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Comprehensive Microbiological and Metagenomic Analysis of the Guillain–Barré Syndrome Outbreak in Lima, 2019

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Abstract: In 2018/2019, two large Guillain–Barré Syndrome (GBS) outbreaks took place in Peru. Here, we report a comprehensive analysis of biological samples from GBS patients from the 2019 outbreak. We applied metagenomic, microbiologic, and serological analyses to different biological samples collected from GBS patients. Further phenotypic and genomic characterization was conducted on *Campylobacter jejuni* isolates from GBS samples. Microbiologic and metagenomic analyses revealed several patients with multiple co-infections, yet no common infectious agents were found other than *C. jejuni*. Four *C. jejuni* isolates were isolated from rectal swabs. Twenty-one patients had detectable IgG serum antibodies related to *C. jejuni*, of whom seven had IgM antibodies. Genomic analyses showed that these four strains were clonal (ST2993) and contained the class A lipooligosaccharide biosynthesis locus. These results further support the idea that that *C. jejuni* is the etiological agent that triggered the GBS outbreak in Peru in 2019 and that the strains are not restricted to Peru, hence could be regarded as a broad public health concern. Furthermore, though we cannot delineate the role played by co-infections in GBS development, results obtained herein highlight metagenomic analysis as a potential new tool for depicting a yet unknown area of research in GBS.

Keywords: *Campylobacter jejuni*; Guillain–Barré syndrome; outbreak; metagenomics; genomics; co-infections

1. Introduction

Guillain–Barré syndrome (GBS) is an acute polyradiculoneuropathy characterized by flaccid paralysis and high levels of cerebral spinal fluid (CSF) protein with no increase in cellular count [1]. Approximately 5% of GBS patients die even with early treatment, and ~20% of patients do not fully recover [2]. GBS clinical phenotypes include acute inflammatory demyelinating polyradiculoneuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor sensorial axonal neuropathy (AMSAN), and the ocular variant

Miller Fisher syndrome (MFS) [3]. AIDP is the most common subtype in Europe, North America, and Australia, while AMAN and AMSAN are the most common in Asia and South America [1]. Two-thirds of cases are preceded by a gastrointestinal or respiratory infection 1 to 3 weeks prior to onset of GBS symptoms, thus complicating the isolation or detection of the etiological agent [4], while about a third of gastrointestinal/respiratory infections are asymptomatic [5]. *Campylobacter jejuni* infection is the common associated microorganism (25–40%), followed by cytomegalovirus (6–15%), *Mycoplasma pneumoniae* (3–21%), and *Haemophilus influenzae* (1–9%) [6]. Strikingly, despite evidence indicating various infectious agents might trigger GBS, to our knowledge no large-scale metagenomic surveillance or research has been performed, nor have research studies been conducted, evaluating the role of co-infections in GBS development.

GBS is thought to be initiated by autoantibodies against gangliosides, sialo-glucolipids located on the surface of the plasma membrane of peripheral nerves [4,7,8]. Indeed, GBS was the first autoimmune disease in humans verified to be triggered by molecular mimicry after *C. jejuni* infection [9]. Self-glucolipid epitopes also present in the lipo-oligosaccharides (LOS) of microorganisms can also generate antibodies [10]. Antibodies in the AMAN phenotype of GBS react against gangliosides GM1, GD1a, and GalNAc-GD1a [6], similar to what is observed after *C. jejuni* infection in GBS patients [11–13]. Variations in the glucolipid structure are increased by rearrangements of genomic regions [14] and slipped-strand mispairing due to homopolymeric tracks [15,16], underscoring the importance of evaluating the genomic composition of *C. jejuni* LOS.

GBS outbreaks are uncommon and might be associated with microbial outbreaks, as various microorganisms might trigger GBS [17]. In Latin America and the Caribbean, arbovirus outbreaks caused by the Zika virus (ZIKV) and Chikungunya virus (CHIKV) increased GBS incidence more than two-fold over background rates [18]. Although there are various pathogens that might trigger GBS, a particular presentation of GBS can usually be associated with, or attributable to, certain pathogens [19]. Yet, even in large microbial infection outbreaks (e.g., the Zika virus outbreak in Latin America in the past decade), the largest number of GBS cases in an outbreak was 68 GBS patients [20].

Two major outbreaks took place in Lima, Peru, between May and July of 2018 and 2019, with 174 and 683 GBS cases being reported, respectively [21]. In response, a collaborative study between the Hospital Nacional Cayetano Heredia (HNCH) and the U.S. Naval Medical Research Unit SOUTH (NAMRU S) was initiated to better understand the nature and epidemiology of the 2019 Peru outbreak and to identify the causative agent. Previous work has described the clinical features of these GBS patients [22]. In addition, evidence of a recent infection by *C. jejuni* was found in 65% of patients, with no evidence of recent infection by arbovirus [22]. Further, isolation and genomic characterization of relevant features of *C. jejuni* were described, revealing its role as the potential etiological agent [22]. Here, we build on this knowledge by reporting a multifaceted investigation that was conducted in tandem with the investigation at HNCH. The analyses reported herein include data obtained from microbiologic and metagenomic analyses of a variety of samples from GBS patients as well as a thorough genomic analysis of *C. jejuni* isolates from GBS patients from the 2019 Peru outbreak. In addition, we conducted a comparative genomic analysis of *C. jejuni* isolated in this study and from non-related diarrheal samples from studies conducted after 1997 [23] to 2006 [24], revealing similarities with the *C. jejuni* isolates from the 2019 Peru outbreak. Through these analyses, we determined and now report their antibiotic resistance and virulence factor repertoires; their taxonomic classification and phylogenetic profiles; sequence typing results using pubMLST; and thorough analyses of their lipooligosaccharide (LOS) genes. To the best of our knowledge, we report the first comprehensive metagenomic characterization of biological samples from GBS patients of the 2019 Peru outbreak. The information herein provided revealed an unknown state of co-infection in an endemic region with an unknown role for GBS clinical manifestation; this information may aid in future study designs to improve insights into GBS. Furthermore, genomic characterization of GBS *C. jejuni* isolates revealed its close relationship to regionally

and globally circulating clusters that should be considered by policymakers as determinants in the formulation and decision-making process to prevent recurrent outbreaks.

2. Materials and Methods

2.1. Biological Samples

Blood (n = 29), rectal swab (n = 37), stool (n = 6), cerebrospinal fluid (n = 2), and oropharyngeal swab (n = 28) samples were collected during June 2019 from 37 patients who were clinically diagnosed with GBS at HNCH. Most patients presented with the AMAN (43%) phenotype, and 18% presented the AIDP phenotype [22]. Samples were immediately transported under refrigerated conditions in Universal Transport Media to NAMRU S for analysis.

2.1.1. Rectal Swabs

Rectal swabs were streaked on McConkey, Thiosulfate–Citrate–Bile Salts–Sucrose (TCBS), Salmonella–Shigella (SS), Hektoen, and Charcoal Cefoperozone Deoxycholate (CCDA) agar media cultures. McConkey, TCBS, SS, and Hektoen agar plates were incubated at 37 °C for 24 h, and CCDA plates were incubated in microaerobic conditions (85% N_2, 10% CO_2, and 5% O_2) at 42 °C for 72 h. Lysine iron agar, Kligler's iron agar, citrate agar, motility indole ornithine, oxidase, and catalase biochemical tests were performed for lactose negative colonies that were grown on McConkey, SS, and Hektoen agar plates. Individual lactose positive colonies (LPC) were sub-cultured on McConkey agar. Total nucleic acid (TNA) was extracted from LPC by thermal shock and tested by multiplex PCR for identification of diarrheagenic *Escherichia coli* (DEC), as previously described [25].

Colonies suspected to be *Campylobacter* spp. were further tested by catalase and peroxidase activity as well as Gram-staining. Multiple colonies with a typical morphology of *Campylobacter* spp. were sub-cultured in thioglycolate broth (TGB) and incubated at 42 °C for 48–72 h in microaerobic conditions. TGB with a typical growth for *Campylobacter* sp. was sub-cultured on Columbia blood agar (CBA) plates by the filter technique [26]. Pure single colonies were replicated on CBA plates for further analysis. Identification of *Campylobacter spp*. and capsule (CPS) typing were performed by a multiplex PCR [27]. Susceptibility to ciprofloxacin was evaluated by an E-test to determine the minimum inhibitory concentration (MIC) (BioMérieux, Marcy-l'Étoile, France). Tetracycline and erythromycin susceptibility testing was performed by disk diffusion according to the Clinical and Laboratory Standards Institute guidelines (CLSI) [28].

2.1.2. Blood and Oropharyngeal Samples

Twenty-eight and twenty-nine serum samples were cultured in African Green Monkey (Vero-76), Madin-Darby Canine Kidney (MDCK), human epithelial type-2 (HEp-2), epithelial carcinoma A549, and *Aedes albopictus* C6/36 cells (ATCC CRL-1660) and evaluated for the presence of cytopathic effect (CPE) daily for 10 days [29,30]. Cultures were harvested and tested by indirect immunofluorescence assay (IFA) once CPE was observed or 10 days after inoculation, as previously described [31]. Viral antigens were detected using hyperimmune mouse ascitic fluid (HMAF) for Flavivirus, Bunyavirus and Alphavirus produced at NAMRU S. A set of positive control slides was used for each IFA run. Respiratory viral antigens were visualized with the D3 Ultra 8 DFA Respiratory virus screening and ID kit (Diagnostic Hybrids, Inc., Athens, OH, USA).

Quantification of specific anti-*C. jejuni* IgG and IgM from human sera was performed on Nunc Maxisorp flat bottom plates. Plates were coated with *C. jejuni* 81176 glycine extract (Naval Medical Research Center, San Diego, CA, USA) at 1 µg/mL in carbonate buffer at 37 °C for 1 h. Human sera were two-fold serially diluted with a starting dilution of 1:500. Horseradish peroxidase-conjugated secondary antibodies, at a concentration of 0.125 µg/mL, were used for detection of goat anti-human IgM (µ) (KPL, USA) and goat anti-human IgG (H + L) (KPL, USA). The endpoint titers were assigned as the interpolated dilutions of the samples, giving an absorbance value of 405 nm of 0.4 optical density

(OD) units above background. Titers were represented using log10 values. For statistical purposes, samples below the 1:500 detection limit were given a value of 2.4 (log10 = 250).

Four samples, OBT12373, OBT12374, OBT12375, and OBT12376, were tested for the presence of circulating IgM antibodies against Dengue (DENV), Zika (ZIKV), and Chikungunya (CHIKV) using in-house developed IgM-capture enzyme-linked immunosorbent assays (ELISA) [32,33]. Commercial normal human serum (ThermoFisher Scientific, Waltham, MA, USA) as well as de-identified previously confirmed positive samples were used as negative and positive controls, respectively.

2.2. Genomic and Metagenomic Sequencing

To minimize sample and exogenous laboratory cross-contamination, a unidirectional workflow was maintained. Nucleic acid extraction, pre-PCR, and post-PCR workspaces were located in different rooms. Also, continuous decontamination of biosafety cabinets and workbenches was performed prior to the experiment. Potential contamination was monitored by the processing of negative water and normal human serum control in parallel with samples for each sequencing run.

2.2.1. Genomic Sequencing

Twenty-two HS41 *C. jejuni* archived isolates from four different sources were refreshed, and genomic DNA was extracted for sequencing, including six isolates from the Loreto region; eight from Lima that were isolated from diarrheal samples in previous studies conducted in Peru [34]; four from the GBS Lima outbreak in 2019 that were isolated and provided by the Instituto Nacional de Salud (INS) [35]; and four isolates from this study. Genomic DNA was extracted from pure cultures using the DNeasy Blood & Tissue kit (Qiagen, Venlo, The Netherlands), following manufacturer's protocol, and quantified using a Qubit 2.0 Fluorometer (Invitrogen Life Technologies, Carlsbad, CA, USA) with the dsDNA High Sensitivity Kit (ThermoFisher Scientific, USA). DNA was diluted to 0.2 ng/µL for library preparation using the Illumina Nextera XT DNA library prep kit (Illumina, San Diego, CA, USA). Barcoded libraries were quantified and pooled for sequencing. The pool was then sequenced using the MiniSeq High Output Kit (300-cycle) on the MiniSeq platform (Illumina, USA).

2.2.2. Metagenomic Sequencing

TNA was extracted from a total of 65 biological samples (blood (n = 29), oral swabs (n = 28), cerebrospinal fluid (n = 2), and stool (n = 6)) using the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) according to manufacturer's instructions. TNA was divided into two fractions, and one fraction was stored at −20 °C and retained for use as DNA input. The other fraction was reverse transcribed into double-stranded cDNA using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific, USA), followed by the NEBNext Ultra II (New England Biolabs, Waltham, MA, USA) Non-Directional RNA Second Strand Synthesis Module according to the manufacturer's instructions. Both were quantified using a Qubit 2.0 Fluorometer (Invitrogen Life Technologies, USA) with the dsDNA High Sensitivity Kit (ThermoFisher Scientific, USA). The quantified cDNA and DNA fractions were mixed and input in equal parts to the Nextera XT DNA library prep kit and sequenced using a MiSeq platform (Illumina, USA) with the v3 MiSeq Reagent Kit (600-cycle) according to the manufacturer's instructions.

2.3. Bioinformatic Analysis

This component of the study focused primarily on bacterial and viral agents previously linked to GBS [6,21].

2.3.1. Bacterial Genome Analysis

MetaDetector, an in-house pipeline, was used to perform quality control (QC), assembly, and taxonomic classification of the sequencing data. Initially, raw reads were

evaluated using FASTQC [36] and subsequently quality-trimmed to a Q20 threshold using BBDuk (v38.49) [37]. The trimmed reads were then assembled using SPAdes (v3.13.1) [38]. Taxonomic classification was performed on the trimmed reads and contigs against a non-redundant protein database (nr, NCBI) using DIAMOND (v0.9.24.125) [39] with the blastx option, and the results were formatted as RMA6 files for visualization in MEGAN (v6.12) [40]. The reads were also assembled by Unicycler (v0.4.7) [41] with SPAdes (v3.12.0) [38]. The resulting contigs were then manually closed using Bandage (v0.8.1) [42].

The completed [43] genomes underwent validation through read mapping and basic variant calling using CLC Genomics Workbench (CLC) (v12.0.1) to confirm assembly accuracy. High-quality draft [43] genomes were further characterized using EDGE Bioinformatics (v2.3) [44] where they were annotated with Prokka (v1.13) [45].

Antibiotic resistance (AR) genes and virulence factors (VF) were identified using RGI (v2.4.4) [46] and ShortBRED (0.9.4M) [47], respectively. Whole-genome comparisons were performed using BRIG (v0.95) [48] to utilize genomic similarities and divergencies and Mauve (v2015_02_25) [49] to align genomes, accounting for rearrangements and providing insights into synteny and evolutionary relationships. Gene-specific maximum likelihood phylogenies were inferred using CLC. Closely related genomes were identified using BLAST against an internal nucleotide database and used to reconstruct whole-genome maximum likelihood phylogenies using PhaME [50], which integrates RAxML (v8.0.26) [51] within EDGE Bioinformatics (v2.3) [44].

Following a phylogenetic analysis, sequence typing was performed using the pubMLST [52] *Campylobacter jejuni/coli* database [53]. Using pubMLST, we performed the following two types of multi-locus sequence typing (MLST) analyses: (1) traditional MLST, which uses seven different loci in *C. jejuni* and *C. coli* to assign a sequence type (ST); and (2) *C. jejuni/C. coli* cgMLST (v1.0), which uses over 1300 loci to determine the closest profile based on the percentage of loci matched.

2.3.2. Metagenomic Analysis

The study focused primarily on bacterial organisms previously linked to GBS, such as *C. jejuni*, *Mycoplasma pneumoniae*, and *Haemophilus influenzae*, and viruses such as Zika, Chikungunya, Influenza, Human Herpesvirus 4 and 5, and HIV viruses [54–59]. Raw sequence data were processed to three characterization pipelines, namely MetaDetector, EDGE Bioinformatics tool suites [44], and VirusSeeker [60], to perform a QC of sequencing reads, genome assembly, and to identify the overall metagenomics contents of each sample. In addition to MetaDetector and EDGE [44], VirusSeeker [60] was employed specifically to determine the type and abundance of both known and novel viral sequences. Further bioinformatic analyses, including custom BLAST searches, CLC Genomics Workbench NGS core tools, visualization using MEGAN [40], and in-house programming scripts, were conducted as needed to further characterize and compare all samples.

Raw sequence data were first analyzed through the MetaDetector pipeline, where raw reads were assessed using FASTQC [36] and quality-trimmed to a Q20 threshold using BBDuk (v38.49) [37]. The trimmed reads were aligned against the human genome reference sequence, common lab contaminants, and common microbiota rRNA databases, with matched reads being removed to obtain "cleaned" reads. The cleaned reads were assembled using metaSPAdes [61], and the trimmed reads were mapped back to the resulting contigs. For taxonomic profiling, both cleaned reads and contigs were searched using DIAMOND (v0.9.24.125) [39], with the resulting BLAST output files being formatted and visualized in MEGAN (v6.12) [40].

Microbial classifications were validated by aligning filtered reads to the putative candidate reference genome using CLCBio Genomics Workbench or BBMap [62]. Any inaccurate classifications identified at this stage were subsequently discarded. Quality-controlled reads were also submitted to EDGE Bioinformatics (v2.3) [44], where heat maps were generated to compare taxonomic profiling results among samples. Raw reads were

processed with VirusSeeker [60] to determine the type and abundance of both known and novel viral sequences present in the samples. The results from VirusSeeker were parsed using an in-house script to ensure an accurate counting of reads corresponding to each virus.

Read mapping was performed using BBSplit [62], a multi-kmer-seed-and-extend aligner that bins reads by mapping them to multiple references simultaneously. Reads were assigned to the bin of the reference to which they mapped best. To be considered "mapped to a reference", a read required a minimum alignment identity of 0.76 over the length of the read compared to the reference. For maximum specificity, ambiguously mapped reads (i.e., those that mapped to multiple references) were excluded from the mapping statistics.

3. Results

3.1. Bacterial and Viral Isolation and Characterization

Diffusely adherent *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) were identified in 9 out of 37 samples using standard microbiological and molecular techniques. No other enteric bacterial pathogens were detected in rectal swabs through these standard techniques. The Respiratory Syncytial Virus (RSV) and Herpes Simplex Virus 2 (HSV) were identified in samples OBT12237 and OBT12388, respectively. CPE was observed in samples OBT12374 and OBT12377, although both tested negative by IFA. Additionally, no IgM antibodies against Chikungunya, Zika, or the Dengue viruses were detected in the tested samples.

3.2. Campylobacter jejuni Isolation and Characterization

Out of the 37 rectal swab cultures, *C. jejuni* was successfully isolated from the following four samples: OBT12377, OBT12386, OBT12390, and OBT12393. All *C. jejuni* isolates were CPS type HS41 and exhibited resistance to ciprofloxacin, but they remained sensitive to tetracycline and erythromycin. Specific IgM and IgG antibodies against *C. jejuni* were detected in 7/28 and 21/28 samples, respectively (Figure 1).

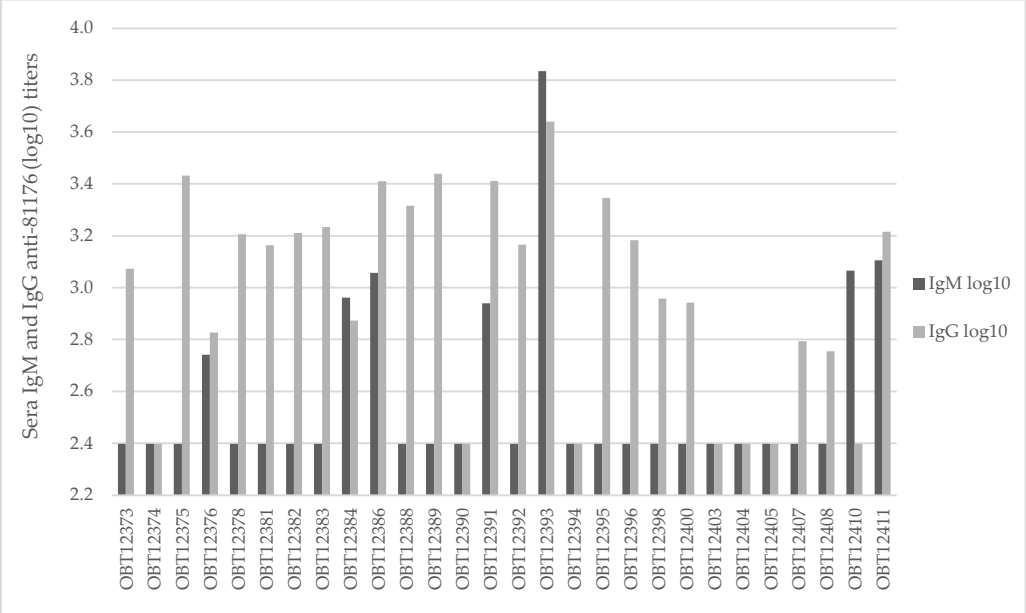

Figure 1. Anti-*C. jejuni* IgM and IgG antibodies log10 titers in sera samples from GBS patients. Sample values above the cut off (0.4 O.D. units above the background at 1:500 dilution (log10 = 2.4)) were considered positive.

Genomic Characterization

The whole-genome maximum likelihood phylogenetic tree (Figure 2), which includes seven closely related *C. jejuni* genomes along with four isolates from the 2019 Peru outbreak (OBT12377, OBT12386, OBT12390, and OBT12393), demonstrates that these four Lima isolates are closely related to strains isolated from GBS patients in China [13] and South Africa [63]. In each of these isolates, five AR genes were identified using RGI, and 125 VF genes were identified using ShortBRED. Genomic analysis revealed that *C. jejuni* was resistant to fluoroquinolones due to a single point mutation in the *gyrA* gene. Additionally, the analysis identified four genes encoding resistance-nodulation-cell division (RND) antibiotic efflux pumps. Moreover, the genomic analysis revealed the presence of various virulence factors, including adherence genes (n = 3), colonization and immune evasion genes (n = 18), glycosylation system genes (n = 20), immune evasion and molecular mimicry genes (n = 19), an invasion gene (n = 1), motility and export apparatus genes (n = 36), toxin-encoding genes (n = 3), and other virulence factor genes (n = 25).

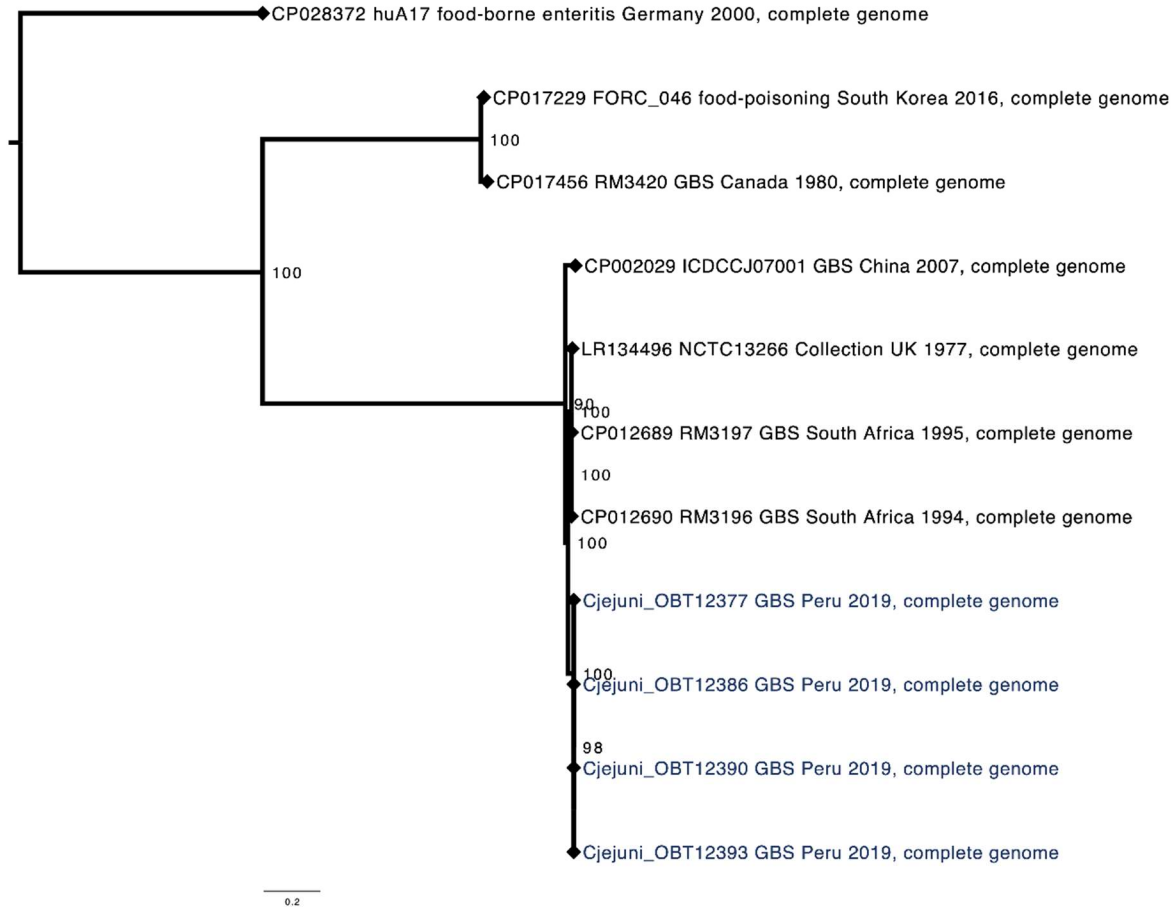

Figure 2. Whole-genome maximum likelihood tree constructed using the seven closest genomes. *C. jejuni* isolates from this study are closely related to strains previously isolated from GBS patients in South Africa and China.

These isolates were clonal, identified as sequence type (ST) 2993 and clonal complex (CC) ST362. The closest publicly available reference genome, identified through BLAST analysis, was *C. jejuni* ICDCCJ07001 (accession number CP012390) [64]. A large deletion of approximately 37 kb, corresponding to a prophage, was observed in all four isolates when compared to the reference genome (Figure S1).

Multiple genome alignment with *C. jejuni* reference genomes RM3196 (accession number CP002029.1) [63] and IDCCJ07001 [64] revealed unique indels in each of the four Lima genomes. Notably, OBT12386 contained a single nucleotide deletion in a homopolymeric

A tract of the *cgtA* gene, which encodes for an outer core glycosyltransferase [65]. This deletion results in a truncated gene (Table S2).

Given the initial finding that all *C. jejuni* isolates from GBS patients were CPS type HS41, we aimed to determine whether circulating HS41 *C. jejuni* isolates from previous studies [34] were related to the strains isolated during the 2019 Peru outbreak. To achieve this, we sequenced samples from the previously circulating HS41 *C. jejuni* isolates, obtaining eight improved high-quality draft genomes and ten high-quality draft genomes. The *C. jejuni* genomes and their corresponding accession numbers at GenBank are shown in Table S1. Despite differences in genome length and numbers of coding sequences, they all contained very similar numbers of tRNA, VF, and AR (Table 1).

Table 1. Statistics of *Campylobacter jejuni* genomes.

Genome	Genome Length (bp)	# CDS	GC%	# tRNA	# VF	# AR	% Reads Mapped	Average Coverage Depth (X)
OBT12377 *	1,627,623	1629	30.6	44	125	5	99.7	1333
OBT12386 *	1,627,622	1625	30.6	44	125	5	99.7	932
OBT12390 *	1,627,621	1626	30.6	44	125	5	99.6	1156
OBT12393 *	1,627,618	1626	30.6	44	125	5	100.0	595
6-1083-19	1,627,620	1625	30.6	44	125	5	97.5	134
6-1197-19	1,627,619	1629	30.6	44	125	5	99.4	168
6-897-19	1,628,038	1627	30.3	44	122	5	98.4	162
6-1198-19	1,638,300	1634	30.4	46	123	5	96.4	144
CMP10190	1,667,545	1681	30.6	49	124	4	98.4	89
CMP10201	1,667,160	1681	30.6	45	124	4	99.1	115
CMP4596	1,653,172	1706	30.4	42	133	6	96.5	169
CMP4850	1,664,740	1683	30.5	44	123	4	97.4	147
CMP4924	1,703,186	1769	30.2	42	134	5	92.4	164
CMP9838	1,667,235	1681	30.6	45	119	5	99.3	181
CMP9934	1,666,416	1681	30.6	45	119	5	99.2	111
CMP10096	1,661,894	1683	30.6	41	117	5	91.5	111
SHI3134	1,666,121	1676	30.5	44	125	3	98.9	179
SHI4648	1,666,109	1679	30.5	44	125	3	95.4	157
SHI5328	1,701,559	1736	30.3	44	131	4	99.8	176
SHI9672	1,666,509	1676	30.5	48	125	4	99.2	96
SHI3157	1,666,090	1680	30.4	42	123	4	97.3	200
SHI5837	1,642,933	1646	29.9	45	131	5	96.5	129

Abbreviations: CDS, coding sequence; tRNA, transfer RNA; VF, virulence factor; AR, antibiotic resistance. * Genomes were deposited in GeneBank (accession numbers: CP086166, CP086165, CP086164, and CP086163).

MLST analysis revealed that 59.1% of these samples belonged to ST2993 and cgST-29793, while 22.7% were identified as ST41 (Table 2). Notably, ST2993 has been previously reported to be associated with GBS [64]. The cgMLST analysis indicated the samples identified as ST2993 shared 90% similarity with ST2993 loci, whereas those identified as ST41 shared 37.8% similarity with ST41 loci. Other sequence types observed were ST353, ST354, and ST3874, with cgMLST loci matching rates ranging from 70 to 80%.

A detailed analysis of the LOS region was conducted for all sequenced genomes. LOS regions were extracted from each finished or draft genome and characterized by comparison to the reference *C. jejuni subsp. jejuni* 81–176 (GenBank accession: CP000538.1) (Table S3). At the end of the LOS region, three genes (*waaV*, *waaF*, and *gmhA-1)* were identified, with a varying number of glycosyltransferases between *waaF* and *gmhA-1*. Sialic acid biosynthesis and translocation genes (*cst-II*, *neuB*, *neuC*, *neuA*) were present only in the sequence types ST2993 and ST41. These two sequence types showed high similarity throughout the entire LOS region, with ST2993 having three extra glycosyltransferase genes (Figure 3).

Table 2. Sequence types of *C. jejuni* genomes from the 2019 GBS outbreak and selected genomes from two regions in Peru.

Sample	MLST-Designated Sequence Type	Diseases Caused by MLST-Designated Sequence Type	Closest Profile (cgMLST)	Loci Matched to Closest Profile
OBT12377	2993	GBS, gastroenteritis	cgST-29793	90.2
OBT12386	2993	GBS, gastroenteritis	cgST-29793	90.2
OBT12390	2993	GBS, gastroenteritis	cgST-29793	90.2
OBT12393	2993	GBS, gastroenteritis	cgST-29793	90.2
6-897-19	2993	GBS, gastroenteritis	cgST-29793	90.2
6-1083-19	2993	GBS, gastroenteritis	cgST-29793	90.2
6-1197-19	2993	GBS, gastroenteritis	cgST-29793	90.2
6-1198-19	2993	GBS, gastroenteritis	cgST-29793	90.1
CMP4596	354	Gastroenteritis, systemic disease	cgST-30119	80.7
CMP4850	2993	GBS, gastroenteritis	cgST-29793	90.3
CMP4924	3874	Gastroenteritis	cgST-17417	73.9
CMP9838	41	Gastroenteritis	cgST-4301	37.8
CMP9934	41	Gastroenteritis	cgST-4301	37.8
CMP10096	41	Gastroenteritis	cgST-4301	37.8
CMP10190	41	Gastroenteritis	cgST-4301	37.8
CMP10201	41	Gastroenteritis	cgST-4301	37.8
SHI3134	2993	GBS, gastroenteritis	cgST-29793	89.9
SHI3157	2993	GBS, gastroenteritis	cgST-29793	89.8
SHI4648	2993	GBS, gastroenteritis	cgST-29793	90.3
SHI5328	353	Gastroenteritis, systemic disease	cgST-30854	73.9
SHI5837	353	Gastroenteritis, systemic disease	cgST-30854	72.6
SHI9672	2993	GBS, gastroenteritis	cgST-29793	90.4

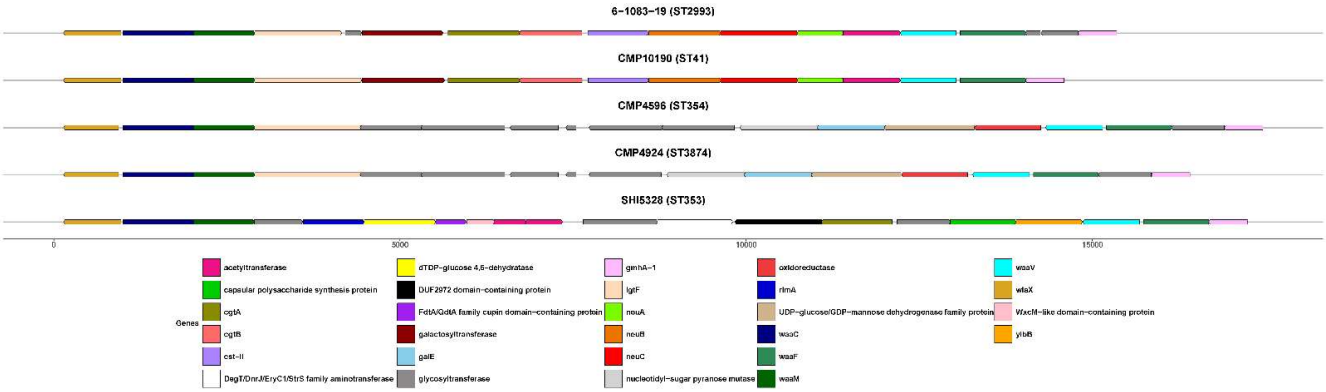

Figure 3. Composition of LOS regions for Sequence Type Representatives. The LOS regions for all five groups begin with the same three genes: *wlaX*, *waaC*, and *waaM*. The presence of the genes *cst-II*, *neuB*, *neuC*, and *neuA* is associated with the biosynthesis of sialylated LOS, which mimic human gangliosides and have the potential to trigger GBS.

ST354 and ST3874 also exhibited a high degree of similarity, with ST354 containing a single additional glycosyltransferase gene in the middle of the LOS region. Notably, the *cgtB* gene was found exclusively in ST2993 and ST41-like samples, while *cgtA* was also present in ST353. ST354 and ST3874 were similar, with ST354 having an extra glycosyltransferase gene near the middle of the LOS region (Table 3).

Table 3. LOS regions in the *C. jejuni* genomes.

Sample	Length of LOS (bp)	# of CDS	Closest Reference (NCBI Accession #)	Percent Identity
6-897-19 [1]	15,370	18	OBT12390 (CP059160.1)	100.0
6-1083-19 [1]	15,370	18		100.0
6-1197-19 [1]	15,370	18		100.0
6-1198-19 *	15,370	18		99.0
SHI3134 [1]	15,370	18		100.0
SHI3157 [1]	15,370	18		100.0
SHI4648 [1]	15,370	18		100.0
SHI9672 [1]	15,370	18		100.0
CMP4850 [1]	15,370	18		99.0
CMP9838 [2]	14,610	15	HF5-4A-4 (CP007188.1)	97.7
CMP9934	14,610	15		97.7
CMP10096	14,610	15		97.7
CMP10190 [2]	14,610	15		97.7
CMP10201 [2]	14,610	15		97.7
CMP4596	17,482	18	CLB104 (CP034393.1)	99.8
CMP4924	16,427	17	AR-0412 (CP044173.1)	97.3
SHI5328	17,255	20	AR-0414 (CP044169.1)	100.0
SHI5837	17,257	20		100.0

[1] Identical LOS regions, sequence type 2993. [2] Identical LOS regions, sequence type 41. * Sample 6-1198-19 is the only sample in this report whose LOS region contains ambiguous bases (Ns). These ambiguous bases are in a non-coding region between the galactosyltransferase and *cgtA* gene.

3.3. Metagenomic Analysis

Most of the sequences were classified as bacterial or eukaryotic. The most abundant groups were Proteobacteria, Terrabacteria, and the Fibrobacteres, Chlorobi, and Bacteriodetes group (Figure S2). No common bacterial pathogens typically associated with GBS were detected in the metagenomic sequence data from fecal samples. However, sequences suggesting the presence of some GBS-causing organisms were identified in other sample types, specifically *Campylobacter concisus* and *H. influenzae* were detected at various levels.

Host-removed reads were mapped against reference genomes from various Campylobacter species, including *C. avium*, *C. coli*, *C. concisus*, *C. cuniculorum*, *C. curvus*, *C. fetus*, *C. gracilis*, *C. helveticus*, *C. hepaticus*, *C. hyointestinalis*, *C. iguaniorum*, *C. insulaenigrae*, *C. jejuni*, *C. lari*, *C. showae*, *C. sputorum*, *C. subantarticus*, *C. ureolyticus*, and *C. volucris*. Sample OBT12376 mapped to the C. concisus 13826 reference genome with 78.5% coverage, while samples OBT12389, OBT12390, and OBT12375 showed lower coverage (15.0%, 19.3%, and 37.0%, respectively) (see Table S4 and Figure S3). No other Campylobacter species were detected.

In addition to *Campylobacter spp.*, sequences derived from *H. influenzae* were also detected. A high percentage of reads from the oropharyngeal swabs of patients OBT12377 (55.0%), OBT12390 (24.1%), OBT12393 (28.4%), OBT12396 (45.2%), and OBT12398 (23.6%) mapped to the *H. influenzae* KR494 reference genome. Samples OBT12378 (4.4%), OBT12381 (7.4%), OBT12389 (16.5%), and OBT12392 (6.7%), also contained reads that mapped to the *H. influenzae*, though at lower proportions.

Plasmodium vivax was detected in reads from blood samples collected from 15 GBS patients, although coverage was limited, with most reads mapping against the ribosomal RNA region.

Human Herpesvirus 4 (Epstein–Barr Virus, EBV) sequences were detected in several patient samples, but were unequivocally confirmed only in an oropharyngeal swab sample from patient OBT12412. Notably, EBV was observed in swabs from patients OBT12375 and OBT12403, but the results for the OBT12412 were more convincing because (1) EBV was detected in this patient's sample by all three bioinformatic pipelines and (2) VirusSeeker

assembled 20 contigs from this sample, comprising 22.6% of the 173 kb viral genome. In contrast, the Zika, Dengue, Chikungunya, HIV, and Influenza viruses were not detected in any GBS sample.

In summary, we isolated and characterized the *C. jejuni* ST2993 genotype from four rectal swabs from GBS patients. Additionally, we also isolated and/or detected the presence of various pathogenic microorganisms by applying microbiological and metagenomic sequencing techniques from different biological samples from GBS patients. Figure 4 illustrates the pathogens detected and/or isolated, either as single infections or co-infections, from samples obtained from these patients.

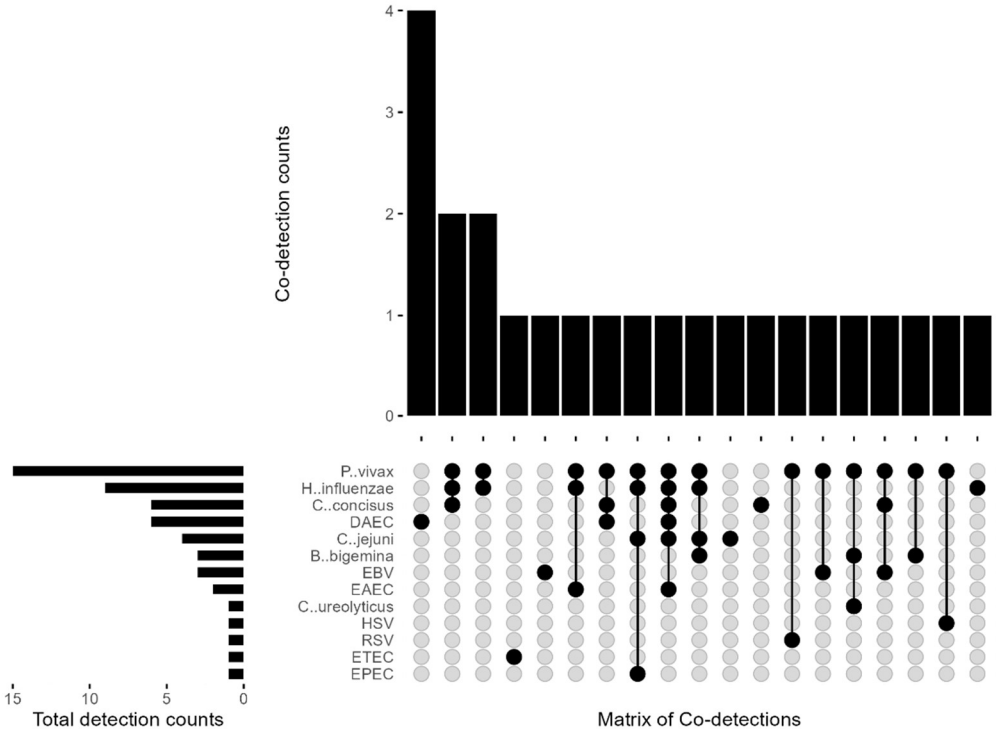

Figure 4. Upset plot illustrating co-infections and co-detections of pathogens in samples from GBS patients. The pathogens detected or isolated are listed on the lower left, with the horizontal bar sizes representing the detection frequency of each pathogen. The vertical bars indicate the count of each set interaction, while the black dots and connecting lines represent the detection of the corresponding pathogen and its co-occurrence with other pathogens.

4. Discussion

In this study, we applied multiple orthogonal approaches to perform phenotypic and genotypic characterizations of the microbial content of numerous clinical sample types from GBS patients from the 2019 Peru GBS outbreak [22]. We isolated four *C. jejuni* from rectal swabs from GBS patients and conducted a genomic characterization of these *C. jejuni* isolates alongside a metagenomic analysis of biological samples to further confirm *C. jejuni* as the potential triggering agent of the GBS outbreak. However, our study has certain limitations. Beyond the limited sample size, the absence of healthy controls in the microbiologic and metagenomic analyses is a notable limitation. Despite these constraints, our findings provide strong evidence supporting *C. jejuni* as the causative agent of the 2019 Peru outbreak.

C. jejuni is the most commonly isolated pathogen from GBS patients [3]. Although *C. jejuni* is frequently found in fecal samples from GBS patients, it can be challenging to isolate since campylobacteriosis is usually a self-limiting infection and the onset of GBS symptoms is approximately two weeks post-infection [66]. In Peru, *C. jejuni* is a common enteric pathogen, so its isolation in both symptomatic and asymptomatic individuals is

not surprising [67,68]. In this study, we isolated four *C. jejuni* specimens, all bearing CPS type HS41, a capsule type that has been previously associated with GBS patients in South Africa [63,69] and China [64] and might potentially contribute to GBS susceptibility [69]. Moreover, MLST characterization revealed that all four samples were clonal, identified as ST2993 and cgST-29793, which have also been found in diarrheal samples in Peru [24,34] and Bangladesh [70,71], as well as in a GBS outbreak in China [64]. Importantly, phylogenetic analysis of the whole-genomic sequence showed that all four strains are closely related to RM3196, RM3197, NCTC13266, and ICDCCJ07001, which were isolated from GBS patients in different regions around the world [13,63]. Remarkably, the phylogenetic analysis also revealed that closely related *C. jejuni* strains, isolated from diarrheal samples, have been circulating in the Lima and Loreto regions of Peru since 1997 and 2000, respectively [34], both with the potential to trigger GBS. Similarly, Quino et al. described the closely related circulating *C. jejuni* ST2993 from human and chicken origin in Peru [35]. Our results, along with those from Quino et al. [35], suggest that closely related *C. jejuni* strains have been circulating since 2003 in Peru and may not be restricted to regional circulation, as previously suggested by in silico MLST analysis. This widespread distribution strengthens the potential of these strains to be associated with future GBS outbreaks. These findings, combined with the presence of ST2993 in poultry, as described by Quino et al. [35], underscore that this bacterial lineage could represent a broader public health concern.

The LOS biosynthetic region of *C. jejuni* can be classified into 23 classes according to genetic composition [72]. Notably, LOS classes A, B, C, M, and R have the potential to generate LOS outer cores that structurally mimic human gangliosides [73]. Indeed, *C. jejuni* isolates bearing LOS class A have been associated with GBS cases in Bangladesh [70] and GBS outbreaks in South Africa [63] and China [64]. Specifically, LOS class A encodes GM1/GM1a, GM1b, GD1a, and GD1b-like human gangliosides [73]. In our study, the analysis of the LOS coding region revealed that the genetic composition closely resembles the class A locus [72]. Significantly, whole-genome sequencing and bioinformatic analysis of *C. jejuni* isolates were conducted in parallel at Johns Hopkins University, Maryland, U.S.A. [22], and at NAMRU SOUTH, Lima, Peru, with bioinformatics analyses of the latter sequence data being conducted at the Naval Medical Research Center-Frederick, Maryland, U.S.A. Interestingly, Ramos et al. reported a Asn51 polymorphism at *cstII* gene [22] which was not observed in our analysis. However, our study identified a homopolymeric A tract of the *cgtA* gene, resulting in a truncated gene in OBT12386, a finding not reported by Ramos et al. [22]. The *cgtA* and *cstII* genes play a crucial role in LOS sialylation, which directly impacts the development of GBS. Furthermore, the differences observed between our findings and those of Ramos et al. [22] may be attributed to stochastic sequencing errors and/or differences in bioinformatic pipelines.

We also showed evidence that 24.1% and 72.4% sera from GBS patients were positive for specific IgM and IgG against *C. jejuni*, respectively. Our results are in agreement with Ramos et al. [22], who reported strong positivity for antibodies against GM1 and GT1a in the same cohort of patients, similar to what has been previously reported in GBS patients that were infected by *C. jejuni* [73]. Furthermore, the four *C. jejuni* isolates from the INS were identical to the isolates from this study, further supporting the hypothesis that the GBS cases resulted post-infection with *C. jejuni* [21].

We hypothesize that co-infections may have played a contributing role in the development of Guillain–Barré Syndrome (GBS), especially considering that several of the agents commonly associated with GBS are endemic to Peru, reflecting the challenges of inadequate sanitation and living conditions [74]. To explore this hypothesis, we aimed to conduct a comprehensive microbiological and metagenomic analysis of samples from GBS patients. Initially, we employed standard microbiologic and immunologic techniques to search for viral particles, bacterial agents, or evidence of recent infections. While we found no consistent evidence of viral infections or recent arbovirus infections, bacterial cultures identified *E. coli* pathotypes in rectal swabs. However, few studies have reported the presence of *E. coli* pathotypes in samples from GBS patients [75,76], and it remains

unclear whether *E. coli* can indeed trigger GBS. Given their endemicity, *E. coli* pathotypes are commonly reported in Peru and other similar low-to-middle-income countries, adding a layer of complexity to our analysis [67,68].

After conducting a metagenomic analysis, we found varying levels of evidence for pathogens that have been rarely associated with GBS. After *C. jejuni*, *H. influenzae*, *M. pneumoniae*, EBV, cytomegalovirus, and the Influenza A virus are among the most frequent pathogens correlated to GBS [5]. Metagenomic sequencing analysis showed that no other potentially bacterial agents commonly linked to GBS other than *H. influenzae* was found in nine oral swabs samples, three of which were co-infected with *C. jejuni*. The GBS phenotype as a consequence of *H. influenzae* infection is similar to *C. jejuni* [77]. However, *H. influenzae* reads mapped to *H. influenza* KR494 strain, which has not been involved in GBS but to septic shock and necrotizing myositis [78]. Furthermore, although co-infections of *C. jejuni* and *H. influenzae* in GBS patients have been reported, it has been demonstrated that *H. influenzae* did not trigger GBS [79]. A limitation in our study was that we did not search for *H. influenzae* by PCR nor an ELISA to detect the cross-reactivity of sera from GBS patients against *H. influenzae* LOS. Additionally, metagenomic analysis conducted on fecal samples did not detect enteric pathogens; however, we had access to only a small set of fecal samples for metagenomic analysis.

In our dataset, we detected the presence of *P. vivax* in fifteen GBS patients. There were several reports of GBS following malaria [80–84], with three cases being associated with *P. vivax* [82,85] and twenty with *P. falciparum* [80]. Except for one report [81], none of the authors described any other co-infections. Lack of information might be attributed to an incomplete search for commonly associated GBS pathogens. The predominant GBS phenotype associated with malaria is AIDP [80]. Conversely, in our dataset, most cases were the pure motor axonal variant of GBS [22]. Interestingly, in all *P. vivax* detected in GBS patients, we detected and/or isolated up-to six different pathogens, making it difficult to assign a role for GBS development. We also detected *B. bigemina* in three samples, and this is the first time, to our knowledge, that this parasite has been reported in GBS patients. Nevertheless, one could hypothesize that multiple co-infections might play a role in triggering GBS.

Arboviruses have been reported on various occasions as being associated with GBS [86–88]. In fact, the ZIKV and CHIKV outbreaks in 2016 were associated with an increasing incidence rate of GBS cases in Latin America and the Caribbean [18]. Given that ZIKV, CHIKV, and other arboviruses are endemic in Peru [89,90], we searched for these viruses as potential triggers of the GBS outbreak in Lima. However, after metagenomic sequencing and conducting virus cultures we found no evidence of arbovirus infections in the samples tested. Nonetheless, we cannot rule out the possibility that the infecting viruses were eliminated before the onset of neurological symptoms.

Interestingly, metagenomic analysis also showed multiple reads assigned to various pathogens, from one to six other pathogens per sample. Additionally, we also found unequivocal evidence of EBV in one sample via metagenomic sequencing, yet we did not detect EBV by multiplex PCR in that same sample nor any other sample from GBS patients. Hence, although interesting, given its low prevalence in our dataset, we cannot conclude that EBV played a role in this GBS outbreak.

We hypothesize that the true incidence and potential consequences of such co-infections and their impact on GBS may be poorly understood, representing a potential area for further investigation. For instance, clinical samples are not routinely subjected to metagenomic sequencing, so all pathogens in a sample may not always be identified. All the samples in this study came from patients who presented with GBS, so we cannot assess whether co-infection increases the likelihood of developing GBS or the severity of the syndrome. However, this hypothesis is worthy of further exploration using a different study design that includes healthy controls, assessing the severity of the syndrome, testing of various biological samples by immunological, microbiological, and genomic techniques.

In Peru, multiple GBS outbreaks have occurred since 2018, primarily during the summer and autumn months, particularly in Lima and the northern regions [91–93]. The first reported outbreak in 2018 was believed to be triggered by enterovirus [92]. Subsequently, in 2020, an increase in GBS cases was observed, although no specific etiological agent was identified [91]. In 2023, another outbreak occurred in Peru, during which the *C. jejuni* ST2993 genotype was isolated from fecal samples of GBS patients [91]. Notably, despite the anticipated changes in hygiene practices due to the COVID-19 pandemic, subsequent outbreaks were still observed in Peru. The findings presented in this study, along with those of Quino et al. [35], Pachas et al. [93], and Ramos et al. [22], further support the *C. jejuni* ST2993 genotype as the etiological agent for the 2019 GBS outbreak and potentially the 2023 outbreak as well [91]. Based on the accumulated evidence, we recommend implementing comprehensive surveillance, including microbiological, immunological, and metagenomic analyses of a wide range of human and environmental samples. Adopting a one-health approach, along with adequate public health policies, could increase the likelihood of detecting potential GBS-triggering agents and thereby help prevent future outbreaks.

5. Conclusions

The cumulative evidence presented in this study further supports that *C. jejuni* was the etiological agent responsible for the 2019 Peru outbreak. Additionally, we identified several patients co-infected with various pathogens. However, the impact of co-infections on GBS remains poorly understood, warranting further investigation, particularly since clinical samples are not routinely sequenced for all pathogens. As a result, we cannot definitively determine whether co-infections increase the likelihood or severity of GBS, but this remains a promising hypothesis for future research. Finally, we underscore the importance of employing advanced sequencing techniques to comprehensively identify all potential pathogens in clinical samples. Understanding the role of co-infections in GBS could be pivotal to developing effective prevention and treatment strategies for this syndrome, as well as in mitigating future outbreaks.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres15030122/s1. Figure S1: CLC read mapping of OBT12386 reads against CP012690; Figure S2: Metagenomic content visualized in MEGAN tool; Figure S3: Mapping of cleaned reads against *C. concisus* 13826. Table S1: *Campylobacter jejuni* genomes and the corresponding accession numbers available at GenBank; Table S2: Insertions-deletions (indels) and single nucleotide variations in specific genes of *C. jejuni* isolates; Table S3: LOS regions in the *C. jejuni* genomes; Table S4: Reference mapping statistics to the six *Campylobacter* species.

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Abbreviations

AIDP	Acute inflammatory demyelinating polyradiculoneuropathy.
AMAN	Acute motor axonal neuropathy.
AR	Antibiotic resistance
ATCC	American-Type Culture Collection.
CBA	Columbia blood agar.
CCDA	Charcoal Cefoperazone Deoxycholate Agar.
cgMLST	Core genome multi-locus sequence typing.
CHIKV	Chikungunya virus.
CLSI	Clinical and Laboratory Standards Institute.
CSF	Cerebral spinal fluid.
DENV	Dengue virus.
DNA	Deoxyribonucleic acid.
EBV	Epstein–Barr Virus.
ELISA	Enzyme-linked immunosorbent assay.
GBS	Guillain–Barré syndrome
HEp-2	Human epithelial type-2 cell line.
HMAF	Hyperimmune mouse ascitic fluid
HIV	Human Immunodeficiency Virus
HNCH	Hospital Nacional Cayetano Heredia
IF	Immunofluorescence assay
LPC	Lactose positive colonies
LOS	Lipo-oligosaccharides
MDCK	Madin-Darby Canine Kidney cell line
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing.
MFS	Miller Fisher syndrome.
NAMRU S	U.S. Naval Medical Research Unit SOUTH.
OD	Optical density.
PCR	Polymerase chain reaction.
QC	Quality control.
RNA	Ribonucleic acid.
SS	Salmonella–Shigella.
ST	Sequence type.
TCBS	Thiosulfate–Citrate–Bile Salts–Sucrose.
TGB	Thioglycolate broth.
TNA	Total nucleic acid.
VF	Virulence factor.
Vero-76	African Green Monkey cell line.
ZIKV	Zika virus.

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