CHAPTER 6. ISOLATION, IDENTIFICATION, AND ENUMERATION OF *CAMPYLOBACTER JEJUNI/COLI* FROM MEAT AND POULTRY PRODUCTS

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6.1 Introduction

Procedures for the recovery of *Campylobacter* spp. from foods are evolving and no single method can be recommended for testing a wide variety of foods. Isolation of *Campylobacter jejuni* and *Campylobacter coli* is achieved both with and without selective broth enrichment. The procedures outlined below are among the most promising for the isolation and enumeration of these bacteria from raw/cooked meat and poultry products.

Campylobacters are sensitive to freezing and die off at room temperature. Samples intended for *Campylobacter* examination should be transported and held at 4°C. Sample analysis should begin as soon as possible since campylobacters can be overgrown by contaminating psychrotrophic bacteria. If freezing of samples cannot be avoided, cryoprotective agents should be used. Stern and Kotula, 1982, reported improved recovery of *C. jejuni* from ground beef stored frozen in 10% dimethyl sulfoxide or glycerol. Blankenship et al., 1983, found that brucella broth supplemented with 10% polyvinyl pyrrolidine was suitable for transporting frozen swab samples (from freshly processed poultry carcasses) to a central laboratory for analysis.

Campylobacters are microaerophilic and certain environmental stresses such as exposure to air, drying, low pH, and prolonged storage can be detrimental to their survival. Use of oxygen-quenching agents, a microaerobic atmosphere, and antibiotics that suppress competitors, significantly improve *Campylobacter* recovery.

6.2 Equipment, Reagents, and Media

6.21 Equipment

a. Phase-contrast microscope with 100X oil immersion objective
b. Agitating incubator(s)/water bath(s) at 37 ± 1.0°C and 42 ± 1.0°C
c. 42 ± 1.0°C incubator (static)
d. Balance, sensitivity of 0.1 g
e. Quart-size Qwik Seal® bags (Reynolds Metals Co., Richmond, VA; # RS78)
f. Anaerobic jars (vented or non-vented)

g. CampyPak Plus™ (BBL 71045)
or
Gas Generating Kits for *Campylobacter* (Oxoid BR56 for 3.0-3.5 liter jars, or BR60 for 2.5-3.0 liter jars)
h. Vacuum pump and gauge with appropriate tubing and connectors for evacuation of vented anaerobic jars
i. Gas cylinder containing a mixture of 5% O_2, 10% CO_2, and 85% N_2 with appropriate tubing and connectors for gassing vented anaerobic jars and Qwik Seal® bags
j. Regulator for gas cylinder compatible with Compressed Gas Association (CGA) connection on cylinder
k. Filter paper (for glycerol humectant and oxidase test)
l. Petri dishes (100 x 15 mm disposable)
m. Platinum or sterile plastic inoculating loops and needles
n. Microscope slides, cover slips, and immersion oil
o. 0.2 μm sterile membrane filters
p. 16 x 150 mm and 16 x 125 mm screw-cap test tubes
q. 250-ml screw-cap bottles
r. Sterile swabs or bent glass rods ("hockey sticks")
s. Sterile forceps and scissors
t. Sterile pipettes
u. Large sterile plastic bags
v. Stomacher™ 400, and Stamacher™ 400 bags
w. Centrifuge, rotor, and 250-ml sterile centrifuge bottles
x. Sterile cheesecloth-lined funnels

6.22 Reagents

a. Glycerol
b. 3% Hydrogen peroxide solution
c. Cephalothin antibiotic susceptibility discs (30 μg)
d. Nalidixic acid antibiotic susceptibility discs (30 μg)
e. Oxidase reagent (1% Tetramethyl-p-phenylenediamine dihydrochloride solution)
f. *Campylobacter* latex test kit (optional presumptive identification)

6.23 Media

a. Hunt Enrichment Broth (HEB)
b. 0.1% peptone water
c. Modified *Campylobacter* Charcoal Differential Agar (MCCDA)
d. Brucella-FBP (BFBP) Broth
e. Semisolid Brucella Glucose Medium
f. Brucella-FBP (BFBP) Agar
g. Enriched Semisolid Brucella Medium (optional)

6.3 Isolation and Enumeration

a. Place 25 g meat or swab samples into 100 ml of HEB in a Reynolds quart-size Qwik Seal® bag. Place the Qwik Seal® bag inside a Stomacher™ 400 bag for reinforcement and stomach for 2 minutes. Flatten the Qwik Seal® bag against the lab bench edge to remove as much air as possible without spilling the contents, then seal the bag, leaving a 1/2 inch opening at one end. Aseptically insert the tip-end of a sterile 10 ml pipette (or equivalent) into the bag through this opening. Be sure that the mouth-end of the pipette contains a sterile cotton filter. Connect the mouth-end of the pipette to the microaerobic Campy gas mixture (5% O_2, 10% CO_2, and 85% N_2) with sterile rubber tubing equipped with a sterile filter (a sterile filter can be made out of an autoclaved, shortened 25 ml volumetric pipette stuffed with glass wool). Slowly inflate the bag to capacity with the Campy gas mixture and continue to fill until excess gas flows from the bag. Then allow a small amount of gas to escape to provide for expansion, before securing the remainder of the seal. Proceed to step d.

b. Place a raw whole chicken carcass or meat pieces (up to 3 lb) in a large sterile plastic bag such as a Stomacher™ 3500 bag, and add 200 ml 0.1% peptone water. Twist bag to seal and shake contents for 2 minutes. Tilt the bag and hold back the meat pieces, allowing the rinse liquid to flow to one corner. Sanitize bag corner with 1000 ppm hypochlorite solution or 70% ethanol, then rinse in sterile distilled water. Aseptically cut the corner of the bag and pour the rinse through a sterile cheesecloth-lined funnel into a sterile 250 ml centrifuge bottle. Centrifuge at 16,000 x g for 15 minutes. Discard the supernatant and suspend the pellet in 10 ml 0.1% peptone water. For detection, inoculate 1 ml of rinse concentrate into 100 ml HEB in a Qwik Seal® bag. Then follow gassing steps as outlined, beginning with the third sentence of step a. above.

c. If enumeration is desired, prepare a three tube MPN series using HEB. Choose test dilutions and HEB volumes based on the expected numbers of campylobacters in the meat species being tested. For example, for poultry rinse samples (prior to centrifuging) begin by adding three 10 ml portions of the rinse to three 90 ml bottles of HEB. (Alternatively, Qwik Seal® bags may be used here [see step a. above]). Then add 1 ml portions of the rinse to each of three 9 ml tubes of HEB. Prepare serial dilutions of the rinse in 0.1% peptone water. Prepare subsequent MPN tubes by transferring 1 ml portions of the

decimal dilutions into 9 ml tubes of HEB in triplicate. Place all bottles and tubes in anaerobic jars. See step g. for jar gassing methods. Follow incubation steps beginning with step d. below. Use tubes or bottles found to contain confirmed *Campylobacter* to calculate MPN (refer to appropriate tables).

d. Incubate gassed Qwik Seal® bags or anaerobic jars containing bottles or tubes at 37 ± 1.0°C, shaking at 100 rpm for 4 h.

e. After the 4 h incubation at 37 ± 1.0°C, aseptically add additional sterile cefoperazone solution to bring the final concentration in each enrichment vessel to 30 mg/L. Reestablish the microaerobic atmosphere and increase the temperature to 42 ± 1.0°C. Continue the incubation for 20 h shaking at 100 rpm.

f. Swab/streak enrichments directly and at a 1:100 dilution onto MCCDA plates (for cooked products, a 1:50 dilution may be plated). Prepare the dilution by swirling a swab in the broth and twisting it against the side of the vessel to remove excess liquid. Break off the swab tip into a tube containing 9.9 ml of 0.1% peptone water and vortex. Inoculate the plates by placing a swab into the enrichment or dilution and removing excess liquid as above. Swab approximately 40% of the MCCDA plate, then streak from the swabbed area to yield isolated colonies. Alternatively, 0.1 ml portions of the enrichments or dilutions may be plated by spreading with a sterile bent glass rod. This plating technique may be used provided isolated colonies result.

g. Incubate the MCCDA plates at 42 ± 1.0°C for 24 h in an anaerobic jar under microaerobic conditions. Add about 4 drops of a humectant such as glycerol to a filter paper and place it in the jar to diminish typical confluent and swarming growth of *Campylobacter*. If no growth is achieved after 24 h, reincubate the plates for an additional 24 to 48 h to attempt recovery. The microaerobic conditions can be achieved in the jar by either of the following methods:

   i. Evacuate the air from a vented anaerobic jar to a partial vacuum of 20 inches of Hg and fill the jar with a gas mixture of 5% O_2, 10% CO_2, and 85% N_2. Repeat the evacuation-replacement procedure a total of three times to assure proper atmospheric conditions.

ii. CampyPak Plus™ (BBL) or Gas Generating Kits for *Campylobacter* (Oxoid). Follow the manufacturer's instructions on use and disposal of the kit materials. Keep jars away from flames when opening.

NOTE: Gas generator envelopes should be used if non-vented anaerobic jars are the only type available. Evacuation-replacement gassing of vented anaerobic jars is very economical.

To facilitate lid removal from a vented anaerobic jar, first release pressure by opening clamped tubing on port or by depressing the valve stem.

6.4 Identification of *Campylobacter*

Campylobacter colonies on MCCDA are smooth, shiny, and convex with a defined edge, or flat, transparent or translucent, and spreading with an irregular edge; colorless to grayish or light cream; and usually 1 to 2 mm in diameter but may be pinpoint to several mm in diameter. Plates of *Campylobacter* colonies may be stored up to 48 h refrigerated under microaerobic conditions if isolates cannot be picked immediately.

Use a platinum or plastic needle to pick three suspect *Campylobacter* colonies for each sample from the MCCDA plates and transfer each to 10 ml of brucella-FBP (BFBP) broth. Since campylobacters can vary greatly in colonial morphology, it is advisable to similarly culture at least one or all colony types present on the plates to assure the target is not overlooked. Alternatively, direct screening of colonies by phase-contrast microscopy can be done prior to picking isolates. To culture isolates, incubate the BFBP tubes with caps loosened for 24 to 48 h at 42 ± 1.0°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Do not vortex culture tubes of *Campylobacter*, this will introduce oxygen into the media.

Perform the following identification tests on each BFBP broth culture:

a. Examine a wet-mount preparation of the BFBP broth culture with a phase-contrast microscope using a 100X oil immersion objective. Young cells of *Campylobacter* appear as narrow curved rods (0.2 to 0.8 μm wide by 1.5 to 5 μm long). The organisms show rapid movement with darting or corkscrew-like motility. Pairs of cells can resemble the silhouette of a gull's wing span or the letter S. Longer chains can appear helically curved, and multispiralled filamentous elongated forms may exist. Cells grown for more than 72 h may become non-culturable and coccoid. Campylobacters are Gram negative, but Gram staining may

be omitted since cell morphology and motility are more significant in the identification of these organisms. (Carbol fuchsin [0.5%] is used instead of safranin as a counter stain to improve Gram stain results.) Continue confirmation of those BFBP cultures that exhibit typical *Campylobacter* morphology.

b. Inoculate the top 10 mm layer of a tube of semisolid brucella glucose medium with several drops of the above BFBP broth culture. Incubate tubes with caps loosened in an anaerobic jar under microaerobic conditions at 42 ± 1.0°C for 1 to 3 days.

   i. Glucose fermentation test: Campylobacters are nonfermentative, so the color of the medium will remain red-orange. A positive reaction shows a yellow color (acid with phenol red indicator) in the semisolid brucella glucose medium.

   ii. Catalase test: After reading the results of the glucose fermentation test, add 1 ml of 3% hydrogen peroxide to the semisolid brucella glucose medium culture, let sit for two to three minutes, then gently invert the tube to distribute the reagent. Examine after 1 to 10 minutes for formation of bubbles, indicating a positive reaction. *C. jejuni* and *C. coli* are catalase positive.

c. Add about six drops of the BFBP broth culture to a BFBP agar plate, and spread the inoculum over the surface with a sterile swab or a bent glass rod. Aseptically place a disc of nalidixic acid (30 μg) and a disc of cephalothin (30 μg) on each plate. Press each disc with sterile forceps to adhere it to the agar surface. Incubate the plates in an anaerobic jar at 42 ± 1.0°C for 1 to 3 days in a microaerobic atmosphere.

   i. Susceptibility to nalidixic acid and cephalothin: Observe the growth patterns surrounding the antibiotic impregnated discs. *C. jejuni* and *C. coli* are sensitive to nalidixic acid, and a clear zone of inhibition will exist around the disc. A zone of any size indicates sensitivity. The organisms are both resistant to cephalothin, so growth will be present right up to the disc. Lawns of *Campylobacter* growth may be very light and can be difficult to see, so it is helpful to tilt the plate at an angle under a light for viewing.

   ii. Oxidase test: Place a 2 cm square piece of filter

paper in an empty petri dish and add 1 to 2 drops of oxidase reagent to the paper. Heavily smear cells from the above BFBP agar plate onto the reagent-impregnated paper in a spot 3 to 5 mm in diameter using a platinum or plastic loop. The test is positive if the cell mass turns dark purple within 30 seconds. Alternatively, the Difco DrySlide™ oxidase test may be used. Campylobacters are oxidase positive.

d. Optional tests

Other biochemical tests useful for differentiation of catalase-positive campylobacters include nitrate and nitrite reduction, H_2S production, growth in 1% glycine, growth in 3.5% NaCl, and growth at 25, 30.5, 37, and 42°C. *C. jejuni/coli* grow well at 42°C and are curved or S-shaped with darting, corkscrew-like motility. Biochemically, they are catalase positive, oxidase positive, nonfermentative, nalidixic acid sensitive, and cephalothin resistant. Distinguishing between *C. jejuni* and *C. coli* is usually not necessary in a food microbiology laboratory since both are causes of human campylobacteriosis. The few existing tests to separate these species are not dependable. Hippurate hydrolysis appears to be the most reliable and useful test for this purpose. A convenient rapid disk method is available (Cacho et al., 1989). *C. jejuni* is positive for this test, while *C. coli* yields a negative reaction.

6.5 Multiple Start Days

Analysis should begin on a Monday, Tuesday, Wednesday, or Thursday to avoid weekend work. Samples received on a Friday should be analyzed immediately or begun on Saturday; starting either day will require weekend work. Follow the table below according to the day analysis is to begin.

	Analysis To Be Done On Days				
Starting Date	Enrichment	Plating	Pick Colonies	Inoculate Biochemicals	Read/ Perform Tests
MON	MON	TUE	WED	THU	FRI
TUE	TUE	WED	THU	FRI	MON
WED	WED	THU	FRI	MON	WED
THU	THU	FRI	MON	TUE	THU
FRI	FRI	SAT	MON	TUE	THU
SAT	SAT	SUN	MON	TUE	THU

6.6 Storage and Transport of Stock Cultures

Inoculate overnight BFBP broth cultures into tubes of Brucella broth with 0.15% agar. Loosen the screw-caps and incubate for 24 to 48 h at 42 ± 1.0°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Store refrigerated under this atmosphere for up to a month without serial passage. Cultures in this medium can be transported by mail. Seal tightened caps with adhesive tape to prevent leakage during shipment.

Cultures grown in enriched semisolid brucella medium may be stored under atmospheric conditions at room temperature with caps tightened, for at least three weeks. This medium is also suitable for transporting cultures by mail.

Cultures may also be preserved frozen. To prepare these stocks, swab 6 drops of a 24 h BFBP broth culture onto a BFBP agar plate and incubate microaerobically at 42 ± 1.0°C for 24 to 48 h. Then remove the plate growth with a swab and suspend the cells in 4 ml of Brucella broth with 15% sterile glycerol. The suspension can be stored frozen at -70°C in 1 ml portions for 6 months or longer. Thawing and refreezing these stocks will usually result in loss of viability.

6.7 Media Quality Control

Pay strict attention when preparing all media to assure proper supplement additions. Ingredients, reagents, and media that are past expiration date should be discarded. It is important to discard all unused liquid media more than one month old and all plating media more than two weeks old, since absorbed oxygen will generate peroxides which can be detrimental to campylobacters. Store all media refrigerated, tightly sealed, and shielded from light.

Inoculated media controls should be incubated with each batch of tests to assure proper media formulation and atmospheric conditions. When enriching, include a Qwik Seal® bag of HEB inoculated with an actively growing BFBP broth culture of *C. jejuni* as a control. Similarly, in each anaerobic jar, include an appropriate agar plate or broth inoculated with a known *C. jejuni* strain. Use of positive and negative controls for all biochemical tests is also recommended. An uninoculated control of all test media should also be included to allow assessment of sterility and any changes that may occur in the medium.

Listed below are some recommended controls for the *Campylobacter* biochemical tests:

a. Glucose fermentation test:
   Inoculate a semisolid brucella glucose tube with an *Escherichia coli* strain and incubate aerobically to generate a positive reaction. Inoculate a *C. jejuni* strain and incubate microaerobically to yield a negative reaction.

b. Catalase test:
   Use a *C. jejuni* strain as a positive control and a *Streptococcus* spp. as a negative control.

c. Susceptibility to nalidixic acid and cephalothin:
   Use a *C. jejuni* strain to demonstrate the desired sensitive/resistant pattern.

d. Oxidase Test:
   Use a *C. jejuni* strain as a positive control and an *E. coli* strain as a negative control.

6.8 Selected References

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