidase production test for the rapid identification of type B strains (Carter and Chengappa 1981).

5.9.1 Hyaluronidase production test

The production of the enzyme hyaluronidase is a characteristic of many species of gram-positive bacteria such as staphylococci, streptococci and clostridia. Among the gram-negative bacteria, type B strains of *P. multocida* have been found to produce appreciable amounts of hyaluronidase. Two methods have been used to demonstrate the production of this enzyme. In the first method, a hyaluronic acid producing culture of *Streptococcus equi* is streaked across a freshly prepared blood agar plate.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antigen</th>
<th>Result</th>
</tr>
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<tbody>
<tr>
<td>Asian (6:B)</td>
<td>Asian</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>African</td>
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<td>Asian</td>
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<td>Australian (11 B)</td>
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</table>
The pasteurella cultures to be tested are streaked across at right angles. Several streaks can be made across, in a single plate. The plates are then incubated at 37°C for 18 hours. Hyaluronidase production is indicated by a reduction in size of the streptococcal growth adjacent to the pasteurella streak. Better results are obtained by using dextrose starch agar medium and a humidified incubator, where the production of hyaluronic acid is enhanced. *P. multocida* type A, which produces large mucoid, capsulated colonies containing hyaluronic acid, may be used instead of *S. equi*.

In the second method, test cultures are spotted onto a medium containing sodium hyaluronidate and bovine albumin fraction V. The test pasteurella is grown in brain–heart infusion broth for 18 hours at 37°C. The plates are then inoculated with the pasteurella broth culture, incubated at 37°C for 18 hours and flooded with acetic acid. The nondegraded substrate precipitates with the albumin, leaving a clear zone around the pasteurella growth that produced hyaluronidase.

Of 74 test cultures of *P. multocida* used by Carter and Chengappa (1980), only 13 type B strains were found to be positive, whilst type A, D and E strains were negative. On subsequent investigation, it was found that within type B strains, the property of hyaluronidase production is restricted to serogroup B:2, which is the classical Asian serotype. Other type B strains such as B:3,4 were negative. De Alwis et al. (1996) reported a type B:2 mutant that was of low virulence for mice, and failed to produce HS, but was hyaluronidase positive.

### 5.10 Molecular Techniques

With recent advances in molecular biology, a number of new techniques have been developed to analyse the genome (DNA) of organisms. Some of these techniques have been used in the diagnosis of HS. These not only differentiate the serotypes that cause HS but also help in the differentiation of strains within serotypes. They are therefore of diagnostic as well as of epidemiological significance. These methods are described in Appendixes 4 and 5.

#### 5.10.1 Polymerase chain reaction

Polymerase chain reaction (PCR) methods amplify minute quantities of DNA present in samples and allow accurate detection of specific genetic sequences, including bacteria. PCR tests have been developed for the diagnosis of HS and used by many workers (Thomas 1996; Natalia 1996; Brickell 1996; Townsend et al. 1998; see Appendix 4).

An appropriately designed PCR test has the distinct advantages of being able to be performed directly on clinical material even with low numbers of bacteria and of giving rapid results. Natalia (1996) tested 100 tonsillar swabs of cattle taken from abattoirs in Indonesia using a PCR test; the results were consistent with those obtained using a standard bacterial culture method. The specificity and sensitivity of the test were shown to be valuable for detecting *P. multocida* type B:2 from field specimens.

In a PCR test, primer sequences are designed to enable identification of the pathogen concerned at any level of specificity (i.e. strain, serotype, species etc.), thus bringing some flexibility into the test. In a given test, only the specific agent for which the test is designed will be detected. For example, if a test is designed to specifically identify serotype B:2, any other serotype such as E:2 or B:3,4 that may be present in specimens in similar circumstances will not be detected. In this regard, a conventional mouse inoculation test coupled with isolation of the agent and serotyping will allow the detection of any other serotype, as well as any other pathogen such as anthrax, which often occurs in similar situations to HS.

Townsend et al. (1996) identified clones using genomic subtractive hybridisation of closely related *P. multocida* isolates. These were useful in differentiating type B strains that cause HS from type E and other type B strains that cause similar septicaemic disease. Further analysis of these clones resulted in the development of a type-specific PCR test for the identification of HS-causing type B serotypes of *P. multocida*, namely B:2, B:5 or B:2,5 (Townsend et al. 1998).

Townsend et al. (1997a) analysed HS-causing isolates of *P. multocida* by a PCR-based fingerprinting method known as repetitive extragenic palindromic (REP) PCR.
Multiple genomic DNA fragments are amplified by outwardly facing primers based upon the REP consensus sequence, generating complex profiles useful in strain differentiation. The analysis of HS-causing *P. multocida* strains provided evidence of a disease-associated REP profile with a high degree of homogeneity observed among the strains, regardless of whether the capsular serotype was B or E. These profiles were clearly distinct from serologically similar strains that do not cause HS, but exhibited a degree of relatedness to strains that cause clinically similar septicæmic disease such as B:1, B:2, B:3, B:4. These findings have paved the way for a disease-specific test rather than a serotype-specific test.

5.10.2 Ribotyping and field alternation gel electrophoresis

Ribotyping and field alternation gel electrophoresis (FAGE) have also been used to analyse the DNA of strains of *P. multocida* that cause HS, after digestion with restriction enzymes (Adamson et al. 1993; Townsend et al. 1997b; see Appendix 5).

Analysis of the DNA restriction patterns using these methods has proved useful in differentiating bacterial strains that have been denoted as identical by all previous typing methods. Restriction profiles demonstrated by Asian strains showed a remarkable degree of homogeneity. Asian isolates displayed similar ribotype and FAGE patterns irrespective of the restriction enzyme used, thus indicating that they are epidemiologically related both genetically and phenotypically. FAGE displayed a greater degree of discrimination between strains, compared with ribotyping. It was particularly useful in distinguishing the North American type B:2 strains that produced HS from the Asian strains.

Both ribotyping and FAGE analysis also helped to differentiate between the classical Asian B:2 strains that cause HS and the reportedly avirulent strain designated *Izathagar* 25, isolated from cases of paraplegia among cattle in India (Dhanda and Nilakanthan 1961). This strain has not been serotyped by the Carter–Heddleston method but has been found to belong to Roberts type I, and has been biochemically differentiated from other strains by its ability to ferment xylose (Dhanda and Sen 1972).

5.10.3 Restriction endonuclease analysis

Restriction endonuclease analysis (REA) of a wide range of cultures of *P. multocida* associated with HS has been carried out (Wilson et al. 1992; Rimler 1997)(see Appendix 5). The DNA was digested with *Hhal* and *Hpall* endonuclease, and examined by agarose gel electrophoresis. Gels were stained with ethidium bromide, and the band patterns (DNA fingerprint profiles) were viewed and photographed in ultraviolet light. Reverse negative photographs of gels were made and scanned to create an image profile. Images of fingerprint profiles were analysed by computer program.

Forty-two different DNA fingerprint profiles were recognised among 190 serotype B:2 strains using *Hhal* endonuclease. Twelve DNA fingerprint profiles were found among 35 serotype B:3,4 strains with the same enzyme. Further distinction could be made among B:2 or B:3,4 strains with *Hpall* endonuclease. For example, treatment with *Hpall* resulted in seven different DNA fingerprint profiles within 56 serotype B:2 strains that produced a profile designated *Hhal* 0018. Thus, a strain could be assigned a descriptive identification epithet (DIE) code such as B:2 / *Hhal* 0018 / *Hpall* 0016 based on the REA results. Strains were analysed on the basis of associated species and country. Among 64 serotype B:2 strains from Sri Lanka, 24 fingerprint profiles were defined using *Hhal* endonuclease. Further, among 17 strains designated B:2/ *Hhal* 0018, originating from animals such as cattle, buffalo, swine and elephant, six *Hpall* profiles were identified. Similar profiles were found among isolates from swine and buffaloes in India.

5.11 Antibody Detection in Host Animals

As mentioned elsewhere, HS occurs mostly in situations with poor husbandry conditions. In such circumstances, disease-reporting systems are also poorly developed. Deaths usually occur quickly and situations are likely to arise in which, by the time veterinary personnel arrive at the scene, no clinically affected animals or carcasses are available for examination.
Ample evidence has now been presented to show that, following exposure to natural infection, surviving animals develop high antibody levels as measured by the IHA test, using the 56°C/30 minute supernatant as antigen to coat erythrocytes (De Alwis 1982b, De Alwis et al. 1986). De Alwis (1982b) was able to specifically demonstrate a steep rise in antibody level within two to four weeks of such exposure. Such high levels of antibody have not been attained by administration of conventional vaccines. Hence, the presence of such levels of IHA antibody following recent deaths in a herd provides reasonable circumstantial evidence that deaths were due to HS.

The test used to detect such an antibody response is important. The HS-antibody ELISA (Johnson et al. 1989; see Appendix 3) may not detect such antibody, since the test uses boiled supernatant as antigen. This was borne out in experiments with a live vaccine when, following vaccination, IHA antibodies continued to rise, as in natural exposure, but the ELISA titres did not (M.C.L. De Alwis, unpublished data).

A single radial haemolysis test for measuring HS antibodies has been described (Rahman et al. 1987; Rahman and Ashfaque 1991). This test was carried out using a sonicated antigen. In a comparison of this test with the conventional IHA, a high degree of correlation \( r=0.792 \) was observed. There is no information on the use of this test for detecting naturally acquired immunity resulting from natural exposure.

### 5.12 Choice of Diagnostic Tests

The choice of diagnostic tests must take into account the nature of the material available, the facilities available in the laboratory and the proximity of the laboratory to the outbreak of disease. Any modest diagnostic laboratory charged with the responsibility of HS diagnosis should have a basic facility for microscopic examination and bacterial culture. It should also have a supply of hyperimmune rabbit antisera and, ideally, a laboratory animal facility with access to a mouse colony. The most reliable procedure is the mouse inoculation and mouse blood culture followed by rapid slide agglutination. Serotyping by the IHA test and AGPT (Heddleston) may be subsequently carried out in the same laboratory, or the culture may be passed on to the next higher grade of laboratory where such a capability exists. In situations where serotypes other than B:2 or E:2 are present (such as B:1, B:4, B:3,4), AGPT is the definitive test. The procedure, starting with the mouse inoculation, detects even small numbers of organisms, even in the presence of contaminants.

Where a PCR facility and technical know-how is available, stale material containing small numbers of organisms can still be used. However, PCR will pick up only the target organism for which the test is designed, and will fail to detect any related serotypes, or other bacteria, such as *Bacillus anthracis*, which are likely to cause similar sudden death syndromes.

Rapid serological tests such as AGPT using the 56°C/30 minute supernatant as antigen and counter-immunoelectrophoresis detect the capsular serogroup, but not the specific somatic type. The hyaluronidase test is also specific for *P. multocida* B:2 strains that cause HS, and serves as an additional complementary test.

Other molecular tests, besides PCR, such as ribotyping, REA and FAGE, are useful for identifying strains within serotypes. They are therefore useful as markers in epidemiological studies and as research and investigatory tools rather than as routine diagnostic tests.
Overview
Haemorrhagic septicaemia (HS) is classified as a List B disease by the Office International des Epizooties. Control and treatment of the disease are therefore an important issue in countries where the disease is endemic.

Treatment
Treatment with antibiotics is of limited value unless carried out in the very early stages. Most field cases are not detected in the early stages, making treatment ineffective. In an organised farm incontact animals can be checked regularly and animals showing an increased temperature can be separated and treated with antibiotics.

Prevention and control
Vaccination is the most effective method of control, in conjunction with other measures. Three broad categories of activities are recommended for prevention: prophylactic measures in endemic countries; measures needed in the event of an outbreak; and measures needed to prevent the spread of disease from endemic to nonendemic areas.

Eradication
Eradication of haemorrhagic septicaemia is difficult to achieve because of the existence of latent carriers.

6.1 International Classification
As described in Section 1.4.1, the Office International des Epizooties (OIE), or World Organisation for Animal Health, has classified haemorrhagic septicaemia (HS) as a List B disease (along with most other bacterial and parasitic diseases that are considered to be of socioeconomic and/or public health importance within countries, and also of significance to the international trade in animals and animal products). List A diseases, on the other hand, are mainly viral diseases, and include those that spread rapidly and have the scope to spread beyond national borders. The classification presumably influences the priority status given to the disease by governments in endemic countries and the allocation of resources for treatment and control.

6.2 Treatment
As HS is a primary bacterial disease with no other biological agents involved, treatment may appear simple using the wide range of antibiotics currently available. In reality, however, treatment is constrained by a host of practical considerations. It has been found in practice that animals can only be cured if they are treated in the very early stages of the disease. However, as the disease occurs mainly in situations with primitive husbandry practices most field cases escape detection in the early stages, thus rendering treatment ineffective.

In organised farms, however, a practical method of achieving early detection and successfully treating animals is to check the rectal temperatures of all incontact animals regularly once an outbreak has been detected. Any animals showing an increased temperature can be separated and treated with a course of an appropriate antibiotic.

Although not documented, there is considerable information from reports of field outbreaks indicating
that antibiotic treatment in the terminal stages accelerates death. This may be because administration of antibiotics to an animal with septicaemia precipitates an endotoxin shock due to release of free endotoxin from the killed bacteria.

### 6.2.1 Antibiotic therapy

The oldest therapy recommended was intravenous treatment with sulfonamides. Intravenous infusion of sulfadimidine sodium 33.33% at a dosage of 1 mL per 5 pounds bodyweight (about 2.3 kg) has been practised. However, the large volume of drug to be injected, the practical difficulties of intravenous therapy in the type of animal involved and the consequences of leakage of the drug into the surrounding tissues all weigh heavily against this treatment regimen (De Alwis 1984). De Alwis (1984) observed that intramuscular treatment with streptomycin or oxytetracycline was very effective.

Resistance to antibiotics in HS strains of *P. multocida* has not been a major problem. When 10 strains obtained from Malaysia, Indonesia, Thailand, Myanmar, India and Sri Lanka were tested against 10 commonly used antibiotics (penicillin, ampicillin, streptomycin, tetracycline, chloramphenicol, erythromycin, neomycin, sulfadiazine and a sulfonamide–trimethoprim combination), no resistance was recorded apart from partial resistance of the Thai strains to streptomycin (De Alwis 1984).

Bandopadhyay et al. (1991) tested 16 isolates obtained from different outbreaks in the Gujarat State (India) against 19 antibiotics. They found that 14 isolates (87.5%) were resistant to compound sulfonamides. Resistance was also observed to vancomycin (75%), tetracycline (50%), colistin (37.5%), streptomycin and novobiocin (25%), and nitrofurantoin and carbenicillin (6.25%). All isolates were sensitive to penicillin, ampicillin, chloramphenicol, erythromycin, gentamicin, caphalothin, polymyxin B and tilmicosin.

In an outbreak of disease in pigs in India caused by the HS serotype B:2, isolates were found to be sensitive to erythromycin, gentamicin, nalidixic acid and neomycin (Verma 1988). In Pakistan, from the results of a questionnaire survey, it has been concluded that preparations containing amoxicillin, sulfapyrazole, gentamicin and chloramphenicol produced positive clinical results (Sheikh et al. 1996). Abeynayake et al. (1992) tested 27 Sri Lankan isolates against 17 antimicrobial agents. These were mainly strains derived from field outbreaks from cattle and buffaloes during the period 1985–90. Also included were a few strains isolated from clinically normal carrier animals, isolates from species other than cattle and buffaloes, and national reference strains. All of these were of serogroup B:2. All strains tested were sensitive to penicillin, ampicillin, enrofloxacin, chloramphenicol and nitrofurantoin. A majority of isolates were also sensitive to neomycin (26/27), gentamicin (26/27), oxytetracycline (25/26), streptomycin (24/25) and sulfonamide–trimethoprim (25/27). Variable degrees of resistance were shown to some antibiotics, the number of sensitive strains being oxacillin (17/27), spiramycin (16/27), clindamycin (17/27) and sulfamethoxazole (12/27).

Thus, of the antibacterial compounds that are recommended for use in cattle and buffaloes, and that can be conveniently and economically administered, penicillin, ampicillin and oxytetracycline appear to be the most useful. The sensitivity patterns, however, are likely to vary from one country to another depending on the prevailing drug usage practices; a knowledge of the sensitivity patterns of local strains will be useful in deciding the drug of choice. It is interesting to note the observation of Bain et al. (1982) that while penicillin was effective against the organisms in vitro, it was ineffective in vivo, a point that must be noted in the choice of an antibiotic.

### 6.2.2 Serum therapy

Serum therapy is only of theoretical interest. Kheng and Phay (1963) used 60–100 mL of hyperimmune serum for experimental administration to two-year-old (400 lb [about 180 kg]) buffaloes at varying periods from six hours before to 18 hours after infection. No significant therapeutic effect was recorded.
6.3 Prevention and Control

There are three categories of measures for prevention and control of HS:

- measures to be adopted in endemic countries on a prophylactic basis;
- measures to be taken in the event of an outbreak; and
- measures necessary for prevention of spread across regional or national borders.

6.3.1 Prophylactic measures in endemic countries

Taking into consideration available information on the nature of the disease, the organism, its survival outside the animal, resistance to external agents and relevant epidemiological parameters, the preventive measures shown below should be taken within a country or region where HS is endemic.

- **Vaccinate on a routine prophylactic basis.** Vaccination is best done two to three months before the high-risk season (in areas where seasonality occurs) so as to ensure peak immunity during the period of maximum risk.

- **Establish a good reporting system.** This will enable information on suspected outbreaks to reach animal health authorities as quickly as possible. In most endemic countries HS is listed as a notifiable disease.

- **Create awareness of the disease among farmers.** Educate farmers to recognise signs of the disease.

- **Prevent mixing of animals from endemic and nonendemic areas.** In endemic areas, a significant proportion of animals are latent carriers and are potential sources of infection. In nonendemic areas, animals are not regularly exposed to infection, lack naturally acquired immunity, are not usually vaccinated, and are highly susceptible. An outbreak originating from an activated carrier in such an instance can be explosive. If contact between such animals is unavoidable, it is of utmost importance that susceptible animals from nonendemic areas are vaccinated at least two weeks before contact with animals from an endemic area.

6.3.2 Preventive measures during an outbreak

In the event of an outbreak of disease occurring, there are equally important measures that should be taken to control further spread.

- **Continue vaccination programs.** Vaccination is recommended even in the face of an outbreak. In such situations broth bacterins or the alum-precipitated (or aluminium hydroxide gel) vaccine is preferred. Broth bacterin and the oil adjuvant vaccine may be administered at different sites, simultaneously.

- **Isolate and treat animals showing clinical signs with a parenteral broad-spectrum antibiotic** (relatively easy in organised farms or herds that are paddocked at night or for part of the day).

- **Check the rectal temperature of all immediate incontact animals in the herd.** This should be done at least once every morning; those animals showing increased temperatures should be treated as above.

- **Search daily for sick animals or carcases of dead animals** (free-roaming, nomadic herds).

- **Confine herds as much as possible, and prevent movement of animals in and out of diseased premises or villages.**

- **Take immediate action to carry out postmortem examinations (local veterinarians). Make a tentative diagnosis.**

- **Dispatch specimens to the nearest diagnostic laboratory.** Specimens should be stored and transported under appropriate conditions (see Chapter 5).

- **Dispose of carcases of dead animals properly.** Deep burial or effective incineration is recommended. Often, after animals die, carcases are disowned by farmers. Stray dogs and other scavenging animals can disseminate infection by carrying away portions of infected carcases, and carcases dumped into streams and waterways are important sources of infection.

- **Properly dispose of unconsumed fodder, bedding etc. from infected premises.** Deep burial or drying and burning should be carried out within the
premises. Effluent from cattle sheds, dung etc. should be prevented from being washed away from the premises. Drains carrying such material should be led into a deep protected pit within the premises, or subjected to disinfectant treatment.

- **Closely monitor or stop rain-associated activities.** Activities such as ploughing and preparation of fields for rice cultivation cause considerable movement of draught cattle and buffaloes. HS often breaks out during wet seasons.

### 6.3.3 Prevention of spread across borders

Where animals are moved from an endemic country or region to a nonendemic country or region, such as during imports, certain practical procedures can be observed to eliminate or at least minimise the transfer of the disease.

- **Ensure that the animals originate from a region where no outbreaks of HS have occurred for a minimum period of one year.** How extensive this disease-free ‘region’ should be will depend on the system of management practised in the region. If animals are confined to farm premises, the region may be as narrow as a radius of half a kilometre. On the other hand, where the animals are on a free-range system, the radius of the disease-free zone may have to be several kilometres.

- **Bleed a random sample of animals.** This should include the herd or farm of origin and/or other incontact animals.

- **Test for the presence of antibody by the IHA test.** The presence of high indirect haemagglutination (IHA) antibody titres is an indication of recent exposure to disease and therefore the presence of disease-causing agent in the locality.

- **Hold animals under observation for two to three weeks before transport.** During this time repeated attempts should be made to check nasopharyngeal swabs for pasteurellae by mouse inoculation and culture. Blood collected at the beginning and end of such a period should be checked for antibody by the IHA test. Animals harbouring type B pasteurellae and/or showing IHA titres should be eliminated. The detection of any carriers or animals with antibody titres would justify an extended period of pretransport observation for other incontact animals in the group.

- **Quarantine animals after transport to the new location.** The animals can be held for a similar period of time to that used before transport. During this time, the same procedures should be carried out.

- **Vaccinate animals from disease-free locations in endemic countries.** A dose of oil adjuvant vaccine may be given at the end of the quarantine period, followed by a booster three months later. It is equally important to vaccinate all animals in the country of import that are likely to come into contact with animals introduced from endemic countries or regions.

Vaccination is the most important control procedure adopted in all countries where the disease is endemic. Details of the vaccines in use and vaccination procedures are given in Chapters 7, 8 and 9.

### 6.4 Eradication

No country has ever attempted to eradicate HS. This reflects the belief that the existence of carriers makes eradication too hard. This has been strengthened by recent findings indicating that a larger proportion of animals are carriers than was originally thought and that for most of the time the disease remains latent. The existence of carriers among feral ruminants may be a further factor that will make eradication difficult.

The only known attempt at eradication was made by the Government of Indonesia on a pilot scale on the island of Lombok with a cattle population of 300 000. The program started in 1978 with intensive vaccination campaigns that were targeted to achieve the highest possible coverage in all susceptible species. The coverage actually achieved over a three-year period was 89% in cattle, 94% in buffaloes, 82% in goats, 93% in sheep and 80% in pigs. The program was evaluated in 1981, after three annual vaccinations using the oil adjuvant vaccine. During this period, 53 cases of HS were reported. Culture of pharyngeal mucous membrane samples from 220 animals slaughtered in an abattoir yielded positive isolations.
from five animals (2.2%). Mass vaccination was continued, and a second evaluation was made in 1985. A total of 450 abattoir samples from cattle, buffaloes, goats and pigs were negative. Also, 103 specimens from animals suspected of HS were negative. This island was declared free of the disease in 1985. However, subsequent evaluations based on culture, serology and field reports indicate that HS is still present in Lombok (Darmadi 1991; Syamsudin 1993).

These observations provide useful indicators of the complexities involved in attempting a total eradication program for HS even on a small island; the implications are much more complex in countries with land borders and a large wildlife population.
Chapter 7

Vaccines

Overview

Vaccine production
Vaccine production consists of four important steps: selection and maintenance of seed culture, production of bulk dense culture, inactivation of dense culture and formulation of vaccine. Simplified methods of vaccine production as well as sophisticated technologies are available.

Types of vaccine
The types of vaccine used against HS are bacterins, alum precipitated vaccine, aluminium hydroxide gel vaccine, and oil adjuvant vaccine.

Quality testing
All HS vaccines have to be tested for purity, stability and sterility. Potency tests are usually carried out in mice. The production technique must be validated in an experiment using natural host animals.

7.1 Vaccine Production

Haemorrhagic septicaemia (HS) is preventable using vaccines containing the causative bacterial agent. However, pasteurella is a poor immunogen and a large amount of antigen (usually whole bacteria) therefore has to be injected. This procedure occasionally leads to endotoxic shock. One way of overcoming this problem is to use a suitable adjuvant. Adjuvants potentiate the action of the immunogen, and provide a depot effect, delaying absorption by a slow release mechanism that simulates the administration of multiple doses.

HS vaccine production involves the following stages:

- selection and maintenance of a seed culture;
- preparation of a bulk dense culture;
- inactivation of the dense culture; and
- formulation of the vaccine.

7.1.1 Selection of a seed culture

It is generally agreed that vaccines produced from fresh field isolates are more effective than those produced from seed cultures propagated in vitro in laboratories over long periods. Most countries have therefore used local isolates as seed culture. Bain (1979b) carried out active protection tests in mice using Pasteurella multocida propagated in bovine cells and as laboratory subcultures. Bacteria grown in vivo gave cross-protection between Asian strains (B:2), African strains (E:2) and the Australian strain 989 (B:3,4), whereas laboratory subcultures gave only homologous protection.

There has been some speculation as to whether strains exist in nature that have special immunogenic merit. The Burmese 'Katha' strain and the Indian P52 strain have been used even in countries other than that of their origin because they were believed to have special immunogenic properties. In Malaysia, vaccines were
produced from five different strains, derived from five regions within the country, presumably as a safeguard in case different strains had different immunogenic properties, although there is no evidence for this. De Alwis (1984) carried out a comparative study of strains originating from Malaysia, Indonesia, Thailand, Myanmar, India and Sri Lanka. He grew strains under the same conditions and then immunised mice with suspensions of bacteria standardised on a dry weight basis to examine cross-protection. The results showed that the density of growth, and therefore the dry weight yield of whole bacteria, varied between strains and that no consistent immunogenic differences were demonstrable when the mice were challenged with a local isolate. On the basis of dry weight yield, the Malaysian strain tested (C 82) consistently gave the highest yields.

The variation in dry matter yields when different seed strains are grown under the same conditions was also shown in the study of Arawwawela et al. (1981) in Sri Lanka. It is likely that strains that are well capsulated and therefore contain a full complement of antigens give higher dry matter yields, and may therefore be better immunogenic strains for vaccine production.

Considering the above information, it appears that it is best to select as the vaccine seed an isolate of known serotype designation which has been tested and proven to be immunogenic and which gives high dry weight yields. Once a relationship between dry weight and turbidity of growth is established, turbidimeter readings can be used as an index of dry weight (see Appendix 7).

### 7.1.2 Storage and maintenance of seed cultures

Once the seed culture has been selected, it is good practice to passage it in a natural host (a calf) at least once a year. Before calf passage, the LD50 for mice can be determined to ensure its virulence. It is best to collect infected blood from either the heart or the main blood vessels two to three hours after death; by this time there will have been a reasonable multiplication of the pasteurella without postmortem invaders. In practice, this procedure is difficult because the time of death of the infected animal is unpredictable and may occur at night. An alternative practical method is to collect blood from the jugular vein of the dead calf, culture on a suitable medium, pick one or two well capsulated colonies and inoculate them onto defibrinated ox blood or ox blood collected aseptically into a sterilised vessel containing sodium citrate or ethylenediamine tetraacetic acid (EDTA) as anticoagulant. The ox blood should not contain HS antibodies. The inoculated blood is incubated for six to eight hours (or even overnight). A smear is examined for purity and for the presence of pasteurella and the blood is dispensed in 1-mL aliquots into small bottles that are screw-capped and stored in a deep freezer below -20°C or in liquid nitrogen. Alternatively, the blood may be divided into 0.2-0.5-mL aliquots, lyophilised and stored at 3–8°C in a refrigerator. Most laboratories stock a year's supply and the process is repeated each year. For each batch of vaccine, a new seed vial, or one to two subcultures in a blood-containing medium, should be used.

### 7.1.3 Bulk culture media

Various media have been developed in different countries to grow dense cultures. Dense suspensions of bacteria can be obtained by growing the cultures in a solid agar medium in Roux flasks and harvesting the bacteria in physiological saline. This is a laborious process and before harvesting and pooling each flask has to be examined, at least visually, for contaminants. The method has therefore not been popular, although it has been used in India and Iraq (FAO 1979). In India only 2.8% of the total vaccine production uses the agar-wash method.

Liquid media are the most commonly used, because they are less time consuming to process. Liquid media consist of a protein as a source of amino acids (digest of casein, beef extract etc.), a fermentable sugar, and growth promoters such as yeast extract or autodigest of pancreas etc., in a phosphate buffered medium. Examples of liquid media are given in Appendix 7.

In Sri Lanka, Arawwawela et al. (1981) have produced a simple and economic medium for dense culture production. They found that the protein content of the medium could be reduced to one-tenth of the quantity used before without significantly affecting growth; that yeast extract was highly effective in increasing the yields; and that the presence of buffer and the addition of sucrose (or refined cane sugar) increased yields. The study led to production of a medium that cost about one-third as much as the conventional medium previously used.
7.1.4 Culture methods

Vaccine production requires bulk production of dense cultures. Either liquid or solid media can be used for this purpose, but seeding and harvesting from solid media are neither practical nor economical in large-scale production. Consequently, the following sections focus on liquid culture. Different workers have used different methods to suit the scale of operation within the available facilities, but it is important to maintain an optimum temperature and pH, and a steady supply of air through an aeration process. The main aeration methods used are vortex aeration and sparger aeration.

Vortex aeration

This method uses a vortex tank. A compressor (usually around 0.5 hp) delivers a stream of air at 10–15 pounds pressure through a filter candle or any modern type of commercial inline filter. This stream of sterilised air is passed through the vortex tank, which contains only around 50% of its volume of liquid medium with the necessary nutrient factors for optimal growth and which is sufficiently buffered to minimise pH changes. Rotation of a propeller connected to a motor at the bottom of the tank produces a vortex current in the liquid; the air passes through the vessel, but not through the liquid medium.

The tanks are sterilised with the medium and the air sterilisation assembly in an autoclave at 120°C for about 45 minutes. They are seeded by injection through a rubber diaphragm on the side of the upper portion of the tank. It is best to seed the cultures after aeration has started, so that the positive pressure within the tank prevents air from being sucked in and contaminating the culture. The entire tank is placed in a 37°C incubator room.

Contamination can occur if the tank is understerilised or if air leaks through the seals at the point of entry of the propeller shaft. The former is easily overcome by adequate sterilisation; the latter is more difficult to overcome, because the air in the 37°C room is normally highly charged with bacteria and even a small volume of air leaking in can provide a significant dose of contaminants when the seed culture is introduced. Such contamination can be minimised by building up a positive pressure within the tank before seeding.

Sparger aeration

In sparger aeration systems, a current of sterile (filtered) air is dispersed through the liquid medium using a filter candle, a block of pumice stone or a fish tank aerator. It is the experience of the author that the finer the dispersion of the air, the better the growth. In such systems, a propeller arrangement may be used to churn the liquid, but it is better to avoid this: a vigorous sparger aeration itself will churn the medium sufficiently.

One of the problems with sparger aeration is the development of froth, which tends to clog the air outlets. Various antifoam agents are available but there is little information on the effect of such agents on the density and quality of growth. Alcohols, oils and silicones may be toxic or inhibitory to growth. Silicones and esters of fatty acids are effective and nontoxic, but limited experiments by Bain (unpublished) indicated that sparger cultures treated with silicone or linseed oil were less immunogenic than cultures prepared using other methods. More information in this regard is required.

Two common bulk culture methods use sparger aeration. One uses a simple culture system with a large vessel and the other makes use of a commercial ‘fermentor’ as the culture vessel and requires more specialised procedures and trained personnel.

Simple culture system. A simple and effective sparger aeration system has been developed in Sri Lanka. A 40-litre vessel containing the medium is placed in a water bath at 37°C, thereby eliminating the need to use an incubator room. Sparger aeration is controlled and excessive froth is collected in a froth trap, while outflowing air is bubbled through a formalin trap. A detailed description of this equipment and its use, which is within reach of even the most modest vaccine-producing laboratory, is described in Appendix 7. The equipment is simple enough for use in a laboratory with very modest facilities. It has, for example, been installed and used successfully in a vaccine laboratory in Cambodia, where it is housed in a shipping container 40 feet long with a width and height of 8 feet (about 12 x 2.5 x 2.5 metres). Bacterial yields of at least 1.5 mg/mL dry weight can be obtained in this system, and the yield compares favourably with that obtained in a sophisticated fermentor.
**Fermentors.** Many Asian countries now make use of industrial fermentation equipment to produce bulk cultures. A fermentor provides a bulk culture vessel in which a constant temperature, pH and oxygen tension can be maintained throughout the growth period with churning of the medium and aeration. It is a closed system, where the medium is sterilised and cooled to 37°C in situ and where seeding can be done and samples withdrawn for testing at any stage without opening the vessel.

Commercially available fermentors range in working capacity from 50 to 500 litres and allow control of temperature, oxygen pressure etc. The manufacturer can build in any special features. A detailed description of the use of a fermentor for vaccine production is given in Appendix 7.

Any laboratory purchasing a fermentor should first determine precisely the specific requirements and scale of operation. It is vital that spare parts, servicing and maintenance facilities are available as well as the general infrastructure facilities needed to operate the fermentor. These factors should be taken into account when preparing specifications for a fermentor. It is also important to train the technicians before a fermentor is purchased.

**7.1.5 Continuous and batch cultures**

Bulk cultures may be produced by batch culture or continuous culture using the simple sparger aeration system or a fermentor. For batch culture, fresh medium and fresh seed is used for every batch. For continuous cultures, a predetermined volume of harvest is removed, and an equal volume of fresh medium introduced aseptically once the maximum turbidity is reached. Generally, around 25% of the total volume can be harvested every hour and the process can go on for several days unless contamination sets in. In a sense, continuous culture is a form of repeated subculture from the original seed, which is grown in blood. It is the experience of the author that when the process is continued for more than two to three days, the density of the harvest begins to diminish, presumably through loss of capsular material. Batch cultures are therefore preferred.

Contamination occurs with most systems; one method of avoiding it is to use a relatively large inoculum and a short incubation. Batch cultures inoculated at 50 mL per litre of medium reach a maximum turbidity in 15–18 hours, at which time growth can be terminated and the entire batch harvested. This enables the pasteurellae to grow rapidly and overcome any contaminants that may be present in small numbers.

**7.1.6 Inactivation of dense cultures and production of vaccines**

Cultures produced in the way described above are inactivated by the addition of 0.5% formalin (36–40% formaldehyde solution). Once a dense suspension of inactivated bacteria is obtained, it is used to prepare one of the four types of vaccine used against HS (see Section 7.2).

**7.2 Types of Vaccine**

There are four different types of vaccine used against HS: broth bacterins; alum precipitated vaccine; aluminium hydroxide gel vaccine; and oil adjuvant vaccines.

**7.2.1 Bacterins**

Bacterins are the simplest form of vaccine and consist of a suspension of whole cells. The common bacterins consist of inactivated broth cultures and are referred to as broth bacterins. Rarely, agar wash bacterins are produced by harvesting the growth from agar plates or from Roux flasks.

If dense, formalinised suspensions are injected, shock reactions occur in a small percentage of animals, which is presumably due to free endotoxin present in the preparation. The formalinised bacterial suspension (bacterin) should therefore be diluted so as to contain not more than 0.5 mg bacteria (dry weight) per mL and a 3-mL dose may be administered. Another disadvantage is that the antibody response to plain bacterin is poor and only provides rapid immunity for about six weeks. Repeated booster doses therefore need to be given.