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Aetiology of diarrhoea among hospitalised children in Blantyre, Malawi following rotavirus vaccine introduction: a case-control study

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summary: Despite health gains achieved through rotavirus vaccination, rotavirus remains the most common cause of severe, hospitalised diarrhoea among children in Malawi. There is a pressing need to identify strategies to improve rotavirus vaccine performance in high disease burden, low-income countries."

ABSTRACT

Despite rotavirus vaccination, diarrhoea remains a leading cause of child mortality. We collected stool from 684 children <5 years of age hospitalised with diarrhoea (cases) and 527 asymptomatic community controls, for four years post rotavirus vaccine introduction in Malawi, and examined for 29 pathogens using PCR. Three or more pathogens were detected in 71% of cases and 48% of controls. Pathogens significantly associated with diarrhoea (cases/controls) included rotavirus (34.7%/1.5%), enteric adenovirus (29.1%/2.7%), *Cryptosporidium (*27.8%/8.2%), heat-stable enterotoxin-producing *E.coli* (21.2%/8.5%), typical enteropathogenic *E.coli* (18.0%/8.3%), and *Shigella*/enteroinvasive *E.coli (*15.8%/5.7%). Additional interventions are required to prevent diarrhoea due to rotavirus and other leading pathogens.

Keywords: Gastroenteritis, diarrhoea, children, PCR, rotavirus, case-control, Malawi

BACKGROUND

The introduction of rotavirus vaccines into childhood immunisation programmes across Africa has reduced rotavirus hospitalisations, with a recent population-based study from Malawi providing the first evidence of vaccine impact on infant diarrhoeal deaths [1]. However, the contribution of other viral, bacterial, and parasitic causes of diarrhoea among rotavirus-vaccinated African populations is not well understood [2,3].

Malawi is a low-income country in sub-Saharan Africa with one of the highest adult prevalence rates for HIV/AIDS, globally; 17.8% of women 15-49 years of age living in urban Malawi are HIV-infected (https://dhsprogram.com/pubs/pdf/SR237/SR237.pdf).

In children under 5 years of age, the mortality rate is 55 per 1000 live births with a high prevalence of stunting (37%). At the Malawi-Liverpool-Wellcome Trust Clinical Research Programme in Blantyre, Malawi, we have examined the effectiveness against hospitalised rotavirus diarrhoea of monovalent human rotavirus vaccine [Rotarix®], which was introduced into Malawi's national immunisation schedule in October 2012 [4]. Our diarrhoea surveillance platform is centred on a large urban hospital (Queen Elizabeth Central Hospital, QECH) and includes collection of clinical, demographic, and socioeconomic data combined with documentation of rotavirus vaccine status and stool collection.

We aimed to understand the aetiology of severe acute childhood gastroenteritis following rotavirus vaccine introduction in Malawi. We therefore undertook a case-control study to examine the prevalence of enteric pathogens among hospitalised children <5 years of age with diarrhoea, compared to diarrhoea-free children in the community.

METHODS

Study setting

The study was conducted in Blantyre, a district in Southern Malawi with a population of approximately 1.3 million people. The QECH provides free healthcare to residents of urban and rural areas of Blantyre district and is the only free/government inpatient referral centre in Southern Malawi. Monovalent rotavirus vaccine is administered according to the WHO-recommended schedule of 6 and 10 weeks. Vaccine coverage increased rapidly following introduction, and has exceeded 90% among age-eligible children since 2014 [1,5].

Symptomatic hospitalised cases

Subject enrolment and specimen collection was undertaken as part of our existing research programme [4]. Stool specimens were collected from November 2012 to December 2015 from children <5 years of age with acute gastroenteritis admitted to QECH (cases). Acute gastroenteritis was defined as the passage of three or more loose stools within a 24-hour period commencing <14 days prior to enrolment. We obtained demographic, clinical, and anthropometric data through parental interview, physical examination, and review of medical notes. Maternal HIV testing was undertaken according to national guidelines, as previously reported [4]. Disease severity was measured using the Vesikari score; a score of ≥11 (out of 20) indicates severe disease.

Asymptomatic community controls

Stool specimens were collected from children in the community who reported no diarrhoea for at least 7 days. These comprised a random sample of community controls recruited between April 2013 and December 2016 into rotavirus vaccine effectiveness case-control studies, in which controls were matched to individual rotavirus confirmed cases by age- and district-of-residence [4,5]. .

Laboratory testing

Faecal specimens were stored at -80^{O}C. We tested for 29 different enteric pathogens in faecal specimens using a 384-well singleplex real-time PCR platform, the enteric Taqman Array Cards (TAC) [6]. The TAC method performance has been previously described; samples were classified as pathogen positive at a cycle threshold (C_T) of <35 [6]. Detection above this threshold is not reproducible and is unlikely to be clinically significant [6].

Analysis

Since the introduction of rotavirus vaccination, norovirus has gained increasing recognition as a significant pathogen associated with diarrhoea in children [7]. We therefore based our sample size calculation on norovirus prevalence. Assuming a prevalence of 10% among hospitalised gastroenteritis cases and 5% among asymptomatic community controls [8], we calculated that for 90% power, 621 samples per group were needed to show a significant difference in prevalence between cases and controls (α=0.05). Pathogen prevalence among cases and controls was compared using χ^2-test or Fisher's exact test. Differences between continuous variables were tested using Student's t-test or Wilcoxon rank-sum test.

Adjusted attributable fractions (AFs) were calculated for pathogens which had a greater prevalence in cases compared to controls. Using a similar approach to the Global Enteric Multicentre Study (GEMS) [9], multivariable logistic regression was used to calculate odds ratios (ORs) for each pathogen detected, adjusting for presence of other pathogens, age in months and month of recruitment. Case or control status was the outcome variable and the predictors were indicator variables representing the presence or absence of each pathogen. We then calculated AFs for pathogens that were significant (p<0.05) in multivariable logistic regression. Calculation of AFs follows the formula:

$$AF_i = P_i \times \left(1 - \frac{1}{OR_i}\right)$$

where P = prevalence of pathogen i among cases. Analyses were performed using R version 3.3.5 (R Development Core Team, Vienna, Austria), with AFs and associated confidence intervals (CIs) calculated using bootstrap resampling in the 'attribrisk' package.

Ethics

Approval was obtained from the Malawi National Health Sciences Research Committee (Protocol #837) and from the University of Liverpool Research Ethics Committee (RETH #490).

RESULTS

Study population

A total of 1,211 faecal samples were analysed, comprising 684 samples from symptomatic hospitalised cases and 527 samples from asymptomatic community controls (Supplementary Table 1). Median age was 10.7 months (IQR: 7.9-15.4) and 12 months (IQR: 9.2-18.7) for cases and controls, respectively. Rotavirus vaccine status was established for 419/451 vaccine age-eligible cases (of whom 97% had received one dose or more), and in 375/400 vaccine age-eligible controls (of whom 95% were vaccinated). Maternal HIV status was determined for 665 (97%) of 684 cases, of whom 18% were HIV exposed. Mid-upper arm circumference (MUAC) was lower in cases (median=13.0 cm) compared with controls (median=15.3 cm). Among cases, 60 (9%) were severely malnourished (MUAC<11.5cm) compared with two (0.4%) controls. Vesikari score among cases ranged from mild (score of 1) to very severe (score of 20) (Median=13; IQR 10-15).

Pathogen prevalence

A positive PCR result for at least one pathogen was returned in 641 samples (94%) from cases and in 390 (74%) from controls (p<0.001) (Supplementary Table 1). Pathogens that were more frequently identified in cases than in controls included, in decreasing order of prevalence (cases/controls): rotavirus (34.7%/1.5%), adenovirus 40/41 (29.1%/2.7%), *Cryptosporidium* (27.8%/8.2%), heat-stable

enterotoxin-producing *E.coli* ([ST-ETEC] 21.2%/8.5%), typical enteropathogenic *E.coli* ([typical EPEC]18.0%/8.3%), *Shigella/*enteroinvasive *E. coli* (EIEC) (15.8%/5.7%), *Salmonella typhimurium* (2.3%/0.4%) and *Vibrio cholerae* (1.3%/0%) (Table 1). Pathogen prevalence by age group is presented in Supplementary Table 2. *Giardia* was less frequently detected in cases compared with controls (7.3%/13.9%). Enteroaggregative *E. coli* (EAEC), *Campylobacter* and norovirus were frequently detected in both cases and controls. Pathogens with the highest AFs ascribed to them were rotavirus, adenovirus 40/41 and *Cryptosporidium* (Table 1). Median Vesikari scores by pathogen ranged from 11 to 14.5 (Supplementary Figure 1).

Pathogen age distribution

The median age of *Giardia*-positive cases (15.6 months) was significantly higher compared to *Giardia*-positive controls (11.7 months) (p=0.014). For *Campylobacter* (10.9 (in cases)/13.8 (in controls) months: p<0.001), LT-ETEC (10.8/12.0 months: p=0.044) and atypical EPEC (11.3/12.4 months: p=0.030), median age of cases was significantly lower compared to controls. There was no difference in age between cases and controls for the remaining pathogens (Supplementary Figure 2).

Mixed infections

Among pathogen-positive samples, a single pathogen was detected in 78 (11%) and in 42 (8%) of samples from cases and controls, respectively. Cases had a significantly higher number of pathogens (mean=3.65, *SD*=2.11) compared to controls (mean=2.42, *SD*=1.96), with three or more pathogens detected in 71% of cases compared with 48% of controls. Enteroaggregative *E.coli* (EAEC) was identified in 51.8% of cases and in 47.8% of controls. The most frequent co-infections involving EAEC were with rotavirus, adenovirus 40/41, *Cryptosporidium,* ST-ETEC and typical EPEC among diarrhoea cases; and with *Campylobacter*, atypical EPEC, *Giardia*, and LT-ETEC among asymptomatic controls (Figure 1).

DISCUSSION

We have shown that following rotavirus vaccine introduction in Malawi, rotavirus remains the leading pathogen detected in children <5 years of age hospitalised due to diarrhoea. This is in agreement with data from two smaller studies, including one in Tanzania that assessed 146 diarrhoea cases in 2015, and another reported by the Global Rotavirus Surveillance Network (GRSN) that analysed a total of 327 samples collected from 10 African countries between 2013 and 2014 [2,3]. The prevalence of rotavirus shedding in asymptomatic children reported in this study (1.5%) is lower than reported previously in Malawi [10]. This may be the consequence of high rotavirus vaccine coverage reducing community rotavirus transmission, and could potentially lead to indirect benefits [5].

Adenovirus 40/41 was significantly associated with diarrhoea hospitalisation, in contrast with data from Tanzania and the GRSN that reported a modest AF for this pathogen [2,3]; however, the AFs calculated by these studies relied upon asymptomatic community samples collected by the GEMS study prior to rotavirus vaccine introduction [9]. Furthermore, pathogen-specific AFs should be interpreted with caution, since the relationship between organism load, disease severity and duration of shedding are not well defined for pathogens other than rotavirus. Nevertheless, the high prevalence of adenovirus 40/41 in cases, combined with a low prevalence in asymptomatic children, suggests an important role for this pathogen in severe diarrhoea in Malawian children. Our data are supported by recent reanalysis of the GEMS and MAL-ED (The Aetiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health) studies, which highlighted the importance of adenovirus 40/41 among children with diarrhoea [11,12].

Cryptosporidium was frequently associated with hospitalised diarrhoea in Malawian children, with a higher prevalence than reported in studies conducted elsewhere [2,3,9,11,12]. *Cryptosporidium* diarrhoea is associated with 4.2 million disability-adjusted life-years (DALYs) lost globally, and 7.85

million DALYs after accounting for the negative impact it has on growth [13]. These data highlight the need to prioritise effective treatment and prevention of *Cryptosporidium* infection in Malawi and other low-income countries that have high rates of stunting and HIV/AIDS, in order to reduce the burden of diarrhoeal disease and its associated long-term health consequences.

We also identified ST-ETEC, typical EPEC and *Shigella*/EIEC as important pathogens associated with hospitalised diarrhoeal disease, in line with previous reports [2,11,12]. Other pathogenic *E. coli* were also commonly detected. EAEC was found in nearly half of the infants regardless of presence of diarrhoea, and was frequently identified in co-infections among both cases and controls. Although EAEC does not appear to be associated with acute diarrhoea in this or previous studies [2,3,9], its presence with two or more other pathogens may have a negative impact on infant growth [14]. Similarly, despite a lack of association with hospitalised diarrhoea, the high prevalence of *Campylobacter* and *Giardia* in asymptomatic children in Malawi may result in growth faltering [15].

In some high-income settings, norovirus has emerged as the leading cause of severe diarrhoea in children following rotavirus vaccine introduction [14]. However, we did not document any change in norovirus prevalence since rotavirus vaccine introduction in Malawi; norovirus was identified in 12.1% of children hospitalized with diarrhoea compared to 11.3% in the pre-vaccine period [15].

Our study has limitations. Firstly, cases were slightly younger than controls; we therefore adjusted for age and month of recruitment in the analyses. We calculated AFs based on the binary presence or absence of pathogens, similar to the original GEMS study [9]. This method differs from those used for the reanalysis of GEMS and MAL-ED studies [12,15], where pathogen quantity was included in the model as a quadratic term. We chose not to use this approach because, unlike longitudinal studies, the duration of pathogen shedding is not quantifiable in case-control studies, and pathogen quantity will be variably associated with disease. A further consideration is our inclusion of cases from Nov 2012 to March 2013 prior to control recruitment, in order to achieve the required sample

size. In sensitivity analysis, excluding the early cases did not significantly alter the point prevalence of any pathogen (data not shown).

Rotavirus remains the most common cause of severe, hospitalised diarrhoea among children in Malawi, highlighting the importance of identifying strategies to improve rotavirus vaccine performance in this and similar high disease burden, low-income countries. Adenovirus 40/41, *Cryptosporidium*, ST-ETEC, typical EPEC and *Shigella*/EIEC are significant additional contributors to the burden of gastrointestinal disease in this population. Public health efforts, including vaccine development, should target these pathogens.

FOOTNOTES

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Potential conflicts of interest

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Figure 1: Pairwise co-infections in hospitalised diarrhoea cases and asymptomatic community controls. Percentage of infections is shown and was calculated by dividing the number of infections with both pathogen *A* (row) and *B* (column) by the denominator for the relevant group (i.e either total cases or total controls). EAEC=Enteroaggregative *E. coli*. EIEC=enteroinvasive *E.coli*. EPEC=enteropathogenic *E.coli*.LT-ETEC=heat-labile enterotoxin-producing *E.coli*. ST-ETEC=STh or STp-producing enterotoxigenic *E.coli*. STEC= shiga toxin-producing *E.coli*.

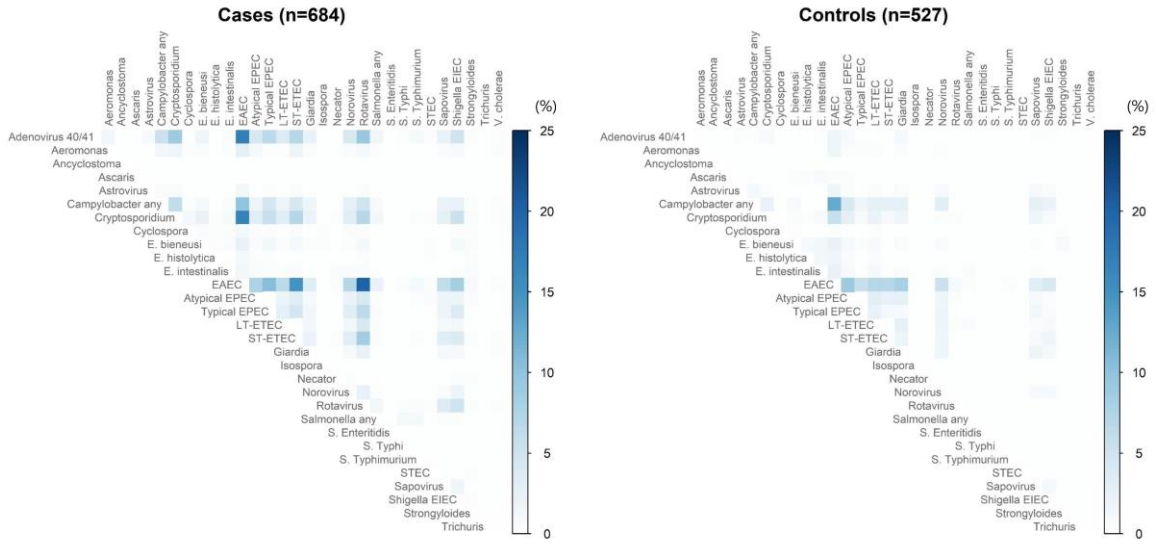

Table 1: Prevalence and attributable fractions of enteric pathogens in hospitalised diarrhoea cases and asymptomatic community controls. Pathogens are listed in order of decreasing prevalence among cases.

	Cases	Controls	p-value	AF	SE	CI
	n=684 (%)	n=527 (%)				
EAEC	354 (51.8)	252 (47.8)	0.193			
Rotavirus	237 (34.6)	8 (1.5)	<0.001	34.2%	0.018	(31.1-38.0)
Adenovirus 40/41	199 (29.1)	14 (2.7)	<0.001	27.7%	0.018	(24.5-31.4)
Cryptosporidium	190 (27.8)	43 (8.2)	<0.001	22.3%	0.023	(18.2-27.2)
ST-ETEC	145 (21.2)	45 (8.5)	<0.001	12.7%	0.030	(7.2-18.1)
Typical EPEC	123 (18.0)	44 (8.3)	<0.001	6.6%	0.037	(0-12.2)
***Campylobacter* any**	113 (16.5)	102 (19.4)	0.229			
Shigella/EIEC	108 (15.8)	30 (5.7)	<0.001	10.8%	0.021	(6.5-14.9)
Norovirus	83 (12.1)	45 (8.5)	0.054	7.0%	0.019	(2.8-10.6)
atypical EPEC	77 (11.3)	80 (15.2)	0.054			
LT-ETEC	68 (9.9)	71 (13.5)	0.069			
Sapovirus	64 (9.4)	34 (6.5)	0.083			
Giardia	50 (7.3)	73 (13.9)	<0.001			
Enterocytozoon bieneusi	31 (4.5)	21 (4.0)	0.747			
Salmonella any	30 (4.4)	5 (0.9)	0.001			
Aeromonas	27 (3.9)	10 (1.9)	0.059			

Salmonella Typhimurium	16 (2.3)	2 (0.4)	0.007	1.8%	0.009	(0.0-3.2)
Encephalitozoon intestinalis	15 (2.2)	20 (3.8)	0.14			
Cyclospora	13 (1.9)	4 (0.8)	0.138			
Astrovirus	12 (1.8)	13 (2.5)	0.509			
Entamoeba histolytica	10 (1.5)	8 (1.5)	1			
Vibrio cholerae	9 (1.3)	0 (0.0)	0.006			
Salmonella Typhi	8 (1.2)	2 (0.4)	0.201			
Strongyloides	7 (1.3)	10 (1.5)	0.845			
Necator	5 (0.7)	1 (0.2)	0.241			
STEC	5 (0.7)	1 (0.2)	0.241			
Ascaris	4 (0.6)	5 (0.9)	0.514			
Isospora	3 (0.4)	1 (0.2)	0.637			
Salmonella Enteritidis	0 (0.0)	0 (0.0)	NA			
Trichuris	0 (0.0)	1 (0.2)	0.435			
Ancyclostoma	0 (0.0)	0 (0.0)	NA			

Ancyclostoma, Salmonella Enteritidis and Trichuris were not included in the analysis because they not detected in either cases or controls. AF=adjusted attributable fraction; SE=standard error; LCI=lower confidence interval; UCI=upper confidence interval; EAEC=Enteroaggregative *E. coli*; EIEC=enteroinvasive *E.coli*; EPEC=enteropathogenic *E.coli*.LT-ETEC=heat-labile enterotoxin-producing *E.coli*; ST-ETEC=STh or STp-producing enterotoxigenic *E.coli*; STEC= shiga toxin-producing *E.coli*.