The National Antimicrobial Resistance Monitoring System

Manual of Laboratory Methods

SECOND EDITION
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Table of Contents

Introduction

The National Antimicrobial Resistance Monitoring System – Enteric Bacteria (NARMS) was established in 1996 as a collaborative effort between the Food and Drug Administration's Center for Veterinary Medicine (FDA-CVM), U.S. Department of Agriculture Food Safety Inspection Service (USDA-FSIS) and Agricultural Research Service (USDA-ARS), and the Centers for Disease Control and Prevention (CDC). The NARMS program monitors select enteric bacteria in humans, animals, and retail meats for changes in resistance to various antimicrobial drugs of human and veterinary importance.

The primary objectives of NARMS are to:

1. Monitor trends in antimicrobial resistance among enteric bacteria from humans, retail meats, and animals
2. Disseminate timely information on antimicrobial resistance to stakeholders in the U.S. and abroad to promote interventions that reduce resistance among foodborne bacteria
3. Conduct research to better understand the emergence, persistence, and spread of antimicrobial resistance
4. Provide data that assist the FDA in making decisions related to the approval of safe and effective antimicrobial drugs for animals

Additionally, NARMS provides a national source of bacterial isolates that are valuable for research such as diagnostic test development, discovering new genes and molecular mechanisms associated with resistance, studying mobile gene elements, virulence and other studies.

Point of Contact:
Dr. Patrick McDermott, Ph.D.
Director, NARMS
U.S. Food and Drug Administration
Center for Veterinary Medicine
Patrick.McDermott@fda.hhs.gov

Equipment and Materials

1. Equipment and Supplies

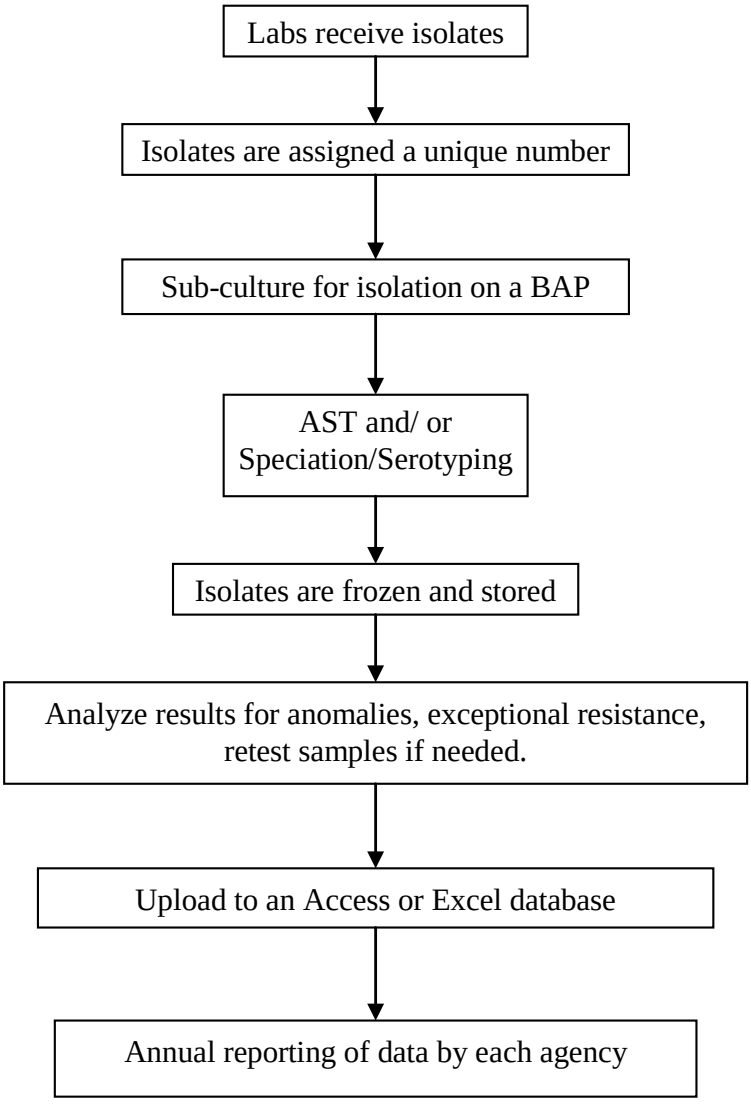
Microaerobic incubators 36±1°C and/or 42°C
Aerobic incubator, 36±1°C
Anaerobe jars (Mart Microbiology B.V.)
Anoxomat® automatic anaerobic system (Spiral Biotech)
Bax® Q7 Instrument
Cryovials
Dry Ice
Extended length pipette tips
Light Cycler (Roche)
Thermal Cycler
Microaerobic gas mixture (85% nitrogen, 10% carbon dioxide, 5% oxygen)
Re-sealable (Ziploc-type) bags
Vitek 2 Compact (V2C) (bioMérieux)
Densicheck (bioMérieux)
Sensititre AutoInoculator (Nephelometer & Dispenser) (ThermoFisher/Trek Diagnostics)
Vizion plate reader (ThermoFisher/Trek Diagnostics)
AIM AutoInoculator (ThermoFisher/Trek Diagnostics)
Sensitouch Manual plate reader with SWIN computer (ThermoFisher/Trek Diagnostics)
ARIS with auto reader (ThermoFisher/Trek Diagnostics)
Campylobacter MIC panels (CAMPY) and seals (ThermoFisher/
Trek Diagnostics)
Gram positive MIC panels and seals (CMV#AGPF) (ThermoFisher/ Trek Diagnostics)
Gram negative MIC panels and seals (CMV#AGNF) (ThermoFisher/ Trek Diagnostics)
Dosing Heads (ThermoFisher/Trek Diagnostics)
Vortex mixer
Mirror viewing device
Sterile plate spreaders

2. Media and Reagents

95% Ethanol
Bactidrop ninhydrin (Remel)
Bax® System Real-time PCR Assay Kits (Dupont Nutrition and Health)
Biochemicals (arginine, arabinose, sucrose, MDGP, motility)
Bolton's *Campylobacter* Enrichment Broth (Oxoid)
Brain Heart Infusion (BHI) agar

Brain Heart Infusion broth
Brucella broth with 20% glycerol
Campy-Cefex Agar (Hardy Diagnostics)
Campy-CVA Agar (Remel)
Demineralized water – 5ml (ThermoFisher/Trek Diagnostics)
Cation-Adjusted Mueller-Hinton broth +TES -5 ml (ThermoFisher/Trek Diagnostics)
Cation-Adjusted Mueller-Hinton broth + TES -11 ml (ThermoFisher/Trek Diagnostics)
MacConkey Agar (MAC)
Mueller-Hinton broth + TES + lysed horse blood -11mL (ThermoFisher/Trek Diagnostics)
Sensititre 0.5 McFarland polymer turbidity standard (ThermoFisher/Trek Diagnostics)
Defibrinated sheep blood
Hydrogen peroxide
Indoxyl acetate (Remel)
Luria Broth (LB) with 30% glycerol
Nutrient Agar slants (NA)
Oxidase identification sticks (Oxoid)
Phosphate buffered saline or water
Salmonella antisera
Sodium Hippurate, 96% (Alfa Aesar)
Trypticase Soy Agar (TSA) slants
Trypticase Soy Agar (TSA) with 5% defibrinated sheep blood (BAP)
Trypticase Soy Broth with 20% Glycerol
Wang's *Campylobacter* Freezing Media

Figure 1. Overview of Isolate Processing



Salmonella & Escherichia coli

A. Receipt of Isolates

Isolates are received from human, retail meats, and food animal sources. They are received either on agar slants or frozen in transport vials. Submission sheets and/or electronic files are included with shipments. Because each agency works with *Escherichia coli* and *Salmonella* from different sources, the acquisition of isolates is different for each agency. For additional information please consult each agency's website.

CDC - Human *Salmonella* isolates are received from public health laboratories in all 50 states. (http://www.cdc.gov/narms).

FDA - Retail meats (ground beef, pork chops) and poultry (ground turkey, chicken parts) are cultured for *E. coli* and *Salmonella* by FoodNet sites. Frozen isolates are then forwarded to FDA. (http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm)

USDA FSIS - Primary *Salmonella* and *E. coli* isolates are done on site at the USDA FSIS NARMS lab from dairy cattle, beef cattle, swine, broilers and turkeys collected from FSIS inspected establishment plants nationwide. The isolates are received in the FSIS-Eastern lab on Sheep Blood Agar plates (SBA) for analysis. **(**FSIS Microbiology Laboratory Guidebook)

B. Subculturing for Isolate Purity

Isolates are subcultured for purity on a BAP or MAC and incubated overnight at 35 ±1 ºC. If a culture appears contaminated or contains more than one strain type, additional selective media are used or additional isolation steps may be taken. If any isolate appears non-viable upon initial recovery, enrichment media may be used to promote the growth of the target organism. Once a pure culture is obtained, isolates are tested using the V2C, serology, or other identification methods.

C. Isolate Storage

For long-term storage of *E. coli* and *Salmonella*, CDC prepares a suspension from a BAP in the appropriate medium and freeze. The original isolate is either stored or discarded depending on the agency. Since FDA receives and stores all isolates frozen, the isolate is streaked for purity twice, tested and refrozen. All isolates are stored in a -70 to -80ºC freezer.

USDA FSIS Eastern lab sends isolates in cryovials containing BHI broth with glycerol to FDA/CVM Office of Research in Laurel, MD for storage and for any further testing. No isolates are maintained in the Eastern lab repository after confirmed positive isolates have been sent to FDA.

D. Identification and Speciation of *Salmonella* and *E. coli*

CDC - *Salmonella* are identified at the primary site (state level) using standard biochemical methods, and serotyped according to the Kauffman-White scheme. Mixed or questionable isolates are confirmed by a CDC reference laboratory.

FDA - *Salmonella* and *E. coli* isolates are confirmed on the V2C. *Salmonella* are serotyped according to the Kauffman-White scheme.

USDA FSIS - *Salmonella* isolates are confirmed using the Vitek2 and serotyped on site by FSIS using molecular typing methods. Questionable isolates are sent to the National Veterinary Service Laboratory (NVSL, Ames, IA) for serotyping. *Escherichia coli* are also isolated on site at the USDA FSIS using selective media and V2C.

E. Antimicrobial Susceptibility Testing of *Salmonella* and *E. coli*

1. Gently invert a Sensititre 0.5 McFarland Polymer Turbidity Standard several times and place it in the nephelometer.
2. Press CALIBRATE on the keypad. This action will standardize the nephelometer to the center green LED light on the auto-inoculator.
3. Prepare a gram negative MIC panel for each isolate according to the manufacturer instructions. Ensure that the desiccant inside the package is orange. If the desiccant is green, discard the panel. Label each panel with appropriate identifying information. Place the panel in the auto-inoculator.

Steps 4 through 10 should be completed within 15 minutes.

4. Prepare the inoculum by selecting a few colonies from the BAP and suspend in a pre-labeled tube of 5 ml demineralized water. Vortex well.
5. Adjust to a 0.5 McFarland equivalent by using the Sensititre nephelometer.
6. Transfer 10 µl of the suspension to a sterile 11 ml tube of Sensititre Cation-Adjusted Mueller-Hinton Broth with TES. This transfer is performed with a sterile, extra long pipette tip. Vortex well.
7. Aseptically replace the MHB tube cap with a disposable dosing head.
8. Open the dosing head clamp, invert the inoculated MHB tube and insert it in the clamp.

9. In response to the auto-inoculator display “Enter Pattern #”, press “001”, and then ENTER to dispense 50 µl into each well of the gram negative MIC panel. For the AIM, choose the plate configuration for 50 µl.
10. Press enter again to start dispensing.
11. Once the MIC panel is inoculated, remove the panel from the auto-inoculator and cover it with the provided clear plastic adhesive seal. Ensure that all wells are covered.
12. Repeat steps 3 through 11 (excluding step 9) for the rest of the test isolates and controls. ***Note: The testing panel should be inoculated and placed in the incubator within 15 minutes of preparing the cell suspension.***
13. At least weekly, perform colony counts from the panel’s positive control well (see colony counts, p. 22).
14. Incubate panels in the ARIS with the auto-reader or an aerobic incubator (stack no more than 4 high) at 35 ±1ºC for 18 hours.
15. Panels should be read by the ARIS auto reader or visually using the Sensitouch or Vizion at 18 hours. ARIS procedures are provided in the ARIS manual from ThermoFisher/Trek Diagnostics.
16. In a timely manner, review the minimum inhibitory concentration (MIC) results for abnormalities as outlined in the section *Criteria for Repeat Testing*. For antimicrobials where no MIC is detected by the ARIS, the plates should be visually inspected. MIC’s for the folate pathway inhibitors should be confirmed manually using a mirror viewing device.

F. Interpretive Criteria

For categorizing susceptibility, breakpoints were adopted from the most recent version of the CLSI document M100 or based on NARMS data when needed (e.g., streptomycin). Refer to the NARMS Integrated Report Data Tables for a reference table showing *Salmonella* and *E. coli* breakpoints.

G. Quality Control (QC) for testing *Salmonella* and *E. coli*

1. Antimicrobial susceptibility testing
    a. **Control strains** – *E. coli* ATCC 25922*, Enterococcus faecalis* ATCC 29212*, Staphylococcus aureus* ATCC 29213*, and Pseudomonas aeruginosa* ATCC 27853.
    b. **Frequency**-The QC strains are tested either daily or weekly and whenever a new lot of sterile water, MHB, or panels are used.

c. **Control strain storage-** Maintain stock cultures at -20ºC or below for prolong storage. Store subcultured isolates at 2 to 8 ºC as appropriate for the organism type. The control strain is plated from the freezer onto a BAP at least monthly and then is subcultured once prior to testing.

d. **Media-** For each new lot number of panels, uninoculated MHB is incubated for a sterility test.

e. **Criteria for repeat testing-** If more than 1 out of 20 or 3 out of 30 MICs for each antimicrobial agent/QC organism combination is outside of acceptable MIC limits, it is repeated for 5 consecutive test runs. CLSI guidelines (M07-A10) are followed in the event of further QC failures.

2. Inter-Agency Quality Assurance

   a. Each agency has the option to participate in the WHO External Quality Assurance System (EQAS) annually to test samples for identification, antimicrobial susceptibility and serotyping. The results are recorded and posted on the WHO EQAS website.

   b. All final data are audited internally within each individual agency to ensure the accurate reporting of results.

Culturing Carcass Rinses for *E. coli*

1. Inoculate 1 ml of the carcass rinsate in Buffer Peptone broth (9 ml) and incubate at 36±1°C for approximately 16-20 hours.
2. To isolate *E. coli*, place a cotton swab in the inoculated broth suspension and make a primary streak onto Chromagar ECC selective medium. Streak the plate with a sterile loop for isolation and incubate the culture for 16-20 hours at 36±1°C.
3. A blue-green colony is a presumptive positive test for *E. coli*. Pick a single colony and streak to a second Chomagar plate. Incubate for 16-20 hours at 36±1°C.
4. Transfer a few colonies to a TSA slant and incubate for 16-20 hrs at 36±1°C.
5. The identity of 10 random isolates is confirmed on the V2C monthly.

Campylobacter

A. Receipt of Isolates

Isolates are received from human, retail meats, and food animal sources. They may be received on agar slants, frozen in transport vials or by primary isolation from chicken carcass rinses and turkey sponges collected by the Food Safety and Inspection Service (FSIS). Submission sheets and/or electronic files are included with the isolate shipments. Each agency works with *Campylobacter* from different sources; therefore, the acquisition of isolates is different for each group. A brief summary is given below. A more detailed description can be found at each agency's website.

CDC - Human *Campylobacter* isolates are received from ten state health departments that participate in the Foodborne Diseases Active Surveillance Network (FoodNet). Each isolate is assigned a unique CDC/NARMS number prior to analysis. (http://www.cdc.gov/narms)

FDA - Retail meat isolates of *Campylobacter* are received frozen by the FDA/CVM/NARMS laboratory from retail meat FoodNet surveillance sites. Each isolate is assigned a unique CVM/NARMS number prior to analysis. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm

USDA FSIS - Primary *Campylobacter* isolation is done on-site at the FSIS laboratory from broiler chicken carcass rinses and turkey sponges received from the FSIS national compliance sampling program (http://www.ars.usda.gov/Main/docs.htm?docid=6750)

B. Subculturing for Isolate Purity

1. Sub-culture isolate onto a BAP and streak for isolation. Incubate plate under microaerophilic conditions (85% N2, 10% CO2, 5% O2) for 24 hours at 42°C. Alternatively, plates can be incubated at 36+1°C for 36-48 hours.
2. If there is typical *Campylobacter* growth, select a single, well-isolated colony and streak for isolation on another BAP and incubate as above.
3. If there is sufficient growth of a pure culture, the isolate is processed for identification, susceptibility testing, and freezing. Additional plates may be inoculated if more growth is needed.
4. For contaminated or mixed cultures, sub-culture on Campy-Cefex agar or a selective Campy agar until a pure isolate is obtained.
5. Cultures remain viable up to one week on plates stored at 4°C in a microaerophilic atmosphere.

C. Recovery of Isolates Showing No Growth

1. Prepare Bolton broth with 5% lysed horse blood (no selective supplement) and aseptically dispense 2 ml into sterile 15 ml disposable centrifuge tubes (or dispense 20-25 ml into 50 ml tubes).
2. For frozen stock, thaw the entire culture vial and add it to the prepared tube of broth. For plated stock, use a sterile cotton swab to remove all growth from a plate and suspend it in broth.
3. With the screw cap loose, incubate the inoculated broth tube at 36±1°C in a microaerophilic environment for 18-24 hours.
4. After incubation, use a swab (or dispense 75 µl) to transfer broth to a BAP. Use a loop to streak the remainder of the plate for isolation. Incubate the plate as above.
5. Re-incubate the Bolton broth at 42°C for an additional 1-2 days and streak again onto a BAP in case the initial plate fails to produce growth.
6. If one of the plates above shows typical *Campylobacter* colonies, proceed with bacterial subculturing as previously described.
7. If there is no growth or the growth is obviously atypical, record as a non-viable sample and, if possible, request the FoodNet site to re-submit the original isolate.

D. Isolate Storage

For long term storage of a *Campylobacter* isolates, use a fresh (< 48 h/ 36±1°C or < 24 h/42°C) culture on a BAP to prepare a heavy homogeneous suspension in a cryoprotective medium and store at -80°C. Two isolate storage options are recommended:

1. Prepare cell suspension in defibrinated sheep blood. Flash-freeze tubes in an ethanol/dry ice bath before transferring to -80°C.
2. Prepare cell suspension in Brucella broth with 20% glycerol or Wang's storage medium (Brucella broth, 10% sheep blood, 15% glycerol). Transition tubes at 4°C for approximately 30 min before transferring to -80°C.
3. USDA FSIS Eastern lab sends isolates in Wang's freezing/storage medium to FDA/CVM Office of Research in Laurel, MD for storage and any further testing. No isolates will be maintained in the Eastern lab repository after confirmed positive isolates have been sent to FDA.

E. Identification and Speciation of *Campylobacter*

Currently, each agency uses different methods for final isolate identification and speciation. To ensure comparability, each laboratory participates in several quality assurance programs (see Quality Control section). A brief description of each agency's protocol is below.

CDC

1. After isolates are confirmed *Campylobacter* by a genus specific PCR, they are screened for *jejuni/coli* species using PCR (Linton, et al. 1997; Pruckler, J. et al. 2006)
2. Isolates that are positive by a genus specific PCR and *jejuni/coli* negative are screened using a series of biochemical tests. Tests include hippurate hydrolysis, catalase and indoxyl acetate. The results of the biochemical tests determine which species-specific PCR will be used to identify the isolate..

FDA

1. Isolates are identified by PCR using the HIP and CC primers (Linton, et al. 1997; Zhao, et al. 2001). DNA templates are prepared using a heated cell lysate.
2. If speciation is not determined, PCR is repeated using HIP and CC primers with a new DNA prep prepared by using the MoBio Laboratories Ultra Clean kit (catalog# 12224).
3. If PCR fails to provide a definitive species, the assay is repeated using additional primers (Burnett, et al. 2002; Gonzales, et al. 1997; Wang, et al. 2002).

USDA FSIS

1. Isolates are identified using the BAX® System Q7 instrument and real-time *Campylobacter* identification kits (DuPont Nutrition and Health) which detect *C. coli*, *C. lari*, and *C. jejuni*.

F. Antimicrobial Susceptibility Testing of *Campylobacter*

1. Set up a rack with one 5 ml Mueller-Hinton broth tube and one 11 ml Mueller-Hinton broth containing lysed horse blood for each isolate to be tested.
2. Prepare Sensititre™ CAMPY panels by removing the foil wrapper and labeling with the isolate number. Make sure the desiccant inside the wrapper is orange. Do not use the panel if desiccant is green.
3. Calibrate nephelometer using the 0.5 McFarland turbidity standard. When using the Sensititre™ nephelometer, calibration is complete when the center green nephelometer LED is lit.

Steps 4 through 10 should be completed within 30 minutes.

4. Use a sterile swab to pick several colonies from a BAP incubated for 48 h/36±1°C or 24 h/42°C. Suspend colonies into 5 ml Mueller-Hinton broth, vortex, and adjust to a 0.5 McFarland standard.
5. Transfer 100 µl of 0.5 Mueller Hinton suspension above (see Determining Colony Counts below) to obtain a final concentration of 5.0 x 10 ^5 CFU/mL into 11 ml Mueller Hinton with lysed horse blood using an extra long tip, then vortex.

    Determining Colony Counts

    - From the positive control well, transfer 10 µl using a pipettor to 10 ml water, broth or appropriate diluent; vortex.
    - Spread plate 100 µl from the inoculated water, broth or diluent to each of two BAPs. Ensure there is not excessive moisture on the plate surface prior to plating the suspension.
    - Incubate colony count plates with MIC panels.
    - Average the number of colonies on the two plates and then multiply by 10^4 to obtain the total number of CFU/ml.
    - The inoculum should be between $1 \times 10^5 - 1 \times 10^6$ CFU/ml.
6. Open the dosing head clamp, invert the inoculated Mueller-Hinton broth with lysed horse blood and set inside the clamp.
7. Transfer 100 µl to each well of the panel by using the auto-inoculator (Program #011-ENTER). For the AIM, choose plate configuration for 100 µl.
8. Press ENTER again to start dispensing.
9. Remove the panel once the dispensing has completed and cover the panel with the provided perforated seal making sure a perforated hole is situated above each well.
10. Incubate panels in a microaerophilic atmosphere (85% N_2, 10% CO_2, 5% O_2) at 36±1°C for 48 h or at 42°C for 24 h. Do not stack panels more than four high.
11. Perform colony counts from the panel's positive control well on at least a weekly basis as described above.
12. After incubation, read the panels by visual inspection with ample light preferably using a magnifying plate reader. Growth appears as a deposit of cells at the bottom of a well or as turbid growth. The minimum inhibitory concentration (MIC) is recorded as the lowest concentration of antimicrobial that inhibits growth. Occasionally, a precipitate or amorphous "speck" may be seen in a well and should not be considered as bacterial growth when determining the MIC.

13. After results are read, check the QC strain to make sure the QC ranges are within acceptable limits. If they are not, follow corrective procedures recommended by the Clinical and Laboratory Standards Institute (CLSI Document M07-A10).
14. In a timely, manner, review the minimum inhibitory concentration (MIC) results for abnormalities as outlined in the section Criteria for Repeat Testing.

G. Interpretive Criteria

For categorizing susceptibility, breakpoints were adopted by epidemiological cut-off values. Refer to the NARMS Integrated Report Data Tables for a reference table of interpretive criteria used for *Campylobacter*.

H. Quality Control (QC) for testing *Campylobacter*

1. Antimicrobial susceptibility testing

a. **Control strain***: C. jejuni* ATCC 33560

b. **Frequency**: The control strain is included with every batch of testing

c. **Control strain storage**: The control strain is plated from the freezer onto a BAP at least weekly and then is subcultured at least once prior to testing.

d. **Media**: For each new lot number of panels, uninoculated Mueller-Hinton broth, and Mueller-Hinton broth with blood is incubated for a sterility test.

2. Equipment

AutoInoculator

All equipment is serviced by a preventative maintenance agreement. In the event problems arise with equipment, service calls are placed to ThermoFisher/TREK Diagnostics. Loaner equipment is sent to the lab while the equipment is being serviced.

3. Inter-Agency QC

a. Each agency has the option to participate annually in the World Health Organization (WHO) External Quality Assurance System (EQAS) to test samples for identification and antimicrobial susceptibility. The results are recorded and posted on the WHO EQAS website.

b. Each agency has the option to participate in the National *Campylobacter* and *Helicobacter* Reference Laboratory Quality Assurance Program for *Campylobacter* spp. Identification sponsored by the CDC.

c. NARMS and CIPARS Quality Assurance of Campy (NCQAC) – Each agency (CDC, CIPARS, FDA, and FSIS) bi-annually sends *Campylobacter* isolates to each other for identification and susceptibility testing. Results are analyzed for comparability.

4. All final data are audited internally within each individual agency to ensure the accurate reporting of results.

Enterococcus

A. Receipt of Isolates

Isolates are received from human, retail meats, and food animal sources. They are received either on agar slants or frozen in transport vials; or they are recovered by culturing chicken carcass rinse samples collected by FSIS. Submission sheets and/or electronic files are included with shipments. Because each agency works with *Enterococcus* from different sources, the acquisition of isolates is different for each group.

FDA - Retail meat isolates are received frozen by the FDA/CVM/NARMS laboratory from the retail meat FoodNet surveillance sites. Each isolate is assigned a unique CVM/NARMS number prior to analysis. (http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm)

USDA FSIS - Animal cecal isolates are received in the Food Safety Inspection Service (FSIS)-Eastern Lab on Nutrient Agar slants (NA) after being processed by FSIS NARMS lab. Each isolates is assigned a unique FSIS/NARMS number prior to analysis. **(**FSIS Microbiology Laboratory Guidebook)

B. Subculturing for Isolate Purity

1. Subculture isolate onto a BAP.
2. Incubate the BAP overnight at 36±1°C for 18-24 hours.
3. Pick a single colony and subculture to 2nd BAP, incubate as above.

If there is no growth on the plate after 24 hours, re-incubate the plate for 48 hours. If there is no growth after 48 hours, go back to the original culture and re-streak for isolation. If there is still no growth, record no growth and ask site to resend. If an isolate displays contamination/mixed culture, a selective media (Enterococcosel Agar) may be used. Streak for purity until *Enterococcus* is isolated.

C. Isolate Storage

CDC - Isolates are stored at room temperature (if frozen, stored in -70°C freezer) until they are tested. After AST and identification, isolates are frozen in defibrinated sheep's blood and stored at -70°C.

FDA - Isolates are stored at -80°C until they are tested. After AST and identification, isolates are discarded. The original isolates are retained. Isolates that were selected from a mixed culture are frozen in Brucella broth with 20% glycerol, and stored at -80°C.

USDA FSIS - Isolates are stored on NA until they are tested. After testing, they are stored in Brain Heart Infusion broth with glycerol and are sent to FDA/CVM Office of Research in Laurel, MD for storage and any further testing.

D. Identification and Speciation of *Enterococcus*

CDC - All isolates are speciated using a multiplex PCR assay. If PCR does not result in a definitive identification, isolates are tested using conventional biochemicals. Isolates may be sent to a reference laboratory if additional biochemical or molecular testing is needed.

FDA - Isolates are identified using the Vitek 2 Compact (V2C) using an isolated colony from the 2nd BAP plate. If the V2C fails to produce a definitive identification, additional testing is done using molecular identification methods.

USDA FSIS - All isolates are identified using the Vitek2 Compact.

E. Antimicrobial Susceptibility Testing of *Enterococcus*

1. Gently invert several times a Sensititre 0.5 McFarland Polymer Turbidity Standard and place it in the Sensititre nephelometer.
2. Press CALIBRATE on the keypad. Calibration is complete when the center green nephelometer LED is lit.
3. Label a Sensititre panel with the isolate number. Ensure the desiccant is orange. Do not use the panel if desiccant is green.

Steps 4 through 10 should be completed within 30 minutes.

4. Using a sterile swab, transfer several colonies from a pure culture on an 18-24 hr blood agar plate to a sterile 5 ml tube of demineralized water. Vortex well.
5. Adjust the suspension to a 0.5 McFarland equivalent by using the Sensititre nephelometer.

6. Transfer 10 µl of the suspension to a sterile 11 ml tube of Sensititre Cation-Adjusted Mueller-Hinton Broth with TES. This transfer is performed with a sterile, extra long pipette tip. Vortex well.
7. Aseptically replace the Mueller-Hinton tube cap with a disposable dosing head.
8. Place the panel in the Sensititre autoinoculator.
9. Open the dosing head clamp, invert the inoculated Mueller-Hinton tube and insert it in the clamp.
10. In response to the autoinoculator display "Enter Pattern #", press "001", and then ENTER to dispense 50µl into each well of a Gram positive MIC panel. For the AIM, choose the plate configuration for 50 µl.
11. Press ENTER again to start dispensing.
12. Remove the panel and seal it with a clear plastic adhesive seal. Ensure that all wells are covered.
13. Colony counts are performed at least quarterly (per CLSI recommendations, M100)
14. Repeat steps 3-12 for each additional test isolate and QC isolate to be tested.
15. Incubate panels in the ARIS with the auto-reader or an aerobic incubator (stack no more than 4 high) at 36 ±1ºC for 18 hours.
16. Panels should be read by the ARIS auto reader or visually using the Sensitouch at 18 hours. ARIS procedures are provided in the ARIS manual from ThermoFisher/Trek Diagnostics.
17. If the ARIS is used, Linezolid and Nitrofurantoin should be read manually at 18 hours and Vancomycin should be read manually at 24 hours.
18. After results are read, check QC strain results to make sure they are within limits. If they are not, follow corrective procedures recommended by CLSI (M07-A10).
19. In a timely manner, review the minimum inhibitory concentration (MIC) results for abnormalities as outlined in the section Criteria for Repeat Testing.

F. Interpretive Criteria

For categorizing susceptibility, breakpoints were adopted from CLSI, when available (M100-S22). Refer to the NARMS Integrated Report Data Tables for a reference table showing *Enterococcus* breakpoints.

G. Quality Control (QC) for testing *Enterococcus*

1. Antimicrobial Susceptibility Testing

a. **Control Strains-** *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 are used with every MIC test run.

b. **Control strain storage**- Maintain stock cultures at -80°C for prolonged storage. The control strain is plated from the freezer onto a BAP. A second pass on BAP is completed.

c. **Media**- For each new lot number of panels, uninoculated MHB is incubated for a sterility test.

2. Inter-Agency

AST and identification methods are exchanged bi-annually among agencies for comparability of results.

3. All final data are audited internally within each individual agency to ensure the accurate reporting of results.

Quality Controls for Antimicrobial Susceptibility Testing

Quality Control

The overall performance of the test system should be monitored by running QC each day the test is performed or, if satisfactory performance is documented (see CLSI criteria in Document M07-A10, Chapter 4), testing may be done weekly. Testing should be done at least weekly. Colony counts should also be performed weekly to ensure the final inoculum concentration is accurate.

Performance is satisfactory if no more than 3 out of 30 consecutive results for a given antimicrobial/QC organism combination are outside of the acceptable limits (shown in Table 4 of CLSI Document M100-S19 and Table 18B of CLSI Document M45-A2 for *Campylobacter*).

If more than 3/30 results are out of control, try to determine the cause of the error and immediately retest the antimicrobial/QC strain combination for a total of 5 consecutive test days.

- If all 5 results are satisfactory, no further action is required.
- If any of the 5 results are unsatisfactory, additional sources of error should be investigated and the other NARMS laboratories should be contacted. It may be necessary to obtain a new QC strain or test materials, review test procedures, or obtain instrument service. Until the problem is resolved, reporting results and continued testing will depend on a careful assessment of the facts in consultation with the other NARMS laboratories.

In addition to routine QC testing, the CLSI (Document M07-A10, Chapter 4) directs that QC testing should be run on each new batch of reagents and panels and that one panel should be incubated uninoculated to check for sterility. If the QC fails, the lot should be rejected.

The frozen stocks of quality control strains used for in vitro testing are obtained directly from the American Type Culture Collection (ATCC). Since repeated passage in vitro can alter strain characteristics, in-house QC strain stocks should be replenished only by purchase of fresh stock from a strain repository such as the ATCC.

Equipment Quality Control

Auto Inoculator or AIM:

To ensure the equipment is well maintained, annual preventative maintenance is performed on each piece of equipment by a ThermoFisher/Trek service technician.

Colony Counts

1. Colony counts are recorded for each test run. At minimum, colony counts are performed on the first test isolate and at least one QC organism each day antimicrobial susceptibility testing is performed.
2. Transfer 10 µl from one of the positive control wells of the inoculated panel into 10 ml of water, broth or other appropriate diluent and mix by vortexing.
3. Transfer 100 µl of the diluted sample onto two separate BAPs and spread using a sterile plate spreader. Incubate overnight at appropriate temperature for that organism.
4. The colonies are counted, averaged and multiplied by 10^4 CFU/ml to obtain a final inoculum concentration

Criteria for Retesting of Isolates

Repeat testing of an isolate must be done when one or more of the following conditions occur:

- No growth on panel
- Growth in all wells
- Multiple skip patterns
- Apparent contamination in wells or isolate preparation
- Unlikely or discordant susceptibility results (Table 1, see also the footnote)

If an isolate is retested, data for <u>all</u> antibiotics should be replaced with the new test results. *Categorical changes may require a third test (and may indicate a mixed culture).*

Uncommon test results (Table 4) may represent emerging resistance phenotypes. Retesting is encouraged.

Table 1. Unlikely or Discordant Resistance Phenotypes

Organism(s)	Resistance Phenotype	Comments
Salmonella* and *E. coli	Nalidixic Acid^{S} (≤16) AND Ciprofloxacin^{R} (≥1)-*Salmonella* Nalidixic Acid^{S} (≤16) AND Ciprofloxacin^{R} (≥4*)- E.coli*	The stepwise selection of mutations in the QRDR does not support this phenotype
	Ceftiofur^{R} (≥8) OR Ceftriaxone^{S} (≤1) AND Ampicillin^{S} (≤8)	The presence of an ESBL or AmpC beta-lactamase should confer resistance to Ampicillin.
	Ceftiofur^{R} (≥8) AND Ceftriaxone^{S} (≤1) OR Ceftiofur^{S} (≤2) AND Ceftriaxone^{R} (≥4)	
	Ampicillin^{S} (≤8) AND Amoxicillin-Clavulanic Acid^{R} (≥32/16)	
	Sulfisoxazole^{S} (≤256) AND Trimethoprim-Sulfamethoxazole^{R} (≥4/76)	
Campylobacter jejuni* and *coli	Nalidixic Acid^{S} (≤16) AND Ciprofloxacin^{R} (≥1)	In *Campylobacter*, one mutation is sufficient to confer resistance to both Nalidixic Acid and Ciprofloxacin
	Nalidixic Acid^{R} (≥32) AND Ciprofloxacin^{S} (≤0.5)	
Campylobacter jejuni	Erythromycin^{S} (≤4) AND Azithromycin^{R} (≥0.5)	Erythromycin is class representative for 14- and 15-membered macrolides (Azithromycin, Clarithromycin, Roxithromycin, and Dirithromycin)
	Erythromycin^{R} (≥8) AND Azithromycin^{S} (≤0.25)	
Campylobacter coli	Erythromycin^{S} (≤8) AND Azithromycin^{R} (≥1)	
Campylobacter fetus* and *lari	Erythromycin^{R} (≥16) AND Azithromycin^{S} (≤0.5)	
	For *C. fetus* and *C. lari* isolates: Nalidixic Acid^{S} (≤16) OR Ciprofloxicin^{S} (≤1)	*C. fetus* and *C. lari* are intrinsically resistant to quinolones. Consider likelihood of misidentification

Table 2. Uncommon Resistance Phenotypes

Organism	Exceptional Resistance Phenotype
Salmonella and *E. coli*	• Pan-resistance • Resistance to Azithromycin (>16) • Resistance to Ceftriaxone (≥4) and/or Ceftiofur (≥8) AND Ciprofloxacin (≥4) and/or Nalidixic Acid (≥32)
Campylobacter	• Pan-resistance • Resistance to Gentamicin • Not susceptible to Florfenicol (>4)
Enterococcus	• Pan-resistance • Resistance to Linezolid (≥8) • For *E. faecium:* not susceptible to Daptomycin (≥8) • Not susceptible to Tigecycline (≥0.5) • For *E. faecium* and *E. faecalis* isolates: resistance to Vancomycin (≥32) • For *E. faecalis*[a]*:* Lincomycin^{S} (≤2) OR Quinopristin-Dalfopristin^{S} (≤1) *(E. faecalis* is intrinsically resistant to Lincomycin and Quinopristin-Dalfopristin. Consider likelihood of misidentification) • For *E. faecalis*[a]*:* Penicillin^{R} (≥16) (Penicillin resistance is mainly detected in *E. faecium.* Consider likelihood of misidentification) • For *E. faecium*[a]*:* Quinopristin-Dalfopristin^{R} (≥4) (Quinopristin-Dalfopristin resistance is mainly detected in *E. faecalis.* Consider likelihood of misidentification, especially if also susceptible to Penicillin (≤8)) • For *E. gallinarum* and *E. casseliflavus*[a]*:* Lincomycin^{S} (≤2), Vancomycin^{S} (≤4), OR Quinopristin-Dalfopristin^{S} (≤1) (*E. gallinarum* and *E. casseliflavus* are intrinsically resistant to Lincomycin, Vancomycin, and Quinopristin-Dalfopristin. Consider likelihood of misidentification)

[a] These phenotypes are specific to CDC due to their medical importance. They may not be relevant to food or animals strains of *Enterococcus*.

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