Campylobacter jejuni capsule types in a Peruvian birth cohort and associations with diarrhoeal disease severity

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Abstract

Campylobacter jejuni is a leading cause of bacterial diarrhoea worldwide. The objective of this study was to examine the association between *C. jejuni* capsule types and clinical signs and symptoms of diarrhoeal disease in a well-defined birth cohort in Peru. Children were enrolled in the study at birth and followed until 2 years of age as part of the Malnutrition and Enteric Infections birth cohort. Associations between capsule type and clinical outcomes were assessed using the Pearson's χ^2 and the Kruskal–Wallis test statistics. A total of 318 *C. jejuni* samples (30% from symptomatic cases) were included in this analysis. There were 22 different *C. jejuni* capsule types identified with five accounting for 49.1% of all isolates. The most common capsule types among the total number of isolates were HS4 complex ($n = 52$, 14.8%), HS5/31 complex ($n = 42$, 11.9%), HS15 ($n = 29$, 8.2%), HS2 ($n = 26$, 7.4%) and HS10 ($n = 24$, 6.8%). These five capsule types accounted for the majority of *C. jejuni* infections; however, there was no significant difference in prevalence between symptomatic and asymptomatic infection (all $p > 0.05$). The majority of isolates ($n = 291$, 82.7%) were predicted to express a heptose-containing capsule. The predicted presence of methyl phosphoramidate, heptose or deoxyheptose on the capsule was common.

Introduction

Diarrhoeal disease is the leading infectious disease cause of morbidity and the second leading infectious cause of death globally in children under 5 years of age [1]. Morbidity resulting from enteric infection can have significant consequences, including acquired malnutrition and linear growth deficits from repeated enteric infections before the age of two [2, 3]. Malnutrition in early childhood may predispose children to more severe and prolonged infections, and can result in impaired cognitive development that yield negative societal outcomes and long-term health effects including cardiovascular and metabolic diseases [3–5].

One of the leading causes of bacterial diarrhoea and enteric infection worldwide is *Campylobacter jejuni* [6, 7]. In developed regions of the world, *C. jejuni* is a common food-borne pathogen associated with poultry and contaminated dairy products. Infection can cause an acute dysentery and/or febrile illness in children and adults [6]. In developing countries, *C. jejuni* is endemic. Poor hygiene, lack of sanitation and living in close proximity to animals contribute to recurrent infection. *C. jejuni* is frequently isolated in the stools of healthy children and the rates of infection between symptomatic and asymptomatic cases are often similar. Clinical disease from *C. jejuni* in developing countries primarily affects the paediatric population and typically results in acute watery diarrhoea with concomitant signs and symptoms such as fever, abdominal pain and vomiting [6, 7]. Additionally, recent studies have also found an association of *Campylobacter* infection with malnutrition and growth stunting in paediatric populations in the developing world [7].

To date only a few virulence factors have been characterised in *C. jejuni*. Nevertheless, the polysaccharide capsule (CPS) present on the surface of the bacterium has been demonstrated to be one of the most important virulence determinants [8]. The capsule is composed of repeating saccharide units attached to the outer membrane via to a phospholipid anchor [9]. Many of *C. jejuni* CPSs are characterised by the presence of heptoses in unusual configurations (e.g. altro, ido, gulo) and non-stoichiometric modifications to the sugars, including ethanolamine and methyl phosphoramidate (MeOPN). The levels of MeOPN are non-stoichiometric due to phase variation of the genes encoding MeOPN transferases. Recent studies have shown that MeOPN contributes to complement resistance [8, 10, 11].

Capsule is the major serodeterminant of the Penner serotyping scheme, of which there are 47 serotypes of *C. jejuni* [12]. However, genomic analyses of the variable regions of the CPS

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loci of the 47 Penner serotypes indicated that there are 35 distinct CPS types [13]. Strains belonging to the same serotype group are predicted to express the identical CPS structures. Nevertheless, difference in the Penner serotype can be also due to differences in lipooligosaccharide (LOS) structures; the LOSs have been suggested to be a minor serodeterminant in the Penner typing scheme [14]. The LOSs are additional saccharide structure present at the surface of the bacterium. Compared with the CPS, LOSs are composed only of a few repeating saccharide units and are anchored in the membrane via a different family of lipid. In this study, we used a multiplex polymerase chain reaction (PCR) method for determination of CPS types. This method can discriminate the 35 CPS types, shown in Table 1. Unlike the Penner typing scheme, the capsule multiplex PCR method is not subjected to LOS interference for the attribution of CPS type. Nonetheless, the attribution is based on the presence of gene sequences and does not provide information on the modulation of structure and expression level of the capsule. *C. jejuni* present homopolymeric GC track in the nucleic sequence of genes involved the capsule biosynthesis. During replication, insertion/deletion of G or C nucleotide(s) induces a frame shift that can cause the enzyme to be non-functional. This mechanism adds an additional layer of complexity to the modulation of the capsule structure [15]. It is believed that these modulations confer an advantage in escaping the host immune response.

Currently, there are no vaccines against *C. jejuni*; however, efforts are ongoing to develop a CPS conjugate vaccine [16, 17]. Capsule-based vaccines have been successfully developed for other encapsulated mucosal pathogens including type B *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*. Given these successes, and the fact that the CPS is a major virulence determinant, a capsule conjugate *C. jejuni* vaccine is a rationale strategy that could significantly reduce the global burden of disease.

The Interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development (MAL-ED) project was established in 2009 as a worldwide collaboration to further investigate the impact of enteric infection, including *C. jejuni*, on child health outcomes, growth and development [5]. The MAL-ED network collected data on diarrhoeal illness in poverty stricken communities in eight developing countries: Peru, Brazil, Bangladesh, India, Pakistan, Tanzania, South Africa and Nepal. Initial results from the global MAL-ED project revealed *Campylobacter* as the most frequently isolated pathogen, and highlight *Campylobacter* as a cause of significant morbidity in children in Loreto, Peru [7, 18]. This study also demonstrated an association between *Campylobacter* infection and linear growth shortfalls and increased intestinal permeability and inflammation in children [19]. Here we apply the multiplex method of determination of CPS types to *C. jejuni* strains isolated as part of the MAL-ED project in Peru.

Materials and methods

Clinical data were collected from three rural communities in the Department of Loreto in Peru: Santa Clara de Nanay, Santo Tomás and La Unión as previously described [20]. Briefly, 198 Peruvian children were enrolled within 17 days after birth and followed until 2 years of age. Field researchers visited the participant's home twice a week and collected information from the mother or caregiver regarding the child's dietary intake, general health information and surveillance for infectious diseases since the previous visit [20]. Stool samples were collected during diarrhoeal episodes, classified as three or more loose stools in a 24-h period if onset was after two or more diarrhoea-free days. Staff members obtained data related to diarrhoeal illness, including associated symptoms and whether any treatment or hospitalisations were needed. Associated symptoms included the presence and duration of dehydration, fever >37.5 °C, anorexia, vomiting, dysentery, the need for oral rehydration therapy or hospitalisation and any episode with four or more semi-liquid/liquid stools, among others. Routine, non-diarrhoeal stool samples were also obtained at monthly surveillance visits to assess for asymptomatic shedding during the first year of life and quarterly during the second year of life.

Definitions

Diarrhoea was defined as greater than or equal to three loose stools in a 24-h period or the presence of blood in at least one stool sample [20]. Two diarrhoeal episodes were considered to be distinct if separated by at least 2 days of normal stool. *C. jejuni* diarrhoeal illness was defined as any diarrhoeal episode in which *C. jejuni* was isolated. Asymptomatic infection was defined as a non-diarrhoeal stool sample that was positive for *C. jejuni*. The first *C. jejuni* positive sample, obtained from either a symptomatic or asymptomatic sample, was considered to be the first infection. Dysentery was defined as mother's observation of blood in a stool during an episode of diarrhoea.

Microbiology

Campylobacter strains were isolated as previously described and underwent phenotypic testing utilising oxidase and catalase phenotypical testing for differentiation between *C. jejuni* and *C. coli* [7]. Isolates were stored at −80 °C in Mueller–Hinton (MH) broth containing 15% glycerol. Strains were revived by plating onto MH agar plates and incubation under a microaerobic atmosphere (85% N_2, 10% CO_2 and 5% O_2) at 37 °C for 24–72 h.

Capsule typing

DNA from the revived strains was extracted using the DNeasy extraction kit (Qiagen, USA). Capsule typing was performed using a multiplex PCR assay following the Poly *et al.* protocol. The typing system is able to discriminate all of the 35 CPS types described above for *C. jejuni*. It also includes a *C. jejuni*-specific positive primer set to confirm speciation [13]. Samples that tested negative with this species-specific primer set were excluded from analysis. Non-typeable *C. jejuni* isolates were those confirmed by multiplex PCR as *C. jejuni*, but with an unidentifiable capsule type. Samples that contained multiple *C. jejuni* CPS types were counted within the total number of isolates for each CPS type. These individuals were assumed to be co-infected.

Analyses

Analyses were performed based on capsule phenotype across the total number of infections and the number of first infections. Descriptive analyses were performed to assess the prevalence of each capsule type for all, first and subsequent total infections and diarrhoea-associated infections. Diarrhoeal illness characteristics were assessed across capsule types for all and first

Table 1. Summary of CPS types identified by multiplex PCR

Serotype	Reference for CPS structure	MeOPN genes	Heptose genes	Deoxy-Hep gene	Determined heptose structure
HS6/7	Muldoon 2002	−	−	−	N/A
HS9	ND	−	−	−	N/A
HS21	ND	−	−	−	N/A
HS27	ND	−	−	−	N/A
HS38	ND	−	−	−	N/A
HS55	ND	−	−	−	N/A
HS57	ND	−	−	−	N/A
HS1	McNally 2005	+	−	−	N/A
HS18	ND	+	−	−	N/A
HS19	ND	+	−	−	N/A
HS22	ND	+	−	−	N/A
HS37	ND	+	−	−	N/A
HS63	ND	+	−	−	N/A
HS2	Karlyshev 2005; McNally 2007	+	+	−	6-O-Me-D-*glycero*-L-*gluco*-Hep$p$
HS33/35	ND	+	+	−	ND
HS8/17	ND	−	+	+	ND
HS11	ND	−	+	+	ND
HS12	ND	−	+	+	ND
HS32	ND	−	+	+	ND
HS40	ND	−	+	+	ND
HS41	Hannify 1999	−	+	+	6-Deoxy-β-D-*altro*-Hep$p$
HS42	ND	−	+	+	ND
HS45	ND	−	+	+	ND
HS53	Gilbert 2007	−	+	+	6-Deoxy-α and β-D-*manno*-Hep$p$
HS58	ND	−	+	+	ND
HS60	ND	−	+	+	ND
HS3	Aspinall 1995	+	+	+	6-Deoxy-α-D-*ido*-Hep$p$
HS4 complex	Chen 2008	+	+	+	6-Deoxy-β-D-*ido*-Hep$p$
HS5/31	ND	+	+	+	ND
HS10	ND	+	+	+	ND
HS15	Bertolo 2013	+	+	+	6-Deoxy-α-*gulo*-Hep$f$
HS29	ND	+	+	+	ND
HS23/36	Aspinall 1992	+	+	+	6-Deoxy-α-D-*altro*-Hep$p$ 6-Deoxy-3Me-α-D-*altro*-Hep$p$ D-*glycero*-α-D-*altro*-Hep$p$ 3Me-D-*glycero*-α-D-*altro*-Hep$p$
HS44	ND	+	+	+	ND
HS52	ND	+	+	+	ND

ND, not determined; N/A, not applicable; Hep, heptose; p, pyranose configuration; f, furanose configuration.

symptomatic infections. To determine if specific *C. jejuni* capsule structures were associated with diarrhoeal disease severity, we compared the prevalence of multiple clinical parameters by infection with specific capsule structures. Statistical comparisons were made using Pearson's χ^2 tests with a two-sided $\alpha = 0.05$. All statistical analyses were performed using SAS version 9.3 (Cary, NC).

This study was approved by the Naval Medical Research Center Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects. The Institutional Review Board of Johns Hopkins Bloomberg School of Public Health and the ethics committee of Asociacion Benefica PRISMA in Lima, Peru approved the paediatric study.

Results

A total of 318 *C. jejuni* infections were identified, of which 171 (53.8%) were characterised as 'first infections' (Table 2). Approximately 70% of all infections were asymptomatic with no significant differences in the proportion of diarrhoeal cases between the first and subsequent infections (29.2% *vs.* 32.0%, respectively; *p* = 0.6). From the 318 infections, 352 *C. jejuni* isolates were identified due to co-infection with two *C. jejuni* CPS types in 30 subjects and three CPS types in two subjects. Co-infection with multiple *Campylobacter* isolates was equally prevalent among first and subsequent infections (11.4% and 7.5%, respectively; *p* = 0.2).

The top five most common serotypes among all infections included the HS4 complex (14.8%), the HS5/31 complex (11.9%), HS15 (8.2%), HS2 (7.4%) and HS10 (6.8%) with no significant differences in the frequency of isolation by the presence or absence of clinical illness (Figs 1 and 2). The HS4 complex, which encompasses eight related CPS types, was the most common capsule type identified among all (14.8%), first (16.1%) and subsequent (13.2%) infections. The HS4 complex was also the most common among total diarrhoea-associated infections (18.2%). However, the HS5/31 complex was the most common capsule type identified in diarrhoea-associated first infections (15.0%).

The majority of children (95/172; 55.2%) had between two and up to seven *C. jejuni* infections during the 2 years of the study. The remaining children (77/172; 44.8%) had a single *C. jejuni* infection. It is interesting to note that in the group with more than one infection, 97.9% of the subsequent infections were of a distinct CPS type. In the few cases in which a child shed a strain of the same CPS type seen in a previous infection, this may have been due to re-infection with a new strain or recrudescent infection with the original strain [21].

The data were stratified by individual features of the different CPS types as shown in Table 1. Then, we examined whether the presence (or predicted presence based on gene content) of MeOPN, heptose or deoxyheptose residues was associated with disease outcome. Specifically, across all symptomatic infections, 52.0% of children infected with a *C. jejuni* strain with a capsule containing MeOPN, heptose and deoxyheptose suffered from fever, 57.5% developed anorexia, 50.0% had vomiting, 56.2% needed oral rehydration therapy, 54.8% had at least 1 day with greater than or equal to four semi-liquid/liquid stools and 46.2% suffered from dysentery (Table 3). When comparing CPS types that contained MeOPN, heptose and deoxyheptose *vs.* those that did not contain one of those three structures, there were no significant differences across all signs, symptoms and management for all or first symptomatic infections (all *p*-values >0.1). Furthermore, there were no significant differences in the duration of any of the diarrhoeal signs and symptoms across capsule types (data not shown).

The top five most common serotypes responsible for re-infection were as follows: HS4 complex (*n* = 21, 13.2%), the HS5/31 complex (*n* = 18, 11.3%), the HS8/17 complex (*n* = 15, 9.4%), the HS6/7 complex (*n* = 13, 8.2%) and HS2 (*n* = 12, 7.6%). In comparison with all infections, while the HS4 complex and HS5/31 complex remained the most prevalent, the HS6/7 complex and the HS8/17 complex were responsible for a larger proportion of subsequent infections than initial infections. When limited to subsequent infections that were symptomatic, 57.1% (*n* = 12) of infections caused by the HS4 complex resulted in diarrhoea, whereas only 33.3% (*n* = 6) of the infections caused by the HS5/31 complex were symptomatic.

Discussion

The most common serotypes identified in this cohort of subjects were the HS4 complex, HS5/31 complex, HS15, HS2 and HS10 which collectively accounted for ~50% of all isolates. To our knowledge, these data represent the first report of CPS types of *C. jejuni* strains from South America. We assessed the association between capsule type and signs and symptoms of clinical disease among all, first and subsequent infections and found only minor, non-statistically significant variations, suggesting little to no variability in the proportion of isolates responsible for infections across a range of epidemiologically important strata. In a recent systemic review on global *C. jejuni* Penner serotype distribution, which covered >21 000 sporadic cases of *C. jejuni* diarrhoea, eight *C. jejuni* serotypes accounted for >50% of all isolates globally [22]. Interestingly, the major CPS/serotype found worldwide and in the current study was the HS4 complex, and HS2, HS5/31, HS15 and HS10 were also among the most frequent. These data also support the current understanding that the majority of *C. jejuni* infections are attributed to a limited number of CPS types [16, 22].

The overwhelming majority of isolates in our sample population (*n* = 291, 82.7%) are predicted to contain a heptose-containing CPS. We observed a trend towards more disease signs, symptoms and need for clinical management in CPS types with heptose; however, this association is likely due to the high proportion of *C. jejuni* CPS types with the potential to express heptose in our sample. Our findings do not further explain the contribution of heptose in the pathogenesis of diarrhoeal disease, which to date remains elusive [23].

While we were unable to determine if specific CPS types were more virulent than others, we observed trends in increased disease severity when subjects were infected with serotypes that contained MeOPN, heptose or deoxyheptose. Overall, our results do not appear to reveal any capsule-specific variability in disease signs, symptoms or need for treatment.

Approximately 70% of all infections in this study were asymptomatic with no significant difference in the proportion of cases or capsule type distribution between the first and subsequent infections. This supports previous studies that have also found asymptomatic *C. jejuni* infection to be common in children living in developing countries around the world [19, 24]. Infection with or without diarrhoeal illness is an important factor in the development of short- and long-term sequelae [19]. Evidence suggests acquired immunity and resistance to *C. jejuni* colonisation is possible after several exposures to various capsule types and increasing age [17, 25]. However, the low incidence of re-infection with the same CPS type described in this study is consistent with a role for CPS in natural immunity.

Efforts to develop a *C. jejuni* vaccine are underway and a capsule-conjugate vaccine based on the polysaccharide CPS has yielded promising results [16]. Early studies with a recently developed monovalent capsule conjugate vaccine were shown to be 100% effective against *Campylobacter*-associated diarrhoeal disease in primates. This form of the vaccine eliminates the risk of several chronic sequelae, including Guillain–Barré syndrome, seen with other forms of vaccination such as oral whole cell vaccines [17]. However, a licensable capsule-based conjugate vaccine will have to be multivalent, targeting the most common and

Table 2. The number of total, first and subsequent infections by the sample type

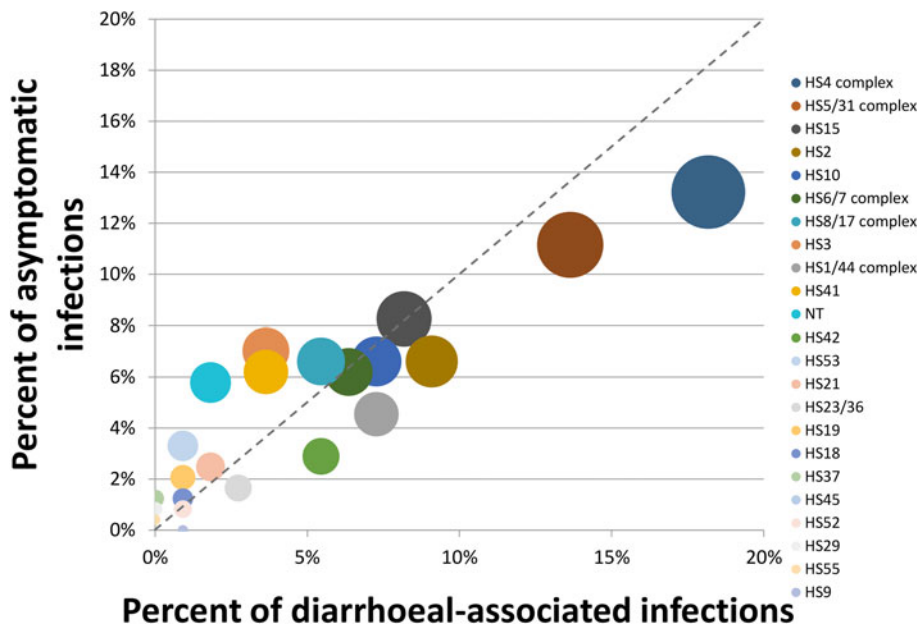| Sample type	Total infections		First infections		Subsequent infections	
	n	%	*n*	%	*n*	%
Asymptomatic	221	69.5	121	70.8	100	68.0
Diarrhoea	97	30.5	50	29.2	47	32.0
Total	318	100.0	171	100.0	147	100.0

Fig. 1. Proportion of asymptomatic *vs.* symptomatic diarrhoeal-associated infections. The size of the circle is proportional to the overall prevalence of the capsule type. The legend is sorted from most to least prevalent capsule types.
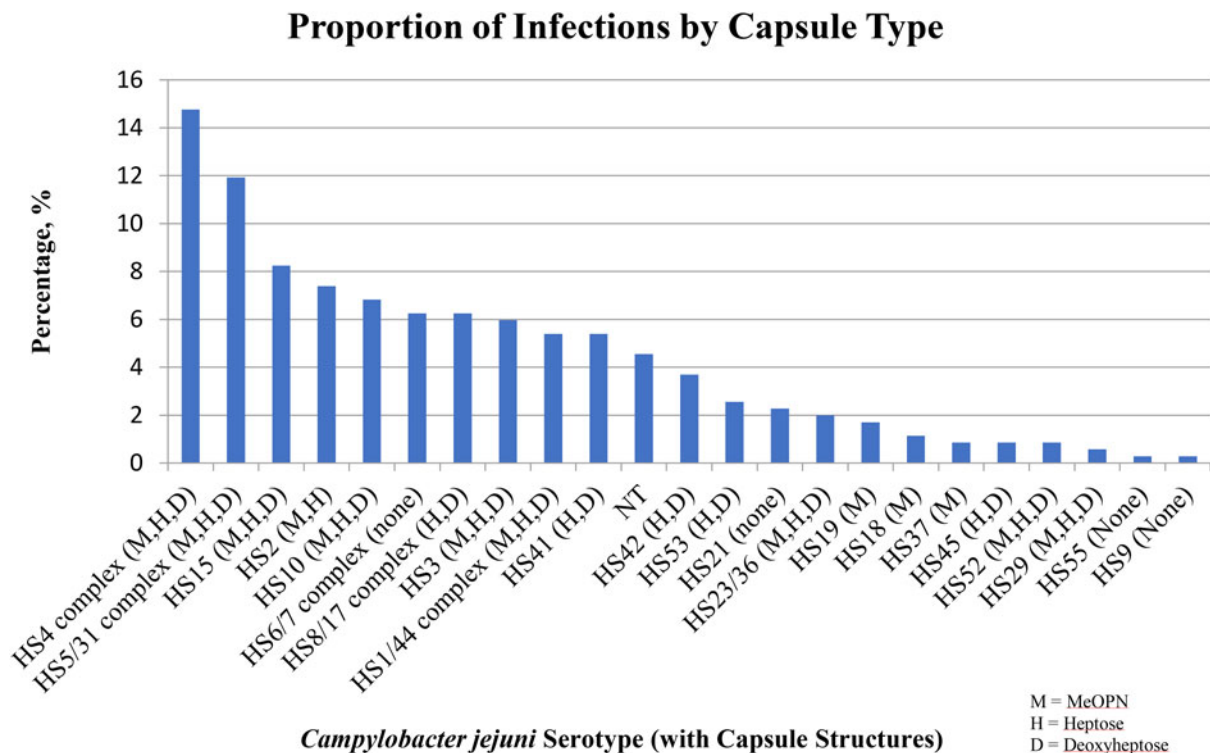

Fig. 2. The percentage of serotypes (with corresponding capsule structures) among all infections.

Table 3. Presence [*n* (%)] of diarrhoeal disease signs, symptoms and management by capsule type for ALL symptomatic infections

Capsule structures	Dehydration *n* (%)	Fever *n* (%)	Anorexia *n* (%)	Vomiting *n* (%)	ORT *n* (%)	Stool consistency *n* (%)	Dysentery *n* (%)
No MeOPN, no heptose, no deoxyheptose	1 (14.3)	3 (12.0)	3 (7.5)	2 (16.7)	2 (12.5)	7 (9.6)	2 (15.4)
MeOPN	1 (14.3)	2 (8.0)	4 (10.0)	0 (0.0)	2 (12.5)	5 (6.9)	1 (7.7)
MeOPN + heptose	1 (14.3)	1 (4.0)	3 (7.5)	1 (8.3)	2 (12.5)	8 (10.9)	2 (15.4)
Heptose + deoxyheptose	2 (28.6)	6 (24.0)	7 (17.5)	3 (25.0)	1 (6.3)	12 (16.4)	2 (15.4)
MeOPN + heptose + deoxyheptose	2 (28.6)	13 (52.0)	23 (57.5)	6 (50.0)	9 (56.2)	40 (54.8)	6 (46.2)
Non-typeable	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.3)	0 (0.0)
Total	7	25	40	12	16	73	13

ORT, oral rehydration therapy.

pathogenic capsule types [17]. These data contribute to the global understanding of CPS diversity and facilitate the prioritisation of CPS targets. If developed, a vaccine could be marketable to populations living in endemic regions and travellers from the developed countries, with the goal of significantly reducing the overwhelming burden of disease.

The results presented here are based on the genotypic characterisation of *C. jejuni* without confirmation of phenotypic expression of specific epitopes. In particular, capsule structure data are based on the genes present in specific capsules. Based on this testing, we assumed that certain phenotypic characteristics were present on the *C. jejuni* capsule when specific serotypes were detected by the multiplex PCR assay. However, biological variability may modify if and how these structures are expressed during infection and during clinical illness.

This study adds to the increasing understanding of the distribution of the *C. jejuni* capsule types associated with acute infection. These data, along with the increased recognition of *Campylobacter* as a global cause of morbidity in paediatric populations in low-middle income countries and as a causative agent of travellers' diarrhoea, highlight the potential utility of a capsule-based *C. jejuni* vaccine as one measure in reducing the burden of enteric infections.

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