

## Research Note

# Use of Cellulose Filters To Isolate *Campylobacter* spp. from Naturally Contaminated Retail Broiler Meat

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MS 09-212: Received 9 May 2009/Accepted 31 July 2009

### ABSTRACT

Membrane filtration has been used to isolate *Campylobacter* spp. from feces, although ~5 log CFU/g must be present in the sample. Few studies have attempted to use filter membranes for the isolation of *Campylobacter* from foods. We investigated the minimum number of thermotolerant *Campylobacter* cells that pass through cellulose filters, the effect of different cell conditions on the rate of passage, and the minimum number of cells that could pass the filters from enriched broiler meat naturally contaminated with *Campylobacter* spp. Cellulose filters with 0.65- $\mu$ m pore sizes retained fewer cells and were more effective than filters with 0.45- $\mu$ m pore sizes. Scanning electron microscopy revealed that 15 min of contact of the filters with agar plates allowed for the passage of most bacteria. The minimum number of bacteria required to pass through the filters was contingent on cell conditions; nonmotile cells were retained more than motile cells ( $P < 0.05$ ). The minimum number of motile bacteria from 24-h cultures and centrifuged cells were 2.2 and 2.1 log CFU, respectively, while the number of coccoid and nonmotile (*flaA/B*<sup>-</sup> mutant) cells were 4.1 and 3.4 log CFU, respectively. Broiler meat samples enriched in Bolton's broth supplemented with 5% lysed blood showed that approximately 1.7 log CFU of *Campylobacter* can be filtered to pure colonies on agar plates. These results demonstrate that the motility of the bacteria influences passage through cellulose filters and that 0.65- $\mu$ m-pore-size filters on agar plates help obtain pure *Campylobacter* colonies from enriched food samples.

The method of choice to isolate *Campylobacter* from contaminated food samples is the combination of enrichment broth with selective plating or direct plating on selective agars. However, due to the slow growth of *Campylobacter* spp., many isolates are lost to competition by contaminant bacteria naturally present in foods. The filtration method was first used to isolate *Campylobacter fetus* (formerly *Vibrio fetus*) from bulls (11) and has been used in direct isolation of *Campylobacter* spp. from human stools on agar plates (12); filters are applied directly on the surface of nonselective agar plates, and fecal samples from patients with diarrhea are applied on top of the filters (7, 12). The membrane filtration technique has allowed for more successful recovery of *Campylobacter* isolates from clinical samples. Cellulose nitrate, cellulose triacetate, or cellulose acetate filters of 0.45- $\mu$ m or 0.65- $\mu$ m pore sizes have been used to isolate *Campylobacter* spp. from fecal samples (2, 8, 12, 15). Filters with pore sizes of 0.45  $\mu$ m have been shown to result in less contamination than filters with pore sizes of 0.65  $\mu$ m. However, *Campylobacter* numbers of fewer than 5 log CFU/g of feces could not be detected by 0.45- $\mu$ m-pore-size filters (5).

The filter technique has also been used to isolate *Campylobacter* spp. from food samples (1). An isolation procedure that uses hydrophobic grid membrane filters applied on semisolid media takes advantage of the differential motility of *C. jejuni* and *C. coli* in order to isolate these bacteria from chicken and turkey samples (13). Studies indicate that a large number of bacterial cells are required for *Campylobacter* detection and that cell motility is essential for the passage of the cells through the filters (4, 12, 15). Capillary action has also been suggested as a way in which *Campylobacter* cells pass through cellulose filters (3). Yet, the parameters that influence the rate of passage of *Campylobacter* through filters have not been systematically addressed.

The aim of the present study was to determine the parameters that influence the efficient use of cellulose filters to isolate *Campylobacter* from retail broiler meat and to isolate *Campylobacter* spp. from contaminated cultures. We tested cellulose filters because they are the most commonly cited filters for the isolation of *Campylobacter* species in the literature (2, 8, 12, 15). Preliminary experiments evaluated the rate of passage of cells through filters with 0.45- and 0.65- $\mu$ m pore sizes by the visualization of scanning electron micrographs. The influence of the age of the culture, the motility of the bacterial cells, and the minimum number of bacteria required for detection were studied by the direct application of filters on selective agar plates. Finally, we

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studied the impact of competing bacteria present in naturally contaminated enriched meat samples.

## MATERIALS AND METHODS

**Strain and growth conditions.** *C. jejuni* ATCC 35918 and a nonflagellated *C. jejuni* *flaA/B*<sup>-</sup> mutant (strain 1543) were recovered from -80°C stock cultures and grown on modified Campy-Cefex (mCC) supplemented with 5% sterile, lysed horse blood (10). Cultures were incubated at 42°C under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>) generated using a MACSmics Jar Gassing System (Microbiology International, Frederick, MD) for 24 h.

**Filters.** Initial experiments included 0.45- and 0.65- $\mu$ m-pore-size filters from GE Water & Process Technologies (no. E06WP04700, Fisher Scientific, Trevose, PA), Millipore Corporation (no. DAWP04700, Fisher Scientific, Billerica, MA), and Whatman (no. 10-401-512, Fisher Scientific, Dassel, Germany). However, filters with 0.65- $\mu$ m pore diameter from Millipore were more readily available, had the lowest cost, and were therefore used throughout the experiments.

**Enrichment of retail broiler samples.** Broiler retail samples (boneless breast meat) were bought from local retail stores and were enriched by stomaching 25 g of chicken meat with 100 ml of Bolton broth supplemented with 5% sterile, lysed horse blood in Whirl-Pak bags (Nasco, Fort Atkinson, WI) (9). Samples were incubated at 42°C under microaerobic conditions for 48 h. Broiler meat samples were screened for *Campylobacter* in advance, and only 12 positive samples were used in these experiments. Enriched samples were transferred to mCC plates using the filters as described for spike samples. An isolate from each sample was stored at -80°C.

**Minimum number of cells and conditions that affect the passage through the filter.** *Campylobacter* cultures (ATCC 35918) were grown on mCC plates under microaerobic conditions at 42°C for 24 h. The conditions evaluated included very motile, 24-h growth of *C. jejuni* ATCC 35918. For this purpose, cells were dissolved in phosphate buffer solution (PBS) to achieve an optical density at 600 nm of 0.14 to 0.15. An aliquot of this cell suspension was also used for the second treatment, in which bacterial cells were centrifuged to reduce their motility. Cells were centrifuged at 16,000  $\times$  *g* in PBS at 25°C for 10 min; the supernatant was discarded, and the pellet was resuspended in fresh PBS. This centrifugation step was repeated three times (14). Another experiment included the induction of coccoid cells (ATCC 35918) by leaving 24-h cultures on mCC at 25°C under aerobic conditions for 24 h. The fourth experiment was composed of 24-h growth of a nonflagellated *flaA/B*<sup>-</sup> mutant dissolved in PBS to an optical density at 600 nm of 0.14 to 0.15. The fifth treatment experiment included enrichment of broiler meat samples spiked with *C. jejuni* ATCC 35918. Samples were enriched at 42°C under microaerobic conditions for 48 h. Each experiment was performed in triplicate and plated in duplicate. The CFU per milliliter was recorded for the last countable spread plate and filter plate from each plating set of dilution. Only the data for the minimum number of cells was recorded from each treatment (minimum number of cells needed for detectable filter plate = logarithm of spread plate - logarithm of filter plate).

To standardize the conditions, all plates were dried in an airflow safety cabinet for 5 h prior to use. For each treatment, a 10-fold serial dilution in sterile PBS was performed and spread plated, while five 20- $\mu$ l drops (100  $\mu$ l total) were applied to the filter

surface. The filter was left in contact with the surface of the agar plate for 15 min. mCC plates were incubated at 42°C under microaerobic conditions for 48 h.

**SEM studies to calculate the rate of cell passage.** Four bacterial cell treatments were used for scanning electron microscopy (SEM): 24-h growth, centrifuged cells, coccoid cells, and cells from enriched cultures. For each treatment, filters were placed on the surface of an empty Petri dish and also directly onto the surface of several mCC agar plates. The treatment-specific inoculum was applied as five 20- $\mu$ l drops (100  $\mu$ l total) per filter. Filters inoculated with sterile PBS were used as controls. At 0, 5, 10, and 15 min, a filter was removed from a Petri dish and one from a mCC plate. These filters were fixed in osmium tetroxide vapor for 2 h. This step was repeated for each treatment. Segments of each filter were mounted onto aluminum support stubs with double-stick carbon tape and coated with gold using an EMS 550  $\times$  Auto Sputter Coating Device (Electron Microscopy Sciences, Hatfield, PA). Samples were analyzed with a Zeiss EVO 50 Variable Pressure SEM (Carl Zeiss SMT, Inc., New York) operated at 20 kV. For standardization purposes, all scanning electron micrographs shown were captured at 6.50 K magnification.

**Fluorescence confocal microscopy studies.** Fluorescence confocal microscopy was used to corroborate the passage of cells through the filters and to validate SEM findings. Cells from *C. jejuni* ATCC 35918 (24-h growth) were dissolved in PBS to achieve an optical density at 600 nm of 0.14 to 0.15. One milliliter of this cell suspension was combined with 100  $\mu$ l (0.4 to 0.5 mg of protein) of biotinylated polyclonal antibody from rabbit serum (AbD Serotec, Raleigh, NC) and 50  $\mu$ l (1/100 working stock) of streptavidin labeled with tetramethyl rhodamine isothiocyanate. The mixture was incubated for 10 min at 42°C. Ten microliters of the sample was applied to a filter and mounted on a glass slide with a cover slip. For controls, 10  $\mu$ l of the sample and a piece of sterile filter were similarly mounted. The transfer time between the preparation laboratory and the equipment laboratory was approximately 10 min. Samples were analyzed with a MRC 1024 Confocal Scanning Laser Microscope (Carl Zeiss).

**Statistical analysis.** Experiments to determine the minimum number of cells that pass through the filter were run in triplicate. CFU counts were transformed into log CFU values. The analysis of variance was done with SAS version 9.1 (SAS Institute Incorporated, Cary, NC), with separation of means using Duncan's test. Statistical differences were set at  $P < 0.05$ .

## RESULTS

In an initial comparison of three brands of filters, those from Millipore and Whatman gave consistently similar results. However, filters from GE Water & Process Technologies were inconsistent with inoculated PBS, and their use was discontinued in the experiments. Note that the filters from Whatman (ME 26), which used to be sold by Schleicher & Schuell, are the filters described in the Cape Town protocol (7).

SEM studies showed a large variation in pore sizes in 0.65- $\mu$ l cellulose filters (Fig. 1A). A 15-min contact between the inoculated filters and the agar plates was found to be sufficient to allow the majority of the bacterial cells to pass through the filter. This time was used as standard for the following experiments. No differences in the minimum

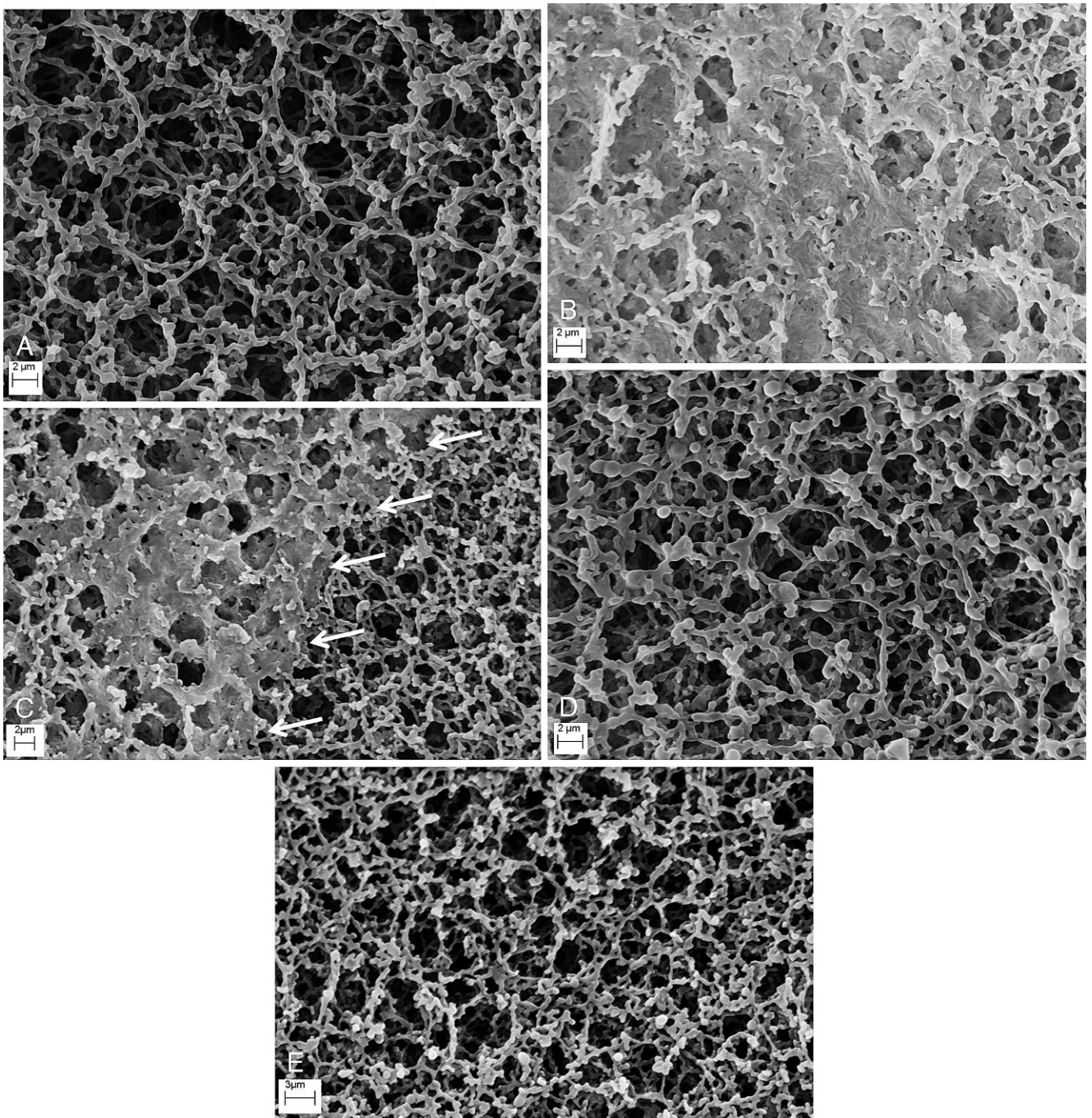


FIGURE 1. SEM images of 0.65- $\mu\text{m}$ -pore-size filters (Millipore Corporation) showing the pore clearance over 15 min. (A) Filter control, (B) *Campylobacter* cells air dried for 0 min, (C) cells air dried for 5 min, (D) cells air dried for 10 min, and (E) cells air dried for 15 min. White arrows in C show the area where the filter has the *Campylobacter* culture (left of the arrows) and where the filter is empty (right of the arrows).

number of bacterial cells needed to pass through filters were found between 15 and 20 min, and SEM micrographs showed that less than 15 min was not sufficient for passage of the bacterial cells (Fig. 1, compare B with C, D, and E).

The studies with fluorescence confocal microscopy showed fluorescently labeled *Campylobacter* cells immediately after inoculation with the filters. At 15 to 20 min after the inoculation, most of the *Campylobacter* cells had already passed through the filter (data not shown). The nitrocellulose filter used as control also yielded a small amount of background fluorescence, as noted by the manufacturer ([http://www.](http://www.millipore.com/faqs/tech1/69vtv9)

[millipore.com/faqs/tech1/69vtv9](http://www.millipore.com/faqs/tech1/69vtv9)). With this technique, we corroborated that 15 to 20 min is enough for the inoculated cells to pass through filters.

The minimum number of bacterial cells required to pass through the filters was dependent on motility. Nonmotile cells (coccoid and *flaA/B*<sup>-</sup> mutants) were retained by the filters more ( $P < 0.05$ ) than were motile wild-type bacteria (24-h and centrifuged). Surprisingly, the lowest number of cells needed to go through the filters came from enriched broiler meat naturally contaminated with *Campylobacter* cells and enriched in Bolton broth. Table 1 shows the

TABLE 1. The minimum number of *Campylobacter* cells needed to pass through the filter for detection on agar plates

Treatment	Mean log CFU/ml	± SEM <sup>a</sup>
24-h growth	2.2	±0.16 A <sup>b</sup>
Centrifuged	2.1	±0.12 A
Enriched	1.7	±0.35 A
Cocoid	4.1	±0.49 B
Mutant	3.4	±0.32 B

<sup>a</sup> SEM, standard error of the mean.

<sup>b</sup> Values in the same column not followed by the same letter are significantly different ( $P < 0.05$ ).

calculated number of bacterial cells needed to pass through the filters for each of the conditions tested.

In the studies of the enriched broiler meat samples, several components of the broth (meat, blood, etc.) collected on top of the filter, blocked the pores, and hampered the visualization of the *Campylobacter* cells by SEM (Fig. 2). Yet the rate of passage of *Campylobacter* cells was not affected by the presence of these substances. In fact, enriched broiler meat samples showed the highest sensitivity for isolation of *Campylobacter* using filters (Table 1). The enriched samples were not prone to contamination from the naturally occurring competing bacteria. After filtration, the enriched samples were pure and often appeared as isolated colonies atypical from this swarming-type bacterium.

## DISCUSSION

In our first set of experiments, we found that filters with 0.45- $\mu$ m pore size retained too many bacterial cells and had low sensitivity for efficient *Campylobacter* isolation. In general, more strains of *C. jejuni* and *C. coli* have been isolated using 0.65- $\mu$ m rather than 0.45- $\mu$ m-pore-size filters (2). To offset the limited sensitivity of 0.45- $\mu$ m-pore-size filters, a short enrichment step prior to filtration has been suggested to increase the isolation frequency in samples with low numbers of *Campylobacter* spp. (8, 15). Steele and McDermott (12) calculated that 90% of the cells are retained by filters with a pore diameter of 0.45  $\mu$ m. The large retention rate by these filters and the large amount of competing microflora may explain the low sensitivity found with these filters when trying to isolate *Campylobacter* from feces (6).

We noticed that filters placed on agar plates with high moisture content soaked in the moisture, which did not allow the inoculum placed on top of the filter to dry in less than 20 min. Also, any volume of liquid above 100  $\mu$ l took longer to go through the filter. When there was still liquid on top of the filters at the time of their removal from the agar plates, chances increased for mishandling the filters and letting unfiltered liquid end up on the surface of the agar, which could lead to contamination of the plate. Amounts of liquid larger than 200  $\mu$ l flooded the filter and spilled over the filter onto the agar. Therefore, we standardized the drying of the agar plates for 5 h in a laminar flow. We also found that 100  $\mu$ l distributed in five 20- $\mu$ l drops on top of the filter

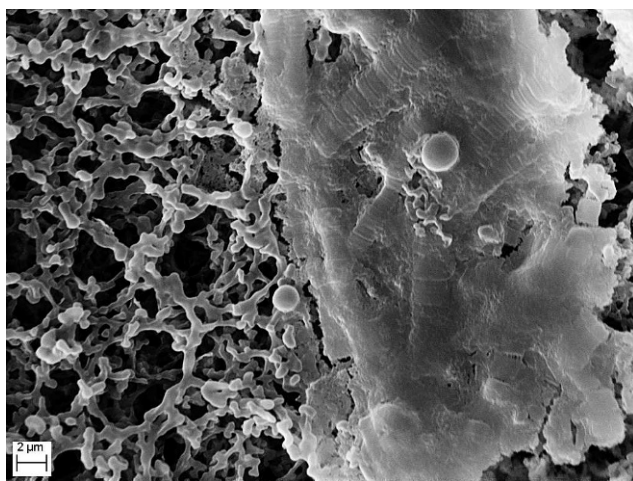


FIGURE 2. A SEM reveals the diversity of components present in an enriched food sample applied to a filter. The different substances drying on top of the filters made it difficult to observe *Campylobacter* cells.

provided a practical solution to obtain isolated *Campylobacter* colonies.

It is pertinent to mention that dried agar plates will absorb any liquid much more quickly, and that a shorter time, perhaps 10 min, would suffice for the liquid (and the cells) to pass through the filter. Yet very dry agar plates may not be conducive for *Campylobacter* growth and may result in lower sensitivity when using cellulose filters. The exact drying time for the plates is difficult to predict. In general, the storage time and the handling of the plates influence the time needed to obtain the optimal dryness of the plates. Because these parameters vary from laboratory to laboratory but can be standardized in a given environment, the best method is to establish the optimal parameters to provide for a plate that is dry enough and that can absorb the liquid from the filter within 15 min.

The centrifuged bacterial cells were less motile, the cocoid were nonmotile in more than 90% of the cells, and the mutants were spiral-shaped but entirely nonmotile. From observations under phase-contrast microscope, the centrifugation process used in our experiments decreased the activity of *Campylobacter* cells. However, they were still more active and motile than the cocoid bacteria or the nonflagellated mutants. Remarkably, we have found that naturally occurring *Campylobacter* strains in enriched broiler samples exhibited the highest rate of motility of the different groups described in our experiments. Therefore, cell motility appears to play a crucial role in the passage of *Campylobacter* through the filter, but the absence of motility does not completely hinder cell passage. These assessments are somewhat surprising, and we are continuing our research to further investigate these findings.

The spiral morphology of *Campylobacter* was difficult to distinguish from the fiber background of the filters in SEM micrographs. Despite these limitations, SEM micrographs allowed us to determine the rate of emptying of the filter pores, which was used as an indication that *Campylobacter* cells transferred from the filter surface to the agar plates. A comparison between the results from streaking mCC agar

plates and from the filtration method from enriched samples resulted in a correlation of 100%, meaning that every time a positive sample was found in plates, the sample was also positive after filtration. However, one of the main advantages of using the filtration technique was that *Campylobacter* was rarely contaminated, so the pure culture was retained and stored more successfully. Therefore, the samples were not outcompeted by naturally occurring bacteria that were retained in the filter.

In summary, we have defined some of the parameters pertaining to the use of direct plating filtration. Filters with 0.65- $\mu$ m pore diameter are the best choice when trying to isolate the highest number of *Campylobacter* from food samples. The motility of the bacteria plays a role in the sensitivity of the filter. However, a bacterial cell can be stressed or injured and still pass through the filter and grow on selective media. We also observed isolated colonies when samples were placed on dried plate media. Dry plates appear to hinder cell motility across the plate surface; therefore, isolated colonies on agar plates can be found more easily.

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