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Testing Protocol

SAM 602

Supplemental Assay Method for the Evaluation of *Salmonella pullorum* Antigens

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1. Introduction

This Supplemental Assay Method (SAM) describes how *Salmonella pullorum* antigens are analyzed by using the following procedures: a percent concentration of formalin by spectrophotometric determination; hydrogen ion concentration by a pH meter; bacterial density by spectrophotometric determination; sensitivity by a rapid agglutination test; and homogeneity by microscopic examination. These procedures are applicable to *S. pullorum* stained K polyvalent antigens, as required in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.407.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Spectrophotometer or colorimeter

2.1.2 Micropipettors

2.1.3 Pipette tips

2.1.4 Sterile serological pipettes

2.1.5 pH meter

2.1.6 Graduated cylinder, 100-mL

2.1.7 Timers or stop clocks with 1 second subdivisions

2.1.8 Minnesota testing box containing a glass plate with perpendicular etched lines forming 3-cm x 3-cm squares

2.1.9 Aluminum mixing device designed for use with the slide agglutination test

2.1.10 Glass slides and cover slips

2.1.11 Light microscope

2.1.12 Vortex mixer

2.1.13 Cuvettes

2.1.14 Centrifuge tubes, 15-mL and 50-mL

2.1.15 Volumetric flasks, 10-mL and 100-mL

2.1.16 Centrifuge

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 10% Formalin solution (Fisher SSF98-4) - **Must be non-neutral buffered**

Note: According to 29 CFR 1910.1048, precautions for the use of formaldehyde are as follows: Toxic by inhalation and if swallowed. Irritating to the eyes, respiratory system and the skin. May cause sensitization by inhalation or by skin contact. Risk of serious damage to eyes. Potential cancer hazard; repeated or prolonged exposure increases the risk.

2.2.2 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) (Sigma #129739)

2.2.3 Ferric chloride/sulphamic acid solution

10 g Ferric chloride
16 g Sulphamic acid
Q.S. to 1000 mL with distilled water

Store at 2º- 8ºC.

2.2.4 pH buffer solutions

2.2.5 1N Hydrochloric acid

82.8 mL Hydrochloric acid
917.2 mL distilled water

Store at 20º- 25ºC.

2.2.6 **Distilled** water (H_2O)

2.2.7 *S. pullorum* serums--A total of at least 12 positive serums shall be used. This shall include 3 definitely positive (high titer) serums--regular (R), intermediate (I), and variant (V), 3 weakly positive (low titer) serums--R, I, and V, and 3

negative serums A panel of 12 positive and negative serums are produced at the Center for Veterinary Biologics (CVB), and can be requested for this assay.

2.2.8 *S. pullorum* stained K antigen reference to compare product on test to a known reference antigen. Available by request from the CVB.

2.2.9 McFarland standard No. 1 (Scientific Device Laboratory #2350)

2.2.10 Immersion oil

3. Preparation for the Test

3.1 Personnel qualifications/training

Laboratory personnel must be trained in the operation of instruments and equipment used prior to performing this test. Personnel must be familiar with proper use of test reagents and biological materials, and have knowledge of safe operating procedures and polices.

3.2 Preparation of equipment/instrumentation

Turn on the spectrophotometer to 'warm up' for at least 10 minutes.

3.3 Preparation of reagents/control procedures

Allow samples, serums, and reagents to warm to room temperature before conducting the tests.

3.4 Preparation of the samples

Samples are *S. pullorum* stained K polyvalent antigens.

4. Performance of the Test

4.1 Formalin content

4.1.1 Prepare a 0.5N HCL solution from 1N HCL solution and distilled water.

4.1.2 Prepare a 0.5g/L solution of MBTH

4.1.3 Centrifuge 5 mL of reagent (sample) at 3000 rpm for 30 minutes in a 15-mL centrifuge tube.

4.1.4 Remove supernatant and place in a new 15-mL centrifuge tube.

4.1.5 Dilute supernatant of sample 1:4 with 0.5N HCL and allow to decolorize.

4.1.6 Prepare standards (std) in 10-mL volumetric flasks. Add the following amounts of 10% formalin to appropriately labeled flasks. Q.S. to volume with 0.5N HCL.

std 1 120 µL
std 2 180 µL
std 3 240 µL
std 4 300 µL
std 5 360 µL

4.1.7 Label 15-mL centrifuge tubes for standards, blank and samples.

4.1.8 Add 5.0 mL 0.5g/L MBTH solution to each tube.

4.1.9 Add 500 µL of standards and samples to appropriately labeled tubes. Add 500 µL of 0.5N HCL to the blank tube.

4.1.10 Allow tubes to stand for 60 minutes.

4.1.11 Add 1.0 mL of ferric chloride/sulphamic acid solution to each tube.

4.1.12 Allow tubes to stand for 15 minutes.

4.1.13 With a spectrophotometer measure the absorbance of all tubes at 628 nm. Subtract the blank from each of the standard and sample readings.

4.1.14 Record results.

4.2 Hydrogen ion concentration

4.2.1 Standardize the pH meter with standard buffer solution pH 4.0 and pH 7.0 according to the equipment manual.

4.2.2 Dispense 5 mL of product into a 50-mL screw-cap centrifuge tube.

4.2.3 Read product pH by inserting electrode into the tube containing the product.

4.2.4 Record results.

4.3 Density

4.3.1 Allow all reagents to warm to room temperature. Prepare a 0.5N HCL solution.

4.3.2 Vortex sample for 10 seconds.

4.3.3 Dilute sample 1:50 with 0.5N HCL (1 mL sample + 49 mL HCL) in the 100-mL volumetric flask. Mix well. Allow to decolorize for 2 hours.

4.3.4 Add 10 mL of distilled water to the sample for a 1:60 dilution. Mix well.

4.3.5 Zero the spectrophotometer using a wavelength of 420 nm and adjust the transmittance to 100% with distilled water.

4.3.6 Record transmittance of McFarland Standard No. 1.

4.3.7 Read and record transmittance of 1:60 diluted sample.

4.3.8 Add 1 mL of distilled water to the volumetric flask, mix well, and read and record transmittance.

4.3.9 Continue to add distilled water 1-5 mL at a time to the volumetric flask until the transmittance of the diluted sample is equivalent to the McFarland Standard No. 1 ± 3%.

4.3.10 Record results.

4.4 Sensitivity

4.4.1 Allow all reagents to warm to room temperature.

4.4.2 Vortex each *S. pullorum* serum and the *S. pullorum* antigen sample for 10 seconds.

4.4.3 Set the electronic timer for 2 minutes.

4.4.4 Using a micropipette, place 20 µL of each *S. pullorum* serum within 2 separate squares on the Minnesota box.

4.4.5 Add 1 drop of the sample in each square next to the serum.

4.4.6 Quickly mix the serum and sample with the aluminum mixing device using a circular motion, wiping the mixing device between each set of samples.

4.4.7 Start the timer.

4.4.8 At the end of the incubation, gently swirl the liquid in a circular motion to read and record the result.

4.5 Homogeneity

4.5.1 Prepare a wet mount slide.

4.5.2 Examine the slide under the 100x objective.

4.5.3 Record all observations.

5. Interpretation of the Test Results

5.1 Formalin content calculation

A formalin content of 1.0 ± 0.2% is a satisfactory result. The absorbance reading shall fall between the readings of std 2 and std 4.

5.2 Hydrogen ion concentration determination

Stained K antigens must have a pH of 4.6 ± 0.4.

5.3 Density calculation

5.3.1 The density is defined as the reciprocal of the amount of distilled water required to dilute the product to the equivalent of McFarland Standard No. 1.

5.3.2 The density must be 80 ± 15 times McFarland Standard No. 1.

5.4 Sensitivity determination

5.4.1 Agglutination (definite clumping with clearing of fluid) shall occur in at least 5 of the 6 positive serums.

5.4.2 No agglutination shall be observed in any of the 6 negative serums.

5.5 Homogeneity determination

5.5.1 There shall be no autoagglutination.

5.5.2 There shall be no unusual appearance such as the presence of flakes, specks or a preponderance of filament forms.

6. Report of Test Results

6.1 Formalin content

Record the spectrophotometic reading of the sample and the readings of the two standards the sample reading fall inbetween, and final test conclusion.

6.2 Hydrogen ion concentration

Record the pH of the product tested, and the final test conclusion.

6.3 Density

Record the density results, in terms of number of times the McFarland Std 1, and the final test conclusion.

6.4 Sensitivity

Record the number of negative serums that were negative and the number of positive serums that were positive, and the final test conclusion.

6.5 Homogeneity

Record the result as homogeneous or heterogeneous, and the status of autoagglutination, and the final test conclusion.

7. References

Code of Federal Regulations, Title 9, Part 113.407, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While content changed significantly, no significant changes were made that impact the outcome of the test.