

DETERMINING THE SCOPE OF *CAMPYLOBACTER* INFECTION AND ITS POST-INFECTIOUS
SYMPTOMS

by

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ABSTRACT

Campylobacter species are the most common bacterial cause of diarrheal illness in the United States and are estimated to cause up to 1.5 million cases of illness per year in the U.S. alone. There has been increasing evidence that after an acute infection by *Campylobacter spp.*, some symptoms may persist and develop further comorbidities such as post-infectious irritable bowel syndrome (PI-IBS). This prospective cohort study aims to determine the relationship between acute symptoms and the incidence of PI-IBS after a diagnosis of campylobacteriosis. Within 6-months of follow-up, of 119 patients with laboratory confirmed *Campylobacter* infection, there were 19 cases with symptoms fitting the criteria of PI-IBS leading to an incidence of 16% in this population. No acute symptoms were shown to be significant except the presence of chronic conditions before acute infection, which showed a positive association with developing PI-IBS (RR: 2.37, 95% CI: 1.00, 5.58). Further characterization of acute symptoms of campylobacteriosis as risk factors for PI-IBS is an important step to understanding the next steps of preventing its occurrence. The sporadic nature of *Campylobacter* infections leads to an underreporting to our public health agencies and thus an under-estimate of their true burden. The analysis of epidemiological interview data with genomic data will increase the likelihood of finding clusters of cases that were not detected through public health surveillance alone. 2018-2019 data from the FoodNet catchment area in the Denver, Colorado area was analyzed via whole genome sequencing (WGS) for clusters of *Campylobacter* cases, where six clusters were identified that were not originally linked to each other through the epidemiologic data alone. Three clusters had strains with the same gene sequence type (ST) and core genome multi-locus sequence type (cgMLST). WGS may prove critical in accurately identifying previously unidentified *Campylobacter* clusters when supported by epidemiological data.

INTRODUCTION

Explanation of the Problem

Campylobacter species are the most common bacterial cause of diarrheal illness in the United States and are estimated to cause around 800,000 cases and potentially up to 1.5 million cases per year in the U.S. alone^{1,2,8}. An incidence rate of 19.5 per 100,000 cases in 2019 was estimated to occur within FoodNet locations, which are active surveillance sites supported by the Centers for Disease Control and Prevention (CDC). This rate followed a 13% increase in incidence from 2016-2018³. Economically, the burden of this pathogen in the U.S. has been reported to be \$1.7 to \$1.9 billion per year and ranks within the top five bacterial causes of foodborne illnesses^{2,4}. Globally, *Campylobacter spp.* infections are endemic in many regions of the world and are speculated to be increasing^{8,10}. However, many reports of campylobacteriosis go unreported each year since cases are sporadic and often are not linked to any recognized outbreaks which can mask the true burden of disease. Detecting cases or outbreaks are challenging because food may become contaminated by multiple pathogens, transmission can

occur by non-food mechanisms, and only a small proportion of illnesses are confirmed by laboratory testing and reported to public health agencies⁵. Being able to detect clusters of campylobacteriosis cases from sporadic cases would lead to increased information about the source of infection. This would decrease further propagation of infection and healthcare costs.

There has been increasing evidence that even after the acute onset of symptoms by *Campylobacter* infection, some symptoms may persist and lead to further comorbidities^{1,2,9}. Post-infectious irritable bowel syndrome (PI-IBS) is a growing concern along with Guillain-Barré Syndrome (GBS) and reactive arthritis (ReA). These long-term outcomes can cause further complications in people's daily lives and additional medical costs^{2,4}. PI-IBS, in particular, has been shown to occur around the world with an estimated prevalence rate of around 4-36% after initial infection, depending on the population^{14,15}. The mechanism of initial infection to persisting symptomology is not fully understood and requires more research to identify the risk factors that drive it.

I. Specific Aims: A Pilot Cohort on *Campylobacter* spp. and Post-Infectious IBS

Goal: To conduct a pilot cohort as a continuation of a previous pilot study to evaluate the incidence of PI-IBS following an acute infection with *Campylobacter*.

Study: We enrolled participants into a follow-up study to record their symptoms at initial onset, 6 weeks, 3 months, and 6 months. The presence of certain symptoms will be used to define the occurrence of PI-IBS and analyzed to investigate if there are any associations that may increase the risk of disease. Based on the identified associations, a better understanding of risk factors for PI-IBS can be used to inform prevention methods.

II. Specific Aims: Linking Epidemiologic and Genomic Data for *Campylobacter* Isolates in Colorado

Goal: To analyze epidemiological and genomic *Campylobacter* spp., case patient data from the Colorado Department of Public Health and Environment (CDPHE) to identify clusters of cases that were not detected through public health surveillance alone.

Study: This was accomplished by analyzing sequencing data of *Campylobacter* isolates from patient stool samples and existing interview data to determine if any associations are present. Associations within the clusters will be analyzed to identify any correlations between the participant's interview data and genomic data to better understand how the different types of data can supplement and improve one another.

REVIEW OF THE LITERATURE

I. *Campylobacter*

Campylobacter species are gram negative, rod-shaped or curved, microaerophilic bacteria with a flagellum or flagella. They require decreased amounts of oxygen to thrive and most of them are chemoorganotrophs, meaning they require amino acids or tricarboxylic-acid cycle intermediates to generate energy^{8,17-19}. They live as commensal organisms in many birds and mammals and are able to thrive without causing disease to the host. Chickens make up approximately 80% of the zoonotic transmissions with humans^{17,18}. For human foodborne illnesses, the species *C. jejuni* and *C. coli*, account for the majority of infections²⁴. The sources of *Campylobacter* infection in humans can range from raw or undercooked meats such as poultry, to untreated water and contact with puppies^{7,17}. Since the route of transmission is fecal-oral, prevention can be as simple as washing your hands and practicing safe food preparation. However, given the potentially very low infectious dose of >360 colony forming units (CFU)⁸,

infections can be easily acquired. Symptoms usually begin 2-5 days after initial infection and persist an average of 6 days^{1,8}. For reference, infected chicken feces could contain up to 10^5 - 10^8 CFU/g¹⁷. Common symptoms include diarrhea, bloody diarrhea, fever, and stomach pain and may be accompanied with nausea or vomiting^{8,17}. Treatment of campylobacteriosis can consist of using antibiotics such as azithromycin, erythromycin, and ciprofloxacin prescribed by a healthcare provider although most infections will resolve without treatment⁸.

Reports of campylobacteriosis often go unreported and mask the true burden of disease. Scallan et.al. estimate that for every one case that is reported to public health, an additional 30 cases are unreported⁵. This is due to a variety of reasons such as the multiple stages of reporting that must occur before it reaches the health department (see Figure 1) and the relative mild severity of symptoms for most cases. The Foodborne Diseases Active Surveillance Network (FoodNet) conducts enhanced surveillance in specific sites for *Campylobacter* infections and encompasses about 15% of the U.S. population. Established in July 1995 by the Centers for Disease Control and Prevention (CDC), it collaborates with 10 state health departments along with the U.S. Department of Agriculture (USDA) and Food and Drug Administration (FDA)²⁹. To reach the state health department or FoodNet, the reporting of foodborne illnesses must go through multiple steps which is represented by the burden of illness model (Figure 1). The burden of illness model illustrates seven steps starting from acute infection by the pathogen to the health department being notified, assuming correct diagnosis and procedure taken by health professionals. This decreases the number of cases that can be reported and increases the amount of time until the health department receives the report.

II. Campylobacter and IBS

Irritable Bowel Syndrome (IBS) is a common disorder with symptoms such as abdominal pain, bloating, and diarrhea or constipation. Sporadic IBS and PI-IBS are clinically indistinguishable but mainly arise from different sources. IBS occurs globally with a pooled prevalence rate of 11.2% with some regions having a rate as high as 45%²⁸. There are 4 categories of IBS subtypes that depend on the frequency of stool types: IBS with predominant constipation (IBS-C), IBS with predominant diarrhea (IBS-D), mixed IBS (IBS-M), and un-subtyped IBS (IBS-U)²⁸. To have a particular predominant subtype, that stool type must be prevalent more than 25% of the time while the other stool type is prevalent less than 25% of the time spent for bowel movements. Stool types can be categorized into Bristol stool types (Figure 2) where there are 7 types, and each are distinctly different²⁷. The diagnosis for IBS is primarily based on the presence of certain symptoms, namely persistent abdominal pain, and exclusion of other diseases. There is no definitive biomarker or lab test that can be used to reliably diagnose IBS, as of yet²⁸. The condition is usually chronic, and symptoms often need to be managed with medication and changes in diet and lifestyle as there is no definitive treatment. Risk factors for developing IBS include younger age, female sex, family history, and psychological factors²⁸. Risk factors for developing PI-IBS are similar and include younger age, female sex, severity and persistency of acute infection, psychological factors, and the infectious agent¹²⁻¹⁵. Review studies of PI-IBS have found higher incidence following parasitic infections (6.9 - 80.5%), followed by bacterial (4.3 - 45.8%) and viral agents (0.4 - 22.4%)¹⁵.

Several studies have shown that persistent inflammation from the lymphatic system in the gut may also be responsible for the ongoing symptoms. Changes in the gut microbiota in IBS patients have also been noted in some studies, but more investigation is required to determine which bacteria are largely responsible^{14,28}. A possible theory involves cytolethal distending toxin (CDT) and vinculin where immune recognition of CDT secreted by *Campylobacter spp.* causes vinculin, a cytoskeletal protein in human epithelial cells, to be ‘attacked’ mistakenly by the immune system. This can lead to chronic inflammation in the gut as epithelial cells are affected. Another possible theory involves *Campylobacter spp.* being able to ‘slip’ through tight junctions between gut epithelial cells and cause increased permeability for other bacteria to permeate the epithelial barrier³³. These mechanisms whereby an infection with *Campylobacter* may lead to PI-IBS are still being investigated and further evidence is needed to confirm and understand these associations.

III. Genetic Epidemiology of *Campylobacter*

Antimicrobial resistance in *Campylobacter spp.* has dramatically increased since the beginning of the 2000s. Studies show that fluoroquinolone resistance increased from approximately 22% to 63.2%, while tetracyclines and macrolides have also been speculated to be increasing in resistance, such that the emergence of multi-drug resistant (MDR) strains has become a problem^{8,18,21}. In Europe and the U.S., antibiotic resistance rates in *C. coli* is higher than in *C. jejuni*. The emergence of antibiotic resistance can be attributed to the routine/growing

use of antibiotics in the treatment of infections among poultry. This resistance is most pronounced against fluoroquinolones¹⁷.

There are many virulence factors that *Campylobacter spp.* employ to invade the gut epithelium, including various adhesion proteins and flagella. *cadF* and *capA* genes both code for adhesion proteins that are used for binding to human epithelial cells. A mutation in these genes has shown to decrease the ability to bind and invade into cells¹⁸. Genes that code for flagellar proteins include *flaA*, *flaB*, *flgB*, *flaC*, and *flgE* and a mutation in these will also decrease the pathogen's ability to form a flagellum and invade. The *Campylobacter* invasion antigen genes (*cia* genes) along with *flaC* are different in that they are transported into the host cell for invasion as opposed to forming the subunits of flagella^{18,32}. There is only one confirmed toxin produced by *C. jejuni*, cytolethal distending toxin that is made up of 3 subunits coded by the *cdtA*, *cdtB*, and *cdtC* genes. This protein specifically inhibits the process of mitosis in the invaded host cell until cell death occurs¹⁸.

Sequencing of the *Campylobacter spp.* genome has been on the rise and has provided much more information on the details of the pathogen's virulence factors, pathogenesis and physiology. Several studies have turned to whole genome sequencing (WGS) to find hidden clusters in seemingly sporadic cases of campylobacteriosis²¹⁻²³. The ability to look at the entire genome of a strain and compare them to other strains is a more accurate and precise method compared to earlier technologies such as pulsed field gel electrophoresis (PFGE). WGS requires more computational analysis, but is very cost-effective. Estimates of cost and time for around 20 bacterial isolates are approximately \$240 and results are available within 2.5-3 days³⁰. Compared

to previous methods, WGS had increased discriminatory power but required more time and was more efficient with larger batches, in 2017³⁰. Today, WGS has started to become more efficient in sequencing smaller batches so that research labs are able to run comparisons for cases in a shorter amount of time. This implication could mean that public health surveillance for diseases could have decreased hand-off time and increase the likelihood of detection for acute infections. The CDC has already started to implement WGS use in a similar agency to FoodNet known as PulseNet which was established in 1996 and set out to have labs within each state to perform routine foodborne surveillance³⁴.

Note: From here on out, language indicating IBS will be synonymous to PI-IBS since they are clinically indistinguishable by symptoms and all participants in the studies had an acute infection by *Campylobacter* species.

METHODS

I. *Campylobacter* Pilot Cohort

Study participants were recruited following routine foodborne disease case investigations with the Maricopa County Department of Public Health (MCDPH). Interviewers were part of the Student Aid for Field Epidemiology (SAFER) course at the University of Arizona and are trained to conduct routine surveys and enrollment procedures²⁵. As part of standard public health surveillance, people with laboratory confirmed infections with *Campylobacter* spp. are contacted over the phone and interviewed using a standardized MCDPH Qualtrics survey regarding symptoms and risk factors, such as specific foods, animal exposure, and animal product exposure. Following the surveillance interview, case patients were asked if they were interested in learning about a longer-term study related to their infection. If they said yes, consent forms were completed for every participant over the phone, through email, or through mail upon request. For children who have been infected (<18 yrs. old), consent from their parent/guardian

was needed to participate in the study as well as assent from the child themselves. People who had co-infections from other pathogens at the same time were excluded, except for *E. coli* that only included one case. Once recruited into the study, a baseline assessment on their general health (mobility, mental health, etc.) was taken, followed by the same 10-to-15-minute survey given over the phone at the 6-week, 3-month, and 6-month follow-up from their initial symptom onset date. The survey included questions about any gastrointestinal symptoms, neurological symptoms, and joint symptoms along with their general quality of health. The surveys were available in both English and Spanish and participants could choose their language preference. For children aged 14-17 years old, the parent was asked permission to speak to the case directly. The survey was given to the parent/guardian if a child was not able or comfortable enough to answer the questions. For younger children below 14 years old, the parent or guardian completed the interview. If participants had at least 5 call attempts without a 6-week or a 3-month survey, or if they had a 6-week and/or a 3-month survey and were not able to be reached for a 6-month survey after 5 call attempts, they were considered lost to follow-up. Recruitment for the study started in February 2019 and ended in September 2020 with the last 6 months follow-up survey completed in January 2021.

A total of 86 surveys at the 6-month mark were completed after exclusions, refusals, no consent signed, and lost to follow-up from the 303 initially recruited cases of campylobacteriosis case patients, leading to a completion rate 28.4% (Figure 3). Rome IV criteria for IBS were used to evaluate the presence of IBS-related symptoms²⁷. The criteria included recurring abdominal pain for at least 1 day/week in the last 3 months and needed to be accompanied by two of the

following symptoms at least: symptoms related to defecation, a change in frequency of stool, and a change in the form of the stool²⁷.

A secondary goal of this study was to determine if participants would reliably provide a stool sample following enrollment. After initial infection, shedding of the pathogen in the stool decreases over time so a stool sample immediately following acute infection would have a greater chance to contain the pathogen. In August 2020, participants at the 3-month and 6-month mark were asked if they would consent to providing a stool sample for a \$25 Amazon gift card incentive. Only 10 were consented (out of around 30 cases) and only 4 stool sample kits were received by the lab. Further stool analysis and sequencing has been delayed due to COVID-19 circumstances.

Statistical Analysis:

Descriptive statistics were used to describe the demographics and symptoms of participants including counts and frequencies. Relative risks were calculated to analyze which demographic and symptomology variables at time of acute infection would lead to an increased or decreased risk of developing PI-IBS. A two-sample t-test was also used to analyze the mean difference in diarrhea duration. Those with prior IBS diagnosis to initial infection were excluded from analysis. Further analysis was completed only on those who had a completed 3-month survey and/or those who had a completed 6-month survey. Since PI-IBS requires at least 3 months of symptoms, those who were surveyed at the 3-month mark were included, even if they did not complete a 6-month survey. Relative risks were also calculated for each symptomology

variable at the 3-month and 6-month follow-ups. In total, there were 33 additional individuals that were added from the 3-month survey period that had valid consent and interview data. All statistical analysis was performed using SAS University Edition.

II. Linking of *Campylobacter* and Genomic Analysis

Interview data and stool isolate information from the Colorado Department of Public Health and Environment (CDPHE) was used to conduct this analysis. This secondary data analysis includes *Campylobacter* cases reported in the Denver FoodNet catchment area from 2018-2019 along with a subsample of WGS isolate data. For each of the 50 strains, genomic analyses included a two-pronged approach: single nucleotide polymorphism (SNP) analysis and comparative genomics using a variety of web-based programs to compare the genomes. Web-based analysis employed several websites for rapid computational analysis of each strain for specific genomic characteristics of the different strains. Online-based tools included: Center for Genomic Epidemiology (CGE) (<http://www.genomicepidemiology.org/>)³⁵, VFAnalyzer (<http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFAnalyzer>)³⁶, PHASTER (<http://phaster.ca/>)³⁷, and PubMLST (https://pubmlst.org/bigdb?db=pubmlst_campylobacter_seqdef&page=batchSequenceQuery)³⁸. The web-based variables for every strain included acquired resistance genes (settings included *Campylobacter* species, 90% threshold with 60% minimum length), known/unknown chromosomal mutations for antibiotic resistance like fluoroquinolones (settings included *Campylobacter* species, 90% threshold with 60% minimum length), k-mer based search for antibiotic resistance genes (default settings), prophages present in the genome using phage search tool enhanced release (PHASTER) results (default settings), virulence profile of each strain including missing virulence factors, lipooligosaccharide (LOS) genes, capsule genes, and total virulence factors (settings included *Campylobacter*, raw FASTA sequences of a draft genome, and all *C. jejuni* genomes for comparison), matched pathogenic family gene numbers (settings included ϵ -proteobacteria, and assembled genome/contigs), 7 gene multilocus sequence

typing (MLST), core genome multilocus sequence typing (cgMLST), and clonal complexes (settings included *C. jejuni/C. coli* cgMLST v1.0 locus/scheme, ordered by locus). Core genome analysis was completed using Geneious Prime³⁹, a program that includes a suite of different bioinformatic tools for comparative genomic analysis. Using the *Campylobacter jejuni* subsp. *jejuni* strain NCTC11168 genome as a reference genome, 102 core genes in all strains were identified using BLAST analysis (>95% identity) in Geneious Prime. Due to missing sequence data in the draft genome sequences, a total of 36 core genes were identified in all the genomes and used for phylogenetic analysis of the strains. Geneious Prime was used to concatenate the core genes together for each of the strains, a MUSCLE alignment was conducted using the Geneious Prime plugin, and a maximum-likelihood tree was generated using MEGA X software⁴⁰ with 1000 pseudoreplicates for statistical comparison. After compiling all the information, epidemiological and genomic comparisons could be made for all the strains. Two phylogenetic trees were generated using either SNP differences or the set of core genes, and were found to be similar.

Statistical Analysis:

Information from the web-based genomic programs were compiled into a dataset and merged with the epidemiological data using SAS University Edition and checked for duplicates of which there were 4 in total. These duplicates arose since 4 of the case patients had 2 stool sample isolates for each of them, resulting in duplicates when crossed with the epidemiological data. Descriptive statistics were used to describe the demographics and symptoms including counts and frequencies. Chi-squared analysis was used to compare across different categorical variables along with the use of additional genetic software. All analyses were completed using STATA 16 (Stata Corp., College Station, TX, USA), SAS University Edition, and additional genetic software. All tests were two-sided, and an alpha-level was established at 5% for statistical significance.

RESULTS

I. Campylobacter Pilot Cohort

There were 119 total cases who had campylobacteriosis at baseline and completed the 3-month and/or 6-month surveys. Based on the inclusion and exclusion criteria shown in Figure 4, there were 19 participants with the symptomology of IBS (IBS cases) and 100 participants who did not meet the criteria (non-IBS comparison group). The incidence rate was thus 16%. Of the 19 IBS cases, 11.5% met the definition of incident IBS at 3-months (n=12) and 9.3% at 6-months (n=8), with one case having IBS symptoms at both time points. At time of acute infection, factors such as age, spoken language, and case classification, were not significantly different between the IBS cases and controls (Table 1). Prescription of antibiotics and type of antibiotic used were also not associated with an increased risk for PI-IBS. Relative risk for race was not calculated as the population was predominantly white.

Table 1. Demographic Characteristics of Incident IBS Cases and Controls at Time of Acute *Campylobacter* Infection

	Baseline (N=119)		
	Cases with IBS N=19	Non-Cases N=100	
	N (%)	N (%)	RR (95% CI)
Age (yrs. old)			
0-18	6 (31.6)	30 (30.0)	1.50 (0.41, 5.47)
19-29	0	9 (9.0)	0.40 (0.02, 7.08)
30-39	3 (15.8)	9 (9.0)	2.25 (0.53, 9.58)
40-49	2 (10.5)	13 (13.0)	1.20 (0.22, 6.40)
50-59	5 (26.3)	15 (15.0)	2.25 (0.61, 8.33)
≥60	3 (15.8)	24 (24.0)	Reference
Gender			
Female	10 (52.6)	48 (48.0)	1.69 (0.51, 2.67)
Race			
White	16 (84.2)	71 (71.0)	
African American	0	1 (1.0)	
American Indian	0	2 (2.0)	
Other (Multi-racial)	2 (10.5)	25 (25.0)	
Ethnicity			
Hispanic	7 (36.8)	34 (34.0)	1.11 (0.47, 2.60)
Spoken Language			
English	15 (79.0)	76 (76.0)	1.07 (0.39, 2.95)
Spanish	4 (21.0)	22 (22.0)	
Case Classification			
Confirmed	11 (57.9)	65 (65.0)	0.78 (0.34, 1.78)
Probable	8 (42.1)	35 (35.0)	
Took antibiotic for infection	16 (84.2)	80 (80.0)	1.28 (0.41, 4.02)
Antibiotic used*			
Azithromycin	9 (47.4)	33 (33.0)	1.81 (0.73, 4.46)
Other (Erythromycin, Ciprofloxacin, Metronidazole)	3 (15.8)	15 (15.0)	1.28 (0.29, 5.59)
Took antibiotic 30 days before infection	2 (10.5)	9 (9.0)	1.16 (0.31, 4.36)
Hospitalized or ED/ Urgent Care	12 (63.2)	48 (48.0)	1.69 (0.71, 3.98)
Owns a pet	17 (89.5)	80 (80.0)	1.93 (0.48, 7.74)
Reptile exposure	2 (10.5)	6 (6.0)	1.63 (0.46, 5.86)

*Reference group were those who did not take antibiotics

Most of the 119 Campylobacteriosis case patients had at least one symptom at the initial onset of campylobacteriosis (Table 2) and was with an average age of ~40 (range 0-89) and with a nearly equal number of males (51.3%) and females (48.7%). The population was predominantly White with only a few participants being African American, American Indian, or multi-racial backgrounds. Among the symptoms assessed on the surveys, none were statistically significantly associated with an increased risk of developing PI-IBS. Chronic conditions showed a positive association with developing PI-IBS, but it was not statistically significant (RR: 2.37, 95% CI: 1.00, 5.58). Diarrhea duration was also analyzed, but a t-test showed no significant difference between the non-IBS cases and the IBS cases. Of those that completed a 6-month survey and/or a 3-month survey: there were 69 surveys at 6-weeks, 104 surveys at 3-months, and 86 surveys at 6-months post initial infection (70 people completed both 3-month and 6-month surveys). At the 6-week survey, the most prevalent symptom was loose stools (46.4%) followed by the feeling of 'feeling full quickly' (30.4%) and diarrhea (29%). Interestingly, loose stools were present in as many as 41.3% of the non-IBS cases at 3-months and 34.6% of the non-IBS cases at 6-months (Table 3). At the 3-month mark, the most prevalent symptom in non-IBS cases was loose stools (41.3%) with acid reflux (31.5%) here in non-IBS cases being the most prevalent among the three follow-up times. Abdominal pain and at least two of the following symptoms: loose stools, constipation, and diarrhea were all used to define the PI-IBS cases, so their prevalence is high in the IBS cases, but they are much higher than the other symptoms even among just the IBS cases. Acid reflux and the feeling of feeling full quickly also remained high among the IBS cases at the 3 and 6-month marks. Anxiety and depression were also significantly and positively associated with risk of PI-IBS at the 6-month follow-up with a RR of 3.78 (95%

CI: 1.05, 13.65) along with the presence of other symptoms not previously listed, such as hair loss, dizziness, and fatigue (RR: 6.17, 95% CI: 1.78, 21.41).

Table 2. Symptoms of Incident IBS Cases and Non-Cases at Time of Acute Infection

	Baseline (N=119)		RR (95% CI)
	Cases with IBS N=19	Non-Cases N=100	
	N (%)	N (%)	
Diarrhea	18 (94.7)	99 (99.0)	0.31 (0.07,1.31)
Fever	14 (73.7)	83 (83.0)	0.64 (0.26, 1.58)
Nausea	11 (57.9)	54 (54.0)	1.14 (0.50, 2.64)
Vomiting	5 (27.8)	38 (38.0)	0.63 (0.24, 1.63)
Abdominal Pain	18 (94.7)	80 (80.0)	3.86 (0.54, 27.32)
Headache	11 (57.9)	56 (56.0)	1.07 (0.46, 2.46)
Chronic conditions Arthritis, Diabetes, others)	12 (63.2)	38 (38.0)	2.37 (1.00, 5.58)
Bloody Diarrhea	8 (42.1)	37 (37.0)	1.20 (0.52, 2.75)
Diarrhea Duration Days	Mean 8.50	Mean 9.17	t-test p-value 0.67

Table 3. Symptoms of IBS Cases Stratified by Interview Time Points							
	6 Weeks (N=69)	3 Months (N=104)			6 Months (N=86)		
		IBS Cases N=12	Non- Cases N=92	RR (95% CI)	IBS Cases N=8	Non- Cases N=78	RR (95% CI)
Abdominal Pain*	18 (26.1)	12 (100)	17 (18.5)	63.33 (3.87, 1036.28)	8 (100)	12 (15.4)	54.24 (3.27, 900.70)
Diarrhea*	20 (29.0)	9 (75.0)	24 (26.1)	6.45 (1.87, 22.30)	8 (100)	18 (23.1)	38.41 (2.30, 641.79)
Loose stool*	32 (46.4)	12 (100)	38 (41.3)	26.96 (1.64, 443.78)	8 (100)	27 (34.6)	24.56 (1.46, 412.12)
Constipation*	10 (14.5)	8 (66.7)	22 (23.9)	4.93 (1.61, 15.16)	4 (50.0)	14 (18.0)	3.78 (1.05, 13.65)
Bloating	15 (22.1)	8 (66.7)	28 (30.4)	3.78 (1.22, 11.70)	7 (87.5)	15 (19.2)	20.36 (2.65, 156.38)
Blood in Stool	2 (2.9)	0	6 (6.5)	0.57 (0.04, 8.59)	1 (12.5)	1 (1.3)	6.00 (1.26, 28.46)
Blood in Toilet Paper	10 (14.5)	1 (8.3)	9 (9.8)	0.85 (0.13, 5.95)	1 (12.5)	9 (11.5)	1.09 (0.15, 7.93)
Feeling of not Emptying Bowels	17 (24.6)	5 (41.7)	24 (26.1)	1.85 (0.64, 5.36)	4 (50.0)	15 (19.2)	3.53 (0.97, 12.80)
Waking in Middle of Night for Toilet	8 (11.6)	6 (50.0)	8 (8.7)	6.43 (2.41, 17.16)	2 (25.0)	11 (14.1)	1.87 (0.42, 8.29)
Difficulty Swallowing	3 (4.4)	1 (8.3)	7 (7.6)	1.09 (0.16, 7.41)	2 (25.0)	0	14.0 (6.48, 30.27)
Feeling Full Quickly	21 (30.4)	5 (41.7)	23 (25.0)	1.94 (0.67, 5.61)	6 (75.0)	13 (16.7)	10.58 (2.32, 48.23)
Acid Reflux	13 (18.8)	6 (50.0)	29 (31.5)	1.97 (0.69, 5.67)	5 (62.5)	21 (26.9)	3.85 (0.99, 14.91)
Weight loss>10lbs.	3 (4.9)	1 (8.3)	3 (3.5)	2.14 (0.36, 12.74)	0	2 (2.6)	1.67 (0.12, 22.65)
Anxiety or Depression	11 (18.3)	4 (33.3)	18 (20.9)	1.73 (0.57, 5.20)	4 (50.0)	14 (18.0)	3.78 (1.05, 13.65)
Arthritic Symptoms	15 (21.7)	3 (25.0)	20 (21.7)	1.17 (0.34, 3.98)	2 (25.0)	13 (16.6)	1.58 (0.35, 7.07)
Neurological Symptoms	10 (14.5)	4 (33.3)	13 (14.1)	2.56 (0.87, 7.55)	4 (50.0)	11 (14.1)	4.73 (1.33, 16.84)
Other Symptoms	19 (27.5)	2 (16.7)	20 (21.7)	0.75 (0.18, 3.16)	4 (50.0)	8 (10.3)	6.17 (1.78, 21.41)

*Abdominal pain, constipation, loose stools, and diarrhea were all used in the definition of incident-IBS
** Bold font indicates p-value<0.05 and statistical significance

II. Linking of *Campylobacter* and Genomic Analysis

One hundred fifteen campylobacteriosis cases from the Colorado study had epidemiological interview exposure data, and 50 of the 115 were matched to a bacterial isolate recovered from a stool sample collected during the time-period. All cases were included in the analyses. Most cases were 18-49 years old, male (54.8%), non-Hispanic (76.5%), and White (78.3%). Many cases resided in Denver, Arapahoe, Jefferson, and Boulder counties. Chi-square tests showed no significant differences in demographic characteristics between those matched with an isolate compared to those who were not (Table 4).

The majority of cases with epidemiological data were confirmed cases as defined by CDPHE, and not hospitalized (93.0%). Over half (55.7%) used antibiotics, but most cases were unsure of which antibiotic was used (46.1%) or were prescribed Azithromycin (30.4%). The majority of cases had diarrhea (90.4%), which was not bloody (43.5%), and fever (55.7%). Most cases did not travel 7 days prior to their illness onset (60.0%). For cases matched with an isolate, the same trends were seen, and chi-square analyses showed no significant differences in clinical data between those matched with an isolate compared to those without, except for case status (Table 5). This is to be expected because an isolate from a stool sample is needed to meet the confirmed case definition by CDPHE. Table 6 shows the specific genomic profiles of each stool isolate with the associated symptoms and sequence types.

Table 4. Demographic characteristics of Colorado FoodNet Cases

	All Epi data N=115	Epi + Genomic data N=50
	N (%)	N (%)
Age (yr)		
0-4	11 (9.6)	5 (10)
5-17	13 (11.3)	6 (12)
18-49	62 (53.9)	27 (54)
50-64	18 (15.7)	7 (14)
≥65	10 (8.7)	5 (10)
Gender		
Male	63 (54.8)	26 (52)
Female	52 (45.2)	24 (48)
Race		
White	90 (78.3)	39 (78)
Black	5 (4.3)	1 (2)
Asian	3 (2.6)	2 (4)
Multi-racial	3 (2.6)	1 (2)
Other	7 (6.1)	2 (4)
Ethnicity		
Hispanic	18 (15.7)	9 (18)
Non-Hispanic	88 (76.5)	37 (74)
Linked to recognized outbreak		
Yes	3 (2.6)	0
No	69 (60)	33 (66)
County of residence		
Adams	11 (9.6)	3 (6)
Arapahoe	18 (15.7)	8 (16)
Boulder	13 (11.3)	4 (8)
Broomfield	3 (2.6)	0
Denver	29 (25.2)	11 (22)
Douglas	8 (7.0)	2 (4)
Jefferson	18 (15.7)	7 (14)
El Paso	7 (6.1)	7 (14)
Fremont	1 (0.9)	1 (2)
Grand	3 (2.6)	3 (6)
Morgan	1 (0.9)	1 (2)
Phillips	1 (0.9)	1 (2)
Weld	2 (1.7)	2 (4)

*Chi-Squared analysis was performed to find any statistically significant difference between the two groups of data, there were none.

Table 5. Clinical Characteristics of Colorado FoodNet Cases

	All Epi data N=115	Epi + Genomic data N=50
	N (%)	N (%)
Case Status		
Confirmed	91 (79.1)	50 (100)
Probable	10 (8.7)	0
Hospitalized		
Yes	3 (2.6)	1 (2)
No	107 (93.0)	45 (90)
Any antibiotic(s) used		
Yes	64 (55.7)	22 (44)
No	24 (20.9)	10 (20)
Antibiotics used		
Amoxicillin	1 (0.9)	0
Azithromycin/Z-Pak	35 (30.4)	11 (22)
Bactrim	1 (0.9)	0
Ciprofloxacin	13 (11.3)	7 (14)
Levofloxacin	1 (0.9)	1 (2)
Metronidazole	5 (4.3)	2 (4)
Flagyl	1 (0.9)	0
Erythromycin	1 (0.9)	0
Other/Unknown	53 (46.1)	30 (60)
Diarrhea		
Yes	104 (90.4)	46 (92)
No	2 (1.7)	1 (2)
Bloody diarrhea		
Yes	46 (40)	21 (42)
No	50 (43.5)	23 (46)
Fever		
Yes	64 (55.7)	31 (62)
No	38 (33.0)	14 (28)
Travelled internationally 7 days prior		
Yes	30 (26.1)	15 (30)
No	69 (60)	28 (56)

*Chi-Squared analysis was performed to find any statistically significant difference between the two groups of data, there were none except in the Case Status which is to be expected due to only confirmed cases having stool samples.

Figure 5 in the Appendix shows the generated phylogenetic tree from the matched gene analysis comprising 36 genes, and was compared to the SNP tree and found to be nearly identical. The tree shows 6 clusters with one cluster consisting of 5 strains and 5 other clusters consisting of just two strains. These clusters did not have individuals that had multiple isolates sequenced except for one cluster which had different strains from the same individual. Of the 6 clusters, 3 of the two-strain clusters had the same sequence type (ST) and core genome multi-locus sequence type (cgMLST). Further investigation into the epidemiological data showed that out of these 3 two-strain clusters, one cluster showed possible familial relatedness, which could mean their initial infection came from the same source, while the other two clusters did not seem to show any familial relatedness, due to different symptom onset times and lack of interview information indicating similar travel patterns. Figure 6 in the appendix shows each strain's virulence factors and does not appear to have any patterns of missing virulence factors that are associated with bloody diarrhea or fever.

Table 6. Genomic and Disease Characteristics of Colorado *Campylobacter* Isolate Strains

Strain	Genome Size (bp)	Age (yrs.)	Sex	International Travel 7 days prior?	Diarrhea?	Fever?	Bloody Diarrhea?	MLST	cgMLST
KKC276	1,678,707	1	F	No	Yes	Yes	Yes	ST48	cgST-23444
KKC277	1,711,711	41	F	Yes	Yes	No	No	ST1030	cgST-34147
KKC278	1,669,122	49	M	Yes	Yes	Yes	Yes	ST572	cgST-3412
KKC280	1,671,650	30	M	No	Yes	Yes	No	Unknown	cgST-11387
KKC281	1,741,597	29	M	Yes	Yes	No	Yes	ST1038	cgST-3295
KKC282	1,749,302	41	M	Yes	Yes	Unknown	No	ST1212	cgST-23240
KKC284	1,736,080	83	F	No	Yes	No	No	ST3262	cgST-5670
KKC285	1,617,662	Unkn own	Unk now n	Unknown	Unknown	Unknown	Unknown	ST918	cgST-23190
KKC286	1,635,117	22	M	Unknown	Yes	No	Yes	ST918	cgST-23190
KKC287	1,739,631	74	F	Yes	Yes	No	No	ST827	cgST-34323
KKC288	1,620,790	52	F	No	Yes	No	Yes	ST6647	cgST-26927
KKC289	1,693,138	35	F	Unknown	Yes	Unknown	Unknown	ST899	cgST-30242
KKC290	1,644,655	14	M	Unknown	Unknown	Unknown	Unknown	ST48	cgST-22502

KKC291	1,603,476	Unkn own	Unk now n	Unknown	Unknown	Unknown	Unknown	ST22	cgST-26891
KKC292	1,689,270	73	M	Unknown	Unknown	Unknown	Unknown	ST50	cgST-26459
KKC293	1,723,864	73	M	Yes	Yes	No	Yes	ST929	cgST-25922
KKC294	1,599,866	27	F	No	Yes	Yes	No	ST22	cgST-30878
KKC295	1,605,337	70	M	No	Yes	Yes	No	ST22	cgST-32553
KKC296	1,639,407	44	M	No	Yes	Yes	No	Unknown	Unknown
KKC297	1,663,170	21	F	Yes	Yes	Yes	No	ST3874	cgST-17417
KKC298	1,608,188	40	M	Yes	Yes	Yes	No	ST469	cgST-30758
KKC299	1,643,900	16	F	Yes	Yes	Yes	Yes	ST22	cgST-24887
KKC300	1,695,985	8	M	No	Yes	No	No	ST4069	cgST-12605
KKC302	1,600,906	21	F	No	Yes	Yes	Yes	ST22	cgST-30497
KKC303	1,639,583	47	M	No	Yes	Yes	No	ST459	cgST-31149
KKC304	1,761,833	47	M	No	Yes	Yes	No	ST459	cgST-30789
KKC305	1,657,926	1	F	Unknown	Yes	Yes	No	ST148	cgST-24820
KKC307	1,734,040	20	F	Yes	Yes	Yes	Yes	ST862	cgST-19920

KKC314	1,683,483	22	M	Yes	Yes	Yes	No	ST2036	cgST-6642
KKC315	1,687,204	22	M	Yes	Yes	Yes	No	ST2036	cgST-6642
KKC316	1,804,707	42	M	No	Yes	Yes	Yes	ST7632	cgST-17257
KKC317	1,676,050	42	M	No	Yes	Yes	Yes	ST2036	cgST-6642
KKC320	1,684,803	21	M	Unknown	Unknown	Unknown	Unknown	ST7634	cgST-33630
KKC321	1,619,187	57	M	No	Yes	No	No	ST8	cgST-28067
KKC323	1,667,646	21	M	No	Yes	Yes	Yes	ST441	cgST-30480
KKC324	1,733,300	27	F	Yes	Yes	Yes	No	ST5	cgST-2868
KKC325	1,627,470	63	F	No	Yes	Yes	No	ST52	cgST-25296
KKC328	1,595,951	50	F	No	Yes	No	Yes	ST52	cgST-24366
KKC329	1,632,312	25	F	Yes	Yes	Yes	Yes	ST536	cgST-25694
KKC330	1,666,011	23	M	Unknown	Yes	Yes	Yes	Unknown	cgST-30152
KKC331	1,824,608	18	M	No	Yes	Yes	Yes	ST939	cgST-5966
KKC332	1,737,890	3	M	No	Yes	No	Yes	ST806	cgST-27856
KKC333	1,695,095	3	F	No	Yes	Yes	Yes	ST508	cgST-24281
KKC335	1,737,149	8	M	No	Yes	Yes	Yes	ST806	cgST-27856

*Bolded font indicates that strain is part of a cluster

DISCUSSION

I. Campylobacter Pilot Cohort

19 cases of incident IBS symptomology were reported from the 119 total cases that completed a follow-up survey at 3 and/or 6 months following acute infection, resulting in a PI-IBS incidence of 16.0%. This is within the range reported by the CDC¹. Using the prospective cohort study design, we were able to follow-up with cases and further investigate the incidence of PI-IBS. From inception to the 6-month follow-up of the last enrolled case, this study took approximately two-years to complete and was a pilot study stemming off a previous pilot study conducted at the University of Arizona²⁶.

A major strength of this study was the prospective cohort study design which allowed us to assess symptoms in relation to time. Having the temporality of the symptoms allows relative risk to be calculated and participants to be followed to see if they develop further symptoms or comorbidities such as PI-IBS. A limitation of this study may be the possibility of recall bias as their symptoms were self-reported. Many of the associations with symptoms at 3-months and 6-

months had wide confidence intervals as the sample size was small, making it difficult to assess their significance. Many of the relative risk calculations will have to be interpreted with caution, as overreporting and/or underreporting of symptoms also may have led to a masking of the true measure. Selection bias would have also occurred as the cohort was sicker overall with 48% of non-IBS cases having been hospitalized or visited the Emergency Department or urgent care for their acute infection. According to Scallan et. al., the hospitalization rate for campylobacteriosis is usually around 17%⁵. 80% of the non-IBS cases also reported having taken antibiotics for their initial infection, showing that their symptoms were most likely more severe than normal. There was also a transition to a new database which made the transfer of data such as surveys and consent forms complicated. There was one participant that had to be excluded due to a missing 6-month survey. They had been consented and surveyed but the survey was not found. The circumstances of 2020, owing to the COVID-19 pandemic, also had an effect as many participants had to work from home and there seemed to be more of a response when phoned. Participants seemed to be more perceptive of their health state and mentioned other unrelated symptoms which showed a positive association although anxiety and depression had increased in later surveys and may point to an increase in anxiety or depression during the pandemic, as seen in Choi et. al⁴¹.

It would be interesting to further study the effects that the pandemic had on incident PI-IBS, as people would have been in closer contact with one another and eating similar foods. There may have been an increase in unreported *Campylobacter* infections, but it would be difficult to record. The stool sample pipeline that we were trying to establish was in an early stage and 40% of the stool kits were returned, even if it was a small sample. The next stage for

our investigation includes the analysis and characterization of the bacterial genomes in the sample and linking of the virulence factors and other genetic markers to the symptoms, to assess the state of the microbiome, post-infection. An early collection of the stool sample during the initial onset of campylobacteriosis would have an increased chance of being able to assess a more recent state of the microbiome and could provide additional information on predicting the delayed onset of PI-IBS.

II. Linking of *Campylobacter* and Genomic Analysis

Outbreaks of *Campylobacter spp.* are difficult to detect as their sporadic nature leads to them being relatively underestimated in analysis and detection, when compared to other pathogens. It can be difficult to ascertain the source from which the infection occurs as there are many ways to contract campylobacteriosis. By combining demographic data and symptomology from interviews with genomic methods, clusters of *Campylobacter* cases can be increasingly identified even when initially thought to be isolated cases. Finding clusters will help investigators to pinpoint the source of the infection more easily and prevent future infections. Similar studies have noticed the potential of using WGS to gather genomic data on stool isolates to find clusters of *Campylobacter* cases^{21-23,31}.

A limitation of this study was that it was not possible to clean and sequence all the strains. There were some strains with contamination that had to be re-cleaned or left out of the analyses. Sequencing delays due to the COVID-19 pandemic has also affected the analysis as there are fewer isolates to work with and compare. This decreases the power of the study as a sample size of 54 unique isolates is not large to begin with. There were also further decreases in sample size since some isolates were not able to be merged with the epidemiological data due to non-matching state identifications (n=7).

Future work on this topic should include more stool samples and possibly widening the catchment area to compare different populations and the clusters that may be hidden. Looking at structural risk factors between states or agencies that could decrease the likelihood of finding a cluster of cases would also be a useful line of future research.

CONCLUSION

Campylobacter species are the most common bacterial cause of diarrheal illness in the United States and are estimated to cause around 800,000 cases and potentially up to 1.5 million cases per year in the U.S. alone^{1,2,8}. Globally, *Campylobacter spp.* infections are endemic in many regions of the world and are speculated to be increasing^{8,10}. Thousands of campylobacteriosis cases go unreported each year since cases of *Campylobacter* infection are sporadic, relatively mild, and often are not linked to any recognized outbreaks. Being able to detect outbreaks of cases is crucial and calls for an increase in the use of genomic data from WGS on stool isolates. Clusters of cases can be found using genomic data coupled with the epidemiological data so that our detection rate is increased. Increasing evidence of persistent symptoms and PI-IBS after the acute infection by *Campylobacter spp.* needs to be investigated to understand the mechanisms and risk factors that are associated with PI-IBS. By using a cohort study design, the incidence of PI-IBS in this population was found to be 16% with risk factors ranging from pre-existing chronic conditions at acute infection to symptoms of anxiety and depression at follow-up time points.

FIGURES

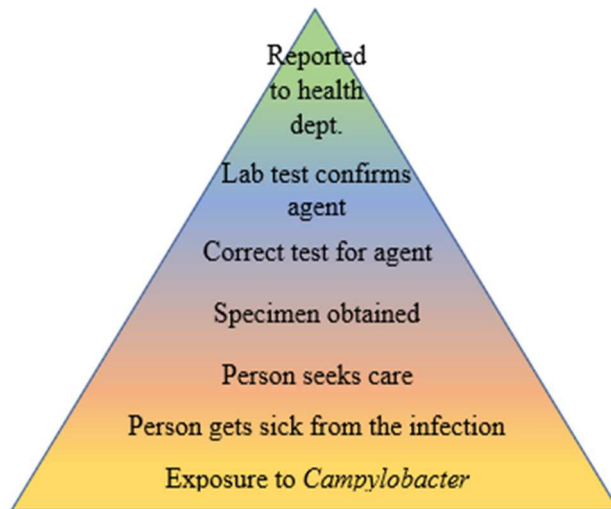


Figure 1. Burden of Illness Model

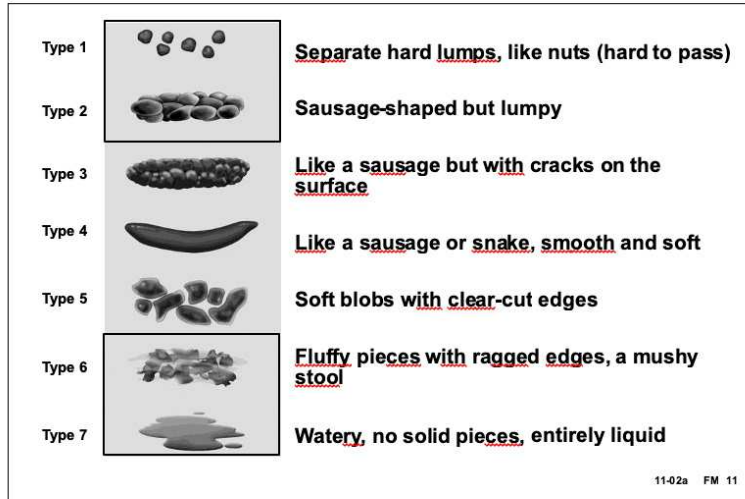
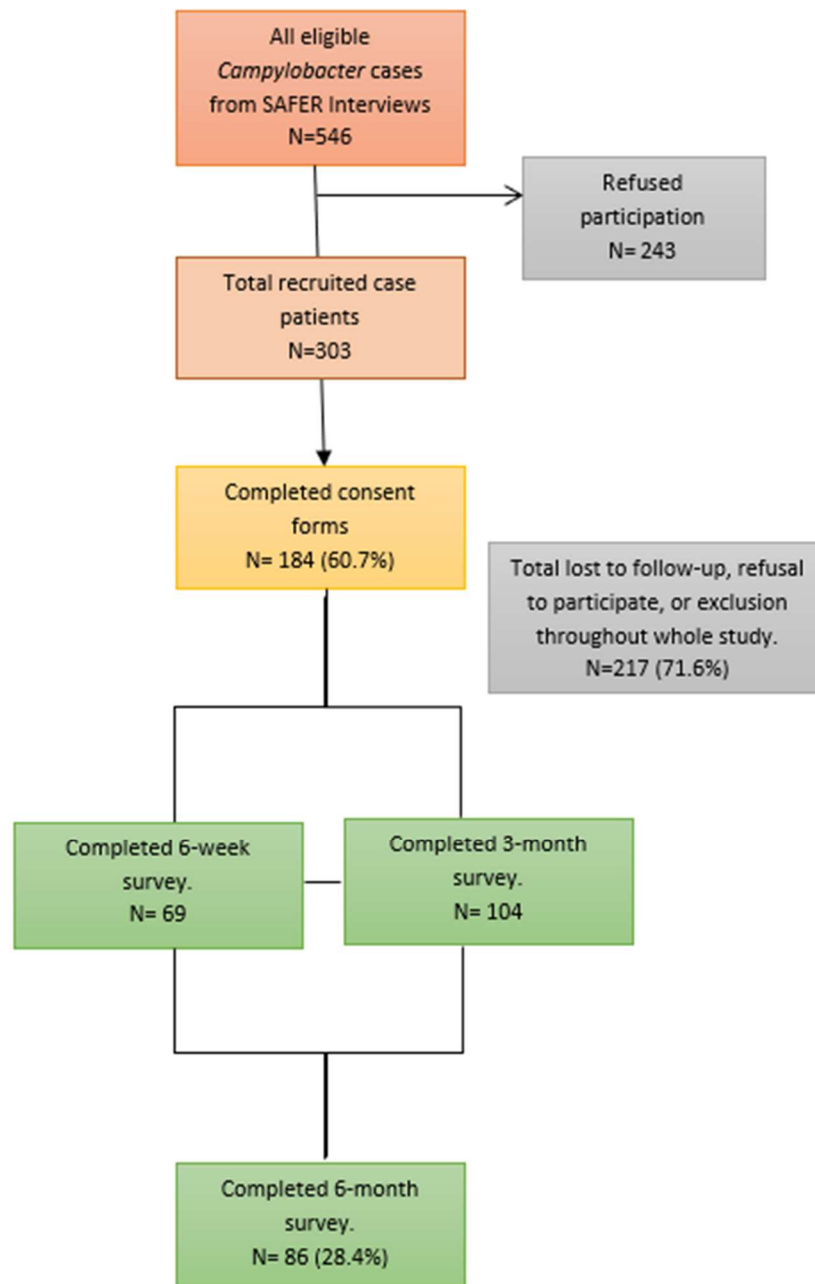


Figure 2. Bristol Stool Chart²⁷

Figure 3. Flowchart of participation in the study

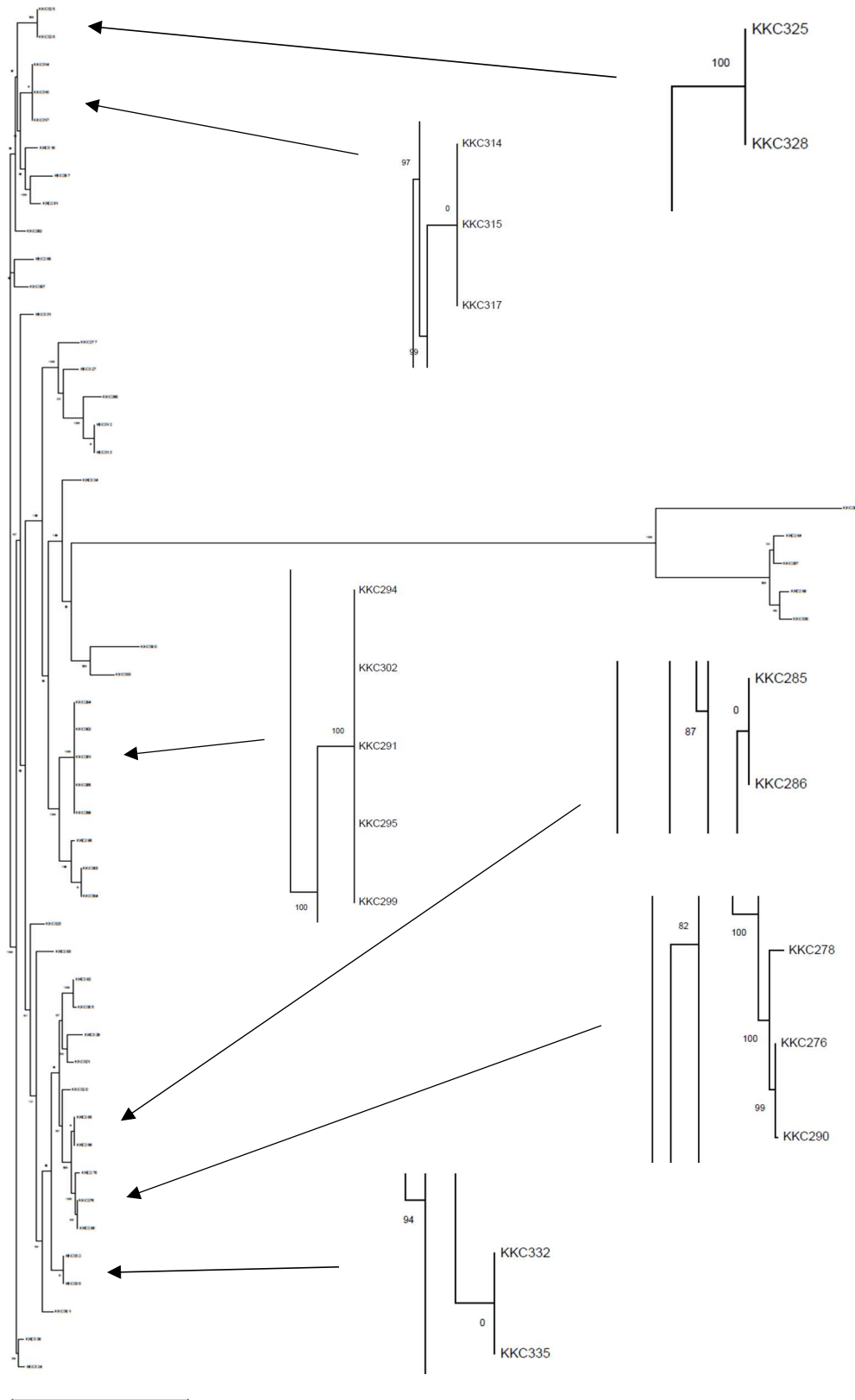


Inclusion Criteria for an Incident-PI-IBS Case each survey
<ul style="list-style-type: none">● Abdominal Pain needs to be present (at least 1 day/week) = needs to indicate at least 4 days per month in survey● Needs two of the following symptoms also present:● Diarrhea, loose stools, or constipation
Exclusion Criteria for a PI-IBS Case
<ul style="list-style-type: none">● Prior diagnosis of IBS before baseline/initial infection

Figure 4. Inclusion Criteria for PI-IBS case definition

APPENDIX

Figure 5.
Phylogenetic
Tree of core
genes
(clusters
enhanced)



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