
**NATIONAL ADVISORY COMMITTEE ON
MICROBIOLOGICAL CRITERIA FOR FOODS**

Analytical Utility of Campylobacter Methodologies

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EXECUTIVE SUMMARY

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF, or the Committee) was asked to address six questions posed to the Committee regarding *Campylobacter*. These questions relate to the analytical utility of *Campylobacter* methodologies in preparation for an upcoming Food Safety and Inspection Service (FSIS) baseline study to enumerate *Campylobacter* spp. from broiler carcass rinse samples.

To address the FSIS questions, the Committee reviewed the available literature regarding *Campylobacter* spp. methodologies, consulted four US experts on *Campylobacter* research, and examined the current method being used in an on-going United States Department of Agriculture (USDA) collaborative study between FSIS and the Agricultural Research Service (ARS), hereinafter referred to as the ARS/FSIS Broiler Rinse Study, for possible use in the upcoming FSIS baseline study on broilers.

As described more fully in this report, the Committee acknowledges that *Campylobacter* spp. are a leading cause of foodborne illness in the United States and that poultry is a primary reservoir of this pathogen. In addition, the Committee recognizes that the majority of human campylobacteriosis cases are caused by *C. jejuni*, followed by *C. coli*, and other species. Finally, the Committee understands that FSIS is awaiting scientific recommendations from NACMCF prior to initiating a nationwide baseline study to determine the prevalence and numbers of *Campylobacter* spp. in broiler carcasses at Federally-inspected establishments as a basis for developing risk management strategies to reduce human exposure to *Campylobacter* spp.

A general summary of the recommendations of the Committee, based on the six questions posed by FSIS, follows:

- The FSIS microbiological baseline study design for broiler carcasses should be based on the species of *Campylobacter* causing the majority of human illness, namely *C. jejuni* and *C. coli*;
- FSIS should partner with other researchers to develop methodologies and conduct surveillance studies to sample poultry products for *Campylobacter* species other than *C. jejuni* and *C. coli* since their prevalence and human illness impact is presently unknown;
- FSIS must clearly state the objectives and potential uses of the baseline data;
- FSIS should determine if analyses from a single carcass rinse for generic *Escherichia coli*, *Salmonella*, and *Campylobacter* would strengthen the evaluation of process control for FSIS and industry;
- The direct plating enumeration methodology currently used in the ARS/FSIS Broiler Rinse Study should be adapted for the upcoming FSIS baseline study, with modifications as indicated throughout this report;
- Individuals conducting sample collection and microbial analyses should be adequately trained; and
- FSIS should speciate *Campylobacter* spp. to differentiate *C. jejuni* and *C. coli*.

I. INTRODUCTION

Campylobacter spp. are major bacterial agents of human foodborne gastroenteritis. Poultry is a primary reservoir of *Campylobacter* spp. and studies show that prevalence may be more than 80% in commercial chicken carcasses (19, 25). Data show that 95% of human illnesses associated with campylobacteriosis are caused by *C. jejuni*, followed by *C. coli* comprising 4%, and other species comprising 1% (23).

For clarity, NACMCF defines the term “broilers” in the same manner as the 2004 NACMCF report “Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Broilers (Young Chickens)” (15). In this report, a broiler is defined as a young chicken of either sex usually under 13 weeks of age. FSIS has proposed to reduce this age requirement to under 10 weeks.

In the past, FSIS has conducted baseline studies of *Campylobacter* spp. enumeration from broiler carcass rinses using a labor intensive Most Probable Number Method (MPN) (25, 26); however, not all of these studies have been published. FSIS is planning to initiate a new *Campylobacter* spp. baseline study and asked NACMCF to advise FSIS in developing the upcoming methodology. The Committee was asked to evaluate a direct plating method currently being used for enumerating *Campylobacter* spp. in a joint ARS/FSIS Broiler Rinse Study (Appendix I.), for its utility in the upcoming FSIS baseline study.

II. PURPOSE OF THE DOCUMENT

The purpose of this document is to summarize the past Committee deliberations on *Campylobacter*, including previous questions posed to the Committee, and to address new questions posed by FSIS to the Committee at the July 12, 2005, public meeting.

Since a formal report specifically addressing *Campylobacter* methods had not previously been developed by NACMCF, the Committee is using this report to compile all past and current activities associated with *Campylobacter*.

III. ORIGINAL WORK CHARGE AND BACKGROUND

Background

Previous NACMCF Activity: The Committee deliberated on the issue of *Campylobacter* as an emerging pathogen in 1993, and in December 1994, published a review of *Campylobacter jejuni/coli* (13). In May 1999, the National Advisory Committee on Meat and Poultry Inspection (NACMPI) asked NACMCF to evaluate options for defining a *Campylobacter* performance standard or, in the alternative, a performance standard that would accomplish the same public health objective. In response, a subcommittee of NACMCF (i.e., at that time, the Meat and Poultry Subcommittee worked on the issue) made a series of recommendations that were never formally adopted by the full Committee but were pursued for further action by both NACMPI and FSIS (12).

At the August 28, 2002 NACMCF meeting, a work charge relating to *Campylobacter* was introduced by FSIS. The presentation consisted of three talks outlining the following: the methods and resulting data from previous *Campylobacter* baseline studies performed in 1994-1995 and 1999-2000 by FSIS; available laboratory methods for *Campylobacter* spp.; and *Campylobacter* cell aggregation (14). The charge to NACMCF at the 2002 public meeting included three elements:

- Review and compare the methodologies used for *Campylobacter* detection in the 1994-1995 and 1999-2000 baseline studies in young chickens;

- Evaluate them for the accuracy and precision that they provide in assessing the prevalence and quantity of *Campylobacter* on chicken carcasses; and
- Compare the methodologies used in the two studies with recent methodological advances for their ability to provide data on the presence and quantity of *Campylobacter* for application in risk assessment and the establishment of baselines.

Although the 2002 NACMCF *Campylobacter* Subcommittee met and discussed the FSIS charge, no formal report was adopted. In addition, the FSIS baseline data from 1999-2000 have never been released due to *Campylobacter* methodology concerns expressed by FSIS.

FSIS Activity: Prior to 2004, FSIS used a labor intensive and resource consuming MPN method for the detection and enumeration of *Campylobacter* spp. (26). Current literature indicates that when *Campylobacter* spp. are present, numbers per ml of carcass rinse can vary from 1 to 3 log CFU (19) and thus direct enumeration on agar plates can be an alternative to MPN methods. The Food and Drug Administration (29), the Centers for Disease Control and Prevention (5), and industry constituents currently use methods incorporating selective plating to detect and/or enumerate *Campylobacter* spp. from clinical and food samples. Since 2004, FSIS, in cooperation with ARS researcher, Dr. J. Stanley Bailey, is conducting a special project that includes *Campylobacter* spp. detection and enumeration. The current ARS method (Appendix I) calls for collection of aseptic whole bird rinses with 100 ml of Buffered Peptone Water (BPW) followed by storage and overnight transport of rinses at $4^{\circ} \pm 4^{\circ}$ C to the laboratory for analysis. The rinses are serially diluted and plated onto Campy-Cefex agar (with Bolton broth enrichment and selective agar plate streaks as a backup) for presence/absence

determination using a customized atmosphere of 5% O₂, 10% CO₂, and 85% N₂ in sealed bags. Presumptive colonies are examined microscopically and confirmed using a serological latex agglutination test confirmatory for *C. jejuni*, *C. coli*, and *C. lari*. Confirmed cultures are then stored at -80°C in *Brucella* broth with 15% glycerol for possible subsequent subtyping.

Present Charge to the Committee: In the near future, FSIS will conduct a baseline study to determine the prevalence of and to enumerate *Campylobacter* spp. of known importance on poultry (possibly including carcasses, parts, and ground product).

It is currently proposed that the study will focus on thermotolerant species, *C. jejuni* and *C. coli*, because these human pathogens account for the vast majority of laboratory-confirmed *Campylobacter* infections. An additional justification for this focus is supported by the numerous microbiological studies of poultry products that indicate that these two species are the only species of *Campylobacter* routinely isolated from chickens. Although some of the other 16 named *Campylobacter* species are reported to rarely cause human illness, the burden of human illness is low and poultry have not been shown to be a reservoir. Many of these other *Campylobacter* species require specialized growth conditions, such as atmospheres containing 5% H₂ (i.e., non-thermotolerant campylobacters) or growth media other than Campy-Cefex agar, which contains the antimicrobial cefoperazone that inhibits growth of *C. upsaliensis*.

FSIS seeks advice on the proposed *Campylobacter* methodology, as well as any other relevant methodology that may be of equal or greater value which should be considered for the upcoming baseline study.

The questions to be addressed are:

1. What additional circumstances should be considered in order for FSIS to conclude that the poultry baseline study should address more than the two principal *Campylobacter* species of *C. jejuni* and *C. coli*?
2. How can the ARS method be most successfully used for high volume analysis in the conduct of a baseline study of *Campylobacter* presence and enumeration on poultry (chicken, turkey, goose, etc.) carcasses, parts, and ground product that may lead to a potential performance standard or guideline for the regulated industry? What if any modifications should be made as a result of discussing this method in comparison with others presented to the Committee? Please consider whether the above described atmospheric conditions, media, pre-enrichment, and storage media are acceptable for the objective of this baseline study.
3. To utilize FSIS resources efficiently and effectively, FSIS expects to maintain as much continuity as possible between the current broiler rinse sampling for *Salmonella* and the proposed sampling for *Campylobacter* spp. What concerns regarding the *Campylobacter* spp. sampling method need to be attended to in order to properly address post-chill injured *Campylobacter* spp. cells as well as viable non-culturable (coccoid) cells?
4. What further subtyping methods should be performed on confirmed cultures (restriction fragment length polymorphism (RFLP), amplified

fragment-length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), ribosomal DNA sequencing, antibiotic susceptibility, etc.), and what, if any, limitations do any of these methods pose?

5. What effect would *in situ* *Campylobacter* spp. cell aggregation have on the accuracy and reproducibility of enumeration counts and is there any remedy to address this issue?
6. Occasionally non-thermophilic *Campylobacter* species cause human illness. It is unclear whether livestock and poultry are reservoirs for these species, or if they are present on meat and poultry products following slaughter and processing. Current methodologies use selective agents and incubation conditions which may reduce their detection. If a pilot study was conducted to ascertain the presence of these species on meat and poultry products, what methodologies would best detect these species?

IV. RESPONSE TO QUESTIONS

Question 1. What additional circumstances should be considered in order for FSIS to conclude that the poultry baseline study should address more than the two principal *Campylobacter* species of *C. jejuni* and *C. coli*?

The Committee stated that *Campylobacter* species that cause the majority of human illness from meat and poultry products should drive the testing for particular species in developing a baseline study. Those two target species are *C. jejuni* and *C. coli* at present. However a certain percentage of samples should also be analyzed in a

separate surveillance research project to estimate the prevalence of other *Campylobacter* spp. No etiological agent is ever attributed to the vast majority of foodborne illnesses; this fact underscores the importance of such an additional study, as this would provide valuable information.

To strengthen the case that FSIS should focus the baseline study on *C. jejuni* and *C. coli*, an analogy was made using the verification testing of certain meat products for *E. coli* O157:H7. *E. coli* O157:H7 was first recognized in 1982 following two outbreaks of hemorrhagic colitis (20). In the years following, *E. coli* O157:H7 has been associated with numerous cases of hemorrhagic colitis and has rapidly become the most studied member of the enterohemorrhagic group (7). Over 25 non-O157 Shiga-like toxin-producing *E. coli* serotypes have been isolated but *E. coli* O157:H7 remains the most common enterohemorrhagic serotype in the United States (30). The epidemiological association of *E. coli* O157:H7 with ground beef along with its low infectious dose necessitated that *E. coli* O157:H7 be the focus of FSIS' intervention efforts to reduce illness due to hemorrhagic colitis. At present, *C. jejuni* and *C. coli* are the leading causes of human campylobacteriosis from poultry; therefore, the baseline should address these two species.

Question 2. How can the ARS method [used presently in the ARS/FSIS Broiler Rinse Study] be most successfully used for high volume analysis in the conduct of a baseline study of *Campylobacter* presence and enumeration on poultry (chicken, turkey, goose, etc.) carcasses, parts, and ground product that may lead to a potential performance standard or guideline for the regulated industry? What if any modifications should be made as a result of discussing this method in comparison

with others presented to the Committee? Please consider whether the above described atmospheric conditions, media, pre-enrichment, and storage media are acceptable for the objective of this baseline study.

The Committee chose to alter the question to reflect the specific ARS *Campylobacter* enumeration method presently being used in the joint ARS/FSIS Broiler Rinse Study (Appendix I.) since there are several methods being used by various ARS researchers. In the above question, the Committee inserted the clarification within the brackets.

In initial discussions regarding a baseline study for *Campylobacter* on poultry, the Committee recommends that FSIS must clearly state the objectives and potential uses of the data. Specifically, the Committee suggests that FSIS consider whether the results of the baseline study will be used to examine multiple points along the poultry processing line and identify interventions that the industry could further develop into points in the process where interventions are needed, and or “best practices”; whether FSIS will look at overall numbers of *Campylobacter* spp. on products in the inspected plants to ascertain the success of intervention strategies; and whether FSIS will use the data in a future risk assessment.

The Committee also suggests that FSIS consider testing for generic *E. coli*, *Salmonella*, and *Campylobacter* from the same carcass rinse to obtain information in relation to the utility of an indicator organism for the poultry industry. This would require that a standardized protocol with a neutralizing rinse broth be determined for quantitative and qualitative analysis of selected microorganisms. To ensure that data can be utilized for evaluating the suitability of indicator organisms, the sensitivities of the

methods for both indicators and target pathogens must be addressed. Methods for indicator organisms and pathogens should have equal sensitivities.

The Committee stated that FSIS should choose a *Campylobacter* method for its upcoming poultry baseline study that can be validated and easily used with a high sample throughput. The method chosen should be widely available to industry constituents for comparison sample analysis. The Committee recommends that for enumeration of campylobacters a direct plating method would presently best fit expected criteria for a baseline study over the previous labor intensive MPN method. The Committee acknowledges that a traditional method validation usually entails comparative evaluation against a “gold standard” method. In this case, it appears there is no “gold standard” *Campylobacter* enumeration method and there would be little value in comparing the new method against the FSIS MPN method. However, if a direct plating method is used, well trained technicians, proficient in colony identification are needed since identification of *Campylobacter* spp. can be difficult; they are non-fermenters and produce translucent colonies on Campy-Cefex agar. It was pointed out by the Committee that properly trained technicians would be essential no matter what direct plating method was chosen. The Committee also recommends that FSIS consult with other entities, such as other national governments, other US Federal agencies, and other private and state research institutions to correlate *Campylobacter* methodologies when possible. For example, the European Union is currently designing a monitoring scheme for *Campylobacter* in broilers (4), and the Nordic Committee on Food Analysis has recently developed methodological standards for the detection and enumeration of thermotolerant *Campylobacter* in foods (18).

The Committee discussed the current ARS/FSIS Broiler Rinse Study *Campylobacter* methodology at length with Dr. J. Stanley Bailey, the principal ARS researcher whose laboratory is performing the analyses. Direct plating was discussed as a method of choice. The Committee saw value in the 1-ml inoculation over four agar plates to achieve plating of a 10^0 dilution.

In further discussions with Dr. Bailey, the Committee determined that a back-up enrichment is not recommended. This was based on this principal researcher's description of preliminary data, using 100-ml carcass rinses, which indicates that back-up enrichments would only provide a 1-2% increase in the number of positive samples, and would also present additional challenges to assigning values to samples that were negative by direct plating (i.e., not detected) but were positive by back-up enrichment. It is noted that previous FSIS work indicated that a back-up enrichment in conjunction with a 400 ml rinse and MPN enumeration procedure increased positives by approximately 17% (27). The need for back-up enrichment to supplement direct plating for *Campylobacter* spp. was further analyzed by the FSIS, Office of Public Health Science, Risk Assessment Division staff members. This internal work acknowledged the above NACMCF recommendation to not perform back-up enrichment with direct plating of *Campylobacter* spp., and agreed with it, based on their FSIS evaluation (6).

In addition to the Campy-Cefex media used in the current ARS/FSIS Broiler Rinse Study, the Committee discussed at length the advantages and disadvantages of various direct plating agars available for *Campylobacter* with the subject matter experts, and their review of literature, with particular attention given to method comparison studies from Line et. al. (8); Oyarzabal et al. (19); and Siragusa et. al. (21). The

Committee discussed comparisons between Campy-Line and Campy-Cefex agars concluding that even though colonies on Campy-Line agar are easier to distinguish, it has additional selective agents which could reduce positive samples by up to 20% over Campy-Cefex agar. The Committee also pointed out that other organisms can produce colonies on Campy-Line agar with the same morphology as *Campylobacter* spp. The Committee also discussed various other media such as Modified *Campylobacter* Charcoal Differential Agar and a commercial Simplate® method for enumeration. As a result of extensive discussion, comparing media preparation, costs of media, and comparable recoveries on available solid plating media (including a modification to the Campy-Cefex (m-Campy-Cefex) media using lysed horse blood in the place of laked horse blood, and a substituted antifungal (19), the Committee recommends that either Campy-Cefex agar or m-Campy-Cefex would be a sensitive, cost effective choice.

Incubation temperatures were also discussed and $42 \pm 1^\circ\text{C}$ for 48 h as used in the current ARS/FSIS Broiler Rinse Study was recommended. The optimal growth temperature for *C. jejuni* ranges between 42 and 45°C. Under appropriate atmospheric and nutritional conditions, *C. jejuni* will grow at temperatures above 30 and at or below 45°C (22). A two-stage 37/42°C incubation was discussed, but these methods were deemed cumbersome and were originally used with broth media. The Committee charged itself with following up on whether there were any documented studies regarding staging incubation temperatures with solid media, and will make the updated information known to FSIS.

NACMCF has, in the past, addressed the parameters important in designing baseline studies and the Committee recommends that FSIS consult the NACMCF reports

entitled: “Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Broilers (Young Chickens)” (15), “Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Raw Ground Chicken” (16), and “Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Raw Ground Turkey” (17).

NACMCF is aware that FSIS has received funding for ongoing baseline studies and that FSIS intends to begin a broiler baseline study in 2006. In any scientific study, the sampling and data collection methods employed, as well as the study design parameters, are critical in assessing the validity, interpretability and generalizability of the results. Therefore, in addition to addressing study parameters, it is important that NACMCF address statistical and data collection issues that should be considered in designing any future baseline studies. NACMCF recommends that the agency come back with a charge to the Committee to review the statistical aspects as well as the data collection methodologies of any future baseline study designs.

Question 3. To utilize FSIS resources efficiently and effectively, FSIS expects to maintain as much continuity as possible between the current broiler rinse sampling for *Salmonella* and the proposed sampling for *Campylobacter* spp. What concerns regarding the *Campylobacter* spp. sampling method need to be attended to in order to properly address post-chill injured *Campylobacter* spp. cells as well as viable non-culturable (coccoid) cells?

As discussed previously, sampling and data collection methods are critical in assessing the validity, interpretability and generalizability of the study results. Therefore, in determining the sampling and data collection methods used in the baseline studies,

several statistical considerations should be addressed. Foremost, the study objective(s) should be clearly stated, the population of interest should be identified and the sampling unit selected should be representative of that population. Sampling methods should also consider other potential factors such as seasonal and regional differences as well as inter-flock and inter-plant correlation. In addition, there should be some statistical justification to the sample size selected for the study. The Committee recommends that FSIS consider the statistical power in selecting the number of plants, number of carcasses and frequency of sampling for the baseline study, and FSIS should create a power calculation matrix to determine the optimal sample size. Further, insofar as possible, samples should be randomly selected and the sampling and data collection methods should be consistent throughout the study. Specifically, FSIS should define how carcasses will be randomly chosen at establishments and at what point(s) in the process they will be selected for rinsing. Sample handling factors such as rinse methods (i.e. type of neutralizing diluent, rinse solution), temperature conditions during shipment, and microbiological testing procedures, should be specified and uniform throughout the study. To assure consistency in sample as well as data collection, it is recommended that a sample and data collection protocol is developed and those involved in carrying out the protocol are trained with a common format.

The choice of validated neutralizing diluent for carcass rinsing and rinse volume (400 ml versus 100 ml) is important when designing a baseline study for *Campylobacter* spp. The desirable features of a rinse diluent include: gives maximum buffering capacity, aids in injured cell recovery but does not promote cell growth during refrigerated transportation and/or result in false negatives due to improper neutralization and

sampling. When carcasses are chemically treated as an intervention, there is a need to document this information on the sampling form using standardized language. Information related to such chemical treatments must be collected to ensure sample integrity and would not be used to measure the effect of the treatments; although, the information may be used for generating hypotheses or informing the design of future studies specifically addressing interventions. If chemical treatments are used, proper neutralization procedures need to be performed with sampling. Proper training and supplies are essential for sample collectors. Post-chill antimicrobial carcass dipping is a practice currently being utilized in industry. Therefore proper carcass draining practices in addition to using non-antimicrobial neutralizing additives, tailored to each chemical treatment, should be developed to maximize *Campylobacter* spp. recovery, as well for generic *E. coli* and *Salmonella* being tested for under the current regulations.

Presently, FSIS uses BPW for *Salmonella* rinse sample collection and has used it for *Campylobacter* sample collection (25), even though it is considered a pre-enrichment broth for *Salmonella* (2). FSIS could also use Butterfield's phosphate diluent, which is not considered to be a pre-enrichment broth, for collecting carcass rinse samples. The Committee recommends using the smallest rinse volume needed to cover all surfaces of the broiler and to perform microbial analysis of *Campylobacter* and other organisms. Researchers conducting the present ARS/FSIS Broiler Rinse Study determined a 100 ml volume of BPW was sufficient, and NACMCF recommends this volume of rinse be validated. Note, it was pointed out by Dr. Bailey during discussions with the NACMCF subcommittee, that based on preliminary results from the ARS/FSIS Broiler Rinse Study, the higher volume of rinse used in the FSIS HACCP verification program (FSIS uses 400

ml BPW, ARS method calls for a 100 ml) may contribute to a lower observed *Campylobacter* spp. count for broiler rinses, as compared to what is being observed in the ARS project.

FSIS should determine the specific volume and type of rinse to be used, taking into account any additional microbiological assays being performed as part of the baseline, and provide scientific justification for the volume chosen. Referencing statistically valid studies/documents comparing different rinse volumes should be included. Rinse solutions should be at 4°C before rinsing, and rinsate should be immediately placed on ice.

In addition, sample shipment temperature conditions were discussed. FSIS baseline studies and the current ARS/FSIS Broiler Rinse Study require a temperature of between 0°-10°C for samples on arrival at the FSIS laboratories. The Committee recommends overnight shipping, and suggests a study be performed to determine the number of ice packs and/or volume of ice needed to maintain temperature, given anticipated ambient temperature extremes.

The Committee discussed microaerobic conditions needed for incubation. For a large volume of samples, as would be generated in a large long-term study such as a baseline, a tri-gas incubator is recommended. However, guidance should be issued on alternative ways to achieve microaerobic conditions if such an incubator is unavailable. Specific details of any gas-filled bag protocols, such as whether bags are to be heat sealed, should be provided, and FSIS should validate the specific methodology for using gas-filled bags.

The Committee recommends that FSIS speciate especially between *C. jejuni* and *C. coli*. Methods such as latex agglutination and multiplex PCR can be used. In collaborating with research partners, FSIS should explore developing and validating molecular technologies such as microarray for speciation and subtyping of *Campylobacter*.

The Committee recognizes that the advantages of phase contrast microscopic examination of a wet-mount for characteristic morphology/motility, are that the test is quick, and that it provides instant feedback. However, a wet-mount exam is not a confirmatory test. FSIS should address the training of laboratory technicians to achieve a high level of proficiency in identifying presumptive *Campylobacter* colonies. A minimum of five colonies, up to a total of 10% of the typical colonies on a countable (or lowest dilution) plate, representing each colony morphology, should be picked for semi-confirmatory testing by cellular morphology and motility on a wet-mount using phase contrast microscopy. Each isolate demonstrating typical *Campylobacter* morphology and motility will be further confirmed using latex agglutination, and then also speciated.

The Committee recommends that FSIS use consistent microbiological methods and procedures, outlining defined parameters for drying agar plates, storage and shelf life of plates, and report enumeration data as CFU/ml rinse when whole carcass rinsates are tested. A subject matter expert noted that a number of researchers from industry and other laboratories have been trained by ARS laboratories in Athens, GA in *Campylobacter* methodology, indicating that this laboratory would be a good resource.

If FSIS determines that classes of poultry other than broilers will be assessed in the future (e.g., turkeys), FSIS should partner with appropriate researchers to develop

methodologies and conduct surveillance studies to sample these products possibly for other *Campylobacter* species in addition to *C. jejuni* and *C. coli*. Turkeys, due to their size and weight, also pose unique sample collection challenges beyond a simple broiler rinse. FSIS should consult research studies such as those by McEvoy et al. (9) and Bodnaruk et al. (1), along with research partners to optimize turkey sample collection techniques. This is a topic that could possibly be brought before the Committee in the future, should FSIS require more guidance.

For ground product, the Committee recommends the use of 25g of product in a filtered stomacher bag with diluent of choice with stomaching for 1 min followed by serial dilution and plating.

The possible importance of viable nonculturable *Campylobacter* strains is not known. This topic could be brought before the Committee again by FSIS when more information becomes available.

Question 4. What further subtyping methods should be performed on confirmed cultures (restriction fragment length polymorphism (RFLP), amplified fragment-length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), ribosomal DNA sequencing, antibiotic susceptibility, etc.), and what, if any, limitations do any of these methods pose?

The Committee discussed a number of subtyping methods. To maximize correlation among government entities, the utility of PFGE was recognized since the method is used by the Centers for Disease Control and Prevention PulseNet (28) to track human illness isolates and by ARS VetNet (24) to track animal diagnostic isolates. The Committee recognizes that PFGE is more readily available than some of the other

methods discussed, such as multilocus sequence typing (MLST). The Committee, in consultation with subject matter experts, discussed the MLST method. Data from the ARS *Campylobacter* research laboratory in Albany, CA using MLST have shown that *C. jejuni* and *C. coli* exchange genetic material (swap genes) making speciation difficult (11). The Committee pointed out that in certain circumstances where PFGE has not provided useful information, MLST has been used successfully.

In addition, the Committee recommends that FSIS explore the feasibility and value of serotyping *Campylobacter* as well as investigate the feasibility of *flaA* sequence comparisons in subtyping *Campylobacter* which has been used at ARS in Athens, GA (10).

Because antibiotic resistance among *Campylobacter* spp. is a public health problem, and there are interagency-established protocols for resistance testing, the Committee recommends that a defined subset of isolates be tested for antibiotic resistance. The results can be used in analyses to help develop hypotheses about how resistant *Campylobacter* spp. enter a facility and move through production lines, and whether some resistant strains are maintained in facilities.

Finally, while a number of subtyping methods have been performed on *Campylobacter* spp. (e.g., serotyping, antibiotic resistance, MLST, PFGE, *flaA* sequencing, etc.), none have yet been sufficiently discriminatory to be generally applicable as a gold standard. A combination of two or more subtyping methods can often increase discriminatory power. However, continued subtyping studies are essential, since with refinement these methods have been of crucial importance in tracking other pathogens to their source. Therefore, the Committee recommends that research on these

methods be continued because of their value in gaining epidemiologically significant information. As part of ongoing sampling, isolates should be preserved in storage for further molecular characterization, but such characterization should not be part of an initial baseline study.

Question 5. What effect would *in situ* *Campylobacter* spp. cell aggregation have on the accuracy and reproducibility of enumeration counts and is there any remedy to address this issue?

The Committee acknowledges that *Campylobacter* spp. cell aggregation is a real phenomenon, but whether it causes significant differences in counts has not been shown. Further research is necessary in this area.

Question 6. Occasionally non-thermophilic *Campylobacter* species cause human illness. It is unclear whether livestock and poultry are reservoirs for these species, or if they are present on meat and poultry products following slaughter and processing. Current methodologies use selective agents and incubation conditions which may reduce their detection. If a pilot study was conducted to ascertain the presence of these species on meat and poultry products, what methodologies would best detect these species?

The Committee recommends that FSIS partner with appropriate researchers to conduct surveillance studies to sample poultry products for analysis of *Campylobacter* species other than *C. jejuni* and *C. coli*. FSIS should examine the findings of the European Campycheck research initiative (3) and consult with other research partners in development of protocols to analyze for other *Campylobacter* species as part of a surveillance study. The surveillance data could inform FSIS regarding whether, in the

future, to expand species testing if other *Campylobacter* species become significant with regard to human illness from FSIS-regulated products. The Committee suggests that FSIS may be able to benefit from the geographical proximity of the FSIS Western Field Service Laboratory and the ARS *Campylobacter* research laboratory in Albany, CA, in that these two facilities can split and share collected rinse samples to maximize the testing performed on these rinses.

V. CONCLUSIONS

Target organisms selected for a microbiological baseline study should be based on species causing the majority of human illness. In designing the FSIS *Campylobacter* spp. from broiler carcass baseline study, presently the two target species are *C. jejuni* and *C. coli*. FSIS should however partner with appropriate researchers to develop methodologies and conduct surveillance studies to sample poultry products for other *Campylobacter* species. Surveillance data could then be used to direct expansion of *Campylobacter* testing in the future if necessary.

In designing the upcoming FSIS baseline for *Campylobacter* spp. enumeration of broilers, and any future baseline studies, FSIS must clearly state the objectives and potential uses of the data. FSIS can achieve maximum data utilization if the objectives are set before sample collection begins. Possible objectives may be to sample at multiple points along the poultry processing line and identify interventions that the industry can use as “best practices” and/or determine the overall numbers of *Campylobacter* spp. leaving establishments to ascertain if regulatory policies are successful and/or develop data to be used in future risk assessments. Consideration should be given to the need to also collect generic *E. coli* and *Salmonella* data simultaneously from whole bird carcass

rinses. To ensure the validity, interpretability and generalizability of the study results, sampling and data collection methods should be evaluated, and a document that details the study protocol should be developed and made available.

The direct plating *Campylobacter* spp. enumeration methodology currently being performed in the ARS/FSIS Broiler Rinse Study should be the basis for developing an upcoming baseline study with modifications as indicated throughout this report. See Figure 1. for the NACMCF suggested *Campylobacter* analysis protocol for FSIS baseline studies for poultry. This method would be widely available to industry constituents and easily used with high numbers of samples that is impractical with MPN methods. It would be paramount to develop and adequately train individuals conducting sample collection and microbiological analysis to maximize data accuracy thus allowing the creation of a data set that could be used to develop FSIS risk management policy with regard to *Campylobacter* spp. contamination on poultry products.

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VII. REFERENCES

1. Bodnaruk, P. W., J. L. Schmelder, P. J. Krakar, and R. B. Tompkin. 1998. A comparison of *Escherichia coli* levels at four sampling sites on turkey carcasses. *Poul. Sci.* 77:1531-1533.
2. Difco Manual. 1998. Culture media and ingredients, dehydrated, p. 99-100. Difco Laboratories, Division of Becton Dickinson and Company, Sparks, MD.
3. European Commission. CAMPYCHECK Research Project. Available at: <http://www.campycheck.org/>. Accessed 10 April 2006.
4. European Union. 2005. Draft technical specifications for an EU monitoring scheme for *Campylobacter* in broiler chickens. Unpublished report.
5. Fitzgerald, C. 14 July 2005. Personal communication [E-mail: chf3@cdc.gov].
6. Golden, N. 13 September, 2005. Personal communication [E-mail: neal.golden@fsis.usda.gov].
7. Griffin, P. M. and R. V. Tauxe. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol. Rev.* 13:60-98.
8. Line, J., N. Stern, C. Lattuada, and S. Benson. 2001. Comparison of methods for recovery and enumeration of *Campylobacter* from freshly processed broilers. *J. Food Prot.* 64:982-986.
9. McEvoy, J. M., C. W. Nde, J. S. Sherwood, and C. M. Logue. 2005. An evaluation of sampling methods for the detection of *Escherichia coli* and *Salmonella* on turkey carcasses. *J. Food Prot.* 68:34-39.

10. Meinersmann, R.J., Phillips, R.W., Hiett, K.L., and Cray, P.J. 2005. Differentiation of *Campylobacter* populations demonstrated by flagellin short variable region sequences. *Appl. Environ. Microbiol.* 71:6368-6374.
11. Miller, W.G., On, S., Wang, G., Fontanoz, S., Lastovica, and A., Mandrell, R.E. 2005. Extended multilocus sequence typing system for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. *J. Clin. Microbiol.*43:2315-2329.
12. National Advisory Committee on Meat and Poultry Inspection. 2000. Briefing on Agency's Efforts with Respect to *Campylobacter*: National Advisory Committee on Meat and Poultry Inspection. Available at: <http://www.fsis.usda.gov/OPPDE/NACMPI/May2000/nacmpicampy.html>. Accessed October 12, 2005.
13. National Advisory Committee on Microbiological Criteria for Foods. 1994. *Campylobacter jejuni/coli*. *J. Food Prot.* 57:1101-1121.
14. National Advisory Committee on Microbiological Criteria for Foods. 2002. Plenary Session (Transcript). Available at: <http://www.fsis.usda.gov/ophs/nacmcf/2002/transcript082802.pdf>. Accessed October 12, 2005.
15. National Advisory Committee on Microbiological Criteria for Foods 2004. Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Broilers (Young Chickens). Available at: http://www.fsis.usda.gov/OPHS/NACMCF/2004/NACMCF_broiler_4_13_04.pdf. Accessed October 12, 2005.

16. National Advisory Committee on Microbiological Criteria for Foods. 2004. Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Raw Ground Chicken. Available at: http://www.fsis.usda.gov/ophs/nacmcf/2004/NACMCF_Ground_Chicken_082704.pdf. Accessed October 14, 2005.
17. National Advisory Committee on Microbiological Criteria for Foods 2004. Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Raw Ground Turkey. Available at: http://www.fsis.usda.gov/ophs/nacmcf/2004/NACMCF_Ground_Turkey_082704.pdf. Accessed October 14, 2005.
18. Nordic Committee on Food Analysis. April 2005. Thermotolerant *Campylobacter*. Detection and enumeration in foods.
19. Oyarzabal, O., K. Macklin, J. Barbaree, and R. Miller. 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. *Appl. Environ. Microbiol.* 71:3351-3354.
20. Riley L.W., R.S. Remis, S.D. Helgerson, H.B. McGee, J.G. Wells, B.R. Davis, R.J. Hebert, E.S. Olcott, L.M. Johnson, N.T. Hargrett, P.A. Blake, and M.L. Cohen. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med.* 308:681-685.
21. Siragusa, G., J. Line, L. Brooks, T. Hutchinson, J. Laster, and R. Apple. 2004. Serological methods and selective agars to enumerate *Campylobacter* from broiler carcasses: data from inter- and intralaboratory analyses. *J. Food Prot.* 67:901-907.

22. Stern, N. J., and S. U. Kazmi. 1998. *Campylobacter jejuni*. p 77. In M. P. Doyle, (ed), Foodborne Bacterial Pathogens. Marcel Dekker, Inc. New York.
23. Tauxe, Robert V. 2005. Human Campylobacteriosis Risk Factors. At South East Poultry Association Annual Meeting. Atlanta, GA February 23, 2005.
24. U.S. Department of Agriculture, Agricultural Research Service. 2005. Antimicrobial Resistance in Pathogenic and Commensal Bacteria of Food Animals: Narms, Vetnet and Cahfse. Available at: http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=191019&pf=1. Accessed 19 April 2006.
25. U.S. Department of Agriculture, Food Safety and Inspection Service. 1994-1995. Nationwide Broiler Chicken Microbiological Baseline Data Collection Program (July 1994-June 1995. Available at: www.fsis.usda.gov/OPHS/baseline/broiler2.pdf. Accessed at 10 April 2006.
26. U.S. Department of Agriculture, Food Safety and Inspection Service. 1998. Isolation, Identification, and Enumeration of *Campylobacter jejuni/coli* from Meat and Poultry Products. Available at: <http://www.fsis.usda.gov/ophs/Microlab/Mlgchp6.pdf>. Accessed 10 April 2006.
27. U.S. Department of Agriculture, Food Safety and Inspection Service. 1998. Preliminary summary report for enumerating *Campylobacter* spp., *E. coli*, and *Salmonella* spp. in broiler carcass rinses before and after simulated transport in artificial ice for 24 h. Unpublished report.

28. U. S. Department of Health and Human Services. Centers for Disease Control and Prevention. PulseNet. Available at: <http://www.cdc.gov/pulsenet/>. Accessed 10 April 2006.
29. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. 2001. Bacteriological Analytical Manual Online: *Campylobacter*. Available at: <http://www.cfsan.fda.gov/~ebam/bam-7.html>. Accessed 10 April 2006.
30. Woody, J., J. Stevenson, R. Wilson, and S. Knabel. 1998. Comparison of the Difco EZ Coli™ Rapid Detection System and Petrifilm™ Test Kit-HEC for detection of *Escherichia coli* O157:H7 in fresh and frozen ground beef. *J. Food Prot.* 61:110-112.

**APPENDIX I. ARS/FSIS BROILER RINSE STUDY *CAMPYLOBACTER*
SPP. ENUMERATION METHOD**

Aseptically collect whole bird rinses with 100 ml of BPW, and then ship overnight at $4^{\circ} \pm 4^{\circ}$ C to the laboratory for analysis. Direct plate carcass rinsates to enumerate *Campylobacter* spp. To obtain CFU/ml of rinse from carcasses (where low numbers (countable plates) are expected) apply 1 ml to a total of four Campy-Cefex agar plates (Hardy Diagnostics, Santa Maria, CA), 0.25 ml per plate. Spread each aliquot on the agar surface with a sterile plastic hockey stick; allow plates to remain upright until dried (approximately 15 min). Incubate Campy-Cefex agar plates at 42°C for 48 h in sealable bags flushed to produce a microaerobic gas environment (5% O₂, 10% CO₂, 85% N₂). As a group of four, designate these plates as the 10⁰ dilution per ml. (Note: Because these plates receive a 0.25-ml volume, the agar needs to be pre-dried to facilitate soaking up the inoculum. Achieve dried agar plates by leaving them on a laboratory bench at ambient temperature and humidity (protected from light) for 24 h prior to plating.)

To allow enumeration of higher numbers of *Campylobacter* spp. per ml, prepare 10-fold serial dilutions of carcass rinsates in phosphate buffered saline. Spread the undilute rinsates (0.1 ml) and aliquots from serial dilutions (0.1 ml) on the surface of duplicate Campy-Cefex agar plates with a sterile plastic hockey stick. Incubate Campy-Cefex agar plates at 42°C for 48 h in sealable bags flushed to produce a microaerobic gas environment (5% O₂, 10% CO₂, 85% N₂).

Following incubation, count colonies characteristic of *Campylobacter* spp. Calculate the number of *Campylobacter* spp. CFU/ml rinsate using either a total number

from all four 10^0 dilution plates, or an average of the duplicate counts at higher dilutions, as appropriate. Characterize each colony type counted as *Campylobacter* spp. from each sample as a member of the genus *Campylobacter* by examination of cellular morphology and motility on a wet-mount under phase contrast microscopy. Further confirm each colony type as species *jejuni*, *coli*, *lari* by a latex agglutination test kit (Med-Ox Diagnostics, Inc.; Ogdensburg, NY).

Figure 1. NACMCF suggested *Campylobacter* analysis protocol for FSIS baseline studies for poultry

