Aetiology and epidemiology of human cryptosporidiosis cases in Galicia (NW Spain), 2000–2008

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SUMMARY

Cryptosporidium infects millions of people worldwide causing acute gastroenteritis, but despite its remarkable epidemiological and economic impact, information on the epidemiological trends of human cryptosporidiosis is still scarce in most countries. Here we investigate a panel of 486 cases collected in Galicia (NW Iberian Peninsula) between 2000 and 2008, which sheds new light on the epidemiology in this region of the South Atlantic European façade. Incidence rates in Galicia are one order of magnitude higher than those reported in other regions of Spain, suggesting that this parasite remains largely underdiagnosed in this country, and are also larger than those typical of other European countries with available data. Two species dominate our dataset, *Cryptosporidium hominis* (65%) and *C. parvum* (34%). The sex ratio of patients infected by either species was 0·5, but *C. hominis* was significantly more common in younger males. *C. parvum* infections were more acute and required more specialized medical attention, which suggests a differential adaptation of each species to human hosts. The parasites display strong seasonal and geographical variation. *C. parvum* incidence peaked during summer and was mainly detected in rural areas while *C. hominis* infections were more frequent in autumn and exhibited a more even geographical distribution. Such differences probably reflect their distinct sources of infection – *C. parvum* is mainly zoonotic and *C. hominis* anthroponotic – and the effects of climatic variables, like temperature and rainfall.

Key words: *Cryptosporidium*, molecular epidemiology, seasonal variation, virulence.

INTRODUCTION

Cryptosporidium is a genus of protozoan parasites that infect many vertebrate species and cause diarrhoeal diseases [1]. They affect humans worldwide and the

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increasing number of cases reported in the last decades has prompted the recognition of cryptosporidiosis as an emerging disease by the WHO [1–4]. This pathogen has a faecal–oral transmission route, and propagates through the ingestion of oocysts present in contaminated water supplies or food [2, 5–7]. Most human infections are caused by *Cryptosporidium hominis* and *C. parvum*, while other species are found only rarely [3, 8]. *C. hominis* is almost

exclusively anthroponotic, in contrast to *C. parvum*, which is commonly detected in domestic animals such as cows and sheep [3].

Cryptosporidiosis is a matter of public health concern in many countries and has inspired a growing number of epidemiological surveys aimed at understanding its demographic and clinical features [9–14]. However, there is little data on the worldwide incidence of this parasite in humans and only a few South European countries have reported studies on its incidence and prevalence [15, 16]. Spain, in common with most countries, lacks a specific cryptosporidiosis surveillance system (notification of cases to the health authorities was not mandatory until 2009 except for outbreaks), which means that the disease is likely to be underdiagnosed and underreported. Thus, although there are some surveys involving Spanish human samples, most of them include a small number of patients and are devoted to the analysis of particular cases or isolated outbreaks [17–19].

In order to improve our understanding of cryptosporidiosis in Europe, we present the results of a large-scale epidemiological study performed on a panel of samples corresponding to all patients with gastrointestinal illness in the Santiago de Compostela health area (Galicia, NW Iberian Peninsula), over a 9-year period. All faecal samples received at the Servizo de Microbioloxía of the Complexo Hospitalario Universitario de Santiago (CHUS) were routinely checked for cryptosporidiosis and subsequently characterized at the species and subtype levels [20]. In order to ascertain relevant epidemiological patterns of cryptosporidiosis in the study region, incidence rates were combined with genetic (species and subtype), environmental (temperature, rainfall, humidity) geographical (location; rural *vs.* urban area), demographic (sex and age of patients) and clinical (medical care required, oocyst counts and stool consistency) data, and time-series analyses were performed. Our results can be taken as a proxy for the epidemiology of cryptosporidiosis in the NW Iberian Peninsula, and represent the largest-scale study of the whole South-Western European façade.

MATERIALS AND METHODS
Sample genotyping

Stool samples were collected from all patients suffering from diarrhoeal disease between January 2000 and December 2008 in the health district of Santiago de Compostela (Galicia, NW Iberian Peninsula), an area with 515 700 inhabitants (2004). Diarrhoea was defined as the passage of three unformed stools in 8 h or of >3 loose stools in 24 h in addition to the presence of one or more symptoms of an enteric infection [21]. Samples were checked for enteric pathogens by routine microbiological methods at the Microbiology and Parasitology Laboratory of CHUS. The presence of viral antigens (adenovirus, astrovirus, rotavirus, norovirus) was assessed by applying a commercial immunochromatography test (CerTest Biotec faecal antigen rapid test; CerTest Biotec, Spain), bacteria (*Campylobacter, Escherichia, Salmonella, Shigella, Yersinia*) were identified using conventional coproculture techniques [22] and protozoan parasites were visualized by microscope examination after concentrating the samples with a modification of the Ritchie method [23]. Coccidian oocysts were detected by fluorescence microscopy (100–400x magnification) after staining stool smears with phenol-auramine [22, 24]. The identification was conducted on the basis of size and shape (for details see the microscopic identification module in http://water.epa.gov/lawsregs/rulesregs/sdwa/lt2/lab_home.cfm#training) by two experienced microscopists from the Microbiology and Parasitology Laboratory of CHUS.

A total of 822 cases of cryptosporidiosis were reported during the study period. Those with enough stool to perform DNA extractions (486 cases) were genotyped by means of iPLEX™ technology (Agena Bioscience, USA) to determine the *Cryptosporidium* species and subtype present in each sample [20]. This method is based on the use of single base extension of a panel of selected single nucleotide variants (SNVs) [25], used as species and subtype markers, followed by sequencing by mass spectrometry (MALDI-TOF) [26].

Demographic and clinical data

All relevant demographic (municipality, age and sex) and clinical (observed co-infections, associated symptoms and immunocompromised status) data were collected from each patient's record. Medical records were kept confidential pursuant to current legislation. Other variables, like specimen collection date, reported outbreaks, recent foreign travel and patient's close contacts affected by enteric problems, i.e. relatives and classmates, were also compiled.

The stage of the parasitization process and clinical profile of each case were assessed by means of three

parameters: (i) excretion rate, (ii) sought medical care and (iii) stool consistency. (i) The excretion rate, or parasitic load, was quantified by scoring the number of oocysts observed per microscope field in stained faecal smears using 100x magnification, and used to typify the stage of the illness [27]. Medical treatment is usually sought after the onset of the first symptoms, mainly within the first week of the infection. In this initial, or acute, stage there is an exponential growth of the parasite population, and oocysts are shed in large numbers (>50 oocysts/field). In the second stage of the infection, usually within the second and third weeks, the parasite population usually reaches a plateau and experiences a progressive reduction, along with the oocyst count (20–50 oocysts/field), and symptoms ameliorate. In the third stage, patients may still shed oocysts (5–20 oocysts/field) for a long period of time (up to 2 months after infection), prior to the final non-proliferative stage (<5 oocysts/field) (J. Llovo, unpublished data). These data were available for 482 samples (99% of the genotyped samples), which were divided into four groups (1–4) according to oocyst counts (<5, 5–20, 20–50, >50, respectively) [28]. (ii) Medical care, as defined according to the kind of medical assistance required by patients, was available for 480 samples (98% of the genotyped samples). It was classified as primary health care (PHC), emergency ward (EW) or hospital admission (HA) [29], following a criterion of increasing symptomatic seriousness. Patients treated at PHC are assumed to present less virulent infections than those who use the EW or are admitted into hospital, which obviously are the most severe cases. (iii) Stool consistency, categorized as formed, pasty, and liquid stools, was evaluated in 369 samples (76% of the genotyped samples) [30]. This parameter can be used as a proxy for the stage of the illness, since the production of loose faeces is more frequent in the acute phase of the infection [31], and it is also expected to be associated with the clinical profile, as watery diarrhoea has a stronger impact on patients (dehydration, weight loss, etc.) than the excretion of pasty or formed stools.

Environmental and climatic analyses

The health district of CHUS is distributed in 14 administrative regions called *comarcas* (analogous to shires; Fig. 1, Table 1), which include 58 lesser administrative units or parishes. A rural–urban index (RUI) was devised to represent the global environment of each *comarca* by combining four socioeconomic factors: (i) human density (population/km^2), (ii) livestock abundance (number of cattle), (iii) availability of public water supply and (iv) sewerage services (fraction of the population with access to these services). Data were obtained from the Instituto Galego de Estatística (IGE; http://www.ige.eu/). To categorize each *comarca*, average values for each variable were compared with their corresponding average value at Galician level. Variables above the Galician average were counted as 0 (otherwise counted as 1), except for livestock abundance, where values were assigned the other way around. Finally the index for each *comarca* was estimated as the sum of the four variables. Thus, this index ranges from 0, which defines an urban *comarca* (high population density, low presence of cattle and high percentage of population with access to public water services) to 4, a rural *comarca*.

Climatic data (i.e. monthly averages for humidity, rainfall and temperature [32]) were collected from the IGE and the Spanish National Institute of Statistics (INE; http://www.ine.es/) at two different weather stations located in Santiago de Compostela (Fig. 1, region 12), which were considered representative of all *comarcas*. The monthly data for each variable was calculated as the mean of the values from the two stations. For each variable, months were sorted according to the value of the variable and grouped into equal-sized bins (36 months in each bin), and the corresponding numbers of parasitized cases was obtained for each bin.

Statistical analyses

Time-series analyses were performed to characterize the seasonal component of the number of patients infected by *C. parvum* or *C. hominis*. The seasonal variation index (SVI), which quantifies the deviation of the number of cryptosporidiosis cases recorded each season relative to the overall mean, was estimated by applying the ratio-to-moving average method under a multiplicative time-series model. These calculations were performed with the aid of IBM SPSS software v. 20·0 (IBM Corp., USA).

Associations between *Cryptosporidium* species – and their corresponding subtypes – and demographical (age, sex, geographical distribution), clinical (excretion rates, medical care, stool consistency or symptoms) and environmental (seasonality, rainfall, humidity, temperature) parameters were evaluated by means of χ^2 tests of homogeneity [33]. These were
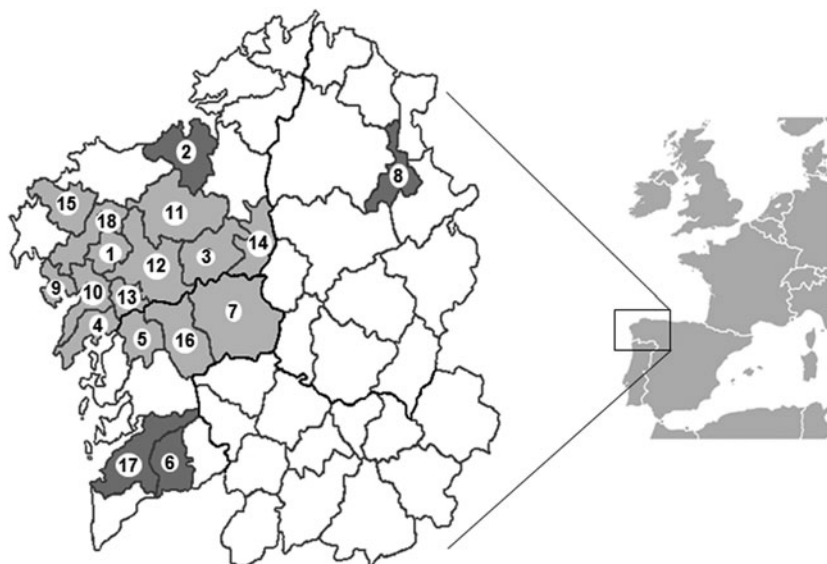
Fig. 1. *Comarcas* included in Santiago de Compostela health district. Several patients from *comarcas* 2, 6, 8 and 17, which are not part of this health area (depicted in dark grey), received medical assistance at CHUS and were included in the analyses. *Comarcas* are: 1, A Barcala; 2, A Coruña; 3, Arzúa; 4, Barbanza; 5, Caldas; 6, Condado; 7, Deza; 8, Meira; 9, Muros; 10, Noia; 11, Ordes; 12, Santiago; 13, Sar; 14, T. Melide; 15, T. Soneira; 16, Tabeirós; 17, Vigo; 18, Xallas.

also used to investigate associations between clinical parameters, such as excretion rate, medical care and stool consistency. The distribution of parasites' species in age groups was assessed by means of a Mann–Whitney U test [33]. To do this, patients were grouped into 10-year bins. Given the large number of children, these were further divided into two age groups (0–3 and 4–9 years, respectively [10]). Spearman's rho (ρ) correlation coefficient [33] was used to evaluate associations between *Cryptosporidium* species and the regional development index (RUI). All statistical analyses were performed using Microsoft Excel (Microsoft Corp., USA) and IBM SPSS software v. 20·0 (IBM Corp., USA). In all cases, P values $\leqslant 0·05$ were considered significant.

RESULTS

The incidence rates of cryptosporidiosis in terms of number of cases per 100 000 population per year in the Santiago de Compostela health area (Galicia, NW Iberian Peninsula) were 9·44 (2000), 9·25 (2001), 17·57 (2002), 29·18 (2003), 13·96 (2004), 20·31 (2005), 27·64 (2006), 5·79 (2007) and 25·77 (2008), with an average (±s.e.) rate of $17·65\pm2·88$, and $91·33 \pm 7·44$ cryptosporidiosis cases per year.

The epidemiological patterns were studied in a subset of these cases ($N = 486$), which were genotyped at the species and subtype levels [20]. Of these, 318 (65·43%) samples were confirmed as *C. hominis*, 164 (33·95%) as *C. parvum*, three (0·62%) as *C. meleagridis* and one (0·21%) as *C. felis*. The *C. hominis* samples corresponded to *gp60* subtypes Ib ($n = 289$), Id ($n = 23$), Ia and Ie ($n = 3$, each). *C. parvum* samples were assigned to subtypes IIa ($n = 146$), IIn ($n = 14$), IId ($n = 3$) and one undetermined subtype. Of the three *C. meleagridis* samples, two were of subtype IIIf and one IIIb. Only the main species (*C. hominis* and *C. parvum*) and subtypes (IIa, IIn, Ib, Id) were used in the analyses (Table 1).

Demographic data analysis

In our sample, *Cryptosporidium* mainly affects young children. The mean age of *C. hominis* patients (9·34 ± 17·58 years, range 1 month to 91 years, median 2 years, mode 1 year) was slightly higher than that of those infected by *C. parvum* (7·53±15·07 years, range 5 months to 80 years, median 2 years, mode 1 year), although the observed difference was not statistically significant by Mann–Whitney U test. No significant variation of the relative frequency of the two parasite species was observed across age bins (Table 1, Fig. 2).

The sex ratio of the patients was nearly 0·5 (239 females and 247 males, including three cases of parasitization with *C. meleagridis* and one with *C. felis*)

Table 1. *Demographic data*

Variable	Outcome	Cryptosporidium species			C. hominis subtypes			C. parvum subtypes		
		C. hominis	C. parvum	P	Ib	Id	P	IIa	IIn	P
Comarca*	1. A Barcala (3)	2	7	<10⁻⁴	2	—	0·83	7	—	0·51
	2. A Coruña (0)	—	1		—	—		1	—	
	3. Arzúa (4)	3	10		1	1		8	2	
	4. Barbanza (1)	22	7		17	3		3	3	
	5. Caldas (0)	6	1		6	—		1	—	
	6. Condado (1)	1	—		1	—		—	—	
	7. Deza (3)	3	10		2	1		8	1	
	8. Meira (3)	—	1		—	—		1	—	
	9. Muros (1)	5	2		4	—		2	—	
	10. Noia (2)	11	5		10	1		5	—	
	11. Ordes (4)	8	24		7	1		20	3	
	12. Santiago (1)	213	62		199	13		57	4	
	13. Sar (2)	8	3		8	—		3	—	
	14. T. Melide (4)	2	7		2	—		7	—	
	15. T. Soneira (3)	1	—		1	—		—	—	
	16. Tabeirós (3)	29	5		26	2		4	1	
	17. Vigo (0)	1	—		1	—		—	—	
	18. Xallas (4)	2	17		2	—		17	—	
Age groups† (years)	1–3	201	107	0·99	184	14	0·98	95	10	0·73
	4–9	54	33		46	6		31	2	
	10–19	18	7		17	1		5	—	
	20–29	13	5		12	1		4	1	
	30–39	8	3		7	—		3	—	
	40–49	5	2		5	—		2	—	
	50–59	3	1		3	—		—	1	
	60–69	5	2		5	—		2	—	
	70–79	8	3		7	1		3	—	
	⩾80	2	1		2	—		1	—	
Sex	Male	164	82	0·74	149	13	0·65	72	8	0·58
	Female	154	82		140	10		74	6	

All statistical analyses were performed using the χ^2 test of homogeneity.
P values obtained with Yates' correction are in italics.
Values in parentheses are the rural–urban index for each *comarca*.
* Unknown in three cases.
† Unknown in one case.

and the distribution of parasite species across patients' sex did not depart from random expectations ($P = 0.74$, χ^2 test; Table 1). But, if age groups are also considered, a differential incidence of *C. hominis* in males and females was detected (Fig. 2): there were more *C. hominis* cases in boys aged <3 years (115 cases) than in girls of the same age (84 cases), whereas this ratio was inverted in patients aged ⩾4 years (49 in males *vs.* 69 in females, respectively; $P < 0.01$, χ^2 test; Fig. 2). This effect was not detected in *C. parvum* patients, with 54 and 52 cases in boys and girls, and 28 and 30 cases in older males and females, respectively ($P = 0.74$, χ^2 test). No deviation from randomness was observed in the distribution of the major

subtypes (Ib, Id, IIa, IIn) across sexes, considering their relative abundances (Table 1).

Clinical analysis

Seriousness of the infection

To check if the pathological course of the infections was in any way associated with the parasite species, we searched for associations between the abundance of the two *Cryptosporidium* species and three parameters which were used as proxies for the stage of the infection and its symptomatic seriousness: the type of medical attention required, the consistency of the stools
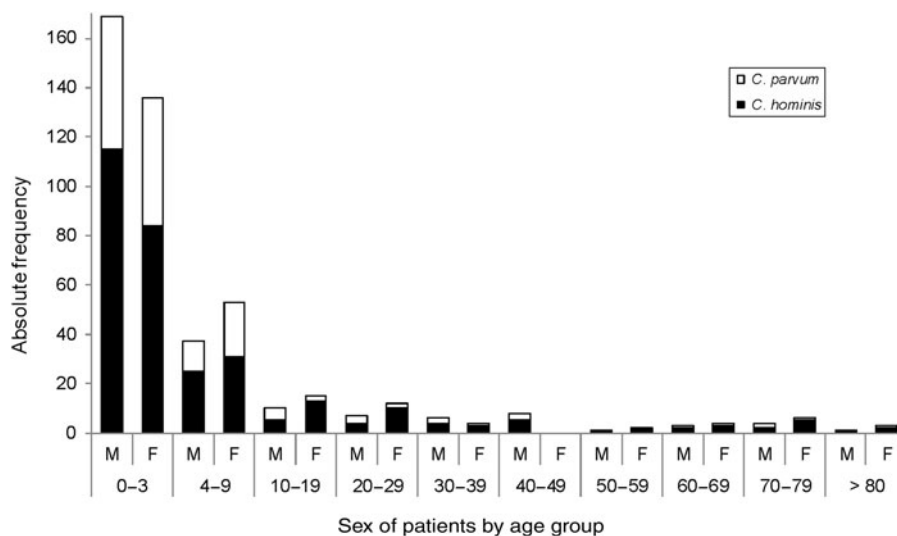
Fig. 2. Absolute frequencies of *Cryptosporidium* cases ranked by the sex and age group of patients.

and the parasitic load. Indeed, (i) *C. parvum* infections required HA in a larger proportion of cases than those caused by *C. hominis* (34·36% *vs.* 17·46%, respectively; $P < 0.0001$, χ^2 test; Table 2), which were more often dealt with at PHC (67·62% of the *C. hominis* cases). No significant differences in required treatment were observed in within-species subtypes. (ii) Although *Cryptosporidium* infections usually presented pasty faeces (more than 50% of cases; Table 2), *C. parvum* were more often associated with liquid stools than *C. hominis* (41·23% *vs.* 25·59%, respectively; $P < 0.01$, χ^2 test). This is interesting, because patients with formed or pasty stools were usually assisted at PHC, while those suffering from watery diarrhoea were mainly treated in the EW or were admitted to hospital ($P < 0.0001$, χ^2 test; Fig. 3a, Supplementary Table S1). This was also observed within *Cryptosporidium* species when they were individually assessed (*C. hominis*, $P < 0.01$ and *C. parvum*, $P < 0.05$, χ^2 test; Fig. 3a, Supplementary Table S1), which suggests that the association between stool consistency and medical care is independent of the infecting species. The same pattern was found for *C. hominis* subtype Ib ($P < 0.01$, χ^2 test, data not shown).

Parasite loads (measured in terms of oocyst excretion rates) did not vary significantly across parasite species (Table 2). Patients with high loads required HA more frequently than those with lower excretion rates ($P < 0.01$, χ^2 test, Fig. 3b, Supplementary Table S2). Markedly, this effect seems to be restricted to *C. parvum* infections ($P < 0.0001$, χ^2 test), and it was not observed in *C. hominis* patients ($P = 0.23$, χ^2 test; Fig. 3b, Supplementary Table S2). The main

C. parvum subtype (IIa) displayed a similar pattern ($P < 0.01$, χ^2 test, data not shown).

No significant association was found between oocyst excretion rates and stool consistency, neither for the whole dataset ($P = 0.24$, χ^2 test; Fig. 3c, Supplementary Table S3) nor within either species (*C. hominis*, $P = 0.32$ and *C. parvum*, $P = 0.49$, χ^2 test; Supplementary Table S3).

Associated co-infections

Enteropathogens other than *Cryptosporidium* were detected in 80 out of the 486 samples, including 11 different organisms. Eight samples had more than one concurrent pathogen. There were three cases of co-infection with either *Endolimax nana*, *Cystoisospora belli* or *Pentatrichomonas hominis*, two with noroviruses, two with *Entamoeba histolytica*, seven with rotaviruses, eight with adenoviruses, nine with *Salmonella enterica*, 13 with *Campylobacter jejuni*, 19 with *Blastocystis hominis* and 26 with *Giardia lamblia*. Some of these pathogens had previously been described as co-infective with *Cryptosporidium* [2, 34, 35]. Co-infections were more often associated with *C. hominis* (77·5% of the co-infection cases, $P < 0.05$, χ^2 test; Table 2). No differences in subtypes were found.

Clinical symptoms, outbreaks and other factors

The classical symptoms of cryptosporidiosis are nausea, fever, abdominal pain, vomiting and mucus in the stool [36]. The latter two were more often associated with *C. parvum* infections ($P < 0.05$ and

Table 2. *Clinical data*

Variable	Outcome		*Cryptosporidium* species			*C. hominis* subtypes			*C. parvum* subtypes		
			C. hominis	*C. parvum*	*P*	Ib	Id	*P*	IIa	IIn	*P*
Excretion rate*		1	70	44	0·34	63	5	0·93	40	3	*0·58*
		2	76	43		69	6		36	6	
		3	70	37		66	4		35	1	
		4	99	39		88	8		34	4	
Stool consistency†		Formed	46	13	<0·01	39	5	0·39	10	3	*0·32*
		Pasty	143	54		135	6		50	3	
		Liquid	65	47		59	5		41	3	
Medical care‡		PHC	213	62	<10⁻⁴	196	12	0·14	52	9	*0·10*
		EW	47	45		44	3		39	4	
		HA	55	56		46	8		54	1	
Co-infections		Yes	62	18	<0·05	55	6	0·58	16	1	*1·00*
		No	256	146		234	17		130	13	
Symptoms	Abdominal pain	Yes	11	5	0·81	11	—	*0·71*	4	—	*0·79*
		No	307	159		278	23		142	14	
	Fever	Yes	14	9	0·60	11	2	*0·56*	8	—	*0·80*
		No	304	155		278	21		138	14	
	Vomiting	Yes	8	11	<0·05	8	—	*0·90*	11	—	*0·61*
		No	310	153		281	23		135	14	
	Nausea	Yes	2	—	*0·79*	1	1	*0·34*	—	—	—
		No	316	164		288	22		146	14	
	Mucoid stools	Yes	5	13	<0·01	4	2	*0·09*	12	1	*0·71*
		No	313	151		285	21		134	13	
	Other symptoms§	Yes	6	3	*0·76*	5	1	*0·93*	4	—	*0·79*
		No	312	161		284	22		142	14	
Immunocompromised		Yes	6	4	*0·94*	6	—	*0·93*	4	—	*0·79*
		No	312	160		283	23		142	14	
Outbreaks		Yes	16	—	<0·01	16	—	*0·50*	—	—	—
		No	302	164		273	23		146	14	
Travel history		Yes	6	1	0·48	2	4	*<10⁻⁴*	1	—	*0·14*
		No	312	163		287	19		145	14	
Family and school cases		Yes	22	11	0·93	19	3	*0·45*	11	—	*0·61*
		No	296	153		270	20		135	14	

PHC, Primary health care; EW, emergency ward; HA, hospital admission.

All statistical analyses were performed by using the χ^2 test of homogeneity.

P values obtained with Yates' correction are in italics.

* Data available for 482 samples.

† Data available for 480 samples.

‡ Data available for 369 samples.

§ Weight loss, dehydration and food rejection.

$P < 0.01$, respectively, χ^2 test; Table 2). No differences were found in subtypes.

Immunocompromised patients, who required HA in 90% of the cases, did not show significant differences in the predominance of any *Cryptosporidium* species or subtype (Table 2).

Samples from a *Cryptosporidium* outbreak in the *comarca* of Tabeirós in autumn 2003, were genotyped as part of our survey. The outbreak was caused exclusively by *C. hominis* subtype Ib and affected hundreds of people, although only 16 cases were included in the study (Table 2).
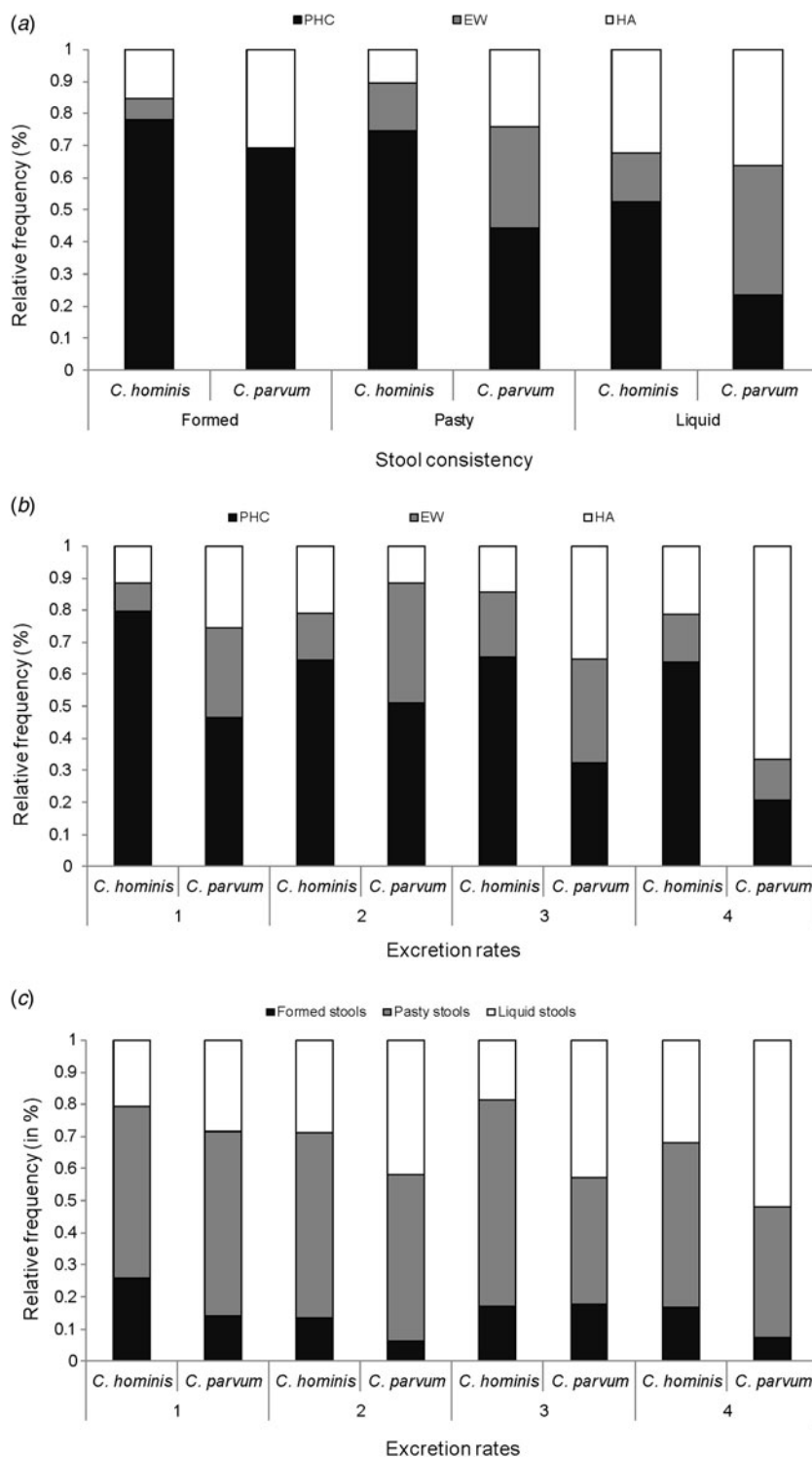
Fig. 3. Relationships in the three variables used to measure the virulence of each infection. (a) Stool consistency and type of medical assistance required by patients infected by *C. hominis* or *C. parvum*. (b) Excretion rates (parasitic load) and type of medical care required by patients infected by *C. hominis* or *C. parvum*. (c) Excretion rates (parasitic load) and stool consistency of patients infected by *C. hominis* or *C. parvum*.

The few cryptosporidiosis patients that reported a recent travel abroad or foreign origin were gathered and studied as a single group. These people came mainly from Africa (Ethiopia) and South America (Mexico, Bolivia, Ecuador). No significant inter-specific differences were reported, although subtype
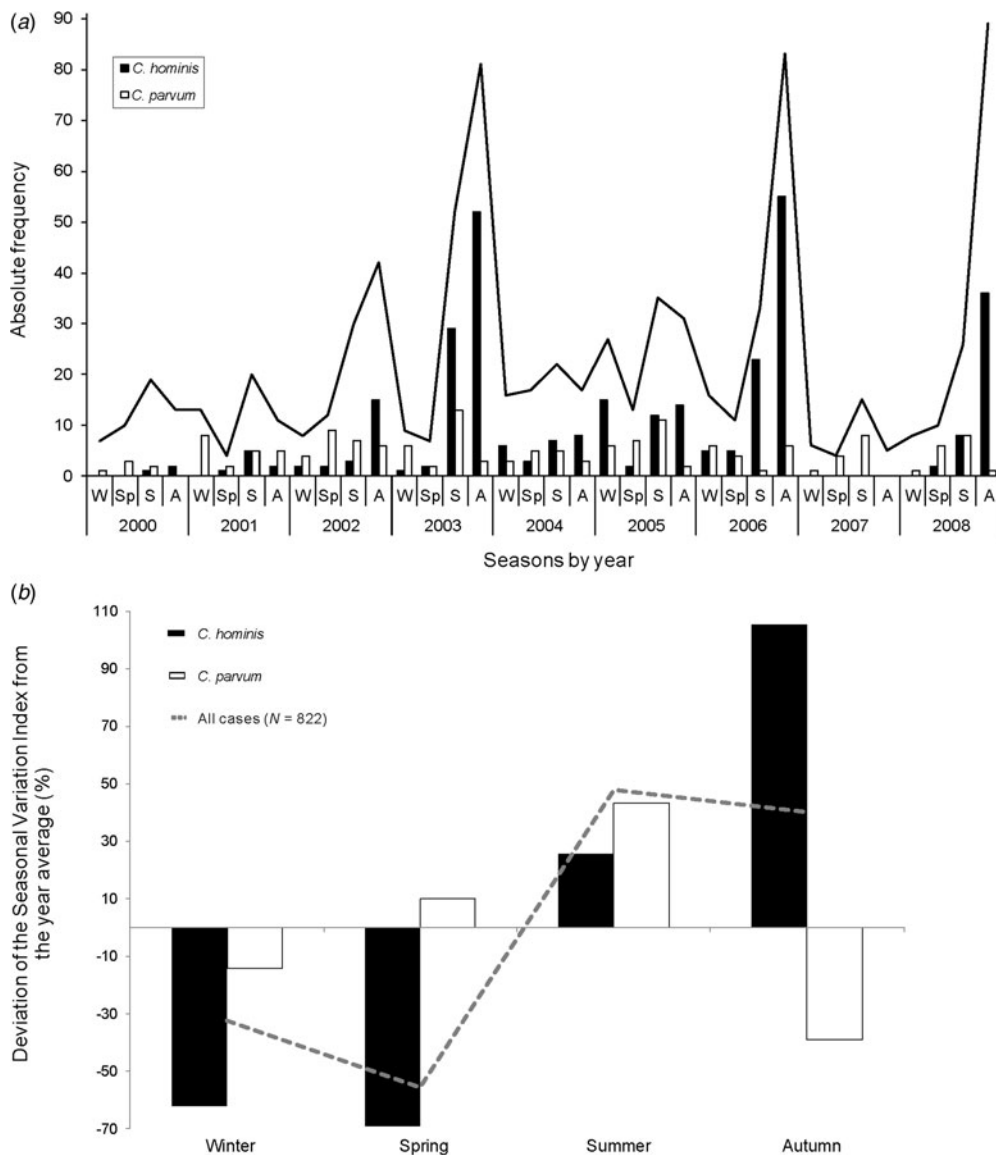
Fig. 4. Seasonality of *Cryptosporidium* incidence. (*a*) Absolute frequencies of cryptosporidiosis cases across the study period. The continuous line represents the total number of cryptosporidiosis cases. (*b*) Seasonal patterns of variation of incidence rates (W, winter; Sp, spring, S, summer, A, autumn).

Id was significantly more frequent in this group of patients ($P < 0.0001$, χ^2 test; Table 2).

Finally, we observed that patients with *C. hominis* presented twice as many members of the same household or schoolmates with a gastroenteric problem than those suffering from *C. parvum* infections, although this difference was not statistically significant (Table 2).

Geographical distribution and environmental analysis

Spatial distribution of cases was different between species ($P < 0.0001$, χ^2 test; Table 1). *C. parvum* incidence

was positively associated with the RUI (Spearman's ρ correlation $\rho_{\text{RUI-}C.parvum} = 0.552$, $P < 0.05$), which indicates a significant association of this species with rural areas, while *C. hominis* infections were not associated with any particular social environment.

Seasonality and climatic analyses

Time-series analyses of the whole dataset revealed a strong seasonal pattern of cryptosporidiosis incidence, with higher numbers in summer and particularly in autumn, as opposed to winter and spring, when incidence rates regularly fell below the yearly average

Table 3. *Environmental data*

Variable*	Outcome	Cryptosporidium species			C. hominis subtypes			C. parvum subtypes		
		C. hominis	C. parvum	P	Ib	Id	P	IIa	IIn	P
Season	Winter	29	36	$<10^{-4}$	28	—	0·07	29	4	0·87
	Spring	17	42		25	2		37	5	
	Summer	88	60		64	11		55	4	
	Autumn	184	26		172	10		25	1	
Humidity (%)	<74	95	51	$<10^{-4}$	95	—	<0·01	43	8	0·22
(73·6 ± 12·9)	74–79·9	74	67		59	12		61	4	
	>80	149	46		135	11		42	2	
Rainfall (mm³)	<60	61	71	$<10^{-4}$	47	11	<0·01	61	8	0·57
(136·5 ± 132·6)	60–140	102	49		98	3		45	2	
	>140	155	44		144	9		40	4	
Temperature (°C)	<10·4	60	35	0·39	57	1	0·13	29	3	0·98
(13·2 ± 4·1)	10·4–15·8	125	54		115	9		49	5	
	>15·8	133	75		117	13		68	6	
Max. temperature (°C)	<22	62	38	<0·01	59	1	<0·05	31	4	0·92
(25·6 ± 6·8)	22–29·4	165	57		154	10		52	5	
	>29·5	91	69		76	12		63	5	
Min. temperature (°C)	<1	87	45	0·90	82	3	0·13	40	3	0·70
(3·6 ± 4·2)	1–6	97	47		90	6		40	6	
	>6	134	72		117	14		66	5	

All statistical analyses were performed by using the χ^2 test of homogeneity.
P values obtained with Yates' correction are typed in italics.
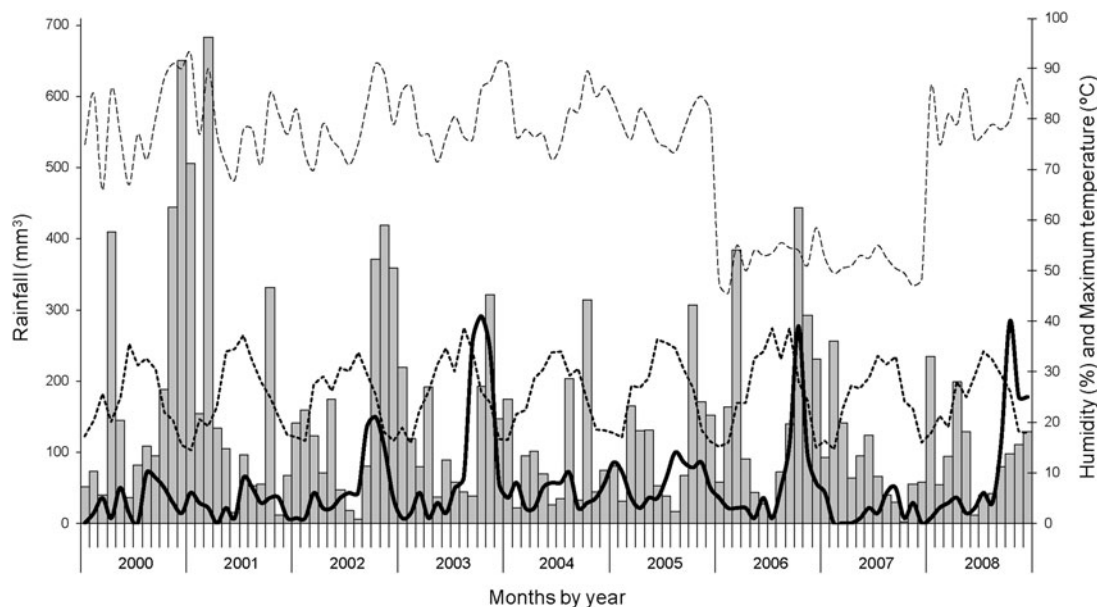* Average monthly values ± standard error for climatic each variable are given in parentheses.

Fig. 5. Cryptosporidiosis cases reported (continuous line, $N = 822$) and monthly averages of rainfall (bars), maximum temperature (dotted line) and humidity (segmented line). All lines follow the right axis.

(Fig. 4a). The two species displayed different seasonal structuration: the number of C. parvum cases experienced moderate seasonal variation, with a large increase in summer (SVI = 43%, Fig. 4b) and a sharp reduction in autumn (SVI = −39%). By contrast, seasonal variation of C. hominis was much

greater, with a peak of cases in autumn (SVI = 105%), and below average incidences in winter and spring (−62% and −70%, respectively).

Potential associations of temporal patterns with climate parameters such as temperature, rainfall and humidity distributions were also investigated (Table 3). Monthly temperature was found not to be associated with the incidence of cryptosporidiosis at the species and subtype levels. However, since Galicia has a temperate climate, a role for monthly extreme temperatures (maximum and minimum) was also assessed [32]. Indeed, maximum monthly temperatures were associated with a higher incidence of both species ($P <$ 0·01, χ^2 test; Table 3). Humidity and rainfall also covaried with the incidence rates of both species, although they showed different patterns. *C. hominis* infections increased with rainfall and/or humidity (Figs 4*a*, 5), and the opposite pattern was observed for *C. parvum*, which was more common after dry periods ($P <$ 0·0001, χ^2 test; Table 3). In good agreement with these observations, only *C. parvum* cases were reported in 2007, which showed particularly low levels of rainfall in summer and autumn (seasons that usually display the highest number of *C. hominis* cases) and was one of the years with the lowest level of humidity reported (Figs 4*a*, 5). On the other hand, an increase in the number of infections caused by *C. hominis* was observed in 2003 and 2006, the warmest years of the temporal series (especially their summers and autumns) that also showed high rainfall rates (Figs 4*a*, 5).

DISCUSSION

This is one of the broadest epidemiological studies on cryptosporidiosis conducted in Southern Europe to date. The 822 cases reported in the 9-year period of analysis represent half of the cases reported elsewhere in Spain between 1995 and 2009 [17]. Considering that the population in the Santiago health area represents just about the 1·2% of the Spanish population, it seems clear that cryptosporidiosis cases were severely underdiagnosed in Spain during this period. In fact, the Microbiology and Parasitology Laboratory of CHUS is one of the few centres in Spain were all faecal samples from patients with enteric problems are routinely analysed for the presence of this and other pathogens. This high level of screening is reflected in the high incidence rates observed in our health area (17·65 ± 2·88 cases per 100000 population per year), that are far greater than those previously reported in most other large-scale surveys conducted in Europe

[16, 17, 37, 38] or in the USA [12, 13]. In fact, only 2007 presented a lower incidence rate than those reported in Ireland (14·17), Great Britain (6·05) – countries that share climatic characteristics with Galicia – and some USA states [13, 38].

In the current dataset there was a strong predominance of *C. hominis* and *C. parvum* infections, involving the most common subtypes in humans (Ib and IIa, respectively) [3, 15, 39] and affecting mainly young children, as described worldwide [8, 10, 11]. As previously reported [11, 40], the number of cases decreased with age and no significant differences in the incidence of both species were observed.

In good agreement with data from other European countries, parasite species were equally distributed across sexes [10, 11]. However, in contrast to *C. parvum*, the fraction of *C. hominis* cases was usually larger in adult females than in males, suggesting human-to-human transmission that can probably be attributed to their greater involvement in childcare.

The analyses of various clinical variables showed differences in virulence between species; however, by contrast with previous studies [41–43], our results revealed that infections produced by *C. parvum* were more harmful than those caused by *C. hominis*, as they were significantly associated with more severe symptoms (vomiting or presence of mucous stools) that often required HA. These disparities in virulence suggest that humans could be better adapted to *C. hominis* infections, which seemed to have a milder effect on patient's physiology. Unlike previous studies [41, 42], the subtype families detected in our health area showed no significant differences in virulence.

Cryptosporidiosis caused by *C. hominis* exhibited clinical characteristics somewhat different to those caused by *C. parvum*. For instance, more than 75% of cases with concomitant enteropathogens were *C. hominis* infections. Moreover, the fact that *C. hominis*, and particularly its subtype Ib, is responsible for the majority of the outbreaks reported in Europe [3], as the one detected in Tabeirós in 2003, confirms the high infectivity of this species and its great capability of spreading both through rural or urban regions. On the contrary, subtype Id, which was found in a much smaller proportion in our panel of samples, was significantly more frequent in patients reporting recent foreign travel, reinforcing the assumption that it is not a typical European subtype [3, 15, 41, 42].

The results of the geographical and seasonal distribution of the Galician samples are concordant with

previous studies showing that *C. hominis* displays a widespread distribution with seasonal peaks in autumn [e.g. 6, 10, 11, 40], while *C. parvum* was significantly more prevalent in rural areas [3, 11], with increments in the number of cases that coincided with lambing and calving activity peaks (usually in spring and summer). This could be the reason for the increase in oocyst concentrations observed in drinking water obtained from the river Tambre – the main water source that flows through the study area – in spring and summer of 2007 [44]. In fact, *C. parvum* subtypes identified in human samples were commonly detected in livestock stools, which further supports their zoonotic origin [20, 45].

Finally, it has been proposed that climate variables such as those analysed here modulate the incidence rate of cryptosporidiosis and, thus, are likely to play a role in the seasonal distribution of the species [32, 46, 47]. Regarding temperature, the intervals of highest incidence occurred consistently after the warmest periods [47], as described in other mid-latitude and temperate regions [46]. On the other hand, variations of rainfall and/or humidity might also contribute to the different incidence rates observed between species. A negative association between rainfall and the risk of infection by *Cryptosporidium*, such as the one observed for *C. parvum*, has been attributed to a reduction of oocyst concentration in the soil and surface water after rainy periods [48, 49]. However, this hypothesis does not seem to apply to *C. hominis*. An explanation of this might reside in the mechanisms of transmission of the two species. *C. parvum* is mainly zoonotic and is commonly found in rural areas, thus the spread of its oocysts is probably associated with rain levels in contrast to person-to-person transmission of *C. hominis*, which is expected to be more affected by seasonal changes in human activity, such as use of recreational water and consumption of fresh vegetables in warmer seasons, or initiation of nursery-primary school attendance of children in late summer.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268815000163.

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