Chapter 3.3.9.

Fowl cholera

SUMMARY

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is distributed world-wide. Fowl cholera outbreaks often manifest as an acute fatal septicaemia, primarily in adult birds. Chronic and ~~asymptomatic~~ subclinical infections also occur. Diagnosis depends on isolation and identification of the causative bacterium, Pasteurella multocida. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of myriad bacteria in blood smears, or impression smears of tissues such as liver or spleen. Mild or chronic forms of the disease also occur where the disease is endemic, with localised infection primarily of the respiratory and skeletal systems.

**Identification of the agent:** Pasteurella multocida is readily isolated, often in pure culture, from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, or from the caseous exudate characteristic of chronic fowl cholera lesions. It is a facultative anaerobic bacterium that grows best at 37°C. Primary isolation is usually accomplished using media such as dextrose starch agar, blood agar, and trypticase–soy agar. Isolation may be improved by the addition of 5% heat-inactivated serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation and are discrete, circular, convex, translucent, and butyraceous. The cells are coccobacillary or short rod-shaped, 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Bipolar staining is evident with Wright or Giemsa stains.

Identification of P. multocida ~~is~~ has been traditionally based on the results of biochemical tests, which include carbohydrate fermentation, enzyme production, and selected metabolite production. In recent times, polymerase chain reaction (PCR)-based assays have been widely adopted.

Serological characterisation of strains of P. multocida includes capsular (Carter) serogrouping and somatic (Heddleston) serotyping. A PCR ~~polymerase chain reaction~~-based method~~s~~ is now the accepted method for Carter capsular typing. A PCR method that recognises eight genotypes (L1-L8) based on the lipopolysaccharide (LPS) outer core biosynthesis locus ~~present within the 16 Heddleston somatic serovars~~ is now preferred to conventional Heddleston serotyping ~~also allow capsular and somatic typing~~. DNA fingerprinting and, most recently, whole genome sequencing can differentiate among P. multocida having the same capsular serogroup and somatic ~~serotype~~ serovar. These characterisations require a specialised laboratory with appropriate diagnostic reagents.

**Serological tests:** Serological tests are rarely used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis through isolation and identification of the causative organism generally precludes the need for serodiagnosis.

**Requirements for vaccines:** The P. multocida vaccines in general use are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple ~~serotypes~~ serovars. Two doses of the killed vaccine are typically required. It is now known that a killed vaccine provides protection only against isolates with the identical or near to identical LPS structure, with multiple structures being possible within a Heddleston serovar. Live culture vaccines tend to impart greater protective immunity and do not have the same requirement of an exact LPS structure to provide protection and can even provide cross serovar protection. Some live vaccines have been associated with ~~but are used less frequently because of~~ potential post-vaccinal sequelae such as pneumonitis and arthritis. Multivalent vaccines typically incorporate somatic ~~serotypes~~ serovars 1, 3, and 4 as they are among the more commonly isolated avian ~~serotypes~~ serovars. Safety and potency testing of bacterins usually use the host animal. Final containers of live cultures are tested for potency by bacterial counts.

A. INTRODUCTION

Fowl cholera (also known as avian cholera, avian pasteurellosis and avian haemorrhagic septicaemia) is a contagious bacterial disease of domesticated and wild avian species ~~caused by infection with~~ *~~Pasteurella multocida~~*. ~~It~~ The disease typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality in older birds. Chronic infections also occur with clinical signs and lesions related to localised infections. The pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection. The causative agent is *Pasteurella multocida,* a member of the family *Pasteurellaceae.* While *P. multocida* is a pathogen of humans, this association is typically linked with animal bites. ~~Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic septicaemia.~~ Fowl cholera isolates of *P. multocida* are ~~is~~ not considered to have zoonotic potential as avian isolates are generally nonpathogenic in mammals exposed by the oral or subcutaneous routes. However, laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Other bacterial diseases, including salmonellosis, colibacillosis, and listeriosis in chickens, and pseudotuberculosis, erysipelas, and chlamydiosis in turkeys, may present with clinical signs and lesions similar to fowl cholera. Differentiation is traditionally based on isolation and identification, as *P. multocida* is readily cultured from cases of fowl cholera. Direct testing of clinical material with molecular tools has also been validated.

B. DIAGNOSTIC TECHNIQUES

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is often fatal (~~Derieux, 1978; Glisson et al. 2013~~ Blackall & Hofacre, 2020). In the peracute form, fowl cholera is one of the most virulent and infectious diseases of poultry. Diagnosis depends on identification of the causative bacterium, *P. multocida*, following isolation from birds with signs and lesions consistent with this disease or direct detection of the organism with specific molecular assays. Presumptive diagnosis may be based on the observance of typical signs and lesions and/or on the microscopic demonstration of bacteria showing bipolar staining in smears of tissues, such as blood, liver, or spleen. Mild forms of the disease may occur.

All avian species are susceptible to *P. multocida*, although turkeys may be the most severely affected. Birds older than 16 weeks are primarily affected. Often the first sign of disease is dead birds. Other signs include: fever, anorexia, depression, mucus discharge from the mouth, diarrhoea, ruffled feathers, drop in egg production coupled with smaller eggs, increased respiratory rate, and cyanosis at the time of death. Lesions that are often observed include: congested organs with serosal haemorrhages, enlarged liver and spleen, multiple small necrotic areas in the liver and/or spleen, pneumonia, and mild ascites and pericardial oedema. Birds that survive the acute septicaemic stage or those infected with organisms of low virulence may develop chronic fowl cholera, characterised by localised infections. These infections often involve joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by bacterial colonisation with necrosis, fibrino-suppurative exudate, and degrees of fibroplasia.

Traditionally, diagnosis has depended on the isolation and identification of the causative organism.

Table 1. Test methods available for the diagnosis of fowl cholera and their purpose

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Method | **Purpose** | | | | | |
| Population freedom from infection | Individual animal freedom from infection prior to movement | Contribute to eradication policies | Confirmation of clinical cases | Prevalence of infection –surveillance | Immune status in individual animals or populations post-vaccination |
| **Detection of the agent(a)** | | | | | | |
| **Culture** | – | – | – | +++ | – | – |
| **PCR methods** | – | – | – | +++ | – | – |
| **Detection of immune response** | | | | | | |
| **~~Serological~~ ELISA** | – | – | – | – | – | ++ |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;   
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.  
(a)A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. *In-vitro* culture

*Pasteurella multocida* is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished using media such as blood agar, trypticase–soy agar or dextrose starch agar, and isolation may be improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not require supplemental serum. Colonies range from 1 to 3mm in diameter after 18–24hours of incubation. They usually are discrete, circular, convex, translucent, and butyraceous. Capsulated organisms usually produce larger colonies than those of noncapsulated organisms. Watery mucoid colonies, often observed with mammalian respiratory tract isolates, are very rare with avian isolates. The cells are coccobacillary or short rod-shaped, usually 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found in tissue smears show bipolar staining with Wright or Giemsa stains or methylene blue, and are usually encapsulated.

Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that succumb to the acute form of the disease, and from exudative lesions of birds with the chronic form of the disease, is generally easily accomplished. Isolation from those chronically affected birds that have no evidence of disease other than emaciation and lethargy is often difficult. In this condition or when host decomposition has occurred, bone marrow is the tissue of choice for isolation attempts. The surface of the tissue to be cultured is seared with a hot spatula and a specimen is obtained by inserting a sterile cotton swab, wire or plastic loop through the heat-sterilised surface. Alternatively the sterilised surface can be cut with sterile scissors/scalpel and the swab or loop inserted into the cut without touching the outer surface. The specimen is inoculated directly on to agar medium or into tryptose or another broth medium, incubated for 2–3 hours, transferred to agar medium, and incubated again.

Identification ~~is~~ has traditionally been based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential. Those carbohydrates that are fermented include: glucose, mannose, galactose, fructose, and sucrose. Those not fermented include: rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, m-inositol, and salicin. Mannitol is usually fermented. Arabinose, maltose, lactose, and dextrin are usually not fermented. High quality maltose with minimal glucose contamination must be used to avoid false positive reactions from the glucose contamination present in lower quality maltose preparations. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. Avian isolates of *Pasteurella multocida* do~~es~~ not cause haemolysis on sheep blood agar, ~~is~~ are not motile and only rarely grow~~s~~ on MacConkey agar. ~~It~~ The organism produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, ß-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; indole and hydrogen sulphide are produced, and methyl red and Voges–Proskauer tests are negative. ~~Detection of hydrogen sulphide production may require lead acetate-laden paper strips suspended above a modified H~~~~2~~~~S liquid medium (Glisson,~~ *~~et al~~*~~., 2008)~~ Full details are provided by Glisson *et al*. (2008; 2013).

Phenotypic ~~Commercial biochemical test kits are available. Polymerase chain reaction (PCR) based methods may enable rapid identification of~~ *~~P. multocida~~* ~~colonies. However, no absolutely specific DNA-based test for the identification of~~ *~~P. multocida~~* ~~has been published (Miflin & Blackall, 2001).~~ differentiation of *P. multocida* from other similar avian *~~Pasteurella~~* ~~spp.~~ organisms ~~and~~ *~~Riemerella~~* ~~(~~*~~Pasteurella~~*~~)~~ *~~anatipestifer~~*can usually be accomplished using the tests and results indicated in Table 2. Laboratory experience has shown that *P. multocida* is most easily identified by its colony morphology and appearance in Gram stains. Positive reactions to oxidase, indole and ornithine decarboxylase are the ~~most~~ useful biochemical indications.

Commercial biochemical test kits are available but have not been recommended (Blackall & Norskov-Lauritsen, 2008). The use of matrix-assisted adsorption ionisation – time of flight (MALDI-TOF) mass spectrometry has been evaluated in just two studies to date with both studies indicating that the technology correctly identified all tested *P. multocida* isolates (Kuhnert *et al.,* 2012; Zangenah *et al.*, 2013).

***Table******2****. Tests used to differentiate* Pasteurellamultocida *from other similar organisms found in avian hosts\**~~Pasteurella~~ *~~species and~~* ~~Riemerella~~~~anatipestifer~~

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Test\*** | ***Pasteurella multocida*** | ***Avibacterium gallinarum*** | ***Gallibacterium anatis* biovar haemolytica** | ***Riemerella anatipestifer*** |
| Haemolysis on sheep blood agar | – | – | + | V |
| Growth on MacConkey’s agar | – | – | v | – |
| Indole production | + | – | – | – |
| Gelatin liquefaction | – | – | – | +u |
| Catalase production | + | + | + | + |
| Urease production | – | – | – | v |
| Glucose fermentation | + | + | + | – |
| Lactose fermentation | –u | – | V | – |
| Sucrose fermentation | + | + | + | – |
| Maltose fermentation | –u | + | V | – |
| Ornithine decarboxylase | + | – | – | – |

\**Avibacterium gallinarum* was once known as[*Pasteurell*a] *gallinarum*, *Gallibacterium anatis* biovar haemolytica was once known as [*Pasteurella*] *haemolytica* and *Riemerella anatipestifer* was once known as [*Pasteurella*] *anatipestifer.* Test reaction results: – = no reaction; + = reaction; v = variable reactions; –u = usually no reaction; +u usually a reaction.

1.2. Animal inoculation

Mouse inoculation has been a traditional approach for isolating *P. multocida,* particularly from non-sterile sites and a full description of the methodology is provided by Muhairwa *et al.* (2001). This approach selectively isolates those clones that are pathogenic for mice. Most importantly, the technique is no longer regarded as a suitable alternative given the issues of animal welfare and the need to replace the use of animals in diagnostic techniques.

1.3. Antigenic characterisation

Antigenic characterisation of *P. multocida* ~~is~~ has traditionally been accomplished by capsular serogrouping and somatic serotyping. Capsular serogroups are determined by a passive haemagglutination test (Carter, 1955; 1972). Capsular serogroups are A, B, D, E, and F. All but serogroup E have been isolated from avian hosts. A nonserological disk diffusion test that uses specific mucopolysaccharidases to differentiate serogroups A, D, and F has been developed (Rimler, 1994). ~~A specific multiplex capsular PCR assay has been developed that allows for rapid and specific capsular typing (Townsend~~ *~~et al.,~~* ~~2001).~~ A multiplex polymerase chain reaction (PCR) that allows rapid and specific capsule typing has been developed (Townsend *et al.,* 2001) and thus the traditional serological-based capsule typing has been largely replaced.

Somatic ~~serotypes are usually~~ serovars have been traditionally determined by an agar gel immunodiffusion (AGID) test and focus on the lipopolysaccharide (LPS) antigens (Heddleston, 1962; Heddleston *et al*., 1972). ~~Serotypes~~ Serovars 1 through 16 have been reported~~;~~ with all 16 ~~serotypes~~ serovars having been isolated from avian hosts (Blackall & Hofacre, 2020 ~~Glisson~~ *~~et al~~*~~. 2013~~). However, a comparison of typing achieved by a full chemical characterisation of the LPS structure, the Heddleston somatic serovar as determined by AGID and a new molecular assay that focuses on the LPS biosynthetic loci (discussed below) revealed that traditional serotyping has a high error rate (Harper *et al.*, 2015). When combined with the difficulty in producing high titre antisera, the traditional Heddleston somatic serotyping scheme can no longer be recommended as a suitable typing methodology. ~~The most effective characterisation involves determination of both serogroup and serotype. These determinations require a specialised laboratory with appropriate diagnostic reagents. To determine the serotype, the laboratory prepares the unknown bacterial culture as antigen for the AGID test and then must test it against all 16 serotype-specific antisera. Antigens present in a single isolate may react with multiple serotype-specific antisera resulting in bi- or trinomial serotypes, as illustrated by the 3, 4 and 3, 4, 12 strains (Glisson~~ *~~et al~~*~~. 2013). A highly specific multiplex PCR assay allows for differentiation among the 16 somatic serotypes. It has proven more accurate and less laborious than conventional typing (Harper~~ *~~et al.,~~* ~~2015).~~

~~1.1. Somatic typing procedure using the gel diffusion precipitin test~~

~~1.1.1. Test procedure~~

~~i) Inoculate a dextrose starch agar (DSA) plate (20 × 150 mm containing 70 ml of medium or two 15 × 100 mm plates containing 20 ml of medium per plate) with cells from a pure culture of~~ *~~P. multocida~~* ~~by using a sterile cotton swab. Swab the entire surface of the plate(s). Incubate the plate(s) in a 37°C incubator for 18–24 hours. This procedure is used to produce antigen for positive control purposes or to prepare antigen from diagnostic cultures.~~

~~ii) Harvest the cells from the plate(s) using 2.5 ml of 0.85% saline with 0.6% formaldehyde and a sterile stick. Place the cells in a tube using a sterile pipette.~~

~~iii) Autoclave the cells at 100°C for 1 hour.~~

~~iv) Centrifuge the cell suspension mixture at 13,300~~ ***~~g~~*** ~~for 20 minutes.~~

~~v) Remove the supernatant and place in a sterile tube.~~

~~vi) Prepare the agar gel for use in the gel diffusion precipitin test (GDPT) by placing 17.0 g of NaCl, 1.8 g of Noble agar, and 200 ml of distilled water into a 500 ml flask. Microwave the contents of the flask with the cap loose for 2.5 minutes. Swirl the contents of the flask and microwave again for 2.5 minutes. Allow the agar to cool slightly for 10–15 minutes. Do not prepare less than 200 ml of agar in a microwave. Dehydration during the microwave process can increase the agar concentration and negatively impact or inhibit diffusion.~~

~~vii) Place 5 ml of melted agar onto the surface of a 75 × 25 mm plain glass microscope slide. It is important that the slides are level prior to dispensing the agar. Allow the agar to cool (approximately 30 minutes) completely.~~

~~viii) Cut wells in the agar bed. The wells are 3 mm in diameter and 3 mm apart from edge-to-edge. Frequently an Ouchterlony template is used to create two or three replicates of wells per slide. Each replicate has a centre well and is surrounded by four wells located at 90° angles (from centre).~~

~~ix) Always place reference antiserum in the centre well (of a replicate). Place antigen from a diagnostic or reference culture in one of the surrounding wells within a replicate. Fill each well to capacity.~~

~~x) Incubate the slides within a moist chamber in a 37°C incubator for 48 hours. Precipitin lines of a reaction can be best observed with subdued lighting from underneath the slide. When present, reactions should occur between the centre and surrounding well(s) as an arc of precipitin. Sometimes these reactions are close to the edge of a well. Examine the slides carefully. Diagnostic cultures can react to more than one reference somatic antiserum.~~

~~xi) Use positive controls. Test reference antiserum against reference antigen each time the test is performed.~~

~~Somatic typing by a multiplex PCR assay is based on the LPS (lipopolysaccharide) genes expressed by the different Heddleston type strains and offers a reliable and fast assay for somatic typing.~~

1.4. Molecular methods – detection of nucleic acids

A number of PCR assays developed for the confirmatory identification of suspect *P. multocida* isolates have been compared (Adhikary *et al.,* 2013). The PCR developed by Townsend *et al.* (1998) was shown to have a sensitivity of 100% and a specificity of 92% when tested with 85 *P. multocida* isolates and 13 strains of related taxa (Adhikary *et al.,* 2013) and is the recommended molecular assay for the identification of *P. multocida* isolates. This assay targets a cloned sequence known as KMT1. The Townsend *et al.* (1998) PCR uses the following primers:

5’-GCT-GTA-AAC-GAA-CTC-GCC-AC-3’ (KMT1SP6)

5’-ATC-CGC-TAT-TTA-CCC-AGT-GG-3’ (KMT1T7)

The PCR amplification mix contains 1.65 µM of each primer, dNTPs (each at 200 µM), 1× Expand High Fidelity buffer with 1.5 mM MgCl2, and 1 U of *Taq* polymerase. The cycle conditions are as follows: initial denaturation at 95°C for 4 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 9 minutes. A 460 bp product is produced by isolates of *P. multocida.*

Few assays have been validated for direct detection of *P. multocida* in clinical material. The real-time PCR described by Corney *et al.* (2007) has been shown to perform at least as well as culture when used directly on swabs from birds. The Corney *et al.* (2007) assay targets the 16S rRNA gene and uses the following primers and a minor groove binder (MGB) probe:

forward primer PMA2f, 5’-ATA-ACT-GTG-GGA-AAC-TGC-AGC-TAA-3’

reverse primer PMA2r, 5’-GGT-CCC-ACC-CTT-T(A/C)-CTC-CTC-3’

MGB probe PMA2, 5’-6FAM-CCG-CGT-A(A/T)-TCT-CT-MGBNFQ-3’

The assay uses a primer concentration of 0.2 µM for the primers and 0.3 µM concentration of probe and a commercial real-time mastermix. The cycle conditions are 15 seconds at 95°C, 60 seconds at 60°C for 50 cycles.

A specific multiplex capsular PCR assay has been developed that allows for rapid and specific capsular typing (Townsend *et al.,* 2001). This assay is now widely used in place of the traditional capsular serotyping assay or the non-serological methods. Details of the primers are provided in Table 3.

***Table*** *3. Primers and target gene details of the* Pasteurellamultocida *multiplex capsular PCR typing assay of Townsend* et al. (2001)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Serogroup** | **Gene** | **Primer** | **Sequence (5’–3’)** | **Amplicon size (bp)** |
| All | KMT1 | KMT1T7 | ATC-CGC-TAT-TTA-CCC-AGT-GG | 460 |
|  |  | KMT1SP6 | GCT-GTA-AAC-GAA-CTC-GCC-AC |  |
| A | *hyaD-hyaC* | CAPA-FWD | TGC-CAA-AAT-CGC-AGT-CAG | 1044 |
|  |  | CAPA-REV | TTG-CCA-TCA-TTG-TCA-GTG |  |
| B | *bcbD* | CAPB-FWD | CAT-TTA-TCC-AAG-CTC-CAC-C | 760 |
|  |  | CAPB-REV | GCC-CGA-GAG-TTT-CAA-TCC |  |
| D | *dcbF* | CAPD-FWD | TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC | 657 |
|  |  | CAPD-REV | CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG |  |
| E | *echJ* | CAPE-FWD | TCC-GCA-GAA-AAT-TAT-TGA-CTC | 511 |
|  |  | CAPE-REV | GCT-TGC-TGC-TTG-ATT-TTG-TC |  |
| F | *fcbD* | CAPF-FWD | AAT-CGG-AGA-ACG-CAG-AAA-TCA-G | 851 |
|  |  | CAPF-REV | TTC-CGC-CGT-CAA-TTA-CTC-TG |  |

The multiple PCR mixture contained each primer (3.2 µM) of the six primer sets, 1 U of *Taq* DNA polymerase, 2 mM MgCl2, and dNTPs (each at a concentration of 200 µM). The cycling conditions are as follows: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes (Townsend *et al.*, 2001).

As noted in the previous section, the molecular characterisation of the LPS genotype is now recognised as being far more reliable than the traditional serological typing scheme based on the LPS antigens – the Heddleston scheme (Harper *et al.*, 2015). The LPS multiplex PCR developed by Harper *et al.* (2015) targets the LPS outer core biosynthesis locus and recognises eight LPS genotypes – termed L1 to L8. The original 16 Heddleston LPS serovar reference strains are assigned as follows to the eight LPS genotypes: serovars 1 and 14 – LPS L1; serovars 2 and 5 – LPS L2; serovars 3 and 4 – LPS L3; serovars 6 and 7 – LPS L4; serovar 9 – LPS L5; serovars 10, 11, 12 and 15 – LPS L6; serovars 8 and 13 – LPS L7; serovar 16 – LPS L8. The details of the primers used in the LPS genotyping assay are shown in Table 4.

***Table******4****. Primers and target gene details of the* Pasteurellamultocida *multiplex   
LPS genotyping assay of Harper* et al. (2015)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **LPS Locus** | **Primer location** | **Primer\*** | **Sequence (5’–3’)** | **Amplicon size (bp)** |
| L1 | *pcgD* | BAP6119 (f) | ACA-TTC-CAG-ATA-ATA-CAC-CCG | 1307 |
|  | *pcgB* | BAP6120 (r) | ATT-GGA-GCA-CCT-AGT-AAC-CC |  |
| L2 | *nctA* | BAP6121 (f) | CTT-AAA-GTA-ACA-CTC-GCT-ATT-GC | 810 |
|  |  | BAP6122 (r) | TTT-GAT-TTC-CCT-TGG-GAT-AGC |  |
| L3 | *gatF* | BAP7213 (f) | TGC-AGG-CGA-GAG-TTG-ATA-AAC-CAT-C | 474 |
|  |  | BAP7214 (r) | CAA-AGA-TTG-GTT-CCA-AAT-CTG-AAT-GGA |  |
| L4 | *latB* | BAP6125 (r) | TTT-CCA-TAG-ATT-AGC-AAT-GCC-G | 550 |
|  |  | BAP6126 (f) | CTT-TAT-TTG-GTC-TTT-ATA-TAT-ACC |  |
| L5 | *rmlA* | BAP6129 (f) | AGA-TTG-CAT-GGC-GAA-ATG-GC | 1175 |
|  | *rmlC* | BAP6130 (r) | CAA-TCC-TCG-TAA-GAC-CCC-C |  |
| L6 | *nctB* | BAP7292 (f) | TCT-TTA-TAA-TTA-TAC-TCT-CCC-AAG-G | 668 |
|  |  | BAP7293 (r) | AAT-GAA-GGT-TTA-AAA-GAG-ATA-GCT-GGA-G |  |
| L7 | *ppgB* | BAP6127 (f) | CCT-ATA-TTT-ATA-TCT-CCT-CCC-C | 931 |
|  |  | BAP6128 (r) | CTA-ATA-TAT-AAA-CCA-TCC-AAC-GC |  |
| L8 | *natG* | BAP6133 (f) | GAG-AGT-TAC-AAA-AAT-GAT-CGG-C | 255 |
|  |  | BAP6134 (r) | TCC-TGG-TTC-ATA-TAT-AGG-TAG-G |  |

\* f = forward primer, r = reverse primer

The multiplex LPS PCR was performed in a 50 µl volume and consisted of 0.4 µM of each primer, 0.2 mM dNTPs and 1.7 U *Taq* in a commercial buffer. The cycling conditions (when using a DNA extract) were 96°C for 5 minutes, followed by 30 cycles of 96°C for 30 seconds, 52°C for 30 seconds, and 72°C for 2.5 minutes, with a final extension at 72°C for 5 minutes. For PCR using direct colony material, the only change to the cycling conditions was that the initial denaturation step at 96°C was increased to 10 minutes.

A range of molecular methods have been used in epidemiological studies of fowl cholera outbreaks e.g. DNA fingerprinting of *P. multocida* by restriction endonuclease analysis (REA) (Wilson *et al*., 1992), enterobacterial repetitive insertion consensus (ERIC)-PCR (Singh *et al.,* 2014) and multi-locus sequence typing (MLST) (Singh *et al.*, 2013). However, these methods are now being replaced by whole genome sequencing and bioinformatic analysis (LeCount *et al.*, 2018; Omaleki *et al.,* 2020). It is now clear that the WGS/bioinformatic analysis provides a more in-depth and accurate strain tracking as well as providing *in silico* LPS typing and MLST (LeCount *et al.,* 2018; Omaleki *et al.,* 2020). The LPS loci can also be examined to identify variants within the LPS genotype. This sequence information can then be used to predict the LPS structure produced by these variants, e.g. due to introduced stop codons and frame shifts (Omaleki *et al.*, 2020). This predicted LPS structure can then be compared with the predicted structure of the killed fowl cholera vaccine in use or planned for use. This ability to predict the LPS structure is critical as it is now known that a killed fowl cholera vaccine only provides protection against field isolates of the identical or near to identical LPS structure (Harper *et al.,* 2016).~~has proved valuable in epidemiological investigations of fowl cholera in poultry flocks. Isolates of~~ *~~P. multocida~~* ~~having both capsular serogroup and somatic serotype in common may be distinguished by REA. Ethidium-bromide-stained agarose gels are analysed following electrophoresis of DNA digested with either~~ *~~Hha~~*~~l or~~ *~~Hpa~~*~~ll endonuclease (Wilson~~ *~~et al~~*~~., 1992).~~

2. Serological tests

Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for serodiagnosis. Serological tests, such as agglutination, AGID, and passive haemagglutination, have been used experimentally to demonstrate antibody against *P. multocida* in serum from avian hosts; none were highly sensitive. Determinations of antibody titres using enzyme-linked immunosorbent assays have been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but not for diagnosis.

C. REQUIREMENTS FOR VACCINES

C1. Inactivated vaccine

1. Background

1.1. Rationale and intended use of the product

Fowl cholera may be caused by any of 16 Heddleston ~~serotypes~~ serovars of *P. multocida*, although certain ~~serotypes~~ serovars appear to be more often associated with disease. The *P. multocida* vaccines in general use are inactivated, containing aluminium hydroxide or oil adjuvant, prepared from cells of ~~serotypes~~ serovars selected on the basis of epidemiological information. Commercial vaccines are usually composed of ~~serotypes~~ serovars 1, 3, and 4. Vaccination plays a significant role in the control of this disease. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Heddleston *et al.* (1970) showed that a killed fowl cholera vaccine could protect against the homologous strain but not a strain from a heterologous serovar. This finding has long been assumed to mean that killed fowl cholera vaccines provide protection that is limited to the somatic serovars of the strains present in the vaccine. However, the recently gained knowledge of the LPS biosynthetic genes has provided a far more subtle and informed understanding of the protection provided by killed fowl cholera vaccines.

An inactivated vaccine is normally administered by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are typically administered at 2- to 4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

All strains of *P. multocida* to be incorporated into a vaccine must be well characterised, of known ~~serotype~~ serovar, pure, safe and immunogenic. See chapter 1.1.8 for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

*Pasteurella multocida* seeds must be pure culture and free from extraneous bacteria and fungi (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

2.1.3. Validation as a vaccine strain

Suitability as a vaccine strain is demonstrated in efficacy and safety trials.

2.2. Method of manufacture

2.2.1. Procedure

Production cultures of each bacterial isolate to be included in the final product are prepared separately. *Pasteurella* *multocida* cultures may be grown in a suitable broth media or initially grown on agar media and scaled up to broth media. Cultures are sub-passaged until the desired volume is prepared. Cultures are harvested when they reach a suitable density, frequently measured by spectrophotometry (optical density).

Cultures are then inactivated by formaldehyde or other suitable ~~inactivant~~ inactivating agent. The inactivated ~~harvest~~ cells may be concentrated, typically by centrifugation or filtration, or diluted to reach the proper concentration for blending into completed product. All the standardised component cultures are mixed, and usually blended with an adjuvant, prior to filling sterile final containers.

2.2.2. Requirements for ingredients

See chapter 1.1.8.

2.2.3. In-process controls

The purity of the cultures is determined at each stage of production prior to inactivation. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) or by culture. Killed cultures are tested for completeness of inactivation. Analytical assays to determine the levels of formaldehyde or other preservatives are done on bulk vaccine and must be within specified limits. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used for efficacy studies.

2.2.4. Final product batch tests

i) Sterility/purity

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.26 (CFR USDA, 2013). (See also Chapter 1.1.9.)

ii) Identity

The identity of the antigens in inactivated products is typically ensured through the master seed concept and good manufacturing controls. Separate identity testing on completed product batches is not required in the USA, but procedures may differ in other countries.

iii) Safety

Safety testing is conducted on each bulk or filled vaccine lot and may be assessed in birds vaccinated for batch potency tests.

Certain countries or regions, such as the European Union (EU), also may require the testing of each batch for endotoxin content.

iv) Batch potency

In the USA, inactivated vaccines are typically tested for batch potency in a vaccination–challenge trial, such as described in 9 CFR Parts 113.116-118 (USDA, 2013). Separate groups of birds (20 vaccinates, 10 controls) are challenged with each of the ~~serotypes~~ serovars of *P. multocida* for which protection is claimed. Vaccines are administered according to the dose and route recommended on the label, and all birds are challenged 2 weeks after the second dose. The birds are observed for 14 days after challenge. For a satisfactory test according to 9 CFR, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die.

In the EU, a serological test or other validated method may be used for batch potency after a batch of minimum permissible potency is initially tested in a vaccination–challenge trial (European Pharmacopoeia, 2008).

v) Formaldehyde content

Vaccines inactivated with formaldehyde are tested for residual formaldehyde (VICH, 2003a).

2.3. Requirements for regulatory approval ~~authorisation/registration/licensing~~

The following section is based on the requirements for inactivated *P. multocida* vaccines in the USA. Other countries may have slightly different requirements.

2.3.1. Manufacturing process

The general method for production of manufacturers should demonstrate that the procedure used to inactivate bacteria is sufficient for complete inactivation. A test should be developed to confirm inactivation of each bacterial culture.

2.3.2. Safety requirements

i) Target and non-target animal safety

Inactivated vaccines should pose no hazard to non-target species. Safety in target animals may be evaluated according to harmonised requirements in VICH GL44 (VICH, 2009). The EU and USA recommend vaccinating at least 20 non-immune, unexposed birds according to label recommendations and evaluating daily for adverse reactions. The EU monitors for 21 days. In the USA, target animal safety is evaluated during the pre-challenge period of the efficacy study, which is typically 5 weeks.

Safety also should be evaluated in a field setting prior to ~~product licensure or registration~~ regulatory approval. This evaluation typically involves multiple geographical locations or husbandry conditions and much larger numbers of birds.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Not applicable.

iii) Precautions (hazards)

Vaccines prepared with aluminium-based adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate problems, but medical advice should be sought as there is a risk of infection via a contaminated needle.

Vaccines prepared with oil-based adjuvants may cause more severe reactions at the site of injection, which may manifest as large nodules. Care should be taken to administer these vaccines correctly. Operator self-injection requires immediate medical attention, involving prompt incision and irrigation of the site.

2.3.3. Efficacy requirements

Products prepared from candidate master seeds should be shown to be effective against challenge infection. Efficacy should be demonstrated in each animal species (e.g., chickens, turkeys) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. Birds used in efficacy studies should be immunologically naïve to fowl cholera and at the minimum age recommended for product use. The lot of product used to demonstrate efficacy should be produced from the highest allowable passage of master seed.

In the USA and EU, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 (USA) or 21 (EU) days after vaccination and are observed for 14 days after challenge. In the USA, mortality is measured, and a satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive (USDA, 2013). In the EU, birds are expected to remain free from severe signs of disease, and a satisfactory test requires at least 70% of the control birds to be affected while at least 70% of the vaccinates remain free from disease (European Pharmacopoeia, 2008).

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to this disease.

2.3.5. Duration of immunity

Formal duration of immunity studies are not typically required, although it is important to check the requirements of individual countries. Revaccination recommendations, beyond the primary vaccination series, are more often determined empirically.

2.3.6. Stability

Vaccine stability should be confirmed by testing the product for potency at periodic intervals through the dating period. In the USA, at least three lots of vaccine are tested and must pass established potency requirements at the end of dating. Vaccines are typically stored at 2–7°C and protected from freezing. Partly used containers should be discarded at the end of a day’s operations.

C2. Live vaccine

1. Background

1.1. Rationale and intended use of the product

Live vaccines containing modified *P. multocida* are ~~not generally~~ used ~~except~~ in some regions of the world, e.g. North America and Australia. Live vaccines are typically administered in the drinking water or wing web. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

A key feature of live fowl cholera vaccines is that the protective efficacy of these vaccines is independent of the LPS outer core structure (Harper *et al.*, 2016).

2. Outline of production and minimum requirements for vaccines

Guidelines for the production of the veterinary vaccines are given in chapter 1.1.8.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

All strains of *P. multocida* to be incorporated into a vaccine must be well characterised, of known serotype, pure, safe and immunogenic. See chapter 1.1.8 for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

*Pasteurella* *multocida* seeds must be pure culture and free from extraneous bacteria and fungi.

2.1.3. Validation as a vaccine strain

Suitability as a vaccine strain is demonstrated in efficacy and safety trials. In addition, seeds used in live vaccines must be genetically and phenotypically stable upon repeated *in-vivo* passage. Ideally, they should not persist in the vaccinated animal and any shedding of the vaccine organism from vaccinated birds should be of limited magnitude and duration.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic

Many countries have mechanisms for provisional acceptance in the event of an epizootic in which commercially available vaccines are not effective. As inactivated fowl cholera vaccines are typically effective and pose less safety risk, however, it is more likely that an inactivated vaccine would be considered for a fowl cholera epizootic.

2.2. Method of manufacture

2.2.1. Procedure

Production cultures of each bacterial isolate to be included in the final product are prepared separately. *Pasteurella* *multocida* cultures may be grown in a suitable broth media or initially grown on agar media and scaled up to broth media. Cultures are sub-passaged until the desired volume is prepared. Cultures are harvested when they reach a suitable density, frequently measured by spectrophotometry (optical density).

Each component culture may be standardised, by concentration or dilution, to a desired concentration. All of the standardised component cultures are mixed prior to filling sterile final containers. Live vaccines are typically lyophilised, to be reconstituted with sterile diluent immediately prior to use.

2.2.2. Requirements for ingredients

See chapter 1.1.8.

2.2.3. In-process controls

The purity of the cultures is determined at each stage of production. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) or by culture. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used for efficacy studies.

2.2.4. Final product batch tests

i) Sterility

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.27 (CFR USDA, 2013). (See also chapter 1.1.9.)

ii) Purity

Each batch shall pass a test for purity carried out using sold media and ignoring the growth of the vaccinal bacterium, for example as detailed in the 9 CFR Part 113.27 (CFR USDA, 2013). (See also chapter 1.1.9.)

iii) Identity

Each batch of live vaccine in the USA is tested for identity. Requirements of other countries may vary. This is most commonly accomplished by characterising the bacteria *in vitro*.

iv) Safety

Live vaccines may be tested according to the method described in Section C1.2.3.2.i, except that frequently only one representative animal species is required.

Certain countries (e.g. EU) also may require testing each batch for endotoxin content (European Pharmacopoeia, 2008).

v) Batch potency

The potency of live vaccine lots is determined by a bacterial count performed on reconstituted lyophilised product in its final container. In the USA, the mean bacterial count of any vaccine lot at the time of preparation must be sufficiently high to ensure that at any time prior to product expiration, the count is at least twice the immunogenicity standard. The EU requires a count that is at least equal to the immunogenicity standard.

vi) Moisture content

Lyophilised vaccine is tested for moisture content. Harmonised requirements for testing moisture by a gravimetric method are found in VICH GL26 (VICH, 2003b). Typically moisture is expected to be less than 5%.

2.3. Requirements for regulatory approval ~~authorisation/registration/licensing~~

2.3.1. Manufacturing process

See chapter 1.1.8.

2.3.2. Safety requirements

i) Target and non-target animal safety

The safety of master seeds used in the production of live vaccines must be evaluated prior to ~~licensing~~ regulatory approval. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Harmonised VICH GL44 (VICH, ~~2006~~ 2007) is available for target animal safety.

Overdose studies are typically required for live vaccines. For example, each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. If unfavourable reactions are seen, this finding should be included in a risk assessment, and it may be appropriate to designate maximum permissible serial potency requirements.

The master seed is also tested in representative non-target species (e.g. rodents or non-target avian species) that may be expected to come into contact with vaccine bacteria shed by vaccinated birds. Master Seed bacteria should be administered to the most sensitive species at the most sensitive age, by the route (e.g. oral) expected to occur in the field.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Master seed bacteria for live vaccines should be evaluated for their stability with repeated passage in vivo. The seed should remain avirulent and genotypically stable after multiple passages. Harmonised requirements for reversion to virulence studies are described in VICH ~~GL40~~ GL41 (VICH, ~~2006~~ 2007).

Seeds for live vaccines also should be tested for their potential to shed from vaccinated animals and persist and spread in the environment. Ideally vaccine organisms should shed no more than briefly and should not persist in the environment. Exceptions from the ideal should be addressed in a risk assessment for the product.

iii) Precautions (hazards)

Inadvertent human exposure to the vaccine organism should be reported to a physician.

2.3.3. Efficacy requirements

Products prepared from candidate master seeds should be shown to be effective against challenge infection. Efficacy should be demonstrated in each animal species (e.g. chickens, turkeys) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. Birds used in efficacy studies should be immunologically naïve to fowl cholera and at the minimum age recommended for product use. The lot of product used to demonstrate efficacy should be produced from the highest allowable passage of master seed.

For live avian *Pasteurella* vaccines in the USA, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 days after vaccination and are observed for 10 days after challenge. A satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive.

The arithmetic mean count of colony-forming units in the lot of product that is used to demonstrate efficacy is used as the minimum standard (immunogenicity standard) for all subsequent production lots of vaccine.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable

2.3.5. Duration of immunity

Formal duration of immunity studies are not typically required, although it is important to check the requirements of individual countries. Revaccination recommendations, beyond the primary vaccination series, are more often determined empirically.

2.3.6. Stability

Vaccine stability should be confirmed by testing the product for potency at periodic intervals through the dating period. In the USA, batches of vaccine are tested until a statistically valid stability record is established. Each lot must pass established potency requirements at the end of dating. Live vaccines should be used promptly upon opening.

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**NB:** At the time of publication (2022) there was no OIE Reference Laboratory for fowl cholera  
(see OIE Web site for the most up-to-date list:   
<https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

**NB**: First adopted in 1990. Most recent updates adopted in 2015.