II. Sampling and Testing Methods

A. Samples

The *Salmonella* isolates included in this report were recovered by FSIS from carcass rinsates (chickens), carcass swabs (turkeys, cattle, and swine), and ground products (chickens, turkeys, and beef). *Campylobacter, E. coli* and *Enterococcus* isolates included in this report were recovered by BEAR from FSIS Eastern Lab carcass rinsates (chickens).

Sampling methods used by FSIS for the PR/HACCP *Salmonella* verification testing program have changed since NARMS animal testing began. Before June of 2006, there were two phases of the FSIS regulatory program for *Salmonella* in raw products: non-targeted and targeted testing. Non-targeted samples were collected randomly from eligible federally inspected establishments, with a goal of scheduling every eligible establishment at least once a year. Targeted samples were collected from establishments that had a previously failed sample set. Beginning in June of 2006, sampling was scheduled using risk-based criteria designed to focus FSIS resources on establishments with the most samples positive for *Salmonella* and the greatest number of samples with serotypes most frequently associated with human salmonellosis.[1,2] Once the establishments presenting the greatest risk are sampled, FSIS prioritizes sampling at the establishments that have not been sampled within the last two years.

B. Isolation and Identification

1. *Salmonella*: Isolation from slaughter samples was conducted by FSIS at all three FSIS Regulatory Field Services Laboratories [Eastern (Athens, GA), Midwestern (St. Louis, MO) and Western (Alameda, CA)] following the "Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg" procedures as described in the Microbiology Laboratory Guidebook, section 4.[3,4] Each FSIS laboratory processes samples collected throughout the U.S. Isolates were forwarded by FSIS to the National Veterinary Services Laboratories, Ames, IA (NVSL) for serotyping and a duplicate isolate was sent to BEAR for susceptibility testing and Pulsed Field Gel Electrophoresis (PFGE). Serotype results were subsequently sent to the BEAR unit as they became available.

2. *Campylobacter*: From 1998 to 2000, *Campylobacter* was isolated by all FSIS laboratories as part of the chicken monitoring baseline programs using the method described in the FSIS Microbiology Laboratory

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[1] USDA/FSIS. 2008. Serotypes Profile of Salmonella Isolates from Meat and Poultry Products. Available at http://www.fsis.usda.gov/Science/Serotypes_Profile_Salmonella_Isolates/index.asp.
[2] USDA/FSIS. FSIS Scheduling Criteria for Salmonella Sets in Raw Classes of Product. Available at http://www.fsis.usda.gov/PDF/Scheduling_Criteria_Salmonella_Sets.pdf.
[3] USDA/FSIS. 2004. Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg Products. Microbiological Lab Guidebook 4.03. Available at http://www.fsis.usda.gov/PDF/MLG_4_03.pdf.
[4] USDA/FSIS. 2010. Laboratories and Procedures. Available a.t http://www.fsis.usda.gov/Science/Laboratories_&_Procedures/index.asp.

Guidebook.[5] Following presumptive identification, isolates were sent to BEAR for final confirmation and susceptibility testing as described below. Upon review of susceptibility data and isolation methods, it was determined that use of nalidixic acid as part of the culture selection criteria may have resulted in recovery of isolates more likely to be resistant to quinolones. A comparative study was initiated by BEAR in 2001.

For the first half of 2001, BEAR pilot tested several isolation methods for *Campylobacter* prior to adopting a new method in July. Since that time, only rinsates from the FSIS Eastern Lab containing $\geq$ 10 ml have been used. Thus, all rinsates tested for *Salmonella* were not processed for *Campylobacter*, *E. coli* or *Enterococcus*. Also important to note is that when the FSIS *Campylobacter* baseline testing ended in 2000, rinsates were no longer temperature controlled during shipment which may have affected isolate recovery. For *Campylobacter* isolation, 10 mls of rinsate was enriched in an equal volume of Campylobacter Enrichment Broth without blood under microaerobic conditions for 48 h at 42°C. Aliquots were struck onto Campy Cefex agar and plates were incubated as above. Final confirmation and speciation of *Campylobacter* isolates were obtained using the BAX® System Q7 (DuPont Qualicon; Wilmington, DE). This real-time PCR assay is able to detect *C. coli*, *C. jejuni*, and *C. lari* and was performed according to manufacturer's directions.

3. *Escherichia coli*: BEAR started isolating generic *E. coli* from the same rinsates used for *Campylobacter* isolation in 2000. A sample of the rinsate was enriched overnight before streaking onto a CHROMAgar™ ECC plate (DRG International; Mountainside, NJ). Plates were incubated at 36°C ± 1°C for 18-24 h as described by the manufacturer. Blue-green colonies, typical of generic *E. coli,* were selected for susceptibility testing and confirmed as *E. coli* using the Vitek (bioMérieux, Inc; Durham, NC).

4. *Enterococcus*: In 2003, isolation of Enterococcus began using the same rinsates used for *Campylobacter* and *E. coli* isolation. An aliquot of each rinsate was enriched for 48 h at 37°C in Enterococcosel broth. Aliquots were taken from enriched broths exhibiting a color change and struck to Enterococcosel agar which was then incubated overnight at 37°C.

A species-specific multiplex PCR was performed on presumptive *Enterococcus* isolates which provided a simultaneous genus and species identification of 23 species of enterococci.[6] Confirmed *Enterococcus* isolates of other species not identified with this procedure were labeled as *'Enterococcus* species*'.*

C. Antimicrobial Susceptibility

In 2010, *Salmonella*, *Campylobacter*, *E. coli* and *Enterococcus* were tested using a semi-automated broth microdilution system (Sensitire®, Trek Diagnostic Systems, Inc., Westlake, Ohio) and a custom made 96-well panel of antimicrobials (catalog no. CMV1AGNF for *Salmonella* and *E. coli;* catalog no. CAMPY for *Campylobacter* and catalog no. CMV3AGPF for *Enterococcus*) to determine the minimum inhibitory

[5] USDA/FSIS. 1998. Isolation, Identification, And Enumeration Of Campylobacter jejuni/coli From Meat And Poultry Products. Microbiology Laboratory Guidebook, chapter 6. Available at http://www.fsis.usda.gov/ophs/Microlab/Mlgchp6.pdf.

[6] Jackson, C. 2004. Use of a Genus- and Species-Specific Multiplex PCR for Identification of *Enterococci*. Journal of Clinical Microbiology, *42*(8):3558-65

concentration (MIC) of antimicrobials important in both human and veterinary medicine. Tables 1, 2 and 3 list the antimicrobials tested, including the breakpoints for *Salmonella*/*E. coli*, *Campylobacter*, and *Enterococcus*, respectively. From 1998-2004, MICs for *Campylobacter* isolates were determined using Etest® (AB Biodisk; Solna, Sweden) as per manufacturer's direction with the exception that MICs were not rounded up prior to categorization. In 2005, the animal arm of NARMS switched to using the Sensititre® broth microdilution system for *Campylobacter*.[7]

Regardless of the susceptibility testing method used, antimicrobial resistance was determined using Clinical and Laboratory Standards Institute (CLSI) breakpoints, when available.[8,9,10]

For antimicrobial agents without CLSI approved breakpoints, interpretive criteria established by the NARMS working group were used.

Quality control strains used for *Salmonella* and *E. coli* susceptibility testing included *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213. *Campylobacter jejuni* ATCC 33560 was used as a control for *Campylobacter* susceptibility testing. For *Enterococcu*s testing, *Enterococcus faecalis* ATCC 29212 and ATCC 51299 were used.

[7] CLSI. 2006. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline. CLSI document M45-A. CLSI, Wayne, PA

[8] CLSI. 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard—Third Edition. CLSI document M31-A3. CLSI, Wayne, PA.

[9] CLSI. 2010. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline- Second Edition. CLSI document M45-A2. CLSI, Wayne, PA.

[10] CLSI. 2011. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-first Informational Supplement. CLSI document M100-S21. CLSI, Wayne, PA.

Table 1. *Salmonella* and *E. coli* Interpretive Criteria (breakpoints)[11]

CLSI Antimicrobial Class[12]	Antimicrobial Agent	Breakpoints (µg/ml)		
		Susceptible	Intermediate	Resistant
Aminoglycosides	Amikacin	≤ 16	32	≥ 64
	Gentamicin	≤ 4	8	≥ 16
	Kanamycin	≤ 16	32	≥ 64
	Streptomycin[13]	≤ 32	Not Applicable	≥ 64
β-Lactam/β-Lactamase Inhibitor Combinations	Amoxicillin–Clavulanic Acid	≤ 8 / 4	16/8	≥ 32 / 16
Cephems	Cefoxitin	≤ 8	16	≥ 32
	Ceftiofur	≤ 2	4	≥ 8
	Ceftriaxone	≤ 1	2	≥ 4
	Cephalothin	≤ 8	16	≥ 32
Folate Pathway Inhibitors	Sulfonamides[14]	≤ 256	Not Applicable	≥ 512
	Trimethoprim–Sulfamethoxazole	≤ 2 / 38	Not Applicable	≥ 4 / 76
Penicillins	Ampicillin	≤ 8	16	≥ 32
Phenicols	Chloramphenicol	≤ 8	16	≥ 32
Quinolones	Ciprofloxacin	≤ 1	2	≥ 4
	Nalidixic acid	≤ 16	Not Applicable	≥ 32
Tetracyclines	Tetracycline	≤ 4	8	≥ 16

[11] Breakpoints established by CLSI (Clinical and Laboratory Standards Institute) were used when available.
[12] According to CLSI M100 document
[13] There are no CLSI breakpoints for streptomycin; breakpoints established by NARMS
[14] From 1997 through 2003, sulfamethoxazole was tested. Sulfisoxazole replaced sulfamethoxazole beginning in 2004

Table 2. *Campylobacter* Interpretive Criteria (breakpoints)[15]

CLSI Antimicrobial Class[16]	Antimicrobial Agent	Breakpoints (µg/ml) Etest (1998-2004)			Breakpoints (µg/ml) Broth Microdilution (2005-2010)		
		Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Aminoglycosides	Gentamicin	≤ 4	8	≥ 16	≤ 2	4	≥ 8
Lincosamides	Clindamycin	≤ 0.5	1 - 2	≥ 4	≤ 2	4	≥ 8
Macrolides	Azithromycin	≤ 0.25	0.5 - 1	≥ 2	≤ 2	4	≥ 8
	Erythromycin	≤ 0.5	1 - 4	≥ 8	≤ 8	16	≥ 32
Ketolides	Telithromycin	Not Tested	Not Tested	Not Tested	≤ 4	8	≥ 16
Phenicols	Florfenicol	Not Tested	Not Tested	Not Tested	≤ 4	Not Applicable	Not Applicable
	Chloramphenicol	≤ 8	16	≥ 32	Not Tested	Not Tested	Not Tested
Fluoroquinolones	Ciprofloxacin	≤ 1	2	≥ 4	≤ 1	2	≥ 4
Quinolones	Nalidixic acid	≤ 16	Not Applicable	≥ 32	≤ 16	32	≥ 64
Tetracyclines	Tetracycline	≤ 4	8	≥ 16	≤ 4	8	≥ 16

[15] Breakpoints established by CLSI (Clinical and Laboratory Standards Institute) were used when available. CLSI breakpoints are available only for erythromycin, ciprofloxacin, and tetracycline. All other breakpoints were established by NARMS

[16] According to CLSI M100 document

Table 3. *Enterococcus* Interpretive Criteria (breakpoints)[17]

CLSI Subclass[18]	Antimicrobial Agent	Breakpoints (µg/ml)		
		Susceptible	Intermediate	Resistant
Aminoglycoside[19]	Gentamicin	≤ 500	N/A	> 500
	Kanamycin	≤ 512	N/A	≥ 1024
	Streptomycin	≤ 1000	N/A	> 1000
Glycopeptide	Vancomycin	≤ 4	8 - 16	≥ 32
Glycylcycline	Tigecycline[20]	≤ 0.25	N/A	N/A[4]
Lincosamides	Lincomycin	≤ 2	4	≥ 8
Lipopeptide	Daptomycin[21]	≤ 4	N/A	N/A[5]
Macrolide	Erythromycin	≤ 0.5	1 - 4	≥ 8
	Tylosin	≤ 8	16	≥32
Nitrofuran	Nitrofurantoin	≤ 32	64	≥128
Oxazolidinones	Linezolid	≤ 2	4	≥ 8
Penicillin	Penicillin	≤ 8	N/A	≥ 16
Phenicol	Chloramphenicol	≤ 8	16	≥ 32
Phosphoglycolipid	Flavomycin	≤ 8	16	≥ 32
Quinolone	Ciprofloxacin	≤ 1	2	≥ 4
Streptogramin	Quinupristin/Dalfoprisitin	≤ 1	2	≥ 4
Tetracycline	Tetracycline	≤ 4	8	≥ 16

D. Phage Typing

Salmonella Typhimurium and *S.* Typhimurium variant 5- isolates with resistance to at least ampicillin, chloramphenicol, sulfisoxazole and tetracycline (ACSuT) were submitted to NVSL for phage typing.

[17] Breakpoints established by CLSI (Clinical and Laboratory Standards Institute) were used when available. CLSI breakpoints are not available for Kanamycin, Lincomycin, Tylosin and Flavomycin and were established by NARMS

[18] According to CLSI M100 document

[19] For the aminoglycosides, breakpoints refer to high-level aminoglycoside resistance

[20] For Tigecycline, only a susceptible breakpoint (≤0.25 µg/ml) has been established. In this report, isolates with an MIC ≥0.5 µg/ml are categorized as resistant

[21] For Daptomycin, only a susceptible breakpoint (≤4 µg/ml) has been established. In this report, isolates with an MIC ≥8 µg/ml are reported as resistant