United States Department of Agriculture
Food Safety and Inspection Service (FSIS), Office of Public Health Science (OPHS)

Detection and enumeration method for *Campylobacter jejuni/coli* from poultry rinses and sponge samples

Introduction

This direct plating procedure is a rapid and effective alternative to the MPN method for the enumeration of *Campylobacter jejuni, Campylobacter coli* and other possible *Campylobacter* spp. The procedure was developed by the Agricultural Research Service, USDA and recommended by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) (1). This guidance protocol is based on methodology used for the current FSIS Young Chicken Baseline Study (YCBS) and Young Turkey Baseline Study (YTBS) for whole chicken rinses and turkey sponge samples.

Campylobacter spp. are microaerophilic and are vulnerable to environmental stresses such as exposure to air, drying, low pH, and prolonged storage, all of which can impact their survival. Use of oxygen-quenching agents, a microaerobic atmosphere, and antibiotics that suppress competitors, significantly improve *Campylobacter* recovery. Furthermore *Campylobacter* spp are very sensitive to freezing and can die off at room temperature as well. Sample analysis should be initiated as soon as possible after receipt in the laboratory. Only samples received within the temperature range of 0-15°C should be analyzed. Upon receipt in the lab, 1 ml of the sample is dispensed across 4 Campy-Cefex plates (0.25ml/plate) and 0.1 ml is dispensed onto each of 2 additional plates (duplicates). These plates are incubated in a microaerobic atmosphere at 42°C. *Campylobacter* colonies are enumerated after 48 hours of incubation.

An enrichment step with Blood-Free Bolton's enrichment broth, (BF-BEB) may be used to supplement the direct plating for increased sensitivity of qualitative detection for low levels of potentially injured cells. After BF-BEB is incubated at 42°C under microaerobic conditions for 24-48 h, the broth is streaked onto Campy-Cefex agar for isolation of *Campylobacter* colonies. The plates are incubated as above and examined for typical *Campylobacter* growth. Typical colonies are confirmed by microscopy and serology.

Although the numerical values given in this method may be expressed as exact values, such as those given for weight, volume, pH, time and temperature to achieve optimum results, it should be clearly understood that an acceptable range exists within which the optimum results can be expected. The following allowable ranges for the given parameters are considered to be acceptable and applicable:

- Weight and volume measures: ± 1% (± 5% for serological pipettes and graduated cylinders)
- pH: ± 0.2 units
- Time: hours ± 2 hour; minutes ± 1%
- Temperature: ± 1°C

The microbial density of air in the working area should be monitored, measured in fallout pour plates taken during plating. It should not exceed 15 colonies/plate during a 15 minute exposure period.

Facility, Equipment and Materials

1. Work area, level table with ample surface in room that is clean, well-lighted, well-ventilated, and reasonably free of dust and drafts.
2. Storage space, free of dust and insects and adequate for protection of equipment and supplies.
3. Petri dishes, glass or plastic (15 x 100 mm)
4. Rainin P100-P200 and P1000, or equivalent, microliter pipette
5. Equipment and materials for ensuring proper atmospheric conditions for *Campylobacter* growth; options include:
   - 42 ± 1.0°C Tri-gas incubator (static) charged with 5% O_2, 10% CO_2, and 85% N_2 (Contact incubator manufacturer to assist you with the initial setup, including an alarm system for improper gas mixture).
   - Gas cylinders containing appropriate gas mixtures (certified by the supplier) to achieve 5% O_2, 10% CO_2, and 85% N_2 with regulators compatible with Compressed Gas Association (CGA) connection on the cylinder.
   - Commercially available gas packs intended for *Campylobacter* testing.
   - Bags or other containers capable of maintaining the atmosphere during incubation.
6. Glass slides with cover slips for wet mount preparations
7. Sterile culture tubes, 16 x 150 mm
8. Sterile bent glass rods, flexible plastic hockey sticks, or equivalent
9. Vortex Mixer
10. Autoclave bags, ~ 24" x 36"
11. Microscope (phase contrast capability recommended)
12. DrySpot *Campylobacter* test-Oxoid LTD., Basingstoke, UK, INDX Campy (jcl) [Integrated Diagnostics, Inc., Baltimore, MD), or equivalent validated procedure.
13. Refrigerator, to maintain samples at 2-8°C.
14. Vented, t-75cm^2 tissue culture flasks for incubating BF-BEB under microaerophilic conditions.
15. Thermometers, appropriate range, accuracy checked with a thermometer certified by NIST (National Institute of Standards and Technology).

Media and Reagents:

1. Buffered Peptone Water (BPW)

2. Campy-Cefex agar- Appendix I
3. BF-BEB (2X)- Appendix I

Prior Preparations for *Campylobacter* Method Implementation

Drying plating media:
Drying Campy-Cefex plates is critical for preventing spreading of colonies. Care should be taken to ensure that agar media are dried appropriately prior to use. Plates should be dried in an area that minimizes light exposure because light can possibly affect the growth and selection of *Campylobacter*.

Atmospheric requirements: *Campylobacter* spp. is microaerophilic and, in order to grow requires an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Add 4 drops of a humectant such as glycerol to a filter paper and place it in the bag or jar to diminish possible confluent and swarming growth of *Campylobacter*.

Use of control cultures: After media preparation, a positive control culture is streaked onto Campy-Cefex agar to determine whether the batch of medium supports growth and typical colonial morphology (media control). In addition, a positive control culture (such as *C. jejuni* ATCC 49943 or 29428) and an uninoculated media control should be analyzed concurrently with each group of daily samples to document proper performance of the media during the course of sample analysis.

Procedure

Sanitation
All instruments must be sterilized in a manner consistent with the specific instrument to render the instruments free of viable organisms. All work surfaces must be cleaned with appropriate disinfectants both prior to and after sample collection, to include sanitizing between samples. The outside surfaces of sample containers must be carefully disinfected prior to sampling being careful not to introduce disinfectants to the sample. Prior to opening containers, aseptically remove residual disinfectant. Bare hands should never touch the sample and the interior of the sample container.

Sample preparation, plating and enrichment
Chicken rinsate sample:
Under the FSIS baseline-sampling program, a chicken carcass is rinsed with 400 ml of Buffered Peptone Water (BPW). For qualitative testing, a 30 ml test portion of this 400 ml is enriched as described below with 30 ml of 2X BF-BEB. For quantitative testing, 1 ml of the 400 ml is distributed across four plates to represent this 10^0 dilution, and subsequent dilutions are prepared as described below.

Turkey sponge sample:
Under the FSIS baseline-sampling program a single sponge is used to sample 50cm^2 back and 50cm^2 thigh representing 100cm^2 on one turkey carcass. The sponge sample is hydrated with BPW to a total volume of 25 ml. For quantitative testing, 1 ml of the 25 ml is distributed across four plates to represent this 10^0 dilution, and subsequent dilutions are prepared as described below. For qualitative testing, the entire remaining sponge and diluent is enriched as described below in an equivalent volume of 2X BF-BEB.

To ensure even distribution, mix the test samples thoroughly by gentle shaking of the collected carcass rinsate for ~1 minute or by squeezing the sponge several times before taking the analytical unit from the sample.

Aseptically dispense 250 ± 10 µl of the sample using a Rainin P1000 or equivalent microliter pipette onto each of four Campy-Cefex plates representing 1 ml sample or 10^0 dilution of the sample. Dispense 100 ± 4 µl sample aliquot onto each of two Campy-Cefex plates representing 0.1 ml or 10^{-1} dilution. Prepare a 1:10 dilution using 1000 ± 40 µl in 9.0 mL of BPW. Using the P100 pipette with a fresh tip, dispense 100 ± 4 µl of the 1:10 dilution of the sample onto each of two Campy-Cefex plates. These are duplicate plates representing 0.01 ml or 10^{-2} dilution of the sample. Similarly any additional dilutions (10^{-3}, 10^{-4}, etcetera) are dispensed using a fresh tip on duplicate plates. Beginning with the highest diluted plates and working back through the more concentrated dilutions, use a single sterile hockey stick, or equivalent, to spread the inoculum evenly over the entire surface of the agar avoiding contact with the plate.

Incubate both enrichment broth and plates at 42°C for 48 hours using a tri-gas incubator flushed with 5% O_2, 10% CO_2, and 85% N_2, or equivalent.

Plate Examination:
After incubation, examine all plates to determine the relative proportion of various suspect colony types that are present in the sample. Often there is a single colony type, but there may be multiple colony types. Suspect colonies are translucent and wet, flat or slightly raised, and may vary significantly in size.

Selecting Dilution for Confirmatory Testing

1. When suspected colonies are found the following criteria are used to select the appropriate dilution for colony confirmation. Pick five colonies (if available) proportionally representative of all suspect colony types from one or more plates for confirmation: Pick from the dilution that averages 15-150 suspect colony forming units (CFU) per plate.
2. If there are no dilutions that average 15-150 suspect CFU per plate, pick from the dilution that averages < 15 CFU per plate.
3. If there are no dilutions meeting the above criteria (*i.e.*, counts exceed 150 CFU) select well isolated colonies from the dilution that contain counts up to 300 CFU.
4. If all dilutions are > 300 CFU then select well isolated colonies from the highest dilution.

If there are mixed confirmation results among the colonies of one perceived colony type (i.e., the colonies look the same but some confirm and others do not) pick several more colonies for a total of 10 colonies representing that type.

If the last dilution has an average of > 50 suspect colonies per plate, pick 10% of the average number of colonies up to a maximum of 10 colonies from that dilution for confirmatory testing.

Confirmation

For any suspect or typical growth perform the following confirmatory steps:

1. Microscopy: Touch a portion of the suspect colony and suspend in a drop of sterile 0.85% saline on a microscope slide. Cover with a glass cover slip and examine using oil immersion phase contrast microscopy. Suspensions demonstrating typical *Campylobacter* corkscrew morphology and darting motility are regarded as presumptively positive.
2. Latex agglutination immunoassay: Each presumptively positive colony is confirmed by testing an additional portion of the colony using the DrySpot *Campylobacter* Test (Oxoid Ltd., Basingstoke, UK) or INDX Campy (jcl) [Integrated Diagnostics, Inc., Baltimore, MD), or equivalent validated procedure.

Calculating:

Count all colonies representing types that have been confirmed above on all plates for each dilution with an average count of 15-150 CFU. Perform additional counts for plates that are not in the countable range by following the criteria listed below:

1. If there are plates with > 150 colonies in the same dilution with plates < 150, count plates with up to 300 CFU.
2. If plates with > 150 colonies represent the final dilution where counts from all other dilutions are > 300 CFU, count plates with up to 300 CFU.
3. If all plates and all dilutions appear to be > 300 CFU, estimate the count of the final dilution using the "4 blocks" method on a colony counter (*i.e.*, average count from 4 blocks multiplied by total number of blocks per plate).

Computing and Recording Counts:

1. If only one dilution averages 15-150 colonies per plate, the CFU/ml will be the mean of all plates of that dilution multiplied by the dilution factor or the sum of the 4 plates from the 10^0 dilution whichever applies.
2. If two separate dilutions average 15-150 colonies per plate the CFU/ml will be determined by calculating the average for each dilution as above, then the CFU/ml for each relevant dilution will be averaged together.
3. For the plates representing the final dilution, count up to 300 colonies/plate for an estimated CFU/ml.
4. If the final dilution is > 300 CFU, it may be necessary to report "TNTC" (Too Numerous To Count). Future analyses should employ additional dilutions to prevent TNTC results.

If all colonies of specific morphology were confirmed, 100% of colonies with that morphology are included in the count. If there are mixed confirmation results among

colonies representing one specific colony type and 10 colonies representing that type were picked for confirmation the total count for that colony type must be multiplied by the percentage of colonies of that type that confirmed.

Qualitative Enrichment for Post-Chill Rinsates:
Qualitative assessment for *Campylobacter* spp. is reported as either positive or negative based on the presence or absence of confirmed *Campylobacter* spp., respectively.

Appendix I

Campylobacter Media

NOTE: The instructions below apply to media preparation situations where individual ingredients are used. These media are also available from commercial sources; in these circumstances, follow the manufacturer's instructions for media preparation and storage.

1) Campy-Cefex Agar

Note: Considering the data of Oyarzabal *et al*. (2) and NACMCF recommendations (1) to employ a cost-effective modified Campy-Cefex formulation, the USDA-ARS semi-modified formulation of Campy-Cefex specified below is both cost-effective and proven.

Ingredient	**Amount**
1. Brucella agar	43 g/L
2. Ferrous sulfate	0.5 g/L
3. Sodium bisulfite	0.2 g/L
4. Sodium pyruvate	0.5 g/L
5. Deionized (DI) water	950 ml
Supplements	**Amount**
Lysed horse blood	50 ml/L
Cefoperazone	33 mg/L
Cycloheximide	0.2 g/L

Directions: Mix ingredients 1, 2, 3, 4 and 5 in a large flask and heat to boiling. Autoclave for 15 minutes at 121ºC and cool it to 50ºC. Add supplements after cooling to 50ºC. Dispense 20-23 ml per plate. Final pH = 6.8-7.2. Store and dry as mentioned above in the 'Prior preparations for *Campylobacter* method implementation' section.

2) Blood-free Bolton's enrichment broth (2X BF-BEB)

Ingredient	**Amount**
1) Meat peptone	20 g
2) Lactalbumin hydrolysate	10g
3) Yeast extract	10 g
4) Sodium chloride	10 g
5) Sodium pyruvate	1.0 g
6) α Ketoglutamic acid	2.0 g
7) Sodium metabisulfite	1.0 g
8) Sodium carbonate	1.2 g
9) Haemin	0.02 g
Distilled or DI water	950 mL
Supplement	Amount

Cefoperazone	40 mg
Vancomycin	40 mg
Trimethoprim	40 mg
Cycloheximide	50 mg

Directions: Add basal ingredients 1-8 to water for a 2X broth solution and bring to a boil to dissolve completely. Autoclave for 15 minutes at 121-124°C. Cool to 50°C. Add supplements. Each above mentioned supplement comes in a vial that is sufficient for 500ml. Therefore for 1000 ml 2X BF-BEB add four vials of each supplement. After supplement addition, medium is stable for approximately 48 hours.
Note: This 2X BF-BEB formulation is twice as concentrated as the traditional BF-BEB formulation to meet the specific needs for diluting 1:2 with a 30 ml test portion; *i.e.,* 30 ml of 2X BF-BEB plus 30 ml sample makes 60 ml of 1X BF-BEB sample enrichment for incubation, as was used for FSIS baseline testing.

Appendix II

Campylobacter Storage and Transport Media

Storage and Maintenance of *Campylobacter* Culture

1) Wang's Storage Medium

Ingredient	**Amount**
1. Brucella broth powder	28 g
2. Glycerol	200 ml
3. DI water	750 ml
Supplement	**Amount**
Lysed horse blood	5 ml/L

Directions: Add Brucella broth powder to water and bring to a boil to dissolve completely. Add 200 mL of glycerol to the homogenous mixture and mix well. Dispense 95 mL of the mixture into individual 100 mL bottles. Autoclave for 15 minutes at 121ºC and cool it to 50ºC. Add 5 mL lysed horse blood into each bottle and mix thoroughly. Dispense 1 mL Wang's storage medium into a 2 ml cryovial.
Note: Once prepared, Wang's storage medium's shelf life is 2-3 weeks at 2-8^{o}C regardless if maintained in a flask or dispensed into cryovials.

Directions for *Campylobacter* spp. cultures storage:

1. When a pure culture is obtained, streak the culture onto three to four trypticase soy agar with 5% sheep blood (SBA) plates. Place plates in a tri-gas incubator, or equivalent, to flush with gaseous mix of 5% O_2, 10% CO_2, and 85% N_2. Incubate

plates at 42 ± 1.0ºC for 24 hours. Do not streak for isolation; streak the plate whereby the culture completely covers the plate with a lawn of growth.
2. Using a sterile cotton swab, plastic loop or equivalent, dispense the entire lawn of bacteria from the three-four SBA plates into a single cryovial.
3. Vortex the mixture until bacterial cells have dispersed. This mixture will be thick due to high bacterial cell numbers in the cryovial.
4. Initially, place the cryovials in a 2-8ºC refrigerator for 20 minutes, and then transfer to the ≤ minus 70ºC freezer for permanent storage. Note: Isolates are initially placed in the refrigerator to avoid sudden shock to the ≤ minus 70ºC freezer temperature.

Directions for recovering the *Campylobacter* spp cultures from ≤ minus 70°C freezer:

1. After removing the cryovial from ≤ minus 70ºC freezer, immediately scrape a small amount of the ice crystals from the inoculum, transfer and streak to the appropriate plating media for isolation.
2. Immediately return the cryovial to the ≤ minus 70ºC freezer. Do not allow the culture to thaw.
3. These streaked plates should be placed in a tri-gas incubator or equivalent, flushed with the gaseous mixture of 5% O_2, 10% CO_2, and 85% N_2, and placed in a 42 ± 1.0ºC incubator for 24 – 48 hours.

Transport of *Campylobacter* Culture

2) <u>Wang's Transport Medium (Semisolid)</u>

Ingredient	**Amount**
1. Purified grade agar	4 g
2. Brucella broth powder	28 g
3. DI water	950 ml
Supplement	**Amount**
Lysed horse blood	5 ml/L

Directions: Add Brucella broth powder and purified grade agar to water and bring to a boil to dissolve completely. Dispense 95 mL of the mixture into individual 100 mL bottles. Autoclave for 15 minutes at 121ºC and cool it to 50ºC. Add 5 mL lysed horse blood into each bottle and mix thoroughly. Dispense 1 mL Wang's transport medium into a 2 ml cryovial.
Note: Once prepared, Wang's transport medium's shelf life is 2-3 weeks at 2-8°C regardless if maintained in a flask or dispensed into cryovials.

Directions for *Campylobacter* spp. cultures transport:

1. When preparing for shipping, streak the culture onto three to four SBA plates. Do not streak for isolation; streak the plate whereby the culture completely covers the plate. These plates should be placed in a tri-gas incubator or equivalent, flushed

with the gaseous mixture of 5% O_2, 10% CO_2, and 85% N_2, and placed in a 42 ± 1.0ºC incubator for 24 hours.

2. On the day of shipping, remove the transport medium from the 2-8^{o}C refrigerator and allow the cryovial to come to room temperature.
3. Using a sterile cotton swab, plastic loop or equivalent, dispense the entire lawn of bacteria from the three-four SBA plates into a single, appropriately labeled cryovial.
4. Ship isolates to destination with ice packs to keep cool within 24–48 hours of packing. Shipping shall comply with Department of Transportation (DOT) regulations. Typically, isolates shipped in Wang's transport medium can last seven days.
5. The receiver should immediately recover the cultures, store and freeze following the procedures for Storage and Maintenance of *Campylobacter* Culture as previously described.

References

1. National Advisory Committee on Microbiological Criteria for Foods. 2007. Analytical utility of *Campylobacter* methodologies. J. Food Prot 70: 241-250.

2. 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.