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Culture Methods to Determine the Limit of Detection and Survival in Transport Media of Campylobacter Jejuni in Human Fecal Specimens

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Corresponding Author:	Janice E Buss, Ph.D. TECHLAB Inc Blacksburg, Virginia UNITED STATES
Corresponding Author's Institution:	TECHLAB Inc
Corresponding Author E-Mail:	jbuss@techlab.com
Order of Authors:	Janice E Buss, Ph.D.
	Elizabeth Thacker
	Michelle Santiago
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TITLE:

Culture Methods to Determine the Limit of Detection and Survival in Transport Media of *Campylobacter Jejuni* in Human Fecal Specimens

AUTHORS AND AFFILIATIONS:

Janice E. Buss[1], Elizabeth Thacker[1,2], Michelle Santiago[1]

[1]TECHLAB, Inc., Blacksburg, VA, USA
[2]Current Address: Duke University School of Medicine, Raleigh-Durham, NC, USA

Corresponding Author:

Janice Buss (jbuss@techlab.com)

Email Addresses of Co-Authors:

Elizabeth Thacker (ethacker12@gmail.com)
Michelle Santiago (msantiago@techlab.com)

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SUMMARY:

Although stool culture for *Campylobacter* is imprecise, it is still considered the gold standard for identification. Methods to determine the limit of detection and survival in transport media of *C. jejuni* in human stool are described and compared with a new immunoassay with better accuracy.

ABSTRACT:

A culture from human stool for diagnosis of *Campylobacter*-based intestinal illness takes several days, a wait that taxes the fortitude of the physician and the patient. A culture is also prone to false negative results from random loss of viability during specimen handling, overgrowth of other fecal flora, and poor growth of several pathogenic *Campylobacter* species on traditional media. These problems can confound clinical decisions on patient treatment and have limited the field from answering fundamental questions on *Campylobacter* growth and infections. We describe a procedure that estimates the lower limit of bacterial numbers that can be detected by a culture and a method for quantifying survival of *C. jejuni* in media used for transport of this fragile organism. Knowing this information, it becomes possible to set clinically relevant detection thresholds for diagnostic tests and address unstudied issues of whether non-symptomatic colonization is prevalent, if co-infection with other enteric pathogens is common, or if bacterial load correlates with symptoms or serious sequelae. The study also included testing of 1,552 prospectively collected patient diarrheal fecal specimens that were initially classified by conventional culture and further tested by a new enzyme immunoassay. Positive and discrepant specimens were then screened by four molecular methods to assign true-positive or true-negative status. The 5 non-culture methods showed complete agreement on all 48 positive and discrepant specimens, while the culture mis-identified 14 (28%). The specimens that were

incorrectly identified by culture included 13 false negative and 1 false positive sample. This basic protocol can be used with multiple *Campylobacter* spp. and will allow the numbers of *Campylobacter* bacteria that produce symptoms of gastroenteritis in humans to be determined and for prevalence rates to be updated.

INTRODUCTION:

The United States Centers for Disease Control (CDC) recently published that the Foodborne Diseases Active Surveillance Network (FoodNet) surveillance program reported 9,723 cases of laboratory-diagnosed *Campylobacter* infections in 2018[1]. This represents a 12% increase in *Campylobacter* case reports over 2015−2017[1]. Worldwide, *Campylobacter* spp. are among the most common bacterial intestinal infections[2]. Nevertheless, the numbers of *Campylobacter*-based intestinal illnesses that occur each year are suspected to be underreported[3]. This underestimation is predictable because most patients can recover with only moderate discomfort and no medical treatment. However, for patients with more severe symptoms or who are at higher risk for serious disease, and who then seek medical care, stool culture is the most common method for assessing whether *Campylobacter* is the pathogen that is causing their distress[4].

For *Campylobacter* spp., stool culture is particularly troublesome. The most common pathogenic organisms, *C. jejuni, C. coli, C. upsaliensis,* and *C. lari,* are microaerophilic[5]. This means that the bacteria will die at random, unknown rates once exposed to air. The time between specimen collection and culture setup thus becomes an uncontrolled variable in the ability to detect viable *Campylobacter* spp. by culture.

For direct culture of fecal specimens, the slow growth of *Campylobacter* is also a problem. *Campylobacter* colonies are very small even after 48 h of incubation and can easily be covered by competing organisms in the fecal matrix. Plates that contain antibiotics to which most strains of *C. jejuni* and *C. coli* are resistant are widely used, as the antibiotics inhibit growth of many (but not all) competing fecal bacteria, allowing better visualization of *Campylobacter* colonies[6]. However, other *Campylobacter* species such as *C. lari* and *C. upsaliensis* are sensitive to some of these antibiotics, and either grow poorly or not at all. This contributes to the underreporting of *Campylobacter* infections from these antibiotic-sensitive species[7].

There is a third reason why a culture for *Campylobacter* may be inaccurate. The bacteria, when stressed, may remain viable but can become "non-culturable"[8]. This by definition means that the culture will not detect the bacteria present in the sample. How often this occurs is not known[8].

Given these potential issues with culture, we used multiple comparison reference methods so that faulty culture results did not make a single comparator assay appear inaccurate[9]. The culture methods used (e.g., *Campylobacter*-selective plates, transport medium, gas-generating sachets) were chosen because they are used widely in clinical laboratories for stool specimen culture[10].

The culture protocols described here were developed because the lowest number of *Campylobacter jejuni* that could be detected by culture in human stool was not known. Although

estimates have been published for numbers of colony forming units (CFU) present in poultry feces[11], these results cannot be equated to human stool, as *Campylobacter* spp. are commensals in chickens, and do not cause diarrhea. This fundamental information is needed to establish the numbers of *Campylobacter* bacteria that will produce symptoms of gastroenteritis in humans and to compare virulence between strains or species.

PROTOCOL:

1. Enumeration of *Campylobacter* in contrived human fecal specimens

NOTE: All steps are carried out using sterile technique and materials on a disposable protective sheet within a disinfected laminar flow safety hood.

CAUTION: Live *Campylobacter* are infectious and can cause illness, including diarrhea. Wear gloves, a lab coat and safety glasses whenever handling bacteria. Do not mouth pipet. Dispose of all material that has contacted bacteria in proper biohazard containers.

1.1. Growth of stock culture of bacteria

1.1.1. Obtain strains of *C. jejuni* (ATCC-33560) or *C. coli* (ATCC 33559) (**Table of Materials**) as dried or frozen cultures and rehydrate or thaw bacteria according to manufacturer's instructions. Streak the rehydrated bacteria onto a *Campylobacter*-specific agar plate to start the culture. Incubate the plate 48 h at 37 °C in an anaerobic jar containing a microaerobic atmosphere gas-generating sachet.

1.1.2. On the following day, prepare 100 mL of brain-heart infusion (BHI) growth broth containing 0.5% trypticase, 0.5% protease peptone, 0.0125% sodium pyruvate, and 0.0125% sodium bisulfite.

1.1.3. Prereduce the BHI broth by covering the flask loosely and placing it in an anaerobic jar with a sachet that will produce a microaerophilic environment. Allow broth to prereduce overnight at 37 °C. Similarly, prereduce *Campylobacter*-specific plates to be used for colony counts in steps 1.1.10 and 1.2.2.

1.1.4. As *Campylobacter* are sensitive to air, gather all materials before inoculating broth and do not dawdle while handling cultures. When ready to inoculate, add fetal bovine serum (FBS) to prereduced broth to 4% of total volume. Retain 1 mL of prereduced broth to serve as a blank in measurements of optical density at 600 nm (OD_{600}).

1.1.5. Remove 3 mL of prereduced broth containing FBS and use broth to scrape the starter plate containing the *Campylobacter* culture. Gently scrape the plate with an inoculating loop, and then transfer the bacterial slurry to a sterile tube.

1.1.6. Inoculate the 100 mL of prereduced broth with approximately 3 mL of bacterial slurry and

incubate with moderate shaking at 115 rpm at 37 °C in an anaerobic jar containing a gas-generating sachet.

1.1.7. Monitor growth of the bacteria spectrophotometrically by turbidity at OD_{600}. Use the reserved broth as a blank. If the anaerobic jar is opened, replace the gas-generating sachet.

1.1.8. Stop broth incubation after 48−72 h or before the OD_{600} value reaches ~0.4.

NOTE: This OD_{600} typically equates to 10^7 to 10^8 CFU/mL. See **Table 1** for typical results.

1.1.9. To establish number of bacteria in pure stock culture, perform eight 10-fold dilution series of 100 µL of broth in 900 µL of dilution buffer (**Table of Materials**). After the 100 µL of broth has been removed for the first dilution, return flask to anaerobic jar with fresh gas-generating sachet to await use in spiking fecal pool.

1.1.10. Use sterile plating beads to spread 100 µL of the 10^{-5} to 10^{-7} dilutions on duplicate prereduced *Campylobacter*-specific plates from step 1.1.3. Label plates with dilution used, place them in a second anaerobic jar with gas-generating sachet, and incubate at 37 °C for 48−72 h.

NOTE: See **Figure 1** and **Figure 2** for dilution scheme and photographs of colonies.

1.1.11. After growth, choose the plate with between 30−300 colonies to count. Utilize the counts to determine the CFU/mL of the stock broth culture using **Equation 1**:

CFU/mL in stock **=** Average # of colonies on chosen (duplicate) analytical plates ÷ (mL plated x dilution of plate) **[Equation 1]**

1.2. Preparation and enumeration of contrived clinical fecal specimens

1.2.1. Immediately after the plates for analytical counts are prepared in step 1.1.10, make a second set of stock broth dilutions by preparing 10 serial 2-fold dilutions from the stock broth and a *Campylobacter*-negative fecal pool (NFP). For example, prepare the first dilution by mixing equal volumes of broth and NFP (e.g., 0.1 mL each) and make subsequent dilutions by transferring a designated volume of broth and NFP mixture into a tube with an equal designated volume NFP. Add a control plate with broth containing no *Campylobacter* added to the fecal pool to help identify non-*Campylobacter* colonies.

1.2.1.1. Make the NFP from de-identified, diarrheal patient surveillance specimens or healthy donor stools that have previously been tested and found to be *Campylobacter*-negative by methods such as a *Campylobacter* enzyme immunoassay and by 16S rRNA qPCR.

1.2.2. T-streak 10 µL of each *Campylobacter*/stool dilution on duplicate prereduced *Campylobacter*-specific agar plates. Place plates in the anaerobic jar with a gas-generating sachet and incubate at 37 °C for 48 ± 2 h.

1.2.3. Examine the streaked plates visually for colonies resembling those from pure *Campylobacter* cultures.

NOTE: The third quadrant is typically where these will be found. See **Figure 1** and **Figure 2** for dilution scheme and images of colony size, color and morphology.

1.2.5. Select multiple *Campylobacter*-like colonies and Gram stain. Using microscopy with an oil immersion lens, examine a thinly streaked area for gram-negative curved, spiral, or cigar-shaped small bacteria.

NOTE: *Campylobacter* are Gram-negative and require basic fuchsin as a counterstain (instead of the typical safranin) to be visualized accurately. Classic gull-winged bacteria may be seen but are not a requirement. See **Figure 2** for representative micrograph.

1.2.6. If either of the duplicate plates at a specific dilution has 1 or more *Campylobacter* colonies present, consider that dilution fecal-culture positive.

1.2.7. Consider the last dilution that contains a visible *Campylobacter*-like gram-negative colony the limit of culture detection. Use **Equation 2** to calculate the CFU/mL of the positive dilution in contrived clinical fecal specimen:

CFU/mL in fecal sample = Analytical CFU/mL ÷ Dilution with last positive colony **[Equation 2]**

NOTE: See **Table 2** for typical results.

2. Viability determination of *Campylobacter* stored in transport media

2.1. Mix 1 mL of *Campylobacter* broth culture (step 1.1.8) with 1 mL of NFP and prepare 10 duplicate two-fold serial dilutions in NFP. Further dilute each dilution an additional 1:4 in Cary-Blair media, just as a clinical specimen prepared in transport media is treated.

2.2. Store the 20 dilution tubes and a negative control in Cary-Blair medium in capped tubes at 2−8 °C for 96 h and count colonies from each dilution occurring at time zero and every 24 h. For colony counting, sample the broth:fecal tubes and setup fecal culture for colony counting of each dilution, in duplicate.

2.3. Each day plate 10 µL portions of the fecal dilutions on *Campylobacter*-selective agar and incubate at 37 °C for 48 h, as described above in steps 1.1.9−1.2.7.

2.4. Perform a simultaneous analytical plate count of the original bacterial stock (from step 1.1.8 or a freshly grown broth stock) as described above (steps 1.1.9−1.1.11). Calculate the CFU/mL of the original bacterial stock (**Equation 1**) to calculate the concentration of bacteria in the transport media fecal sample and its dilutions (**Equation 2**).

3. Non-culture assays for verifying culture results

3.1. Use an enzyme immunoassay (EIA) that gives minimal false positive results[12] and perform according to package insert instructions to verify culture results.

3.2. Use a molecular assay that can detect the 16S rRNA gene or other gene of a broad range of *Campylobacter* species[13]. Confirm that the molecular assay reacts with species such as *C. upsaliensis* or *C. lari* that grow poorly on standard antibiotic-containing agar[14]. Follow manufacturer's instructions for extraction of DNA from fecal samples and performing the test.

NOTE: Bidirectional DNA sequencing of the 16S amplicon can be used to confirm the species of *Campylobacter* in a positive specimen. Species-specific PCR (see **Table 3** for target genes) can also be used to identify species present in discrepant or positive specimens[15].

REPRESENTATIVE RESULTS:

Identifying *Campylobacter* spp. colonies among competing fecal flora requires keen eyesight and considerable judgement. The lowest number of colonies that can be detected by culture has not been studied, although specimens from patients have been estimated to harbor 10^6–10^9 CFU/mL[16,17]. However, patient samples cannot be used quantitatively as there is no independent method to establish accurate bacterial numbers. To overcome this limitation, two simultaneous measurements are made with one bacterial stock. One test is used for visual detection of *Campylobacter* colonies from serial dilutions of the stock bacteria in a fecal matrix, simulating clinical specimens; the other is used analytically to quantify the CFU/mL present in the bacterial stock culture used for spiking (**Figure 2A**).

The detection thresholds for *Campylobacter* will not be defined values. This is to be expected because each fecal matrix is complex and unique, and growth of bacteria is variable. A key parameter for success is identifying the pinpoint size colonies amongst the competing fecal flora. A representative plate of spiked stool culture is shown in **Figure 2C** and **Figure 2D**. The negative control plate without added *Campylobacter* is important to help identify other fecal flora. Gram staining many candidates also trains the eye to distinguish the correct glossy colonies and the intermediate pink color of fuchsin-stained gram-negative bacteria and confirms the morphology of the bacteria in the selected colonies (**Figure 2B**). Seven independent experiments were performed, using 5 *C. jejuni* and 2 *C. coli* broths, and gave thresholds that overlapped and spanned from 0.3−5 x 10^6 CFU/mL. See **Table 2** for typical data. The detection limits averaged 2 x 10^6 for *C. jejuni* and 1.2 x 10^6 CFU/mL for *C. coli*. This indicates that culture can likely detect ~1−2 x 10^6 *C. jejuni* or *C. coli* per gram of fecal specimen on standard antibiotic-containing *Campylobacter*-specific agar used by many clinical laboratories. There are multiple specialized agars with different antibiotics that may give different thresholds for colony detection. The methods described here should encourage more quantitative and comparative studies to improve the accuracy of culture and broaden the versatility of new media. For example, 152 colonies were counted on the first 10^{-5} plate and 144 colonies on the second 10^{-5} plate. The average between the two plates is 148 colonies. The plates were inoculated with 0.1 mL (100 µL)

of 10^{-5} dilution, which by **Equation 1** equates to 148 x 10^6 (14.8 x 10^7) CFU/mL in the pure culture stock. When the fecal dilutions were made, the culture was spiked into negative fecal pool at a 1:1 ratio. Therefore, by **Equation 2**, the first point (plate "a") on the fecal curve corresponds to 14.8 x 10^7 divided by 2 and equals 7.4 x 10^7 CFU/mL. This "a" tube is used to make 9 additional dilutions. In **Figure 1**, the last dilution with one visible gram-negative colony with *Campylobacter*-like morphology is on plate "g". This equates to 1.1 x 10^6 CFU/mL for the fecal culture threshold of detection in this example.

Even though sustained viability is key to culture's accuracy, retention of viability of *Campylobacter* spp. during handling and shipment of specimens from patients to clinics to reference labs is problematic. Typical storage is to refrigerate specimens in ordinary capped tubes with air exposure and with no special atmosphere. Specimens in transport media (also known as preserved samples) are thought to have better survival, but there are few reports that provide quantitative data[18].

The combination of analytical and contrived sample methods shown above was used again to obtain viability and survival time estimates of *C. jejuni* in transport media. A bacterial stock broth was used to prepare ten duplicate 2-fold to 1024-fold sample dilutions in fecal matrix. The initial broth was found by the analytical counts to have a concentration of 4.8 x 10^7 CFU/mL. On plates made on day 0, *C. jejuni* was detected (2 days later) on the plate streaked with the 32-fold dilution, equivalent to 1.5 x 10^6 CFU/mL. However, on the plates made after refrigerating the Cary Blair fecal sample for 24 hours, only the 2-fold dilution (equivalent to 2.4 x 10^7 CFU/mL) grew visible colonies. No further loss of viability was seen out to 96 hours, when the study was stopped. This loss of viability equates to a 16-fold (94%) loss of culturable organisms in less than 24 hours and indicates that, even with refrigeration, stool in Cary Blair medium with less than 10^7 CFU/mL *C. jejuni* may be missed by culture.

In contrast to the results of culture, the EIA detected the presence of *C. jejuni* at the 256-fold dilution at the initial time point and throughout the 4-day testing period. The *C. jejuni* detection threshold for this EIA using spiked fecal samples is 8.4 x 10^4 CFU/mL. This threshold is below that of fecal culture and allows more sensitive and stable detection of *C. jejuni*.

To test the ability of culture to detect *Campylobacter* spp. in an actual clinical setting, 1552 clinical stool specimens were characterized by 6 procedures: fecal culture, a new immunoassay for *Campylobacter* spp., and 4 molecular methods. All samples were prospectively collected and initially classified by conventional culture at 3 laboratories in the United States, and then cross-checked by EIA. Any culture-positive or EIA/culture-discrepant specimens were then screened by the molecular methods[12]. Specimens were assigned a true-positive or true-negative status based on the results of the 5 non-culture methods. The 5 non-culture methods showed complete agreement on all 48 positive and discrepant specimens, while culture mis-identified 14 (28%). The specimens that were incorrectly identified by culture included 13 false negative and 1 false positive sample.

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme for simultaneous preparation of analytical and spiked fecal samples.

Figure 2: Identification of *C. jejuni* colonies from pure and fecal cultures. (**A**) Photograph of *C. jejuni* colonies from pure bacterial culture after 72 hours incubation. (**B**) Gram stain of *C. jejuni* from pure bacterial culture, oil-immersion 400x magnification. (**C**) Photograph of *C. jejuni*-positive spiked fecal culture after 48 h incubation. (**D**) Enlarged area in box in (**C**), 10x magnification. White arrows indicate pin-point size gram-negative *C. jejuni* colonies. The black arrowhead indicates a colony that is slightly larger, gram-positive, and not *C. jejuni*.

Table 1: Typical growth and CFU/mL of *C. jejuni* and *C. coli* stocks. [1]*C. jejuni* culture was stopped after 48 h of incubation. *C. coli* culture was stopped after 54 h of incubation.

Table 2: Typical numbers of colonies on plates of spiked fecal samples. [1]Gram negative colonies among *Campylobacter*-like colonies, [2]nd = not determined, [3]Data in bold type indicates last positive dilution.

Table 3: Genes useful for detection of individual *Campylobacter* species qPCR.

DISCUSSION:

The culture methods described here are built on simple, widely used techniques and materials available in most laboratories[10]. It is the combination of analytical and contrived samples that provide new information of a clinically relevant detection threshold for fecal cultures. Additionally, the adjudication of culture results with 5 separate assays strengthens the conclusions that *Campylobacter* fecal culture mis-identifies a significant portion of patient specimens. The EIA and molecular assays are useful as controls because they are each based on a different principle (antigen interaction with antibody vs. DNA amplification) and, importantly, do not rely on viability of bacteria. Note that the EIA assay used for these studies is well-validated and has been shown to agree fully with 4 molecular tests[12].

Culture of *Campylobacter* spp. is particularly troublesome, with sensitivity reported to range from 60−76%[19,20], and as evident from its ~30% rate of failure to detect true-positive specimens here. Personnel can expect that control EIA and molecular tests will frequently produce positive results when culture data are negative.

The most critical step in the protocol is the identification of pin-point colonies among competing fecal flora. It is not unusual, as dilutions near the detection threshold, to have alternating zero and non-zero colony count estimates (e.g., 2, 0, 1, 0, 0). It is important to recognize that culture thresholds will be a range of concentrations, not a specific CFU/mL. Nevertheless, the estimate of ~1 x 10^6 CFU/mL feces as a lower limit for culture detection compares well with reports that infected humans shed 10^6 to 10^9 *Campylobacter* per gram feces[21]. Changes in antibiotics or agar plates and variations inevitable in individual fecal specimens will undoubtedly change threshold values. This protocol should enable improvements in growth media.

This first information on a limit for culture detection makes it possible to set clinically relevant thresholds for diagnostic tests, and lays the microbiological foundation which is needed to address unstudied issues of non-symptomatic carriage[22,23] by *Campylobacter*, or if bacterial load correlates with symptoms or serious sequelae.

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DISCLOSURES:

The authors are employees of TECHLAB, Inc. that produces the QUIK CHEK™ kit used as a comparator in this article.

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Figure

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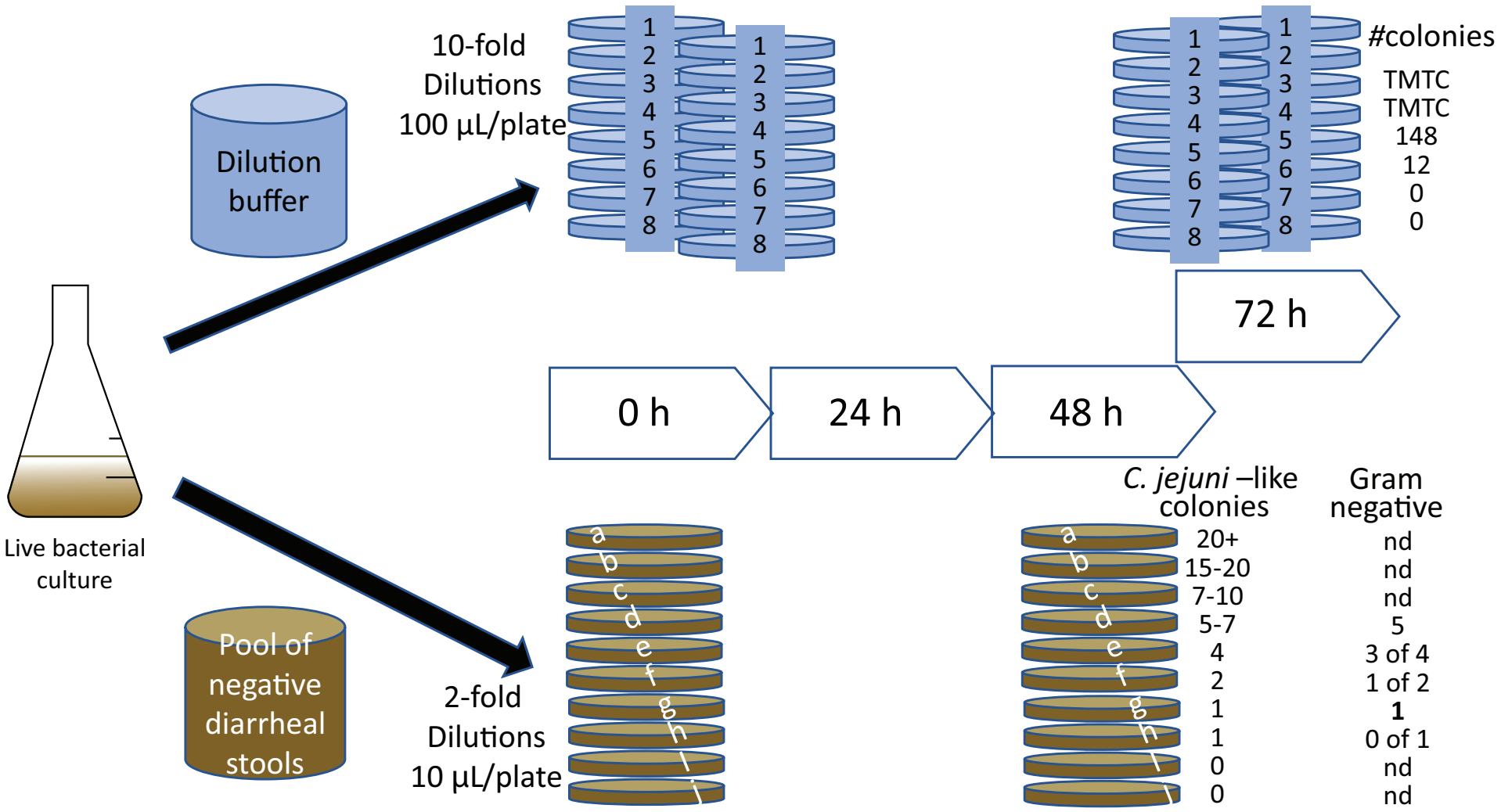

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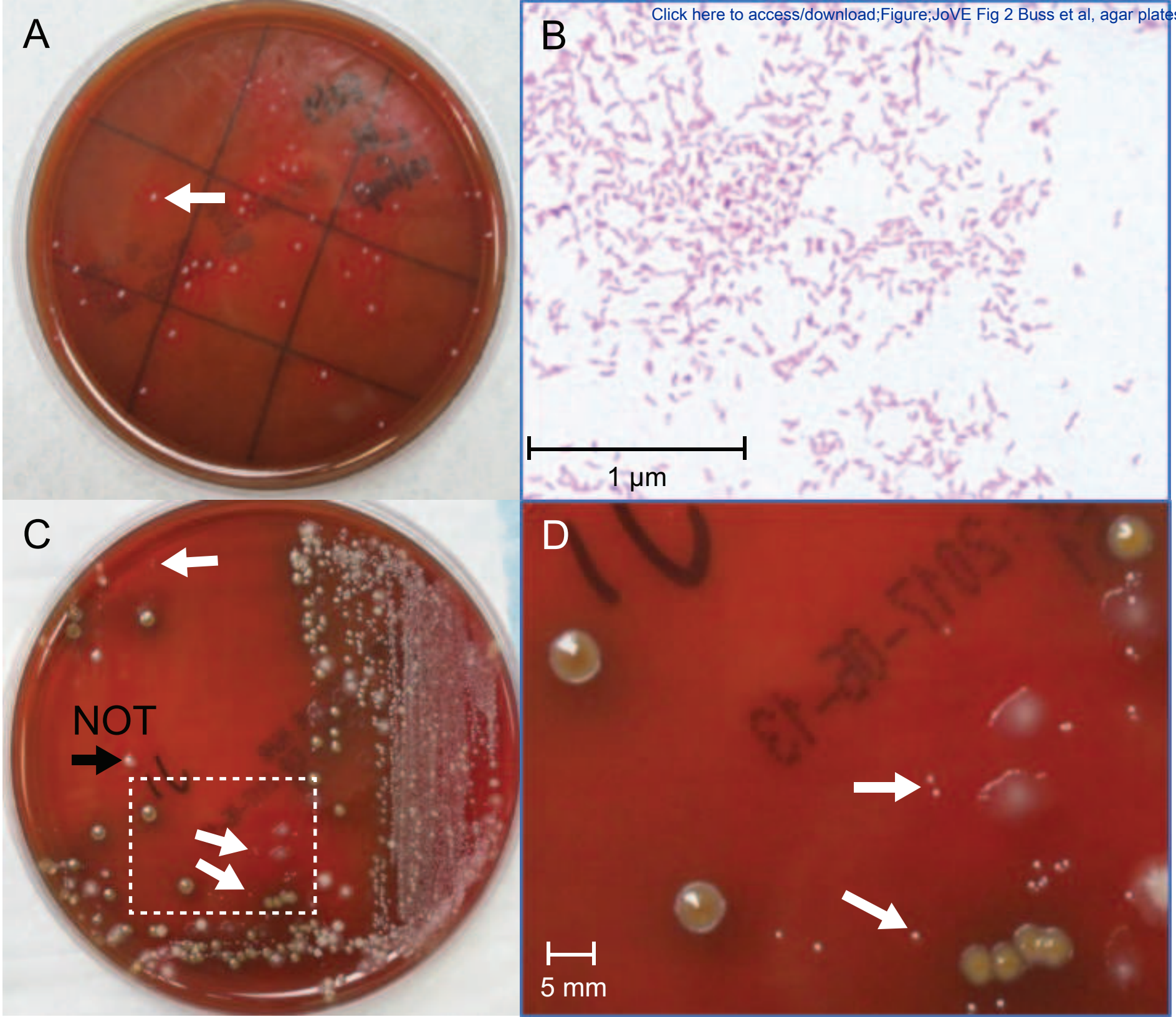

Table 1

Cultures	OD_{600} @ T0	OD_{600} @ T Final[1]	Final CFU/mL
C. jejuni	0.146	0.321	1.28×10^7
C. coli	0.245	0.508	4.50×10^8

Table 2

Click here to access/download;Table;JoVE Table 2 Colony Counts- Buss, Thacker, Santiago.xlsx

	Dilution tube for spiked fecal sample	Number of Campylobacter-like colonies	Number of Gram-negative colonies[1]	Culture positive?
C. jejuni (1.28×10^8 CFU/mL stock)	(2-fold) a	Dense	nd[2]	yes
	(4-fold) b	20+	nd	yes
	(8-fold) c	4-10	nd	yes
	(16-fold) d		nd	yes
	(32-fold) e		nd	yes
	(64-fold) f	1-3	1 of 2	yes
	(128-fold) g		2 of 3	yes
	[3](256-fold) h		**1 of 2**	**yes**
	(512-fold) i		0 of 1	no
	(1024-fold) j	0	nd	no
C. coli (4.50×10^8 CFU/mL stock)	(2-fold) a	Dense	nd	yes
	(4-fold) b		nd	yes
	(8-fold) c	50+	nd	yes
	(16-fold) d	30+	nd	yes
	(32-fold) e	10+	nd	yes
	(64-fold) f	3-8	nd	yes
	(128-fold) g		nd	yes
	[3](256-fold) h	1-3	**1 of 3**	**yes**
	(512-fold) i		0 of 1	no
	(1024-fold) j	0	nd	no

Calculated CFU/mL of spiked sample
6.40×10^7
3.20×10^7
1.60×10^7
8.00×10^6
4.00×10^6
2.00×10^6
1.00×10^6
5.00×10^5
2.50×10^5
NFP
2.25×10^8
1.13×10^8
5.63×10^7
2.81×10^7
1.41×10^7
7.03×10^6
3.52×10^6
1.76×10^6
8.79×10^5
NFP

Table 3.

Species	Gene target
C. jejuni	*hipO*
C. coli	*cadF*
C. upsaliensis	*cpn60*
C. lari	*cpn60*
C. helveticus	*cpn60*
C. fetus	*cpn60*
C. hyointestinalis	*cpn60*
C. concisus	*cpn60*

Table of Materials

Name of Material/Equipment	Supplier
Anaerobic 3.5L Jar	Thermo Fisher
AnaeroGRO Campylobacter Selective Agar	Hardy Diagnostics
Bacto Brain Heart Infusion	BD Biosciences
Bacto Protease Peptone	Life Technologies Corp
Basic Fuchsin	Fisher Scientific
BBL Trypticase Peptone	Life Technologies Corp
C. coli Type strain	ATCC
C. jejuni Type strain	ATCC
CampyGen gas generating system sachet	Thermo Fisher
Campylobacter QUIK CHEK	TechLab, Inc.
Cary-Blair transport medium	Fisher Scientific
Coli Roller Sterile plating beads	Millipore Sigma
Dilution Buffer	Anaerobe Systems
Fetal bovine serum	Equitech-Bio, Inc
Sodium bisulfite	Sigma-Aldrich
Sodium pyruvate	Sigma-Aldrich
Spectrophotometer cuvettes	USA Scientific

Supplier Catalog number
HP0031A
AG701
237500
211684
B12544
211921
33559
33560
CN0025A
T5047 / T31025
23-005-47
71013
AS-908
SFBM30
243973
P2256
9090-0460

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GI DISEASE DIAGNOSTICS

DATE: December 2, 2019
TO: JoVE
FROM: Janice E. Buss, Ph.D., Corresponding Author
RE: Revision of manuscript JoVE 60457

To the Editor:

We have now completed a second revision of our manuscript entitled "Culture methods to determine the limit of detection and survival in transport media of *Campylobacter jejuni* in human fecal specimens" in response to the comments by the editor and two reviewers. Changes and rationale are detailed below.

We appreciate the comments of the reviewers and hope that the revised version of the manuscript will be acceptable for publication.

Sincerely,

Janice E. Buss, Ph.D., Corresponding Author
TechLab, Inc., 2001 Kraft Drive, Blacksburg, VA 24060
Email: jbuss@techlab.com; Phone: 540-953-1664; FAX: 540-953-1665

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Thank you, manuscript has been proofed.

2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution. No tissues were used in this study. De-identified patient specimens not designed for this study, or healthy donor stools were used to prepare a negative fecal pool. Thus, no patient consent was needed. This information has been inserted in paragraph 1.2.1.1.

3. 1.1.2: Please list an approximate volume of BHI broth to prepare. "100 mL" added to sentence.

4. 2.1: From which step is the Campylobacter broth culture obtained? Please specify. Step 1.1.7 was divided to give 1.1.8 (which number was missing). This 1.1.8 was inserted into Step 2.1.

5. Figure 1: Please change the time unit "hr" to "h". Figure revised as requested.

6. Figure 2: Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend. Images and Figure legend changed as requested.

7. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name. Table of Materials sorted alphabetically.

- Please note that an additional sentence has been added at the end of the Abstract to more clearly state the usefulness of this protocol.

Reviewers' comments:
Reviewer #3:

Manuscript Summary:

1. The authors spiked Campylobacter-free fecal samples with C. jejuni and C. coli, and plated on one commercial Campylobacter-selective plate in a gas system produced by gas-generating sachets to determine the lowest CFU this method can detect.

2. The authors used these spiked samples and one commercial transport medium and the same plating method as above to determine the viability of Campylobacter in transport medium for 4 days.

3. The authors used the same method as above to test for Campylobacters in clinical samples, and used non-culture methods for multiple Campylobacter species (including species that cannot grow on the Campylobacter-selective media) for comparison.

Major Concerns:

1. As a method description and comparison paper, the authors used only one Campylobacter-selective plate, one gas system, and one transport medium, which we are not told whether these are commonly used in other detection labs. The purpose of this paper was not comparison or optimization of culture methods, but documentation of *Campylobacter* viability in conditions often used in clinical laboratories. A new reference (#10- M'ikanatha, N. M. *et al.*) has been added to the penultimate paragraph of the Introduction as a source for typical information on laboratory practices.

Also, the manuscript lacks descriptions of the media and gas used in the experiments to emphasize what is important for various Campylobacters to grow, for example, H2 is necessary for some Campylobacters. The media broth used was brain-heart infusion (BHI) and is noted in paragraph 1.1.2. The specific gas-generating packets and selective agar used are noted in the **Table of Materials**. These reagents are appropriate for the *C. jejuni* and *C. coli* studied here.

2. To isolate Campylobacter from fecal samples, the filter method is a more reasonable choice since the Campylobacter-selective plates are not intended for all Campylobacter species. The two studies on

Campylobacter viability did not isolate *Campylobacter* spp, but instead enumerated viable *C. jejuni* or *C. coli* from pure cultures spiked within a fecal matrix, using a selective agar. The clinical studies utilized the validated methods already in place in each laboratory. The filter method would certainly have been appropriate should isolates have been desired.

Minor Concerns:

1. Not enough strain information for the Campylobacter strains used in this study. The ATCC numbers for the Type strains of *C. jejuni* and *C. coli* used in this study have been added to paragraph 1.1.1. They are also presented in the Table of Materials.

2. In the introduction, the authors mention that other Campylobacter species do not grow on the Campylobacter-selective plates, but it is not addressed in the experimental design. This study reports results from deliberate addition of *C. jejuni* and *C. coli* (the two *Campylobacter* species most commonly identified in diarrheal stool) to fecal matrix. The clinical studies utilized the validated methods already in place in each laboratory which included selective agars. That the broad use of selective agars may constrain growth of other species is mentioned to note this limitation for current estimates of species' prevalence, and to inform future studies of detection limits of such species.

3. Some statements need references, such as line 306-307. Reference added (the first sentence in Discussion).

Reviewer #4:

Manuscript Summary:
This protocol compares culture based detection to molecular methods to establish a lower limit of detection for Campylobacter in spiked human stool specimens, and then compares the detection of Campylobacter in culture of clinical specimens to detection by molecular methods. The studies provide import information regarding the lower limits of detection, and the survival of Campylobacter in media such as Cary-Blair transport media that are often used in field studies.

Major Concerns:
In the introduction the statement that "World-wide, Campylobacter spp. are the most common bacterial intestinal infections" is probably misleading. Campylobacter infections appears to be somewhat population dependent. Using molecular detection methodology, it is the most commonly isolated pathogen in some regions, while it falls behind other major diarrheal pathogens (e.g., Shigella, enterotoxigenic E. coli, etc.) in others. There is no question that Campylobacter is frequently overlooked

by culture based methods, and it is a common cause of diarrheal disease worldwide, but saying that it THE most common bacterial intestinal infection may be overstating the case. The reviewer has a good point. We have modified the sentence (Line 53) to repair the overstatement.

Minor Concerns:

Some suggestions to improve readability of the manuscript:

1. include false positive/false negative detection rate for stool culture compared to molecular methods in the abstract rather than simply stating that 28% were mis-identified. Data included in Abstract. Last paragraph of Results also mentions these fp/fn rates.
2. reference to the table of materials might be helpful early in the protocol. Added to 1.1.1.

3. on page 7 perhaps a well-validated assay rather than an EIA that gives minimal false positives? Line 322 improved.

4. line 323 page 11: It seems likely that based on the authors' findings that what was previously reported about the shedding of Campylobacter in stool may not reflect reality and that shedding probably goes undetected in nearly a third of patients. We happily agree.