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Original article

Clinical relevance of enteropathogen co-infections in preschool children—a population-based repeated cross-sectional study

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ABSTRACT

Objectives: This study aimed to (i) determine risk factors for enteropathogen co-infections, (ii) determine whether enteropathogen co-infections influence gastroenteritis risk, and (iii) determine whether enteropathogen co-infection occurred randomly in preschool children.

Methods: A monthly-repeated cross-sectional survey in Dutch children aged 0–48 months was conducted during October 2012 to October 2014. A total of 981 stool samples were collected along with questionnaires collecting data on gastrointestinal symptoms and potential risk factors; 822 samples were successfully tested for 19 enteropathogens using real-time multiplex PCRs. Logistic regression analysis assessed co-infections in relation to gastroenteritis and potential risk factors.

Results: In all, 598/822 (72.7%) stool samples tested positive for at least one enteropathogen, of which 290 (48.5%) were positive for two or more enteropathogens. Risk factors for two or more enteropathogen co-infections were young age (<12 months, OR 1.9, 95% CI 1.1–3.3; 13–36 months, OR 1.7, 95% CI 1.1–2.5, versus 37–48 months), day-care attendance (OR 1.8, 95% CI 1.3–2.5), households with three or more children versus those with one child (OR 1.7, 95% CI 1.1–2.8). Stool samples collected in spring less often had two or more enteropathogens versus summer (OR 0.4, 95% CI 0.2–0.7). Food allergy was a risk factor for three or more enteropathogen co-infections (OR 3.2, 95% CI 1.1–8.9). The frequency of co-infection was higher than expected for norovirus GI/norovirus GII, *Clostridium difficile*/norovirus GI, *C. difficile*/rotavirus, astrovirus/*Dientamoeba fragilis*, atypical enteropathogenic *Escherichia coli*/adenovirus, typical enteropathogenic *E. coli*/adenovirus, and enteroaggregative *E. coli*/astrovirus. No co-infection was associated with increased gastroenteritis risk.

Conclusions: Risk factors for enteropathogen co-infections were identified and specific enteropathogens co-occurred significantly more often than expected by chance. Enteropathogen co-infections were not associated with increased gastroenteritis risk, calling into question their clinical relevance in preschool children. **R. Pijnacker, Clin Microbiol Infect 2019;25:1039.e7–1039.e13**

Introduction

Gastroenteritis (GE) is one of the most common diseases in developing countries and causes significant morbidity in developed countries as well, particularly among preschool children [1]. Among children hospitalized for GE in the Netherlands, co-infections of viruses, bacteria and parasites were detected in 40% [2]. Other European countries report widely varying rates of co-infections in children hospitalized for GE [3–7]. Available data are limited and inhomogeneous, however, testing for different panels of enteropathogens. Furthermore, it is unclear whether children with co-infections have increased risk for (severe) GE, as the literature shows conflicting results.

A study among children admitted to hospital for GE in Italy and children with GE in hospital emergency rooms in Spain found that those with co-infections had more severe clinical pictures [3,6]. This is in contrast with other studies among hospitalized children

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in Italy, France and Brazil [7–9]. In the Netherlands, mixed infections were not associated with specific symptoms in children hospitalized for GE [2]. Because these studies focus on children hospitalized for GE, they can only associate co-infections with disease severity, but cannot assess whether children with co-infections are more likely to have GE *per se*. Although this requires studies on co-infections in children with and without GE, these are scarce in the literature. Furthermore, if enteropathogens could occur independently of each other, they would occur at random in all children. However, possible interactions between enteropathogens have been described but are poorly understood [10].

Focusing on preschool children in the general population, the objectives of this study were to (i) identify risk factors for enteropathogen co-infections in children in the general population, (ii) determine whether enteropathogen co-infections influence GE risk, and (iii) determine whether specific combinations of co-infecting enteropathogens occur significantly more or less often than expected.

Methods

Study design and population

We used data from a monthly-repeated cross-sectional survey in the Netherlands conducted during October 2012 to October 2014. For a detailed description of its design, we refer to previously published papers [1,11]. In short, 2000 families with preschool children <4 years old were randomly selected from Dutch municipal population registries every month for a period of 25 months. They were invited to complete a web-based questionnaire regarding household characteristics and health status in the past 4 weeks for the sampled child and one freely chosen parent in the household. If they were willing to submit a stool sample, we provided a sample collection kit with an additional questionnaire to check whether new symptoms had occurred in the 2 weeks before stool sampling.

Gastroenteritis case definition

A GE case was defined as a child with three or more diarrhoeal discharges in 24 hours or any 'clinically relevant' vomiting during the previous 2 weeks before the stool sampling, according to a standard GE case definition [12]. By 'clinically relevant' vomiting we refer to vomiting events other than regurgitation, vomiting due to motion sickness/vertigo, intense exercise, traumatic or nauseous events.

Detection of bacteria, parasites and viruses

Stool samples were tested for several viruses, bacteria and parasites using internally controlled quantitative real-time multiplex PCRs as described previously [13–16]. Viruses tested for were norovirus genogroups I (GI) and II (GII), sapovirus, astrovirus, rotavirus, adenovirus and adenovirus type 41; bacteria were *Salmonella* spp., *Yersinia enterocolitica*, *Shigella* spp./enteroinvasive *Escherichia coli*, *Campylobacter jejuni/coli* and *Clostridium difficile* toxin A/B. Bacterial pathogenicity genes tested for were Shiga toxins *stx*1 and *stx*2, *bfpA*, *escV* and *aggR*. *Stx* is a common characteristic of Shiga toxin-producing *E. coli*. *BfpA* is a marker gene for enteropathogenic *E. coli* (EPEC), *escV* is a marker gene for atypical enteropathogenic *E. coli* (a-EPEC) and *aggR* for enteroaggregative *E. coli*. Parasites tested for were *Dientamoeba fragilis*, *Giardia lamblia*, *Cryptospordium* spp. and *Entamoeba histolytica*.

Data analysis

For each enteropathogen, we determined the observed and expected frequencies of co-infection with any of the other enteropathogens in the same children. The expected frequency was calculated as the weighted sum of the products of the age-specific (0–12, 13–36 and 37–48 months) prevalence rates of these two enteropathogens. We tested for differences between the observed and expected co-infection prevalence rates using two-sample Z-tests for proportions. To minimize spurious associations between enteropathogens due to shared risk factors, those with a p-value <0.10 were entered in a multivariable logistic regression model (one enteropathogen as the binary dependent variable and the other as the binary independent variable) to be adjusted for shared risk factors. These factors were selected based on previous risk analyses on the same data set [11]. Correlations between enteropathogens were visualized using principal component analysis (see Supplementary material, Fig. S1).

We examined for each enteropathogen whether co-infection with each of the other enteropathogens was associated with GE.

Table 1
Demographics and household characteristics of the study population ($n = 822$)

	n	%
Demographics		
Age		
<12 months	120	14.6
13–36 months	470	57.2
37–48 months	232	28.2
Female	408	49.7
Non-Western migration background[a]	37	4.5
Enteropathies	68	8.3
Food allergy	36	4.4
Reflux	34	4.1
Bowel disorder	6	0.7
Day-care attendance	434	52.9
Use of gastric acid inhibitors[b]	6	0.7
Use of antibiotics[b]	66	8.0
Hospitalization[c]	0	0
Having gastroenteritis	185	22.5
Season[d]		
Spring	145	17.6
Summer	353	42.9
Autumn	144	17.5
Winter	180	21.9
Household characteristics		
Number of children in household		
1	251	30.6
2	397	48.4
≥3	173	21.1
Breastfeeding		
None	181	22.1
<3 months	179	21.8
3–6 months	234	28.5
>6 months	223	27.2
Unknown	4	0.5
Degree of ubanization		
<500 addresses/km^2	108	13.2
500–2500 addresses/km^2	502	61.3
>2500 addresses/km^2	209	25.5
Having a pet dog	137	16.7
Having a pet cat	222	27.0
Having farm animals	89	10.8
Having a sandpit	478	58.2

[a] The child or one of its parents is not born in Europe (excluding Turkey), North America, Oceania, Indonesia or Japan.
[b] In the past 6 months.
[c] In the past 4 months due to gastroenteritis.
[d] Spring: March to May, summer: June to August, autumn: September to November, winter: December to February.

A separate multivariable logistic regression model was built for each pair of enteropathogens, including GE as the dependent variable and the two enteropathogens as interaction term. Children with no enteropathogens detected were the reference group. If the combination of two enteropathogens was significantly associated with GE ($p < 0.05$), we also compared the combination with single infections of both enteropathogens. All analyses were adjusted for age and underlying enteropathies. Because *C. difficile* infections are more often asymptomatic in children <2 years, we performed age-stratified analyses for *C. difficile* co-infections [17].

Finally, we built univariate logistic regression models to examine associations between demographics and household characteristics (listed in Table 1) with co-infection of two or more enteropathogens compared with only one enteropathogen. We did the same for co-infection of three or more enteropathogens. Variables with a p-value of <0.10 were entered in a multivariable model built using backward stepwise selection. In multivariable analyses, a p-value of <0.05 was considered to be statistically significant. Analyses were performed using STATA version 14.2 (College Station, TX, USA).

Ethics statement

This study received ethics approval from the Medical Research Ethics Committee of the University Medical Centre, Utrecht (WAG/om/14/012490). Informed consent was obtained from all participants. All participants gave consent and in the case of children, parents gave consent.

Results

In total, 49 732 households were invited and 10 109 (20.3%) filled in the questionnaire. Of these, 946 (9.5%) were excluded because the child was older than 48 months, the parent had reported data for a child other than the one invited, or the questionnaire was inconsistently filled in. Faecal samples were obtained from 981 children (9.7%) and enteropathogen detection (positive or negative) was successfully performed for the entire panel of enteropathogens in 822 samples (83.3%). The median age of children was 28 months (interquartile range 17–38 months) and 408 (49.7%) were female (Table 1). A total of 185 children (22.5%) experienced GE within 2 weeks of stool sampling.

Co-infection

In 224 children (27.3%), no enteropathogens were detected, in 308 children (37.5%) one enteropathogen, in 202 children (24.6%) two enteropathogens, in 69 children (8.4%) three enteropathogens and in 19 children (2.2%) four or more enteropathogens were detected. Of 290 children with at least two enteropathogens, 219 (75.5%) had at least one virus, 191 (65.9%) had a bacterium and 178 (61.4%) had a parasite (Table 2). Most children with four or more enteropathogens were female (15/19, 78.9%) and all except one attended day-care (18/19, 94.7%). After adjusting for shared exposures, the observed frequency of co-infection was significantly higher than expected for norovirus GI and norovirus GII (expected 0.30% versus observed 0.76%, p 0.016), *C. difficile* and norovirus GI (expected 0.34% versus observed 1.16%, p <0.001), *C. difficile* and rotavirus (expected 0.14% versus observed 0.47%, p 0.011), astrovirus and *D. fragilis* (expected 0.48% versus observed 1.50%, p <0.001), a-EPEC and adenovirus (expected 7.0% versus observed 8.8%, p 0.046), typical EPEC (t-EPEC) and adenovirus (expected 1.1% versus observed 2.5%, p <0.001) and enteroaggregative *E. coli* and astrovirus (expected 0.08% versus expected 0.31%, p 0.020) (Table 3). The frequency of co-infection was not significantly lower than expected after adjusting for shared exposures for any of the enteropathogens. The same co-infection patterns were observed in the principal component analysis (see Supplementary material, Fig. S1).

Gastrointestinal illness

In children with no enteropathogens detected, 50/224 (22.3%) had GE, compared with 68/308 (22.1%, χ^2 test, p 0.947) in children with one enteropathogen, 43/202 (21.3%, χ^2 test, p 0.796) in children with two enteropathogens, and 24/88 (27.3%, χ^2 test, p 0.309) in children with three or more enteropathogens, but differences were not significant. Co-infections of adenovirus with norovirus GII and of a-EPEC with norovirus GII were significantly associated with GE (12/25, 48.0%; OR 3.0, 95% CI 1.3–7.1 and 8/18, 44.4%; OR 2.7, 95% CI 1.0–7.3, respectively), compared with children with no enteropathogens detected (Table 4; and see Supplementary material, Table S1). This was probably due to the increased risk for GE of norovirus GII alone, because neither co-infection was associated with GE when compared with children with norovirus GII alone (OR 1.3, 95% CI 0.4–3.6 and OR 1.0, 95% CI 0.3–3.1, respectively).

Risk factors for co-infection

Having two or more enteropathogens compared with a single enteropathogen was associated with being <12 months old (OR 1.9, 95% CI 1.1–3.3) or 13–36 months old (OR 1.7, 95% CI 1.1–2.5), compared with being 37–48 months old, day-care attendance (OR 1.8, 95% CI 1.3–2.5) and living in a household with at least three children compared with living in a household with one child (OR 1.7, 95% CI 1.1–2.8) (Table 5). Stool samples taken during spring less often had two or more enteropathogens compared with those taken in summer (OR 0.4, 95% CI 0.2–0.7).

Having three or more enteropathogens compared with a single enteropathogen was associated with having a food allergy (OR 3.2, 95% CI 1.1–8.9) and day-care attendance (OR 2.0, 95% CI 1.2–3.4). Stool samples taken during spring were less likely to have three or more enteropathogens compared with those taken during summer (OR 0.3, 95% CI 0.1–0.7)

Discussion

This study identified several risk factors for co-infection in preschool children. Co-infections were detected in 290 (48.5%) of 598 children who had at least one enteropathogen, but were not associated with increased GE risk. We also observed that several enteropathogens co-occurred more frequently than would be expected by chance.

Norovirus GI and norovirus GII co-infection occurred more often than expected. This could be due to their largely overlapping epidemiology, even when adjusting for shared exposures. Another explanation may be that around 20% of Dutch inhabitants do not have the FUT2 allele required for histo-blood group antigen expression at the gut surface, to which norovirus binds [18,19]. Therefore, they have near-total protection against some norovirus genotypes, including the dominant norovirus GII.4 in the Netherlands [18,20–23]. Hence, norovirus GI and GII co-infection may not occur randomly but more often in histo-blood group antigen secretors. However, other molecules and cell types binding to norovirus may also play a role, but are not well understood [19]. Co-infection of *C. difficile* with norovirus GI and rotavirus also occurred more frequently than expected. A US study reported higher faecal *C. difficile* ribotype 027 concentrations in children with viral co-infections compared with children without co-infections [24]. They stated that viruses may create favourable conditions for *C. difficile* to multiply, possibly through disruption of the intestinal

Table 2
Number of single infections and co-infections of enteropathogens in children aged 0–48 months, and the percentage of children with single infections that experienced gastroenteritis in the 2 weeks before stool sampling ($n = 822$)

	Total detections	% GE	Single occurrence	Viral co-infection	Bacterial co-infection	Parasitic co-infection	Norovirus GI	Norovirus GII	Sapovirus	Astrovirus	Rotavirus	Adenovirus	Adenivurus 41	*Yersinia*	*Campylobacter*	*C. difficile*	STEC	a-EPEC	t-EPEC	EAEC	*D. fragilis*	*G. lamblia*
Virus																						
Nororivus GI	38	24	7	16	26	16	—	6	1	1		12				6		14	3	3	14	4
Norovirus GII	57	44	9	28	26	22	6	—	1	2	2	25	1			7	1	18	3	2	22	2
Sapovirus	34	18	16	12	0	8	1	1	—	1	3	9				3		3			8	
Astrovirus	14	50	4	4	5	8	1	2	1	—		3						2		4	8	1
Rotavirus	12	33	2	7	3	2		2	3		—	5				3					2	
Adenovirus	228	25	65	45	103	76	12	25	9	3	5	—		1		27	8	75	16	10	74	10
Adenovirus type 41	6	67	3	1	2	1		1					—			1	1	1			1	1
Bacteria																						
Yersinia enterocolitica	1	0	0	1	0	0						1		—							1	
Campylobacter	2	100	1	0	0	1															1	
Clostridium difficile	69	23	26	37	14	3	6	7	3		3	27	1			—	2	12	5	3	3	
Salmonella	0	—	—	—	—	—																
Shigella spp./EIEC	0	—	—	—	—	—																
STEC (*stx* 1/2)	23	13	2	8	2[a]			1				8	1				—	18		2	8	
a-EPEC (*escV*)	195	22	41	93	12[a]		14	18	3	2		75	1			12	18	—	32	10	69	11
t-EPEC (*bfpA*)	32	34	0	17	5[a]		3	3				16				5		32	—	3	14	3
EAEC (*aggR*)	41	24	11	14	3[a]		3	2		4		10				3	2	10	3	—	19	3
Parasite																						
Dientamoeba fragilis	272	20	101	109	90	27	14	22	8	8	2	74	1	1	1	3	8	69	14	19	—	27
Giardia lamblia	39	13	5	14	14	27	4	2		1		10	1					11	3	3	27	—
Entamoeba histolytica	0	—	—	—	—	—																
Cryptosporidium	1	100	1	—	—	—																

a-EPEC, atypical enteropathogenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; STEC, Shiga toxin-producing *Escherichia coli*; t-EPEC, typical enteropathogenic *E. coli*.
[a] Excluding co-infections between pathogenicity genes *stx 1/2* (STEC), *escV* (a-EPEC), *bfpA* (t-EPEC), *aggR* (EAEC).

Table 3
Observed and expected % prevalence of co-infection of each enteropathogen with the other enteropathogens, with and without adjusting for shared exposure ($n = 822$)

		Norovirus GI	Norovirus GII	Sapovirus	Astrovirus	Rotavirus	Adenovirus	*C. difficile*	STEC	a-EPEC	t-EPEC	EAEC	*D. fragilis*	*G. lamblia*
Norovirus GI	Expected %		0.30					0.34						0.22
	Observed %		0.73					0.73						0.49
Norovirus GII	Expected %	0.30					1.98							
	Observed %	0.76[a]					3.04							
Sapovirus	Expected %					0.08				1.06				
	Observed %					0.36				0.36				
Astrovirus	Expected %											0.08	0.48	
	Observed %											0.49	0.97	
Rotavirus	Expected %			0.08				0.14		0.39				
	Observed %			0.19[b]				0.36		0.0				
Adenovirus	Expected %									7.0	1.1			
	Observed %									9.1	2.0			
Clostridium difficile	Expected %	0.34				0.14							1.45	
	Observed %	1.16[c]				0.47[d]							0.36	
STEC	Expected %													
	Observed %													
a-EPEC	Expected %			1.06		0.39	7.0							
	Observed %			0.54[e]		0.0	8.8[f]							
t-EPEC	Expected %						1.1							
	Observed %						2.5[g]							
EAEC	Expected %				0.08									
	Observed %				0.31[h]									
Dientamoeba fragilis	Expected %				0.48			1.45						1.82
	Observed %				1.39[i]			0.82[j]						3.28
Giardia lamblia	Expected %	0.22											1.82	
	Observed %	0.48[k]											2.37[l]	

a-EPEC, atypical enteropathogenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*; STEC, Shiga toxins-producing *Escherichia coli*; t-EPEC, typical enteropathogenic *E. coli*

Two-sample Z-tests for proportions were used to compare the observed and expected % prevalence; red ($p < 0.01$), orange ($p < 0.05$), yellow ($p < 0.10$), grey ($p \geq 0.10$). Only those with a p-value <0.10 are shown.

The observed prevalence was adjusted for age[a–f, i–k], gender[a,c,k] season[a,b,d–g,i], day-care attendance[a,b,c,k,l], degree of urbanization[a,c,h–k], number of children in the household[c,d,h,i,j,l], presence of farm animals[a,c,k] or cats[k,l], or dogs[i,j], or sandpits[k,l] in the household, presence of horses, ponies or donkeys in the household or in the day-care centre.[h]

microbiota or host defences, which was also hypothesized in two case reports [25,26]. However, evidence is scarce, and ribotyping was not performed in our study, questioning the comparability with the US study. Co-infections of astrovirus with enteroaggregative *E. coli* and *D. fragilis*, and adenovirus with t-EPEC and a-EPEC, were found more often than expected. To our knowledge, there is no literature describing possible interactions between them. Previous research based on the same study population found that astrovirus was significantly associated with GE and t-EPEC, which pointed towards an association, albeit non-significant [11]. We speculate that GE might flush out other enteropathogens as well, increasing their detection chance, possibly explaining why these co-infections were observed more than expected.

Only co-infections of norovirus GII with adenovirus and norovirus GII with a-EPEC were associated with GE. However, this was probably a result of the increased risk for GE of norovirus GII alone [11]. Indeed, when comparing both co-infections with norovirus GII single infections, no increased GE risk was observed. Comparing our results with other studies is difficult because they mostly focus on children admitted to hospital and can therefore only examine GE severity [3,6–9,27,28]. However, they show conflicting results regarding the clinical relevance of co-infections. Two studies did compare children with and without GE. A study in Mexico compared children hospitalized for GE with children without GE from surrounding schools, and found that co-infections were more prevalent in those who were hospitalized [29]. However, they only associated co-infection as a whole with GE and not specific enteropathogens, and focused on a different panel of enteropathogens. Co-infections were not associated with GE in a Korean study comparing hospitalized children with and without GE, but did they aggravate GE symptoms [30]. We were unable to assess GE severity because no data were collected on duration and frequency of symptoms, which are the most common criteria to define GE severity.

Younger children more often had co-infections, which could be because almost all co-infections included at least one virus, which were more prevalent in younger children, as were bacteria [11].

Table 4
Multivariable logistic regression model of the association between gastroenteritis and combinations of enteropathogens, compared with children that were negative for all enteropathogens that were tested

	n	% GE[b]	aOR[c]	95% CI	p
Adenovirus × norovirus GII					
None[a]	244	22	Ref		
Adenovirus	203	22	0.9	0.6–1.4	0.616
Norovirus GII	32	41	2.4	1.1–5.2	0.029
Adenovirus × norovirus GII	25	48	3.0	1.3–7.1	0.011
a-EPEC × norovirus GII					
None[a]	244	22	Ref		
a-EPEC	177	19	0.8	0.5–1.3	0.350
Norovirus GII	39	44	2.7	1.3–5.6	0.006
a-EPEC × norovirus GII	18	44	2.7	1.0–7.3	0.048

a-EPEC, atypical enteropathogenic *Escherichia coli*; aOR, adjusted odds ratio.
Models are only shown where the combination of enteropathogens was significantly associated ($p < 0.05$) with gastroenteritis. An overview of all models can be found in the Supplementary material, Table S1.

[a] Negative for all enteropathogens that were tested.
[b] Gastroenteritis.
[c] Odds ratio, adjusted for underlying enteropathies and age.

Table 5
Multivariable logistic regression model of risk factors in children with more than one enteropathogen and more than two enteropathogens compared with children with one enteropathogen

	1 enteropathogen ($n = 308$)		>1 enteropathogen ($n = 290$)				>2 enteropathogens ($n = 88$)			
	n	%	*n*	%	aOR[a]	95% CI	*n*	%	aOR[a]	95% CI
Child's age										
37–48 months	95	30.8	67	23.1	Ref		16	18.2	Ref	
13–36 months	170	55.2	179	61.7	**1.7**	**1.1–2.5**	59	67.1	**2.2**	**1.2–4.1**
≤12 months	43	14.0	44	15.2	**1.9**	**1.1–3.3**	13	14.8	**2.5**	**1.0–5.8**
Food allergy	9	2.9	16	5.5	ns	-	8	9.1	**3.2**	**1.1–8.9**
Day-care attendance	165	53.8	187	64.5	**1.8**	**1.3–2.5**	60	68.1	**2.0**	**1.2–3.4**
No. of children in the household										
1	91	29.6	77	26.6	Ref		23	26.1	Ref	
2	148	48.2	134	46.2	1.1	0.8–1.7	42	47.7	ns	-
≥3	68	22.2	79	27.2	**1.7**	**1.1–2.8**	23	26.1	ns	-
Season										
Summer	115	37.3	130	44.8	Ref		43	48.9	Ref	
Autumn	65	21.1	55	19.0	0.7	0.5–1.2	21	23.9	0.8	0.4–1.6
Winter	62	20.1	73	25.0	1.0	0.7–1.6	15	17.1	0.6	0.3–1.2
Spring	66	21.4	32	11.0	**0.4**	**0.2–0.7**	9	10.2	**0.3**	**0.1–0.7**

ns, not significant ($p < 0.05$) and therefore not included in the model.
The odds ratio's expressed in bold had a p-value <0.05 in multivariable analyses.
[a] Adjusted odds ratio.

Furthermore, day-care-attending children were more often co-infected, probably reflecting increased enteropathogen exposure in the day-care centre compared with those children who were home-cared [11]. Co-infections were also more prevalent in children living in households with two or more children compared with children being the only child, probably reflecting increased transmission between children [31]. We observed that stool samples collected during spring had fewer co-infections than those in summer, possibly because viruses were mostly detected during autumn and winter and bacteria and parasites during autumn [11]. Lastly, children with a food allergy had more co-infections with three or more enteropathogens. Children with food allergy may be more likely to have gastrointestinal symptoms, leading to a disturbed normal gut flora and thereby increased vulnerability to infections [32]. Moreover, an immature mucosal immune system has been described as a risk factor for food allergy, probably also increasing the chance for co-infections [32].

This study has several limitations. Due to its cross-sectional design, the chronological order of the infections caused by the different enteropathogens involved was unknown. This makes it more difficult to understand the underlying mechanism(s) of possible enteropathogen interactions. Furthermore, although the current study only focused on enteropathogens, complex relationships between enteropathogens and non-pathogenic micro-organisms probably also influence the outcome of infection. When assessing the effect of co-infection on GE, children with no enteropathogens detected were the reference group, possibly making the results less generalizable to areas with different testing capabilities. However, results were identical when single infections of both enteropathogens in question were the reference groups (results not shown). Because symptoms in children were self-reported by their parents, misclassification of GE may have occurred due to diarrhoea or vomiting that occurred as a result of non-infectious causes. Furthermore, the presence of enteropathogens in stool was determined based on the detection of genetic material. This could have led to an overestimation, as this can be detected in faeces up to several weeks after infection [33]. Lastly, although we adjusted for shared exposure when analysing whether co-infections occurred more or less often than expected, it is unlikely that we were able to fully adjust for it.

In conclusion, we observed that several enteropathogens co-occur in the guts of preschool children non-randomly and independently of exposure to common risk factors. We hypothesize possible enteropathogen interactions that would need to be tested further, for example in experimental settings. Co-infections were associated with crowding in households, use of day-care centres and younger age. Yet, co-infections did not lead to a significantly increased GE risk in our study population, questioning the clinical relevance of such co-infections in preschool children in industrialized countries.

Transparency declaration

The authors declare that they have no conflict of interests.

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Contribution of authors

WvP participated in the design and coordination of the study; HV was responsible for the virological laboratory tests. RP and LMG were involved in the statistical analyses under advice of WvP; RP, LMG, WvP, RAC, JAW, HV, LMK, DN and EF participated in the interpretation of data and in drafting and reviewing the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2018.11.029.

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