AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

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# Pullorum Disease

## *Bacteriology and Serology*

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## 1. Introduction

The incidence of pullorum disease in Australia has remained at a low level since 1950. A slight increase occurred during 1964–68 when the variant strain appeared in Australia (McDougall, 1967) and was inefficiently detected by the antigens available at that time (Jackson *et al.*, 1968; Whittem, 1968).

Outbreaks of mortality in chickens caused by pullorum disease have not occurred for a number of years. The disease is now considered eradicated from the commercial industry.

This paper describes standard techniques for the bacteriological examination of chickens, reactor birds and dead-in-the-shell embryos for pullorum disease. As well, standard techniques for serological examinations based on the standard tube agglutination test are described.

We recommend that specimens from all early chick mortalities should be submitted to the procedure outlined below to indicate whether *S. pullorum* or another Salmonella serotype is responsible for the mortality. This procedure is to be carried out also in specimens submitted for early microbiological monitoring (EMM).

In all cases where *S. pullorum* is isolated, the isolates should be forwarded to the Australian Salmonella Reference Laboratory (SRL, Institute of Medical and Veterinary Science, PO Box 14, Rundle Mall, Adelaide, SA 5000, Australia. Tel. (08) 228 7365; Fax (08) 228 7538) for antigenic typing.

## 2. Bacteriological Examination of Chickens for Pullorum Disease

(a) The following technique is recommended for chickens up to about two weeks of age. Where chickens are being submitted for EMM a minimum sample of 15 is required from parent flocks and a minimum sample of 20 for grandparent flocks. Older chickens are rarely cultured for salmonellas but the method is generally applicable. Tissues may be examined separately or pooled. It is desirable that highly contaminated material be examined separately. Hence liver, heart (blood) and yolk sac may be pooled but caecal contents handled separately. Pools may consist of tissues from up to five chickens but no more.

(b) If examined separately each sample should be cultured directly onto a non-inhibitory medium, e.g. sheep blood agar and onto a selective medium, e.g. brilliant green agar and each tissue also inoculated into a liquid selective medium (Muller–Kauffman tetrathionate broth, Rappaport–Vassiliadis broth or selenite broth would be suitable). After overnight incubation at 37°C the broths are plated onto blood agar and brilliant green agar.

(c) The composite samples of tissue (10 g per 90 mL medium) should be inoculated into selective broths and after incubation plated onto blood agar and brilliant green agar.

(d) Suspicious single colonies should be subcultured on Kohns media or triple sugar iron (TSI) agar slants and incubated overnight at 37°C. An alternative is to inoculate suspect colonies into a Urea broth and incubate for 4–16 hours at 37°C. Isolates giving suspicious reactions in Kohns or TSI or giving a urea negative broth are submitted to further biochemical and serological tests. Any culture suspected to be *S. pullorum* should be subjected to a slide agglutination test by using group D antiserum and subjected to further biochemical tests if in doubt.

(e) All salmonella cultures isolated should be sent to the SRL for serological typing to indicate species. If confirmed as *S. pullorum* the SRL can also determine whether they are standard, intermediate or variant strains.

The following are the characteristics of *S. pullorum*: Gram negative non-motile bacillus, producing acid and small amounts of gas in glucose and mannitol, no fermentation of maltose, dulcitol, lactose or sucrose, indole and urease negative, colony size much smaller than that of other salmonellas (about 1.0 mm), and reacts with group D antiserum (antigens 9 and 12 ).

*N.B.*

(a) *S. gallinarum*, the cause of fowl typhoid, is not known to occur in Australia. It is distinguished from *S. pullorum* by large colony size, fermentation of maltose, negative ornithine decarboxylase reaction and growth on Christensen's citrate agar.

(b) Bile salt sensitive strains of *S. pullorum* have been reported, hence the recommendation to use both non-inhibitory as well as selective media.

(c) Mannitol selenite broth has been reported as a more satisfactory enrichment medium than tetrathionate broth.

(d) Commercial identification kits may not specifically identify *S. pullorum* and should be used with considerable caution.

(e) After overnight incubation at 37°C, colonies on brilliant green agar will be better recognised if plates are left at room temperature for two to four hours.

## 3. Bacteriological Examination

The description of this procedure has been adapted from the United States Department of Agriculture (1965).

(a) The pericardial sac, peritoneum, oviduct and any visible pathological tissues should be cultured on beef extract agar or tryptose agar or brilliant green agar by means of

sterile swabs. Sterile technique should be followed. (Primary culture of these organs in a suitable nutrient broth and transfer to a suitable nutrient agar is optional).

(b) The following organs should be aseptically collected for culture:
  (i) Heart (apex, pericardial sac, and contents if present);
  ii) Liver (portions exhibiting lesions or in grossly normal organs,the drained gall-bladder and adjacent liver tissues);
  (iii) Ovary–testes (entire inactive ovary or testes but if ovary is active use own judgement and include any atypical ova);
  (iv) Oviduct (if active include any debris and dehydrated ova);
  (v) Pancreas;
  (vi) Spleen.

(c) A composite sample of the organs listed in (b) should be ground in a sterile mortar or suitable blender, e.g. Stomacher. Individual organs may be used if desired. Nutrient broth should be added as a diluent. This suspension (10 mL) should be inoculated into 90 mL of mannitol selenite broth or Muller-Kauffman tetrathionate broth or Rappaport–Vassiliadis broth and into 90 mL of a suitable non-inhibitory broth, e.g. peptone water.

(e) After overnight incubation at 37°C a loopful of the cultures from each flask should be streaked on a suitable non-inhibitory medium, such as blood agar, and on a selective medium, e.g. brilliant green agar. If no suspicious colonies are observed after overnight incubation the enrichment broths should be restreaked on solid media after a further two to four days incubation.

(e) A portion of the crop wall and intestine to include the caecal tonsils are put into either mannitol selenite broth or Muller-Kauffman tetrathionate broth or Rappaport–Vassiliadis broth. Transfers should be made for the broth onto agar plates as indicated in (d).

(f) Suspicious single colonies should be identified as set out in 2(d).

(g) All salmonella cultures isolated should be sent to the SRL for serological typing to indicate species. If *S. pullorum*, the SRL can also determine whether they are standard, intermediate or variant strains of *S. pullorum*.

## 4. Bacteriological Examination of Dead-in-shell Embryos for Pullorum Disease

(a) A random sample of suitable [see 4.(b)] dead-in-shell embryos should be collected at the hatchery from eggs of each fertile egg supply flock requiring examination.

(b) All cracked eggs, infertile eggs and eggs containing embryos less than 18 days old should be discarded. It is recommended that a minimum of 15 eggs be examined from parent flocks and 20 from grandparent flocks.

(c) Eggs should be washed in warm soapy water, dipped in an ethanol ($C_2H_5OH$) solution.

(d) Eggs should be opened aseptically and the total contents placed in a homogeniser or Stomacher and ground to a paste. Each egg can be handled separately or they can be pooled in groups of not more than five eggs.

(e) A loopful of the paste from each egg or pool should be plated onto blood agar or other non-inhibitory medium and about 10 mL of this suspension should be inoculated into 90 mL of mannitol selenite broth or Muller-Kauffman tetrathionate broth or Rappaport–Vassiliadis broth and incubated at 37°C overnight.

(f) The procedure is then identical as for chickens for steps 2(c)–(e).

## 5. Serological Examination for Pullorum Disease

### *5.1. The Standard Tube Agglutination Test*

The description of this procedure has been adapted from the United States Department of Agriculture (1965).

It is recommended that sera be tested against antigens made from both standard and variant strains. These may be combined but more information is obtained by testing sera against each antigen separately.

#### 5.1.1. Collection of Blood Samples

(a) The blood samples should be labelled and preferably in containers provided by the laboratory.

(b) Sufficient blood should be procured by making a small incision in the large median wing vein with a small sharp lancet and allowing the blood to run into the tube, or by the use of a small syringe (with 20 or 21 gauge needle) which is properly cleansed between bleedings with physiological saline solution. To facilitate the separation of the serum the tubes should be placed in a slanted position until the blood has solidified. After separation of the clot, the sample should be kept cool but not frozen and delivered to the laboratory as soon as possible. Haemolysed or contaminated samples are usually unsatisfactory.

#### 5.1.2. *Salmonella pullorum* Antigen

The antigen shall consist of representative strains of *S. pullorum* which are of known antigenic composition, high agglutinability, but are not sensitive to negative and non-specific sera. The stock cultures may be maintained satisfactorily by transferring to new agar slopes at least

once a month and keeping at 18–25°C (average room temperature) in a dark closet or chest, following incubation for from 24–36 hours at 37°C. The antigenic composition and purity of the stock cultures should be checked consistently.

### 5.1.3. Growth Medium for *Salmonella pullorum*

A satisfactory medium which has been used for a long time has the following composition:

| | |
|---|---|
| Water | 1 L |
| Difco beef extract | 4 g |
| Difco Bacto-peptone | 10 g |
| Difco dry-granular agar | 20 g |

pH 6.8–7.2

Large 25 mm test tubes or Roux bottles should be streaked liberally over the entire agar surface with inoculum from 48 hour slant cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated for 48 hours at 37°C and the surface growth washed off with sufficient physiological saline (0.85%, 0.15 mol/L) solution containing 0.5% of phenol ($C_6H_5OH$) to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigens of the separate strains may be combined in equal volume-density and stored in the refrigerator (5–10°C) in tightly stoppered bottles.

### 5.1.4. Thiosulfate–Glycerin (TG) Medium

Thiosulfate–glycerin medium may be used as an alternate medium for the preparation of the tube agglutination antigen. The TG medium, formerly used for the preparation of stained, whole-blood antigen, is described in more detail in the article by MacDonald (1941). The medium provides a tube antigen of excellent specificity and greatly increases the yield of antigen from a given amount of medium. The TG medium has the following composition:

| | |
|---|---|
| Beef infusion | 1 L |
| Difco Bacto-peptone | 20 g |
| Sodium thiosulfate, $Na_2S_2O_3$ | 5 g |
| Ammonium chloride, $NH_4Cl$ | 5 g |
| Glycerin, 95% | 20 mL |
| Difco dry-granular agar | 30 g |

pH 6.8 to 7.2

Large 25 mm test tubes or Roux bottles should be seeded over the entire agar surface with inoculum from 24-hour beef infusion broth cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated for 96 hours at 37°C and the surface growth washed off with sufficient physiological saline (0.85%) solution containing 0.5% of phenol to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigen should be then centrifuged. The mass of bacteria should be removed from the centrifuge tubes or bowl and resuspended in physiological saline (0.85%) solution containing 0.5% of phenol. After the bacterial mass has been uniformly suspended in the diluent it should be again passed through a cotton pad in a Buchner funnel without the aid of suction. The antigens of the separate strains may be combined in equal volume–density and stored in the refrigerator (5–10°C) in tightly stoppered bottles.

### 5.1.5. Preparation of Diluted Antigen

The diluted antigen to be used in the routine testing should be prepared from the stock antigen by dilution of the latter with physiological (0.85%) saline solution containing 0.25% of phenol to a turbidity corresponding to 0.7–1.00 on the McFarland nephelometer scale (40% transmittance at 600 nm on Bausch & Lomb Spectronic 20 spectrophotometer). The hydrogen ion concentration of the diluted antigen should be corrected to pH 8.2–8.5 by the addition of dilute sodium hydroxide. New diluted antigen should be prepared each day and kept cold. The diluted antigen may be employed in 2 mL quantities in 4 x 1/2 inch test tubes or 1 mL quantities in smaller tubes in which the final serum–antigen mixtures are made and incubated.

### 5.1.6. Serum Dilutions

In all official reports on the blood test the serum dilutions shall be indicated. Sera showing complete agglutination at 1/25 or above final dilution after addition of antigen should be regarded as positive. The sera should be introduced into the agglutination tubes in the desired amounts with well-cleaned serological pipettes or special serum-delivery devices which do not permit the mixing of different sera. The antigen and serum should be well mixed before incubation. The serum and antigen mixture must be incubated for at least 20 hours at 37°C.

### 5.1.7. Coding of Results

The results for 1/25 dilution of serum plus antigen shall be recorded as in Table 1.

Some allowance must always be made for the difference in sensitivity of different antigens and different set-ups, and, therefore, a certain amount of independent, intelligent judgement must be exercised at all times. Also, the histories of the flocks require consideration. In flocks where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of *S. pullorum*.

If titres are desired, doubling dilutions for final dilutions of 1/12.5 to 1/450 may be prepared by mixing 0.84 mL saline with 0.16 mL serum and transferring 0.5 mL of the mixture into 0.5 mL of physiological saline (0.85%) solution containing 0.5% of phenol repeatedly for five dilutions. Antigen (0.5 mL) is added to the remaining 0.5 mL of diluted serum in each tube.

**Table 1.** Coding of results of blood tests

| | | |
|---|---|---|
| N | – | Negative. Serum–antigen mixture remains uniformly turbid. |
| Y | + | Positive. A distinct clumping of the antigen and the liquid between the agglutinated particles is clear. |
| S | ? | Suspicious. The agglutination is only partial or incomplete. |
| M | Missing | Samples listed on the original record sheet are missing. |
| H | Haemolysed | Blood samples are haemolysed and cannot be tested. |
| B | Broken | Sample tubes are broken and no serum can be obtained. |

## 6. Media

The various media mentioned in this document are available from several commercial sources such as those listed in Table 2. The list is neither meant to be exhaustive nor to imply any recommendation of any particular commercial source.

### *6.1. Concerns Over the Safety of Selenite Broths*

Selenium compounds are potential mutagens (Noda *et al.*, 1979) and, hence, selenite broths are not a preferred enrichment medium. If used, it is advisable that all the precautionary measures indicated by the commercial supplier of the medium should be strictly followed.

**Table 2.** Suppliers and catalogue numbers of media used in the bacteriology and serology of pullorum disease

| *Medium* | *BBL* | *Difco* | *Oxoid* |
|---|---|---|---|
| Brilliant green agar | 11073 | 0285-01-5 | CM263 |
| Muller-Kauffman tetrathionate broth | 11706 | 0104-17-6 | CM29 |
| Rappaport-Vassiliadis broth | — | — | CM866 |
| Selenite broth | 11608 | 0275-01-7 | CM395 +L121 |
| TSI medium | 11749 | 0265-01-9 | CM277 |

## 7. References


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