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Isolation and Whole-Genome Sequencing of Environmental *Campylobacter*

Campylobacter Isolation and Whole-Genome Sequencing

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Significance Statement

Campylobacter is the leading cause of bacterial-derived gastroenteritis in the world. The ability of *Campylobacter* to reside in various agricultural and environmental reservoirs plays a critical role in the prevalence of human infections, highlighting the importance of performing environmental source-tracking for this pathogen. This protocol focuses on isolating *Campylobacter* from agricultural and environmental sources, as well as clinical specimens. After isolating *Campylobacter* from these sources, this protocol provides information for the extraction and preparation of genomic DNA for whole-genome sequencing (WGS). Additionally, this protocol provides initial parameters for preparing raw sequencing reads for downstream bioinformatics analyses.

ABSTRACT

As a leading cause of bacterial derived gastroenteritis worldwide, *Campylobacter* has a significant impact on human health. In the developed world, most campylobacteriosis cases are attributed to the consumption of undercooked, contaminated poultry; however, it has been shown that *Campylobacter* can be transmitted to humans through contaminated water and other types of food, including beef and milk. As such, high-resolution microbial source-tracking is essential for health department officials to determine the source(s) of *Campylobacter* outbreaks. For these reasons, this protocol provides the techniques needed for isolation *Campylobacter* from agricultural and environmental sources, as well as human clinical specimens. Additionally, we describe a simple method for preparing high-quality genomic DNA that can be used for whole-genome sequencing (WGS) and downstream bioinformatic analyses of *Campylobacter* genotypes.

Keywords: *Campylobacter*, Environmental Isolation, Whole-genome Sequencing

INTRODUCTION

Campylobacters are microaerophilic bacterial pathogens that can commensally colonize various hosts, but induce severe gastroenteritis in humans (Johnson et al., 2016; Wilson et al., 2008). While the majority of infections in the developed world are attributed to the consumption of undercooked, contaminated poultry meat (Hermans et al., 2012; Skarp et al., 2016), several other sources have been identified, including beef. In addition, infections in the developing world likely come from diverse sources, including contaminated water or livestock (Coker et al., 2002; Kaakoush et al., 2015). Unfortunately, many sources are not identified due to the difficulty of analyzing *Campylobacter* genotypes with older methods like pulse-field gel electrophoresis. With the significant impact *Campylobacter* has on human health and the rate at which it is becoming antibiotic resistant, there is increasing urgency to definitively identify the sources of human infections.

In terms of potential reservoirs to investigate, it is well known that a majority of commercial poultry flocks are colonized with *Campylobacter* (Newell and Fearnley, 2003; Evans and Sayers, 2000), but the organism is regularly isolated from other agricultural sources, including cattle, pigs, and sheep (Huang et al., 2015; Stanley and Jones, 2003; Zhao et al., 2001). Additionally, *Campylobacter* has the ability to survive under various environmental conditions, particularly those that occur in water sources. To begin isolating from these diverse sources, we evaluated several methods and determined that the methods provided in this protocol are inexpensive, straightforward, and allow us to broadly sample agricultural and environmental sources.

Upon isolation of *Campylobacter* from these sources, the ability to analyze and compare the isolates on a genome-wide scale is extremely beneficial. The use of whole-genome sequencing as a method for comparing *Campylobacter* isolates has only been employed within the last few years, allowing for high-resolution analysis of diverse isolates to the nucleotide level (Cody et al., 2013; Zhao et al., 2016; Cantero et al., 2017). Utilizing reads from an Illumina

platform, this protocol also includes a method for evaluating the quality of the reads and assembling them into genomes for subsequent annotation and downstream bioinformatics analyses.

BASIC PROTOCOL 1

ISOLATION OF *CAMPYLOBACTER* FROM ANIMAL FECAL SAMPLES

It is understood that poultry are regularly colonized with *Campylobacter*, but other agricultural animals can also transmit *Campylobacter* (Stanley and Jones, 2003). To establish a microbial source-tracking pipeline, we developed the following method as a means to select for *Campylobacter* from animal fecal samples. This method also allows for the enumeration of *Campylobacter* through the use of serial dilution plating.

Materials

Approximately 2 g fresh fecal sample in 15-ml conical tube
1X PBS, sterile
Fresh CS plates (see recipe)
MH broth with 20% glycerol (see recipe)

15-ml sterile conical tubes (Fisher – 05-539-4)
Wooden tongue depressors , sterile
Parafilm
96-well plates, deep well (Fisher - 12566611), sterile
Glass beads (4 mm), sterile
37°C incubator, 10% CO_2, 5% O_2 (Thermo – Model 4130)

Protocol steps—*Step annotations*

1. Aliquot 900 µl sterile 1X PBS into each well in rows A-F of a deep 96-well plate and cover the plate with parafilm.

   This should be done ahead of time to ensure the dilution and plating steps occur as quickly as possible once the sample is weighed out in step 2. This is the recommended number of dilutions to achieve countable results.

   Plates with unused rows should not be saved for future use.

2. Using a sterile tongue depressor, weigh out 200 mg of the fecal sample into a sterile 15-ml conical tube with a screw cap. Use one tongue depressor per sample, and discard used tongue depressors in a biohazard bag.

   The most efficient method for weighing out fecal samples is to place the tube in a small tube rack on the scale and tare with the tube, tube lid, and rack before adding any sample.

3. Add 2 ml sterile 1X PBS to each 200 mg sample, cap, and vortex 20 seconds.

   The addition of the 2 ml PBS creates a 1:10 dilution and this will be considered when labeling plates for the dilution series.

   Ensure the cap is screwed tightly before mixng to ensure the sample does not leak. The standard time to vortex is 20 seconds, but may vary depending on the consistency of the sample. Samples that are dense or dried out may take slightly longer to homogenize.

4. Serially dilute each sample: Transfer 100 µl of sample into the top well of a column in the 96-well plate. Mix well by pipetting up and down several times. Using the same tip, transfer 100 µl from the top well into the well below, and discard the pipette tip. Using a fresh tip, mix the contents of the second well, then transfer 100 µl to the next well below. Repeat for each well, to serially dilute the sample down the column.

   The plate should be labeled down the side with the dilution series. Example: -3, -4, -5, -6, -7, and -8.

   For accurate enumeration, pipette tips should be changed between each dilution step.

   It is useful to label the plate with indicators of each sample to keep track of samples once diluted.

5. Once dilutions are complete, pipette 100 µl of each dilution, including the sample in the 15-ml tube, onto appropriately labeled CS plates. Proceed quickly to step 6.

   This can be done using a single pipette tip by starting from most dilute (-8) and moving up the column to the least dilute (-3), and finally the sample in the 15ml tube.

   Make sure to work with one sample (column) at a time to prevent cross-contamination and to ensure an efficient workflow.

6. Aseptically pour approximately 10 sterile glass beads onto each CS plate. Recap plates, shake briskly for 15 seconds, and invert plates so beads settle in the lid and can be dumped out.

   It is easier to shake the plates if placed in stacks. Make sure the beads do not run around the edge of the plates as this will not efficiently spread the sample on the plate. The beads should bounce around on the plate and sound like a maraca.

   After inverting the plates, make sure to tap once or twice to ensure no beads stick to the surface of the plates.

   Keep the plates inverted while dumping the beads from the lid into a capture container with 95% ethanol.

7. Incubate inverted plates in a microaerophilic environment (10% CO_2, 5% O_2) for 48 hours at 37°C.

8. Count the colonies on the plates, then pick a single colony from each sample and streak on fresh CS plates to stock. Incubate as in step 7.

    Sterile inoculating loops should be used to streak colonies.

9. Pass a sterile inoculating loop 2-3 times across the growth on the CS plates. Suspend the loop-full of bacteria in 500 µl MH broth with 20% glycerol and store at -80°C for future use.

    It is only necessary to pass the loop across the plate 2-3 times before resuspension in the MH solution. It may be necessary to cap the tube and vortex for a few seconds to ensure re-suspension. The cryogenic vial should be stored in the freezer soon after adding the bacteria.

ALTERNATE PROTOCOL 1

ISOLATION OF *CAMPYLOBACTER* FROM WATER SAMPLES

Although it is known that *Campylobacter* can survive in water sources, it is not well understood how the organism can do so under such varying environmental conditions (Bronowski et al., 2017; Murphy et al., 2006). Many human infections in the developing world are the result contaminated water, so there may be potential for human infections due to contaminated water in the developed world as well (Ashbolt, 2004). For these reasons, the ability to isolate *Campylobacter* from water samples is important for tracking the flow of *Campylobacter* through various environmental sources. This is done through the use of vacuum filtration of water samples and selective plating to isolate *Campylobacter* alone. This method also utilizes a dilution plating scheme in order to enumerate the *Campylobacter* load of various water sources.

Additional Materials *(also see Basic Protocol 1)*

100-ml water samples in sterile glass bottles
95% Ethanol

125-ml vacuum filtration units with 0.22µm filter membranes (Fisher – 565-0020)
Sterile forceps
Bunsen burner

Protocol steps—*Step annotations*

1. Aliquot 900 µl sterile 1X PBS into each well in rows A-F of a deep 96-well plate and cover with parafilm.

This should be done ahead of time to ensure the dilution and plating steps occur as quickly as possible once the sample is weighed out in step 2.

Plates with unused rows should not be saved for future use.

2. Using a vacuum filtration unit hooked to a vacuum pump, filter water samples through the 0.22 µm filter.

   The entire water sample will be filtered through the membrane. A larger apparatus may be used if the initial water sample is greater than 100 ml.

3. Use sterile forceps to gently remove the membrane from the filter unit and place it in a sterile 15-ml conical tube filled with 5 ml 1X PBS, cap the tube, and vortex for 20 seconds.

   Care should be taken to not drop the membrane or to allow it to contact other surfaces to prevent contamination. It may be useful to tear the membrane into smaller pieces using the forceps so it fits in the 15 ml tube more easily.

   Ensure the cap is screwed tightly before vortexing to ensure the sample does not leak. The standard time to vortex is 20 seconds, but this may vary depending on the amount of particulates present in the sample. With the tube still closed, examine the membrane after 20 seconds. If particulate is still present on the membrane, vortex for another 10-15 seconds until large particulates are removed from the membrane.

   Forceps may be autoclaved initially, but can be sterilized between samples by dipping in 95% ethanol and passing over a Bunsen burner to burn off the ethanol. Care must be taken, however, as the membranes in the filtration units are quite flammable.

4. Follow steps 4-9 of Basic Protocol 1.

ALTERNATE PROTOCOL 2

ISOLATION OF *CAMPYLOBACTER* FROM FOOD SAMPLES

Contaminated food as a source of *Campylobacter* infections is well known, with contaminated chicken meat and raw milk being the most frequently implicated (Mungai, et al., 2015; Skarp et al., 2016). Other foods such as vegetables and fruit may also become contaminated either in the field or through cross contamination in the kitchen (Guévremont et al., 2015). To determine if a food sample is contaminated with *Campylobacter,* similar methods as those described for water and fecal samples may be employed. Because *Campylobacter* loads on food are usually less than that of fecal or water samples, an initial enrichment step is necessary to aid in the detection of *Campylobacter*. The resulting enrichment can then be serially diluted and plated on selective media in a manner as described above.

Additional Materials *(also see Basic Protocol 1)*

Food samples

Razor blade or scalpel
Cutting board

Protocol steps—*Step annotations*

1. Aliquot 900 µl sterile 1X PBS into each well in rows A-F of a deep 96-well plate and cover with parafilm.

   This should be done ahead of time to ensure the dilution and plating steps occur as quickly as possible once the sample is weighed out in step 2.

   Well plates with rows that are not used should not be saved for future use.

2. Clean a razor and cutting board with 95% ethanol in preparation for dicing food samples.

   A scalpel may be used in place of a razor. Exercise caution when using either, for the safety of the researcher.

3. Cut food sample into pieces no larger than 3 cm^3. For each sample, place several cubes into an appropriately labeled 50-ml tube, cover with 5-ml MH broth, cap tube securely, and vortex 20 seconds.

   Ensure the cap is screwed tightly before vortexing to ensure the sample does not leak. The standard time to vortex is 20 seconds, but this may vary as larger pieces may need more time to ensure through rinsing. The amount of MH broth necessary to cover the food samples may vary as well.

   Make sure to clean both the razor blade and the cutting board with 95% ethanol between each food sample.

4. Loosely cap each 50-ml tube and place in a tube rack secured to an orbital shaker in a 37°C microaerophilic incubator. Allow to shake overnight.

   When loosely attaching the screw cap, small pieces of lab tape may be used to prevent the cap from falling off while the tubes shake. However, it is important to not completely seal the lid to allow oxygen flow.

5. Follow steps 4-9 of Basic Protocol 1.

ALTERNATE PROTOCOL 3

ISOLATION OF *CAMPYLOBACTER* FROM HUMAN CLINICAL SAMPLES

An important aspect of *Campylobacter* transmission is the ability of the organism to infect susceptible hosts, like humans; humans may present with inflammatory diarrhea for several days when infected by *Campylobacter* (Tribble et al., 2010). For this reason, the ability

to isolate *Campylobacter* from human fecal samples is important for health departments in order to determine potential sources of infection. Typically, health departments receive either a slant, plate, or broth inoculated with a sample from an individual presenting with signs of *Campylobacter* infection. The growth from the initial media is then used to inoculate selective media before being subjected to various biochemical tests, including hippurate, catalase, and oxidase tests. Based on these results, the public health lab can determine whether the sample is positive for *Campylobacter* and can notify the clinic.

Additional Materials *(also see Basic Protocol 1)*

Human clinical sample on slant or plate or in broth
CCDA Selective media (CCDA) – Sigma Aldrich
GasPak EZ Campy sachets – BD 260680
GasPak EZ Airtight container – BD 260671
Gram stain supplies: Crystal Violet, Gram's Iodine, 95% Ethanol, and Safranin (Fisher – R40058)
Hippurate Hydrolysis Reagent – Sigma 53275-500G
Ninhydrin Reagent – Sigma-Aldrich N7285
Kovacs Oxidase Reagent – Acros Organics
3% Hydrogen Peroxide – BDH
Clean glass slide
Indoxyl Acetate Disk – Remel R21087

Protocol steps – *Step annotations*

1. Upon receipt of clinical sample on slant or plate or in broth, streak for isolation on a CCDA plate.

   Samples may be obtained from clinical labs in various forms. These vary between clinical labs and will depend on the standard protocol for the laboratory.

2. Incubate CCDA plates in a GasPak airtight container with a Campy satchet for 48 hours at 42 C.

   This incubation step more closely replicates the isolation procedure utilized by clinical laboratories when working with human samples.

3. Pick isolated colonies from the CCDA plate, and streak to a blood agar plate. Incubate as in step 2.

   The blood agar plates should also be used to streak for isolation. This should be done with several colonies (5) from each CCDA plate for each sample.

4. Perform a Gram stain, following a standard protocol (Moyes, Reynolds, & Breakwell, 2009).

Campylobacter is a Gram-negative rod, with a slight crook in the rod shape. A Gram-negative result will appear pink, while a Gram-positive result will appear purple. If there seems to be a mix of Gram-positive and -negative, another slide should be prepared. If the mix persists, the growth on the blood agar plate should be restreaked for isolation.

5. After performing a Gram stain, perform a Hippurate hydrolysis test to differentiate between *C. jejuni* and *C. coli.*

   Heavily inoculating a tube containing 0.2 ml water with the bacterial culture from the blood agar plate.

   Incubating 2 hours at 37°C.

   Add 2 drops ninhydrin reagent to each tube, and incubate 30 minutes at 37°C.

   Check tubes often for color change. A dark blue color is Hippurate-positive and indicates C. jejuni, while no color change is Hippurate-negative and indicates C. coli.

6. Using a small amount of bacterial culture, perform a catalase test using 3% hydrogen peroxide.

   Campylobacter is catalase-positive, so the addition of hydrogen peroxide to the bacterial culture on the glass slide should result in the formation of bubbles.

7. Following the directions included with the Indoxyl Acetate disks, perform an indole test using colonies from the blood agar plate.

   The indole test differentiates Campylobacter from other enteric pathogens. Campylobacter can hydrolyze the indoxyl within the disk, resulting in a blue color (i.e., Campylobacter-positive).

8. Following the directions included with the Kovacs oxidase reagent, conduct an oxidase test using culture from the blood agar plate.

   Campylobacter is oxidase-positive and a will result in a blue color change.

9. If the oxidase test is positive, the sample can then be sent for MALDI-TOF analysis to identify *Campylobacter* species.

   This step is commonly utilized by medical diagnostic and clinical labs, but species can also be determined by PCR, as in Basic Protocol 3. Because Basic Protocol 3 focuses on C. jejuni and C. coli, MALDI-TOF may be necessary to identify other species of interest.

10. Perform a PCR analysis of isolates, as described in Basic Protocol 3, to confirm species of the *Campylobacter*-positive sample.

BASIC PROTOCOL 2

ISOLATION OF *CAMPYLOBACTER* GENOMIC DNA

When isolating *Campylobacter* from an environmental sample, it is necessary to use molecular methods to confirm the bacteria isolated on selective media is in fact *Campylobacter.* The most common method utilized for confirmation is PCR of genomic DNA with primers specific for the *Campylobacter* species of interest (Platts-Mills et al., 2014). In order to utilize this approach and to sequence the genomic DNA, it is necessary to extract high-quality DNA from the suspected *Campylobacter* isolates.

Materials

Genomic Lysis buffer (see recipe)
Campylobacter isolate streaked on CS plate
Protein Precipitation solution (Promega – A7951)
2-proponal
75% Ethanol
Ultrapure water (Thermo Fisher – 10977-015)

Sterile 1.5 ml microcentrifuge tubes
Set of adjustable micropipettes
Sterile pipette tips
Sterile inoculating loops
Ice
Microcentrifuge (Eppendorf – 5424)
Kim wipes

Protocol steps—*Step annotations*

1. Pipette 300 µl genomic lysis buffer into sterile 1.5 ml Eppendorf tubes labeled with the appropriate sample ID.

   To save time and prevent the plated Campylobacter from sitting out for too long, it is best to aliquot the genomic lysis buffer and label all tubes before beginning the extraction process.

2. Using a sterile loop, make 2-3 quick passes across the growth on CS plate and resuspend this in the genomic lysis buffer.

   Only a small amount is necessary for successful purification of Campylobacter gDNA. If there is heavy growth on the plate, one pass may suffice.

3. Pipette 150 µl protein precipitate solution into each tube and securely close.

4. Vortex each tube for 20 seconds, then incubate on ice for 5 minutes.
5. Centrifuge at 18,000 x *g* for 15 minutes. While the tubes are spinning, label another set of 1.5 ml tubes with the appropriate sample names.
6. Remove the tubes from the centrifuge and carefully pour the supernatant into a fresh, labeled 1.5 ml tube.
7. Pipette 750 µl 2-propanol into each tube with the supernatant from the previous step.
8. Cap tightly and mix gently by inverting 35-40 times. Do not vortex.
9. Centrifuge for 5 minutes at 18,000 x *g*.
10. Remove tubes from centrifuge and carefully pour off supernatant.
11. Pipette 500 µl 75% ethanol into each tube, and centrifuge for 5 minutes at 18,000 x *g*.
12. Pour off supernatant, and repeat wash by adding 500 µl 75% ethanol to each tube and centrifuging for 5 minutes at 18,000 x *g*.
13. After the final wash step, pour off supernatant and use a pipette to carefully remove the remaining ethanol.
14. Invert tubes on a kimwipe for 10 minutes to dry the pellets.
15. Suspend each pellet in 100 µl ultrapure water and store at -20°C.

BASIC PROTOCOL 3

PCR CONFIRMATION OF *CAMPYLOBACTER* GENOMIC DNA

While there are several common species of *Campylobacter* found in the environment, the majority of this work focuses on *C. jejuni* and *C. coli* as these are the two most common species isolated from agricultural sources, food, and human infections (Wagenaar et al., 2014). A common method for determining the species *Campylobacter* is by performing PCR with genomic DNA and primers specific for either *C. jejuni* or *C. coli.* To identify *C. jejuni*, primers for the *mapA* gene are commonly used, while primers for the *ceuE* gene are used to identify *C. coli* (Rodgers et al., 2017; Vidal et al., 2016). These primers are then used in a standard PCR along with a species standard. Samples that are not positive for *C. jejuni* or *C. coli* may be another species of *Campylobacter* that can be confirmed via PCR, but those primers are not included in this protocol.

Materials

Purified genomic DNA from suspected *Campylobacter* isolates
Purified *C. jejuni* (81-176) genomic DNA

Purified *C. coli* genomic DNA
Ultrapure water (Invitrogen – 10977-015)
DreamTaq (ThermoScientific – K1081)
MapA F primer – 5'- TCAATGCAGTTCTTGTGAAA -3'
MapA R primer – 5'- TTCAGAGATTAAACTAGCTGC -3'
CeuE F primer – 5'- ATGAAAAAATATTTAGTTTTTGCA -3'
CeuE R primer – 5'- ATTTTATTATTTGTAGCAGCG -3'
Ultrapure water (Thermo Fisher – 10977-015) 10 mg/ml ethidium bromide

PCR tubes
Thermocycler (Applied Biosystems – A24812)
Agarose electrophoresis rig (ThermoScientific – EasyCast B2)
UV light box (UVP – ChemiDoc-It2)

Protocol steps—*Step annotations*

1. Thaw genomic DNA, DreamTaq, primers, and primers on ice while labeling PCR tubes.

2. Prepare each sample according to the following recipe: 2 µl gDNA, 1 µl Forward primer, 1 µl Reverse primer, 8.5 µl water, and 12.5 µl DreamTaq.

   A positive and negative control should be run for each PCR. The positive control uses gDNA from either C. jejuni or C. coli, depending on the primer pairs used. The negative control uses water in place of gDNA.

3. Run a standard PCR under the following conditions: 95°C for 5', 95°C for 30s, 45°C for 30s, 72°C for 1', 72°C for 7', and hold at 4°C.

   If the samples will not be run on a gel right away, tubes should be placed in a freezer.

4. Once PCR is completed, run 10 µl samples of each PCR sample on a 1% agarose gel containing 0.01% (v/v) ethidium bromide gel at 100 V for 1 hour.

5. Visualize DNA in the gel using a UV light box.

   The expected amplicon sizes are as follows: map!, 0.5 kb and ceuE, 0.9 kb (Fig. 1).

BASIC PROTOCOL 4

PREPARATION OF *CAMPYLOBACTER* DNA FOR WHOLE-GENOME SEQUENCING

Whole-genome sequencing as a means for comparison of *Campylobacter* isolates is a relatively new technique. Previously, *Campylobacter* isolates were characterized using Multi-locus sequence typing (MLST) or pulse-field gel electrophoresis (PFGE) (Sheppard et al., 2009; Kittl et al., 2013). Recently, however, there has been a shift towards WGS as a means for source tracking *Campylobacter* isolates (Clark et al., 2016; van Rensburg et al., 2016). To prepare samples for sequencing, the genomic DNA of confirmed *Campylobacter* samples must

be treated to remove contaminating RNA. This is achieved by treating the genomic DNA with RNase to degrade any RNA present before cleaning with a commercial kit. The resulting RNase-treated genomic DNA can then be sent to a core facility for library preparation and whole-genome sequencing.

Materials

Purified genomic DNA of confirmed *Campylobacter* isolates
RNase (NEB – M0243L)
NEBuffer 3 (NEB – B7003S)
Zymo genomic DNA clean and concentrator kit (Zymo – D4011)
37°C and 70°C heat blocks
Nano-drop spectrophotometer
DNA gel loading dye (NEB – B7025S)

Protocol steps – *Step annotations*

1. Thaw genomic DNA samples on ice while labeling 1.5 ml tubes for each sample.
2. Prepare RNase treatment for each sample according to the following recipe: 44 µl gDNA, 1 µl RNase, and 5 µl NEBuffer 3.
3. Incubate gDNA and RNase for 1 hour in a 37°C heat block.
4. Inactivate RNase by incubating for 20 minutes in a 70°C heat block.
5. Following the direction enclosed in the Zymo genomic DNA clean and concentrator kit, clean the RNase-treated sample.
6. Measure the concentration of each sample using a nano-drop spectrophotometer.
7. Run 3 µl of each cleaned sample with 7 µl dye on a 1% agarose gel to check the quality.

   The standard volume of DNA to run on a gel is 10 µl, regardless of the concentration. Once confirmed as Campylobacter-positive, the concentration of a sample will be utilized when preparing for sequencing.

   When observing the gels, lanes with a single, bright band of the appropriate size (mapA=0.5 kb; ceuE=0.9 kb) are considered good quality. Samples with fainter bands may be positive, but the sample may need to be prepared again if the concentration is low (<20 ng/µl) in order to get a higher quality sample.
8. After checking the quality of the DNA, samples can be sent to a core facility for whole-genome sequencing utilizing the Illumina platform.

BASIC PROTOCOL 5

PREPARATION OF *CAMPYLOBACTER* SEQUENCING LIBRARIES

Several sequencing platforms are available to produce genomic data for bioinformatics analysis, but a commonly utilized method for whole-genome sequencing is the Illumina platform (Kaas et al., 2014; Kwong et al., 2015). While different methods can provide variable levels of read length and depth, the Illumina platform produces shorter reads with higher base pair coverage within a genome (Besser et al., 2017). For this reason, the Illumina platform was chosen for higher read depth with shorter reads for downstream *de novo* assembly. Several machines with varying levels of sequencing power are available, however NextSeq machines as described in this protocol as most commonly found in core facilities rather than individual research labs. The RNase-treated genomic DNA from the previous step was sent to a core facility for standard Illumina library preparation, as described below, resulting in 150 bp X 150 bp paired-end reads with 100X coverage produced for each sample of genomic DNA

Materials

RNAse-treated and cleaned genomic DNA
NEXTflex-Rapid-DNA-Seq kit (Bioo Scientific – 514402)
NEXTflex-DNA-Barcode-12 kit (Bioo Scientific – 514102)
Double-sided beadcut
Qubit3 fluorometer (ThermoFisher)
2200 TapeStation bioanalyzer (Agilent Technologies)
NextSeq 500 (Illumina)
NextSeq 150 Mid Output v2 kit (Illumina – FC-404-2001)
Bcl2fastq (Software version 1.4.1.2)

Protocol steps – *Step annotations*

1. Upon receipt of RNAse-treated and cleaned genomic DNA, NextSeq libraries are prepared according to manufacturer's protocol from the NEXTflex-Rapid-DNA-Seq kit.

2. Multiplex sequencing barcodes are added to the libraries during construction following manufacturer's protocol from the NEXTflex-DNA-Barcode-12 kit.

3. The barcoded libraries are cleaned with double-sided beadcut, following manufacturer's protocol.

4. The cleaned libraries are verified with a Qubit3 fluorometer and 2200 TapeStation bioanalyzer following manufacturer's protocols.

5. The cleaned and verified libraries are then pooled together and sequenced on an Illumina NextSeq 500 machine, following the protocol included in the NExtSeq150 Mid Output v2 kit.

6. Resulting paired-end read sequences were de-multiplexed using bcl2fastq software. These reads are then utilized for analysis in Basic Protocol 6.

BASIC PROTOCOL 6

PREPARATION OF *CAMPYLOBACTER* SEQUENCES FOR BIOINFORMATIC ANALYSES

As previously mentioned, whole-genome sequencing for analysis and comparison of *Campylobacter* isolates, especially from environmental sources, is still relatively novel and the potential of WGS as a means of source tracking *Campylobacter* is a technique of interest to many health agencies (Allard et al., 2016; Cody et al., 2017). For these reasons, the ability to appropriately analyze sequencing reads is important. The protocol described below is an outline for preliminary assembly of Illumina reads into a *de novo* genome. Illumina sequence reads for each sample are checked for quality and trimmed of adapters. A series of programs are then utilized for the assembly and annotation of the genomes for each *Campylobacter* sample. While this is a standard analysis pipeline, it may be adjusted based on the type of information that is of interest to the researcher.

Materials

150 X 150 paired end Illumina sequencing reads
Atropos
Prodigal genome annotation software package
Prokka
PanOCT

Protocol steps – *Step annotations*

1. For each sample, quality and adapter trimming of Illumina sequence reads with will be performed using Atropos.
2. Resulting reads are assembled *de novo* using the latest version of SPADES.
3. Upon assembly, protein-coding genes are predicted with Prodigal and RNA genes are predicted using RNAmmer and tRNAscan-SE.

   These programs are all part of the Prokka genome annotation software package.
4. Proteins are annotated using Prokka, as well as hmmscan vs. the PFAM and TIGRFAM databases.
5. Genes are clustered into orthologous groups using PanOCT and labeled as pan- and core- genes as appropriate.
6. A presence/absence matrix can then be constructed for each identified group.

REAGENTS AND SOLUTIONS

Campylobacter Specific (CS) Plates

Suspend 38 grams Mueller Hinton agar in 1 liter of water in a 2 liter Erlenmeyer flask. Autoclave following standard liquid cycle parameters and allow to cool in a 50C warm water bath after autoclaving. Before pouring plates, aseptically add 1 ml of each of the following antimicrobial agent solutions: 40 mg/ml Cefoperazone, 100 mg/ml Cyclohexamide, 10 mg/ml Trimethoprim, 100 mg/ml Vancomycin. Aseptically add 100 ml defibrinated sheep blood and swirl to mix before pouring plates.

Mueller Hinton Agar – (Criterion – C6421)
Cefoperazone – (Alfa Aesar – J65185)
Cyclohexamide – (Acros – 357420250)
Trimethoprim – (RPI – T59000-5.0)
Vancomycin – (VWR – 0990-5G)
Defibrinated Sheep Blood – (Hemostat Labs – CC/153289)

Genomic Lysis Buffer

50 mM Tris Base (pH 7.5) – (Fisher – BP152-1)
50 mM EDTA (Acros – 118430010)
1% SDS (Fisher – BP166-500)
10 mM NaCl
*Filter sterilize final solution before use.

COMMENTARY

Background Information

While several methods exist for the isolation of *Campylobacter* from various sample sources, the majority utilize selective media as a means to prevent other organisms from overgrowing *Campylobacter.* Historically, *Campylobacter* selective media was comprised of a mix of antibiotics and supplemented in some cases with either sheep or horse blood (Bolton et al., 1984; Queiroz et al., 1987). In some cases, a broth enrichment step was included before plating (Baylis et al., 2000; Berndtson et al., 1992), while direct plating of samples was utilized by others (Musgrove et al., 2001). Other methods also use vacuum filtration, but plate filters directly (Steele et al., 1984) rather than the rinsing approach described here. The sample preparation and plating methods described in this protocol are standard among the field and can be adapted for a variety of samples. The methods presented for the characterization of samples via PCR are also standard in the field. However, other methods such as agglutination tests and multiplex PCR are also utilized (Linton et al, 1997; Lior 2018; Wang et al, 2002). While whole-genome sequencing analysis of *Campylobacter* isolates is still relatively novel, the majority of previous genomic classification of *Campylobacter* comes from multi-locus sequence typing and pulse-field gel electrophoresis (Cody et al., 2013; Kittl et al., 2013). The methods described in this protocol provide a pipeline for the analysis and comparison of environmental and human *Campylobacter* isolates that may provide useful information for microbial source-tracking.

Critical Parameters

It is important that samples be prepared and plated as soon as possible after collection to increase the chances of successfully isolating *Campylobacter*. Additionally, when working with isolates streaked on CS plates, the amount of time outside of a microaerophilic environment should be minimized to maintain viability of organisms on the plates.

Troubleshooting

In some cases, such as with poultry fecal samples, an abundance of *Campylobacter* may be present resulting in lawns of bacteria on the dilution plates. When this occurs, it is necessary to use a very small amount of bacteria and a sterile loop to streak for isolation on fresh CS plates. This should provide isolated colonies for further analysis. Additionally, cattle fecal samples may result in the growth of a fibrous white mold when diluted on CS plates. Plates with this mold should not be used for further analysis in order to prevent contamination. It may be useful to streak suspected *Campylobacter* colonies on fresh CS plates before further work to ensure pure colonies.

When conducting PCR confirmation of *Campylobacter,* the positive control may not result in a visible band when run on an acrylamide gel. While this is not common, it may occur and the solution is to ensure the genomic DNA utilized has not been thawed and refrozen multiple times. If this becomes a problem, new genomic DNA should be prepared for each control.

Understanding Results

When performing PCR confirmation for *Campylobacter* environmental isolates, the positive control should result in a band for either *mapA* (~0.5 kb) or *ceuE* (~0.9 kb). These control bands should be utilized to confirm whether an organism is either *Campylobacter jejuni (mapA)* or *Campylobacter coli (ceuE).* This protocol only focuses on the isolation and analysis of these two species, but it is possibly to use primers for other species as well, if others are of interest. Additionally, the selective media used in this protocol may not completely prevent the growth of non-*Campylobacter* organisms in all cases, as *Roseobacter* has been found to grow on CS plates. This demonstrates the importance of verifying isolates as *Campylobacter* via PCR.

Ideal sequencing results should include total reads in the millions for each sample. This will also provide for an average of 100X coverage for all nucleotides in the genome. The %GC content of the resulting reads should average ~31%, as *Campylobacter* is AT-rich and has a relatively low GC content.

After quality control and trimming of reads, the *de novo* assembly of reads for each isolate should result in a genome of ~1.7Mb. This can vary slightly between species, but *Campylobacter* averages 1.7 Mb in the genome.

Time Considerations

- Sample preparation for water and fecal samples – 1 day for plating
- Sample preparation for food samples – 18-24 hours for enrichment, 1 day for plating
- Incubation period for all diluted samples – 48 hours
- Genomic DNA preparation – 2 hours (depends on number of samples, >20 samples will take longer)
- PCR – 1.5 hours
- Running PCR samples on a gel – 1 hour
- RNase treatment – 2 hours (depends on number of samples, >20 samples will take longer)
- Cleaning RNase-treated samples – 1 hour (depends on number of samples, may be more or less)
- Shipment to core facility – overnighted
- Library preparation – 1-2 days, depending on number of samples
- Sequencing – 3-4 days, depending on number of samples and efficiency of machines
- Quality control and trimming of sequencing reads – 1 week (depends on number of isolates and computing power, may be more or less)
- *de novo* assembly – 1 week (depends on number of samples and computing power, may be more or less)
- Genome annotation – 1-2 weeks
- Clustering of genes – 1-2 weeks

Further time considerations for bioinformatics analysis will vary based on the types of analysis performed and the research interests of the investigator.

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INTERNET RESOURCES (optional)

Atropos - https://github.com/jdidion/atropos

SPADES – http://cab.spbu.ru/software/spades

Prodigal – http://prodigal.ornl.gov

Prokka - http://www.vicbioinformatics.com/software.prokka.shtml

Hmmscan - https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan

Pfam - https://pfam.xfam.org

TIGRFAM - http://www.jcvi.org/cgi-bin/tigrfams/index.cgi

PanOCT - https://www.jcvi.org/gcid/project/panoct

FIGURE LEGENDS

Figure 1. An agarose gel of various environmental isolates obtained through the isolation techniques described previously. Reactions containing primers for *mapA* are on top while reactions for *ceuE* are on bottom. The positive control for each sample is the last lane on the right and labeled (+).