

NARMS Retail Meat, 2002

Experimental Design and Procedures:

Microbiological analysis:

In the laboratory, samples were refrigerated at 4°C and processed no later than 96 hours after purchase. After microbiological examination, recordings were made on the log sheets whether or not the meat and poultry samples were presumptively positive for *Salmonella*, *Campylobacter*, *E. coli*, and *Enterococcus*. Each laboratory used essentially the same procedure for sample collection. Retail meat and poultry packages were kept intact until they were aseptically opened in the laboratory at the start of examination. For chicken and pork samples, one piece of meat was examined, whereas, 25 g of ground product was examined for ground beef and ground turkey samples. The analytical portions from each sample were placed in separate sterile plastic bags, 250 mL of buffered peptone water was added to each bag, and the bags were vigorously shaken. Fifty mL of the rinsate from each sample was transferred to separate sterile flasks (or other suitable sterile containers) for isolation and identification of *Salmonella*, *Campylobacter*, *E. coli*, or *Enterococcus* using standard microbiological procedures. Once isolated and identified, bacterial isolates were sent to FDA's CVM Office of Research for further characterization including species confirmation, antimicrobial susceptibility testing and PFGE analysis (*Salmonella* and *Campylobacter* only).

Salmonella isolation:

Fifty mL of double strength lactose broth was added to each flask containing the 50 mL of rinsate to be used for *Salmonella* isolation. The contents were mixed thoroughly and incubated at 35°C for 24 hours. From each flask, 0.1 ml was then transferred to 9.9 mL tubes of RVR10 medium. The tubes of RVR10 medium were incubated in a water bath at 42°C for 16-20 hours before transferring one ml to pre-warmed (35-37°C) 10 mL tubes of M Broth. The

inoculated M Broth tubes were incubated in a water bath at 35-37°C for 6-8 hours. From each M Broth culture, one ml was heated at 100°C for 15 minutes, and the remaining portion was refrigerated. The heated portion from each culture was cooled to room temperature and tested using the TECRA *Salmonella* Visual Immunoassay kit (International BioProducts, Bothell, WA) or the VIDAS® *Salmonella* Immunoassay kit (bioMérieux, Hazelwood, MO) according to the manufacturers' instructions. If the TECRA or VIDAS assay was negative, the sample was considered negative for *Salmonella*. If the TECRA or VIDAS assay was positive, a loopful of the corresponding, unheated M Broth culture was streaked for isolation onto a XLD agar plate. The inoculated plate was incubated at 35°C for 24 hours. Each XLD agar plate was examined for typical *Salmonella* colonies (pink colonies with or without black centers). If no *Salmonella* like growth was observed on a XLD agar, the sample was considered negative and the appropriate documentation was made on the log sheet accompanying the sample. When *Salmonella* like growth was observed, one well-isolated colony was streaked for isolation onto a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (BAP). The BAP(s) were incubated at 35°C for 18-24 hours before sub-culturing an isolated colony for further biochemical identification and serotyping using the FoodNet laboratory's standard procedures. *Salmonella* isolates were subsequently frozen at -60 to -80°C in Brucella broth with 20% glycerol and shipped in cryo-vials on dry ice to FDA-CVM. Upon arrival at CVM, every isolate was streaked for purity on a BAP before being confirmed as *Salmonella* using the Vitek microbial identification system (bioMérieux, Hazelwood, MO). These isolates were further serotyped for O and H antigens using either commercially available (Difco-Becton Dickinson, Sparks, MD) or CDC antisera.

Campylobacter isolation:

Fifty mL of double strength Bolton broth was added to each flask containing the 50 mL

of rinsate to be used for *Campylobacter* isolation. The broth and rinsate were mixed thoroughly, but gently to avoid aeration, and incubated at 42°C for 24 hours in a reduced oxygen atmosphere that was obtained using a Campy Pak (BBL-Becton Dickinson, Sparks, MD) or a gas mixture containing 85% nitrogen, 10% carbon dioxide, and 5% oxygen. Using a swab, the first quadrant of a CCA Plate was inoculated with the incubated Bolton broth culture. The remainder of each plate was then streaked with a loop to obtain isolated colonies, and the CCA plates were incubated at 42°C in the above atmosphere for 24 to 48 hours. Each CCA plate was examined for typical *Campylobacter* colonies (round to irregular with smooth edges; thick translucent white growth to spreading, film-like transparent growth). If no *Campylobacter* like growth was observed on a CCA plate, the sample was considered negative and the appropriate documentation was made on the log sheet accompanying the sample. When *Campylobacter* like growth was observed, one typical well-isolated *Campylobacter* like colony from each positive CCA plate was sub-cultured to a BAP and incubated as described for the CCA plates. Following incubation, one typical well-isolated *Campylobacter* like colony was gram stained and tested using a smear catalase, oxidase, hippurate and/or motility test. If the Gram stain showed small, Gram- negative, curved rods, and the isolate was positive with the other test(s) that were conducted, a sample was considered presumptively positive for *Campylobacter*. If the CCA plates or BAPs had no typical colonies or isolate testing was inconsistent with *Campylobacter*, a sample was considered negative. All isolates presumptively identified as *Campylobacter* were frozen at -60 to -80°C in Brucella broth with 20% glycerol and shipped in cryo-vials on dry ice to FDA-CVM. Upon arrival at CVM, isolates were streaked for purity on a BAP twice before being confirmed as *Campylobacter* using a repeat Gram stain and an AccuProbe *Campylobacter* Identification Test (Gen-Probe, San Diego, CA). *Campylobacter* species were determined using a multiplex PCR assay previously described (3,7).

E. coli isolation (Georgia, Maryland, Oregon and Tennessee)

Fifty mL of double strength MacConkey broth was added to each flask containing the 50 mL of rinsate to be used for *E. coli* isolation. The contents were mixed thoroughly and incubated at 35°C for 24 hours. One loopful from each flask was then transferred to an EMB agar plate and streaked for isolation. Agar plates were then incubated at 35°C for 24 hours in ambient air and examined for typical *E. coli* colonies (colonies having a dark center and usually a green metallic sheen). If no typical growth was observed on an EMB agar plate, the sample was considered negative and the appropriate documentation was made on the log sheet accompanying the sample. When *E. coli*-like growth was present, one typical, well-isolated colony was streaked for isolation onto a BAP. The BAPs were incubated at 35°C for 24 hours in ambient air and examined for purity. One typical, well-isolated colony was subcultured for indole and oxidase tests. Indole positive and oxidase negative isolates were considered presumptively positive as *E. coli*. Presumptive *E. coli* isolates were subsequently frozen at -60 to -80°C in Brucella broth with 20% glycerol and shipped in cryo-vials on dry ice to FDA-CVM. Upon arrival at CVM, every isolate was streaked for purity on a BAP before being confirmed as *E. coli* using the Vitek microbial identification system (bioMérieux, Hazelwood, MO).

Enterococcus isolation (Georgia, Maryland, Oregon and Tennessee)

Fifty mL of double strength Enterococcosel broth was added to each flask containing the 50 ml of rinsate to be used for *Enterococcus* isolation. The contents were mixed thoroughly and incubated at 45°C for 24 hours in ambient air. If no typical growth or blackening was observed in the flask, the sample was considered negative and the appropriate documentation was made on the log sheet accompanying the sample. If blackening of the broth was observed, a loopful was streaked onto an EAP for isolation. The plates were then incubated at 35°C for 24 hours in ambient air and examined for enterococci-like colonies (small colonies surrounded by a

blackening of the agar). If no typical growth was observed on the EAP, the sample was considered negative and the appropriate documentation was made on the log sheet accompanying the sample. If enterococci-like growth was present, one well-isolated colony was streaked for isolation onto a BAP, and incubated at 35°C for 24 hours in ambient air. Presumptive *Enterococcus* isolates were subsequently frozen at -60 to -80°C in Brucella broth with 20% glycerol and shipped in cryo-vials on dry ice to FDA-CVM. Upon arrival at CVM, every isolate was streaked for purity on a BAP before being confirmed as *Enterococcus* using the Vitek microbial identification system (bioMérieux, Hazelwood, MO).

Antimicrobial Susceptibility Testing:

For *E. coli*, *Enterococcus*, and *Salmonella*, antimicrobial MICs were determined using a 96 well broth microdilution method (Sensititre, Trek Diagnostic Systems, Westlake, OH) according to NCCLS standards (4,5,6). *Salmonella* and *E. coli* isolates were tested using a custom plate developed for Gram negative bacteria, catalog # CMV6CNCD; *Enterococcus* isolates were tested using a custom plate developed for Gram positive bacteria, catalog # CMV5ACDC (Table 1). NCCLS recommended QC organisms were used each time that antimicrobial susceptibility testing was performed. The QC organisms included *Escherichia coli* ATCC 25922 and 35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 (4,5,6).

For isolates confirmed as *Campylobacter*, the NCCLS approved agar dilution procedure was used to determine MICs to ciprofloxacin, doxycycline, erythromycin, gentamicin, and meropenem. (4,5). The NCCLS recommended quality control organism *Campylobacter jejuni* ATCC 33560 was used each time that antimicrobial susceptibility testing was performed (5). As there are no NCCLS-approved interpretive criteria for *Campylobacter*, tentative breakpoints used by NARMS are shown in Table 1. All of the resistant breakpoints with the exception of

meropenem, have been used previously in the absence of NCCLS approved interpretive criteria (2). All antimicrobial susceptibility testing was conducted in the laboratories of the Division of Animal and Food Microbiology, CVM-FDA, Laurel, MD.

Pulsed Field Gel Electrophoresis (PFGE):

Pulsed-field gel electrophoresis was used to assess genetic relatedness among *Salmonella* and *Campylobacter* isolates. The PFGE was performed according to protocols developed by CDC (1). Agarose-embedded DNA was digested with the enzyme *Xba*I for *Salmonella* isolates and *Sma*II for *Campylobacter* isolates. DNA restriction fragments were separated by electrophoresis using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA). Genomic-DNA profiles or “fingerprints” were analyzed using BioNumerics software (Applied-Maths, Kortrijk, Belgium), and banding patterns were compared using Dice coefficients with a 1.5% band position tolerance. PFGE analysis was conducted in the laboratories of the Division of Animal and Food Microbiology, CVM-FDA, Laurel, MD.

References

1. Center for Disease Control and Prevention. 2002. Standardized molecular subtyping of foodborne bacterial pathogens by pulsed-field gel electrophoresis. Center for Disease Control and Prevention. Atlanta, GA.
2. Ge, B., S. Bodeis, R.D. Walker, D.G. White, S. Zhao, P.F. McDermott, and J. Meng. 2002. Comparison of the Etest and agar dilution for in vitro antimicrobial susceptibility testing of *Campylobacter*. *J. Antimicrob. Chemother.* 50:487-494.
3. Linton, D., A. J. Lawson, R. J. Owen, and J. Stanley. 1997. PCR detection, identification

- to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J. Clin. Microbiol. 35:2568-2572
4. National Committee for Clinical Laboratory Standards. 2003. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; 2nd edition. NCCLS M31-A2. NCCLS, Wayne, Pa.
 5. National Committee for Clinical Laboratory Standards. 2004. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; first information supplement. NCCLS M31-S1. NCCLS, Wayne, Pa.
 6. National Committee for Clinical Laboratory Standards. 2004. Performance standards for antimicrobial susceptibility testing; fourteenth information supplement. NCCLS M100-S14. NCCLS, Wayne, Pa.
 7. Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. White, D. Wagner, and J. Meng. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. Appl. Environ. Microbiol. 67:5431-5436