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Original Article

Correlation of Clinical Outcomes With Multiplex Molecular Testing of Stool From Children Admitted to Hospital With Gastroenteritis in Botswana

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Background. Diarrheal disease is a leading cause of death for young children. Most pediatric gastroenteritis is caused by viral pathogens; consequently, current recommendations advocate against routine antibacterial therapy if children present without bloody stools.

Methods. In this prospective cohort study, we enrolled children with severe acute gastroenteritis admitted to hospital in Botswana. Details of presenting history, physical examination, and course in the hospital were recorded. Stool samples were characterized using a 15 pathogen polymerase chain reaction assay.

Results. There were 671 participants with a median age of 8.3 months; 77 (11%) had severe acute malnutrition. Only 74 children had bloody stools, of whom 48 (65%) had a detectable bacterial pathogen, compared to 195 of 592 (33%) of those without. There were 26 deaths (3.9%). Covariates associated with death in multivariable logistic regression were the detection of any of *Campylobacter/Shigella*/enterotoxigenic *Escherichia coli* (odds ratio [OR] 2.57, 95% confidence interval [CI] 1.07–6.17), severe acute malnutrition (OR 4.34, 95% CI 1.79–10.5), and antibiotic therapy (OR 8.82, 95% CI 2.03–38.2). There was no significant association between bloody stools and death, and the effect of *Campylobacter/Shigella*/enterotoxigenic *E. coli* infection on death was not modified by the presence of bloody stools.

Conclusions. Detection of bacterial enteropathogens is associated with increased mortality in children in sub-Saharan Africa. Unfortunately, most children with these infections do not have bloody stools, and bloody dysentery was not found to be associated with worse outcomes. Clinical trials evaluating outcomes associated with more aggressive diagnostic strategies in children presenting with severe acute gastroenteritis in sub-Saharan Africa should be undertaken.

Key words. diagnosis; dysentery; gastroenteritis; polymerase chain reaction; treatment.

Diarrheal disease is the second-leading cause of death for children under the age of 5 years in the world today [1]. The majority of these deaths are in lower-income countries, with almost half occurring in sub-Saharan Africa [2]. A recent multicountry study following children presenting to health centers with moderate-to-severe diarrhea documented case-fatality rates of 1.1–7.5% [3].

It has long been accepted that most episodes of nondysenteric pediatric gastroenteritis are attributable to viral pathogens. Consequently, the World Health Organization (WHO) advocates against routine antimicrobial therapy if children presenting with acute gastroenteritis do not have blood in the stools [4]. However, it is now known that bacterial and parasitic pathogens can often be detected in enteric specimens taken from children living in many African and Asian regions experiencing acute nondysenteric diarrheal disease; though it is difficult to definitively ascribe causation, it is reasonable to hypothesize that many of these episodes of

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gastroenteritis might be attenuated with appropriate antimicrobial therapy [3].

The objective of this study was to describe the enteropathogens detected in children hospitalized with acute gastroenteritis in 2 major referral hospitals in Botswana and to correlate these findings with clinical outcomes.

METHODS

Study Population

Enrollment took place on the pediatric ward of Princess Marina Hospital, Gaborone, Botswana, from May 2011 to April 2013 and on the pediatric ward of Nyangabgwe Referral Hospital, Francistown, Botswana, from February 2011 to July 2011. All inpatients less than 13 years of age were eligible for inclusion if they had diarrhea at admission present for less than 14 days and had had at least 3 loose stools, or a single loose stool with at least 2 episodes of emesis, in any 24-hour period prior to enrollment. Children who developed diarrhea >48 hours after admission to the hospital or who had been discharged within 7 days of diarrhea onset were excluded. Those with additional infective diagnoses at admission to the hospital (e.g., bacterial sepsis, tuberculosis) were not excluded; none of the study participants had malaria. Note that universal rotavirus immunization in Botswana began in July 2012.

Interventions

The research protocol was approved by the research ethics boards of the Botswana Ministry of Health, Princess Marina Hospital, Nyangabgwe Referral Hospital, University of Pennsylvania, and McMaster University, Hamilton, Canada. All study participants were contacted on the ward by a research nurse as soon as possible after admission, and informed consent was obtained from the caregiver. Bulk stool samples were obtained from all participants. The Vesikari 20-point scale was used to quantify acute gastroenteritis severity in children [5]. The presence of bloody stool was assessed by caregiver report.

Microbiologic Testing

Stool samples were collected, transported, and processed as described previously [6]. Reverse transcription, amplification, and detection of 15 pathogen targets (3 viruses, 3 parasites, and 9 bacteria) was performed using the Gastrointestinal Pathogen Panel (GPP) assay (Luminex Molecular Diagnostics, Toronto, Canada) on the MAGPIX™ system. This assay has previously been evaluated [7, 8] and will simultaneously detect the following: *Giardia, Cryptosporidium, Entamoeba histolytica, Yersinia enterocolitica, Salmonella, Escherichia coli* heat-stable (ST) enterotoxin, *E. coli* heat-labile (LT) enterotoxin, *Shigella*, *Clostridium difficile* toxin A, *C. difficile* toxin B, *Campylobacter*, *Vibrio cholerae, E. coli* O157, Shiga toxin 1, Shiga toxin 2, norovirus GI, norovirus GII, rotavirus A, and adenovirus 40/41. Aliquots of MS2 bacteriophage (Luminex Molecular Diagnostics, Cat. Number 0820002) were added prior to pretreatment as RNA internal positive control. Negative controls were included with each assay run to account for the possibility of crossover contamination.

Statistical Analysis

Study data were collected and managed using REDCap electronic data capture tools, hosted at McMaster University. Baseline characteristics were analyzed using descriptive statistics reported as median (first quartile, third quartile) for continuous variables and count (percent) for categorical variables. Associations between categorical variables were described as risk ratios with 95% confidence intervals (CI), using χ^2 or Fisher's exact testing to determine statistical significance; evidence of effect modification by other covariates was investigated using the Mantel-Haenszel test. A multivariable logistic regression model was constructed to identify factors associated with death. *A priori* covariates of interest were as follows: infection with treatable bacterial pathogens (*Campylobacter, Shigella,* and enterotoxigenic *E. coli* [ETEC]), infection with viral pathogens, *Cryptosporidium* infection, presence of co-infections, severe acute malnutrition, human immunodeficiency virus (HIV) status, age category, and bloody stools; covariates with P values <.2 in bivariate analysis were also trialed in the model. A forward regression model was created adding covariates of interest 1 at a time; covariates that were associated with a statistically significant drop in the -2 log likelihood were retained, regardless of individual Wald P-value. A less statistically robust model containing all microbiologic variables and age category was also created, to better permit comparisons. Time-to-discharge analysis was also performed to investigate covariates associated with length of stay in the hospital; statistical significance for bivariate analyses was done using the log-rank test. Statistical significance was set as $P < .05$ for all comparisons. Analysis was done using STATA version 11.2 (Statacorp, College Station, TX).

RESULTS

There were 671 participants who had stool-testing results available (Table 1). The median participant age was 8.3 months (quartile 1 to 3: 5.0–13.1 months) and 42% of participants were female. There were 23 (3.4%) children with edematous malnutrition (kwashiorkor) at admission and another 62 (9.1%) with wasting (weight-for-length < −3 SD), for a total of 77 participants (11%) with severe

acute malnutrition. The majority of children were not HIV infected: 326 (49%) were HIV negative with no perinatal exposure, 203 (30%) were HIV-exposed perinatally but were known to be uninfected (HEU), 32 (4.8%) had unknown HIV status and possible perinatal exposure, 15 (2.2%) had mothers who had declined testing for both their child and for themselves, 55 (8.2%) had unknown HIV status but known perinatal exposure, and 40 (6.0%) of study participants were documented to be HIV positive. Of the children under 6 months of age with feeding data available, 13% were exclusively breastfed, 76% were exclusively formula-fed, and the remainder were fed with a mixture of breast milk, formula, and solid food. Almost two thirds of the participants (64%) had been given oral rehydration solution prior to hospital admission, though only a minority (29%) had received treatment with zinc. The median Vesikari score of study participants was 12 (Q1–3: 11–14) and 81% had "severe" gastroenteritis (Vesikari score $\geq$11) [9].

Seventy-four children (11%) had bloody stools at admission. Approximately half of study participants (328 of 630 with pharmacy data available, 52%) were started on antibiotics after hospitalization. Almost two thirds of children with bloody diarrhea (n = 47, 64%) received any sort of antimicrobial therapy, as did half of those without bloody diarrhea (n = 278, 50%).

There were 26 participants (3.9%) who died prior to discharge. Fourteen percent of those with severe acute malnutrition (and 26% of those with edematous malnutrition) died, compared to 2.5% of those without (risk ratio 5.64, 95% CI 2.69–11.8, $P < .001$). Antimicrobial therapy was also associated with death; 6.7% of those who were prescribed antibiotic therapy died, as compared to 0.66% of those who did not (risk ratio 10.1, 95% CI 2.40–42.7, $P < .001$). Of the 26 participants who died, 16 had pharmacy data available; of those 16, 4 received broad gram-negative coverage (e.g., amoxicillin + clavulanic acid, nalidixic acid + gentamicin), 7 received cefotaxime, 2 received ampicillin + gentamicin, 1 received ampicillin, and 2 received only antimicrobials other than those already listed (e.g., metronidazole, trimethoprim–sulfamethoxazole). There was no relationship between Vesikari score and death; the mean Vesikari score was slightly higher among those who survived to discharge. A statistically greater proportion of children who were potentially HIV positive died (11.1%, $P = .001$) than those who were HEU (2.0%) or HIV unexposed (3.4%), though there were no fatalities among the 39 participants known to be HIV positive. There was no statistically significant difference in the mortality seen in the different age groups (4.6% in infants, 4.0% in children aged 12–24 months, and 2.0% in children aged >24 months).

Only 115 children (17%) did not have an enteropathogen isolated from stool. Almost half the participants (n = 328, 49%) had 1 pathogen found, 160 participants (24%) had 2 pathogens detected, 52 participants (7.8%) had 3 pathogens found, 12 participants (1.8%) had 4 pathogens detected, and 4 participants (0.60%) had 5 pathogens isolated (Table 2).

The distribution of these pathogens in the study population was affected by age. Rotavirus was more commonly detected in younger children (42% of infants, 32% of children aged 12–24 months, and 12% of children aged >24 months, $P < .001$), as was *Campylobacter* (15% of infants, 16% of children aged 12–24 months, and 2.0% of children aged >24 months, $P = .016$). In contrast, *Shigella* was more often found in older children (9.6% of infants, 24% of children aged 12–24 months, and 47% of children aged >24 months, $P < .001$), as was *Salmonella* (5.5% of infants, 15% of children aged 12–24 months, and 12% of children aged >24 months, $P = .001$). The detection of ETEC, norovirus, and *Cryptosporidium* was not statistically associated with a specific age category.

Of the 74 children presenting with bloody diarrhea, 48 (65%) had a detectable bacterial pathogen in the stool (*C. difficile* was not judged a "pathogen" in children under the age of 2 years). Of the remaining 26 children, 6 had parasitic infections (2 had parasitic–viral co-infections)

Table 1. Characteristics of Study Participants

	Frequency[a] (%)
Age category	
<12 mo	437 (71)
12–24 mo	127 (21)
>24 mo	49 (8)
Gender	
Female	280 (42)
Severe acute malnutrition	
Wasting type	62 (9)
Edematous type	23 (3)
HIV status summary	
HIV negative, no exposure	326 (48)
HIV exposed but uninfected	203 (30)
HIV unknown, possible exposure	32 (5)
HIV testing declined by mother	15 (2)
HIV unknown, known exposure	55 (8)
HIV positive	40 (6)
Feeding method (<6 mo of age)	
Exclusive breast	19 (13)
Exclusive formula	115 (76)
Mixed breast-formula	10 (6)
Treatment with oral rehydration solution prior to admission	295 (64)
Treatment with zinc prior to admission	131 (29)
Vesikari score	Median 12 (Q1-Q3 11-14)
Blood in stool	74 (11)
Treatment with antimicrobials	328 (52)
Serum creatinine	Median 32 μmol/L (Q1-Q3 23-50)

[a]Note that, because of missing data, frequencies do not always total 671.

Table 2. Frequency of Pathogen Identification

Pathogen	Number of Detections (% of Each Stool Category)	P^a	Number of Detections, Multipathogen infections Excluded (% of Each Stool Category)	P^a
Campylobacter				
Nonbloody stools	81 (14%)	0.24	15 (3.8%)	0.46
Bloody stools	14 (19%)		2 (5.1%)	
Total	95 (14%)		17 (3.9%)	
Shigella				
Nonbloody stools	80 (14%)	<0.001	21 (5.3%)	<0.001
Bloody stools	29 (39%)		10 (26%)	
Total	109 (17%)		31 (7.1%)	
ETEC LT/ST[b]				
Nonbloody stools	55 (9.5%)	0.98	7 (1.8%)	0.53
Bloody stools	7 (9.5%)		1 (2.6%)	
Total	62 (9.4%)		8 (1.8%)	
Yersinia				
Nonbloody stools	0	0.11	0	0.09
Bloody stools	1 (1.4%)		1 (2.6%)	
Total	1 (0.2%)		1 (0.2%)	
Salmonella				
Nonbloody stools	50 (8.5%)	0.90	8 (2.0%)	0.57
Bloody stools	6 (8.1%)		1 (2.6%)	
Total	56 (8.5%)		9 (2.1%)	
Escherichia coli O157				
Nonbloody stools	12 (2.0%)	0.56	0	n/a
Bloody stools	1 (1.4%)		0	
Total	13 (2.0%)		0	
STEC[c]				
Nonbloody stools	11 (1.9%)	0.44	1 (0.25%)	0.17
Bloody stools	2 (2.7%)		1 (2.6%)	
Total	13 (2.0%)		2 (0.46%)	
Clostridium difficile				
Nonbloody stools	25 (4.3%)	0.61	7 (1.8%)	0.19
Bloody stools	3 (4.0%)		2 (5.1%)	
Total	28 (4.2%)		9 (2.1%)	
Cryptosporidium				
Nonbloody stools	44 (7.5%)	0.03	8 (2.0%)	0.57
Bloody stools	11 (15%)		1 (2.6%)	
Total	55 (8.3%)		9 (2.1%)	
Giardia				
Nonbloody stools	24 (4.1%)	<0.001	4 (1.0%)	0.093
Bloody stools	11 (14.9%)		2 (5.1%)	
Total	35 (5.3%)		6 (1.4%)	
Rotavirus				
Nonbloody stools	222 (38%)	<0.001	158 (40%)	0.001
Bloody stools	12 (16%)		6 (15%)	
Total	234 (36%)		164 (38%)	
Adenovirus				
Nonbloody stools	58 (9.9%)	0.54	26 (6.6%)	0.28
Bloody stools	9 (12%)		4 (10%)	
Total	67 (10%)		30 (7%)	
Norovirus GI/GII				
Nonbloody stools	79 (13%)	0.52	35 (8.8%)	0.33
Bloody stools	12 (16%)		2 (5.1%)	
Total	91 (14%)		37 (8.5%)	

The total number of stool samples was 660. There were a total of 74 stool samples with gross blood present. Eleven samples were not included in this table because data regarding presence/absence of blood in stool was not present. Only 435 of 660 stools did not have multiple pathogens detected; of these, 39 were bloody.
Abbreviation: n/a, not applicable.
[a]Comparison for proportion of positive testing results between participants with and without bloody stools. Chi-square tests used for all except *Yersinia*, *Salmonella*, STEC, and *C. difficile*.
[b]Enterotoxigenic *E. coli*, toxins LT or ST detected.
[c]Shiga toxin–producing *E. coli*, stx1 or stx2 detected.

and 11 had only viral pathogens isolated in the stool; 9 children with bloody diarrhea had no enteropathogens detected. There were 195 of 592 children (33%) without bloody stools who harbored a treatable enteric pathogen (*Campylobacter, Shigella,* ETEC, or *Cryptosporidium*) and an additional 23 participants (3.9%) without bloody diarrhea had *Salmonella* spp. detected in the stools. *Shigella* infections were strongly associated with bloody diarrhea and rotavirus infections were strongly associated with the absence of blood in the stools; no other

enteropathogens were found to be statistically associated with the presence or absence of bloody diarrhea when co-infections were excluded (Table 2).

There was no statistically significant association between bloody diarrhea and death (risk ratio 1.41, 95% CI 0.50–4.00); this estimate was not statistically modified when adjusted for antimicrobial treatment. However, statistically more children died who had any of *Campylobacter/Shigella*/ETEC in the stools (risk ratio 2.61, 95% CI 1.22–5.58, $P = .01$). Stratifying by the presence of bloody stool did not lead to any change in this risk ratio. There was no association seen between *Cryptosporidium* stool positivity and death (risk ratio 0.956, 95% CI 0.232–3.93). Less than half of children with a viral gastroenteritis died compared to those without (risk ratio 0.462, 95% CI 0.213–1.00, $P = .045$); adjusting for age did not find any statistically significant effect modification ($P = .11$). There was no statistically significant association between co-infection and death (risk ratio 1.65, 95% CI 0.776–3.51), and this relationship was not different when restricted solely to the subgroup of children with severe acute malnutrition.

The covariates that displayed the strongest associations with mortality in the multivariable logistic regression model were the detection of *Campylobacter/Shigella/*ETEC in the stools (odds ratio [OR] 2.57, 95% CI 1.07–6.17, $P = .03$), the presence of severe acute malnutrition (OR 4.34, 95% CI 1.79–10.5, $P = .001$), and the provision of antimicrobials at admission (OR 8.82, 95% CI 2.03–38.2, $P = .004$). When age category, viral infection, and *Cryptosporidium* detection covariates were included, they did not lead to any statistical improvement in model fit, and the point estimates for the above ORs were not significantly changed. When the regression analysis with the covariates for severe acute malnutrition and antibiotic therapy was re-run with each of the 3 bacterial pathogens separately, ETEC detection (OR 4.59, 95% CI 1.70–12.4) seemed to contribute proportionately more to mortality than the detection of *Shigella* (OR 1.66, 95% CI 0.645–4.32) or *Campylobacter* (OR 1.13, 95% CI 0.391–3.28). The OR of death associated with *Shigella* detection increased 35% (from approximately 1.7 to 2.3) when adjusted for age; the point estimates for the ORs of death associated with ETEC and *Campylobacter* did not change appreciably when adjusted for age.

The median length of stay was 4 days (Q1–Q3: 2–7 days) but the distribution was highly skewed, with 10% of the participants staying in hospital for >12 days. Length of stay was significantly prolonged in those with severe acute malnutrition and those treated with antibiotics ($P < .0001$ for both). Age category and the presence of *Campylobacter/Shigella/*ETEC in the stools were not associated with an increased length of stay, even among those children with dysentery; in contrast, *Campylobacter* positivity was associated with a longer length of stay ($P = .02$), and children with viral infections were discharged sooner ($P < .001$). Participants with bloody diarrhea did not have a statistically significant increased length of stay ($P = .07$). Children who were HIV negative had a significantly decreased length of stay compared to those who were HEU or those who were possibly or known to be HIV positive ($P < .001$).

DISCUSSION

In this report, we describe one of the largest cohorts of young children presenting with acute gastroenteritis in sub-Saharan Africa for which both comprehensive clinical data and extensive stool microbiologic data were collected. In contrast to other similar studies, our study population included a significant proportion of children with either severe acute malnutrition or HIV infection/exposure. The in-hospital mortality rate in our cohort was 3.9%, which is relatively high even for sub-Saharan Africa.

The current standard of care in many countries is to treat children presenting with bloody diarrhea for presumed dysentery with empiric antimicrobials, and to refrain from using antimicrobials when managing children with nonbloody diarrhea. In our cohort, *Shigella* infection was statistically more common in children with bloody stools, but this was not true for any other bacterial pathogen. We note that 219 of 586 children (37%) with nonbloody stools were found to be positive for *Shigella, Salmonella, Campylobacter,* ETEC, or *Cryptosporidium;* furthermore, we did not test for enteroaggregative *E. coli*, which suggests that the true proportion of children with severe gastroenteritis with nonbloody stools who harbored a potentially treatable pathogen was even higher. Children with bloody stools did not have increased mortality, even among only those with *Shigella* or *Campylobacter* infection, and those with bloody stools were not kept in the hospital longer than those without bloody stools. Adherence to antimicrobial therapy for dysentery was suboptimal, as just over one third of those with bloody stools were not treated, but this only serves to strengthen the argument that the presence of blood in the stool is not a useful diagnostic or prognostic factor for young children presenting with acute gastroenteritis in Botswana. Overall, our results would suggest that the current WHO treatment algorithm may not be effective in Botswana, where many children with acute gastroenteritis harboring a potentially treatable pathogen do not have bloody stools, and bacterial dysentery

does not appear to be associated with worse outcomes than bacterial enteritis without blood in the stool, even when adjusted for antibiotic use.

It is more important to determine which factors are in fact associated with poorer outcomes in children with acute gastroenteritis. In our cohort, detection of *Campylobacter*/*Shigella*/ETEC was associated with a statistically significantly increased odds of death. As described earlier, the odds of death prior to hospital discharge appeared to be highest in those children infected with ETEC; this is consistent with other reports suggesting ETEC is associated with increased virulence/pathogenicity [3, 10]. The association of death with severe acute malnutrition was even more marked, which is unsurprising given the extreme fragility of malnourished children. Children prescribed antimicrobials had an 8-fold increased odds of death, but we do not believe that antibacterial therapy itself confers a mortality risk; the association seen is almost certainly confounding by indication, as it is standard practice at the study hospitals to start empiric antibiotics to any child presenting with sepsis. It is important to consider that many study participants received antimicrobials without good activity against bacterial enteropathogens or *Cryptosporidium*, such as ampicillin or trimethoprim–sulfamethoxazole; furthermore, the molecular platform used does not predict antimicrobial susceptibility, so we cannot make judgments about the prevalence of antibiotic-resistant pathogens in the study population.

It should be noted that we made numerous comparisons and did not adjust our threshold for statistical "significance"; furthermore, though the mortality rate in our cohort was much higher than we would like, the absolute number of fatalities was too low to properly conduct a logistic regression including more than 2–3 important covariates. However, this study was exploratory, designed more to describe the epidemiology of childhood gastroenteritis in Botswana than to definitively answer a particular clinical question. We also note that our most important conclusions—that only *Shigella* infections were statistically associated with bloody diarrhea, that many children without bloody stools were infected with treatable enteropathogens, and that children with bloody stools did not have increased mortality nor an increased length of stay in the hospital—would be unaffected regardless of the method selected to correct for multiple comparisons.

It has been repeatedly demonstrated that children in lower-income settings can harbor enteropathogens without evidence of overt gastroenteritis [3, 10–12]; some investigators have utilized longitudinal follow-up with serial quantitative stool testing to more reliably identify the etiologic cause of a given diarrheal episode [13]. Consequently, we cannot be certain that the pathogen(s) identified in the children in our cohort were the cause of the illnesses that resulted in their admission to the hospital; however, the positive predictive value of a single-stool polymerase chain reaction test is undoubtedly higher in children with severe gastroenteritis requiring hospitalization. It is also not practical to suggest that all children living in low-income settings have serial stool microbiologic testing to facilitate evaluation in case they require admission to the hospital because of severe diarrheal disease. Some investigators have sought to increase specificity of stool molecular testing using stricter threshold cycle values [10]; we did not capture these data, but this approach bears further study in diverse populations. We note, however, that it is not yet clear whether higher amounts of specific enteropathogen nucleic acid correlate with disease *severity*. A recent analysis of lower respiratory tract infection in children in Kenya did not find a correlation between disease severity and quantitative load of pathogen nucleic acid for many respiratory pathogens [14]. It will be important to conduct randomized trials to verify whether treatment of selected bacterial and/or parasitic pathogens actually leads to improvement in clinical outcomes.

In conclusion, our study has provided additional data describing the etiology of acute gastroenteritis in a sub-Saharan African pediatric population with a high prevalence of HIV infection. We have demonstrated that the presence of bloody diarrhea does not predict infection with potentially treatable pathogens (aside from *Shigella*) and it does not appear to be associated with poorer outcomes; consequently, the adequacy of current WHO recommendations relating to the decision to start antimicrobial therapy should be revisited. Further studies should investigate the potential utility of rapid stool testing and/or the refinement of clinical assessment of gastroenteritis responsive to medical treatment.

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