

Supplement

Analytical Utility of *Campylobacter* Methodologies[†]

ADOPTED 28 SEPTEMBER 2005, CORAL GABLES, FLA.

NATIONAL ADVISORY COMMITTEE ON MICROBIOLOGICAL CRITERIA FOR FOODS

NACMCF Executive Secretariat,* U.S. Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science, Room 333
Aerospace Center, 1400 Independence Avenue S.W., Washington, D.C. 20250-3700, USA

MS 06-361: Received 5 July 2006/Accepted 20 July 2006

TABLE OF CONTENTS

- Executive Summary
- I. Introduction
- II. Purpose of the Document
- III. Original Work Charge and Background
- IV. Response to Questions
 1. What additional circumstances should be considered in order for the Food Safety and Inspection Service (FSIS) to conclude that the poultry baseline study should address more than the two principal *Campylobacter* species, *Campylobacter jejuni* and *Campylobacter coli*?
 2. How can the Agricultural Research Service (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, growth media, preenrichment conditions, and storage media are acceptable to meet the objective of this baseline study.
 3. To utilize FSIS resources efficiently and effectively, the FSIS expects to maintain as much continuity as possible between the current broiler rinse sampling protocol for *Salmonella* and the proposed sampling protocol for *Campylobacter* spp. What concerns regarding the *Campylobacter* spp. sampling method need to be attended to in order to properly address postchill-injured *Campylobacter* spp. cells as well as viable but nonculturable (coccolid) cells?
 4. What further subtyping methods should be performed on confirmed cultures (restriction fragment length polymorphism, amplified fragment length polymorphism, pulsed-field gel electrophoresis, ribosomal DNA sequencing, antibiotic susceptibility, etc.), and what, if any, limitations do any of these methods have?
 5. What effect would in situ *Campylobacter* cell aggregation have on the accuracy and reproducibility of enumerations, and is there any remedy to address this issue?
 6. Occasionally, nonthermophilic *Campylobacter* species cause human illness. It is unclear whether livestock and poultry are reservoirs for these species and whether these pathogens are present on meat and poultry products following slaughter and processing. Current methodologies include use of selective agents and incubation conditions that may reduce the detection of these pathogens. If a pilot study was conducted to ascertain the presence of these *Campylobacter* species on meat and poultry products, what methodologies would be most effective for detecting these species?
- V. Conclusions
- VI. Acknowledgments
- VII. References
- Appendix I. ARS/FSIS Broiler Rinse Study *Campylobacter* spp. Enumeration Method

EXECUTIVE SUMMARY

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF, or the Committee) was asked

to address six questions regarding *Campylobacter*. These questions relate to the analytical utility of *Campylobacter* methodologies in preparation for an upcoming U.S. 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 U.S. experts on *Campylobacter* research, and examined the current method being used in an on-going U.S. Department of Agriculture collaborative study between the FSIS and the Agricultural

* Author for correspondence: NACMCF Executive Secretariat. Tel: 202-690-6600; Fax: 202-690-6364; E-mail: gerri.ransom@fsis.usda.gov.

† Sponsored by the U.S. Department of Agriculture, Food Safety and Inspection Service; U.S. Department of Health and Human Services, Food and Drug Administration, and Centers for Disease Control and Prevention; U.S. Department of Commerce, National Marine Fisheries Service; and U.S. Department of Defense, Veterinary Service Activity. This article may be reproduced without prior permission.

Research Service (ARS), hereinafter referred to as the ARS/FSIS Broiler Rinse Study, for possible use in the upcoming FSIS baseline study of broilers.

As described more fully in this report, the Committee acknowledges that *Campylobacter* species 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 *Campylobacter jejuni*, followed by *Campylobacter coli* and other species. Finally, the Committee understands that the FSIS is awaiting scientific recommendations from the 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*.
- The 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.
- The FSIS must clearly state the objectives and potential uses of the baseline data.
- The 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 the 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.
- The FSIS should identify *Campylobacter* to species to differentiate *C. jejuni* and *C. coli*.

I. INTRODUCTION

Campylobacter species are major bacterial agents of human foodborne gastroenteritis. Poultry is a primary reservoir of *Campylobacter* species, 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* causing 4% of these illnesses, and other species causing 1% (23).

For clarity, the NACMCF has defined the term “broilers” in the same manner as they did in the 2004 NACMCF report “Response to the Questions Posed by FSIS Regarding Performance Standards with Particular Reference to Broilers (Young Chickens)” (15). In that report, a broiler

was defined as a young chicken of either sex that is usually under 13 weeks of age. The FSIS has proposed to reduce this age requirement to younger than 10 weeks.

In the past, the FSIS has conducted baseline studies of *Campylobacter* cell enumeration from broiler carcass rinses using a labor-intensive most-probable-number (MPN) method (25, 26); however, not all of these studies have been published. The FSIS is planning to initiate a new *Campylobacter* spp. baseline study and asked the NACMCF to advise the FSIS in developing the methodology. The Committee was asked to evaluate a direct plating method currently being used for enumerating *Campylobacter* cells 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 the FSIS to the Committee at the 12 July 2005 public meeting. Since a formal report specifically addressing *Campylobacter* methods was not previously developed by the NACMCF, the Committee used this report to compile all past and current activities associated with *Campylobacter*.

III. ORIGINAL WORK CHARGE AND 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 the NACMCF to evaluate options for defining a *Campylobacter* performance standard or, as an alternative, a performance standard that would accomplish the same public health objective. In response, a subcommittee of the NACMCF (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 the NACMPI and the FSIS (12).

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

- Review and compare the methodologies used for *Campylobacter* detection in the 1994 to 1995 and the 1999 to 2000 baseline studies in young chickens;
- Evaluate these methodologies for the accuracy and precision that they provide for 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 issued. In addition, the FSIS baseline data from 1999 to 2000 have never been released because of *Campylobacter* methodology concerns expressed by the FSIS.

FSIS activity. Prior to 2004, the FSIS used a labor-intensive and resource-consuming MPN method for the detection and enumeration of *Campylobacter* cells (26). Current literature indicates that when *Campylobacter* spp. are present, numbers per milliliter 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 U.S. 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. in clinical and food samples. Since 2004, the FSIS in cooperation with ARS researcher Dr. J. Stanley Bailey has been 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 $4 \pm 4^\circ\text{C}$ 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 under 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 *Campylobacter 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, the FSIS will conduct a baseline study to determine the prevalence of *Campylobacter* spp. and to enumerate cells of those species of known importance on poultry (possibly including carcasses, parts, and ground product). It is currently proposed that the study will focus on the 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 comes from results of 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., nonthermotolerant campylobacters) or growth media

other than Campy-Cefex agar, which contains the antimicrobial cefoperazone that inhibits growth of *Campylobacter upsaliensis*.

The FSIS seeks advice on the proposed *Campylobacter* methodology, as well as any other relevant methodology that may be of equal or greater value, that should be considered when designing the upcoming baseline study. Six questions are to be addressed.

1. What additional circumstances should be considered in order for the FSIS to conclude that the poultry baseline study should address more than the two principal *Campylobacter* species, *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, growth media, pre-enrichment conditions, and storage media are acceptable to meet the objective of this baseline study.
3. To utilize FSIS resources efficiently and effectively, the FSIS expects to maintain as much continuity as possible between the current broiler rinse sampling protocol for *Salmonella* and the proposed sampling protocol for *Campylobacter* spp. What concerns regarding the *Campylobacter* spp. sampling method need to be attended to in order to properly address postchill-injured *Campylobacter* spp. cells as well as viable but nonculturable (cocoid) cells?
4. What further subtyping methods should be performed on confirmed cultures (restriction fragment length polymorphism, amplified fragment length polymorphism, pulsed-field gel electrophoresis [PFGE], ribosomal DNA sequencing, antibiotic susceptibility, etc.), and what, if any, limitations do any of these methods have?
5. What effect would in situ *Campylobacter* cell aggregation have on the accuracy and reproducibility of enumerations, and is there any remedy available to address this issue?
6. Occasionally, nonthermophilic *Campylobacter* species cause human illness. It is unclear whether livestock and poultry are reservoirs for these species and whether these pathogens are present on meat and poultry products following slaughter and processing. Current methodologies include use of selective agents and incubation conditions that may reduce the detection of these pathogens. If a pilot study was conducted to ascertain the presence of these *Campylobacter* species on meat and poultry products, what methodologies would be most effective for detecting these species?

IV. RESPONSE TO QUESTIONS

1. **What additional circumstances should be considered in order for the FSIS to conclude that the poultry**

baseline study should address more than the two principal *Campylobacter* species, *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. At present, those two target species are *C. jejuni* and *C. coli*. However, a certain percentage of samples should also be analyzed in a separate surveillance research project to estimate the prevalence of other *Campylobacter* species. No etiological agent is ever identified in the vast majority of foodborne illnesses; this fact underscores the importance of such an additional study, as it would provide valuable information.

To strengthen the case that the 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 study should address these two species.

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, growth media, preenrichment conditions, and storage media are acceptable to meet 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 given within the brackets.

In initial discussions regarding a baseline study of *Campylobacter* on poultry, the Committee recommended that the FSIS must clearly state the objectives and potential uses of the data. Specifically, the Committee suggested that

the FSIS consider (i) 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 at points in the process where interventions are needed, and/or for defining “best practices,” (ii) whether the FSIS will look at overall numbers of *Campylobacter* spp. on products in the inspected plants to ascertain the success of intervention strategies, and (iii) whether the FSIS will use the data in a future risk assessment.

The Committee also suggested that the 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 approach would require that a standardized protocol with a neutralizing rinse broth be developed 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 determined and should be equal.

The Committee stated that for its upcoming poultry baseline study the FSIS should choose a *Campylobacter* method 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 recommended that for enumeration of campylobacters a direct plating method would better fit expected criteria for a baseline study than would the labor-intensive MPN method. The Committee acknowledged that a traditional method validation usually entails comparative evaluation against a “gold standard” method. In this case, it appears that there is no gold standard *Campylobacter* enumeration method, and there would be little value in comparing the new method with 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; these bacteria are nonfermenters 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 recommended that the FSIS consult with other entities, such as other national governments, other U.S. agencies, and 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 10⁰ dilution.

In further discussions with Dr. Bailey, the Committee determined that a backup enrichment is not necessary. This decision was based on the principal researcher's description of preliminary data obtained using 100-ml carcass rinses, which indicates that backup enrichments would provide only a 1 to 2% increase in the number of positive samples and would present additional challenges to assigning values to samples that were negative by direct plating (i.e., not detected) but positive by backup enrichment. Previous FSIS work indicated that a backup enrichment in conjunction with a 400-ml rinse and MPN enumeration procedure increased positive results by approximately 17% (27). The need for backup 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 use backup enrichment with direct plating of *Campylobacter* spp. and agreed with it, based on their FSIS evaluation (6).

In addition to the Campy-Cefex medium used in the current ARS/FSIS Broiler Rinse Study, the Committee discussed with the subject matter experts the advantages and disadvantages of various direct plating agars available for *Campylobacter* and their review of the literature, with particular attention given to method comparison studies of Line et al. (8), Oyarzabal et al. (19), and Siragusa et al. (21). The Committee discussed comparisons between Campy-Line and Campy-Cefex agars and concluded that even though colonies on Campy-Line agar are easier to distinguish, this medium has additional selective agents that could reduce the number of positive samples by up to 20% compared with Campy-Cefex agar. The Committee also pointed out that other organisms grown on Campy-Line agar can produce colonies with the same morphology as those of *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) medium using lysed horse blood in the place of laked horse blood and a different antifungal preparation (19), the Committee recommended that either Campy-Cefex agar or m-Campy-Cefex would be a sensitive, cost-effective choice.

Incubation temperatures were also discussed, and 42 ± 1°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 and 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 report the updated information to the FSIS.

The NACMCF has, in the past, addressed the parameters important for designing baseline studies, and the Com-

mittee recommended that the 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).

The NACMCF is aware that the FSIS has received funding for ongoing baseline studies and that the 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 the NACMCF address statistical and data collection issues that should be considered when designing any future baseline studies. The NACMCF recommended 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.

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

As discussed previously, sampling and data collection methods are critical in assessing the validity, interpretability, and generalizability of the study results. Therefore, when 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 interflock and interplant correlations. In addition, there should be some statistical justification for the sample size selected for the study. The Committee recommended that the FSIS consider the statistical power when selecting the number of plants, number of carcasses, and frequency of sampling for the baseline study, and the FSIS should create a power calculation matrix to determine the optimal sample size. Insofar as possible, samples should be randomly selected, and the sampling and data collection methods should be consistent throughout the study. Specifically, the 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 and 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, a sample and data collection protocol should be developed, and those involved in carrying out the protocol should be 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 the following: gives maximum buffering capacity, aids in injured cell recovery but does not promote cell growth during refrigerated transportation, and does not produce false-negative results 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 followed with sampling. Proper training and supplies are essential for sample collectors. Postchill antimicrobial carcass dipping is a practice currently being utilized in the industry. Therefore, proper carcass draining practices in addition to use of nonantimicrobial neutralizing additives tailored to each chemical treatment should be developed to maximize recovery of *Campylobacter* spp. as well as for generic *E. coli* and *Salmonella* being tested for under the current regulations.

Presently, the FSIS uses BPW for *Salmonella* rinse sample collection and has used it for *Campylobacter* sample collection (25), even though it is considered a preenrichment broth for *Salmonella* (2). The FSIS could also use Butterfield's phosphate diluent, which is not considered to be a preenrichment broth, for collecting carcass rinse samples. The Committee recommended 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 the NACMCF recommended this volume of rinse be validated. During discussions with the NACMCF subcommittee, Dr. Bailey pointed out that based on preliminary results from the ARS/FSIS Broiler Rinse Study the higher volume of rinse used in the FSIS hazard analysis critical control point verification program (FSIS uses 400 ml of BPW, but the ARS method calls for 100 ml) may contribute to a lower observed *Campylobacter* count for broiler rinses compared with that found in the ARS study.

The 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 should provide scientific justification for the volume chosen. References to statistically valid studies and documents comparing different rinse volumes should be in-

cluded. Rinse solutions should be at 4°C before rinsing, and the 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 and 10°C for samples on arrival at the FSIS laboratories. The Committee recommended overnight shipping and suggested a study be performed to determine the number of ice packs and/or the volume of ice needed to maintain the correct 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 study, a tri-gas incubator was recommended. However, guidance should be issued on alternative ways to achieve microaerobic conditions when 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 the FSIS should validate the specific methodology for using gas-filled bags.

The Committee recommended that the FSIS identify *Campylobacter* isolates to species, especially *C. jejuni* and *C. coli*. Methods such as latex agglutination and multiplex PCR assays can be used. In collaborating with research partners, the FSIS should explore the development and validation of molecular technologies such as microarrays for species identification and subtyping of *Campylobacter* isolates.

The Committee recognized the advantages of phase contrast microscopic examination of a wet mount for characteristic morphology and motility: this method is quick and provides instant feedback. However, a wet mount exam is not a confirmatory test. The 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 semiconfirmatory testing based on cell morphology and motility on a wet mount examined with phase contrast microscopy. Each isolate demonstrating typical *Campylobacter* morphology and motility would be further confirmed with a latex agglutination test and then identified to species.

The Committee recommended that the FSIS use consistent microbiological methods and procedures, outlining defined parameters for drying agar plates, storage, and the shelf life of plates, and report enumeration data as CFUs per milliliters of 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 in ARS laboratories in Athens, Ga., in *Campylobacter* methodology, indicating that these laboratories would be good resources.

If the FSIS determines that classes of poultry other than broilers will be assessed in the future (e.g., turkeys), the 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. The 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 the FSIS require more guidance.

For ground product, the Committee recommended stomaching 25 g of product with the diluent of choice for 1 min in a filtered stomacher bag 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 the FSIS when more information becomes available.

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

The Committee discussed a number of subtyping methods. To maximize correlation of results 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 recognized that PFGE is more readily available than some of the other methods discussed, such as multi-locus sequence typing (MLST). The Committee, in consultation with subject matter experts, discussed the MLST method. Data from the ARS *Campylobacter* research laboratory in Albany, Calif., collected using MLST have shown that *C. jejuni* and *C. coli* exchange genetic material (swap genes), making species identification 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 recommended that the FSIS explore the feasibility and value of serotyping *Campylobacter* as well as investigate the feasibility of *flaA* sequence comparisons in subtyping *Campylobacter*; this method has been used at the ARS in Athens, Ga. (10).

Because antibiotic resistance among *Campylobacter* species is a public health problem and there are interagency-established protocols for resistance testing, the Committee recommended 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* species 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 used with *Campylobacter* species (e.g., serotyping, antibiotic resistance, MLST, PFGE, *flaA* sequencing, etc.), none have yet been sufficiently discriminatory to be gen-

erally 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 recommended 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.

5. What effect would in situ *Campylobacter* cell aggregation have on the accuracy and reproducibility of enumerations, and is there any remedy available to address this issue?

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

6. Occasionally, nonthermophilic *Campylobacter* species cause human illness. It is unclear whether livestock and poultry are reservoirs for these species and whether these pathogens are present on meat and poultry products following slaughter and processing. Current methodologies include use of selective agents and incubation conditions that may reduce the detection of these pathogens. If a pilot study was conducted to ascertain the presence of these *Campylobacter* species on meat and poultry products, what methodologies would be most effective for detecting these species?

The Committee recommended that the 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*. The FSIS should examine the findings of the European Campycheck research initiative (3) and consult with other research partners in development of protocols to identify other *Campylobacter* species as part of a surveillance study. The surveillance data could inform the FSIS regarding whether, in the future, to expand species testing if other *Campylobacter* species become significant with regard to human illness associated with FSIS-regulated products. The Committee suggested 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, Calif., in that these two facilities can split and share collected rinse samples to maximize the testing performed on these samples.

V. CONCLUSIONS

Target organisms selected for a microbiological baseline study should be species causing the majority of human illness. The present design of the FSIS baseline study of *Campylobacter* species from broiler carcasses includes two target species, *C. jejuni* and *C. coli*. The FSIS should how-

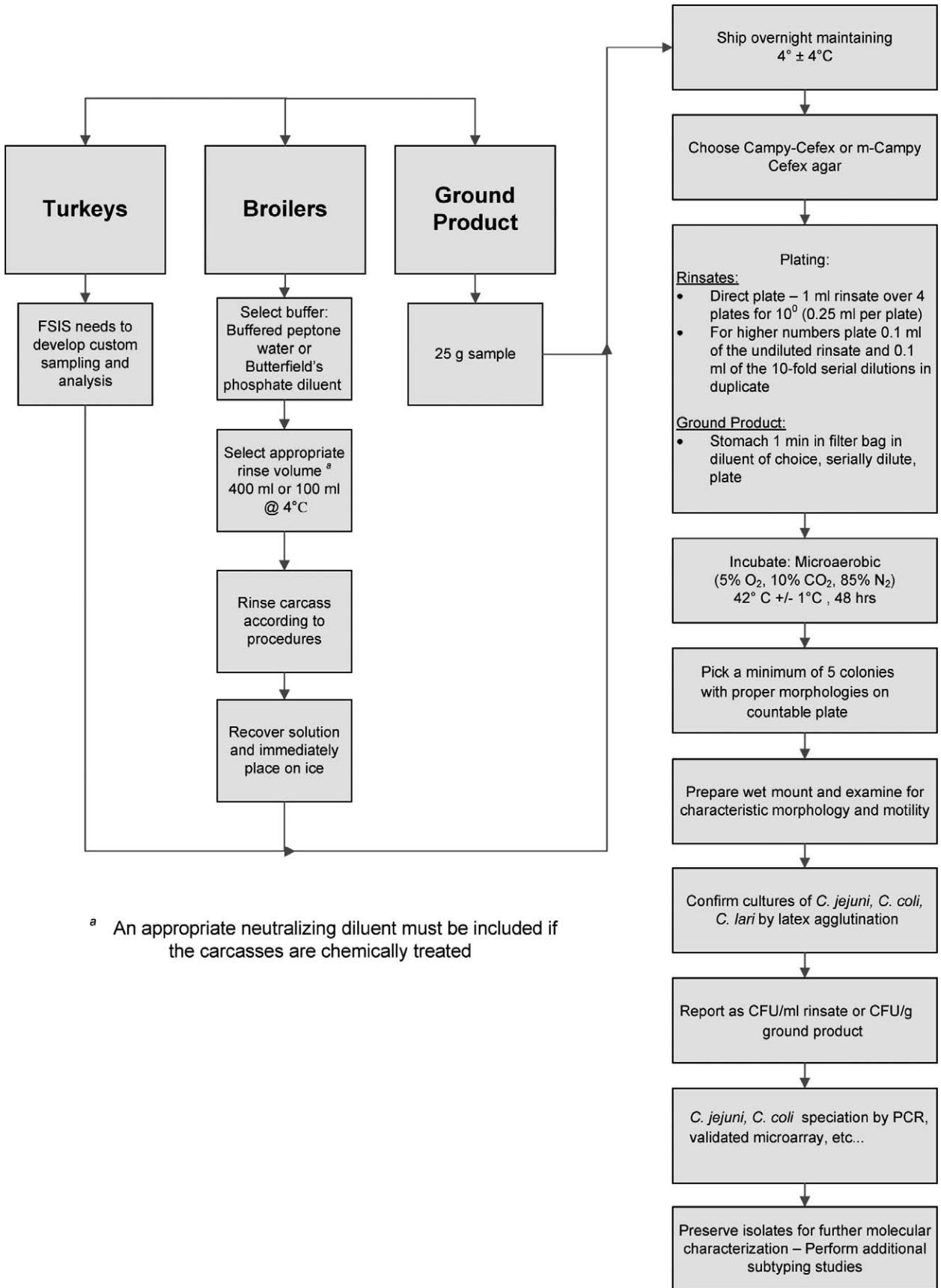


FIGURE 1. Campylobacter analysis protocol suggested by the NACMCF for FSIS baseline studies in poultry.

ever 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 study for enumeration of *Campylobacter* cells on broilers and any future baseline studies, the FSIS must clearly state the objectives and potential uses of the data. The 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, to identify interventions that the industry can use as “best practices,” to determine the overall numbers of *Campylobacter* spp. leaving establishments to ascertain if regulatory policies are successful, and/or to 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 used in the ARS/FSIS Broiler Rinse Study should be the basis for developing the upcoming baseline study, with modifications as indicated throughout this report. Figure 1 depicts the *Campylobacter* analysis protocol suggested by the NACMCF for FSIS baseline studies for poultry. This method would be widely available to industry constituents and could be easily used with high numbers of samples, which are impractical to analyze with MPN methods. It would be of paramount importance 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.

VI. ACKNOWLEDGMENTS

The NACMCF acknowledges and thanks the following *Campylobacter* research experts for sharing their expertise with the subcommittee in support of this report: Dr. J. Stanley Bailey (ARS, Athens, Ga.; ARS/FSIS Broiler Study), Dr. Eric Line (ARS, Athens, Ga.), Dr. Robert Mandrell (ARS, Albany, Calif.), and Dr. Mark Berrang (ARS, Athens, Ga.). The NACMCF acknowledges the hard work and efforts of the following individuals in the creation of this document: Drs. William Shaw, Celine Nadon, and Evelyne Mbandi.

VII. REFERENCES

- 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. *Poult. Sci.* 77:1531–1533.
- Difco Laboratories. 1998. Culture media and ingredients, dehydrated, p. 99–100. In Difco manual. Difco Laboratories, Becton Dickinson, Sparks, Md.
- European Commission. CAMPYCHECK Research Project. Available at: <http://www.campycheck.org/>. Accessed 10 April 2006.
- European Union. 2005. Draft technical specifications for an EU monitoring scheme for *Campylobacter* in broiler chickens. Unpublished report.
- Fitzgerald, C. (chf3@cdc.gov). 2005. Personal communication.
- Golden, N. (neal.golden@fsis.usda.gov). 13 September 2005. Personal communication.
- 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.
- 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.
- 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.
- Meinersmann, R. J., R. W. Phillips, K. L. Hiatt, and P. J. Cray. 2005. Differentiation of *Campylobacter* populations demonstrated by flagellin short variable region sequences. *Appl. Environ. Microbiol.* 71:6368–6374.
- Miller, W. G., S. On, G. Wang, S. Fontanoz, A. Lastovica, and R. E. Mandrell. 2005. Extended multilocus sequence typing system for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. *J. Clin. Microbiol.* 43:2315–2329.
- 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/nacpicampy.html>. Accessed 12 October 2005.
- National Advisory Committee on Microbiological Criteria for Foods. 1994. *Campylobacter jejuni/coli*. *J. Food Prot.* 57:1101–1121.
- 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 12 October 2005.
- 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 12 October 2005.
- 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 14 October 2005.
- 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 14 October 2005.
- Nordic Committee on Food Analysis. April 2005. Thermotolerant *Campylobacter*. Detection and enumeration in foods. Nordic Committee on Food Analysis, Veterinary Institute, Oslo.
- 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.
- 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.
- 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.
- Stern, N. J., and S. U. Kazmi. 1998. *Campylobacter jejuni*, p. 77. In M. P. Doyle (ed.), *Foodborne bacterial pathogens*. Marcel Dekker, New York.
- Tauxe, R. V. 2005. Human campylobacteriosis risk factors. Presented at the South East Poultry Association Annual Meeting, Atlanta, Ga., 23 February 2005.
- U.S. Department of Agriculture, Agricultural Research Service.

2005. Narms, Vetnet and Cahfse. Available at: http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=191019. 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 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/MIgchp6.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 samples overnight at $4 \pm 4^{\circ}\text{C}$ to the laboratory for analysis. Direct plate carcass rinsates to enumerate *Campylobacter*

spp. To obtain CFU per milliliter 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, Calif.), at 0.25 ml per plate. Spread each aliquot on the agar surface with a sterile plastic hockey stick, and 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% O_2 , 10% CO_2 , 85% N_2). As a group of four, designate these plates as the 10^0 CFU/ml dilution. (Note: Because these plates receive a 0.25-ml volume, the agar needs to be predried to allow it to soak up the inoculum. Dry the agar plates by leaving them on a laboratory bench at ambient temperature and humidity in the dark for 24 h prior to plating.)

To allow enumeration of high numbers of *Campylobacter* spp. per milliliter, prepare 10-fold serial dilutions of carcass rinsates in phosphate-buffered saline. Spread the undiluted 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% O_2 , 10% CO_2 , 85% N_2).

Following incubation, count colonies characteristic of *Campylobacter* spp. Calculate the number of *Campylobacter* CFU per milliliter of 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 with phase contrast microscopy. Further confirm each colony type as *C. jejuni*, *C. coli*, or *C. lari* with a latex agglutination test kit (Med-Ox Diagnostics, Inc., Ogdensburg, N.Y.).