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HISTO-BLOOD GROUP ANTIGENS, ENTEROPATHOGEN CARRIAGE AND ENVIRONMENTAL ENTEROPATHY IN STUNTED ZAMBIAN CHILDREN

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Abstract

Objectives: Stunting, the most common form of childhood undernutrition, is associated with environmental enteropathy (EE). Enteric infections are believed to play a role in the pathophysiology of EE and stunting though the exact mechanism remains undetermined. The FUT2 (secretor) and FUT3 (Lewis) genes have been shown to be associated with some symptomatic enteric infections in both children and adults. These genes are responsible for the presence of histo-blood group antigens (HBGAs) in various secretions and epithelial surfaces.

We evaluated whether the secretor and Lewis status influences asymptomatic enteric infections and thus EE severity on duodenal biopsies of stunted children.

Methods: In this case-control study, we used saliva samples to determine the secretor and Lewis status of stunted children (cases, n=113) enrolled in a nutritional rehabilitation program and from

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their well-nourished counterparts (controls, n=42). Where available, saliva was also collected from the mothers. Baseline stool samples were used to detect asymptomatic enteropathogen carriage. Duodenal biopsies were collected from a subgroup of stunted children (n=77) who had an upper GI endoscopy done as part of the evaluation process for their non-response to nutritional therapy.

Results: The proportion of secretors was similar between the cases and the controls (82% vs 81%, p=0.81). The stunted children had significantly higher rates of carrying multiple enteropathogens, but this was not associated with their sector status nor that of their mothers. The secretor status was also not associated with mucosal morphometry of duodenal biopsies.

Conclusion: This case-control analysis in Zambian children does not support the hypothesis that fucosylation status determines asymptomatic enteropathogen carriage in stunting.

Keywords

Secretor status; Lewis status; *FUT* genes; Malnutrition

Introduction

Childhood undernutrition remains a global problem that mainly affects children under the age of five years in low- and middle-income countries (LMICs) of Southern Asia and sub-Saharan Africa; these two regions bear the brunt of this burden(1). Worldwide, about 45 million suffer from acute malnutrition (wasting) and 149 million are chronically malnourished (stunted), making stunting the most common form of childhood undernutrition). Almost 50% of the deaths in children below the age of 5 years are at least partly attributable to undernutrition, a condition which puts these children at risk of severe manifestations of common infections (2,3).

The causes of stunting are multifactorial but environmental enteropathy (EE) and diarrhoea have been receiving a lot of attention in trying to understand its pathophysiology (2). EE is a subclinical disorder of the gut that is almost universal in adults and children living in impoverished communities. It is characterised by blunted intestinal villi, microbial translocation from the gut lumen into the systemic circulation, immune dysregulation and undernutrition (4). Even though the exact aetiology of EE is not yet known, it is associated with stunting and enteropathogens operating on a background of undernutrition are believed to be important contributors (5–7). It is however not completely understood why certain children are predisposed to recurrent enteric infections despite living in the same environment as other children who do not experience such infections.

Various factors influence susceptibility to enteric infections. The ABO blood group system genes, with its histo-blood group antigens (HBGAs), is one such factor associated with enteric infections as some enteropathogens attach to epithelial surface carbohydrates in the process of establishing infections (8). HBGAs are found on different types of epithelial cells and in secretions such as saliva, mucus and breast milk (as human milk oligosaccharides-HMOs) (9–11). The presence of HBGAs on epithelial cells and in secretions depends on a functional secretor gene (fucosyltransferase-2, *FUT2*) and the closely related Lewis gene (fucosyltransferase-3, *FUT3*). Individuals with a functional *FUT2* gene are referred to as

"secretors" while those with a non-functional gene are referred to as "non-secretors". The non-secretor phenotype is characterised by the absence of fucosylated HBGAs on epithelial cells, breast milk, saliva and mucus (12,13). The prevalence of the secretor phenotype varies by geographical location and ethnicity, ranging from 65-85% in Africa to more than 90% in North and South America (10,14,15). Susceptibility to infection by some pathogens associated with childhood diarrhoea is influenced by HBGAs. These include *Campylobacter jejuni*, enterotoxigenic *Escherichia coli (ETEC), Vibrio cholerae* O1, rotavirus and norovirus (GII.4 and GII.6). By acting as decoys or epithelial attachment factors, HBGAs influence susceptibility to these gut pathogens (16–21).

We set out to describe the relationship between the secretor phenotype and enteropathogen carriage in stunted Zambian children coming from EE prevalent communities. It is hoped that understanding of this relationship may further our understanding of both EE and childhood undernutrition.

Methods

This was a prospective case-control study conducted in southern part of Lusaka, Zambia. It was nested within the Biomarkers of Environmental Enteropathy in Children (BEECH) study that was conducted between August 2016 and June 2019 (22). The study site was St. Augustine clinic which has a catchment area that covers impoverished, high-density communities of Misisi, Kuku, John Laing and Chawama.

Study design and child recruitment

The BEECH study was an observational study whose objectives were to identify children from birth to 18 months of age with malnutrition through community screening. Children with evidence of growth faltering indicated by Weight-for-Age (WAZ), Weight-for-Length (WLZ) and Length-for Age (LAZ) z-scores of ≤−2 SD, were enrolled after obtaining informed, written consent from the parents/caregiver. The children were given a daily ration of corn-soy blend, micronutrient sprinkle (Nutrimix, Hexagon Nutrition, Mumbai) and an egg. They were followed up to the age of 24months. If the LAZ and/or WLZ persisted to be ≤−2 SD after 3-6 months of follow-up, the children were re-evaluated to identify possible causes of their non-response. This re-evaluation included ruling out infections such as tuberculosis, and an upper GI endoscopy with duodenal biopsies if no cause was identified (22,23). Children with complicated SAM were referred for in-patient management at the Children's Hospital. A subset of healthy children (0-18months) with no evidence of growth faltering were also enrolled from the same communities and served as controls. The study was approved by the National Health Research Authority and the University of Zambia Biomedical and Research Committee (UNZABREC reference number 006-02-16, 31st May, 2016).

Anthropometry

Anthropometric measurements were done in triplicates and averaged. The instruments used included infant and mobile taring scales (SECA 384 & 874, Hamburg Germany) for weight, infantometer (SECA 416, Hamburg Germany) and UNICEF stadiometer for length.

WHOAnthro (v3.2.2) software was used for calculation of precise WAZ, LAZ and WLZ scores according to the WHO child growth standards (24)(WHO, 2006).

Saliva analysis

Saliva was collected at baseline from the child (and mother if available) using an Oracol swab (Malvern Medical Developments S10) for phenotyping and Oragene-DNA kit (DNA Genotek Inc, Canada) for genotyping. The saliva for phenotyping was centrifuged, aliquoted and stored at −80°C before undergoing analysis for the presence of HBGAs (A, B, H, Le^a and Le^b) by ELISA according to a protocol adapted from Robert L. Atmar for the Kosek/Yori laboratory. In brief, on day 1, 1.5μL of boiled saliva sample was diluted in 750μL of phosphate buffered-saline (PBS). A 100μL aliquot of each diluted sample was then added to a well on a platethen 10% non-fat dry milk (nfdm) was added after a 4-hour incubation. The plates were then incubated overnight at 4°C in a humidity chamber.

On day 2, the plates were washed with 0.05% Tween-20/PBS wash solution. The corresponding primary antibody in 1% nfdm or 1% nfdm alone was added to the wells as appropriate. Thereafter, the plates were incubated before the secondary antibody or primary H-antibody was added to the different columns. After a further 1-hour incubation at 37°C, the plates were washed with 0.05% PBST. A mixture of 3,3',5,5'-tetramethylbenzidine and peroxidase substrate solution-B was added to the wells followed by 1 M H_3PO_4. The plates were finally read at 450nm using the Biotek-EL800 plate reader.

The presence of A, B, H or Le^b antigens in saliva meant that the participant was a secretor. If only the Le^a antigen was present, the participant was classified as a non-secretor. Lewis positivity (*FUT3*) was indicated by the presence of Le^a or Le^b antigens, absence of both with the detection of A, B or H indicated a Lewis negative status. If negative for all antigens, the sample was classified as undetermined and genotyping was used to determine the secretor status.

Genotyping

Genotyping of salivary DNA was used to clarify the secretor status of participants with undetermined results as well as to confirm the phenotyping results obtained by ELISA. This was done using the H3Africa chip (https://chipinfo.h3abionet.org/) at Cedars-Sinai Medical Center, California, USA. The H3Africa chip/array was designed by the H3Africa consortium and Illumina to study associations of genomic variants and diseases in African populations.

(https://h3africa.org/index.php/2019/12/12/h3africa-chip-faq/?mode=list). We used 3 SNPs (rs602662, rs492602 and rs516246) that have a strong linkage disequilibrium (AFR r^2>0.99) with rs601338 (428 G>A nonsense mutation) to identify secretors and non-secretors (25–27).

Stool analysis

Stool samples were collected from children without diarrhoea at baseline and stored at −80°C before being sent to the Center for Infectious Disease Research in Zambia laboratory in Lusaka for identification of enteropathogens using the Magpix xTAG®

Gastrointestinal Pathogen Panel (GPP). The panel was used to qualitatively identify viral (adenovirus, norovirus, rotavirus), bacterial (*Campylobacter*, ETEC, *Salmonella* spp, *Shigella, Clostridium difficile, vibrio cholerae*) and parasitic nucleic acids (*Cryptosporidium* spp, *Giardia intestinalis*)

Endoscopy and duodenal biopsy

The endoscopy and biopsy collection process for non-responders has been described in other publications (23). In brief, 3 biopsies were collected from the second part of the duodenum and immediately placed in saline and orientated under a microscope. They were then placed in formalin for fixation, processed into wax blocks, then sectioned at 3μm and stained using haematoxylin and eosin prior to mounting with DPX. An Olympus VS120-S6-107 scanning microscope was used to scan the slides and measure villus height (VH), crypt depth (CD), and epithelial surface area (Supplemental digital content 1) (28).

Data analysis and statistical considerations

Precise z-scores were generated using WHOanthro software (version 3.2.2.); STATA 15.1 was used for further analysis. Normally distributed variables were summarised in terms of means and standard deviation while medians and interquartile ranges (IQR) were used for variables not normally distributed. Fishers exact test was used to compare proportions among the different groups. Kruskal-Wallis was used to analyse differences in continuous variables among the groups.

Results

We analysed data for 113 stunted and 42 well-nourished children. The stunted children were significantly older and were more likely to have a history of diarrhoea in the 2 weeks preceding enrolment compared to their mainly exclusively breastfed, well-nourished counterparts (Table 1). Other parameters such as sex distribution, HIV status and area of residence were similar between the groups.

Prevalence of secretor phenotype in stunted and well-nourished children

The secretor status of 99/113 stunted and 36/42 well-nourished participants was determined by phenotyping with a total of 20 (14 cases, 6 controls) showing undetermined results. Genotyping identified the secretor status of these undetermined samples and provided confirmation of the phenotyping results for the rest of the cohort. This brought the total number with confirmed secretor status to 155 (113 cases, 42 controls). There was good concordance between the phenotyping and genotyping results except for one participant who was identified as a non-secretor by phenotyping but as a secretor by genotyping and was thus analysed as a secretor (Table 2). The Lewis status was determined by phenotyping only.

There was no statistical difference in the proportion of secretors between the cases and controls (82% vs 81%, p=0.81). The same trend was noted for the Lewis phenotype (56% vs 53%, p=0.79). All the mothers of the children with normal growth were secretors compared to 78% among the mothers of stunted children, but this difference was not statistically

significant (Table 2). Most of the secretors were heterozygous (69% cases, 62% controls) at all the 3 SNP loci, i.e., rs602662, rs492602 and rs516246.

Secretor Phenotype and carriage of enteropathogens in malnourished and well-nourished children

We then looked at asymptomatic carriage of enteric pathogens and compared the proportions of secretors and Lewis positive children between the cases (n=70) vs controls (n=12) (Table 3) and among stunted children (Table 4). The rate of enteropathogen carriage was significantly higher among the cases as compared to the controls among whom no rotavirus or *Clostridium difficile* was identified. Thirty percent (30%, 21/70) of the stunted children were positive for rotavirus despite more than 80% of them having documentation showing receipt of the two rotavirus vaccine (*Rotarix*, GSK) doses by 10 weeks of age (Supplemental digital content 3). Only one child (stunted) among the entire cohort had asymptomatic *Vibrio cholerae* infection. The most frequently identified enteropathogens in the stunted children were *Salmonella* spp (83%), *Giardia intestinalis* (76%), ETEC (74%) and *Shigella* (73%); among the controls, *Giardia intestinalis* (58%) and *Salmonella* spp (50%) were the most common, followed by *Shigella*, ETEC and norovirus (all 25%). On average, 5 enteropathogens were identified from cases with the maximum being 10 pathogens identified in 6 of the children, compared to an average of 1 pathogen among the controls. There was no statistical difference in the rates of pathogen carriage between the secretors and non-secretors for all the pathogens identified, including rotavirus and norovirus.

Mucosal morphometry

Of the 113 stunted children in this group, 77 (68%) had an upper GI endoscopy and well oriented duodenal biopsies that were suitable for villus morphometry. All the stunted children had features of enteropathy on gross inspection during endoscopy as well as on histology. There were no statistical differences between the secretors and non-secretors in terms of villus height (VH), crypt depth (CD) and other measurements made on the biopsies. Lewis positive children did however have significantly larger CD (Table 4 and Supplemental digital content 1).

Discussion

Stunting continues to affect millions of children in LMICs, putting them at higher risk of mortality as well as of poor neurocognitive development (1,3,29). We set out to test the hypothesis that fucosylation (or HBGAs) could in part explain the carriage of enteropathogens, and thereby severity of enteropathy, in stunted children living in an EE endemic Zambian community.

The prevalence of secretors (82%) and non-secretors (18%) in our cohort (cases and controls) is similar to that published in literature for both adults and children in other countries (14,15,30). The proportion of secretors did not significantly differ among the stunted vs. well-nourished children (82% stunted, 81% normal growth: p=0.81), with the majority being heterozygous secretors. The distribution of Lewis (*FUT3*) positive phenotype was also similar between the two groups (56% stunted, 53% normal growth: p=1.00).

Our findings are similar to those of a study in neighbouring Malawi which found the proportion of secretors to be around 88% among rotavirus infected children and 72% among community controls (31).

Our finding of asymptomatic carriage of enteropathogens in stunted children is consistent with other studies (32–35). These enteropathogens were significantly higher in the cases compared to the younger controls (10 months vs 4 months) who were mainly exclusively breastfed. The stunted children would have also had a higher chance of exposure to pathogens through complementary feeding practices while the controls may have benefited from the protective effects of fucosylated-HMOs that are found in the breast milk of secretor mothers (36,37). None of the pathogens investigated was significantly associated with the secretor or Lewis status of the child or the mother (Table 1 and Supplemental digital content 2)

Some studies have found that HBGAs were associated with infection by pathogens such as rotavirus and norovirus (17,30,38–41), while others have found no association (31,42,43). Most of the evidence in support of an association have come from studies involving children with symptomatic infections as opposed to asymptomatic infections. Our findings in this cohort of asymptomatic children are similar to those obtained by Pollock *et al* in Malawi (31). Other possible explanations for our findings may be differences in the genotypes of the pathogens under study. Some studies have shown, for example, that norovirus GII.4 mainly infects secretors while non GII.4 has a predisposition for non-secretors (41). We however did not separately analyse the rotavirus or norovirus infections based on their genotypes. The surprisingly high frequency of detection of rotavirus DNA in stool samples despite a well-established vaccination programme suggests that vaccines have reduced the incidence of symptomatic and severe infections but have not prevented viral circulation in the community; this asymptomatic circulation may still contribute (together with other pathogens) to EE. The P[8] strain was dominant before *Rotarix* (GSK) vaccine introduction in Zambia, but the post-vaccine era has been dominated by the P[4] and P[6] strains (44). It is unclear whether ever-changing viral genotypes could explain our findings; further investigation is needed with a larger sample size and strain identification

Similar to our previous findings of EE in this community, all the stunted children who had an upper GI endoscopy had evidence of EE on gross examination of the intestinal mucosa (45). The mucosal morphometry was comparable to that of children with SAM and persistent diarrhoea even though the stunted children in the current cohort were asymptomatic at the time of sample collection. The morphometry findings and the highly prevalent carriage of enteropathogens are consistent with the hypothesis that enteric infections contribute to EE and undernutrition. However, there were no significant differences in the VH, CD and surface area of the secretors compared to non-secretors. In this study, the secretor status was not associated with either asymptomatic enteropathogen carriage nor mucosal morphometry.

Apart from the enteropathogens, the gut microbiome is also influenced by the presence of fucosylated HBGAs found in the guts and breastmilk of secretors (11,37)(Bode., 2012 & 2009). It is possible that secretor phenotype may influence EE and stunting via the

microbiota. Analysis of the microbiota, enteropathogens, mucosal histology and secretor status of both stunted and well-nourished children could shed more light on the role of this phenotype in EE and stunting (16). We are well aware of the ethical challenges of obtaining duodenal samples from age-matched, well-nourished controls in study settings.

Our findings add to the evidence that enteric infections are common in children from low-resource communities which also suffer from undernutrition. We did not find any association between the secretor status and asymptomatic enteropathogens carriage or mucosal morphometry. Lewis positive children had a 22% greater crypt depth than Lewis negative children, but we can only speculate if this is related to infectious burden, altered immune responses, or the chemical composition of secreted mucins or trefoil factors or composition of the microbiota. Further studies are needed to confirm this finding and determine how this effect might operate.

Conclusion

The prevalence of secretor phenotype was similar between the stunted and well-nourished children in this Zambian cohort. Stunting was associated with asymptomatic carriage of multiple enteropathogens with evidence of EE. The carriage of these enteropathogens was however not associated with the secretor status of the children or their mothers. All the biopsies collected from the stunted children had similar features of EE regardless of the secretor status of the child or the mother.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is Known

- Stunting, the most form of childhood undernutrition, is associated with environmental enteropathy (EE)
- Enteric infections are believed to play a major role in the pathogenesis of EE.
- Fucosylated histo-blood group antigens influence susceptibility to many enteric infections

What is New

- Stunted Zambian children have multiple asymptomatic enteropathogen infections
- The secretor or Lewis status is not associated with asymptomatic enteropathogen infections in stunted children
- EE histopathology scores of duodenal biopsies from stunted children are not associated with the secretor/Lewis status of the child or their mother

Table 1.

Demographic characteristics of stunted (cases) and well-nourished (control) children

	CASES (n=113)	CONTROLS (n=42)	*P* value
Sex			
Female	56 (50%)	24 (57%)	0.47 (Fisher's exact)
Age (months)	10	4	**<0.001** (Kruskal Wallis))
HIV status			
Negative	76 (67%)	36(86%)	
Exposed but Uninfected	35 (31%)	6 (14%)	0.08 (Fisher's exact)
Positive	2 (2%)	0	
Breastfeeding *	94/111 (85%)	41 (98%)	**0.04** (Fisher's exact)
Diarrhoea in the past 2 weeks	40/105 (38%)	5/38 (13%)	**0.004** (Fisher's exact)
Baseline Anthropometry ** (median (IQR))			
Weight-for-Age z-score	−2.6 (−3.0;−2.2)	0 (−0.5;0.3)	
Weight-for-Length z-score	−1.1 (−1.7;−0.5)	0.79 (0.2;1.2)	
Length-for-Age z-score	−3.1 (−3.7;−2.5)	−0.9 (−1.1;−0.4)	
Undernutrition Phenotypes			
Wasting	7 (6%)	0	
Stunting	92 (81%)	0	
Stunting/Wasting	14 (12%)	0	
Birth weight (Kg)	2.7	3.1	**<0.001** (Kruskal-Wallis)
Rotavirus vaccination			
None	6 (5%)	8 (19%)	
1st dose only	8(7%)	4 (10%)	**0.02**
1st and 2nd dose	99 (88%)	30 (71%)	

* Breastfeeding status at enrolment includes both exclusive breastfeeding and mixed feeding, and is consequently dependent on age.

** Statistical tests were not done for baseline anthropometry as this was the basis on which the children were enrolled as either cases or controls

Table 2.

Secretor status of stunted and well-nourished children.

	Cases n=113	Control n=42	*P* value
Secretor *(FUT2)* *			
Child	93 (82%)	34 (81%)	0.81
Homozygous secretor	29/92 (31%)	13/ (38%)	0.40
Heterozygous secretor	64/92 (69%)	21/34 (62%)	
Mother	32/41 (78%)	13/13 (100%)	0.1
Lewis positive (*FUT3*)	n=52	n=19	
Child	29 (56%)	10 (53%)	1.00
Mother	27/41 (66%)	6/11 (55%)	0.5

* The secretor status was determined by phenotyping and confirmed by genotyping. The Lewis status was determined by phenotyping only.

Table 3:

Enteropathogen carriage in stunted children and controls

	Cases (stunted) N=70	Controls (Well nourished) N=12	*P*-value (Fisher's exact)
Campylobacter	36 (51%)	1 (8%)	**0.01**
ETEC	52 (74%)	3 (25%)	**0.002**
Rotavirus A	21 (30%)	0	**0.03**
Adenovirus	22 (13%)	1 (7%)	1.00
Norovirus	47 (67%)	3 (25%)	**0.01**
Clostridium difficile	2 (3%)	0	1.00
Salmonella	58 (83%)	6 (50%)	**0.02**
Shigella	51 (73%)	3 (25%)	**0.002**
Cryptosporidium	22 (31%)	2 (17%)	0.5
Giardia	53 (76%)	7 (58%)	0.3
Number of pathogens (median (IQR)	5 (3-7)	1.5 (0.5-3.5)	**<0.001**

Table 4:

Enteropathogens and duodenal mucosal morphometry in stunted children grouped by secretor and Lewis status

	Stunted children					
	Secretor N=56	**Non-Secretor N=14**	***P* value**	**Lewis positive N=18**	**Lewis Negative N=14**	***P* value**
Enteropathogens						
Campylobacter	29 (52%)	7 (50%)	1.00	6 (33%)	7 (50%)	0.47
ETEC	40 (71%)	12 (86%)	0.5	15 (83%)	9 (64%)	0.25
Rotavirus	16 (29%)	5 (36%)	0.75	4 (22%)	3 (21 %)	1.00
Adenovirus	7 (12%)	3/12 (21%)	0.4	4 (21%)	1 (7%)	0.37
Norovirus	38 (69%)	9 (64%)	1.00	11 (61%)	12 (85%)	0.24
Clostridium difficile	2 (4%)	0	1.00	1 (6%)	1 (7%)	1.00
Salmonella	47 (84%)	11 (79%)	0.7	16 (89%)	13 (93%)	1.00
Shigella	39 (70%)	12 (86%)	0.32	15 (83%)	8 (57%)	0.13
Cryptosporidium	18 (32%)	4 (29%)	1.00	6 (33%)	3 (21%)	0.7
Giardia	42 (75%)	11 (79%)	1.00	14 (78%)	8 (57%)	0.27
Mucosal morphometry	N=60	N=17		N=20	N=17	
Villus height (VH)	180μm (145-214)	168μm (133-202)	0.6	185μm (152-225)	175μm (157-194)	0.6
Crypt depth (CD)	175μm (146-203)	180μm (166-200)	0.23	200μm (166-234)	157μm (139-178)	**0.01**
VH:CD	1.06	0.90	0.15	0.93	1.12	0.15
Villus epithelial surface area	532μm (462-590)	487μm (374-549)	0.13	514μm (469-584)	575μm (517-590)	0.2