CHAPTER 33. DETECTION OF ANTIMICROBIAL RESIDUES IN MEAT AND POULTRY TISSUE BY SCREEN TESTS

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33.1 Introduction

Rapid microbiological screen tests are used in slaughter establishments to detect the presence of antimicrobial residues in food animal tissues. The Swab Test on Premises (STOP) is used for all red meat species except bob veal calves, where the Calf Antibiotic and Sulfa Test (CAST) is used. The Fast Antimicrobial Screen Test (FAST) developed recently and tested on bovine tissue, has been found to have greater sensitivity than STOP and CAST. The test is being conducted in bovine slaughter establishments on a limited basis. The FAST procedure is presently being tested in swine.

These microbial inhibition tests are simple to perform, cost effective and allow routine testing and release of large numbers of food animal carcasses in the shortest possible time. Use of these screen tests permit FSIS to analyze only those carcasses which were found to contain antimicrobial compounds by in-plant tests.

PART A

33.2 DETECTION OF ANTIMICROBIAL RESIDUES BY SWAB TEST ON PREMISES (STOP)

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33.21 Background

The Swab Test on Premises (STOP) was developed for tentative detection of antimicrobial residues in carcasses. It is performed by inserting a sterile cotton swab into the kidney sample of a carcass. After 30 minutes, the tissue fluid soaked top, one-fourth portion of the swab is transferred to an agar plate seeded with *Bacillus subtilis* spores. After incubation for 16-18 h at 29°C, plates are examined for a zone of inhibition (ZI) around the swab. If no inhibition is seen, the carcass is free of antimicrobial residue at detectable levels. In case of inhibition, presence of antimicrobial residues is suspected and muscle, liver and kidney tissues from the suspect carcass are collected and submitted for confirmation and identification at FSIS laboratories.
In 1980, a modified version of the original test was introduced in slaughter establishments. The agar plates and vials of spores are

separately supplied. In the modified version, prior to performing the test, the plates are surface streaked with the spore suspension by sterile swabs. The rest of the procedure is similar to the original test. The supplies are now commercially available and are stable for 6 months when stored at either room or refrigerated temperatures.

Initially when the test was developed, it used tissues from kidney, liver, muscle, and injection site. However, at present kidney is the target tissue. The sensitivity of the STOP test for sulfonamide detection is unsuitable for regulatory purposes.

33.22 Equipment, Reagents and Supplies

33.221 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34, and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Centrifuge must be able to operate at 20,000 x G at a constant 5°C. It should also operate with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators, one maintaining 37°C and the other 29°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906) or equivalent

33.222 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water.

* Do not use deionized water.

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* Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 per liter of

distilled water. If necessary, adjust pH by dropwise addition of either 0.1 N HCl or NaOH depending upon pH reading. Sterilize by autoclaving at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals) Sterilize in a covered beaker by autoclaving prior to use.

e. Butterfield's Phosphate Buffer, sterile

33.223 Supplies

a. Sterile Roux bottles
b. Sterile glass beads, 4 mm diameter
c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes, 10 ml and 1 ml graduated to the tip
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 51 x 15 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antimicrobial sensitivity discs containing 5 mcg of the antibiotic neomycin (N5)
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 mm diameter X 15 mm petri plates (Falcon Cat. # 1007 or equivalent)

33.23 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize (121°C for 15 minutes)
b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC)
c. Antibiotic Agar No. 5 (Streptomycin Assay Agar)
d. Mueller-Hinton Agar

e. A-K Sporulating Agar No. 2

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with an extra 0.5% Purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare as slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with an extra 0.5% Purified Agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

33.24 Test Organism

Bacillus subtilis ATCC 6633 (American Type Culture Collection, Rockville, MD)

33.241 Purity and Biochemical Properties of *Bacillus subtilis*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture, incubate at 37°C for 18 h and check for culture purity.

b. For isolation, streak the culture onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for the presence of catalase. *Bacillus* sp. are catalase positive.

f. Use colonies from the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h. The biochemical patterns of *B. subtilis* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	+	V

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid; (V) = variable.

g. If the organism does not meet all the above criteria, replace with a new ATCC culture of the organism.

33.242 Preparation of *B. subtilis* spores

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. To each agar slant, add 4-6 sterile glass beads and 2-3 ml sterile distilled water and gently shake for 2 minutes to dislodge bacterial growth.

c. Aseptically transfer the slant suspensions to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of the glass beads. Multiple cultures may be prepared and pooled for transferring.

d. Incubate the Roux bottles horizontally for 18-24 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by adding 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate bottles to dislodge bacterial growth. (Care must be taken not to break the agar during harvesting).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 minutes.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

   i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
   ii. Pour off supernatant.
   iii. Resuspend the pellet in 20 ml sterile distilled water.
   iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

   Mix 34.1 ml of sterile phosphate buffer and 11.8 g of sterile polyethylene glycol in a 100 ml sterile glass stoppered volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the

pellet in 20 ml of sterile distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.243 Enumeration of *B. subtilis* Spores in Working Suspension

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 50 ± 1°C) into each plate. Mix by swirling or tilting plates to evenly disperse the inoculum throughout the medium. Incubate for 48 h at 37 ± 1°C.

d. Count colonies (30-300) in triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml in each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock spore suspension, use the following formula:

$$\text{Concentration of stock spore suspension (cfu/ml)} = \text{Dilution factor} \times \text{Desired concentration of working spore suspension (cfu/ml)}$$

Example:

Stock spore suspension = 1 x 10^9 spores/ml
Desired concentration of working spore suspension = 1 x 10^6 spores/ml:

$$(1 \times 10^9 \text{ cfu/ml}) = (x)\ (1 \times 10^6 \text{ cfu/ml})$$

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

x = 1000

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.244 *Packaging of *B. subtilis* Spore Suspension (for Field Use)

a. Dispense 4.0 ml of the final (working) spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into sterile 51 x 15 mm clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label, or equivalent:

i. "STOP spores"
ii. *B. subtilis* ATCC 6633
iii. Lot Number
iv. Packaging Date

NOTE: *B. subtilis* spores (1 x 10^6 or 1 x 10^7 cfu/ml) can also be obtained from EDITEK, Burlington, NC, by special order.

33.25 *Preparation of STOP Plates (for Field Use)

a. Add 25.5 g of Antibiotic Agar No. 5 (Streptomycin Assay Agar) powder into 1 L of glass distilled water. Heat while stirring and bring to a boil. Sterilize at 121°C for 15 min. Cool and mix the medium thoroughly in a 48°C water bath. Continue mixing during cooling and dispensing.

b. Aseptically add 6.0 ml of the agar to each 60 x 15 mm plate and distribute evenly. Place plates on a flat level surface and allow agar to harden.

*NOTE: Under FSIS contract, STOP spores (1 x 10^6 cfu/ml) and plates are now produced commercially and are routinely available for use. After they meet all quality control specifications they are used in

slaughter plants.

c. Label the lid of each plate with the following information:

i. "STOP PLATE"
ii. Lot Number
iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

33.251 Preparation of STOP Plates (Used in Laboratory)

a. Add 25.5 g of Antibiotic Agar No. 5 (Streptomycin Assay Agar) powder to 1 L of distilled water. Heat while stirring and bring to a boil. Sterilize at 121°C for 15 minutes. Cool and mix the medium thoroughly in a 48°C water bath.

b. Aseptically add 1 ml of 1 x 10^7 cfu/ml *B. subtilis* spore suspension per 100 ml of the agar. Mix thoroughly. Pipette 8 ml of the agar into each 100 x 15 mm plate and tilt plates to insure even distribution. Allow the plates to harden on a flat, level surface.

c. Label the lid of each plate with the following information:

i. "STOP PLATE"
ii. Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 10 working days.

33.26 Performing the STOP Test

33.261 Sample Condition

a. Assure that the samples are cold, 4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: Presently STOP is used only on kidney tissue of all classes of animals, i.e., bovine, swine, sheep/goat, and horses with the exception of bob veal calves.

33.262 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into each kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab and insert the cotton tip into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissue for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes before streaking. Check each plate for absence of contamination, cracking of agar or dryness.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at the 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Check for seal integrity of vials containing spores.

i. Shake the ***B. subtilis*** spore vial (1×10^6 cfu/ml) and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

j. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

k. Repeat this streaking process 2 more times. Finally turn the plate 1/2 turn and streak. (Use a separate swab for each plate)

NOTE: Above applies only for plates used in the plant. The plates used in laboratories are seeded at a different

concentration level and therefore should not be surface streaked.

l. Place a neomycin 5 μg disc on the agar surface near the vertical line on a plate.

m. Remove the swab from the tissue, break the shaft approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to inserting.

n. Gently place the swab on the surface of the plate with the broken end of the shaft near the neomycin 5 μg disc making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Swabs from two kidney tissues from two different carcasses can be placed on each plate provided they are properly identified on the plate.

If two tissue swabs are used per plate, place cotton tips in "rabbit ears" configuration (Fig. 1)

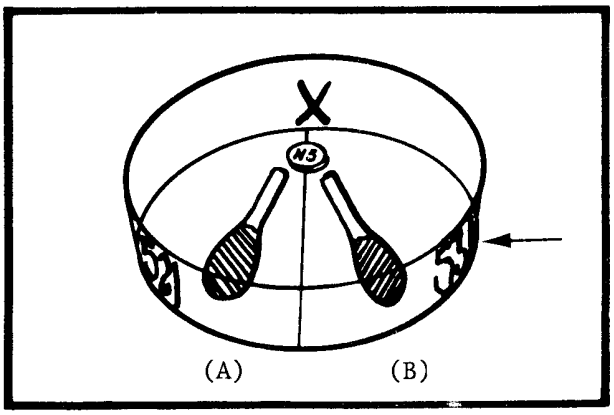

Figure 1. Swab placement on plate

o. Incubate the plates upright at 29 ± 1°C for 16-18 h.

p. Store samples under refrigeration until the test is completed.

33.27 Results and Interpretation

a. Remove the incubated plates from incubator and remove swabs.

b. Measure the ZI by the N5 disc with a mm ruler or with an antibiotic zone reader. The zone should be 20-26 mm wide. If the zone is not 20-26 mm in width, the test is inconclusive and should be repeated.

c. Observe the plates for inhibition of *B. subtilis* growth surrounding the swabs.

i. If a zone of inhibition is observed, the test is positive. Measure the length and the width of the zone and record results.

ii. If no zone of inhibition is observed, the test is negative. Record the result.

33.28 Selected References

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PART B

33.3 DETECTION OF ANTIMICROBIAL RESIDUE IN CALVES BY CALF ANTIBIOTIC AND SULFONAMIDE TEST (CAST)

Clarence A. White, B. P. Dey and Richard H. Reamer

33.31 Introduction

The Calf Antibiotic and Sulfa Test (CAST) is a modified form of the Sulfa Swab Technique (SST). Sulfonamides are frequently used in bob veal calves, a class of animals weighing under 150 pounds and less than three weeks old. This test is used to detect antibiotic and sulfonamide residues in bob veal calves at slaughter.

The inspectors performing the test at slaughter plants are supplied with agar plates and vials containing an alcohol suspension of spores. To perform the test, a sterile cotton tipped applicator (swab) is inserted into the kidney sample of a bob veal calf and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked by sterile swabs with the supplied *Bacillus megaterium* spore suspension. The swab is removed from the kidney, broken as close to the cotton tip as possible, and placed on to the agar plate streaked with spores. After 16-18 h incubation at 44°C, plates are examined for a zone of inhibition (ZI) around the swab. If no inhibition is seen, the carcass is free of antimicrobial residues at a detectable level. All carcasses presenting inhibition are subjected to laboratory confirmation.

33.32 Equipment, Reagents and supplies

33.321 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34, and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Centrifuge must be able to operate at 20,000 x G at a constant 5°C. It should also operate with a swinging bucket rotor at 1,500 x G at room temperature.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators 37°C and 44 + 1°C
g. Precision water bath (temperature 48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906) or equivalent

33.322 Reagents

a. **Distilled water:**

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. **Phosphate buffer (3 M, pH 7.1)**

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of either 0.1 N HCl or NaOH depending on pH reading. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. **Ethyl alcohol (USP grade, 200 proof)**

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. **Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.**

e. **Butterfield's Phosphate Buffer, sterile**

33.323 Supplies

a. **Sterile Roux bottles**
b. **Sterile glass beads, 4 mm diameter**
c. **Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks**
d. **Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)**
e. **Sterile pipettes, 10 ml and 1 ml graduated to the tip**
f. **Sterile, clear glass vials 51 x 15 mm with deep seated screw caps**
g. **Pressure sensitive labels not to exceed 2" x 1/2"**
h. **Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material**

*** Resins of some systems produce quaternary ammonium compounds**

which interfere with the analysis.

i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm petri plates (Falcon Cat. No. 1007 or equivalent)

33.33 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize at 121°C for 15 minutes.

b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC).

c. A-K Sporulating agar No. 2.

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with extra 0.5% Purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with extra 0.5% purified Agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

d. Mueller-Hinton Agar (Acumedia Manufacturers Inc., Baltimore, MD); reconstitute according to manufacturer's directions, dispense 100 ml/flask and sterilize (121°C for 15 minutes).

33.34 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.341 Purity and Biochemical Properties of *Bacillus megaterium*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture, incubate at 37°C for 18 h and check for culture purity.
b. Streak the culture for isolation onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C

for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for presence of catalase. *Bacillus* are catalase positive.

f. Use colonies from the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h.

The biochemical patterns of *B. megaterium* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	-	A

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid.

g. If the test organism does not meet all the above criteria, replace with a new ATCC culture of the test organism.

33.342 Preparation of *Bacillus megaterium* Spore Suspension

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. After incubation, put 4-6 sterile glass beads and 2-3 ml sterile distilled water into each tube and gently shake for 2 minutes to dislodge organisms from agar slants.

c. Aseptically transfer the suspension from slants to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of glass beads. (Multiple cultures may be prepared and pooled for transfer to Roux

bottles).

d. Incubate the Roux bottles horizontally for 18-24 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by the use of 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate bottles to dislodge bacterial growth. (While harvesting care must be taken not to break the agar).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 minutes.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
ii. Pour off supernatant.
iii. Resuspend the pellet in 20 ml sterile distilled water.
iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

Mix 34.1 ml of sterile phosphate buffer and sterile 11.8 g of polyethylene glycol in a 100 ml glass stoppered volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with sterile distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall

RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the pellet in 20 ml of sterile distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.343 Preparation of Working Spore Suspension of *B. megaterium*

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 48 $\pm$ 1°C) into each plate. Mix by swirling or tilting plates to disperse the inoculum evenly throughout the agar. Incubate for 48 h at 37 $\pm$ 1°C.

d. Count colonies (30-300) in triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml for each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock

spore suspension, use the following formula:

Concentration of stock spore suspension (cfu/ml)	=	Dilution factor	X	Desired concentration of working spore suspension (cfu/ml)

Example:

Stock spore suspension = 1×10^9 spores/ml
Desired concentration of working spore suspension = 1×10^6 spores/ml:

$(1 \times 10^9 \text{ cfu/ml}) = (x)\ (1 \times 10^6 \text{ cfu/ml})$

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

$x = 1000$

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.344 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into each (51 x 15 mm) clear glass vial with leak-proof screw caps.

b. After capping the vials, seal with shrink-seal, or equivalent material to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label, or equivalent:

i. "CAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Lot Number
iv. Packaging Date

NOTE: Under FSIS contract, CAST spores are produced commercially. After these spores meet all quality control specifications they are used in slaughter plants.

33.35 Preparation of CAST Plates

a. Weigh and add 38 g of Mueller-Hinton Agar powder to 1 L distilled water. Heat while stirring and bring to a boil. Sterilize the medium at 121°C for 15 minutes and then mix it thoroughly. Allow agar to cool to 48°C in a water bath. Continue mixing during cooling and dispensing.

b. Using a sterile agar delivery system, deliver 6.0 ml of the agar to each 60 x 15 mm plate. Distribute the agar evenly to cover entire surface of the plate. Allow the agar to harden on a flat, level surface.

c. Label the lid of each plate using a label containing the following information:

   i. "CAST PLATE"
   ii. Lot Number
   iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

NOTE: Under FSIS contract, CAST plates are produced commercially. After these plates meet all quality control specifications they are used in slaughter plants.

33.36 Performing the CAST Test

33.361 Sample Condition

a. Assure that the samples are received at a temperature of 4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: CAST test should only be used on kidney tissue of bob veal calves.

33.362 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and

insert the sharp end of the swab about 1/2" to 3/4" into the kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab and insert the cotton tip into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissues for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes before streaking. Check each plate for absence of contamination, cracking of agar or dryness.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Check for seal integrity of vials containing spores.

i. Shake the *B. megaterium* spore vial and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

j. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

k. Repeat this streaking process 2 more times. Finally turn the plate 1/2 turn and streak. (Use a separate swab for each plate)

l. Place a neomycin 5 μg disc on the agar surface near the vertical line on a plate.

m. Remove the swab from the tissue, break the shaft approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue

and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

n. Gently place the swab on the surface of the plate with the broken end of the shaft near the neomycin 5 μg disc making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Swabs from two different tissues or carcasses may be placed on each plate provided they are properly identified as to location on the plate.

If two tissue swabs are used per plate, place cotton tips in a "rabbit ears" configuration (Fig. 2).

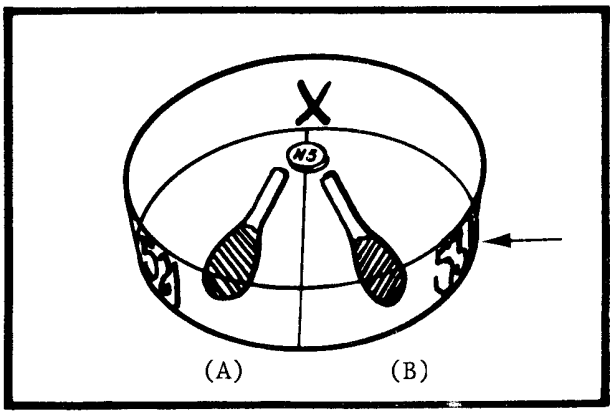


Figure 2. Swab placement on plate

o. Incubate the plates upright at 44 ± 1°C for 16-18 h.

p. Store samples under refrigeration until the test is completed.

33.363 Results and Interpretation

a. Remove plates from the incubator and remove the swabs.

b. Measure the zone of inhibition around the N5 disc with a mm ruler. The ZI should be 24-29 mm wide. If the ZI is not 24-29 mm in width, the test is inconclusive and

should be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs.

i. If a zone of inhibition is observed, the test is positive. Measure the width of the zone and record results.

ii. If no zone of inhibition is observed, the test is negative. Record the result.

33.37 Selected References

Johnston, R. W., R. H. Reamer, E. W. Harris, H. G. Fugate, and B. Schwab. 1981. A new screening method for the detection of antibiotic residues in meat and poultry tissues. J. Food Prot. 44:828-831.

Kramer, J., G. G. Carter, B. Arret, J. Wilner, W. W. Wright, and A. Kirshbaum. 1968. Item 344-837 (4008). Antibiotic Residues in Milk, Dairy Products and Animal Tissues: Methods, Reports and Protocols. Food and Drug Administration, Government Printing Office, Washington, DC.

Read, R. B., J. G. Bradshaw, A. A. Swatzentruber, and A. R. Brazis. 1971. Detection of sulfa drugs and antibiotics in milk. Appl. Microbiol. 21:806-808.

United States Department of Agriculture. 1982. The shelf stable swab test system for detecting antibiotic residues in tissues. Laboratory Communication No. 31. Food Safety and Inspection Service, S&T, Microbiology Division, Washington, D.C.

United States Department of Agriculture. 1984. Performing the Calf Antibiotic and Sulfa Test. Food Safety and Inspection Service, Administrative Management, Training and Development Division, College Station, TX.

PART C

33.4 TENTATIVE CONFIRMATION OF CAST RESULTS FOR SULFONAMIDE RESIDUES IN MEAT AND POULTRY TISSUE

B. P. Dey, Sandra L. Kamosa and Clarence A. White

33.41 Background

The Calf Antibiotic and Sulfa Test (CAST) is presently being used for detecting sulfonamide residues in bob veal calves. The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. A *Bacillus megaterium* spore suspension is applied to CAST agar plates by a sterile swab. The swab from the kidney is then placed on the agar plate and incubated at 44°C for 16-24 h. The plate is then examined for a zone of inhibition (ZI) around the swab. In the case of an 18 mm or greater zone of inhibition, the carcass is subjected to further laboratory analysis. The muscle, liver and kidney tissues from the suspect carcass are sent to the laboratories for analysis. This procedure describes a modified CAST method with sensitivity equal or better than commercial CAST for verifying field results in 5-6 h with inclusion of another plate for confirming the presence of sulfonamide residues in suspected samples at the same time.

33.42 Equipment, Reagents and supplies

33.421 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 37°C and the other 44 ± 1°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906)

33.422 Reagents

a. **Distilled water:**

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. **Phosphate buffer (3 M, pH 7.1)**

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filter through a 0.2 μm filter.

c. **Ethyl alcohol (USP grade, 200 proof)**

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. **Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.**

e. **Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.**

f. **Bacto-Dextrose (Difco, Detroit, MI; Cat. No. 0156-17-4)**

g. **p-aminobenzoic acid (Fisher Scientific Co. NJ; Cat. No A-41-70522)**

h. **Butterfield's Phosphate Buffer, sterile**

33.423 Supplies

a. **Sterile Roux bottles**
b. **Sterile glass beads, 4 mm diameter**
c. **Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks**

*

Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes graduated to the tip, 10 and 1 ml
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 51 x 15 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. # 1007 or equivalent)

33.43 Media

Proceed exactly as that described in Section 33.33.

33.44 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.441 Purity and Biochemical Properties of *Bacillus megaterium*

Proceed exactly as that described in Section 33.341.

33.442 Preparation of *B. megaterium* Spore Suspension

Proceed exactly as that described in Section 33.342

33.443 Enumeration of *B. megaterium* Spores in Working Suspension

Proceed exactly as that described in Section 33.343 except prepare the final spore suspension such that it contains 1 x 10^7 cfu/ml.

33.444 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^7 cfu/ml in 50% ethyl alcohol) into sterile 51 x 15 mm clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "CAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

NOTE: *B. megaterium* spores (1×10^7 cfu/ml) can be obtained from EDITEK, Burlington, NC, by special order.

33.45 Preparation of Plates

33.451 Preparation of Modified CAST (M-CAST) Plates

a. Weigh and add 38 g of Mueller-Hinton Agar (Acumedia) powder to each liter of glass distilled water. Weigh and add 8 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to boil.

b. Cool to 48°C and adjust the pH to 7.2 ± 0.1. Sterilize at 121°C for 15 minutes and mix thoroughly. Allow the agar medium to cool to 48°C in a water bath.

c. Continue mixing during cooling.

d. Add 1 ml of *B. megaterium* spore suspension (1×10^7 cfu/ml) to every 100 ml of the medium and mix thoroughly.

e. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

f. Distribute the agar evenly to cover entire surface of the plate. Place plates on a flat, level surface and allow the agar to harden.

g. Label the side of each plate with a marker with the following information:

i. "M-CAST PLATE"
ii. Date

h. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 15 working days.

33.452 Preparation of Modified CAST Plus (M-CAST+) Plates

a. Weigh and add 38 g of Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Weigh and add 8 g dextrose to the mixture. Add 70 milliliters of Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to a boil.

b. Add 200 mg of p-aminobenzoic acid to the medium.

c. Cool to 48°C and adjust pH to 7.2 ± 0.1. Sterilize at 121°C for 15 minutes and mix thoroughly. Cool the medium in a 48°C water bath.

d. Continue mixing during cooling.

e. Add 1 ml of *B. megaterium* spore suspension (1 x 10^7/ml) to every 100 ml of the medium and mix thoroughly.

f. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

g. Distribute the agar evenly to cover entire surface of the plate. Place plates on a flat surface and allow the agar to harden.

h. Label the side of each plate with a marker with the following information:

   i. "M-CAST+ PLATE"
   ii. Date

i. Refrigerate plates in sealed plastic (Ziplock®) bags to prevent moisture evaporation. These plates can be used for a period of 15 working days.

33.46 Performing the Test

33.461 Sample Condition

a. Assure that the samples are received at a temperature of 4°C or below.

b. Identify samples according to standard operating procedures.

33.462 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that

ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove both swabs, and insert the sharp end of the swabs shaft 1/2" to 3/4" into the kidney tissue.

c. Move the swab shafts back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shafts.

d. Reverse the swabs and insert the cotton tips into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissue for a minimum of 30 minutes.

f. Leave refrigerated plates (M-CAST and M-CAST+) at room temperature for about 20-30 minutes to warm up. Discard plates which are contaminated, dried or cracked.

g. Place a neomycin 5 μg (N5) disc and a sulfamethazine 2 μg (S2) disc on separate M-CAST and M-CAST+ plates (control plates) in use each day the test is performed. Make sure that the distance between the two discs is 35-40 mm.

h. Remove the swabs from the tissue, break the shafts approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

i. Gently place one of the swabs on an M-CAST plate and the other swab on an M-CAST+ plate making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Properly identified, four (4) swabs from 4 samples can be placed on each plate provided the cotton tip end of one lies next to the shaft of another as shown in Fig. 1.

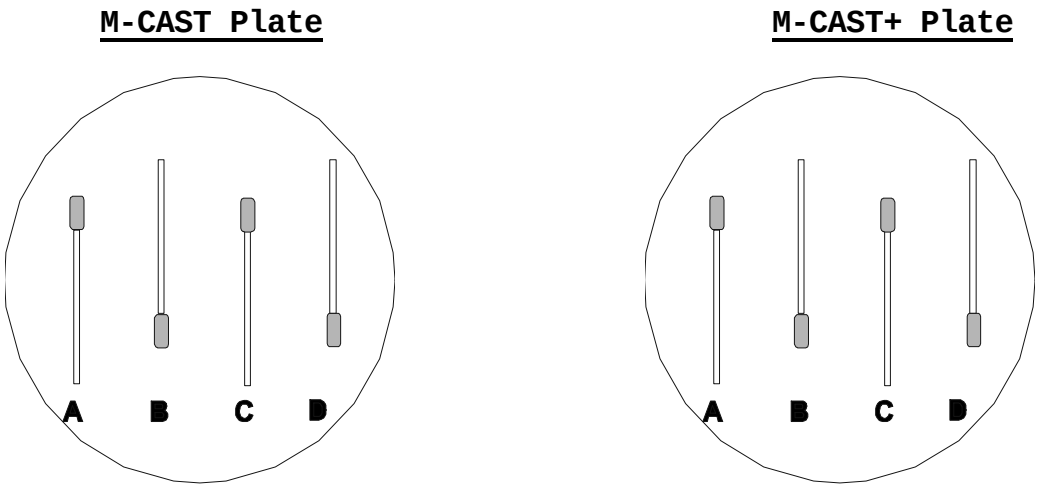


Fig. 1 . Placement of swabs on M-CAST and M-CAST+ plate

j. Incubate plates with sample swabs and the control discs (N5 and S2) upright at 44 ± 1°C for 5-6 h.

k. Refrigerate sample until the test is complete.

33.47 Results and Interpretation

a. Remove the plates from incubator and remove swabs.

b. Measure the ZI around the N5 and S2 discs on the control plates with a mm ruler or by a zone reader. The N5 zone should measure between 20-26 mm on both M-CAST and M-CAST+ plates. There should be a 16-19 mm zone by the S2 disc on the M-CAST plate only, where as there will be no zone by the S2 disc on the M-CAST+ plate. If the observed ZI are not in agreement with the above, repeat the test.

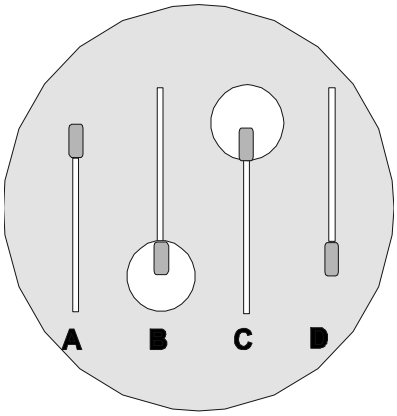
c. Measure the zone of inhibition surrounding each swab corresponding to a sample on each plate (from right to left).

NOTE: It is essential to read test results within 6 h. As the inhibitory effect by bacteriostatic drugs such as sulfonamides diminishes, organisms temporarily inhibited recover over time causing reduction in the zone of inhibition as incubation time increases.

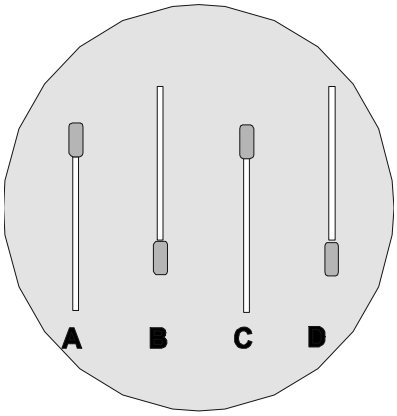
d. i. Samples with sulfonamide residue appear as illustrated below:

M-CAST plate: Zone of inhibition (Samples B and C)
M-CAST+ plate: No zone of Inhibition (Samples B and C)

M-CAST Plate



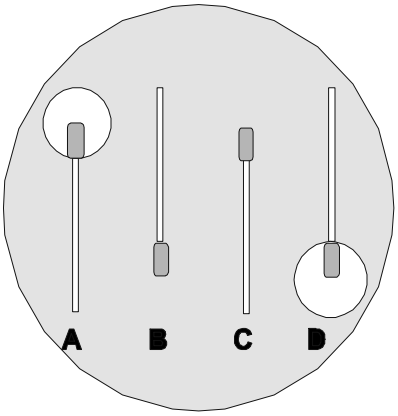
M-CAST+ Plate



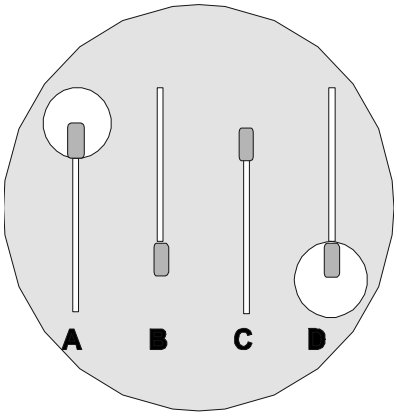
ii. Samples free of sulfonamide residue, but containing antibiotics, appear as illustrated below:

M-CAST plate: Zone of inhibition (Samples A and D)
M-CAST+ plate: Zone of Inhibition (Samples A and D)

M-CAST Plate



M-CAST+ Plate



33.48 Quality Control

a. Test organism must be evaluated for purity and proper biochemical patterns.

b. Freshly prepared plates must be tested with the N5 and S2 discs to assure proper performance.

c. Plates must not be used for more than 15 working days past preparation.

d. Extreme caution should be taken in adding para-amino benzoic acid because the chemical at a higher concentration than the recommended level is toxic to the test organism.

e. New chemicals/reagents and agar should be checked to assure quality.

33.49 Selected References

Dey, B. P., S. Kamosa, and Clarence White. 1995. Tentative confirmation of CAST results for sulfonamide residues in meat and poultry tissue. Laboratory Communication No. 78. USDA, Food Safety and Inspection Service, S&T, Microbiology Division, Washington, D.C.

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United States Department of Agriculture. 1982. The shelf stable swab test system for detecting antibiotic residues in tissues. Laboratory Communication No. 31. Food Safety and Inspection Service, S&T, Microbiology Division, Washington, D.C.

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PART D

33.5 DETECTION OF ANTIMICROBIAL RESIDUE BY FAST ANTIMICROBIAL SCREEN TEST (FAST)

B. P. Dey, Clarence A. White and Nitin H. Thaker

33.51 Introduction

The Fast Antimicrobial Screen Test (FAST), an in-plant screen test, was developed in 1989 to improve the capability of the Antibiotic Residue Detection Program. FAST has higher sensitivity and can detect a wider range of antibiotics and sulfonamides than STOP and CAST. The test has been introduced in 50 bovine slaughter establishments. It is also being evaluated in swine species. If found suitable in both bovine and swine, it may be used in all species of food animals for detecting antimicrobial residues. Besides improving efficiency, this test would be used uniformly for detecting antibiotic and sulfonamide residues in food animal carcasses.

The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked with *Bacillus megaterium* spore suspension on a sterile cotton swab. The swab from the kidney is removed, broken as close to the cotton tip as possible, and placed onto the agar plate and incubated at 44°C. The plate is examined for a zone of inhibition (ZI) around the swab at 6 and 18 h. In the case of inhibition at 6 h, the plate is further examined at 18 h for confirmation. If there is clear inhibition, muscle, liver and kidney tissues from the suspect carcass are collected and further analyzed for confirmation at an FSIS laboratory. When no inhibition is seen at 6 h, the carcass is free of antimicrobial residues at detectable levels. The test allows screening and releasing a large number of residue free carcasses within a work shift.

33.52 Equipment, Reagents and Supplies

33.521 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent

d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 35°C and the other at 44 ± 0.5°C
g. Precision water bath (with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Cat.# 07-906)

33.522 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.

e. Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.

f. Bacto-Dextrose (Difco, Detroit, MI; Cat. # 0156-17-4) or equivalent

g. Butterfield's Phosphate Buffer, sterile

*** Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.**

33.523 Supplies

a. Sterile Roux bottles
b. Sterile glass beads, 4 mm diameter
c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes, 10 ml and 1 ml graduated to the tip
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. No. 1007 or equivalent)

33.524 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize (121°C for 15 min).

b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC).

c. A-K Sporulating Agar No. 2.

   i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with extra 0.5% purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare slants.

   ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with extra 0.5% purified agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

d. Mueller-Hinton Agar (Acumedia Manufacturers Inc., Baltimore, MD); reconstitute according to manufacturer's

directions, dispense as desired and sterilize (121°C for 15 minutes).

33.53 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.531 Purity and Biochemical Properties of *Bacillus megaterium*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture and incubate plates at 37°C for 18 h. After incubation check for culture purity.

b. Streak the culture for isolation onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for presence of catalase. *Bacillus* are catalase positive.

f. Use the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h.

The biochemical patterns of *B. megaterium* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	-	A

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid.

g. If the organism does not meet all the above criteria, replace with a new ATCC culture of the test organism.

33.532 Preparation of *Bacillus megaterium* Spore Suspension

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. Add 4-6 sterile glass beads and 2-3 ml sterile distilled water to each slant and gently shake for 2 minutes to dislodge organisms.

c. Aseptically transfer the slant suspensions to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of sterile glass beads. Multiple cultures may be prepared and pooled.

d. Incubate the Roux bottles horizontally for 18 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by adding 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate each bottle to dislodge bacterial growth. (Care must be taken not to break the agar during harvesting).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 min.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

   i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
   ii. Pour off supernatant.
   iii. Resuspend the pellet in 20 ml sterile distilled water.
   iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

   Mix 34.1 ml of sterile phosphate buffer and 11.8 g of polyethylene glycol in a 100 ml glass stoppered sterile volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a

Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the pellet in 20 ml of distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.533 Enumeration of *B. megaterium* Spores in Stock Suspension

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 48 ± 1°C) to each plate. Mix by swirling and tilting plates for even dispersal of the inoculum. Incubate the plates at 37 ± 1°C for 48 h.

d. Count colonies (30-300) of triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml in each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock spore suspension, use the following formula:

Concentration of stock suspension (cfu/ml)	=	Dilution factor	X	Desired concentration of working spore suspension (cfu/ml)

Example:

Stock spore suspension = 1 x 10^9 spores/ml

Desired concentration of spore suspension = 1 x 10^6 spores/ml:

(1 x 10^9 cfu/ml) = (x) (1 x 10^6 cfu/ml)

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

x = 1000

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.534 Packaging of *B. megaterium* Spore Suspension (Field Use)

a. Dispense 4.0 ml of the working spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into sterile (15 mm diameter x 51 mm height) clear, glass vials with deep seated, leak-proof screw caps.

NOTE: Under FSIS contract, FAST spores are produced commercially. After they meet all quality control specifications they are used in slaughter plants.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "FAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

33.54 Preparation FAST Plates (Used in the Plant)

a. Weigh and add 38 g Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Add 7 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to boil. After sterilizing at 121°C for 15 minutes, mix the medium thoroughly, and cool it in a 48°C water bath. Continue mixing during cooling and dispensing.

b. Using a sterile agar delivery system, deliver 6.0 ml agar to each 60 x 15 mm plate. Distribute the agar evenly to cover entire surface of the plate. Place plates on flat level surface and allow the agar to harden.

c. Label the lid of each plate using a label, containing the following information:

i. "FAST PLATE"
ii. Lot Number
iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

NOTE: Under FSIS contract, FAST plates are produced commercially. After these plates meet all quality control specifications they are used in slaughter plants.

33.55 Performing the FAST Test

33.551 Tissue Sample and Conditions

a. The kidney is the target tissue for FAST

b. The kidney and other tissue samples should be received at 4°C or below and identified properly.

33.552 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into the kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab, insert the cotton tip into the tissue opening and twist to make sure that the cotton tip is in contact with the macerated tissue.

e. *Allow swabs to remain in the tissues for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes. Check plates for contamination, cracking or dryness of agar.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Shake the *B. megaterium* spore vial and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

i. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

j. Repeat this streaking process 2 more times. Finally turn

the plate 1/2 turn and streak. (Use a separate swab for each plate)

*NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

k. As a control, place a neomycin (N5) 5 μg disc one half inch from the edge of the plate on the agar surface.

l. Remove swab from the tissue and break the shaft approximately two inches from the swab end.

m. Gently place the swab on the agar without breaking the surface. Make sure that swab has uniform contact with the surface.

NOTE: Two (2) swabs from two samples can be placed on one plate as illustrated below in (Figure 1).

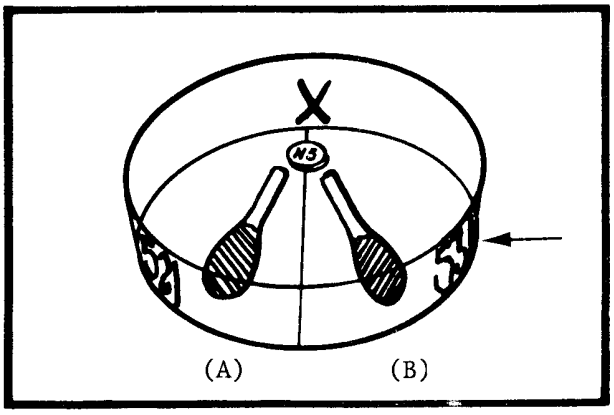

Figure 1. Swab placement on plate

n. Incubate the plates upright at 44 ± 0.5°C for 6 h, up to a maximum of 16-18 h.

o. Store samples in refrigerator until the test is completed.

33.56 Results and Interpretation

a. Remove plates from the incubator and remove the swabs.

b. Measure the ZI around the N5 disc with a mm ruler or a zone reader. The zone should be 20-26 mm wide. If not, the test must be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs (Figure 2).

i. Samples with Antimicrobial Chemical Residue

Zone of inhibition around swab "A": Sample A may contain antimicrobial residue, and must be subjected to confirmatory testing procedures.

ii. Samples without Antimicrobial Chemical Residue

No Zone of Inhibition around swab "B" : Sample B is free of antimicrobial residue.

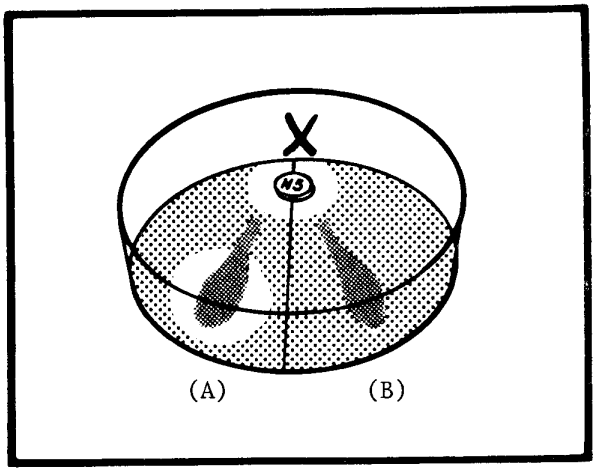

Figure 2. Inhibition of microorganism by swab

33.57 Quality Assurance

a. The FAST plates can be stored at room temperature protected from extremes of heat, cold and moisture.

b. Store spore suspensions under refrigeration condition with cap tightly closed.

c. Store neomycin disc vial in a plastic bag in refrigerator.

d. Do not use outdated plates, spores or N5 discs.
e. Shake the spore vial for even dispersal of spores.

f. Check plates before use for contamination, cracking or drying of agar.

g. Do not to break the agar surface while placing the neomycin disc and the swab.

h. Allow swabs to remain in the tissues for 30 minutes.

i. Read plates any time after 6 h of incubation, up to a maximum of 18 h.

j. Make sure that the incubator temperature is 44 ± 0.5°C.

33.58 Selected References

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PART E

33.6 EVALUATION OF ANTIMICROBIAL RESIDUES IN MEAT AND POULTRY TISSUE BY A MODIFIED FAST ANTIMICROBIAL SCREEN TEST (M-FAST)

B. P. Dey, Richard H. Reamer and Sandra L. Kamosa

33.61 Introduction

The Fast Antimicrobial Screen Test (FAST) developed in 1989, is presently being used in selected bovine slaughter plants. It is expected that the test will be used universally in plants for the detection antimicrobial residue in all species of food animal carcasses. The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked with *Bacillus megaterium* spore suspension using a sterile cotton swab. The swab from the kidney is removed, broken as close to the cotton tip as possible, and placed onto the agar plate and incubated at 44°C. The plate is examined for a zone of inhibition (ZI) around the swab at 6 and 18 h. In the case of inhibition at 6 h, the plate is further examined at 18 h for confirmation. If there is no inhibition at 6 h, the carcasses is released. The test allows screening and releasing a large number of residue free carcasses within a work shift. If there is clear zone of inhibition, muscle, liver and kidney tissues from the suspect carcass are collected and further analyzed for confirmation at an FSIS laboratory. The method described here is a modified FAST procedure for verifying field test results in 6 h with sensitivity equal to the commercial FAST at comparable incubation times.

33.62 Equipment, Reagents and Supplies

33.622 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 37°C and the other 44 ± 0.5°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or

equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906)

33.623 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.

e. Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.

f. Dextrose (Bacto Dextrose-Difco, Detroit, MI; Cat. No. 0156-17-4) or equivalent.

g. Butterfield's Phosphate Buffer, sterile

33.624 Supplies

a. Sterile Roux bottles.
b. Sterile glass beads, 4 mm diameter

*

Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes graduated to the tip, 10 and 1 ml.
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin (N5)- 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. No. 1007 or equivalent)

33.63 Media

Proceed exactly as that described in Section 33.524.

33.64 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.641 Purity and Biochemical Properties of *Bacillus megaterium*

Proceed exactly as that described in Section 33.531.

33.642 Preparation of *B. megaterium* Spore Suspension

Proceed exactly as that described in Section 33.532.

33.643 Enumeration of *B. megaterium* Spores in Stock Suspension

Proceed exactly as that described in Section 33.533 except prepare the final spore suspension such that it contains 1 x 10^7 cfu/ml.

33.644 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^7 cfu/ml in 50% ethyl alcohol) into sterile (51 x 15 mm) clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

NOTE: *B. megaterium* spore (1×10^7 cfu/ml) can be obtained by special order from EDITEK, Burlington, N.C.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "FAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

33.65 Preparation of Plates

a. Weigh and add 38 g Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Add 8 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.4%) to the mixture. Heat while stirring and bring to boil. Cool (48°C waterbath). Adjust the pH to 7.2 ± 1. After the medium has been sterilized at 121°C for 15 minutes, mix the medium thoroughly.

b. Keep mixing the medium while cooling in a 48°C water bath.

c. Add 1 ml of *B. megaterium* spore suspension (1×10^7/ml) to every 100 ml of the medium and mix thoroughly.

d. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

e. Distribute the agar evenly over the entire plate. Place plate on a flat, level surface and allow agar to harden.

f. Label the lid of each plate using a label containing the following information:

i. "M-FAST PLATE"
ii. Expiration Date

g. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of up to 15 working days.

33.66 Performing the Test

33.661 Sample Condition

a. Assure that the samples are received at a temperature of

4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: FAST test is used on the kidney tissue of all bovine species where implemented.

33.662 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab, insert the cotton tip into the tissue opening and twist to make sure that the cotton tip is in contact with the macerated tissue.

e. Leave the swab in the tissues for a minimum of 30 minutes.

f. Allow the plates to warm at room temperature for about 20 minutes. Check plates for contamination, cracking and dryness of agar.

g. As a positive control place a neomycin 5 μg (N5) disc in the center of a plate from the same batch used in the analysis.

h. Remove the swab from the tissues, break the shaft approximately two inches from the swab end.

NOTE: If a swab appears dry, reinsert and squeeze the tissue around the swab to absorb fluid. For a dry muscle tissue, moisten the swab with distilled water prior to insertion.

i. Place the swab on the agar surface gently with uniform contact with the surface.

NOTE: Properly identified, four (4) swabs from 4 samples can

be placed on each plate provided the cotton tip end of one lies next to the shaft of another as shown in Fig. 1.

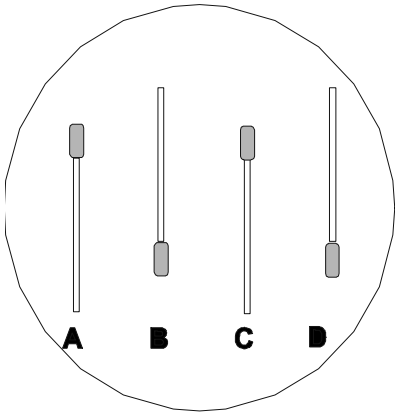

Figure 1. Swab placement on plate.

j. Incubate the plates upright at 44 ± 0.5°C for 6 hours, up to a maximum of 16-18 h.

k. Store samples in refrigerator until the test is completed.

33.663 Results and Interpretation

a. Remove the control and test plates with swabs from incubator and remove swabs.

b. Measure the ZI around the N5 disc on the control plate with a mm ruler or a zone reader. The zone should be 20-26 mm wide. If not, the test should be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs (Figure 2).

i. Samples Free of Antimicrobial Chemical Residue

If no zone of inhibition is observed around a swab, the test is negative, .i.e. the samples (A, B, C and D) do not contain an antimicrobial residue (Fig. 2).

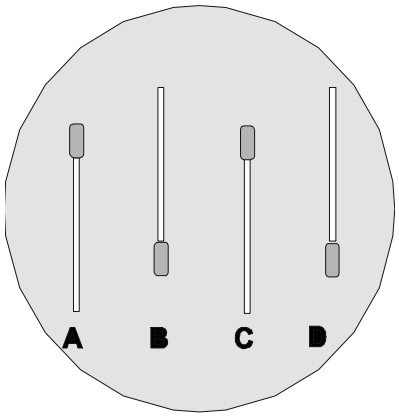

Figure 2. Swabs with no zone of inhibition.

ii. Samples with Antimicrobial Residue

If a zone of inhibition is observed around a swab, the test is positive, i.e. the samples (A and D) may have an antimicrobial residue. Measure the width of the zone.

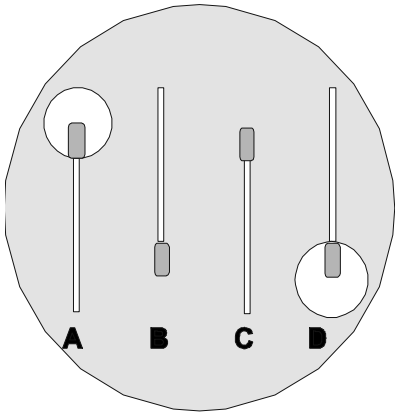

Figure. 3. Positive samples illustrating zone of inhibition around the swabs (samples) A and D.

d. Record and compare result with the field result. Positive samples must be subjected to confirmatory testing.

33.67 Quality Assurance

a. The M-FAST plates wrapped in plastic bags should be stored at refrigerator temperature (4-8°C).

b. Spore suspensions in tightly closed container should be stored at refrigerator temperature (4-8°C).

c. Neomycin disc vial wrapped in a plastic bag should be stored at refrigerator temperature (4-8°C).

d. Observe expiration date of plates. More than 2 week old plates should be discarded.

e. The spore vial should be shaken thoroughly before use.

f. Incubate 1 plate each day at 44°C as control.

g. Check plates before use for contamination, drying or cracking of agar.

h. Allow enough room for each swab placed on a plate.

i. Be careful not to break the agar surface while placing the neomycin disc and the swabs.

j. Leave swabs in the tissues for a minimum of 30 minutes.

k. Read plates any time after 6 h of incubation, up to a maximum of 18 h.

l. Stabilize the incubator temperature at 44 ± 0.5°C.

33.68 Selected References

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