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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,

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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.