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Original article

Polymicrobial enteric infections in African infants with diarrhoea—results from a longitudinal prospective case–control study

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ABSTRACT

Objectives: This longitudinal case–control study aimed to determine the frequency of polymicrobial enteric detections in Ghanaian infants with and without diarrhoea.
Methods: Infants aged 1–12 months with and without diarrhoea attending the outpatient department of a peri-urban Ghanaian hospital were prospectively assessed and stool samples were collected on days 0, 6 and 28 and analysed for 18 enteric pathogens with PCR.
Results: At least one enteric pathogen was detected in 100 of 107 cases with diarrhoea (93%) and in 82 of 97 controls (85%). The number of pathogens was higher in cases than in controls (median three versus two pathogens, p 0.001). The adjusted attributable fraction (AF) for diarrhoea was highest for enterotoxigenic *Escherichia coli* (7.2%, 95% CI −2.0% to 16.3%), rotavirus (4.1%, 95% CI 0.6%–7.5%), *Giardia lamblia* (2.3%, 95% CI −0.7 to 5.3%) and astrovirus (2.3%, 95% CI −2.9 to 7.5%). In cases, a higher pathogen number was significantly associated with watery stool consistency (median 3, interquartile range (IQR) 2–5 versus median 2.5, IQR 1–4, p 0.014), stool frequency five or more per day (median 4, IQR 3–5 versus median 3, IQR 2–4, p 0.048) and vomiting (median 4, IQR 3–5 versus median 3, IQR 2–4, p 0.025). During follow-up, 94% (78/83) of cases and 85% (67/79) of controls had acquired at least one new pathogen without developing a new episode of diarrhoea.
Conclusion: Enteric pathogens could be identified in the stool of the vast majority of Ghanaian infants, whereby pathogens were very frequently acquired without resulting in new episodes of diarrhoea during follow-up. A higher number of co-occurring pathogens may increase the risk of diarrhoea and disease severity. **Melina Heinemann, Clin Microbiol Infect 2021;27:1792**

Introduction

Despite considerable progress, diarrhoea remains the second leading cause of mortality in children aged 1 month to 5 years worldwide [1,2]. Studies have revealed that detection of multiple diarrhoeal pathogens in the stool of an individual at one time is common rather than an exception in developing countries [2–4]. However, the clinical relevance of polymicrobial carriage, frequent

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(re-)exposure and shedding of enteric pathogens is not well understood, because the few longitudinal studies on enteric pathogens in children with diarrhoea focused on one or few pathogens only or lacked a control group without diarrhoea [5]. Furthermore, the association of polymicrobial infection and severity of acute diarrhoea is controversial [6,7] and needs to be further investigated. Responding to this need, this prospective study compared the presence of a set of 18 viral, bacterial and parasitic enteric pathogens in stool samples from Ghanaian infants in a case–control study at three time-points over a period of 4 weeks. Our aims were to assess the clinical importance of single pathogens as well as polymicrobial carriage, the association of polymicrobial detection with disease severity as well as clearance and new acquisition rates of enteric pathogens and their clinical impact during follow-up.

Materials and methods

Study population

This prospective case–control study was conducted at the St Michael's Catholic Hospital in Pramso, a 121-bed facility in the suburbs of Kumasi, the regional capital of the Ashanti region. All infants aged between 1 and 12 months attending the outpatient department between August 2014 and July 2015 were screened for eligibility. After informed consent was obtained from the legal guardian, the infants were matched to the case or control group: cases were defined as patients with acute diarrhoea for $\leq$5 days and controls as infants with any medical condition except diarrhoea and vomiting for $\leq$5 days. Diarrhoea was defined as an acute change of stool consistency and frequency with three or more loose stools within 24 hours. Infants who required urgent medical attention, had a chronic enteric disorder or could not provide any stool sample within 24 hours after enrolment were excluded. To avoid logistic challenges for follow-up, only infants living $\leq$30 km around the hospital were included.

Recruitment and sample collection

Infants were recruited after attending the outpatient department (day 0) and followed up in their guardians' households after 6 days (day 6 ± 48 hours) and after 28 days (day 28 ± 48 hours). A clinical examination was conducted by the medical doctor on duty at admission. Treatment was administered by the physician in charge based on clinical reasoning. The medical history was obtained by a study team member at the time of inclusion and both follow-up time-points using a comprehensive questionnaire.

On day 0 and the designated follow-up visits stool samples were collected at the outpatient department and at the infants' households. Several studies have shown that detection rates for PCR-based identification of enteric pathogens are comparable between stool samples and rectal swabs [8,9], so a rectal swab (stored in Amies media) was taken if a child was not able to provide fresh stool. All samples were transported to the hospital, stored at 2–8°C and processed on the same day.

Sample processing and microbiological analyses

Extraction of the RNA/DNA from whole stool samples was performed with commercially available kits (innuPREP Virus DNA/RNA kit, Analytik Jena, Jena, Germany) and according to the manufacturer's instructions. The resulting eluate was aliquoted and stored at −80°C until shipment to Germany, where a real-time PCR with TaqMan™-Oligonucleotide probe linked fluorescence dyes was performed (for details see Supplementary material, Appendix S1 and Table S1).

Statistical analysis

Details of the statistical analyses are descried in the Supplementary material (Appendix S1). In brief, sample size and power calculations were performed using G*Power [10]. Binary logistic regression was used to analyse the association of pathogens with positive PCR and diarrhoea, adjusted for variables that were significantly associated with diarrhoea in univariate analyses. Model-based estimation of the population attributable fraction (AF) used the R package 'AF' (version 0.1.5). The AF indicates the proportional reduction of a disease in a population that would occur by eliminating the exposure to a risk factor.

Ethical statement

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and consistent with Good Clinical Practice. Ethical approval was obtained from the Ethics committee of the School of Medical Science, Kwame Nkrumah University of Science and Technology, Kumasi (CHRPE/RC/161/14).

Results

Characteristics of study population

Two-hundred-and-four of 218 infants enrolled between August 2014 and July 2015 were considered for further analyses: 107 (52%) cases and 97 (48%) controls (Fig. 1). Demographic and socio-economic characteristics of the study population are presented in Table 1. Ninety-seven of 107 cases (91%) and 75 of 97 controls (77%) suffered from various symptoms. Disease characteristics, antibiotic intake before admission and treatment started after inclusion are shown in Table 2.

Predictors of diarrhoea

With 60 of 107 cases (56%) versus 40 of 97 controls (41%, p 0.048) being female, a significant association between female sex and diarrhoea was detected by univariate analysis. Age, household size or close contact with animals were not significantly associated with diarrhoea. A higher number of pathogens was detected in the case compared with the control group (medians 3 versus 2, p 0.001) (Table 1).

Enteric pathogens and burden of polymicrobial carriage

At least one enteric pathogen was detected in 100 of 107 cases (93%) and in 82 of 97 controls (85%). In descending order, the following pathogens were most frequently found on day 0: enteroaggregative *Escherichia coli* (EAEC) in 55 of 107 (51%) and enterotoxigenic *E. coli* (ETEC) as well as enteropathogenic *E. coli* (EPEC) in 40 of 107 (37%) cases each. In the 97 controls, EAEC (n = 45; 46%) was followed by enterovirus (n = 35; 36%) and EPEC (n = 30; 31%).

Results of univariate and multivariate analyses assessing the association of single pathogens with diarrhoea are shown in Table 3. After adjusting for sex and co-occurring pathogens only rotavirus showed an independent association with diarrhoea.

The AF adjusted for sex and number of pathogens was highest for ETEC (7.2%, 95% CI −2.0% to 16.3%, p 0.124), followed by rotavirus (4.1%, 95% CI 0.6%–7.5%, p 0.02), *Giardia lamblia* (2.3%, 95% CI −0.7% to 5.3%, p 0.131), astrovirus (2.3%, 95% CI −2.9% to 7.5%, p 0.379) and

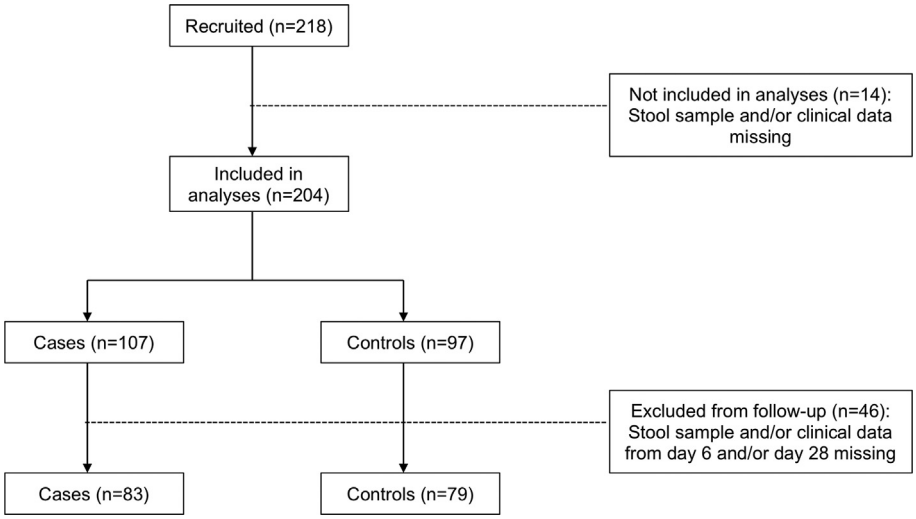

Fig. 1. Tree diagram of exclusion and inclusion of patients.

Cryptosporidium spp. (2.2%, 95% CI −1.6% to 6.0%, p 0.257). The Cycle threshold (Ct) value of the pathogens was not associated with the presence of diarrhoea, except for *Cryptosporidium* spp., for which a lower Ct value was observed in cases (see Supplementary material, Table S2). The optimal Ct cut-off to distinguish cases from controls was ≤25 for *Cryptosporidium*.

One pathogen was detected in only 19 of 107 cases (18%) and 16 of 97 controls (16%), two or more pathogens were detected in the stool from 81 cases (76%) and 66 (68%) controls. Among cases, the highest co-occurrence rate was observed for EAEC + ETEC (31/64, 48%) (Fig. 2a). In controls, the highest co-occurrence rate was observed for EAEC + EPEC (19/56, 34%) (Fig. 2b).

Clinical impact of polymicrobial detections in cases

In the subgroup of 100 of 107 cases (93%) with detection of at least one pathogen, a higher number of pathogens was associated with several criteria of disease severity. A higher number of pathogens (median 3, interquartile range (IQR) 2–5) was observed in the 67 cases (67%) with watery stool compared with the 32 cases (32%) with mushy stool (median 2.5, IQR 1–4, p 0.014). The 54 cases (54%) with a stool frequency of three or four per day had a median number of three pathogens (IQR 2–4), a median of four pathogens was observed in the 43 cases (43%) with a stool frequency of five or more per day (IQR 3–5, p 0.048). The 43 cases (43%) with vomiting excreted a higher number of stool pathogens (median 4, IQR 3–5) compared with the 57 cases (57%) without vomiting (median 3, IQR 2–4, p 0.025). Fever (temperature ≥38.0°C versus <38.0°C) was observed in 67 cases (67%) and not associated with the number of detected pathogens (median 3, IQR 2–5 versus median 3, IQR 1–4, p 0.116).

Follow-up

For a total of 162 infants, including 83 cases and 79 controls, stool samples and clinical data on presence of diarrhoea from all three time-points (days 0, 6 and 28) were available (Fig. 1). Within this subgroup, at least one pathogen was detected in 78 of 83 cases (94%) and 66 of 79 controls (84%) on day 0. Seven of 83 cases (8%) still suffered from diarrhoea on day 6, whereas none of the controls had developed diarrhoea. None of the cases and controls suffered from diarrhoea on day 28. On day 6 at least one of the initially detected pathogens was still detected in 63 of 78 cases (81%) and 53 of 66 controls (80%, persistence rate). On day 28, at least one enteric pathogen was persistently detected in 49 of 78 cases (63%) and 29 of 66 controls (44%). Detection of at least one new enteric pathogen was observed in 78 of 83 cases (94%) and 67 of 79 controls (85%) during the whole follow-up period (i.e. on day 6, day 28 or both).

The persistence rate and the rate of newly detected pathogens for the single pathogens in cases (Figs. 2c,e) and controls (Figs. 2d,f) during follow-up were demonstrated. The median persistence rate of the 18 different pathogens over the first 6 days was 61% (IQR 43–79) in cases and 50% (IQR 33–59) in controls. On day 28, the median persistence rate (with reference to day 0) of the different pathogens was 25% (IQR 7–38) in cases and 20% (IQR 0–31) in controls. The median rate of new detections among the 18 different

Table 1
Demographic and socio-economic characteristics of study participants ($n = 204$)

Potential predictor	Cases ($n = 107$)[a]	Controls ($n = 97$)[a]	OR[b]	95% CI	p value
Demographics					
Age (months)	7 (5–9)	7 (4–9)	1.02	0.97–1.07	0.505
Female	60 (56%)	40 (41%)	1.81	1.01–3.3	0.048
Living conditions					
Household members	6 (4–11)	6 (4–10)	1.01	0.98–1.04	0.775
Own bathroom	70 (65%)	63 (66%)	0.99	0.53–1.84	1
Contact with animals	66 (62%)	61 (63%)	0.95	0.52–1.74	0.974
Pathogen number at admission	3 (2–4)	2 (1–3)	1.3	1.11–1.54	0.001

[a] Median and interquartile range are given for continuous variables.
[b] Crude ORs are reported. ORs for continuous variables were calculated based on logistic regression. Increments were 6 months for age, one person for household size and one pathogen for pathogen number.

Table 2
Disease characteristics and therapy (day 0)

Characteristics	Cases (% of $n = 107$)	Controls (% of $n = 97$)
Symptoms at admission		
Diarrhoea	107 (100%)	0
Vomiting	47 (44%)	0
Obstipation	0	5 (5%)
History of fever	71 (66%)	72 (74%)
Fever on admission (>38.0°C)	25 (23%)	20 (21%)
Cough	51 (48%)	64 (66%)
Breathing difficulty	22 (21%)	30 (31%)
Running nose	50 (47%)	61 (63%)
Blocked nose	46 (43%)	52 (54%)
Ear problem	1 (1%)	2 (2%)
Eye problem	7 (6%)	12 (12%)
Apathy	12 (11%)	1 (1%)
Inability to suck	2 (2%)	1 (1%)
Diagnosis of malaria[a]	33 (36%)	29 (33%)
Antibiotic intake before admission[b]	10 (9%)	7 (7%)
Treatment[c]		
Antibiotics (systemic)	76 (71%)	69 (71%)
Zinc	65 (61%)	1 (1%)
Oral rehydration solution	63 (59%)	0

[a] Twenty-five patients (12%) not tested for malaria.
[b] Twenty-one patients (10%) with missing information.
[c] Twenty-six patients (12%) with missing information.

pathogens was 3% (IQR 1–7) in cases and 2% (IQR 0–4) in controls on day 6. On day 28, the cumulative new detection rate (day 6 and/or 28 with reference to day 0) was 10% (IQR 3–17) in cases and 6% (IQR 0–15) in controls.

Discussion

A high level of pathogen burden was observed in the present study, and in only 7% of cases and 15% of controls was no enteric pathogen detected at baseline. A higher number of enteric pathogens detected at a time was significantly associated with diarrhoea. In infants with diarrhoea and detection of at least one pathogen, a higher number of pathogens detected was predictive for several parameters of disease severity. Interestingly, infants eliminated and also very frequently acquired new enteric pathogens during follow-up without developing episodes of diarrhoea.

After adjusting for sex and the number of pathogens detected, only the presence of rotavirus was significantly associated with diarrhoea. This result must be regarded with caution, because the PCR applied could not differentiate between wild-type rotavirus and vaccine-derived rotavirus strains. Ghana introduced rotavirus vaccination in May 2012 and achieved >85% coverage within a few months [11,12]. The adjusted AF of 4.1% observed for rotavirus is in line with one of the large multi-centre studies, where rotavirus had an AF of 4.8% in infants in the first two years of life [2], and suggests that rotavirus burden still poses a clinically relevant risk to African children [13–15].

We detected high frequencies of enteric pathogens in all infants independent of acute diarrhoea in accordance with earlier investigations [3,16,17]. Apart from rotavirus, not a single pathogen was associated with diarrhoea in multivariate analysis. Of interest, *Tropheryma whipplei* was found equally in cases and controls.

Table 3
Enteric pathogens detected at baseline (day 0)

Pathogens	Cases		Controls		Crude OR	Crude 95% CI	Adjusted OR[a]	Adjusted 95% CI[a]
	n	%	*n*	%				
Adenovirus	16	15%	19	20%	0.72	0.32–1.6	0.45	0.2–1
Astrovirus	17	16%	9	9%	1.84	0.73–4.95	1.54	0.63–3.96
Enterovirus	39	36%	35	36%	1.02	0.55–1.87	0.65	0.33–1.23
Norovirus I	2	2%	2	2%	0.91	0.06–12.71	1.15	0.13–10.26
Norovirus II	12	11%	6	6%	1.91	0.63–6.48	1.07	0.36–3.39
Rotavirus	12	11%	2	2%	5.96	1.27–56.17	5.32	1.37–35.22
Sapovirus	9	8%	4	4%	2.13	0.57–9.78	1.82	0.53–7.25
EAEC	55	51%	45	46%	1.22	0.68–2.2	0.87	0.45–1.64
EHEC	12	11%	8	8%	1.4	0.5–4.15	0.61	0.2–1.84
EPEC	40	37%	30	31%	1.33	0.72–2.49	0.85	0.43–1.68
ETEC	40	37%	18	19%	2.61	1.32–5.31	1.8	0.87–3.81
Salmonella	0	—	1	1%	0	—	—	—
Shigella/EIEC	8	8%	3	3%	2.52	0.58–15.19	1.79	0.47–8.75
Campylobacter	28	26%	18	19%	1.55	0.76–3.24	0.9	0.42–1.92
Tropheryma whipplei	13	12%	13	13%	0.89	0.36–2.22	0.53	0.21–1.32
Entamoeba histolytica	0	—	0	—	—	—	—	—
Giardia lamblia	10	9%	2	2%	4.86	1–46.82	3.16	0.75–21.66
Cryptosporidium	13	12%	4	4%	3.2	0.94–13.96	1.99	0.61–7.82

Abbreviations: EAEC, enteroaggregative *Escherichia coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*.
[a] Adjusted for gender and number of pathogens.

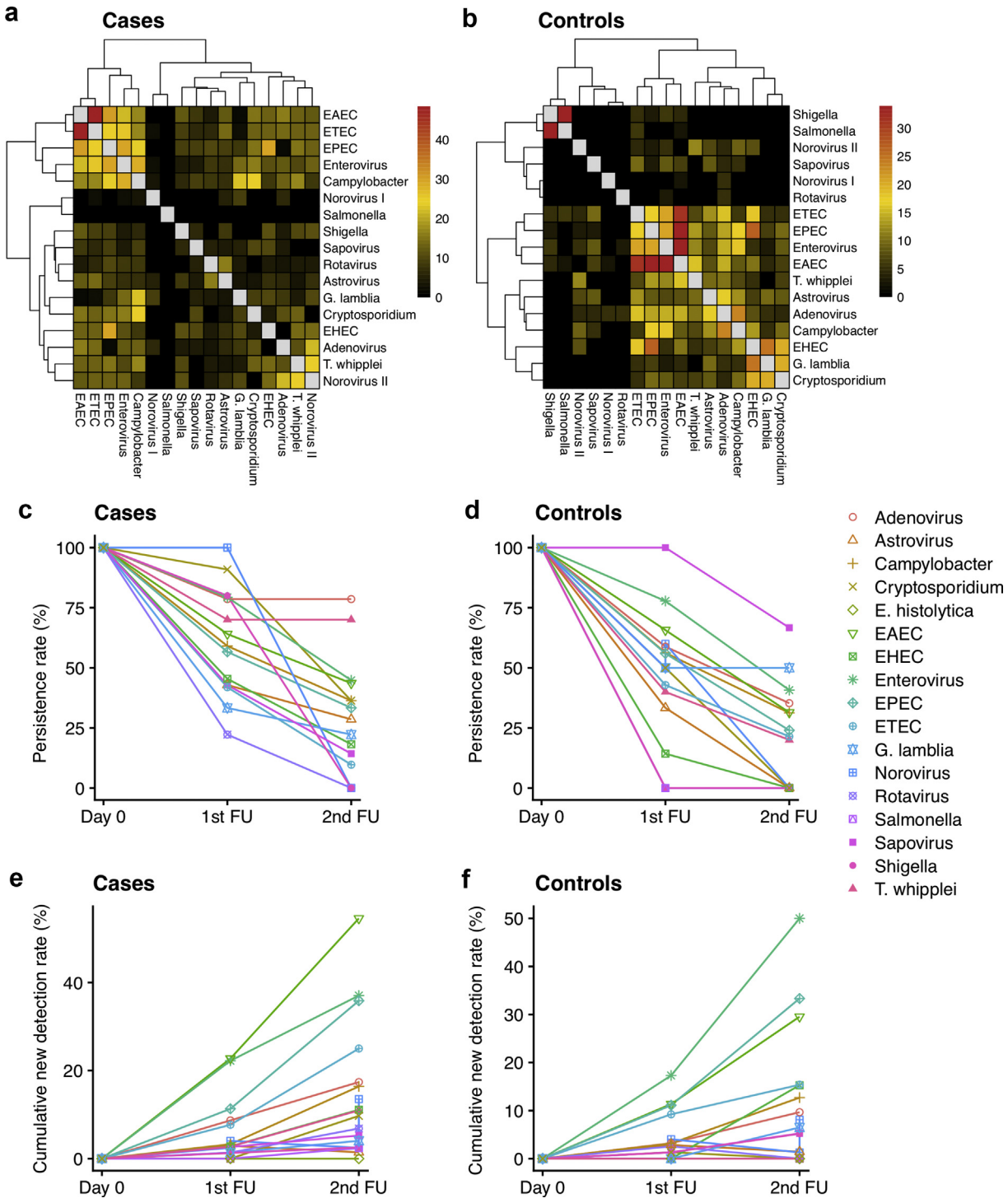

Fig. 2. Co-occurrence of stool pathogens at admission and persistence and cumulative new detection rate of pathogens during follow-up. (a,b) Heatmap with co-occurrence of stool pathogens at admission (day 0) in cases (a) and controls (b). Co-prevalence was calculated as the percentage of infants where both pathogens were detected among infants where at least one of two pathogens was prevalent. (c–f) Median persistence rate for each pathogen on day 6 (first follow-up) and day 28 (second follow-up) in cases (c) and controls (d). Median rate of newly detected pathogens on day 6 (first follow-up) and day 28 (second follow-up; i.e. day 6 and/or 28 with reference to day 0) in cases (e) and controls (f). Abbreviations: EAEC, enteroaggregative *Escherichia coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; FU, follow-up; G, *Giardia*; T., *Tropheryma.*

Earlier studies indicated that individuals might develop mild diarrhoea when infected [18–20]. Except for *Cryptosporidium* spp., application of Ct value as a measure of enteropathogen quantity failed to predict diarrhoea, although earlier studies suggested Ct values as a useful marker [4,17,21–23].

As reported previously [2,3], detection of a higher number of enteric pathogens was associated with the occurrence of diarrhoea. Our results highlight that, in the subgroup of infants with diarrhoea and the detection of at least one pathogen, a higher number of co-occurring pathogens was associated with several parameters of

disease severity including vomiting, watery stool consistency and higher stool frequency.

Even during follow-up, new acquisition of pathogens in cases and controls was common (94% of cases and 85% of controls, cumulatively, including days 6 and 28). Surprisingly, none of these infants developed new diarrhoea, even after (new) acquisition of rotavirus, *Campylobacter* or ETEC, and diarrhoea was absent in all infants on day 28. The period of pathogen persistence in the gut of individuals after initial detection is known to be variable [24]. On the last follow-up (day 28), persistence of at least one enteric pathogen was observed in 63% of cases and 44% of controls. In a previous study including 127 children with acute diarrhoea, clearance rates of 34%–100% were demonstrated for the different pathogens after 14 days of follow-up [5]. However, the respective study did not include a control group without diarrhoea, comprised only one follow-up and assessed fewer pathogens than the recent study. In the present study, the median persistence rate on day 28 was 25% (IQR 7%–38%) in cases and 20% (IQR 0%–31%) in controls for the different pathogens. Therefore, most pathogens were cleared by the last follow-up. Taken together our findings are highly suggestive that more complex mechanisms are involved in the development of paediatric diarrhoea in low- and middle-income countries than simple acquisition of a certain enteric pathogen. Some authors hypothesized that specific co-occurring pathogens may act synergistically and result in higher pathogenicity [6,7,25]. The recent transition from a narrow focus on individual organisms to polymicrobial carriage by the usage of multiplex PCR platforms bears important implications for emerging preventive and therapeutic approaches. The current challenge to determine the contribution of individual pathogens to diarrhoea makes treatment decisions difficult and might lead to over-utilization of antibiotics with adverse events and antimicrobial resistance [26]. Further technological refinement of such platforms is expected to significantly advance the field. Recent studies have shown that interindividual variation of the gut microbiome composition is crucial in conferring colonization resistance against *Vibrio cholerae* [27] and specific members of the commensal microbiome may protect against enteric rotavirus infection [28]. Future studies should attempt to combine polymicrobial detection of pathogens and microbiome profiling in the context of prospective clinical studies to facilitate a deeper understanding of the interactions of pathogens and commensals.

This study has some limitations. An additional control group of healthy infants would have further strengthened the study. Human immunodeficiency virus (HIV) infection might be associated with co-infection with diarrhoeal pathogens, even some that were not assessed in this study. We did not test infants for HIV infection, but HIV incidence in Ghana is comparably low [29]. For pathogens with low prevalence, the study lacks sufficient power to show significant difference.

In conclusion, infants from low- and middle-income countries are frequently exposed to numerous enteric pathogens, but data from this study exaggerates the causal relation to occurrence of diarrhoea. Rapid elimination and acquisition of enteric pathogens as observed during the follow-up appear to be independent from diarrhoeal episodes. Polymicrobial carriage may increase the risk of developing diarrhoea and a higher number of co-occurring pathogens accounts for certain parameters of disease severity.

Authors' contributions

TR, JPC and CDV designed the study. MH, CS, EOD, TR and CDV collected samples and clinical data. MH, ML, MÄ, EMA, EOD, JPC and CDV performed the experiments. MH, TR and CVD analysed and interpreted the data. MH and CDV drafted the manuscript. CS, ML, MÄ, EMA, EOD, TR and JPC read and revised the manuscript. All authors approved the final version.

Transparency declaration

The authors declare that they have no conflicts of interests, financial or otherwise, related to the publication of this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2021.03.020.

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