

ORIGINAL ARTICLE

INFECTIOUS AGENTS IN BIOLOGICAL SAMPLES FROM PATIENTS WITH GUILAIN-BARRÉ SYNDROME IN PERU, 2018-2019

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ABSTRACT

Objective: To describe the results of laboratory tests performed on biological samples from patients with Guillain-Barré syndrome (GBS) submitted to the Instituto Nacional de Salud (INS) between 2018 and 2019. **Materials and methods:** We conducted an observational study on patients with GBS, by using data from the epidemiological surveillance system. Biological samples, previously analyzed at the INS, were obtained to study arboviruses, respiratory viruses, enteroviruses and enterobacteria, among others.

Results: A total of 2,051 specimens were obtained from 906 patients with GBS. Three patients tested positive for dengue and three for Zika. In 19 patients, the stool culture was positive for *Campylobacter jejuni*. Phylogenetic analysis of 10 *Campylobacter jejuni* strains classified them as genotype ST2993, which was previously reported in China and associated to a GBS outbreak. Twelve cerebrospinal fluid samples tested positive for enterovirus by PCR in 2018, but none could be verified by culture or complete genome sequencing during the study. One patient was positive for influenza A, two for influenza B, two for adenovirus, five for respiratory syncytial virus, and ten for rhinovirus. **Conclusion:** Several pathogens were found in samples from patients with GBS. However, we found that the genotype ST2993 of *Campylobacter jejuni* was the most likely causal agent, a pathogen that is related to GBS outbreaks in different continents. It is necessary to confirm this hypothesis with additional analytical studies and it is important to describe the transmission mechanism of *C. jejuni* genotype ST2993 in order to implement prevention and control measures.

Keywords: Guillain-Barre Syndrome; *Campylobacter jejuni*; Enterovirus Infections (Source: MeSH NLM).

INTRODUCTION

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Guillain-Barré syndrome (GBS) is an acute polyneuropathy that manifests as an acute ascending flaccid paralysis. Its diagnosis is mainly clinical, based on the anamnesis and findings during physical examination, complemented by electromyographic results and the typical changes observed in the cerebrospinal fluid (CSF)⁽¹⁾. The physiopathology of GBS is based on an immunological phenomenon, generally triggered by an infectious process. It has been postulated that this immune response is initiated by a wide variety of antigens, such as infections by virus or bacteria, particularly *Campylobacter jejuni*⁽²⁾. A systematic review has estimated that between 40 and 70% of the cases of GBS are preceded by an upper respiratory infection or an infection of the gastrointestinal tract; mainly due to *C. jejuni*⁽³⁾ which is the cause of GBS in up to 31% of cases⁽²⁾. In a meta-analysis, inactivated monovalent influenza A (H1N1) virus vaccine was reported to increase the risk of GBS by 1.6 cases per million doses applied, a small increase⁽⁴⁾.

Zika is one of the viruses reported as possible etiological agents of GBS. Outbreaks of Zika associated with GBS have been reported studies carried out in French Polynesia⁽⁵⁾, Brazil,

Colombia⁽⁶⁾, Dominican Republic, El Salvador, Honduras, Suriname, and Venezuela⁽⁷⁾. A bias in all these studies is that no tests have been conducted to rule out etiological agents other than Zika, dengue, and chikungunya.

Viruses from the genus *Enterovirus* are associated with acute flaccid paralysis (AFP), with a clinical picture like GBS. This genus has 12 species including enteroviruses A to J and rhinoviruses A, B and C. The transmission route depends on the serotype, some are transmitted by the fecal-oral route and others by the respiratory tract. Many enteroviruses are neurotropic and can affect the central or peripheral nervous system. For example, Coxsackievirus and echovirus can cause viral meningitis; *Coxsackie*, *enterovirus A71* and *echovirus 9* can produce meningoencephalitis⁽⁸⁾.

Poliovirus and enteroviruses D68 and A71 can produce AFP. In 2014, an increase in acute respiratory infection caused by EV-D68 was observed in the United States as well as an AFP outbreak associated with EV-68⁽⁹⁾. Other viruses, such as Coxsackie and echovirus, were reported to cause AFP in Korea, with a low incidence⁽¹⁰⁾. GBS incidence varies with age, seasonality, and the place where measurements are made. Considering all age groups, the incidence varies from 0.16 to 3×10^5 person-years, is higher in men and increases with age⁽³⁾. Peru has had an unusual increase of GBS cases since 2018, with 1,378 cases reported and an incidence of 4.59×10^5 inhabitants, compared to previous years, which was approximately 0.91×10^5 inhabitants⁽¹¹⁾. In 2019, until the epidemiological week number 47, a total of 1,341 cases were reported⁽¹²⁾. A high percentage of the cases occurred in the northern and central departments of the country (Lambayeque, La Libertad, Piura, and Lima). However, almost all of Peru's departments were affected by the outbreak. Several hypotheses have been proposed regarding possible infectious agents associated with GBS⁽¹³⁾; however, information about affected patients and the results of the tests conducted have not been systematized.

The aim of this study is to describe the results of laboratory tests performed on biological samples from patients diagnosed with GBS received at Instituto Nacional de Salud between 2018 and 2019.

MATERIALS AND METHODS

Type of study and population

We carried out an observational and descriptive study on patients with GBS treated in the hospital networks of

KEY MESSAGES

Motivation for the study: Due to the unusual increase in cases of Guillain-Barré syndrome (GBS) between 2018 and 2019, we conducted a search for the possible etiologic agent that triggers the autoimmune process that causes this neurological syndrome.

Main findings: We identified several pathogens, among which we observed a variant of *Campylobacter jejuni* associated with GBS outbreaks in other countries. We propose this pathogen as a probable causal agent of the outbreaks in Peru.

Implications: These findings open the opportunity to identify the infectious agent that triggers GBS and will help to implement prevention and control measures to cut the chain of infection of *Campylobacter jejuni*.

Ministerio de Salud (MINSA), Seguro Social (EsSalud) and Sanidad de las Fuerzas Armadas y Policiales, from which biological samples were obtained to be analyzed in the Instituto Nacional de Salud (INS) or processed in the Regional Reference Laboratories. To contextualize the outbreak, we reviewed the reports on the website of the Centro Nacional de Epidemiología, Prevención y Control de Enfermedades (CDC-Peru) on the number of GBS cases reported per epidemiological week since the beginning of 2018.

The INS is the state agency in charge of public health research and specialized diagnosis. INS and CDC-Peru work on the investigation of unusual health occurrences and epidemic outbreaks. As part of the investigation of an outbreak, immunological, chemical, phenotypic and molecular laboratory tests, among others, are performed to explore the association with infectious agents, chemicals, or both.

We included patients from the epidemiological surveillance system who sent biological samples to the Regional Reference Laboratories of Lima, Piura, Lambayeque, La Libertad, Cajamarca, Junín or to the INS to rule out infectious diseases. We also described the report of a private laboratory that processed biological samples from eight patients and sent the results to the INS.

In Peru, since 2016, the GBS has been a notifiable disease for the Epidemiological Surveillance System, in all public and

private health facilities, according to the emergency health protocol for GBS surveillance ⁽¹⁴⁾. This standard defines a clinically confirmed case of GBS (level 3 of diagnostic certainty, according to the Brighton criteria) when the following criteria are met: bilateral flaccid muscle weakness in the extremities; diminished or absent deep osteotendinous reflexes in the weakened extremities; monophasic disease, with an interval between the onset and nadir of the weakness between 12 hours and 28 days, followed by a clinical plateau; and absence of alternative diagnosis to explain the weakness. GBS may or may not have electrophysiological findings in the CSF regarding albumin-cytological dissociation. In June 2019, CDC-Peru, in response to the increase in GBS cases in northern Peru, issued an epidemiological alert notifying of all clinical cases of GBS along with the recollection of the following biological samples: serum, feces, nasopharyngeal swabs, CSF, and urine ⁽¹⁵⁾.

Procedure

Serum tests used were IgM and IgG ELISA for Dengue, Zika and Chikungunya viruses; and quantitative polymerase chain reaction (qPCR) for Dengue and Zika viruses. IgM ELISA and polymerase chain reaction (PCR) in CSF were used for Dengue and Zika viruses; PCR for enterovirus, as well as the FilmArray® meningoencephalitis/encephalitis panel. We performed real-time reverse transcription polymerase chain reaction (qRT-PCR) on nasopharyngeal swab samples to detect influenza A and B viruses, rhinovirus, respiratory syncytial virus, and adenovirus. We used direct immunofluorescence for influenza A and B viruses, respiratory syncytial virus, adenovirus, parainfluenza virus 1, parainfluenza virus 2, parainfluenza virus 3, and metapneumovirus. We used rectal swabs and stool cultures to search for enteropathogenic bacteria and enteroviruses, as well as PCR for enteroviruses, *Campylobacter* spp., diarrheagenic *Escherichia coli* and *Shigella* spp.

Any PCR result that indicated the presence of genetic material from any of the mentioned microorganisms was considered positive, as well as the predetermined values of ELISA results for each etiological agent ⁽¹⁶⁾. In the case of results submitted to the INS by other laboratories, the method mentioned in the report was shown.

We selected the infectious agents according to their frequency to be subjected to sequencing of the entire genome. In the case of viruses, the sequencing was done with the Sanger method ⁽¹⁷⁾. The sequencing result was obtained in the ABI format, and the sequences were assembled and analyzed using

the Geneious V8 software. Likewise, the Blast and Kallisto algorithms were used to detect the most similar transcripts ⁽¹⁸⁾.

Four CSF samples and three stool samples collected in 2018, which tested positive for enterovirus at the INS, were sent to the Center of Diseases Control of the United States of America (CDC-USA), where PCR was performed for enterovirus and human parechovirus.

Statistical analysis

We obtained the laboratory results from the NetLab application, the INS laboratory results reporting system, and exported them to an Excel database (Microsoft Office®). Categorical variables were described using relative and absolute frequencies. For the numerical variables, their distribution was determined by means of the Shapiro-Wilk test and they were described in means and standard deviations or medians and interquartile range, respectively, if they fulfilled or not the normality criterion. The analysis was performed in Stata version 9 (StataCorp, College Station, Texas, USA).

Ethical aspects

This study is part of the investigation of the GBS outbreak in Peru. NetLab registry codes were used without identifying the patients. Laboratory results were only used in the context of this epidemiological situation, so it was not necessary to submit them to an institutional ethics committee for approval.

RESULTS

The INS received a total of 2,051 biological samples including serum (738), CSF (247), nasopharyngeal swab (475), urine (278), rectal swab and/or stool (313). These samples were obtained from a total of 906 patients with a clinical diagnosis of GBS from April 27, 2018 to November 5, 2019, of which 26 (2.9%) samples were obtained in 2018 and 880 (97.1%) in 2019. Figure 1 shows the number of patients with GBS reported by the CDC-Peru and the number of patients whose biological samples have been sent to the INS (included in this study) in the mentioned period.

Most patients were from Lima, Piura, La Libertad, and Junín (Table 1), and from the following hospitals: 52 (5.9%) patients were from the Hospital de Apoyo Santa Rosa, Piura; 49 (5.6%) from Hospital Nacional Almanzor Aguinaga Asenjo, Chiclayo; 48 (5.5%) from Hospital Edgardo Rebagliati Martins, Lima; and 43 (4.9%) from Hospital Nacional Daniel Alcides Carrión, Junín.

From the 738 serum samples, 735 had negative serology or PCR results for arbovirus; 3 patients were positive for dengue and came from Lambayeque, La Libertad, and Lima. Likewise, 3 patients had positive PCR in urine for Zika virus. Of the CSF samples, 12 were positive by PCR for enterovirus; however, all were from 2018 and all cultures were negative. Five of these patients also had positive PCR for enterovirus in their stool. Of the 220 CSF samples from 2019 analyzed by PCR and culture for enterovirus, none were positive; the 90 samples processed by the FilmArray technique were negative for the 14 pathogens identified by the test. Regarding respiratory viruses, 1 sample was positive for influenza A virus, 2 for influenza B virus, 2 for adenovirus, five for respiratory syncytial virus, and ten for rhinovirus. Finally, in 24 patients, stool/rectal swab culture was positive for *Campylobacter* spp. and 69 for diarrhoeagenic *E. coli* (Table 2).

A private laboratory in Lima reported to INS the results obtained by the FilmArray® gastrointestinal panel, from 8 patients with GBS reported in 2018. There were 5 positive results for enteropathogenic *E. coli*, 4 for *Campylobacter* spp., and 3 for human rhinovirus/enterovirus, *Shigella* spp., enteroinvasive *E. coli*, and enteroaggregative *E. coli*.

We carried out the molecular study of CSF and stool

samples received by the INS with positive PCR results for enterovirus. First, the 5' UTR region was amplified and identified as enterovirus. Then, the complete genome was sequenced; however, the number of fragments detected was less than 10% of the total virus genome, so it was not possible to identify the species. Out of 4 CSF samples and 3 stool samples with positive PCR for enterovirus sent to the CDC-USA, only 1 stool sample was positive for *echovirus* 1.

Similarly, the complete genome of the 10 strains isolated from *Campylobacter* spp. was sequenced: 6 biological samples received at the INS (two from Piura and four from Junín) and 4 strains isolated by the Naval Medical Research Unit 6 (NAMRU-6). All specimens were from 2019. We proceeded according to the methods previously described and identified the *C. jejuni* species. The phylogenetic analysis of this strain's genomic sequence identified it as genotype ST2993, a genotype never reported in Peru. The ten isolates are from the same clone, which means, they share the same genetic information. As part of the genomic analysis, we also identified the presence of *cst-II* and *cst-III* genes, which code for lipooligosaccharides associated with GBS. Likewise, we observed the presence of a specific point mutation in the quinolone resistance

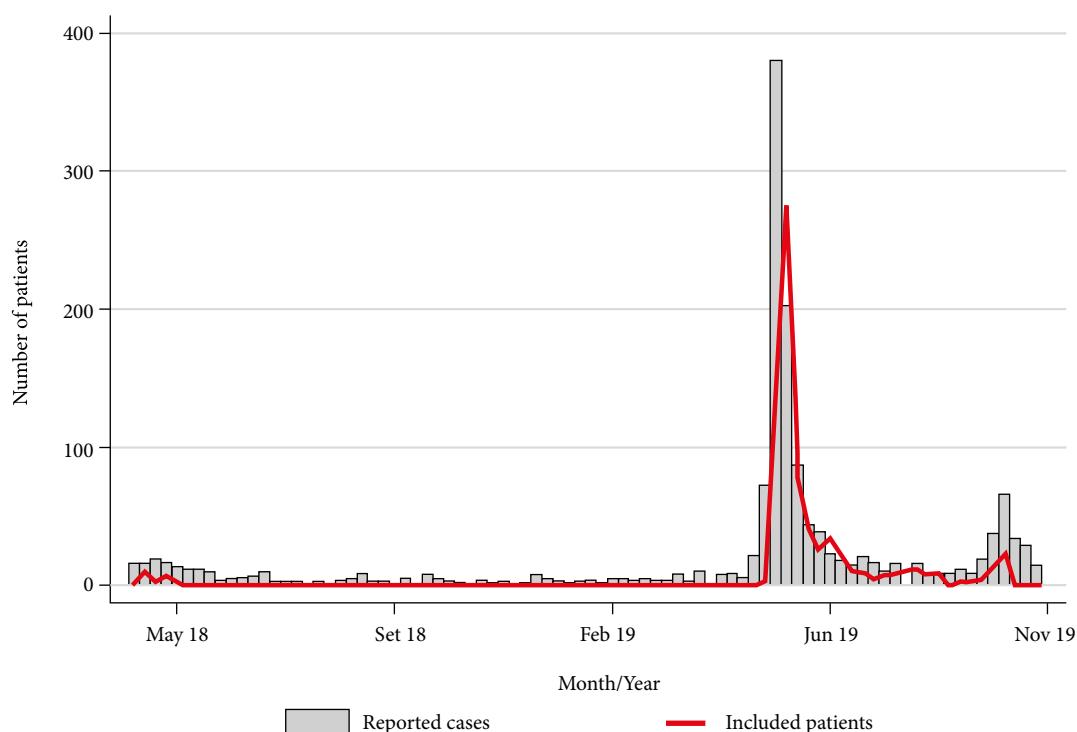


Figure 1. Cases of Guillain-Barré syndrome reported by Centro Nacional de Epidemiología, Prevención y Control de Enfermedades and patients with biological samples sent to Instituto Nacional de Salud, Peru, 2018-2019.

Table 1. Demographic characteristics and provenance of Guillain-Barré syndrome cases with biological samples sent to Instituto Nacional de Salud, 2018-2019 (n = 906).

General characteristics	n (%)
Median age (IQR)	40 (24-56)
0-14	112 (12.7)
15-24	113 (12.8)
25-40	207 (23.4)
41-60	271 (30.7)
>60	181 (20.5)
No data	22 (2.4)
Male sex	539 (59.5)
Provenance	
Lima	358 (39.5)
Piura	199 (22.0)
La Libertad	98 (10.8)
Junin	82 (9.1)
Lambayeque	61 (6.7)
Cajamarca	48 (5.3)
Others	60 (6.6)

IQR: Interquartile Range

determining region (QRDR) of the *gyrA* gene (Thr86Ile), which is related to resistance to fluoroquinolones.

DISCUSSION

We were able to systematize the laboratory results of patients diagnosed with GBS in Peru whose biological samples were analyzed at the INS between 2018 and 2019, as well as those whose results were submitted to the INS. Medical literature reports that up to 75% of GBS cases have a history of previous respiratory or digestive infection in the last six weeks; however, in many of them, the infection had already been controlled by the time the etiological agent began to be searched, thus it couldn't be isolated⁽¹⁹⁾. The presence of *C. jejuni* (6.5%) stands out among all the infectious agents isolated or identified in this study. Other agents we identified are the following: arbovirus (1.1%), enterovirus (2%), diarrheagenic *E. coli* (22.9%) and respiratory viruses as rhinovirus in 5.6% and RSV in 2.8%.

This is the first report of isolation of *C. jejuni* ST2993 in Peru and America. A strain of *C. jejuni* of the same genotype ST2993 was reported in 2007 in China and was associated to a GBS outbreak⁽²⁰⁾. Previous reports of genetic sequencing of Peruvian isolates of *Campylobacter* show strains genetically

quite different from this new strain⁽²¹⁾. Ten isolated strains were genetically sequenced, and the results were indistinguishable from each other, so we conclude that it is the same clone with a wide distribution since it was identified in the regions of Piura, Junín, and Lima. There are multiple reports associating *C. jejuni* with GBS^(2,20,21). Activated macrophages, T lymphocytes and serum antibodies against human gangliosides are related to the physiopathology of GBS and it is believed that the lipooligosaccharides of *C. jejuni* antigenically mimic these gangliosides, triggering the immune reaction⁽²²⁾. Therefore, this finding is significant and urges to investigate the chain of infection of *C. jejuni*, which is usually associated with poultry, dairy food consumption, water, meat, among others.

We identified diarrheagenic *E. coli* in stool/rectal swab samples from 69 patients. However, these strains that showed high heterogeneity corresponded to different pathotypes. There is little information on the association between this bacterium and the occurrence of GBS, and previous reports refer predominantly to urinary tract infections rather than enteric infections⁽²³⁾. We have not found reports of GBS outbreaks with this etiology, so the association with the outbreak in Peru seems unlikely, considering that *E. coli* is a germ that is part of the human gastrointestinal tract microbiome.

Regarding arboviruses, we identified infection by dengue virus in three patients (two by IgM antibodies and one by serum PCR) and infection by Zika virus also in three patients (positive PCR in urine). Additionally, two patients had positive IgM antibodies against chikungunya virus; one was from Piura and the other from Cajamarca (province of Jaén). There are several reports in the literature about the association between GBS and Zika virus infection⁽²⁴⁾. However, in our study there were only three cases which reflects the usual transmission in endemic areas of this virus. The same is true for chikungunya, an increase of GBS cases was reported during an outbreak of chikungunya in French Polynesia as well as other reports of sporadic cases⁽²⁵⁾. However, it should be considered that this outbreak of GBS has even affected areas of the central and northern highlands of Peru, where there is no transmission of these viruses. Recently, the role of Zika virus in these outbreaks has been questioned because most of the studies on this subject do not use a control group which does not allow to determine association⁽²⁶⁾.

Few patients test positive for respiratory viruses. Although the association between GBS and influenza virus is documented⁽²⁷⁾, we believe that due to the low frequency observed in this group of

Table 2. Laboratory results of biological samples sent to Instituto Nacional de Salud, from patients with Guillain-Barré syndrome, Peru, 2018-2019.

Sample type	Diagnostic test	Positives n (%)	Total of processed samples
Serum	Dengue IgM ELISA	2 (0.5)	369
	qRT-PCR Dengue	1 (1.9)	54
	qRT-PCR Zika	0 (0)	372
	Zika IgMELISA	0 (0)	597
	Chikungunya IgM ELISA	2 (0.3)	581
	qRT-PCR Chikungunya	0 (0)	15
Urine	qRT-PCR Zika	3 (1.1)	278
CSF	qRT-PCR Zika	0 (0)	82
	IgM Zika ELISA	0 (0)	64
	qRT-PCR Dengue	0 (0)	5
	ELISA IgM Dengue	0 (0)	1
	RT-PCR Enterovirus	12 (5.1)	234
	Culture of Enterovirus	0 (0)	222
Swab	FilmArray® Meningitis/ Encephalitis panel	0 (0)	90
	DIF Influenza A virus	0 (0)	293
	qRT-PCR Influenza A virus	1 (0.2)	475
	DIF Influenza B virus	1 (0.3)	304
	qRT-PCR Influenza B virus	1 (0.2)	464
	qRT-PCR Rhinovirus	10 (5.6)	179
	qRT-PCR RSV	5 (2.8)	177
	Dif RSV	0 (0)	171
	qRT- PCR Adenovirus	2 (1.1)	176
	DIF Adenovirus	0 (0)	171
	DIF Parainfluenza 1	0 (0)	171
	DIF Parainfluenza 2	0 (0)	171
	DIF Parainfluenza 3	0 (0)	171
	DIF Metapneumovirus	0 (0)	171
Rectal swap	RT-PCR Enterovirus	5 (2.0)	250
	Campylobacter spp (by PCR and culture)	24 (6.5)	367
	Diarrheagenic <i>E. coli</i>	69 (22.9)	302

CSF: cerebrospinal fluid, qRT-PCR: real-time quantitative reverse transcription polymerase chain reaction; RT-PCR: reverse transcription polymerase chain reaction; PCR: polymerase chain reaction; DIF: direct immunofluorescence.

patients these viruses might not be relevant to the development of GBS in the outbreak in Peru.

The CDC-US laboratories (*echovirus* 1) and one Peruvian private laboratory identified enteroviruses in CSF of patients diagnosed with GBS in 2018, these results were consistent with the INS findings. However, all viral cultures for enteroviruses were negative, possibly because of an inadequate sample or some problem that may have affected the viability of the virus. Furthermore, this finding was limited to 2018. During the 2019 outbreak, no genetic material for enteroviruses was isolated or identified in any patient's stool or CSF samples. There are reports in the literature associating enteroviruses with acute neurological syndrome, specifically enterovirus D68⁽⁹⁾. However, these reports refer to acute flaccid paralysis with a neurological picture that affects the motor neuron located in the anterior horn of the spinal cord, a clinical and electrophysiological picture different from GBS. In the GBS the peripheral nerves are affected in each of the different forms of presentation⁽²²⁾, this has been reported in Peru. Based on these findings, we ruled out the epidemiological link of enterovirus with this GBS outbreak.

Papers on the study of the phylogeny of digestive pathogens in Peru are scarce and the few that exist have been conducted on children under five years old, which limits our knowledge about the microorganisms that circulate in our country. At a time when technological advances allow access to genome sequencing in public health, this knowledge gap must be quickly resolved with research or, ideally, with the implementation of specific sentinel surveillance.

There are some limitations to our study. It uses a descriptive design and does not have a control group, consequently, the association between GBS and a potential etiologic agent was not demonstrated; however, our hypothesis is that *C. jejuni* is the potential etiologic agent, which should be demonstrated with further analytical studies. The tests were carried out by regional laboratories and by the INS, under the usual parameters of quality control, without additional studies of concordance between laboratories. Only one stool sample or rectal swab was collected for stool culture to isolate *Campylobacter* spp., therefore the sensitivity of this test may be reduced. One study reported a sensitivity of 100% with three consecutive stool samples⁽²⁸⁾. In Peru, self-medication with antibiotics is relatively frequent, and can range from 34% in the general population⁽²⁹⁾ to 70% in university students⁽³⁰⁾; without this information about

the patients, the sensitivity of stool tests could be reduced. The time between the collection of stool samples and the processing to carry out the stool culture in many cases was more than 24 hours, which reduces the sensitivity to isolate *C. jejuni*. The collection of biological samples was not carried out according to the surveillance system; samples such as CSF were collected only in 25.8%, urine in 30.7% and stool or rectal swab in 40.5% of the cases. Finally, serum samples were usually collected after the treatment had already started, which could alter the serological tests with false positive results in patients who were given immunoglobulins, or false negatives if plasmapheresis were used.

In conclusion, various pathogens were identified in biological samples from patients with GBS in the outbreaks that occurred in Peru between 2018 and 2019. The most outstanding finding from 2018 was the identification of enteroviruses by PCR at the INS; however, this finding could not be confirmed by genome sequencing and, in addition, of the four samples sent to the CDC-USA only one was positive for echovirus. All CSF and stool samples were negative for enteroviruses by culture and PCR in 2019. On the other hand, it was possible to isolate *C. jejuni* genotype ST2993 in 2019, a pathogen widely related to GBS in several continents. This finding opens new research lines to carry out analytical studies, identify the reservoir and the source of infection to implement the most effective prevention measures and cut the chain of infection. Likewise, it opens a whole new field of clinical research, such as the genetic factors associated to the susceptibility to this syndrome, the prognostic factors to present complications or to die, the effectiveness of the different treatments, among others.

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Contributions of the authors: PP, FD and LS participated in the conception of the study, analysis and interpretation of the data and writing of the article. WQ, MV and CC participated in the interpretation of the data and writing of the article. RG, MG, MH, FP and FV processed the laboratory samples. All the authors approved the final version and declare themselves responsible for its content.

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