While false positives may occur with isolates from pneumonic cattle and pigs, *P. multocida* and *P. canis* biovar 2 can be distinguished on the basis of indole and mannitol fermentation.

Primer sequences

KMT1SP6 5' – GCTGTAAACGAACTCGCCAC – 3'

KMT1T7 5' – ATCCGCTATTTACCCAGTGG – 3'

PCR analysis

The 25-μL PCR reaction mixture consists of a template of DNA (<1 colony, 1 μL culture, or 5 μL boiled culture supernatant), 3.2 pmol of each primer, 200 μM of each dNTP, 1 X PCR buffer containing 2 mM $MgCl_2$ and 0.5 Units Taq DNA polymerase with oil overlay.

The thermal cycling parameters are as follows: initial denaturation at 94°C for 5 minutes; 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and a final extension of 72°C for 9 minutes.

Analysis of PCR products

Amplified products are separated by electrophoresis in a 2% agarose gel in 1 × TAE at 4 V/cm for 1 hour, stained with 0.1 μg/mL ethidium bromide and visualised by UV illumination.

Detection of *P. multocida-specific* DNA in turkeys

Kasten et al. (1997) have described detection of *P. multocida*-specific DNA in turkey flocks using PCR. *P. multocida* is known to contain a gene known as psl (for P6-like) that encodes a protein unique to *P. multocida* and *Haemophilus influenzae*. Because *H. influenzae* is not normally isolated from poultry, it was proposed that a PCR assay based on this gene could be used to detect *P. multocida* from oropharyngeal samples obtained from these birds. While this hypothesis is correct, the identification of *H. influenzae* limits the assay to veterinary specimens and excludes its use in human diagnostic pathology. A further limitation is that achieving the specificity of 10 *P. multocida* organisms (24 fg purified DNA) requires hybridisation of membrane-bound PCR products with the psl gene. Although the number of laboratories with PCR technology is increasing in HS-endemic countries, routine diagnostic laboratories in these countries are unlikely to have the equipment required for Southern hybridisation.

Primer sequences

(includes BamHI sites at each end)

FWD
5' – TCTGGATCCATGAAAAAACTAACTAAAGTC – 3'

REV
5' – AAGGATCCTTAGTATGCTAACACAGCACGACG – 3'

PCR analysis

The PCR reaction mixture consists of 5 μL template DNA, 25 pmol of each primer, 2.5 mM of each dNTP, 1 × PCR buffer containing 1 mM $MgCl_2$ and 1.25 Units Taq DNA polymerase.

The thermal cycling parameters are: initial denaturation at 94°C for 2 minutes; followed by 35 cycles of 94°C for 1 minute, 50°C for 30 seconds, and 72°C for 3 minutes; and a final extension of 72°C for 7 minutes.

Analysis of PCR products

Amplified products are separated by electrophoresis in a 2% agarose gel in 1 × TAE, at 74 V for 2 hours, stained with ethidium bromide and visualised by UV illumination.

C. PCR assay for HS-associated type B serotypes of *P. multocida*

Townsend et al. (1998) described the development of PCR assays for species- and type-specific identification of *P. multocida* isolates (see above). Oligonucleotide primers designed during the sequencing of an HS-associated type B specific clone isolated by genomic subtractive hybridisation formed the basis of a type-specific PCR assay for the detection of HS-associated type B serotypes of *P. multocida*. The primer pair KTSP61–KTT72 specifically amplifies a product of approximately 560 bp in all *P. multocida* isolates possessing the type B capsular antigen and either type 2 or 5 as the dominant somatic antigen. The clone, originally isolated from a reportedly avirulent HS culture, demonstrated significant nucleotide sequence identity to a region in the *H. influenzae* Rd genome flanking DNA sequences tenuously associated with bacteriophage Mu genes. While this proposed identity indicates that there is potential for cross-reactivity, evidence of nonspecific amplification has not been observed. At present, the assay retains 100% specificity for HS-associated serotypes of *P. multocida* following examination of a diverse range of bacterial species.

Primer sequences

KTSP61 5' – ATCCGCTAACACACTCTC – 3'

KTT72 5' – AGGCTCGTTTGGATTATGAAG – 3'

PCR analysis

The 25-µL PCR reaction mixture consists of the following: template DNA (< 1 colony, 1 µL culture, or 5 µL boiled culture supernatant), 3.2 pmol of each primer, 200 µM of each dNTP, 1 × PCR buffer containing 2 mM $MgCl_2$ and 0.5 Units Taq DNA polymerase with oil overlay.

The thermal cycling parameters are as follows: initial denaturation at 94°C for 5 minutes; followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and a final extension of 72°C for 9 minutes.

Analysis of PCR products

Amplified products are separated by electrophoresis in a 2% agarose gel in 1 × TAE at 4V/cm for 1 hour, stained with 0.1 µg/mL ethidium bromide and visualised by UV illumination.

The HS-PCR method can also be used in a multiplex reaction with the PM-PCR (described above in *P. multocida*-specific PCR assays). To achieve comparable intensities of amplified products from both PCR assays, the concentration of the HS-PCR primers should be doubled to 6.4 µM. All other parameters are as previously described for each individual assay. The multiplex PM/HS-PCR assay will provide a rapid, efficient and sensitive method for the specific detection of HS-associated type B serotypes of *P. multocida*.

D. PCR assay for the gene region associated with the pathogenicity of *P. multocida* serotype B:2

Brickell et al. (1998) have developed a PCR test based on a gene region associated with the pathogenicity of *P. multocida* serotype B:2, the causal agent of HS in Asia. Primers designed from a 16S–23S rRNA intergenic spacer region PCR product unique to type B:2 *P. multocida* formed the basis of a PCR assay for diagnosis of HS in cattle and buffalo. While this assay generally appears to identify type B:2 P. *multocida*, inconsistent correlation between PCR amplification and serological designation indicates that further optimisation is required before the assay is incorporated into routine diagnosis in HS-endemic countries. The assay cannot be regarded as 'B:2 specific' since a product was amplified from isolate 0350 (E:2). Nor can it be viewed as 'HS-specific', as 100% detection of all HS-associated serotypes was not demonstrated.

It is also difficult to support the suggestion that sequences adjacent to the unique fragment will identify the phage insertion site and phage-associated virulence determinants if the genetic organisation of this region in *P. multocida* is similar to that of *H. influenzae* Rd. The associations between the *H. influenzae* Rd predicted coding regions and the Mu proteins are tenuous, and do not appear to encode a complete prophage. It is therefore unlikely that any hypothetical virulence-associated determinants once associated with the Mu phage would have survived the integration into the *P. multocida* genome intact. There is thus no evidence for the claim that this region is in any way virulence associated.

Primer sequences

IPFWD 5' – CGAAAGAAACCCAAGGCGAA – 3'

IPREV 5' – ACAATCGAATAACCGTGAGAC – 3'

PCR analysis

The 20-µL PCR reaction mixture consists of: template DNA (1 µL bacterial culture), 0.25 µM of each primer, 200 µM of each dNTP, 1 × PCR buffer containing 1.5 µM $MgCl_2$ and 2.5 Units Tth DNA polymerase with oil overlay.

The thermal cycling parameters are as follows: 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

Analysis of PCR products

Amplified products are separated by electrophoresis in a 1.0% agarose gel in 1 × Tris-boric acid (EDTA), stained with 0.1 μg/mL ethidium bromide and visualised by UV illumination. Positive reactions should produce a product of 334 bp in size.

E. PCR fingerprinting of *P. multocida* by REP–PCR

Townsend et al. (1997a) have described repetitive extragenic palindromic (REP)–PCR analysis of *P. multocida* isolates that cause HS. Amplification of multiple genomic DNA fragments from *P. multocida* isolates by outwardly-directed primers based on the REP consensus sequence generates comparative profiles in a PCR-based fingerprinting method known as REP–PCR. A high degree of homogeneity was observed among HS strains of serotypes B and E, indicating evidence of a disease-associated REP profile that may serve as a novel method for the identification of HS-associated isolates regardless of serotype. REP–PCR profiles of other *P. multocida* serotypes were highly variable, illustrating the value of this technique for molecular fingerprinting in outbreaks of fowl cholera or atrophic rhinitis. This technique has recently been utilised in a study of *P. multocida* strains isolated from cases diagnosed as acute septicaemic pasteurellosis in Vietnam (Townsend et al. 1998), with all HS-associated serotypes again displaying homogeneous profiles.

Primer sequences

REP1R-IDt 3' – CGGNCTACNGCNGCNNNN – 5' (←)

REP2-IDt 5' – NCGNCTTATCNGGCCTAC – 3' (→)

PCR analysis

The 25-μL PCR reaction mixture consists of the following: template DNA (<1 colony, 1 μL culture, or 5 μL boiled culture supernatant), 6.4 pmol of each primer, 200 μM of each dNTP, × PCR buffer containing 4 mM $MgCl_2$ and 1.0 Units Taq DNA polymerase.

The thermal cycling parameters are as follows: initial denaturation at 95°C for 7 minutes; followed by 30 cycles of 94°C for 1 minute, 42°C for 1 minute, and 65°C for 8 minutes; and a final extension of 65°C for 16 minutes.

Analysis of PCR products

Amplified products are separated by electrophoresis in a 2.0% agarose gel in 1 × TAE at 2 V/cm for 3 hours, stained with 0.1 μg/mL ethidium bromide and visualised by UV illumination.

Appendix 5 DNA Fingerprinting Methods

Conventional techniques used to classify *Pasteurella multocida* have been reliant on the phenotypic characteristics of the organism, and have frequently been found to confound rather than clarify the relationship between strains. Molecular techniques have overcome the restrictions of phenotypic classifications by providing an insight into the genetic 'fingerprint' of each strain, and have been used successfully to distinguish phenotypically similar isolates.

Three methods are described in this appendix:

A. restriction endonuclease analysis
B. ribotyping analysis
C. field alternation gel electrophoresis

A. Restriction endonuclease analysis

Restriction endonuclease analysis (REA) involves the cleavage of genomic DNA by restriction endonucleases at specific nucleotide sequences, producing a set of DNA fragments that, when separated by electrophoresis, provide a characteristic banding pattern or fingerprint. Characterisation of the genome by REA has proved effective for accurate identification and epidemiology studies of *P. multocida* (Snipes et al. 1989; Kim and Nagaraja 1990; Wilson et al. 1992; Wilson et al. 1993). The method involves extraction and purification of genomic DNA, digestion with restriction endonucleases and analysis and visualisation of the DNA fragments ('fingerprint').

Equipment

Standard equipment

As for PCR (see Appendix 4)

Consumables/reagents

- Culture media
  - Sheep blood agar (SBA)
  - Tryptone soya broth (TSB)
  - Trypticase soy broth containing 1.25% tryptose (TST)
  - Heart infusion broth (HIB)
- Cetyltrimethylammonium bromide (CTAB)/NaCl solution
- 5 M sodium chloride (NaCl)
- Tris–EDTA (TE) buffer, pH 8.0
- Lysozyme (10 mg/mL)
- 10% sodium dodecyl sulfate (SDS)
- RNase (100 mg/mL)
- Proteinase K solution (20 mg/mL)
- Equilibrated phenol, pH 7.0
- Phenol–chloroform–isoamyl alcohol (25:24:1 vol/vol)
- Chloroform–isoamyl alcohol (24:1 vol/vol)
- Sodium acetate (3 M)
- Isopropanol
- Ethanol
- Restriction endonucleases and restriction buffers
- Tris–acetic acid–EDTA (TAE) or Tris–boric acid–EDTA (TBE) buffers
- Stop mixture (0.25% bromophenol, 0.25% xylene cyanole, 25% Ficoll 400)
- Agarose
- Ethidium bromide

Note: Details of buffers and solutions required are shown in Appendix 6.

Special equipment for extensive analysis

Computerised restriction fragment length polymorphism equipment for the comparative analysis of a DNA fingerprint database is needed. The database is created through the amalgamation of calibrated and standardised fragment data from multiple photographic images.

Comparison of DNA fingerprints and somatic serotypes of *P. multocida*

Wilson et al. (1992) compared DNA fingerprints and somatic serotypes of serogroup B and E *P. multocida* isolates using REA. This study remains the single most comprehensive characterisation of serogroup B and E isolates of *P. multocida* using both serologic typing and DNA fingerprinting, with *Hha*I and *Hpa*II determined to be the most informative restriction enzymes for fingerprinting analysis. Thirteen *Hha*I profiles were recognised among the 54 isolates designated as classic haemorrhagic septicaemia (HS)-causing *P. multocida* (serotype B:2 or B:2,5). All 13 serogroup E isolates had identical somatic serotypes and *Hha*I DNA fingerprint profiles; however, the *Hha*I profile did not match any fingerprint profile of the reference somatic serotype strains. DNA profiling with the *Hpa*II endonuclease allowed differentiation of the serogroup E isolates, with five distinct profiles observed. It is evident that the HhaI endonuclease yields discriminatory profiles among serogroup B strains; however, comparison among all *P. multocida* serotypes requires the use of both *Hha*I and *Hpa*II DNA profiles to provide definitive classification.

A rapid DNA extraction method was developed to allow the efficient processing of isolates, reducing the time taken to obtain 16 purified DNA samples to 2.5 hours. While discrimination of *Hpa*II and *Hha*I profiles could be accomplished with the naked eye, the use of computerised restriction fragment length polymorphism equipment enabled precise comparison of multiple genomic profiles with increased resolution of minor fragment differences.

DNA extraction

1. Inoculate a single colony into 5 mL of TST and incubate at 37°C for 18–24 hours.
2. Centrifuge a 1.5-mL aliquot of the 24-hour TST broth culture at 16,000 × g for 4 minutes.
3. Discard the supernatant and resuspend the pellet in 1 mL of TE, pH 8.0.
4. Centrifuge as above and decant all but 50 µL of the supernatant. The residual supernatant and pellet is stored at –70°C until required.
5. Thaw the frozen pellet by adding 350 µL of TE, and vortex to resuspend cells.
6. Add 150 µL of freshly prepared lysozyme solution (10 mg of lysozyme per mL of H_2O (preferably double distilled or sterile distilled) to the mixture and place on ice for 15 minutes to lyse the cells.
7. After lysis, add 40 µL of 10% SDS and mix for 1 minute, or until the suspension clears.
8. Add 8 µL of RNase solution (100 mg/mL) to the cleared suspension and mix by inversion for 1 minute.
9. Add 60 µL of proteinase K solution (20 mg/mL), mix and incubate at 37°C for 30 minutes.
10. Extract DNA by adding 0.8 mL of equilibrated phenol (pH 7.0) to the mixture. Invert vigorously until a white emulsion is formed, then centrifuge at 16 000 × g for 1 minute.
11. Transfer 600 µL of aqueous phase to a new microfuge tube containing 150 µL TE.
12. Add 0.7 mL of a 1:1 mixture of phenol (pH 7.0) and chloroform–isoamyl alcohol (25:1 vol/vol). Invert vigorously, then centrifuge at 16 000 × g for 1 minute.
13. Transfer 600 µl of aqueous phase to a new microfuge tube, and add 0.8 mL of chloroform–isoamyl alcohol (25:1 vol/vol). Mix by inversion and centrifuge at 16 000 × g for 1 minute.
14. Transfer 425 µL of aqueous phase to a tube containing 75 µL 3 M sodium acetate and mix briefly.
15. Add 1 mL of ethanol (25°C) and invert the tube several times before placing on ice for 10 minutes.
16. Pellet the precipitated DNA by centrifugation at 16 000 × g for 15 minutes; decant and discard the supernatant.

17. Air-dry the pellet or dry in a vacuum concentrator and suspend in 50 µL of TE.

Another method used for the recovery of high molecular weight DNA removes cell wall debris, polysaccharides and proteins by selective precipitation with CTAB. The CTAB–protein–polysaccharide complex precipitates pellets more effectively than residual protein–polysaccharides in other methods, and therefore will produce a cleaner DNA preparation.

1. Grow the bacterial strain of interest in a 5-mL liquid medium and conditions appropriate for that strain (for *P. multocida*, inoculate 5 mL of TSB or brain–heart infusion with the strain of interest and grow overnight at 37°C).

2. Spin 1.5 mL of culture in a microcentrifuge for 2 minutes and discard the supernatant.

3. Resuspend the pellet in 567 µL TE buffer by repeated pipetting. Add 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K to give a final concentration of 100 µg/mL proteinase K in 0.5% SDS. Mix thoroughly and incubate for 1 hour at 37°C. The solution should become viscous as the detergent SDS lyses the bacterial cells.

4. Add 100 µL of 5 M NaCl and mix thoroughly. It is important to keep the salt concentration above 0.5 M at room temperature to prevent the formation of a CTAB–nucleic acid precipitate.

5. Add 80 µL of CTAB–NACl solution. Mix thoroughly and incubate at 65°C for 10 minutes.

6. Add an approximately equal volume (0.7 to 0.8 mL) of chloroform–isoamyl alcohol, mix thoroughly and spin for 4–5 minutes in a microfuge.

7. Remove aqueous, viscous supernatant to a fresh microfuge tube, leaving the interface behind, and add an equal volume of phenol–chloroform–isoamyl alcohol. Extract thoroughly and spin in a microfuge for 5 minutes.

8. Transfer the supernatant to a fresh tube and add 0.6 volume isopropanol to precipitate the nucleic acids. Invert the tube several times until a stringy white DNA precipitate becomes visible.

9. Pellet the precipitate by spinning in a microfuge briefly at room temperature.

10. Wash the pellet with 70% ethanol to remove residual CTAB and spin for 5 minutes at room temperature. Carefully remove the supernatant and discard. Either air-dry the pellet or briefly dry in a lyophiliser.

11. Redissolve the pellet in 100 µL of TE buffer.

REA analysis

1. Genomic DNA is digested with each restriction endonuclease as recommended by the manufacturer.

2. Stop the reaction after 3 hours by adding 5 µL of stop mixture (0.25% bromophenol, 0.25% xylene cyanole, 25% Ficoll 400).

3. Electrophorese DNA fragments in a 0.7% agarose gel in 1 × TBE buffer for 17 hours at 2.4 V/cm.

4. Stain the gel with ethidium bromide, and visualise by UV illumination.

B. Ribotyping analysis

Ribotyping involves the use of Southern blot analysis to detect restriction fragment length polymorphisms associated with bacterial ribosomal operon(s). This method has proved valuable for the discrimination of *P. multocida* isolates of similar serotype, particularly among avian strains isolated from fowl cholera outbreaks (Blackall et al. 1995). While serogroup B and E isolates have been analysed by REA, this application is limited by its dependence on computerised analysis of restriction fragment length polymorphisms. The use of labelled ribosomal probes to highlight variation within ribosomal RNA (rRNA) genes simplifies the profiles obtained by REA, and allows visual comparison of banding profiles (Townsend et al. 1997b).

Equipment

Standard equipment

As described for REA, plus

- Hybridisation oven and bottles (or a 65°C circulating water bath)
- Boiling water bath
- PC2 laboratory with radioactive material safety

standards, and staff trained in the use and handling of radioactive materials (if radioactive labelling of DNA probe is required)

Consumables/reagents

- Culture media
  - Luria-Bertani media (for propagation of *Escherichia coli*)
- Ampicillin (or tetracycline, depending on plasmid)
- Nylon membrane (e.g. Hybond-N+, Amersham)
- Plasmid DNA extraction and purification kit (Promega, QIAGEN, Boehringer Mannheim) or use conventional methods (alkaline lysis/polyethylene glycol precipitation)
- For conventional plasmid purification:
  - glucose buffer (25 mM Tris pH 8.0, 50 mM glucose, 10 mM EDTA)
  - lysozyme solution (8 mg/mL in glucose buffer)
  - isopropanol
  - lysis buffer (0.2 M NaOH with 1% SDS, make fresh each time)
  - potassium acetate solution
  - phenol
  - chloroform
  - 3 M sodium acetate pH 7.4
  - TE
- Restriction endonucleases (according to plasmid/insert requirements)
- Nucleic acid labelling system (radioactive or non-radioactive) (e.g. Prime-a-Gene Labeling System, Promega)
- DIG-High Prime DNA labelling and detection kit (Boehringer Mannheim)
- Easytides™ dCTP [α-^{32}P] 370 Mbq/mL (DuPont, NEN Research Products, MA, USA)
- Salmon sperm DNA (type III sodium salt)
- Sephadex G-50 (fine)
- Spermidine
- X-ray film

Analysis of HS-causing isolates of *P. multocida* by ribotyping

Townsend et al. (1997b) have described the analysis of HS-causing isolates of *P. multocida* by ribotyping. Examination of ribosomal hybridisation profiles of *P. multocida* isolates confirmed the homogeneity of HS isolates originating from Asia described by Johnson et al. (1991), and provided greater discrimination than previous typing methods.

Classification of HS isolates by ribotyping can be generally correlated with geographical origin, with particular regard to Asian and North American HS strains. The limited ability of the phenotypic classification systems to identify individual strains is evident among Carter type E isolates, in which three distinct ribotypes were detected. Interestingly, ribotype classification of African HS isolates varied with restriction endonuclease; however, the degree of diversity remained the same. While the choice of restriction enzyme did not appear to influence Asian HS ribotype determination, *Pst*I ribotype analysis of Asian HS strains did reveal correlation with virulence, with the reportedly avirulent 0131 possessing a distinct profile. Ribotyping analysis also demonstrated discrepancies among the typing systems, as a lack of correlation between reportedly equivalent reference type strains was observed.

DNA preparation for ribotyping

Extraction and restriction enzyme digestion of DNA for ribotyping was performed as described for REA, above.

Southern blotting

1. Separate DNA restriction fragments by agarose gel electrophoresis (for DNA extraction, digestion and electrophoresis see above methods in REA). Soak agarose gel containing DNA fragments in 0.25 M HCl for 15 minutes, then in 0.4 M NaOH for 30 minutes with gentle shaking to depurinate and denature the DNA.
2. Transfer the DNA fragments to a nylon membrane (Hybond-N+, Amersham, UK), by either vacuum or capillary transfer. For vacuum transfer, a TE 80

Transvac™ Vacuum Blotter (Hoeffer Scientific Instruments, San Francisco USA) was used at 1–3 psi for 45 minutes. Capillary transfer is performed according to Sambrook et al. (1989). Briefly, place gel inverted on a support wrapped in wet blotting (3MM) papers in a container with transfer buffer: 10 × salt sodium citrate (SSC) or 10 × salt sodium phosphate/EDTA (SSPE); see Appendix 6. Surround, but do not cover, gel with Parafilm or Saran Wrap. Place a wet nylon membrane on top of the gel, making sure there are no air bubbles between the membrane and the gel. Wet two pieces of 3MM paper in 2 × SSC and place on top of the wet membrane. Place a stack of paper towels on top, and weigh down with a 500-g weight.

3. Following transfer, immerse the membrane in 0.2 M Tris-HCl pH 7.0/2 × SSC for 10 minutes to neutralise the membrane.
4. Air-dry the membrane, and use for hybridisation or store until required at 4°C, wrapped in plastic to prevent drying.

Alkaline lysis/polyethylene glycol (PEG) precipitation of plasmid DNA

1. Grow transformed *E. coli* (*E. coli* containing the desired plasmid) in 20 mL Luria broth (Luria-Bertani media) supplemented with appropriate antibiotic overnight at 37°C (use increased proportional volumes for large-scale preparation). Appropriate antibiotics are ampicillin (50 μg/mL of medium) or tetracycline (12.5 μg/mL of medium) depending on the location of the insert in the plasmid used.
2. Centrifuge cells for 10 minutes at 2000 × g.
3. Resuspend the pellet in 450 μL of glucose buffer.
4. Add 150 μL of lysozyme solution and incubate for 5 minutes at room temperature.
5. Transfer the solution to a 15-mL Corex centrifuge tube.
6. Add 1.2 mL of lysis solution (0.2 M NaOH/1% SDS), mix by inversion, place on ice for 5 minutes and mix.
7. Add 900 μL of ice-cold potassium acetate solution and mix.
8. Centrifuge for 10 minutes at 12 000 × g at 4°C.
9. Pour supernatant into a new Corex tube, being careful not to transfer any white pellet. Discard the pellet.
10. Add 1.5 mL of isopropanol and mix by vortexing.
11. Place in a –20°C freezer for 15 minutes.
12. Centrifuge for 15 minutes at 12 000 × g at 4°C.
13. Discard the supernatant and resuspend the pellet in 400 μL of TE buffer.
14. Transfer the solution to a 1.5-mL microfuge tube.

Plasmid digestion and purification of DNA probe

Digest the plasmid with appropriate restriction endonuclease to generate the fragment to be used as a DNA probe. Prepare and perform the digestion reaction according to the manufacturer's instructions. Following the reaction, electrophorese the sample in a 0.8% agarose gel made with 1 × TAE, containing 1% ethidium bromide. After electrophoresis, DNA fragments are visualised by UV illumination. Excise the fragment of interest and purify using either glass wool or a commercially available purification kit (e.g. QIAquick Gel Extraction Kit (QIAGEN) or BRESA-CLEAN™ kit (Bresatec Ltd).

Purify DNA fragments with glass wool, as follows.

1. Cut off the top of an Eppendorf tube, creating a tube approximately two-thirds of the original size.
2. Pierce a hole with a 19-gauge needle in the bottom of the tube, and insert a 3-mm glass wool plug in the bottom.
3. Place this tube into a second Eppendorf tube, and load the agarose pieces in the top tube.
4. Microfuge the tube at 6000 rpm for 10 minutes, collect the effluent and store at –20°C until required.

Random primer DNA labelling using α-^{32}P dCTP

cDNA probes are labelled using commercial random primer labelling kits (e.g. Promega's Prime-a-Gene® labelling system). All reagents are supplied at working concentrations and stored at –20°C as directed by the manufacturer. To prepare the nonradioactive dNTP mix, add together equal volumes of each of dATP, dGTP and dTTP. Before the labelling reaction, cDNA template is denatured at 95°C for 5–10 minutes, and then placed immediately on ice. The following reaction mixture is then prepared.

- 30 μL of denatured DNA (25 ng)
- 10 μL of 5 × labelling buffer (includes random hexadeoxynucleotides)
- 2 μL of unlabelled dNTP mix
- 2 μL of nuclease-free bovine serum albumin (BSA)
- 10 units Klenow fragment (2 μL)
- 40 μCi [α-^{32}P] dCTP (DuPont)

Incubate the reaction at room temperature for 1 hour, then terminate the reaction by adding 2 μL of 0.5 M EDTA. Before use, purify the radiolabelled probe using a Sephadex G-50 spin column prepared as follows:

1. Insert a 3-mm glass wool plug at the base of a 1-mL disposable syringe, and carefully fill the syringe with autoclaved Sephadex G-50 gel slurry avoiding the insertion of air bubbles.
2. Centrifuge the column encased in a 10-mL centrifuge tube at 1600 rpm for 4 minutes.
3. Discard the eluant, and again fill the column with Sephadex G-50 gel slurry.
4. Centrifuge at 1600 rpm for 4 minutes and discard the eluant.
5. At this stage, spin columns can be kept at 4°C with the tip submerged in 10 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA buffer (STE) and the other end sealed with parafilm.
6. To purify radiolabelled probes, first wash the spin column with 100 μL STE and centrifuge at 1600 rpm for 4 minutes.
7. Discard the eluant, and add 50 μL to the labelled probe, mix and then add the sample to the top of the column.
8. Centrifuge at 1600 rpm for 4 minutes and collect the labelled probe from the bottom of the 10-mL centrifuge tube.
9. Denature the eluted fluid at 100°C for 5 minutes, and chill on ice prior to hybridisation with membrane-bound DNA.

DNA:DNA hybridisation using a hybridisation oven

1. Pre-wet the DNA-bound membrane and mesh of similar size in a container with 2 × SSC.
2. Ensure that the membrane overlays the mesh, roll both into a roll and place into a hybridisation bottle (Hybaid Limited, UK).
3. Add 30 mL of 2 × SSC to the bottle and unwind the membrane/mesh by gently rolling the bottle along a flat surface. Release air bubbles trapped between the mesh and membrane by gently tapping the bottle in an upright position.
4. Remove the 2 × SSC and replace with 15 mL of hybridisation buffer (Appendix 6). Use 15 mL for a single membrane in a large bottle, 20 mL for two membranes. Incubate for 1–4 hours at 65°C in a Hybaid™ hybridisation oven (Hybaid Limited, UK).
5. Denature salmon sperm DNA by boiling for 5 minutes, and chill on ice for 7 minutes before adding to hybridisation fluid.
6. Denature the radioactively labelled probe by boiling, and cool for 5 minutes at room temperature. Then add to the hybridisation fluid and hybridise overnight at 65°C.
7. Following hybridisation, wash the membrane twice at 65°C in Wash solution (0.1 × SSPE/0.1% SDS) with vigorous shaking for 10 minutes.
8. Blot dry and wrap the membrane in clear plastic film ('cling wrap').
9. Analyse by autoradiography by placing the membrane and a sheet of Cronex IV Medical X-ray film against a Quanta III Cronex intensifying screen (DuPont) in an X-ray cassette.

DNA:DNA hybridisation using a water bath

(If a hybridisation oven is not available, DNA:DNA hybridisation can be performed in heat-sealable bags in a 65°C water bath.)

1. Prepare the prehybridisation solution (about 0.2 mL of fluid is required for each square centimetre of membrane).

2. Pre-wet the DNA-bound membrane in a container with 6 × SSC.

3. Slip the wet membrane into a heat-sealable bag and add the appropriate volume of prehybridisation solution. Squeeze as much air as possible out of the bag, and seal the open end of the bag with a heat sealer.

4. Incubate the bag for 1–2 hours submerged in a 65°C water bath.

5. Denature the DNA probe by boiling, and chill rapidly in ice water.

6. Working quickly, remove the bag from the water bath, and open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridisation solution and then squeeze as much air as possible from the bag.

7. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag.

8. To avoid radioactive contamination of the water bath, the resealed bag can be sealed inside a second, noncontaminated, bag. Incubate the bag submerged in the 65°C water bath overnight.

9. Following hybridsation, wash the membrane twice at 65°C in Wash solution (0.1 × SSPE/0.1% SDS) with vigorous shaking for 10 minutes.

10. Blot dry and wrap the membrane in clear plastic film ('cling wrap').

11. Analyse by autoradiography by placing the membrane and a sheet of Cronex IV Medical X-ray film against a Quanta III Cronex intensifying screen (DuPont) in an X-ray cassette.

C. Field alternation gel electrophoresis

Field alternation gel electrophoresis (FAGE) involves the analysis of large chromosomal DNA fragments (up to 10 megabase pairs in length). This is achieved by suspending and lysing bacterial cells within a solid support (agarose), which provides stability for the chromosomal DNA preventing it from shearing into small fragments. The method is capable of demonstrating heterogeneity throughout the entire bacterial genome, providing a complete picture of strain variation. Townsend and Dawkins (1993) published an extensive review of the technique and its applications, illustrating the versatility and increasing importance of FAGE in the field of molecular biology.

The combination of FAGE and restriction endonuclease digestion of chromosomal DNA can provide important information regarding genetic variability between strains, allowing the examination of DNA heterogeneity throughout the entire bacterial genome without the complexity of REA profiles. FAGE has also been used successfully to construct physical maps of bacterial genomes, and to provide definitive estimation of genomic size. However, the greatest worth of this technique is as an epidemiological tool to determine the clonality of outbreak strains, because FAGE analysis has demonstrated greater discrimination than ribotyping or REA. The spectrum of bacterial species examined by FAGE has increased dramatically in the past decade, and the technique has become an integral component of bacterial genetics and epidemiology.

Equipment

Special equipment:

- Pulsed field gel electrophoresis system (PFGE):
  - CHEF-DRII or DRIII with Pulsewave switcher (Bio-Rad) (preferred) or
  - Gene Navigator (Pharmacia LKB)

Consumables:

- Culture media (SBA and heart infusion broth [HIB])
- Agarose (low melt preparative grade, Bio-Rad)
- Agarose (ultra pure DNA grade, Bio-Rad)
- Molecular weight markers (Promega or Bio-Rad)
- Lambda concatemers or *Saccharomyces cerevisiae* chromosomes
- EDTA
- Lysozyme
- Chromosomal proteinase K buffer (see Appendix 6)
- Restriction endonucleases and buffers (low- to medium-frequency cutters such as *Apa*I, *Sma*I, or *Not*I)

- BSA (1 mg/mL)
- TAE or TBE
- Ethidium bromide

Analysis of HS-causing isolates of *P. multocida* by FAGE

Townsend et al. (1997b) described a method for FAGE analysis of HS-causing isolates of *P. multocida*. FAGE analysis of *P. multocida* isolates has confirmed the homogeneity of Asian HS-associated serotypes observed following examination of protein, lipopolysaccharides (LPS), ribosomal and repetitive extragenic palindromic (REP)–PCR profiles (Johnson et al. 1991; Townsend et al. 1997a; see Appendix 4). FAGE analysis also exhibited greater discriminatory power than ribotyping by further distinguishing North American isolates of similar ribotype. These cultures were thought to represent re-isolations from the original Buffalo 'B' strain following in vivo passage to retain virulence (Gochenour 1924). Studies by ribotyping (Townsend et al. 1997b) and REA (Wilson et al. 1992) have been unable to distinguish these isolates; however, FAGE analysis demonstrated relatedness but not identity between the cultures.

Preparation of agarose plugs for FAGE analysis

1. Grow bacteria to log phase in HIB at 37°C.
2. Pellet bacteria by centrifugation at 3000 rpm for 10 minutes.
3. Resuspend and wash the pellet in 0.05 M EDTA, pH 8.0.
4. Discard the supernatant, and resuspend with an equal volume of 0.05 M EDTA, pH 8.0.
5. Prepare molten 2.4% low melt temperature agarose (in 0.125 M EDTA, pH 7.5) and cool to 50°C.
6. Mix the agarose and cell suspension in a 1:1 ratio, pipette the mixture into a plug mould and allow to set at room temperature for 30 minutes.
7. Remove samples from the mould and place into chromosomal proteinase K buffer (0.5 M EDTA, pH 8.0; 0.001 M Tris, pH 8.0; 1% N-laurylsarcosine, 1 mg proteinase K/mL of buffer).
8. Incubate overnight at 50°C.
9. Wash the plugs with 0.05 M EDTA (pH 8.0) three times at room temperature for 30 minutes each.
10. Store the plugs at 4°C in 0.05 M EDTA, pH 8.0 until required.

Restriction digestion and electrophoresis

1. Rinse plugs twice in double distilled water for 30 minutes at room temperature.
2. Wash twice with 50 × volume of TE pH 8.0 for 30 minutes at room temperature.
3. Wash once in ice-cold double distilled water on ice for 30 minutes.
4. Wash once in 250 µL 1 × restriction buffer on ice for 30 minutes.
5. Add 40 µL restriction enzyme and 2 µL BSA (1 mg/mL) and stand on ice for 30 minutes.
6. Incubate overnight at an appropriate temperature for the enzyme.
7. Add 12 µL 0.5 M EDTA pH 8.0 to terminate the enzyme reaction.
8. Place plugs and molecular weight standards into wells of a 1% agarose gel, 0.5 × TAE.
9. Remove any trapped air bubbles and seal wells with 0.8% molten agarose.
10. Place gel in an electrophoresis chamber and equilibrate the gel and buffer (0.5 × TAE) at running temperature (14°C) for 30 minutes before the run.
11. Use the following conditions:

    – for 7–400 kilobases: 0.5–35.5-second switch ramp, 170 V, 24 hours

    – for 0.1–1 megabases:10–150-second switch ramp, 180 V, 24 hours
12. Following electrophoresis, stain the gel in 0.5 × TAE with ethidium bromide (1 µg/mL) for 30 minutes. Destain in double distilled water for 30 minutes, and then visualise with UV illumination.

Appendix 6 Preparation of Buffers and Solutions

When preparing and using buffers and solutions:

- use the highest grade of reagents available
- prepare all solutions with double-distilled, deionised water
- where possible, sterilise all solutions by autoclaving or by filtration.

Phosphate buffered saline (PBS)

For 1 litre, use:

NaCl	8.5 g
Na_2HPO_4	1.07 g
NaH_2PO_4 (anhydrous)	0.39 g

Make to volume with double distilled water; adjust pH to 7.2–7.4 and autoclave.

Citrate buffer (0.1 M)

For 1 litre use:

Citric acid	21.01 g

Make up to volume with double distilled water; adjust pH to 4.2 with 10 M NaOH and autoclave.

EDTA (0.5 M)

For 1 litre, use:

Ethylenediaminetetraacetic acid (EDTA).$2H_2O$ (MW 372.2)	186.1 g
NaOH pellets	~20 g
Double distilled water	800 mL

Adjust the pH to 8.0 with NaOH; make up to volume with double distilled water and autoclave.

Tris–EDTA (TE), pH 8.0

For 500 mL, use:

0.5 M EDTA, pH 8.0 (1 mM EDTA)	1 mL
1 M Tris-HCl, pH 8.0 (10 mM Tris-HCl)	5 mL

Make up to volume with double distilled water; autoclave or filter-sterilise.

Tris–acetic acid–EDTA (TAE)

Make stock solution as 50 × TAE

For 1 litre use:

Tris base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA, pH 8.0	100 mL

Make up to volume with double distilled water and autoclave; store at room temperature or 4°C.

Tris–boric acid–EDTA (TBE)

Make stock solution as 10 × TBE

For 1 litre, use:

Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8.0	40 mL

Make up to volume with double distilled water and autoclave; store at room temperature or 4°C.

CTAB/NaCl solution

10% cetyltrimethylammonium bromide (CTAB)/0.7 M NaCl. Dissolve 4.1 g NaCl in 80 mL H_2O and slowly add 10 g CTAB while heating and stirring. If necessary heat to 65°C to dissolve. Adjust to final volume of 100 mL.

Salt sodium citrate buffer (SSC)

Make stock solution as 20 × SSC
For 1 litre, use:

NaCl (3 M)	175.3 g
Na_3-citrate.$2H_2O$ (0.3 M)	88.2 g

Adjust pH to 7.0 with 1 M HCl; make up to volume with double distilled water and autoclave.

Salt sodium phosphate /EDTA buffer (SSPE)

Make stock solution as 20 x SSPE

For 2 L, use:

NaCl	248 g
$NaH_2PO_4.H_2O$ (or 62.4 g $NaH_2PO_4.2H_2O$)	55.2 g
EDTA	14.8 g

Add about 1600 mL double distilled water; adjust pH to 7.4 with NaOH (about 6.5 mL of a 10 M solution); make up to volume and autoclave.

Tris-HCl (0.2 M), pH 7.0/2 × SSC

For 2 L, use:

1 M Tris-HCl, pH 7.0	400 mL
20 x SSC	200 mL

Make up to volume with double distilled water.

DNase-free RNase

For 10 mL, use:

Ribonuclease powder (usually 1 vial)	100 mg
1 M Tris-HCl, pH 7.5 (10 mM Tris-HCl)	100 µL
1 M NaCl (15 mM NaCl)	150 µL

Make up to volume with sterile double distilled water, and heat at 100°C for 15 minutes. Allow to cool slowly to room temperature, dispense into aliquots and store at –20°C.

Type IV gel loading buffer

For 20 mL, use:

Bromophenol blue	0.25% (0.05 g)
Sucrose	40% (wt/vol) (8 g)

Make up to volume and store at 4°C in 1-mL aliquots.

Chromosomal proteinase K buffer

For 100 mL, use:

1 M Tris-HCl, pH 8.0 (0.01 M)	1 mL
Sarkosyl	1% (wt/vol) (1 g)

Make to volume with 0.5 M EDTA, pH 8.0; add proteinase K (final concentration, 1 mg/mL) to an aliquot of chromosomal proteinase K buffer when required.

Salmon sperm DNA preparation

1. Dissolve 500 mg of salmon sperm DNA in sterile double distilled water to 50 mL (concentration 10 mg/mL).
2. Stir the solution on a magnetic stirrer for 2–4 hours at room temperature to allow the DNA to dissolve.
3. Shear the DNA by sonication for 15 minutes at 5-minute bursts (i.e. three bursts).
4. Check that the shearing process is complete by electrophoresis of 1 µL of single-stranded (ss) DNA. Sheared DNA should be between 400 and 700 base pairs (bp). If incomplete, sonicate again.
5. When completely sheared, denature the DNA by boiling for 10 minutes, filter through a 0.22-µm filter to sterilise, and store at –20°C in 1-mL aliquots.
6. Just before use, boil the ssDNA for 5 minutes, then chill quickly on ice (ssDNA needs to be reboiled and denatured before each use).

Appendix 7 Vaccine Production and Quality Testing

Vaccine production and quality testing involve the following steps.

- *Production:*
  - seed management;
  - dense culture production;
  - culture inactivation; and
  - vaccine formulation.
- *Quality control:*
  - validation of the production technique;
  - in-process controls; and
  - testing of the finished product.

Seed management

Usually, a local isolate from an outbreak of haemorrhagic septicaemia (HS) is used. A good seed culture has to be well capsulated and grow rapidly, producing large translucent colonies of around 2 mm diameter on casein–sucrose–yeast (CSY) blood agar in 24 hours. Colony size is often a good indicator of capsulation. The colony has to be stable, and consistent in its characteristics. Cultures should be stored in semisolid nutrient agar slants at room temperature or as deep frozen or freeze-dried infected blood.

Periodically (say once a year) a mouse-passaged seed culture is inoculated to a young antibody-free calf by the subcutaneous route. Ideally, blood should be collected from the jugular vein of this calf when the calf is moribund and about to die or within 1–2 hours of death. The blood is collected aseptically into a sterile vessel containing an anticoagulent. It is often not possible to observe this stage, which may arise at night. In such situations, the blood is collected within a few hours of death by puncture of the jugular vein. Following culture of the blood, a few good capsulated colonies are tested for purity and agglutinability and portions of these are inoculated onto either sterile defibrinated ox blood or ox blood containing an anticoagulant. Either the infected blood directly obtained from the calf, or the ox blood inoculated with pasteurellae and incubated overnight, is tested for purity by blood smear examination; if pure, it is dispensed in 1.0-mL aliquots in sterile screw-capped bottles and stored frozen at –20°C or below. Alternatively, for long-term storage, the blood may be freeze-dried.

It is best to use a fresh bottle of blood for every batch of vaccine. One or two subcultures may be used, provided there is no sign of dissociation as evidenced by reduction in colony size. The thawed frozen blood or reconstituted lyophilised blood is plated out and single colonies are transferred onto a suitable liquid medium in 10-mL volumes in McCartney bottles and incubated at 37°C for 6–8 hours. These bottles are then transferred aseptically on to 500-mL volumes of the same liquid medium in 1-litre flasks. These flasks, incubated overnight, will constitute the inoculum for bulk culture production.

Bulk culture production

Media

A variety of liquid media have been recommended in different countries:

Medium described by Bain et al. (1982)

The medium described by Bain et al. (1982) consists basically of three components: (A) acid digest of casein; (B) autodigest of pancreas; and (C) yeast extract. Complete medium is prepared by adding these components to a basic medium (D), as described below.

(A) Acid digest of casein

1. Weigh out 200 g of commercial casein powder and place in a dry 1-litre conical flask.
2. Prepare a mixture of 170 mL concentrated (10 M) HCl and 110 mL of distilled water.
3. Pour this over the casein and stir briskly with a glass rod to ensure thorough mixing before the casein swells and hardens.

4. Invert a small beaker over the neck of the flask. Do not plug with cotton wool, as it will be destroyed by the acid on heating.

5. Put the flask in the autoclave, raise the pressure to 15 psi at 121°C and maintain at this level for 45 minutes.

6. On removal from the autoclave, cool the digest to room temperature and transfer to a 3-litre pyrex beaker.

7. Using cold 5% NaOH solution, adjust the pH of the digest to 7.0. When adding the NaOH, put the beaker in an ice bath, as the temperature of the digest must not be allowed to rise above 30°C. The volume of fluid at this stage is approximately 2 litres.

8. Add 30 g of activated decolourising charcoal and transfer to a suitable Florence flask fitted with a reflux condenser. Boil for 20 minutes.

9. Filter to remove the charcoal.

10. Add another 30 g of charcoal and repeat reflux boiling for a further 20 minutes.

11. Filter through a clarifying pad to remove the charcoal.

12. The pale amber fluid obtained is the stock casein acid hydrolysate. If it is to be stored for later use, add 1% chloroform as a preservative.

The acid hydrolysate can be obtained in dry form as 'casamino acids'. The process destroys the aromatic amino acids, which are added in the form of an enzymatic digest of casein.

The scale of production depends on the apparatus available. A digest from 400 g of casein is sufficient base material for 24 litres of medium. Ordinary commercial grades of casein are adequate, but dried milk powder cannot be used. The acid digest is easy to make and filter. No difficulties should be experienced, provided a good decolourising charcoal is used. The final product should be a clear amber colour without any suggestion of blackness.

(B) Autodigest of pancreas

1. Take about 5 kg of pancreas (pig, sheep, ox or buffalo seem to be equally effective). Trim off fat and loose connective tissue. Mince or (better still) disperse finely in a blender or disintegrator. Measure the volume and add half the quantity of tap water. Adjust the pH to 9 with (10 M) NaOH and place in a water bath at 45°C. Correct the pH every half hour (it falls considerably during the first few hours). Use an automatic stirrer and leave for 5 hours to digest. At the end, there should be little left other than fibrous connective tissue.

2. Remove from the bath and adjust the pH to 4 with HCl. Filter through muslin. Heat the filtrate to boiling and leave in the boiling water bath for 5 minutes. Cool and store in the refrigerator overnight.

3. Heat to 80°C and filter through paper. Adjust the pH to 7.4, heat to boiling and leave for 5 minutes in the boiling water bath.

(C) Yeast extract

This may be prepared in the laboratory from dried inactivated yeast powder that is available commercially wherever yeasts are produced for industrial purposes.

1. Weigh out 450 g of yeast powder.

2. Suspend evenly in 5 litres of distilled water, avoiding lumps. A large vessel such as a 20-litre stainless steel bucket should be used, as the yeast suspension froths greatly when boiled.

3. Bring the suspension to the boil and continue to simmer for 5 minutes with constant stirring to prevent boiling over.

4. Cool.

5. Recover the fluid extract of yeast either by centrifuging or by allowing to stand, decanting the supernatant and filtering through paper pulp on a Buchner funnel. About 3 litres should be obtained.

6. The clear straw-coloured fluid is the stock yeast extract ready for use in the medium. It may be preserved for storage by the addition of 1% chloroform.

(D) Basic medium

Tryptone or casitone	120 g
Sucrose	48 g
Disodium hydrogen phosphate ($Na_2 HPO_4 \cdot 12 H_2O$)	172 g
Potassium dihydrogen phosphate ($KH_2 PO_4 \cdot 7 H_2O$)	44 g
Magnesium sulfate ($Mg SO_4.7H_2O$)	24 g
Distilled water	5 litres

Complete medium

1. Mix items A, C and D (above) in a large vessel in the following proportions:

   A. acid digest from 400 g of casein (approximately 4 litres)

   C. 5 litres of home made yeast extract

   D. 5 litres of basic medium.

   Make up the volume to 24 litres with distilled water.

2. Adjust the pH to 7.6 with 10% NaOH solution.
3. Autoclave at 18 psi for 10 minutes.
4. Remove from the autoclave and filter through paper or pulp.
5. Adjust to pH 7.0 with 10% HCl solution
6. Transfer to a vortex tank and steam for 15 minutes. After cooling, 1 litre of pancreatic digest (item B) is added via a seitz filter and the medium is ready for inoculation.

Note: The production of components A, B and C is laborious and is not practised in modern production laboratories. A range of dehydrated media of bacteriological grade are available instead. Commercial 'casamino acids' (an acid digest of casein, supplemented with enzymatic digest of casein) may be used, together with commercial dehydrated yeast extract of bacteriological grade. There is no simplified method of preparing item B (autodigest of pancreas). This ingredient, however is not used in many modern media, because substitutes that provide comparable growth promoting effects, such as yeast extract, are available. Furthermore, the use of material of animal origin is not encouraged, as this could result in transmission of pathogens from the species of origin of the material to the species on which the vaccine is to be used.

Hence, items A and C can be replaced by 500 g of casamino acids and 100 g of dehydrated yeast extract, respectively. Item B can be omitted because adequate yields can be obtained without it.

Medium used in Malaysia (Thomas 1968)

Peptone (Oxoid)	150 g
Lab Lemco (Oxoid)	150 g
Sodium chloride	75 g
Sodium bicarbonate	75 g
Glucose	30 g
Yeast extract	60 g
Autodigest of pancreas (seitz filtered)	750 mL
Distilled water up to	15 litres

Medium used in Thailand (Pakchong vaccine laboratory, 1988–89)

Tryptose	20 g
Yeast extract	3 g
Sodium chloride	5 g
Dextrose	1 g
Distilled water	1 litre
pH	7.4

Medium used in Egypt (Geneidy et al. 1967)

Casamino acids	500 g
Casitone (Difco) or tryptone (Oxoid)	120 g
Yeast extract	100 g
Sucrose	45 g
Magnesium sulfate	25 g
Phosphate buffer	
Na_2HPO_4	175 g
KH_2PO_4	45 g
Distilled water	25 litres

CSY broth medium developed in Sri Lanka (Arawwawela et al. 1981)

Acid hydrolysate of casein	2 g
Sucrose (refined cane sugar)	6 g
Yeast extract	6 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate (K_2HPO_4, anhydrous)	8.6 g
Potassium dihydrogen phosphate (KH_2PO_4, anhydrous)	1.36 g
Distilled water	1 litre

The first three ingredients are prepared as a concentrate, filter-sterilised and added to the bulk tank containing the other ingredients, which have been heat sterilised.

Bulk culture methods

Various methods are adopted for the production of dense cultures in bulk. These include the use of vortex tanks with vortex aeration and sparger aeration systems. The former system is now obsolete, and vortex tanks are no longer manufactured. Sparger aeration can be done using simple, improvised systems, or by the use of automated fermentors.

Simple sparger aeration system

The inoculum is prepared in 500-mL volumes in 1-litre flasks. The system requires at least 4–6 such flasks tested and certified for purity. The medium used is the CSY broth medium.

A diagrammatic representation of the layout of the equipment is shown in Figure A7–1. The growth vessel consists of a 40-litre glass vessel (F), placed in a locally manufactured water bath (G) consisting of an aluminium vessel fitted with a heating element and a thermostat. The three glass tubes passing through the tight-fitting rubber stopper consist of a filtered air inlet (D), air outlet (H) and opening (N) for introduction of presterilised liquids and inoculum. All openings are controlled by stainless steel clips. The air outlet tube passes into a foam trap (I) that collects the froth that passes through the outlet tube; the outlet stream of air passes out through tube J and bubbles through a 10% formalin solution in flask K (to prevent infective aerosols) before passing to the exterior through tube L.

The current of air from a compressor is delivered through tube A. Tube B, controlled by opening or closure of the clip, helps to control the flow of air through the in-line filter C before passing through tube D to be dispersed into small bubbles through a filter candle E, which serves as a sparger. Tube M, which also passes through an in-line air filter, may be opened only when an additional current of air is required to pump the sterile filtrate (yeast, casein hydrolysate and sugar solution) into the tank (F) through tube N and also the inoculum. If the aeration of the tank is started before the above operation, the positive pressure built up within the tank will prevent the entry of an air current through tube N when opened, and will help to avoid contamination.

It is important that the whole assembly, including the filtration set-up, is adequately sterilised in a large autoclave at 121°C for 45 minutes; if contamination occurs, it is usually due to understerilisation. The use of a large inoculum (2–3 litres of 6–8-hour culture in the same medium for 40 litres) and early harvesting (18–20 hours) help further to obtain a dense growth in a short time, with no chance for any contaminants to multiply. The entire process should be carried out in a relatively clean room.

Preparation of the bulk culture

The inoculum prepared with the seed culture is introduced into the medium in the 40-litre vessel through tube N by building up a positive pressure in the flask by introducing sterile air through in-line filter M. The volume of inoculum (i.e. the number of 500-mL culture flasks used) is about 10% of the volume of medium used in the bulk culture production.

The temperature of the water bath (G) containing the 40-litre culture vessel is maintained at 37°C. The introduced culture in the 40-litre vessel is incubated for 16–18 hours with adequate fine aeration through the sparger (E).

At the completion of the incubation, the bulk culture harvest is obtained. A sample of the harvest is collected for quality control. The bulk harvest is inactivated by introducing a calculated volume of formalin to give a final concentration of 0.5%, by opening the mouth of the vessel while aerating. During this process the

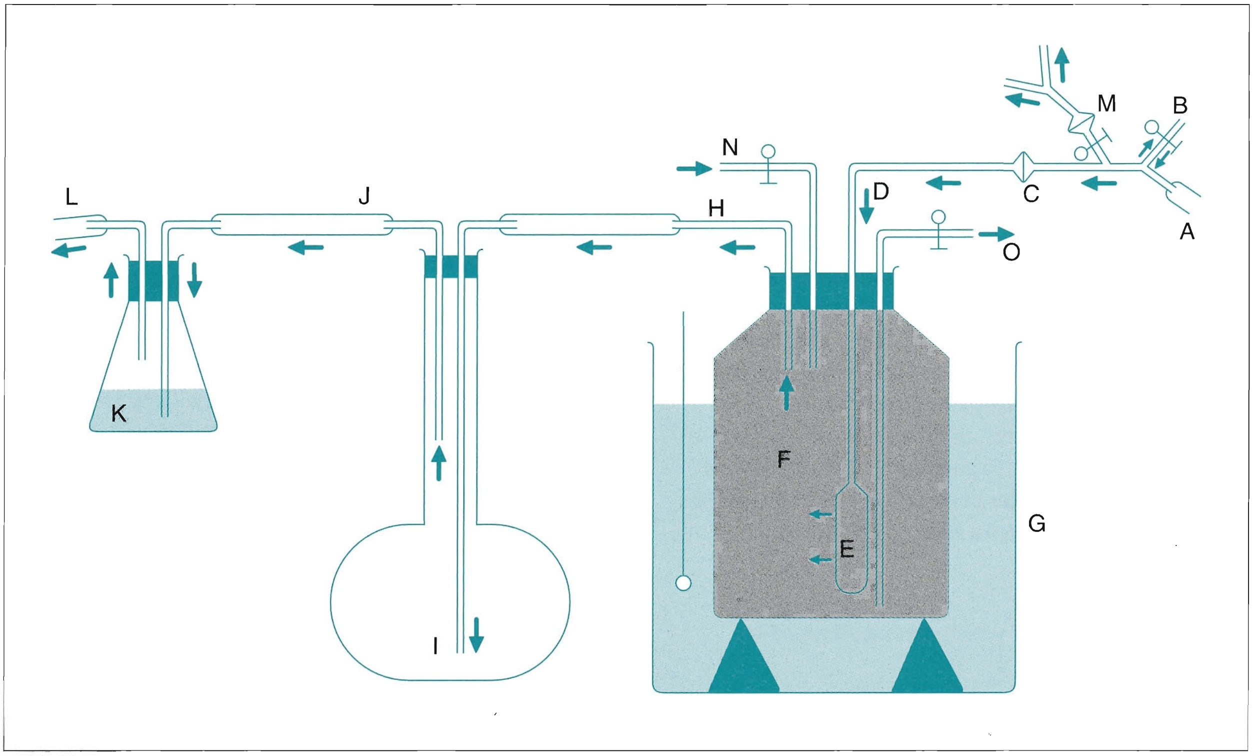

Figure A7-1. *'Fermentor' apparatus for bulk culture of P. multocida (see text for explanation of letters A–O).*

sterility in the vessel is maintained due to the positive pressure built up in the vessel. The added formalin is thoroughly mixed with the harvest by aeration of the vessel for another 30 minutes.

The inactivated harvest is then transferred into a 50-litre sterile can through tube O of the 40-litre vessel. The collected harvest is allowed to stand at room temperature for 24 hours to complete the inactivation process and then stored at 4°C until formulation of the vaccine.

Use of fermentor

A 100-litre fermentor will have a working capacity of about 80 litres. Assuming that the growth gives just the required density, a batch will yield 80 litres of harvest, giving rise to 160 litres of oil adjuvant vaccine (equivalent to approximately 53 000 doses of vaccine). Often, the density is higher, and the harvest can be diluted, thereby yielding more vaccine per batch. Most manufacturers of fermentors will modify basic fermentors to suit customer requirements.

Desirable features in a fermentor meant for this purpose are:

- an in situ sterilisation chamber, of adequate capacity for the scale of operation, with ports for inoculation, introduction of filter-sterilised fluids and harvesting, and a few extra ports for use if necessary;
- rapid (30-minute) cooling system from 121°C to 37°C;
- automatic temperature and pH control systems;
- manual air flow control;
- panel indicating all relevant parameters;
- built-in fluid filtration system; and
- steam generator and compressor to match.

Preparation of the medium

Heat-sterilised components

The heat-sterilised components (sterilised in the chamber in situ) consist of the following:

Dipotassium hydrogen phosphate (K_2HPO_4, anhydrous)	688 g
Potassium dihydrogen phosphate (KH_2PO_4, anhydrous)	108.8 g
Sodium chloride (anhydrous NaCl)	400 g
Distilled water	72 litres

The mixture is sterilised in situ in the fermentor chamber at 121°C for 1 hour. The sterilisation phase is as follows. The chamber temperature is set at 121°C and stirrer speed at 200 rpm. It takes around 45 minutes for this temperature to be reached. This temperature is maintained for 60 minutes, at the end of which it is reset at 37°C. The time taken to cool from 121°C to 37°C is approximately 30 minutes in most fermentors. Thus the sterilisation phase requires a total of 2.25 hours.

Other components

The other components, which are not sterilised by heat, are as follows:

Yeast extract	480 g
Cane sugar	480 g
Casein hydrolysate	160 g

These are added to 8 litres of distilled water, heated to 65°C and stirred until dissolved.

The mixture is then sterilised by filtration using a built-in sterilisation system with a 10-litre vessel and cartridge-type in-line filters or a 10-litre capacity filtration vessel for positive pressure filtration using seitz D9 filter pads or equivalent membrane or cartridge-type filtration systems.

The 8 litres of filter-sterilised solution is transferred to the chamber through a separate port after the chamber temperature has dropped to 37°C.

Inoculation of the medium

Eight litres of the inoculum (pretested for purity and agglutinability) is introduced into the chamber through a separate port under positive pressure. The process takes about 15 minutes. The total volume in the chamber after inoculation and the commencement of incubation is approximately 90 litres.

Incubation phase

The chamber is now at 37°C, containing the additives and the inoculum. The stirrer speed is set at 50 rpm. and the air flow is adjusted so as to permit adequate bubbling through the liquid, without undue formation of froth. The total incubation time is 18 hours and the bacterial harvest is obtained at the end of this incubation period.

Inactivation

The bacterial culture is inactivated in the harvest by adding 0.5% formalin (36–40% formaldehyde solution) to a final concentration of 0.5% and stirring at 50 rpm for 5–10 minutes. The harvest is then held at room temperature for 24 hours.

An air pressure of 1 bar is built up in the chamber. A sterile tubing system is connected to the harvesting port. The harvesting port valve is opened and the harvest is collected in sterilised stock cans.

Formulation of the vaccine

The type of formulation depends on what type of vaccine is required. It is important to ensure that the required mass of whole bacteria is present in the vaccine. Each dose of vaccine should contain 2 mg of bacteria (dry weight). The inactivated harvest must therefore be standardised. First, the dry bacterial mass of a dense harvest must be determined. After this initial determination, a relationship can be determined between dry bacterial mass and the turbidity of the bacterial suspension, and dilutions of it, measured using any standard turbidity measuring system. Thus, if broth bacterin, alum precipitated or aluminium hydroxide gel vaccine is to be prepared, the harvest is standardised to contain 2 mg in 3 mL. For the oil adjuvant vaccine, 2 mg should be contained in 1.5 mL of standardised harvest since a 3-mL dose will contain only half its volume of bacterin.

Alum precipitated vaccine

A 10% hot potash alum solution (i.e 1 litre of potash alum to 99 litres of bacterin) is added to the harvest to give a final concentration of 1%. The pH is adjusted to 6.5 and the solution allowed to stand overnight.

Aluminium hydroxide gel vaccine.

To the standardised harvest, 3% aluminium hydroxide gel is added to give a final concentration of 0.3%.

Oil adjuvant vaccine

The original formulation (Bain and Jones 1955) consisted of equal volumes of bacterin and mineral oil with approximately 10% anhydrous lanoline. This vaccine has a high viscosity. Newer formulations that enable less viscous emulsions to be produced contain either lower concentrations of lanoline or other modern adjuvants and emulsifying agents.

Some of the formulations of the oil adjuvant vaccine currently in use are given below.

Formulation used in Sri Lanka

Mineral oil	48 parts
Anhydrous lanoline (BP grade)	4 parts
Bacterin	48 parts

Formulation developed in Thailand (Neramitmansook 1993)

Mineral oil (Marcol 52)	55 parts
Arlacel 'A'	4 parts
Tween 80	1 part
Bacterin	40 parts

Experimental formulations developed in Pakistan (Muneer et al. 1993)

(i)	Mineral oil (Marcol 52)	60 parts
	Montanide 888	10 parts
	Bacterin	30 parts
(ii)	Mineral oil (Marcol 52)	63 parts
	Arlacel 'A'	7 parts
	Bacterin (containing 5% Tween 80)	30 parts
(iii)	(medium used by Shah et al. 1997)	
	Mineral oil (Marcol 52)	9 parts
	Bacterin	8 parts
	Emulsifier	1 part

The emulsifier used is a blend of Span 85 (sorbitantriolate) and Tween 85 (polyoxyethylene 20 sorbitan triolate) in the ratio of 54:46 (vol/vol).

Emulsification process

Emulsification is carried out using a turbo emulsifier. The oil and emulsifying agent mixture are added to the emusifying vessel and sterilised by heat. The emulsifier is then switched on, and the bacterin is added slowly through a port on a side under positive pressure. If a 40-litre vessel is used with 20 litres of oil-emulsifier mixture, 20 litres of bacterin is added over a period of 8–10 minutes. Emulsification is continued for a further 10 minutes, with sufficient formalin added to give a final concentration of 0.5%. The stability of the emulsion is improved if the cans are left overnight at room temperature and emulsified for a further 10 minutes before bottling. It is also recommended that the bottles of emulsified vaccine are stored for 14 days at 4–8°C, before release for testing.

Quality testing of vaccines

Quality testing can be broadly divided into three categories: validation of the production technique, in-process control tests, and tests with the finished product.

Validation of technique

HS vaccines produced in different countries vary in their production methodology. Variations occur in the composition of the growth medium, the seed culture used, the formulation and the bacterial content. The composition of the growth medium is more important than was recognised in the early years of vaccine production; it is not only the bacterial mass that matters, but also the quality of bacterial growth. There is insufficient information to define the growth medium that facilitates the expression of a full complement of important immunogens, but once a medium is proven to produce effective vaccine, it is important to adhere to it without deviations. Micronutrients in the medium may also play a role in the type of growth and antigens expressed, so it is desirable to standardise even the grades and brands of chemicals and media used.

The complete production technique, which consists of growth medium, seed culture and formulation, should be tested for stability, potency (including duration of immunity) and shelf life. Tests for stability should include storage at 37°C, and measurement of electrical conductivity. Potency should be tested in mice, and there should be a duration-of-immunity test in cattle and buffaloes. As a measure of shelf life, the above tests should be carried out after different periods of storage at the desired temperatures (4°C and tropical room temperatures). If any change is made to the technique, the new technique must be validated in regard to the above parameters.

In-process control tests

The seed culture plate, seed culture flasks and final harvest are tested for purity by smear examination.

As an additional test, the agglutinability of each of the above is tested using rabbit antiserum. Testing for inactivation is carried out with the harvest after addition of formalin and standing for 24 hours. The turbidity of the harvest should be matched against a standard equal to 1.5 mg/mL and adjusted.

Tests with the finished product

Tests for sterility

Tests for sterility and inactivation of the bacterial agent are carried out by plating out 0.1 mL of the vaccine on blood agar and Sabouraud's agar and incubating for 24 hours.

Safety test

Ten mice are inoculated, each with 0.5 mL of the vaccine by the subcutaneous route, and observed for 7 days. All mice should survive without showing any adverse effects.

Potency test

Selected batches (approximately one in five) are tested using an active mouse protection test (AMPT). Of 100 mice, 6–8 weeks of age, 50 are given 0.5 mL of vaccine by the subcutaneous route. After 14 days, the vaccination is repeated. One week after the second vaccination, the 50 vaccinated mice as well as the 50 unvaccinated mice are divided into 10 groups of five each. A 6–8 hour culture from a local field strain of *Pasteurella multocida* is serially diluted to give 10-fold dilutions. For each dilution a group of vaccinated (5) and a group of control (5) mice are given 1.0-mL by the intraperitoneal route. All mice are observed for 7 days.

The median lethal dose (LD50), measured as the dilution of the *P. multocida* suspension tested, for vaccinated and unvaccinated mice is calculated by the method of Karber (1931) as follows:

$$\log LD50 = 0.5 + \log H - \frac{\text{sum of } A}{100}$$

where:

H = the highest bacterial concentration (eg –1, if the highest concentration tested was 10^{-1})

A= the sum of the death rates (%) at each dilution

A minimum difference of 4 log units is required between log LD50 values for vaccinated and control mice. For example, if the log LD50 is calculated to be –1.9 (ie 50% of mice die when the bacterial suspension is diluted $10^{-1.9}$), the log LD50 for control mice should be at least –5.9 (ie 50% of the mice die when the bacterial concentration is diluted $10^{-5.9}$).

When the approximate LD50 is known, the number of mice used can be reduced slightly. The AMPT is preferred over the alternative passive mouse protection test (PMPT) because the latter, which involves vaccination of five cattle or buffaloes and inoculation of mice with vaccinated cattle serum (five mice for each animal vaccinated plus five controls), is both cumbersome and time consuming.

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