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 acute fatal septicaemia, primarily in adult birds. Chronic and asymptomatic 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 based on the results of biochemical tests, which include carbohydrate fermentation, enzyme production, and selected metabolite production.*

Serological characterisation of strains of P. multocida *includes capsular serogrouping and somatic serotyping. Polymerase chain reaction-based methods also allow capsular and somatic typing. DNA fingerprinting can differentiate among* P. multocida *having the same capsular serogroup and somatic serotype. 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. Two doses of the killed vaccine are typically required. Live culture vaccines tend to impart greater protective immunity, but are used less frequently because of potential post-vaccinal sequelae such as pneumonitis and arthritis. Multivalent vaccines typically incorporate somatic serotypes 1, 3, and 4 as they are among the more commonly isolated avian serotypes. 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 is a contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida*. It 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. Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic

septicaemia. Fowl cholera is not considered to have zoonotic potential as avian isolates are generally nonpathogenic in mammals exposed by the oral or subcutaneous routes. 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 based on isolation and identification, as *P. multocida* is readily cultured from cases of fowl cholera.

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). 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. 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.

Diagnosis depends on 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
Agent identification[1]						
Culture	–	–	–	+++	–	–
Detection of immune response						
Serological ELISA	–	–	–	–	–	++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

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 3 mm in diameter after 18–24 hours 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

1 A combination of agent identification methods applied on the same clinical sample is recommended.

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 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. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. *Pasteurella multocida* does not cause haemolysis, is not motile and only rarely grows on MacConkey agar. It produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, beta-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_2S liquid medium (Glisson, *et al*., 2008). 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 avian *Pasteurella* spp. 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 indole and ornithine decarboxylase are the most useful biochemical indications.

Table 2. *Tests used to differentiate* Pasteurella multocida *from other avian* Pasteurella *species and* Riemerella anatipestifer

Test*	*Pasteurella*		*Riemerella anatipestifer*
	multocida	*gallinarum*	
Haemolysis on blood agar	–*	–	v
Growth on MacConkey's agar	–	–	–
Indole production	+	–	–
Gelatin liquefaction	–	–	+u
Catalase production	+	+	+
Urease production	–	–	v
Glucose fermentation	+	+	–
Lactose fermentation	–u	–	–
Sucrose fermentation	+	+	–
Maltose fermentation	–u	+	–
Ornithine decarboxylase	+	–	–

*Test reaction results: – = no reaction; + = reaction; v = variable reactions; –u = usually no reaction; +u usually a reaction.

Antigenic characterisation of *P. multocida* is 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).

Somatic serotypes are usually determined by an agar gel immunodiffusion (AGID) test (Heddleston, 1962; Heddleston *et al*., 1972). Serotypes 1 through 16 have been reported; all 16 serotypes have been isolated from avian hosts (Glisson *et al*. 2013). 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.

DNA fingerprinting of *P. multocida* by restriction endonuclease analysis (REA) 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*I or *Hpa*II 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 of *P. multocida*, although certain serotypes 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 selected on the basis of epidemiological information. Commercial vaccines are usually composed of serotypes 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.

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, 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 subpassaged 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. The inactivated harvest 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 testing 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 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.

2.3. Requirements for 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. 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 North America. 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.

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 subpassaged 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, 2003). Typically moisture is expected to be less than 5%.

2.3. Requirements for 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. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Harmonised VICH GL44 (VICH, 2006) 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 (VICH, 2006).

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: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2015.